Physicochemical and bioactive properties of whey protein hydrolysates produced using gastric and pancreatic proteinases

Thesis

presented by

Margaret Maria Mullally, B.Sc. (Biotech)

Teagasc*

Chemistry and Microbiology Department

National Dairy Products Research Centre

Moorepark

Fermoy Co. Cork

and

School of Biological Sciences†

Dublin City University

Glasnevin Dublin 9.

Research Supervisor: Dr. Richard J. FitzGerald*

Academic Ph.D. Supervisor: Dr. John P. Dalton†

Head: Professor Richard O'Kennedy†

---

for Degree of

Doctor of Philosophy

January 1996

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed Margaret Mullally **ID No.: 91700990**

Date: 12-1-96

Table of contents

page

Contents of Literature Review

page

Tables in Literature Review

page

Figures in Literature Review

Abbreviations

ACE	Angiotensin I converting enzyme
α-La	α-Lactalbumin
ACTH	Adrenocorticotrophic hormone
AMC	Amino-methyl-coumarin
BSA	Bovine serum albumin
β-Lg	β-Lactoglobulin
CM	Carboxymethyl cellulose
CMA	Cows milk allergy
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B
CSTR	Continuous stirred tank reactor
DH	Degrees of hydrolysis
DIFP	Diisopropylphosphofluoridate
DEAE	Diethylaminoethyl
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FABMS	Fast atom bombardment mass spectrometry
FPLC	Fast protein liquid chromatography
GP-HPLC	Gel permeation - HPLC
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
LAP	Leucine aminopeptidase
PMSF	Phenylmethylsulphonylfluoride
PPDA	Post proline dipeptidylaminopeptidase
PCPA	Procarboxypeptidase A
PCPB	Procarboxypeptidase B
RP-HPLC	Reverse phase-HPLC
SAS	Saturated ammonium sulphate
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TLCK	N-tosyl-l-lysine chloromethyl ketone
TPCK	N-tosyl-l-phenylalanine chloromethyl ketone
UHT	Ultra high temperature
WPC	Whey protein concentrate

Abstract

Physicochemical and bioactive properties of whey protein hydrolysates produced using gastric and pancreatic proteinases

Margaret M. Mullally

This project investigated the contribution of gastric and pancreatic enzymes to some physicochemical and biological activity characteristics of whey protein hydrolysates. Depending on the method of isolation, and particularly on the zymogen activation conditions, it was shown in this study that the endoproteinase and exopeptidase activities in pancreatic preparations can exist in different ratios. Furthermore, it has been shown that certain physicochemical characteristics of whey protein hydrolysates can be altered by manipulation of the zymogen activation conditions of pancreatic protease preparations. Novel whey protein derived peptide inhibitors of angiotensin I converting enzyme (ACE), generated using pancreatic proteinases, have been identified in this study. Differential scanning calorimetry (DSC) was used to study the thermal stability of an enriched fraction of β-lactoglobulin. The susceptibility of β-lactoglobulin to hydrolysis by gastric and pancreatic proteinases was related to the thermal stability of the protein. A pilot scale (tangential crossflow plate and frame) ultrafiltration system was investigated with the view to developing a procedure for the fractionation and continuous hydrolysis of whey proteins.

Acknowledgements

I would like to thank Dr. J.F. Connolly, Head of the Chemistry and Microbiology Department and Dr. W.J. Donnelly, Director, National Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, for allowing me the opportunity to carry out this research for degree of philosophy.

I am indebted to Dr. Richard FitzGerald for his invaluable help and guidance throughout this project and for his patient attention to detail. I would also like to thank Dr. John Dalton of Dublin City University, Dr. Dan O'Callaghan formerly of Moorepark, and Dr. Hans Meisel of the Institut für Chemie und Physik, Keil, Germany, for helpful discussions. My sincere appreciation goes to Dr. Raj Mehra of the Chemistry Dept. and Jim Kelly of Technology Dept., Moorepark for their expert words of advice throughout the 'membrane days' and for showing me the balance between work and good-humour. Have I finally become a student of life?

I would also like to thank the people in the Chemistry Dept. for their ever willingness to help in moments of need, for their good humour and their friendship. Special words of thanks to Helen Slattery, for her readiness to share her technical expertise.

My thanks to Siobhan Keating, who is always helpful as our librarian. She is also a true artist and I am proud to be owner of a 'Keating' original !

and without further proper punctuation capitals or commas of any kind I would like to thank all the friends ive made over the past four years only a few have shared the whole of my time here in moorepark and these will now be impossible to forget those reliable and steadfast in their friendships and some of whom have flown away but are still close to mind thanks to those that I can call on at a moments notice to partake in just the one sociable drink to solve the problems of the world my thanks too to some of those bloody foreigners (clogs, frogs and swedes) ive met for making a difference we'll meet again who knows where, who knows when

to those ive broken bread with, sang with, stayed up late and hung out clothes with especially the ravers Nora, Deirdre, Carmel, Una, Helen, Gemma, Ciaran, Jimmy, Maria, both Siobhans and Sheila. I will call on ye again! thanks to the 'famly' for those wonderful 'holdays' in the Weist !! words of appreciation to the proprietors of

the forge and caesars to murphys breweries its sometimes a blur but thanks anyway to all in the queue at lunch time I think that covers all the really important people to philosophers and my thanks to quentin tarantino for the inspiration throughout the pulp fiction write up thanks for the ideas quentin wishful thinking im trying real hard to be a shepherd

Dank je wel to Jaap for the many encouraging and entertaining letters and for his friendship.

Most importantly, I must express my gratitude to my Mother, my sisters Liz and Bernie and to my Aunt, whose clothes I've often worn - for their support and encouragment throughout my student years - suppose I had better get a job now finally!

Dedicated to the memory of my brother James (1980-1991)

Literature Review

1 Gastric and pancreatic proteinases

Proteolytic enzyme preparations find many applications in the food industry, such as the baking, brewing or cheese-making industries. More specifically, proteolytic preparations have been extensively used in the hydrolysis of milk proteins. Table 1.1 lists proteinases used in the food industry (printed in boldface), in addition to their source, their optimal pH range and their preferential specificity. More detailed information on the individual enzymes is given by Godfrey and Reichelt (1983) and in a recent book concerning enzymes in food processing edited by Nagodawithana and Reed (1993).

The gastric enzyme pepsin and the proteolytic enzymes of the pancreas, trypsin, chymotrypsin, elastase, carboxypeptidase A and B are reviewed herein. Only pertinent aspects of the literature, which is extensive, are examined in detail. The focus is mainly on factors which may lead to a greater understanding of the action of crude proteolytic preparations (such as those used in industry) on protein hydrolysis. These factors include biochemical characteristics, purification, activation, autolysis, stability, inhibition and denaturation. Throughout this section attention has been paid to species differences (porcine or bovine), in the production of different proportions of proteinases (Marchis-Mouren, 1965), on the mechanism of action (Vithayathil *et al.*, 1961; Buck *et al.*, 1962); the stability (Vestling *et al.*, 1990) and activation of the enzymes (Desnuelle and Rovery, 1961), with a view to how these differences in the proteinase preparations could affect the production of milk protein hydrolysates with desired functional and/or bioactive characteristics (Thibault, 1990; Jost and Monti, 1977).

1.1 Classification of Proteinases

Proteinases are classified according to their source (animal, plant, microbial), their catalytic action (endoproteinase or exopeptidase), and the nature of the catalytic site. In the Enzyme Commission (EC) system for enzyme nomenclature, all proteinases (or peptide hydrolyases) are in subclass 3.4. This sub-class is further divided into the exopeptidases, 3.4.11-19 and the endo-peptidases or proteinases,

Table 1.1 Protease enzymes finding application in the food industry.

Type of protease	Source	Common names, tradenames	Typical pH range	Preferential specificity
Serine protease	bovine / porcine pancreas	**Trypsin**	pH 7-9	Lys-, Arg-COOH
		Chymotrypsin	pH 8-9	Phe-, Tyr-, Trp-COOH
	Bacillus licheniformis	**Subtilisin Carlsberg, Alcalase,** Maxatase, Optimase.	pH 6-10	Broad specificity, mainly hydrophobic-COOH
	Bacillus amyoliquefaciens (Bacillus subsilis)	Subtilisin NOVO, subtilisin BPN'	pH 6-10	" " " " " " " "
	Bacillus sp. alkalophilic	Subtilisin Esperase, subtilisin Savinase		" " " "
Cysteine protease	Papaya latex	**Papain**	pH 5-8	Broad specificity, mainly hydrophobic-COOH
	Pineapple stem	**Bromelain**	pH 5-8	" " " "
	Fig latex	**Ficin**	pH 5-8	" " " "
Aspartic protease	bovine, porcine	**Pepsin,** pepsin A	pH 1-4	Mainly hydrophobic-COOH and NH_2
	Calf	**Chymosin,** rennin	pH 4-6	Rennet specificity on casein;
	Mucor miehei	**Rennilase,** Fromase, Marzyme, Morcurd	pH 4-6	mainly hydrophobic -COOH and NH_2 on protein substrates
	Mucor pusillus	**Emporase,** Meito rennet, Noury lab	pH 4-6	in general
	Endothia parasitica	Surecurd, Suparen	pH 4-6	Also Glx-COOH
	Aspergillus niger (Aspergillus saitoi)	Aspergillopeptidase A (pure aspartic protease), Sumyzyme AP, Proctase, Molsin, Pamprosin (mixed with carboxypeptidase)	pH 2-5	Pure aspartic protease: like pepsin; mixed preparations: broad specificity.
	Rhizopus sp.	Sumyzyme RP, Newlase	pH 3-6	Like pepsin
Metalloprotease	*Bacillus thermoproteolyticus*	Thermolysin, Termoase	pH 7-9	Ile-, Leu-, Val-, Phe-NH_2
	B. amyloliquefaciens (B. subtilis)	**Neutrase**	pH 6-8	Leu-, Phe-NH_2

(continues on next page)

(continued from previous page)

Type of protease	Source	Common names, tradenames	Typical pH range	Preferential specificity
Mixture of trypsin chymotrypsin, elastase, carboxypeptidase A or B	Bovine, porcine	Pancreatin	pH 7-9	Very broad specificity
Mixture of papain, chymopapain, and lysozyme	Papaya fruit	Papain, crude	pH 5-9	Broad specificity
Mixture of *B. amyloliquefaciens* serine and metalloprotease	*B. amyloliquefaciens*	Biophrase, Nagase, Rapidermase, Rhozyme P 53, MKC protease	pH 6-9	Broad specificity
Mixture of aspartic protease, metalloprotease, serine protease and carboxypeptidase	*Aspergillus oryzae*	***A. oryzae* protease**, Takadiastase, Sumyzyme LP, Veron P, Panazyme, Prozyme, Biozyme A, Sanzyme	pH 4-8	Very broad specificity
Mixture of alkaline and neutral protease plus aminopeptidase and carboxypeptidase	*Streptomyces griseus*	Pronase	pH 7-9	Very broad specificity
Aspartic protease, with some carboxypeptidase	*Penicillium duponti*	*P. duponti* protease	pH 2-5	Broad specificity

taken from Adler-Nissen (1993).

3.4.21-24. Endoproteinases are the proteinases most commonly used in food processing, but in some cases their action is supplemented with exopeptidases.

Endoproteinases cleave polypeptides at susceptible bonds distributed within the polypeptide chain, whereas exopeptidases sequentially hydrolyse one amino acid from either the N terminus (aminopeptidases; or dipeptidases, in the case of 3.4.13-15) or the C terminus (carboxypeptidases).

1.2 Endoproteinases

Endoproteinases can be divided into four major classes, i.e. serine proteinases (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23), and metalloproteinases (EC 3.4.24).

As the names imply, serine (trypsin, chymotrypsin, proteinases from bacteria and moulds which are active under alkaline conditions), cysteine (papain, bromelain, ficin, and certain enzymes from micro-organisms), and aspartic proteinases have serine, cysteine, and aspartic acid side chains, respectively, as an essential part of the catalytic site. Modification or blocking of these amino acids usually leads to complete inactivation of the enzyme, and is a standard way of determining the nature of an unknown proteinase.

The serine proteinases have maximum activity at alkaline pH. Those proteinases that contain serine residues at their active centres are deactivated by diisopropyl fluorophosphate (DIFP) which reacts with this residue. The closely related cysteine proteinases usually show maximum activity at more neutral pH values. These enzymes are inhibited by oxidising agents and heavy metals. The aspartic proteinases generally have maximum catalytic activity at acid pH. Acidic proteinases of the pepsin group have asparagyl or glutamyl residues at their active centres. Apart from pepsin, a number of proteinases from moulds, which are active under acidic conditions also belong to this category. The metalloproteinases contain an essential metal atom, usually Zn, and have optimum activity near neutral pH. Ca^{2+} stabilises these enzymes and strong chelating agents, such as ethylenediaminetetraacetic acid (EDTA) act as inhibitors.

Among human digestive enzymes, the aspartic proteinase, pepsin, is secreted in the stomach and the serine proteinases, trypsin and chymotrypsin, are excreted in the duodenum.

1.3 Exopeptidases

The aminopeptidases (EC 3.4.1 1) are ubiquitous in nature, but are less readily available in commercial products, since many are intracellular or are membrane bound. The commercial enzyme preparation, Pronase, isolated from *Streptomyces griseus,* contains endoproteinase, aminopeptidase and carboxypeptidase activities. Pronase is often used in the laboratory to achieve complete hydrolysis of proteins. However, the preparation is too costly for general food use. Animal tissue homogenates also have been applied on a semi-industrial scale as a source of aminopeptidase activity (Clegg *et al.*, 1974).

Carboxypeptidases are subdivided into serine carboxypeptidases (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17) and cysteine carboxypeptidases (EC 3.4.18) according to the nature of the catalytic site. Many commercial proteinases, in particular from fungi, contain appreciable amounts of carboxypeptidase activity. In the digestive tract, the metallocarboxypeptidases A and B are excreted in conjunction with the major serine digestive proteinases (trypsin, chymotrypsin, and elastase). Therefore, they occur in the impure, commercially available preparation, known as pancreatin, which is usually obtained by dehydration of the pancreas (Gerhartz, 1990).

The exopeptidase subgroup also includes the dipeptide hydrolases (EC 3.4.13-15) which are specific for dipeptide substrates and, consequently, cannot be classified as amino- or carboxypeptidases.

From a food-processing perspective, the exopeptidases find utility as a means of debittering protein hydrolysates. The most systematic debittering studies have been carried out on hydrolysates of casein, which is particularly prone to yielding bitter peptides. Aminopeptidases (Clegg *et al.*, 1974; Minagawa *et al.,* 1989) and carboxypeptidases (Umetsu *et al.,* 1983) have been applied successfully to debitter casein hydrolysates.

1.4 Activation and inhibition

Mammalian digestive enzymes are generally secreted in a proenzyme form. This proenzyme or zymogen form must be activated, generally by cleavage of specific bonds to release the active form of the enzyme. The gastric enzyme pepsin, in addition to the pancreatic enzymes trypsin, chymotrypsin, elastase and carboxypeptidases are all secreted in inactive zymogen forms.

Enzyme inhibition can be reversible or irreversible. Irreversible inhibition generally involves the formation of a stable adduct with the enzyme by covalent bonding with an amino acid residue at the active centre. Reversible inhibition can be described as, (a) Competitive, where the inhibitor competes with the substrate for the active site by forming an enzyme-inhibitor complex. In most cases the chemical structure of the inhibitor resembles that of the substrate; (b) Non-competitive, where the inhibitor decreases the catalytic activity of an enzyme without influencing the binding relationship between the substrate and enzyme. The inhibition is dependant solely on the concentration of the inhibitor; (c) Uncompetitive, where the inhibitor reacts only with the intermediate enzyme-substrate complex; (d) Substrate inhibition, where a high concentration of substrate or coenzyme may decrease the catalytic activity of the enzyme or (e) End-product inhibition which usually occurs in multi-enzyme systems where the end-product acts as a specific inhibitor of the enzyme at, or near, the beginning of the enzyme sequence. Comprehensive reviews of enzyme interactions with proteinase inhibitors are given by Laskowski and Kato (1980) and by Birk (1987).

1.5 Pepsin

The purification, molecular properties, kinetics and mechanism of action of pepsinogen and pepsin have been reviewed (Ryle, 1970; Fruton, 1971, 1987). The main features of this aspartic proteinase are as follows:

1.5.1 Biochemical characteristics of pepsin

Pepsin is a gastric proteinase, composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35 kDa (Edeloch, 1957). The amino acid

composition was determined both for the zymogen and active pepsin (Rajagopalan *et al.*, 1966) and it is known that two carboxyl groups are involved in the active site. The tertiary structure of pepsin is stabilised in part by three disulphide bridges and a phosphate group and it is active in acid, in the pH range of 1-5. However, the phosphate group which is attached to the hydroxyl group of a seryl residue, can be removed without loss of enzymatic activity. The three dimensional structure of the hexagonal crystal form of porcine pepsin at a resolution of 23 Å has been determined (Cooper *et al.*, 1990).

Pepsin shows a broad specificity for peptide bonds, favouring those involving carbonyl groups of aromatic amino acids such as tryptophan, tyrosine, phenylalanine, leucine and isoleucine (Carrey, 1989). Quantitatively, the most important food use of pepsin is in a mixture with chymosin in rennet preparations (Fruton, 1987). The kinetics of different active forms of pepsin and pepsin-like proteinases, such as rennin (chymosin) or cathepsins have been compared and contrasted, in addition to the influence of secondary structure on the proposed mechanism of action of pepsin (Fruton, 1971, 1987).

1.5.2 Purification of pepsin

In contrast to the extremely low isoelectric point of pepsin, i.e. between 2-3, that of pepsinogen is about 3.7 (Herriott, 1938). This difference is consistent with the cationic character of the peptides removed from the zymogen upon its conversion to pepsin. The significant difference in net charge between zymogen and active pepsin forms means that ion-exchange chromatography is commonly used to separate the zymogen from the active form of pepsin (Fruton, 1971, 1987).

1.5.3 Activation of pepsinogen

Pepsin is secreted in an inactive zymogen form, pepsinogen (40.4 kDa), by the cells of the mucosa of the stomach lining. Pepsin is converted from the inactive zymogen to the active enzyme by an autocatalytic process, not unlike that of trypsin, in the presence of HCl. Conversion of pepsinogen to active pepsin involves cleavage of a 41-residue amino terminal portion of the zymogen at pH 2. Activation of the zymogen form of pepsin also results in the formation of several peptides of low molecular

weight. The heterogeneity of the peptic secretion is due mainly to autodigestion, as in the case of chymotrypsin (α, β, etc.). The differences in amino acid sequences between pepsin from different species gives rise to corresponding differences in chromatographic and electrophoretic mobility. Peptides are derived from the released 41 amino acid sequence at the amino terminal portion of pepsinogen. One of these peptides, with 29 amino acid residues, acts as a pepsin inhibitor above pH 5 by forming a pepsin-inhibitor complex. These fragments have been shown to inhibit further autodigestion and also the proteolytic activity toward haemoglobin (Determann *et al.*, 1969). At pH values below pH 5, this complex is readily dissociated and active pepsin is formed (Herriott, 1962).

1.5.4 Stability and denaturation of pepsin

Porcine pepsinogen can undergo reversible denaturation following heating to 60 °C at pH 7 or after treatment with alkali (up to pH 11). Pepsin is quite stable between pH 2-5, but in contrast to its zymogen, it rapidly loses activity above pH 5.0 due to denaturation. The pH optimum for peptic hydrolysis of proteins is about pH 2 but on synthetic substrates it is around pH 4.0. When hydrophobic groups sensitive to pepsin are exposed on the surface of proteins through denaturation, the nature of these groups may affect the optimal pH and hydrolysis rate of the enzyme. Hydrophobic interaction has been shown to increase pepsin stability to heat, i.e. pepsin activity was unaffected at 60 °C and treatment with 4 M urea or 3 M guanidinium (Edeloch, 1958; Fruton, 1971). The pH dependence of the kinetic parameters of pepsin, and its active-site hydrogen bond mutants have been determined (Lin *et al.*, 1992).

1.6 Trypsin

1.6.1 Biochemical characteristics of trypsin

Bovine trypsin was first isolated from pancreatic acinar cells, where an inactive zymogen, trypsinogen, is synthesised and subsequently converted to an active form (Northrop *et al.*, 1948). Trypsin is commonly isolated in conjunction with insulin by extraction from bovine or porcine pancreatic glands. The commercial availability of trypsin has, therefore, been linked to the market for insulin for many years. Trypsin

catalyses the hydrolysis of ester and peptide bonds involving the carboxyl group of the basic amino acids, arginine and lysine, and in which a serine and a histidine residue participate in the catalytic mechanism (Desnuelle, 1960). Bovine pancreatic trypsin consists of a single chain of 233 amino acid residues with six disulphide bonds (Balls, 1965). Its structure has been established by X-ray crystallography at 1.8 Å resolution (Keil, 1971; Bode and Schwager, 1975). Trypsin is known to possess two binding sites close to each other, one to which charged molecules bind preferentially and the other to which neutral molecules bind (Sanborn and Bryan, 1968). The chemical, physical and enzymatic properties of the serine proteinase, trypsin have been reviewed (Desnuelle, 1960; Cunningham, 1965).

The differences between the proportions of enzymes present in bovine and porcine pancreatic juice have been reviewed (Desnuelle and Rovery, 1961; Marchis-Mouren, 1965). Although bovine, and porcine trypsins have similar pH and temperature optima, Michaelis-Menten constants and kinetics of esterolytic activity, a number of important differences exist. Some differences which are reviewed here, must be considered during purification and activation of trypsin from a particular species.

Porcine trypsin differs from bovine in its electrophoretic behaviour and stability in alkaline media, although both have similar ion-exchange chromatographic profiles using carboxymethyl (CM) or diethylaminoethyl (DEAE) -cellulose as adsorbents (Vithayathil *et al.*, 1961; Buck *et al.*, 1962; Hakim *et al.*, 1962; Van Melle *et al.*, 1963). Porcine trypsin has a lower isoelectric point, PI = 10.2, than bovine trypsin, PI = 10.5 (Buck *et al.*, 1962). Magnesium and calcium ions stabilise bovine trypsin but porcine trypsin is less sensitive to the cationic nature of the surrounding medium. These differences in electrophoretic behaviour and the stability of porcine trypsin point to the fact that structural differences exist between the porcine enzyme and trypsins of other species. There are significant differences in amino acid composition between bovine and porcine species (Travis and Leiner, 1965). For instance, the NH_2-terminal residue of the porcine zymogen is phenylalanine instead of valine in the bovine counterpart and the existence of C-terminal asparagine residues is characteristic of porcine trypsin (Charles *et al.*, 1963). In addition, bovine and porcine trypsins have different immunological properties (Uriel and Avrameas, 1965). Species variations,

however, will be found only in the general protein characteristics of the enzyme and not in the properties directly dependant on the active site (Buck *et al.,* 1962).

1.6.2 Purification of trypsin

The classical purification of bovine trypsinogen as described by Northrop *et al.* (1948), involves acid extraction of fresh pancreas, ammonium sulphate fractionation and removal of α-chymotrypsinogen as a crystalline by-product (Laskowski, 1956). Subsequent purification and crystallisation of trypsinogen involves treatment of the liquor and washings from the crystallisation of chymotrypsinogen with acidic 70 % saturated ammonium sulphate (SAS) to form a precipitate of crude trypsinogen. Two crystallisation methods have been described, one involving $MgSO_4$ at pH 8.0 (Laskowski, 1956), the other crystallisation in ethanol-water mixtures at 4 °C (Balls, 1965). There are significant differences in the isolation and crystallisation procedures of porcine trypsin in comparison to bovine trypsin (Van Melle *et al.,* 1963; Travis and Leiner, 1965). Porcine trypsinogen is less soluble than its bovine homologue, since it precipitates at 40 % SAS (Charles *et al.,* 1963).

Commercial trypsin is treated with the inhibitor N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) to inhibit traces of contaminating chymotrypsin activity. Methods such as ion-exchange chromatography have been used to separate chymotrypsin and inert proteins from different active forms of trypsin (Schroeder and Shaw, 1968). In addition, a simple and rapid purification of commercial trypsin and chymotrypsin by reverse phase-high performance liquid chromatography (RP-HPLC) has been described (Titani *et al.,* 1982). More recently, large-scale continuous purification processes, using a combination of affinity chromatography and ultrafiltration have been developed for the purification of trypsin (Luong *et al.,* 1988; Powers *et al.*, 1990). For example, trypsin was selectively bound by a water-soluble high molecular weight polymer, bearing a potent and specific trypsin inhibitor, para-aminobenzamidine. The trypsin macroligand complex was then retained by an ultrafiltration membrane. The bound trypsin was eluted with a solution of arginine or benzamidine (Luong *et al.,* 1988).

1.6.3 Activation of trypsinogen

Control of the rate of activation of the zymogen can be achieved by manipulating temperature, pH or by the inclusion of calcium ions or specific trypsin inhibitors. The mechanism of activation of trypsinogen in the presence of calcium ions has been studied (Abita *et al.,* 1969). Activation at pH 8 is catalysed by small amounts of trypsin in the presence of calcium (McDonald and Kunitz, 1946), or by enterokinase (Yamashina, 1956). Addition of calcium speeds up the activation of bovine trypsinogen but is less essential for activation of porcine trypsinogen (Vithayathil *et al.,* 1961).

The predominant single chain product is β-trypsin, resulting from the release of a hexapeptide (at the Lys_6-Ile_7 sequence) from bovine trypsinogen while an octopeptide is released from the porcine zymogen (Charles *et al.,* 1963). Subsequent cleavage of the Lys_{131}-Ser_{132} bond at the N-terminal end of the molecule of bovine trypsinogen leads to α-trypsin (Schroeder and Shaw, 1968) which is a two-chain structure held together by disulphide bonds. A further cleavage of α-trypsin at Lys_{176}-Asp_{177} yields an active bovine form termed pseudotrypsin (Smith and Shaw, 1969).

1.6.4 Stability, denaturation, autolysis and inhibition of trypsinogen and trypsin

Porcine trypsin has a superior resistance to self-digestion in neutral or alkaline solutions in comparison to bovine trypsin (Vithayathil *et al.,* 1961). Porcine trypsin has been shown to be more stable than bovine trypsin to thermal, alkaline or acidic denaturation (Buck *et al.,* 1962; Lazdunski and Delaage, 1965). Trypsinogen is stable at pH 2 to 4, but at neutral or alkaline pH auotactivation to trypsin takes place. Even lyophilised samples undergo slow autodigestion during storage at 4 °C. Immobilisation of trypsin improved its stability to autolysis (Swaisgood and Catignani, 1987) and allowed the use of trypsin at elevated temperatures which is advantageous in minimising microbial growth during the hydrolysis of whey proteins (Section 2 and 3).

Trypsin is fully active in urea solutions up to 6.5 M (Lazdunski and Delaage, 1965) and in 30 % (v/v) ethanol (Schwert and Eisenberg, 1949). Trypsin can be reversibly heat-denatured below pH 8 and the effect of pH on temperature-induced reversible denaturation of both bovine and porcine trypsinogen and trypsin has been studied. In an investigation of the susceptibility of the different forms of trypsin to

urea denaturation, it was shown that substrates or competitive inhibitors can protect trypsin from denaturation (Lazdunski and Delaage, 1965).

Since trypsin contains 14 lysines and 2 arginines residues, it is a substrate for its own action, and this autolysis has long been recognised as a potential source of artifacts in structural studies (Andrews and Dixon, 1989). The autolysis products produced by trypsin can be detected using fast atom bombardment mass spectrometry (FABMS) when the product mixture is analysed directly and when it is fractionated by HPLC. The autolysis of porcine pancreatic trypsin produces a different set of peptides from that of bovine trypsin (Vestling *et al.,* 1990) and it was shown that there is a slower proteolysis rate for porcine trypsin at pH 8.5 in 0.1 M NH_2HCO_3 and 0.1 mM $CaCl_2$ buffer (Vestling *et al.,* 1990). The formation of inactive products during the activation of bovine trypsinogen can be prevented by the addition of 0.02-0.10 M calcium which speeds up the selective splitting of Lys_6-Ile_7 by a factor of more than ten, thus promoting β-trypsin formation and also preventing further non-specific cleavage (Abita *et al.,* 1969) by protecting some of the bonds from tryptic attack. The protective effects of calcium ions is more pronounced in bovine than in porcine trypsin (Lazdunski and Delaage, 1965; Abita *et al.,* 1969). Spontaneous autocatalytic activation of trypsinogen can also be prevented by low molecular weight peptide inhibitors such as para-amino benzamidine and benzamidine or by natural inhibitors of trypsin e.g. soybean or cereal inhibitors (Mares-Guia and Shaw, 1965; Laskowski and Kato 1980; Kroghdahl and Holm, 1983).

Several inhibitors of trypsin have been characterised and reviewed (Keil, 1971). Trypsin is inhibited by DIFP and phenylmethyl-sulphonylfluoride (PMSF), which interact with the active centre by binding covalently to Ser_{183} (Speakman and Yarwood, 1966; Keil, 1971). Many compounds with a positively charged group and hydrophobic moiety such as amidines and guanidines, (Lorand and Rule, 1961; Mares-Guia and Shaw, 1965), benzoyl and tosyl arginine (D'Albis and Béchet, 1967) and aliphatic and aromatic amines (Inagami, 1964), have also been used to study the active site of trypsin. For example, the structure of the complex formed by bovine trypsin and the bovine pancreatic trypsin inhibitor has been studied (Huber *et al.,* 1974). Trypsin inhibitors have relatively low molecular weights (6000-8000 Da) and their tertiary structure is very compact owing to a high content of disulphide bridges.

These inhibitors are therefore very stable to denaturation or to the action of proteolytic enzymes. The most well known proteinase inhibitors are probably the trypsin inhibitors of soybean, e.g. Kunitz soybean trypsin inhibitor (KSBI) and Bowman-Birk inhibitor (BBI). Only rarely do natural trypsin inhibitors inhibit trypsin exclusively. In most cases they are polyvalent and also inhibit thrombin, chymotrypsin and kallikrein (Keil, 1971).

1.7 Chymotrypsin

1.7.1 Biochemical characteristics of chymotrypsin

The serine proteinase chymotrypsin is also secreted by the acinar cells of the pancreas. Two forms of the bovine zymogen are present, chymotrypsinogen A, with a molecular weight of 25 kDa and an isoelectric point of 9.1, and chymotrypsinogen B, of similar weight, with an isoelectric point of 5.2. The isoelectric points of the porcine zymogen A is pH 7.2, notably lower than the isoelectric point of the bovine zymogen. An additional zymogen form, chymotrypsinogen C was isolated from porcine pancreas (Folk and Schirmer, 1963). This zymogen was found in a complex with procarboxypeptidase A (PCPA) and proelastase E. Chymotrypsinogen C is often known as Subunit II in this complex (Pétra, 1970). The separation of this zymogen from the other components of the complex has been detailed (Puigserver *et al.,* 1986; Cambillau *et al.,* 1988).

Although porcine chymotrypsinogen B has similar biochemical characteristics to the bovine zymogen, it differs in that it is denatured at low pH and cannot be isolated by acid extraction of porcine pancreas. Acid lability is also a property of the third zymogen chymotrypsinogen C.

Chymotrypsin preferentially catalyses the hydrolysis of peptide bonds involving tyrosine, phenylalanine and tryptophan. In addition to bonds involving aromatic amino acids, chymotrypsin catalyses, at a high rate, the hydrolysis of bonds of leucine, methionine, asparagine and glutamine residues, along with the amides and esters of the above amino acids (Cunningham, 1965; Ardelt and Laskowski, 1985). Chymotrypsinogen C has been found to attack the carbonyl side of leucine in a large variety of simple substrates (substrates composed of a repeating unit) and larger

polypeptides. Chymotrypsin C is specific for the α-amino acids already mentioned but more especially for leucine residues.

The structure of bovine α–chymotrypsin from X-ray diffraction studies at a resolution of 2 Å has been studied (Sigler *et al.,* 1968; Blow, 1971). The amino acid sequence of bovine chymotrypsinogen A has been determined (Meloun *et al.,* 1966). It contains 241 amino acids residues and the amino acid residues known to participate in the mechanism of catalysis are His_{97}, Asp_{102}, and Ser_{195} (Hess, 1971).

The purification, activation procedures, physical and chemical properties of both bovine and porcine chymotrypsin have been reviewed by a number of groups (Desnuelle and Rovery, 1961; Folk, 1970a; Wilcox, 1970; Hess, 1971). In its specificity and pH-activity profiles, chymotrypsin resembles the subtilisins (e.g. Alcalase) rather than trypsin (Adler-Nissen, 1993).

1.7.2 Purification of chymotrypsin

The classical purification procedure of chymotrypsins A and B has been detailed (Laskowski, 1956). Briefly, the zymogen of chymotrypsin A is first isolated from an acid extract of pancreatic tissue and purified by recrystallisation from an ammonium sulphate solution. The pure zymogen is then activated with bovine trypsin under slow activation conditions and the enzyme is isolated by crystallisation at about pH 4 from ammonium sulphate. Salt is removed by dialysis and the product is lyophilised. Ion-exchange chromatography using CM-cellulose and Sephadex-G25 has been used to separate α-chymotrypsin from different active forms, i.e. the π, δ, and γ forms and the neochymotrypsinogen impurities which are produced under different activation conditions. Addition of DIFP inhibits chymotrypsin activation of the zymogen during the purification process. The purification and properties of porcine chymotrypsinogen A have been outlined (Wilcox, 1970) and this zymogen is similar to its bovine counterpart.

Purification of porcine chymotrypsinogens differs from bovine chymotrypsinogen, due to their acid labile nature and involves extraction of acetone powders of pancreatic tissue followed by ammonium sulphate fractionation and DEAE-cellulose chromatography (Folk, 1970a and Wilcox, 1970).

1.7.3 Activation of chymotrypsinogens

The different zymogens of bovine chymotrypsin A and B have several active forms, i.e α, β, δ, γ, π chymotrypsin, depending on the purification procedures and activation conditions used. Tryptic cleavage of a single peptide bond between Arg_{15} and Ile_{16} results in a fully active enzyme (Wilcox, 1970). However, under conditions of slow activation i.e. at low temperatures and in the absence of calcium ions, chymotryptic digestion of the zymogen produces neochymotrypsinogens, which are then activated by trypsin to various active forms. The different activation mechanisms, properties and types of active chymotrypsinogen A and B have been reviewed (Desnuelle and Rovery, 1961; Sigler *et al.,* 1968). It has been noted that the duration of activation and presence or absence of ions such as calcium dictates the forms, activities and stabilities of the resulting enzymes (Wilcox, 1970). Calcium ions act to stabilise the conformation of both the zymogen and active forms during the activation process and prevent autolysis. These ions, however, are not essential in the activation of the zymogen form by trypsin to release active chymotrypsin (Wilcox, 1970).

The differences which exist between activation of zymogens from the porcine species in comparison to the bovine counterpart, in addition to the types of active enzymes formed have been reviewed (Wilcox, 1970). The specific activity of chymotrypsin changes during the activation process and further autolytic modification. Some of this change is due to inherent differences among the different species of chymotrypsin, some due to inhibition by end-products of autolysis and some due to formation of inert protein fragments (Wilcox, 1970). It is known that differences in specific activities of the different forms of chymotrypsin is due to heterogeneity of the active forms of this enzyme (Wilcox, 1970).

1.7.4 Stability and inhibitors of chymotrypsins

Chymotrypsin A is most stable at pH 3, nevertheless, even at this pH autolysis proceeds, but at a slower rate than at higher pH values (Wilcox, 1970). At pH 6.5 chymotrypsins aggregate to high molecular weight oligomers. The enzyme is also denatured by urea at concentrations above 3.5 M (Harris, 1956). Porcine chymotrypsin C can be stored at 4 °C and pH 6 as a suspension in 65 % saturated ammonium sulphate, as a salt free solution for up to 4 months. Immobilisation has been used as a

method of stabilisation of α-chymotrypsin against different denaturing agents such as heat, urea and organic solvents (Guisán *et al.,* 1991).

Natural inhibitors (Krogdahl and Holm, 1983), in addition to PMSF include, heavy metals such as Cu^{2+}, Hg^{2+} and Ag^{2+}, organophosphorus compounds, α-chloroketone and phenothiazine-N-carbonyl chloride also inhibit chymotrypsin activity.

1.8 Elastase

1.8.1 Biochemical characteristics of elastase

Elastase, an endoproteinase, is secreted in the acinar cells of the pancreas in the proenzyme form and is activated by trypsin. Pure elastase was first isolated from porcine pancreas and the important physicochemical characteristics of this enzyme were established (Lewis *et al.*, 1956, 1959). Elastase has a broad substrate specificity and is involved in hydrolysis of peptide bonds involving the carbonyl groups of amino acids with uncharged non-aromatic side chains i.e. alanine, valine, leucine, isoleucine, glycine and serine. It has a molecular weight of approximately 26.5 kDa (Lewis *et al.,* 1956). It exists as a single peptide chain containing 240 amino acids cross-linked by four disulphide bridges and has an isoelectric point of pH 9.5. It has been shown that elastase splits peptide bonds in elastin (Partridge and Davis, 1955) and also hydrolyses the A and B chains of oxidised insulin (Naughton and Sanger, 1961). Many of the chemical, physical and kinetic properties in addition to the primary and tertiary structure of elastase have been reviewed in detail (Shotton, 1970 and Hartley and Shotton, 1971).

Two families of elastase are known to exist in the porcine pancreas. One family, represented by porcine elastase I (Ureil and Aurameas, 1965 and Powers *et al.,* 1977), is characterised by a general specificity for small amino acid side chains such as isoleucine, valine and alanine (Powers *et al.,* 1977). Porcine elastase I has a very basic isoelectric point in the region of pH 10.7. The second family, represented by porcine elastase 2 and human elastase 2 (Largmann *et al.,* 1976; Gertler *et al.,* 1977), is characterised by a specificity for bulky hydrophobic amino acids (Del Mar *et al.,* 1980). The porcine elastase II family have a low basic isoelectric point of

approximately pH 9.6 (Largmann, 1983). Details of the primary and tertiary structure of porcine elastase have been reviewed (Hartley and Shotton, 1971). Structural analysis of the active site of porcine pancreatic elastase based on the X-ray crystal structures of complexes with trifluoroacetyl-dipeptide-analide inhibitors has been recently carried out (Mattos *et al.*, 1995).

1.8.2 Purification of elastase

A purification procedure widely used involves ammonium sulphate precipitation of a sodium acetate powder of pancreatic tissue and often involves ethanol fractionation (Lewis *et al.,* 1956) followed by dialysis against water to yield a euglobulin precipitate (Hall, 1957). A variety of materials including elastin, alumina gel (Hall, 1957) and various ion-exchange materials have also been used with some success to adsorb elastase from crude pancreatic extracts. Elastase can be easily crystallised from dilute salt solutions in which the contaminating proteins are fully soluble. This has lead to a simple and selective procedure for obtaining pure recrystallised elastase (Shotton *et al.,* 1968). Various methods have been used to purify the euglobulin precipitate (Lewis *et al.,* 1959, Naughton and Sanger, 1961 and Gertler and Birk, 1970). Details of these methods have been reviewed (Shotton, 1970). Other purification methods have combined techniques such as affinity chromatography with immobilised proteinase inhibitors and CM-Sephadex chromatography (Gertler *et al.,* 1977; Largmann, 1983) to isolate the different types of pancreatic elastase from within the two families previously mentioned.

1.8.3 Activation and inhibition of elastase

The activation and inhibition of elastase has been extensively reviewed (Shotton, 1970; and Hartley and Shotton, 1971). The elastolytic activity of elastase is appreciably affected by salt. Sodium chloride (50-70 mM) caused 50 % inhibition of elastase activity and similar effects were observed with potassium chloride, ammonium sulphate and sodium cyanide (Lewis *et al.,* 1956). However, the activity of elastase towards the specific substrate benzoyl-L-alanine methyl ester (BAME), while being inhibited 14 % by 100 mM sodium chloride, is greatly stimulated by 1.2 M sodium sulphate or by 10 mM trishydroxymethylamine (Shotton, 1970). Elastase as in the case

of the serine protease enzymes, trypsin and chymotrypsin is irreversibly inhibited by DIFP. Similarly covalent inhibition has been achieved with sulfonyl fluorides and with p-dinitrophenoldiethyl phosphate (Shotton, 1970).

1.8.4 Stability of elastase

Elastase is readily soluble in water and dilute salt solutions at concentrations up to 50 mg/ml between pH 4 and 10.5. At 2 °C elastase solutions are stable for prolonged periods below pH 6 and reasonably stable at higher pH values. Brief pH-induced conformational changes are reversible but prolonged incubation at acid pH leads to irreversible denaturation with loss of enzymatic activity (Lewis *et al.,* 1956). However, it is a powerful proteolytic enzyme and will rapidly autolyse to a mixture of peptides if incubated at room temperature at or near its optimum pH of 8.8. At pH 5.0 elastase solutions can be stored or used at room temperature with little autolysis. Crystals of elastase are stable indefinitely in 1.2 M sodium sulphate, buffered at pH 5.0 with sodium acetate. Freeze-dried elastase prepared following dialysis against 1 mM acetic acid is stable indefinitely at -10 °C with no loss of activity (Shotton, 1970).

1.9 Carboxypeptidases (CP) A and B

1.9.1 Biochemical characteristics of carboxypeptidases

The pancreatic exopeptidases have been reviewed by a number of groups (Folk, 1970b; Hartsuck and Lipscomb, 1971 and Puigserver *et al.,* 1986). Carboxypeptidases (CP) A and B are exopeptidase, zinc-containing peptidases, which are specific for the C-terminal amino acid residues of substrates and are secreted in the zymogen (proenzyme) form. Both procarboxypeptidases (PCPs) amount to 20 % of the total pancreatic secretory proteins (Puigserver *et al.*, 1986). Both enzymes also catalyse the hydrolysis of esters and therefore have dual specifies. The zymogen forms have been shown to have intrinsic esterolytic activity. For example, when PCPA was assayed with the ester substrate hippuryl-DL-phenylacetate, this activity was about 5 % of that observed after tryptic activation of the zymogen (Puigserver *et al.*, 1986).

CPA is secreted in a precursor form, procarboxypeptidase A (PCPA). Porcine pancreatic tissue is a rich source of PCPA (Marchis-Mouren *et al.,* 1961). In most

species pancreatic PCPA is either monomeric (Marchis-Mouren *et al.,* 1961) or is associated with a functionally different protein, described in pig as a proelastase-like zymogen of proteinase E which is also a serine proteinase (Kobayashi *et al.,* 1978 and Martínez *et al.,* 1981). The fundamental properties of bovine procarboxypeptidases have been characterised and have been compared with the porcine pancreatic zymogens (Martínez *et al.,* 1981 and Vendrell *et al.,* 1982). Carboxypeptidases of porcine pancreas are much more water soluble than bovine euglobin CPA and hence are easier to separate (Folk and Schirmer, 1963; Venrell *et al.,* 1982). CPA was first crystallised from beef pancreas (Anson, 1937). Porcine CPA has also been isolated and the relationships of the three forms of this enzyme (α, β, γ) have been established (Folk and Schimer, 1963; Pétra, 1970). They have a molecular weight of approximately 35.3 kDa (Bradshaw *et al.,* 1969). CPA has been sequenced and is composed of a single polypeptide chain of 307 amino acid residues (Bradshaw *et al.,* 1969). Several kinetic constants and some of the physical properties of porcine CPA have been compared with those of bovine CPA (Folk and Schirmer, 1963). The kinetics of PCPA and CPA highlight differences between the zymogen and active form of the enzyme. The three dimensional structure of CPA and CPB and the structure of activation segment have been studied at 2 Å resolution. (Guasch *et al.,* 1992).

The isolation of PCPB from pancreatic juice or aqueous extracts of pancreatic acetone powder has been described (Winterberger *et al.,* 1962). The amino acid sequence of PCPB and CPB has been established (Cox *et al.,* 1962; Folk, 1971). CPB was first isolated from bovine pancreas (Waldschmidt-Leitz *et al.,* 1931). It has a molecular weight of 34.3 kDa and requires zinc ions for activation (Wintersberger *et al.,* 1962). It displays esterase activity towards carboxyl terminal argininic acid (Folk and Gladner, 1958). Many of the physical and chemical properties of CPB, its activation and kinetics of activity have been reviewed (Folk, 1970b).

1.9.2 Differences between CPA and CPB

Ternary complexes of the zymogen, PCPA, with chymotrypsinogen C and proteinase E have been characterised in ruminant species, i.e. goat, sheep and cow (Kerfelec *et al.,* 1985). It has been suggested that differences in the three-dimensional structure of the A and B zymogens may explain the differences in intrinsic activity and

different rates of proteolytic activation of each zymogen (Guasch *et al.,* 1992). CPA and CPB are similar in amino acid sequence and mechanism of reaction, however, they differ in substrate specificity. CPA preferentially cleaves substrates with aromatic or large aliphatic carboxy terminal residues, whereas CPB acts on aromatic and basic amino acid such as lysine, arginine, ornithine and homoarginine. Histidine in a C-terminal position is not hydrolysed by CPB (Folk, 1970b). These differences may reflect the different roles of the enzymes. CPB acts on the products of trypsin digestion. CPA, on the other hand, acts on the products of chymotrypsin activity. CPA will not release peptide-bound proline, (proline will stop the hydrolysis reaction) arginine or glycine residues and acidic amino acids are only released slowly (Guasch *et al.*, 1992). The preparation, properties and uses of CPA and CPB for end-group (protein sequencing) analysis have been reviewed (Ambler, 1972).

1.9.3 Purification of carboxypeptidases

The purification of PCPA by extraction of acetone powder of pancreatic tissue, chromatography on DEAE-cellulose followed by ammonium sulphate precipitation serves as a general purification scheme for this procarboxypeptidase (Anson, 1937). Methods involving soya-bean trypsin inhibitor followed by DEAE-cellulose, Biogel R150 chromatography, or combinations of both have also been used to purify PCPA (Martínez *et al.,* 1981). The properties and methods of crystallisation of the PCPA complex have been reviewed (Pétra, 1970).

CPB is isolated from acetone powders of porcine pancreas in a process involving fractionation with ammonium sulphate followed by chromatography on DEAE-cellulose (Folk, 1971). Bovine CPB and porcine CPB have been crystallised following tryptic activation of either purified or partially purified proenzyme preparations (Winterberger *et al.*, 1962). The purification and properties of three PCPA and two PCPB forms of human procarboxypeptidase by anion-exchange HPLC has been demonstrated (Pascual *et al.,* 1989).

1.9.4 Activation of CPA and CPB zymogens

CPA and CPB are secreted as zymogens (PCPA and PCPB) from acinar pancreatic cells and are activated by trypsin in a two-step cascade system, producing

the active form of the enzyme. The sequence of events during activation by proteolysis is different for PCPA and PCPB. Transformation of PCPB to CPB by trypsin is a faster process than the activation of PCPA. In the activation of PCPB, a 95 residue activation segment is released giving rise to intermediates and to a proteolytically resistant activation fragment of 81 residues (Pascual *et al.*, 1989; Burgos *et al.*, 1991). In the tryptic conversion of PCPA to active CPA, the activation segment remains bound to the enzyme until a second trypsin cleavage occurs (Vendrell *et al.*, 1990). The kinetics involved in zymogen activation have been reviewed (Guasch *et al.*, 1992).

1.9.5 Stability and inhibitors of carboxypeptidases

The inhibition and activation kinetics have been reviewed for both CPA and CPB (Folk, 1970b; Puigserver *et al.*, 1986). Both CPA and CPB retain stability when stored in 0.01 M Tris/HCl pH 8.0 at 4 °C for several years (Wintersberger *et al.*, 1962). For example, concentrated solutions of porcine CPB in dilute Tris buffer pH 7.5 or suspensions of crystals of the enzyme in water have been stored at -10 °C for periods of up to one year without detectable loss in enzymatic activity (Folk, 1971). Substantial losses (25-45 %) in activity occurred upon lyophilisation of either solutions or crystalline suspensions of CPB. CPA is insoluble at low ionic strength and stable between pH 7 and 10 (Wintersberger *et al.*, 1962). Different allelomorphs of CPA have been isolated and there are differences in their thermal stability (Pétra, 1970).

Many substances inhibit CPA and CPB, including metal chelating agents such as 1,10 phenanthroline (Smith and Hanson, 1949), substrate analogs and products of CP hydrolysis. Removing the Zn^{2+} atom inhibits both peptidase and esterase activities of the zymogen and active forms of CPA and CPB. CPB is also inhibited by pyrophosphate and borate buffers above pH 8 (Wolff *et al.*, 1962). Butanol and several other alcohols influence CPB activity and it was shown in the presence of 0.3 M butanol that the rate of hydrolysis of hippuryl-l-arginine hydrolysis was increased (by 100 %) but rates of esterolysis were decreased (by 85 %) (Folk *et al.*, 1962).

2 β-Lactoglobulin and other whey proteins

Nutritionally, whey proteins are rich in essential amino acids and have a high biological value. Therefore, they are suitable for the supplementation of various foods and are, for example, often incorporated into infant formulae (Renner, 1983). Whey proteins represent 20 % of the total protein in milk. The literature on the manufacture, functional properties and uses of whey proteins has been extensively reviewed (Marshall and Harper, 1988; Sienkiewicz and Riedel, 1990; Zadow, 1992). In addition, the chemistry and physiochemical properties of the whey proteins has been reviewed (McKenzie, 1971; Whitney, 1988; Fox, 1992).

Whey has been defined as 'the product obtained by using acids, rennet and/or chemico-physical processes during cheese and casein production' (Sienkiewicz and Riedel, 1990). Depending on the method of recovery whey may be classified into acid or rennet wheys. Acid whey (pH 5.1) is 'the milk serum obtained by separation of casein, effected predominantly by acid, but also by β-galactosidase'. Rennet whey (pH 5.6), also known as sweet whey, is 'the milk serum obtained by separation of casein, effected predominantly by rennet, but also by β-galactosidase'. The types of whey, the technology used in the manufacture of various whey powders and the utilisation of established whey products have been extensively reviewed (Sienkiewicz and Riedel, 1990). Potential routes for the industrial scale processing of whey are given in Figure 2.1.

Whey proteins can have a high degree of functionality. Functionality encompasses such properties as solubility over a wide pH range, water-binding and water retention capacity, i.e. ability to form heat-induced gels and stable foams, and emulsifying properties (Sienkiewicz and Riedel, 1990). A number of processing variables, such as heat treatment used in the extraction of whey protein concentrates can affect the functional properties of whey (Schmidt *et al.,* 1984; deWit and Klarenbeek, 1984; Gauthier *et al.,* 1993). For example, heat treated whey proteins frequently lose their ability to form stable gels and to be soluble over a broad pH range. Heat or alkali treatment may also generate hydrolysis-resistant intermolecular and intramolecular covalent bonds, which in turn, may affect the nutritional quality of whey protein or its hydrolysis products (Erbersdobler, 1983).

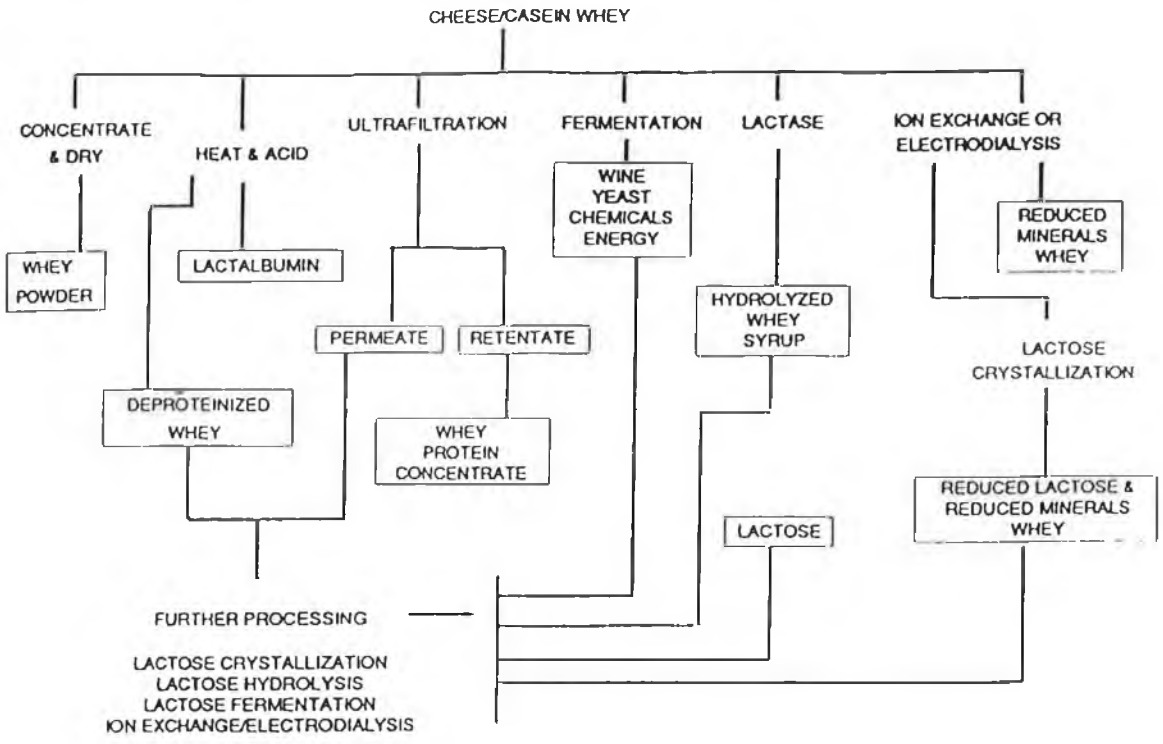


Figure 2.1: Potential routes for the industrial scale processing of whey (taken from Morr, 1982).

2.1 Manufacture of whey protein concentrates and lactalbumin

Whey protein powders (10-15 % protein) are usually produced using evaporation or spray-drying, while whey protein concentrates, (WPCs 25-95 % protein) are produced using ultrafiltration in combination with precipitation and/or chromatographic separation. The production of WPC is often coupled with demineralisation and lactose crystallisation steps (Marshall and Harper, 1988; Sienkiewicz and Riedel, 1990).

Lactalbumin is prepared by washing, in the presence of calcium ions, and drying, the proteins precipitated from heat-treated whey (Roger *et al.*, 1981). Lactalbumin generally has a protein content greater than 77 % (w/w). The lactose, lipid and ash content of lactalbumin varies with the degree of mineral washing during the manufacturing process and with the lactation stage of the milk used for

manufacture (Robinson *et al.*, 1976). The protocol for the manufacture of lactalbumin is given in Figure 2.2.

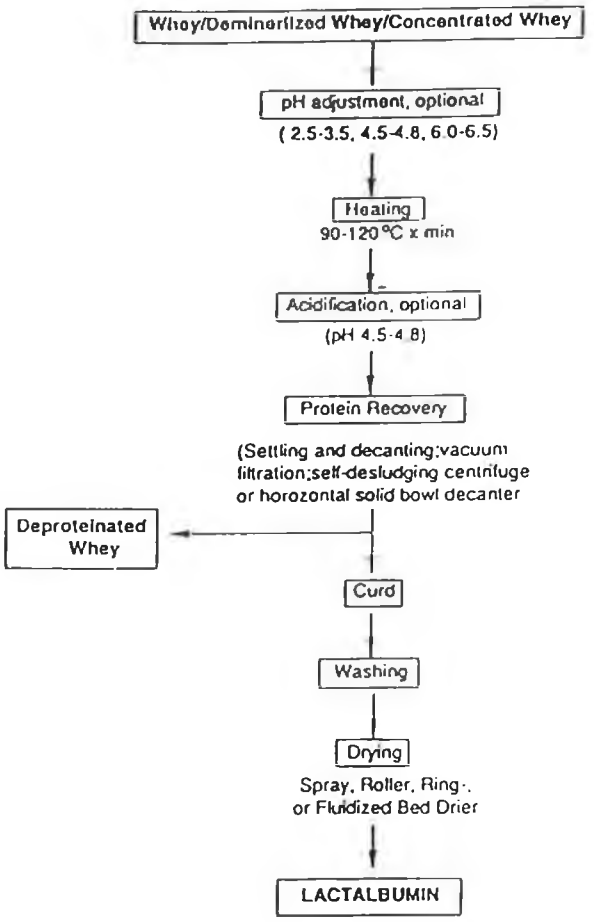


Figure 2.2 The protocol for the manufacture of lactalbumin

(taken from Mulvihill, 1992)

Lactalbumin is a gritty and insoluble whey protein powder, having a high water binding capacity (Short, 1980). It has been shown that heating whey at 88 to 90 °C between pH 2.2 to 3.5 and readjusting to pH 4.6 to precipitate the protein gives a lactalbumin product with improved solubility (Modler and Harwalker, 1981). Even though it has limited functionality, lactalbumin is of a high nutritional value (Wingerd, 1970). Solubilisation of lactalbumin occurs at an alkaline pH, i.e. pH values >9.4 (Smith *et al.*, 1989). However, it has been shown that the solubility of lactalbumin at

lower pH values was greatly improved by enzymatic hydrolysis (Smith *et al.*, 1989). Because of its nutritional value, lactalbumin is often used to fortify foods containing cereal proteins (Towler, 1982), in foods where solubility is not an important factor e.g. biscuits (Short *et al.*, 1978) and as a meat extender (Jelen, 1975).

The use of enzymatic hydrolysis to improve lactalbumin functionality is reviewed in Section 3. The manufacture, properties and uses of lactalbumin have been reviewed elsewhere (Robinson *et al.*, 1976).

2.2 The individual whey proteins

A number of industrially feasible methods have been developed to fractionate the major protein components, i.e. β-lactoglobulin (β-lg) and α-lactalbumin (α-la) from whey (Amundson *et al.*, 1982; Slack *et al.*, 1986; Maubois *et al.*, 1987; Pearce, 1987). The major whey proteins, which are also designated serum proteins, are given in Table 2.1. The major whey proteins are globular and contain a significant number of sulphydryl groups which in turn can be involved in protein/protein interactions during processing of whey protein as well as interacting with other proteins in complex food systems.

Minor whey proteins are the iron-binding proteins, (lactoferrin and transferrin), lactollin, β_2-microglobulin, the milk fat globule membrane proteins and hydrolysis products of caseins (Walstra and Jenness, 1984; Fox, 1992). In addition, another constituent of rennet and cheese whey is glycomacropeptide, consisting of 64 amino acids, a component released from κ-casein by the hydrolytic action of rennin.

Traces of various enzymes of primary origin (plasmin, alkaline phosphatase, lactoperoxidase, catalase, lipases and proteinases) and are also present in whey and their characteristics have been reviewed (Fox, 1992). Peroxidase is the milk enzyme present in the greatest proportion (1 % of the total whey protein content).

Table 2.1: Concentration and physicochemical properties of the whey proteins of bovine milk

Protein	% of whey protein	Concentration ($g\ l^{-1}$)	Molecular mass (kDa)	Isoelectric point (pI)
β-Lactoglobulin	55-65	3.3	18.4	5.35-5.49
α-Lactalbumin	15-25	1.2	14.2	4.2-4.5
Serum albumin	5-6	0.3	66.3	5.1
Immunoglobulins	10-15	0.5	80-900	5.5-8.3
Proteose peptones	10-20	0.2	4.1-80	5.1-6.0

(taken from Evans, 1980 and Marshall and Harper, 1988)

2.3 ß-Lactoglobulin

The globular protein β-lg is an abundant component of the whey fraction of the milk of several animal species and has been characterised both chemically and physically (McKenzie, 1971; Whitney, 1988; Hambling *et al.,* 1992). In the bovine species, it represents 55-65 % of the total whey proteins (Table 2.1). ß-Lg has a high content of essential amino acids (McKenzie, 1971; Hambraeus, 1982) which makes it a potentially nutritious protein source. But apart from being very nutritious, the molecular properties, particularly the acid solubility and ligand binding capability of β-lg leads to the supposition that some other, as yet unclear, more specific function exists for this protein. The possible biological functions of β-lg have been reviewed (Pérez and Calvo, 1995).

The structure, genetic variants, stability to various pHs and temperatures and the biological significance of β-lg will be dealt with in this review. Particular attention will be given to how enzymatic hydrolysis has been used to modify the protein. It is known that changes in protein conformation brought about by, for example, pH adjustment, heat treatment, or ligand binding, can alter the rate of proteolysis (Mihalyi,

1978; Swaisgood and Catignani, 1987; Puyol *et al.,* 1994). The effects of heating, esterification, pressurisation or ligand binding on the susceptibility to enzymatic hydrolysis will also be reviewed.

2.3.1 β-*Lg structure*

The molecular weight of bovine ß-lg B (monomeric form) is estimated to be 18.3 kDa corresponding to a polypeptide chain of 162 amino acid residues (Table 2.1). The molecule contains two disulphide bridges as well as one free cysteine. The free thiol group is of significance in the processing of milk, as it can interact with other proteins such as κ-casein (Sawyer, 1969) and α-la (Hunziker and Tarassuk, 1965). β-Lg also exists in dimer form. ß-Lg contains four tryptophan groups per dimer (McKenzie, 1971). The primary structure and function of β-lg has been reviewed (Godovac-Zimmermann and Braunitzer, 1987).

In terms of secondary structure, ß-lg in solution possesses approximately 10 % α-helix, 43 % antiparallel ß-pleated sheet and 47 % unordered structure (Creamer *et al.,* 1983). A core structural motif, a hydrophobic pocket, consisting of an eight-stranded ß-barrel with another ß-strand and α-helix on the surface has been identified in β-lg by X-ray crystallography (Green *et al.*, 1979; Monaco *et al.*, 1987). β-Lg has a structural similarities to the plasma retinol binding protein (Papiz *et al.*, 1986; Cho *et al.*, 1994). The structural stability of β-lg has been studied using circular dichroism spectra analysis (Alexander and Pace, 1971; Chen *et al.*, 1993) and by differential scanning calorimetry (DSC, deWit and Swinkels, 1980; Puyol, *et al.,* 1994). In addition, studies using trypsin hydrolysis are frequently used to investigate ß-lg structure (Dalgalarrondo *et al.,* 1990; Chobert *et al.,* 1991; Chen *et al.*, 1993).

2.3.2 Genetic variants

At least seven genetic variants of ß-lg , designated β-lg A, B, C, D, E, F and G, differing in overall composition by amino acid substitutions have been reported (McKenzie, 1971; Eigel *et al.,* 1984; Hambling *et al.,* 1992). The major variants are β-lg A and B which differ at positions 64 and 118, where Asp and Val in variant A are substituted by Gly and Ala, respectively in variant B. β-Lg A and B both have 17 potential tryptic cleavage sites, however, comparative differences exist in a number

physiochemical properties (Townend *et al.,* 1967; Alexander and Pace, 1971), as outlined in section 2.3.3.2. For example, the thermal stability of the A and B variants differ as detected using differential scanning calorimetry (deWit and Swinkels, 1980; Imafidon *et al.,* 1991; Huang *et al.,* 1994a).

2.3.3 Factors affecting susceptibility of β-*lg to enzymatic hydrolysis*

A number of modifications which change the susceptibility of β-lg to enzymatic hydrolysis are as follows: (1) pH and temperature changes; (2) genetic variants; (3) ligand binding; (4) pressurisation and (5) chemical modification. Each of these modifications indirectly imparts different physicochemical properties to the hydrolysate produced by alteration of the number and type of cleavage points available to the digestive enzymes. Because of their globular and mostly stable structure, only slight modification in the conformation of the protein can produce significantly different digestion products. The correlation between the susceptibility of a peptide bond to proteolysis with the flexibility of the residues surrounding that bond has been well documented (Mihalyi, 1978; Swaisgood and Catignani, 1987).

2.3.3.1 pH and temperature changes

β-Lg exhibits a unique self-association pattern which is pH dependent (Zimmermann *et al.,* 1970). At neutral pH ß-lg exists as a dimer. Below pH 3.7 and above pH 5.2 dimers are favoured that increasingly dissociate to monomers as the pH decreases or increases, respectively. Between pH 3.7 and 5.2, the dimer undergoes a progressive reversible tetramerization (Kumosinski and Timasheff, 1966). A reversible conformational change above pH 7.5, causes the exposure of tryptophan and tyrosyl residues to solvent (Tanford *et al.*, 1959; Townend *et al.*, 1967, 1969; Zimmermann *et al.,* 1970). This pH induced dissociation during the so called "Tanford transition", exposes a carboxyl group and a sulphydryl group but is not accompanied by gross changes in molecular conformation (Timasheff *et al.,* 1966; Mills and Creamer, 1975; Teller *et al.,* 1979). It has been suggested that this transition in ß-lg structure associated with pH changes is reflected in its susceptibility to proteolysis (Monnot and Yon, 1964).

In addition to the influence of pH, ß-lg undergoes temperature - dependent thermodenaturation and conformational changes (Brunner, 1977). ß-Lg is known to dissociate to the monomer in the pH region of 5.2-5.9, and as the temperature is increased from 20 to 45 °C. β-Lg aggregates above 65 °C with increasing holding time involving SH oxidation and disulphide interchange (McKenzie, 1971; Gotham *et al.*, 1992). Dupont (1965) and deWit and Swinkels (1980) showed that reversible conformational changes can occur to β-lg below 70 °C, however, above this temperature irreversible denaturation takes place. The monomer-dimer dissociation constant varies with the genetic variant and is increased with increasing temperature (Imafidon *et al.,* 1991). Heating steps such as pasteurisation, sterilisation and evaporation, as used in the preparation of many whey products, and the influence of heating on the characteristics of β-lg have been reviewed (McKenzie, 1971). The thermal denaturation and gelation of ß-lg has been studied (Paulsson and Elofsson, 1994) using DSC and dynamic rheology.

Modification, of ß-lg by heating and pH changes makes this protein more susceptible to proteolysis by digestive enzymes (Schmidt and van Markwijk, 1993). For example, heat treatment followed by enzymatic hydrolysis has been used to eliminate the allergenicity induced by this protein (see 2.3.4.1).

2.3.3.2 Variants

It has been previously reported that tryptic hydrolysis of ß-lg A is 3.5 times faster than ß-lg B (Nakamura *et al.*, 1993b). The difference in hydrolysis rates of ß-lg A and B by trypsin might be ascribed to the substitution of Asp_{64} in ß-lg A with Gly in ß-lg B (Dalgalarrondo *et al.*, 1990) and resultant differences in structural stability (Huang *et al.,* 1994a). It was suggested that the variant related differences in chymotryptic hydrolysis of ß-lg could also be attributed to the amino acid substitution at position 64 (Van Willige and FitzGerald, 1995). It has been shown that the variants differ in their gelling properties (Huang *et al.,* 1994a, b) and heat stability, in addition to resistance to enzymatic hydrolysis (Imafidon *et al.,* 1991; Huang *et al.,* 1994c).

2.3.3.3 Ligand binding

It is suspected that ß-lg has a role in nutrition in the transport of essential fatty acids, triglycerides, alkanes, minerals such as calcium ions and the vitamin, retinol (Puyol *et al.,* 1994; Pérez and Calvo, 1995). It has been suggested that the biological role of ß-lg is related to digestion and/or transport of fatty acids or retinol to specific receptors in the duodenum of new born animals (Pervaiz and Brew, 1985; Puyol *et al.*, 1993).

ß-Lg belongs to a family of proteins that interact with small volatile hydrophobic ligands (Hambling *et al.,* 1992; Puyol *et al.,* 1994). These proteins share a similar three dimensional structure containing an internal hydrophobic pocket (Sawyer *et al.*, 1985). Both the centre of the ß-barrel (Papiz *et al.*, 1986) and a surface hydrophobic pocket (Monaco *et al.*, 1987) of β-lg have been suggested as possible high affinity binding sites for retinol and vitamin A. Ruminant ß-lg binds fatty acids (Diaz de Villegas *et al.*, 1987; Spector and Fletcher, 1970) and retinol (Fugate and Song, 1980) *in vitro*. Fatty acids compete *in vitro* with the binding of retinol to ß-lg (Puyol *et al.*, 1991). It has been shown that the binding of fatty acids to ß-lg increases its conformational stability and thus resistance to trypsin degradation (Puyol *et al.,* 1993). Differential scanning calorimetry (DSC) has been used to study the thermal stability of bovine ß-lg as influenced by binding of palmitic acid or retinol (Puyol *et al.,* 1994). The findings concluded that binding of fatty acids to ß-lg may play an important role in the stabilisation of ß-lg structure.

2.3.3.4 Pressurisation and its influence on enzymatic hydrolysis

Pressurisation has been used as a method of modification of ß-lg which alters the three-dimensional (3D) structure of the protein during enzymatic hydrolysis and increases the enzyme activities. This effect which can in turn promote the reduction of allergenicity (Nakamura *et al.,* 1993a, b). The susceptibility of the ß-lg variants A, B, AB to enzymatic hydrolysis at ambient and high pressure has been described (Van Willige and FitzGerald, 1995).

2.3.3.5 Chemical modification and hydrolysis of β-lg

Chemical modification through cleavage of S-S bonds using permissible food additives such as sulphite has been used to improve the digestibility of ß-lg (Otani, 1981; Reddy *et al.,* 1988). Modification of ß-lg by esterification using alcohol followed by tryptic (Briand *et al.,* 1994) or peptic hydrolysis (Dalgalarrondo *et al.,* 1995) has also been investigated. It has been suggested that the peptides produced following hydrolysis may have different biological or physiological properties. Esterification with alcohols blocks exposed carboxyl groups in proteins neutralising negative charges of carboxylates and increases protein hydrophobicity.

2.3.4 Properties of enzymatically hydrolysed β-lg

The applications of proteinases in the hydrolysis of whey proteins are given in Section 3. Some properties related to β-lg hydrolysates are mentioned briefly here:

2.3.4.1 Reduction of allergenicity

ß-lg is absent from human and rodent milk and its inclusion in baby milk formulae has focused attention on its potential allergenic properties in infants (Nakamura *et al.,* 1993a, b). Since bovine milk contains ß-lg, which is a potential allergen, it is desirable, in some instances, to selectively eliminate ß-lg from whey (Okamoto *et al.*, 1991) for inclusion in infant or milk protein formulae. Heat treatment has been used to reduce allergenicity of β-lg. However, this has been shown to produce new compounds between ß-lg and lactose by the Maillard reaction and that these compounds in turn show a greater allergenic response than native ß-lg. The second method used to reduce allergenicity employs enzymatic hydrolysis. Hydrolysis results in an increase of charged groups and hydrophilicity, a decrease in molecular weight and alterations in molecular conformation. β-Lg was hydrolysed by trypsin and chymotrypsin or porcine pancreatin to give a reduction in its allergenic properties (Asselin *et al.,* 1989). High pressure treatments prior to proteolysis have also been used to produce hypoallergenic hydrolysates (Okamato *et al.*, 1991; Nakamura *et al.* 1993a, b). A brief review of the application of enzymatic hydrolysis to produce low allergenic hydrolysates is given in Section 3.1.2.

2.3.4.2 Bioactive peptide hydrolysates

There are two known bioactive peptides derived from the enzymatic hydrolysis of β-lg using digestive proteinases. An opioid peptide, β-lactorphin (Tyr-Leu-Leu-Phe) was produced using pepsin and trypsin or a combination of trypsin and chymotrypsin hydrolysis of native or heat-treated ß-lg (Antila *et al.,* 1991). Another tetrapeptide, known as β-lactotensin (His-Ile-Arg-Leu), which acts on smooth muscle, was released from the internal sequence of β-lg by the action of chymotrypsin or a combination of pepsin and chymotrypsin (Pihlänto-Leppälä *et al.*, 1994a). Details of milk derived bioactive peptides will be dealt with in Section 4.

2.3.4.3 Emulsification and foaming properties

β-Lg in particular displays a low surface hydrophobicity, but on enzymatic hydrolysis, the exposed hydrophobic residues become available for interaction with oil surfaces. Therefore, limited hydrolysis of β-lg improves emulsification and foaming properties. For example, peptide fragments isolated from β-lg after tryptic hydrolysis in an ultrafiltration type membrane reactor, showed enhanced interfacial activities (Turgeon *et al.*, 1992). These hydrolysates were suitable for improvement of food emulsions and foams. Most research has been carried out on emulsification and foaming properties of β-lg in the presence of the other whey proteins, i.e. in lactalbumin and WPC, and this research is outlined in Section 3.1.1.2.

2.3.4.4 Gelation

Besides the influence of the inclusion of salts such as sodium chloride and calcium chloride on the gelation of ß-lg (Mulvihill and Kinsella, 1988; Paulsson and Elofsson, 1994; Li *et al.*, 1994), the role of limited enzymatic hydrolysis by immobilised trypsin on the gelation of β-lg has also been investigated (Chen *et al.,* 1994). It has been shown that a hydrolysate giving superior gelling properties to the native protein can be produced by limited hydrolysis of β-lg (Chen *et al.,* 1994). Partially hydrolysed ß-lg had a lower gel point and gelled more rapidly than native ß-lg at 80 °C. Polypeptide fragments which retain their secondary and tertiary structure after limited hydrolysis and which are derived from the core ß-barrel domain are suspected to be crucial in this gelling process (Chen *et al.,* 1993; Chen *et al.,* 1994).

2.4 α-Lactalbumin

α-La represents 15-25 % of the total whey proteins in bovine milk (Table 2.1). α-La is nutritionally important because it is the principal whey protein of human milk (Heine *et al.,* 1991). This protein is also involved in the final stage of lactose biosynthesis (Brodbeck and Ebner, 1966). It functions by promoting the binding of glucose to glactosyltransferase and has a role in the formation of lactose from glucose and galactose. The physical and chemical properties of α-la have been reviewed (McKenzie, 1967; Gordon, 1971; Brew and Grobler, 1992).

α-La consists of a single polypeptide chain of 123 amino acids, (14.2 kDa) and contains eight cysteines which exist as intramolecular disulphides (Eigel *et al.*, 1984). The four disulphide bridges, which are intrinsic to α-la structure contribute to the thermal stability and the very high renaturability of the molecule. Upon heating at pH 6.5, α-la unfolds at temperatures as low as 62 °C, but on cooling the molecule reverts to its native structure (Pfeil, 1981). The cysteine residues, which occur as four disulphide bridges, are mainly responsible for this reversible change. α-La is the most heat stable whey protein. Of all the normal methods for the heat treatment of liquid milk, only indirect ultra high temperature (UHT) heating (15s above 100 °C, maximum 141 °C) effects an irreversible denaturation on α-la. α-La is a metalloprotein, capable of binding calcium, zinc and other metals (Stuart *et al.,* 1986). The removal of calcium by EDTA reduces its heat stability (Bernal and Jelen, 1984). A conformational change occurs in α-la around pH 4.0, where the molecule loses the Ca^{2+} which is tightly bound at higher pH values. The thermal stability of α-la in comparison to the other whey proteins has been determined (Pfeil, 1981) The 3D structure of α-la has been determined (Acharya *et al.*, 1991) and the metal binding and physicochemical properties of α-la have been studied (Kronman, 1989; FitzGerald and Swaisgood, 1989; Brew and Grobler, 1992).

There is a large amount of sequence homology between α-la and type-c lysozymes (Brew and Campell, 1967; Hill and Brew, 1975). α-La secondary structure includes approximately 26 % α-helix, 14 % β-sheet and 60 % unordered structure which includes β-turns (Hall *et al.*, 1987). Three genetic variants of α-la have been identified in Western bovine breeds (Fox, 1989). It was suggested that the protective

effect of certain lipophilic compounds to α-la may have physiological significance (Hirai *et al.*, 1992).

