

**The use of chemically stabilised proteolytic
enzymes in peptide synthesis**

By

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Philosophy**

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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.

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ABBREVIATIONS

ϵ :	Molar absorption coefficient
AA:	Amino acid
ΔA :	Change in absorbance
Å:	Angstrom
Abs:	Absorbance
Ala:	Alanine
APPE:	N-acetyl-L-phenylalanine ethyl ester
Arg:	Arginine
Asn:	Asparagine
Asp:	Aspartic Acid
ATP:	Adenosine triphosphate
BAEE:	N $_{\alpha}$ -Benzoyl-L-Arginine ethyl ester
BL-GSE:	Glutamic acid-specific endopeptidase from <i>Bacillus licheniformis</i>
Cys:	Cysteine
Da:	Dalton
DEAE:	Diethylaminoethyl
DNA:	Deoxyribonucleic acid
EDC:	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl
EG-NHS:	Ethylene glycol-bis (succinic acid N-hydroxy-succinimide ester).
Gln:	Glutamine
Gly:	Glycine
His:	Histidine
HPLC:	High performance liquid chromatography
k_{cat} :	Catalytic constant
kDa:	kilodaltons
K_m :	Michaelis Menten constant
Lys:	Lysine
M:	Molar

mM:	Millimolar
Met:	Methionine
Moz:	<i>p</i> -Methoxybenzyl carbonyl
NHS:	N-hydroxysuccinimide
PEG:	Polyethylene glycol
Phe:	Phenylalanine
Pro:	Proline
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
Ser:	Serine
SucAAPF:	3-Carboxy-propionyl-(Ala) ₂ -Pro-Phe
SucAAPF-SBn:	3-Carboxy-propionyl-(Ala) ₂ -Pro-Phe thiobenzyl ester
T _m :	Temperature at which the endothermic unfolding transition is maximal
Trp:	Tryptophan
Tyr:	Tyrosine
UV/Vis:	Ultra violet/Visible spectroscopy
v/v:	Volume per volume
Val:	Valine
V _{max} :	Maximum velocity
w/v:	Weight per volume
Z:	Benzyloxycarbonyl protecting group

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ABSTRACT

Abstract:

The aim of this project was to study various serine proteases, both native and chemically modified, with a view to their application in peptide synthesis. Various chemical modifications of these were carried out to improve their stability before peptide synthesis.

Porcine trypsin was stabilised by reaction with ethylene glycol bis-(succinic acid N-hydroxy-succinimide ester) (EG). The enhanced stability is likely due to intramolecular crosslink(s) being formed in the enzyme. EG-trypsin retained 100% amidase activity at 55°C for up to 150 minutes compared to 10 minutes for the native. EG-trypsin also had a 5-fold greater esterase/amidase k_{cat}/K_m value than the native, in an aqueous solution (Esterase/amidase ratio indicates potential usefulness in peptide synthesis).

Literature reports indicate that formylation of trypsin's tryptophan residues retains esterase activity while eliminating amidase activity. Modification of the tryptophan residues in porcine trypsin to l-formyltryptophan was undertaken. The formylated trypsin retained 100% esterase activity up to 50°C. The T_{50} value was found to be 55°C (compared to 45°C for the native porcine trypsin). This increased thermal stability was unexpected. As had previously been shown, no amidase activity was detected but the residual esterase activity was too low for practical use.

Next to be investigated were subtilisin Carlsberg and alcalase, an industrial alkaline protease. Alcalase had a 20-fold higher esterase/amidase ratio than subtilisin Carlsberg in an aqueous solution whereas in a 50% (v/v) DMF solution the subtilisin Carlsberg demonstrated a 3-fold higher ratio compared to the alcalase. These were also stabilised by reaction with EG. Subtilisin Carlsberg demonstrated 50% amidase activity after 12.5 minutes at 65°C whereas the modified form retained the same activity for up to 56 minutes. Alcalase was more stable, with the native showing 50% amidase activity after 56 minutes and the modified showing the same activity at 100 minutes.

Both the native and EG-modified forms of alcalase and subtilisin Carlsberg were then used to make the tripeptide Tyr-Gly-Gly. Native subtilisin Carlsberg showed the greatest rate of peptide synthesis with the optimum temperature being 25°C. Native and EG-trypsin were used in the synthesis of the peptide Bz-Arg-Leu-NH₂ with the EG-trypsin synthesising twice as much peptide as the native.

CHAPTER 1

INTRODUCTION

1.1 Introduction:

This literature survey deals with the topic of enzymatic peptide synthesis and alterations to enzyme molecules for this purpose. It focuses mainly on trypsin and on variants of subtilisin, as these were the enzymes chosen for experimental study, although other enzymes are mentioned where appropriate. This was to avoid excessive length.

1.2 Introduction to protein synthesis:

Proteins are present in every living cell and possess a variety of biochemical activities. They can be found in muscle, skin and hair. In nature, the following series of reactions occurs to form proteins.

DNA \longrightarrow mRNA \longrightarrow Protein

DNA, the archive of genetic information, dictates a proteins amino acid sequence DNA is used as a model for the synthesis of a smaller molecule called mRNA; the process of copying mRNA from DNA is known as transcription mRNA then finds its way from the nucleus to a ribosome, where large molecules called tRNAs change or translate the information on the mRNA into a protein in a step-wise manner.

A second type of natural protein synthesis, often referred to as the non-ribosome method, also occurs where microorganisms synthesise peptides without using the transcription/translation process (Murphy and Ó'Fágáin, 1996a). Fungi, bacteria and soil organisms provide many of our antibiotics, e.g. vancomycin, using this method. Kleinkauf and von-Döhren (1990) have reviewed this topic extensively and have determined that it is due to an enzymatic scheme known as the 'thiotemplate

multienzymic mechanism'. These polyenzymes or peptide synthetases use amino acids as the substrates to form peptides. Kallow et al. (2002) have investigated how ACVS (δ -(L- α -Aminoadipyl)-L-cysteinyL-D-valine synthetase) catalyses the nonribosomal biosynthesis of the penicillin and cephalosporin precursor tripeptide ACV (δ -(L- α -Aminoadipyl)-L-cysteinyL-D-valine). They state that the polyenzymes or peptide synthetases display a modular organisation, the number and sequence of the modules depending on the amino acid constituents required for the peptide product. Kallow et al. (2002) state that the modules are divided into specific groups according to their partial reaction. They discovered the position of a second ATP binding site on the ACVS enzyme, which would be used in the protein thiotemplate mechanism.

Peptide synthesis can also be achieved synthetically either by:

(1) Chemical synthesis - The two methods that will be discussed briefly here are solid phase peptide synthesis and solution (classical) synthesis. For a more detailed review Wilken and Kent, 1998 and Murphy and Ó'Fágáin, 1996a should be referred to.

(2) Enzymatic synthesis - The use of proteolytic enzymes namely the serine and cysteine proteases are used in this type of peptide synthesis. Trypsin and subtilisin are reviewed here in terms of their ability to synthesis peptides. Various reaction conditions are looked at to see their effects on the rate of peptide synthesis. These include the effects of organic solvents and various methods of altering protein function including crosslinking, hydrophilization, protein coupling to polyethylene glycol, protein engineering and immobilization.

(3) Recombinant DNA technology - The biosynthesis of a foreign gene (protein) in an organism relies on a recombination of the genetic material of the micro organism with the DNA fragment encoding for the desired protein. This technology comprises the following steps (Sewald and Jakubke, 2002):

- (i) Isolation of the encoding DNA fragment from the donor organism.
- (ii) Isolation of the DNA into a vector.
- (iii) Transfection of the vector into the host organism.
- (iv) Cultivation of the host organism (cloning), which leads to gene amplification, mRNA synthesis and protein synthesis.
- (v) Isolation of the recombinant protein.

For an extensive review of this topic see Dawson et al. (1996).

1.3 Chemical peptide synthesis:

(1) Solid phase peptide synthesis-

Bruce Merrifield developed this type of synthesis in 1963. Using this method the first amino acid, which is protected at N^α, is attached via its carboxy group to an anchoring group (linker), which is in turn attached to an insoluble polymer. Then the protecting group is removed and the next amino acid is added and so on until the desired peptide is synthesised. Finally the bond between the linker group and the peptide can be cleaved and the polymeric support can be separated from the solution by filtration leaving the synthesised peptide.

(2) Solution or classical synthesis-

The carboxy group of an N- α -protected amino acid is activated and a second amino acid attacks the activated carboxy component in a nucleophilic attack with the formation of an amide bond (i.e. a peptide) and the elimination of water. It is important when using this method that all functional groups not involved in the peptide bond formation be blocked both temporarily and reversibly. Two of the most common protecting groups are the Fmoc (9-Fluorenylmethoxycarbonyl) group (Figure 1.1 (A)) and Boc (tertiary-butyloxycarbonyl or tBoc-Cl) (Figure 1.1 (B)).

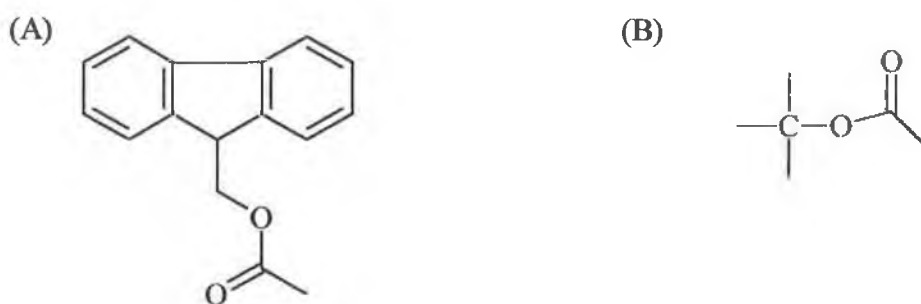


Figure 1.1: Some common protecting groups used in peptide synthesis.

(A) *Fmoc-Cl (9-Fluorenylmethoxycarbonyl) and*

(B) *Boc (tertiary-butyloxycarbonyl or tBoc-Cl).*

1.4 Enzymatic Peptide Synthesis

Proteases can catalyse peptide synthesis. Van't Hoff in 1898 predicted that the "reverse potential of proteases could be used as catalysts for peptide bond formation".

Enzymatic synthesis is cheaper, as there is no need for side chain protection and there is less need for hazardous organic solvents. Peptide bond formation will be enhanced if the rate of hydrolysis is reduced relative to the rate of aminolysis (See section 1.4.2 below).

There are two types of enzymatic peptide synthesis:

- (i) Thermodynamically controlled and
- (ii) Kinetically controlled peptide synthesis.

These will be discussed in detail in section 1.4.2.

Gill et al. (1996) have also stated the important advantages of using enzymatic peptide synthesis as compared to classical methods:

- (A) Reactions take place under very mild conditions
- (B) The high regiospecificity of proteases allows the use of minimally protected substrates which are often inexpensive and readily available
- (C) The synthesis is simplified as there is no need for intermediate protection/deprotection steps
- (D) Reactions are stereospecific and no racemization is observed.

1.4.1 Classes of proteases

Proteases are globular water-soluble proteins that function as enzymes; their Enzyme Commission numbers are in the 3.4.X.X range. They are a specific group of proteins that cleave, cut or degrade other proteins by hydrolysing peptide bonds. However, in certain reaction conditions they will synthesise peptide bonds rather than break them; this will be discussed further in section 1.4.2.

Proteases can be classified into four groups depending on the structure of their active sites: serine, cysteine or thiol, metallo- and aspartic proteases.

Serine proteases – this group is made up of two distinct classes:

1. The chymotrypsin family, which includes mammalian enzymes such as trypsin
2. The subtilisin family, which includes bacterial enzymes such as subtilisin.

The structures of their active sites are highly conserved. The active site arrangement is a His⁵⁷-Asp¹⁰²-Ser¹⁹⁵ triad. The enzyme (E) and the peptide substrate (S) combine to form a tetrahedral transition state, [ES] (Figure 1.2). The acyl - enzyme intermediate ([ES']) is then produced by the reaction of the hydroxyl group of the reactive serine with the carbonyl carbon of the scissile peptide bond (the bond that is cleaved during the reaction). There is also a release of the C-terminal peptide (P_c in Figure 1.2). The reverse reaction then occurs, where the water replaces the enzyme as the nucleophile and the N-terminal peptide (P_N) acts as the leaving group.



Figure 1.2: A schematic diagram of the reaction catalysed by serine proteases leading to the formation of an acyl-enzyme intermediate. Then the breakdown of this acyl-enzyme (deacylation) occurs by the enzyme-catalysed attack of water to obtain the peptide product.

Cysteine or thiol proteases – this group of proteases feature the covalent attack of a polarised cysteine side-chain and the formation of an acyl-enzyme intermediate whose breakdown also involves the enzyme catalysed attack of water as with the serine proteases (Figure 1.2). The active site arrangement is Cys-His charge transfer. Examples are papain and caspases.

The next two groups of proteases cause the cleavage of peptide bonds without the use of nucleophilic attack by a functional group of the enzyme.

Metallo protease - here, the scissile bond undergoes attack as a result of a polarised water molecule by an enzyme co-ordinated to a metal, which is normally zinc. An example is thermolysin where the active site consists of a co-ordinated Zn^{2+} ion.

Aspartic protease - attack takes place via a water molecule polarised by enzyme aspartic acid side-chains. The active site is composed of two aspartate residues, one charged and one uncharged and they engage in a general acid-base catalysis mechanism. Examples are pepsin and chymosin.

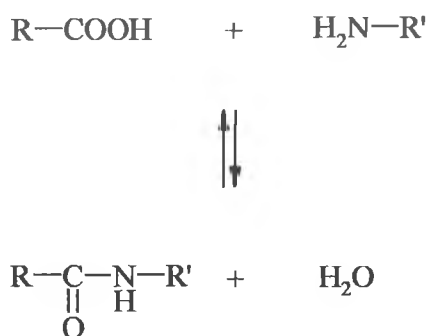
1.4.2 Thermodynamically and kinetically controlled peptide synthesis:

Protease-catalysed peptide bond formation can be classified into two basic strategies: thermodynamic (equilibrium) approach and kinetic approach. The type of synthesis is dependent on the type of carboxyl component being used in the reaction. If the carboxyl group is free (RCOOH) then the reaction will proceed by the thermodynamic (equilibrium) approach and if the carboxyl group has been activated chemically (RCOOX) then the reaction will be through the kinetic pathway.

(1) Thermodynamic (equilibrium) approach:

The protease is used only to increase the rate with which the equilibrium is established; the enzyme cannot influence this equilibrium (Figure 1.3).

Thermodynamic approach.



Kinetic approach.

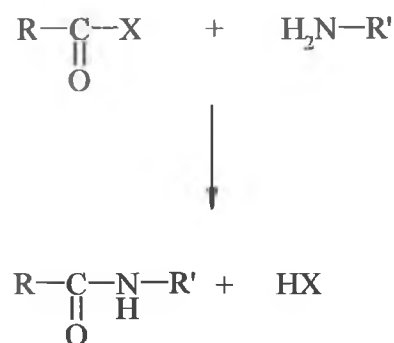


Figure 1.3: A schematic diagram of the difference between the thermodynamic and kinetic approach.

(2) Kinetic approach (Figure 1.3):

For this approach, it is the rate of product formation relative to the rate of peptide hydrolysis, which is important. As can be seen from Figure 1.4, the acyl-enzyme complex can undergo two routes either aminolysis to form the required product or hydrolysis to form an acylamino acid. The aim is to have the greatest k_4/k_3 ratio possible so that the reaction will go in the direction of the peptide synthesis. This may be done by providing an amino acid or peptide, which is a more powerful nucleophile than water in accepting a peptide unit from an enzyme-peptide intermediate. An activated substrate (peptide or amino acid) is used and the enzyme catalyses the transfer of the acyl group to a nucleophile (amino acid or peptide). The product yield depends on the properties of the enzyme used and on its substrate specificity.

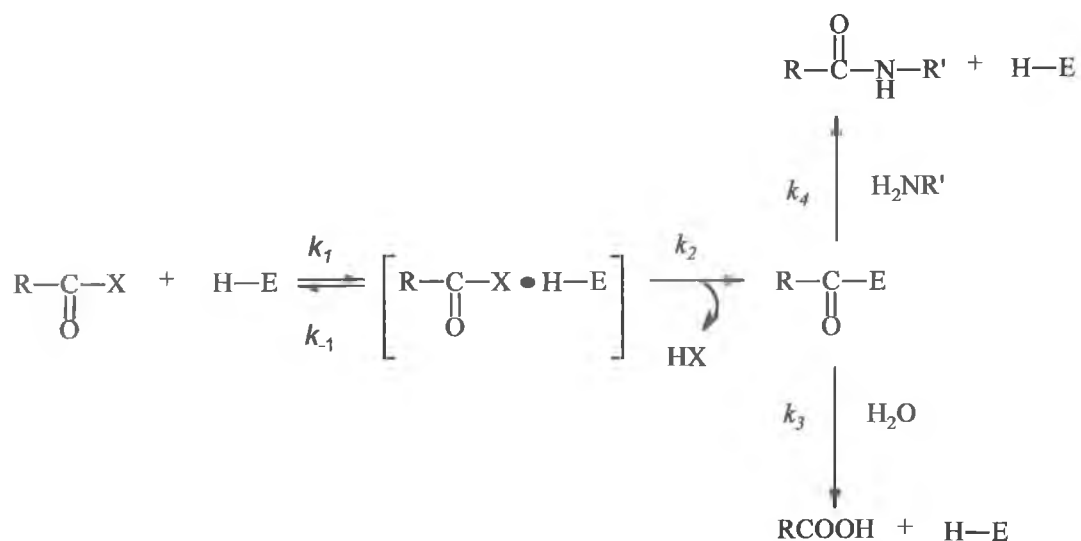


Figure 1.4: Kinetically controlled peptide synthesis catalysed by serine and cysteine proteases (H-E). Where R-CO-X is the carboxyl component, X is OAlk, H₂N-R' is the amino component, R-CO-NH-R' is the peptide product, RCO-E is the acyl-enzyme complex, k₁ is the equilibrium constant for the E-S complex formation and k₋₁ the equilibrium constant for the reverse reaction, k₂ is the rate constant for the formation of the acyl-enzyme intermediate, k₃ is the rate constant for the hydrolysis of the acyl-enzyme intermediate and k₄ is for the synthesis of the peptide product.

Equilibrium controlled synthesis can be performed using all proteases whereas kinetically controlled synthesis can be performed with serine and thiol proteases only, as it requires the formation of an acyl-enzyme intermediate. Blanco et al. (1991) compared the synthesis of the dipeptide benzoylarginine leucinamide using both the kinetically (KCS) and thermodynamically (TCS) controlled synthesis. For the KCS benzoyl arginine ethyl ester and leucinamide were used as substrates and for the TCS benzoyl arginine and leucinamide were used. They used an immobilised form of trypsin, a trypsin-agarose derivative, as the catalyst for both reactions. They found a much higher

reaction rate of 5000 μmol peptide per hour per mg of trypsin for the KCS compared to 9 μmol peptide per hour per mg of enzyme for the TCS.

Many people have investigated the use of organic solvents in peptide synthesis (Table 1.1). In particular the advantages of using organic solvents in peptide synthesis have been reported. Among these advantages are the shift in reaction equilibrium in favour of peptide synthesis, the suppression of hydrolytic side reactions, the increased solubility of amino acid derivatives in organic solvents and the greater thermostability of many enzymes in low-water media.

Miyazawa et al. (1998) has investigated the effect of various esters as acyl donors. Generally, methyl esters are used but they have discovered that using esters such as 2,2,2-trifluoroethyl or carbamoylmethyl esters increases the yield considerably in the α -chymotrypsin catalysed synthesis involving a low-reactive substrate e.g. alanine. After 48 hours they obtained a 4.2% yield for the Z-Ala-Leu-NH₂ peptide using a methyl ester whereas the carbamoylmethyl ester produced a yield of 88.4% over the same length of time.

Table 1.1: Examples of peptide synthesis where organic solvents have been used.

Enzyme	Peptide synthesised	% (v/v) Solvent / aq.buffer	% Yield	Reference
Trypsin	Bz-Arg-Leu-NH ₂	95% ACN	90	Čeřovsky (1990)
	Ala-Gly-Ala-pNa	50% DMSO	91	Sekizaki et al. (1996)
Alcalase	Moz-Phe-Leu-NH ₂	100% 2-Me-2-propanol	95	Chen et al. (1992)
α - Chymotrypsin immobilised on celite.	Ac-Phe-Arg-NH ₂	90% ACN	86	Jónsson et al. (1996)
Subtilisin	Z-Phe-Gly-NH ₂	50% DMF	92	Khuntaveeporn et al. (1999)

Sekizaki et al. (1996, 1997) also investigated the effect of using different types of acyl donors. They used various p-Guanidinophenyl esters as "inverse substrates" in trypsin catalysed peptide synthesis reactions. Inverse substrates behave as specific substrates for the enzyme and they allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site. This characteristic feature of inverse substrates suggest that they would be extremely useful in peptide synthesis as it would remove the greatest disadvantage of enzymatic peptide synthesis i.e. the narrow specificity of the enzyme. Schellenberger et al. (1991) defined inverse substrates as "a substrate which has a cationic centre included in the leaving group instead of being in the acyl moiety". They have shown that the use of Z-Ala-OMe i.e. a normal unspecific

substrate, led to trypsin activities that were lower by 3 to 4 orders of magnitude in comparison to Z-Ala-OGP. They demonstrated that inverse substrates allow the trypsin-catalysed synthesis of peptide bonds that cannot be split by the enzyme.

Many people have also investigated the effect of temperature on the yield of peptide synthesis. Chen et al. (1994) determined that the % yield of Moz-Phe-Phe-OBu^t in an alcalase (a commercial subtilisin-like alkaline protease) catalysed reaction changed from 45% at 30°C to 80% at 5°C. It is thought that the low reaction temperature depressed the rate of hydrolysis thereby increasing the rate of peptide bond formation.

Other research has been carried out to find enzyme derivatives, which remain very active and stable in even simpler synthetic conditions and more severe experimental conditions. Blanco et al. (1991) found that trypsin immobilised through multipoint covalent attachments formed very stable trypsin-agarose derivatives. They then used this immobilised trypsin as a catalyst to form the peptide benzoyl arginyl leucinamide both by kinetically controlled and thermodynamically controlled synthesis. Murphy and Ó'Fágáin (1997) used AA-trypsin (trypsin acetylated with acetic acid N-hydroxysuccinimide ester) in 95% acetonitrile to obtain a yield of 90% of the dipeptide Bzl-Arg-Leu-NH₂. There was very little difference between the native and AA-trypsin at low acetonitrile concentrations with respect to peptide synthesis but at high acetonitrile concentrations (95%) that the AA-trypsin had a better rate of peptide synthesis.

Chen et al. (1997) investigated the effect of microwave irradiation on peptide synthesis. They used 0.3mmol Pro-NH₂ as a nucleophile and 0.1mmol Cbz-Ala-Phe-OMe as an acyl donor in 2-methyl-2-propanol with alcalase as the catalyst. They synthesised the tripeptide Cbz-Ala-Phe-Pro-NH₂ with an 85% yield in just 20 minutes. Previously this

reaction had been reported to take 4 days and only obtain a 59% yield. They suggest that the effects caused by microwave irradiation are due to its molecular stirring mechanism. Microwaves are electromagnetic radiation that changes molecules into dipole rotation without causing rearrangement of their structures. So the irradiated molecules would accelerate the rate of reaction, as this irradiation would probably cause a kinetic effect of agitating all molecules in the system.

Recent developments in this area have led to the enzymatic synthesis of a tripeptide RGD i.e. Bz-Arg-Gly-Asp(-Ome)-OH (So et al., 2000). RGD is a small hydrophilic tripeptide, which is a recognition factor for a range of cellular adhesion molecules. Recently RGD has been investigated to be the active ingredient in a new drug for injury such as heavy burns, ulcer etc. (Chen et al., 1998). The tripeptide was synthesised in two steps with a 70% yield. Firstly, the dipeptide Bz-Arg-Gly-OEt was synthesised with trypsin and then H-Asp(-OMe)₂ was inserted into the previously synthesised dipeptide i.e. Bz-Arg-Gly-OEt using chymopapain. It has been suggested that this enzymatic method is much quicker and cheaper than the solution-phase or solid-phase chemical synthesis. Zhang et al. (2001a) synthesised a RGD diamide using porcine pancreas lipase (PPL) in a water-miscible organic cosolvent system. They obtained a 76% yield in 50% DMF and a 71.7% yield in 50% DMSO. This is an unusual reaction as lipase is not a protease and so instead of performing peptide synthesis through the reversion of hydrolysis the peptide synthesis is completed through transesterification (conversion of one ester to another).

Recent work has been undertaken to combine enzymatic and chemical peptide synthesis. Ulijn et al. (2002) has coupled phenylalanine to a poly- (ethylene glycol)-acrylamide

support and then the protease thermolysin catalysed a variety of peptide synthesis reactions with various acyl donors. This will be discussed in further detail in chapter seven.

1.5 Organic solvents.

In an aqueous system, enzymes fold in such a way that the hydrophobic (non-polar) residues lie on the inside of the structure and the hydrophilic (polar) residues lie on the exterior of the enzyme, where they can interact with the water. The water present can be divided into two categories: (A) >98% is known as the bulk or true solvent and (B) <2% being the tightly attached enzyme bound water (Krisha, 2002). Fitzpatrick et al. (1993) have determined that even when a protein is placed in a water-miscible organic solvent, the protein still retains some or all of the enzyme-bound water and this would indicate that the bound water is an important part of the structure of the protein. The amount of enzyme bound water needed to retain its catalytic activity is enzyme dependent e.g. α -chymotrypsin needs only 50 molecules of water per molecule to remain active while polyphenol oxidase requires the presence of about 3.5×10^7 molecules of water (Krisha, 2002). There is always a layer of water molecules tightly bound to the enzyme and without this water enzymatic catalysis would be impossible.

Traditionally it is the aqueous system that has been studied, as it was thought that organic solvents would reduce the polarity of the solution and, as "like attracts like," non-polar residues on the inside would try to get to the outside of the molecule and the enzyme would unfold and become deactivated (Fukui and Tanaka, 1985). Some commercially important substrates are water-unstable and/or water-insoluble and so it is

desirable to carry out the reaction in the presence of organic solvent (s). So, extensive research has been carried out to find appropriate methods to either stabilize the enzyme or to keep it in isolation from the organic solvents to prevent inactivation. Carrea and Riva (2000) have carried out an extensive review of this topic.

Schulze and Klivanov (1991) and Gorman and Dordick (1992) suggested that organic solvents strip water off enzymes as they physically disrupt the enzyme-bound water and result in deactivation of the enzyme. This is known as "water stripping". However, Fitzpatrick et al. (1993) showed that subtilisin Carlsberg had the same three-dimensional structure in anhydrous acetonitrile as in water. Ninety-nine of the one hundred and nineteen enzyme-bound water molecules have such a great affinity to subtilisin that they are not displaced even in anhydrous acetonitrile. Of the twelve enzyme-bound acetonitrile molecules, only four displace water-bound molecules and eight of them bind where no water has been observed. Klivanov (2001) states that the reason for the stability of the enzymes in various anhydrous solvents is that, in the absence of water, which acts as a molecular lubricant, enzymes are very rigid and so find it extremely difficult to unfold.

Ogino and Ishikawa (2001) have shown that, for some enzymes, solvent molecules can replace some enzyme bound water molecules and this can actually stabilise the structure of the enzyme. For example, the half-life of lipase in η -Decane is >100 days whereas in water it is 12.5 days.

The enantioselectivity of enzymes can also be improved by using organic solvents in the reaction media (enantiomers are two forms of a compound which have molecular structures that are mirror images of each other). Carrea et al. (1995) described the

enantioselective performance of enzymes as the enantiomeric ratio, which is the ratio of the catalytic constant, k_{cat}/K_m , for the two enantiomers. The higher the enantiomeric ratio, the higher the enantiomeric excess (i.e. the optical purity) of the product and this is important for the synthesis of enantiomerically pure drugs. Sometimes only one enantiomer of a drug may be physiologically useful. An example is thalidomide, which was synthesised by chemical means. The R (+) enantiomer has a sedative effect while the S (-) enantiomer is teratogenic (causes abnormalities in the unborn child).

Sakurai et al. (1988) showed that the enantioselectivity of an enzyme could be controlled by the solvent system. Subtilisin Carlsberg was reacted with various substrates in both water and butyl ether. An enantiomer excess of 1800 was determined for Subtilisin Carlsberg in water compared to 4.4 in butyl ether. Sakurai et al. (1988) showed that the enzyme's enantioselectivity decreases, as the hydrophobicity of the solvent increases. Fitzpatrick et al. (1992) confirmed this using the x-ray crystal structure of the enzyme and a computer modelling system which constructed the structures of the reactive enzyme-substrate complex. Kawashiro et al. (1997) showed that when anhydrous acetonitrile was used as a solvent, the L-amino acid derivative was the preferred enantiomer for *Aspergillus oryzae* protease and for two forms of subtilisin (BPN' and Carlsberg). The enantiomer excess for *Aspergillus oryzae* protease was 92% and was 83% for the subtilisin with anhydrous acetonitrile as solvent. However, the enantioselectivity in the other solvents tested (DMF, toluene, THF) were very poor.

Ke and Klibanov (1999) have shown that by temporarily enlarging the substrate by forming a salt with a bulky counterion further enhancement of the enantioselectivity of an enzyme can occur. They have shown that the less reactive enantiomer exerts a greater

steric hindrance. By complexing the substrate with a bulky agent, the steric difficulties should be exaggerated and this should increase the enzymatic enantioselectivity. Ke and Klibanov (1999) have shown that subtilisin Carlsberg in *tert*-amyl alcohol demonstrated an enantiomer excess of 8.1 ± 0.6 when cross-linked with 4-(4-chlorobenzoyl)pyridine compared to 1.5 ± 0.1 for the native.

One important thing to note is that the activity of native and modified enzymes varies greatly from one organic solvent to another. Khmel'nitsky et al. (1991) developed a Denaturation capacity (DC) scale of organic solvents, which could be used to predict the maximum concentration of various organic solvents that native proteins can withstand before they start to lose their activity and become denatured. The denaturation scale was developed on the basis of the relationship between the physiochemical properties of organic solvents and their denaturing strength (Figure 1.5).

However Rosell et al. (1995) have developed a simpler scale for the prediction of the tendency of organic solvents to denature proteins. The scale is based on the hydrophobic effect of various solvents on naphthalene. The relative solubilising effects of different solvents on naphthalene correspond with the solvents' known tendency to denature proteins. For example, cytochrome *c* showed a C_{50} value of 12.7 in 1,2-Ethanediole on the DC scale (Khmel'nitsky et al. 1991) and a C_{50} value of 13.9 using the scale developed by Rosell et al. (1995).

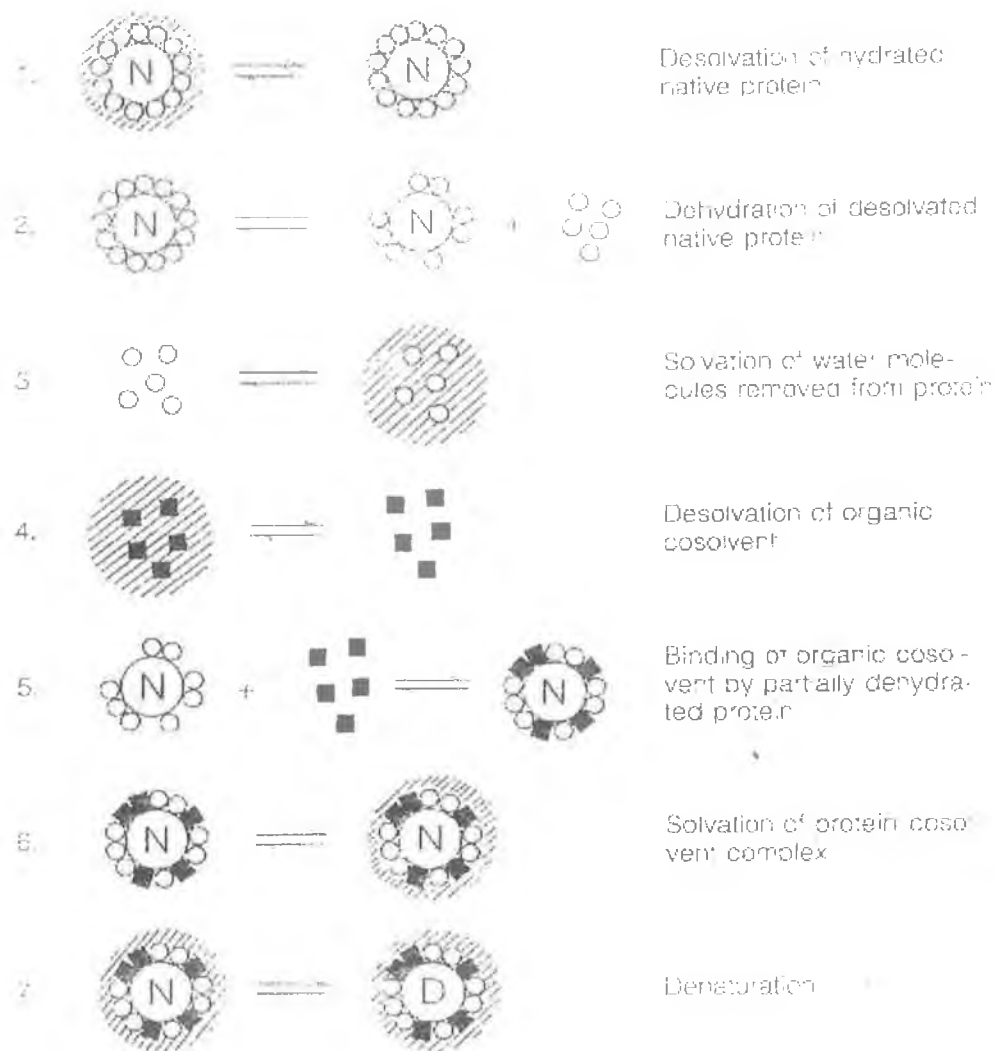


Figure 1.5: A schematic representation of molecular steps involved in the process of reversible protein denaturation by organic solvents (Khemelnitsky et al., 1991).

○ Water molecule, ■ organic cosolvent molecule, ▣ surrounding solvent.

Colombo and Carrea (2002) have shown through computer simulations that enzymes in non-aqueous media demonstrate increased intramolecular interactions (e.g. hydrogen bonds, ion pair interactions) and reduced ratio of surface area to volume, thereby improving conformational rigidity. This explains the lower catalytic efficiency of many

enzymes in nonaqueous solutions, as if it is more rigid, it will be less able to adapt to the substrates entering the active site pocket. It also explains the higher thermostability of many enzymes in nonaqueous media: if there is very little water present in the reaction system the structure of the enzyme is more inflexible and so is not as prone to denaturation. Volkin et al. (1991) determined the thermal unfolding (T_m) of native ribonuclease in an aqueous solution to be 61°C whereas the corresponding value was 124°C when the enzyme (containing 6% water in the enzyme powder) was placed in anhydrous nonane.

Guo and Clark (2001) showed that it was also possible to have an enzyme, which retained its activation in nonaqueous media if subtilisin Carlsberg and horseradish peroxidase that had been previously lyophilised from aqueous urea solutions (even up to 8M) were used. Activity assays and active site titrations indicated that the enzymes' activity might result from a combination of lyoprotection and partial unfolding of the enzyme's active sites. Horseradish peroxidase (HRP) lyophilised from 8 M urea demonstrated approximately 56 times greater activity in 97% acetone than HRP lyophilised from aqueous buffer. Sears et al. (1999) discovered that when subtilisin BPN was placed into organic solvents the enzyme's activity was more dependent on the lyophilisation and suspension conditions than to the mutations they had made (Table 1.2 for an example of the results). However no pH values were given for the buffers used so it is difficult to compare these results. Ru et al. (1999, 2001) has carried out research on the effect of salts and lyophilisation on the activity of various enzymes. They have observed that by including simple inorganic salts e.g. KCl and NaCH₃COO, in the solution prior to lyophilisation, the activities of the enzymes are increased by several

orders of magnitude in organic solvents. Native subtilisin Carlsberg demonstrated a V_{max}/K_m value of 0.013mMol of APPE/min/mg of enzyme compared to 24.1mMol of APPE/min/mg of enzyme for subtilisin Carlsberg that had 98% KCl added prior to lyophilisation.

Table 1.2: The effect of various aqueous buffers on the $T_{50\%}$ of subtilisin BPN in 99% DMF (Sears et al., 1999).

99% DMF		
	* $T_{50\%}$	% activity remaining after 50 hours.
Tris-HCl	<1 minute	<0.1%
Sodium citrate	240 hours	76%
Sodium phosphate	6 hours	16%
Sodium borate	80 hours	59%
Sodium carbonate	2 minutes	<0.1%

* $T_{50\%}$ = length of time required for the enzyme to deactivate to 50% of its original activity.

Getun et al. (1997) synthesised an enzyme complex, which was highly soluble in polar organic solvents. The enzyme complex consisted of subtilisin 72, which had formed hydrophobic ion pairs with the anionic detergent, sodium dodecyl sulfate (SDS). They used this complex in peptide synthesis to form the peptide Z-Ala-Ala-Leu-Phe-pNa in 96 % ethanol with a yield of 87% after 2 hours. The native enzyme is only slightly soluble in ethanol and so would not be able to catalyse a reaction under these conditions. Getun et al., 2001 carried out further research on the efficiency of this complex in the

synthesis of peptides containing mostly hydrophobic amino acid residues in the P₁- and P'₁- positions. However, this type of modification would not be suitable for all enzymes. Sweadner (1991) determined that when trypsin was allowed to react with SDS the enzyme lost all of its activity. It was discovered that in order to maintain the activity of the enzyme, it must be reacted first with an inhibitor. Both chymotrypsin and elastase didn't need an inhibitor and showed normal catalytic activity when reacted with SDS.

Only some natural enzymes are organic solvent-stable. If enzymes are not stable in organic solvents, several physical and chemical methods of stabilisation have been developed to overcome this (Arnold, 1990). Krishna (2002), in a review on trends in enzyme catalysis, states that enzymes have been used in the following states in organic systems: - native enzymes, suspended enzyme powder, solid enzyme absorbed on support, polyethylene glycol-modified enzymes soluble in organic solvents, enzyme entrapped within a gel, microemulsion or reversed micelle and immobilised enzyme.

Site directed mutagenesis and directed evolution have also been used to obtain organic solvent-stable enzymes (Ogino and Ishikawa, 2001). One such strategy that has been used to modify enzymes for use in organic solvents is multiple steps of random mutagenesis and screening in continually higher concentrations of organic solvent (Chen et al., 1991). Zhang et al. (1991) used site directed mutagenesis to prepare a mutant of subtilisin BPN' that was more organostable in DMF. They engineered a variant with the following amino acid substitutions:

Asn 218 Ser, Gly 169 Ala, Met 50 Phe, Gln 206 Cys and Asn 76 Asp.

This new variant called 8397 had a half-life of 350 hours in DMF at 25°C compared to the native's half-life of 20 minutes under the same conditions. The 8397 variant also

demonstrated increased stability in aqueous solutions, with the half-life for the native enzyme being 15 hours compared to 1600 hours for the mutant. Wong (1992), based on the work carried out by Zhang et al. (1991), stated that the following changes must be made to make enzymes more stable and active in organic solvents:

1. Minimization of surface charges to reduce solvation energy
2. Enhancement of internal polar interactions e.g. hydrogen bonding
3. Optimisation of internal hydrophobic interactions
4. Introduction of conformational restrictions to reduce the tendency of the protein to denature.

Recently research has been carried out to investigate the effect of ionic liquids on enzyme activity. Ionic liquids are compounds that consist only of ions and are liquid at room temperature. Rantwijk et al. (2003) discovered that ionic liquids are similar to organic solvents in that some enzymes tolerate the liquids well while other enzymes do so to a much lesser extent.

Some advantages of using ionic liquids are their lack of vapour pressure making them a safe solution to work with, their thermal stability and their widely tuneable properties. These properties include polarity, hydrophobicity and solvent miscibility and can be changed depending on the cation or anion used. Some of the liquids studied so far are composed of a 1,3-dialkylimidazolium or N-alkylpyridinium cation. Erbeldinger et al. (2000) were the first to use ionic liquids in peptide synthesis; the dipeptide aspartate-phenylalanine was synthesised in a thermolysin-catalyzed reaction in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate. They also observed that the enzyme

demonstrated a high level of stability in the ionic liquid that would normally require some type of chemical modification.

The use of organic solvent-stable enzymes is effective for peptide synthesis reactions: the addition of organic solvents to the reaction mixture increases the product yield due to a shift in the thermodynamic equilibrium in favour of peptide synthesis (Ogino and Ishikawa, 2001). This has been discussed in detail in section 1.2.2.

1.6 Stabilization of Proteases

Enzyme stability is the degree to which an enzyme stays intact and retains its catalytic activity when exposed to different environmental conditions, e.g. extremes of pH or temperature. Enzymes can lose their activity when they are denatured. Denaturation is defined as a major change from the original native state without alteration of the molecule's primary structure to an inactive conformation. Most globular proteins show complicated three-dimensional folding described as secondary, tertiary and quaternary structures and denaturation causes the unfolding of the protein. There are many complex interactions involved in maintaining the protein structure; these include hydrogen bonding, electrostatic forces, hydrophobic effects, packing contributions and solvation (Sowdhamini and Balaram, 1993). When the protein is exposed to strong acids or bases, high concentrations of inorganic salts or organic solvents, heat or radiation, denaturation occurs. Inactivation also causes a loss in activity of the enzyme and it is due to a change in the actual structure of the protein (and, ultimately, a change in the active site configuration).

Mozhaev (1993) described the process of inactivation as:



where N represents the native, D is the denatured (reversibly unfolded state) and I stands for the irreversibly inactivated forms of the protein. As can be seen from the equation, the reversible denaturation of the enzyme is a two-state transition and the irreversible inactivation is a multi-step process. The reversible denaturation is also called thermodynamic stability and is a measure of the resistance of the protein to denaturation whereas the irreversible inactivation is a measure of the protein's long-term stability (Ó'Fágáin, 1997). When the degree of denaturation is being investigated, the studies involve either variation of temperature (heat or cold denaturation) or addition of chaotropic agents as a means of inducing unfolding. Extensive research has been carried to obtain a chemical modification that increases the thermal stability of protein structures.

Tyagi and Gupta (1993) define chemical modification as "any chemical alteration of protein structure with or without accompanying changes in biological function". Chemical modification is a very efficient method for fine-tuning an enzyme's chemical and physical properties and has many advantages. These include modifications which produce more efficient catalysts for peptide synthesis, increase an enzyme's stability, improve their solubility, mask antigenicity, alter patterns of inhibition and activation and change pH optima or substrate specificity (Hilvert, 1991). Chemical modification includes the following types of reaction, which will be discussed in detail later in this chapter:

- (i) Chemical modification with monofunctional reagents

- (ii) Crosslinking
- (iii) Hydrophilization
- (iv) Immobilisation
- (v) Chemical aggregation
- (vi) Coupling to PEG.

1.6.1 Chemical modification with monofunctional reagents.

Chemical modification with monofunctional reagents is the reaction of the free functional groups of the protein with monofunctional (small molecular weight) reagents. Ito et al. (1993) modified trypsin with a hydrophobic synthetic polymer, polystyrene. The free amines in the trypsin were coupled with azobis (4-cyanovaleric acid) and this molecule was then irradiated with a mercury lamp in the presence of styrene. This caused the decomposition of the azo structure, which allowed free radical initiation of styrene polymerisation to take place. Therefore, the trypsin became part of the end group of the polymer. This modified enzyme was then used to catalyse the synthesis of three peptides in chloroform. The native trypsin showed no activity in the same environment. This type of modification could also be considered to be a type of immobilisation (section 1.5.4).

Elsner et al., (2000) chemically modified lysine residues in trypsin by succinylation and guanylation. They observed that the succinyl-trypsin was more resistant to autodigestion following the introduction of additional negative charges into the protein. The succinyl-trypsin in particular retained 100% amidase activity over 250 minutes compared to the native, which retained only 5% amidase activity over the same time span. The activity of

the modified trypsin was unaffected and so the modification had decreased autolysis. The reduction in amidase activity of the enzyme was due to the enzyme starting to auto-digest.

1.6.2 Crosslinking

Crosslinking reagents contain two reactive groups, allowing them to link two target groups. In homobifunctional crosslinking reagents (the most frequently used), the two reactive groups are identical. Examples of these reagents include glutaraldehyde, dimidoesters, diisocyanates, dianhydrides and succinimide esters (Gleich et al., 1992). In heterobifunctional crosslinking reagents the reactive groups have dissimilar chemistry, allowing the formation of crosslinks between unlike functional groups. Rajput and Gupta (1987a) used this method to crosslink trypsin and chymotrypsin using N-succinimidyl pyridyl dithiopropionate. They also crosslinked trypsin with alkaline phosphatase (1988) using the oxidized carbohydrate moiety of alkaline phosphatase and the free amino groups of the trypsin. They found that the trypsin-chymotrypsin insoluble co-aggregate has been prepared and found to contain both enzyme activities. The aggregate was also more stable to autolysis, retaining >80% activity for six hours compared to native which retained only 5% over six hours.

Murphy and Ó'Fágáin (1998) chemically modified trypsin with ethylene glycol bis (N-hydroxysuccinimide ester). This derivative (EG-trypsin) showed a half-life of 25 min at 55 °C, which was fivefold greater than native trypsin. The EG-trypsin also synthesised the dipeptide Bzl-Arg-Leu-NH₂ at higher rates than native trypsin in 95% acetonitrile.

Rajput and Gupta (1987b) crosslinked trypsin with glutaraldehyde and bisimidoesters. Bisimidoesters were the better crosslinker for trypsin as they caused the enzyme to be more stable. Esterase activity of the crosslinked trypsin was approximately 71% of the native. However, while native enzyme lost 55% of its activity, the treated enzyme lost only 25% of its activity owing to autolysis. Venkatesh and Sundaram (1993) showed that trypsin crosslinked with glutaraldehyde was much more stable in organic solvents (such as acetonitrile and methanol) than was native trypsin. In addition, the crosslinked form could withstand greater temperatures than the native could. The temperature optimum of the modified trypsin was 76°C whereas the native's was 45°C, while the T_{50} changed from 54°C for the native to 76°C for the modified. In further work, Venkatesh and Sundaram (1998) converted the normally mesophilic trypsin to a form resembling a thermophilic enzyme, by chemical modification with various reagents such as monomeric glutaraldehyde, polymeric glutaraldehyde, oxidized sucrose and oxidized sucrose polymers. The temperature optimum for native trypsin was found to be 45°C, whereas the trypsin modified with an oxidised sucrose polymer, molecular weight 400KDa, demonstrated an optimum temperature of 76°C.

Kobayashi and Takaatsu (1994) crosslinked trypsin with dextran-dialdehyde and they found that the modified trypsin showed an increase in thermal and pH stabilities. The crosslinked trypsin retained approximately 95% of its residual activity after 100 minutes at pH 9.5 compared to the native, which retained approximately 25% of its residual activity in the same environment.

Tafertshofer and Talsky (1989) crosslinked chymotrypsin with dianhydrides and N-hydroxysuccinimide esters. They found both inter- as well as intramolecular crosslinking

in the reaction solution. They found that native chymotrypsin showed a half-life of one hour, whereas the cross-linked trypsin had a half-life of 22.2 hours at 50°C. Gleich et al. (1992) crosslinked trypsin with N-hydroxysuccinimide esters of five different dicarboxylic acids. In all cases, stability increased greatly, at 50°C, trypsin modified with the NHS-ester of acetylglutamic acid was 40 times more stable than the native trypsin at the same temperature.

1.6.3 Hydrophilisation

Another way to stabilize proteins is the artificial hydrophilisation of the surface of the protein. This involves the preferential modification of $-NH_2$ groups on the surface of the protein by either alkylation or acylation i.e. into $-NHCH_2COOH$ structures (Melik-Nubarov et al., 1987). This artificial hydrophilisation can be achieved in two ways (Figure 1.6).

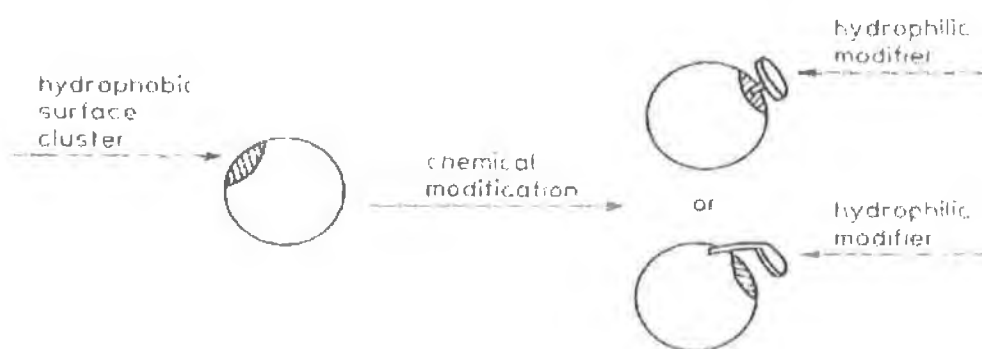


Figure 1.6: A schematic presentation of the approach to hydrophilization of nonpolar surface areas in a protein globule by chemical modification (Mozhaev et al., 1988).

Mozhaev et al. (1988) used this method to stabilize both trypsin and α -chymotrypsin. The hydrophobic amino acid residues of the trypsin were directly modified by the hydrophilic reagent (Figure 1.7). The modified trypsin became up to 100 times more stable to thermoinactivation. The extent of the stabilization was determined by the number of surface Tyrosine residues of trypsin that were converted to aminotyrosine; up to four Tyrosines could be converted. There are ten tyrosine residues in beef trypsin but only four are entirely exposed to the solvent and can undergo chemical modification. Native trypsin showed 50% residual activity after 30 minutes at 56°C whereas the modified showed 50% residual activity after three hours. The modified trypsin was extremely stable up to temperatures of 98°C.

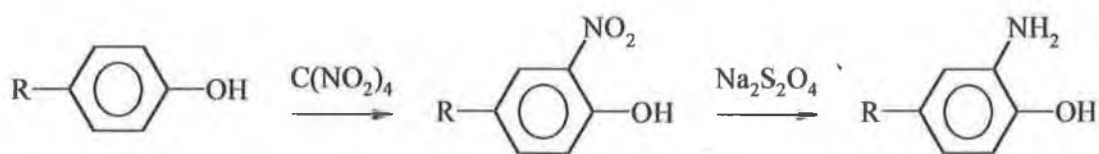


Figure 1.7: The introduction of an amino group into the tyrosine residues of trypsin (Mozhaev et al., 1988).

In a separate experiment the amino groups of α -chymotrypsin were acylated by cyclic anhydrides of pyromellitic and mellitic acids (Figure 1.8). Mozhaev et al. (1988) found that these modified forms show nearly the same thermostability as proteolytic enzymes from extremely thermophilic bacteria.

Murphy and Ó'Fágáin (1996b) neutralised the positive charges on the lysine residues of bovine trypsin by modifying it with acetic acid N-hydroxy-succinimide ester (AANHS). Approximately eight of the fourteen lysine residues were modified. The T_m value of the

modified trypsin was 51°C compared with 46°C for the native. The modified trypsin had a half-life of 8.7 minutes compared to 4.3 minutes for the native at 55°C.

Plou et al. (1994) modified subtilisin Carlsberg by acylation of its amino groups with long chain fatty acid residues e.g. palmitoyl chloride, octanoyl chloride. They then investigated the effect of this modification on the activity and stability of the enzyme. The $t_{1/2}$ (hours) of the native subtilisin at 45°C was determined to be 2.4 whereas the corresponding value for the palmitoyl-subtilisin had a value of 37.2.

Fernández et al. (2002) chemically modified bovine trypsin with monoamine derivatives of cyclodextrins using EDC as the coupling agent. The complexes prepared were more stable against thermal incubation than the native; the half-life of the native trypsin was 60 minutes at 45°C whereas the trypsin- γ CD conjugate showed a half-life of 186 minutes. Also, the modified forms were more resistant to autolysis than the native enzyme at pH 9.0. They hypothesised that this was due to the steric hindrance caused by the bulky cyclodextrin derivatives to the cleavage sites in the enzyme.

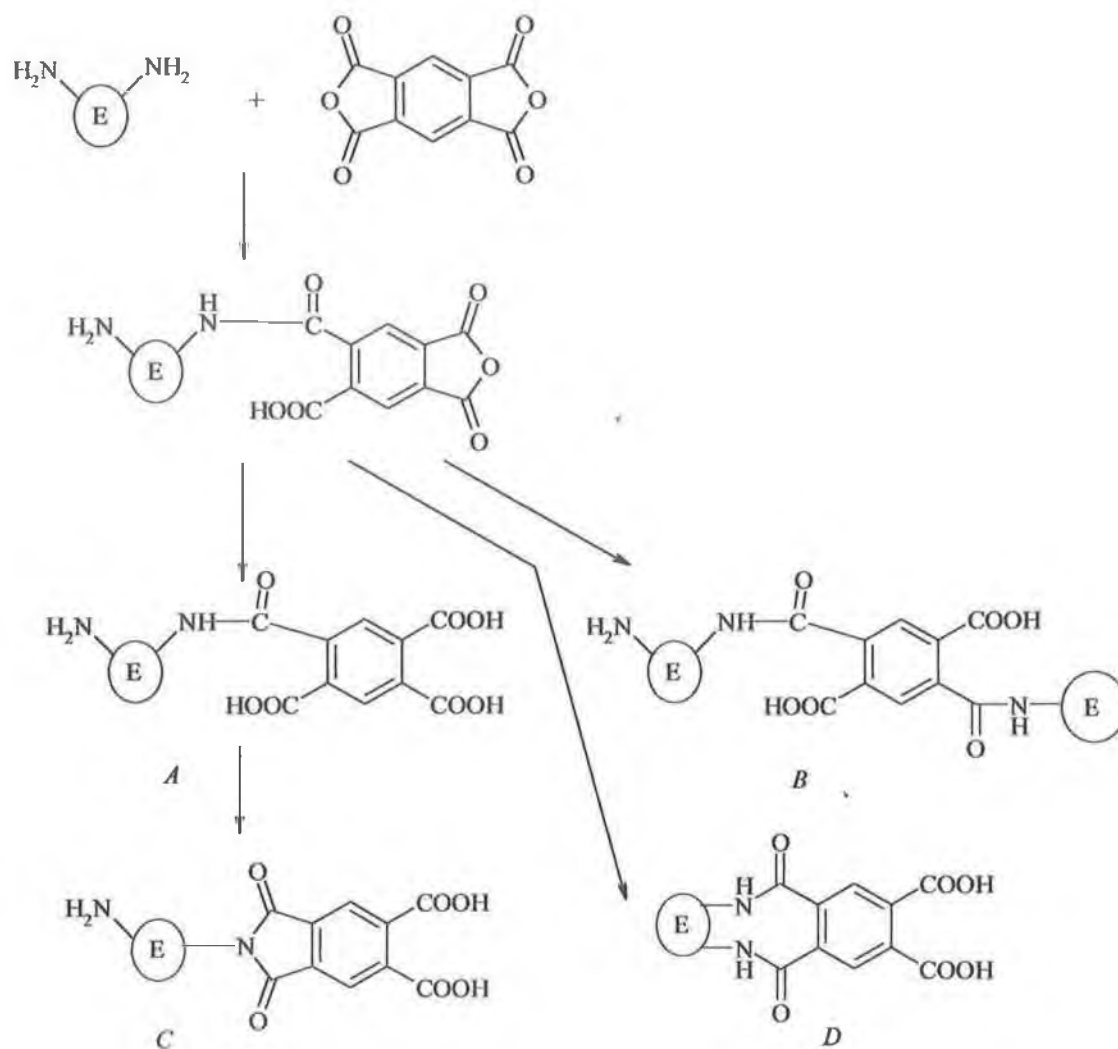


Figure 1.8: The possible reaction of the modification of amino groups in α -chymotrypsin by pyromellitic dianhydride (Mozhaev et al., 1988).