The effects of the proteolytic digestion of α-la by trypsin, chymotrypsin and pepsin have been studied, with respect to the influence of ion binding on the stability of this protein to hydrolysis (Schmidt and van Markwijk, 1993). α-La has good emulsifying and foaming properties, although the native molecule reveals low surface hydrophobicity. The increased susceptibility of α-la to surface denaturation at oil-water or air-water interfaces is involved in its emulsifying and foaming properties (Fox, 1989).

Only one bioactive peptide derived from α-la has been characterised to date. An opioid peptide known as α-lactorphin (Tyr-Gly-Leu-Phe) was isolated from a proteolytic digest of native α-la using pepsin or a combination of pepsin and trypsin (Antila *et al.*, 1991).

α-La is also a potential allergen (Gjesing *et al.*, 1986) and it has been shown that the allergenicity of α-la can be reduced using enzymatic hydrolysis by trypsin and chymotrypsin (Asselin *et al.*, 1989).

2.5 Serum Albumin

The concentration of serum albumin in milk is approximately 0.3 g l^{-1}, corresponding to approximately 5-6 % of the total whey proteins (Table 2.1). Bovine serum albumin (BSA) consists of 582 amino acid residues, has a molecular weight of 66.3 kDa and contains 17 intramolecular disulphide bridges and one free thiol group. This protein binds several ligands, including fatty acids, which enhance its heat stability (Gumpen *et al.*, 1979). The physiochemical properties of BSA have been reviewed (Peters, 1985). It has been suggested that the rigid, disulphide structure of this protein acts as a shielding effect against enzymatic hydrolysis. There is no apparent function for BSA in milk and it is assumed to be a leakage protein from blood.

Like β-lg and α-la, BSA can also elicit allergic responses, indicated by the production of IgE antibodies against this protein (Gjesing *et al.*, 1986). It has been reported that BSA was poorly hydrolysed by pancreatin or trypsin (Jost *et al.*, 1987;

Maubois *et al.*, 1981) during the production of hypoallergenic whey protein hydrolysates (Section 3).

2.6 Immunoglobulins and proteose peptones

Immunoglobulins (Igs) and proteose peptones are known to pass into the milk from the blood serum. Immunoglobulins are high molecular weight glycoproteins synthesised by the cells of the reticuloendothelial system (Roitt, 1984). They represent approximately 10 % of the total whey proteins (Fox, 1989) and serve to transfer passive immunity to the neonate (Larson, 1992). The production of whey protein fractions rich in immunoglobulins as colostrum supplements has been investigated (Haines *et al.*, 1990). The literature on the three classes of Igs (IgG, IgA and IgM) in bovine milk has been reviewed (Larson, 1992).

The proteose peptone fraction, also originating from blood serum, has been defined as a mixture of various phosphoglycoproteins that are soluble at pH 4.6 after heating at 95 °C for 20 min which can then be precipitated by 12 % trichloroacetic acid. These proteins are very stable with respect to heat and acidification (pH 4.6-4.8) (Swaisgood, 1982).

3 The application of pancreatic proteinases in the production of specialised whey protein hydrolysates

The literature on enzymatic hydrolysis of food proteins is vast and therefore this review is confined to hydrolysis of whey proteins with food grade proteinases, with particular emphasis on proteinases of gastric and pancreatic origin. The uses of milk protein hydrolysates are extensive. Limited enzymatic hydrolysis of milk proteins has been used to produce changes in solubility, emulsification and foaming properties (Ennis and Harper, 1986) and has been used to produce different flavours and textures in food (Jost and Monti, 1977; Monti and Jost, 1978; Jolly, 1978). In addition, hydrolysates for use in cosmetics (Hidalgo and Jost, 1980) and hair care products have been developed. Hydrolysates have been used in nutritional fortification, for example, in beverages (Jolly, 1978; Driessen and Van den Berg, 1988) and in microbiological media such as peptone broths and in peptoned milk tryptone broth (Marshall *et al.*, 1982). Hydrolysates can also be used for clinical or pharmaceutical purposes i.e. pre-digested diets for pre- or post operative patients and in infant foods.

3.1 Properties of whey protein hydrolysates

3.1.1 Functionality of whey protein hydrolysates

The functional properties of proteins denote any physicochemical property which affects the processing and behaviour of proteins in food systems, as judged by the final attributes of the product. The functional characteristics and properties of hydrolysates can be correlated with percentage degree of hydrolysis (DH), (Ennis and Harper, 1986; Smith *et al.*, 1989), enzyme specificity and hydrolysate hydrophobicity (Chaplin and Andrews, 1989; Turgeon *et al.*, 1992; Mahmoud *et al.,* 1992). Peptide size distribution is another important factor in the functionality of protein hydrolysates (Adler-Nissen, 1986; Turgeon *et al.,* 1991, 1992). Factors known to affect the size distribution of peptides within hydrolysates are enzyme specificity, enzyme to substrate ratio (E:S), reaction pH, duration of hydrolysis and ionic strength (Jost and Monti, 1982; Chobert *et al.,* 1988; Turgeon *et al.,* 1992). Membrane processing of hydrolysates is often employed to control the size distribution of peptides in the

permeate (Olofoson *et al.,* 1980; Gauthier *et al.,* 1993) during ultrafiltration, reviewed in Section 5, and membrane processing has been employed to improve the emulsification (Turgeon *et al.*, 1991) or non allergenic properties of hydrolysates (Van Beresteijn *et al.,* 1994) as reviewed in 3.1.2.

It is often the case that a number of endoproteinases and exopeptidases must be used in conjunction with one another to reach the desired change in protein functionality/solubility/flavour (Clegg *et al.*, 1974; Adler-Nissen, 1976; Maeda *et al.*, 1987). For example, in the production of a non-bitter hydrolysate, it is desirable to use an endoproteinase, followed by an exopeptidase (Clegg *et al.*, 1974) In the case of enzymatic hydrolysis of milk proteins, mixtures of enzymes which have complementary specificities to each other may be used to attain the required degree of hydrolysis. The relative proportions of the endoproteinases and the presence or absence of large amounts of exopeptidases in a hydrolysis system is important in the production of most hydrolysates (Thibault, 1991). In general, studies on hydrolysate functionality using limited digestion by endoproteinases and exopeptidases, has focused on efforts to improve solubility, emulsification and foaming and to eliminate off-flavours, such as bitterness (Clegg and McMillan, 1974).

3.1.1.1 Solubility of whey hydrolysates

Enzymatic hydrolysis produces peptides of smaller molecular size than the native proteins. Thus, functional properties of such peptides are different in comparison to the parent protein. Generally following hydrolysis there is increased solubility over a pH range (Jost and Monti, 1977; Olofoson *et al.*, 1980), in addition to decreased viscosity (Hooker *et al.*, 1982). Insoluble, whey proteins such as lactalbumin (Section 2.1) have been digested using porcine trypsin (Adler-Nissen, 1976; Jost and Monti, 1977; Monti and Jost, 1978), papain (Monti and Jost, 1978), Alcalase (Jolly, 1978; Ennis and Harper, 1986; Smith *et al.*, 1989; Perea *et al.,* 1993) and Esperase (Kuehler and Stine, 1974; O'Keefe and Kelly, 1981), producing hydrolysates that are soluble over a wide pH range. During these studies, the effects of temperature, hydrolysis time, pH, and enzyme dosage (at a constant substrate concentration) on the final hydrolysate composition and functional properties were monitored (Kuehler and Stine, 1974; Ennis and Harper, 1986). Commercial tryptic and

chymotryptic preparations, such as PTN 3.0S, PEM 2500S (Perea *et al.,* 1993) and Corolase PP (Samuelsson and Poulsen, 1987), have also been used to alter the functional properties of whey proteins. However, reaction progress curves of solubilization versus time for lactalbumin are very unusual with a maximum solubilization of 90 % occurring at DH values of 11-12 %. Further hydrolysis produces a decrease in solubility to 67 %, followed by a further gradual increase in solubilization on further hydrolysis (Smith *et al.,* 1989).

3.1.1.2 Emulsification and foaming properties of whey protein hydrolysates

Emulsifying capacity is influenced by solubility (Ennis and Harper, 1986; Turgeon *et al.*, 1992) and hydrophobicity (Mahmoud *et al.,* 1992) of the protein hydrolysate. Emulsification capacity is related to the interfacial area that can be coated by the protein (Pearce and Kinsella, 1978). Peptide chain length is an important factor, for example, in emulsification, as enzymatic hydrolysis is used to expose hydrophobic residues for interaction with the oil-water interface. It has been suggested that in order to have good emulsifying properties that the molecular weight of a peptide should not be less than 5 kDa (Chobert *et al.*, 1988). Partial hydrolysis is advantageous in producing peptides of such an average size (Thibault, 1991) which in turn display good emulsification characteristics (Jost and Monti, 1982). Tryptic hydrolysis of whey proteins produces a higher content of larger peptides than chymotryptic hydrolysis (Chobert *et al.*, 1988; Turgeon *et al.*, 1991). Chymotrypsin produced peptides with a wider range of molecular weights having a slightly higher proportion of small-size digestion products. Chymotryptic hydrolysis of WPC resulted in hydrolysates with lower emulsification activity than tryptic hydrolysates in the acid pH range. Moreover, tryptic peptides can have internal hydrophobic residues which can enhance interaction with oil, depending on their conformation (Turgeon *et al.*, 1992).

A low degree of hydrolysis as obtained by peptic hydrolysis, has also been used to improved the foam expansion and emulsifying capacity characteristics of WPC hydrolysates (Kuehler and Stine, 1974; Nakai and Li-Chan, 1989). However, at higher DH values foam expansion decreased and foam stability was reduced (Kuehler and Stine, 1974). It has been shown that hydrolysis of WPC or lactalbumin by other commercial proteinases showed significant changes in foaming or emulsification

properties (Kuehler and Stine, 1974; O'Keefe and Kelly, 1981; To *et al.,* 1985) depending of percentage degrees of hydrolysis.

3.1.2 Hypoallergenic hydrolysates

There is a difference in composition between human and bovine milks, and particularly the ratio of whey protein to casein (Brockbank, 1984). Human milk is rich in whey protein but does not contain β-lg which is the predominant whey protein in bovine milk. This protein is usually associated with cow milk allergy (CMA), although any component of bovine milk can potentially cause an allergenic response. For example, in addition to IgE antibodies to β-lg, IgE antibodies were found to α-la, BSA, and the four caseins (αs_1, αs_2, β, and κ) in the sera of infants allergenic to cows milk (Gjesing *et al.*, 1986; Otani *et al.*, 1989). The incidence, clinical features, diagnosis and treatment of CMA have been reviewed (Freier and Kletter, 1970).

Enzymatic hydrolysis is used to reduce the allergenicity of native bovine milk proteins by the elimination of antigenic determinants responsible for creating hypersensitivity in some infants (Pahud *et al.*, 1985; Asselin *et al.,* 1988; Jost *et al.*, 1991). The immunogenicity and allergenicity of whey protein hydrolysates has been demonstrated (Jost *et al.*, 1987, 1991; Asselin *et al.*, 1988, 1989) and the use of enzymatic treatment to reduce allergenicity of whey proteins has been studied by many groups (Asselin *et al.*, 1989; Akita and Nakai, 1990).

Specific amino acid concentrations and specific peptide profiles on gel permeation FPLC were shown to be desirable for certain hypoallergenic hydrolysates (Thibault, 1991). This requirement of uniformity is important where hydrolysates are used for inclusion in non-allergenic foods or for therapeutic purposes. For example, pancreatic enzyme mixtures such as PEM 2500S (Novo) which contains trypsin, chymotrypsin and porcine elastase 2, have been employed to produce non-allergenic hydrolysates with desired levels of certain amino acids and peptide chain lengths to allow maximum tolerance and absorption in the human diet (Thibault, 1991; Kahn *et al.*, 1991). In addition, trypsin (Asselin *et al.*, 1989), or Corolase PP (Poulsen, 1987), chymotrypsin or a pepsin-pancreatin digestion step (Olofoson *et al.*, 1980; Jost *et al.*, 1988; Akita and Nakai, 1990) or combinations of pure trypsin and chymotrypsin in specific ratios (Thibault, 1990), have been used to reduce the allergenicity of whey

proteins. Several patented processes have been developed to produce dietetic foods having reduced allergenicity (Heine and Wutzke, 1990; Kahn *et al.,* 1991).

The minimum molecular mass to elicit immunogenicity and allergenicity of whey protein hydrolysates has been shown in some cases to be between 3 kDa and 5 kDa (Van Beresteijn *et al.,* 1994). This is an important consideration when choosing an enzyme preparation and/or a membrane for the production of hydrolysates intended for use in nonallergenic foods (Section 5). Membrane processing using ultrafiltration has been used in many cases to remove larger peptides and aggregates, which were deemed to be the causative agents in allergenic responses (Olofoson *et al.*, 1980; Poulsen, 1987; Kahn *et al.*, 1991; Thibault, 1991; Van Beresteijn *et al.,* 1994).

Non-allergenic hydrolysates are often used for infants having gastro-intestinal disease (Merritt *et al.*, 1990), reduced digestive or absorptive capacity, extensive intestinal inflammatory disease, in addition to those who are allergic to cow's milk (Asselin *et al.*, 1989; Buzinco *et al.,* 1989). Examples of the above conditions include enteropathic conditions; short bowel syndrome; cystic fibrosis (Clegg and McMillan, 1974), Crohn's disease and atrophy and other minor immunodeficiences which result in allergic responses to dietary components.

3.1.3 Bioactive hydrolysates

Biologically active peptides can be produced by hydrolysis of whey proteins and their production and the related literature is reviewed in Section 4.

3.1.4 Therapeutic peptides from whey protein hydrolysates.

Hydrolysates are used in clinical situations where there are digestive disorders, limited nutrient absorptive capacity or diminished luminal hydrolysis in the small intestine (Cordano *et al.*, 1988). Therapeutic hydrolysates can be described as those hydrolysates which are used in defined formulae diets, enteral nutrition, parental nutrition or in preparations which enhance the absorption of minerals (i.e. phosphopeptides). Enzymatic hydrolysis of whey protein can produce a product with peptides of a critical chain length in dipeptide or tripeptide form which allows rapid *in vivo* absorption (Grimble *et al.,* 1986). These hydrolysates can therefore be used for pre- or post- operative patients, as they are absorbed via a separate and more efficient

transport system than free amino acids (Grimble and Silk, 1989). Because these hydrolysates are more efficient due to their high absorptive capacity and lower osmolality they are often used for patients with limited absorptive capacity or pancreatic insufficiency in the form of a pre-digested diet (see 3.1.4.1). Whey protein hydrolysates have also been used in the treatment of specific metabolic disorders (see 3.1.4.2).

The use of pancreatic proteinases, for hydrolysis of lactalbumin has been employed by a number of groups to produce a hydrolysate suitable for dietetic purposes and the procedures to obtain such hydrolysates have been outlined (Olofoson *et al.*, 1980; Maubois *et al.*, 1981). The hydrolysis of α-la and β–lg by trypsin, chymotrypsin and pancreatin in the production of low molecular weight peptides for dietetic and therapeutic applications has also been examined (Roger *et al.*, 1981).

3.1.4.1 Peptide absorption from whey protein hydrolysates

Generally, intact peptides do not enter the bloodstream, small peptides entering absorptive cells are hydrolysed completely within the cells and enter the blood as free amino acids (Matthews and Payne, 1980). Alternatively, small peptide fragments (approximately 3-4 amino acid residues) may be absorbed after brush-border membrane hydrolysis of the peptide. It is known that separate transport mechanisms exist for absorption of free amino acids and small peptides (2-4 amino acid residues). Free amino acids are absorbed by at least 4 group specific transport systems (Gray and Cooper, 1971) while peptide uptake is independent of the mechanism of free amino acid absorption (Silk *et al.*, 1985). Di- and tri peptides probably share a common transport mechanism (Sleisenger *et al.*, 1976). Peptides containing four or more amino acid residues are likely therefore, to be hydrolysed by brush border peptidases before absorption, but there is evidence to suggest that some tetrapeptides are absorbed (Smithson and Gray, 1977; Chung *et al.*, 1979). Because of the independence of the mechanism of peptide uptake there is no competition between peptides and amino acids for absorption sites and therefore the efficiency of peptide transport is high (Rérat *et al.*, 1988). The mechanism of peptide absorption and nature of peptide transport may be of considerable consequence when, for example, physiological aspects of therapeutic peptides, such as bioactive peptides are discussed (Section 4).

The size distribution of peptides present in a whey protein hydrolysate is of importance, when efficient absorption in the gut is to be taken into consideration. The intestinal absorption of protein hydrolysates has been reviewed previously (Webb, 1990). In many cases, membrane processing is used following protein hydrolysis by digestive proteinases. For example, pancreatic proteinases such as pancreatin were used to hydrolyse WPC and the resultant hydrolysate was ultrafiltered using a membrane with molecular weight cut-off of < 10 kDa (Olofoson *et al.*, 1980) or < 5 kDa (Maubois *et al.,* 1981). The permeates of these hydrolysates were intended for enteral nutrition.

In specific examples of the use of whey protein hydrolysates as therapeutic agents, it has been shown that amino acids that were poorly absorbed from mixtures of free amino acids, were absorbed to a substantially greater extent in the form of a lactalbumin hydrolysate in the human jejunum (Grimble *et al.,* 1986). As a nitrogen source, there appeared to be no significant difference between peptide containing hydrolysates or free amino acid mixtures. However, it has been suggested that the lower osmolality of the peptide diet in comparison to an amino acid diet would be advantageous in patients with impaired fluid and electrolyte absorption as less fluid would be secreted into the lower part of the proximal intestine due to the peptide diet (Silk *et al.*, 1979). The efficacy of enterally administered protein hydrolysates and peptide fractions has been reviewed (Webb, 1990; Mobarhan and Trumbore, 1991).

3.1.4.2 Clinical applications of whey protein hydrolysates

There are a number of inborn errors of amino acid metabolism for which hydrolysates are used as therapeutic agents. The production of a low phenylalanine peptide foodstuff suitable for treatment of phenylketonuria has been examined using peptic hydrolysate of β-lg, followed by hydrolysis by an exopeptidase preparation, i.e. Pronase (Maeda *et al.*, 1987). Phenylketonuria is a metabolic disorder resulting from a deficiency of the enzyme, phenylalanine hydroxylase, which is responsible for the conversion of phenylalanine to tyrosine (Davidson *et al.*, 1979). In addition, milk protein hydrolysates, free of specific amino acids (Phe, Tyr, Met) are used in the treatment of metabolic disorders such as homocystinuria and tyrosinemia. The production of a hydrolysate for human consumption often involves the operation of a

hydrolysis procedure which attempts to mimic human digestion (Silk *et al.*, 1973). Many groups incorporate a gastric digestion step (Silk *et al.*, 1973) of whey proteins such as α–la or β-lg by pepsin followed by digestion with a mixtures of enzymes (Haddad *et al.*, 1979; Maeda *et al.*, 1987; Asselin *et al.,* 1989). The clinical uses of protein hydrolysates have been reviewed (Milla, 1991).

4 Biologically active peptides derived by enzymatic hydrolysis of milk proteins

There are a number of the naturally occurring regulatory peptides in milk such as the thyro-parathyroid and hypothal-hypophyseal hormone groups, growth factors, and gastrointestinal regulatory peptides (Fiat and Jollès, 1989; Koldovsky, 1992; Yamauchi, 1992). However, there are various bioactive peptides which can be released, from within the sequence of milk proteins, as a result of enzymatic hydrolysis. A number of bioactive peptides derived from α-, β-, κ-casein have been characterised and are summarised in Tables 4.1, 4.2 and 4.3, respectively. There are a smaller number of bioactive peptides which can be derived from whey proteins (Table 4.4). The structure, physiological significance and analytical aspects of milk derived bioactive peptides has been reviewed previously (Maubois and Léonil, 1989; Schlimme and Meisel, 1993; Fiat *et al.,* 1993; Shimizu, 1994). This review concentrates on the types of bioactive peptides which can be derived from bovine casein and whey protein, in addition to the physiological significance of these peptides *in vivo*.

4.1 Types of bioactive peptides derived from milk proteins

4.1.1 Opioid peptides

Many of the endogenous bioactive peptides sequences studied to date are opioid peptides. Opioid receptors are classified into subtypes, and the physiological response following binding and can be either of an agonistic or antagonistic nature. Individual receptors are responsible for specific physiological effects. For example, the μ-receptor for analgesia and suppression of intestinal motility, the δ-receptor for emotional behaviour and κ-receptor for sedation and control of food intake (Chiba and Yoshikawa, 1986). The typical endogenous ligands of opioid receptors have a definite N-terminal sequence of the form Tyr-x-x-Phe, (where x = any amino acid residue such as Gly), and are derived from precursor molecules, e.g. pro-enkephalin, pro-dynorphin and pro-opiomelanocortin (Höllt, 1983). Opioids having a receptor ligand with agonistic activity, may have a peptide or alkaloid structure (Teschemacher *et al.,* 1994

Table 4.1: Biological functions of peptides derived from α_{s1}-casein.

Fragment	Sequence	Function	Reference
f (23-34)	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	ACE inhibition	Maruyama and Suzuki,1982
f (23-27)	Phe-Phe-Val-Ala-Pro	ACE inhibition	Maruyama *et al*.,1985
f(24-27)	Phe-Val-Ala-Pro	ACE inhibition	Maruyama *et al*.,1987 (a)
f (25-27)	Val-Ala-Pro	ACE inhibition	Maruyama *et al*.,1987 (a)
f(27-30)	Pro-Phe-Pro-Glu	ACE inhibition	Maruyama *et al*.,1987 (a)
f(28-34)	Phe-Pro-Glu-Val-Phe-Gly-Lys	ACE inhibition	Maruyama *et al*.,1987 (a)
f(32-34)	Phe-Gly-Lys	ACE inhibition	Maruyama *et al*.,1987 (a)
f(43-47)	Asp-Ile-Glu-Ser-Glu	mineral absorp	Lorenzen *et al*.,1991; Meisel and Schlimme, 1993, 1994
f(59-79)	Gln-Met-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn-Ser-Val-Glu-Gln-Lys	mineral absorp	Lorenzen *et al*.,1991; Meisel and Schlimme, 1993, 1995
f(66-74)	Ser-Ser-Ser-Glu-Glu-Ile-Val-Pro-Asn	mineral absorp	Lorenzen *et al*.,1991; Meisel and Schlimme, 1993, 1996
f(90-95)	Arg-Tyr-Leu-Gly-Tyr-Leu	opioid agonist	Zioudrou *et al*.,1979; Chiba *et al*.,1989
f(90-96)	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu	opioid agonist	Zioudrou *et al*.,1979; Chiba *et al*.,1990
f(143-148)	Ala-Tyr-Phe-Tyr-Pro-Glu	ACE inhibition	Yamamoto *et al*.,1994
f (194-199)	Thr-Thr-Met-Pro-Leu-Trp	ACE inhibition; immuno	Maruyama *et al*.,1987 (b); Fiat *et al*.,1993
f(197-199)	Pro-Leu-Trp	ACE inhibition	Maruyama *et al*.,1987 (b)
f(198-199)	Leu-Trp	ACE inhibition	Maruyama *et al*.,1987 (b)

Immuno; peptides which modulated the immune system; mineral absorp; peptides which stimulate mineral absorption; ACE; Angiotensin I converting enzyme.

Table 4.2: Biological functions of peptides derived from β-casein.

Fragment	Name	Sequence	Function	Reference
f(1-25)	-	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Gly Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg	mineral absorp	Meisel and Frister, 1988; Meisel and Schlimme, 1993
f(60-62)	β-casomorphin-3	Tyr-Pro-Phe	Opioid agonist	Meisel and Schlimme, 1990
f(60-63)	β-casomorphin-4	Tyr-Pro-Phe-Pro	Opioid agonist	Meisel and Schlimme, 1990
f(60-63)	β-casomorphin-4	amide; morphiceptin	Opioid agonist	Chung *et al*.,1985
f(60-64)	β-casomorphin -5	Tyr-Pro-Phe-Pro-Gly	Opioid agonist; ACE inhibition	Brantl *et al*.,1979
f(60-65)	β-casomorphin-6	Tyr-Pro-Phe-Pro-Gly-Pro	Opioid agonist	Meisel and Schlimme, 1993
f(60-66)	β-casomorphin -7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	Opioid agonist	Brantl *et al*.,1979; Meisel and Schlimme, 1994
f(60-67)	β-casomorphin-8	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	Opioid agonist	Meisel and Schlimme, 1993
f(60-70)	β-casomorphin -11	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	Opioid agonist	Meisel and Frister, 1989
f(63-68)	-	Pro-Gly-Pro-Ile-Pro-Asn	Immuno	Parker *et al*.,1984
f(74-76)	-	Ile-Pro-Pro	ACE inhibition	Nakamura *et al*.,1995
f(84-86)	-	Val-Pro-Pro	ACE inhibition	Nakamura *et al*.,1995
f(177-181)	-	Ala-Val-Pro-Tyr-Pro	ACE inhibition	Maruyama *et al*. ,1987 (a)
f(177-183)	-	Ala-Val-Pro-Tyr-Pro-Gln-Arg	ACE inhibition; Immuno; G stim	Maruyama *et al*.,1987 (a); Fiat *et al*.,1994
f(177-179)	-	Ala-Val-Pro	ACE inhibition	Maruyama *et al*. ,1987 (a)
f(179-181)	-	Pro-Tyr-Pro	ACE inhibition	Maruyama *et al*. ,1987 (a)
f(181-183)	-	Pro-Gln-Arg	ACE inhibition	Maruyama *et al*. ,1987 (a)
f(191-193)	-	Leu-Leu-Tyr	Immuno	Fiat *et al*.,1994
	-	Ser-Leu-Val-Leu-Pro-Val-Pro-Glu	ACE inhibition	Yamamoto *et al*.,1994
f(193-202)	–	Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg	ACE inhibition	Meisel and Schlimme, 1994

Mineral absorp; peptides that stimulate mineral absorption; Immuno; peptides which modulated the immune system; G stim; peptides which had growth stimulating properties, ACE; Angiotensin I converting enzyme.

Table 4.3: Biological functions of peptides derived from κ-casein.

Fragment	Name	Sequence	Function	Reference
f(25-34)	Casoxin C	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg	opioid antagonist; ACE inhibition;ileum contraction	Chiba *et al*.,1989
f(33-38)	-	Ser-Arg-Tyr-Pro-Ser-Tyr	opioid antagonist	Chiba and Yoshikawa, 1986
f(34-38)	-	Arg-Tyr-Pro-Ser-Tyr	opioid antagonist	Chiba and Yoshikawa, 1986
f(35-41)	Casoxin A	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr	opioid antagonist; ACE inhibition	Chiba *et al*.,1989
f(35-38)	-	Tyr-Pro-Ser-Tyr	opioid antagonist	Chiba and Yoshikawa, 1986
f(58-61)	Casoxin B	Tyr-Pro-Tyr-Tyr	opioid antagonist	Chiba *et al*.,1989
f(103-111)	-	Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys Asn-Gln-Asp-Lys	antithrombic	Fiat *et al*.,1993
f(106-116)	-	Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Glu-Asp-Lys	mineral absorp; antithrombic	Fiat *et al*.,1989; Chabance *et al*.,1995
f(106-112)	-	Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Leu-Lys-Lys	antithrombic	Fiat *et al*.,1993
f(113-116)	-	Asn-Gln-Asp-Lys	antithrombic	Fiat *et al*.,1993

mineral absorp; peptides that stimulate mineral absorption, ACE; Angiotensin I converting enzyme.

Table 4.4: Biological functions of peptides derived from whey proteins

Milk protein Whey	Fragment	Name	Sequence	Function	Reference
β-lactoglobulin	f(102-105)	β-lactorphin	Tyr-Leu-Leu-Phe	Ileum contraction;Opioid (agonist)	Yoshikawa *et al*.,1986; Antila *et al*.,1991
β-lactoglobulin	f(146-149)	β-lactotensin	His-Ile-Arg-Leu	Opioid (agonist)	Pihlanto-Leppala *et al*.,1994
α-lactalbumin	f(50-53)	α-lactorphin	Tyr-Gly-Leu-Phe	Opioid (agonist)	Yoshikawa *et al*.,1986; Antila *et al*.,1991
BSA	f(208-216)	albutensin A	Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg	Ileum contraction; ACE inhibition	Chiba and Yoshikawa, 1991
BSA	f(399-404)	serorphin	Tyr-Gly-Phe-Gln-Asn-Ala	Opioid (agonist)	Tani *et al*.,1993

ACE; Angiotensin I converting enzyme.

In general, opioid agonistic peptides derived from milk proteins are selective for μ receptors. Some of the peptides with opioid antagonistic activity show affinity for κ-type opioid receptors in addition to affinity for μ receptors. Opioid peptides from caseins are called casomorphins (agonists) and casoxins (antagonists) while those from whey proteins are called lactorphins (agonists). Opioid peptides which are released from the internal structure of milk protein sequences are referred to as exorphins to distinguish them from the naturally-occurring opioid peptides, i.e. enkephalins, endorphins and dynorphins (Hamosh, 1989). Recent developments in research concerning milk derived opioid peptides and their functional significance have been reviewed (Chiba and Yoshikawa, 1986; Teschemacher and Scheffler, 1992). The existence of opioid peptides has been described in partial digests for many food proteins (Zioudrou *et al.,* 1979; Loukas *et al.,* 1983; Ariyoshi, 1993).

4.1.1.1 Agonists

β-Casein derived opioid peptides, known as β-casomorphins, have been extensively studied (Brantl *et al.,* 1979, 1981; Hamosh, 1989; Maubois and Léonil, 1989; Yamauchi, 1992; Schlimme and Meisel, 1993; Pihlänto-Leppälä *et al.* ,1994b). For example, two opioid peptides from bovine ß-casein, fragment f(60-64) and f(60-66) were first found in peptone (Brantl *et al.,* 1979) and were termed β-casomorphin 5 and 7, respectively. The opioid activity of sequences corresponding to human-casomorphins has also been reported (Yoshikawa *et al.*, 1986; Koch *et al.*, 1988). It was found that the pentapeptide, casomorphin-5 and the amidated form of the tetrapeptide, morphiceptin (β-casomorphin-4-amide) both isolated from *in vitro* casein digests (Chang *et al.,* 1985) were found to be the most potent of the β-casomorphins characterised to date (Table 4.2). β-Casomorphin f(60-70) has been shown to be released *in vivo* by digestive enzymes having been found in the duodenal chyme of minipigs (Meisel, 1986) and this opioid peptide was later identified as ß-casomorphin-11 (Meisel and Frister, 1989). In addition, opioid peptides have also been located in newborn calf plasma (Umbach *et al.,* 1985), after the ingestion of bovine milk.

Similarly, opioid peptides have been derived from a peptic hydrolysis of α_{s1}-casein, corresponding to f(90-95) and f(90-96) and were termed α-casein exorphins

or α-casomorphins (Zioudrou *et al.*, 1979; Loukas *et al.*, 1983; Chiba *et al.,* 1989) and these peptides (Table 4.1) were resistant to tryptic hydrolysis (Zioudrou *et al.*, 1979; Loukas *et al.*, 1983).

Whey protein derived synthetic peptide sequences (lactorphins), which were terminally amidated, were found to possess opioid activity (Yoshikawa *et al.,* 1986). α–Lactorphin, corresponded to f(50-53) of α-la in both human and bovine species. ß-Lactorphin f(102-105) was derived from bovine ß-lg. In addition, α-lactorphin and β-lactorphin were shown to be released *in vitro* using enzymatic hydrolysis of the parent protein. α-Lactorphin (Tyr-Gly-Leu-Phe) was isolated from a proteolytic digest of native α-la using pepsin or a combination of pepsin and trypsin (Antila *et al.*, 1991). This appears to be the only bioactive peptide derived from α-la which has been characterised to date. β-Lactorphin (Tyr-Leu-Leu-Phe) was produced using pepsin and trypsin or a combination of trypsin and chymotrypsin hydrolysis of native or heat-treated ß-lg (Antila *et al.,* 1991). The pharmacological activities of the synthetically produced tetrapeptides were characterised *in vitro* using a receptor assay with rat brain homogenates and an assay using coaxially stimulated guinea pig ileum. These assays showed that these peptides could displace/compete with Naloxone®, a potent synthetic opioid antagonist (Antila *et al.,* 1991). Another opioid peptide known as serorphin f(399-404) was found in serum albumin (Tani *et al.,* 1994).

Opioidergic receptors are located in the nervous, endocrine and immune systems. In the context of these systems, casomorphins as opioid ligands have been shown to modulate social behaviour, e.g. after intracerebroventricular administration to experimental animals (Panksepp *et al.,* 1984; Paroli, 1988). For example, a variety of physiological activities have been reported for β-casomorphin-7. It regulates the plasma levels of the pancreatic polypeptide (Schusdziarra *et al.,* 1983a) and a plasma somatostatin-like substance (Schusdziarra *et al.,* 1983b). Furthermore, it induces the accumulation of amino acids at the intestinal brush border (Ermisch *et al.,* 1989), increases the short circuit current of the intestinal layer (Tomé *et al.,* 1987) and induces histamine release from human peritoneal lympocytes (Kurek *et al.,* 1992). In other examples, β-casomorphins have been shown to influence motor behaviour, increase analgesic activity (Paroli, 1988), regulate gastrointestinal motility, exert anti-diarrhoeal action (Daniel and Erll, 1992) and influence postprandial metabolism by stimulating

the secretion of insulin and somatostatin (Maubois and Léonil, 1989; Meisel *et al.*, 1989; Fiat *et al.*, 1993; Schlimme and Meisel, 1993; Shimizu, 1994; Meisel and Schlimme, 1994).

4.1.1.3. Antagonists

Casein derived opioid antagonistic peptides are known as casoxins and these are generally derived from κ-casein (Table 4.3). Opioid antagonists are ligands which suppress the agonistic activity of enkephalin, and function in the same manner as Naloxone®. Casoxins have been produced *in vitro* by peptic or tryptic hydrolysis of caseins. For example, casoxin C was isolated from a tryptic digest of bovine κ-casein (Chiba *et al.*, 1989) and was shown to induce contraction of guinea pig ileum longitudinal muscle (Loukas *et al.*, 1983). Other antagonist peptides corresponding to f(33-38), f(34-38) and f(35-38) of κ-casein have been described (Table 4.3).

Furthermore, peptides derived from a peptic hydrolysis of human lactoferrin were demonstrated to have opioid antagonistic activity (Yoshikawa *et al.*, 1988).

4.1.1.2 Peptides acting on smooth muscle

A peptide sequence acting on smooth muscle, known as albutensin A, corresponding to f(208-216) was found in bovine serum albumin (Chiba and Yoshikawa, 1991). Another tetrapeptide, known as β-lactotensin f(146-149), which acts on smooth muscle (Chiba and Yoshikawa, 1991), was released from the internal sequence of β-lg by the action of chymotrypsin or a combination of pepsin and chymotrypsin (Pihlänto-Leppälä *et al.*, 1994a). Although these peptides had no affinity with opioid receptors, they induced contraction of guinea pig ileum longitudinal muscle when the test was performed without electrical stimulation in the absence of agonist.

4.1.2 Immunomodulating peptides

The properties of immunomodulating bioactive peptides derived from milk proteins have been reviewed (Parker *et al.*, 1984; Migliore-Samour *et al.*, 1989; Fiat and Jollès, 1989; Fiat *et al.*, 1993). These immunostimulants corresponded to f(194-199) of α_{s1}-casein and f(63-68) and f(191-193) of β-casein. The hexapeptide derived

from β-casein corresponds to the COOH-terminal part of β-casomorphin-11. Casein derived immunomodulating peptides were shown to stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages. In addition, casein derived peptides were shown to exert a protective effect against *Klebsiella pneumoniae* infection in mice following intravenous treatment (Parker *et al.*, 1984).

Several other milk protein derived peptides were shown to act on immunocompetent cells i.e. T and B lymphocytes or macrophages (Jollès *et al.*, 1986; Chabance *et al.*, 1995). It has been suggested that these peptides may play a role in the stimulation of the immune system (Portier *et al.*, 1994).

4. 1.3 Angiotensin-I-converting enzyme (ACE) inhibitory peptides

ACE is a dipeptide-liberating exopeptidase and has been classically associated with the renin-angiotensin system, regulating peripheral blood pressure. ACE which removes the two amino acids from the C- terminus of angiotensin I to form angiotensin II, a very hypertensive compound, is inhibited by certain peptides. Angiotensin II reduces blood flow and thereby decreases the renal excretion of fluid and salts. ACE also catalyses the degradation of bradykinin and enkephalins (Aoki *et al.*, 1984). Therefore, the inhibition of ACE decreases the activity of angiotensin II but increases bradykinin and enkephalin activities, thus lowering blood pressure in hypertensive humans and animals (Ondetti *et al.*, 1977; Koike *et al.*, 1980). Several endogenous peptides such as enkephalins, ß-endorphin, substance P and adrenocorticotrophic (ACTH) were reported to be substrates and inhibitors of ACE (Philips, 1987). Various ACE inhibitory peptides have been identified in hydrolysates of different food proteins including fish, yeast, α-zein, maize and gelatin (Ariyoshi, 1993) in addition to various fermented foods (Okamoto *et al.*, 1995).

Bioactive peptides, which inhibit ACE, have been were isolated from enzymatic casein hydrolysates (Maruyama *et al.*, 1982, 1985, 1987a, b; Kohmura *et al.*, 1990; Chiba and Yoshikawa, 1991; Schlimme and Meisel, 1993, Meisel and Schlimme, 1994). These potential anti-hypertensive peptides are known as casokinins (Table 4.1) and their function as inhibitors of ACE has been reviewed (Meisel, 1992, 1993a,b). The most potent casokinins were found in the region corresponding to f(39-52) in human ß-casein and f(63-65) in human κ–casein. Bovine α_{s1}-casein

f(23-27) is one of the most potent inhibitors of ACE having an IC_{50} of 6.0 μM (Maruyama *et al.*, 1985; Ariyoshi, 1993). ACE inhibitory activity (IC_{50} = 3.4 μm) was also observed for albutensin A f(208-216), derived from serum albumin (Chiba and Yoshikawa, 1991). In addition, numerous antihypertensive peptides have been synthesised corresponding to fragments of human caseins (Kohmura *et al.*, 1990). No whey protein derived ACE inhibitors have been characterised to date.

ACE has been identified as a brush border-bound enzyme of the human jejunum (Stevens *et al.*, 1988). Casein derived ACE inhibitors could act on ACE localised in the intestinal mucosa and at the luminal surface of the vascular endothelium. In this case, the affected functions may include jejunal electrolyte and water transport as a result of the decrease of the activity of angiotensin II. Since ACE is a multifunctional enzyme, exogenous ACE inhibitors derived from food proteins may affect different regulatory systems involved in modulating blood pressure, immune defence and nervous system activity (Meisel, 1992). As already stated ACE catalyses the activation of angiotensin II and inactivates bradykinin. The latter is able to stimulate lymphocyte migration and to increase secretion of lymphokines (Paegelow and Werner, 1986). Inhibitors of ACE would favour bradykinin and thus potentially act as immunomodulators.

The physiological significance, i.e. the hypotensive effects of ACE inhibitors derived from sour milk has been shown (Nakamura *et al.*, 1995b). In addition, a reduction in arterial blood pressure was observed in rat (Yamamoto *et al.*, 1994) using casein derived ACE inhibitory peptides. Although, in some studies relatively high doses of the peptide were required to have the effect by oral administration (Karaki *et al.*, 1990; Yamamoto *et al.*, 1994). The anti-hypertensive effects of a tryptic hydrolysate of casein were also studied in human volunteers with normal and mild hypertension (Sekiya *et al.*, 1992). Their blood pressure and pulse rates were monitored after oral administration of the hydrolysate. A study was carried out with 18 subjects suffering mild hypertension and receiving 10g of the tryptic casein hydrolysate twice a day. It was shown that blood pressure was significantly ($p < 0.01$) reduced by 4.6 mmHg/6.6 mmHg. However, the pulse rate did not change. It was concluded that the tryptic hydrolysate could be used as a physiological functional food to prevent

hypertension (Sekiya *et al.*, 1992). In addition, the hypotensive effects of sake derived peptides were sustained for up to 30h in murine models (Saito *et al.*, 1994).

4.1.4 Anti- thrombic peptides

Casein derived anti-thrombic peptides are known as casoplatelins and are inhibitors of both aggregation of ADP-activated platelets and binding of human fibrinogen γ-chain to a specific receptor region on the platelet surface (Fiat *et al.*, 1989). The interacting region of the fibrinogen γ-chain in platelet aggregation is at the C-terminal dodecapeptide sequence which itself possesses similar inhibiting effects as the bioactive sequences isolated from a tryptic hydrolysate of κ-casein (Kloczewiak *et al.*, 1984). For example, the sequence of the κ-casein peptide f(106-116) is homologous to that of the fibrinogen γ-chain dodecapeptide f(400-411). Another fragment of κ-casein f(103-111) has been shown to inhibit platelet aggregation but not fibrinogen binding to ADP-treated platelets (Fiat *et al.*, 1993).

4.1.5 Mineral binding peptides

These are generally phosphopeptides which bind calcium or trace elements such as iron and zinc and are generated by limited hydrolysis of casein by trypsin (Schlimme and Meisel, 1993; Lorenzen *et al.*, 1994). Phosphopeptides (Table 4.1 and 4.2) derived from α_{s1}-casein f(43-79) and from β-casein f(1-25) contain a serine phosphate cluster and glutamyl as well as aspartyl residues in their sequences (Eigel *et al.*, 1984). It is known that phosphopeptides bind calcium preventing formation of insoluble calcium phoshate complexes under alkaline conditions. Thus, these peptides can increase the soluble calcium concentration in the intestine, this in turn may facilitate passive absorption of calcium (Sato *et al.*, 1991) and other minerals, such as iron or zinc (Brulé *et al.*, 1982). In addition, the *in vivo* formation of an α_{s1}-casein derived phosphopeptide has been shown (Meisel and Frister, 1989; Kasai *et al.*, 1995). Because casein phosphopeptides form salts with certain trace elements, they have found interesting applications in dietary supplements and as medicines (Brulé *et al.*, 1982).

4.1.6 Growth stimulating peptides

Peptides derived from tryptic digests of ß-casein were shown to stimulate the growth of Balb/c 3T3 cells (Nagaune *et al.,* 1989). The induction of antigen-driven T cell proliferation by a C-terminal peptide from ß-casein was also reported (Coste and Tomé, 1991). Stimulation of the growth of *Lactobacillus lactis* by a tryptic digest of caseinmacropeptide derived from κ-casein has been described (Bouhallab *et al.,* 1993).

4.1.7 Milk derived peptides with other bioactivities

Casein derived peptides have also been described as exogenous prohormones. For example, peptides cleaved from β-casein were demonstrated to stimulate mitosis of different cell types (Nagaune *et al.,* 1989; Coste *et al.,* 1992). Another casein derived peptide was shown to inhibit protein synthesis and proteolysis and to enhance ureogenesis (Takenaka *et al.,* 1991).

A two-chain polypeptide consisting of 71 amino acid residues, corresponding to f(155-143) and f(144-184), was isolated from tryptic digests of bovine serum albumin and had insulin-stimulating activity (Ueno *et al.,* 1985). These peptides which were connected to each other by a disulphide bond enhanced the action of insulin in fatty acid synthesis and CO_2 production from glucose in a rat adipose tissue preparation.

A bacteriocidal activity was found in a peptic hydrolysate of bovine lactoferrin f(17-41) which was more potent than intact lactoferrin (Tomita *et al.,* 1991; Bellamy *et al.,* 1992). Bacteriocidal activity was found against *Eschericha coli* and *Candida albicans*, but not against bifidobacterium. It was suggested that this peptide may play a role in the colonisation of the intestine by bifidobacteria in milk suckling infants. In addition, chymotrypsin inhibitors derived from the C-terminal region of para κ-casein have been characterised (Fiat *et al.,* 1989).

4. 2 Multifunctional aspects of certain bioactive peptides

The multifunctional properties of certain peptides have been discussed (Shimizu, 1994) and are indicated in Tables 4.1-4.4. Since the immunomodulating peptide, β-casein f(63-68), overlaps the β-casomorphin-7, (f60-66), it has been

suggested that a 'strategic zone' i.e. f(60-70) exists within the β-casein sequence (Migliore-Samour and Jollès, 1988). Since T lymphocytes have μ receptors on the surface, it is conceivable that opioid peptides with high affinity for μ receptors could affect T cell function (Migliore-Samour and Jollès, 1988). Longer peptides containing β-casomorphins and immunomodulating sequences have been detected as β-casomorphin f(60-66) or f(60-67) immunoreactive peptides in the plasma of calves following milk ingestion (Umbach *et al.,* 1985). In addition, these peptides have been found in the plasma of lactating women (Koch *et al.,* 1988).

β-Casein f(177-183) has been shown to have an ACE inhibitory, immunomodulating and cell growth-promoting activity (Table 4.2). Casoxin C, showed ACE-inhibitory activity, vasorelaxing activity as well as opioid antagonist activity (Chiba and Yoshikawa, 1991).

In addition to the opioid peptides of β- and κ–casein, certain ACE inhibitors are also implicated in immunodulation (Migliore -Samour *et al.,* 1989). Albutensin A (Chiba and Yoshikawa, 1991) is multifunctional, showing ileum-contracting and vasorelaxing activities in addition to ACE-inhibitory activity (Chiba and Yoshikawa, 1991). In addition, α_{s1}-casein f(194-199) was reported to have both ACE-inhibitory and immunomodulating activities (Maruyama *et al.*, 1987b; Fiat *et al.*, 1993).

An antithrombic peptide from κ- casein f(106-116), which was observed to inhibit ADP - induced aggregation and fibrinogen receptor binding to platelet surface, overlaps with a sequence involved in the stimulation of mineral absorption (Fiat *et al.,* 1989).

4. 3 The relationship between bioactive peptide structure and physiological function

In order to be of physiological significance, the bioactive effect for a given peptide must be demonstrated *in vivo*. Peptides may be inactivated in the gastro-intestinal tract by gastrointestinal proteinases or brush-border enzymes and thereby may never reach their potential site of action. For example, morphiceptin, the amidated β-casomorphin, was unable to cross the epithelial layer because of degradation by brush-border dipeptidylpeptidases (Mahé *et al.*, 1989). Unless the

bioactive peptides act inside the intestinal tract or act upon intestinal epithelial cells directly from the luminal side, they must be transferred from the luminal (mucosal) side to the serosal side across the intestinal epithelial cell layer. After absorption, the intact peptides must then reach their target organs. It has been suggested by Zioudrou *et al.* (1979) that in order for exorphins to function *in vivo* as opioid peptides in the central nervous system they must (a) generally be produced by gastro-intestinal tract digestive proteinases (b) survive further degradation by intestinal proteinases (c) be absorbed, without degradation, into the bloodstream, (d) cross the blood - brain barrier and thereby reach the central opiate receptors and (e) interact as opiates with these receptors. It has to be assumed that there are similar criteria for all bioactive peptides.

Bioactive peptides therefore, are often generated using digestive proteinases. For example, combinations of pepsin with trypsin or chymotrypsin were used to produce *in vitro* the opioid peptides from α-la and ß-lg (Antila *et al.,* 1991) and from caseins (Pihlänto-Leppälä *et al.,* 1994b). Pre-treatment with pepsin prior to enzymatic hydrolysis with trypsin or chymotrypsin has been used to mimic *in vivo* digestion and to produce peptides which would have a greater chance of being resistant to further digestion in the digestive tract.

4.3.1 Bioactive peptide structure

Many bioactive peptides are resistant to degradation by gastric or pancreatic proteinases (Zioudrou *et al.,* 1979). Regarding the physiological function of the β-casomorphins it is known that they are relatively resistant to further proteolytic attack by digestive enzymes (Mellander, 1963; Henschen *et al.,* 1979; Brantl *et al.,* 1979; Meisel, 1986; Kasai *et al.*, 1995). In general, these peptides are rich in hydrophobic residues or have a high proline content in their sequences. For example, the high content of proline in the β-casomorphins appears to make them resistant to further proteolysis by gastro-intestinal proteinases (Brantl *et al.,* 1981). It has been suggested that these peptides may then be absorbed as inactive long-chain precursors which may be further hydrolysed by oligoaminopeptidases at the brush border into small bioactive peptides (Meisel, 1990). Because, in general β-casomorphins are very hydrophobic, it has also been suggested that they could in principle cross the blood-brain barrier (Meisel, 1990).

The structure-activity relationship of ACE-inhibitory peptides has not yet been fully established, however, a number of common features have been proposed with respect to the C-terminal amino acids, conformation states and the ACE binding sites (Cheung *et al.,* 1980; Meisel, 1993a, b). The C-terminal dipeptide has been suggested to play a major role in the inhibitory activity of the peptides (Cheung *et al.,* 1980). It has been suggested that ACE inhibitory peptides are rich in hydrophobic amino acids, but contain no acidic residues. For example, with the exception of ß-casomorphin-7, all casein derived peptide inhibitors of ACE have Pro, Lys, or Arg as the C-terminal residue (Meisel, 1993a, b). Other groups have suggested that hydrophobic residues such as tyrosine, proline, phenylalanine are related to the potency of ACE inhibitory peptides (Cheung *et al.,* 1980; Kohmura *et al.,* 1990; Saito *et al.,* 1994; Nakamura *et al.,* 1995a).

4.3.2 Bioactive peptide size

As can be seen from the Tables 4.1-4.4, the bioactive peptides characterised to date range from di- and tripeptides which are involved in ACE inhibition, to very long peptides of 21 to 26 amino acid residues which are involved in mineral absorption. In order to exert a physiological effect, these bioactive peptides must be absorbed intact and must reach their target organ. Certain of these peptides derived from milk can evolve naturally during digestion in the human digestive tract. For example, β-casomorphin-7 was detected in the intestinal contents of human volunteers who drank bovine milk (Svedberg *et al.*, 1985).

Since many bioactive peptides characterised to date are di- or tri-peptides, they may be transported into the epithelial cells via specific peptide transporters (Fei *et al.,* 1994). The small amount of peptides and proteins which are absorbed intact and which enter the circulation were reviewed (Gardner, 1988; Webb, 1990). It would be expected that peptides greater than 3 or 4 amino acids in length would be degraded by digestive proteinases, or by brush-border oligoaminopeptidases. However, β-casomorphins have been located in the plasma of newborn calves (Umbach *et al.*, 1985). In addition, eleven-mer antithrombic peptides from κ-casein (caseinoglycopeptides) were detected in the plasma of 5-day-old infants after ingestion of cow-milk based formula or human milk. It was suggested that these bioactive

peptides are released from milk proteins during digestion (Chabance *et al.*, 1995). Much work is obviously required to confirm the physiological significance of bioactive peptides known to be active *in vitro* by carrying out extensive *in vivo* studies with animal models.

5 Application of ultrafiltration in processing of whey protein hydrolysates

The principle of ultrafiltration (UF) has been used in many processes for the purposes of concentration, diafiltration or fractionation of molecules which differ in molecular size (Glover, 1985; Hallström *et al.*, 1989). UF has been used in the fractionation of milk proteins hydrolysates to produce hydrolysates which contain a certain molecular mass distribution of peptides (Section 3.1.1 and Section 3.1.4.1). In most cases UF is used to process batch hydrolysate products. However, UF membranes can also be used to physically retain (immobilise) an enzymatic activity if the molecular weight cut-off of the membrane is lower than the molecular weight of the active enzyme used in the hydrolysate reaction.

Immobilised enzymes are defined as 'enzymes physically confined or localised in a certain defined region of space with retention of their catalytic activities, which can be used repeatedly and continuously' (Chibata, 1978). This definition is applicable to enzymes, cellular organelles, microbial cells, plant cells and animal cells. In some cases, these biocatalysts are bound to, or within, insoluble support materials (carriers) by chemical or physical interaction. In other cases, biocatalysts are free, but confined to limited domains or spaces of supporting materials (entrapment). With the advent of immobilisation techniques for enzymes and cells, a certain control of biological processes is made possible, as substrates and products can easily be separated from catalysts or growing cells respectively. Methods of immobilisation include carrier binding, covalent binding, ionic binding, physical adsorption, cross-linking, in addition to entrapment. Immobilisation of enzymes to solid supports for the hydrolysis of whey proteins and other milk proteins has been used previously (Jost and Monti, 1977; Monti and Jost, 1978; Swaisgood and Catignani, 1985; Guisán *et al.*, 1991; Haque, 1993; Turgeon *et al.*, 1991; Lorenzen *et al.*, 1994). In addition, the method of immobilisation which employs affinity ligand chromatography has also been employed to recover the active enzyme after hydrolysis of whey protein (Monti and Jost, 1978). The method of enzyme entrapment/retention in membrane reactors is of most interest in this review and has been employed by several groups in the hydrolysis of milk proteins (Monti and Jost , 1978; Roger *et al.*, 1981; Haque, 1993; Turgeon *et al.*, 1991; Mannheim and Cheryan, 1990; Deeslie and Cheryan, 1981, 1982, 1988, 1991), as seen

in Table 5.1. The industrial operation of membranes and bioreactors has also been reviewed (Daniels, 1987; Belfort, 1989).

The application of UF type membranes to retain the enzyme during a hydrolysis reaction is reviewed here. The substrate (milk protein) is fed to the ultrafiltration unit and hydrolysed to low molecular weight products, which can permeate through the membrane. Other groups have also reviewed the application of this technology within the food industry (Hallström *et al.*, 1989; Trägårdh, 1988). The molecular weight distribution of hydrolysed products from a continuous membrane hydrolysis reactor depends upon membrane pore size, mean membrane pressure, enzyme to substrate ratio, temperature and residence time of substrate in the reactor (Butterworth *et al.,* 1970). Therefore, careful assessment of the above factors would allow control over the final molecular characteristics of the hydrolysate.

5.1 Definitions related to ultrafiltration and its application to processing of enzymatic hydrolysates.

There are several definitions which are related solely to UF membrane technology. The definitions which are used in this review are given below:

5.1.1 Flux

Flux is usually given in units of permeation of the product per unit membrane area over time i.e. (l m^{-2} h^{-1}). Flux must be optimised for each membrane system and this requires that concentration polarisation (see below) be minimised. The interrelated factors which influence flux and the permeation of the hydrolysate product are as follows: (a) mean membrane pressure; (b) feed crossflow (l h^{-1}) over the membrane; (c). process temperature; (d) concentration of retained substrate and products and (e) membrane cleanliness and fouling characteristics of the feed. These factors are outlined in Chapter 6. The performance of a membrane during processing is usually represented by permeate flux curves i.e. a plot of flux against the mean membrane pressure or against the concentration of a component such as protein under stated conditions.

Table 5.1: Summary of literature reports on continuous protein hydrolysis systems

Substrate	Enzyme	Membrane System	Reference
Casein	Alcalase[1]	Polysulphone hollow fibre ultrafiltration (A/G Technology MWCO 5-10 k Da)	Mannheim & Cheryan (1990)
Casein	Alcalase (glutaraldehye cross-linked)	Thin channel ultrafiltration cell (Amicon) MWCO-30 kDa	Boudrant & Cheftel (1976a,b)
Caseinomacro - peptide	Trypsin	Spiral-Wound UF cartridge	Bouhallab & Touzé (1995)
Soybean protein	Pronase[2]	Hollow fibre ultrafiltration (Amicon) MWCO-10 kDa	Deeslie & Cheryan (1981)
Soybean protein	Pronase	Hollow fibre ultrafiltration (Amicon) MWCO 10 kDa	Deeslie & Cheryan (1982)
Soybean protein	Pronase	Hollow fibre ultrafiltration (Amicon) MWCO 10 kDa	Deeslie & Cheryan (1988)
Soybean protein	Pronase	Hollow fibre ultrafiltration (Amicon) MWCO 10 kDa	Deeslie & Cheryan (1991)
Soybean protein	Acid proteinase[3]	Laminar flow cellulose acetate membrane MWCO 10-20 kDa	Iacobucci *et al.* (1974)
Blood protein	Alcalase	Hollow fibre ultrafiltration (Amicon) MWCO 10 kDa	Bressolier *et al.* (1988)
Fish protein	Pronase	Dead end ultrafiltration cell	Cheftel (1972)
Fish protein	Trypsin	Dead end ultrafiltration cell	Bhumiratana *et al.* (1977)
Alfalfa leaf protein	Trypsin	Dead end ultrafiltration cell	Payne *et al.* (1978)

[1]Alcalase: from *Bacillus lincheniformis* (Novo)
[2]Pronase: from *Streptomyces griseus* (Calbiochem-Behring Corp)
[3]Acid proteinase: from *Penicillium duponti* (Kikhoman Shoyu Co.)
MWCO: molecular weight cut-off

5.1.2 Concentration polarisation

Concentration polarisation is defined as the build up of feed components on the surface of the membrane leading to reduced flux. The degree of concentration polarisation in turn is dependant on the type of membrane used, the feed composition, crossflow velocity and mean membrane pressure.

5.1.3 Crossflow

Flow of retentate across the membrane or flow through the module is a concept of filtration known as cross-flow or tangential flow. Crossflow allows for long term processing with little decline in permeate flux over the course of the processing run e.g. in tangential flow UF modules. With conventional filtration, the retained particles on the filter media accumulate to a point where no liquid will pass through the filter i.e. in dead-end filtration. As the feed cross flow ($l\ h^{-1}$) is increased, concentration polarisation effects are alleviated due to the 'sweeping' action of the feed across the membrane. Cross-flow filtration, therefore, creates a turbulent environment on the membrane surface which can prevent accumulation of retained particles.

5.1.4 Fouling

Membrane fouling can be defined as the accumulation of thin molecular layers on the surface of the membrane that cannot be removed by increasing the flowrate of the feed across the membrane. Fouling may be reversible where periodic cleanings remove this layer and restore performance of the membrane to original levels. The response of a membrane system to fouling is manifested as a productivity decline in the first hour of the process which is followed by a gradual decline to the end of the run.

Macromolecular adsorption and fouling problems during ultrafiltration and their relationship to concentration polarisation has been studied (Matthiasson, 1984; Nilsson, 1989; Van der Horst, 1995). The fouling layer properties are known to depend on the degree of protein adsorption, hydrophobic interactions between the proteins and the membrane and membrane ionic interactions. The composition of the feed solution is of particular importance (i.e. pH, ionic strength, calcium content and degree of protein aggregation, presence of lipids and other particulates) in the formation of the fouling layer (Marshall and Daufin, 1995). A comprehensive

discussion of the physico-chemical aspects of membrane fouling, strategies to minimise fouling and the cleaning of membrane systems has been published recently (IDF Bulletin, 1995).

5.2.5 Mean membrane pressure

This is the driving force across the membrane (in the pressure controlled region), and is expressed as follows:

$$\Delta P_t = P_i + P_0/ 2 - P_p$$

where P_i = inlet pressure; P_0 = outlet pressure; P_p= permeate back pressure.
By increasing the mean membrane pressure, the fouling phenomena can be counteracted by the increase in hydraulic pressure on the membrane.

5.1.6 Area to volume ratio

Normal membrane fouling can be compensated for by increasing the mean membrane pressure or by adding membrane area, i.e. increasing the area of membrane to volume reaction ratio. Increasing the area to volume ratio, implies that the capacity of the membrane is increased, thus, decreasing transmembrane pressure and minimising concentration polarisation and fouling.

5.1.7 Residence time /Space time

The residence time/space time refers to the contact time between the substrate and the enzyme during an hydrolysis reaction in a continuous stirred tank reactor (CSTR). Space time has an effect on the molecular mass distribution profiles of peptides in an hydrolysate and the rate of decay of the enzyme in the reactor. These parameters can be controlled by the manipulation of space time factors (Deeslie and Cheryan, 1982). Space-time (τ) is defined as

$$\tau = (E\ V)/ (S_0^{1} J)$$

where E = enzyme concentration in a reactor (mg of N(nitrogen)/ml); V = volume of the substrate in reactor (ml); J = permeate flux (ml/min); S_0'= S_0-P_0 (mg of N/ml), where S_0 is the initial concentration of the substrate and P_0 is the initial concentration of the product. At higher space-time values the rate of build-up of unreacted substrate in the reactor is lower (Deeslie and Cheryan, 1982). By manipulation of space-time variables, higher conversions of substrate to product for longer time periods can be produced (Deeslie and Cheryan, 1981, 1982).

5.1.8 Dilution rate

Dilution rate is defined as follows:

$$D = J / V$$

where J = flux (ml/min) and V = reactor volume (ml).

5.2 Enzymatic hydrolysis in a continuous stirred tank reactor (CSTR)

Conventional batch protein hydrolysis has several disadvantages in comparison to continuous hydrolysis systems which use the principle of enzyme retention by a membrane. The disadvantages of the batch system of protein hydrolysis are as follows (a) high cost of using large quantities of commercial enzymes, (b) difficulty in controlling the extent of reaction that can result in nonhomogenous products, consisting of fractions of varying molecular weight, (c) low yields per unit enzyme activity, and (d) the need to inactivate enzymes by using a heat treatment or pH adjustment at the end of the reaction, which adds further to processing costs (Cheryan, 1986; Deeslie and Cheryan, 1988) and potentially changes the nutritional characteristics of the resultant hydrolysate (Erbersdobler, 1983).

However, confinement of the enzyme in a membrane reactor gives some of the advantages of enzyme immobilisation which include the following (a) improved efficiency, due to the fact that the enzyme can be reused, as it is retained by the membrane and is not heat inactivated or denatured after each run; (b) increased productivity per unit enzyme; (c) continuous systems allow for removal of substrate/product inhibition, and (d) a control of the molecular weight distribution of

the peptides produced in the hydrolysate fractions, within the limits of the molecular weight cut-off of the membrane (Deeslie and Cheryan, 1988).

The principles of ultrafiltration, types of membranes, membrane modules, plant configurations and the physical and chemical properties of milk proteins relevant to membrane processing have been reviewed (Glover, 1985). There are a number of interrelated factors which are important in the development of UF membrane enzymatic hydrolysis systems. These are (a) configuration of the membrane, (b) types of membranes, (c) membrane pore size and (d) flux characteristics of the system.

5.2.1 Membrane configurations used in continuous hydrolysis of food proteins

The primary consideration in developing continuous enzyme reactors should be, in addition to those advantages already mentioned, to obtain a high conversion of substrate to product and to rapidly establish a steady-state operation. The former is controlled by the reaction kinetics of the system i.e. type, specificity and activity of the enzyme, E:S ratio and enzyme stability during continual usage. The latter is controlled by the operating conditions of the UF system, such as control of concentration polarisation, area to volume ratio of the membrane module and the fouling characteristics of the system.

In the hydrolysis of food proteins, two membrane configuration types have been generally used, the dead-end cell and the continuous stirred tank reactor (CSTR). The first description of a laboratory experiment using a membrane reactor for protein hydrolysis appears to be the work of Blatt *et al.* (1968) for the hydrolysis of whey protein. However, the reactor configuration used in this and many later investigations is the so-called 'dead-end' design with no cross-flow over the membrane. In the dead end type of membrane reactor a cell fitted with a membrane at one end is loaded with the appropriate enzyme solution and substrate is continuously fed in under pressure while the permeate is continuously drawn through the membranes (i.e. the UF cell is operated in the continuous diafiltration mode). Agitation is necessary to minimise concentration polarisation effects and this is generally provided with a built in magnetic stirrer. However, this mode generally gives a higher concentration polarisation and a lower flux than for example, CSTR configurations.

A different configuration was proposed by Porter and Michaels (1972) in which a continuous stirred tank reactor (CSTR) is coupled in a semi-loop configuration through a hollow fibre UF membrane. Figure 5.1 gives a schematic representation of a CSTR in this configuration.

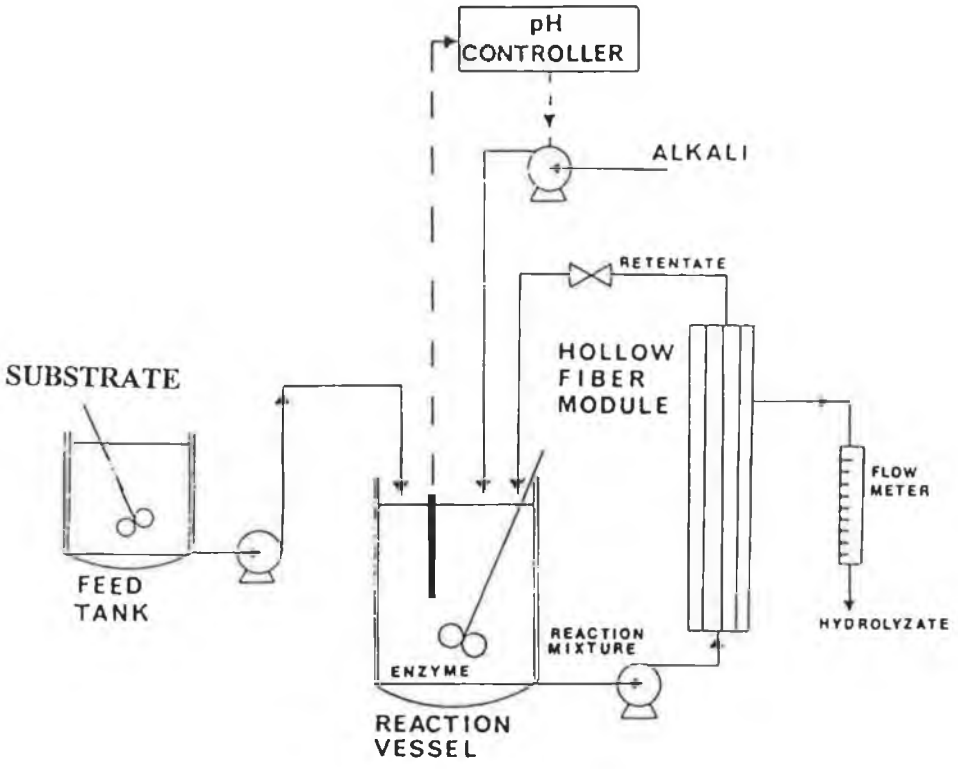


Figure 5.1 Schematic representation of a continuous stirred tank reactor (CSTR) in a semi-closed loop configuration

(taken from Mannheim and Cheryan, 1990).

In the CSTR configuration, the reaction mixture, consisting of enzyme, unreacted and partially hydrolysed protein and hydrolysed protein is pumped through the membrane module under pressure. Hydrolysate containing peptides of a particular molecular size distribution, smaller than the cut-off of the membrane, pass through the ultrafiltration membrane and are collected as permeate. The rest of the reaction mixture, consisting of enzyme and partially hydrolysed protein are returned to the CSTR. The residence time of the substrate in the enzymatic reactor is controlled by the system parameters such as flux. By careful design and selection of operating parameters, a steady state operation can be achieved within 30-60 min of start up. This design has been applied in the more industrially oriented work on membrane reactors for protein hydrolysis, as well as for the concentration and purification of proteins where the engineering problems are similar (Olsen, 1978). In particular this configuration has been used with hollow fibre membranes (Deeslie and Cheryan, 1981,1988, 1991; Bressolier *et al.*, 1988; Mannheim and Cheryan, 1990).

5.2.2 Membranes used in entrapment of enzymes during food protein hydrolysis

5.2.2.1 Types and geometry of membranes

The membranes first employed in ultrafiltration of milk proteins were made of organic polymers, such as cellulose. However, these membranes were subject to many limitations. They were susceptible to hydrolysis, had a limited operational pH range i.e. 3-7 and an upper temperature limit of 35 °C. Therefore, efficient cleaning and sterilisation was constrained. Other polymers for use in membranes such as polyvinyl chloride, polyacrilonitrile, ploycarbonate, polyamide and polysulphone were thus developed. These membranes have proved to be stable to large changes in pH, temperature and stable to stringent cleaning procedures. For example, polysulphone type membranes, having a repeating $(C_6\ H_5)_2\ SO_2$ unit, can withstand temperatures between 50-80 °C and pH values between pH 2-12. Membrane geometry can be tubular or flat (rectangular, oval or spirally wound). It is known that flat membranes are difficult to clean if badly fouled (Glover, 1985). The membrane types used for continuous hydrolysis are normally hollow fibre, although use of a spiral membrane has been reported (Bouhallab *et al.*, 1992).

5.2.2.2 Membrane pore size

For continuous processing of milk protein hydrolysates, an ultrafiltration membrane having a defined molecular weight cut-off (MWCO) value can separate a product from the substrate-enzyme reaction mixture, thus controlling the molecular size of the peptides product in the permeate. Control of the molecular properties of hydrolysate products is one of the major advantages of using UF type membrane reactors. This control of molecular size is important where the hydrolysate product is exploited for its functional properties, such as emulsification and foaming properties (Adler-Nissen, 1986; Deeslie and Cheryan, 1991; Chobert *et al.*, 1991). The importance of control of peptide size to final product functionality has been outlined previously (Section 3). It must be remembered, however, that ultrafiltration membranes currently available do not have a sharp cut-off or pores of a specific and uniform size hence certain molecules would also permeate through, depending on shape and size of the peptides/molecules (Merin and Cheryan, 1980, Deeslie and Cheryan, 1982). Hence, peptide size distribution of hydrolysate products is referred to in most cases. Table 5.1 gives the type of membranes and their molecular mass cut-off values, which have been used during continuous hydrolysis of food proteins.

5.2.2.3 Flux characteristics of a UF membrane system

The flux characteristics of a UF membrane system are dictated by a number of factors as mentioned in section 5.1.1 These factors have an influence on the membrane flux and permeation characteristics of the hydrolysate product.

5.3 Kinetics of enzymatic hydrolysis reactions in UF membrane type systems

Kinetic models have long been established for the batch reactor system. The normal kinetic model adopted is that of Michaelis-Menten (1913). A number of kinetic models have been developed for the continuous hydrolysis of proteins utilising UF membranes. Most continuous models demand that certain assumptions be made initially and Michaelis-Menten kinetics are then applied to describe the reaction when a steady-state is reach, i.e. the solute concentration crossing over the membrane (retentate) is equal to the solute concentration in the permeate.

Most of the literature on kinetics of hydrolysis in UF reactors up to 1979 has been thoroughly reviewed by Cheryan and Deeslie (1980). Other later publications deal mainly with kinetic investigations of protein hydrolysis in membrane reactors and the establishment of mathematical models for batch and continuous enzymatic hydrolysis of food proteins (Sejr Olsen and Adler-Nissen, 1981; Deeslie and Cheryan, 1981, 1982; Alford *et al.*, 1984). The only other work in relation to the mathematical modelling of protein hydrolysis in membrane reactors previous to this was carried out by Iacobucci *et al.* (1974) and Myers *et al.* (1974). These groups hydrolysed soya protein isolate in a membrane reactor with a semi-closed loop configuration, using membranes with a nominal molecular mass cut-off value of 10 kDa. Other groups hydrolysed casein using Alcalase in a batch and continuous mode CSTR, using a hollow fibre membrane with a 10 kDa cut-off (Mannheim and Cheryan, 1990).

All of the above groups developed mathematical models for the behaviour of the membrane reactor. All of these models were based on mass balance considerations in steady state, but they differed with regard to the underlying kinetics. Iacobucci *et al.* (1974), Myers *et al.* (1974) and Deeslie and Cheryan (1981, 1982) used Michaelis-Menten kinetics to determine a rate constant, k, which is proportional to the enzyme concentration. First-order reaction kinetics are assumed in these models. The rate constant is determined under steady-state conditions in the membrane reactors, and is found, as expected, to be much lower than the corresponding initial reaction rate constant obtained in batch hydrolysis experiments. The assumptions of the models were used to describe the hydrolysis of casein by Alcalase (Mannheim and Cheryan, 1990).

A quantitative kinetic model for the continuous hydrolysis of soy protein has been described which used a different approach (Sejr Olsen and Adler-Nissen, 1981). The rate constant k was derived from an empirical batch hydrolysis curve, for which zero-order kinetics have been established. By expressing k as a function of DH, however, k is transformed into a pseudo first-order rate constant.

Ultimately, all the above groups arrive at expressions for the conversion percentage in steady state which are roughly mathematically equivalent at high substrate to product conversions. The conversion percentage, i.e. the ratio between the

nitrogen concentration in the permeate and the feed, is a function of the feed concentration, the reactor volume (confined mass), the flux and the rate constant, *k*.

The economical advantage of using a membrane reactor process depends critically on the ability to purge the reactor. Usually, a small fraction of the substrate cannot be converted to hydrolysate and when the reactor is run continuously, this residue will gradually build up in the reactor. This necessitates that a small part of the hydrolysate is bled continuously from the concentrate side of the membrane (Iacobucci *et al.*, 1974). However, the concomitant loss of enzyme may then in certain cases lead to overall enzyme consumption close to that of the batch hydrolysis process (Sejr Olsen and Adler-Nissen, 1981).

5. 4 Enzymes used in semi- and continuous membrane systems for the hydrolysis of food proteins

A summary of some of the enzymes used in the hydrolysis of food proteins in continuous systems is given in Table 5.1. Some of the factors controlling high conversion rate of substrate to product are (a) the enzyme to substrate ratio and (b) type, specificity and stability of the enzymes used in the reaction. The enzyme to substrate ratio will dictate the rate of reaction and the type of product obtained within a specific time of hydrolysis (Mullally *et al.*, 1994). The type, specificity and stability of, for example, gastric and pancreatic enzymes have been discussed in Section 1. It has been suggested that one of the key factors is that the enzyme should have high activity and as broad a specificity as possible to prevent build-up of unhydrolysable material in the system, particularly when complex protein systems (e.g. milk proteins) are used in enzymatic hydrolysis reactions. This may mean having to use a mixture of exo- and endopeptidases activities. Problems of enzyme stability has been shown for soy protein hydrolysis using Pronase and Alcalase (Deeslie and Cheryan, 1988). It was suggested that should one enzyme decrease in activity at a faster rate than the others, that this would lead to problems in the hydrolysis reaction, such as those related to enzyme decay (Deeslie and Cheryan, 1988).

5.4.1 Enzyme decay within a membrane reactor.

Problems which arise due to decay of enzyme in a continuous system are as follows: (i) build up of unhydrolysed material and (ii) the rate of conversion of substrate to products which permeate through the UF membrane is decreased. Enzyme decay during an hydrolysis reaction can cause a shift in molecular weight distribution to larger peptides. In addition, solubility the substrate decreases as enzyme decay increases and there can be an increase in turbidity. The mean membrane pressure in the system may rise above an acceptable level or flux rate may drop. This has been shown for soy protein hydrolysis with Pronase and Alcalase (Deeslie and Cheryan, 1988). A number of reasons for enzyme decay were shown for hydrolysis of soy protein by Pronase (Deeslie and Cheryan, 1982), which may be monitored within a particular system and controlled in many cases. (a) Thermal inactivation of the enzyme may occur over time of the reaction. It has been shown, for example, in the Alcalase-casein system, that it is generally advisable to operate at lower temperature (35 °C), if long-term enzyme stability is required (Boudrant and Cheftel, 1976a, b). However, higher hydrolysis temperatures (40-50 °C) reduce microbial contamination of the product. It is known that in the presence of the substrate that certain enzyme preparations may be more thermostable. This is the case for porcine trypsin in the presence of whey protein (Jost and Monti, 1977). It is known that porcine trypsin is more thermostable than bovine trypsin (Lazdunski and Delaage, 1965), however at 55 °C and over the enzyme is denatured, even in the presence of calcium ions. It has been shown that in the presence of whey protein as substrate, the enzyme trypsin retains over 70 % activity after 1 hour at 55 °C (Buck *et al.,* 1962; Deeslie and Cheryan, 1982). This demonstrates the use of a mesophilic enzyme at thermophilic digestion temperature due to the protective effect of the whey protein substrate (Jost and Monti, 1977). Most control experiments, i.e. in the absence of substrate, may give a false impression of enzyme stability or denaturation. (b) Depending on the membrane type, enzyme adsorption to the membrane may occur. Low protein binding membranes are used to eliminate this problem, such as certain polysulphone membranes (c) Mechanical shearing of the enzyme may occur over time within a continuous system. (d) In certain cases, the enzyme may be inhibited by the substrate or products. However, in a continuous system, where product is continually removed and fresh

substrate is added, this problem should not occur. (e) Loss of activators such as calcium through the membrane was shown to lead to loss of Pronase enzymatic activity in the hydrolysis of soy protein (Deeslie and Cheryan, 1982). (f) Enzyme leakage at 50 °C was shown to be higher than at 37 °C for a Pronase/soy protein hydrolysis system (Deeslie and Cheryan, 1982). Lowering the operating temperatures reduced the rate of enzyme leakage due to a general effect of lower solute (enzyme) mass flux at lower temperature, a common ultrafiltration phenomena. Enzyme leakage has been shown to occur in the first 1-2 h of hydrolysis operation (Deeslie and Cheryan, 1982). Generally, it was found that lowering the dilution rate of the system reduced the loss of activity within the reactor by lowering flow through of the product and rate of enzyme leakage (Deeslie and Cheryan, 1982). However, also it was suggested that product or unreacted substrate may act to stabilise the enzyme and a high dilution rate would reduce the stabilising factor (Deeslie and Cheryan, 1982). (g) Finally, certain enzymes are specific for amino acid residues within their own sequence, which may lead to autodigestion and loss of overall enzymatic activity.

5. 5 Hydrolysates which have been produced by continuous hydrolysis of milk proteins and the application of UF technology.

Hydrolysis in a continuous stirred tank membrane reactor (CSTR) which links enzymatic hydrolysis with simultaneous separation of products from a reaction mixture is a process which has been applied to continuous and extensive hydrolysis of proteins from various sources. For example, the application of ultrafiltration to recover low molecular weight products from enzymatic hydrolysis of whey protein such as lactalbumin has been described (Olofoson *et al.,* 1980; Turgeon *et al.*, 1991). In addition, the hydrolysis of casein with a number of proteinases, including glutaraldehyde-stabilised Alcalase has been investigated by Boudrant and Cheftel (1976a, b).

A considerable number of publications on the development of continuous enzymatic hydrolysis of other food proteins in membrane reactors has appeared in the last two decades. Hydrolysis of soya protein has been described by Roozen and Pilnik (1973,1979), Iacobucci *et al.* (1974) together with Myers *et al.* (1974), Vieth *et al.*

(1977), Sejr Olsen and Adler-Nissen (1981), Cheryan and Deeslie (1980, 1983), Deeslie and Cheryan (1981, 1982). Other proteins which have been hydrolysed in membrane reactors are fish protein concentrate (Bhumiratana *et al.*, 1977), cottonseed protein (Cunningham *et al.*, 1978), leaf protein (Payne *et al.*, 1978) and potato protein (Roozen and Pilnik, 1979).

Several other groups have employed continuous enzymatic hydrolysis of protein in a membrane reactor (Boudrant and Cheftel, 1976a, b; Bressollier *et al.,* 1988; Mannheim and Cheryan, 1990), to produce hydrolysates with improved nutritional and functional properties (Brulé *et al.,* 1980; Cheryan and Mehaia, 1986). A recent example of the use of CSTR is in the production of bioactive peptides from a tryptic hydrolysate of caseinomacropeptide. The continuous system was compared to the batch process for the production of an antithrombic peptide and it was found that the productivity of the continuous process was 3 times higher after 3.5 h of hydrolysis. A continuous hydrolysis process has also been described in the production of gram quantities of this anti-thrombic peptide during the hydrolysis of caseinomacropeptide (Bouhallab *et al.,* 1992; Bouhallab and Touzé, 1995).

PROPOSED INVESTIGATION

The objectives of this project were as follows:

- To quantify the protease and peptidase activities within commercially available pancreatic proteolytic preparations and to characterise the influence of these activities on some physicochemical properties of whey protein hydrolysates.
- To investigate the effect of different large scale isolation and activation conditions for pancreatic zymogens on the ratios of proteolytic activities within an enzymatic preparation. As a follow on to the above, to study the effects of different zymogen activation procedures on the development of certain molecular characteristics in whey protein hydrolysates.
- To investigate the angiotensin-I-converting enzyme (ACE) inhibitory activity of synthetic peptides corresponding to whey protein sequences known to possess opioid activity.
- To characterise the development of ACE inhibitory activity from whey protein hydrolysates generated with a range of gastric and pancreatic enzyme activities. To develop a fractionation scheme for the identification of specific β-lactoglobulin derived ACE inhibitory peptides.
- To perform preliminary studies using differential scanning calorimetry to investigate the relationship between the thermal stability of β-lactoglobulin and its susceptibility to enzymatic hydrolysis.
- Finally, to perform preliminary studies to determine the efficacy of a tangential flow ultrafiltration membrane system for the fractionation of a whey protein hydrolysate.

CHAPTER 1

Proteolytic and peptidolytic activities in commercial pancreatic protease preparations and their relationship to some whey protein hydrolysate characteristics[1]

[1] This chapter was published in the Journal of Agriculture and Food Chemistry,(1994) Vol 42 (12), p2973-2981.

1.1 Summary

Endoproteinase and exopeptidase activities in the commercially available pancreatic protease preparations Corolase PP, PTN 3.0S, pancreatin, PEM 2500S, PEM 2700S and PEM 800S were quantified using synthetic peptide substrates. These preparations were generally found to be low in aminopeptidase and dipeptidase activity. Trypsin and chymotrypsin, albeit in different ratios, were present in all pancreatic preparations. Elastase was present only in Corolase PP and pancreatin. The ability of these protease preparations to hydrolyse the insoluble heat denatured whey protein, lactalbumin, was compared and contrasted with that of crystalline trypsin, chymotrypsin, and elastase in addition to a commercial exopeptidase preparation, Debitrase DBP.20. When the source, number, or ratio of endoprotease activity changes, there are distinctive differences in products produced with respect to percentage degrees of hydrolysis, gel permeation profile, solubility, and free amino acids present in the hydrolysate.

1.2 Introduction

Proteolytic enzyme preparations are economically the most important group of enzymes and their use is well established in the food industry (Godfrey and Reichert, 1983). Commercial protease preparations for use in large scale processes may consist of mixtures of proteinases and peptidases that are isolated from animal tissues, plants or micro-organisms. Proteinases split protein molecules into peptides, while exopeptidases remove amino acids either from the N-terminus, i.e. aminopeptidases (Sanderink *et al.*, 1988) or from the C-terminus i.e. carboxypeptidases (Pétra, 1970), of peptides. The purification, and physical and chemical properties of pancreatic proteinases have been reviewed: trypsin (Northrop *et al.*, 1948; Desnuelle, 1960; Hakim *et al.*, 1962; Vestling *et al.*, 1990); chymotrypsin (Wilcox, 1970), elastase (Hartley and Shotton, 1971; Gertler *et al.*, 1977); and protease E (Kobayashi *et al.*, 1981). The exopeptidases of the pancreas include carboxypeptidases A and B (Puigserver *et al.*, 1986) in addition to a number of aminopeptidases and dipeptidases.

Enzymatic modification of proteins using selected proteases to hydrolyse specific peptide bonds is widely used (Adler-Nissen, 1986; Fox, 1991; Arai and Fujimaki, 1991). Whey proteins enzymatically hydrolysed with trypsin and chymotrypsin showed superior solubility and *in vitro* digestibility as compared to their chemically treated counterparts (Lakkis and Villota, 1992). The choice of substrate, proteases employed and the degree to which the protein is hydrolysed are factors known to affect the physicochemical properties of resulting hydrolysates. Manipulating the reaction conditions during enzymatic hydrolysis of milk proteins, produces hydrolysates with different solubility and emulsifying characteristics (Turgeon *et al.*, 1992), foaming properties (Kuehler and Stine, 1974), or taste characteristics (Murray and Baker, 1952; Poulsen, 1987; Vegarud and Langsrud, 1989).

The degree to which milk proteins are hydrolysed depends on the intended use for the hydrolysate. Low degrees of hydrolysis are desirable for maintaining functional properties, while extensive hydrolysis is necessary when the hydrolysate is to be used in, for example, chemically defined or hypoallergenic infant formulas (Merritt *et al.*, 1990; Thibault, 1991). Mixtures of proteases having complementary specificities may

be used to attain the required degree of hydrolysis. The proportions of different proteinases, such as trypsin to chymotrypsin, and the presence or absence of exopeptidases, such as carboxypeptidase B, may be significant for production of specific hydrolysates such as hypoallergenic hydrolysates (Thibault, 1991) and/or hydrolysates with reduced bitterness (Fullbrook *et al.*, 1987; Plainer and Sproβler, 1990).

To date little information has been published which links the proteinase/peptidase activity of commercial proteases used for hydrolysis of milk proteins to the physicochemical characteristics of the final hydrolysate. Although many groups have devised processes for the production of milk protein hydrolysates using commercial pancreatic proteases for clinical nutrition (Grimble and Silk, 1989; Maubois and Léonil, 1989), hypoallergenic and special dietetic products (Jost *et al.*, 1988; Asselin *et al.*, 1988, 1989; Thibault, 1991), the choice of enzyme is usually based on empirical screening or by random selection.

The specificity of the pancreatic proteolytic enzymes dictates the nature of peptides and free amino acids found in the hydrolysate. The tissue source, bovine or porcine, in addition to the methods of purification used, influences the composition and stability of protease preparations. A knowledge of the proteinase and exopeptidase activities in commercial protease preparations would be a useful guide in the selection of a particular protease to generate a desired hydrolysate. The objective of this study was to determine the proteinase and peptidase activities in pancreatic protease preparations and to characterise the physicochemical properties of lactalbumin hydrolysates produced using these preparations.

1.3 Materials and Methods

1.3.1 Enzymes

Commercial pancreatic proteases were received as gifts from manufacturers. Corolase PP (porcine) was supplied by Rohm GmbH, Kirschenallee D6100, Darmstadt, Germany; pancreatin (porcine) by Chemicon Ltd., Dublin, Ireland. PEM 2500S (porcine/bovine), PEM 800S (bovine), PEM 2700S (bovine) and PTN 3.0S (porcine) were supplied by Novo Nordisk A/S, Bagsvaerd, Denmark, and Debitrase DBP.20 (*Lactococcus lactis, Aspergillus oryzae*), was supplied by Imperial Biotechnology Ltd., London, U.K. Bovine trypsin (type XII), bovine chymotrypsin (type II), porcine carboxypeptidase A, porcine carboxypeptidase B (Type I) and porcine elastase (type I) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

1.3.2 Substrates

Aminomethylcoumarin (AMC) and fluorogenic substrates Alanine-AMC, Asparagine-AMC, Arginine-AMC, Glycine-AMC, Histidine-AMC, Proline-AMC, Arg-Arg-AMC, Gly-Arg-AMC, Gly-Pro-AMC, Lys-Ala-AMC, acetyl-Ala-Ala-Pro-Ala-AMC, N-Suc-Leu-Leu-Val-Tyr-AMC and N-benzoyl-L-Arg-AMC were obtained from Bachem, Bubendorf, Switzerland. Carboxypeptidase substrates, hippuryl-L-lysine, hippuryl-L-phenylalanine and N-tosyl-l-lysine chloromethyl ketone (TLCK) were supplied by Sigma. Lactalbumin (Alatal 560) was from New Zealand Dairy Board, Wellington, New Zealand; it is an insoluble heat denatured whey protein, and its manufacture has been outlined by a number of groups (Robinson *et al.*, 1976; Mulvihill, 1992). Reagents for amino acid analysis were obtained from Beckman Instruments, High Wycombe, U.K. All other reagents were of analytical grade unless otherwise specified.

1.3.3 Quantification of Enzyme activity

Enzyme activities were assayed using a modification of the fluorogenic (AMC) assay (Zimmerman *et al.*, 1977) as follows. Proteases (10 mg ml^{-1}) were centrifuged at 14,400 *g* (Microcentaur, MSE, U.K.) for 10 min and 20 µl of supernant was added to

980 µl of 0.01 M Tris-HCl, pH 7.0, containing 0.02 mM specific substrate. The substrate-enzyme mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 ml of 1.5 M acetic acid and fluorescence developed was measured on a spectrofluorometer (Perkin Elmer 1000, Beaconsfield, Bucks, England) at excitation and emission wavelengths of 360 nm and 440 nm, respectively. When the substrates Arg-Arg-AMC, Gly-Arg-AMC, Arg-Arg-AMC were used, the protease preparation was incubated with the trypsin inhibitor TLCK (1mg ml^{-1}) in the ratio 1:5 (v/v) at 37 °C for 10 min, before the assay. One unit of activity is defined as that amount of enzyme which will give 1 µmole AMC min^{-1} $(mg\ protein)^{-1}$.

For quantification of carboxypeptidase A and B activities, samples (10 mg/ml of powder) were filtered through a 0.45 µm PS Whatman syringe filter, to remove aggregated material, and assayed using procedures outlined by Worthington Diagnostic Systems Inc., Freehold NJ. One unit of carboxypeptidase A activity was defined as that amount of enzyme which catalyses the hydrolysis of 1 µmol N-α-hippuryl-L-phenylalanine min^{-1} at pH 7.50 and 25 °C. One unit of carboxypeptidase B activity was defined as that amount of enzyme which catalyses the hydrolysis of 1 µmol N-α-hippuryl-L-lysine min^{-1} at pH 7.65 and 25 °C.

1.3.4 Preparation of Hydrolysates

A 25 ml solution of lactalbumin, 8 % (w/v) protein, was hydrolysed with individual proteases at 50 °C for 240 min. The pH was maintained at constant pH 8.0 by continuous addition of 0.5 M NaOH using a pH-stat (Metrohm Ltd., Herisau, Switzerland.). The degree of hydrolysis (DH, %), defined as the percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Following hydrolysis, proteases were inactivated by heating at 80 °C for 30 min, cooled, and stored at -20 °C for further analysis. The enzyme (E) to substrate (S) ratio (0.003) was calculated on the basis of total protein content in the enzyme preparations and lactalbumin. The DH was calculated as follows:

$$DH, \% = B\ (M_b)\ (1/\alpha)(1/MP)(1/h_{tot}) \times 100$$

where B = volume of NaOH consumed (mL); M_b = molarity of NaOH; α is the average degree of dissociation of the α-NH_2 groups at pH 8.0 and 50 °C; MP is the mass of protein (g); h_{tot} is the total number of peptide bonds in the protein substrate (mequiv/g protein). The h_{tot} value, 8.8 mequiv/g protein, and values of $1/\alpha$ for various pH-temperature combinations were those given by Adler-Nissen (1986).

1.3.5 Characterisation of Hydrolysates

1.3.5.1 Protein (Nitrogen) Determination

Total protein (N x 6.25) of pancreatic preparations and of whey protein (N x 6.38) was determined by micro-Kjeldahl (AOAC, 1980).

1.3.5.2 Protein (Nitrogen) Solubility

Hydrolysates were adjusted to pH 6.6 with 1M HCl and centrifuged at 1300 *g* (Mistral 6000, MSE Scientific Instruments, West Sussex, England.) for 15 min at 20 °C. The supernatants were then filtered through Whatman no.1 filter paper; N content was determined as described above and expressed as a percentage of total N in the hydrolysate.

1.3.5.3 Molecular size distribution of peptides in lactalbumin hydrolysates

A fast protein liquid chromatograph (FPLC) fitted with a Superose 12 gel permeation column (Pharmacia LKB Biotechnology Ltd., Upsalla, Sweden.) was used to monitor the size distribution of peptides in lactalbumin hydrolysates. The column was eluted at 1 ml min^{-1} with 0.1 M Tris-HCl buffer containing 0.1 M NaCl and 10 % (v/v) methanol. Hydrolysates were diluted in elution buffer to 0.25 % (w/v) protein, filtered through a Whatman 0.45 μm PS syringe filter and 100 μl was applied to the column. Analyses were performed in duplicate. Eluate was continually assayed at 214 nm or 280 nm using two single path monitors (UV-1, Pharmacia) and recorded using a Minichrom© data handling system (VG, Data Systems, Altrincham, Cheshire, U.K). A calibration curve was prepared from the average retention volume (R_v) of standard proteins and peptides.

1.3.5.4 Free amino acid analysis of hydrolysates

Hydrolysates were deproteinised by mixing equal volumes of 24 % (w/v) TCA and sample, which was allowed to stand for 30 min before centrifuging at 14400*g* (Microcentaur, MSE, U.K.) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2, to give approximately 25 nmol of each amino acid residue per 50 µl injection volume, and then analysed on a 120 x 4 mm cation exchange column (Na^+ form) using a Beckman 6300 amino acid analyser (Beckman Instruments Ltd., High Wycombe, U.K).

1.4 Results

1.4.1 Characterisation of proteolytic activity in commercial pancreatic proteases

1.4.1.1 Aminopeptidase activity

Aminopeptidase activity in the bovine and porcine protease preparations tested was generally very low (Table 1). Corolase PP had activity against all the aminopeptidase substrates except Asp-AMC. Interestingly, there was essentially no activity against Asp-AMC in any preparation except Debitrase DBP.20. Debitrase DBP.20, was used as a positive control, as it is known to contain high exopeptidase activity as shown in Table 1. Apart from Corolase PP and to a lesser degree pancreatin, there was no activity against Ala-AMC in any of the pancreatic preparations. As expected, the two crystalline proteinases, trypsin and chymotrypsin, showed least aminopeptidase activity; however, activity was observed against His-AMC and Leu-AMC in both preparations. While activity towards Arg-AMC was detected in both crystalline trypsin and chymotrypsin, it was eliminated by addition of trypsin inhibitor, TLCK. The activity against Arg-AMC was decreased to 70-75 % of total activity in all preparations in the presence of TLCK.

1.4.1.2 Carboxypeptidase activity

Carboxypeptidase A was present in the porcine preparations Corolase PP, PTN 3.0S, and pancreatin but was absent in the bovine preparations (Table 1). Carboxypeptidase B activity was detected in Corolase PP [1.74 units min^{-1} (mg

preparation)$^{-1}$] and pancreatin [14.76 units min^{-1} (mg preparation)$^{-1}$]. Carboxypeptidase A or B activity was not detected in Debitrase DBP.20.

1.4.1.3 Dipeptidase activity

Corolase PP was the only pancreatic preparation having dipeptidase activity for all four of the dipeptidase substrates tested (Table I). Pancreatin, Corolase PP and PTN 3.0S were the only pancreatic preparations showing Gly-Pro-AMC activity, which decreased in the order pancreatin > Corolase PP > PTN 3.0S. There was activity in all preparations towards Gly-Arg-AMC and Arg-Arg-AMC, but inhibitor studies using TLCK showed this activity to be largely due to trypsin (Table 1). Addition of TLCK reduced tryptic activity for Arg-Arg-AMC by ~ 33 % in the case of crystalline trypsin. Crystalline chymotrypsin had little or no dipeptidase activity. The dipeptidase activity of PTN 3.0S towards Gly-Arg-AMC or Arg-Arg-AMC was reduced by 41 and 30 %, respectively, in the presence of TLCK.

Debitrase DBP.20 had activity on all dipeptidase substrates tested; highest levels of activity were obtained with Gly-Pro-AMC as substrate [97.12 μmoles min^{-1} (mg protein)$^{-1}$].

1.4.1.4 Endoproteinase activity

Trypsin activity, measured by hydrolysis of N-benzoyl-L-Arg-AMC, for bovine crystalline trypsin and pancreatic proteases are given in Table 1. The specific activities (micromoles of AMC per minute per milligram of protein) for each of the preparations in order of decreasing magnitude were as follows: Trypsin > PTN 3.0S > Corolase PP > PEM 2500S > PEM 2700S > PEM 800S > pancreatin. Crystalline chymotrypsin showed no activity against this substrate as expected.

All of the commercial preparations tested showed chymotrypsin activity as measured by hydrolysis of N-Suc-Leu-Leu-Val-Tyr-AMC (Table 1). Specific activities (micromoles of AMC per minute per milligram of protein) in order of decreasing magnitude were as follows: PEM 800S > PEM 2500S > pancreatin > PEM 2700S > PTN 3.0S > Corolase PP. Crystalline bovine chymotrypsin showed the highest specific activity [555.56 μmolesAMC min^{-1} (mg protein)$^{-1}$], as expected. Low elastase activities were observed in PTN 3.0S, PEM 2500S and PEM 800S; however,

Table 1: Aminopeptidase activity (nmoles AMC/min/mg protein), carboxypeptidase activity (units/min/mg powder) and dipeptidase activity (nmoles AMC/min/ mg protein) and endopeptidase activities including trypsin, chymotrypsin and elastase activities (μmoles AMC/min/mg protein).

Enzyme preparation	Corolase PP	PTN 3.0S	Pancreatin	PEM2500S	PEM800S	PEM2700S	Debitrase	Trypsin	Chymotrypsin
Aminopeptidase substrates									
Ala-AMC	1764.00	0.00	9.49	0.00	0.00	0.00	501.25	0.00	0.00
Arg-AMC	2954.50	3705.85	1021.85	1056.90	616.23	1216.84	2976.20	28.57	30.00
Arg-AMC + TLCK	2090.00	3190.00	740.00	790.00	470.00	880.00	160.00	0.00	0.00
Asp-AMC	0.00	0.00	0.00	0.00	3.08	0.00	181.70	0.00	0.00
Gly-AMC	86.90	19.33	21.90	0.00	2.30	2.64	20.68	0.00	0.00
His-AMC	152.10	8.38	29.20	27.00	26.56	20.28	37.59	1238.10	34.79
Leu-AMC	2520.00	184.30	208.70	104.92	212.50	111.55	64223.00	38.10	330.55
Pro-AMC	34.76	61.87	70.05	12.34	12.40	7.10	25.06	7.62	6.09
Carboxypeptidase substrates									
hippuryl-L-phenyalanine	6.73	3.37	4.21	-	-	-	-	-	-
hippuryl-L-lysine	1.74	-	14.76	-	-	-	-	-	-
Dipeptidase substrates									
Arg-arg-AMC	9558.57	8700.70	6567.43	1974.94	2231.19	2433.68	4699.25	10952.38	34.79
Arg-arg-AMC + TLCK	6600.00	6090.00	4600.00	1380.00	1560.00	1700.00	3290.00	7670.00	20.00
Gly-arg-AMC	7038.58	5317.09	1824.28	1357.77	1460.90	1886.10	839.60	5142.86	26.10
Gly-arg-AMC +TLCK	1880.00	3130.00	240.00	600.00	320.00	610.00	530.00	100.00	0.00
Gly-pro-AMC	225.93	19.33	620.26	0.00	0.00	0.00	97117.79	0.00	0.00
Lys-ala-AMC	391.03	0.00	0.00	0.00	0.00	0.00	313.28	0.00	0.00
Endoproteinase substrates									
N-benzoyl-L-arg-AMC	58.65	70.89	6.75	51.84	15.67	41.27	0.00	89.05	0.00
N-suc-leu-leu-val-tyr-AMC	157.80	172.40	226.21	279.27	449.78	185.57	2.19	1.98	555.56
Acetyl-ala-ala-pro-ala-AMC	189.43	0.70	80.27	0.49	0.52	0.11	0.01	0.01	0.03

- not determinable (i.e activity values < 1nmole AMC/min/mg protein using flurogenic substrates or < 0.0001 unit/min/mg protein for carboxypeptidases)

Corolase PP and pancreatin displayed much higher elastase activities, having values of 187.43 and 80.27 μmoles AMC $min^{-1}(mg\ protein)^{-1}$, respectively, as measured by hydrolysis of acetyl-Ala-Ala-Pro-Ala-AMC.

1.4.2 Characterisation of Lactalbumin Hydrolysates

1.4.2.1 Degrees of hydrolysis

The hydrolysis curves (DH,%, vs time, min) obtained by incubation of lactalbumin with commercial proteases are shown in Figure 1. Debitrase DBP.20, as expected, had low activity on lactalbumin as seen from the low degree of hydrolysis obtained after incubation for 240 min. The curves obtained for crystalline chymotrypsin and trypsin began to plateau after ~ 60 min, at DH, 2.77 and 5.23 %, respectively, while the DH values after 240 min were 3.93 and 6.04 % respectively. The curve obtained for crystalline elastase indicated low activity in the early stages of the hydrolysis reaction, but activity continued to increase over the incubation period.

Protease preparations which contained more than one activity (Table 1) hydrolysed lactalbumin to higher DH % values than the crystalline preparations, as seen for PEM 2500S, PEM 800S, PEM 2700S, Corolase PP and pancreatin. The DH curves obtained for Corolase PP and Pancreatin were distinctly different from all other proteases and indicated high activity towards lactalbumin. Unlike the other preparations, Corolase PP and pancreatin continued to hydrolyse lactalbumin as incubation continued, giving DH values of 15.82 and 13.22 %, respectively after 240 min.

1.4.2.2 Nitrogen solubility of lactalbumin hydrolysates

Incubation of lactalbumin with bovine crystalline trypsin, chymotrypsin or pancreatin produced hydrolysates with high solubility at pH 6.6, i.e., ~ 90-100 % of total N (Table 2), while protease preparations such as PEM 2500S, PEM 800S and PEM 2700S produced hydrolysates that had similiar nitrogen solubility values, i.e, in the range 78-81 % of total N. The hydrolysate produced using Debitrase DBP.20 had a very low degree of solubility i.e., 0.15 % of total N.

Figure 1: Enzymatic hydrolysis of lactalbumin by commercial proteases as a function of time: (△) Corolase PP; (tilted square right solid) pancreatin; (□) PTN 3.0S; (♦) PEM 2500S; (▽) PEM 2700S; (◪) PEM 800S; (◊) trypsin; (O) chymotrypsin; (⊞) elastase; (⊕) Debitrase. Reaction conditions: substrate concentration; 8% (w/v); E:S, 0.003; pH 8.0; 50 °C; time 240 min.

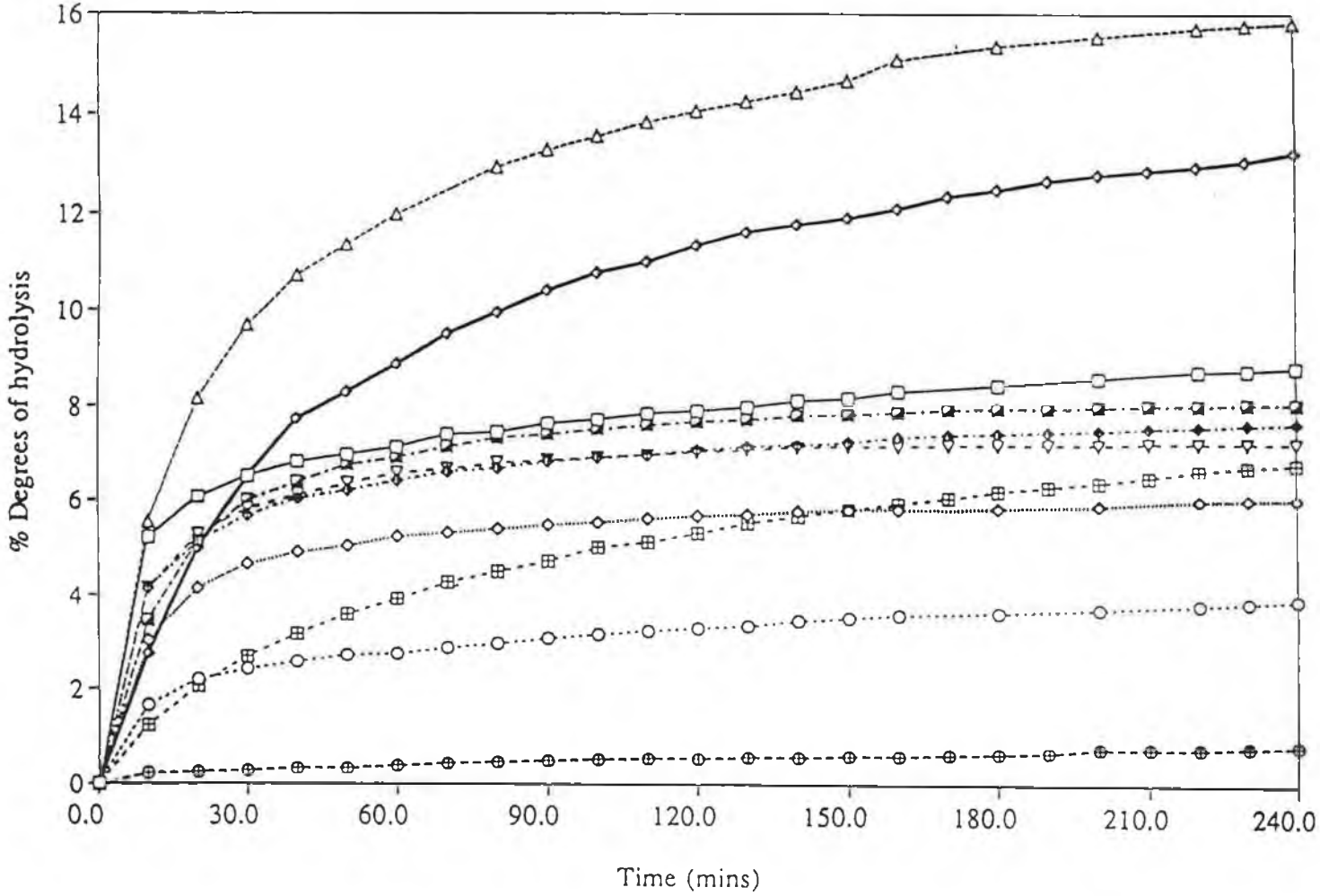


Table 2: Soluble nitrogen as percentage of total N (pH 6.6) and degree of hydrolysis (DH %) in the lactalbumin hydrolysates after incubation with commercial proteases for 20 min.

Protease	Soluble N	DH %
PTN 3.0S	89	8.83
Trypsin (bovine)	100	6.04
Chymotrypsin (bovine)	93	3.93
Elastase (porcine)	78	6.78
Corolase PP	81	15.82
PEM 2500S	78	7.66
Debitrase DBP.20	0.15	0.83
PEM 2700S	78.5	7.22
PEM800S	78.7	8.08
Pancreatin	100	13.22

1.4.2.3 Production of free amino acids

The concentration of amino acids in free solution produced by incubation of lactalbumin with commercial proteases are shown in Table 3. Concentrations less than 0.005 mg/ml were not included in the table. All the commercial pancreatic protease preparations produced hydrolysates with Lys, Arg, Ala and Leu in free solution with the exception of the hydrolysates produced by PTN 3.0S, which did not contain Lys, and PEM 800S, which did not contain Arg. The hydrolysates produced by Corolase PP, pancreatin, PEM 2500S and elastase contained Tyr and Ser free in solution. All of the hydrolysates with the exception of Debitrase DBP.20 are deficient in residues Asp and Glu and Gly. Tryptophan was not detected. Proline was not found free in solution for any of the hydrolysates examined. The elastase hydrolysate had Ala, Val, Leu, Phe, Ser and Tyr, in common with the hydrolysates produced by Corolase PP and pancreatin. Phenylalanine appeared to be produced only by Corolase PP, pancreatin and PEM 2500S. Both Corolase PP and pancreatin produced hydrolysates which contained relatively high levels of free amino acids. The amino acids present in hydrolysates produced from both of these proteases were lysine > leucine > arginine > tyrosine > phenalanine > valine > isoleucine > methionine > serine > histidine > alanine > threonine.

Debitrase, as an exopeptidase preparation, showed only limited hydrolysis of the lactalbumin substrate (Figure 1). However in the hydrolysate produced, all amino acids were found.

Table 3: Free Amino Acids (milligrams per gram of protein) present in Lactalbumin hydrolysates produced by commercial protease preparations

Amino Acid	Corolase PP	PTN 3.0S	Pancreatin	PEM 2500S	PEM800S	PEM2700S	Trypsin	Chymotrypsin	Elastase	Debitrase
Asp	-	-	-	-	-	-	-	-	-	0.09
Thr	1.11	-	1.49	-	-	-	-	-	-	0.76
Ser	2.33	-	2.80	0.11	-	-	-	-	0.08	0.63
Glu	-	-	-	-	-	-	-	-	-	0.46
Pro	-	-	-	-	-	-	-	-	-	-
Gly	-	-	-	-	-	-	-	-	-	0.07
Ala	1.38	0.29	2.04	0.28	0.30	0.02	0.30	0.30	0.29	1.01
Met	3.47	-	3.99	-	-	-	-	-	-	0.65
Val	8.82	-	9.45	-	-	-	-	-	0.79	0.73
Ileu	5.00	-	5.49	-	-	-	-	-	0.04	1.33
Leu	24.07	0.49	25.71	0.60	0.44	0.01	-	-	0.27	1.95
Tyr	12.30	-	17.80	0.62	0.22	-	-	-	0.56	-
Phe	10.74	-	12.25	0.27	-	-	-	-	0.72	0.37
His	2.01	-	2.17	-	-	0.07	0.63	-	0.40	0.40
Lys	38.86	6.37	30.07	5.05	1.31	0.17	2.06	0.09	1.76	0.66
Trp	-	-	-	-	-	-	-	-	-	-
Arg	22.87	0.12	22.60	1.07	-	0.12	-	-	-	-

a - not detected

1.4.2.4 Molecular size distribution of peptides in lactalbumin hydrolysates

The chromatograms obtained on Superose 12, assayed at 280 or 214nm, of lactalbumin hydrolysates are shown in Figures 2-4. The void volume (V_o) of the column corresponds to 2 x 10^6 Da, while the total column volume (V_t) corresponded to 174 Da. The molecular size distributions of peptides in hydrolysates produced by crystalline elastase or chymotrypsin (Figure 2 (a) and (b) respectively) were similiar, i.e., between 150,000 and 14,700 Da, while crystalline trypsin (Figure 2 (c)) produced a distinctly different profile, particularly in this region. There was a strong similiarity in the chromatograms of hydrolysates produced by PTN 3.0S, PEM 2500S, PEM 2700S and PEM 800S (Figure 3 and Figure 4 (a)). However, hydrolysates produced by Corolase PP or pancreatin (Figure 4 (b) and (c) respectively) were substantially different, showing increased peak area corresponding to low molecular weight peptides, and reduced peak area corresponding to V_o.

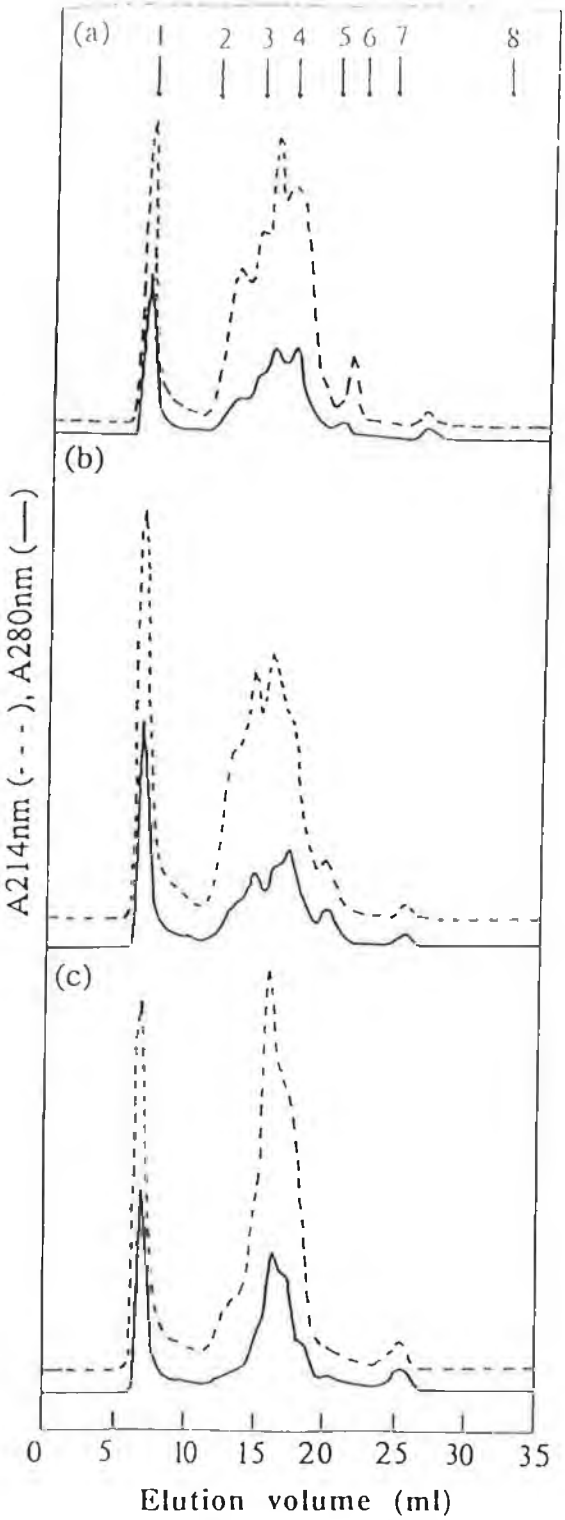


Figure 2: Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 nm (- - - -) of (a) elastase, (b) chymotrypsin, or (c) trypsin. Conditions: elution buffer (pH 7.0), 0.1 M Tris-HCl-0.1 M NaCl-10 % methanol; flowrate, 0.5 ml min^{-1}. (1) Blue dextran (2 000 000 Da); (2) immunoglobulin G (150 000); (3) β-lactoglobulin (36 000); (4) α-lactalbumin (14 700); (5) insulin chain B (3494); (6) L-arginine (174); (7) L-tyrosine (181); (8) DL-tryptophan (204).

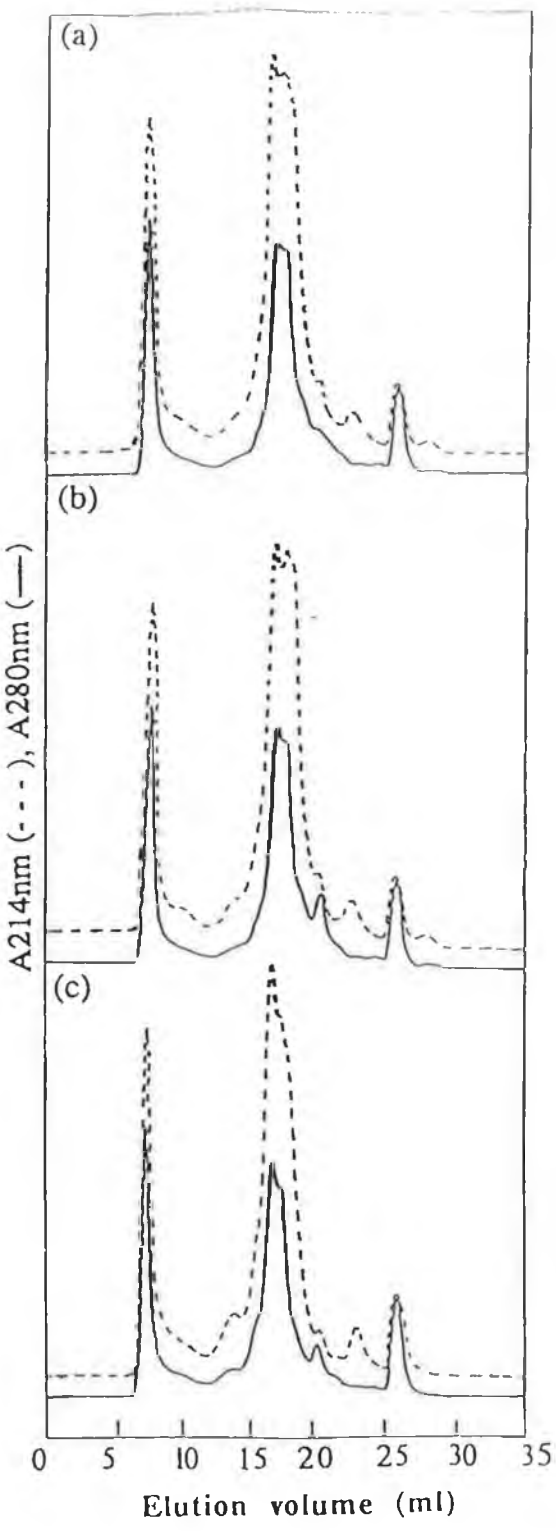


Figure 3: Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 nm (- - - -) of (a) PTN 3.0S,(b) PEM 2500S (c) PEM 2700S. Conditions as in Figure 2.

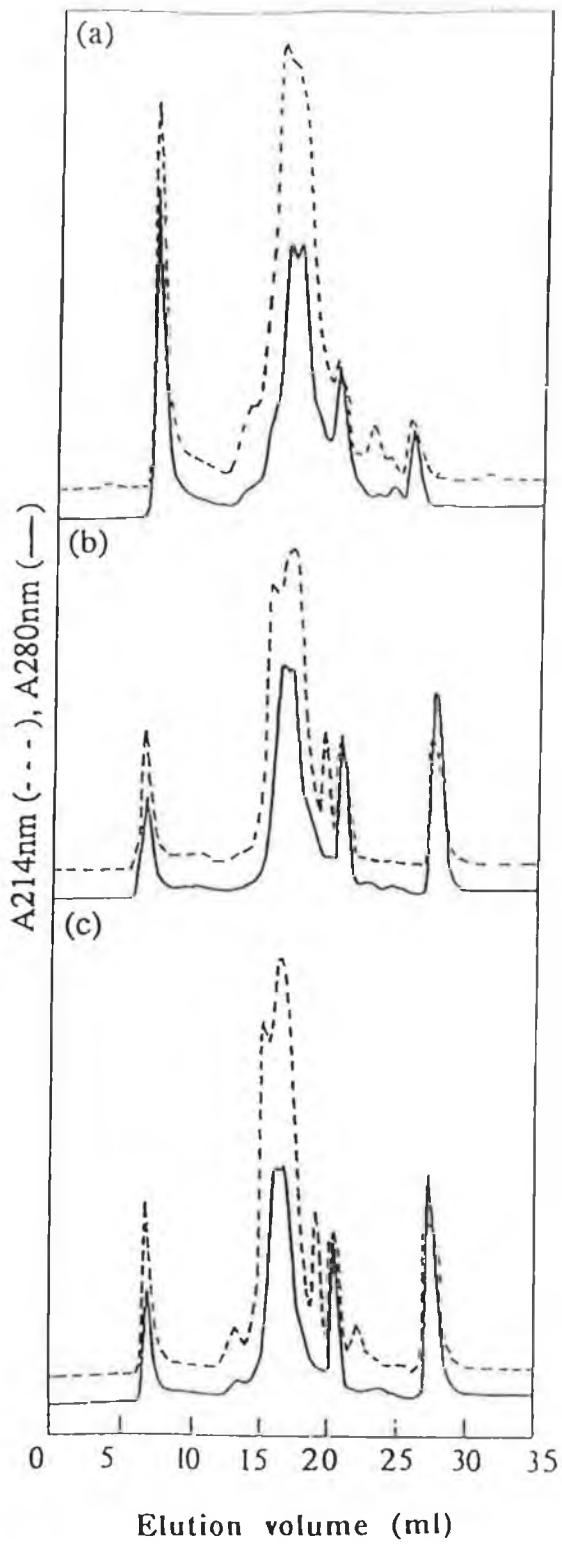


Figure 4 Chromatograms from FPLC Superose 12 column, assayed by absorbance at 280 (—) and 214 nm (- - - -) of (a) PEM 800S,(b) pancreatin (c) Corolase PP . Conditions as in Figure 2.

1.5 Discussion

There were low levels of aminopeptidase and dipeptidase activity in PEM 2500S, PTN 3.0S, PEM 800S and PEM 2700S, unlike Corolase PP and pancreatin which had higher levels of these activities. However, for all of the pancreatic preparations examined the ratios of the principal endoproteinases trypsin, chymotrypsin and elastase were different. Limited hydrolysis of the lactalbumin substrate resulted when Debitrase DBP.20 was used, as is seen by the low percentage degrees of hydrolysis; this is because it contains only aminopeptidase and dipeptidase activities (Figure 1). The influence the detected activities may have on hydrolysis of lactalbumin was determined by characterising hydrolysates obtained after incubation with individual protease preparations.

The ratio of proteinases in a pancreatic enzyme preparation is related not alone to the degree of purification but also to the source of pancreatic tissue (Marchis-Mouren, 1965). In this study, trypsin- like activity was highest in preparations of porcine origin, i.e. Corolase PP, PTN 3.0S and PEM 2500S, and may be attributed to the superior stability of porcine trypsin over the bovine counterpart (Buck *et al.*, 1962; Lazdunski and Delaage, 1965; Vithayathil *et al.*, 1961). PEM 2500S is sourced from porcine and bovine pancreas, although it is not clear at what stage of the purification that the two sources were mixed or if the bovine and porcine tissues were extracted separately and proteases then mixed in a defined ratio. In this study the bovine trypsin preparations hydrolysed lactalbumin to a lower degree than those preparations which contained porcine trypsin (Table 2 and Figure 1). The most obvious reason for this may be the better thermostability of porcine trypsin in comparison to bovine trypsin (Buck *et al.*, 1962), particularly in the presence of a whey protein substrate (Jost and Monti, 1977).

The low specific activity for chymotrypsin in Corolase PP, PTN 3.0S, and pancreatin, which are of porcine origin, may be due to the labile nature of the porcine chymotrypsins B and C during acidic extraction (Desnuelle and Rovery, 1961; Northrop *et al.*, 1948). Chymotrypsin also had activity towards Leu-AMC which may be attributed to the presence of chymotrypsin C in porcine preparations (Folk, 1970a). All preparations displayed arginine aminopeptidase activity, although TLCK inclusion had shown this to be largely associated with tryptic activity. There may, however, be a

specific arginine aminopeptidase associated solely with porcine preparations which would explain the high levels of arginine found in hydrolysates produced by such preparations (Table 3). The levels of tyrosine and phenylalanine in hydrolysates produced by pancreatic preparations could be attributed to carboxypeptidase A activity, particularly in preparations of porcine origin (Table 1 and Table 3) as this carboxypeptidase acts on the products of chymotryptic digestion (Guash *et al.*, 1992). Carboxypeptidase B acts on the products of trypsin digestion and therefore the high levels of arginine and lysine found in hydrolysates are not unexpected, particularly in hydrolysates produced by Corolase PP or pancreatin which had high levels of carboxypeptidase B activity (Table 1).

Similarities in the peptidase activities between Debitrase DBP.20 and the pancreatic preparations Corolase PP and pancreatin are evident both from activity analysis using fluorogenic substrates and also from the type and concentration of amino acids liberated in the hydrolysates produced by these preparations. Debitrase DBP.20, is a protease preparation from *Lactococcus lactis* and *Aspergillus oryzae* and had significantly more aminopeptidase activity than the pancreatic preparations (Table 1). The increase in area and number of peaks corresponding to peptides of low molecular weight in chromatograms of Corolase PP and pancreatin hydrolysates probably reflects the contribution of aminopeptidase, dipeptidase and elastase activities in these preparations (Figures 1 and 4).

Characteristic products of hydrolysis are obtained following treatment of lactalbumin with crystalline pancreatic enzymes. These characteristics are related to the specificities of the enzyme towards lactalbumin and can be seen in the degrees of hydrolysis (Figure 1), molecular weight profile of the peptides, within the range of 150,000-14,700 Da (Figure 2), and free amino acid profiles (Table 3). To maintain or improve functionality, generally low degrees of hydrolysis are necessary (Kilara, 1985). Trypsin is specific for lysine and arginine residues; therefore, characteristic peptides and concentration of amino acids are expected after a specific hydrolysis period. In the case of chymotrypsin and elastase, the existence of a number of families of each enzyme increases the range of specificities of these enzymes, as is evident from the existence of a number of peaks corresponding to peptides, within the same range of 150,000-14,700 Da, of intermediate chain length in the gel permeation profiles at 280

nm and 214 nm (Figure2 (a) and (b)). Trypsin, because of its narrow specificity, produces larger peptides than chymotrypsin, and these peptides are reported to have greater emulsifying properties (Jost and Monti, 1982; Turgeon *et al.*, 1991, 1992).

There were very distinct differences in molecular mass profiles of peptides in lactalbumin hydrolysates produced using Corolase PP or pancreatin (Figure 4 (b) and (c)) in comparison to the hydrolysates produced using PTN 3.0S, PEM 2500S, PEM 2700S and PEM 800S (Figure 3 and Figure 4 (a)). Generally, PTN 3.0S, PEM 2500S, PEM 2700S and PEM 800S produced hydrolysates of similar molecular mass profiles, and with low DH values, while Corolase PP and pancreatin gave more extensive hydrolysis.

Pancreatic preparations, PTN 3.0S, PEM 2500S, PEM 2700S, and PEM 800S, which contain only trypsin and chymotrypsin as the principal endoproteinase activity produce hydrolysates with products within a narrow molecular mass range (Figure 3 Figure 4 a) seen by lower percentage degrees of hydrolysis than that obtained with Corolase PP and pancreatin (Table 2). Interestingly, a similiar DH value (~ 8 %) was obtained after 240 min of hydrolysis for the enzymes PTN 3.0S, PEM 2500S, PEM 2700S and PEM 800S, eventhough the ratio of trypsin to chymotrypsin was different in each (Table 1). The lactalbumin hydrolysates produced with these preparations had very similar molecular mass profiles as determined by absorbance at 280 and 214 nm. The differences between preparations exist mainly in the molecular mass range of 150,000-36,000 Da. It appears that as the ratio of chymotrypsin to trypsin decreased, there was less breakdown of large molecular mass peptides such that the size of the peak no. 2 eluted within the molecular weight range of 150,000 Da is less substantial. Evidence of this can be seen when PTN 3.0S is compared with PEM 2500S, PEM 800S and PEM 2700S. This peak does not exist in PTN 3.0S, but as the ratio of chymotrypsin is increased, the peak becomes more prominent. This may be attributed to the broader specificity of chymotrypsin (Folk, 1970a), the low levels of exopeptidase activities (Table 1), or the presence of carboxypeptidase A or B in Corolase PP, pancreatin and PTN 3.0S. It is known that the levels of carboxypeptidases are higher in porcine tissues (Desnuelle and Rovery, 1961). The chromatograms of hydrolysates produced using PTN 3.0S, PEM 2500S and PEM 2700S were similar; this may be due to presence of only two endoproteolytic activities,

trypsin and chymotrypsin, with minimal elastase activity (Table 1). Thibault (1991) suggested that a specific ratio of trypsin to chymotrypsin was necessary for hydrolysis of whey proteins to achieve a particular FPLC molecular mass profile and reduction in antigenicity, while the individual enzymes did not produce the desired product characteristics. Kahn *et al.* (1991) also used a specific ratio of trypsin, chymotrypsin and porcine elastase to reduce the allergenicity of whey protein. Therefore, incubation of substrates with proteinases containing different ratios of endoproteolytic activities influences the hydrolysate characteristics.

There was no correlation between final degree of hydrolysis and solubility at pH 6.6 for the hydrolysates produced in this study. Elastase produced a hydrolysate that had a high solubility and was comparable to hydrolysates produced by commercial preparations containing mixtures of trypsin and chymotrypsin (Table 2). Preparations such as Corolase PP and pancreatin produced hydrolysates of higher solubility than hydrolysates produced by PTN 3.0S, PEM 2500S, PEM 2700S and PEM 800S. This may be explained by the presence of an additional endoproteolytic activity, elastase, and also to the additional exopeptidase activities in the former preparations. Debitrase DBP.20, being a preparation that had predominantly exopeptidase activities, produced a hydrolysate of low solubility.

The presence or absence of exopeptidases in commercial proteases may be of consequence for enzymatic hydrolysis of food proteins; for instance, in some applications, the level of free amino acids (Table 3) should be controlled so as to maintain low osmolality (Duke *et al.*, 1976) in the hydrolysate. Proteolytic preparations that contain exopeptidase activities produce less bitter hydrolysates than specific proteinases, such as trypsin (Clegg and McMillan, 1974; Cogan *et al.*, 1981; Vergarud and Langsrud, 1989; Moll, 1990). Interestingly, analysis of amino acids in free solution in the hydrolysates produced using pancreatic preparations (Table 3) did not reflect all of the aminopeptidase activities detected using fluorogenic substrates. The apparently low levels of free amino acids (Table 3) observed in hydrolysates from preparations containing exopeptidase activity (Table 1) may be due to the thermolability of the exopeptidases at 50 °C for 240 min. Furthermore, endoproteolytic digestion of lactalbumin may not result in peptides having N- or C-terminal sequences suitable for subsequent digestion by specific exopeptidases.

Pancreatin and Corolase PP, like Debitrase DBP.20, showed some activity towards the Gly-Pro-AMC substrate in addition to high activity for leucine aminopeptidase. Post proline dipeptidylaminopeptidase (PPDA), detected using Gly-Pro-AMC, has been used in synergy with leucine aminopeptidase (LAP) in the removal of bitterness from casein hydrolysates (Whitty, 1993). It could be suggested, therefore, that Corolase PP and pancreatin could have the capacity to produce hydrolysates of reduced bitterness as shown by other groups (Samuelsson and Poulsen, 1987; Lalasidas *et al.*, 1978; Chiang *et al.*, 1982). It may be that the cruder pancreatic preparations such as Corolase PP and Pancreatin retain some of the dipeptidase activity, as they may have not been subjected to multiple purification steps such as acid extraction, which is used in preparation of chymotrypsin (Northrop *et al.*, 1948). The TCA soluble hydrolysates produced by Corolase PP and pancreatin are high in hydrophobic amino acid residues, free in solution, such as valine, leucine and phenylalanine. The removal of these amino acids from peptides is often correlated with debittering of protein hydrolysates (Umetsu *et al.*, 1983).

From this study, it can be shown that depending on the number and ratio of activities within a protease preparation, that the molecular characteristics such as molecular mass of the peptides, DH values, and concentration of amino acids in hydrolysates, differ. As the range of enzymatic activities within commercial preparations is increased, the hydrolysate became more complex. The commercial proteases in this study can be classified into two distinct groups (Table 4). Group II proteases contain only trypsin and chymotrypsin, whereas Group I preparations contain exopeptidases, in addition to endoprotease activities.

Table 4: Commercial pancreatic protease preparations, principal activities, and potential uses.

	Commercial protease	Principle activities	Potential uses	References
Group I	Corolase PP Pancreatin	Trypsin Chymotrypsin Elastase Leucine aminopeptidase Diaminopeptidases	enteral feeds microbial growth media dietetic foods removal of bitterness	Lalasidas *et al*. (1978) Jost *et al.* (1988) (patent) Poulsen, 1987 (patent) Sameulsson and Poulsen, 1987 (patent)
Group II	PTN 3.0S PEM 2500S PEM 800S PEM 2700S	Trypsin Chymotrypsin	functional food ingredients dietetic foods hypoallergenic foods	Perea *et al*., 1993 Kahn *et al.*,1991 (patent) Thibault,1991 (patent).

CHAPTER 2

Zymogen activation in pancreatic endoproteolytic preparations and influence on some whey protein hydrolysate characteristics.[1]

[1] This chapter was published in the Journal of Food Science, (1995), 60 (2), 227-233.

2.1 Summary

A proteolytic preparation from porcine pancreas was isolated. Trypsin, chymotrypsin and elastase were characterised and their time-dependent stability at 37 °C was studied. The supernatant of a 30 % (w/v) saturated ammonium sulphate precipitation of a pancreatic extract (30S) was developed to pilot-scale level. The influence of zymogen activation time on molecular characteristics of whey protein hydrolysates produced by 30S and the commercial pancreatic preparation Corolase PP were compared. Amino acid analysis and gel permeation chromatography were used to characterise lactalbumin hydrolysates produced. Physicochemical characteristics of whey protein hydrolysates could be altered by manipulation of zymogen activation conditions in pancreatic proteinase preparations to be used during subsequent protein hydrolysis.

2.2 Introduction

Enzymatic modification of proteins using selected proteolytic enzyme preparations to split specific peptide bonds is widely used in the food industry (Godfrey and Reichelt, 1983; Adler-Nissen, 1986; Fox, 1991; Arai and Fujimaki, 1991). Whey proteins enzymatically hydrolysed with trypsin and chymotrypsin show better solubility and *in vitro* digestibility than their chemically treated counterparts (Lakkis and Villota, 1992). The choice of substrate, protease employed and degree to which the protein is hydrolysed affect physicochemical properties of resulting hydrolysates. By controlling reaction conditions during enzymatic hydrolysis of milk proteins, hydrolysates with different solubility and emulsifying characteristics (Turgeon *et al.*, 1992), foaming properties (Kuehler and Stine, 1974) or taste (Murray and Baker, 1952; Poulsen, 1987; Vegarud and Langsrud, 1989) have been produced.

The degree to which milk proteins are hydrolysed depends on the intended use for the hydrolysate. Low degrees of hydrolysis are desirable for maintaining functional properties. Extensive hydrolysis may be necessary when the hydrolysate is to be used for specific purpoese such as chemically defined or hypoallergenic infant formula (Merritt *et al.*, 1990; Thibault, 1991). Mixtures of proteases which have complementary specificities may be used to attain a required degree of hydrolysis. The number and ratio of proteolytic activities is critical to the final hydrolysate characteristics. The proportions of different proteinases, such as trypsin to chymotrypsin and the presence or absence of exopeptidases, such as carboxypeptidase B, may be important in the production of hypoallergenic hydrolysates (Thibault, 1991), or those with reduced bitterness (Fullbrook *et al.*, 1987; Plainer and Sproβler, 1990).

Little information has been published which relates critical ratios of proteinase/peptidase activity of commercial proteases used for hydrolysis of milk proteins to physicochemical characteristics of the final hydrolysate. Many groups have devised processes for production of milk protein hydrolysates using commercial pancreatic proteases, i.e. for clinical nutrition (Grimble and Silk, 1989; Maubois and Léonil, 1989), hypoallergenic and special dietetic products (Jost *et al.*, 1988; Asselin

et al., 1988, 1989; Thibault, 1991). However, the choice of enzyme preparation has usually been based on empirical screening or random selection.

Published methods for purification of pancreatic proteinases have mainly focused on the isolation of individual enzymes with elimination of other activities (Bergmann and Fruton, 1941; Northrop *et al.*, 1948; Lewis *et al.*, 1959; Balls, 1965). Purification, physical and chemical properties of both the zymogen, and active forms of pancreatic proteinases have been reviewed, i.e. trypsin (Northrop *et al.*, 1948; Desnuelle and Rovery, 1961; Hakim *et al.*, 1962; Vestling *et al.*, 1990), chymotrypsin (Wilcox, 1970), elastase (Hartley and Shotton, 1971; Gertler *et al.*, 1977). Limited studies have been published on the enrichment of a mixture of pancreatic enzymes where the activity of all enzymes was retained, or where a desired ratio of proteinases was required to mimic the *in vivo* digestive system. Prior to any pancreatic protease development, factors governing the specific activity of the proteolytic enzymes must be considered. Specific activities can be influenced by many factors. These include activation of one enzyme by another (Desnuelle and Rovery, 1961); autolysis/degradation (Vestling *et al.*, 1990); presence or absence of metal ions; hormonal activators in the tissue source (Padfield and Case, 1987), reverse feed-back mechanisms in diet (Dagorn and Lahaie, 1981) and species differences (Marchis-Mouren, 1965).

Our study considered stability (Vestling *et al.*, 1990; Vithayathil *et al.*, 1961; Buck *et al.*, 1962; Hakim *et al.*, 1962) and activation of the zymogen forms of proteolytic enzymes of porcine pancreas (Desnuelle and Rovery, 1961). This tissue was chosen for this study as it is known that porcine trypsin has better stability with respect to heat, influence of metal ions and pH changes (Buck *et al.*, 1962). Furthermore, porcine pancreatic proteases have similarities with pancreatic proteases of human origin (Clemente *et al.*, 1972; Gertler *et al.*, 1977; Largmann *et al.*, 1976).

Our objective was to develop methods for isolation and activation of porcine pancreatic proteolytic preparations. These preparations were compared with existing commercial proteases. Effects of the enzyme isolation and activation conditions on the development and associated physicochemical properties of whey protein hydrolysates were compared.

2.3 Materials & Methods

2.3.1 Materials

Commercial pancreatic proteases were gifts from manufacturers. Corolase PP (porcine) was supplied by Röhm GmbH, Darmstadt, Germany. PTN 3.0S (porcine) and PEM 2500S (porcine) were supplied by Novo Nordisk A/S, Bagsvaerd, Denmark. Bovine trypsin (Type XII) was obtained from Sigma Chemical Co., Poole, Dorset, UK.

Amino-methyl-coumarin (AMC) and the fluorogenic substrates for elastase, chymotrypsin and trypsin were, acetyl-ala-ala-pro-ala-AMC, N-succinyl-leu-leu-val-tyr-AMC and N-benzoyl-L-arg-AMC respectively, obtained from Bachem, Bubendorf, Switzerland. Lactalbumin (Alatal-560) was from New Zealand Dairy Board, Wellington, New Zealand. It is an insoluble heat denatured whey protein and its manufacture has been described (Robinson *et al.*, 1976; Mulvihill, 1992). The standard proteins and peptides for FPLC were from Sigma and were as follows: Blue dextran (2,000,000 Da), Immunoglobulin G (150,000), bovine serum albumin (67,500), β-lactoglobulin (36,000), α-lactalbumin (14,700), insulin chain B (3,494), DL-tryptophan (204), L-tyrosine (181), L-arginine (174). Reagents and standards for amino acid analysis were obtained from Beckman Instruments, High Wycombe, U.K. All other reagents were of analytical grade unless otherwise specified.

2.3.2 Preparation of pancreatic enzyme extracts

2.3.2.1 Extraction and ammonium sulphate fractionation

Initial steps in the method of Kunitz, as outlined by Laskowski (1956), were used and several modifications were made to account for scale-up. Fresh pancreas (5.25 kg) was from Cappoquin Meats Ltd. (Pork and Bacon Processors, Cappoquin, Co. Waterford) which was stored cold during transit to the laboratory and was then immersed in ice-cold 0.125 M H_2SO_4. Fat was trimmed from pancreatic tissue, which was chopped and then macerated in a Waring blender for 5 sec. The macerated tissue was suspended in 0.125 M H_2SO_4 (1:1 volume ratio of tissue to acid solution). Macerated tissue (≈5L Sample A) was obtained and this was stirred at 4 °C overnight

at 130 rpm in an equal volume of cold 0.125 M H_2SO_4. The suspension (10L) was filtered through one layer of synthetic cheese cloth and the filtrate (5.2 L) was collected. Residual tissue was further macerated, using a large-scale blender, for 3 min and was again stirred overnight in an equal volume of 0.125 M H_2SO_4. The suspension was centrifuged at 1900 x g (Mistral 6000, MSE Scientific Instruments, Crawley, West Sussex, U.K.) for 10 min at 0 °C and the supernatant was combined with the previous filtrate to give a total extract volume of 10.95 L (Sample B). A range of ammonium sulphate precipitations were carried out on 100-ml aliquots of this filtrate for initial studies. Saturated ammonium sulphate (SAS) solution was added to Sample B at 4 °C from a separating funnel at a flow rate of 40 ml / min, with continuous stirring. The mixture was allowed to stand for 30 min and then centrifuged for 20 min at 10,000 x g at 0 °C. The supernatant of the SAS fractionation was designated S (for example, 30S refers to the supernatant of the 30 % ammonium sulphate precipitation of Sample B).

2.3.2.2 Desalting and concentration of protein in 30S preparation

In a larger-scale experiment the supernatant (1000 ml) from the 30 % ammonium sulphate precipitation (30S) was vacuum-filtered on a Büchner funnel through Whatman no. 4 and no. 1 filter papers, consecutively, to remove suspended solids. An Amicon® (CH_2A Concentrator, Amicon Ltd., Stonehouse, Gloucestershire, U.K.) hollow-fiber ultrafiltration system was then used to desalt and concentrate the protein. Inlet pressure was maintained at ≈0.7 kg/cm^2 (10 psi) and the pump speed setting was maintained between 4-5. An Amicon® hollow-fiber membrane with a molecular weight cut-off of 30,000 Da and surface area of 0.06 m^2 was used (HIP 20-30 Serial no. 0032-1A120). A constant permeate flow rate of ≈7 ml / min was maintained during continuous diafiltration with 2.5 L distilled H_2O before the retentate was reduced to 200 ml (i.e. a volume concentration reduction = 5). Samples from the permeate and retentate streams (≈5 ml) were removed at intervals and assayed for trypsin activity. The degree of diafiltration was monitored using continuous conductivity measurements (Philips PW9509 digital conductivity meter, Pye Unicam Ltd., Cambridge, U.K.).

2.3.3 Activation of zymogens

Initial experiments were carried out on Sample B and 30S to determine the activation/de-activation rate of the principal endoproteinases. These fractions were buffered with 0.1 M Tris-HCl, pH 7.0, containing 100 mM $CaCl_2$ taking one volume protein solution and mixing with 9 volumes of Tris buffer. The protein suspension was adjusted to pH 7.0 and further diluted 1.45-fold using Tris buffer. Exogenous trypsin, Sigma type XII (1mg/ml), was then added at 2 % (v/v). A negative control was also used (i.e. sample without added trypsin). Subsamples (300 µl) of both extract fractions were taken at 1-h intervals, frozen immediately and later assayed for trypsin, chymotrypsin and elastase using a modification of the standard fluorogenic assay (Zimmerman *et al.*, 1977). To determine the effects of ammonium sulphate on elastase activity, a sample of 30S was desalted using a PD-10 Sephadex G25 column (Pharmacia LKB, Biotechnology Ltd., Uppsala, Sweden). Further studies were also carried out on desalted, concentrated 30S samples. Aliquots of these were activated, as above, for different times at 37 °C (4, 16, 20, 28, and 44 h), prior to freeze-drying. The enzymatic activity of trypsin, chymotrypsin and elastase was determined in 10 mg/ml solutions of each freeze-dried sample for the different activation times. A concentrated desalted 30S sample was activated for 4 h in the presence and absence of calcium chloride (30Snc) to determine the effects of Ca^{2+} ions on the activation/de-activation of the endoproteinases. Freeze-dried samples were stored in plastic containers at -20 °C.

2.3.4 Pretreatment of Corolase PP preparation

Before hydrolysis of lactalbumin with Corolase PP, aliquots of this preparation (10 mg powder/ml) were incubated at 37 °C for different time intervals (4, 16, 28 and 44 h).

2.3 5Quantification of enzyme activity

2.3.5.1 Specific activity

Enzyme activities were assayed using a modification of the standard fluorogenic assay (Zimmerman *et al.*, 1977). Aqueous solutions of commercial proteases and

freeze-dried 30S (10 mg powder/ml) in addition to pellets (redissolved to 100 ml) and supernatants (brought to 250 ml) from different SAS precipitations were centrifuged at 13,000 rpm (Microcentaur, MSE Scientific Instruments, West Sussex, U.K.) for 10 min. Twenty µl of supernatant was added to 980 µl of 0.01 M Tris-HCl buffer, pH 7.0, containing 0.02 mM specific substrate. The substrate-enzyme mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 ml of 1.5 M acetic acid and fluorescence was measured on a Perkin Elmer 1000 Spectrofluorimeter (Beaconsfield, Bucks, U.K.) at excitation and emission wavelengths of 360 nm and 440 nm, respectively. Activity units were defined as µmoles AMC/min/mg protein or µmoles AMC/min/total volume in each fraction. All enzymatic analysis was carried out in duplicate.

2.3.5.2 Total proteolytic activity

We used a modification of a previous method (Garcia de Fernando and Fox, 1991). Ten ml of 0.4 % (w/v) azocasein in 0.02 M Tris/HCl buffer pH 8.0 was added to 1 ml enzyme solutions (0.01-1.00 mg/ml i.e., sufficient to establish a linear activity response) and incubated at 50 °C for 15 min. The reaction was stopped by adding 2 ml 12 % (w/v) trichloroacetic acid (TCA) to an equal volume of the reaction mixture. The mixture was filtered through Whatman no.40 filter paper and the absorbance at 440 nm of the supernatant was measured using a Cary 1/3 UV-VIS Spectrometer (Varian Ltd., Mulgrave, Victoria, Australia). One unit of activity was defined as that amount of activity which gave a change of 1 absorbance unit at 440 nm/min/mg of enzyme preparation. For the ammonium sulphate precipitation experiments, endoproteolytic activity in the pellet and supernatants was expressed as a percentage of total activity in the starting material, i.e. Sample B.

2.3.6 Preparation of hydrolysates

A 25-ml solution of lactalbumin, 8 % (w/v) protein, was hydrolyzed with individual protease preparations at 50 °C, pH 8.0, for 240 min. The pH was maintained constant by continuous addition of 0.5 M NaOH using a pH-stat (Metrohm Ltd., Herisau, Switzerland). The degree of hydrolysis (DH, %), defined as the

percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Following hydrolysis, proteases were inactivated by heating at 80 °C for 30 min, hydrolysates were then cooled and stored at -20°C for further analysis. The DH was calculated as follows:

$$DH, \% = B \times M_b \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100$$

where B = volume of NaOH consumed (ml); M_b = Molarity of NaOH ; α=average degree of dissociation of the α-NH_2 groups at pH 8.0 and 50 °C; MP = mass of protein (g); h_{tot} = total number of peptide bonds in the protein substrate (mequiv/g protein). The h_{tot} value, 8.8 mequiv/g for whey protein, and values of $1/\alpha$ for various pH-temperature combinations were those given by Adler-Nissen (1986). The enzyme-to-substrate ratio (E:S) for lactalbumin hydrolysis experiments was calculated on the basis of (1) weight of enzyme preparation to weight of protein in the substrate, (2) total proteolytic activity units in the enzymatic preparation to weight of protein in the substrate, (3) tryptic activity (μmoles AMC/min/mg protein) in enzyme preparation to weight of protein in the substrate, or (4) protein in enzyme preparation to weight of protein in the substrate (Table 3).

2.3.7 Characterisation of hydrolysates

All protein analysis, molecular size distribution and amino acid analyses were carried out in duplicate.

2.3.7.1 Protein (nitrogen) determination

Total protein (N x 6.25) of pancreatic preparations and of whey protein (N x 6.38) was determined by the micro-Kjeldahl method (AOAC, 1980).

2.3.7.2 Molecular size distribution of peptides in lactalbumin hydrolysates

A fast protein liquid chromatograph (FPLC) fitted with a Superose 12 gel permeation column (Pharmacia LKB Biotechnology Ltd., Uppsala, Sweden.) was used to monitor the size distribution of peptides in lactalbumin hydrolysates. The column was eluted at 1 ml/min using 0.1 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl and 10 % (v/v) methanol. Hydrolysates were diluted in elution buffer to 0.25 % (w/v)

protein, filtered through a Whatman 0.45 μm PS syringe filter and 100 μl was applied to the column in duplicate. Eluate was continually monitored at 214 or 280 nm using two single-path monitors (UV-1, Pharmacia) and data were recorded using a Minichrom© data handling system (VG, Data Systems, Altrincham, Cheshire, U.K.). A molecular weight calibration curve was prepared from the average retention volumes (Rv) of standard proteins and peptides.

2.3.7.3 Free amino acid analysis of hydrolysates

Hydrolysates were deproteinized by mixing equal volumes of 24 % (w/v) TCA with sample and holding at room temperature for 30 min before centrifuging at 14,400 x g (Microcentaur, MSE Scientific Instruments, Crawley, West Sussex, U.K.) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2, to give ≈25 nmoles of each amino acid residue per 50 μl injection volume. Amino acids were analyzed on a 12 cm x 4 mm cation exchange column (Na^+ form) using a Beckman 6300 amino acid analyzer (Beckman Instruments Ltd., High Wycombe, U.K.). Standard amino acids were used to calibrate the analyzer.

2.4 Results and Discussion

Although porcine trypsin is more thermostable than bovine trypsin (Lazdunski and Delaage, 1965), both are subject to autolysis. Porcine trypsin is denatured at 55 °C, but it has been shown that, in the presence of whey protein as substrate, that the enzyme retains >70 % activity after 1 h at 55 °C (Buck *et al.*, 1962; Jost and Monti, 1977). Trypsin is important, not only for its intrinsic activity but also in its activation of the other zymogens in the pancreas (Desnuelle and Rovery, 1961; Peanasky *et al.*, 1969) and control of its activation can determine the extent of hydrolysis by the pancreatic proteases. Chymotrypsin and elastase can also act as substrates for themselves and for each other. In addition, each pancreatic proteinase can again have different active forms which relates to the activation procedure employed (Wilcox, 1970; Puigserver *et al.*, 1986; Vestling *et al.*, 1990; Guasch *et al.*, 1992; Martínez *et al.*, 1981). With a selected ratio of endoproteolytic activities during the activation

process, in the presence of substrate, (i.e., whey protein) such activities can be stabilised as the protein substrate will act as a protective agent (Jost and Monti, 1977).

2.4.1 Ammonium sulphate fractionation of pancreatic endoproteinases

At 35 % SAS almost 90 % trypsin, 20 % chymotrypsin and > 40 % elastase original activities remain in solution (Table 1). Preliminary experiments showed that only proteolytic solutions with conductivity values less than that corresponding to a 1 % ammonium sulfate solution would freeze-dry efficiently. The recovery of freeze-dried 30S proteolytic preparation was ~ 84g/kg of pancreatic tissue. Trypsin activity was used as an index of activation. When the 30S preparation (10 mg/ml powder) was activated for 4 h, it had 24 times less trypsin activity (μmoles/min/mg protein) than the commercial preparation PTN 3.0S (50.4 μmoles/min/mg protein).

2.4.2 Activation of pancreatic zymogens

The stability of trypsin, chymotrypsin and elastase, in addition to possible exopeptidase activities, appeared to be dependent on the duration of the activation procedure and factors such as storage conditions of the active product. Storage of the zymogen form of any of the proteolytic mixtures in 0.01 M Tris-HCl buffer, pH 7.0, containing 100 mM $CaCl_2$ for 2 days at 4 °C resulted in an increase in trypsin and chymotrypsin activity. However, after incubation at 4 °C over 5-6 days in the same buffer, a decrease in activity of these enzymes was observed. Activation of both trypsinogen and chymotrypsinogen appeared to be enhanced following freezing and thawing at -20 °C. Possibly freeze-thaw action may disrupt cells/granules resulting in release of zymogen form which is then activated or, alternatively, freezing may favour the formation of a specific optical rotatory form which would speed up the activation process (Walsh, 1970; Keil, 1971). We observed that holding extract fraction solutions at 37 °C for 24 h resulted in decreased activity for elastase and chymotrypsin.

In our study, the activation of porcine proteolytic preparations was not dependent on the provision of Ca^{2+} ions. The activation effect of calcium is less pronounced in porcine trypsin then in bovine trypsin (Lazdunski and Delaage, 1965; Abita *et al.*, 1969). Interestingly, 30S preparation activated in the absence of calcium (30Snc) had a

slightly higher DH value at 240 min than the sample activated with these cations (Table 2), though the lactalbumin hydrolysis curves were similar (Figure 2). The 30Snc also produced a hydrolysate with more peptides in the < 570 Da molecular mass range. Though trypsin activity levels in both preparations were the same, inclusion of Ca^{2+} ions in the activation process may influence the formation of certain types of active enzyme products (Abita *et al.*, 1969; Smith and Shaw, 1969; Vestling *et al.*, 1990).

Table 1: Activity[a] of trypsin, chymotrypsin and elastase in supernatants of ammonium sulphate purification fractions, expressed as percentage of total activity in the starting material (Sample B).

Purification Fractions	Total activity (%)		
	Trypsin	Chymotrypsin	Elastase
Sample B	100.00	100.00	100.00
20S	[b] -	70.30	66.70
30S	95.00	33.70	37.80
30S (desalted)	-	-	37.60
35S	87.70	20.00	41.70
40S	73.60	19.70	0.00
30P[c]	-	-	47.40

a using fluorogenic assays as described in Materials and methods;
b not determined;
c P refers to pellet fraction.