1.6.4 Immobilization

The term immobilised enzyme means 'enzymes physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously', as defined by Ephraim Katchalski-Katzir at the first Enzyme Engineering Conference, at Henniker, New Hampshire, USA, in 1971. The

advantages to carrying out this technique are that, once the product has been removed from the system, the enzyme can be re-used, better quality products are obtained (as there should be little enzyme in the final product) and it makes continuous manufacturing processes easier to develop. Enzymes are normally stabilised due to their binding with the solid support. When immobilizing an enzyme to a support, a method that does as little damage as possible to the enzyme must be found, as it is essential that a loss of enzyme activity be avoided.

Enzyme immobilisation techniques can be classified into classes as follows (Katchalski-Katzir, 1993):

1.6.4.1 Carrier-Binding: the binding of enzymes to water-insoluble carriers:

Bryjak and Kolarz (1998) investigated carrier-binding and in particular covalent binding. They showed that the immobilisation of trypsin on various acrylic copolymers caused an increase in the storage and pH-stability of the bound enzyme. At 35.5°C, 50% residual activity remained after thirty hours for the native enzyme compared to 50% residual activity after fifty hours for the immobilised trypsin.

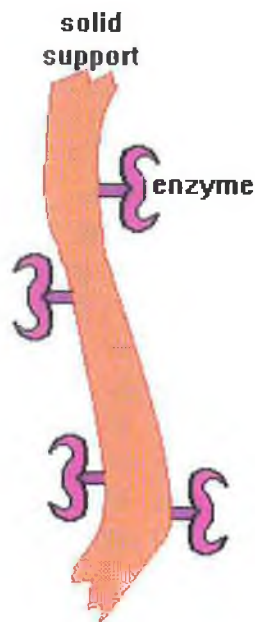


Figure 1.9: A diagram showing the binding of enzymes to water-insoluble carriers (Katchalski-Katzir, 1993).

Malmsten and Larsson (2000) also investigated this method of immobilisation. Trypsin was immobilised on porous glycidyl methacrylate (GMA-GDMA) beads due to covalent binding. It was best to modify the surface of the beads with a hydrophilic and uncharged polymer in order to increase specific activity after immobilisation. Native trypsin showed a specific activity (Mol. of BAEE/mg trypsin) of 3.6 compared to a specific activity of 14.6 following immobilisation onto hydrophilised GMA-GDMA beads coated with Dextran T40. Bahadur and Bahadur (1985) describe the immobilisation of trypsin on poly (methylmethacrylate-co-acrylic acid) latex. The native trypsin had a T_{50} of 38°C whereas the immobilised had a T_{50} of 50°C. Yasui et al. (1997) developed a method whereby they attached trypsin to latex particles using a PNIPAM (poly (N-isopropylacrylamide) spacer. Differential scanning calorimetry (DSC) revealed that this

method exhibited a drastic phase transition, and that the transition temperature was considerably elevated when the enzyme was immobilised.

Jiang et al. (2000) immobilised trypsin onto a fibre membrane surface by covalent binding. Glutaraldehyde was used as the linker between the enzyme and the membrane, where one aldehyde group from the glutaraldehyde reacts with an amine group from the hexyl diamine on the fibre and the other aldehyde group reacts with the amine of the trypsin. They then used a novel method of analysis, high-performance frontal analysis "to investigate the activity and reaction kinetics of the immobilised enzyme". High-performance frontal analysis is a procedure in which the sample liquid or gas is fed continuously into the chromatographic column with the result that only the least-sorbed compound, which moves at the fastest rate, is obtained in a pure state. They determined that the relative activity of the immobilised enzyme (versus the equivalent free enzyme) was 55.6%.

Ferreira et al. (2003) have looked at the immobilisation of alcalase 2T, a commercial preparation of subtilisin Carlsberg onto a silica support, where glutaraldehyde was used as the activating agent (Figure 1.10). They found that multipoint covalent attachment of the alcalase preparation enhanced the thermal, operational and storage stability of immobilised enzyme compared to the native. The immobilised form was stable at 50°C and showed minimal deactivation up to 2 hours compared to a 50% activity loss by the native over the same period of time.

Huang et al. (1997) immobilised trypsin onto the surfaces of two derivatives of Celite™. Firstly the Celite™ was derivatized with organosilane to give aminopropyl-Celite (APC) and then some of this derivative was succinylated to produce succinamidopropyl-Celite

(SAPC). They carried out studies on both the immobilised forms and then they were compared to native trypsin. The derivatives demonstrated a broader pH range for optimal activity than the native. Both immobilised forms showed 50% activity at a pH of 6.5 whereas the native showed the same activity at pH 7.0. Huang et al. (1997) argue that this shift in pH could be advantageous in bioprocesses e.g. limited proteolysis of cheese whey or milk could be performed without pH adjustment. The trypsin immobilised on the SAPC derivative showed 100% relative activity at 40°C compared to the native, which had only 65% activity at the same temperature.

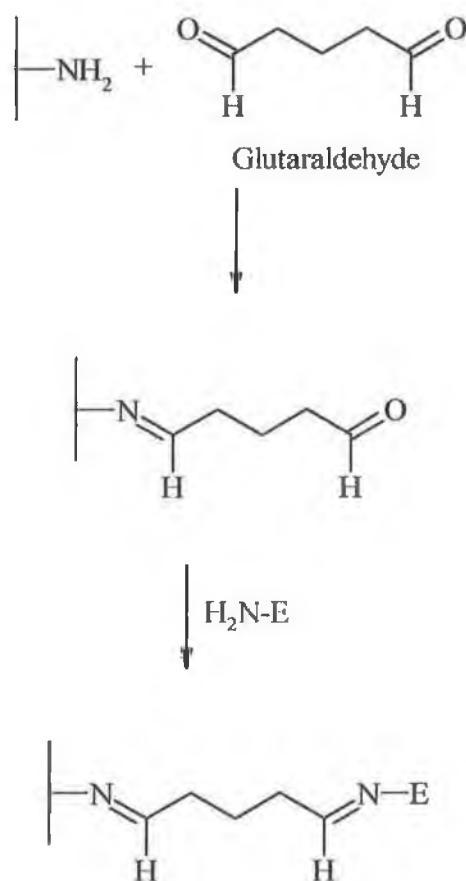


Figure 1.10: The stepwise reaction in the attachment of glutaraldehyde to a silica support followed by its attachment to the enzyme, alcalase 2T (Ferreira et al., 2003).

1.6.4.2 Cross-Linking: intermolecular cross-linking of enzymes by bi-functional or multi-functional reagents:

St. Clair and Navia (1992) developed cross-linked enzyme microcrystals (CLECs; these are microcrystals grown from aqueous solution and crosslinked with a bifunctional agent such as glutaraldehyde) that displayed high activities and stabilities in harsh conditions including high temperature, pH extremes and organic solvents (Figure 1.11). Their mechanical robustness, resistance to autolysis and ease of handling and reuse make them very attractive as industrial catalysts. Perischetti et al. (1995) developed CLECs of thermolysin, which showed an increased stability in nearly-anhydrous organic solvents, high temperatures and aqueous-organic solvent mixtures. Native thermolysin demonstrated 50% residual activity after 2 hours in DMF compared to the CLECs of thermolysin, which retained 100% activity after 5 days in DMF. These CLECs were used as catalysts for the synthesis of peptides.

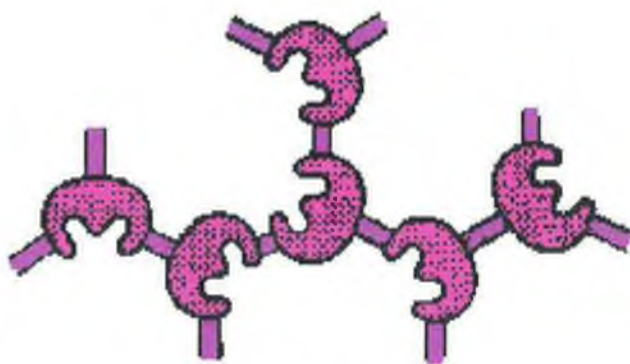


Figure 1.11: A schematic diagram showing the effect of crosslinking on an enzyme (Katchalski-Katzir, 1993).

Van Unen et al. (1998) discovered that CLECs of subtilisin Carlsberg showed increased catalytic activity by pre-treatment with crown ethers via a soaking and drying procedure.

No increase in activity was observed when the crown ether was added directly to the reaction solution. They determined the catalytic activity by observing the rate of peptide bond formation between N-acetyl-L-Phe-OC₂H₄Cl and L-Phe-NH₂. When subtilisin Carlsberg CLECs were added to an acetonitrile solution with 18-crown-6 and the solvent allowed to evaporate, the resulting enzyme crystals showed an approximately tenfold increase in the rate of synthesis of the dipeptide N α -L-Phe-Phe compared to using the 'ordinary' CLEC of subtilisin Carlsberg. There have been many reviews published on the stability of CLECs of subtilisin Carlsberg. An example is Wang et al. (1997) who showed that the enzyme retained full catalytic activity after seven days at 40°C in either 50% v/v aqueous acetonitrile or DMF or in 100% aqueous buffer at 60°C. The potential use of CLECs in the modification of the catalytic activity or specificity of enzymes has also been investigated. Häring and Schreier, 1999 synthesised CLECs of subtilisin, which they subsequently transformed into a semi synthetic peroxidase "seleno-subtilisin" (Figure 1.12 for the preparation). By introducing the element selenium into the enzyme, its original specificity was changed. This semi synthetic enzyme retained the stability characteristics of the CLEC of subtilisin Carlsberg.

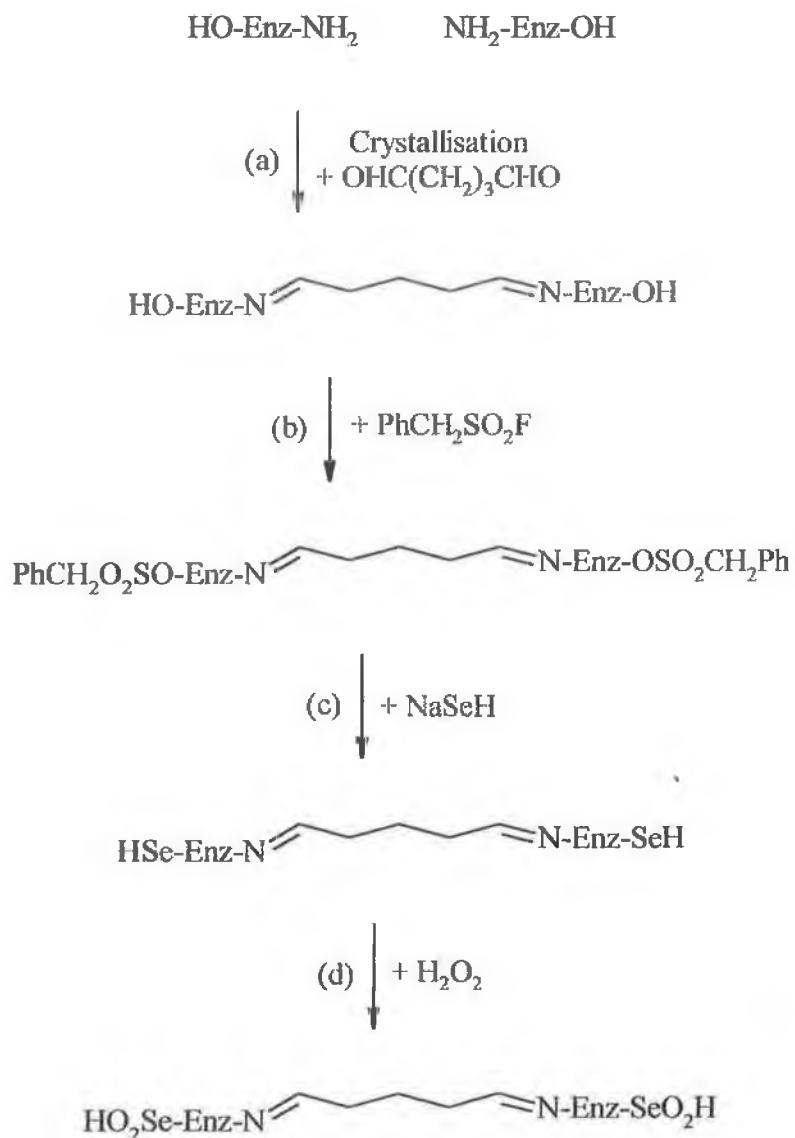


Figure 1.12: The preparation of CLECs seleno-subtilisin (Häring and Schreier, 1999). (a) Batch crystallisation in 12% (w/v) Na_2SO_4 at $16\text{-}18^\circ\text{C}$ was followed by treatment (45 minutes) with glutardialdehyde at $\text{pH}7.5$. (b) 1 hour in 0.11M PMSF at $\text{pH}7.0$. (c) Addition of aqueous sodium hydrogen selenide solution was followed by 45 hours stirring at 45°C . (d) 30 minutes in 10mM H_2O_2 . (Enz = enzyme, Enz - OH = Ser221 of subtilisin, Enz-NH₂ = lysine residues on the surface of the enzyme and Ph = phenyl).

1.6.4.3 Chemical aggregation

Another type of enzyme immobilisation is chemical aggregation with bifunctional or multifunctional agents, if high concentrations of crosslinking reagents are used insoluble aggregates of enzymes form. These enzyme aggregates contain both intramolecular and intermolecular cross-links in the protein molecule. Broun (1977) described a method for the production of a large number of insoluble enzyme aggregates using the bifunctional reagent glutaraldehyde. One advantage of chemical aggregation over the normal methods of immobilisation is that the volume of the reaction is greatly decreased. This is an important consideration for analytical or biomedical applications.

Rajput and Gupta (1988) described the formation of a coaggregate of trypsin and chymotrypsin by extensive cross-linking with glutaraldehyde. This trypsin-chymotrypsin insoluble coaggregate was found to contain both enzyme activities. A characteristic of the coaggregate was that the trypsin component showed a reduction in autolysis.

Tyagi et al. (1999) investigated the effect of heat and aqueous/organic cosolvents on four enzyme aggregates prepared with glutaraldehyde, i.e. polyphenol oxidase, acid phosphatase, β -glucosidase and trypsin. Each showed enhanced thermal stability and greater stability in both acetonitrile and dimethylformamide.

Khan et al. (1985) immobilised ammonium sulfate-precipitated porcine pepsin by chemical aggregation using glutaraldehyde. They hypothesised that electrostatic interaction takes place between the enzyme molecules during the formation of enzyme aggregates and the subsequent treatment with glutaraldehyde results in covalent bond formation. The immobilised enzyme showed 260% relative activity at 60°C compared to

180% for native pepsin in an aqueous buffer. The immobilised enzyme was also more stable to the denaturants urea and guanidine hydrochloride.

Khare et al. (1991) slightly modified the chemical aggregation technique. Initially they placed the enzyme (β -galactosidase, acid phosphatase or trypsin) into Sephadex beads of appropriate sizes and the extensive crosslinking of the enzyme was carried out using glutaraldehyde. The trypsin demonstrated no loss in activity when incubated at 45°C for 2 hours while the native lost 20% of its activity under the same conditions. Similar results were obtained for the other enzymes under investigation.

1.6.4.4 Entrapment: incorporating enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane:

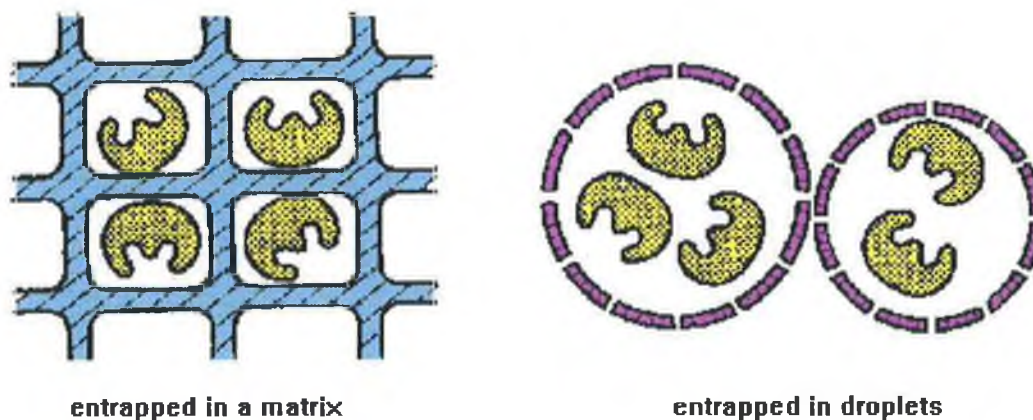


Figure 1.13: A diagram showing how enzymes can be trapped in a semi-permeable gel or how they can be enclosed in a semi-permeable polymer membrane (Katchalski-Katzir, 1993).

Chemical attachment and entrapment methods are the most frequently used techniques.

Mozhaev et al. (1983) developed a method of immobilisation which covalently incorporated an enzyme into polyacrylamide gel. The temperature of maximum activity

for α -chymotrypsin was increased by 25°C and for trypsin an increase of 30°C was determined following incorporation into the polyacrylamide gel. The advantage of this type of immobilization is that the native enzyme structure is kept intact and so its characteristic properties are maintained. Also, the matrix protects the enzyme from harsh experimental conditions. Reddy et al. (1986) immobilised trypsin onto alginic acid-poly(glycidyl methacrylate) graft copolymer (AAGMA) and noted increased thermostability. Immobilised trypsin had an "optimum" temperature of 35°C compared to 30°C for the native and showed activity up to a temperature of 75°C whereas the native retained activity only up to 60°C.

Tyagi et al. (1994) investigated the effect of chemically modifying trypsin by pyromellitic dianhydride (PMDA) before it was immobilised on DEAE-cellulose. It was possible to control the strength of binding by varying the extent of chemical modification. The immobilisation can be reversed by ion exchange chromatography.

Immobilised enzymes are often used in peptide synthesis (Andersen et al., 1991). Nilsson and Mosbach (1984) first immobilised α -chymotrypsin to tressyl chloride-activated Sepharose CL-4B. The enzyme was then used to synthesise Ac-Phe-Ala-NH₂ from Ac-Phe-O-Me and Ala-NH₂. With the immobilised enzyme, 97% of the reactants were converted to the peptide product.

Van Unen et al. (2001) investigated the effects of immobilisation of the three serine proteases, trypsin, subtilisin Carlsberg and α -chymotrypsin in a sol-gel matrix. Sol-gel matrixes are silicon oxide glasses, which are prepared by hydrolytic polymerisation of silicon alkoxide precursors. The activities of trypsin and subtilisin Carlsberg were increased by 437 and 31 times respectively, while α -chymotrypsin activity was forty

three times higher in the transesterification reaction of N-acetyl-L-phenylalanine ethyl ester with 1-propanol in cyclohexane. All three enzymes demonstrated 90% immobilisation.

1.6.5 Protein coupling to water-soluble polymers

One type of chemical modification which is used a lot to stabilise enzymes is the coupling of proteins to water-soluble polymers. Veronese (2001) wrote an extensive review of this subject. PEG is a polymer with the structure $(-\text{CH}_2\text{CH}_2\text{O}-)_n$. The use of many peptides and proteins as a treatment for diseases, e.g. cancer, has been investigated; PEG is the most widely used modifier of proteins with therapeutic potential. PEG is used as a modifier as disadvantages of using the biomolecules without modification i.e. the peptides and proteins are; the low immunogenicity of these products and also their low molecular weights cause them to be eliminated very rapidly from the body through renal excretion.

The advantages of PEG modified enzymes include:

- (1) Increasing the molecular weight of the protein thereby decreasing renal filtration in the body.
- (2) Ability to catalyse reverse hydrolysis, carried out in hydrophobic media
- (3) Increased catalysis of hydrophobic substrates
- (4) Stereospecific synthesis in hydrophobic media
- (5) Synthesis of compounds that are unstable in aqueous media
- (6) Increased thermostability in both hydrophobic and aqueous media.

Inada et al. (1995) used the proteinases - streptokinase, thrombin, trypsin and urokinase in PEG-protein complexes in the treatment of anti-thrombosis. The amphipatic polymer (has both hydrophilic and hydrophobic properties), polyethylene glycol (PEG) chemically modifies the protein by covalently attaching to proteins. Usually an activating agent (e.g. cyanuric chloride) acts as a linker between the PEG and protein. Several PEG molecules may be attached to one molecule of protein. Attachment of PEG allows the enzyme to become soluble in organic solvents, as the PEG confers its amphipatic properties onto the protein. This can be very useful for peptide synthesis. Sakurai et al. (1990) used PEG-trypsin to synthesise the dipeptides Bz-Arg-Ile-NH₂ and Bz-Arg-Phe-NH₂ with high yields (70-85%). PEG-trypsin had 23% of the total amino groups modified and it retained 71% of its original activity. Kwon (1999) modified subtilisin with PEG and AOT (Bis(2-ethylhexyl)sulfosuccinate sodium salt) and found that both modified subtilisins were more active than native subtilisin in organic solvents. For example in isooctane, native subtilisin had a k_{cat}/K_m value of $24 \pm 3 \text{ M}^{-1}\text{s}^{-1}$, PEG-subtilisin had a value of $26 \pm 4 \text{ M}^{-1}\text{s}^{-1}$ and AOT-subtilisin had a value of $517 \pm 20 \text{ M}^{-1}\text{s}^{-1}$. Zalipsky et al. (1992) converted a methoxypolyethylene glycol of molecular weight 5000 [$\text{CH}_3 (\text{-O-CH}_2\text{-CH}_2)_n\text{-OH}$] to a reactive succinimidyl carbonate form (SC-PEG). These PEG-trypsin conjugates had no proteolytic activity, well preserved esterolytic activity and also enhanced activity toward amidolytic substrates i.e. chromogenic p-nitroanilide substrates.

Many workers have used methoxypolyethylene glycol (MPEG) to modify proteins. . Yang et al. (1996) modified subtilisin with MPEG (methoxypolyethylene glycol) which had been previously activated by cyanuric chloride (Figure 1.14) or nitrophenol

carbonate (Figure 1.15) according to the method of DeSantis and Jones (1999) and found that the MPEG-subtilisin was more stable against extremes of temperature and pH. Differential scanning calorimetry was used to compare the thermostabilities of both the native and PEG-modified enzyme.

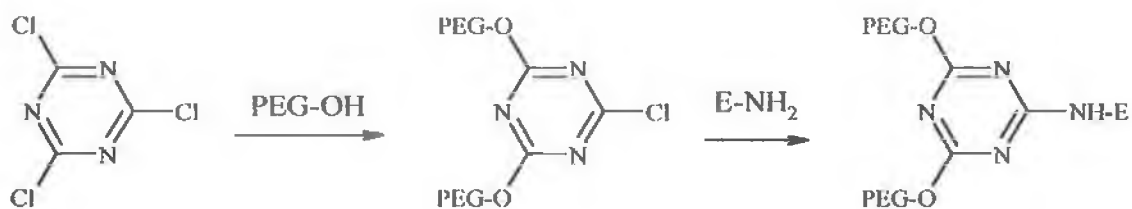


Figure 1.14: The enzyme (E) modification with PEG activated with cyanuric chloride (DeSantis and Jones, 1999).

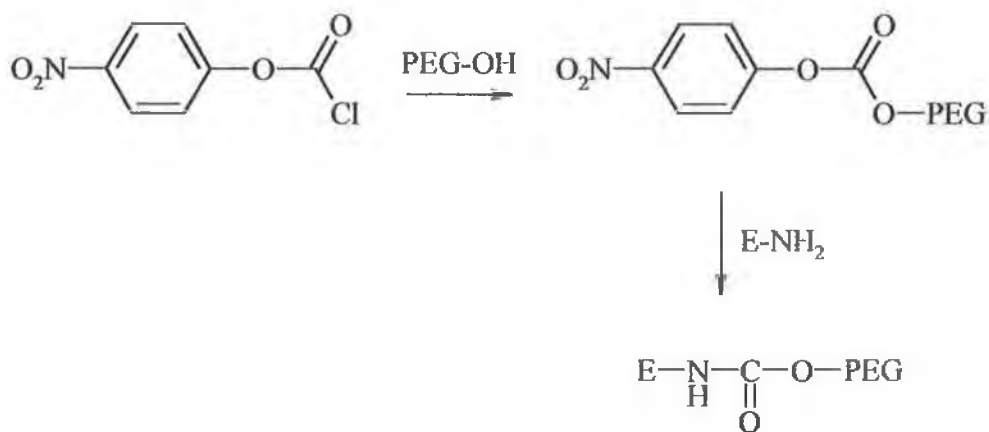


Figure 1.15: A schematic diagram of the reaction involved in the enzyme (E) modification with PEG activated with *p*-nitrophenyl chloroformate (DeSantis and Jones, 1999).

Zhang et al. (2001) modified trypsin with MPEG (methoxypolyethylene glycol) activated by *p*-nitrophenyl chloroformate (NPC) and found that MPEG trypsin was more thermal stable than native. MPEG-trypsin retained 50% residual activity after 43 minutes at 50°C whereas native trypsin activity decreased to 50% after only 22 minutes. He et al. (2000) set up a kinetic model for MPEG trypsin, which took thermal denaturation and autolysis into account. The model accurately described the thermal inactivation process of native and MPEG modified trypsin.