Table 2: Total proteolytic activity, final DH (after 240 min), and E:S ratio for lactalbumin hydrolysis experiments using commercial pancreatic preparations, pretreated Corolase PP and pancreatic extracts after different activation times.

Proteolytic samples	Total proteolytic activity (units/min/mg preparation)	Activation time (h)	Pretreatment time (h)	E:S ratio[a]	Final DH (%)
PTN 3.0S	0.16	-	0	* [c]	8.83
PEM2500S	0.90	-	0	*	8.57
CorolasePP	0.15	-	0	*	13.83
CorolasePP	-	-	4	0.02	13.11
CorolasePP	-	-	16	0.02	10.73
CorolasePP	-	-	28	0.02	9.82
CorolasePP	-	-	44	0.02	6.08
Pancreatic extracts					
Sample B	-	4	0	0.20	4.09
30S nc [b]	-	4	0	0.02	6.31
30S	-	4	0	0.02	5.81
30S	-	16	0	0.02	8.44
30S	-	28	0	0.02	7.47
30S	-	44	0	0.02	7.61

[a] E:S ratio calculated on the basis of weight of preparation to protein in the substrate.

[b] 30S nc - preparation activated in the absence of calcium ions

[c] E:S ratio was on the basis of total proteolytic activity units to protein in the substrate (i.e. 0.16 Azocasein units/g protein).

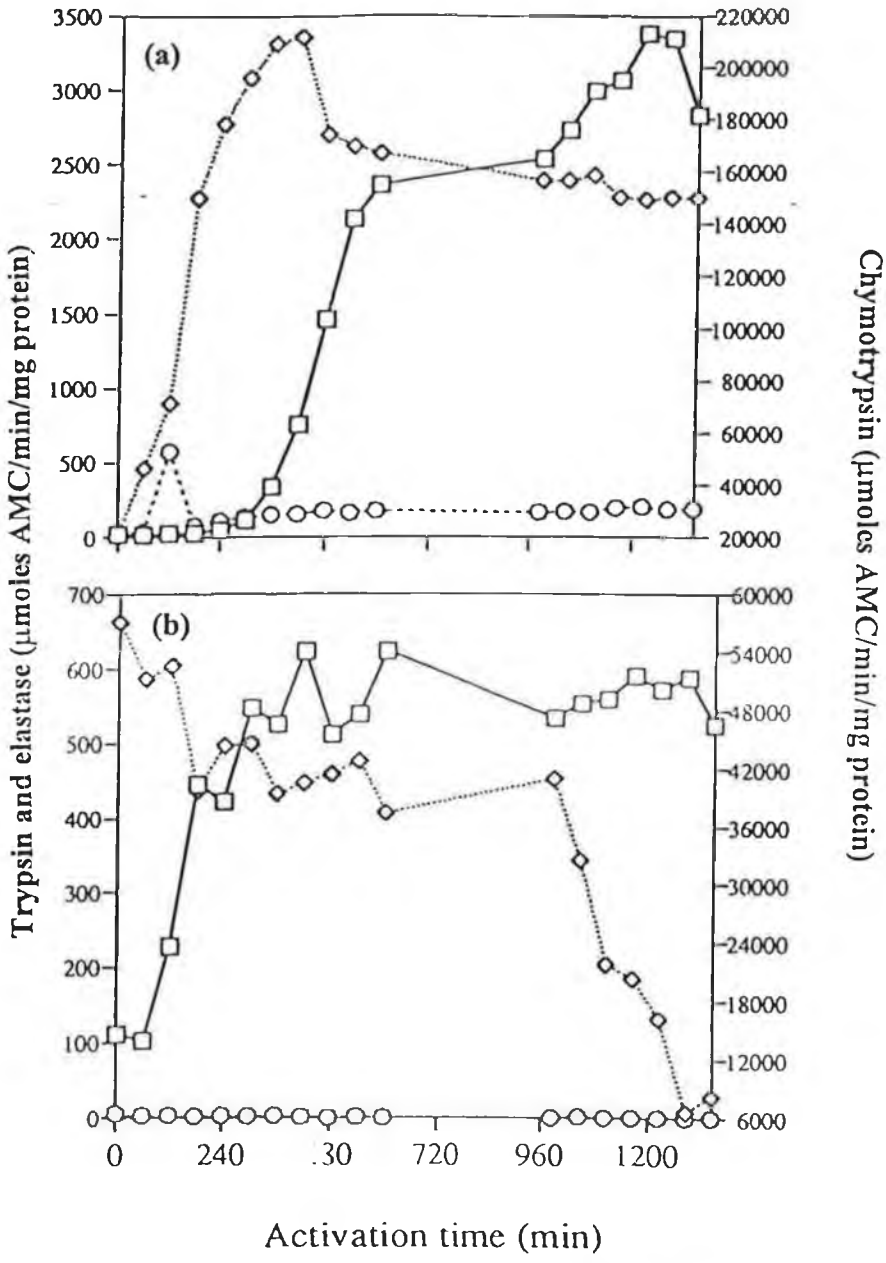
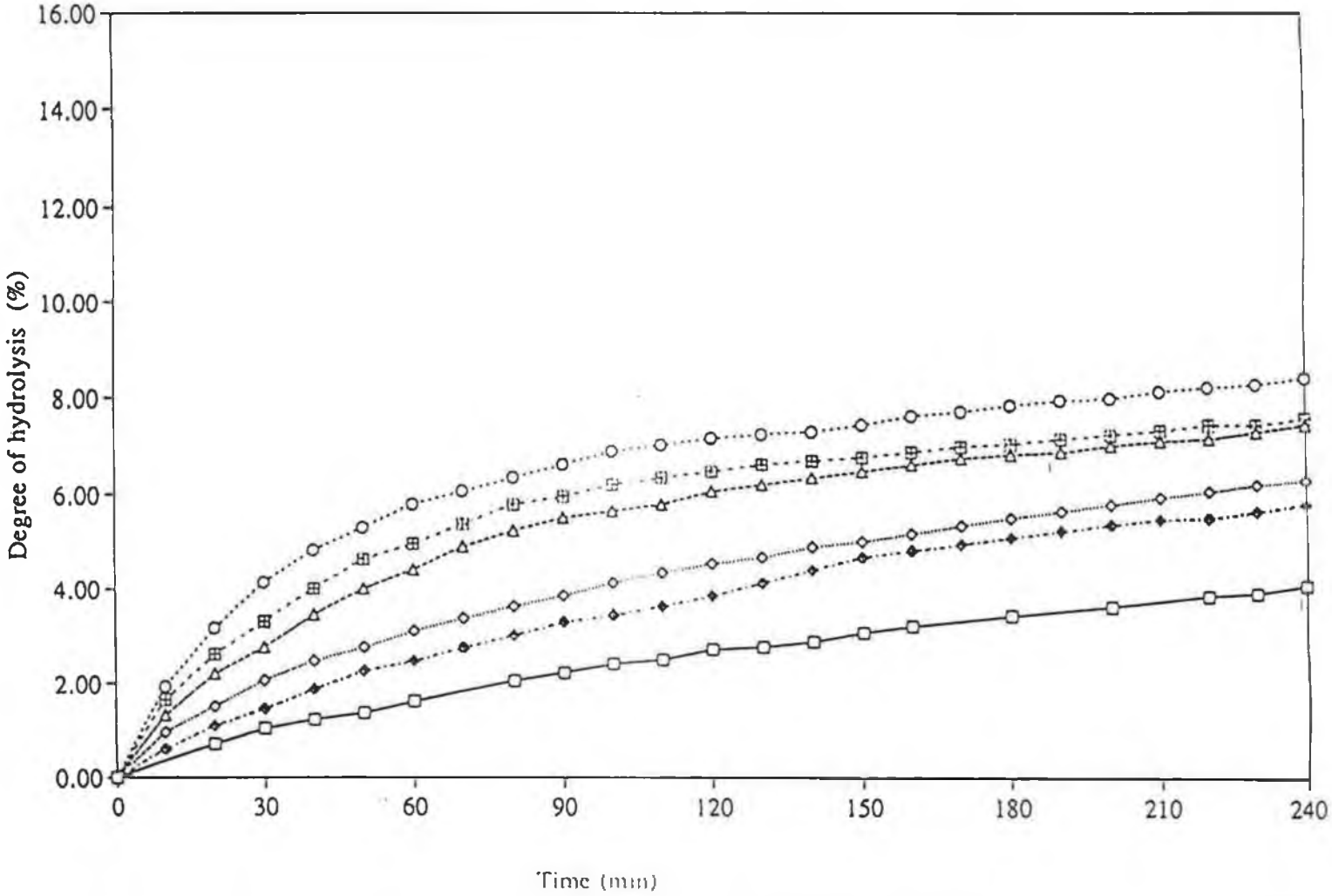


Figure 1: Specific activity of trypsin —□— , chymotrypsin ·····◇····· , and elastase ----○---- , using fluorogenic substrates (µmoles AMC/min/mg protein) in the extraction fractions, Sample B (a) and the dilute form of 30S (b) over time at pH 7.0 and 37 °C.

Activation of pancreatic proteinases was independent of exogenous trypsin addition. However, activation was slower if not initiated by trypsin, as shown previously (Desnuelle and Rovery, 1961). During activation, time-dependent changes occurred in the ratios of pancreatic endoproteinases in Sample B and 30S during 37 °C incubation (Figure 1 (a) and (b)). Each proteinase activity reached a maximum at a different times. In sample B, trypsin activity started to increase in a linear fashion after 300 min incubation, reaching a maximal activity after 1220 min incubation. In the absence of exogenous trypsin, however, this was delayed by 125 min. In sample B, the ratio of trypsin to chymotrypsin and elastase after 1220 min incubation was in the order of 17:740:1 μmole AMC/min/mg protein. After that time, the trypsin activity began to decrease. Chymotrypsin activity reached a maximum after 240 min after which the activity started to decrease reaching 149,235 μmoles /min/mg protein at 1225 min. Elastase activity appeared to remain constant after 245 min. The activity of the endoproteinases differed greatly in the dilute solution of SAS, i.e. 30S (Figure 1 (b)) from that in the crude sample B (Figure 1 (a)). Trypsin activity increased and reached a maximum at ~ 650 units after 600 min and remained stable close to this level over an extended period. Chymotrypsin activity was very unstable during 37 °C activation of the 30S fraction. The activity decreased rapidly from the beginning of the process. After 600 min the endoproteinase activity ratios were as follows: trypsin : chymotrypsin : elastase (625 : 38,000 : 1). Minimal elastase activity was detected.

Chymotrypsin and elastase appeared to autolyse or deactivate at a faster rate than trypsin (Figure 1 (b)) The low levels of elatase (Figure 1 (b)) could be attributed to dilution of stabilizing influences which may prevent autolysis. Ammonium sulphate and other salts are known to inactivate elastase activity (Lewis *et al.*, 1959; Lamy *et al.*, 1961; Gertler and Birk, 1970). In our study, removal of ammonium sulphate by desalting had no effect on elastase activity (Table 1); however, deactivation of elastase activity may have occurred prior to desalting.

Figure 2: Time course hydrolysis of lactalbumin by extraction fraction Sample B and 30S, at different activation levels; Sample B (4 h) —□— ; 30S (4 h)···◆·· ; 30S (16 h)···○···; 30S (28 h) ----△---- ; 30S (44 h) ---⊞--- and 30S nc (4 h) ·····◇····· ,(activated in the absence of Ca^{2+} ions). Reaction conditions: 8 % (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50 °C.



A concentrated, desalted 30S fraction was used for further study on the effects of activation time on endoproteolytic activities. By extension of activation time, the ratios of endoproteinases was changed (Figure 1 and Table 3). Furthermore, the hydrolysis curves (Figure 2) and the products (Table 4 and 5) from these proteolytic preparations used in the hydrolysis of lactalbumin were different for each activation time (Table 4).

2.4.3 Characterisation of Lactalbumin hydrolysates

2.4.3.1 Final percentage degrees of hydrolysis and molecular distribution

The hydrolysis curves obtained on incubation of lactalbumin with sample B (Figure 2) were compared with those using concentrated desalted and activated 30S freeze-dried preparations (Figure 2) and commercial proteases (Figure 3 and 4) under different conditions. The molecular weight distribution data (Table 4) are averages for two elutions of a given hydrolysate. Peak areas for a specific molecular weight range were expressed as percentage of total peak area obtained at 280 nm.

The 30S extract had more activity on lactalbumin than sample B as seen from the higher degree of hydrolysis obtained after incubation for 240 min (Figure 2 and Table 2). Sample B hydrolysate showed a low DH value after 240 min as compared to 30S. However, the E:S ratio for sample B used in hydrolysis was calculated on the basis of protein, whereas the other preparations were used on the basis of weight of powder. Sample B hydrolysates displayed large peak areas corresponding to peptides within the molecular mass ranges of 13, 300-570 Da (28.3 %). Less material was produced in the < 570 Da region. In comparison to the cruder Sample B profile, the 30Snc activated for 4 h showed a reduction in peak area in the 13, 000-570 Da range and an increase in peak area corresponding to < 570 Da region. The peptide distributions for 30S and 30Snc were different. Considerably less peptides occurred in the 13,000-570 Da molecular mass region for 30Snc than for 30S.

Table 3: Proteolytic activity (μmoles AMC/min/mg protein) after different activation times for concentrated, desalted 30S preparation.

	Trypsin	Chymotrypsin	Elastase
Activation time (h)	μmoles AMC/min/mg protein		
0[a]	-	-	-
4	2.10	879.63	0.30
16	39.90	1008.23	0.41
28	52.80	1440.33	0.51
44	84.90	1152.26	0.31

[a] In absence of activation, no endoproteolytic activity was detected.

Table 4: Molecular weight distribution (%)[a] of protein components in lactalbumin hydrolysates prepared using commcercial pancreatic protease preparations and pancreatic extracts activated for different times, E:S ratio as given in Table 2.

		Molecular mass (Da)		
		29,200-13,300	13,300-570	< 570
Commercial preparation	Pretreatment time (h)			
Corolase PP	0	0.26 ± 0.07[b]	6.98 ± 0.67	92.90 ± 0.12
Corolase PP	4	0.68 ± 0.29	17.75 ± 0.10	81.52 ± 0.09
Corolase PP	16	0.94 ± 0.05	21.79 ± 0.36	77.10 ± 0.20
Corolase PP	28	0.58 ± 0.36	24.88 ± 0.03	74.40 ± 0.09
Corolase PP	44	0.38 ± 0.00	32.54 ± 0.14	67.10 ± 0.82
PTN 3.0S	0	0.01 ± 0.01	25.00 ± 1.42	75.01 ± 0.20
PEM 2500S	0	0.00 ± 0.00	23.46 ± 0.22	76.43 ± 0.22
Pancreatic extracts	Activation time (h)			
Sample B	4	0.01 ± 0.01	28.32 ± 0.53	76.13 ± 2.16
30S nc [c]	4	0.01 ± 0.01	15.70 ± 0.01	83.92 ± 0.03
30S TPA	4	0.04 ± 0.01	1.45 ± 0.12	98.12 ± 0.05
30S	4	0.00 ± 0.00	26.95 ± 0.30	72.77 ± 0.11
30S	16	0.00 ± 0.00	22.52 ± 0.16	77.18 ± 0.33
30S	28	0.05 ± 0.05	20.21 ± 0.83	79.75 ± 0.22
30S	44	0.00 ± 0.05	10.01 ± 0.09	89.99 ± 0.56

[a] Integrated peak areas between defined elution volumes corresponding to particular molecular weight distribution was calculated using Minichrom.

[b] Mean value from 2 repetitions +/- standard deviation

[c] Proteolytic extract activated in the absence of calcium ions.

Figure 3: Time course hydrolysis of lactalbumin by Corolase PP preparations pretreated at different times; 0 h —□— ; 4 h--△--;16 h ···○··· ; 28 h -·◇·· ; 44 h - - -⊞- - -(indicated in Table 2). Reaction conditions: 8 % (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50 °C.

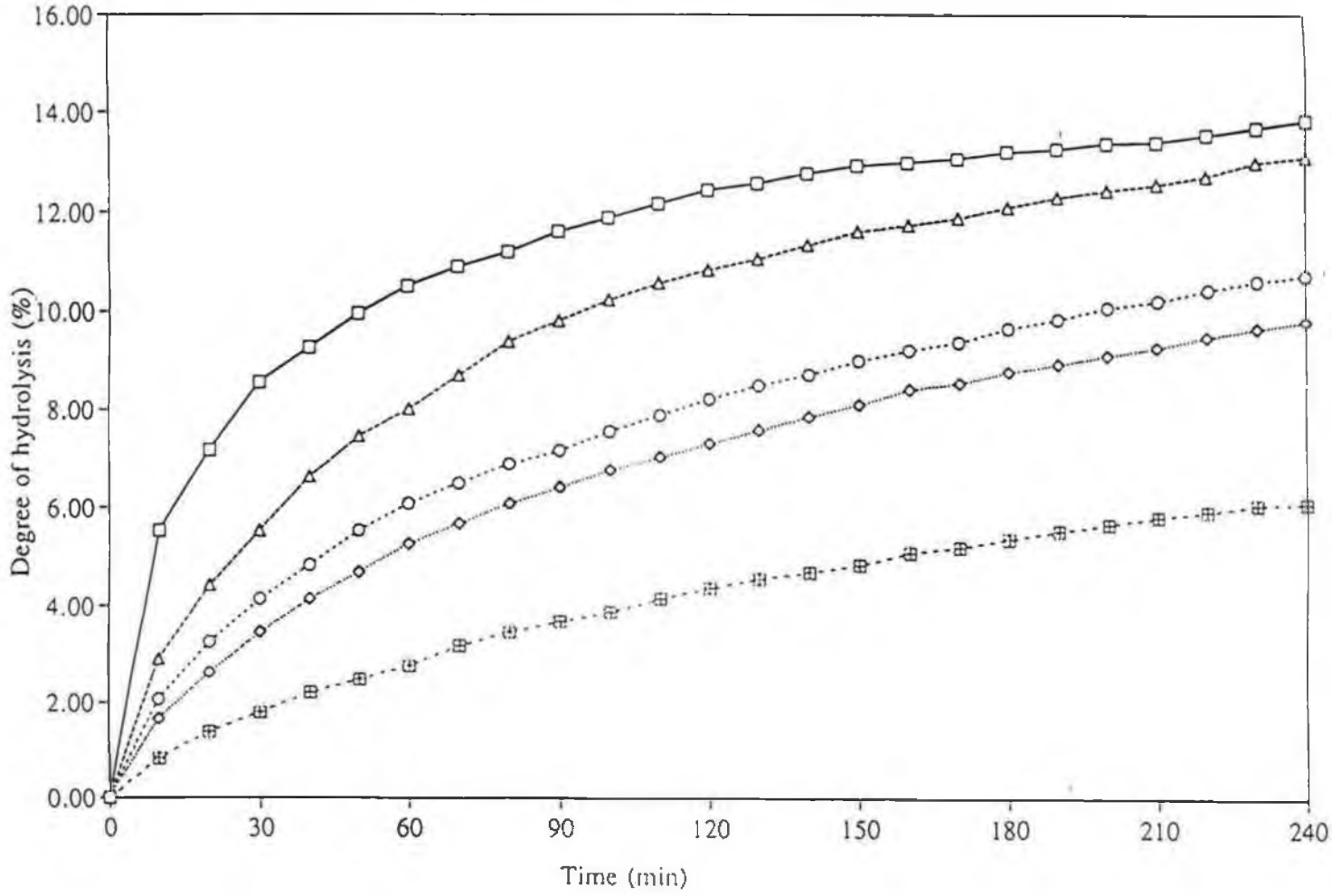
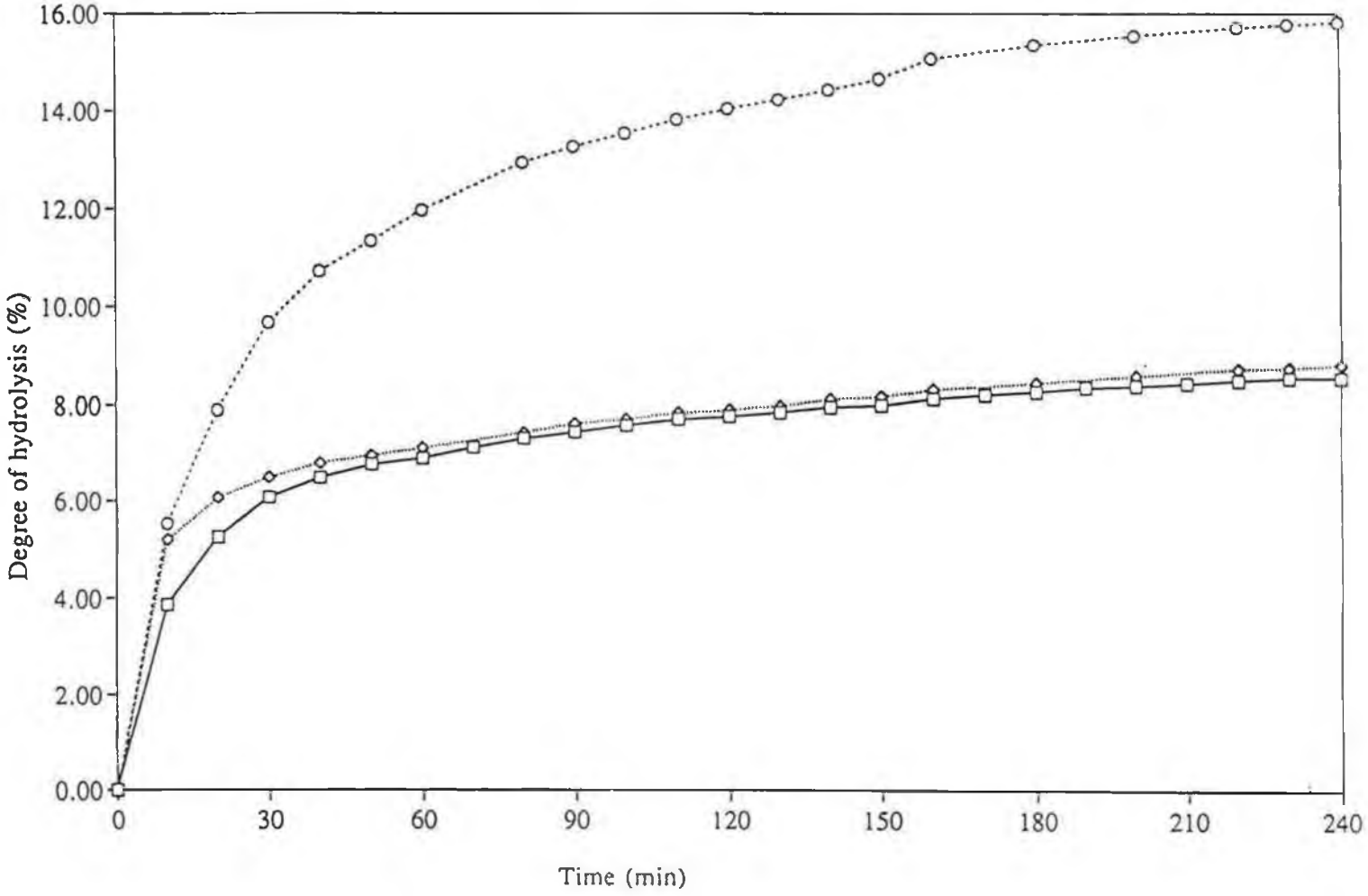


Figure 4: Time course hydrolysis of lactalbumin by commercial preparations without pretreatment Corolase PP ····○····;PTN 3.0S ···◇···;PEM 2500S —□— ; (indicated in Table 2), as a function of time. Reaction conditions: 8 % (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50 °C.



We studied different activation times as a means of producing a specific ratio of endoproteinases. Hydrolysates produced using these preparations revealed significant changes in molecular characteristics, i.e. with respect to DH (Figure 2), molecular weight distribution of peptides (Table 4), and levels of free amino acids generated (Table 5). As activation time was increased the final DH value increased up to 16 h (Table 2). At longer activation times, i.e. 28 and 44 h, the final DH value was decreased. When the 30S activation times were increased, i.e., 16, 28 and 44 h, a corresponding increase in hydrolytic activity occurred (Table 3). Note the small reduction of peak area within the 13,300-570 Da range (Table 4). The high final DH value of 30S (16h) was apparently contradictory to other data (Table 4). The preparations 30S (28 h) and 30S (44 h), which had lower final % DH values, had higher hydrolytic activity in the 13,300-570 Da range. This may indicate the formation of different active forms of proteinases during activation of those preparations (Vestling *et al.*, 1990).

Similarities occurred between 30S (16 h), PEM 2500S and PTN 3.0S as seen from the molecular weight profiles of peptides and hydrolysis curves produced by those preparations (Table 4, Figure 2 and 4, respectively). This may be due to the presence of two main endoproteolytic activities, i.e. trypsin and chymotrypsin, in 30S (16 h) (Table 3), in PEM 2500S and PTN 3.0S (Mullally *et al.*, 1994). This was confirmed by the low concentrations of free amino acids in hydrolysates produced by those preparations (Table 5 and 6). PEM 2500S and PTN 3.0S appeared to have similar activity against lactalbumin (Figure 4) on the basis of total proteolytic activity (Table 2). The curves for the treatment of lactalbumin by PTN 3.0S and PEM 2500S began to plateau after 40-50 min, while the DH values after 240 min were 8.83 and 8.57 %, respectively. The 30S purification sample was presumably approaching the ratios of proteinases in commercial preparations when the zymogen activation time was increased to 16 h, (as indicated by very similar final DH values). The final DH value of 30S sample activated for 16 h was 8.44 % (Table 2).

Table 5: Free amino acids (mg/g protein) in lactalbumin hydrolysates prepared using pancreatic extracts activated for different times.

Amino acid	Sample B	30S nc [b]	30S	30S	30S	30S
	Activation time (h)					
	4	4	4	16	28	44
Asp	[a] -	-	-	-	-	-
Thr	-	-	-	-	-	-
Ser	-	-	-	-	0.15	-
Glu	-	-	-	-	-	-
Pro	-	-	-	-	-	-
Gly	-	-	-	-	-	-
Ala	0.36	0.36	0.34	0.35	0.37	0.32
Met	-	-	-	-	-	-
Val	-	-	-	-	0.10	-
Ile	-	-	-	-	-	-
Leu	-	-	-	0.13	0.14	0.08
Tyr	-	-	-	-	-	-
Phe	-	-	-	-	-	-
His	-	-	-	-	-	-
Lys	-	-	-	1.20	0.59	0.86
Trp	-	-	-	-	-	-
Arg	-	-	-	-	-	-

[a] not detected; [b] 30S nc = 30S preparation activated without calcium ions

Table 6: Free amino acids (mg/g protein) in lactalbumin hydrolysates prepared using commercial preparations and pretreated Corolase PP

Amino acid	PTN 3.0S	PEM 2500S	Corolase PP				
			Pretreatment time (h)				
			0	4	16	28	44
Asp	[a] -	-	0.47	0.43	0.37	0.33	0.23
Thr	-	-	1.08	1.01	0.58	0.49	0.29
Ser	-	-	1.86	2.21	1.18	1.04	0.63
Glu	-	-	-	0.02	0.12	0.10	-
Pro	-	-	-	-	-	-	-
Gly	-	-	-	-	0.22	0.26	-
Ala	0.29	0.29	1.44	1.12	0.84	0.85	0.70
Met	-	-	3.19	30.70	1.87	1.73	0.58
Val	-	-	9.12	7.85	4.56	4.01	3.26
Ile	-	-	4.86	4.38	2.69	2.50	1.31
Leu	0.49	0.47	24.37	19.99	11.75	10.28	6.06
Tyr	-	-	15.91	14.26	10.24	9.11	5.75
Phe	-	-	12.04	10.83	8.73	7.91	5.94
His	-	-	2.06	1.66	1.17	0.99	1.00
Lys	6.36	4.30	36.84	32.37	21.42	17.44	10.07
Trp	-	-	-	-	-	-	-
Arg	0.12	3.16	13.79	12.33	9.47	6.19	6.19

[a] not detected

Pretreatment of a solution (10 mg/ml) of commercial protease preparation (Corolase PP) by holding at 37 °C, prior to hydrolysis of lactalbumin, showed that, as time of preatreatment increased, the degree of hydrolysis decreased (Table 2). Increasing holding times had a progressively negative effect on initial rates of hydrolysis and final DH values after 240 min incubation. Material correspoinding to the 13,300-570 Da and < 570 Da molecular weight region was hydrolysed to a lesser extent with increasing holding times (Table 4). For 16, 28, and 44 h samples a decrease occurred in areas corresponding to low-molecular-weight peptides as holding time was extended.

2.4.3.2 Production of free amino acids

The concentrations of amino acids in free solution following incubation of lactalbumin with pancreatic extracts and commercial preparations were compared (Tables 5 and 6). Concentrations < 0.006 (mg/g) were not included. All preparations produced hydrolysates which contained alanine in free solution. Both sample B and 30S activated for 4h produced hydrolysates which only had alanine free in solution. There was no difference in the hydrolysate produced by the 30S preparation activated for 4h in the presence and absence of Ca^{2+} ions (Table 5). All enzyme preparations when applied to lactalbumin hydrolysis on the basis of total proteolytic activity, produced hydrolysates deficient in glutamic acid and glycine. All hydrolysates contained leucine, with exception of that produced by 30S, activated for 4 h and 16 h, 30S activated in the absence of Ca^{2+} ions and Sample B. As the time of activation of zymogen forms of 30S preparation was increased, several additional free amino acids are released into the hydrolysates, especially lysine and leucine. The hydrolysates produced by Corolase PP contained the following amino acids: lysine > leucine > tyrosine > arginine > phenylalanine > valine > isoleucine > methionine > serine > histidine > alanine > threonine. The concentration of each amino acid was reduced with increasing pretreatment time of the Corolase PP preparation, although the levels of each appeared to remain in proportion (Table 6). The analysis of free amino acids in the hydrolysates of Corolase PP and 30S preparations revealed significant differences. The low levels of free amino acids in hydrolysates produced by 30S indicated an

absence of exopeptidase activity contrary to levels known to be present in Corolase PP (Mullally *et al.*, 1994). Corolase PP also has elastase activity and low levels of this activity in 30S may have contributed to similarities between preparations with respect to shape of DH curves (Figure 2 and 3).

Different published extraction procedures have been used for purification of individual pancreatic proteinases. Acidic extraction has been used in the initial step for trypsin and chymotrypsin recovery, whereas acetone powder extraction is used in protocols for elastase, carboxypeptidase and exopeptidase recovery (Northrop *et al.*, 1948; Laskowski, 1956; Lewis *et al.*, 1959; Folk and Schirmer, 1963). In our study proteolytic preparations Sample B and 30S were prepared using an intial acid extraction step. This may have destroyed exopeptidase activities and certain endoproteinase activities. Porcine chymotrypsin B and C are acid-labile (Folk, 1970a). Incubation of elastase at very low pH values for extended times deactivated the enzyme (Lewis *et al.*, 1956). This would, therefore, explain the absence of most of the free amino acids in the hydrolysates and the extremely low levels of amino acids, such as alanine. Levels of carboxypeptidases are higher in porcine tissues (Desnuelle and Rovery, 1961), although carboxypeptidase B is subject to de-activation at very low pH values and, therefore, the purification procedure employed would not be suitable for its isolation. All these factors considered, it is unlikely that exopeptidase activity was prominent in the 30S preparation.

2.5 Conclusion

The ratio of proteinases in a pancreatic enzyme preparation was related not only to the degree of purification or source of pancreatic tissue but also to the activation procedure employed. Unless an extensive purification protocol involving chromatographic procedures is employed, it seems unlikely that a precise ratio of endoproteinases (trypsin, chymotrypsin and elastase) could be maintained. Zymogen activation alone, however, can control the ratio of proteinases present in an enzymatic preparation, and thus the physicochemical properties of any resultant whey protein hydrolysate. During the hydrolysis of proteins, if a number of enzymatic actvities

work in conjunction with one another, the resulting hydrolysates differ. Properties of hydrolysates are dependent on several factors, such as the number and ratio of proteases, enyme specificity, enzyme stability, and, possibly auto-digestion. To find a desirable ratio of endoproteinases within a proteolytic preparation, a compromise between activation and de-activation must be found.

CHAPTER 3

Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity[1]

[1] This chapter was prepared for submission as a short communication

3.1 Summary

Novel angiotensin I converting enzyme (ACE) inhibitory activities were detected for β-lactoglobulin and α-lactalbumin derived synthetic peptide sequences which are known to possess opioid activity. Using hippuryl-histidyl-leucine as substrate, the tetrapeptides β-lactorphin (Tyr-Leu-Leu-Phe), α-lactorphin (Tyr-Gly-Leu-Phe) and β-lactotensin (His-Ile-Arg-Leu) were shown to have IC_{50} values of 171.8, 733.3 and 1153.2 μmol/l respectively. Related dipeptides also inhibited ACE with Tyr-Leu being the most potent, having an IC_{50} value of 122.1 μ mol/l.

3.2 Introduction

Angiotensin-I-converting enzyme (ACE; peptidyldipeptide hydrolase EC 3.4.15.1) is a dipeptide-liberating exopeptidase which has been classically associated with the renin-angiotensin system regulating peripheral blood pressure. ACE which removes the two amino acids from the C-terminus of angiotensin I to form angiotensin II, a very hypertensive compound, is inhibited by certain peptides. Several endogenous peptides such as enkephalins, β-endorphin, substance P and ACTH are reported to be competitive substrates and inhibitors of ACE. In addition, several food derived peptides inhibit ACE (Ariyoshi, 1993; Meisel, 1993b). Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease in angiotensin II activity and the concomitant increase in bradykinin activities. Sekiya *et al.* (1992) reported a significant reduction in blood pressure in human volunteers with mild hypertension following oral intake of a tryptic casein hydrolysate.

Several casein derived ACE inhibitory peptides having other biological activities have been reported (Meisel and Schlimme, 1994). For instance, it has been shown that the casein derived opioid peptide, β-casomorphin-7, also inhibits ACE (Meisel and Schlimme, 1994). A multifunctional bioactive peptide, albutensin A, derived from bovine serum albumin f(208-216), displaying ileum contracting and relaxing activities in addition to ACE inhibitory activity has been characterised (Chiba and Yoshikawa, 1991). To date, there appears to be no evidence in the literature to suggest that ACE inhibitory peptides have been found in the sequences of the major whey proteins i.e. β-lactoglobulin and α-lactalbumin. In this study we show that bioactive peptide sequences derived from bovine ß-lg and α-la can act as inhibitors of ACE.

3.3 Materials and Methods

The tetrapeptides, i.e. Tyr-Leu-Leu-Phe, Tyr-Gly-Leu-Phe and His-Ile-Arg-Leu were synthesised using Fmoc solid phase synthesis by Bioresearch Ireland University College Cork, Ireland. Mass spectroscopy was used to confirm the purity of these tetrapeptides (Biochemistry Department, University of Nottingham, UK.). Dipeptides were obtained from Bachem (Bubendorf, Switzerland). All other reagents were of analytical grade unless otherwise specified.

3.3.1 Characterisation of ACE inhibition

The assay system used was the standard spectrophotometric assay of Cushman and Cheung, (1971), using rabbit lung tissue as source of ACE, and is based on the liberation of hippuric acid by ACE from hippuryl-histidyl-leucine. Analyses were carried out by Dr. Meisel at the Institut für Chemie und Physik, Bundesanstalt für Milchforschung, Keil, Germany. The IC_{50} values were determined using different dilutions of the solubilised peptides to obtain distinct molar concentrations.

3.4 Results and Discussion

The ACE inhibitory activities (IC_{50} values) of tetra- and dipeptides corresponding to different whey protein sequences are given in Table 1. The IC_{50} values obtained for bradykinin potentiator B and Captopril are in accordance with those reported by Maruyama *et al.* (1985) and Wyvratt and Patchett, (1985), respectively. It has been suggested that peptides which contain hydrophobic amino acids at each of the three C-terminal positions are potent ACE inhibitors (Cheung *et al.*, 1980; Saito *et al.*, 1994). From Table 1 it is seen that the tetrapeptide IC_{50} values appear to reflect this observation i.e. ß-lactorphin (IC_{50} = 171.8 µmol/l) > α-lactorphin (IC_{50} = 733.3 µmol/l) > ß-lactotensin (IC_{50} = 1153.2 µmol/l). The synthetic tetrapeptides in our study corresponding with sequences actually found in whey protein hydrolysates have certain bioactive properties. For example, ß-lactorphin and α-lactorphin possess opioid activity (Antila *et al.*, 1991).

Table 1: ACE inhibitory activity of ß-lactorphin, α-lactorphin, ß-lactotensin and related peptides.

Peptide sequence	Name	IC_{50} (μmol/l)[a]
Tetrapeptides		
Tyr-Leu-Leu-Phe	β-Lactorphin (β-lg (f102-105))[b]	171.8
Tyr-Gly-Leu-Phe	α–Lactorphin (α-la (f50-53))[c]	733.3
His-Ile-Arg-Leu	β-Lactotensin (β-lg (f146-149))	1153.2
Dipeptides		
Tyr-Leu	-	122.1
Leu-Phe	-	349.1
Tyr-Gly	-	1522.6
Ile-Arg	-	695.5
Arg-Leu	-	2438.9
Controls		
-	Captopril	0.006
-	Bradykinin Potentiator B	3.2

[a] IC_{50} is defined as that amount of peptide which causes 50 % inhibition of ACE using hippuryl-histidyl-leucine as substrate. [b] β-lg; β-lactoglobulin.[c] α-la; α-lactalbumin

Not surprisingly, it has been observed that C-terminal dipeptides are generally the more potent fragment of ACE inhibitory peptides (Cheung *et al.*, 1980). The results obtained using dipeptides indicate that the nature of the amino acids and not necessarily peptide size has an effect on ACE inhibitory potency. The Tyr-Leu dipeptide, a peptide related to ß-lactorphin (Tyr-Leu-Leu-Phe), was more potent (IC_{50}= 122.1µmol/l) than the original tetrapeptide (IC_{50} = 171.8 µmol/l). However, another related peptide i.e. Leu-Phe had a potency (IC_{50} = 349.1 µmol/l) which was lower than ß-lactorphin (Table 1). It was interesting to note that the substitution of Leu with Gly weakens the inhibitory potency i.e. Tyr-Gly has a higher IC_{50} value than Tyr-Leu (Table 1). However, for the β-lactotensin related peptides, Ile-Arg (IC_{50} = 695.5 µmol/l) was more potent than Arg-Leu (IC_{50} = 2438.9 µmol/l). Structure-activity data indeed suggest that a positive charge, as on the guanidino group of the C-terminal Arg, contribute substantially to the ACE inhibitory activity of several peptides (Meisel, 1993b). Perhaps the penultimate C-terminal amino acid, in combination with the C-terminal residue has a strong influence on the potency of ACE inhibitory peptides.

It should be noted that peptides derived from food proteins may not have the potency of Captopril (D-3-mercapto-2-methylpropanoyl-l-proline; IC_{50} < 0.03 µmol/l), a drug commonly used in the treatment of hypertension (Wyvratt and Patchett, 1985). However, should peptides derived from whey proteins be shown to have significant physiological effects as is the case for tryptic casein hydrolysates (Sekiya *et al*, 1992), their use in the prevention of hypertension could be favoured since they are naturally derived. Food derived peptides with a potency within the IC_{50} 100-500 µmol/l range are reported to be of nutritive/physiological importance in that they could be active following oral administration (Sekiya *et al.*, 1992). A nutraceutical is any substance that is a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease (DeFelice, 1995). Accordingly, the ACE-inhibitory peptides derived from food proteins, such as bovine whey proteins, represent potential nutraceuticals and may be useful ingredients in functional foods for the prevention of hypertension.

CHAPTER 4

Angiotensin I converting enzyme inhibitory activities derived from whey proteins following hydrolysis with gastric and pancreatic enzymes.

4.1 Summary

There is a strict requirement for enzymatic hydrolysis in the production of angiotensin I converting enzyme (ACE) inhibitory activity from whey proteins. Several whey protein hydrolysates were examined for ACE inhibitory activity. Heat treatment of WPC, α-lactalbumin (α-la) and β-lactoglobulin (β-lg) fractions resulted in higher degrees of hydrolysis after 240 min with gastric and pancreatic enzymes. However, the corresponding indices of ACE inhibition for the hydrolysates from the native and heat-treated proteins did not differ greatly. A tryptic hydrolysate of β-lg was fractionated using solid phase extraction and acetonitrile elution fractions with highest indices of ACE inhibition were further characterised. Mass spectrometry of three acetonitrile elutions showed peptide sequences worthy of further study. One of these peptides (Ala-Leu-Pro-Met-His-Ile-Arg) was synthesised. This peptide, corresponding to a tryptic fragment of β-lg, appeared to be resistant to further hydrolysis by pepsin and had an IC_{50} value of 42.6 μmol/l. This peptide was shown to be a more potent ACE inhibitor than β–lactotensin and two other related peptides examined in a previous study. Enzyme specificity was shown to be more important than percentage degree of hydrolysis in the production of ACE inhibitory peptides. This study shows that angiotensin I converting enzyme inhibitors can be obtained from natural sources, such as whey protein, and as a consequence may have the potential for use in physiological functional foods.

4.2 Introduction

Milk proteins and in particular whey proteins are well recognised for their nutritional properties and for the provision of dietary amino acids (Hambraeus, 1982). However, peptide fragments of milk proteins resulting from the action of the digestive tract proteinases function not only as a source of amino acids, but also as potential physiological regulators (Maubois and Léonil, 1989). There are numerous bioactive peptides which exist in an inactive form within the amino acid sequence of milk proteins. These regulatory peptides can be released during *in vitro* hydrolysis of milk proteins and also *in vivo* by gastric and/or pancreatic enzymes. The significance of milk protein derived oligopeptides in biological regulation suggests an important physiological role for milk proteins. These include peptides with opioid agonist and antagonist activity (Teschemacher and Scheffler, 1992; Tani *et al*, 1994; Pihlänto-Leppälä *et al*., 1994a, b), immunostimulating activity (Fiat *et al*, 1993), anti-thrombic activity (Chabance *et al*, 1995), mineral binding activity and growth stimulating activity (Sato *et al*., 1991; Bouhallab *et al*., 1993). Currently, the majority of milk protein derived bioactive peptides originate from the caseins. However, some whey protein derived bioactive peptides have been described. For example, combinations of pepsin with trypsin or chymotrypsin have previously been used to produce *in vitro* opioid peptides from α-la and β-lg (Antila *et al*., 1991). Furthermore, a vasorelaxing tetrapeptide from β-lg, designated β-lactotensin was produced *in vitro* using chymotrypsin or a combination of pepsin and chymotrypsin (Pihlänto-Leppälä *et al*., 1994a, b). The structure and physiological nature of milk protein derived bioactive peptides has been reviewed (Fiat *et al*., 1993; Schlimme and Meisel, 1993; Shimizu, 1994).

Angiotensin-I-converting enzyme (ACE) is a dipeptide-liberating exopeptidase (peptidyldipeptide hydrolase EC 3.4.15.1) which has been classically associated with the renin-angiotensin system regulating peripheral blood pressure. The enzyme can raise blood pressure by converting angiotensin I (a decapeptide) to the potent vasoconstrictor angiotensin II (an octapeptide). Angiotensin II reduces peripheral blood flow and thereby decreases the renal excretion of fluid and salts. ACE is a

multifunctional enzyme (Figure 1) which also catalyses the degradation of bradykinin, (a vasodilating nonapeptide) and enkephalins (Aoki *et al*, 1984). Several endogenous peptides such as enkephalins, β-endorphin, substance P and ACTH were reported to be substrates and inhibitors of ACE (Maruyama *et al.*, 1987a). Therefore, inhibition of ACE decreases the activity of angiotensin II but increases bradykinin and enkephalin levels, thus lowering blood pressure in humans and animals (Ondetti *et al.*, 1977; Koike *et al.*, 1980). Consequently, ACE inhibitory peptides may exert antihypertensive and immunostimulating effects (Migliore-Samour *et al.*, 1989) and may increase neurotransmitter activity (Figure 1).

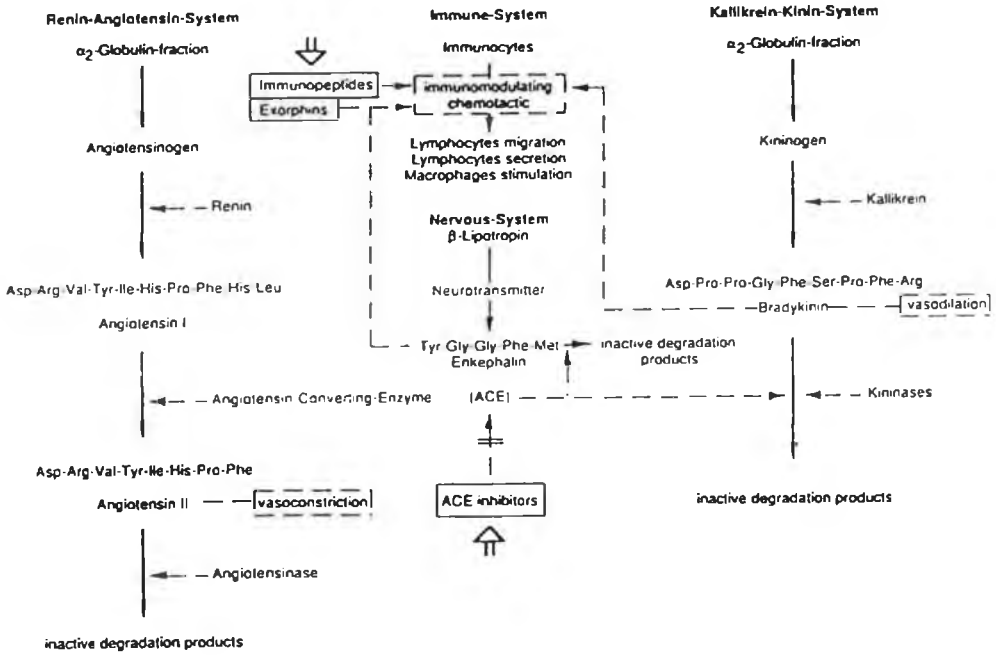


Figure 1: The effects of inhibitory peptides on systems for regulation of blood pressure, immune system and neuroendocrine information transfer in the organism (taken from Meisel, 1992).

Peptides which inhibit ACE have been isolated from many different food sources, including gelatin (Oshima *et al.*, 1979), α-zein (Miyoshi *et al.*, 1991), sardine (Ukeda *et al.*, 1992; Matsufuji *et al.*, 1994), sake and sake lees (Saito *et al.*,

1994) and fermented milk (Nakamura *et al.*, 1995a, b). ACE inhibitory activity has also been found in cheese (Ito *et al.*, 1987), tea (Young *et al.*, 1993) and other fermented foods (Okamoto *et al.*, 1995). Peptides which inhibit ACE, were also isolated from enzymatic casein hydrolysates (Maruyama *et al.*, 1982, 1985, 1987a, b; Chiba and Yoshikawa, 1991; Meisel and Schlimme, 1994; Yamamoto *et al.*, 1994). Fragments of κ-casein which inhibit ACE have also been synthesised (Kohmura *et al.*, 1990). Casein peptides with potential anti-hypertensive activities are known as casokinins and their function as inhibitors of ACE has been reviewed (Meisel, 1992). Only limited studies have been carried out on whey protein derived ACE inhibitors. A peptide, known as albutensin A, derived from serum albumin (Chiba and Yoshikawa, 1991) was to our knowledge the only reported whey protein derived ACE inhibitor prior to our recent findings (Chapter 3). Table 1 and 2 respectively summarise the current status with respect to casein and whey protein derived ACE inhibitory peptides.

The multifunctional aspect/nature of certain bioactive peptides has been described (Schlimme and Meisel, 1993; Shimizu, 1994). For example, the casein derived casoxin C which has been shown to induce contraction of guinea pig ileum longitudinal muscle (Loukas *et al.*, 1983) also has ACE inhibitory activity. In addition, casomorphins can inhibit ACE (Meisel and Schlimme, 1994). Albutensin A f(208-216) is also multifunctional, showing ileum-contracting and vasorelaxing activities in addition to ACE inhibitory activity (Chiba and Yoshikawa, 1991). In an earlier study (Chapter 3), we have shown that opioid peptide sequences derived from the whey proteins, ß-lg, α-la and a muscle acting peptide derived from β-lg, can also act as inhibitors of ACE.

In this study, it was shown that both purified and commercially available proteinase preparations of gastric and pancreatic enzymes can be used to hydrolyse WPC, enriched fractions of β-lg and α-la, and the heated- treated forms of these whey proteins, to generate peptides which are inhibitors of ACE.

Table 1: Summary of casein [a] derived angiotensin I converting enzyme inhibitory peptides.

Milk protein	Fragment	Synthetic peptide	Hydrolysate derived	Name	Sequence	IC_{50} [b] ($\mu mol\ l^{-1}$)	Reference
Casein							
α_{s1} - casein	*f(23-34)*		+	-	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	77.0	Maruyama and Suzuki,1982
	f(23-27)		+	-	Phe-Phe-Val-Ala-Pro	6.0	Maruyama *et al*.,1985
	f(24-27)	+		-	Phe-Val-Ala-Pro	10.0	Maruyama *et al*.,1987 (a)
	f(25-27)	+		-	Val-Ala-Pro	2.0	Maruyama *et al*.,1987 (a)
	f(27-30)	+		-	Pro-Phe-Pro-Glu	>1000	Maruyama *et al*.,1987 (a)
	f(28-34)		+	-	Phe-Pro-Glu-Val-Phe-Gly-Lys	140.0	Maruyama *et al*.,1987 (a)
	f(32-34)	+		-	Phe-Gly-Lys	160.0	Maruyama *et al*.,1987 (a)
	f(194-199)		+	-	Thr-Thr-Met-Pro-Leu-Trp	16.0	Maruyama *et al*.,1987 (b)
	f(197-199)	+		-	Pro-Leu-Trp	36.0	Maruyama *et al*.,1987 (b)
	f(198-199)	+		-	Leu-Trp	50.0	Maruyama *et al*.,1987 (b)
	f(143-148)		+	-	Ala-Tyr-Phe-Tyr-Pro-Glu	106.0 [c]	Yamamoto *et al*.,1994
β-casein	*f(57-64)*		+	-	Ser-Leu-Val-Leu-Pro-Val-Pro-Glu	39.0 [c]	Yamamoto *et al*.,1994
	f(60-66)	+		β-casomorphin -7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	500.0	Meisel and Schlimme, 1994
	f(74-76)		+	–	Ile-Pro-Pro	5.0	Nakamura *et al*.,1995(a)
	f(84-86)		+	–	Val-Pro-Pro	9.0	Nakamura *et al*.,1995 (a)
	f(177-181)	+		-	Ala-Val-Pro-Tyr-Pro	80.0	Maruyama *et al*.,1987 (a)
	f(177-183)		+	-	Ala-Val-Pro-Tyr-Pro-Gln-Arg	15.0	Maruyama *et al*.,1987 (a)
	f(177-179)	+		-	Ala-Val-Pro	340.0	Maruyama *et al*.,1987 (a)
	f(179-181)	+		-	Pro-Tyr-Pro	220.0	Maruyama *et al*.,1987 (a)
	f(181-183)		+	-	Pro-Gln-Arg	>400.0	Maruyama *et al*.,1987 (a)
	f(193-202)	+		β-casokinin-10	Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg	300.0	Meisel and Schlimme, 1994
κ-casein				–			
	f(25-34)	+		Casoxin C	Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg	na	Chiba and Yoshikawa, 1991
	f(35-41)	+		Casoxin A	Tyr-Pro-Ser-Tyr-Gly-Leu-Asn-Tyr	na	Chiba and Yoshikawa, 1991

a - further details of other peptide sequences and/or related sequences of casein which inhibit ACE are given elsewhere (Yamamoto *et al.*,1994; Maruyama *et al.*,1987 a, b); b - Concentration of peptide producing 50 % inhibition of ACE activity; c - IC_{50} value reported in μg/ml; - name not given; na- value not available

Table 2: Whey protein derived angiotensin I converting enzyme inhibitory peptides.

Whey protein	Fragment	Synthetic derived	Hydrolysate derived	Name	Sequence	IC_{50} [a] ($\mu mol\ l^{-1}$)	Reference
β-lactoglobulin	*f (50-51)*	+		–	Tyr-Gly	1522.6	Chapter 3
β-lactoglobulin	*f(102-105)*	+	+	β–lactorphin	Tyr-Leu-Leu-Phe	171.8	Chapter 3
β-lactoglobulin	*f (102-103)*	+		–	Tyr-Leu	122.1	Chapter 3
β-lactoglobulin	*f (104-105)*	+		–	Leu-Phe	349.1	Chapter 3
β-lactoglobulin	*f(146-149)*	+	+	β–lactotensin	His-Ile-Arg-Leu	1153.2	Chapter 3
β-lactoglobulin	*f(147-148)*	+		–	Ile-Arg	695.5	Chapter 3
β-lactoglobulin	*f(148-149)*	+		–	Arg-Leu	2438.9	Chapter 3
α-lactalbumin	*f(50-53)*	+	+	α–lactorphin	Tyr-Gly-Leu-Phe	733.3	Chapter 3
Bovine serum albumin	*f(208-216)*		+	albutensin A	Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg	3.4	Chiba and Yoshikawa, 1991

a - Concentration of peptide producing 50 % inhibition of ACE activity; - name not given.

4.3 Materials and methods

4.3.1 Enzymes

Commercial pancreatic proteases were received as gifts from the manufacturers. Corolase PP (porcine) was supplied by Röhm GmbH, Darmstadt, Germany. PTN 3.0S (porcine) was supplied by Novo Nordisk A/S, Bagsvaerd, Denmark. Porcine trypsin (Type IX, 13,700 BAEE units/mg protein), bovine trypsin-TPCK treated (Type XIII, 12,700 BAEE units/ mg protein), bovine chymotrypsin-TLCK treated (Type II, 40-60 BTEE units/mg protein), porcine elastase (Type I, 25-100 units/mg protein, one unit of elastase will solubilise 1 mg of elastin in 20 min at pH 8.0 at 37 °C) and porcine pepsin A, (3,200-4,500 units/mg protein, one unit will produce an dA_{280} of 0.001 per min at pH 2.0 and 37 °C for TCA soluble products of haemoglobin) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

4.3.2 Substrates

Whey protein concentrate, i.e. Lactalbumin-80 (Soluble UF-WPC) was from Milei GmbH., Leutkirch, Germany and had the following composition: protein (80 %), lactose (4.7 %), minerals (2.8 %), fat (6 %) and moisture (5 %). The enriched fractions of β-lg AB and α-la were isolated according to the method of Maubois *et al.* (1987) on a pilot scale with some modifications. Details of the production and composition of the β-lg and α-la enriched fractions are given by Mehra (1994). The composition of the β-lg enriched fraction was as follows: total solids (95.2 %), total protein (82.7 %), true protein (76.3 %), β-lg (89.8 %), α-la (10.2 %), fat (0.3 %), ash (4.7 %), calcium (0.79 %), sodium (0.68 %) and chloride (0.91 %). The composition of α-la was as follows: total solids (95.2 %), total protein (71.0 %), true protein (69.3 %), β-lg (8.5 %), α-la (~50 %), fat (16.9 %), ash (1.4 %) and calcium (0.2 %). The degree of denaturation for the enriched whey protein fractions was determined on the basis of solubility over a wide range of pH values and details are given in Mehra (1994). The β-lg enriched fraction had approximately <5 % denaturation versus ~60 % for α-la (Mehra, 1994). Purified bovine β-lg AB (3x crystallised and lyophilised) and α-la

(Type III: approx. 85 % by polyacrylamide gel electrophoresis) were also obtained from Sigma Chemical Co. Poole, Dorset, UK.

The β-lg peptide, Ala-Leu-Pro-Met-His-Ile-Arg corresponding to f(142-148) was synthesised using Fmoc solid phase synthesis at Bioresearch Ireland, University College Cork, Ireland. Mass spectroscopy was carried out at the Biochemistry Department, University of Nottingham, UK, to confirm the purity of this peptide (Section 4.3.8.3).

4.3.3 Solid phase extraction columns

Bond Elut® C_{18} columns, (2.8 ml) were from Varian Sample Preparation Products, Harbour City, CA, USA. All other reagents were of analytical grade unless otherwise specified.

4.3.4 Analysis of substrate molecular mass distributions using gel permeation HPLC

Whey protein substrates were analysed by gel permeation (GP) HPLC on a Beckman (High Wycombe, Bucks, UK) Spherogel-TSK 2000 SW column (600 x 7.5 mm), equilibrated and run with 30 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid (TFA) in water. Chromatography was conducted using a Waters™ HPLC detector, autosampler and pumping systems (Millipore Corp. Bedford, MA 01730, USA), at room temperature. The flowrate was 1 ml min^{-1} and the absorbance at 214 nm was recorded. The injection volume was 20 µl.

The column was calibrated with the following standard proteins and peptides: bovine serum albumin (66,000 Da); carbonic anhydrase (29,000 Da); ribonuclease A (13,700 Da); cytochrome C (13,000 Da); aprotinin (6,500 Da); insulin (5,700 Da); bacitracin (1,400 Da); tryptophan (204 Da); phenylalanine (165 Da) and glycine (75 Da). A Minichrom® data handling package (V.G. Data Systems, Manchester, England) was used to record and integrate chromatograms (Chapter 1 and 2).

4.3.5 Substrate pre-treatment

In certain instances the β-lg and α-la substrates were held at 80 °C for 20 min to heat denature the protein. Protein samples which were not heat-treated were designated to have native structure.

4.3.6 Preparation of hydrolysates

Porcine trypsin was used for the generation of all tryptic hydrolysates, with the exception of the experiment in which the enriched fraction of β-lg was hydrolysed for subsequent solid phase extraction fractionation, i.e. in the generation of f(142-148).

4.3.6.1 Laboratory scale whey protein hydrolysis with pepsin, trypsin and commercial proteinase preparations.

A 25 ml solution of substrate, 8 % (w/v) protein, was hydrolysed with individual protease preparations at 50 °C and pH 8.0 for pancreatic proteases or at 37 °C and pH 3.0 for pepsin for a total hydrolysis time of 240 min. Where a combination of individual proteinases were used (i.e. pepsin and trypsin), each enzyme was incubated firstly at 50 or 37 °C for 120 min at the pH optimum of the enzyme. The pH of the reaction was then adjusted to the pH optimum value for the action of the second proteinase (120 min). The pH was maintained constant by continuous addition of 0.5 M NaOH (for tryptic reactions) or 1 M HCl (for peptic reactions) using a pH-stat (Metrohm Ltd, Herisau, Switzerland). The degree of hydrolysis (DH %), defined as the percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Following hydrolysis, proteases were inactivated by heating at 80 °C for 20 min, hydrolysates were then cooled and stored at -20 °C for further analysis. Details for the calculation of DH are given previously (Chapter 1 and 2). The enzyme-to-substrate ratio (E:S), 0.003, was calculated on the basis of total protein content in the enzyme preparations and the substrate. The standard deviation for hydrolysis reactions using this pH-stat system was determined previously to be DH ± 0.1 % (O'Callaghan, 1994).

4.3.6.2 Pilot scale hydrolysis of WPC

WPC Lactalbumin -80 was reconstituted using a Silverson® mixer (Machines Ltd., Waterside, Chesham, Bucks, U.K.). Generally, a 5 l solution of WPC at 8 % (w/v) protein, was hydrolysed with PTN 3.0S at 50 °C in a jacketed vessel, until a defined degree of hydrolysis was reached (i.e. 8 %). The pH was maintained at pH 8.0 by continuous addition of 4 M NaOH using a pH-stat (Metrohm Ltd, Herisau, Switzerland.). The degree of hydrolysis (DH %), was calculated as above. During hydrolysis, samples were taken at various intervals and the protease (5 ml) was inactivated by heating to 80 °C and holding for 20-30 min. These samples were later freeze-dried. Again the enzyme to substrate ratio used (0.003) was calculated on the basis of total protein content in the enzyme preparation and in WPC.

4.3.6.3 Enzymatic hydrolysis of the β-lg derived synthetic peptide f(142-148).

The synthetic peptide of β-lg corresponding to residues 142-148, i.e., Ala-Leu-Pro-Met-His-Ile-Arg was presented to chymotrypsin and pepsin solutions. For each reaction, 500 µl of enzymatic solution (0.1 mg/ml) was added to 500 µl of peptide solution (6 mg/ml), mixed and incubated at 50 °C for 45 min. Enzymes were inactivated by heating at 80 °C for 20 min. Chymotrypsin reactions were buffered using 20 mM Tris-HCl, pH 8.0 while the pepsin reactions were carried out in 0.1 M HCl, pH 3.0. Controls containing peptide, pepsin and chymotrypsin were treated in the same manner as the test sample. Peptide blanks were was also made up in corresponding buffers. The enzyme to substrate ratio (E:S) was 1: 60. Acid-washed glassware was used throughout.

4.3.7 Fractionation of hydrolysate peptides by solid phase extraction

The solid phase extraction column was first conditioned using methanol (2.8 ml), and then washed with distilled water (2.8 ml), followed by 5.6 ml 0.1 % (v/v) TFA. The hydrolysate solution (1 ml at 80 mg/ml (protein)) was then applied to the column. Peptide material was slowly removed from the solid phase extraction column by stepwise elution (i.e. 2.8 ml volumes) with increasing concentrations of acetonitrile (ACN) in 0.1 % TFA. Each fraction was then freeze-dried or analysed directly,

following filtration through a 0.22 μm filter (polysulphone membrane -Whatman Inter. Ltd., Maidstone, England), by reversed-phase HPLC.

4. 3.8 Characterisation of whey protein hydrolysates

4.3.8.1 Reversed-phase high performance liquid chromatography

Peptides from whey protein hydrolysates were separated by reversed-phase (RP) HPLC on a Phenomenex (Phenomenex Ltd., Macclesfield, Chesire, England) C_{18} column (250 x 3.2 mm, 5μm), equilibrated with solvent A (0.1 % TFA in H_2O) and elution was with a linear gradient to 80 % solvent B (60 % acetonitrile, 40 % H_2O, 0.1 % TFA) during the first 50 min, to 90 % in the next 10 min, to 100 % in the next 5 min and the column was re-equilibrated at 0 % solvent B for the last 20 min. Runs were conducted at room temperature using a Shimazu® HPLC system controller, autoinjector, pumps, UV-VIS detector and chromatograph integrator (Shimadzu Corp., Analytical Instruments Nakagyo-ku, Kyoto, Japan); the flowrate was 0.30 ml min^{-1} and the absorbance of the column effluent was recorded at 280 nm and 214 nm. The injection volume was generally 50 μl and the concentration of the peptide applied was generally 0.1 mg/ml. However, peptides from solid phase extraction were applied directly to the column without further dilution. Different injection volumes (15-50 μl) were used in order to have similar peptide concentrations to compare peptide fractions and to determine the efficiency of the solid phase extraction procedure. All samples were filtered through a 0.22 μm filter prior to application to the C_{18} column.

4.3.8.2 Characterisation of ACE inhibition

The assay system used was the standard spectrophotometric assay of Cushman and Cheung (1971), using rabbit lung tissue as source of ACE, and is based on the liberation of hippuric acid by ACE from hippuryl-histidyl-leucine. Analyses were performed at the Institut für Chemie und Physik, Bundesanstalt für Milchforschung, Kiel, Germany (Dr. H. Meisel). In most cases, hydrolysates were in freeze-dried form. Initial screening of whey protein hydrolysates was carried out using a single assay to give an index of ACE inhibition. In general, 50μl of a 10mg/ml solution of

hydrolysate was taken to screen for ACE inhibitory activity. Certain hydrolysate samples with ACE inhibition index values >80 % were selected for determination of an IC_{50} value, i.e. that amount of peptide/hydrolysate which gives 50 % inhibition of ACE activity. The IC_{50} value was expressed in terms of mg freeze-dried hydrolysate/l. The % inhibition versus Log_{10} [hydrolysate] (mg/l) curves or the % inhibition versus Log_{10} [peptide] (μmol/l) curves, in the case of synthesised peptides, were constructed using at least five separate analyses.

4.3.8.3 Mass spectroscopy

The solid phase extraction fractions of a tryptic hydrolysate of the enriched β-lg fraction which gave high indices of ACE inhibition (i.e. peptide fractions eluting between 20-25, 25-30 and 50-60 % ACN) were subjected to mass spectroscopy. Samples were forwarded in ACN and/or in freeze-dried form for analysis to the Biochemistry Department, University of Nottingham, UK.

Peptide masses were determined by plasma desorption mass spectroscopy (PDMS) using a Bio-Ion 20 instrument (Applied Biosystems AB, Uppsala, Sweden). The lyophilised peptides were taken up in 50 % methanol in water. Sample (20-100 pmol of solution) was then applied on a nitrocellulose-covered aluminised mylar target. The sample was then allowed to air dry, before washing with 50 μl of 100 mM ammonium bicarbonate which removes the alkali ions (in order to minimise interference from alkali metal ions). The spectra were recorded for $1x10^6$ fisson events at 15kV acceleration voltage and the spectra calibrated on the H^+ and NO^+ ions. The resolution of the instrument was 0.1 %, i.e. 1 in 1000 Da.

4.4 Results

4.4.1 Gel permeation HPLC of the whey protein substrates

The gel permeation profiles obtained at 214 nm of WPC, Sigma β-lg, the β-lg enriched fraction, Sigma α-la and the α-la enriched fraction are shown in Figure 2. The enriched fraction of β-lg has a profile which resembles that of the purified Sigma fraction. WPC gave a number of peaks as expected corresponding to a combination of whey proteins. The enriched α-la fraction has several peaks corresponding to contamination with other whey proteins.

4.4.2 Hydrolysis of whey proteins using gastric and pancreatic proteinases

Table 3 gives the DH obtained for enzymatic hydrolysis of the native and heat-treated β-lg enriched fraction. Table 4 gives the DH for the hydrolysis of native and heat-treated α-la enriched fraction. Table 5 gives the DH for the hydrolysis of WPC.

In general, the native (i.e. non-heat treated) forms of β-lg had lower DH values than the heat treated forms. However, it appeared that the effect of heat-treatment was not as pronounced for enzymatic hydrolysis of α-la by the individual enzymes, trypsin or pepsin, or for combinations of these enzymes, for 240 min. Trypsin hydrolysed native α-la to a DH of 7.92 % but hydrolysed the heat-treated form to a DH of 7.23 %. In the case where trypsin and pepsin were used in series to hydrolyse α-la the hydrolysis of the heat-treated forms had similar DH values to the native hydrolysis after 240 min. For example, the hydrolysis of the native α-la by trypsin followed by pepsin gave a total DH of 9.95 % after 240 min. A similar value was generated by trypsin followed by pepsin hydrolysis of the heat-treated α-la, which gave a total DH of 10.02 % after 240 min (Table 4).

β-Lg and α-la were hydrolysed by pepsin in the native form (Table 3 and 4) giving DH values of 4.03 and 5.10 % at 50 °C after 240 min hydrolysis. The degree of hydrolysis obtained with this endoproteinase was relatively low, when using the native forms of β-lg, in comparison to the other proteinases (Table 3). α-La, however, was more susceptible to hydrolysis by pepsin than β-lg. Pepsin generated similar DH values after 240 min in comparison to the values of DH obtained by the other

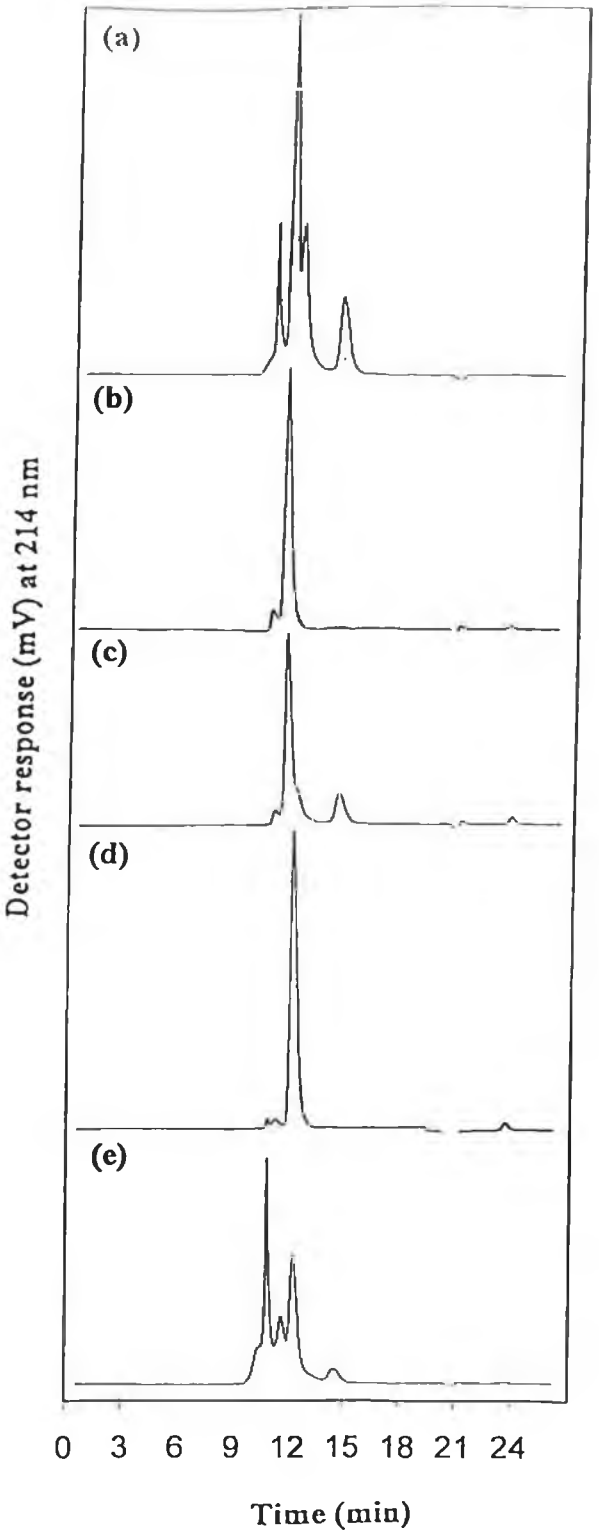


Figure 2: Gel permeation chromatograms from HPLC Spherogel - TSK SW column showing millivolt response, assayed at 214 nm, and retention times of (a) whey protein concentrate (b) Sigma β-lactoglobulin (c) β-lactoglobulin enriched fraction; (d) Sigma α-lactalbumin (e) α-lactalbumin enriched fraction.

Table 3: Native and heat treated β-lactoglobulin, temperature of enzymatic hydrolysis, proteinases used, degrees of hydrolysis (DH%) obtained after 240 min (in case of one enzyme), after 120 min (in the case of two enzymes) and index of ACE inhibition (%) in resulting hydrolysates.

Substrate	Temperature (^{0}C)	Proteinase	% DH (first enzyme)	% DH (second enzyme)	ACE inhibition [a] index (%)
Native ß-lactoglobulin					
Unhydrolysed control	na	na	na	na	9.6
	37	Pepsin	2.69	na	66.7
	50	Pepsin	4.03	na	85.3
	37	Trypsin	5.69	na	80.2
	50	Trypsin	6.52	na	84.3
	50	Chymotrypsin	7.64	na	87.6
	50	Elastase	5.50	na	63.4
	50	PTN 3.0S	11.23	na	87.6
	50	Corolase PP	16.72	na	77.7
	50	Pepsin/PTN 3.0S	nd	nd	84.8
	50	Trypsin+pepsin	7.66	2.48	89.8
	50	Pepsin+trypsin	2.36	7.58	86.1
Heat treated ß-lactoglobulin					
Unhydrolysed control	na	na	na	na	8.9
	50	Pepsin	6.64	na	85.1
	50	Trypsin	7.15	na	81.9
	50	Trypsin+pepsin	5.26	4.06	86.6
	50	Pepsin+trypsin	5.34	5.96	86.4

a - 50 µl of 10 mg/ml solution of hydrolysate (8% protein) was taken for the determination of ACE inhibition;
na-not applicable.

Table 4: Native and heat treated α-lactalbumin, temperature of enzymatic hydrolysis, proteinases used, degrees of hydrolysis (DH%) obtained after 120 min (in the case of one enzyme) and 240 min (in the case of two enzymes) and index of ACE inhibition (%) in resulting hydrolysates.

Substrate	Temperature (^{0}C)	Proteinase	% DH (first enzyme)	% DH (second enzyme)	ACE inhibition index[a] (%)
Native α-lactalbumin					
Unhydrolysed control	na	na	na	na	3.5
	50	Pepsin	5.10	na	84.4
	50	Trypsin	7.92	na	86.9
	50	Chymotrypsin	5.16	na	58.7
	50	Elastase	5.27	na	56.8
	50	PTN 3.0S	11.51	na	79.7
	50	Corolase PP	20.50	na	73.1
	50	Trypsin + Pepsin	6.69	3.26	87.1
	50	Pepsin + Trypsin	1.36	6.68	84.8
Heat treated α-lactalbumin					
Unhydrolysed control	na	na	na	na	7.6
	50	Pepsin	5.48	na	84.5
	50	Trypsin	7.23	na	85.1
	50	Trypsin + Pepsin	7.16	2.86	87.8
	50	Pepsin +Trypsin	2.99	7.39	88.0

a - 50 µl of 10 mg/ml solution of hydrolysate (8% protein) was taken for indices of ACE inhibition determination;
na-not applicable; nd- not detected.

Table 5: Whey protein concentrate, temperature of enzymatic hydrolysis, proteinases used, degrees of hydrolysis (DH%) obtained after 240 min and index of ACE inhibition (%) in resulting hydrolysates.

	Temperature (^{0}C)	Proteinase	% DH (first enzyme)	ACE inhibition index [a] (%)
Whey protein concentrate				
Unhydrolysed control		na	na	7.1
	50	Trypsin	6.71	88.6
	50	Chymotrypsin	5.08	87.7
	50	Elastase	2.49	35.5
	50	PTN 3.0S	10.43	80.8
	50	Corolase PP	19.05	78.2

a - 50 µl of 10 mg/ml solution of hydrolysate (8% protein) was taken for determination of ACE inhibtion; na-not applicable.

individual proteinases for hydrolysis of α-la. For example, the peptic hydrolysis of α-la generated a DH of 5.10 % after 240 min, in comparison to the tryptic or chymotryptic hydrolysis of α-la which gave DH values of 7.92 and 5.16 % , respectively. The DH value for the peptic hydrolysis of heat-treated α-la was only slightly higher, i.e. 5.48 % (Table 4). For the heat-treated form of the enriched fraction of β-lg, however, higher DH values were obtained using pepsin after 120 min, i.e. 5.34 % and after 240 min, i.e. 6.64 % (Table 3).

In most cases there appeared to be no real difference in the total DH obtained after 240 min when pepsin and trypsin were each incubated with the native enriched β-lg fraction in a different sequence for 120 min (i.e. pepsin following by trypsin hydrolysis, or trypsin followed by pepsin hydrolysis). The sequence of addition of pepsin and trypsin gave only slight differences (0.2 %) in the total DH values obtained (i.e., after a total of 240 min) for hydrolysis of native β–lg at 50 °C (Table 3). The total degree of hydrolysis (i.e. after 240 min) was higher when pepsin was used before trypsin in the hydrolysis of heat-treated β–lg. Differences, however, were seen in the hydrolysis of native α-la by pepsin or trypsin in succession. It was seen that a total DH value of 8.04 % was generated after 240 min when pepsin was used to hydrolyse native α-la before trypsin. However, a total DH value of 9.95 was obtained when trypsin was used before pepsin in the hydrolysis of native α-la (Table 4).

The commercial pancreatic proteinases, which contain combinations of trypsin, chymotrypsin and elastase in differing ratios, gave the highest DH values when presented to the native β-lg, α-la or WPC substrates. Corolase PP and PTN 3.0S, when used in the hydrolysis of the native enriched fraction of β–lg for 240 min gave higher DH values after 240 min of 16.72 and 11.23 % respectively than the individual proteinases, trypsin, chymotrypsin or elastase (6.52, 7.64, and 5.50 % respectively) over the same hydrolysis time (Table 3). In addition, Corolase PP and PTN 3.0S when used to hydrolyse α-la generated a DH of 20.50 and 11.51 % respectively (Table 4) and generated a DH of 19.05 and 10.43 % when used to hydrolyse WPC (Table 5). The final DH in all cases showed that combinations of proteinases, as found in PTN 3.0S and Corolase PP, always produced an hydrolysate with a higher final DH than individual proteinases over the same time period (Table 3, 4 and 5).

As expected it was seen that the higher temperature, i.e. 50 °C versus 37 °C, resulted in higher DH values after 240 min. For example, the values obtained for a tryptic hydrolysis of β-lg generated a DH of 5.69 % at 37 °C versus a DH value of 6.52 % at 50°C (Table 3).

4.4.3 Evolution of ACE inhibitory peptides

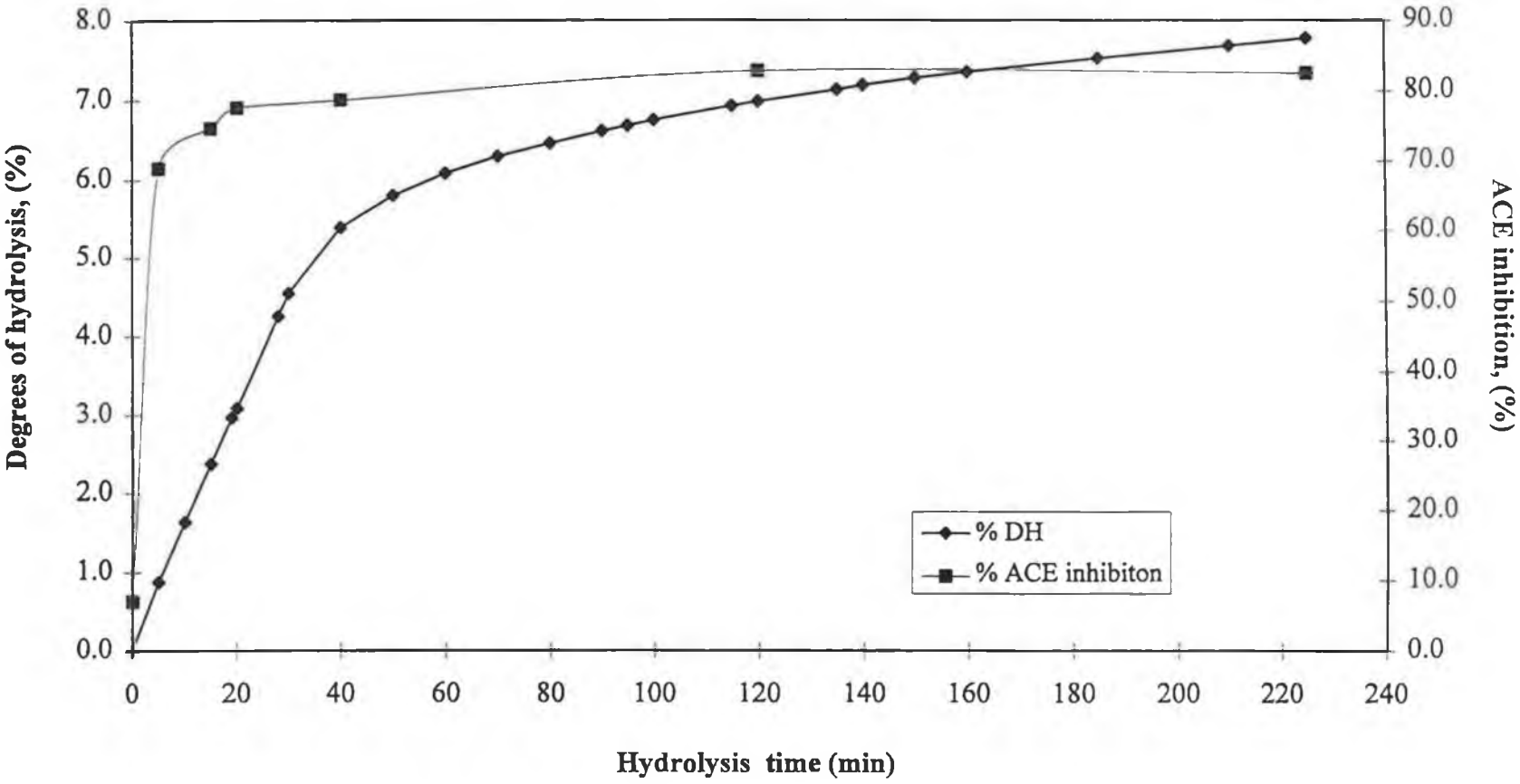
4.4.3.1 ACE inhibitory activity generated with gastric and pancreatic hydrolysis of whey proteins

It was seen from Table 3, 4, and 5 and Figure 3 that hydrolysis of both heat treated and native forms of whey proteins is necessary in the evolution of ACE inhibitory activity. The ACE inhibitory activity was very low for the unhydrolysed substrates (Table 3, 4 and 5), giving ACE inhibition values < 10 %.

Hydrolysis is necessary in order to release ACE inhibitory peptides from an inactive form within the sequence of whey proteins. This requirement for hydrolysis was illustrated for the pilot scale hydrolysis of WPC by PTN 3.0S. In the first 20 min of the hydrolysis reaction it was seen that evolution of > 70 % of the ACE inhibitory activity occurs (Figure 3).

Indices of ACE inhibition are given in Table 3, 4, and 5 for the various hydrolysates tested. These inhibition values while qualitative are nevertheless informative. All hydrolysates of β-lg, α-la and WPC, except for hydrolysates generated using elastase, gave very high indices of ACE inhibition after 240 min hydrolysis. Hydrolysates generated using elastase gave indices of inhibition in the range of 35-63 %, while other proteinases generated hydrolysates which gave indices of inhibition in the range of 57-85 %. Trypsin and combinations of trypsin and pepsin, in addition to the commercial pancreatic preparations, PTN 3.0S and Corolase PP, generated hydrolysates which gave % inhibition values in the range of 78-90 %. Surprisingly, peptic hydrolysates of α-la and β-lg gave indices of inhibition in the range of 84-85 %. The temperature (37 °C or 50 °C) at which the hydrolysates were generated appeared to have no influence on the indices of inhibition obtained after 240 min hydrolysis.

Figure 3: Development of ACE inhibitory activity during hydrolysis of WPC using a pancreatic enzyme preparation



4.4.3.2 ACE inhibitory activity for fractionated hydrolysates

In this study, a tryptic hydrolysate of the enriched β-lg preparation was fractionated using solid phase extraction. Analysis of each fraction on C_{18} RP-HPLC revealed the efficacy of the fractionation procedure (Figure 4 (i) and (ii)). It was seen that there was a shift in the peptide profile from left to right for the peptide fractions eluted at higher ACN concentrations. The more hydrophobic the peptide material, the higher the % ACN required for elution. Table 6 gives the indices of ACE inhibition (%) corresponding to various acetonitrile elutions. In general, at the lower concentrations of ACN, the eluted peptides gave relatively high ACE inhibition values, in comparison to the peptides eluted at higher concentrations of ACN. Three fractions of the tryptic hydrolysate of β-lg were selected for further study i.e., the peptide material eluting between 20-25 , 25-30 and 50-60 % ACN (Figure 4 (i) c, d and 4 (ii) d). The peptides eluting between 20-25, 25-30 and 50-60 % ACN gave corresponding ACE inhibition indices of 93.6, 98.6 and 41.8 %, respectively. It should be noted that for the 50-60 % ACN elution that a 3mg/ml solution was used for the assay instead of the usual 10 mg/ml solution.

4.4.3.3 Mass spectroscopy

Three ACN elutions from the solid phase extraction of the tryptic hydrolysate of β-lg, chosen on the basis of the index of ACE inhibition, were analysed using mass spectroscopy. A number of peptides were seen to dominate in the three fractions examined. Figures 1, 2 and 3 in Appendix A, give the mass spectroscopy profiles obtained for the 20-25, 25-30, and 50-60 % ACN elutions, respectively. The results compared favourably with the theoretically expected masses for tryptic digest products of the β-lg sequence as given in Table 7. From the observed masses of the major peptides in the different ACN elutions it was possible to predict the peptide sequences corresponding to these masses (Table 7 and Figure 5).

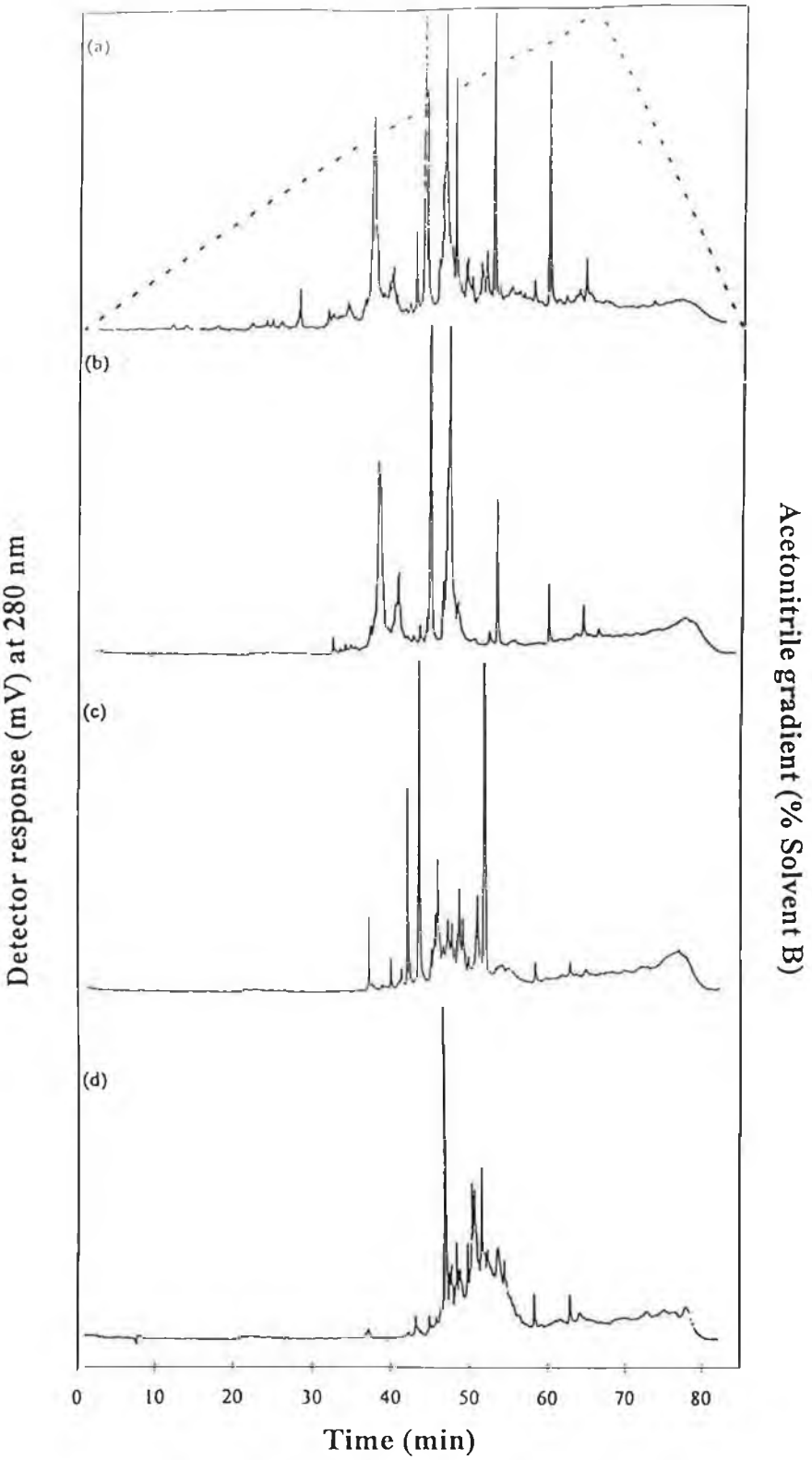


Figure 4 (i): Reversed Phase-HPLC (C_{18}) chromatograms of the peptides in (a) tryptic hydrolysate of enriched β-lactoglobulin fraction and in the solid phase extraction fractions corresponding to (b) 1-20 % Acetonitrile (ACN) elution (c) 20-25 % ACN elution and (d) 25-30% ACN elution. The ACN gradient used throughout is indicated in Fig 4 (i)(a).

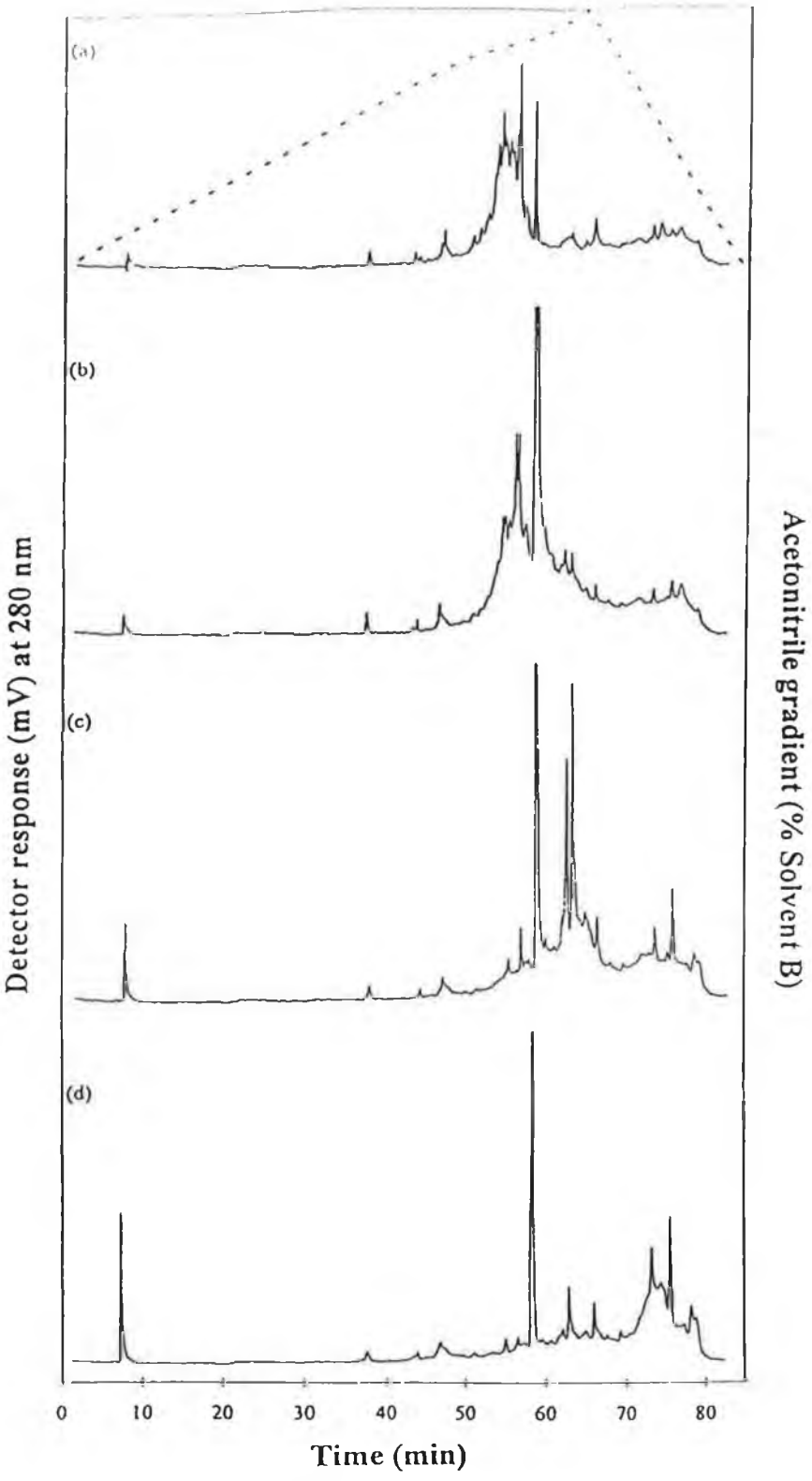
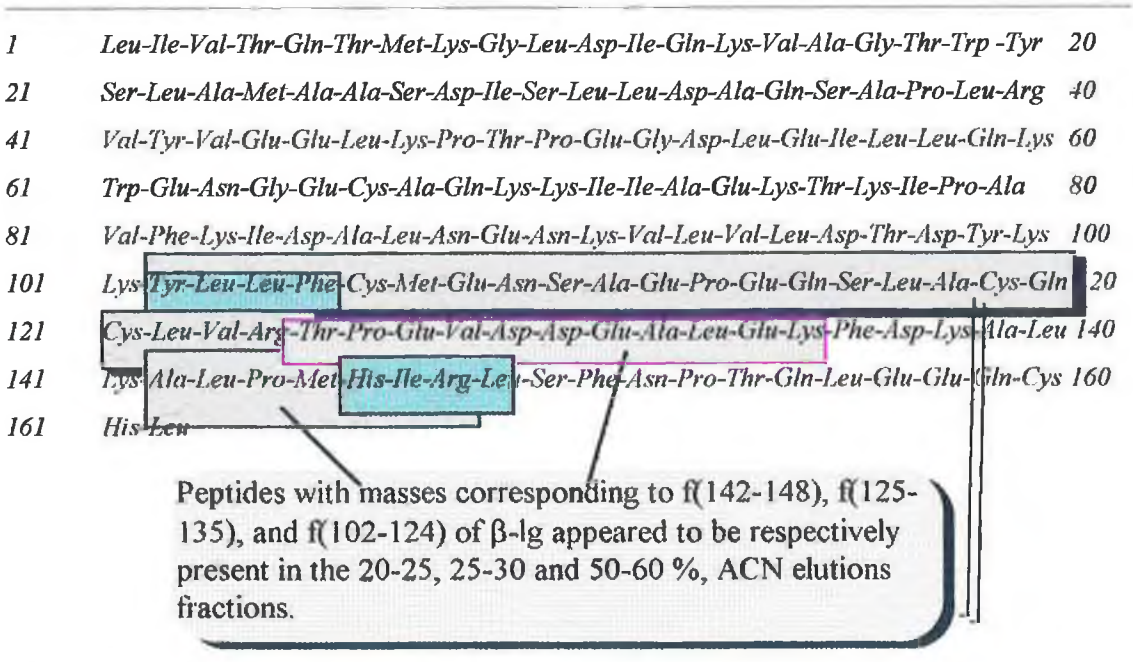


Figure 4 (ii): Reversed Phase-HPLC (C_{18}) chromatograms of the peptides in the solid phase extraction fractions corresponding to (a) 30-35 % ACN elution (b) 35-40 % ACN elution (c) 40-50 % ACN elution and (d) 50-60 % ACN elution. The ACN gradient used throughout is indicated in Fig 4 (ii)(a).

Figure 5: Amino acid sequence of bovine β-lactoglobulin variant B and positions of potential bioactive fragments as indicated using mass spectroscopy.



(from Hill, 1994); variant A Asp [64] Val [118]; variant B (as shown). Shaded ▬ indicates peptide sequences previously characterisated as having opioid/muscle acting peptides.

Table 6: Indices of ACE inhibition (%) corresponding to various sequential acetonitrile elutions from solid phase extraction of a tryptic hydrolysate of β-lactoglobulin

Acetonitrile elution (%)	ACE inhibition indices [a] (%)
1	41.2
20	62.5
25	96.9
26	91.0
27	95.2
28	76.2
29	73.0
30	66.9
35	91.9
40	63.1
50	49.0
51	28.5
52	51.0
53	23.6
54	8.6
55	10.2
56	7.6
57	1.2
58	18.9
59	4.1
60	7.9

a - 50 µl of 10 mg/ml solution of hydrolysate (8% protein) was taken for ACE inhibition determination; (method as described Meisel, 1992)

Table 7: Expected peptide masses corresponding to tryptic fragments of β-lactoglobulin.

Peptide number	Fragment	Expected peptide masses
1	1-8	933.17
2	9-14	672.18
3	15-40	2708.07
4	41-60	2313.67
5	61-69	1063.13
6	70-70	146.19
7	71-75	572.70
8	76-77	247.29
9	78-83	673.85
10	84-91	916.00
11	92-100	1065.23
12	101-101	146.19
13	102-104	2645.05
14	125-135	1245.30
15	136-138	408.45
16	139-141	330.43
17	142-148	837.05
18	149-162	1657.83

In Figure 1, Appendix A, a peptide, giving a charge to mass ratio of 838.4 was observed and this approximates to the theoretical mass (Table 7) of f(142-148) of the β-lg sequence. Figure 2, Appendix A shows a peptide which gave a charge to mass ratio of 1201.4. This peptide may correspond to f(125-135) of the β-lg sequence as indicated in Table 7 and Figure 5. Figure 3, Appendix A, shows a peptide eluting between 50-60 % ACN, which had a charge to mass ratio of 2710.4 and this peptide may correspond to f(102-124), as indicated in Table 7 and Figure 5. The peptide, f(142-148), which was found to be prominent in the fraction eluting between 20-25 % ACN (Figure 1, Appendix A) was synthesised and used for further studies.

4.4.3.4 ACE inhibition-IC_{50} values

In order to obtain an accurate value for the ACE inhibitory potency for a given hydrolysate sample/fraction or peptide it is necessary to generate an activity curve and to calculate precisely an IC_{50} value. A typical activity curve for the determination of an IC_{50} value is given in Figure 6 (courtesy of Dr. H. Meisel). This curve was generated for the peptide material eluting between 20-25 % ACN for a tryptic hydrolysate of β-lg. The IC_{50} value was calculated as 159.8 mg/l. In the same way, an hydrolysate produced from WPC using PTN 3.0S (240 min, Table 5), and a peptic hydrolysate of the enriched β-lg fraction (240 min, Table 3) gave IC_{50} values of 374.0, and 318 mg/l, respectively. The synthetic peptide fragment corresponding to f(142-148) of β-lg gave an IC_{50} value of 42.6 μmol/l.

4.4.5 Treatment of β-lg f(142-148) with peptic and chymotryptic activities

The synthetic peptide corresponding to β-lg f(142-148) was incubated with pepsin and chymotrypsin. Figure 7 (i) gives peptide profiles at 280 nm and Figure 7 (ii) gives peptides profile at 214 nm. Figure 7 (i) and (ii), (a) gives the RP-HPLC profile for peptide f(142-148) of β-lg. Figure 7 (i) and (ii), (b) gives the profile for chymotryptic control which had been incubated at 50 °C for 45 min. Figure 7 (i) and (ii), (c) gives the profile for the chymotryptic incubation with the peptide at 50 °C for 45 min. Figure 7 (i) and (ii), (d) gives the profile for the pepsin control which was incubated at 50 °C for 45 min. Figure 7 (i) and (ii), (e) gives the profile for the pepsin

incubation with the peptide under the same reaction conditions. It appeared from reversed phase HPLC profiles obtained at 280 nm that little or no hydrolysis of f (142-148) occurred using chymotrypsin (Figure 7 (i) a, b and c) or pepsin (Fig 7 (i) a d and e). The profiles produced by pepsin and chymotrypsin alone following incubation at 50 °C for 45 min, revealed the existence of peptide material due possibly to autodigestion of the proteinases. Profiles at 214 nm (Figure 7 (ii), a-e) however, appear to indicate that there is low degrees of hydrolysis by chymotrypsin of f(142-148) and there appears to be no hydrolysis of this peptide by pepsin.

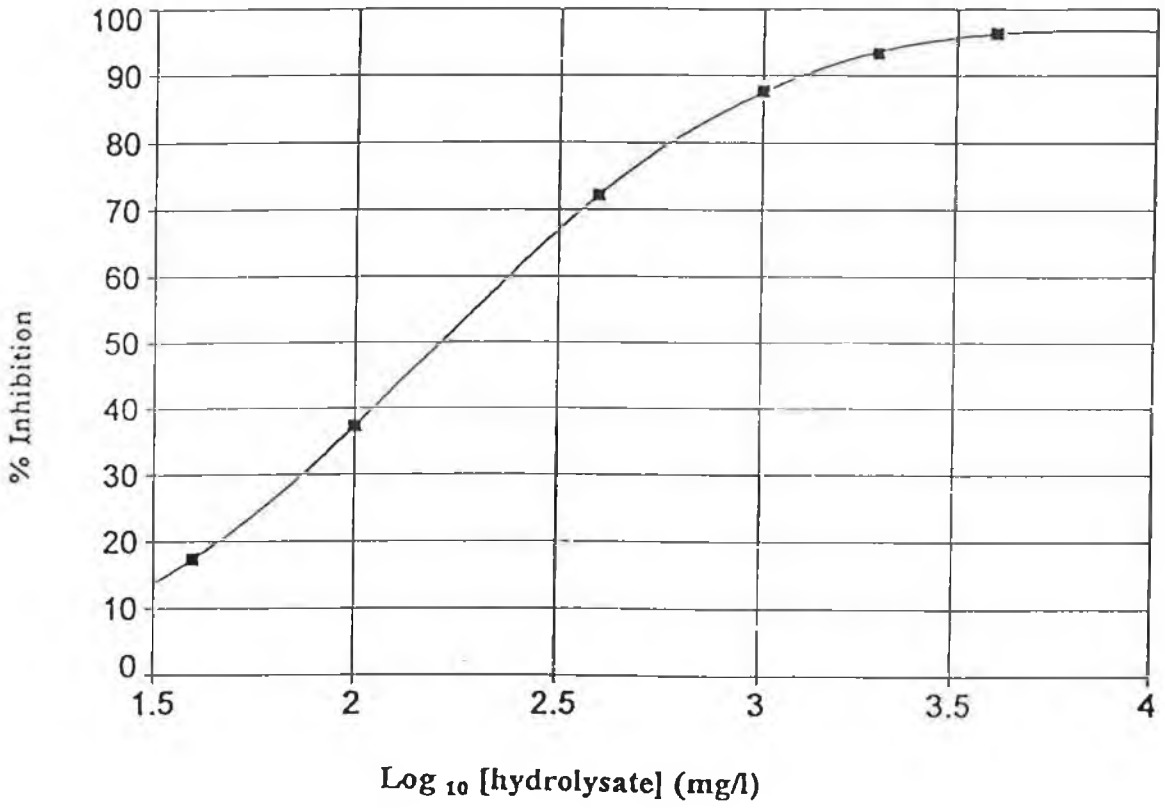


Figure 6 : ACE inhibition curve of peptides eluted between 20-25 % ACN of a tryptic hydrolysate of β-lactoglobulin, IC_{50} = 159.8 mg /l (courtesy of Dr. H Meisel).

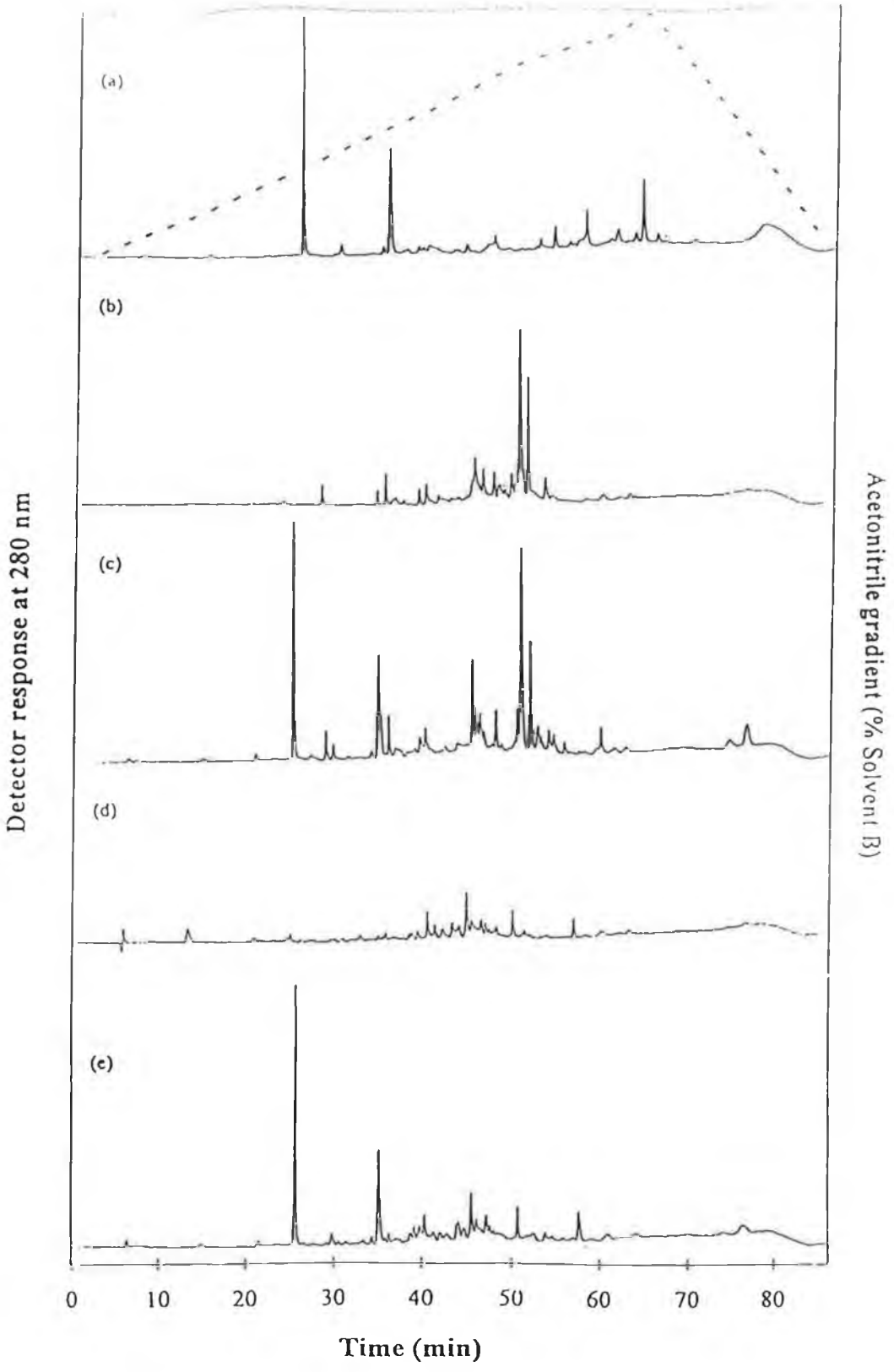


Figure 7 (i): Reversed Phase HPLC (C_{18}) chromatograms detected at 280 nm of (a) peptide f(142-148) of β-lactoglobulin (b) Chymotrypsin (c) Chymotryptic incubation with peptide (d) Pepsin and (e) Peptic incubation with peptide Gradient elution used throughout is indicated in (a).

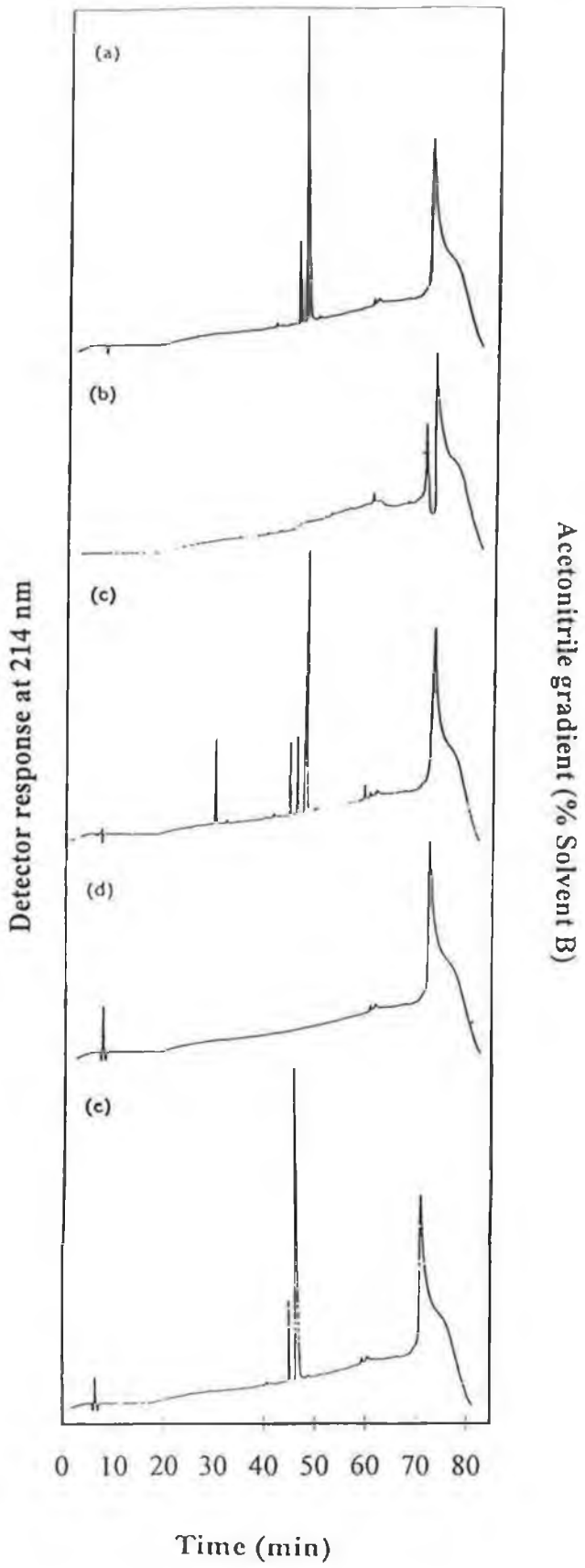


Figure 7 (ii): Reversed Phase HPLC (C_{18}) chromatograms detected at 214 nm of (a) peptide f(142-148) of β-lactoglobulin (b) Chymotrypsin (c) Chymotryptic incubation with peptide (d) Pepsin and (e) Peptic incubation with peptide Gradient elution used throughout is indicated in Figure 7 (i) (a).

4. 5 Discussion

4.5.1 Effect of hydrolysis by gastric and pancreatic proteinases on the release of ACE inhibitory activity.

Initial screening of a range of whey protein hydrolysates revealed that hydrolysis was necessary to release ACE inhibitory peptides from an inactive form within the sequence of the whey proteins. Low endogenous ACE inhibitory activity existed for the unhydrolysed whey protein substrate controls (Table 3, 4, and 5). It can be noted from Table 3, 4, 5, that DH values of 2-3 % can give rise to 70-90 % ACE inhibition indices. In addition, inhibitory activity levels of this magnitude could be produced on a larger scale, i.e. 10 l hydrolysate, as was seen in the hydrolysis of WPC by a pancreatic proteinase preparation, PTN 3.0S, which at DH values of < 5 % resulted in a high level of ACE inhibition, i.e. > 70 % inhibition (Figure 3).

It was not unexpected that heat treatment of the whey protein substrates resulted in higher percentage degrees of hydrolysis, particularly in the case of β-lg. It is known that heat treatment causes protein unfolding and reduction of disulphide bonds thereby making proteins more susceptible to proteolytic attack (Reddy *et al.*, 1988; Mihalyi, 1978). In Chapter 5, it is shown using differential scanning calorimetry that temperature and pH influence the conformation of β-lg and therefore the susceptibility of this protein to hydrolysis. However, heat-treatment did not appear from our initial screening of all the whey protein hydrolysates to have a detrimental effect on the evolution of ACE inhibitory activity.

Furthermore, sequential hydrolysis with combinations of pepsin and trypsin or trypsin and pepsin increased the DH values obtained after 240 min in comparison to the values obtained for the individual proteinases. However, this increase in DH did not appear to affect the indices of ACE inhibition in the resultant hydrolysates. Combinations of enzymes have been used previously to mimic *in vivo* digestion in the evolution of bioactive peptides (Antila *et al.*, 1991; Pihlänto-Leppälä *et al.*, 1994a, b). It was seen from this study, that while the inclusion of pepsin in addition to trypsin, increased the final DH value, it did not appear to affect the evolution of ACE inhibitory peptides. For example, the evolution of f(50-53) from α-la was unaffected when

pepsin was used to hydrolyse the protein before trypsin (Antila *et al.*, 1991). In addition, with the exception of β-lg, the order of usage of individual proteinases did not appear to have any great effect on the final DH values obtained (240 min) when pepsin (120 min hydrolysis) followed by trypsin (120 min hydrolysis) were each presented to the native or denatured whey protein enriched fractions. Although as expected, β-lg appeared to be more resistant to peptic hydrolysis, the treatment of this substrate with pepsin prior to hydrolysis with trypsin did increase the final DH after 240 min. Pepsin treatment has been shown to make the β-lg molecule more susceptible to enzymatic hydrolysis with other proteinases (Porter *et al.*, 1984; Antila *et al.*, 1991).

Corolase PP which contains more than two major proteinase activities (Mullally *et al.*, 1994), hydrolysed the whey protein fractions to a greater extent than PTN 3.0S. The final DH found in all cases showed that a combination of proteinases always produces an hydrolysate with higher final DH values than individual proteinases over the same time (Table 3, 4 and 5).

Overall, the apparent indices of ACE inhibition did not appear to be affected by the use of enzyme combinations, the order of usage of enzymes, or commercial preparations such as PTN 3.0S and Corolase PP. However, the molecular mass distributions of whey protein hydrolysates are greatly dependent on the proteolytic activities used (Mullally *et al.*, 1994). It appeared, that DH could not be directly correlated to indices of inhibition, as low degrees of hydrolysis for one particular substrate/enzyme combination produced inhibition of apparently similar potency to the peptides from a hydrolysate of higher DH. For example, the peptic hydrolysis of β-lg, having a low DH (i.e. 2.69 %) released peptides which had a higher ACE inhibition index than an hydrolysate of β-lg produced using PTN 3.0S (DH of 11.23 %), as seen in Table 3. The ACE inhibition index for the peptic hydrolysate was 92.7 % whereas the index for the PTN 3.0S hydrolysate was 87.6 %. It would be reasonable to assume that the types/size of individual peptides produced depends on the specificity of the proteinase first presented to the substrate. However, evidence of the importance of enzyme specificity was not seen during initial screening of the hydrolysates for indices of ACE inhibition, due perhaps to the complexity of peptides present in each hydrolysate.

4.5.1.1 ACE inhibition in peptide fractions of whey protein hydrolysates.

Solid phase extraction proved to be an efficient method of enriching for ACE inhibitory peptides (Table 6 and Figure 4 (i) and (ii)). Fractionation using solid phase extraction has been used previously as an enrichment step in the purification of casein derived bioactive peptides such as the β-casomorphins (Meisel, 1993a). Solid phase extraction, therefore, allowed for enrichment of peptides with high indices ACE inhibitory activity.

Overall, the IC_{50} values of the fractionated peptides were relatively high indicating a lower potency in comparison to synthetic drugs, such as Captopril (D-3-mercapto-2-methylpropyl-L-proline) which has an IC_{50} value of 0.006 μmol/l (Chapter 3).

However, the IC_{50} values found in this study for whey proteins are comparable to those found for casein hydrolysates (Table 1). The IC_{50} values of freeze-dried hydrolysate material would, however, reflect the total inhibitory effect of more than one peptide. A number of research groups have demonstrated the physiological significance of individual peptides or mixtures of peptides generated as a result of hydrolysis of casein (Maruyama *et al.*, 1987a, b; Sekiya *et al.*, 1992; Yamamoto *et al.*, 1994) or other food proteins such as fish (Suetsuna and Osajima, 1989), α-zein (Miyoshi *et al.*, 1991), sake (Saito *et al.*, 1994), or sour milk (Nakmura *et al.*, 1995b). It was shown that peptides from these sources have significant physiological effects when administered *in vivo*. The hypotensive effects of sake derived peptides were sustained for up to 30 h in murine models (Saito *et al.*, 1994). The hypotensive effect of fish hydrolysate was shown to be effective for up to 6 h after oral administration (Sugiyama *et al.*, 1991). In some studies, however, it was found that relatively high doses of the peptide were required to have an effect following oral administration (Karaki *et al.*, 1990; Yamamoto *et al.*, 1994) in comparison to synthetic drugs. It has been shown that peptides with molecular masses of 1-2 kDa obtained by enzymatic hydrolysis of sardine muscle have a hypotensive effect after oral administration (Suetsuna and Osajima, 1989). It has been stated that peptides/hydrolysates with IC_{50} values within the 100-500 μmol/l range could be physiologically active (Sekiya *et al.*,

1992). A summary of recent findings in relation to physiologically active ACE inhibitory food protein hydrolysates is given in Table 8.

The source of ACE used in this study was from rabbit lung tissue. It is known, that care should be taken when comparing degrees of potency or IC_{50} values, if different sources of ACE are used in determining the inhibition activities of peptides/hydrolysates (Aryioshi, 1993).

4.5.2 Proteinase specificity in the production of angiotensin I converting enzyme inhibitory activities - hydrolysis of β-lg f(142-148).

In order for peptides to exert a physiological effect, they must interact with ACE at various sites around the body. One of the first requirements for this physiological effect is that inhibitory peptides should be resistant to further proteolytic attack by digestive proteinases.

The synthetic fragment of β-lg f(142-148), shown to be present in a tryptic digest of β-lg was incubated in the presence of pepsin and chymotrypsin, in order to investigate the possible formation of further hydrolysis products. It is known that bioactive peptides are often resistant to further degradation by gastric or pancreatic proteinases (Zioudrou *et al.*, 1979; Henschen *et al.*, 1979) as is the case with β-casomorphins (Meisel, 1986). It was therefore of interest to investigate the susceptibility of f(142-148) to further digestion by pepsin and chymotrypsin. Pepsin is a broad spectrum proteinase and possible cleavage sites in β-lg have been suggested by Maeda *et al.* (1987). However, it appeared from C_{18} reversed phase HPLC analysis that no hydrolysis of f(142-148) occurred using pepsin. Chymotrypsin is specific for Tyr-X, Phe-X and Trp-X bonds and is less active against Leu-X, Met-X and Ala-X bonds (Ardelt and Laskowski, 1985). It was seen from RP-HPLC peptide profiles at 214 nm that there was a low degree of hydrolysis of f(142-148) by chymotrypsin. The low degrees of hydrolysis was indicated by two 'extra' peaks in the peptides profile (Figure 7 (ii), c). However, the intensity of the peaks corresponding to the synthetic peptide changed only slightly, indicating a low degree of hydrolysis of f(142-148) with chymotrypsin. There may have been partial autolysis of pepsin and chymotrypsin at 50 °C for 45 min, as these control samples (Figure 7 (i) (b) and (d) respectively), revealed

Table 8: Examples of food derived protein hydrolysates shown to reduce hypertension in animal and human models.

Source	Enzyme	Material	Animal model	Administration format	Reference
Casein	Trypsin	Peptide [b]	SHR	Intravenous	Maruyama *et al*., 1987b
	Trypsin	Hydrolysate	human	Oral [a]	Sekiya *et al*.,1992
	L.helveticus protease	Peptide [d]	SHR	Oral	Yamamoto *et al*.,1994
	L.helveticus protease	Peptide [e]	SHR	Oral	Yamamoto *et al*.,1994
	Trypsin	Hydrolysate	SHR	Oral	Karaki *et al*.,1990
Fish	Alkaline proteinase	Hydrolysate	SHR	Intravenous [c]	Suetsuna and Osajima, 1989
	Alkaline proteinase	Hydrolysate	rabbit	Intravenous	Suetsuna and Osajima, 1989
	Alkaline proteinase	Hydrolysate	SHR	Oral	Sugiyama *et al*.,1991
α-zein	Thermolysin	Peptide	rabbit	Intravenous	Miyoshi *et al*.,1991
Calpis sour milk	*L. helveticus* protease	Peptide	SHR	Oral	Nakamura *et al*.,1995b
	L. helveticus protease	Peptide	SHR	Oral	Nakamura *et al*.,1995b
Sake or Sake lees	Pepsin or pancreatin	Hydrolysate	SHR	Oral	Saito *et al*.,1994

SHR- Spontaneously hypertensive rats; a-oral administration; b- synthetic peptides were administered which corresponded to peptides produced in hydrolysates; c-effect was dose dependent; d- αs_1-casein; e- β-casein.

the presence of peptide material which was detected at 280 nm. The tryptic peptide Ala-Leu-Pro-Met-His-Ile-Arg has sites which would potentially be susceptible to hydrolysis by chymotrypsin. It would be expected that the peptide chain length (i.e. 7 amino acid residues) would be too short for the further hydrolytic action by endoproteinases, e.g. chymotrypsin. However the hydrolysis conditions (as used in the experiment) with an E:S ratio of 1:60 may have forced the hydrolysis of f(142-148) by chymotrypsin. The question remains as to whether the chymotryptic hydrolysis products of f(142-148) are more or less potent in the inhibition of ACE than the parent peptide.

There appeared to be no hydrolysis of f(142-148) by pepsin. It might be concluded that this peptide would be resistant to further hydrolysis in the digestive tract, prior to absorption in the gut. The presence of proline in the sequence of this peptide may also contribute to its resistance to hydrolysis. Proline rich peptides are known to be resistant to enzymatic hydrolysis (Henschen *et al.*, 1979; Brantl *et al.*, 1981; Saito *et al.*, 1994).

It is therefore evident from these findings that the specificity of the proteinases first presented to the substrate will dictate the type and potency of the resulting bioactive peptides formed. In the study by Pihlänto-Leppälä *et al.* (1994a, b), the peptide fragment corresponding to His-Ile-Arg-Leu was formed from β-lg by the action of chymotrypsin. However, His-Ile-Arg did not appear to be released in the present study (as shown by C_{18} RP-HPLC analysis) by the action of chymotrypsin on the tryptic peptide Ala-Leu-Pro-Met-His-Ile-Arg. In general, knowledge of the specificity of the enzyme and information on the sequence of the substrate is sufficient to predict the sequence of peptides formed, only when using purified substrates and enzymes. However, it has been shown *in vitro* that cleavage may occur at sites normally not susceptible to the enzyme. In the production of casomorphins from β-casein, it has been shown that trypsin cleaves the Asn-Ser bond (Meisel, 1992). In the present study on hydrolysis of f(142-148), it was evident that the type of peptides produced in the hydrolysis of β-lg is dependent on the sequence of addition of the enzymes to the substrate. When a number of enzymes are working in conjunction with each other, products which are not predicted may occur. This should be particularly evident where

commercial pancreatic proteinase preparations are used to generate whey protein hydrolysates on a large scale for inclusion into various food products.

4.5.3 Structure-activity relationships and the importance of gastric and pancreatic proteinases in the production of biologically active peptides.

The multifunctional properties of certain peptide sequences has been discussed previously (Shimizu, 1994). A sequence within β–casein, f(60-70), is known as the 'strategic zone' for the production of bioactive peptides (Fiat *et al.*, 1993). There is an overlap of opioid, ACE inhibitory and immunomodulating activities within sequences derived from this fragment. For example, β-casomorphin f(60-64) was shown to have opioid agonist and ACE inhibitory activities (Brantl *et al.*, 1981; Meisel and Schlimme, 1994). In the present study, it was observed that certain peptide sequences, found to be present in the solid phase extraction elutions of the tryptic hydrolysate of β-lg, showed an overlap with other known bioactive sequences (Figure 5). It would be interesting to investigate the possibility of a so-called 'strategic zone' or 'zones' within the sequence of whey proteins. Two such potential 'zones' were seen in this study for β-lg. For example, the mass spectroscopy of the 50-60 % acetonitrile elution (Appendix A, Figure 3) gave a prominent peak which corresponded to f(102-124). This peptide showed an overlap with the opioid peptide, β-lactorphin f(102-105), as seen in Figure 5.

It was also noted that the 'zone' corresponding to f(142-149) had overlapping bioactivies. The tryptic fragment f(142-148) which was prominent in the analysis of the 20-25 % ACN elution showed some overlap with the sequence of β-lactotensin, which is a muscle-acting peptide produced using chymotrypsin and combinations of chymotrypsin and pepsin (Pihlänto-Leppälä *et al.*, 1994a, b). The IC_{50} values of β-lactotensin (His-Ile-Arg-Leu) and related dipeptides Ile-Arg and Arg-Leu were 1153.2, 695.5 and 2438.9 respectively (Chapter 3). The IC_{50} value of the peptide corresponding to f(142-148) with the sequence Ala-Leu-Pro-Met-His-Ile-Arg was more potent (IC_{50} = 42.6 μmol/l) than β-lactotensin, and a related dipeptide (Ile-Arg) corresponding to the C-terminal dipeptide, which gave an IC_{50} = 695.5 μmol/l. Table 9

summarises the recent findings in relation to overlap of sequences of novel ACE inhibitory peptides from this study and Chapter 3.

The structure-activity relationship of ACE-inhibitory peptides has been discussed by an number of groups (Meisel, 1993a, b; Saito *et al.*, 1994). It has been suggested that peptides with hydrophobic amino acids at the C-terminus act as potent ACE inhibitors i.e., those amino acids which interact with the active site. Cheung *et al.* (1980) concluded that the C-terminal dipeptide fragment of peptides was important in inhibition of ACE. This theory would be supported by the fact that ACE is a carboxydipeptidase. The results of a previous study (Chapter 3) appeared to concur with this theory. It was shown that dipeptides related to β-lactotensin, α-lactorphin and β-lactorphin were more potent than the parent tetrapeptides. However, ACE also displays peptidyl tripeptidase and endopeptidase activity on some peptides, such as substance P and luteinizing hormone-releasing hormone (Erdös and Skidgel, 1989). In addition, it has been reported that ACE showed high specificity for the amino acid residue at position 3 from the C-terminal end of peptides, but showed little specificity for amino acids at position 4 (Oshima *et al.*, 1979). It has also has been suggested that the C-terminal tripeptide may by important in the inhibition of ACE (Maruyama *et al.*, 1987b). The results of the present study would contradict some of the suggestions that the C-terminal dipeptide dictates inhibitory potency. It might be suggested, however, that the proline residue may contribute to the potency of f(142-148). Proline residues were shown to contribute to the potency of ACE-inhibitory peptides from food protein sources (Saito *et al.*, 1994; Yamamoto *et al.*, 1994; Nakamura *et al.*, 1995a, b). In addition, the first potent ACE inhibitors, reported from snake venom, also had proline in their C-terminal sequence (Ondetti and Cushman, 1982). It was seen that f(142-148) which has proline in its sequence is more potent than related peptides (Table 9). Different conformational states of peptides must also be considered. For example, bradykinin has an inverse γ-turn conformation around Pro7 (Meisel, 1992) and this may be significant in ACE inhibitory activity.

The DH produced in the generation of ACE inhibitory peptides may be important from the point of view of efficient absorption of the peptides. Peptide chain length is important as it is believed that peptides can be absorbed intact and enter the

circulation (Gardner, 1988; Silk and Grimble, 1992). In the case of di- and tri-peptides, these may be transported into the intestinal epithelial cells via specific peptide transporters (Webb, 1990; Fei *et al.*, 1994).

Several reports exist on the physiological significance of protein hydrolysates from different sources in the reduction of hypertension and these are summarised in Table 8. Further studies on human subjects is obviously required to fully elucidate the physiological significance of the ingestion of food protein hydrolysates/peptides containing ACE inhibitory activity. The fact that the whey protein hydrolysates developed in this study have lower ACE inhibitory potencies than the synthetic antihypertensive drug Captopril (IC_{50} = 0.006 μmol/l) does not negate against the application of whey protein derived peptides in the treatment/prevention of hypertension. It is to be expected that milk protein derived ACE inhibitory peptides, unlike Captopril would have no undesirable side-effects.

As a consequence, whey protein derived ACE inhibitory peptides could find applications as a nutraceutical in various 'physiologically functional foods'.

Table 9: Summary of ACE inhibitory activity within β-lg sequence f(142-149).

Peptide sequence	Fragment	IC_{50} (μmol/l)[a]
Ala-Leu-Pro-Met-His-Ile-Arg	f(142-148)	42.6
His-Ile-Arg-Leu	f(146-149)	1153.2
Ile-Arg	f(147-148)	695.5
Arg-Leu	f(148-149)	2438.9

[a] IC_{50} is defined as that amount of peptide which causes 50 % inhibition of ACE using hippuryl-histidyl-leucine as substrate.

Appendix A

Mass spectroscopy profiles obtained from Dept Biochemistry, University of Nottingham, UK.

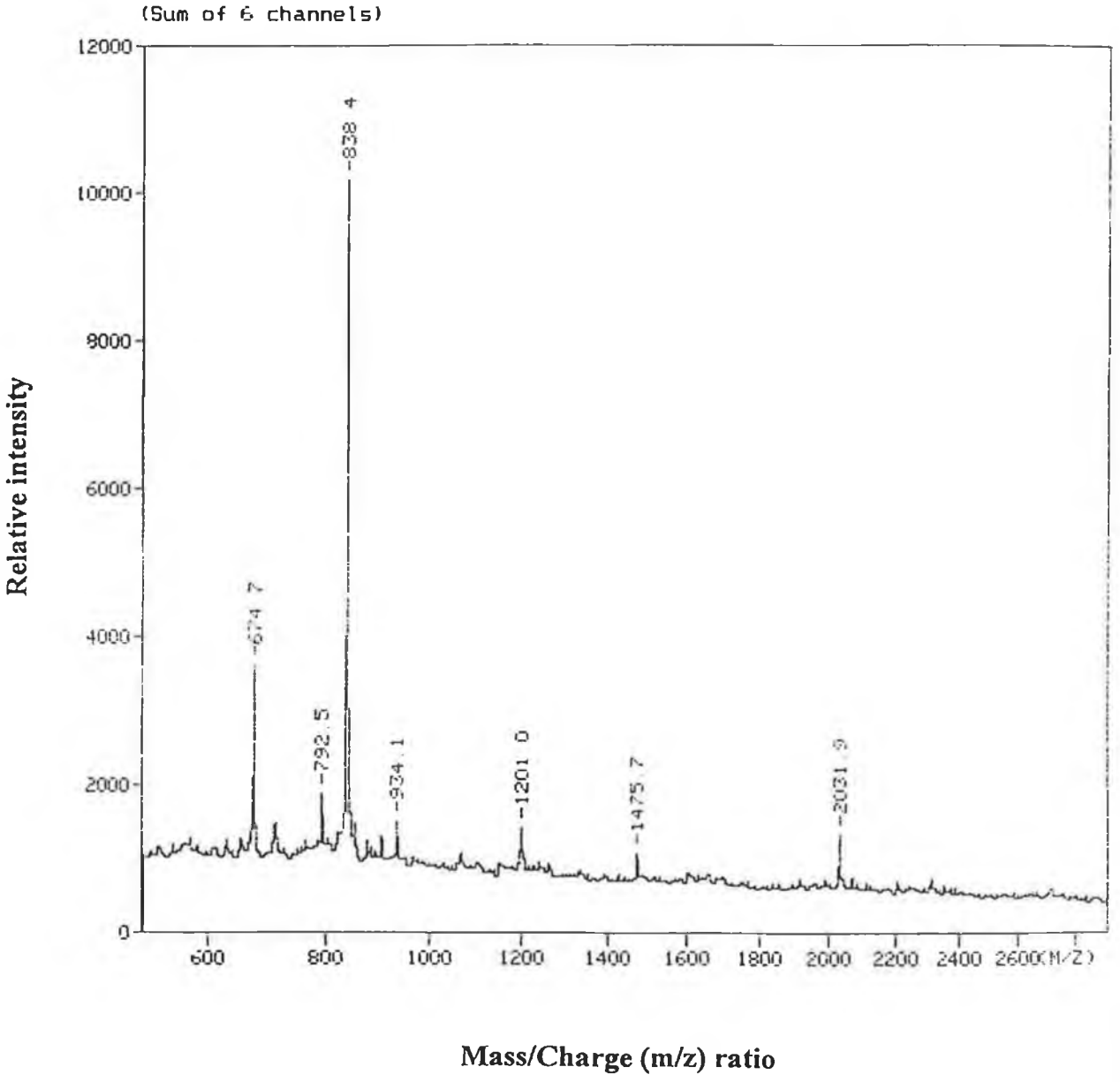


Figure 1: Mass spectroscopy profile for the 20-25 % acetonitrile elution obtained from a solid phase extraction of a tryptic hydrolysate of β-lactoglobulin.

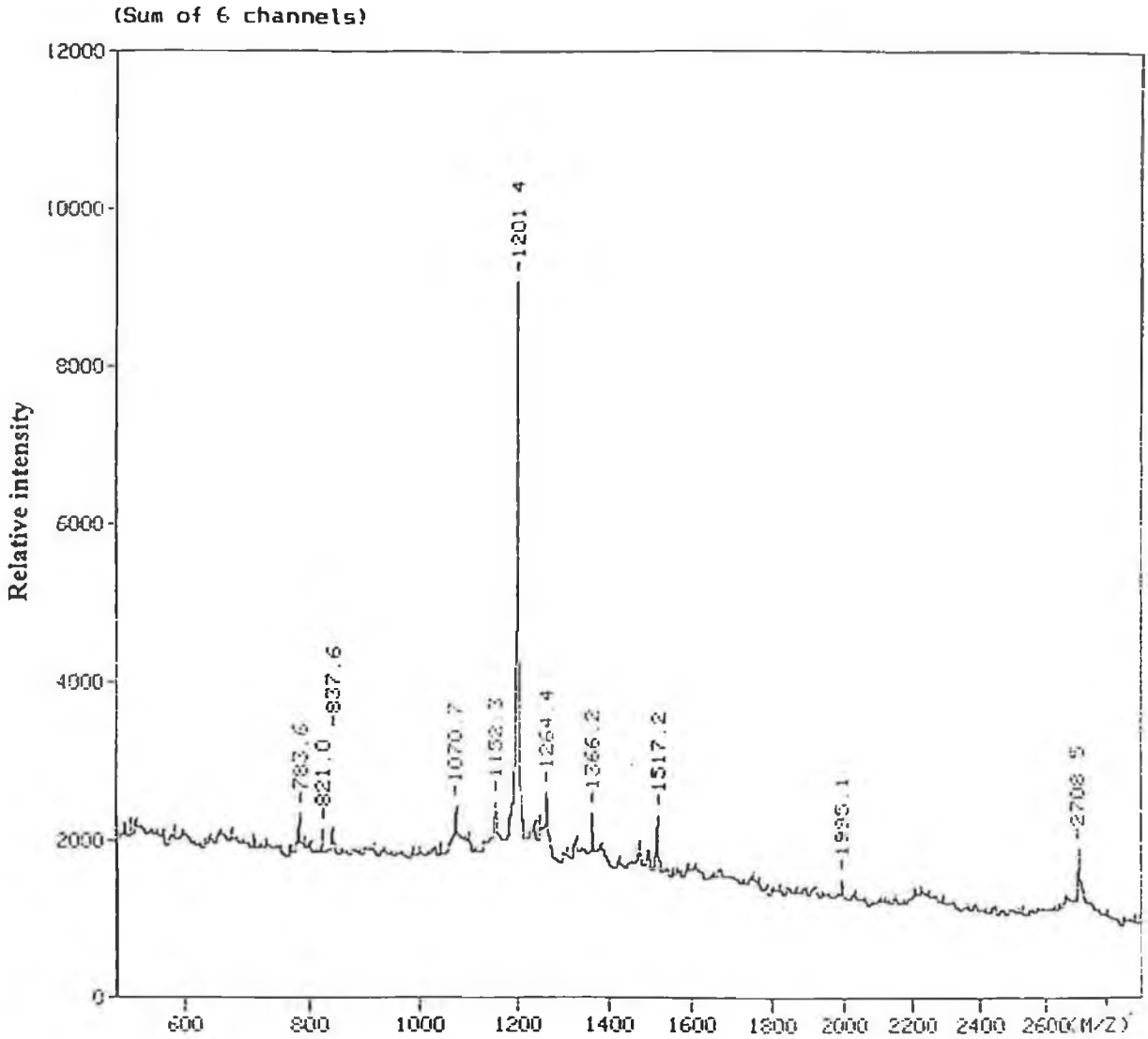


Figure 2: Mass spectroscopy profile for the 25-30 % acetonitrile elution of a tryptic hydrolysate of β-lactoglobulin.

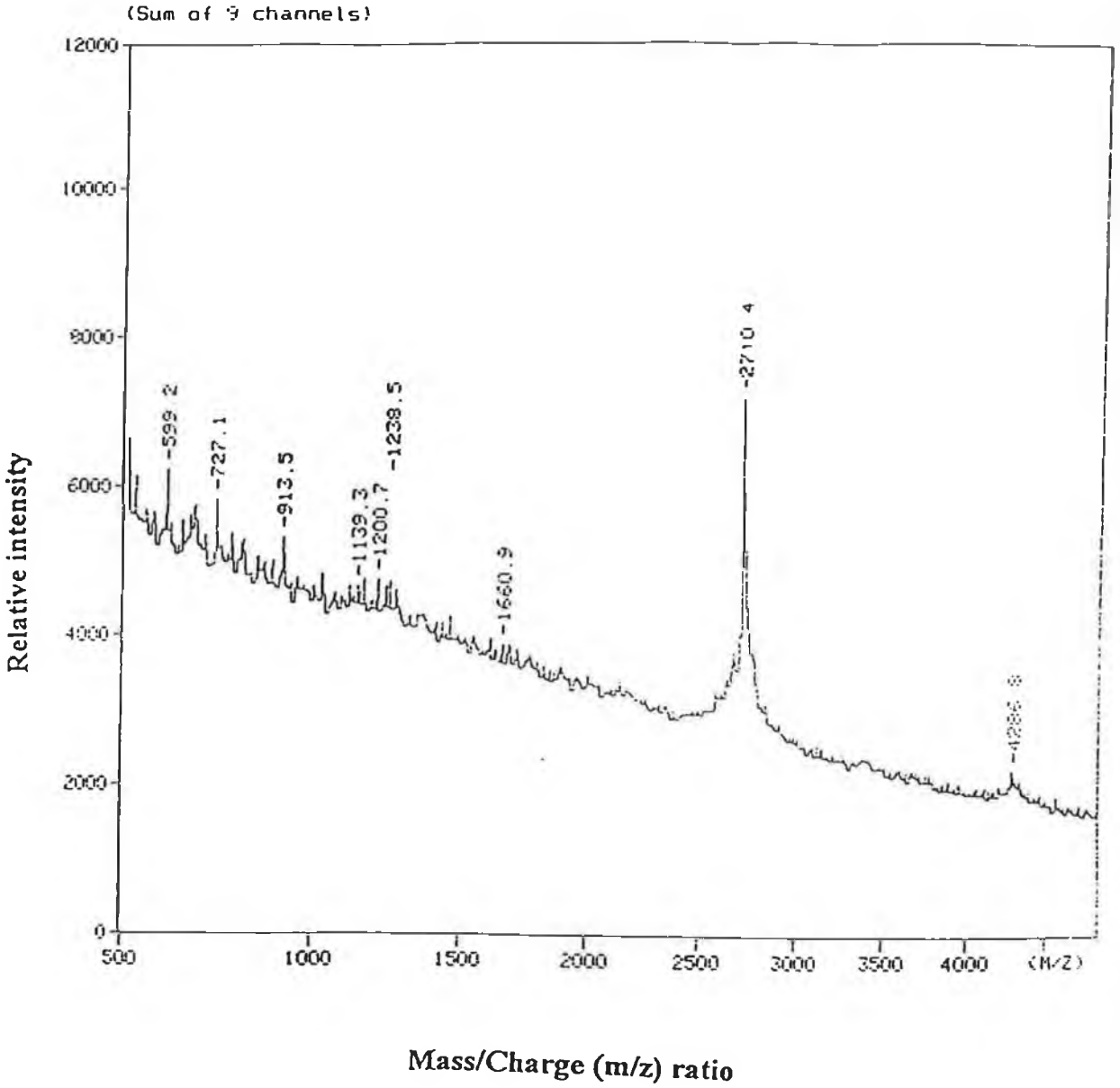


Figure 3: Mass spectroscopy profile for the 50-60 % acetonitrile elution of a tryptic hydrolysate of β-lactoglobulin.

CHAPTER 5

Temperature and pH effects on the conformation and susceptibility of β-lactoglobulin to enzymatic hydrolysis.

5.1 Summary

A protein fraction enriched in bovine β–lactoglobulin (β–lg) variants AB was hydrolysed with pepsin, trypsin, chymotrypsin and elastase activities. In addition, this fraction was hydrolysed with combinations of trypsin and pepsin activities. Hydrolysis was also performed with two commercially available enzyme preparations, i.e. Corolase PP and PTN 3.0S. Peptic hydrolysis was performed at pH 3.00 while hydrolysis with other enzymes was at pH 8.00. In general, the hydrolysis reaction temperature was 50 °C. The enriched β-lg preparation was also heat treated (80 °C for 20 min) prior to hydrolysis with pepsin or trypsin and or with combinations of these enzymes. As expected, the extent of the reaction during the first 20 min of hydrolysis, also referred to as the initial rate (Δ DH/min), and the final degrees of hydrolysis (DH) after 240 min incubation obtained were higher at 50 °C than at 37 °C.

The final DH value obtained after 240 min incubation at 50 °C for a peptic hydrolysate of the native enriched β-lg was lower (i.e. 4.03 %) than for hydrolysates obtained with pancreatic enzymes, i.e. trypsin, chymotrypsin and elastase (6.52, 7.64 and 5.50 % respectively). Combinations of pepsin and trypsin gave higher degrees of hydrolysis after 240 min incubation at 50°C than the individual enzymes. The highest degree of hydrolysis of native β-lg was obtained using the commercial proteinase preparations, Corolase PP and PTN 3.0S. The complexity of the RP-HPLC profiles and the apparent degree of hydrophobicity of the various hydrolysates could be related to the DH values obtained. As the number of enzymatic activities was increased for a given hydrolysis reaction, the appearance of peptides with less hydrophobic characteristics was evident.

Differential scanning calorimetry (DSC) was used to determine the effect of heating on the structure and stability of the enriched β-lg preparation. The DSC thermograms showed that the β-lg preparation had a higher thermal/structural stability at pH 3.00 than at pH 8.00, i.e. pH values which are suitable for peptic or pancreatic hydrolysis reactions, respectively. The protein showed greater resistance to pepsin hydrolysis at pH 3.00 (DH = 2.69 % at 37 °C and 4.03 % at 50° C), due to greater structural and thermal stability of the protein at this pH (Tm = 82.98 C ± 0.62 °C).

Holding the native enriched fraction of β-lg at 50 °C for 120 min, appeared to affect the structural stability of the protein, even at pH 3.00 as shown by the appearance of shallow and diffuse DSC thermograms.

The structure of the β-lg substrate was removed completely after pre-heat treatment (80 °C for 20 min). Pre-heating greatly increased the susceptibility of an enriched fraction of β–lg to enzymatic hydrolysis by pepsin or trypsin. In addition, peptic hydrolysis of the heat-treated form of the enriched β-lg fraction allowed for more efficient hydrolysis, if subsequently followed by a tryptic hydrolysis reaction.

This study confirmed that the susceptibility of β–lg to hydrolysis by gastric or pancreatic enzymes is dependant on the pH and temperature used in the hydrolysis reaction.

5. 2 Introduction

β-Lg is a globular protein which represents approximately 50 % of the total whey protein in bovine milk. The monomer molecular weight is 18.4 kDa and the molecule contains two disulphide bridges and one free thiol group. At physiological pH, β-lg exists as a homodimer and has a molecular weight of 36.8 kDa (Godovac-Zimmermann and Brautnitzer, 1987). β-Lg has been extensively characterised both physically and chemically (McKenzie, 1971; Swaisgood, 1982; Eigel *et al.,* 1984; Hambling *et al.*, 1992).

Structural studies have revealed that β–lg belongs to a family of ligand binding proteins. It is known to bind fatty acids and retinol (Spector and Fletcher, 1970; Fugate and Song, 1980; Puyol *et al.,* 1991; Pérez *et al.,* 1992) and it is thought to have a biological role in the binding and digestion of fatty acids and/or in the transport of retinol (Pérez and Calvo, 1995). It was shown using DSC that palmitic acid and retinol binding to β-lg increases the thermal stability of the protein (Puyol *et al.,* 1994).

The effects of pH and temperature on β-lg unfolding and aggregation have been investigated (deWit and Swinkels, 1980; Park and Lund, 1984). β–Lg is known to have an unusual unfolding pattern and self association equilibria at different pH values. At pH values less than pH 3.5, β–lg is most stable in a monomer state (Mills and Creamer, 1975). Between pH 3.5 and 5.2, the dimer undergoes a progressive reversible tetramerization (Kumosinski and Timasheff, 1966). A transition, referred to as the 'Tanford transition' which involves a reversible conformational change at pH 7.5, causes the exposure of tryptophan and tyrosyl residues to solvent (Tanford *et al.*, 1959; Townend *et al.*, 1969; Zimmermann *et al.,* 1970). At values greater than pH 8.5, β-lg undergoes a polymerisation which progresses with time and which seems to be mainly associated with the oxidation of the sulphydryl groups (McKenzie and Sawyer, 1967). It has been suggested that this transition in ß-lg structure associated with pH changes is reflected in its susceptibility to proteolysis (Monnot and Yon, 1964). Heat treatment causes dimer dissociation, denaturation of the monomer with the exposure of sulphydryl groups and subsequent polymer formation (Caligant *et al.,* 1991). When the disulphide groups are reduced, the protein becomes more susceptible to enzymatic

attack (Otani, 1981; Reddy *et al.,* 1988). There has been a certain degree of controversy in the literature with regard to the kinetics of this denaturation process, i.e. as to whether it is first (deWit and Swinkels, 1980; Puyol *et al.,* 1994) or second order (Park and Lund, 1984; Relkin and Launay, 1990). Recently, a multi-state thermal unfolding and aggregation model for β–lg has been postulated (Gotham *et al.,* 1992; Lin Qi *et al.,* 1995)

Differential scanning calorimetry (DSC) has been used by several groups to study the thermal stability of β–lg or the susceptibility of this protein to enzymatic hydrolysis (deWit and Swinkels, 1980; deWit and Klarenbeek, 1984; Park and Lund, 1984; Paulsson and Dejmek, 1990; Puyol *et al.,* 1993; Chen *et al.,* 1993; Paulsson and Elofsson, 1994). For example, it has been shown using DSC, that β–lg containing bound fatty acids has an increased resistance to tryptic hydrolysis (Puyol *et al.,* 1993). Differences in the relative structural stabilities (Imafidon *et al.,* 1991; Huang *et al.,* 1994a, b) and/or susceptibility to enzymatic hydrolysis (Van Willige and FitzGerald, 1995) has been investigated for the A and B variants of β–lg. It was shown that β-lg A is more thermostable than β-lg B. Although this difference in thermostability is not as yet fully understood, it is thought to be due to differences in structure which may be ascribed to the substitution of Asp_{64} in β–lg A with Gly in β–lg B. It has also been shown, that limited enzymatic hydrolysis improves the gelling properties of the protein, by changing the denaturation characteristics of the protein as indicated using DSC (Chen *et al.,* 1993). In addition, the interaction with other milk proteins and the influence of ionic strength on gelling properties and thermal stability of β–lg have also been examined using DSC (Paulsson and Elofsson, 1994).

This study examined the thermal stability at pH 3.00 and 8.00 (using DSC) and susceptibility of β–lg to enzymatic hydrolysis by gastric and pancreatic proteinases.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Enzymes

Commercial pancreatic proteases were received as gifts from the manufacturers. Corolase PP (porcine) was supplied by Röhm GmbH, Darmstadt, Germany. PTN 3.0S (porcine) was supplied by Novo Nordisk A/S, Bagsvaerd, Denmark. Porcine trypsin (Type IX, 13,700 BAEE units/mg protein), bovine chymotrypsin (Type II, 40-60 BTEE units/mg protein), porcine elastase (Type I, 25-100 units/mg protein, one unit of elastase will solubilise 1 mg of elastin in 20 min at pH 8.0 at 37 °C) and porcine pepsin A, (3,200-4,500 units/mg protein, one unit will produce an dA_{280} of 0.001 per min at pH 2.0 and 37 °C for TCA soluble products of haemoglobin) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

5.3.1.2 Substrate

An enriched fraction of β–lactoglobulin (β–lg) variant AB was isolated according to the method of Maubois *et al.* (1987) on a pilot scale with some modifications. Details of the production and composition of the β–lg enriched fraction are given by Mehra (1994). The details of the composition of the β–lg enriched fraction was given in Chapter 4. The degree of denaturation of the enriched β–lg fraction was determined on the basis of solubility over a wide pH range (Mehra, 1994). The enriched β–lg fraction had approximately < 5 % denaturation (Mehra, 1994) and therefore was designated as a native fraction in this study. Purified bovine β–lg AB (3x crystallised and lyophilised) was obtained from Sigma Chemical Co. Poole, Dorset, UK. The degree of denaturation was not determined for the Sigma protein. All other reagents were of analytical grade unless otherwise specified.

5.3.2 Substrate pre-treatment

In certain instances β–lg was heat treated prior to hydrolysis by holding at 80 °C for 20 min.

5.3.3 Characterisation of whey protein substrates

5.3.3.1 Differential scanning calorimetry

DSC thermograms were recorded on a thermal analyser (model DSC 2910 TA Instruments, Surrey, England) using a thermal analyst 2000 system. Indium standards were used for temperature and energy calibrations of the DSC 2010 cell. Under the experimental conditions, the indium standard gave a melting point of 157.95 °C, and an enthalpy value of 45.85 J/g. Using these values, the instrument was calibrated and a baseline was established. The instrument was calibrated according to the manufacturers instructions (TA Instruments, Leatherhead, England). β–Lg solutions (8 % (w/v) protein) were adjusted to pH 3.00, using 1 N HCl, or to pH 7.00 or 8.00, using 0.5 M NaOH, then equilibrated for 20 min at both 37 or 50 °C. Samples (in the range of 12-18 mg of this solution) were sealed in aluminium pans (provided by the instrument manufacturers) and exact weights were recorded. The reference sample consisted of an identical empty pan. Protein samples were analysed using two different programs, as follows; (1) equilibration of sample for approximately 5 min at 50 °C followed by heating at a rate of 5 °C/min to 100 °C; and (2) equilibration at 50 °C for approximately 5 min, holding at this temperature for 120 min, followed by heating at a rate of 5 °C /min to 100 °C. The second program was set up in an attempt to mimic possible changes in β–lg conformation due to temperature alone over 120 min during the normal course of an enzymatic hydrolysis experiment. Overall enthalpies were calculated by numerical integration of the transition peak obtained from heat capacity versus temperature curves using the instrument manufacturers program. The temperature of maximum deflection from the baseline (known as the transition temperature), was observed when heat flow (dH/dt) is plotted as a function of the sample temperature. The transition enthalpy is a measure of the released heat during the change in protein conformation from the native to the denatured state. All reported values are the means of three independent replicates.

5.3.4 Preparation of hydrolysates

Hydrolysates were prepared according to the method described in Chapter 4 for laboratory scale whey protein hydrolysis with pepsin, trypsin and commercial preparations. (Chapter 4, Section 4.3.6.1). Details of DH calculation were given previously (Chapter 1 and 2). The enzyme-to-substrate (E:S) ratio, 0.003, was calculated on the basis of total protein content in the enzyme preparations and the substrate. The initial rate of change of DH over time (Δ DH/min) was taken as the extent of the hydrolysis reaction achieved following the first 20 min of each hydrolytic reaction. The standard deviation for hydrolysis reactions using this pH-stat system was determined previously to be DH ± 0.1 % (O'Callaghan, 1994).