1.7 Protein Engineering

Protein engineering can be defined as the use of genetic and chemical techniques to change the structure and function of a protein. There are various protein engineering techniques, these include site-directed mutagenesis, random mutagenesis and directed evolution (including DNA shuffling). Sears and Wong, 1996 wrote a review of this area so it will be only briefly discussed here. The most significant method of protein engineering is site-directed mutagenesis. This is achieved through single-stranded DNA carrying the cloned sequence (normally produced using the bacteriophage M13) being hybridised to a synthetic oligonucleotide carrying the desired mutation (Ó'Fágáin, 1997).

Subtilisin has become one of the most widely used models for protein engineering. Bryan (2000) wrote a comprehensive review on this topic and so subtilisin will not be reviewed further here. He has concluded that the main reasons for modifying subtilisin to date have been to investigate the catalytic mechanism, substrate specificity, new

activities, general proteolytic activity, general stability, stability in exotic environments, surface activity and folding mechanisms of the enzyme.

Briand et al. (1997) described the oligonucleotide-directed mutagenesis of lysine 188 to histidine in trypsin. This method introduced a chelating site near the substrate-binding pocket of the trypsin. They showed that the addition of Cu^{2+} to the trypsin K188H caused a 30-100-fold increase in the K_m value for the substrate Suc-Ala-Ala-Pro-Arg-*pNA* while the k_{cat} value scarcely decreased.

Pouvreau et al. (1998) tried to modify the catalytic properties of trypsin by producing three different mutants. They substituted Lys-188 with an aromatic amino acid (Phe, Tyr or Trp). These mutants showed that, in β -casein hydrolysis, in addition to cleaving Arg and Lys residues new sites of cleavage appeared, involving glutamyl and asparaginyl residues. Chobert et al. (1998) found that, depending on the mutant, the optimum pH of activity changes. The mutant K188Y had the greatest activity at pH 10 with the substrate Suc-Ala-Ala-Pro-Arg-*pNA* whereas the native trypsin had the greatest activity at pH 8 with the same substrate.

Graf et al. (1987) investigated the effect on specificity of the conversion of Asp-189 to Lys by site-directed mutagenesis. Asp-189 is found at the base of the substrate-binding pocket of trypsin. This mutant showed no specificity towards basic substrates, i.e. arginyl and lysyl substrates; this was consistent with the presumed crucial role of Asp-189 in binding basic substrates. However, the mutant showed no compensatory specificity toward Asp or Glu residues on either natural or synthetic substrates. Briand et al. (1999) investigated the same characteristic of trypsin in a different way. They investigated the effect on the specificity of trypsin when there was an inversion of the

lysine-188 and aspartic acid-189 residues. The mutant K188D/D189K displayed a large increase in preference for arginine over lysine residues compared with native trypsin. The native trypsin had an Arg/Lys ratio (calculated as the ratio of k_{cat}/K_m values) of 5.9, whereas the mutant had an Arg/Lys ratio of 22.5.

A disadvantage of chemical modification is the lack of control with respect to the extent and regiochemistry of the reaction. The combination of site-directed mutagenesis with chemical modifications is a relatively new area of investigation. This method has been shown to be a rapid, controlled and versatile approach, which yields well-characterised homogenous products. It involves the insertion of a "handle" into a protein by site-directed mutagenesis, which can then be chemically modified to introduce a synthetic amino acid sidechain (De Santis and Jones, 1999a and 1999b). De Santis and Jones (1999a and 1999b) have used this approach to change the catalytic properties of subtilisin *Bacillus lentus* (SBL). Site-directed mutagenesis introduces a cysteine residue at a key active-site position in SBL; this is then chemically modified with methanethiosulfonate (MTS) reagents to give chemically modified mutant (CMMs) enzymes (Figure 1.16). Native SBL contains no cysteine residues so the modification of the cysteine occurs solely at the required site. Plettner et al. (1998) also used this approach to synthesise CMMs and they have developed the first rapid, microscale, combinatorial method for the modification of subtilisin cysteine mutants that evaluates their amidase and esterase activities. The amidase activity, using the substrate Suc-AAPF-pNA, for native subtilisin had a k_{cat}/K_m value of $75 \pm 5 \text{ s}^{-1}\text{mM}^{-1}$ whereas the CMMs showed a value of $113 \pm 18 \text{ s}^{-1}\text{mM}^{-1}$.

The introduction of charge into wild-type enzymes can be used to alter substrate specificity or catalytic activity. Davis et al. (1999) used various charged MTS reagents to chemically modify the cysteine thiols of SBL mutants N62C, S156C, S166C and L217C. All of these changes, however, led to reduced catalytic activity in the hydrolysis of the substrate Suc-AAPF-*p*NA.

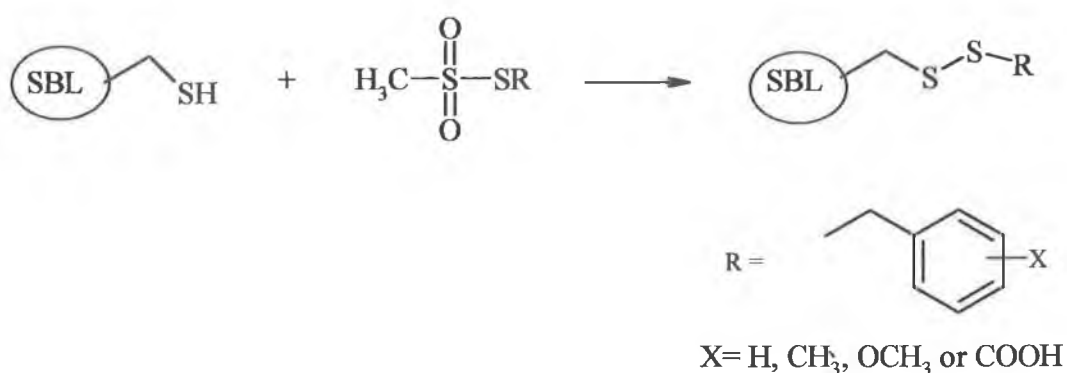


Figure 1.16: The chemical modification of the thiol of subtilisin Bacillus lentus (SBL) cysteine mutants by reaction with MTS reagents to synthesise chemically modified mutant enzymes (DeSantis and Jones, 1999).

Dickman and Jones (2000) also used various charged MTS reagents to chemically modify the cysteine thiols of SBL mutants. This was to investigate differences in catalytic activity between diastereomeric enzymes. Differences up to three fold were found between N62C-(R) and the wild type enzyme and the (R) diastereoisomer was 1.56 times more active than the (S) diastereoisomer. They used amidase and esterase kinetic assays using a low substrate approximation similar to the method of Plettner et al. (1998).

1.8 Conclusion

Proteases have a variety of uses. The enzymes discussed here are for the most part trypsin, subtilisin and a commercial form of subtilisin called Alcalase®. Trypsin has been used in the synthesis of biologically active peptides including a Hepatitis B antigen and in the semisynthesis and modification of proteins such as human insulin analogue (Gill et al., 1996). Alcalase® is a bacterial protease, which is used in the detergent industry to remove protein-based stains. It is also used in the food industry to improve the functional, nutritional and flavouring properties of proteins (milk protein modification) and in the textile industry for silk degumming (www.novozymes.com).

The use of proteases in peptide synthesis is an area of rapid growth and development. However, enzymes have some disadvantages in that they are easily denatured and inactivated and, therefore, lose their catalytic activity. The aim of this project is to make the enzymes trypsin, subtilisin Carlsberg and Alcalase® as stable as possible in regards to extremes of temperature and pH and then to use these modified forms in peptide synthesis. Subtilisins broad specificity for hydrophobic residues complements trypsins narrow specificity for basic amino acids i.e. Arg and Lys.

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipment

Labsystems Multiskan MS microplate reader

Unicam UV/Vis spectrophotometer, UV2

ALC Multispeed Centrifuge PK121.

Metrohm 718 pH-STAT titrino.

Varian HPLC system.

2.2 Materials

The suppliers of materials were as follows:

Sigma-Aldrich (Airton Road, Tallaght, Dublin 24, Ireland).

Hydrochloric acid (HCl), Tris- (hydroxymethyl) aminomethane (Tris), Casein, Bovine pancreatic trypsin, Porcine pancreatic trypsin, sodium hydroxide (NaOH), Acetic acid, dimethylsulphoxide (DMSO), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), thiobenzoyl benzoyloxycarbonyl-L-lysinate (Z-Lys-SBzl), 2-(N-Morpholino)ethanesulfonic acid (MES), calcium chloride (CaCl₂), N- α -p-Toluenesulfonyl-L-arginine methyl ester (TAME), p-Nitrophenyl p'-guanidinobenzoate HCl (NPGB), potassium dihydrogen orthophosphate (KH₂PO₄), Ethylene Glycol-bis(succinic acid N-hydroxysuccinimide ester) (EG-NHS), benzamidine, Sephadex™G-25, dichloromethane, oxalyl chloride, triethylamine, imidazole, L-Lysine methyl ester dihydrochloride, L-Arginine methyl ester dihydrochloride, 3-Carboxy-propionyl-(Ala)₂-Pro-Phe-p-Nitroanilide (SucAAPFp-Na), Subtilisin Carlsberg, Borax, N-trans-Cinnamoylimidazole (NTCl), Sodium acetate, Bradford reagent, L-Arginine methyl ester dihydrochloride, L-leucinamide,

N_{α} -Benzoyl-L-Arginine ethyl ester (BAEE), sodium tetraborate, triethylamine, N-hydroxysuccinimide (NHS), Gly-Gly-amide.

Bachem (UK) Ltd., (17K Westside Industrial Estate, Jackson Street, St. Helens, Merseyside, England).

Benzoyl-L-arginine p-nitroanilide (L-BAPNA), 3-Carboxy-propionyl-(Ala)₂-Pro-Phe-(sucAAPF-SBzl), Z-Tyr-OMe, Arg-ethyl ester, H-(Gly)₅-OH, H-Tyr-Gly-Gly-OH.

AGB Scientific Ltd/Fisher Chemicals (Slaney Close, Dublin Industrial Estate, Dublin 11, Ireland).

Dimethylformamide (DMF), acetonitrile (ACN), 1,4-Dioxan, acetone, tetrahydrofuran (THF), methanol, trifluoroacetic acid (TFA), piperidine, NaCl (sodium chloride).

BDH/Merck/Lennox (John Kennedy Drive, Naas Road, Dublin 12, Ireland).

Potassium chloride (KCl), Formic acid, sodium dihydrogen orthophosphate dihydrate (NaH₂PO₄.2H₂O), sodium barbitone, di-sodium hydrogen orthophosphate dihydrate (Na₂HPO₄.2H₂O), Tween®20.

Gift from Dr. Palle Schneider, Novo Nordisk (2880 Bagsvaerd, Denmark).

Alcalase®.

Pierce (Rockford, Illinois 61105, U.S.A).

Bicinchoninic acid (BCA) Protein Assay kit, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl).

2.3 Methods

2.3.1 General assays - Activity:

2.3.1.1 BCA™ Protein Assay

(Smith et al. 1985)

Principle:

A graph of the change in absorbance versus known concentrations of trypsin was set up using this Protein assay kit. The WR with the unknown protein concentration was then assayed and its concentration could be read off the standard graph.

Method:

The WR (working reagent) was made up by mixing 50 parts BCA Reagent A with 1 part of BCA Reagent B. The working reagent was mixed to give a clear green colour. Each standard or unknown sample (25µl) was put into the appropriate microplate wells. WR (200µl) was added to each well and the microplate was mixed for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. After this incubation, the plate was cooled to room temperature. The absorbance was measured at 560nm on a microplate reader.

2.3.1.2 Amidase Activity

(A)Trypsin

This method is based on the method developed by Erlanger et al. (1961).

Principle:

As a direct result of trypsin hydrolysis of BAPNA, *p*-nitroaniline, a coloured product, is released. This product can be estimated colorimetrically and the amidase activity of the enzyme can therefore be calculated ($\epsilon_{414} = 8581 \text{ M}^{-1}\text{cm}^{-1}$ for *p*-nitroaniline (Plettner et al., 1998)).

Method:

43.5mg of BAPNA were dissolved in 1ml DMSO. The solution was brought to 100ml with 0.05M Tris buffer, pH 8.2, containing 0.02M CaCl_2 . An enzyme stock solution of $0.045\text{mg}\cdot\text{ml}^{-1}$ in 1mM HCl was prepared. Water (0.45ml) and 2.5ml substrate (*L*-BAPNA) solution was added together and equilibrated in a waterbath at 30°C for 5 minutes. At time zero, 0.05ml of enzyme solution ($0.09\text{-}0.45\text{mg}\cdot\text{ml}^{-1}$) was added. The solution was mixed and reaction was run for 15 minutes. Acetic acid (0.5ml of 30% v/v) was added to terminate the reaction. A control containing no enzyme was also included. The absorbance was read at 405nm.

(B) Subtilisin\Alcalase®

This method has been based on that of Plettner et al., (1998)

Principle:

Subtilisin Carlsberg or Alcalase® hydrolysis of the tetrapeptide sucAAPF-pNa releases p-nitroaniline, a coloured product, which can be estimated colorimetrically. The amidase activity of the enzyme can therefore be calculated.

Method:

A stock solution of 0.16mM sucAAPF-pNa in 0.1M Tris\20mM CaCl₂ pH8.0 was prepared. A 0.01mg/ml enzyme solution in 0.1M NaH₂PO₄, which was adjusted to pH8.4 was also made up. 300µl of the substrate solution was transferred to a microplate. 25µl of the enzyme solution was then added. The reaction was allowed to proceed for one minute and the absorbance was read at 405nm. A blank containing no enzyme was also included.

2.3.1.3 Active site titration

(A) Trypsin:

This experiment was carried out after Walsh (1970).

Principle:

The active site titrant, NPGb, is mixed with the trypsin. The NPGb reacts with the active site of the trypsin (in particular the Ser of the Ser-His-Asp catalytic triad) to produce a coloured compound, p-Nitrophenol. The increase of absorbance multiplied by

6.025×10^{-5} gives the molarity of the p-nitrophenol (and therefore the molarity of active trypsin).

Method:

NaH_2PO_4 (acid, 1.56g) and 1.78 g of Na_2HPO_4 (base) were dissolved in 100ml H_2O . The pH was adjusted to 7.0 using 5M HCl. Trypsin (5mg) was added to 5ml 0.1M phosphate buffer. NPGb (1.7mg) was dissolved in 1ml DMF. NPGb solution (10 μ l) was added to 1ml trypsin solution. The absorbance was measured at 410nm every minute over 15 minutes, as the formation of the product causes a yellow colour, which can be observed spectrophotometrically. A control containing 1ml of 0.1M phosphate buffer was also measured. Readings were taken in triplicate.

(B) Subtilisin\Alcalase®:

This experiment was carried out after Zhong et al. (1991) and Bender et al (1966).

Principle:

The active site titrant, NTCL, is mixed with subtilisin\alcalase® and an “initial burst” (ΔA) is produced. This is due to the stoichiometric reaction of NTCL with the enzyme.

Method:

NTCL solution at a concentration of 1mg/ml in acetonitrile and 1mg/ml enzyme solution in 3mM KH_2PO_4 , 0.1M KCl, adjusted to pH8.2 using 5M NaOH were prepared. 975 μ l of 0.1M Tris solution, pH 7.0 containing 0.1M NaCl was transferred into a cuvette. 20 μ l of the substrate solution was added and the absorbance was measured at 335nm after one minute. 35 μ l of the enzyme solution was added to the cuvette and the change in

absorbance was recorded after 5 seconds. A control containing NTCL only was also measured. Readings were taken in triplicate.

2.3.1.4 pH Profile

Principle:

The pH-stat operates on the principle that H^+ ions are produced as the enzymatic reaction proceeds, which the pH electrode detects. As the pH of the sample drops due to the production of carboxyl groups by peptide bond cleavage, the pH-stat adds more NaOH into the solution to keep the pH constant. As a result of this, enzymatic activity can be monitored, as it is proportional to the amount of NaOH added during the reaction.

Method:

2.5g of Casein was dissolved in 100ml of 2mM Tris solution and brought to a temperature of 40°C and maintained at this temperature until the casein was dissolved. It was allowed to cool and adjusted to the required pH (varying from pH 6.3 to 10.8) using 1M HCl. A $1\text{mg}\cdot\text{ml}^{-1}$ trypsin stock solution was prepared in 1mM HCl; CO_2 was removed from the solution by bubbling nitrogen through it.

The following were added to the reaction vessel:

4ml casein substrate

2ml 0.1M KCl

2ml distilled water

200 μl of the trypsin stock solution was added. The enzyme activity was monitored using the pH-stat.

2.3.1.5 Casein digestion

Principle:

See section 2.3.1.4.

Method:

The casein was prepared as described in section 2.3.1.4. It was allowed to cool and adjusted to pH 8.1 using 5M HCl. A 1mg/ml enzyme stock solution in 3mM KH_2PO_4 0.1M KCl pH8.2. Serial dilutions of the substrate solution were then performed. The following were added to the reaction vessel:

4ml Casein substrate

2ml 0.1M KCl

2ml distilled water

The reaction solution was adjusted to pH 8.0 and 100 μ l of the enzyme solution was then added. The enzyme activity was monitored using the pH-stat.

2.3.1.6 Determination of the Arginine\Lysine ratio of trypsin

Principle:

See section 2.3.1.4

Method:

1M KCl, 1mM HCl and 20mM Tris-HCl solutions were prepared. Stock solutions of 0.1M L-Lysine methyl ester dihydrochloride and 0.2M L-Arginine methyl ester dihydrochloride in the 20mM Tris-HCl solution were made and the pH was adjusted to 8.0 using 5M NaOH. Serial dilutions of the two solutions were then performed. A 1mg/ml enzyme solution was prepared in the 1mM HCl solution.

The following were added to the reaction vessel:

4ml substrate solution

2ml 0.1M KCl

2ml distilled water

The reaction solution was adjusted to pH8.0, using 5M HCl and 100 μ l of the enzyme stock solution was added. The enzymatic activity was monitored using the pH-stat every minute for one hour. A control containing no enzyme was also included.

2.3.2 General assays - Stability:

2.3.2.1 Temperature profile

Principle:

The basis of this experiment is that an increase in temperature causes an increase in activity because of the increased kinetic energy (due to collisions between the molecules) in the solution. Aliquots of the enzyme solution were heated in 5°C increments up to a high temperature, the aliquots were chilled on ice and subsequently their amidase activity was recorded. A decrease in activity is observed as the temperature increases further due to thermal denaturation of the enzyme.

Method:

(A) Trypsin:

A stock enzyme solution of 4.5mg.ml⁻¹ in 3mM KH₂PO₄, 0.1M KCl adjusted to pH 8.2 with 5M NaOH was prepared. A blank containing KH₂PO₄ solution, adjusted to pH8.2, only was also prepared. Aliquots of the enzyme solution (70 μ l) were heated to temperatures between 30-80°C (in 5°C increments) for 10 minutes. The aliquots were

then placed on ice and assayed for residual activity using the amidase assay for trypsin in Section 2.3.1.2.

(B) Subtilisin Carlsberg\Alcalase®:

A stock enzyme solution of 0.01mg/ml in 0.1M Borate, 0.1M CaCl₂ solution adjusted to pH8.0 with 5M HCl was prepared. A blank containing the borate buffer only was also prepared. Aliquots of the enzyme solution (70µl) were heated to temperatures between 30-90°C (in 5°C increments) for 10 minutes. The aliquots were then placed on ice and assayed for residual activity using the amidase activity assay for subtilisin\alcalase® in Section 2.3.1.2.

Note:

The experiment was also carried out using 0.1M Borate, 0.1M CaCl₂ solution adjusted to pH9.0 with 5M HCl instead of the 0.1M Borate, 0.1M CaCl₂ solution adjusted to pH8.0.

2.3.2.2 Thermoinactivation of the enzyme:

Principle:

The basis of this experiment is that the enzyme is maintained at a high temperature and at certain time intervals, aliquots are removed from the solution and the amidase activity of the enzyme is monitored. Over time, it is observed that its activity decreases as thermal denaturation occurs.

Method:

(A) Trypsin:

An enzyme stock solution of $4.5\text{mg}\cdot\text{ml}^{-1}$ in $3\text{mM KH}_2\text{PO}_4$, 0.1M KCl adjusted to pH 8.2 using 5M NaOH was prepared. The enzyme solution was heated at 55°C and a blank solution containing the phosphate solution only was heated. $50\mu\text{l}$ aliquots were taken out at suitable intervals. The aliquots were kept on ice and then assayed for residual activity using the amidase assay for trypsin described in Section 2.3.1.2.

(B) Subtilisin Carlsberg/Alcalase®:

An enzyme stock solution of 0.01mg/ml in 0.1M Borate , 0.1M CaCl_2 pH9.0 was prepared. The enzyme solution was heated at 75°C and a blank solution containing the borate solution only was heated. $50\mu\text{l}$ aliquots were taken out at suitable intervals. The aliquots were kept on ice and then assayed for residual activity using the amidase activity assay for subtilisin Carlsberg\alcalase® described in Section 2.3.1.2.

2.3.2.3 Autolysis Assay:

This experiment is based on the methods of Bradford (1976) and of Bickerstaff and Zhou (1993).

Principle:

The Bradford dye binds to the protein, which causes an increase in the absorbance seen at 595nm . The absorption maximum of the dye alone is at 465nm and this will be seen to decrease as the dye binds to the protein and the absorbance at 595nm increases. As the enzyme starts to degrade (auto-digest) the absorbance at 595nm decreases as there will be less protein bound to the dye.

Method:

A 0.1mg/ml enzyme stock solution was prepared in 0.2M NaH₂PO₄, pH7.0. 5µl of the enzyme solution was transferred into a microtiter plate and 250µl of the ready-to-use Bradford reagent was added into the well. The plate was mixed gently on the multiplate reader and the absorbance was read at 620nm (against blank) every hour for five hours.

2.3.2.4 Organotolerance

Principle:

The aim of this experiment was to investigate at what concentration the enzyme loses its catalytic activity and becomes deactivated. In various concentrations of organic solvent, it is observed that the enzyme activity decreases as denaturation occurs.

Method:

(A) Trypsin:

Native or EG-NHS-modified trypsin (0.05mg.ml⁻¹), as described in section 2.3.3.1, in aqueous (3mM KH₂PO₄, 0.1M KCl adjusted to pH 8.2, using 5M NaOH)/organic mixture in the range 0-90% (v/v) organic solvent was incubated at 30°C for 1 hour. The organic solvents investigated were acetonitrile, 1,4-dioxan, acetone, DMF, DMSO and THF.

Residual activity was measured, as described in Section 2.3.1.2. Thermoinactivation at 55°C was then carried out on the 30% (v/v) aqueous acetonitrile as detailed in section 2.3.2.2. Kinetic experiments were also carried out on the 30% solution using a 0.225mg.ml⁻¹ trypsin stock solution (Section 2.3.4.1 and Section 2.3.4.2).

For native trypsin, the stock solution was used for determination of the amidase activity (Section 2.3.4.1) and a 1/6 dilution of the stock solution for the esterase activity (Section 2.3.4.2).

For the EG-modified trypsin, a 1/16 dilution of the enzyme solution was performed for the esterase activity (Section 2.3.4.2) and a 1/2 dilution of the enzyme solution for the amidase activity (Section 2.3.4.1).

(B) Subtilisin Carlsberg\Alcalase®:

Native or EG-NHS-modified enzyme ($0.1\text{mg}\cdot\text{ml}^{-1}$) in aqueous ($0.1\text{M KH}_2\text{PO}_4$ adjusted to pH 8.4, using 5M NaOH)/organic solvent mixture in the range 0-90% (v/v) solvent was incubated at 30°C for 1 hour. The organic solvents used were Acetone, Acetonitrile, 1,4-Dioxan, DMF, DMSO and THF.

Residual activity was measured for each solution, as described in section 2.3.1.2.

2.3.3 Chemical modifications

2.3.3.1 Crosslinking of porcine trypsin with EG-NHS

This method was based on that described by Murphy (1996).

Principle:

The aim of this experiment was to crosslink the lysine residues in trypsin using the homobifunctional reagent EG-NHS. These crosslinkers act like a clamp, making it difficult for the enzyme to unfold and therefore the modified enzyme should be more stable to extremes of temperature and pH.

Method:

(A) Trypsin:

A solution of 3mM KH_2PO_4 , 0.1M KCl, adjusted to pH8.2 using 5M NaOH and containing 3mM benzamidine was prepared. Trypsin (5mg) was dissolved in 2.375ml of the phosphate solution. EG-NHS (5mg) was dissolved in 125 μl DMSO. The EG-NHS solution was added slowly, with stirring, to the enzyme solution. Stirring was maintained for 20 minutes at room temperature. The reaction was terminated by SephadexTMG -25 gel filtration as described below.

Kinetic experiments were then performed on the modified enzyme using a trypsin concentration of 0.225mg/ml for amidase activity (Section 2.3.4.1) and a 1/18 dilution of this stock solution for the esterase activity (Section 2.3.4.2).

Some of the modified enzyme was concentrated down to 0.5ml using centricon tubes at 3000rpm in a ALC Multispeed Centrifuge PK 121 for 15 minutes. This concentrated enzyme solution then underwent formylation according to Section 2.3.3.2.

(B) Subtilisin Carlsberg\Alcalase@:

A solution of 3mM KH_2PO_4 , 0.1M KCl, adjusted to pH 8.2 was prepared. Enzyme (5mg) was dissolved in 2.375ml of the phosphate solution. EG-NHS (5mg) was dissolved in 125 μl DMSO. The EG-NHS solution was added slowly, with stirring, to the enzyme solution. Stirring was maintained for 20minutes at room temperature. The reaction was terminated by SephadexTMG-25 filtration as described below.

Sephadex™ G-25 gel filtration.

(Helmerhost and Stokes, 1980).

Sephadex™G-25 (10g) was swollen in 100ml of 3mM KH₂PO₄, 0.1M KCl adjusted to pH 8.2 using 5M NaOH. The mixture was stirred slowly overnight. Slurry (20ml) was poured into a 10ml column. The buffer was centrifuged out at 1800 rpm in a ALC Multispeed Centrifuge PK 121 for 3 minutes. Modified enzyme solution (2ml) was added to the semi-dry Sephadex and centrifuged at 1800 rpm for 3 minutes.

2.3.3.2 Formylation of the tryptophan residues in trypsin

Combining the methods of Coletti-Previero et al. (1969), Kitagawa et al. (1994) and Villeneuve and Chan (1997) this method was developed.

Principle:

The aim of this experiment was to chemically modify trypsin so that it demonstrates esterase activity but no amidase activity. A high esterase/amidase ratio is very important in peptide synthesis. The tryptophan residues in the trypsin are modified by the N-formyl-imidazole, which had been prepared *in situ* (Figure 6.1).

Method:

Oxalyl chloride (170µl) dissolved in 10ml dichloromethane was added dropwise to an ice-cold, stirred solution of 0.42g imidazole, 0.4g triethylamine and 75µl formic acid in 20ml dichloromethane. This solution was stirred at room temperature for 15 minutes. Then, 0.04g trypsin dissolved in 40 ml dichloromethane was added slowly to the stirring solution. The reaction was left stirring for 90 minutes at room temperature. The solution

was then put on ice and a white precipitate of imidazole hydrochloride formed. The residue was filtered off and the filtrate was left overnight, to allow the solvent to evaporate. Then, 0.005g/ml of the formylated trypsin was dissolved in 8M urea pH 4.0 and a UVVisible scan was performed between 200-800nm. A scan of native trypsin was also performed for comparison. Kinetic experiments (Section 2.3.4.1 and 2.3.4.2 for trypsin) using a concentration of 22.5mg/ml of the formylated trypsin and thermoinactivation (Section 2.3.2.2) experiments were carried out.

2.3.3.3 Deformylation of the l-formyltryptophan residues in trypsin

Principal:

Coletti-Previero et al. (1969) showed that the amidase activity of the formylated trypsin could be returned if the modified enzyme was maintained at a certain pH, i.e. pH 9.5 for approximately sixty minutes using a pH-stat. The esterase activity would also be retained in the deformylated trypsin.

Method:

The formylated trypsin was dissolved in 8M urea adjusted to pH 9.5 at a concentration of 1mg.ml⁻¹. The pH was maintained at 9.5 by the release of 0.1M NaOH in a pH-stat. Samples (1ml) were withdrawn from the reaction solution every 15 minutes for 200 minutes and diluted with acidic 8M urea to a pH of 4.0. The solution was made up to a total volume of 2ml with distilled water and a UV-visible spectrum was scanned from 200-800nm.

2.3.4 Kinetic Studies

2.3.4.1 Amidase Activity

These methods were developed using the technique described by Plettner et al. (1998).

Principle:

Trypsin hydrolysis of BAPNA or subtilisin Carlsberg/Alcalase® hydrolysis of the tetrapeptide sucAAPF-*p*Na, releases *p*-nitroaniline a coloured product, which can be estimated colorimetrically. When the substrate concentrations are kept to a minimum the rate of product formation increases linearly with the substrate concentration. The value for the change in absorbance (the greatest value before the graph started to lose its linearity) was then used to calculate k_{cat}/K_m .

Method:

A stock solution of 2.25mg porcine trypsin in 10ml of 5mM MES/2mM CaCl₂, pH 6.5 was prepared. A substrate stock solution of 2mM L-BAPNA in DMSO was prepared. A 0.1M Tris-HCl solution with 0.005% v/v Tween®20, pH 8.6 was prepared. A microtitre plate with 60µl of the enzyme solution in the rows A-G was set up, while in row H there was MES buffer alone. This was labelled the "loading plate". A second microtitre plate with 180µl Tris solution and 25µl of the L-BAPNA solution was dispensed. This plate was labelled the "assay plate" (Figure 2.1). Enzyme solution (25µl) was transferred with an 8-channel pipette from the loading plate into each well on the assay plate. The absorbance was read at 405nm on a multiplate reader. It was read every 5 seconds over two minutes.

(B) Subtilisin Carlsberg\Alcalase®:

Method:

A 0.5mM sucAAPF-pNa solution was prepared in 0.1M Tris-HCl \ 20mM CaCl₂, pH8.0.

A microtitre plate with 50µl of the enzyme solution (0.001mg/ml solution of enzyme was prepared in 0.1M NaH₂PO₄, pH8.4) in the rows A-G was set up, while in row H there was only 50µl of 0.1M NaH₂PO₄, pH8.4. This was labelled the "loading plate". A second microtitre plate with 300µl of the substrate was set up. This plate was labelled the "assay plate" (Figure 2.1). Enzyme solution (25µl) was transferred from the loading plate into each well on the assay plate. The absorbance was read at 405nm on a multiplate reader. It was read every 30 seconds over five minutes.

2.3.4.2 Esterase activity

These methods were also developed using the method described by Plettner et al. (1998).

(A) Trypsin:

Principle:

Esterase activity was obtained using the thiobenzyl ester substrates, Z-Lys-SBzl for trypsin or sucAAPF-SBn for subtilisin Carlsberg/alcalase®. Both ester substrates lack a chromogenic leaving group, so they are used in conjunction with DTNB (5,5'-dithiobis-2, 2'-nitrobenzoate), which forms a coloured thiophenolate ion with benzyl mercaptan when hydrolysis of Z-Lys-SBzl by trypsin or sucAAPF-SBn by subtilisin Carlsberg/alcalase® occurs. When the substrate concentrations are kept to a minimum, the rate of product formation increases linearly with the substrate concentration. The

value for the change in absorbance (the greatest value before the graph started to lose its linearity) was then used to calculate k_{cat}/K_m .

Method:

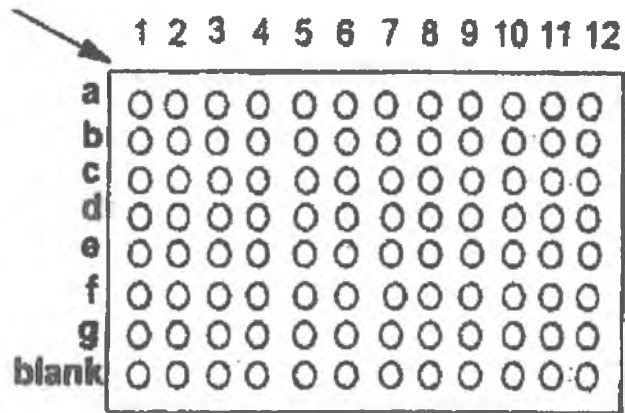
A solution of 0.1M Tris-HCl, pH 8.6, with 0.005% v/v Tween®20 and 0.375mM DTNB was made up. An enzyme stock solution of 2.25mg porcine trypsin in 10ml 5mM MES with 2mM CaCl₂, pH 6.5, was prepared. A 1/12 dilution of the enzyme was prepared. A Z-Lys-SBzl stock solution of 0.6mM in DMSO was prepared. Tris buffer (180µl) and 25µl Z-Lys-SBzl solution were placed into each well of the assay plate. Diluted enzyme solution (40µl) was placed into wells A-G of the loading plate, while MES buffer only was put into row H to act as the blank. Enzyme solution (25µl) was transferred into each well on the assay plate using a multi-channel pipette (Figure 2.1). The absorbance was read at 405nm on a microplate reader. It was read every 5 seconds over one minute.

(B) Subtilisin Carlsberg \ Alcalase®:

Method:

A stock solution of 1mM sucAAPF-SBn in 0.1M NaH₂PO₄ containing 0.375mM DTNB, pH7.2, was made up. A 0.001mg/ml enzyme solution in 0.1M NaH₂PO₄, pH8.4 was prepared. 180µl NaH₂PO₄ solution, pH8.0 and 25µl of sucAAPF-SBn solution were placed into each well of the assay plate. Enzyme solution (40µl) was placed into wells A-G of the loading plate, while 0.1M NaH₂PO₄, pH8.4, solution only was put into row H to act as the blank. Enzyme solution (25µl) was transferred into each well on the assay plate using a multi-channel pipette (Figure 2.1). The absorbance was read at 405nm on a microplate reader. It was read every 5 seconds over one minute.

Loading plate:



Transfer 25 μ l of the enzyme solution from the loading plate into each well on the assay plate.

Assay plate:

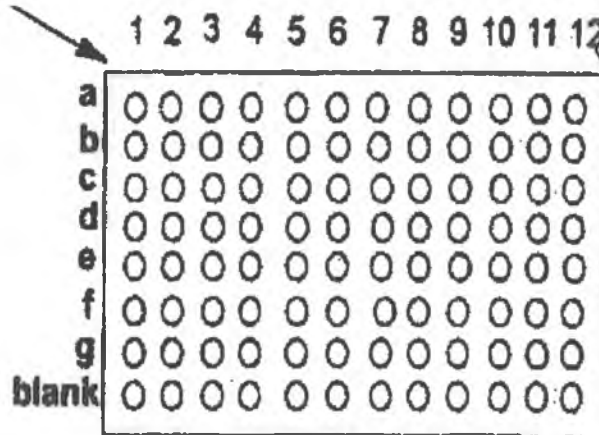


Figure 2.17: A schematic diagram of the set up involved in the amidase and esterase activity assays (Plettner et al. 1998).

2.3.5 Peptide synthesis

2.3.5.1 Model dipeptide synthesis of Bz-Arg-Leu-NH₂:

2.3.5.1.1 Effect of time on rate of peptide synthesis

This method was developed using the methods of Cerovsky (1990) and Blanco et al. (1991).

Principle:

Proteolytic enzymes hydrolyse peptide bonds; however, under certain conditions they can catalyse peptide bond formation. Peptide bond synthesis occurs when the rate of hydrolysis is reduced relative to the rate of aminolysis. Using this principle, the synthesis of the dipeptide Bz-Arg-Leu-NH₂ was attempted catalysed by native trypsin and trypsin that was crosslinked with EG-NHS.

Method:

0.2M leucinamide (33.5mg) and 0.1M BAEE (34.5mg) were dissolved in 950 μ l of 0.1M borate buffer pH9.0. 190 μ l of reaction mixture was removed and equilibrated at 4°C for ten minutes. 10 μ l of enzyme (1mg/ml) was added to the reaction mixture and incubated at 4°C. At regular time intervals 20 μ l aliquots were removed, added to 0.5ml of 50% (v/v) methanol in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC. Analysis was carried out on a Varian HPLC system with a Prostar 410 autosampler, Star 9050 UV\Vis Detector and a Hypersil C18 column (250mm x 4.6mm); flow rate 1ml min⁻¹, detection at 204nm and 230nm, mobile phase 50% (v/v) aqueous

methanol with 0.05% aqueous trifluoroacetic acid. Samples (50µl) were injected onto the column using the autosampler.

2.3.5.1.2 Effect of acetonitrile on the synthesis of Bz-Arg-Leu-NH₂

Principle:

The aim of this experiment was to determine the effect of 30% (v/v) acetonitrile in aqueous solution on the rate of synthesis of Bz-Arg-Leu-NH₂.

Method:

0.2M leucinamide (33.5mg) and 0.1M BAEE (34.5mg) were dissolved in 950µl of 30% (v/v) acetonitrile in 0.1M borate buffer pH9.0. 45µl of triethylamine was added to ensure the reaction solution was alkaline. 190µl of reaction mixture was removed and equilibrated at 4°C for ten minutes.

The reaction was then carried out as described in section 2.3.5.1.1

2.3.5.2 Model peptide synthesis of Tyr-Gly-Gly:

2.3.5.2.1 Effect of time on the rate of peptide synthesis

This method was developed using the methods of Klein et al. (2000) and Chen et al. (1991).

Principle:

The synthesis of the tripeptide Tyr-Gly-Gly catalysed by subtilisin Carlsberg and alcalase® was attempted.

Method:

0.2mM Gly-Gly-NH₂ was prepared in 0.2M sodium barbitone, pH 9.5. 0.1M Z-Tyr-OMe was prepared in DMF and equilibrated at -15°C for 10 minutes. An enzyme solution of 1mg/ml was prepared in 3mM KH₂PO₄, 0.1M KCl pH 8.2. 250µl Z-Tyr-OMe solution was mixed with 245µl Gly-Gly-NH₂. 5M aqueous triethylamine (5µl) was added to the reaction mixture. Then, 5µl of enzyme solution was added to the reaction mixture and the solution incubated at 4°C. At regular time intervals, 50µl aliquots were removed, added to 375µl of 50% (v/v) aqueous acetonitrile and analysed by HPLC. Analysis was carried out on a Varian HPLC system with a Prostar 410 autosampler, Star 9050 UV\Vis Detector and a Hypersil C18 column (250mm x 4.6mm); flow rate 1ml min⁻¹, detection at 280nm, mobile phase 50% (v/v) aqueous acetonitrile. Samples (50µl) were injected onto the column using the autosampler. The substrates and products were compared to their analogous standards.

2.3.5.2.2 Effect of enzyme concentration on peptide synthesis

Principle:

The aim of this experiment was to determine at what enzyme concentration the rate of synthesis of Tyr-Gly-Gly-NH₂ was maximal.

Method:

The reaction solution was prepared as described in Section 2.3.5.2.1 and the solution was incubated at 4°C after the enzyme solution (concentration either 1mg/ml or 2mg/ml)

had been added. At regular intervals, 50µl aliquots were removed, added to 375µl of 50% (v/v) aqueous acetonitrile and analysed by HPLC as described in Section 2.3.5.2.1.

2.3.5.2.3 Effect of temperature on Tyr-Gly-Gly synthesis

Principle:

The aim of this experiment was to determine at what temperature the rate of synthesis of Tyr-Gly-Gly-NH₂ was maximal.

Method:

The reaction solution was prepared as described in Section 2.3.5.2.1 and the solution was incubated at the following temperatures after the enzyme had been added: 4, 25, 37 and 50°C. At regular intervals, 50µl aliquots were removed, added to 375µl of 50% (v/v) aqueous acetonitrile and analysed by HPLC as described in Section 2.3.5.2.1.

CHAPTER 3

STUDIES on NATIVE and MODIFIED TRYPSIN

3.1 Introduction

As porcine trypsin was the focus of this study, a few basic experiments were initially performed. The optimum pH of the enzyme was determined using a pH-stat. This was obtained by observing the proteolytic activity of the enzyme on casein at various pH values.

The amidase activity of the enzyme was monitored using BAPNA as the substrate. A change in colour of the reaction mixture from colourless to yellow indicates trypsin activity. The intensity of the yellow colour is proportional to the concentration of enzyme present (Erlanger et al., 1961).

The specific three-dimensional structure of the active site of an enzyme is what determines the catalytic efficiency of it. Therefore when an enzyme undergoes autolysis (self-digestion) it will retain some of its activity as long as the active site structure is kept intact. Trypsin undergoes autolysis (self-digestion) during all experimental conditions and has a high specificity for hydrolysing peptide bonds adjacent to basic amino acids. So the rate of porcine trypsin autolysis was investigated by recording the enzyme activity lost over a period of time (section 2.3.2.3).

In terms of stabilisation most research has been carried out on proteases susceptible to autolysis e.g. trypsin. The rate of autolysis increases with temperature making such enzymes quite thermoliable and so if they are to have an industrial use they must be modified to remain active at higher temperatures. The modification of free amino groups of trypsin reduces the number of susceptible bonds, effectively decreasing the rate of autolysis. With this in mind, both porcine and bovine trypsin, were treated with the homobifunctional compound EG-NHS (see Figure 3.18). Previous work in this group

had been carried out on the modification of bovine trypsin with acetic acid N-hydroxy-succinimide that neutralises the positive charge on the lysine residues (Murphy and Ó'Fágáin, 1996b). A modified form of trypsin was synthesised which had a T_m value of 51°C compared to 46°C for the native. The half-life of this AA-NHS trypsin at 55°C was double that of the native, 8.7 minutes compared to 4.3 minutes. They then went on to investigate the effect of crosslinking lysine residues with EG-NHS rather than just modifying them with AA-NHS. They found that the EG-trypsin showed an even greater stability to thermal denaturation with the half-life of the modified form being five times greater than the native (Murphy, 1996). It is well known that porcine trypsin is more stable than bovine trypsin and is active down to relatively low pH values (Walsh and Wilcox, 1970; Rose 2000). Therefore this particular type of crosslinking was chosen to investigate whether an even more stable modified enzyme would be possible and to take the reasearch started by Murphy (1996) further.

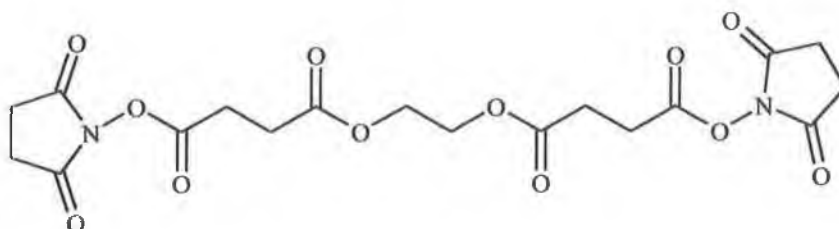


Figure 3.18: Structure of Ethylene glycol-bis (succinic acid N-hydroxy-succinimide ester)

EG-NHS is a homobifunctional crosslinking reagent, i.e. it is a reagent that contains two identical reactive groups, thereby providing a means of covalently linking two target groups. EG-NHS in a sense behaves like a monofunctional reagent as only one reactive group of the EG-NHS reacts with a single functional group of the protein. In this case, the target groups are the lysine residues of trypsin. The maximum molecular linkage

length of EG-NHS is 16 Å (Murphy, 1996). The successful formation of crosslinks is dependent on the target groups of the protein being within the "span" of the crosslinking reagent. Both bovine and porcine trypsin, were reacted with EG-NHS to compare the effects of cross-linking on the same enzyme from different sources. Porcine trypsin contained 11 lysine residues while bovine contains 15 lysine residues. A number of experiments were carried out to distinguish the differences.

A temperature profile was determined. The basis for this experiment is that an increase in temperature causes an increase in activity due to the increased kinetic energy of the molecules in solution. Further collisions occur and this produces more kinetic energy. As the temperature increases further, a decrease in activity is recorded. This is due to thermal denaturation (Section 1.6). The denatured state of a protein isn't a single fixed state. It is a collection of states of very similar energies that are in a very rapid equilibrium with one another. This collection of states is very sensitive to its physical and chemical environment. For each set temperature, residual amidase activity was measured. The half-inactivation temperature T_{50} , where observed activity is 50% maximal, was calculated.

A thermoinactivation study was carried out at 55°C for both trypsins. Trypsin was incubated at 55°C for 80 minutes and the percentage residual amidase activity of samples removed at certain time intervals was recorded.

The effect of various concentrations of different organic solvents on both the native and modified forms of trypsin was assessed with regard to enzyme stability and kinetics. The relationship between enzyme hydration and catalytic activity is well known. Gorman and Dordick (1992) suggested that organic solvents remove the enzyme-bound water in

a process known as "water stripping". However, Fitzpatrick et al. (1993) have shown that the protein retains some of the enzyme-bound water when it is placed in a water-miscible organic solvent. There is a fine balance between the complete deactivation of an enzyme and an increase in catalytic activity. This is due to the dual effect of such a mixture, the enzyme wanting to denature because of the organic component and the aqueous phase allowing it to succumb to denaturation.

The preference of native and modified trypsin for arginine versus lysine residues was also investigated, as the enzyme is known for its narrow specificity toward the bond on the carboxyl side of both of these amino acids (Walsh and Wilcox, 1970). The substrates used were L-Methyl esters of the two amino acids and the catalytic efficiency of the enzyme was measured using a pH-stat.

Generally the hydrolysis of casein is measured by monitoring the formation of aromatic amino acid fragments; observing the absorption at 280nm following TCA precipitation of the intact protein allows this (Rick 1974). Results can be inaccurate as not all the casein fragments contain aromatic amino acids (Reimerdes and Klostermeyer 1974). Therefore, in order to compare and contrast the proteolytic activity of native and modified trypsin, a casein digestion assay using a pH-stat was performed. As the enzyme hydrolysed the casein, the pH of the solution lowered and so the pH-stat added 0.1M NaOH to maintain the pH at 8.0. The enzymatic activity can then be monitored, as it is proportional to the amount of NaOH added during the reaction.

3.2 Results:

3.2.1 Native trypsin.

3.2.1.1 pH-Activity profile.

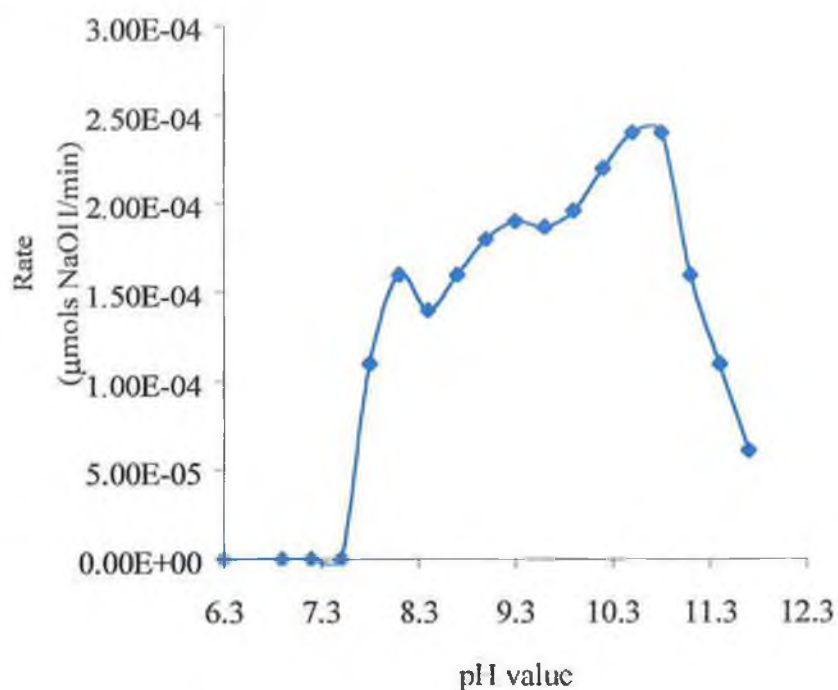


Figure 3.19: pH-Activity profile of porcine trypsin.

The proteolytic activity of trypsin on casein at various pH values was investigated to determine the pH optimum of the enzyme. As can be seen from Figure 3.19, the optimum pH for porcine trypsin was found to be between 7.8 and 10.8. No activity was observed below pH 7.8.

3.2.1.2. Autolysis:

As can be seen from Figure 3.20, the native trypsin had lost 50% of its residual activity after one hour at these particular experimental conditions (Section 2.3.2.3) due to autolysis.

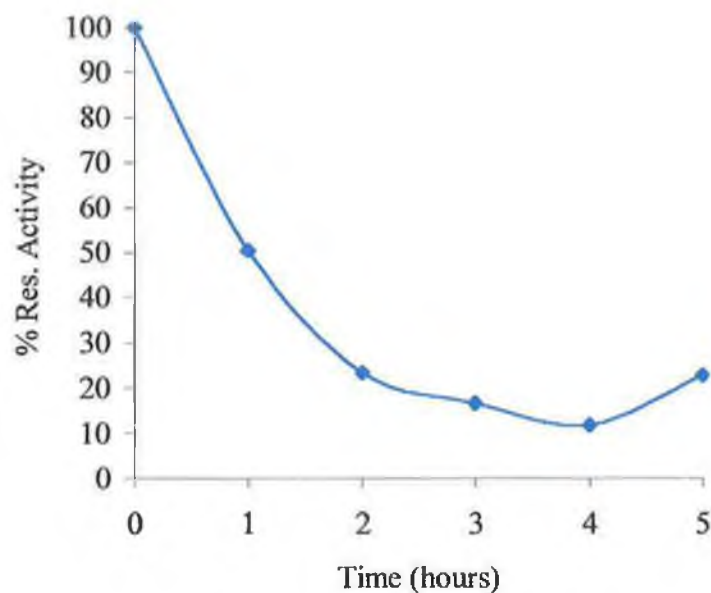


Figure 3.20: Autolysis of trypsin over time.

3.2.1.3 Trypsin microassay.

The assay used is described in Section 2.3.1.2. The assay was optimised with respect to enzyme, substrate concentration and time. The concentration of trypsin versus the rate of hydrolysis of L -BAPNA at a concentration of 1.67mM shows a linear relationship up to $18\mu\text{g enzyme ml}^{-1}$ (Figure 3.21). The optimum time was determined to be ten minutes.

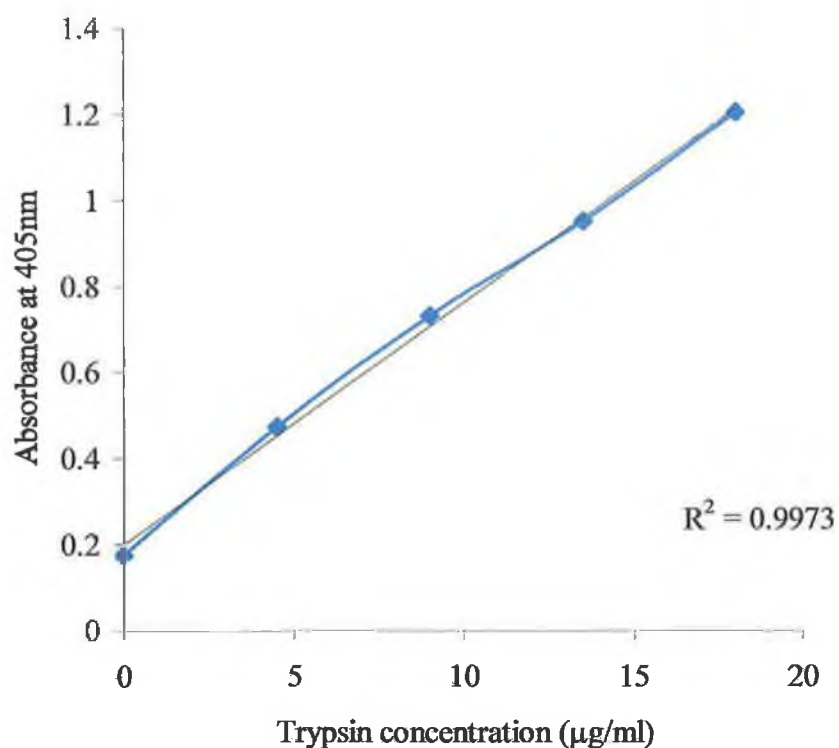


Figure 3.21: A graph of the concentration versus absorbance for native porcine trypsin using L-BAPNA as the substrate.

3.2.1.4 Temperature profile:

The temperature profile (Figure 3.22) showed that native porcine trypsin had a T_{50} value of 45°C while native bovine trypsin had a T_{50} value of 38°C. It should also be noted that the absorbance values obtained for the porcine trypsin were 4-fold greater than those of the bovine trypsin at the same concentrations.

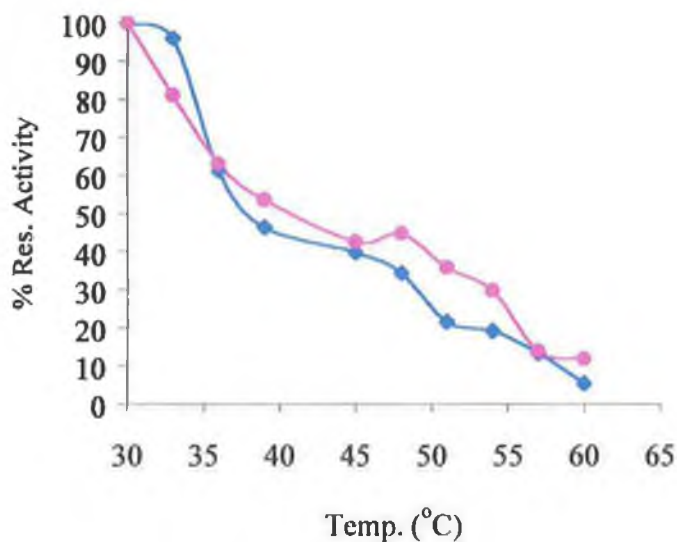


Figure 3.22: A temperature profile to compare the T_{50} values of
 ◆ bovine and ■ porcine trypsin .

3.2.2 EG-NHS modified trypsin.

3.2.2.1 BCA Assay

The protein concentration of the modified trypsin was determined by a BCA assay (Section 2.3.1.1) and was found to be 0.09mg/ml (Figure 3.23). Bovine serum albumin (BSA) was used as the benchmark for the standard curve. The theoretical concentration of protein in the microplate was 0.25mg/ml therefore during the modification process the enzyme retained 36% of its activity.