5.3.5 Characterisation of β-lg hydrolysates

5.3.5.1 Reversed phase high pressure liquid chromatography (RP-HPLC)

Peptides in β-lg hydrolysates were separated by reversed-phase (RP) HPLC according to the method described in Chapter 4. The flowrate was 0.30 ml min^{-1} and the detector response (mV) at 280 nm was recorded. The injection volume was 50 μl and the concentration of the peptide applied was equivalent to a protein concentration of 0.1 mg/ml.

5.4 Results

5.4.1 Degrees of hydrolysis of native and heat-treated β–lg.

Native β–lg was hydrolysed with pepsin at 37 °C and 50 °C (Figure 1 and Table 1), giving DH values of 2.69 and 4.03 % respectively, after 240 min hydrolysis. At 50 °C, peptic hydrolysis of the Sigma fraction of β–lg gave a DH of 2.72 % after the same time. The degree of hydrolysis obtained by pepsin was relatively low, when using the native forms of β-lg, in comparison to the other proteinases (Table 1). In this study, trypsin, chymotrypsin and elastase showed high activity at 50 °C against β–lg with DH values after 240 min of 6.52, 7.64 and 5.50 % respectively (Table 1). For the heat-treated form of the enriched fraction of β-lg, however, higher DH values were obtained for the peptic hydrolysis reactions after 120 min, i.e. 5.34 % and after 240 min, i.e. 6.64 % (Table 1). The same was the case for a tryptic hydrolysis of a heat-treated fraction of β-lg which resulted in a DH value of 7.15 %.

In general, the native (i.e. unheated) form of the enriched fraction of β–lg, had lower DH values after 240 min than the heat treated forms, with the exception of the hydrolysis of native Sigma β–lg by trypsin which gave a DH value of 9.03 % after 240 min hydrolysis at 37 °C.

The initial rate of reaction for the hydrolysis of the enriched native β-lg fraction was slower using pepsin than for trypsin (Table 1). For example, at 50 °C the ΔDH/min value for pepsin was 0.12 versus 0.26 for trypsin. When heat-treated β-lg was used, smaller differences in the ΔDH/min were observed i.e. values of 0.13 for pepsin and 0.22 for trypsin (Table 1). There was a difference, however, in the final DH values obtained for peptic or tryptic hydrolysis after 240 min with heat-treated β-lg, i.e. 6.64 and 7.15 % respectively. No major differences in the DH in the first 120 min of the reaction was evident where pepsin (DH = 5.34 %) and trypsin (DH = 5.26 %) were used in different combinations for the heat-treated β-lg (Table 1, Figure 2 and 3).

The sequence of addition of pepsin and trypsin gave a small difference, i.e. 0.2 % in the total DH values (i.e. after 240 min) obtained for the hydrolysis of native β–lg at 50 °C (Table 1). It was noted that when pepsin was used to hydrolyse the protein for only 120 min, that low degrees of hydrolysis were obtained (i.e. ~ 2.4 %) for the

hydrolysis of native β–lg, regardless of the sequence in which this enzyme was used. However trypsin hydrolysis during the same time period gave a DH value of ~ 7.6 %. The total degree of hydrolysis (i.e. after 240 min) was higher when pepsin was used before trypsin in the hydrolysis of heat-treated β–lg (Table 1). The DH curves generated using pepsin followed by trypsin in the hydrolysis of native and heat-treated β-lg can be seen in Figure 2. It is clearly seen that the final DH values for the heat-treated β-lg were much higher than the corresponding values for the native β-lg. In general, the initial rate of reaction with trypsin was higher than for pepsin (Figure 2 and 3) and Table 1.

Figure 3 shows the DH curves for the hydrolysis of native and heat-treated β-lg when the trypsin was used before pepsin in the hydrolysis reaction. It is surprising to note that the final DH value was higher for the native as opposed to the heat-treated β-lg. The native and heat-treated β-lg gave similar types of hydrolysis curves on digestion with trypsin followed by pepsin (Figure 3).

The commercial pancreatic proteinases, which contain combinations of trypsin, chymotrypsin and elastase in differing ratios gave the highest DH values when presented to the native β-lg substrate. Corolase PP and PTN 3.0S, when used in the hydrolysis of the native β–lg gave higher DH values of 16.72 and 11.23 % respectively, than the individual proteinases, trypsin, chymotrypsin or elastase (6.52, 7.64, and 5.50 % respectively) after 240 min hydrolysis in all cases (Table 1).

It was seen that the higher temperature of 50 °C versus 37 °C increased the initial rate of reaction. For example, the initial rate (ΔDH/min) of hydrolysis of β-lg by trypsin at 37 °C was 0.14 versus 0.26 at 50 °C. In addition, the rate of hydrolysis of native β-lg using pepsin was higher at 50 °C than at 37 °C as seen in Figure 1 for the peptic hydrolysis of β-lg at these temperatures. The initial rate of β-lg hydrolysis was highest when using Corolase PP and PTN 3.0S, with ΔDH/min values of 0.50 and 0.38 respectively (Table 1).

Table 1: Native and heat treated β-lactogloublin, temperature of enzymatic hydrolysis, proteinases used, degrees of hydrolysis (DH%) obtained after 240 min (in case of one enzyme), after 120 min (in the case of two enzymes).

β-Lactoglobulin treatment	Hydrolysis Temperature (° C)	Proteinase	% DH (first enzyme)	% DH (second enzyme)	ΔDH/min
Native ß-lactoglobulin [a]					
	37	Pepsin	2.69	[c] -	0.06
	50	Pepsin	4.03	-	0.12
	37	Trypsin	5.69	-	0.14
	50	Trypsin	6.52	-	0.26
	50	Chymotrypsin	7.64	-	0.12
	50	Elastase	5.50	-	0.08
	50	PTN 3.0S	11.23	-	0.38
	50	Corolase PP	16.72	-	0.50
	50	Trypsin+pepsin	7.66	2.48	0.30 + 0.03
	50	Pepsin+trypsin	2.36	7.58	0.06 + 0.27
Heat treated ß-lactoglobulin [b]					
	50	Pepsin	6.64	-	0.13
	50	Trypsin	7.15	-	0.22
	50	Trypsin+pepsin	5.26	4.06	0.15 + 0.06
	50	Pepsin+trypsin	5.34	5.96	0.13 + 0.18

[a] β-lactoglobulin which was not pretreated prior to hydrolysis; [b] β-lactoglobulin which was heat - treated prior to hydrolysis (80 °C for 20 min); [c] - not applicable

Figure 1: Plot of the degree of hydrolysis (% DH) versus time (min) for the hydrolysis of native β-Lactoglobulin at 37 ^{0}C and 50 ^{0}C with pepsin

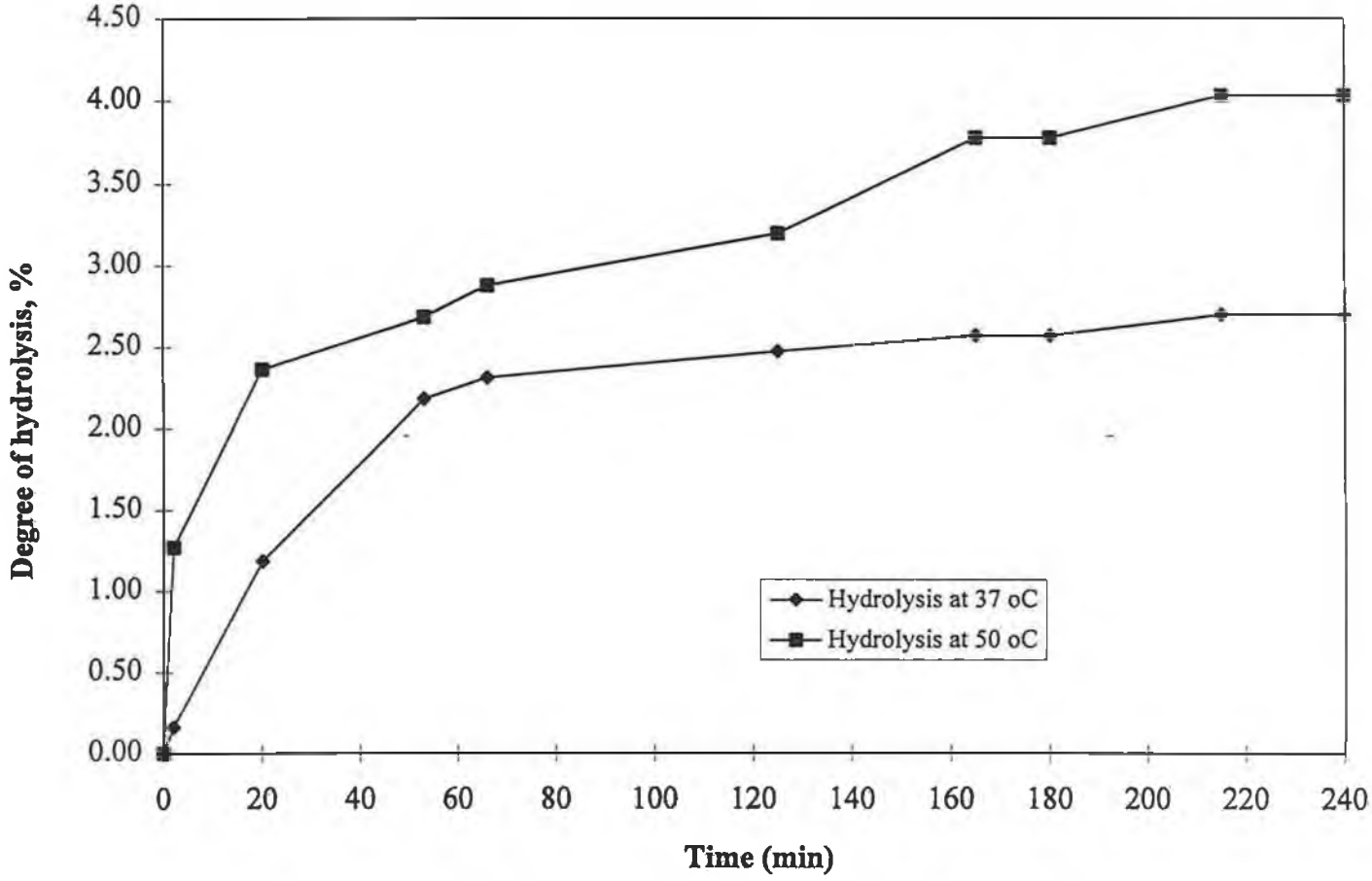


Figure 2: Plot of the degree of hydrolysis (% DH) versus time (min) for the hydrolysis of native and heat-treated β-lactoglobulin at 50 ^{0}C with pepsin and trypsin. The arrow marks the point of addition of the second enzyme.

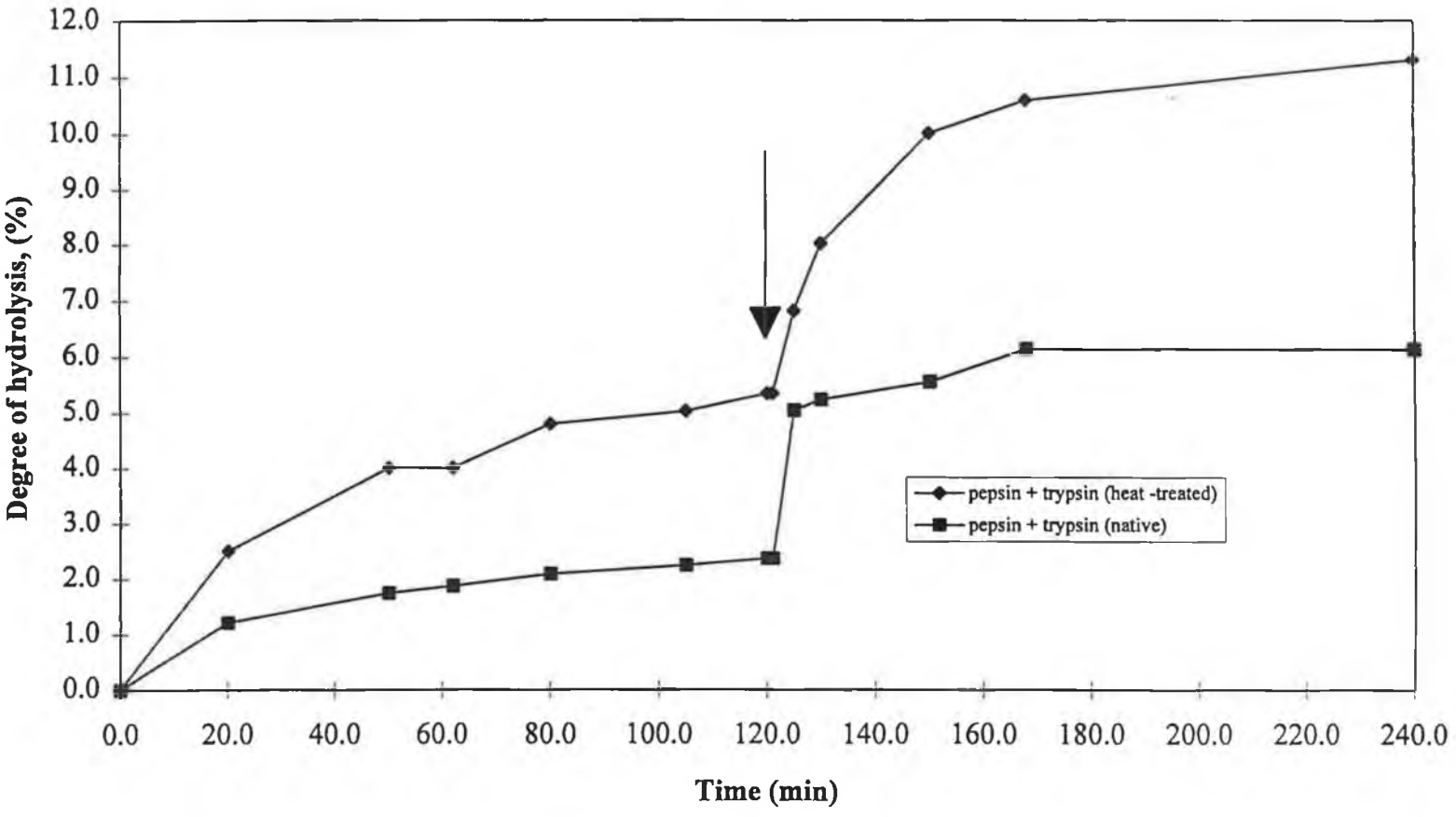
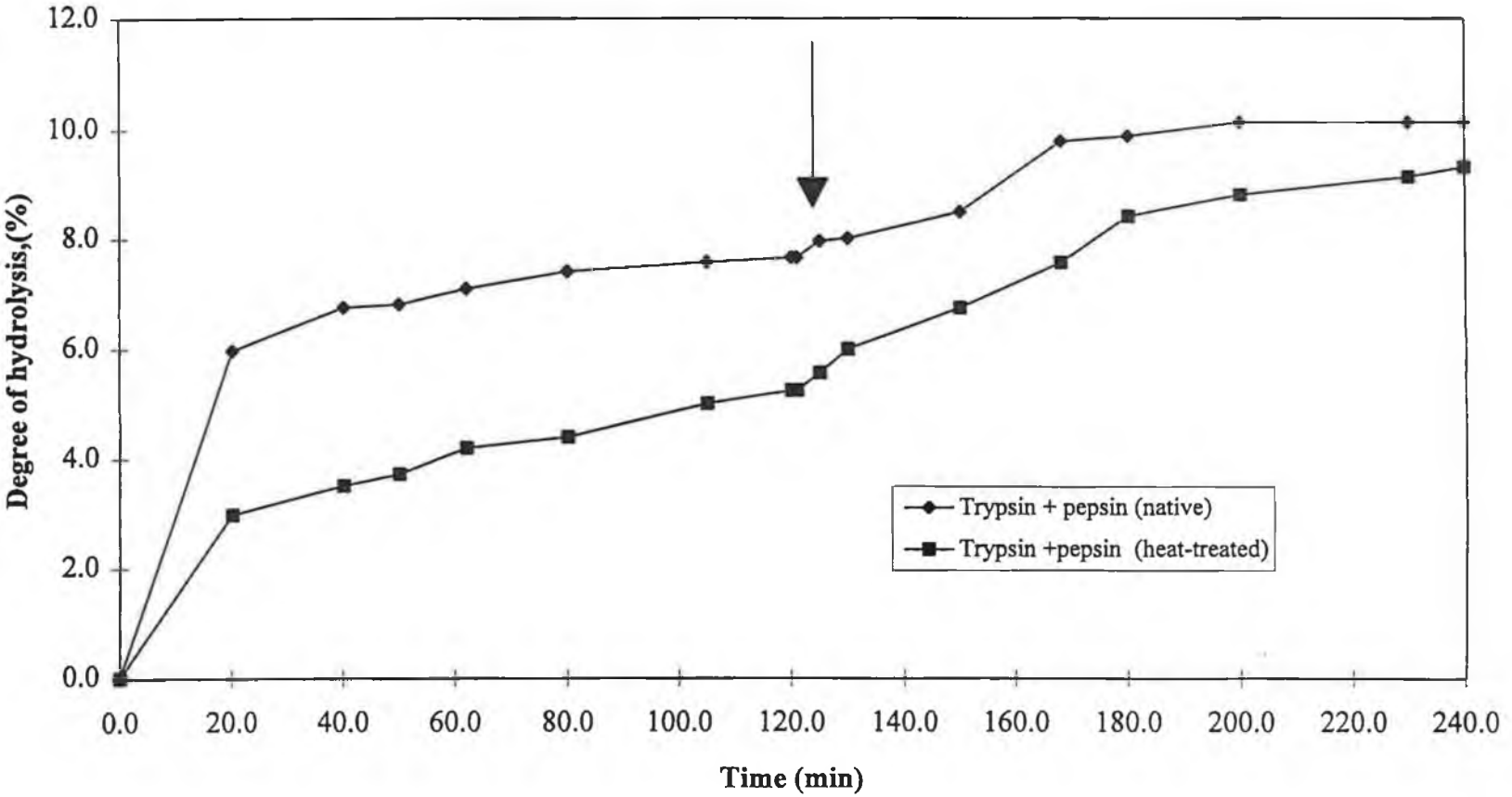


Figure 3: Plot of the degree of hydrolysis (% DH) versus time (min) for the hydrolysis of native and heat-treated β-lactoglobulin at 50^0C with trypsin and pepsin. The arrow marks the point of addition of the second enzyme.



5.4.2 Peptide profiles of hydrolysates analysed by RP-HPLC

In general, a complex profile containing a large number of peptides was obtained for those preparations which contained more than one endoproteinase activity, i.e. Corolase PP and PTN 3.0S (Figure 4 (a) and (b) respectively). The β-lg hydrolysates produced using Corolase PP (Figure 4 (a)) displayed a complex mixture of peptides, the majority of which eluted at a lower acetonitrile concentration than the peptides derived from β-lg using PTN 3.0S (Figure 4 (b). It appeared that PTN 3.0S produces hydrolysates which are more hydrophobic than the peptides produced by Corolase PP.

The peptic hydrolysis of β-lg resulted in low degrees of hydrolysis for the native protein, which corresponded to a large amount of more hydrophobic peptides in the elution profile (Figure 5 (a)). Figure 5 (b) shows the RP-HPLC profile for a peptic hydrolysis of the heat-treated β-lg and shows lower amounts of hydrophobic peptides eluting at the higher ACN concentrations. A large number of peptides were obtained for the hydrolysates produced using a combination of two individual proteinases, such as pepsin and trypsin (Figure 6).

In general, RP-HPLC profiles show that for the hydrolysis of the native β-lg, a greater number of hydrophobic peptides were generated than for heat-treated β-lg. At low DH values and where only one proteinase activity was used to hydrolysis β-lg, the majority of peptides were eluted at higher ACN concentrations. Where combinations of pepsin and trypsin, or commercial preparations, were used, peptides were eluted over a broader range of ACN concentrations.

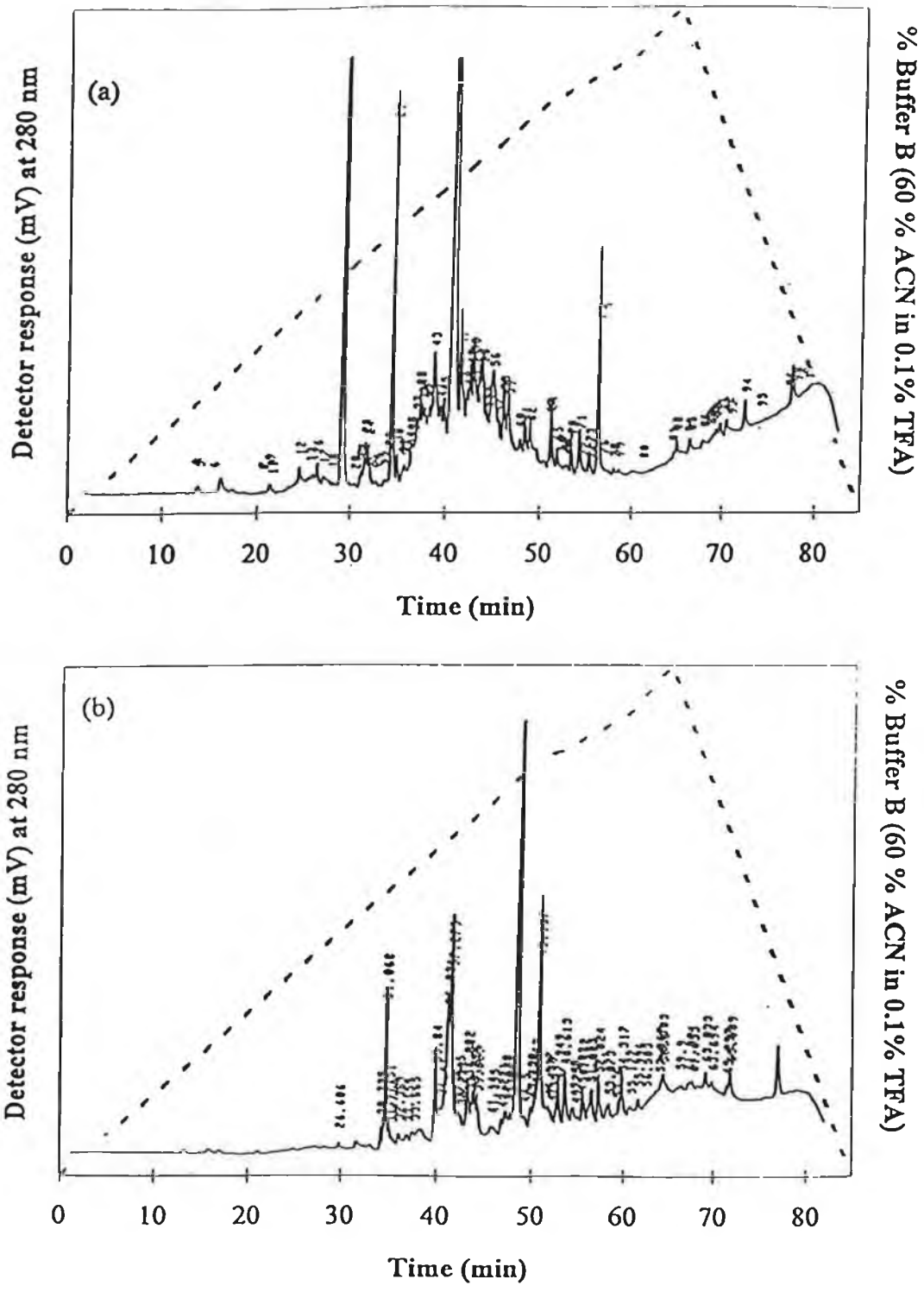


Figure 4 : RP-HPLC chromatograms showing peptide profiles of hydrolysates of native β-lg enriched fractions produced using (a) Corolase PP and (b) PTN 3.0S

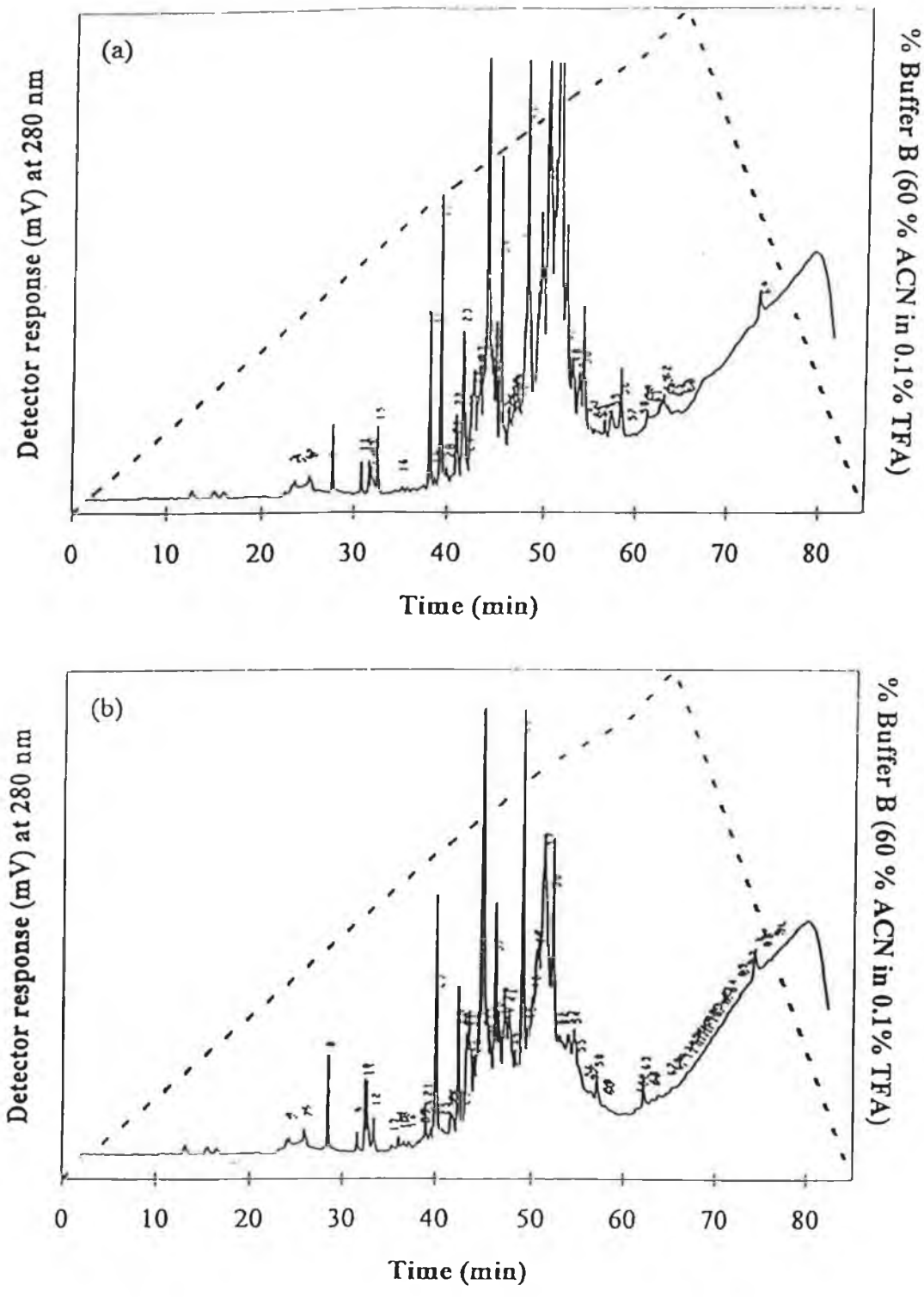


Figure 5 : RP-HPLC chromatograms showing peptide profiles over 85 min gradient elution assayed at 280 nm of peptic hydrolysate of (a) native (b) and heat treated β-lg enriched fraction

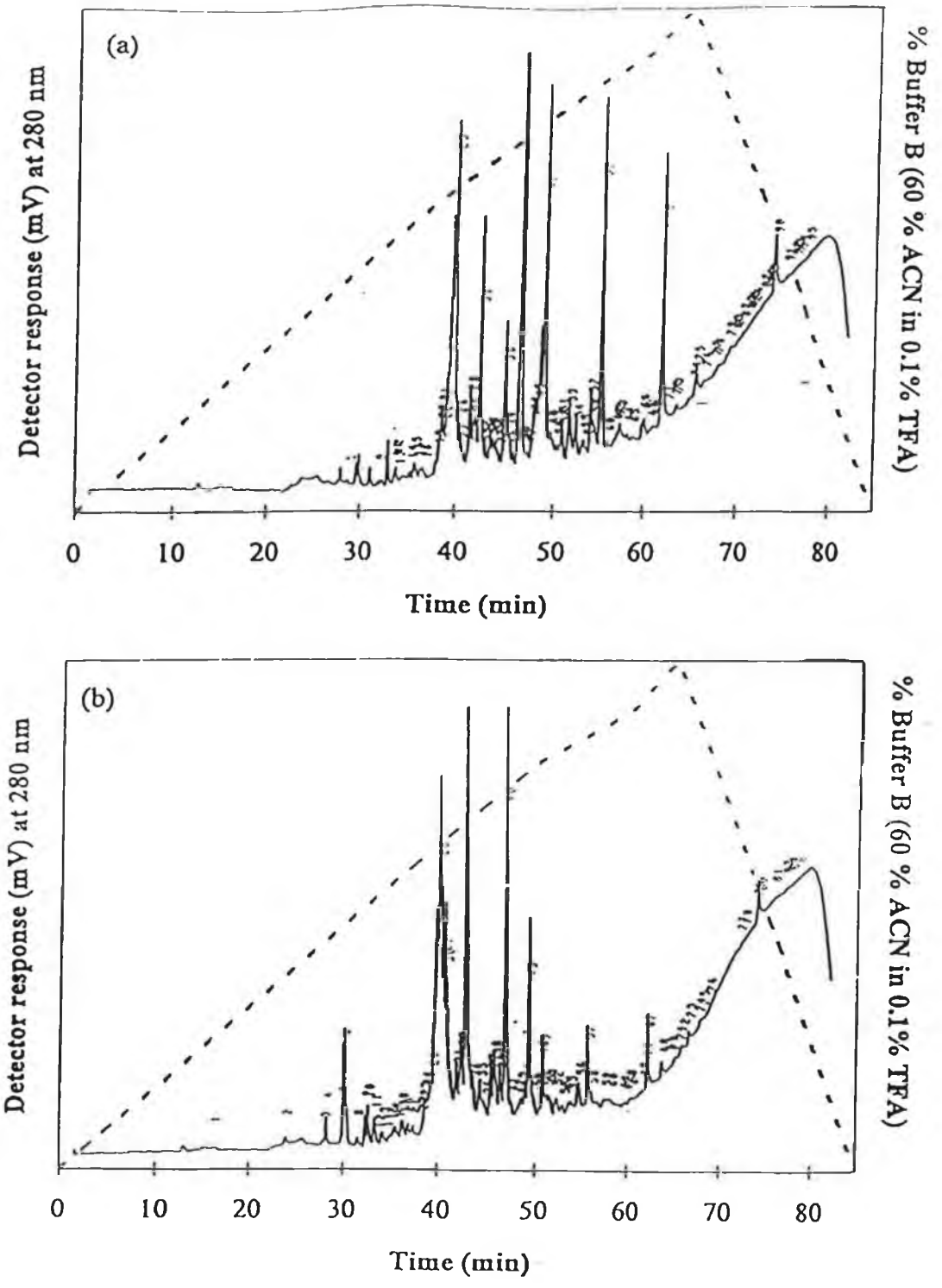


Figure 6 : RP-HPLC chromatograms showing peptide profiles over 85 min gradient elution assayed at 280 nm of peptic followed by a tryptic hydrolysate of (a) native and (b) heat-treated β-lg enriched fraction.

5.4.3 Differential Scanning Calorimetry (DSC)

Two sources of β–lg, both AB variants, were used for DSC studies, i.e. an enriched fraction and a Sigma purified protein. The β-lg enriched fraction has other whey proteins and ions in low concentrations (given in Materials Chapter 4) and this may have had a bearing on the results. The gel permeation chromatograms of the β-lg enriched fraction and the Sigma β–lg proteins were given in Figure 2, Chapter 4. Sigma β-lg has been used by other researchers for DSC studies (Table 2).

It was noted in this study that there was a visible difference in colour for the unhydrolysed β–lg solution (8 % protein) at the three different pH values examined, i.e. pH 3.00, 7.00 and 8.00. At pH 7.00 and 8.00, the β–lg (enriched fraction) solution had a milky appearance. However, at pH 3.00 this solution was less turbid. Sigma β-lg gave clear solutions at the 3 pH values tested. All the protein solutions were at 8 % (w/v).

The transition temperature was dependent on the solution pH. The higher pH values resulted in lower transition temperatures. Typical thermograms (i.e. heat flow versus temperature) obtained for DSC analysis of the enriched fraction β-lg at pH 3.00 and at pH 8.00 are given in Figure 7 (a) and Figure 7 (b) respectively. It was observed that the enriched fraction of β–lg had a transition temperature which was quite similar to the Sigma protein at pH 3.00. The enriched fraction of β–lg had a transition temperature of 82.98 ± 0.62 °C at pH 3.00 and 69.15 ± 7.59 °C at pH 8.00. Sigma purified β–lg was found to have a transition temperature of 83.48 °C ± 0.32 °C at pH 3.00 (Table 2). The enriched fraction of β–lg had a transition temperature of 71.9 °C ± 1.12 °C at pH 7.00 (Table 2).

The shape of the denaturation curves obtained also gave information with regard to the stability of the protein. It appeared at lower pH values, i.e. pH 3.00, (Figure 7 (a)), that the denaturation curve was more symmetrical and more defined than the curve obtained at pH 8.00 (Figure 7 (b)). In addition, for the pH 3.00 thermograms, it was easier to select a position, at which to draw a baseline, for the determination of enthalpy values. The principle reason for the lack of reproducibility between enthalpy values at higher pH values was due to difficulties in selecting a suitable position for the baseline. In this study, higher standard deviations were

observed for the Tm values at higher pH. Transition temperature and enthalpy values were reproducible at pH 3.00. The average enthalpy value obtained for the β-lg enriched fraction at pH 3.00 was 0.9825 J/g. This value was obtained using the program recommended by the instrument manufacturers. Because of problems with reproducibility with respect to enthalpy values at pH 7.00 and 8.00, these values were not reported in this study.

There was no major difference in the Tm at pH 3.00 for the β-lg substrate which was held (i) at 50 °C for 5 min or, (ii) at 50 °C for 125 min, followed by heating at a rate of 5 °C to 100 °C (Figure 7 (a) versus Figure 8 (a)). However, the thermograms obtained at pH 3.00 and pH 8.00 were more diffuse and broad when β-lg samples were held for 125 min at 50 °C. This effect was more pronounced at pH 8.00 (Figure 8 (b)) than at pH 3.00 (Figure 8 (a)). For pH 8.00 incubation at 50 °C for 125 min, a transition temperature 67.30 °C was obtained. Heat treatment of β-lg at 80 °C for 20 min prior to analysis by DSC resulted in the removal of the structure of the protein as indicated by the absence of differential heat flow and a flat line thermogram (data not shown). Figure 8 (b) shows a thermogram which is tending towards a flat line thermogram.

Table 2 summarises the transition temperatures obtained in this study and in those of other workers for β-lg at a range of pH values.

Table 2 : Transition temperatures and pH of solutions of β-lactoglobulin AB from this study in comparison with values from the literature.

Transition Temperature (Tm) (°C) ± SD [a]			Analysis pH	Reference
83.48	±	0.32	3.00	This study [b]
82.98	±	0.62	3.00	This study [c]
81.10	±	[d] -	3.00	Chen *et al.*, 1993 [c]
78.50	±	0.50	6.50	Puyol *et al.*,1994 [j]
70.50	±	0.50	6.50	Puyol *et al.*,1994 [h]
84.0 [f]	±	0.08	6.50	Imafidon *et al.*,1991 [g]
79.6 [e]	±	0.10	6.50	Imafidon *et al.*,1991 [g]
72.00	±	0.40	6.60	Gotham *et al.*,1992 [b]
70.40	±	0.50	6.70	deWit and Swinkels, 1980 [i]
76.70	±	0.31	6.80	Imafidon *et al.*,1991 [g]
71.90	±	1.12	7.00	This study [c]
75.30	±		7.00	Foegeding *et al.*,1992 [b]
77.00	±	0.5-1.0	7.00	Paulsson and Elofsson, 1994 [b]
78.50	±	-	7.00	Chen *et al.*,1994 [b]
69.15	±	7.59	8.00	This study [c]

[a] SD - Standard deviation obtained from 3 different experiments in the present study

[b] Sigma β-lg

[c] Enriched fraction of β-lg

[d] value not available

[e] using sodium phosphate buffer

[f] using Piperazine-N, N'-bis (enthanesulfonic acid)

[g] β-lg AB isolated according to Imafidon (1990).

[h] delipidated β-lg AB

i β-lg isolated according to Fox et al. (1967).

j β-lg with bound palmitic acid

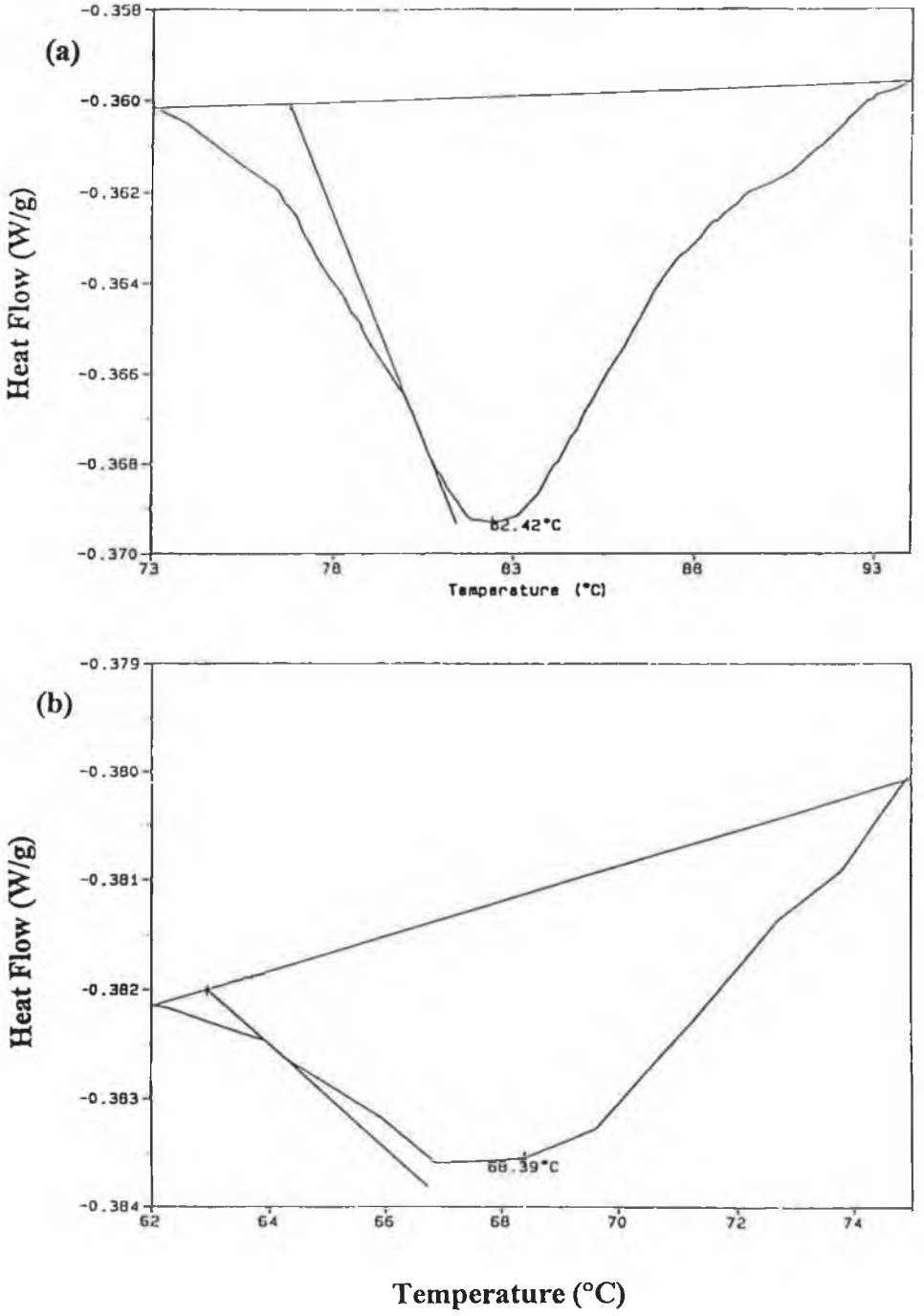


Figure 7: Thermogram of β-lg showing denaturation of the enriched fraction of β-lg at (a) pH 3.00 and (b) pH 8.00 Equilibration was at 50°C, followed by a heating rate of 5°C /min to 100°C.

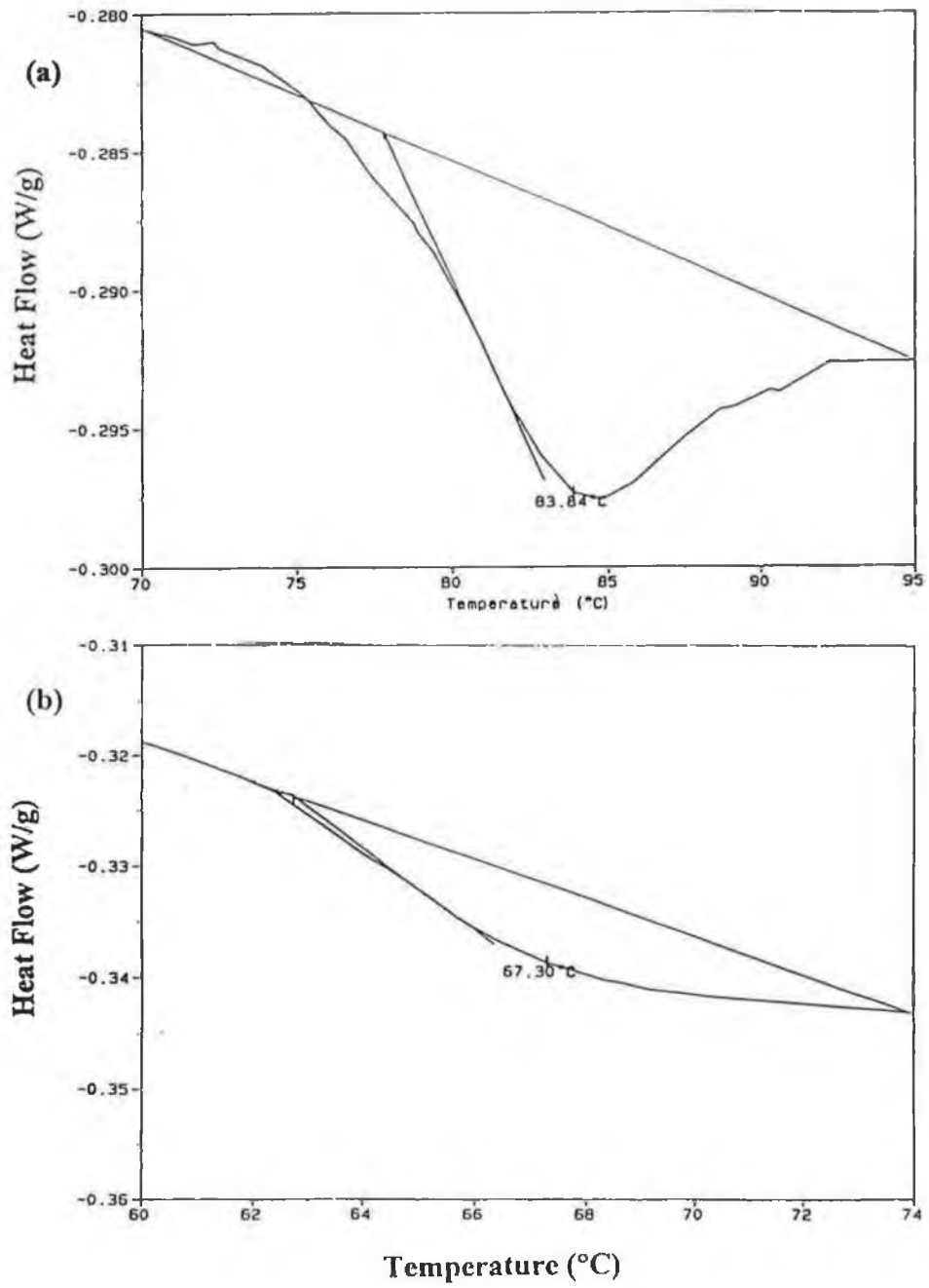


Figure 8 Thermogram of β-lg showing denaturation after equilibration at 50°C, holding at this temperature for 120 min, followed by a heating rate of 5°C /min to 100°C at (a) pH 3.00 and (b) pH 8.00.

5. 5 Discussion

The transition temperature (Tm) is an indicator of the thermal denaturation process of a protein. At the Tm it is thought that protein aggregation begins to dominate and the unfolding reaction become irreversible. It has been shown previously, in the case of β-lg, that thermostability was affected by the source and purity of β–lg, initial degrees of denaturation of β-lg, phenotypes of β–lg, composition and pH of buffers (Imafidon *et al.,* 1991), in addition to the protein concentration used during DSC analysis (Relkin and Launay, 1990; Paulsson and Dejmek, 1990). In this study, therefore, the pH and purity of the β–lg sources used would be expected to exert the greatest influence on the thermal stability of the protein, and thus on their susceptibility to enzymatic hydrolysis, particularly at 50 °C. It should be noted that eventhough the same β-lg variants, i.e. AB was used by all groups in Table 2 that differences may arise between Tm values obtained because of the above mentioned factors, in addition to differences in the heating rates used during DSC analysis.

Our overall findings in this study with respect to transition temperature values for β-lg at pH 3.00 and pH 8.00 correlate with those in the literature (Table 2). In this study, the enriched fraction of β–lg had a higher transition temperature, i.e. 82.98 ± 0.62 °C, at pH 3.00 than at pH 8.00, i.e. Tm value was 69.15 °C. The low DH values obtained in this study when native β–lg was hydrolysed using pepsin at pH 3.00 may be correlated to the thermal stability of the protein at this pH (Table 2 and Figure 7).

The purity of the β-lg source is also known to have an influence on thermal stability of the protein. However unexpectedly, the enriched fraction of β-lg (which contains small amounts of contaminants) had a Tm value of 82.98 ± 0.62 °C at pH 3.00 which was very similar to the value obtained for Sigma β–lg, which had a value of 83.48 ± 0.32 °C (Table 2). Sigma β–lg has been previously shown to have an transition temperature of 77 °C at pH 7.00. (Paulsson and Elofsson, 1994). The enriched fraction of β–lg had a Tm value of 71.9 ± 1.12°C at the same pH in this study. This lower value might reflect the effect of trace impurities in the enriched fraction of β–lg, at higher pH values, as suggested by other groups (Imafidon *et al.*, 1991; Gotham *et al.*, 1992). Discrepancies in Tm of β–lg have been attributed previously to the

presence of other 'contaminating' proteins e.g. α-lactalbumin and lactoferrin (Imafidon *et al.,* 1991). Irregularities in the shape of the denaturation curves at pH 8.00 may also indicate the involvement of contaminating proteins or ions in the unfolding/ aggregation processes. Two individual processes are known to occur during heating (deWit and Klarenbeek, 1984; Gotham *et al.*, 1992), the unfolding step (endothermic) and the aggregation step (exothermic), the combination of which in this study may have become an interfering factor (Gotham *et al.*, 1992) in the production of uniform and symmetrical thermograms. The complexities involved in this unfolding / aggregation process have already been argued by a number of groups (Gotham *et al.*, 1992; Lin Qi *et al.,* 1995). It is obvious, however, from this study that β–lg is thermally less stable at pH 8.00 than at pH 3.00. (Table 2 and Figure 5). When a complex is formed between proteins (depending on pH and other factors such as Ca^{2+} ions), changes in thermostability are known to occur (Imafidon *et al.,* 1991). An indication of the influence of ions such as calcium was seen in the difference in colour of the enriched β–lg fraction at pH 3.00 and pH 8.00. The cloudy colour of the unhydrolysed enriched fraction at pH 8.00 may be due to calcium ions which are tightly bound to β–lg at high pH and may be involved in the aggregation process. The interference by calcium ions may have given the 'broad' and 'diffuse' peak as the unfolding and aggregation steps were combined (Figure 5 (b) and Figure 6 (a) and (b)).

In addition, in this study, the standard deviation of the temperature of transition was higher at high pH values (Table 2). This may also be indicative of the unstable nature of the protein at higher pH values. Observations in this study at pH 8.00 concluded that the enthalpy values at this pH were not reproducible and the values therefore were not reported. The position of the baseline could be changed slightly between samples, without affecting the transition temperature, but such changes had an effect on the calculation of the enthalpy value at pH 8.00. This was seen in particular when samples of β-lg were held for 120 min at 50 °C. The thermograms obtained were more shallow, irregular and diffuse in comparison to those found for the protein which was not held for 120 min at 50 °C (Figure 7 (a) and (b) respectively). It is known that the absolute enthalpy value is very sensitive to the position of the base line (deWit and Swinkels, 1980; Gotham *et al.,* 1992; Puyol *et al.,* 1994). This phenomena was found

during this study. However, at pH 3.00, β-lg was more thermally stable and an enthalpy value of 0.9825 J/g was obtained. This value was about ten fold lower than previously reported by Chen *et al.* (1993) who found a value of 50.5 kcal/mol, i.e. 11.4 J/g. It might be suggested that the difference in values obtained was due to the different concentration of sample (60 µl of 7 mg/ml) used by this group, in addition to a different heating rate (10 °C/min) in comparison to this study which used a heating rate of 5 °C/min. (Note: a monomer molecular weight value of 18,400 was used in the calculation of the enthalpy value in this study).

The DSC results obtained give insight into the thermal stability of β-lg, and thus stability of this protein during the hydrolysis reaction. Our study showed that the pancreatic proteinases, trypsin and chymotrypsin showed high activity against β–lg with final DH values of 6.52 and 7.63 % respectively (Table 1). Hydrolysis by all pancreatic proteinases was at pH 8.00, which was seen from DSC analysis to be the pH value which correlated to a lower thermal stability (Figure 5 (b) and Figure 6 (b)). It is known that β–lg dissociates to monomer and undergoes reversible conformational change above pH 7.5, causing the exposure of tryptophan and tyrosyl residues to solvent (Townend *et al.,* 1969; Zimmermann *et al.,* 1970). Therefore, β–lg would be less resistant to tryptic and chymotryptic hydrolysis at pH 8.00 due to the increased number of exposed cleavage points for these enzymes (Chobert *et al.,* 1991), particularly at 50 °C.

Several literature reports show that β–lg is resistant to tryptic, chymotryptic in addition to peptic digestion (Otani, 1981; Reddy *et al.,* 1988; Porter *et al.,* 1984). However in these studies hydrolysis reactions were carried out at lower temperatures i.e. 20 °C or 37 °C and pH values i.e. ≤ pH 2.00, whereas hydrolysis reactions in this study were carried out at 50 °C and pH 3.00. The resistance of β-lg to hydrolytic attack appears therefore to be only under conditions of low pH and temperature. Kella and Kinsella (1988) suggested that acid stability (pH 2.00) of β–lg could result from increased internal hydrogen bonding that arises between either two titrated carboxyl groups or one amide and one carbonyl group. It has been shown, for instance, that β–lg is resistant to gastric digestion and remains intact after passage through the stomach (Miranda and Pelisser, 1983). These conditions would correspond to approximately

pH 2.00, a physiological temperature of 37 °C, and a transit time of ~ 240 min. Peptic hydrolysis of the native enriched fraction of β–lg was evident in this study at pH 3.00 at both 37 and 50 °C, giving DH values of 2.69 and 4.43 % respectively (Table 1). It was evident from this study that at temperatures ≥ 37 °C, and at pH values > pH 2.00, a certain degree of hydrolysis by pepsin could be expected.

It may be that holding at 50 °C for 120 or 240 min may make the protein more susceptible to enzymatic hydrolysis by pepsin, as indicated by DH of 2.36 and 4.03 % respectively. DSC analysis showed that holding β-lg at 50 °C for 120 min at both pH 3.00 and pH 8.00 resulted in more diffuse and broad thermograms (Figure 8 (a) and (b)). These thermograms suggest that the protein is perhaps slowly unfolding and becoming more unstable over time. In general broad diffuse thermograms reflect thermal instability of proteins due to protein-protein interaction/unfolding (Gotham *et al.*, 1992). The specificity of pepsin is for tyrosine, phenyalanine, leucine, isoleucine and tryptophan residues (Fruton, 1971). β–Lg contains four tryptophan groups per dimer (McKenzie, 1971). Therefore, in the course of the holding time at 50 °C, β-lg become less stable and has a more 'open' structure, allowing hydrolysis by proteinases such as pepsin of susceptible bonds which become exposed.

The conformational resistance of β–lg to peptic hydrolysis at low pH values was removed by heat treatment of the substrate (80 °C for 20 min) as indicated by DH values of 5.34 and 6.64 % for 120 and 240 min hydrolysis times, respectively (Table 1). This was opposed to DH values of 2.36 and 4.03 % for 120 and 240 min peptic hydrolysis of the native β-lg. Heat treatment of β–lg results in denaturation of the protein, reduction of the disulphide groups at high pH values, and thus exposure of mainly hydrophobic amino acid residues which are usually buried within the internal structure of the native molecule (Mihalyi, 1978; Puyol *et al.*, 1994). DSC analysis of heat-treated β-lg samples indicated no differential heat flow and a flat line thermogram was observed (data not shown). Heat treatment, as expected also resulted in the exposure of additional amino acid residues for the action of trypsin. The final % DH of a tryptic hydrolysis of heat treated β-lg (DH = 7.15 %) was higher then that for tryptic hydrolysis of native β-lg (DH = 6.52 %). The native forms of the enriched fraction of β-lg, had a lower % DH after 240 min than the heat treated form (Table 1). This was

not the case however, for the hydrolysis of Sigma purified β–lg by trypsin at 37 °C. The degree of hydrolysis obtained was highly unusual, as DSC analysis indicated that there was little difference between the structural stability of this sample, indicated by the transition temperature, 83.48 ± 0.32, and those values obtained in the literature, 81.10 (Chen *et al.*, 1993) as seen in Table 2. It could be that an overestimated E:S ratio was employed in the hydrolysis reaction, giving higher degrees of hydrolysis.

Higher total DH values (i.e. after 240 min) were obtained, when pepsin was followed by trypsin hydrolysis of heat-treated β-lg (DH = 11.30 %) in comparison to trypsin followed by pepsin hydrolysis of the same protein (DH = 9.32 %) as seen in Table 1. Pepsin is important in the provision of substrates for the action of trypsin during hydrolysis of native β-lg at 50 °C. This is reflected in the DH values obtained during sequential hydrolysis of native β-lg with pepsin and trypsin. (Table 1). It has been established previously that pepsin makes the molecular structure of native β–lg more susceptible to proteolytic attack by other proteinases (Porter *et al.*, 1984; Antila *et al.*, 1991). RP-HPLC chromatograms for the reaction on both native and heat treated β-lg indicated the production of a larger number of peptides, with a broad range of hydrophobicities when both endoproteinases are used in this sequence (Figure 5).

In general, the peptides generated appeared to be more hydrophobic for the native than the heat-treated β-lg hydrolysates. The heat-treated, β-lg had no structural resistance to hydrolysis (as seen using DSC) by either pepsin or trypsin, and therefore, more peptides of lower hydrophobicity are seen to elute on RP-HPLC, i.e. at lower concentrations of acetonitrile.

The commercial proteinase preparations Corolase PP and PTN 3.0S produced complex RP-HPLC peptide profiles (Figures 2 (a) and (b)) which may be indicative of more than one proteinase activity (Chapter 1 and 2). The importance of the specificity and ratio of proteinases within a proteinases preparation in the production of peptides with specific molecular characteristics was discussed previously (Chapter 1 and 2). It was evident that fewer peptides evolved from a hydrolysis reaction in which only one proteinase activity was used, such as the case with pepsin (Figure 6). In addition, higher initial hydrolysis rates, in addition to higher final DH values after 240 min, were obtained for the commercial preparations (Table 1),

This preliminary study showed the utility of DSC analysis in elucidating the thermal stability and as a consequence the susceptibility of β-lg to enzymatic hydrolysis by gastric and pancreatic proteinases. The accessibility of β-lg to enzymatic digestion is dependent not only on the purity and the initial level of denaturation of the substrate, but more specifically on the temperature and pH of the hydrolysis reaction.

CHAPTER 6

Evaluation of an ultrafiltration system for the fractionation of whey protein hydrolysates[1]

[1] This chapter was submitted as a final report for an EU Co-Responsibility funded project-contract no. 1116-92

6.1 Summary

A laboratory scale tangential crossflow plate and frame ultrafiltration (UF) membrane system was evaluated for the processing of a whey protein hydrolysate. Several problems were encountered during the optimisation of the above system. These problems were mainly due to incompatibility of the membrane with the pumps and/ or with the feed solution. Two membranes (10 kDa cut-off) of different surface areas, i.e. 0.093 m^2 and 0.46 m^2, were investigated. It was initially observed that a peristaltic pumping system was unsuitable for processing of WPC hydrolysates using the 0.093 m^2 membrane. The feed channels in the membrane frame holder were narrow, causing high backpressures and resulted in bursting of the peristaltic tubing. Therefore, a centrifugal pump with reinforced tubing was installed to the system containing the 0.093 m^2 surface area membrane. It was found, however, that this assembly was very sensitive to the torque applied during membrane installation. Therefore, a membrane of larger surface area, i.e. 0.46 m^2, was used which was not as sensitive to torque and did not show compressibility problems during operation. It was also observed that the centrifugal pump was sensitive to high total solids content in the feed and to low crossflow. A strategy was thus adopted to control mean membrane pressure for the 0.46 m^2 membrane operated with the centrifugal pump, by adjustment of pump speed setting, whilst leaving both inlet and outlet valves open. For detailed characterisation of the 0.46 m^2 surface area membrane mean membrane pressures of 0.52 and 1.38 bar were used.

WPC hydrolysates were produced using the commercially available pancreatic proteinase preparation, PTN 3.0S. It was shown that the main proteolytic activities in PTN 3.0S are stable over 28 h in the presence of whey protein. For the membrane optimisation procedures, a hydrolysate with a degree of hydrolysis of 8 % and a total solids content of 10 or 5 % (i.e. from 8% to 4% total protein) was used. The lower total solids content in the feed was used to reduce the likelihood of membrane fouling.

The ultrafiltration of whey protein hydrolysates was subject to severe fouling by protein and mineral deposits. The membrane in our study permitted the use of alkaline detergents and sodium hypochlorite to remove deposited proteins and amino acids.

However, it was found that the pump seals were not compatible with various cleaning reagents suitable for efficient membrane cleaning. A cleaning procedure which differed to that recommended by the manufacturers was thus developed.

Overall, the system studied was acceptable for the batch membrane processing of hydrolysates. The membrane allowed for retention of the active enzyme, PTN 3.0S, in addition to fractionation of the WPC hydrolysate to peptides <10 kDa. This UF system would thus have the potential to enrich for hydrolysate peptides of a certain molecular mass distribution. Furthermore, active enzyme could be retained by the membrane allowing for enzyme re-use and thus eliminate the requirement of a heat inactivation step, as in conventional hydrolysate production protocols. The system investigated, however, proved very difficult to efficiently operate due to problems with fouling by the feed. Much work remains to be carried out in order to develop an efficient ultrafiltration system for continuous/ semi-continuous hydrolysis of milk proteins.

6.2 Introduction

Milk protein hydrolysates find application in a wide range of product areas, e.g. in infant formulae, dietetics, sports nutrition (Driessen and Van den Breg, 1988), functional food ingredients (Jost and Monti, 1977; Ennis and Harper, 1986; Chobert *et al.*, 1988), cosmetics (Hidalgo and Jost, 1980), bacteriological growth media (Bouhallab *et al.*, 1993), and as bioactive peptides (Bouhallab and Touzé, 1995).

Traditionally, hydrolysates have been generated enzymatically in batch-type processes (Adler-Nissen, 1986). This involves holding the substrate, such as whey protein, and enzyme in a reaction vessel at a defined temperature and pH for a specified time interval. When a desired degree of hydrolysis (DH, %) has been attained the enzyme is inactivated generally using a high heat treatment step. Significant costs are associated with batch processes due to the requirement for large quantities of enzyme and the high energy inputs, i.e. during enzyme inactivation.

Heat treatment as a means of enzyme inactivation may induce formation of peptide aggregates, the amount and size of which may depend on the degree of hydrolysis and the heating temperature and pH of the solution (deWit and Swinkels, 1980). Solubility is one characteristic which is changed, among others, as a result of this enzyme inactivation heat treatment step (Britten *et al.*, 1994). In addition, heating may destroy some of the functional, or nutritive properties (Erbersdobler, 1983) associated with hydrolysates (especially those at low DH), where some tertiary protein structure may still be associated with stable domains, as in the case of partially hydrolysed β–lactoglobulin (Chen *et al.*, 1994). Heat treatment may also lead to the generation of undesirable off-flavours. Furthermore, re-use of expensive enzyme systems is not possible with batch enzymatic hydrolysate production due to the irreversible inactivation of the enzyme during heat treatment. The output from a batch process, defined as the mass of product produced per unit time, therefore, decreases as the reaction proceeds. This decrease is due to the disappearance of substrates and to the lowering of the concentration of active enzyme either due to inactivation or reversible adsorption to reaction products. Iacobucci *et al.* (1974), showed that there

was a 2 % per hour loss of active enzyme during the hydrolysis of soybean protein isolate using a thermostable fungal acid protease preparation.

The advent of ultrafiltration systems capable of being fitted with membranes having different molecular sieving capabilities eliminates the requirement for heat inactivation of the enzyme in the hydrolysis product after hydrolysis is complete, as the enzyme can be retained by the membrane. Substrates with relatively large molecular masses are enzymatically broken down during batch, semi-continuous or continuous enzymatic hydrolysis to smaller peptide products which are permeable to the membrane.

The application of membrane technology in the food industry has been reviewed (Hallström *et al.*, 1989; Trägårdh, 1988). Ultrafiltration has been used by many groups in the production of specialised protein hydrolysates (Turgeon *et al.*, 1991; Gauthier *et al.*, 1993), such as those having reduced allergenicity (Van Bersteijn *et al.*, 1994) or hydrolysates which contain bioactive peptides (Bouhallab and Touzé, 1995).

Further to the advances in membrane technology, the development of continuous hydrolysis systems offers the advantage of enzyme re-use. This alternative to the batch process often employs the use of enzyme immobilisation on solid supports and/or enzyme entrapment in membrane reactors (Turgeon and Gauthier, 1990; Haque, 1993). The system configurations and kinetics of enzymatic hydrolysis differ between batch and continuous hydrolysis of milk proteins (Sejr Olsen and Adler-Nissen, 1981). Given that enzymes can be re-used in continuous membrane systems, it becomes economically feasible to incorporate a high enzyme concentration in the reactor system. Quantitative and rapid removal of enzyme can also be achieved, a capability of major importance, particularly during the development of products with low degrees of hydrolysis. By choosing suitable membrane pore sizes it is also possible to exercise some control over the molecular size of the product. Continuous/semi-continuous enzyme systems combined with membrane processing allow for high product outputs by (a) keeping the enzyme and substrate concentrations constant and (b) by using large enzyme to substrate ratios taking advantage of the enzyme retention capability of the membrane. In using high enzyme concentrations it is possible to achieve very fast reaction rates giving short residence times for the conversion of substrate into

diffusible products. It is theoretically possible to achieve a steady state at constant flux in turn giving quantitative conversion of substrate for extended periods of time. There have been several reports in the literature describing laboratory-scale continuous and semi-continuous enzyme systems (Table 5.1 in Literature review). Such systems have been described for a variety of hydrolysable protein substrates, e.g. caseins, soybean protein isolate, blood proteins, fish proteins, alfalfa, leaf protein, etc. (Table 5.1).

The general principles of ultrafiltration (UF), UF membranes and UF systems of importance to dairy industry applications have been reviewed (Glover, 1985). Three factors are of utmost importance in the optimisation of any membrane processing system. These are: the membrane type, membrane configuration and permeate flux through the membrane. Flux is usually given in units of permeation of the product over time per unit membrane area, i.e. l m^{-2} h^{-1}. The membrane type is defined by the material the membrane is made of (organic, inorganic, metallic), the configuration by the geometry of the membrane (plate and frame, tubular, spirally wound, hollow fibre), and the permeate flux which is partly controlled by the fouling characteristics of the feed. Interaction of these factors must be carefully considered, especially the influence of feed characteristics on the membrane flux, and feed component retention on permeation characteristics. The performance of a membrane during a processing operation is usually represented by permeate flux curves, i.e. a plot of flux against the mean membrane pressure or against the concentration of a given component such as protein/peptide under stated conditions. Optimisation of permeation (i.e. permeate flux) requires that concentration polarisation, (defined as the build up of feed components on the surface of the membrane leading to reduced flux), be minimised.

Other interrelated factors that influence flux and feed component permeability (Koch Membrane Systems, 1986) are as follows:

6.2.1 Mean membrane pressure

The driving force of an ultrafiltration process is a hydraulic pressure gradient between the feed and permeate sides of the membrane. This pressure gradient is termed the mean membrane pressure. The permeate flux of an ultrafiltrate is dependent on the mean membrane pressure, defined as (Pressure $_{inlet}$ + Pressure $_{outlet}$)/2. As the

mean membrane pressure increases, the flux increases to a point where the hydraulic flow is limited by other membrane phenomena. At low pressure, the membrane usually responds to small changes in mean membrane pressure. As the pressure increases, the flux response to pressure decreases until the flux becomes pressure-independent. At this point, a gel layer or concentration polarisation layer restricts flux. Concentration polarisation and the formation of the 'gel' polarisation layer of resistance at the membrane surface is inherent in each membrane process. This can never be eliminated but should be minimised during optimisation of a membrane process (Glover, 1985). The degree of concentration polarisation in turn is dependant on the type of membrane used, the feed composition, flow velocity and mean membrane pressure. When the pressure increases the gel layer is compressed which in turn provides a greater resistance to the passage of permeate through the membrane. In some cases, the compression of the gel layer under increasing pressure more than offsets the pressure gradient, and fluxes will then decline with added pressure.

6.2.2 Feed crossflow (l h^{-1}) over the membrane

Flow of feed over the membrane or flow through the module is a concept of filtration known as cross-flow or tangential flow. The crossflow technique allows for long term processing with little decline in permeate flux over the course of the process run. With conventional filtration, the retained particles on the filter material accumulate to a point where no liquid will pass through the filter. As the feed cross flow (l h^{-1}) is increased, concentration polarisation effects are alleviated due to the 'sweeping' action of the feed across the membrane. Cross-flow filtration, therefore, creates a turbulent environment on the membrane surface that prevents accumulation of retained particles. A high flow velocity at the membrane surface sweeps particles back into the bulk stream and minimises formation of the protein gel layer. For some process streams, the permeate flux is highly dependent on the flow across the membrane. When flux is flow-dependant, flux is limited by the gel concentration layer. An increase in flow rate will increase the permeate flux. At very high flow rates, a flow-independent stream may become flow-dependent, where other controlling factors such as membrane fouling may take over.

6.2.3 Process temperature

All ultrafiltration processes are temperature-dependent. Several membrane and enzyme systems can be operated at elevated temperatures, e.g., 50 °C. This is advantageous in that higher temperatures can result in faster rates of substrate conversion, lower viscosities of the feed and retarded growth of (mesophilic) microorganisms. In general, the permeate flux will increase with higher temperature due to decreases in viscosity of the feed solution. When processing dairy streams, the relationship of increasing flux with increasing temperature holds only within a certain range. Below 35-39 °C, the fat components of dairy products undergo a phase change and solidify causing membrane fouling. Similarly at high temperatures (50-65 °C), over extended time periods whey proteins denature and this may result in membrane fouling.

6.2.4 Concentration of retained species

The objective of using the selective membrane, e.g. with defined molecular weight cut-offs, is to concentrate some components of the feed stream relative to others. When the concentration of retained species becomes sufficiently high, the process flux is limited. In dairy streams, the protein and fat fraction are flux-controlling species. This concentration dependent flux phenomenon is found in all UF systems operating at high concentration levels. In a batch process, as the total solids concentration is increased on the feed side of the membrane during ultrafiltration, this leads to a decrease in flux and fouling of the membrane occurs. This is accompanied by pressure build-up in the system.

6.2.5 Membrane cleanliness and fouling characteristics of the feed

All ultrafiltration membranes are subject to fouling by components in the feed stream. Membrane fouling can be defined as the accumulation of thin molecular layers on the surface of the membrane that cannot be removed by increasing the flow rate across the membrane. Fouling may be reversible where periodic cleanings remove this layer and restore performance of the membrane to original levels (i.e. > 90 % of the original Normal Water Permeability (NWP)). Irreversible fouling is caused by

unwanted components in the process stream or cleaning water that cannot be removed by normal cleaning cycles. Reversible foulants include protein, fats, calcium salts, polysaccharides and polyphenols. Irreversible foulants include silicone, some surfactants, colouring agents in certain chemicals. The response of a membrane system to fouling is usually manifested as a productivity decline during the first hour of the process followed by a more gradual decline to the end of the run. For some highly fouling streams the flux loss will be more pronounced causing large productivity losses in a short process time. Normal membrane fouling can be compensated for by increasing the mean membrane pressure or by adding membrane area, i.e increasing area to volume ratio. By increasing the mean membrane pressure, the fouling phenomena is counteracted by the increase in hydraulic pressure on the membrane. By adding an additional stage/membrane on line, the new clean membrane offsets the portion of the system lost due to normal membrane fouling.

6.2.6 Pre-treatment conditions of the feed

Pre-treatment of the feed can potentially affect membrane performance by changing the fouling characteristics of the feed. The pre-treatment effect may be due to direct changes in the quality (molecular stability/thermal stability) of protein, fats or calcium in the feed stream or may be due to indirect changes related to e.g. microbiological contamination of the feed. As already stated, an upstream process can affect the performance of the ultrafiltration step. Such upstream process may include the following: product heating and holding at a high temperature for a certain time; fat removal or standardisation of the feed stream; pasteurisation temperature and holding times; chemical treatment of the feed; pH adjustment; prefiltration of the feed (fines removal or suspended solids removal); preconcentration of the feed by evaporation or by osmosis and micro-organism contamination (e.g. lactic acid bacteria and their influence on feed pH; nonlactic acid bacteria; moulds and yeasts).

Commonly used treatments in the processing of WPC are heat treatments which can result in feed component unfolding and aggregation (Britten *et al.*, 1994). The composition of the feed solution has an influence on the flux and susceptibility towards fouling. Whey protein concentrate may have a high content of calcium, which may

result in precipitate formation in the feed and thus expedite fouling during ultrafiltration. Addition of chelators such as EDTA, pH adjustment and prefiltration steps (Haque, 1993) are also used in certain cases, to pretreat the feed prior to ultrafiltration (Pouliot and Jelen, 1995).

6.2.7 pH of the feed

The pH value at which a membrane system is operated can effect the permeate flux. This may be due, for instance, to pH effects on the feed material leading to increased concentration polarisation. In addition, the operating pH may have a contributory affect to the deposition of salts on the membrane surface.

The effect of pH on the permeation of casein hydrolysates and amino acids has been shown previously (Pouliot and Gauthier, 1990; Gourley *et al.*, 1995). It was found that if the pH was increased from 6.0 to 10.0, the rejection characteristics of a polysulphonal membrane increased and permeation was improved (Gourley *et al.*, 1995). The effects of pH on hydrolysate aggregation, especially in the presence of calcium, have been discussed (Brulé *et al.*, 1980; Britten *et al.*, 1994). The solubility of a heated WPC hydrolysate at neutral pH was high, but below pH 7.0 heat induced aggregates were formed (Britten *et al.*, 1994). These aggregates would contribute to rapid formation of the 'gel' layer during membrane processing.

6.2.8 Membrane fouling

While theoretically ultrafiltration/ membrane/ enzyme systems offer many advantages over batch processes there are still several technical/engineering difficulties to be solved for optimal operation. These relate mainly to control of concentration polarisation/fouling. Several approaches have previously been taken to try to overcome problems of concentration polarisation and fouling. By using dead-end ultrafiltration cells, Bhumiratana *et al.* (1977) and Payne *et al.* (1978) showed that periodic pressurisation and depressurisation was required to depolarise the membrane of deposited matter. Iacobucci *et al.* (1974) employed a 'bleeding' facility in their continuous system for soybean protein hydrolysis with acid protease to remove insoluble, non-permeable and non-hydrolysable material building up at the feed side of

their ultrafiltration membrane. These steps may result in a more controlled transmembrane pressure and thus concentration polarisation is minimised. Macromolecular adsorption and fouling problems during ultrafiltration and their relationship to concentration polarisation has been studied (Matthiasson, 1984; Nilsson, 1989; Van der Horst, 1995). A mechanism of whey deposit removal has been proposed for the cleaning of heat-exchangers (Jeurnink and Brinkman, 1994) and deposition models for UF membrane fouling by milk proteins have been reported (Hallström *et al.*, 1989; Jeurnink and Brinkman, 1994). Fouling by whey protein solutions has also been studied by other groups (Belmar-Beiny and Fryer, 1993; Ramachandra Rao *et al.*, 1995). The fouling layer on the membrane surface dominates membrane behaviour. It is made up of both absorbed protein and protein deposited in a multilayer (Marshall and Daufin, 1995). The properties of a membrane fouling layer are known to depend on the degrees of protein adsorption, hydrophobic interactions between the proteins and the membrane, and membrane ionic interactions, resulting in a multi-fouling layer formation. The composition of the feed solution is of particular importance (i.e. pH, ionic strength, calcium content and degree of protein aggregation, presence of lipids and other particulates) in the formation of the fouling layer (Marshall and Daufin, 1995). A comprehensive discussion of the physico-chemical aspects of fouling, strategies to minimise fouling and the cleaning of membrane systems has been published recently (IDF Bulletin, 1995).

This study reports on initial attempts at characterising a tangential flow plate and frame membrane system for subsequent use in continuous/semi-continuous enzymatic hydrolysis of whey protein concentrate.

6.3 Materials and methods

6.3.1. Materials

6.3.1.1 Reagents

The commercial pancreatic protease, PTN 3.0S (porcine), was supplied by Novo Nordisk A/S, Bagsvaerd, Denmark. Amino-Methyl-Coumarin (AMC) and the fluorogenic substrates, N-suc-leu-leu-val-tyr-AMC and N-benzoyl-L-arg-AMC, were obtained from Bachem, Bubendorf, Switzerland. All other reagents were of analytical grade unless otherwise specified.

6.3.1.2 Substrate

Whey protein concentrate Lactalbumin-80 (Soluble UF-WPC) was from Milei GmbH., Leutkinch, Germany and had the following composition: protein (80 %), lactose (4.7 %), minerals (2.8 %), fat (6 %) and moisture (5 %).