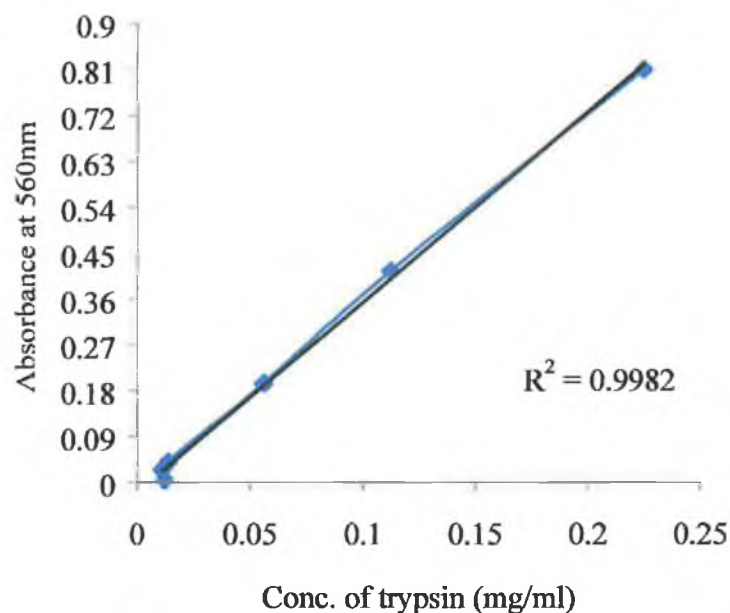


Figure 3.23: A BCA standard assay curve to determine the concentration of protein after EG-modification.

3.2.2.2 Thermoinactivation.

EG NHS-modified porcine trypsin was found to be more thermally stable than native bovine or porcine trypsin at 55°C. It retained 100% of its amidase activity for 150 minutes whereas the native porcine trypsin was completely inactive after 100 minutes. Bovine trypsin was less stable than porcine, as that trypsin showed 50 % loss of activity after 10 minutes (Figure 3.24). The results of % residual activity versus time were analysed using the computer programme Enzfitter.

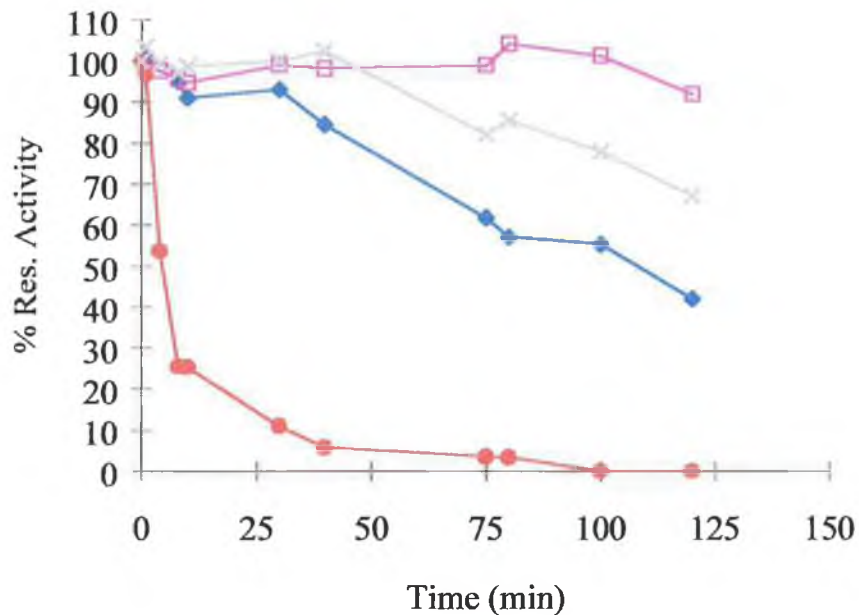


Figure 3.24: A Comparison of the thermo-stability of both native and modified porcine and bovine trypsin at 55°C, where \square is modified porcine trypsin, \blacklozenge is native porcine trypsin, \bullet is native bovine trypsin and \times is modified bovine trypsin.

The data were fitted to the first order exponential decay equation and visual observation of the graphic fit shows that the native bovine (Figure 3.25) and porcine trypsin (Figure 3.26) thermal inactivation's at 55°C obeys first-order rate equations. The bovine trypsin obeys the first-order rate equation over ten minutes compared to the porcine trypsin, which obeys it over 125 minutes. The modified forms of porcine and bovine trypsin by visual observation of the graphic fit didn't obey first-order rate equations (data not shown). The $t_{1/2}$ values obtained for the native bovine trypsin was found to be 4 minutes and porcine trypsin was 110 minutes at 55°C.

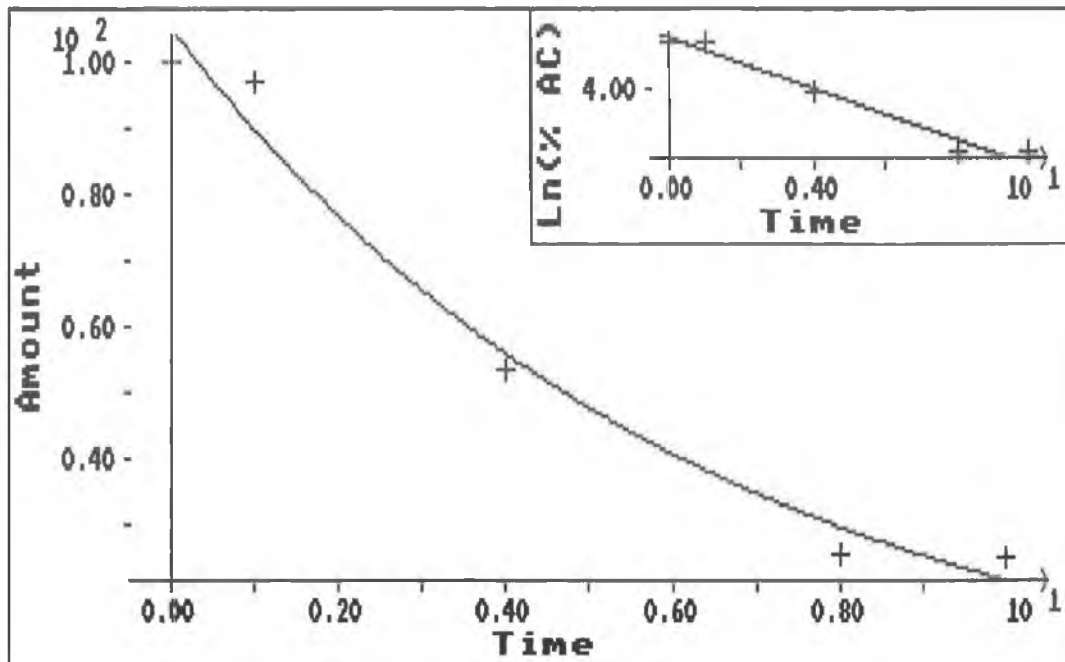


Figure 3.25: Data for bovine trypsin were fitted to the first order exponential decay equation using the computer programme Enzfitter.

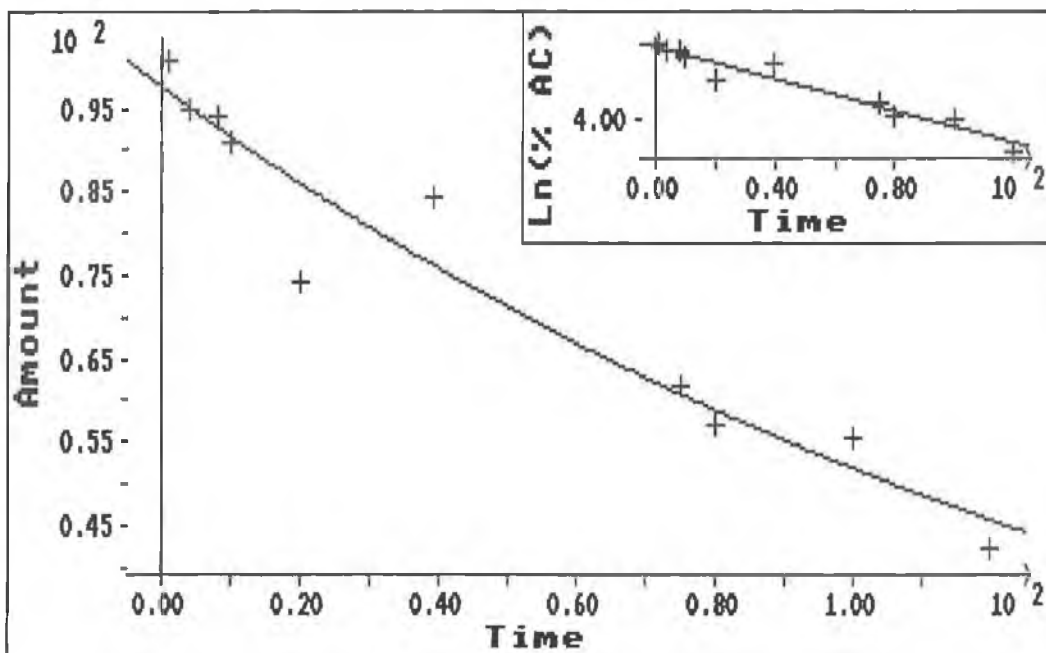


Figure 3.26: Data for porcine trypsin were fitted to the first order exponential decay equation using the computer programme Enzfitter.

3.2.2.3 Organo-tolerance assay

Organo-tolerance assays were carried out on native and EG-trypsin for the following solvents: acetone, acetonitrile, THF, DMF, DMSO and 1,4-Dioxan (Section 2.3.2.4). Graphs of % residual activity versus concentration of solvent were plotted for each solvent and none of the graphs exhibited first-order deactivation kinetics (data not shown). The concentration at which half the amidase activity (C_{50}) was calculated for each form of enzyme in each solvent (Table 3.3).

Acetone showed no denaturing effect on either form of the enzyme as they retained 100% of their amidase activity in up to 90% v/v acetone/aqueous solution. The EG-trypsin also retained 100% amidase activity in 90% v/v THF/aqueous solution while native trypsin lost 50% amidase activity in 70% THF/aqueous solution. The most powerful denaturing solvent of the six tested was DMSO, as this caused the greatest loss of activity-native porcine trypsin lost 50% amidase activity in a 52%v/v acetonitrile/aqueous solution. The difference between the number of active sites (NPGB, Section 2.3.1.3) for the native and modified porcine trypsin in an aqueous and an acetonitrile/aqueous solution was negligible. The porcine trypsin in an aqueous solution was shown to be 40% active whereas the porcine trypsin in acetonitrile was shown to be 32% active.

Table 3.3: Threshold concentrations of organic solvents for native porcine and EG-trypsin.

<u>Solvent</u>	<u>log P^a</u>	<u>C₅₀</u>	
		<u>Native trypsin</u>	<u>EG-trypsin</u>
Tetrahydrofuran	0.46	80	*
Acetone	-0.24	*	*
1,4-Dioxan	-0.27	72	72
Acetonitrile	-0.34	65	72
DMF	-1.01	72	80
DMSO	-1.35	52	58

^a Values taken from Khmelniysky et al., (1991)

* The activity retained > 50% activity at all concentrations.

3.2.2.4 Arg/Lys ratio.

The specificity of the two types of trypsin for arginine versus lysine residues was determined as described in Section 2.3.1.6. The *k_{cat}* and *V_{max}* values were determined using Enzfitter as both forms displayed normal Michaelis-Menten kinetics, see Table 3.4. The *V_{max}* values were converted to *k_{cat}* values by dividing by the active enzyme concentration. This had been previously been determined to be 40% for both native and modified porcine trypsin using the active site titrant NPGB (Section 2.3.1.3). Both native and modified trypsin showed a slightly higher preference for the lysine substrate.

Table 3.4: Determination of the Arg/Lys ratio for native and modified pig trypsin

		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	<u>Arg:Lys</u> Ratio
<u>Native trypsin:</u>	Arg	3768	50.58	75	1 : 1.7
	Lys	5066	38.66	131	
<u>EG-trypsin:</u>	Arg	3309	54.86	60	1 : 1.5
	Lys	1448	15.97	91	

3.2.2.5 Casein digestion.

The rate of hydrolysis of casein by both native porcine trypsin and EG-trypsin was determined as described in section 2.3.1.5. The K_m and V_{max} values (Table 3.5) were determined using the computer programme, Enzfitter, as they followed normal Michaelis-Menten kinetics.

Table 3.5: Casein Digestion

	V_{max} (s^{-1})	K_m (mM)	V_{max}/K_m ($s^{-1}mM^{-1}$)
<u>Native trypsin:</u>	49.8	0.21	237.1
<u>EG-trypsin:</u>	77.6	0.23	337.4

3.3 Discussion:

The pH optimum of porcine trypsin was determined using the pH-stat with casein as the substrate and was found to be in the range pH 7.8 to 10.8, in a Tris-HCl buffer (Section 2.3.1.4), which seems to be similar to that reported in literature (Dallas Johnson et al., 2002). Dallas Johnson et al., 2002 found the optimum pH was 8.5 for porcine trypsin in 25mM glycine-NaOH. They also determined that the pH optimum of the enzyme varies depending on the buffer used, obtaining different pH graphs for glycine-NaOH solution as compared to a sodium phosphate buffer. They also stated that ovine trypsin demonstrated a difference in its optimum pH when the buffer was changed. Outzen et al., 1996 showed that bovine and salmon trypsin showed a pH optimum of 8.5 to 10.5.

Trypsin undergoes autolysis as do many other proteinases. Murphy, 1996 found that bovine trypsin lost only 40% activity over an hour at 50°C, however the pH of the solution used in this case was 8.2. The porcine trypsin solution was allowed to autolyse over six hours and the level of protein remaining in the sample was determined using the Bradford Method (Section 2.3.2.3). Native porcine trypsin had lost 70% of its activity after one hour, the solution used was at pH 7.0 and as this was not an optimal pH for this enzyme at least not for casein digestion (Figure 3.19), this may have increased the rate of autolysis as changes in environmental conditions can change the rate of autolysis. Binggen et al., 1980 have found that under drastic environmental conditions e.g. pH 9.1 the autolysis becomes fast and nonspecific. Interestingly they have also shown, by chromatographic methods, the formation of new active fragments of porcine trypsin when the enzyme was left to auto-digest and so this would explain why enzymes don't lose all their activity instantly once autolysis begins. These pseudotrypsins contained an

intrachain split between Lys₁₄₅ - Ala₁₄₆ and Arg₁₀₅ - Val₁₀₆, which were not found in the original enzyme.

In order to detect the amidase activity L-BAPNA was used as a substrate. This is a direct chromogenic substrate; i.e. it releases a coloured product as a direct result of enzymatic hydrolysis. Trypsin hydrolysis of this substrate releases p-nitroaniline, which is yellow and can be estimated colorimetrically. L-BAPNA does not hydrolyse in the absence of an enzyme. The extent of hydrolysis of L-BAPNA was determined by measurement of p-nitroaniline at 405nm (a filter for 410nm was unavailable); above 400nm there is no absorbance due to the uncleaved substrate, i.e. L-BAPNA (Erlanger et al., 1961). Trypsin concentrations were varied from 0-18.5 µg/ml and a linear response against the rate of hydrolysis of L-BAPNA was shown.

The above microassay was then used to determine percentage residual activity for both temperature profile and thermoinactivation experiments. For a comparison of native porcine and bovine trypsins, a temperature profile was carried out. For the same concentration, the porcine trypsin was shown to be slightly more stable. The T₅₀ value for the porcine trypsin was found to be 45°C, while that of bovine trypsin was 38°C. These results agree with published literature reports that porcine trypsin is more stable than bovine trypsin to thermal denaturation (Walsh and Wilcox, 1970). Murphy (1996) obtained a T₅₀ value of 46°C for bovine trypsin however she used a greater amount of enzyme in her experimental procedure in comparison to the one discussed here.

The chemical modification of enzymes is a widely used technique to increase the catalytic activity and stability of enzymes (Section 1.6.1). On reviewing the literature there is very little work done in terms of chemical modification on porcine trypsin in

comparison to that of bovine trypsin. Elsner et al., 2000 have chemically modified the lysine residues in bovine trypsin. They stated that the reason for targeting the lysine residues was due to the fact that these residues were involved in the proteolytic activity and specificity of the trypsin. They discovered that succinylation lead to about 10-fold better acceptance of basic residues in P₁' position. They also found that the succinyl-trypsin was more stable to autolysis than the native and guanyl-trypsin retained ~100% activity over 250 minutes compared to a loss of 20% activity for the other two forms over the same period of time. It is thought that this is due to the increased negative charge on the surface of the protein. Gleich et al., 1992 modified trypsin with various bifunctional reagents e.g. N-hydroxysuccinimide esters (NHS) of dicarboxylic acids, dianhydrides and bisimidoesters. They found that the half-life of native bovine trypsin at 50°C was 4-5 minutes (a similar result was obtained, see Figure 3.24) and that the trypsin derivative, which had been stabilised with the NHS-ester of acetylglutamic acid, demonstrated a half-life of 200 minutes. This modification was only observed for 125 minutes at 55°C and it retained approx. 78% activity over this period of time (Figure 3.24). Zhang et al., (2001) modified bovine trypsin with methoxypolyethylene glycol (MPEG), of molecular mass 350Da. The t_{1/2} of the native bovine trypsin was 2 fold less stable than that of the modified form at 60°C. In comparison these results show that the EG-NHS modification of bovine trypsin showed >25 fold increase in stability at 55°C compared to that of the native and the EG-NHS modification on the porcine trypsin was superior again with the modified porcine trypsin retaining approximately 100% activity over 125 minutes at 55°C (Figure 3.24). EG-trypsin was thought to be stabilised against reversible unfolding. This stability is likely due to intramolecular crosslink(s) being

formed in the enzyme. These bonds act like a clamp, making it difficult for the enzyme to unfold.

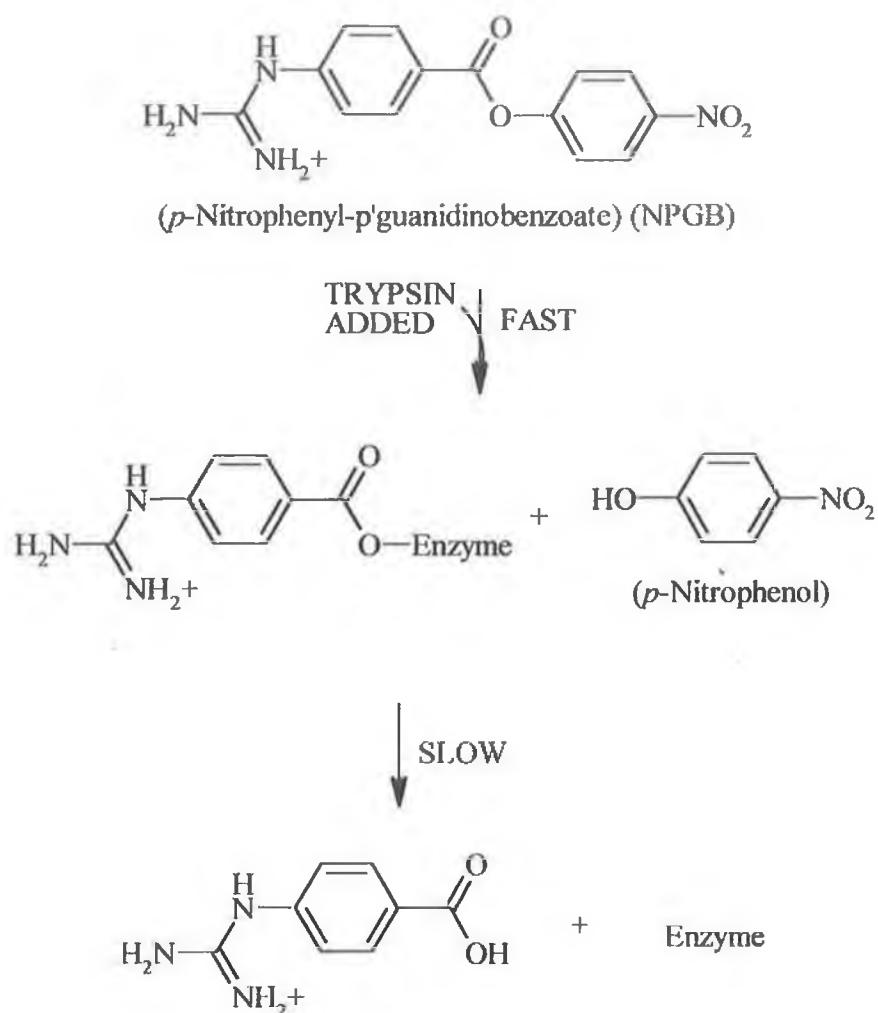


Figure 3.27: A schematic diagram of the reaction involved in determining the active site of porcine trypsin (Walsh, K.A. et al., 1970).

There was no significant difference between the number of active sites of native and EG-modified porcine trypsin. The number of active sites was determined using the assay

described in Section 2.3.1.3. The active site titrant (Figure 3.27) is mixed in excess with the trypsin.

Organic solvents can effect the conformational structure of the enzyme in several ways:

- (i) Disruption of the tightly bound water layer essential for catalysis and folding (ii)

Interfering with the protein structure by reaction with protein solvation sites, either hydrophobic or H-bonding. Of the solvents tested DMF, DMSO, THF, acetonitrile and 1,4-Dioxan are all chemically inert solvents that tend not to react with the enzyme; acetone is a more chemically active solvent. It forms charged molecules in solution as it can interact chemically with the enzyme. DMSO, DMF, acetone and acetonitrile cause a 50% decrease in amidase activity for the native trypsin in 50 - 70% v/v organic solvent/aqueous solution, the EG-trypsin was moderately more stable in each case (Table 3.3).

However, THF (the solvent with the highest denaturation capacity (according to Khmel'nitsky et al., 1991)) showed very little effect on either the native or EG-trypsin; which retained 70% amidase activity in up to 90% v/v THF/aqueous solution. This may be due to the fact that the THF had not been dried before use and therefore the solvent probably contained a large amount of water, giving an inaccurate result. Also, Nurok et al., 1999 state that the effect of various hydrophilic solvents on enzymes is related to their log P value i.e. the lower the log P value the more likely the solvent is to distort the water bound to the enzyme and hence cause a detrimental effect to the catalytic activity of the enzyme. THF has the highest log P value and therefore would be expected to cause the least disturbance to the activity of the enzyme (Table 3.3). It is worth noting

that the EG-trypsin showed increased amidase activity, 120%, in 0-50% v/v THF/aqueous solution.

The EG-trypsin showed the greatest solubility compared to the native in DMF, it retained 100% amidase activity in 70% v/v DMF/aqueous solution compared to 40% v/v for the native at the same concentration. Lozano et al., 1996 attribute the negative effect of DMF at higher concentrations to be due to the interaction of the solvent molecules with the tightly bound water layer of the enzyme. The EG-trypsin could be more stable due to the crosslinkers in the modified form. Even so the native demonstrated a C_{50} value of 82 compared to 72 for the native in DMF. Porcine trypsin is more organotolerant when compared to the results of Murphy (1996) native bovine trypsin had a C_{50} value of 65 compared to 67 for the EG-trypsin in DMF. This increased stability in DMF will be investigated further in terms of catalytic efficiency (Chapter 5) and also the use of EG-trypsin in peptide synthesis (Chapter 7).

The present results agree with those obtained by Tyagi and Gupta, 1998 who also found that trypsin was most stable in DMF with a half-life of 176 hours compared to 36 hours for acetonitrile and 35 hours for DMSO. Schulze and Klivanov, 1991 found that the half-life of subtilisin BPN' in DMF dramatically depends on the pH of the solution from which the enzyme had been lyophilised "pH-memory". The half-life increased from 48 minutes to 20 hours when the pH of the solution was changed from 6.0 to 7.9, it is possible that the trypsin would be affected in the same way.

The native porcine trypsin demonstrated 120% amidase activity in 0-50% v/v 1,4-dioxane/aqueous solution, this is similar to the result obtained by Simon et al., 1998 for bovine trypsin. They also observed that in contrast to trypsin when chymotrypsin was

placed into 1,4-dioxane the enzyme had lost 40% activity in 50% v/v 1,4-dioxane/aqueous solution.

In terms of catalytic activity (k_{cat}/K_m), trypsin demonstrates specificity for arginine and lysine that is 10^5 times greater than for any other amino acid (Halfon and Craik, 1998). Asp189 determines the specificity of trypsin for arginine and lysine residues, which is located at the bottom of the cylindrical shaped binding pocket (S_1). The guanidinium group of the arginine substrate binds directly to the Asp189 whereas the lysine residues bind through indirect hydrogen bonds to a water molecule (Craik et al., 1985 and Briand et al., 1999). Surrounding amino acids in this binding site then determine the preference of the trypsin for arginine or lysine residues. Perona et al., 1993 investigated the Arg/Lys ratio for rat trypsin and they found that depending on the substrate used the ratio varied. They found using different substrates caused a change in the ratio i.e. tos-GPX-AMC (X=Arg or Lys) gave a ratio of 4.5 compared to the substrate Z-X-AMC (X=Arg or Lys) which had a ratio of 5.1. It was found that native porcine trypsin demonstrated a 0.6 Arg/Lys ratio using the substrate L-X-methyl ester dihydrochloride (X= Arg or Lys) and the EG-trypsin showed a slightly higher ratio of 0.7 for the same substrates (Table 3.4). The catalytic efficiency (k_{cat}/K_m) of the EG-trypsin for the hydrolysis of the lysine residue was not that much higher (16% higher) than that of the native trypsin for the lysine.

The proteolysis of the natural substrate casein was investigated as proteolytic hydrolysates have been found to be useful in the food, pharmaceutical and cosmetic industries. The catalytic efficiency (V_{max}/K_m) for the digestion of casein by native porcine trypsin was found to be $237.1 \text{ s}^{-1}\text{mM}^{-1}$ (Table 3.5) while the EG-trypsin had an

increased catalytic efficiency of $337.4 \text{ s}^{-1}\text{mM}^{-1}$. This increase in catalytic efficiency of the enzyme may be due to the enzyme being more stable to the reaction conditions and thereby not being as prone to autolysis as the native.

CHAPTER 4

STUDIES on NATIVE and MODIFIED SUBTILISIN CARLSBERG and ALCALASE

4.1 Introduction

In order to compare trypsin, which has a narrow specificity i.e., arginine and lysine residues in terms of peptide synthesis with an enzyme, which has a broader specificity it was decided to, study subtilisin Carlsberg. Alcalase was also investigated to compare a commercial enzyme with that of the classical enzyme i.e. subtilisin Carlsberg. To compare and contrast these bacterial serine proteases, subtilisin Carlsberg and alcalase, a number of stability and activity assays were carried out. Alcalase is produced by submerged fermentation of "*Bacillus licheniformis*" and is known to contain subtilisin Carlsberg (Philipp and Bender 1983) as well as a glutamic-acid-specific endopeptidase (Svendsen and Breddam 1992).

Temperature profiles were carried out for the two enzymes at pH8.0 and at pH9.0. Alcalase is used in washing detergents with moderate alkalinity and shows optimum activities at pH8.0 between 50-70°C (www.novozymes.com). Therefore, in order to calculate the half-inactivation temperature, T_{50} , where observed activity is 50% maximal, the temperature profiles had to be performed at pH 9.0. A thermoinactivation study was carried out at 75°C. The enzymes were kept at this temperature for up to three hours, cooled and the % residual activity was recorded at certain time intervals.