6.3.1.3 Ultrafiltration equipment

A Pellicon® stainless steel plate and frame system was obtained from Millipore Corp (Millipore, U.K.). Two different systems were used, employing membranes with surfaces areas of (a) 0.093 m^2 and (b) 0.46 m^2. The membranes used had a molecular weight cut-off of 10 kDa and were a low protein binding durapore microporus type (Biomax® 10K polyethersulphone). Two pumping systems were also investigated: (i) a peristaltic pump (Model XX80 2G2 30, Millipore, U.K.) and (ii) a centrifugal pump (Model 260 TXLE) which was from Procon® pumping systems (Munfreesboro, TN, U.S.A). Thermoplastic tubing with polyester braid reinforcement capable of withstanding pressures up to 6.9 bar, fitted with 316SS Triclover sanitary compression swaged terminations was obtained from H+H Equipment Co., Ltd. (Dungourney, Co. Cork.).

6.3.1.4 Membrane cleaning agents

Henkel P3-53 (enzymatic preparation, containing organic and inorganic complexing agents, in addition to emulsifying agents) was from Henkel Chemicals

(Ireland) Ltd., and Esperase® (an enzymatic preparation) was from Novo Nordisk A/S, Bagsvaerd, Denmark.

6.3.2 Methods

6.3.2.1 Quantification of enzyme activity.

Enzyme activities were assayed using a modification of the fluorogenic (AMC) assay as described in Chapter 1 and 2.

6.3. 2.2 Preparation of hydrolysates.

Whey protein concentrate (WPC) was reconstituted using a Silverson® mixer (Machines Ltd., Waterside, Chesham, Bucks, U.K.). Generally, a 5 L solution of WPC at 4 % or 8 % (w/v) protein, was hydrolysed with PTN 3.0S at 50 °C in a jacketed vessel, until a defined degree of hydrolysis was reached (i.e. 8 %). The pH was maintained at pH 8.0 by continuous addition of 4 N NaOH using a pH-stat (Metrohm Ltd, Herisau, Switzerland.). The degree of hydrolysis (DH, %), defined as the percentage of peptide bonds cleaved, was calculated from the volume and normality of NaOH used to maintain constant pH (Adler-Nissen, 1986). Details for the calculation of DH were given previously (Chapter 1 and 2). Following hydrolysis to a DH of 8 %, the protease was inactivated by heating the hydrolysate to 80 °C and holding for 30 min. Samples were taken and stored at -20 °C until analysed. The enzyme (E) to substrate (S) ratio used (0.003) was calculated on the basis of total protein content in the enzyme preparation and WPC. The above hydrolysate was used to characterise the performance of the plate and frame tangential flow membrane system.

6.3.2. 3 Process optimisation using the Millipore tangential flow membrane filtration system

All optimisation experiments were carried out with feed temperature at 50 °C. In accordance with manufacturer specifications, the mean membrane pressure was maintained at less than 2.07 bar for the 0.093 m^2 membrane and at less than 3.45 bar for the 0.46 m^2 membrane. The recommended feed crossflow for the 0.093 m^2

membrane was that value (l h^{-1}) which was obtained when the mean membrane pressure was adjusted to 1.3 bar. The recommended crossflow for the 0.46 m^2 membrane was 360 l h^{-1} ± 50 %.

It should be noted that initial trials for the optimisation of permeate flux were carried out using semi-soft tap water at 50 °C for both membranes (surface areas 0.093 m^2 (System no 1) and 0.46 m^2 (System no. 2)) in batch recycle mode, i.e. where retentate and permeate lines were returned to the feed reservoir. Flux rates (l m^{-2} h^{-1}) were recorded.

6.3.2.3.1 Calculation of Normal Water Permeability (NWP)

The first parameter which is measured with respect to any new membrane system is the normal water permeability (NWP). The normal water permeability (NWP) of a membrane is a good indicator of cleanliness of a membrane following a processing operation and subsequent cleaning steps. The inlet valve was adjusted to give an inlet pressure of 1.38 bar and the outlet valve was adjusted to give an outlet pressure of 0.69 bar, giving a mean membrane pressure of 1.03 bar (using System no. 2 and the 0.46 m^2 surface area membrane). A NWP value of 1.38 l /m^2/h/bar was obtained for the new unused membrane (0.46 m^2 surface area) with tap water at 50 °C. The water permeability value (NWP) for the membrane following each processing and subsequent cleaning was determined and compared to the above initial NWP value. The following equation was used to determine NWP:

$$NWP = R*F / (A*(Pin + Pout)/2)-Pp)$$

where R= Permeate flowrate (l); Pin = Feed inlet pressure (bar); Pout = Feed outlet pressure (bar); Pp = Permeate pressure (which in this case is zero); A = Total membrane surface area (m^2); F= Correction factor used for temperature of water (See appendix no. 1).

6.3.2.3.2 Optimisation experiments with hydrolysate in batch recycle mode

The whey hydrolysate (DH of 8 %, total solids of 5 or 10 % at 50 °C) was processed with membranes with surface areas of 0.093 m^2 and 0.46 m^2, and where the system was driven by either the peristaltic or centrifugal pump. These experiments were carried out in batch recycle mode, i.e. where the permeate and retentate streams were returned to the feed reservoir.

During initial experiments with the 0.093 m^2 surface area membrane, the inlet and outlet valves were adjusted to give the desired inlet and outlet pressures, and thus obtain a specific mean membrane pressure (bar) while keeping feed velocity constant. Later, using the 0.46 m^2 surface area membrane, the membrane system was operated at variable inlet feed pressures by adjusting the velocity setting on the pump. Both inlet and outlet valves remained open throughout this process.

6.3.2.3.3 Optimisation experiments with hydrolysate in concentration mode

The hydrolysate (DH of 8 %, total solids of 5 or 10 % at 50 °C) was fractionated using the tangential flow membrane system using the 0.093 m^2 and 0.46 m^2 surface area membranes, while driven by the centrifugal pump. These experiments were carried out in ultrafiltration mode where retentate was returned to the hydrolysate feed reservoir and permeate was continually removed.

During initial experiments with the membrane of 0.093 m^2 surface area the inlet and outlet valves were adjusted to obtain a specific mean membrane pressure (bar), while the feed velocity was maintained constant. Later, using the 0.46 m^2 surface area membrane, the system was operated at variable inlet feed pressures by adjusting the velocity setting on the pump. Both inlet and outlet valves remained open throughout the process.

6.3.2.4 Membrane cleaning procedures

As recommended by the manufacturers, the Pellicon® membranes were cleaned by recirculating 0.1 N NaOH at 50 °C through the system for 30 min. This was followed by rinsing with water at 50 °C before determining the NWP.

6.3.2.5 Analysis of peptide molecular mass distributions using gel permeation HPLC

Whey protein hydrolysate peptides, i.e. retentate and permeate from the UF system were analysed by gel permeation (GP) HPLC on a Beckman Spherogel-TSK 2000 SW column (600 mm x 7.5 mm), equilibrated and run with 30 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid (TFA) in water. Chromatography was conducted using a Waters™ HPLC detector, autosampler and pumping systems (Millipore, U.K), at room temperature, the flowrate was 1 ml min^{-1} and the absorbance at 214 nm was recorded. The injection volume was 20 μl. The column was calibrated with the following standard proteins and peptides: Bovine serum albumin (66,000 Da); Carbonic anhydrase (29,000 Da); Ribonuclease A (13,700 Da); Cytochrome C (13,000 Da); Aprotinin (6,500 Da); Insulin (5,700 Da); Bacitracin (1,400 Da); Tryptophan (204 Da); Phenyalanine (165 Da) and Glycine (75 Da). A Minichrom® data handling package (V.G. Data Systems, Manchester, England) was used to record and integrate chromatograms (Mullally *et al.*, 1994). The proportion of peptides corresponding to a particular molecular weight, e.g., ≤ 10 kDa was calculated as a percentage of the total area of the profile obtained at 214 nm.

6.4 Results and Discussion

The flux rate (l m^{-2} h^{-1}) of the permeate was the main factor requiring optimisation, since a higher flux results in shorter processing time. However, lower flux rates with minimum membrane fouling may be more desirable in some instances for the development of a semi/continuous mode for hydrolysate processing.

The factors considered during the optimisation of flux in our study were as follows: (a) temperature which was maintained at 50 °C. This temperature was the maximum allowable for the membranes used. Furthermore, at this temperature microbial growth was minimised; (b) mean membrane pressure (c) total % solids concentration of the feed and (d) the torque applied to membrane during installation in the membrane holder. Optimisation trials were carried out using membranes of different surface area, i.e. 0.093 m^2 (System no. 1) and 0.46 m^2 (System no. 2).

6.4.1 System no 1: Using membrane with a surface area of 0. 093 m^2.

6.4.1.1 Optimisation of flux using water in batch recycle mode

The initial NWP using this membrane was 1.167 l /m^2/h/bar obtained using a peristaltic pumping system. However, attempts to characterise this membrane in detail with respect to optimum flux proved impossible. Problems were encountered with increasing backpressure exerted by the feed, causing the connecting tubing to burst. It was concluded that the peristaltic pump was unsuitable for the operation of this membrane system. Therefore, the system was subsequently run with the centrifugal pump and reinforced tubing (capable of withstanding pressures up to 6.90 bar) allowing maximal crossflow across the membrane.

6.4.1.2 Optimisation of flux using whey protein concentrate

The objective was to characterise the system (system no. 1) using the unhydrolysed WPC substrate and a centrifugal pump. The pump speed setting was adjusted to 0.5, and the mean membrane pressure was adjusted to 1.3 bar (i.e. to obtain the recommended feed crossflow (1 h^{-1})).

The feed substrate was unhydrolysed whey protein concentrate at 10 % (w/v) total solids. This substrate was presented to the membrane at 50 °C. Attempts at establishing the flux of the unhydrolysed material using a centrifugal pump resulted in immediate pressure build-up (within 1 min of start up) in the system and fouling of the membrane. The pressure attained was the maximal allowable for the system (i.e. mean membrane pressure of 2.07 bar), and therefore, this experiment was terminated.

It was concluded that the unhydrolysed WPC substrate could not be processed through the membrane as this resulted in immediate concentration polarisation and fouling. It was concluded that a limited degree of hydrolysis is required before the WPC substrate can be processed through the membrane. It is known that whey protein substrate viscosities can be reduced following hydrolysis (O'Keefe and Kelly, 1981; Hooker *et al.*, 1982).

In subsequent experiments, therefore, attempts at characterising the optimum flux for this system were performed with WPC which had been hydrolysed with PTN 3.0S to DH of 8 %. It was still found, however, that there was a lack of reproducibility in the flux values when a WPC hydrolysate was used in attempts to characterise system no. 1. It was found that the NWP changed for this system on disassembly and re-assembly of the membrane. This led to the conclusion that the system was sensitive to differences in the applied torque during membrane installation.

6.4.1.3 Establishment of an optimum torque for UF system assembly

The objective was to establish the optimum torque level which could be applied to the membrane during installation which in turn would result in reproducible NWP values following membrane use, cleaning and reassembly. Both water at 50 °C and a whey protein hydrolysate (5 % total solids, DH of 8 %, at 50 °C) were presented to the membrane. The pump speed setting was 0.5 and the mean membrane pressure was adjusted to give 1.3 bar. The membrane was installed into the membrane holder using different torques, as shown in Table 1. The permeate flux was recorded (Table 1).

There appeared to be no correlation between the torque applied to the membrane during installation and the resultant flux value. For example, it was seen following

three different installations of the membrane at a torque of 200 in lbs that the permeate flux for water at 50 °C varied from 12.34 to 29.03 l m^{-2} h^{-1}.

Table 1: Effect of torque applied on the membrane (surface area 0.093 m^2) during installation, on permeate flux (l m^{-2} h^{-1}).

Torque (in lbs)	Permeate flux (l m^{-2} h^{-1})
Water at 50°C	
	1 st installation
200	12.3
	2 nd installation
150	33.55
200	21.29
160	37.60
	3rd installation
200	29.03
200	27.35
Hydrolysate at 50°C	
100	23.23
200	28.39

It was concluded that the membrane with a surface area of 0.093 m^2 was very sensitive to the torque applied during installation in the holder. During consultation with the membrane manufacturers, it was disclosed that this type of membrane was also shown by other research workers to be susceptible to deformation due to applied torque/pressure, in particular if this pressure was applied in an uneven manner. Compressibility was a further problem as the membrane had the ability to 'recover' after each installation. For example, the first installation with an applied torque of 200 in lbs gave a permeate flux value of 12.34 l m^{-2} h^{-1}. However, after the second installation when the torque was adjusted to 200 in lbs, a permeate flux of 21.29 l m^{-2} h^{-1} was attained using water at 50 °C in both cases. It was conceded by the membrane manufacturers that 'wafer-thin' membranes may display this problem. Following recommendations by the manufacturer and distributor of the Pellicon® system, a

membrane with larger surface area, i.e. 0.46 m^2 was purchased. This cassette proved to be most robust and was not as sensitive to applied torque and did not show compressibility problems during further experiments.

6.4. 2 System no 2: Using membrane with a surface area of 0.46 m^2

6.4.2.1 Optimisation of flux using water in the batch recycle mode

The NWP using this system was initially determined to be 1.410 ± 0.006 l/m^2/h/bar, using a torque of 300 in lbs during installation of the membrane. The value obtained for this system was the average of four separate determinations. This membrane, in general gave more reliable results as compared with the 0.093 m^2 surface area membrane, as it was not subject to compressibility.

6.4.2.2 Optimisation of flux using whey protein hydrolysate (10 % total solids, at 50°C, DH of 8 %) in the concentration(UF) mode.

The objective was to characterise the permeation properties of 0.46 m^2 membrane with a hydrolysed feed at a particular pump speed setting (i.e. velocity). The initial mean membrane pressure was ~ 0.28 bar (i.e. 4 psi) which was obtained by adjustment of inlet (Pin) and outlet (Pout) valves. The initial feed crossflow was ~ 26 l h^{-1} at a pump speed setting no. 1. The final objective was to repeat this characterisation using higher speed settings.

According to the recommended operating conditions, membrane pressure and feed crossflow rate are normally controlled by adjustment of inlet and retentate valves in order to generate standard performance curves of permeate flux versus mean membrane pressure for the ultrafiltration system. From these curves the optimum mean membrane pressure to be used in order to provide maximal permeate flux over a long processing time can be ascertained. Therefore, using a hydrolysate and a constant pump speed setting, i.e. speed setting no. 1, a performance curve for the membrane system was generated (Figure 1).

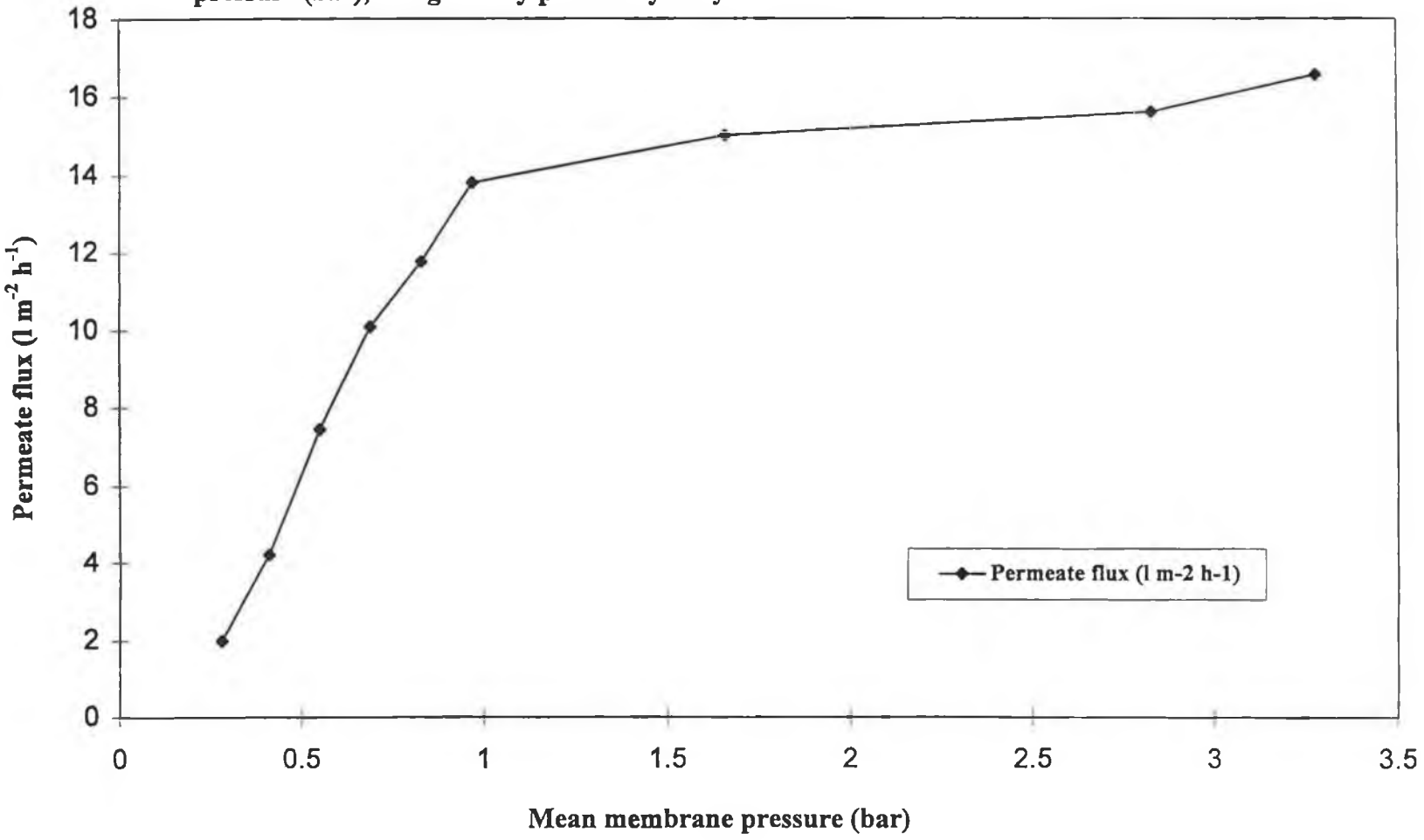
From Figure 1 it was seen that it is possible to generate a performance curve for the membrane system when it was operated in the concentration mode. Figure 1 shows that permeate flux initially increased with increasing mean membrane pressure.

However, a situation was reached where the permeate flux was independent of mean membrane pressure, i.e. at a mean membrane pressure of approximately 1 bar.

It should be noted, during this experiment, that fluctuations were observed in the pressure values which was due to an unusual 'pulsing' action by the pump. Therefore, the curve generated in Figure 1 was generated from the average pressure obtained at the inlet and outlet pressure gauges (i.e. the highest and the lowest pressure value on each pressure dial divided by 2). This fluctuation was ± 0.70-0.14 bar above and below the each average reading initially, however, the fluctuation increased to ± 0.35 bar for each of the higher readings. It appeared, therefore, that the concentration of the feed/substrate used was too high. The pump appeared to malfunction due possibly to the development of high backpressures exerted by the feed as the inlet and outlet pressure valves/gauges were adjusted, for each mean pressure value, during the generation of the standard performance curve.

It was concluded that problems associated with concentration polarisation, membrane fouling and increasing back pressures were found using a whey protein hydrolysate at 10 % total solids during membrane characterisation in the concentration mode. Although the objective would be to increase system productivity by using a high substrate total solids concentration, the results indicated that a lower total solids concentration would be required for optimisation of this membrane system.

Figure 1: Performance curve showing permeate flux (l m^{-2} h^{-1}) as a function of mean membrane pressure (bar), using a whey protein hydrolysate feed.



6.4.2.3 Optimisation of flux using whey protein hydrolysate (5 % total solids, at 50 °C, DH of 8 %) in batch recycle mode.

The objective was to characterise permeation properties of the membrane using a whey protein hydrolysate with a lower total solids concentration. The procedure was as in as in previous experiment (6.4.2.2) except using 5 % total solids concentration in the feed.

It was observed that at low feed crossflow (pump setting no. 1), the centrifugal pump behaved in an irregular manner. This was characterised as a 'pulsing' of pressure over the membrane resulting in irreproducible results. This problem persisted even when using water at 50 °C. The observed crossflow values in this experiment were lower than the manufacturer recommendations, particularly at low mean membrane pressures. The manufacturer recommended crossflow values were 240-480 $l\ h^{-1}$. The values obtained in this experiment were between 60 and 288 $l\ h^{-1}$. The higher crossflow value obtained corresponds to a mean membrane pressure of 2.06 bar (this pressure is approximately 60 % of the maximal allowable pressure for the system). At this time the pump had to be switched off due to the severe 'pulsing' of the pressure across the membrane.

It was concluded that the system could not be operated when the mean membrane pressure and feed crossflow rate were regulated by adjustment of the feed inlet and outlet valves. The strategy subsequently adopted, was to control mean membrane pressure by adjustment of pump speed setting, whilst leaving both inlet and outlet valves open.

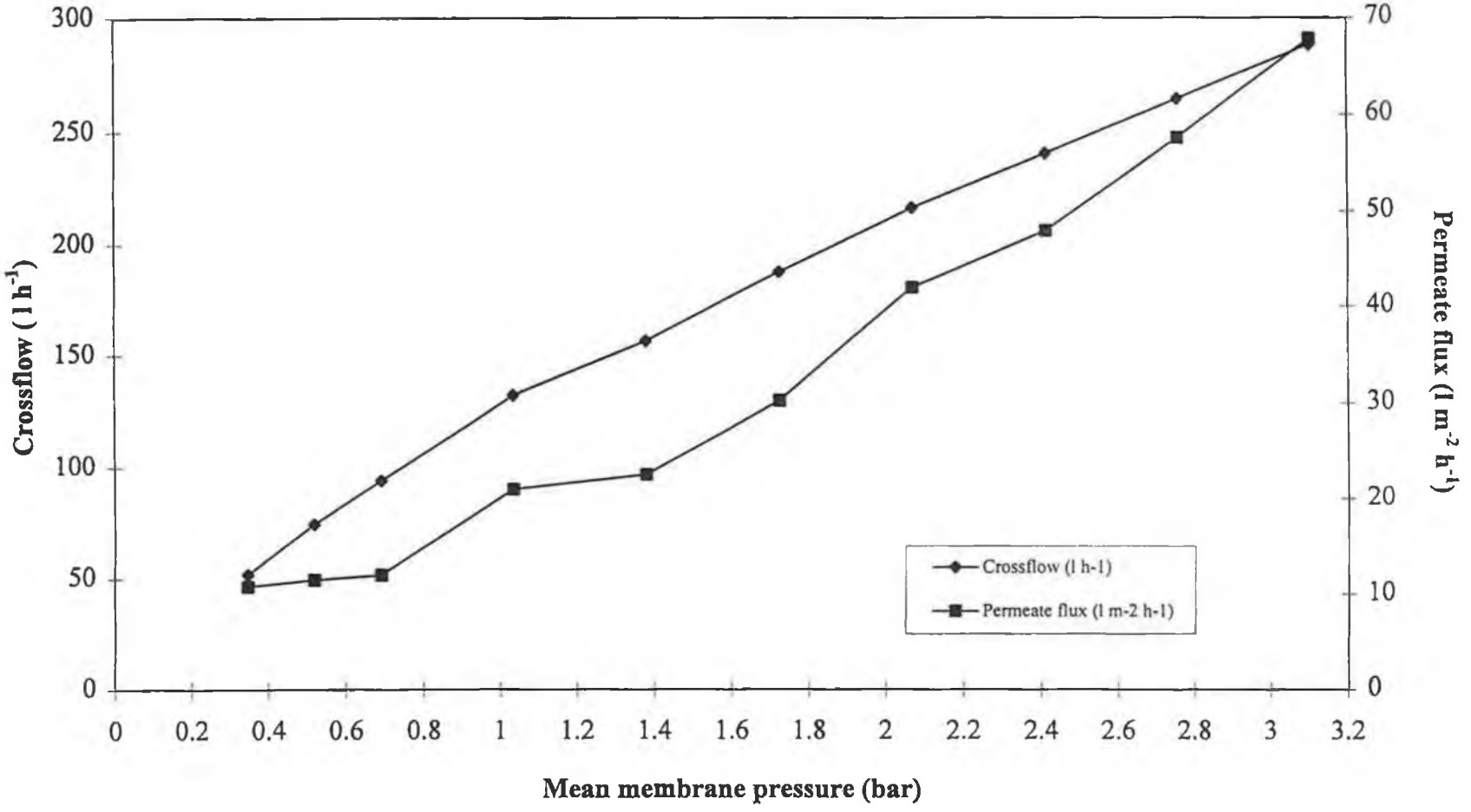
6.4.2.4 Generation of flux performance curves in a batch recycle mode with mean membrane pressure set with variable pump speed control

The objective was to find the relationship between mean membrane pressure, feed crossflow and permeate flux over a broad range of inlet pressures. The procedure was as for the previous experiment (6.4.2.3), except that mean membrane pressure was regulated solely by the velocity setting on the pump, while the inlet and outlet valves remained fully open.

It was shown that as inlet pressure was increased by adjustment of pump speed setting that both permeate flux (l m^{-2} h^{-1}) and feed crossflow (l h^{-1}) increased in a linear fashion (Figure 2).

It was concluded that this new approach for characterisation of the membrane was satisfactory. For further studies feed inlet pressures of 1.03 bar and 2.76 bar were selected. These inlet pressures correspond to mean membrane pressures of 0.52 and 1.38 bar at pump speed settings of 0.1 and 0.8 respectively.

Figure 2: Effect of mean membrane pressure on crossflow (l h^{-1}) and permeate flux (l m^{-2} h^{-1})



6.4.2.5 Characterisation of permeate flux with whey protein hydrolysate in the concentration mode at two selected mean membrane pressures

The objective was to characterise the permeation properties of the 0.46 m^2 membrane using two different mean membrane pressures.

Both the inlet and outlet valves remained open. Two separate trials were carried out, the first using a mean membrane pressure of 0.52 bar and the second using a mean membrane pressure of 1.38 bar. The mean membrane pressure was regulated by the velocity speed setting on the pump. The feed (10 litres) used was a whey protein hydrolysate at DH of 8 %, with a total solids concentration of 5 %. The initial flux and crossflow values were recorded after ten minutes of operation. The initial feed crossflow was 37.5 l h^{-1} for the mean membrane pressure of 0.52 bar. The initial feed crossflow for the mean membrane pressure of 1.38 bar was 218 l h^{-1}.

It was seen that for a mean membrane pressure of 0.52 bar, the permeate flux remained relatively constant for the first 90 min of operation. However, after this time as the total solids concentration increased in the retentate, the flux decreased to 3 l m^{-2} h^{-1} after 120 min (Figure 3). Figure 3 also shows slight increases in mean membrane pressure over time. After 90 min the mean membrane pressure was readjusted (to the initial value, i.e. 0.52 bar), however, this reduced the flux rate. The drop in flux was not proportional to the drop in pressure. This would indicate that concentration polarisation was beginning to dominate and fouling of the membrane was occurring, thus reducing the flux.

For the higher mean membrane pressure of 1.38 bar the time of processing was shorter. Over a 25 min period the flux decreased from approximately 42.5 l m^{-2} h^{-1} to 32 l m^{-2} h^{-1}. At this stage, a large volume of the hydrolysate (i.e. 8.2 l) had been processed (Figure 4). For the lower mean membrane pressure, 1.12 l of the feed was processed in the same time.

It was concluded that the higher the mean membrane pressure the shorter the processing time. For the development of a semi-continuous/ continuous operation, however, this may not be desirable, as a certain reaction time must be allowed for the enzyme and substrate in the reactor. This experiment showed that it is possible to characterise the membrane system while keeping the inlet and outlet valves open.

Figure 3: A performance curve showing permeate flux (l m^{-2} h^{-1}) over time using an inlet pressure of 1.03 bar.

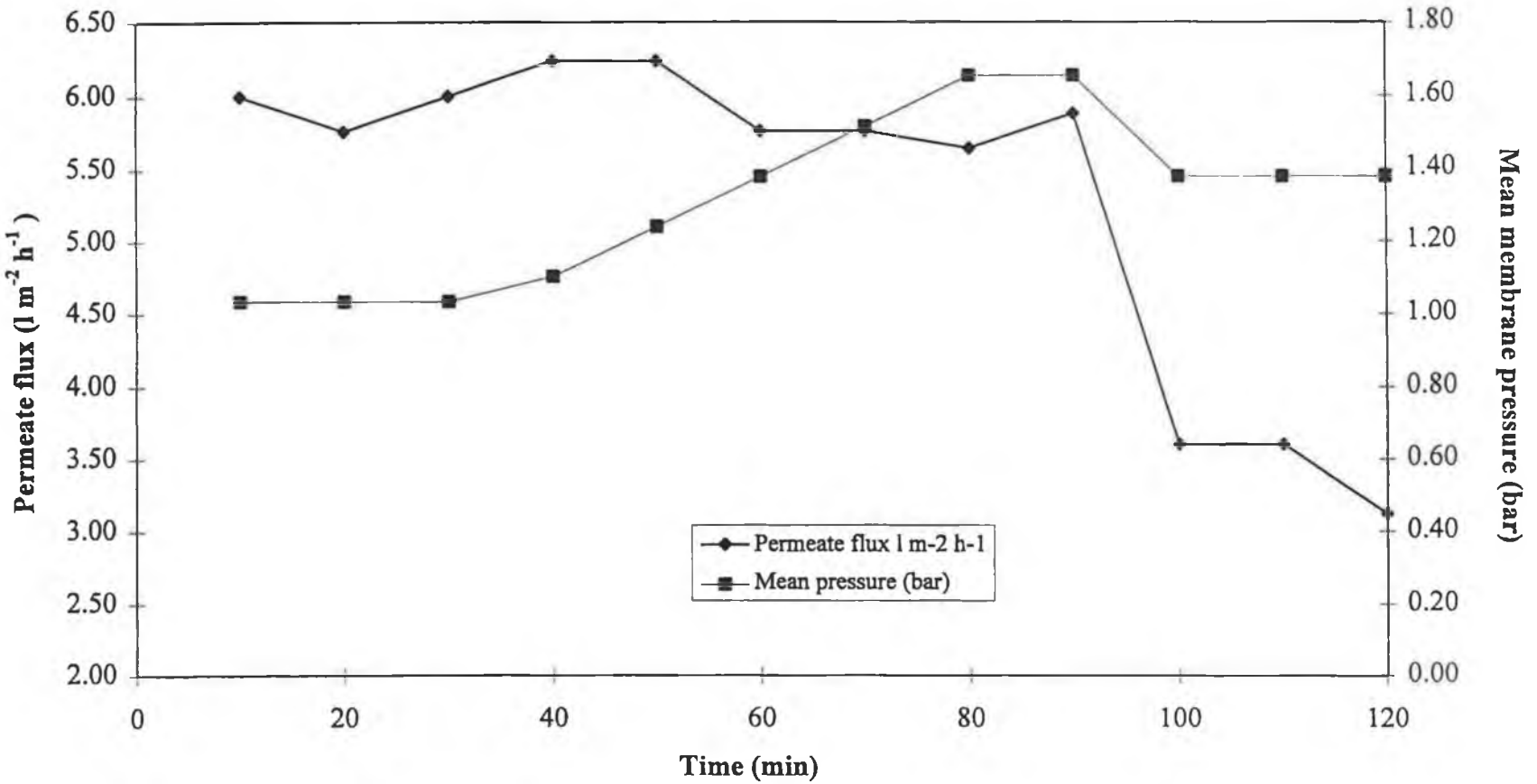
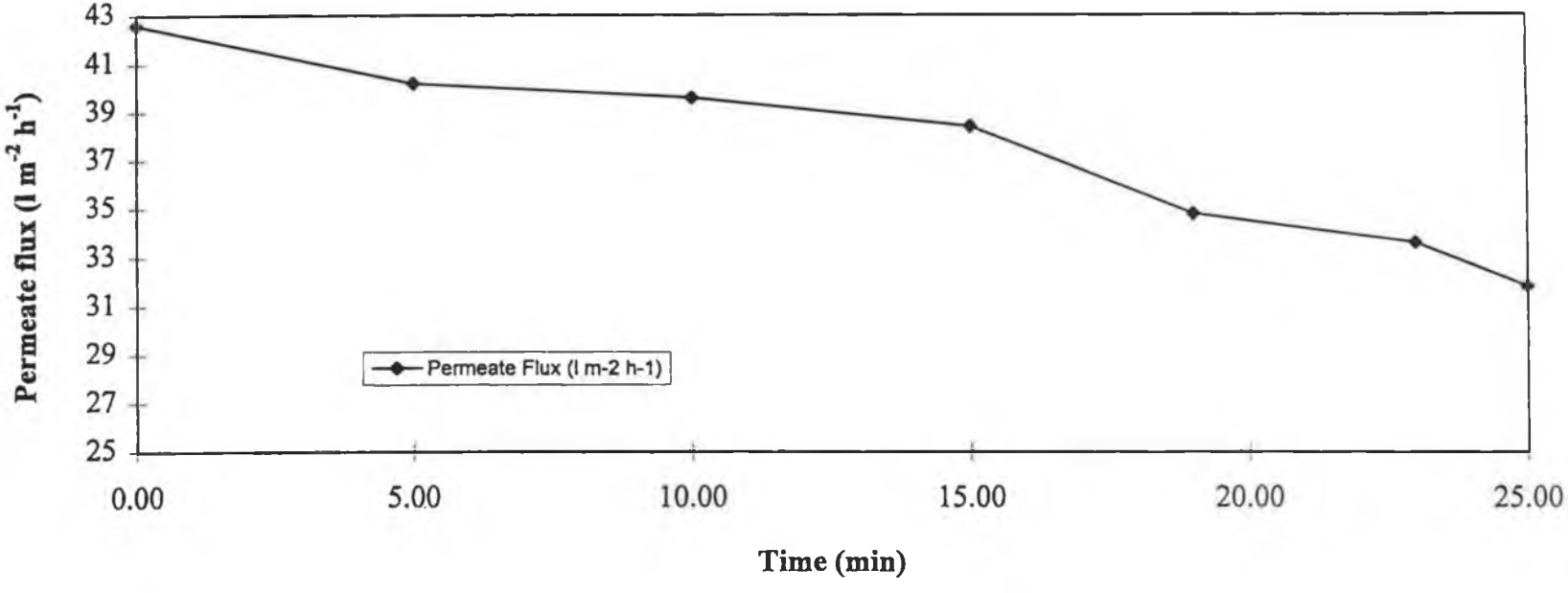


Figure 4: A performance curve showing permeate flux (l m^{-2} h^{-1}) over time using an inlet pressure of 2.76 bar



However, there is less control of the mean membrane pressure over time as seen in Figure 3. It appears that the lower mean membrane pressure value chosen (i.e. 0.52 bar) would favour an extended processing time, in particular for the potential continuous processing of a hydrolysate.

6.4.2.6 Evaluation of suitable cleaning agents for cleaning Millipore membranes

Whey is a well known membrane foulant. Whey protein concentrates such as that used in our study have a high mineral content, in particular calcium and phosphate, which may contribute to protein aggregation and fouling. For optimal productivity an efficient membrane cleaning procedure is required. Periodic cleaning of the membrane removes deposited protein and salts from the membrane pores and should return the membrane to those conditions favouring optimal productivity. The objective was to evaluate an efficient cleaning procedure for the Millipore ultrafiltration membrane.

Cleaning agents/ procedures were evaluated for their ability to restore the NWP value of the membrane to its initial value, i.e. for the new unused membrane. The NWP value established for the 0.46 m^2 surface area Pellicon cassette, using an applied torque of 300 in lbs, was 1.410 $l/m^2/h/bar$.

According to the manufacturers instructions, the membrane should be efficiently cleaned by recirculating 0.1 N NaOH at 50 °C through the system. This procedure alone, however, was not capable of restoring NWP values following ultrafiltration trials with the whey protein hydrolysate. A number of alternative cleaning agents, such as sodium hypochlorite or nitric acid were then recommended by the cassette manufacturers. However, some of the recommended cleaning procedures were not compatible with the stability of the membrane and /or the seals within the pumping system. The differential compatibility of certain cleaning agents with the pumping system had to be taken into consideration when developing an efficient cleaning procedure. Sodium hydroxide (0.1 N) in combination with sodium hypochlorite (200 ppm) was recommended as an efficient cleaning agent for the removal of whey protein foulants. However, sodium hypochlorite is not compatible with the seals in the centrifugal pump and therefore a peristaltic pump was linked to membrane for this cleaning step. In cases of severe fouling, a 1-2 % (v/v) solution of a commercially

available enzymatic membrane cleaning agent, Henkel P3-53, was run through the system at alkaline pH values and at temperatures up to 50 °C. This reagent restored the NWP to between 80-90 % of the original value. Cleaning trials with a second enzymatic cleaning agent, Esperase® at 1-2 % (v/v) concentration was used but lead to immediate severe fouling of the membrane within minutes of start up and the NWP value was reduced to less than 20 % of the original NWP.

It was also observed that over time a grey deposit was formed on the membrane at the feed inlet channels. This suggested the formation of a mineral precipitate. A cationic chelator ethylenediaminetetracetic acid (EDTA) tetrasodium salt, was then used in 0.1 N NaOH at a concentration of 0.5-1 % (w/v) in an attempt to clean the membrane. However, this cleaning step appeared to have only minimal effect on the recovery of NWP. When mineral fouling is a problem in other membrane systems, usually an acid wash is included. Phosphoric acid, at a concentration of 6.6 % (v/v), therefore became the reagent of choice to remove mineral foulants. This acid wash restored the NWP to >90 % of the original value. However, acids including nitric acid, which are usually used in this situation were not compatible with the seals of the centrifugal pumping system. Table 2 summarises the cleaning agents considered and shows their compatibility with the system.

Table 2: Compatibility of various cleaning agents with the centrifugal pump and membrane system

Cleaning agent at 50 °C	Pump	Membrane
0.1 N NaOH	√	√
Henkel P3-53 1 % (w/v)	√	√
Esperase® 1-2 % (w/v)	X	X
EDTA, tetrasodium salt 0.5 % (w/v)	√	√
Sodium hypochlorite (200 ppm in 0.1 N NaOH)	X	√
0.1 N Nitric acid	X	√
6 % (v/v) Phosphoric acid	√	√

where X ≡ not compatible; and where √ ≡ compatible.

During cleaning, the flowrate of the cleaning solution was found to be important, it has been shown that with decreasing flowrate, the rate of cleaning decreases (Bird and Fryer, 1991). However, it appears that low mean pressures are favoured during the rinsing step, because at higher pressures, the permeate stream may exert high compressional forces on the deposited particles, not allowing them to be removed as efficiently as in the case of lower pressures (Kulozik, 1995). In our study, it was difficult to reduce mean pressures, without lowering velocities. Therefore, the maximum allowable flowrate within the pressure limits of the system was used during the cleaning procedure. A number of cleaning steps with NaOH (0.4 % w/v) as used in our study may lead to the build-up of a sodium layer on the membrane from this reagent as seen by others (Timperley and Smeulders, 1988; Bird and Fryer, 1991), thus reducing NWP values. It appeared that the acid washing step was the most efficient, in terms of cleaning the membrane, in particular, immediately after the alkaline cleaning step.

The whey deposit is represented as a complex of protein together with calcium salts, containing mainly calcium phosphate. In the case of concentrated whey, calcium

nitrate deposits have also been found (Jeurnink and Brinkman, 1994). Whey deposit is known to contain considerably more mineral compounds than other milk based deposits (Delsing and Hiddink, 1983), therefore it has been recommended to start the cleaning procedure with an acid cleaning step followed by an alkaline step after processing of whey (Jeurnink and Brinkman, 1994). Fouling by whey protein solutions and deposition models for UF membrane fouling have been studied (Hallström *et al.*, 1989; Belmar-Beiny and Fryer, 1993). A more specific study of an individual whey protein, β–lactoglobulin, by differential scanning calorimetry, showed that the rate of fouling decreased as the pH of the feed solution was increased. However, the thermal stability of β–lactoglobulin as expected decreased as the pH increased, (Gotham *et al.*, 1992).

It was concluded that the following cleaning steps for the 0.46 m^2 surface area membrane, were thus adopted: In all cases an inlet pressure of 1.38 bar and an outlet pressure of 0.35 bar was used, (a) the system was flushed with water at 50 °C (b) 0.1N NaOH was recirculated for 30 min at 50 °C (c) the membrane system was then connected to a peristaltic pump system and a solution of sodium hypochlorite(200 ppm in 0.1N NaOH) was pumped through the system at 50 °C. Step (a) was then repeated. If the NWP was not restored at this point, a 6.6 % (v/v) phosphoric acid solution was recirculated through the system for 30 min at 50 °C. Step (a) was than repeated, followed by (b), before rinsing with water and recording the NWP.

6.4. 3 Gel permeation HPLC of the ultrafiltered whey protein hydrolysate

The objective was to monitor the effect of membrane processing on the fractionation of peptides produced by enzymatic hydrolysis of WPC. The molecular weight distribution of peptides produced during hydrolysis was monitored using gel permeation HPLC to assess the fractionation effectiveness of the membrane, in addition to ascertaining the peptide profile in the permeate. The molecular size distribution of a whey protein hydrolysate can dictate its use as a functional food (Turgeon *et al.*, 1991,1992), as a fraction rich in bioactive peptides (Bouhallab and Touzé, 1995) or for inclusion in hypoallergenic infant foods (Jost *et al.*, 1988).

The procedure was as outlined in methods (section 3.2.5) using hydrolysate of DH = 8 % and the 0.46 m^2 membrane (i.e. System no. 2). Samples were taken during the course of the hydrolysis experiment as outlined in section 3.2.2. (Figure 1).

The gel permeation HPLC peptide profiles of the permeate and retentate following ultrafiltration of the whey protein hydrolysate through the 10 kDa molecular weight cut-off membrane are shown in Figure 5 (a) and (b). Samples permeate and retentate were taken after 30 min and when the system was operated in the concentration mode. The HPLC profiles of the permeate, as expected, revealed that a large proportion of the peptides are <10 kDa. The permeate peptide profile revealed that after processing of the hydrolysate, only 3.9% of the peptides had a molecular weight distribution >10 kDa (Figure 5 (b)). In the retentate (unfractionated hydrolysate) 10.5 % of the peptides were > 10 kDa (Figure 5 (a)). The peptide profile of the retentate therefore gives an indication of the molecular size distribution of the fraction of hydrolysate peptides retained by the membrane, which were produced by hydrolysis of WPC by the pancreatic preparation, PTN 3.0S to DH of 8 % (Figure 5 (a)).

It is well known that peptides of certain size have certain functional properties, or specific properties such as hypoallergenicity and bioactivity. It was concluded that these properties may be enriched within hydrolysates if peptides can be fractionated using membrane processing.

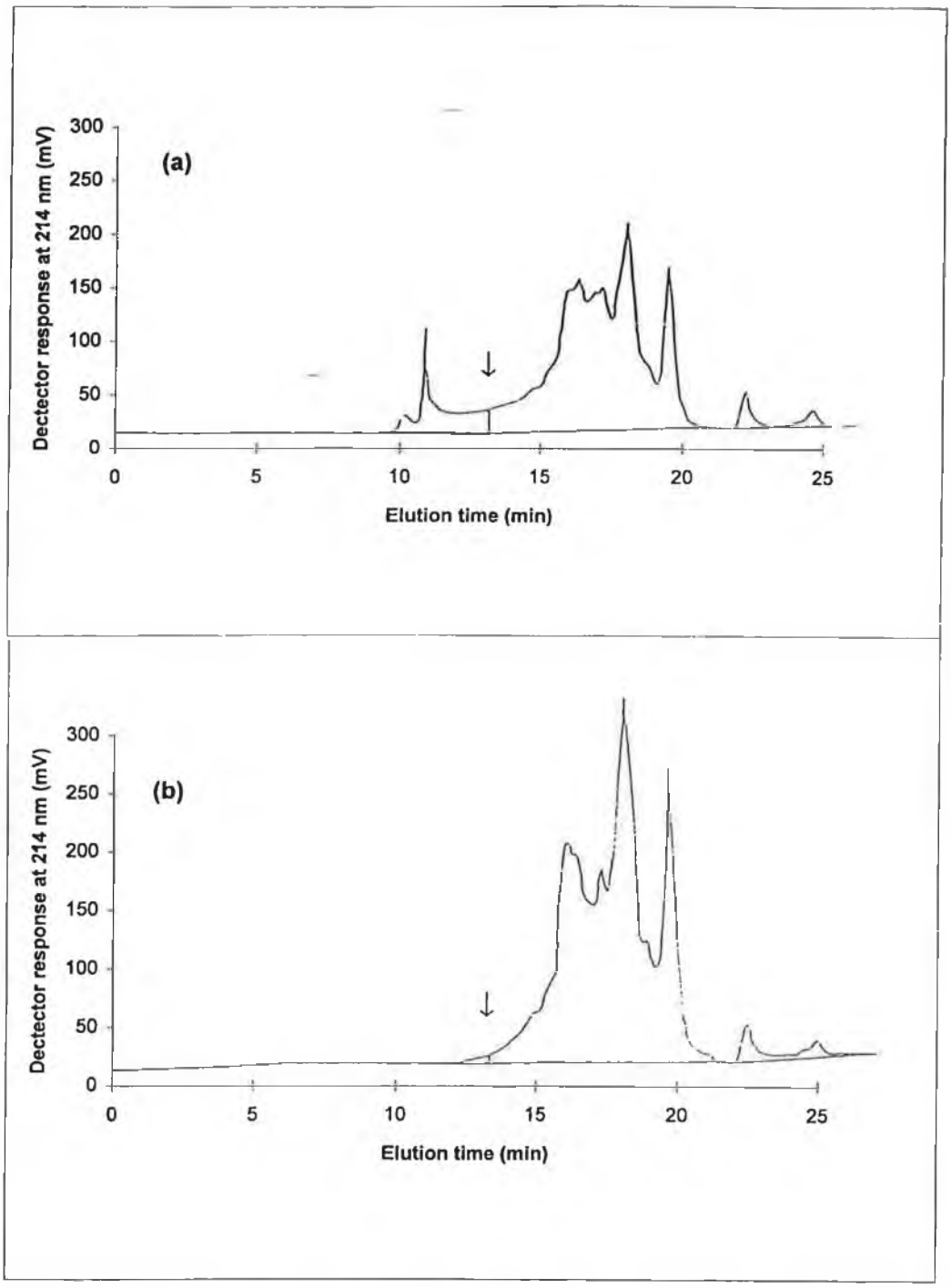


Figure 5: Gel permeation chromatograms from the Spherogel - TSK 2000 SW HPLC column showing millivolt response, assayed by absorbance at 214 nm and elution times of (a) retentate and (b) permeate peptides following ultrafiltration using a 10 kDa cut-off membrane. The arrow marks the elution of peptides at an elution time corresponding to 10 kDa.

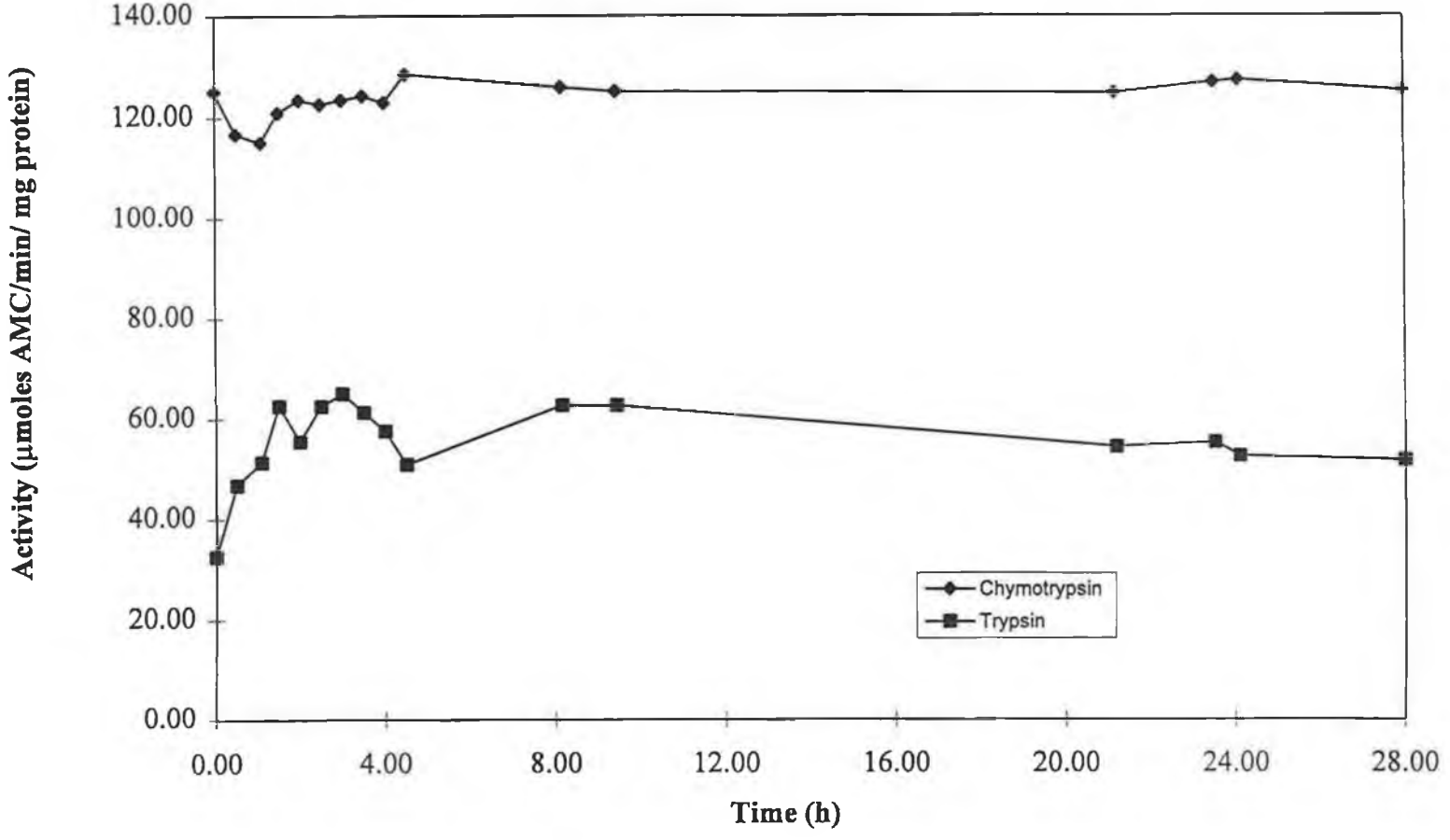
6.4.4 Enzyme stability in the presence of whey protein substrate

The objective was to establish the rate of decay for trypsin and chymotrypsin, enzymes present in the commercial protease preparation, PTN 3.0S which were used in the development of whey protein hydrolysates in this study. The hydrolysis reaction (as outlined in method 3.2.2) was extended for 28 h, during which subsamples of the hydrolysate were taken, frozen at -20 °C, and later assayed for trypsin and chymotrypsin activity.

It was shown that trypsin and chymotrypsin (μmoles AMC/min/mg protein) remained active for extended processing times in the presence of the whey protein substrate/hydrolysate (Figure 6).

It was concluded that the fact that trypsin and chymotrypsin are completely active for 28 h even at 50 °C argues well for their application in a continuous enzyme hydrolysis system. It has previously been shown that whey protein/ hydrolysates appears to stabilise trypsin activity (Jost and Monti, 1977).

Figure 6: Chymotrypsin and trypsin activities (μmoles AMC/min/mg protein) during extended hydrolysis of WPC.



6.5 Conclusions

This study attempted to characterise a tangential crossflow plate and frame membrane system with a view to its use in batch, semi- and continuous processing of milk protein hydrolysates. Two membranes of different surface areas were investigated, i.e. 0.093 m^2 and 0.46 m^2. A number of conclusions were drawn:

It was initially observed that a peristaltic pumping system was unsuitable for processing of WPC hydrolysates using the above membrane system. This was due to the fact that the tubing which linked the pump to the plate and frame membrane unit was not sufficiently reinforced to withstand high backpressures. A centrifugal pump was selected for further experiments to allow maximal crossflow over the membrane. This pump could be equipped with high pressure tubing capable of withstanding high backpressures.

It was found that the unhydrolysed WPC (10 % total solids) substrate could not be processed by the membrane as this resulted in rapid membrane fouling. A certain degree of hydrolysis was required before the WPC substrate could be processed through the membrane. Hydrolysis of the WPC should bring about a reduction in the viscosity of the feed (Hooker *et al.*, 1982). The hydrolysate used therefore for characterisation of the system had a degree of hydrolysis of 8 % (DH = 8 %). Furthermore, the total solids concentration of the hydrolysate was decreased during membrane optimisation experiments from 10 to 5 % (i.e. from 8 % to 4 % total protein). The feed solution was maintained at 50 °C during membrane processing, this temperature would result in a further reduction in the viscosity of the feed, in addition to controlling possible microbial contamination during the course of a membrane processing experiment.

The membrane with a surface area of 0.093 m^2 was very sensitive to the torque applied during installation in the holder. Following recommendations by the manufacturers and distributors of the Pellicon®, a membrane of larger surface area, i.e. 0.46 m^2 was used. This cassette proved to be most robust and was not as sensitive to torque and did not show compressibility problems in further experiments.

Another important consideration during our study was the efficacy of the cleaning and sanitation procedures for the membrane. Membranes are fouled by proteins, and to different extents depending on the concentration of the product reached during processing. The membrane in our study permitted the use of alkaline detergents and sodium hypochlorite to break down proteins and amino acids. Acids could also be used to remove mineral deposits. However, it was found that the pump seals were not compatible with various cleaning reagents suitable for efficient membrane cleaning. A cleaning procedure which differed to that recommended by the manufacturers was thus developed.

The centrifugal pump was sensitive to high feed solids concentrations and low crossflow. Because this system was unsuitable for the use at low cross flow, the strategy adapted was to control mean membrane pressure by adjustment of pump speed setting, whilst leaving both inlet and outlet valves open.

For characterisation of permeation of the 0.46 m^2 membrane, 1.03 bar and 2.76 bar, were selected as the feed inlet pressures, corresponding to mean membrane pressures of 0.52 and 1.38 bar respectively. It was found that the 1.38 bar mean membrane pressure resulted in a shorter processing time.

The stability of the PTN 3.0S enzyme preparation over time in the presence of a whey protein substrate suggests that this preparation is ideally suited to continuous enzymatic hydrolysis over extended periods, even at temperatures up to 50 °C.

It is well known that fractionated peptides, produced using membrane ultrafiltration, of a specific size have certain functional properties (Gauthier *et al.*, 1993), or may have specific properties such as hypoallergenicity (Van Bersteijn *et al.*, 1994) and bioactivity (Bouhallab and Touzé, 1995). These properties are enriched within hydrolysates if peptides can be fractionated using membrane processing. Fractionation of peptides <10 kDa was achieved by membrane processing of the WPC hydrolysate in this study. In addition, if membrane processing was adopted as a method of fractionation of specific peptides, heat inactivation could be eliminated during processing of hydrolysates, as the proteolytic activities could be retained by the membrane. The enzymatic preparation could, potentially be reused. Elimination of the

heating step would also alter the permeation characteristics and solubility of the hydrolysate (Turgeon and Gauthier, 1990; Britten *et al.*, 1994).

However, the Millipore membrane system has not been specifically designed or developed for use in the dairy industry. The primary market to date has been in the pharmaceutical industry, where these membrane systems are used mainly in clarification steps involving low feed protein concentration. Future developments in the processing of milk proteins in the food industry should involve ceramic membranes, as suggested by others (Hallström *et al.,* 1989). Ceramic membranes are in general more robust towards the stringent cleaning required to remove protein and mineral foulants and in general are more suitable for highly viscous feed solutions (Daufin and Merin, 1995). The system investigated proved very difficult to efficiently operate due to problems with fouling by the feed and due to a lack of compatibility of conventional cleaning agents with the pumping systems. Much work remains to be carried out in order to develop an efficient pilot scale ultrafiltration system for continuous/ semi-continuous hydrolysis of milk proteins.

6. 8 Appendices

Appendix B

NORMALIZED WATER PERMEABILITY TEMPERATURE CORRECTION FACTOR (F)*								
T(°F)	T(°C)	F	T(°F)	T(°C)	F	T(°F)	T(°C)	F
125.6	52	0.595	96.8	36	0.793	68.0	20	1.125
123.8	51	0.605	95.0	35	0.808	66.2	19	1.152
122.0	50	0.615	93.2	34	0.825	64.4	18	1.181
120.2	49	0.625	91.4	33	0.842	62.6	17	1.212
118.4	48	0.636	89.6	32	0.859	60.8	16	1.243
116.6	47	0.647	87.8	31	0.877	59.0	15	1.276
114.8	46	0.658	86.0	30	0.896	57.2	14	1.310
113.0	45	0.670	84.2	29	0.915	55.4	13	1.346
111.2	44	0.682	82.4	28	0.935	53.6	12	1.383
109.4	43	0.694	80.6	27	0.956	51.8	11	1.422
107.6	42	0.707	78.8	26	0.978	50.0	10	1.463
105.8	41	0.720	77.0	25	1.000	48.2	9	1.506
104.0	40	0.734	75.2	24	1.023	46.4	8	1.551
102.2	39	0.748	73.4	23	1.047	44.6	7	1.598
100.4	38	0.762	71.6	22	1.072	42.8	6	1.648
98.6	37	0.777	69.8	21	1.098	41.0	5	1.699

* Based on Water Fluidity Relative to 25°C (77°F) Fluidity Value

$F = (\mu_{T°C}/\mu_{25°C})$ or $(\mu_{T°F}/\mu_{77°F})$

Bibliography

Abita, J. P., Delaage, M. and Lazdunski, M. (1969). The mechanism of activation of trypsinogen: The role of the four N-terminal aspartyl residues. Eur. J. Biochem., 8, 314-324.

Acharya, K.-Ravi, Ren, J. and Stuart, D.I. (1991). Crystal structure of human α-lactalbumin at 17 Å resolution. J. Mol. Biol., 221, 571-81.

Adler-Nissen, J. (1976). Enzymatic hydrolysis of proteins for increased solubility. J. Agric. Food. Chem., 24 (6), 1090-1093.

Adler-Nissen, J. (1986). Enzymatic hydrolysis of food proteins. Elsevier Applied Science Publishers, Amsterdam, Holland.

Adler-Nissen, J. (1993). Proteinases. In: Enzymes for food processing. T. Nagodawithana and G. Reed (eds.). 3rd edition, Academic Press Inc., London.

Akita, E. M. and Nakai, S. (1990). Lipophilisation of β-lactoglobulin: effect on allergenicity and digestibility. J. Food Sci., 55, 718-23.

Alexander, S.S. and Pace, C.N. (1971). A comparison of the denaturation of bovine ß-lactoglobulins A and B, and goat ß-lactoglobulin. Biochem., 10, 2738-43.

Alford, E.S., Piriyapan, V., Dill, C.W., Young, C.R., Richter, R.L. and Landmann, W.A. (1984). Effect of succinylation on proteolysis of food proteins. J. Food Sci., 49, 614-616.

Ambler, R. P. (1972). Enzymatic hydrolysis with carboxypeptidases. In: Methods in Enzymology-Vol. XXV. Enzyme structure - Part B. C.H.W. Hirs and S.N. Timasheff (eds.), Academic Press Inc., New York, p143-154.

Amundson, C.H., Watanawanichakorn, S. and Hill, C.G. (1982). Production of enriched protein fractions of β-lactoglobulin and α-lactalbumin from cheese whey. J. Food Processing and Preservation, 6, 55-71.

Andrews, P.C. and Dixon, J.E. (1989). Application of fast atom bombardment mass spectrometry to post-translational modifications of neuropeptides. In: Methods in Enzymology. Academic Press Inc., New York, 168, 72-103.

Anson, M.L. (1937). Carboxypeptidase: I The preparation of crystalline carboxypeptidase. J. Gen. Physiol., 120, 633-669.

Antila, P., Paakkari, I., Järvinen, A., Mattila, M.J., Laukkanen, M., Pihlänto-Leppälä, A., Mäntsälä, P. and Hellman, J. (1991). Opioid peptides derived from *in vitro* proteolysis of bovine whey proteins. Int. Dairy J., 1, 215-229.

AOAC (1980). Official methods of analysis, 13th ed. Association of Official Analytical Chemists, Washington DC.

Aoki. K., Kajiwara, M. and Oka, T. (1984). The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive 'Enkephalinase' in the potency of enkephalins in the guniea pig ileum. Japan J. Pharmacol., 36, 59-65.

Arai, S. and Fujimaki, M. (1991). Enzymatic modification of proteins with special reference to improving their functional properties. In: *Food Enzymology-2.*, P.F. Fox (ed.). Elsevier Applied Science, London, p83-104.

Ardelt, W. and Laskowski, M, Jr. (1985). Turkey ovomucoid third domain inhibits eight different serine proteinases of varied specificity on the same Leu^{18}-Glu^{19} reactive site. Biochem., 24, 5313-5320.

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. Trends Food Sci. and Tech., 4, 139-144.

Asselin, J., Amiot, J., Gauthier, S. F., Mourad, W. and Hebert, J. (1988). Immunogenicity and allergenicity of whey protein hyrolysates. J. Food Sci., 53(4), 1208-1211.

Asselin, J., Hébert, J. and Amiot, J. (1989). Effects in *in vitro* proteolysis on the allergenicity of major whey proteins. J. Food Sci., 54 (4), 1037-1039.

Balls, A. K. (1965). Concerning Trypsinogen. Proc. Natl. Acad. Sci. U.S., 53, 392-397.

Belfort, G. (1989). Membranes and bioreactors: A technical challenge in biotechnology. Biotech. Bioeng., 33, 1047-1066.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. (1992). Identification of the bacterial domain of lactoferrin. Biochim. Biophys. Acta., 1121, 130-136.

Belmar-Beiny, M.T. and Fryer, P.J. (1993). Preliminary stages of fouling from whey protein solutions. J. Dairy Res., 60, 467-483.

Bergmann, M. and Fruton, J. S. (1941). The specificity of proteinases. Advan. Enzymol., 1, 63-98.

Bernal, V. and Jelen, P. (1984). Effect of calcium binding on thermal denaturation of bovine α-lactalbumin. J. Dairy Sci., 67, 2452-2454.

Bhumiratana, S., Hill, C.G. and Amundson, C.H. (1977). Enzymatic solubilisation of fish protein concentrate in membrane reactors. J. Food Sci., 42, 1016-1020.

Bird, M.R. and Fryer, P. (1991). An experimental study of the cleaning of surfaces fouled by whey proteins. Trans I. Chem. Eng., 69 (Part C), 13-21.

Birk, Y. (1987). Proteinase inhibitors. In: Hydrolytic enzymes. A. Neuberger and K. Brocklehurst (eds.). Elsevier Press, Amsterdam, p257-305

Blatt, W.F., Hudson, B.G., Robinson, S.M. and Zipilivan, E.M. (1968). A modified ultrafiltration cell for separating the products of proteolysis. Anal. Biochem., 22, 161-165.

Blow, D.M. (1971). The structure of chymotrypsin. In: The Enzymes-Vol III Hydrolysis:Peptide Bonds. P.D. Boyer (ed.), Academic Press Inc., New York, p185-206.

Bode, W. and Schwager, P. (1975). The refined crystal structure of bovine ß-trypsin at 1.8 Å resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0. J. Mol. Biol., 98, 693-717.

Boudrant, J. and Cheftel, C. (1976a). Continuous proteolysis with a stabilised proteinase. I. Chemical stabilisation of an alkaline proteinase. Biotechnol Bioeng., 18, 1719-1734.

Boudrant, J. and Cheftel, C. (1976b). Continuous proteolysis with a stabilised proteinase. II. Continuous experiments. Biotechnol Bioeng., 18, 1735-1749.

Bouhallab, S. Mollé, D. and Léonil, J. (1992). Tryptic hydrolysis of caseinomacropeptide in membrane reactor: Preparation of bioactive peptides. Biotechnol. Lett., 14 (9), 805-810.

Bouhallab, S., Favrot, C. and Maubois, J.L. (1993). Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactobacillus Lactis* subsp. *lactis*. Lait,73, 73-77.

Bouhallab, S. and Touzé, C. (1995). Continous hydrolysis of caseinomacropeptide in a membrane reactor: Kinetic study and gram-scale production of antithrombic peptides. Lait 75, 251-258.

Bradshaw, R.A., Babin, D.R., Nomoto, M., Srinivasin, N.G., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1969). The amino acid sequence of bovine carboxypeptidase A. II. Tryptic and chymotryptic peptides of cyanogen bromide fragment F_{111}. Biochem., 8, 3859-3871.

Brantl, V., Teschemacher, H., Henschen, A. and Lottspeich, F. (1979). Novel opioid peptides derived from casein (ß-caspmorphins). Hoppe-Sayler's Z. Physiol. Chem. Bd., 360, 1211-1216.

Brantl, V., Teschemacher, H., Henschen, A. and Lottspeich, F. (1981). Opioid activities of ß-casomorphins. Life Sci., 28, 1903-1909.

Bressollier, Ph. P., Petit, J.M. and Julien, R. (1988). Enzyme hydrolysis of plasma proteins in a CSTR ultrafiltration reactor: Performance and modelling. Biotechnol. Bioeng. 31, 650-658.

Brew, K. and Campbell, P.N. (1967). The characterisation of whey proteins of guinea - pig milk. Biochem. J. 102, 258-64.

Brew, K. and Grobler, J.A. (1992). α-Lactalbumin. In: Advanced dairy chemistry - 1, Proteins. P.F. Fox, (ed.), Elsevier Applied Science, London, p191-229.

Briand, L., Chobert, J.M. and Haertlé, T. (1994). Tryptic hydrolysis of esterified ß-casein and ß-lactoglobulin. Milchwissenschaft 49 (7), 367-371.

Britten, M., Giroux, H.J. and Gaudin, V. (1994). Effect of pH during heat processing of partially hydrolysed whey protein. J. Dairy Sci., 77, 676-684.

Brockbank, R. (1984). Uses for dairy ingredients in dietary and nutritional foods. J. Soc. Dairy Technol., 37(4), 127-130.

Brodbeck, U. and Ebner, K.E. (1966). Resolution of a soluble lactose synthetase into two protein components and solubilisation of microsomal lactose synthetase. J. Biol. Chem., 241, 762-4.

Brulé, G., Roger, L., Fauquant, J. and Piot, M. (1980). Procédé de traitement d'une matière à base de caséine contenant des phosphocaséinates de cations monovalents ou leurs dérivés, produits obtenus et applications. French Patent no. 2 474 829

Brulé, G., Roger, L., Fauquant, J. and Piot, M. (1982). Phosphopeptides from casein based material. U.S. Pat. 4, 358,465 9/1982.

Brunner, J.R. (1977). Milk Proteins. In: Food Proteins; J.R. Whitaker and S.R. Tannenbaum, (eds.), AVI Publishing company Inc., Westport, Connecticut., p145-207.

Buck, F.F., Vithayathil, A. J., Bier, M. and Nord, F.F. (1962). On the mechanism of enzyme action. LXXIII. Studies on trypsins from beef, sheep and pig pancreas. Arch. Biochem. Biophys., 97, 417-424.

Burgos, F.J., Salvà, M., Villegas, V., Soriano, F., Mendez, E. and Avilés, F.X. (1991). Analysis of the activation process of porcine procarboxypeptidase B and determination of the sequence of its activation segment. Biochem., 30, 4082-4089.

Butterworth, T.A., Wang, D.I.C. and Sinskey, A.J. (1970). Application of ultrafiltration for enzyme retention during continuous enzymatic reaction. Biotechnol. Bioeng., 12, 615-631.

Buzinco, L., Catani, A., Longhi, M.A. and Giampietro, P.G. (1989). Anaphylatic reactions to a cow's milk whey protein hydrolysate (Alpha - Ré, Nesltlé) in infants with cow's milk allergy. Ann. Allergy, 62, 333-335.

Caligant, A. Dumay, E., Casas Valencia, C., Cuq, J.L. and Cheftel, J.C. (1991). Surface hydrophobicity and aggregation of β–Lactoglobulin heated near neutral pH. J. Agric. Food Chem., 39, 2147-2152.

Cambillau, C., Kerfelec, B., Sciaky, M. and Chapus, C. (1988). Subunit III of ruminant procarboxypeptidase A-S6 complexes and pancreatic proteinases E. FEBS, 323 (1), 91-95.

Carey, E.A. (1989). In: Protein struture- a practical approach. T.E. Creighton (ed.), IRL Press, Oxford, p117.

Chabance, B., Jollès, P., Izquierdo, C., Mazoyer, E., Francoual, C., Drouet, L. and Fiat, A.-M. (1995). Characterization of an antithrombic peptide from κ-casein in newborn plasma after milk ingestion. British J. Nutr., 73, 583-590.

Chang, K. -J., Su, Y.-F., Brent, D.A. and Chang, D.- K. (1985). Isolation of a specific μ-opiate receptor peptide, morphiceptin, from an enzymatic digest of milk proteins. J. Biol. Chem., 260, 9706-9712.

Chaplin, L.C. and Andrews, A.T. (1989). Functional properties of peptides derived from casein proteolysis. J. Dairy Res., 56, 544 -545.

Charles, M. C., Rovery, M., Guidoni, A. and Desnuelle, P. (1963). Sur le trypsinogéne et la trypsine de porc. Biochim. Biophys. Acta., 69, 115-129.

Cheftel, C. (1972). Continuous enzymatic solubilisation of fish protein concentrate. Studies with recycling of enzymes. Annales de Technologie Agricole, 21, 423-433.

Chen, S.X., Hardin, C.C. and Swaisgood, H.E. (1993). Purification and characterization of β-structural domains of β-lactoglobulin liberated by limited proteolysis. J. Prot. Chem., 12 (5), 613-625.

Chen, S.X., Swaisgood, H.E. and Foegeding, E.A. (1994). Gelation of ß-lactoglobulin treated with limited proteolysis by immobilised trypsin. J. Agric. Food Chem., 42, 234-239.

Cheryan, M. and Deeslie, W.D. (1980). Production of protein hydrolysates in ultrafiltration-enzyme reactors. In: Ultrafiltration membranes and applications. A.R. Cooper (ed.), Plenum Press, New York, 591-601.

Cheryan, M and Deeslie, W.D. (1983). Soy protein hydrolysis in membrane reactors. J. Amer. Oil Chem. Soc., 60, 1112-1115.

Cheryan, M. (1986). Process design. In: Ultrafiltration handbook, Ed Technomic Publishing Company, Inc. Pennsylvania, USA, p197-231

Cheryan, M. and Mehaia, M.A. (1986). Membrane bioreactors. In: Membrane separations in biotechnology, C.W. McGregor, Ed. 255-301. Marcel Dekker, Inc., NY.

Cheung, H.-S., Wang, F.-L., Ondetti, M. A., Sabo, E. F. and Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. J. Biol. Chem., 255 (2), 401-407.

Chiang, J.P., Illingworth-Asmus, B.L., Sternberg, M.M. (1982). Method for the preparation of a protein hydrolysate from whey protein. European patent no. 0065 663.

Chiba, H. and Yoshikawa, M. (1986). Biologically functional peptides from food proteins: New opioid peptides from milk proteins. In: Protein tailoring for food and medical uses. R.E. Feeney and J.R. Whitaker (eds.), Marcel Dekker, Inc., New York, p123-153.

Chiba, H., Tani, F. and Yoshikawa, M. (1989). Opioid antagonist peptides derived from κ-casein. J. Dairy Res., 56, 363-366.

Chiba, H. and Yoshikawa, M. (1991). Bioactive peptides derived from food proteins. Kagaku to Seibutsu, 29, 454-458.

Chibata, I. (1978). Immobilised Enzymes, Research and Development, I Chibata (ed.), Kodansha, Tokyo.

Cho, Y., Bah, C.A. and Sawyer, L. (1994). Probing the retinol-binding site of β-lactoglobulin. J. Biol. Chem., 269, 11102-7.

Chobert, J.M., Bertrand-Harb, C. and Nicolas, M.G. (1988). Solubility and emulsifying properties of casein and whey proteins modified enzymatically by trypsin. J. Agric. Food Chem., 36, 883-892.

Chobert, J-M., Dalgalarrondo, M., Dufour, E., Bertrand-Harb, C. and Haertlé, T. (1991). Influence of pH on the structural changes of β-lactoglobulin studied by tryptic hydrolysis. Biochimica et Biophysica Acta., 1077, 31-34.

Chung, Y., Silk, D.B.A. and Kim, Y.S. (1979). Intestinal transport of a tetrapeptide, L-leucylglycylglycylglycine in rat small intestine *in vivo*. Clin. Sci. Mol. Med., 57, 1-11.

Clegg, K.M. and McMillan, A.D. (1974). Dietary enzymatic hyrolysates of protein with reduced bitterness. J. Food. Technol., 9, 21-29.

Clegg, K.M., Lim, C.L. and Manson, W. (1974). The structure of a bitter peptide derived from casein by digestion with papain. J. Dairy Res., 41, 283-287.

Clemente, F., De Caro, A. and Figarella, C. (1972). Composition du suc pancréatique humain. Eur. J. Biochem. 31, 186-193.

Cogan, U., Moshe, M. and Mokady, S. (1981). Debittering and nutritional upgrading of enzymatic casein hydrolysates. J. Sci. Food Agric., 32, 459-466.

Cooper, J.B., Khan, G. and Taylor, G. (1990). X-ray analyses of aspartic proteinases II. Three dimensional structure of the hexagonal crystal form of porcine pepsin at 23 Å resolution. J. Mol. Biol., 214, 199-22.

Cordano, A., Gastanaduy, A.S and Graham, G.G. (1988). Absorption and retention from an iso-osmolal casein hydrolysate infant formulae. Nutr. Res., 8, 1353-1362.

Coste, M. and Tome, D. (1991). Milk proteins with physiological activities. II Opioid and immunostimulating peptides derived from milk proteins. Lait, 71, 241-247

Coste, M., Rochet, V., Léonil, J., Mollé, D., Bouhallab, S. and Tomé, D. (1992). Identification of C-terminal peptides of bovine β-casein that enhance proliferation of rat lymphocytes. Immunol. Lett., 33, 41-46.

Cox, D.J., Wintersberger, E. and Neurath, H. (1962). Bovine pancreatic procarboxypeptidase B. II. Mechanism of activation. Biochem., 1(6), 1078-1083.

Creamer, L.K., Parry, D.A.D. and Malcolm, G.N. (1983). Secondary structure of bovine ß-lactoglobulin B. Arch. Biochem. Biophys., 227, 98-105.

Cunningham, L. (1965). Trypsin. In: Comprehensive Biochemistry. M. Florkin and E.H. Stotz, (ed.). Elsevier Press Amsterdam, 16, 85-100.

Cunningham, S.D., Cater, C.M. and Mattil, K.F. (1978). Cottonseed protein modification in an ultrafiltration cell. J Food Sci., 43, 1477-1480.

Cushman, D.W. and Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmol., 20, 1637-1648.

D'Albis, A and Béchet, J-J. (1967). Effet du pH sur la fixation d'inhibiteurs compétitifs synthéthiques par la trypsine. Biochim. Biophys. Acta., 140, 435-458.

Dagorn, J.C. and Lahaie, R. G. (1981). Dietary regulation of pancreatic protein sythesis. I. Rapid and specific modulation of enzyme synthesis by changes in dietary composition. Biochim. Biophys. Acta, 654, 111-118.

Dalgalarrondo, M., Chobert, J.M., Dufour, E., Bertrand Harb, C., Dumont, J.P. and Haertlé, T. (1990). Characterisation of bovine β-lactoglobulin B tryptic peptides by reverse-phase high performance liquid chromatography, Milchwissenschaft, 45(4), 212-216.

Dalgalarrondo, M., Dufour, E., Chobert, J.-M., Bertrand-Harb, C. and Haertlé, T. (1995). Proteolysis of β–lactoglobulin and β-casein by pepsin in ethanolic media. Int. Dairy J., 5, 1-14.