Native subtilisin Carlsberg is not associated with biological membranes in nature and is naturally a protease that catalyses the hydrolysis of water-soluble proteins in an aqueous solution, so organo-tolerance studies were carried out to see how active the enzyme would be in an organic solvent solution. Khmel'nitsky et al. (1988) determined that the inactivation of an enzyme in certain organic solvents is due to the solvent thirstily absorbing the water and stripping the enzyme of its hydration shell, which is essential

for catalytic activity. However, some enzymes retain their bound water so tightly that a solvent molecule cannot replace the water molecules. Fitzpatrick et al. (1993) demonstrated that subtilisin Carlsberg retained the same three-dimensional crystal structure in anhydrous acetonitrile as it did in water. 99 of 119 structural water molecules were not displaced from the crystal even in anhydrous acetonitrile. However, other hydrophilic organic solvents can cause detrimental effects to the enzyme, as they can strip away the water molecules bound to the enzyme and cause a decrease in catalytic activity (Gorman and Dordick 1992). Chen et al. (1991) found that alcalase had lost 50% activity after 15 minutes in 40% dioxane whereas it retained practically 100% activity for 75 hours in amyl alcohol. Alcalase retained high levels of activity in some anhydrous alcohols. The organo-tolerance of the two enzymes was investigated by measuring the rates of inactivation in the following organic solvents acetone, acetonitrile, 1,4-Dioxan, DMF, DMSO and THF at various concentrations.

As all proteases have the ability to hydrolyse themselves (autolysis), the rate of subtilisin Carlsberg and alcalase self-digestion was investigated over time (using the method developed by Bickerstaff and Zhou 1993). Kawamura et al. (1981) proposed the following simultaneously occurring two-step mechanism for autolysis:

(A) Thermal denaturation:



(B) Autolysis:

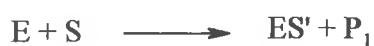


Where:

N is the native enzyme, U is the inactivated form, I is the denatured form and P is the decomposed product.

So looking at the equation, the partially inactivated enzyme is rendered inactive by the hydrolysis of the native enzyme. Therefore the higher the enzyme concentration the higher the rate of autolysis. Castro (1999) showed that subtilisin Carlsberg demonstrated a 44-fold increase in autolysis in water when the reaction temperature was changed from 37°C to 45°C. He has also observed that the rate of autolysis can be decreased further if the enzyme is put into glycerol as opposed to water, after six hours there was no enzymatic activity in water as the enzyme had completely self-digested, but the enzyme showed 30% activity in glycerol after the same length of time.

NTCl was used as the titrant to determine the active site content of subtilisin Carlsberg and alcalase (Figure 4.28). The assay is based on the following equation, which is a stoichiometric reaction with respect to each enzymatic active site (Bender et al., 1966):



Where E is the free active enzyme (which we are measuring), S is the substrate, ES' is the enzyme that is bound to a substrate and P₁ is the product. The possibility that there is another enzymatically active component in the alcalase that reacted with the NTCl has not been investigated.



Figure 4.28: Ribbon diagram of the protein structure of trans-cinnamoyl-subtilisin in water. The catalytic triad (Asp-32, His-64, and Ser-221) and cinnamoyl group (black) are portrayed as sticks. The grey balls depict water molecules (Schmitke et al., 1998).

The proteolytic activity of both subtilisin Carlsberg and alcalase was measured using the pH-stat method with casein as the substrate (section 2.3.1.5). Over the past few years, the hydrolysis of large proteins such as casein into di and tri-peptides has received some attention, as the smaller peptides have very high nutritional value. They can be used for people that require a special diet e.g. premature newborn baby. Morato et al. (2000) found that the highest content of small peptides suitable to be included in a special diet was obtained when the hydrolysis of casein by subtilisin was allowed to proceed with an E:S ratio of 4%, at 40°C and pH7.5.

Previous studies by Svendsen and Breddam (1992) have shown that the alcalase also contains at least one other enzymatically active component BL-GSE. Ferreira et al., 2003 have observed numerous protein bands on an electrophoretogram of alcalase with the band corresponding to subtilisin Carlsberg (which has a molecular weight of 27.3Kda) being <20% of the overall proteins.

Both subtilisin carlsberg and alcalase were reacted with the crosslinking reagent, EG-NHS, which has a span of 16 Å and uses the lysine residues of the enzyme as its target group (Section 3.1). Subtilisin Carlsberg contains nine lysine residues and GSE contains six. Visual observation of the subtilisin Carlsberg structure in the computer programme, Rasmol, indicates that only one of the lysine residues would be out of range of the crosslinker. The GSE has no available structure in this computer programme. A number of experiments were carried out to investigate the effect of crosslinking on both enzymes and to compare and contrast the two enzymes in both their native and modified forms.

4.2 Results:

4.2.1 Native Subtilisin Carlsberg/Alcalase:

4.2.1.1 Temperature profile.

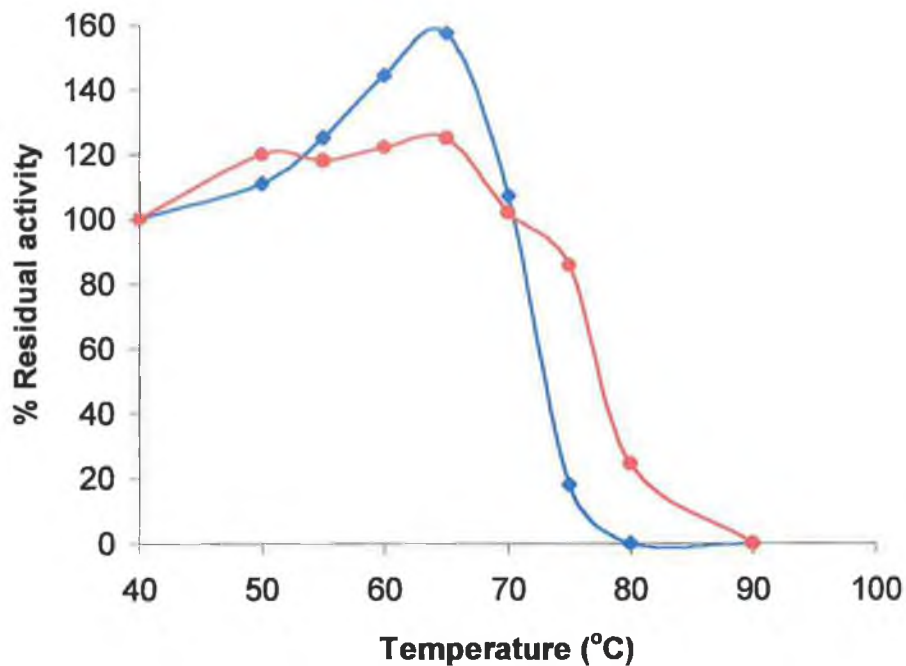


Figure 4.29: A temperature profile to compare the T_{50} values of • subtilisin Carlsberg and ◆ alcalase at pH 8.0.

As can be seen from Figure 4.29, the residual activity of alcalase increases up to 160% at 70°C, pH 8.0 and so the T_{50} value cannot be calculated for this enzyme at this particular pH. An apparent T_{50} is calculated instead and it is 73°C, subtilisin Carlsberg has a T_{50} value of 78°C at pH 8.0. At pH 9.0, subtilisin Carlsberg demonstrated a T_{50} value of 68°C and the alcalase showed a T_{50} value of 72°C (Figure 4.30).

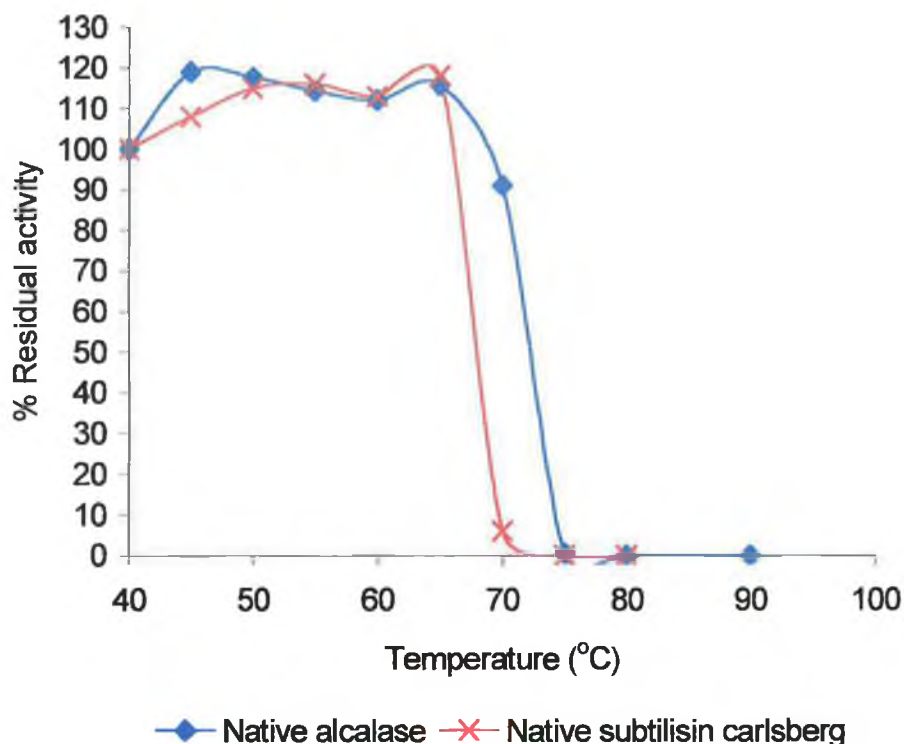


Figure 4.30: A temperature profile to compare the T_{50} values of native subtilisin Carlsberg and alcalase at pH 9.0.

4.2.1.2 Thermoactivation.

The results of % residual activity versus time were analysed using the computer programme, Enzfitter. The data were fitted to the first order exponential decay equation and visual observation of the graphic fit shows that both enzymes' thermal inactivation at 75°C obeys first-order rate equations (Figure 4.31 and Figure 4.32). The $t_{1/2}$ value for alcalase was determined to be 4.6 minutes and the $t_{1/2}$ value for subtilisin Carlsberg was 3.9 minutes at 75°C. At 75°C, both enzymes showed 50% loss in amidase activity after five minutes (Figure 4.33).

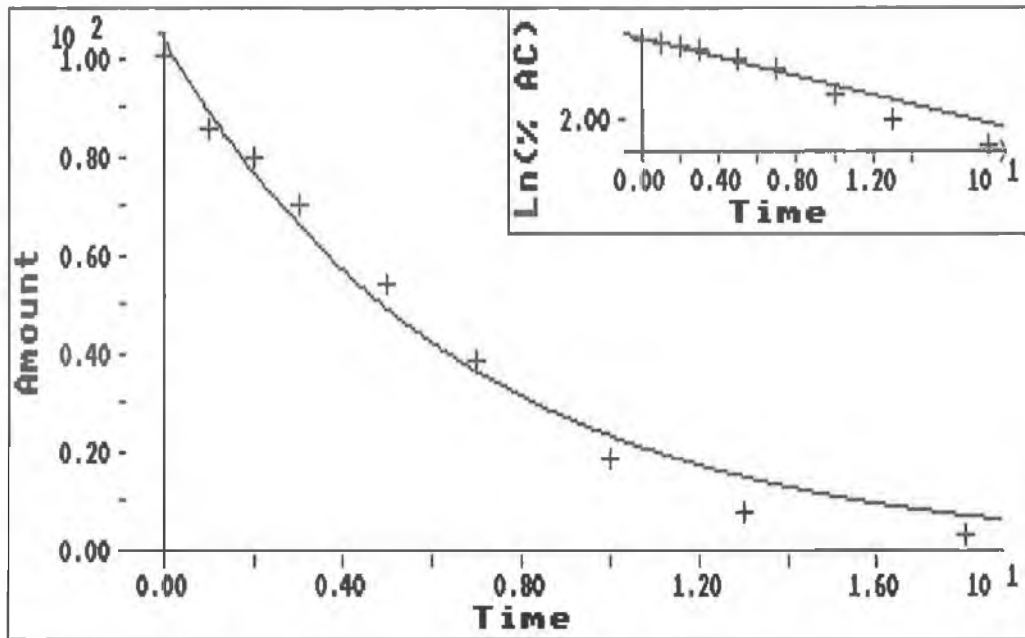


Figure 4.31: Data for alcalase were fitted to the first order exponential decay equation using the computer programme Enzfitter.

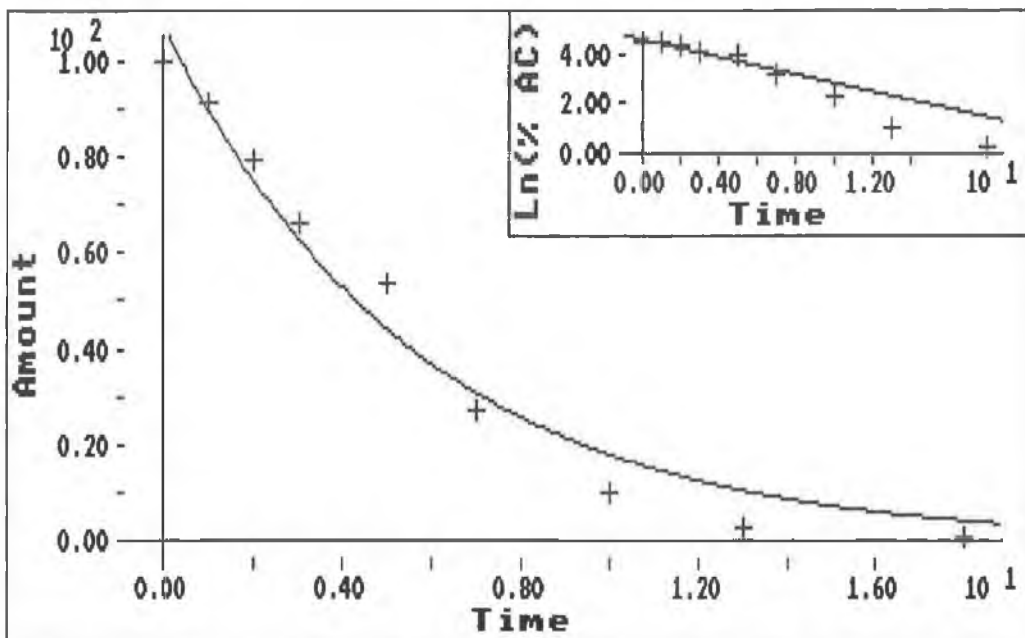


Figure 4.32: Data for subtilisin Carlsberg were fitted to the first order exponential decay equation using the computer programme Enzfitter.

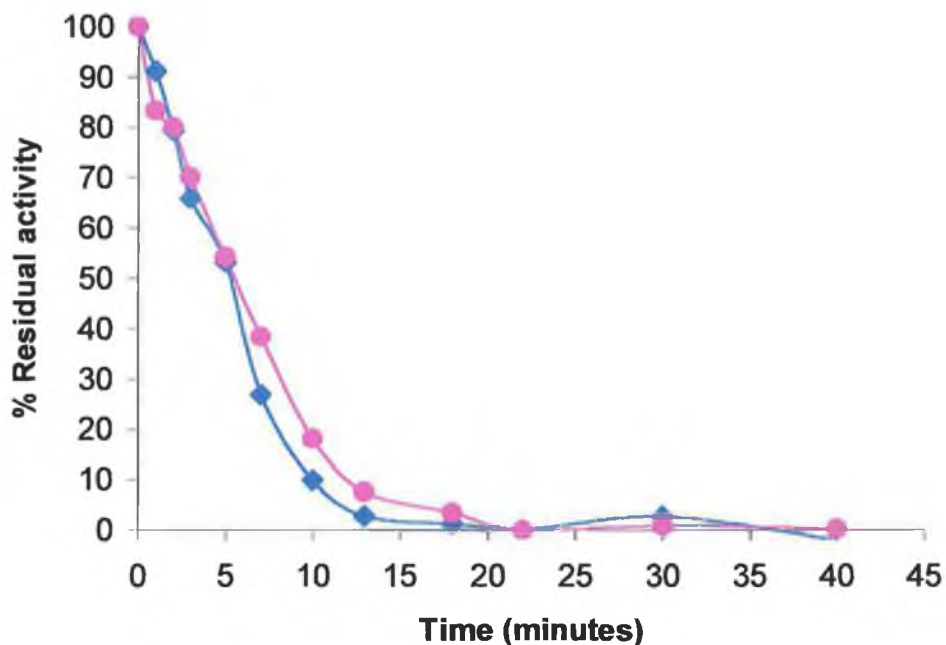


Figure 4.33: A study on the comparison of the thermo-stability of both \blacklozenge subtilisin Carlsberg and \bullet alcalase at 75°C.

4.2.1.3 Organo-tolerance assay.

As can be seen from table 4.6, acetonitrile caused the greatest detrimental effect to subtilisin Carlsberg with 40% v/v acetonitrile/0.1M KH_2PO_4 causing a 50% loss in amidase activity. Alcalase was more stable, losing 50% amidase activity in 72% v/v acetonitrile/0.1M KH_2PO_4 . DMSO caused the greatest adverse effect on the alcalase with 50% loss in amidase activity at a concentration of 48% v/v acetonitrile/0.1M KH_2PO_4 . The same DMSO concentration had similar effects on subtilisin Carlsberg (Table 4.6). Alcalase retained 100% amidase activity in up to 90% v/v 1,4-Dioxan/0.1M KH_2PO_4 .

Table 4.6: The C_{50} value of various organic solvents for subtilisin Carlsberg and alcalase.

Solvent:	S. Carlsberg	Alcalase	Log P ^b
	C_{50} (% v/v)	C_{50} (% v/v)	
Acetone	60	70	-0.24
Acetonitrile	40	72	-0.34
1,4 – Dioxan	68	*	-0.27
DMF	56	58	-1.01
DMSO	48	48	-1.35
THF	60	64	0.46

* The enzyme retained greater than 50 % activity up to 90 % v/v solvent.

^b Values taken from Khmelniysky et al., (1991)

4.2.1.4 Active site titration.

The active enzyme concentrations were calculated (see section 4.3) for both native and modified forms of subtilisin Carlsberg and alcalase and the results were as follows: native subtilisin Carlsberg was 73% active, EG-subtilisin was 37% active, native alcalase was 78% active and EG-alcalase was 36% active.

4.2.1.5 Autolysis.

As can be seen from Figure 4.34, subtilisin Carlsberg and alcalase both showed autolysis. Subtilisin Carlsberg was more liable to self-digestion, as it had lost 40% amidase activity after three hours compared to 20 % amidase activity for the alcalase over the same period of time under these conditions. It should also be noted that the

alcalase showed a much higher absorbance value than the subtilisin Carlsberg at the same enzyme concentration.

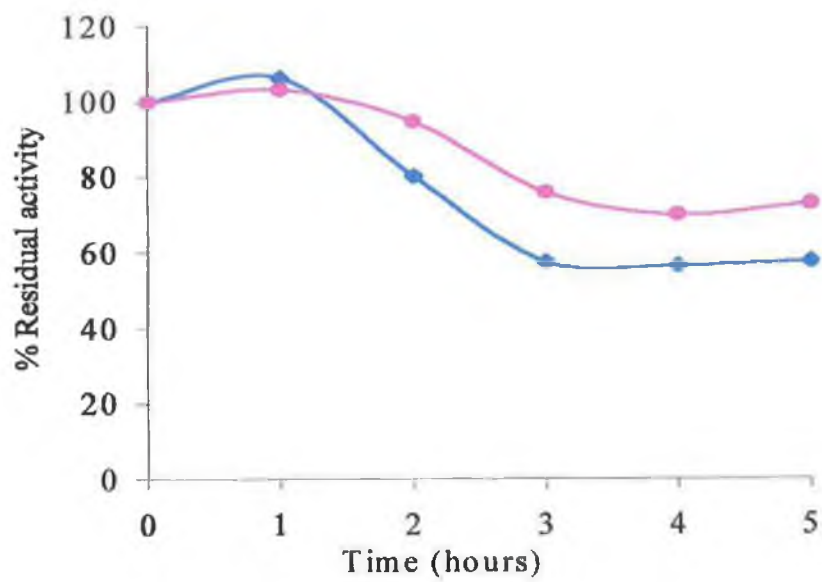


Figure 4.34: Autolysis of \blacklozenge subtilisin Carlsberg and \bullet alcalase.

4.2.2 EG-NHS modified subtilisin Carlsberg/alcalase.

4.2.2.1 BCA Assay

The protein concentration of the modified enzymes was determined by a BCA assay (section 2.3.1.2) and the EG-subtilisin Carlsberg was found to be 1.25mg/ml while the EG-alcalase was 1mg/ml (Figure 4.35). Bovine serum albumin (BSA) was used as a standard.

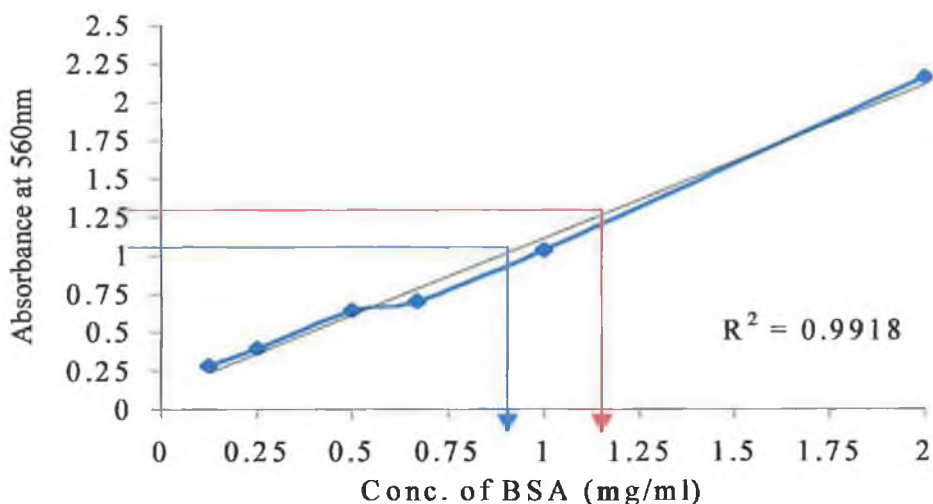


Figure 4.35: A BCA standard assay curve to determine the concentration of the proteins after EG-modification, with \rightarrow corresponding to the subtilisin Carlsberg and \rightarrow corresponding to the alcalase.

4.2.2.2 Thermo-inactivation.

At 65°C, the EG-alcalase was the more stable as it took 100 minutes for the enzyme to lose 50% amidase activity. EG-subtilisin Carlsberg on the other hand took 56 minutes (Figure 4.36). The native subtilisin Carlsberg was easily inactivated at this temperature as it took only 12 minutes to lose 50% activity. The EG-subtilisin Carlsberg and native

alcalase took the same length of time to lose 50% amidase activity, i.e. 56 minutes (Figure 4.36). The results of % residual activity versus time were analysed using the computer programme Enzfitter. The data were fitted to the first order exponential decay equation and visual observation of the graphic fit shows that only the native subtilisin Carlsberg thermal inactivation at 65°C obeys a first-order rate equation (data not shown). The native alcalase may not obey a first-order rate equation because it is less prone to autolysis (Figure 4.34) and therefore is more stable at higher temperatures. This may also be true for the modified forms of both enzymes. The $t_{1/2}$ value obtained for the native subtilisin Carlsberg was found to be 7.58 minutes at 65°C.

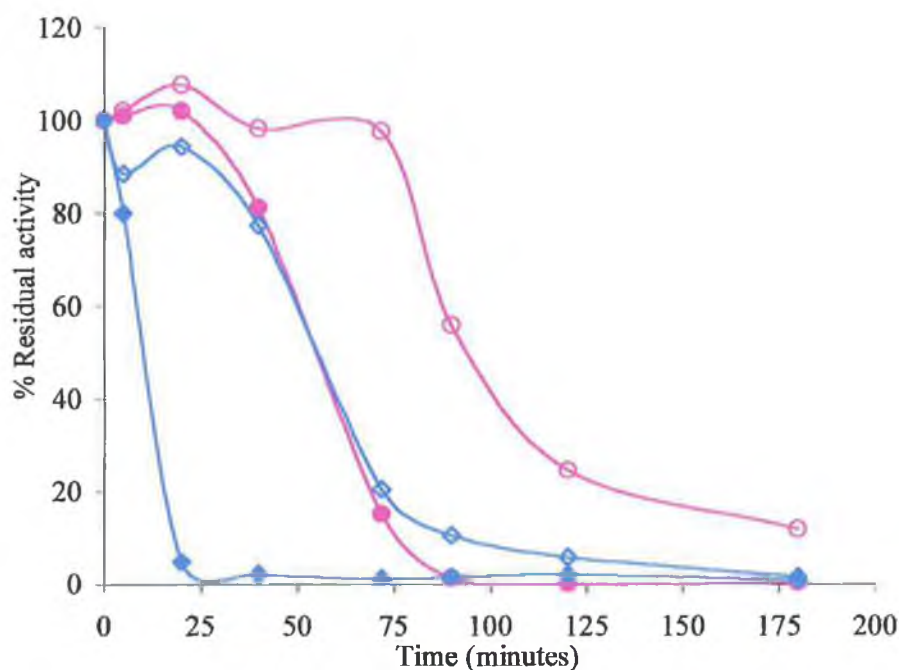


Figure 4.36: Thermoinactivation of the \blacklozenge native and \circ EG-subtilisin Carlsberg and the \bullet native and \diamond EG-alcalase at 65°C.

4.2.1.6 Casein digestion.

The proteolytic efficiency of both native and modified forms of subtilisin Carlsberg and alcalase was determined as described in section 2.3.1.5. The V_{max} and K_m values (Table 4.7) were determined using the computer programme Enzfitter as they followed normal Michaelis-Menten kinetics. The EG-subtilisin Carlsberg was ~3-fold more efficient at hydrolysing the casein than the native form. Surprisingly the native and EG-alcalase demonstrated approximately the same activity in hydrolysing the casein.

Table 4.7: Casein Digestion

	V_{max} (s^{-1})	K_m (mM)	V_{max}/K_m ($s^{-1}mM^{-1}$)
Native subtilisin Carlsberg	37.3	0.1514	246.4
Native alcalase	31.3	0.07	447.0
EG-subtilisin Carlsberg	39.3	0.06	655.0
EG-alcalase	38.9	0.09	432.2

4.3 Discussion:

The temperature profiles obtained for the two enzymes subtilisin Carlsberg and alcalase were as expected. The reaction solution was maintained at pH 8.0 for the first temperature profile and the alcalase showed a bell shaped activity curve, with the amidase activity reaching an optimum of 160% at 65°C (Figure 4.29). Subtilisin Carlsberg did not demonstrate any increased amidase activity at this pH. The amino acid sequence of the subtilisin Carlsberg and alcalase are very different (Figure 4.37) and so they would not be expected to have the same characteristics. Alcalase is a commercially produced enzyme, which was first discovered in the 1960's by NOVO. It was their first enzyme, which was produced by fermentation and it was found to be ideal as a detergent enzyme as it showed increased activity between pH 7-10 and had a pH optimum of 8.0.