Daniel, H. and Erll, G. (1992). β-Casomorphins and gastrointestinal functions. In: New perspectives in Infant nutrition: Symposium, Antwerp. B. Renner and G. Sawatzki (eds.). Thieme Medical Publisher Inc. New York. p153-159.

Daniels, M.J. (1987). Industrial operation of immobilised enzymes. In: Immobilised Enzymes and Cells (Part C). Methods in Enzymology, 136, K. Mosbach, (ed.) Academic Press Inc., London. p371-379.

Daufin, G. and Merin, U. (1995). Fouling of inorganic membranes in filtration processes of dairy products. Fouling and cleaning in pressure driven membrane processes. IDF Special Issue, p53-67. International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium.

Davidson, S., Passmore, R., Brock, J.F. and Truswell, A.S. (1979). Human nutrition and dietetics, 7th edition., Churchill Livingstone, Edinburgh.

Deeslie, W.D. and Cheryan, M. (1981). A CSTR-hollow fiber system for continous hydrolysis of proteins. Performance and kinetics. Biotech. Bioeng., 23, 2257-2271.

Deeslie, W.D. and Cheryan, M. (1982). A CSTR-hollow-fiber system for continuous hydrolysis of proteins. Factors affecting long-term stability of the reactor. Biotech. Bioeng., 24, 69-82.

Deeslie, W.D. and Cheryan, M. (1988). Functional properties of soy protein hydrolysates from a continous ultrafiltration reactor. J. Agric. Food Chem., 36, 26-31.

Deeslie, W.D. and Cheryan, M. (1991). Fractionation of soy protein hydrolysates using ultrafiltration membranes. J. Food Sci., 57 (2), 411-413.

DeFelice, S.L. (1995). The nutraceutical revolution: its impact on food industry Research and Development. Trends in Food Sci. and Technol., 6, 59-61.

Del Mar , E.G., Largman, C., Broderick, J.W., Fassett, M. and Geokas, M.C. (1980). Substrate specificity of human pancreatic elastase 2. Biochem., 19, 468-472.

Delsing, B.M.A. and Hiddink, J. (1983). Fouling of heat transfer surfaces by dairy liquids. Neth. Milk Dairy J., 37 (3), 139-148.

Desnuelle, P. (1960). Trypsin. In: The Enzymes-Vol IV. P.D. Boyer, H. Lardy and K. Myeback (eds.), Academic Press Inc., New York, p119-140.

Desnuelle P. and Rovery, M. (1961). The proteins of the exocrine pancreas. Adv. Prot. Chem., 16, 139-95.

Determann, H., Jaworek, D., Kotitschke, R. and Walch, A. (1969). Die produkte der autolytischen zersetung von pepsin. Z. Physiol. Chem., 350, 379-388.

deWit, J.N. and Swinkels, G.A.M. (1980). A differential scanning calorimetry study of the thermal denaturation of bovine ß-lactoglobulin. Biochim. Biophys. Acta, 624, 40-50.

deWit, J.N. and Klarenbeek, G. (1984). Effects of various heat treatments on structure and solubility of whey proteins. J. Dairy Sci., 67, 2701-2710.

Diaz de Villegas, M.C., Oria, R., Sala, F.J. and Calvo, M. (1987). Lipid binding by ß-lactoglobulin of cow milk. Milchwissenschaft, 42, 357-8.

Driessen, F.M. and Van den Berg, M.G. (1988). New developments in whey drinks. IDF Bulletin, 250, 11-19.

Duke, J. H., Jr., Amen, R. J., Beigler, M. A., Spiller, G. A., Saperstein, S., Bassler, K.H., Fekl, W. and Lang, K. (1976). The use of amino acids and oligopeptides in dietetics. In 3rd International Symposium 'Balanced nutrition and Therapy' with the International Society of Parenteral Nutrition, Erlangen, p17-19.

Dupont, M. (1965). Study of reversible step in the thermodenaturation of bovine β-lactoglobulin A. Biochim. Biophys. Acta., 102, 500-613.

Edeloch, H. (1957). The denaturation of pepsin. I. Macromolecular changes. J. Amer. Chem. Soc., 79, 6100-6109.

Edeloch, H (1958). The denaturation of pepsin II. Hydrogen ion equilibria of native and denatured pepsin. J. Amer. Chem. Soc., 80, 6640-6648.

Eigel, W.N., Butler, J.E., Ernstrom, C.A., Farrell, Jr. H.M., Harwalker, V.R., Jenness, R. and Whitney, R. McL. (1984). Nomenclature of proteins of cow's milk: fifth revision. J. Dairy Sci., 67 (8), 1599-1631.

Ennis, B.M. and Harper,W.J. (1986). Properties of spray-dried lactalbumin treated with a proteinase. N. Z. J. Dairy Sci. Technol., 21, 205-216.

Erbersdobler, H.F. (1983). Protein utilisation and amino acid availability in milk products after technological treatment. In: Role of milk proteins in human nutrition. W. Kaufmann (ed.). Th. Mann. Gelsenkirchen-Buer, p301.

Erdös, E.G and Skidgel, R.A.. (1989). Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB Journal., 3, 145-31.

Ermisch, A., Burst, P. And Brandsch, M. (1989). β–Casomorphins alter the intestinal accumulation of l-leucine. Biochim. Biophys. Acta 982: 79-84.

Evans, E.W. (1980). Whey research. J. Soc. Dairy Technol., 33, 95-100.

Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S.K., Boron, W.F. and Hediger, M.A. (1994). Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature, 368, 563-566.

Fiat, A.-M., Lévq-Toledano, S., Caen, J.P. and Jollès, P. (1989). Biologically active peptides of casein and lactoferrin implicated in platelet function. J. Dairy Res., 56, 351-355.

Fiat, A.- M. and Jollès, P. (1989). Caseins of various origins and biologically active casein peptides and oligosaccharides: Structural and physiological aspects. Mol. Cell. Biochem., 87, 5-30.

Fiat, A.-M., Migliore-Samour, D., Jollès, P., Drouet, L., Bal dit Sollier, C. and Caen, J. (1993). Biologically active peptides from milk proteins with emphasis on two

examples concerning antithrombotic and immunomodulating activities. J. Dairy Sci., 76, 301-310.

FitzGerald, R.J. and Swaisgood, H.E. (1989). Binding of ions and hydrophobic probes to α-lactalbumin and κ-casein as determined by analytical affinity chromatography. Arch. Biochem. Biophys., 268, (1), 239-248.

Foegeding, E.A., Kuhn, P.R. and Hardin, C.C. (1992). Specific divalent cation-induced changes during gelation of β-lactoglobulin. J. Agric. Food Chem., 40, 2092-2097.

Folk, J.E. and Gladner, J.A. (1958). Carboxypeptidase B. 1. Purification of the zymogen and specificity of the enzyme. J. Biol. Chem., 331, 379-391.

Folk, J.E., Wolff, E.C., Schirmer, E.W. and Cornfield, J. (1962). The kinetics of carboxypeptidase B activity. J.Biol. Chem., 10, 3105-3109.

Folk , J. E. and Schirmer, E. W. (1963). The porcine pancreatic carboxypeptidase A system. J. Biol.Chem., 238(12), 3884-3894.

Folk, J.E. (1970a). Individual proteolytic enzymes-chymotrypsin C (porcine pancreas). In: Methods in Enzymology-Vol XIX Proteolytic enzymes. G.E. Perlmann and L. Lorand (eds.), Academic Press Inc., New York, p109-112.

Folk, J. E. (1970b). Carboxypeptidases B (porcine pancreas). In: Methods in Enzymology-Vol XIX Proteinases Specific for releasing COOH-terminal amino acids. G.E. Perlmann and L. Lorand (eds.). Academic Press Inc., New York, p504-508.

Folk, J. E. (1971). Carboxypeptidases B. In: The Enzymes-Vol III Hydrolysis:peptide bonds. P.D. Boyer (ed.). Academic Press Inc., New York, p57-79.

Fox, P.F., Holsinger, V.H., Posati, L.P. and Pallansch, M.J. (1967). Separation of β-lactoglobulin from other milk serum proteins by trichloroacetic acid. J. Dairy Sci., 50, 1363-1367.

Fox, P.F. (1989). The milk protein system. In: Developments in dairy chemistry-4. Functional milk proteins, P.F. Fox (ed.), Elsevier Applied Science Publishers, London, p1-53.

Fox, P. F. (1991). Food Enzymology. Elsevier Applied Science, London.

Fox, P.F. (1992). Advanced dairy chemistry-1. Proteins. Elsevier Applied Science Publishers, London.

Freier, S. and Kletter, B. (1970). Milk allergy in infants and young children. Clin. Pediat., 9, 449-453.

Fruton, J. S. (1971). Pepsin. In: The Enzymes-Vol III Hydrolysis: peptide Bonds. P.D. Boyer (ed.) Academic Press Inc., New York, p120-164.

Fruton, J.S. (1987). Aspartyl proteinases. In: Hydrolytic enzymes. A. Neuberger and K. Brocklehurst (eds.), Elsevier Press, Amsterdam, p1-37.

Fugate, R.D. and Song, P. (1980). Spectroscopic characterisation of ß-lactoglobulin - retinol complex. Biochim. Biophys. Acta 625, 28-42.

Fullbrook, P., Pawlett, D. and Parker, D. (1987). The aminopeptidases: a novel group of enzymes with applications within the food industry. In: Novel Biotechniques and Processes for the Food Industry. On Line Publications, London, p79-85.

García de Fernando, G.D. and Fox, P. F. (1991). Extracellular proteinases from *Micrococcus GF:* Isolation and characterisation. Lait, 71, 371 -382.

Gardner, M.L.G. (1988). Gastrointestinal absorption of intact proteins. Ann. Rev. Nutr., 9, 329-350.

Gauthier, S.F., Paquin, P., Pouliot, Y. and Turgeon, S. (1993). Surface activity and related functional properties of peptides obtained from whey proteins. J. Dairy Sci., 76 (1), 321-328.

Gerhartz, W. (1990). Survey of industrial enzymes. In: Enzymes in Industry. W. Gerhartz (ed.). VCH Publishers, Weinheim, Germany.

Gertler, A. and Birk,Y. (1970). Isolation and characterisation of porcine proelastase. Eur. J. Biochem., 12, 170-176.

Gertler, A., Weiss, Y. and Burstein, Y. (1977). Purification and characterisation of porcine elastase II an investigation of its elastolytic specificity. Biochem., 16 (12), 2709-2716.

Gjesing, B., Osterballe, O., Schwartz, B., Wahn, U. and Lowenstein, H. (1986). Allergen specific IgE antibodies against antigenic components in cow milk and milk substitutes. Allergy, 41, 51-56.

Glover, F.A. (1985). Ultrafiltration and reverse osmosis for the dairy industry. Technical bulletin 5. National Institute for research in dairying (NIRD), Reading, England.

Godfrey, T. and Reichelt, J. (eds.). (1983). Industrial Enzymology. The application of enzymes in industry. Nature Press, New York.

Godovac-Zimmerman, J. and Braunitzer, G. (1987). Modern aspects of the primary structure and function of β-lactoglobulins. Milchwissenschaft, 42 (5), 294-296.

Gordon, W.G. (1971). Alpha-lactalbumin. In: Milk proteins-Vol II, H.A. McKenzie, (ed.), Academic Press, New York, p331-365.

Gotham, S.M., Fryer, P.J. and Pritchard, A.M. (1992). β-lactoglobulin denaturation and aggregation reactions and fouling deposit formation: a DSC study. Inter. J. Food Sci. and Technol., 27, 313-327.

Gourley, L., Gauthier, S.F. and Pouliot, Y. (1995). Separation of casein hydrolysates using polysulphone ultrafiltration membranes with pH and EDTA treatments applied. Lait, 75, 259-269.

Gray, G. M. and Cooper, H. L. (1971). Protein digestion and absorption. Gastroenterology, 61, 535-544.

Green, D.W., Aschaffenburg, R., Camerman, A., Coppola, J.C., Dunnill, P., Simmons, R.M., Komorowski, E.S., Sawyer, L., Turner, E.M.C. and Woods, K.F. (1979). Structure of bovine β-lactoglobulin at 6 Å resolution. J. Mol. Biol., 31, 375-397.

Grimble, G.K., Keohane, P.P., Higgins, B.E., Kaminski, M.V. and Silk, D.B.A. (1986). Effect of peptide chain length on amino acid and nitrogen absorption from two lactalbumin hydrolysates in the normal human jejunum. Clinical Sci., 71, 65-69.

Grimble, G.K. and Silk, D.B.A. (1989). Milk protein and enteral and parenteral feeding in disease. In: Milk proteins: Nutritional, Functional and Technological Aspects, C.A. Barth and E. Schlimme (eds.), Steinkopff Verlag, Darmstadt, Germany, p270-281.

Guasch, A., Coll, M., Avilés F. X. and Huber, R. (1992). Three-dimensional structure of porcine pancreatic procarboxypeptidase A. J. Mol. Biol., 224, 141-157.

Guisán. J. M., Bastida, A. Cuesta, C., Fernandez-Lafuente, R. and Rosell, C. M. (1991). Immobilisation-stabilisation of α-chymotrypsin by covalent attachment to aldehyde-agarose gels. Biotechnol. Bioeng., 38, 1144-1152.

Gumpen, S. Hegg, P.O. and Martens, H. (1979). Thermal stability of fatty acids-bovine serum albumin complexes studied by DSC. Biochim. Biophys. Acta., 574, 189-196.

Haddad, Z.H., Kalra,V. and Verma, S. (1979). IgE antibodies to peptic and peptic-tryptic digests of β-lactoglobulin: significance in food hypersensitivity. Annals of Allergy, 42, 369-371.

Haines, D. M., Chelak, B.J. and Naylor, J.M. (1990). Immunoglobulin concentrations in commercially available colostrum supplements for calves. Can. Vet. J., 31, 36-37.

Hakim, A. A., Peters, R. L., Samsa, E. G. and Van Melle, P. J. (1962). Heterogeneous nature of crystallized trypsins. Enzymologia, 25, 134-154.

Hall, D.A. (1957). The complex nature of the enzyme elastase. Arch. Biochem. Biophys., 67, 366-377.

Hall, L., Emery, D.C., Davies, M.S., Parker, D. and Craig, R.K. (1987). Organisation and sequence of the human α-lactalbumin gene. Biochem. J., 242, 735-742.

Hallström, B., Trägårdh, G. and Nilsson, J.L. (1989). Membrane technology in the food industry. In: Engineering and Food, Vol 3-Advanced processes. W.E.S Spiess and H. Schubert (eds.), Elsevier Applied Science. p194-209.

Hambling, S.G., McAlpine, A.S. and Sawyer, L. (1992). ß-lactoglobulin. In. Advanced dairy chemistry-1, Proteins. P.F. Fox (ed.), Elsevier Applied Science, London, p141-190.

Hambraeus, L. (1982). Nutritional aspects of milk proteins. In: Developments in dairy chemistry-1: P.F. Fox (ed.), Applied Science, New York, p289-313.

Hamosh, M. (1989). ß-casomorphins - milk ß-casein derived opioid peptides. In: Textbook of Gastroenterology and Nutrition in Infancy, 2nd edn., E. Lebenthal, (ed.), Raven Press Ltd., New York. p143-150.

Haque, Z.H. (1993). Influence of milk peptides in determining the functionality of milk proteins. A review. J. Dairy Sci., 76, 311- 320.

Harris, J. I. (1956). Effect of urea on trypsin and alpha-chymotrypsin. Nature, 177, 471-473.

Hartley, B.S. and Shotton, D.M. (1971). Elastase. In: The Enzymes-Vol III Hydrolysis: Peptide Bonds. P.D. Boyer (ed.), Academic Press Inc., New York, p323-373.

Hartsuck, J. A. and Liposcomb, W. N. (1971). Carboxypeptidase A. In: The Enzymes -Vol III Hydrolysis: peptide Bonds. P.D. Boyer (ed.), Academic Press Inc, New York, p1-56.

Heine,W. and Wutzke, K-D. (1990). Process for producing bifidogenic infant and dietetic foods reduced in antigenicity. US Patent no. 4 963 384.

Heine,W.E. Klein, P. D. and Reeds., P.J. (1991). Importance of α-lactalbumin in infant nutrition. J. Nutrition 121(3), 277-283.

Henschen, A., Lottspeich, F., Brantl, V. and Teschemacher, H. (1979). Novel opioid peptides derived from casein (β-casomorphins) II. Structure of active components from bovine casein peptone. Hoppe-Seylers Z. Physiol. Chem., 360, 1217-1224.

Herriott, R.M. (1938). Isolation, crystallization and properties of swine pepsinogen. J. Gen. Physiol., 21, 501-540.

Herriott, R.M., (1962). Pepsinogen and pepsin. J. Gen. Physiol 45 (4), Suppl., 47.57-76.

Hess, G. P. (1971). Chymotrypsin-chemical properties and catalysis. In: The Enzymes Vol III - Hydrolysis:Peptide Bonds 3rd Ed. P.D. Boyer (ed.), Academic Press Inc., New York, 213-247.

Hidalgo, J. and Jost, R. (1980). Skincare composition containing lactalbumin hydrolysate. UK Patent Application 2 046 591 A.

Hill, (1994). The relationship between β-lactoglobulin phenotypes and milk protein composition in New Zealand dairy cows. J. Dairy Sci., 76, 281-286.

Hill, R.L. and Brew, K. (1975). Lactose synthetase. Adv. Enzymol., 43, 411-90.

Hirai, Y., Permyakov, E.A. and Berliner, L.J. (1992). Proteolytic digestion of alpha-lactalbumin: Physiological implications. J. Prot. Chem., 11(1), 51-57.

Höllt, V. (1983). Multiple endogenous opioid peptides. Trends in Neuro. Sci., 1, 24-26.

Hooker, P.H., Munro, P.A. and O'Meara, G.M. (1982). Reduction of the viscosity of sodium caseinate solutions by enzymatic hydrolysis. N.Z. J. Dy. Sci. Technol., 17: 35-40.

Huang, X.L., Catignani, G.L. and Swaisgood, H.E. (1994a). Comparision of the rate and size of formation of peptides released by limited proteolysis of β-lactoglobulins A and B with immobilised trypsin. J. Agric. Food Chem., 42, 1281-1284

Huang, X.L., Catignani, G.L. and Swaisgood, H.E. (1994b). Relative structural stabilities of β-lactoglobulins A and B as determined by proteolytic

susceptibility and differential scanning calorimetry. J. Agric. Food Chem., 42, 1276-1280.

Huang, X.L., Catignani, G.L., Foegeding, E.A. and Swaisgood, H.E. (1994c). Comparison of the gelation properties of ß-lactoglobulin genetic variants A and B. J. Agric. Food Chem., 42, 1064-1067.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J. and Steigemann,W. (1974). Structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor II. Crystallographic refinement at 1.9 Å resolution. J. Mol. Biol., 89, 73-101.

Hunziker, H.G. and Tarassuk, N.P. (1965). Chromatographic evidence for heat induced interaction of α-lactalbumin and β-lactoglobulin. J. Dairy Sci., 48, 733-734.

Iacobucci, G.A., Myers, M.J., Emi, S. and Myers, D.V. (1974). Large scale continuous production of soybean protein hydrolyzate in a constant flux membrane reactor. Proc. Int. Congr. Food Sci. Technol., 5, 83-95.

IDF Special Issue (1995). Fouling and cleaning in pressure driven membrane processes. International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium.

Imafidon, G.I. (1990). Genetic polymorphism and physicochemical properties of milk proteins. Ph. D. Diss., McGill. Univ., Montreal, Canada.

Imafidon, G.I., NG- Kwai - Hang, K.F., Harwalker, V.R. and Ma, C.-Y. (1991). Differential scanning calorimetric study of different genetic variants of ß-lactoglobulin. J. Dairy Sci., 74 (8), 2416-2422.

Inagami, T. (1964). The mechanism of the specificity of trypsin catalysis. J. Biol.Chem., 239(3), 787-791.

Ito, O., Kamata, S., Yamano, S., Kakiichi, N., Hatuoka, M. and Yokoyama, K. (1987). Inhibitor of angiotensin converting enzyme in cheese. Med. Biol. 115, 375-377.

Jelen, P. (1975). Use of coagulated lactalbumin from cheese whey in ground meats. J. Food Sci., 40, 1072-1074.

Jeurnink, T. J. M. and Brinkman, D.W. (1994). The cleaning of heat exchangers and evaporators after processing milk or whey. Int. Dairy J., 4, 347-368.

Jollès, P., Levy-Toledano, S., Fiat, A.M., Soria, C., Gillessen, D., Thomaidis, A., Dunn, F.W. and Caen, J.P. (1986). Analogy between fibrinogen and casein: effect of an undecapeptide isolated from κ-casein on platelet function. Eur. J. Biochem., 158, 379-382

Jolly, R.C. (1978). Modified protein. US Patent no. 4 107 334.

Jost, R. and Monti, J. C. (1977). Partial enzymatic hydrolysis of whey protein by trypsin. J. Dairy Sc., 60 (9), 1387-1393

Jost, R. and Monti, J.C. (1982). Emulgateurs peptidiques obtenues par l'hydrolyse enzymatic partielle de la proteine serique de lait. Lait 62, 521-530.

Jost, R., Monti, J.C. and Pahud, J.J. (1987). Whey protein allergenicity and its reduction by technological means. Food Technol., 10, 118-121.

Jost, R., Meister, N. and Monti, J.C. (1988). Procédé de préparation d'un hydrolysat de protéines de lactosérum et d'un aliment hypoallergénique. European Patent Application no. 0 322 589.

Jost, R., Monti, J.C. and Pahud, J.J. (1991). Reduction of whey protein allergenicity by processing. (Nutritional and toxicological consequences of food processing), Adv. Exp. Med. Biol., 289, 309-320.

Kahn, J-M., Mendy, F. and Roger, L. (1991). Improvements in or relating to organic compounds. European Patent Application no. 0 421 309 -A2.

Karaki, H., Doi, K., Sugano, S., Uchiya, H., Sugai, R., Murakami, U. and Takemoto, S. (1990). Antihypertensive effect of tryptic hydrolysate of milk casein in spontaneously hypertensive rats. Comp. Biochem. Physiol., 96C (2), 367-371.

Kasai, T., Iwasaki, R., Tanaka, M. and Kiriyama, S. (1995). Caseinphosphopeptides (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. Biosci. Biotech. Biochem., 59(1), 26-30.

Keil, B. (1971). Trypsin. In: The Enzymes-Vol III Hydrolysis:Peptide Bonds. P.D. Boyer (ed.), Academic Press Inc., New York, p250-272.

Kella, N.K.D. and Kinsella, J.E. (1988). Enhanced thermodynamic stability of β–Lactoglobulin at low pH. Biochem. J., 255, 133-118.

Kerfelec, B., Chapus, C. and Puigserver, A. (1985). Existence of ternary complexes of procarboxypeptidase A in the pancreas of some ruminant species. Eur. J. Biochem., 151, 515-519.

Kilara, A. (1985). Enzyme-modified protein food ingredients. Process Biochem., 20, 149-157.

Kloczewiak, M., Timmons, S., Lukas, T.J. and Hawiger, J. (1984). Platelet receptor recognition site on human fibrinogen. Structure-function relationship of peptides corresponding to the carboxyl-terminal segment of gamma-chain. Biochem., 23, 1767-1774.

Kobayashi, R., Kobayashi, Y. and Hirs, C.H. W. (1978). Identification of a binary complex of procarboxypeptidase A and a precursor of proteinase E in porcine pancreatic secretion. J. Biol.Chem., 253(15), 5526-5530.

Kobayashi, R., Kobayashi, Y. and Hirs, C. H. W. (1981). The specificity of porcine pancreatic protease E. J. Biol.Chem., 256 (5), 2460-2465.

Koch Membrane Systems (1986). Training Manual. 850 Main St. Wilmington, Ma 01887. USA. p1-1 to 2-19.

Koch, G., Wiedemann, K., Drebes, E., Zimmermann, W., Tink, G. and Teschemacher, H. (1988). Human β-casomorphin-8 immunoreactive materials in the plasma of women during pregnancy and after delivery. Regul. Peptides, 20, 107-117.

Kohmura, M., Nio, N. and Ariyoshi, Y. (1990). Inhibition of angiotensin-converting enzyme by synthetic peptide fragments of human κ-casein. Agric. Biol. Chem., 54(3), 835-836.

Koike, H., Ito, K., Miyamoto, M. and Nishino, H. (1980). Effects of long -term blockade of angiotensin-converting enzyme with captopril (SQ 14, 225) on hemodynamics and circulating blood volume in SHR. Hypertension, 2, 229-303.

Koldovsky, O. (1992). Biologically active peptides in milk. New perspectives in Infant nutrition: Symposium Antwerp. B. Renner and G. Sawatzki (eds.) 1993. Thieme Medical Publisher Inc., New York. p123-130.

Krogdahl, Å. and Holm, H. (1983). Pancreatic proteinases from man, trout, rat,pig, cow, chicken, mink and fox. Enzyme activities and inhibition by soybean and lima bean inhibitors. Comp. Biochem. Physiol., 74B(3), 403-409.

Kronman, M.J. (1989). Metal ion binding and the molecular conformational properties of alpha-lactalbumin. C.R.C. Crit. Rev. Biochem. Mol. Biol., 24, 565-667.

Kuehler, C.A. and Stine, C.M. (1974). Effect of enzymatic hydrolysis on some functional properties of whey protein. J. Food Sci., 39: 379-382.

Kulozik, U. (1995). Cleaning of membrane systems. Chp 9: Water rinsing in reverse osmosis and ultrafiltration. In: Fouling and cleaning in pressure driven membrane processes. IDF Special Issue 9504. International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium., 136-150.

Kumosinski, T.F. and Timasheff, S.N. (1966). Molecular interactions in β-lactoglobulin. X. The stoichiometry of the β-lactoglobulin mixed tetramerisation. J. Am. Chem. Soc., 88, 5635-5642.

Kurek, M., Przybilla, B., Hermann, K. and Ring, J. (1992). A naturally occurring opioid peptide from cow's milk, β-casomorphin-7, is a direct histamine releaser in man. Arch Allergy Immunol., 97, 115-120.

Lakkis, J. and Villota, R. (1992). Comparative performance of chemically and enzymatically modified proteins. Food Chem., 43, 93-105.

Lalasidas, G., Boström, S. and Sjöberg, L.-B. (1978). Low molecular weight enzymatic fish protein hydrolysates: Chemical composition and nutritive value. J. Agric. Food. Chem., 26(3), 751-756.

Lamy, F., Craig, C. and Tauber, S. (1961). Studies on elastase and elastin. I. Assay and properties of elastase. J. Biol. Chem. 236, 86-91.

Largmann, C., Broderick, J. W. and Geokas, M. C. (1976). Purification and characterisation of two human pancreatic elastases. Biochem., 15(11), 2491-2500.

Largmann, C. (1983). Isolation and characterisation of rat pancreatic elastase. Biochem., 22, 3763-3770.

Larson, B. L. (1992). Immunoglobulins of the mammary secretions. In: Advanced Dairy Chemistry- 1. P.F. Fox (ed.), Elsevier Applied Science, London, p231-254.

Laskowski, M. (1956). Chymotrypsinogens and chymotrypsins. In: Methods in Enzymology- Vol II. S.P. Colowick and N.O. Kaplan (eds.), Academic Press, New York, p9-36.

Laskowski, M. Jr. and Kato, I. (1980). Protein inhibitors of proteinases. Ann. Rev, Biochem., 49, 593-626.

Lazdunski, M. and Delaage, M. (1965). Sur la morphologie des trypsins de porc et de boeuf. Etude des dénaturations reversibles. Biochim. Biophys. Acta., 105, 541-561.

Lewis, U.J, Williams, D.E. and Brink, N.G. (1956). Pancreatic elastase: Purification, properties and function. J. Biol. Chem., 222, 705-720.

Lewis, U. J., Williams, D. E. and Brink, N. G. (1959). Pancreatic Elastase : Differentiation from other pancreatic proteinases. J. Biol.Chem., 234 (9), 2304-2307.

Li, H., Hardin, C.C. and Foegeding, E.A. (1994). NMR studies of thermal denaturation and cation-mediated aggregation of β-lactoglobulin. J. Agric. Food Chem., 42, 2411-2420.

Lin Qi, X., Brownlow, S., Holt, C. and Sellers, P. (1995). Multi-state thermal unfolding and aggregation of β–Lactoglobulin A. Biochem. Soc. Trans., 74s, 23

Lin, Y., Fusek, M. and Lin-X. (1992). pH dependence of kinetic parameters of pepsin, rhizopuspepsin, and their active-site hydrogen bond mutants. J. Biol. Chem., 267, 184-18.

Lorand, L. and Rule, N. G. (1961). Inhibition of proteolytic enzymes by decarboxylated amino acid derivatives. Effect of toleunesulphonyl (tosyl)agmatine (4-toluenesulphonamidobutylguanidine) on thrombin and trypsin. Nature, 190 (4777), 722-723.

Lorenzen, P.C., Fischer, H. and Sclimmme, E. (1994). Controlled *in vitro* proteolysis of casein using immobilised trypsin. Influence of variation of the enzyme-substrate ratio and the re-use of the immobilised enzyme on the preparation of phosphopeptide rich fraction. Nahrung, 38 (6), 551-558.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A. and Klee, W.A. (1983). Opioid activities and structures of α–casein-derived exorphins. Biochem., 22, 4567-4573.

Luong, J. H. T., Male, K. B. and Nguyen A. L. (1988). A continuous affinity ultrafiltration process for trypsin. Biotechnol. Bioeng., 31, 513-520.

Maeda, A., Abe, K., Watanabe, M. and Arai, S. (1987). Peptic hydrolysis of bovine ß-lactoglobulin to produce a low-phenylalanine peptide foodstuff for phenylketonuria. Agric. Biol. Chem., 51(6), 1501-1507.

Mahé, S., Tomé, D., Dumontier, A.M. and Desjeux, J.F. (1989). Absorption of intact morphiceptin by DFP-treated rabbit ileum. Peptides, 10, 45-52.

Mahmoud, M.I. Malone, W.T. and Cordle, C.T. (1992). Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. J. Food Sci., 57 (5), 1223-1229.

Mannheim, A. and Cheryan, M. (1990). Continuous hydrolysis of milk protein in a membrane reactor. J. Food Sci., 55(2), 381-385.

Marchis-Mouren, G., Charles, M., Ben Abdeljlil, A. and Desnuelle, P. (1961). Sur l'équipement en enzymes du suc pancréatique de porc et de chien. Biochim. Biophys.Acta., 50, 186-188.

Marchis-Mouren, G. (1965). Protéines: Étude comparée de l'équipement enzymatique du suc pancréatique de diverses espèces. Bull. Soc. Chim. Biol., 47 (12), 2207-2217.

Mares-Guia, M. and Shaw, E. (1965). Studies on the Active Center of Trypsin. J. Biol. Chem., 240(4), 1579-1585.

Marshall, V.M., Cole, W.M. and Vega, J.R. (1982). A yogurt-like product made by fermenting ultrafiltered milk containing elevated whey proteins with *Lactobacillus acidophilus*. J. Dairy Res., 49, 665-670.

Marshall, K.R. and Harper, W.J. (1988). Whey protein concentrates. In: Trends in utilization of whey and whey products. IDF Bulletin 233, p21-32.

Marshall, A.D. and Daufin, G. (1995). Physico-chemical aspects of membrane fouling by dairy fluids. In: Fouling and cleaning in pressure driven membrane processes. IDF Special Issue 9504, 1995. International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium, p8-29.

Martínez, M. C., Avilés., F.X., Sansegundo, B. and Cuchillo, C. M. (1981). Comparison between the monomeric and binary-complex forms of procarboxypeptidase A from whole pig pancreas. Biochem. J. 197, 141-147.

Maruyama, S and Suzuki, H. (1982). A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agric. Biol. Chem. 46, 1393-1394.

Maruyama, S. , Nakagomi, K., Tomizuka, N. and Suzuki, H. (1985). Angiotensin I converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II Isolation of bradykinin-potentiating activity on the uterus and the ileum of rats. Agric. Biol. Chem., 49, 1405-1409.

Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N. and Suzuki, H. (1987a). Studies on the active site and anti-hypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. Agric. Biol. Chem., 51(6), 1581-1586.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizika, N. and Suzuki, H. (1987b). Angiotensin I converting enzyme inhibitory activity of the C-terminal hexapeptide of α_{s1} -casein. Agric. Biol. Chem., 51 (9), 2557-2561.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M. and Osajima, Y. (1994). Angiotensin I converting enzyme inhibitory peptides in an alkaline protease hydrolysate derived from sardine muscle. Biosci. Biotech. Biochem., 58 (12), 2244-2245.

Matthews, D.M. and Payne, J.W. (1980). Transmembrane transport of small peptides. In: Current topics in membranes and transport-Carriers and membrane transport proteins. F. Bronner and A Kleinzeller (eds.), 14, 331-425.

Matthiasson, E. (1984). Macromolecular adsorption and fouling in ultrafiltration and their relationship to concentration polarisation, PhD thesis, Lund, Sweden.

Mattos, C., Giammond, D.A. and Petsko, G.A. (1995). Structural analysis of the site of porcine pancreatic elastase based on the X-ray crystal structures of complexes with trifluoroacetyl-dipeptide-analide inhibitors. Biochem., 34(5), 3193-203.

Maubois, J.L., Roger, L., Brulé, G. and Piot, M. (1981). Hydrolysate enzymatique total de protéines de lactosérum, obtention et application. European Patent Application. no. 0022 019 A1.

Maubois, J.L., Pierre, A., Fruquant, J. and Piot, M. (1987). Industrial fractionation of main whey proteins. International Dairy Federation Bulletin 212, Brussels, Belgium, 154-159.

Maubois, J.L. and Léonil, J. (1989). Peptides du lait a activité biologique. Lait 69, 245-269.

McDonald, M. R. and Kunitz, M. (1946). An improved method for the crystallization of trypsin. J. Gen. Physiol., 29, 155-156.

McKenzie, H.A. (1967). Milk proteins. Adv. Prot. Chem., 22, 56-234.

McKenzie, H.A. (1971). ß-lactoglobulins. In Milk proteins: Chemistry and molecular biology-II, H.A. McKenzie (ed.), Academic Press, New York, p 257-330.

McKenzie, H.A. and Sawyer, W.H. (1967). Effect of pH on β–Lactoglobulins. Nature (London), 214, 1101-1104.

Mehra, R. (1994). Whey protein fractions and their properties. In: Second Food Ingredients Symposium. M.K. Keogh (ed.), National Dairy Products Research Centre, Moorepark, Fermoy, Ireland. p42-52.

Meisel, H. (1986). Chemical characterisation and opioid activity of an exorphin isolated from *in vivo* digests of casein. FEBS 196 (2) 223-227.

Meisel, H., Frister, H. and Schlimme, E. (1989). Biologically active peptides in milk proteins. Z. Ernahrungswiss 28, 267-78.

Meisel, H. and Frister, H. (1989). Chemical characterization of bioactive peptides from *in vivo* digests of casein. J. Dairy Res., 56, 343-349.

Meisel, H. (1990). Milk protein and biological activity. Proc XXIII Inter. Dairy Congr. Montreal, 2, 1534-1549.

Meisel, H. (1992). Casokinins as inhibitors of angiotensin-converting enzyme. In: New perspectives in Infant nutrition: Symposium Antwerp. B. Renner and G. Sawatzki (eds.). Thieme Medical Publisher Inc., New York. p153-159.

Meisel, H. (1993a). Determination of bioactive peptides in milk. In: Safeguarding food quality. H. Sommer, B. Petersen, P. v Wittke, (eds.), Springer-Verlag Berlin, Heidelberg, p97-110.

Meisel, H. (1993b). Casokinins as bioactive peptides in the primary structure of casein: In: Proceedings from the 4th Symposium on Food Proteins - structure Functionality Relationships. K.D. Schwenke and R. Mothes (eds.) VCH Weinheim, New York, p67-75.

Meisel, H. and Schlimme, E. (1994). Inhibitors of angiotensin converting enzyme derived from bovine casein (casopkinins). In: ß-casomorphins and related

peptides recent developments. V. Brantl, V. and H. Teschemacher, H. (eds.) VCH Weinheim, New York p27-33.

Mellander, O. (1963). In: The transfer of calcium and strontium across biological membranes. R.H. Wasserman, R.H. (ed), Acadmeic Press, London. p 265-276.

Meloun, B., Kluh, I., Kostka, V., Morávuek, L., Prusík, Z., Vanecek, J., Keil, B. and Sorm, F. (1966). Covalent structure of bovine chymotrypsinogen A. Biochim. Biophys. Acta., 130, 543-546.

Merin, U. and Cheryan, M. (1980). Factors affecting the mechanism of flux decline during ultrafiltration of cottage cheese whey. J. Food Processing and Preservation 4 (3), 183-198.

Merritt, R J., Carter, M., Haight, M. and Eisenberg, L.D. (1990). Whey protein hydrolysate formula for infants with gastrointestinal intolerance to cow milk and soy protein in infant formulas. J. Pediat. Gastroent. Nutr., 11, 78-82.

Michaelis, L. and Menten, M.L. (1913). Die kinetik der invertinwirkung. Biochem. Zeitshr., 49, 333-369.

Migliore-Samour, D. and Jollés, P. (1988). Casein, a prohormone with an immunomodulating role for the newborn? Experientia, 44, 188-193.

Migliore-Samour, D., Floc'h, F. and Jollès, P. (1989). Biologically active casein peptides inplicated in immunomodulation. J. Dairy Res., 56, 357-362.

Mihalyi, E. (1978). Application of proteolytic enzymes to protein structural studies. Vol 1, CRC: West Palm Beach, FL,1, p153-193.

Milla, P. J. (1991). The clinical uses of protein hydrolysates and soya formulae. Eur. J. Clin. Nutrition., 45(Suppl.1), 23-28.

Mills, O.E and Creamer, L.K. (1975). A conformational change of bovine ß-lactoglobulin at low pH. Biochim. Biophys. Acta., 379, 618-626.

Minagawa, E., Kaminogawa, S., Tsukasaki, F. and Yamauchi, K. (1989). Debittering mechanism in bitter peptides of enzymatic hydrolysates from milk casein by aminopeptidase T. J. Food Sci., 54, 1225-1229.

Miranda, G. and Pelissier, J.-P. (1983). Kinetic studies of in vivo digestion of bovine unheated skim-milk protein in rat stomach. J. Dairy Res., 50, 27-36.

Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H. and Maruyama, S. (1991). Structures and activity of angiotensin-converting enzyme inhibitors in an α-zein hydrolysate. Agric. Biol. Chem., 55, 1313-1318.

Mobarhan, S. and Trumbore, L. S. (1991). Enteral tube feeding: a clinical perspective on recent advances. Nutr. Rev., 49 (5), 129-140.

Modler, H.W. and Harwalker, V.R. (1981). Whey protein concentrate prepared by heating under acidic conditions. 1. Recovery by ultrafiltration and functional properties. Milchwissenschaft, 36, 537-42.

Moll, D. (1990). Manufacturing protein hydrolysates without giving use to a bitter taste. In: Food Ingredients Europe Conference, Expoconsult Publishers: Maarssen, The Netherlands, p257-260.

Monaco, H.L., Zanotti, G., Spadon, P. Bolognesi, M., Sawyer, L. and Eliopoulos, E. (1987). Crystal structure of the trigonal form of bovine ß-lactoglobulin and its complex with retinol at 2.5 Å resolution. J. Mol. Biol., 197, 695-706.

Monnot, M. and Yon, J. (1964). Etude cinétique comparée de l'hydrolyse trypsique des lactoglobulines A et B en fonction du pH. Biochim. Biophys. Acta, 92 105-110.

Monti, J. C. and Jost, R. (1978). Solubilization of cheese whey protein by trypsin and a process to recover the active enzyme from the digest. Biotechnol. Bioeng., 20, 1173-1185.

Morr, C.V. (1982). Functional properties of milk proteins and their use as food ingredients. In: Developments of Dairy Chemistry-1. P.F. Fox, (ed), Elsevier Applied Science Publishers, London, p375-399.

Mullally, M.M., O'Callaghan, D.M., FitzGerald, R.J., Donnelly, W.J. and Dalton, J.P. (1994). Proteolytic and peptidolytic activities in commercial pancreatic proteinase preparations and their relationship to some whey protein hydrolysate characterisitics. J. Agric. Food Chem., 42, 2973-81.

Mulvihill, D.M. and Kinsella, J.E. (1988). Gelation of β-lactoglobulin: Effects of sodium chloride and calcium chloride on the rheological and structural properties of gels. J. Food Sci., 53, 231-236.

Mulvihill, D.M. (1992). Milk protein products. In: Advances dairy chemistry-1, P.F. Fox (ed.), Elsevier Applied Science, London, p369-404.

Murray, T. K. and Baker, B. E. (1952). Studies on protein hydrolysis. 1. Preliminary observations on the taste of enzymatic protein hydrolysates. J. Sci. Food Agric., 3, 470-475.

Myers, D.V., Ricks, E., Myers, M.J., Wilkinson, M. and Iacobucci, G.A. (1974). Chemical and nutritional evaluation of enzymatic soybean hydrolysates. Proc. IV Int. Congr. Food Sci. Technol., Madrid Instituto de Agroquímica y Technología de Alimentos Valencia. Vol. V, pp 96-102.

Nagodawithana, T. and Reed, G. (eds.). (1993). Enzymes in food processing. 3rd edition, Academic Press Inc., London.

Naguane, S., Azuma, N., Ishino, Y., Mori, H., Kaminogama, S. and Yamauchi, K. (1989). DNA - synthesis stimulating peptide from bovine ß-casein. Agric. Biol. Chem., 53, 3275-3278.

Nakai, S. and Li-Chan, E. (1989). Chemical and enzymatic modification of milk proteins. In: Developments in dairy chemistry-4, P.F. Fox, (ed.), Elsevier Applied Science, London, p347-376.

Nakamura, T., Sado, H., and Syukunobe, Y. (1993a). Production of low antigenic whey protein hydrolysates by enzymatic hydrolysis and denaturation with high pressure. Milchwissenschaft 48(3), 141-145.

Nakamura, T., Sado, H., Syukunobe, U and Hirota, T. (1993b). Antigenicity of whey protein hydrolysates prepared by a combination of two proteinases. Milchwissenschaft, 48(12), 667-670.

Nakamura, Y, Yamamoto, N., Sakai, K., Okubo, H., Yamazaki, S. and Takano, T. (1995a). Purification and characterisation of angiotensin - converting enzyme inhibitors from sour milk. J. Dairy Sci., 78, 777-783.

Nakamura, Y, Yamamoto, N., Sakai, K., and Takano, T. (1995b). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors of angiotensin I converting enzyme. J. Dairy Sci., 78, 1253-1257.

Naughton, M. A. and Sanger, F. (1961). Purification and specificity of pancreatic elastase. Biochem. J., 78, 165-163.

Nilsson, J.L. (1989). A study of ultrafiltration membrane fouling, Ph.D. thesis, Lund, Sweden.

Northrop, J.H., Kunitz, M. and Herriott, R.M. (1948). Trypsin. In: Crystalline Enzymes-2nd ed: Columbia Univ Press (New York).

O'Callaghan, D.M. (1994). Physicochemical, functional and sensory properties of milk protein hydrolysates. Ph.D thesis, National University of Ireland.

O'Keeffe, A.M. and Kelly, J. (1981). Solubilization of denatured whey protein. Neth. Milk Dairy J., 35, 292-297.

Okamoto, M., Hayashi, R., Enomoto, A., Kaminogawa, S. and Yamauchi, K. (1991). High pressure proteolytic digestion of food proteins : Selective elimination of ß-lactoglobulin in bovine milk whey concentration. Agric. Biol. Chem., 55 (5), 1253-1257.

Okamoto, A., Hanagata, H., Matsumoto, E. Kawamura, Y., Koizumi, Y. and Yanagida, F. (1995). Angiotensin I converting enzyme inhibitory activities of various fermented foods. Biosci. Biotech. Biochem., 59(6), 1147-1149.

Olofoson, M., Buhler, M. and Wood, R. (1980). A process for the preparation of a purified protein hydrolysate. UK Patent Application no. 2 043 651 A.

Olsen, H.S. (1978). Continuous pilot plant production of bean protein by extraction, centrifugation, ultrafiltration and spray drying. Lewbensm. -Wissu. -Technol. 11, 57-64.

Ondetti, M.A. and Cushman, D.W. (1982). Enzymes of the renin-angiotensin system and their inhibitors. Ann. Rev. Biochem., 51, 283-308.

Ondetti, M.A., Rubin, B. and Cushman, D.W. (1977). Design of specific inhibitors of angiotensin converting enzyme: A new class of orally active antihypertensive agents. Science 196, 441-444.

Oshima, G., Shimabukuro, H. and Nagasawa, K. (1979). Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. Biochim. Biophys. Acta. 566, 128-137.

Otani, H. (1981). Susceptibility of S-carboxymethylated ß-lactoglobulin to peptic, tryptic and chymotryptic digestions. Jpn. J. Zootech. Sci., 52 (9), 689-691.

Otani, H. Karasawa, Y., and Akiyoshi, H. (1989). Production of antibody to bovine α s_1-casein in yolk of chicken eggs. Agric. Biol. Chem., 53, 1725-6.

Padfield, P.J. and Case, R.M. (1987). Release of individual enzymes from guinea pig pancreas following stimulation with CCK-8 or CCK-33. Pancreas, 2, 439-446.

Paegelow, I. and Werner, H. (1986). Immunomodulation by some oligopeptides. Methods Find. Exp. Clin. Pharmacol., 8, 91-95.

Pahud, J. J., Monti, J.C. and Jost, R. (1985). Allergenicity of whey protein: Its modification by tryptic *in vitro* hydrolysis of the protein. J. Pediat. Gastroent. Nutr., 4, 408-413.

Panksepp, J., Normansell, C., Siviy, S., Rossi, J. and Zolovick, A. J. (1984). Casomorphins reduce separation distress in chicks. Peptides 5, 829-831.

Papiz, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C.T., Findlay, J.B.C., Sivaprasadarao, R., Jones, T.A., Newcomes, M.E. and Kraulis, P.J. (1986). The structure of ß-lactoglobulin and its similarity to plasma retinol-binding protein. Nature, 324, 383-385.

Park, K.H. and Lund, D.B. (1984). Calorimetric study of thermal denaturation of β–Lactoglobulin. J. Dairy Sci., 67, 1699-1706.

Parker, F., Migliore-Samour, D., Floc'h, F., Zerial, A., Werner, G.H., Jollés, J., Casaretto, M., Zahn, H. and Jollés, P. (1984). Immunostimulating hexapeptide from human casein: Amino acid sequence, synthesis and biological properties. Eur. J. Biochem., 145, 677-682.

Paroli, E. (1988). Opioid peptides from food (exorphins). World Rev. Nutr. Diet, 55, 58-97.

Partridge, S.M. and Davis, H.F. (1955). The chemistry of connective tissues. 3. Composition of the soluble proteins derived from elastin. Biochem. J., 61, 21-30.

Pascual, R., Burgos, F. J., Salva, M., Soriano, F., Mendez, E. and Aviles, F. X. (1989). Purification and properties of five different forms of human procarboxypeptidases. Eur. J. Biochem., 179, 609-616.

Paulsson, M. and Dejmek, P. (1990). Thermal denaturation of whey proteins in mixtures with caseins studied by differential scanning calorimetry. J. Dairy Sci., 73, 590 -600.

Paulsson, M. and Elofsson, U. (1994). Thermal denaturation and gelation of β-lactoglobulin and lactoferrin. Milchwissenschaft, 49 (10), 547-551.

Payne, R.E., Hill, C.G. and Amundson, C.H. (1978). Enzymatic solubilisation of leaf protein concentrate in membrane reactors. J. Food Sci. 43, 385-389.

Peanasky, R. J., Gratecos, D., Baratti, J. and Rovery, M. (1969). Mode of activation and N-terminal sequence of subunit II in bovine procarboxypeptidase A and of porcine chymotrypsinogen C. Biochim. Biophys. Acta, 181, 82-92.

Pearce, K.N. and Kinsella, J.E. (1978). Emulsifying properties of proteins: evalution of a turbidimetric technique. J. Agric. Food Chem., 26 (3), 716-723.

Pearce, R.J. (1987). Fractionation of whey proteins. In: Trends in whey utilisation. IDF Bulletin 212, 150-153.

Perea, A., Ugalde, U., Rodriguez, I. and Serra, J.L. (1993). Preparation and characterisation of whey protein hydrolysates. Applications in industrial bioconversion processes. Enzyme Microb. Technol., 15, 418-423.

Pérez, M.D., Sáchez, L., Aranda, P., Ena, J.M., Oria, R. and Calvo, M. (1992). Effect of ß-Lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. Biochim. Biophys. Acta., 1123, 151-155.

Pérez, M.D and Calvo, M. (1995). Interaction of β-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. J. Dairy Sci., 78, 978-988.

Pervaiz, S.and Brew, K. (1985). Homology of ß-lactoglobulin, serum retinol-binding protein, and protein HC. Science, 228, 335-7.

Peters, T., Jr. (1985). Serum albumin. Adv. Protein Chem., 37, 161-245.

Pétra, P.H. (1970). Bovine procarboxypeptidase and carboxypeptidase A. In: Methods in Enzymology-Vol XIX. Proteinases specific for releasing COOH-terminal amino acids. G.E. Perlmann and L. Lorand (eds.), Academic Press Inc., New York. p140-477

Pfeil, N. (1981). Thermodynamics of α-lactalbumin unfolding. Biophys. Chem., 13, 181-185.

Philips, M.J. (1987). Functions of angiotensin in the central nervous system. Ann. Rev. Physiol. 49, 413-435.

Pihlanto - Leppälä, A., Pakkari, I., Rinta-Koshi, M. and Antila, P. (1994a). Bioactive peptide derived from *in vitro* proteolysis of bovine β-lactoglobulin. In: Brief Communications, International Dairy Congress Melbourne, Australia, Abstract Gb79.

Pihlänto-Leppälä, A., Antila, P., Mäntsälä, P. and Hellman, J. (1994b). Opioid peptides produced by *in-vitro* proteolysis of bovine caseins. Int. Dairy J., 4, 291-301.

Plainer, H. and Sproßler, B. (1990). Debittering of protein hydrolysates by a new technical exopeptidase preparate. Food Biotechnol., 4 , 366-367.

Porter, D.H., Swaisgood, H.E. and Catignani, G.L. (1984). Characterisation of an immobilised digestive enzyme systems for determination of protein digestibility. J. Agric. Food Chem., 32, 334-339.

Porter, M.C. and Michaelis, A.S. (1972). Membrane ultrafiltration. Applications in food processing. Chem. Tech 1, 59-63.

Portier, A., Boyaka, N.P., Bougoudogo, F., Dubarry, M., Huneau, J.F., Tomé, D., Dodin, A. and Coste, M. (1994). Fermented milks and increased antibody responses against cholera in mice. Inter. J. Immunotherapy, 9, 217-224.

Pouliot, Y. and Gauthier, S.F. (1990). Effect of selected physico-chemical parameters on the flux decline of casein hydrolysate on ploysulfone ultrafiltration membranes. Proc. ICOM, 20-24 August 1990, Chicago, IL, p273-275.

Pouliot, Y. and Jelen, P. (1995). Pretreatments of dairy fluids to minimise long term fouling. In: Fouling and cleaning in pressure driven membrane processes. IDF

Special Issue 9504. International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium. p 80-92

Poulsen, O. (1987). A process for the preparation of a heat resistant non-bitter water soluble peptide product, the product produced by the process, and nutrients, refreshments and dietetics comprising the product. PCT Patent Application, W 0 no. 87 / 03785.

Powers, J. D., Kilpatrick, P. K. and Carbonell, R. G. (1990). Trypsin Purification by Affinity Binding to small unilamellar liposomes. Biotechnol. Bioeng., 36, 506-519.

Powers, J.C., Gupton, B.F., Hartley, A.D., Nishino, N. and Whitney, R.J. (1977). Specificity of porcine pancreatic elastase, human leukocyte elastase and cathepsin G. Biochim. Biophys. Acta., 485, 156-166.

Puigserver, A., Chapus, C. and Kerfelec, B. (1986). Pancreatic Exopeptidases. In: Molecular and Cellular Basis of Digestion. P. Desnuelle, H. Sjostrom and O. Noren (eds.), Elsevier Science Publishers B.V. p235-247.

Puyol, P., Perez, M.D., Ena, J.M. and Calvo, M. (1991). Interaction of bovine ß-lactoglobulin and other bovine and human whey proteins with retinol and fatty acids. Agric. Biol. Chem., 55, 2515-20.

Puyol, P., Perez, M.D., Mata, L., Ena, J.M. and Calvo, M. (1993). Effect of retinol and fatty acid binding by bovine ß-lactoglobulin on its resistance to trypsin digestion. Int. Dairy J., 3 589-597.

Puyol, P., Perez, M.D., Peiro, J.M. and Calvo, M. (1994). Effect of binding of retinol and palmitic acid to bovine ß-lactoglobulin on its resistance to thermal denaturation. J. Dairy Sci., 77, 1494-1502.

Rajagopalan, T.G., Moore, S. and Stein, W.H. (1966). Pepsin from pepsinogen.-Preparation and properties. J. Biol. Chem., 241, 4940-4950.

Ramachandra rao, H.G., Lewis, M.J. and Grandison, A.S. (1995). Effect of pH on flux during ultrafiltration of sweet whey and buttermilk. J. Dairy Res., 62, 441-449.

Reddy, I.M., Kella, N.K.D. and Kinsella, J.E. (1988). Structural and conformational basis of the resistance of ß-lactoglobulin to peptic and chymotryptic digestion. J. Agric. Food Chem., 36, 737-741.

Relkin, P. and Launay, B. (1990). Concentration effects on the kinetics of β–Lactoglobulin heat denaturation: A differential scanning calorimetic study. Food Hydrocolloids, 4, 509-520

Renner, E. (1983). Milk and dairy products in human nutrition, W-GmbH, Volkwirtschaftlicher Verlag, Munich, Germany.

Rérat, A., Vaissade, P. and Vaugelade, P. (1988). Absorption kinetics of dietary hydrolysis products in conscious pigs given diets with different amounts of fish protein. 1. Amino-nitrogen and glucose. The British J. Nutrition, 60, 91-104.

Robinson, B.P., Short, J.L. and Marshall, K.R. (1976). Traditional lactalbumin-manufacture, properties and uses. N. Z. J. Dairy Sci. Technol., 11, 114-126.

Roger, L., Brulé, G. and Maubois, J. L. (1981). Nouvelles voies de valorisation des protéines lactosériques. I - Hydrolyse des protéines de lactosérum Intérêt thérapeutique. La Technique Laitière, 952, 65-67.

Roitt, I. L. (1984). Essential Immunology, 5th edn. Blackwell Scientific Publications, Oxford.

Roozen , J. and Pilnik, W. (1973). Ultrafiltration controlled enzymatic degradation of soy protein. Process Biochem., 8, 24-25.

Roozen, J. and Pilnik, W. (1979). Enzymatic protein hydrolysis in a membrane reactor related to taste properties. Enzyme Microb. Technol., 1, 122-124.

Ryle, A.P. (1970). The porcine pepsins and pepsinogens. In: Methods in Enzymology-Vol XIX-Proteolytic enzymes. G.E. Perlmann and l. Lorand (eds), Academic Press Inc., New York, p316-336.

Saito, Y., Wanezaki (Nakamura), K., Kawato, H. and Imayasu, S. (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. Biosci. Biotech. Biochem., 58 (10), 1767-1771.

Samuelsson, E.G. and Poulsen, O.M. (1987). A peptide preparation, a process for producing it and use of the peptide preparation. European Patent Application no. 0 226 221 A1.

Sanborn, B.M. and Bryan, W. P. (1968). The binding of phenylguanidinium ion and phenol to trypsin. Biochem., 7, 3624-3627.

Sanderink, G.-J., Artur, Y. and Siest, G. (1988). Human aminopeptidases: A review of the literature. J. Clin. Chem. Biochem., 26, 795-807.

Sato, R., Shindo, M., Gunshin, H., Noguchi, T. and Naito, H. (1991). Characterization of phosphopeptide derived from β-casein: An inhibitor of intra-intestinal precipitation of calcium phosphate. Biochim. Biophys. Acta, 1077, 413-415.

Sawyer, W.H. (1969). Complex between ß-lactoglobulin and κ-casein. A review. J. Dairy Sci., 52, 1347-55.

Sawyer, L., Papiz, M.Z., North, A.C.T., Findlay, J.B.C., Sivaprasadarao, R., Jones, T.A., Newcomer, M.E. and Kraulis, P.J. (1985). Structure and function of bovine β-lactoglobulin. Biochem. Soc. Trans., 13, 256-66.

Schlimme, E. and Meisel, H. (1993). Bioactive peptides: Structural, physiological and analytical aspects. International Dairy Federation. 77th Annual Sessions. Newsletter of IDF no. 189 Oct 1993 Supplememt p57-71.

Schmidt, R.H. Packard, V.S. and Morris, H.A. (1984). Effect of processing on whey protein functionality. J. Dairy Sci., 67, 2723-2733.

Schmidt, D.G. and van Markwijk, B.W. (1993). Enzymatic hydrolysis of whey proteins. Influence of heat treatment of α–lactalbumin and β-lactoglobulin on their proteolysis by pepsin and papain. Neth. Milk Dairy J., 47, 15-22.

Schroeder, D. D. and Shaw, E. (1968). Chromatography of trypsin and its derivatives. J. Biol.Chem., 243(11), 2943-2949.

Schusdziarra, V., Schick, R., Holland, A., De La Fuente, A., Specht, J., Maier, V., Brantl, V. and Pfeiffer, E.F. (1983a). Effect of opiate-active substances on pancreatic polypeptide levels in dogs Peptides 4 , 205-210.

Schusdziarra , V., Schick, R., De La Fuente, A., Holland, A., Brantl, V. and Pfeiffer, E.F. (1983b). Effect of β- casomorphins on somatostatin release in dogs. Endocrinology 122, 1948-1951.

Schwert, G. W. and Eisenberg, M. A. (1949). The kinetics of the amidase and esterase activities of trypsin. J. Biol.Chem., 179, 665-672.

Sejr Olsen, H. and Adler-Nissen, J. (1981). Application of ultra- and hyperfiltration during production of enzymatically modified proteins. ACS Symp. Ser., 154, 133-169.

Sekiya, S., Kobayashi, Y., Kita, E., Imamura, Y. and Toyama, S. (1992). Antihypertensive effects of tryptic hydrolysate of casein on normotensive and hypertensive volunteers. J. Jpn. Soc. Nutr. Food Sci., 45, 513-517 (In Japanese. [English abstract]).

Shimizu, M. (1994). Bioactive peptides from milk proteins. In: Proceedings of international dairy congress, Melbourne, Australia.

Short, J.L., Cooper, H.R. and Doughty, R.K. (1978). The effect of manufacturing variables of lactalbumin for use in high protein biscuits. N. Z. J. Dairy Sci. Technol. 13, 43-48.

Short, J.L. (1980). The water absorption capacity of heat precipitated whey proteins. N. Z. J. Dairy Sci. Technol., 15, 167-176.

Shotton, D.M., Hartley, B.S., Camerman, N., Hofmann, T., Nyburg, S.C. and Rao, L. (1968). Crystalline porcine pancreatic elastase. J. Mol. Biol., 32, 155-156.

Shotton, D.M. (1970). Elastase. In: Methods in Enzymology Vol XIX- Proteolytic Enzymes. G.E. Perlmann and L. Lorand (eds), Academic Press Inc., New York, p113-140.

Sienkiewicz, T. and Riedel, C.-L. (1990). Whey and whey utilization., 2nd edn, Verlag Th. Mann, Gelsenkirchen-Buer, Germany.

Sigler, P. B., Blow, D. M., Matthews, B. W. and Henderson, R. (1968). Structure of crystalline α-chymotrypsin. J. Mol. Biol., 35, 143-164.

Silk, D.B.A., Marrs, T.C., Addison, J.M. Burston, D., Clark, M.L. and Matthews, D.M. (1973). Absorption of amino acids from an amino acid mixture simulating casein and tryptic hydrolysate of casein in man. Clin. Sc. Mol. Medicine, 45, 715-719.

Silk, D.B.A., Chung, C., Bergen, K.L., Corley, K., Beigler, M., Sleisenger, M.H., Spiller, A. and Kim, Y.S. (1979). Comparison of oral feeding of peptide and amino acid meals to normal human subjects. Gut, 20, 291-299.

Silk, D.B.A., Grimble, G.K. and Rees, R.G. (1985). Protein digestion and amino acid and peptide absorption. Proc. Nut. Soc., 44, 63-72.

Silk, D.B.A. and Grimble, G.K. (1992). Relevance of physiology of nutrient absorption to formulation of enteral diets. Nutrition-Burbank, 8 (1), 1-12.

Slack, A.W., Amundson, C.H. and Hill, C.G. (1986). Nitrogen solubilities of β–lactoglobulin and α-lactalbumin enriched fractions derived from ultrafiltered cheese whey retentate. J. Food Processing and Preservation, 10, 19-29.

Sleisenger, M.H., Burston, D., Dalrymple, J.A., Wilkinson, S. and Matthews, D.M. (1976). Evidence of a single common carrier for uptake of a dipeptide and tripeptide by hamster jejunum. Gastroent., 71, 76-81.

Smith, E.L. and Hanson, H.T. (1949). The chemical nature and mode of action of pancreatic carboxypeptidase. J. Biol. Chem., 179, 803-813.

Smith, R.L. and Shaw, E. (1969). Pseudotrypsin. A modified bovine trypsin produced by limited autodigestion. J. Biol. Chem., 244 (17), 4704-4712.

Smith, C.G., Munro, P.A., Otter, D.E. and Brauer, R.M. (1989). Solubilisation of spray-dried lactalbumin by Alcalase. J. Dairy Res., 56, 87-95.

Smithson, K.W. and Gray, G.M. (1977). Intestinal assimilation of a tetrapeptide in the rat. Obligate function of brush-border membrane aminopeptidases. J. Clin. Investigation., 60, 665-674.

Speakman, P.T. and Yarwood, R.E. (1966). Trypsin inhibition by phenylmethylsulphonylfluoride with reduced wool as substrate. Nature, 211, 201-202.

Spector, A. and Fletcher, J. (1970). Binding of long chain fatty acids to ß-lactoglobulin. Lipids, 5, 403-11.

Stevens, B.R.; Fernandez, A., Kneer, C., Cerda, J.J., Philips, M.I. and Woodward, E.R. (1988). Human intestinal brush border angiotensin converting enzyme activity and its inhibition by antihypertensive Ramipril. Gastroent., 94, 942-947.

Stuart, D.I., Acharya, K.R. and Walker, N.P.C., Smith, S.G., Lewis, M. and Philips, D.C. (1986). α–Lactalbumin possesses a novel calcium binding loop. Nature, 324, 84-88.

Suetsuna, K. and Osajima, K (1989). Blood pressure reduction and vasodilatory effects *in vivo* of peptides originating from sardine muscle. J Jap. Soc Nutr. Food Sci., 42 (1), 47-54.

Sugiyama, K., Takada, K., Egawa, M., Yamamoto, I., Onzuka, H. and Oba, K. (1991). Hypotensive effects of fish protein hydrolysate. J. Agric. Chem. Soc. Japan, 65 (1), 35-43.

Svedberg, J., De Haas, J., Leimenstoll, G., Paul, F. and Techemacher. (1985). Demonstration of β–casomorphin immunoreactive materials in *in vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. Peptides, 6, 825-830.

Swaisgood,H.E. (1982). Chemistry of milk proteins. In: Developments in dairy chemistry-1. Proteins, P.F. Fox (ed.), Elsevier Applied Science Publishers, London, p63-110.

Swaisgood, H.E. and Catignani, G.L. (1985). Digestibility of modified milk proteins: Nutritional implications. J. Dairy Sci., 68, 2782-2790.

Swaisgood, H.E. and Catignani, G.C. (1987). The use of immobilised proteinases and peptidases to study structural changes in proteins. Methods in Enzymology 135, 596-604.

Takenaka, A., Ohishi, Y., Noguchi, T. and Naito, H. (1991). A tryptic peptide from β-casein depresses protein synthesis and degradation and enhances ureigenesis in primary cultures of rat hepatocytes. Int. J. Biochem., 23, 483-490.

Tanford, C. , Bunville, L.G. and Nozaki, Y. (1959). The reversible trans-formation of β-lactoglobulin at pH 7.5. J. Am. Chem. Soc., 81, 4032-6.

Tani, F., Shiota, A., Chiba, H. and Yoshikawa, M. (1994). Serorphin, an opioid peptide derived from bovine serum albumin. In: β-Casomorphin and related peptides. E. Brantl (ed.), VCH, Verlag, Weinheim, p49-53.

Techemacher, H. and Scheffler, H. (1992). Milk protein derived opioid peptides: History and recent development. New perspectives in Infant nutrition: Symposium Antwerp. B. Renner and G. Sawatzki (eds.) Thieme Medical Publisher Inc. New York. p 131-137.

Techemacher, H., Koch, G. and Brantl, V. (1994). In: Proceedings from the second international symposium on β-casomorphins and related peptides V. Brantl and H. Teschemacher (eds.) VCH, Weinheim, New York.

Teller, D.C., Swanson, E. and DeHaen, C. (1979). The translational friction coefficient of proteins. Methods Enzymol., 61, 103 -124.

Thibault, P.A. (1990). Hydrol partiel des protéines de lactosérum exempt de lactoglobulines et procédé enzymatique pour son obtention. French Patent no. 2 644 986 - A1.

Thibault, P.A. (1991). Partial hydrolysate of whey proteins, enzymatic process for the preparation of this hydrolysate, and hypoallergenic dietetic milk food containing it. US Patent no. 4 981 704.

Timasheff, S.N., Townend, R. and Mescanti, L. (1966). The optical rotatory dispersion of the β-lactoglobulins. J. Biol. Chem., 241, 1863-70.

Timperley, T.A. and Smeulders, C.N.M. (1988). Cleaning of dairy HTST plate heat exchangers: Optimisation of the single-stage procedure. J. Soc. Dairy Technol., 41, 4-7.

Titani, K. Sasagawa, T., Resing, K. and Walsh, K.A. (1982). A simple and rapid purification of commercial trypsin and chymotrypsin by reverse-phase high performance liquid chromatography. In: Proceedings from the first international symposium on high performance liquid chromatography of proteins and peptdes, M.T. Hearn and F. Regnier (eds.), p23-27.

To, B., Helbig, N.B., Nakai, S. and Ma, C.Y. (1985). Modification of whey protein concentrate to simulate whippability and gelation of egg white. Can. Inst. Food Sci. Technol., 18 150-157.

Tomé, D., Dumontier, A.-M., Hautefeuille, M. and Desjeux, J.-F. (1987). Opiate activity and transepithelial passage on intact β-casomorphins in rabbit ileum. Am. J. Physiol., 253, G737-G744.

Tomita, M., Bellamy, W., Takase, M.,Yamauchi, K., Wakabayashi, H. and Kawase, K. (1991). Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci. 74, 4137-4142.

Towler, C. (1982). Utilisation of whey proteins in pasta. N. J. Z. Dairy Sci. Technol., 17, 229-236.

Townend, R., Kumosinski, T.F. and Timasheff, S.N. (1967). The circular dichrosm of variants of β-lactoglobulin. J. Biol. Chem., 242, 4538-4545.

Townend, R., Herskovits, T.T., Timasheff, S.N. and Gorbunoff, M.J. (1969). The state of amino acid residues in ß-lactoglobulin. Arch. Biochem. Biophys., 129, 567-580.

Trägårdh, G. (1988). Application of membrane technology in food industry. Advances in membrane phenomena and processes, Gdansk-Sobieszewo.

Travis, J. and Leiner, I.E. (1965). The crystallisation and partial characterisation of porcine trypsin. J. Biol. Chem., 240 (5), 1962-1966.

Turgeon, S.L. and Gauthier, S.F. (1990). Whey peptide fractions obtained with a two-step ultrafiltration process: Production and characterisation. J. Food Sci., 55, 106-110.

Turgeon, S.L., Gauthier, S.F. and Paquin, P. (1991). Interfacial and emulisifying properties of whey protein fractions obtained with two-step ultrafiltration process. J. Agric. Food Chem., 39 673-676.

Turgeon, S.L., Gauthier, S.F. and Paquin, P. (1992). Emulsifying property of whey peptide fractions as a function of pH and ionic strength. J. Food Sci., 57(3), 601-604.

Ueno, A., Hong, Y.-M., Arakaki, N. and Takeda, Y. (1985). Insulin-stimulating peptide from tryptic digest of bovine serum albumin: Purification and characterisation. J. Biochem., 98, 269-278.

Ukeda, H., Matsuda, H., Osajima, K., Matsufuji, H., Matsui, T. and Osajima, Y. (1992). Peptides from peptic hydrolysate of heated sardine meat that inhibit angiotensin I converting enzyme. J. Agric. Chem. Soc. Japan, 66, 25-29.

Umbach, M., Teschemacher, H., Praetorius, K., Hirschhauser, R., and Bostedt, H. (1985). Demonstration of a β-casomorphin immunoreactive material in the plasma of newborn calves after milk intake. Regul. Peptides, 12, 223-230.

Umetsu, H., Matsuoka, H. and Ichishima, E. (1983). Debittering mechanism of bitter peptides from milk casein by wheat carboxypeptidase. J. Agric. Food Chem., 31, 50-53.

Uriel, J. and Avrameas, S. (1965). Systematic fractionation of swine pancreatic hydrolases. I. Fractionation of enzymes soluble in ammonium sulphate solution at 0.40 satuation. Biochem., 4 (9), 1740-1749.

Van Beresteijn, E.C.H., Peeters, R.A., Kaper, J., Meijer, R.J.G.M., Robben, A.J.P.M. and Schmidt, D.G. (1994). Molecular mass distribution, immunological properties, and nutritive value of whey protein hydrolysates. J. Food Protection, 57 (7), 617-625.

Van der Horst, H.C. (1995). Fouling of organic membranes during processing of dairy liquids. In: Fouling and cleaning in pressure driven membrane processes. IDF Special Issue 9504, International Dairy Federation, 41, Square Vergote, B-1040 Brussels, Belgium p36-52.

Van Melle, P.J., Lewis, S.H., Samsa, E.G. and Westfall, R.J. (1963). Crystallisation of porcine trypsin. Enzymologia, 10(4), 133-139.

Van Willige, R.W.G. and FitzGerald, R.J. (1995). Tryptic and chymotryptic hydrolysis of ß-lactoglobulin A, B and AB at ambient and high pressures. Milchwissenschaft, 50(4), 183-186.

Vegarud, G. E. and Langsrud, T. (1989). The level of bitterness and solubility of hydrolysates produced by controlled hydrolysis of caseins. J. Dairy Res., 56, 375-379.

Vendrell, J. Aviles, F.X., San Segundo, B. and Cuchillo, C.M. (1982). Isolation and re-association of the sub-units from the pro-(carboxypeptidaseA)-pro-(proteinase E) binary complex from pig pancreas. Biochem. J., 205, 449-452.

Vendrell, J. Cuchillo, C.M. and Avilés, F.X. (1990). The tryptic activation pathway of monomeric procarboxypeptidase A. J Biol. Chem., 256, 6949-6953.

Vestling, M.M., Murphy, C.M. and Fenselau, C. (1990). Recognition of trypsin autolysis products by high performance liquid chromatography and mass spectrometry. Anal. Chem., 62, 2391-2394.

Vieth, W.R., Constantinides, A. and Bernarth, F.R. (1977). Enzymatic hydrolysis of vegetable protein. In: Non-conventional proteins and foods. Conference Proceedings, Uni. Madison, Nat., Sci., Found., PB 283965, Washington D.C. p 43-61.

Vithayathil, A.J., Buck, F., Bier, M. and Nord, F.F. (1961). On mechanism of enzyme action. LXXII Comparative studies on trypsins of various origins. Arch. Bioch. Biophys., 92, 532-540.

Waldschmidt-Leitz, E., Ziegler, F., Schäffner, A. and Weil, L. (1931). Uber die struktur der protamine I. Protamine und die produkte ihrer einwirkung auf clupein und salmin. (Funfte mitteilung uber enzymatische proteolyse.). Z. Physiol. Chem. 197 219-237.

Walsh, K. A. (1970). Trypsinogens and trypsins of various species. In: Methods in Enzymology Vol XIX- Proteolytic Enzymes: The serine Proteases. G. E. Perlmann, and L. Lorand (eds.), Academic Press, New York, p41-63.

Walstra, P. and Jenness, R. (1984). Dairy Chemistry and Physics. John Wiley, New York.

Webb, K.E. Jr. (1990). Intestinal absorption of protein hydrolysis products. A review. J. Anim. Sci., 68, 3011-3022.

Whitney, R. McL. (1988). Proteins of milk. In: Fundamentals of Dairy Chemistry, 3rd edn, N.P.Wong (ed.), AVI Publishing, Westport CT, p81-169.

Whitty, A.A. (1993). The role of post proline dipeptidyl peptidase in debittering hydrolysed rennet casein. M. Sc. Thesis. National University of Ireland.

Wilcox, P.E. (1970). Chymotrypsinogens-chymotrypsins. In: Methods in Enzymology Vol XIX-Proteolytic Enzymes:The serine proteinases. G.E. Perlmann and L. Lorand, L. (eds.), Academic Press Inc., New York, p64-112.

Wingerd, W.H. (1970). Lactalbumin as a food ingredient. J. Dairy Sci., 54, 1234-1236.

Wintersberger, E., Cox, D.J. and Neurath, H. (1962). Bovine pancreatic procarboxypeptidase B. I. Isolation properties, and activation. Biochem., 1 (6), 1069-1077.

Wolff, E.C., Schirmer, E.W. and Folk, J.E. (1962). The kinetics of carboxypeptidase B activity. J. Biol. Chem., 237 (10), 3094-3099.

Wyvratt, M.J. and Patchett, A.A. (1985). Recent developments in the design of angiotensin-converting enzyme inhibitors. Med. Res. Rev. *5*, 485-531.

Yamamoto, N., Akino, A. and Takano, T. (1994). Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci., 77 917-922.

Yamashina, I. (1956). The action of enterokinase on trypsinogen. Biochim. Biophys. Acta., 20, 433-434.

Yamauchi, K. (1992). Biologically functional proteins of milk and peptides derived from milk proteins. IDF Bulletin, 272., 51-58.

Yoshikawa, M., Tani, F. and Chiba, H. (1988). Structure activity relationship of opioid antagonist peptides derived from milk proteins. In: Peptide chemistry. Protein Res. Found. Osaka , T. Shiba, and S. Sakakibara (eds.), p473-476.

Yoshikawa, M., Tani, F., Yoshimura, T. and Chiba, H. (1986). Opioid peptides from milk proteins. Agric. Biol. Chem. 50(9), 2419-2421.

Young, J.C., Bong, J.A., and Cheong, C. (1993). Isolation and enzyme inhibition of tannins from Korean green tea. Korean Biochem. J., 26 (3), 216-223.

Zadow, J.G. (1992). Whey and lactose processing. Elsevier Science Publishers, London.

Zimmerman, J.K., Barlow, G.H. and Klotz, I.M. (1970). Dissociation of ß-lactoglobulin near neutral pH. Arch. Biochem. Biophys., 138, 101-109.

Zimmerman, M., Ashe, B., Yurewicz, E. C. and Patel, G. (1977). Sensitive assays for trypsin, elastase, and chymotrypsin using new fluorogenic substrates. Anal. Biochem., 78, 47-51.

Zioudrou, C., Streaty, R.A. and Klee, W.A. (1979). Opioid peptides derived from food proteins. J. Biol. Chem., 254 (7), 2446-2449.