A temperature profile was then carried out with the reaction maintained at pH 9.0, where subtilisin Carlsberg had a T_{50} value of 68°C and the alcalase demonstrated a T_{50} value of 72°C (Figure 4.30). The T_{50} value for subtilisin Carlsberg at pH 8.0 was 78°C compared to 68°C for the enzyme at pH 9.0, so this demonstrates how easily enzymes are affected by pH changes in the reaction conditions. As can be seen from Figure 4.30, subtilisin Carlsberg had 120% amidase activity up to a temperature of 65°C, and then this was followed by a severe loss in activity at 70°C. Hirata et al. (2003) have obtained the same result: they stated that subtilisin Carlsberg is active at room temperature and even up to 60°C, after this the enzyme starts to become denatured, loses activity, and so the hydrolysis of the substrate is decreased.

```

Crl : MTRKESFULEMFAFLMFTMAFSDSASAMQAKNVEKDYIVGEEISGVKTAIVKKLIKESGKVDQRIINAAKAKLDEALAEVKN
Alc : MTSKESVKRGLIIGLIGLSIYSLGMHPAQAAASPHTPVSSDPSYKA-ETEVLYDPKSDQYGLYSKAPTGTGKVNETKEAEKESPAK

Crl : DFDVAYVEEDHVAHAIAQTWPYGIPLIKADQVQAQGFKGANVQVAALDNGIQASHPDLNQVGGASFVLEAYNTDGNHGHVVAHWAA
Alc : AP-----YSIKSVIGS-----DDETRVTNTTAYPYRAIWHIESSIGSCTGWMIGPKTVATAGHC IYDTSSGS---FAGTATV

Crl : LDNTTEVLGVAQSVELIYAVVVLNSKESGTYSGIIVSGIEMATTGG-MQVINMSLGGPSESAMKQAVDNAKARGVWVAAAAGNESSEMT
Alc : SPGRNETS--YPPYGSVKSTPYFIPSE-----DRSGMTNYDYGALELSEPIENTVG--YFGYSYTTSSLVGTTVTISGYPEDK

Crl : NIIIEYPAKYDSVIAVGAVDNSNSNRASFSSVGAEEVMAFGAGVYSYPTSYATVNGTSMASPHVAFAAAAILISKHPNLSAIOVRNRLS
Alc : -TARTQWQHSGPITSETYKLOQ-----AMDYGGQSGSPWFEQSSRTNCSEPCSLAVHTNGVYGGSYNRGTR

Crl : SSTATYLGSSFYYGKGLIQVEAAAQ : 379
Alc : IYKEVFDN-----LIRWKNSAQ : 316

```

Figure 4.37: The amino acid sequence of subtilisin Carlsberg (Crl), accession number P00780 and alcalase (Alc), accession number P80057 with the shaded areas representing the identical amino acid residues that the two enzymes have in common. The sequences were obtained from ClustalW at EBI (www.ebi.ac.uk/clustalw) and the computer programme Genedoc (www.psc.edu/biomed/genedoc) was used to align the two amino acid sequences.

Bakhtiar et al. (2003) have used differential scanning calorimetry to determine that the melting temperature, T_m , for subtilisin Carlsberg is 67.3°C.

Subtilisin Carlsberg and alcalase show stability up to 60°C. One reason for this is a universal characteristic of the subtilisin type proteases, i.e. the presence of calcium binding site(s). Proteases with enhanced calcium binding demonstrate higher thermostability (Briedigkeit and Frömmel, 1989). These workers determined that there are two calcium-binding sites in subtilisin Carlsberg and the removal of any of the Ca^{2+} leads to a significant decrease in stability.

When the thermoinactivation assay was carried out at 75°C, both alcalase and subtilisin Carlsberg had lost all activity after twenty minutes (Figure 4.33). Alcalase showed a slightly higher $t_{1/2}$ value (4.62 minutes) than the subtilisin Carlsberg (3.89 minutes). Bakhtiar et al. (2003) incubated a sample of subtilisin Carlsberg at 50°C for 24 hours and then examined its structure using circular dichroism. The structure of subtilisin Carlsberg changed dramatically after being exposed to 50°C for 24 hours (Figure 4.38). Therefore when the enzyme becomes denatured at high temperatures, its structure changes and it loses its catalytic activity (Figure 4.38).

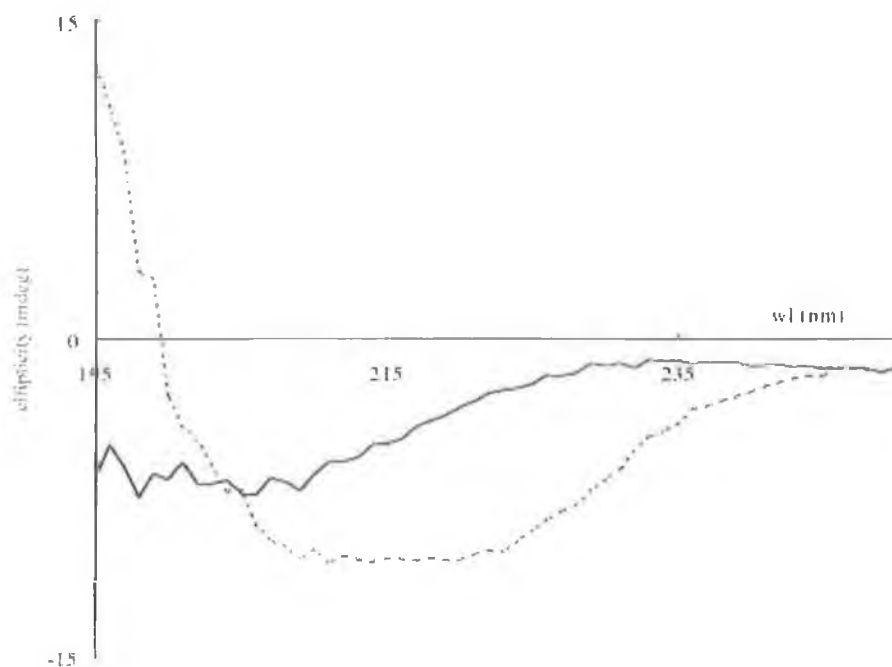


Figure 4.38: A circular dichroism scan of subtilisin Carlsberg before (--) and after (—) storage for 24 hours at 50°C (Bakhtiar et al., 2003).

The C_{50} values of various organic solvents for subtilisin Carlsberg and alcalase are shown in Table 4.6 and also the log P values of these solvents. It is thought that the lower the log P value the greater distortion the solvent will cause to the enzymes' bound water (Nurok et al., 1999). Acetonitrile, DMF and DMSO caused the greatest effects to the subtilisin Carlsberg with each solvent having a C_{50} value less than 60 and these are the three solvents that had the lowest log P values of the six solvents tested (Table 4.6). It is interesting to note that the alcalase didn't behave in the same way; it had a C_{50} value of 72 in acetonitrile compared to 40 for the subtilisin Carlsberg. Both enzymes retained their highest activity in 1,4-Dioxan (Table 4.6). This is unexpected, as it doesn't have the highest log P value. As discussed in section 3.3 one reason why enzymes don't behave as expected in organic solvents is due to their pH memory. Klivanov, 2001 stated that one

of the most important parameters for enzymes in organic solvents is their "pH memory"; this is when the enzymes catalytic activity in organic solvents reflects the pH of the last aqueous solution to which it was exposed. Therefore this "pH memory" maybe a reason for obtaining unexpected C_{50} values.

Zaks and Klivanov, 1988 investigated the effect of anhydrous solvents on the activity of subtilisin Carlsberg (Table 4.8) and they found that the enzyme was most stable in acetone and least stable in DMSO. This change in the activity of subtilisin in these solvents is thought to be due to the fact that the enzyme is unable to unfold when it is placed in an organic solvent and so its native conformation is retained due to high kinetic barriers.

Table 4.8: Solvents listed in decreasing hydrophobicity (Zaks and Klivanov 1988).

Solvent	Subtilisin ($V/K_m \text{ min}^{-1} \times 10^6$)
Acetone	810
Acetonitrile	150
Tetrahydrofuran	120
Dimethylformamide	19
Dioxane	9.2
Dimethylsulfoxide	<0.1

Both subtilisin Carlsberg and alcalase showed signs of autolysis (section 2.3.2.3). Over five hours, subtilisin Carlsberg lost 40% activity compared to 27% activity for alcalase.

Briedigkeit and Frömmel (1989) have shown that the self-digestion of the subtilisin Carlsberg depends on the number of calcium ions bound to it. When the enzyme was in its native state (i.e. with 2 calcium ions per molecule) the rate of autolysis was one-fifth the rate of the enzyme containing no calcium ions. Alcalase was less prone to autolysis, losing only 27% activity over five hours. It is possible that the glutamic acid specific endopeptidase present in alcalase also contains some bound calcium and this increase in calcium ions would increase the enzymes stability.

The active enzyme concentration was determined using the following equation:

$$E (M) = \frac{\Delta A \times 1030}{35 \times \epsilon}$$

Where;

E = active enzyme concentration

ΔA = initial burst of absorbance

$$\epsilon_{335nm} = 13208 \text{ M}^{-1}\text{cm}^{-1}$$

As can be seen from section 4.2.1.4, the native forms of subtilisin Carlsberg and alcalase contained approximately 25% inactive material. Bender et al. (1966) obtained results to show that a typical sample of crystalline subtilisin Carlsberg contained 57% active enzyme and 43%(w/w) inactive material. They concluded that the inert material might be mainly due to autodegradation products that occurred due to autolysis. The EG-NHS modified forms of both enzymes, had approximately 65% inactive material, this would include the EG-NHS crosslinkers.

Subtilisin Carlsberg and alcalase were crosslinked with EG-NHS. Most published literature to date has increased the stability of subtilisin using site-directed mutagenesis

rather than chemical modifications (Bryan et al., 2000). This EG-NHS chemical modification is both cheaper and easier to perform than site-directed mutagenesis. A protein concentration assay was carried out on the modified enzymes, the alcalase retained 50% of its concentration and the subtilisin Carlsberg had 62.5% of its concentration remaining (Figure 4.35).

As can be seen from Figure 4.36, the EG-alcalase was extremely stable retaining 100% residual amidase activity for up to 85 minutes at 65°C whereas the native subtilisin Carlsberg had lost all amidase activity after just 25 minutes at this temperature. The native subtilisin Carlsberg was the only form of the enzyme that obeyed the first-order rate equation and its $t_{1/2}$ value was 7.58 minutes at 65°C, this is twice the value (3.89 minutes) obtained for the subtilisin Carlsberg at 75°C. It would appear that the EG-NHS had crosslinked the enzyme and therefore had caused the enzyme structure to become more resistant to heat inactivation (Figure 4.36). Plou et al., 1994 chemically modified subtilisin Carlsberg by the covalent attachment of various long chain fatty acids to the enzyme. The modified enzyme Octanoyl-subtilisin showed a $t_{1/2}$ of 2.9 hours which was ~2-fold higher than the native enzyme at 65°C, Palmitoyl-subtilisin had a ~3-fold increase in its $t_{1/2}$ value compared to the native. Yang et al. (1996) modified subtilisin Carlsberg with PEG and increased the half-life of the enzyme 28-fold at 30°C but at 60°C there was no difference in the half-life between the native and the modified subtilisin Carlsberg. The present results for EG-subtilisin demonstrated a $t_{1/2}$ value which was superior with a ~4-fold increase in its half-life at 65°C. The EG-alcalase was not as thermostable as it had a 2-fold increase in its half-life at the same temperature.

The proteolytic activities of the two enzymes were measured using casein as the substrate. Casein is the most frequently used substrate for protease activity under neutral and alkaline conditions. Beutel et al. (2002) tested seven different proteases (alcalase, α -chymotrypsin, elastase, esperase, savinase, subtilisin and trypsin) for their activity against casein substrates. Casein layers had been applied over sixty years ago to medieval wall paintings to protect them from peeling due to environmental effects. However, due to ageing of the casein, the paintings began to peel even more drastically. Beutel et al., (2002) wanted to find the most effective protease to remove this casein layer from the paintings. They discovered that alcalase was the most efficient protease, hydrolysing the casein from 2mg/ml to 0.9mg/ml in two minutes. Over the same length of time subtilisin Carlsberg only decreased the casein from 2mg/ml to 1.9mg/ml. These results would agree with those of Beutel et al., 2002 and Morato et al., 2000 who both stated that subtilisin was more efficient in the hydrolysis of casein compared to trypsin. These results show alcalase was approximately twice as efficient as both subtilisin Carlsberg (Table 4.7) and trypsin (Table 3.5) in the hydrolysis of casein. The EG-subtilisin Carlsberg demonstrated a 1.5-fold increase in proteolytic activity compared to that of the native and a 3-fold increase compared to that of the native trypsin. Alcalase showed only slightly more proteolytic activity when crosslinked with EG-NHS (Table 4.7). Svendsen and Breddam (1992) have isolated a glutamic acid specific endopeptidase from a preparation of alcalase. They have determined that this isolated enzyme consists of 222 amino acid residues and is a serine endopeptidase, which is specific for hydrolysis of peptide bonds on the carboxyl side of acidic amino acids, with a strong

preference for glutamic acid. This may indicate a reason for the increased proteolytic efficiency of alcalase over subtilisin Carlsberg.

CHAPTER 5

KINETICS of NATIVE and MODIFIED TRYPSIN, SUBTILISIN CARLSBERG and ALCALASE

5.1 Introduction:

Enzymes are catalysts that act by lowering the activation energy of a chemical reaction. The catalytic activity of an enzyme can be determined by observing the rate of formation of product or the rate of depletion of substrate.

Firstly, the catalytic efficiency of trypsin was determined using methods described in Sections 2.3.5 and 2.3.6 (Plettner et al., 1998). The results are all based on the fact that the rate of product formation increases linearly with the substrate concentration at low concentrations. This is a limiting case of the Michaelis-Menten equation and so $v \cong (k_{\text{cat}}/K_m) [S][E]$ for $[S] \ll K_m$. Thus, an overall value for k_{cat}/K_m is obtained.

Amidase activity was detected using L-BAPNA. The concentration of enzyme present is directly related to the intensity of the yellow colour formed. For the detection of amidase activity, a graph of the change in absorbance versus time showed a linear plot up to 90 seconds. The value for the change in absorbance at this point was then used to calculate k_{cat}/K_m . The data was converted to rates in M s^{-1} using $\epsilon_{414} = 8581 \text{ M}^{-1}\text{cm}^{-1}$ for *p*-nitroaniline (Plettner et al., 1998). The results from the active site titration were also taken into account.

Esterase activity was obtained using the thiobenzyl ester substrate (Z-Lys-SBzl). This ester substrate lacks a chromogenic leaving group, so it is used in conjunction with DTNB (5,5'-dithiobis-2,2'-nitrobenzoate). Trypsin causes the hydrolysis of Z-Lys-SBzl to benzoyloxycarbonyl-L-lysine plus benzyl mercaptan; the latter instantaneously reacts with excess DTNB to form the products (the thiophenolate anion and the mixed disulfide) shown in Figure 5.39. The assay used here is based on that described by Coleman and Green (1981). The thiophenolate ion is a yellow colour. A graph of the

change in absorbance versus time for the esterase activity is linear up until 20 seconds. This value was then taken and converted to rate in $M s^{-1}$ using $\epsilon_{414} = 8708 M^{-1}cm^{-1}$ for the thiophenolate ion (Plettner et al., 1998). Again, the active site titration values were taken into account in calculating trypsin activity.

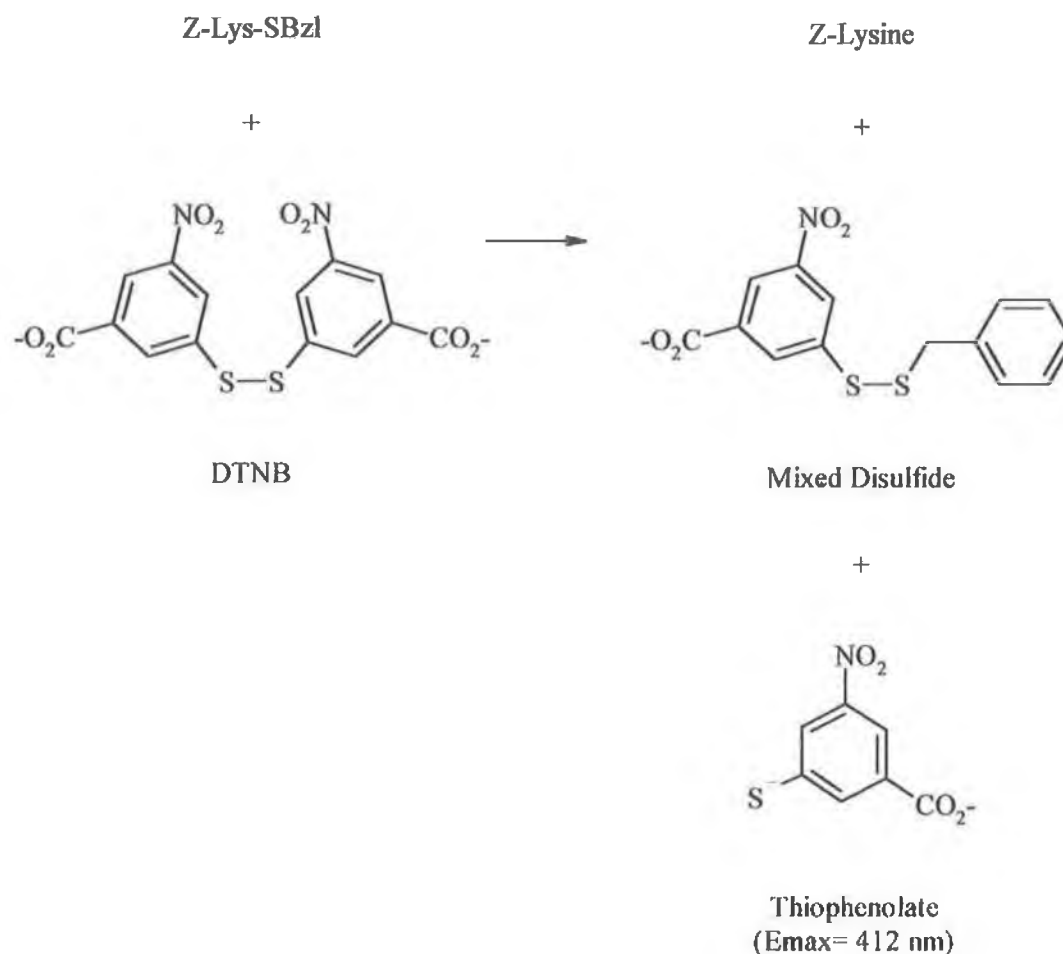


Figure 5.39: The reaction of Z-Lys-SBzl with DTNB to form a coloured product i.e. thiophenolate ion.

It should also be noted that the amide substrate was an Arg derivative while the ester substrate was a Lys compound. The Arg/Lys ratio of native porcine was found to be 0.6

and the EG-trypsin had a ratio of 0.7 (Table 3.4). The reason for this was the commercial availability of substrates.

Kinetic results were obtained for native and EG-trypsin in both aqueous and in 30% (v/v) aqueous/organic solvent systems. The enzyme had been incubated in 30% (v/v) aqueous/acetonitrile solvent at 30°C for one hour before the kinetic experiments. The esterase activity of an enzyme is a useful parameter in peptide synthesis and the aim was to see whether the use of aqueous/acetonitrile solvent systems would affect the esterase/amidase activity.

To determine the difference between the catalytic efficiency of a naturally occurring enzyme and a commercially produced enzyme the kinetics of subtilisin Carlsberg and alcalase were investigated. Monitoring the release of p-nitroaniline from the tetrapeptide sucAAPF-pNa determined the amidase activity of these two enzymes. A yellow colour is produced and a graph of the change in absorbance versus time showed a linear plot up to 540 seconds. At this particular time point the data was converted to rates in $M^{-1}s^{-1}$ using $\epsilon_{414} = 8581 M^{-1}cm^{-1}$ for p-nitroaniline. The results from the active site titrations were also taken into account (section 4.2.1.4), as determined using NTCl by the method described in section 2.3.1.3.

Proteolytic enzymes demonstrate greater catalytic activity towards ester substrates compared to amide substrates (section 5.3) and so the esterase activity was monitored using the corresponding thiobenzyl ester substrate (succAAPF-SBn) so that the activities could be directly correlated. SuccAAPF-SBn liberates a thiobenzyl-leaving group when the enzyme hydrolyses it. The liberated benzyl mercapton reacts with DTNB as described above. These methods are based on those of Plettner et al., 1998. A graph of

the change in absorbance versus time showed a linear plot up to 95 seconds. Again, this particular time point value was used to calculate k_{cat}/K_m . The data was converted to rates in $M s^{-1}$ using $\epsilon_{414} = 8708 M^{-1}cm^{-1}$ for the thiophenolate ion. The results from the active site titrations were also taken into account.

Kinetic results were determined for native subtilisin Carlsberg and alcalase in both an aqueous and 50% (v/v) aqueous/DMF solvent system (section 2.3.2.4). The enzymes were incubated in 50% (v/v) aqueous/DMF solvent systems for one hour at room temperature. The above amidase and esterase kinetic assays (section 2.3.4.1 and 2.3.4.2) were then carried out to determine whether the change in the aqueous environment of the enzymes had any effect on the kinetic values of the subtilisin Carlsberg and the alcalase.

5.2 Results:

5.2.1 Trypsin kinetics.

The amidase activity assay was optimised with respect to both substrate and enzyme concentration (Figure 5.40). The optimum concentrations were determined to be 1.6mM L-BAPNA (0.174mM in the well) and 4.2 μ M porcine trypsin solution. The k_{cat}/K_m value was determined to be 1225 s⁻¹M⁻¹.

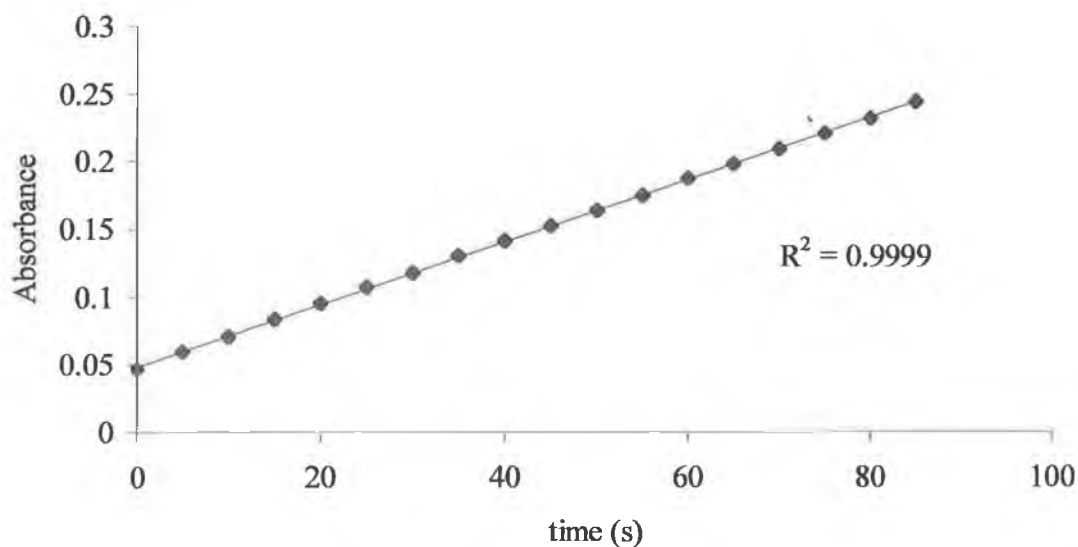


Figure 5.40: A graph of the change in absorbance versus time (seconds) of native porcine trypsin for amidase activity in 0.1M Tris-HCl solution, pH 8.6, at room temperature.

For the esterase assay, the k_{cat}/K_m value was determined to be 4.29*10⁴ s⁻¹M⁻¹. The optimum enzyme concentration was determined to be 3.52*10⁻⁷M and the optimum substrate concentration was 0.065mM (Figure 5.41).

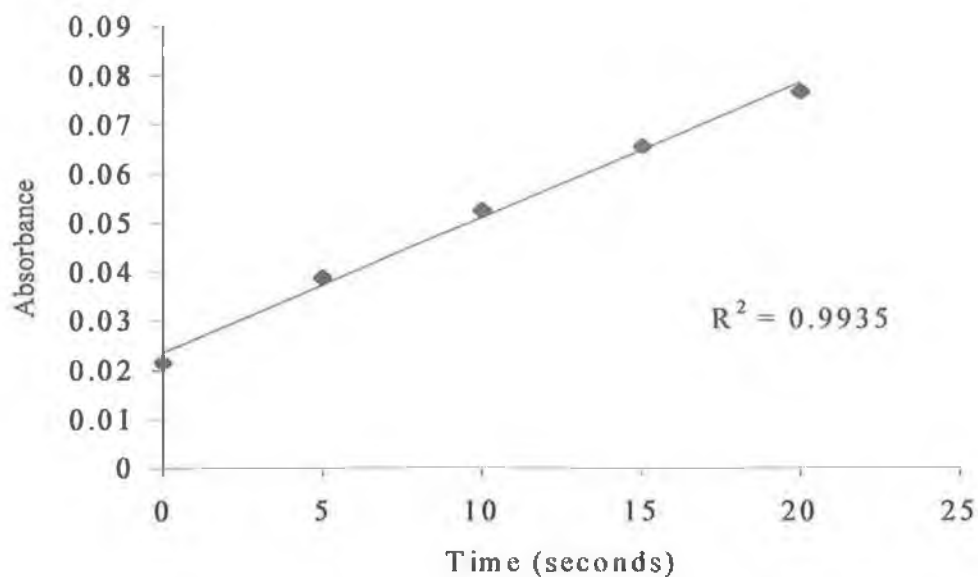


Figure 5.41: A graph of the change in absorbance versus time (seconds) for native porcine trypsin for esterase activity in 0.1M Tris-HCl containing 0.375mM DTNB, pH 8.6, at room temperature.

The above assays were also used to determine the activity of native porcine trypsin in a 30% acetonitrile/aqueous solution and EG-trypsin in both an aqueous solution and a 30% acetonitrile/aqueous solution. All plots of absorbance versus time had $r^2 \geq 0.99$ over the period of the assay for both the amidase and esterase activity. Each assay was performed in triplicate and the average values obtained experimentally for these assays are summarised in Table 5.9 and Table 5.10 using the calculations described in Figure 5.42.

$$k_{cat}/K_m \cong v / [S] * [E]$$

and

$$v = (\Delta \text{ Absorbance per sec}) / \epsilon_{414}$$

Where:

$$v = \text{rate (M s}^{-1}\text{)}$$

$$\epsilon_{414} = 8581 \text{ M}^{-1}\text{cm}^{-1} \text{ for } p\text{-nitroaniline (Plettner et al., 1998)}$$

$$[S] = \text{Substrate concentration} = 0.00017\text{M}$$

$$[E] = \text{Enzyme concentration with active site titration taken into account} =$$

$$(4.2 * 10^{-6}\text{M}) * 40 \% \text{ (determined by the method in section 2.3.1.4)} = 1.7 * 10^{-6}\text{M}$$

Calculations:

$$v = (0.4534 / 60 \text{ seconds}) / 8581 \text{ M}^{-1}\text{cm}^{-1} = \underline{8.8 * 10^{-7} \text{ M s}^{-1}}$$

$$k_{cat}/K_m \cong (8.8 * 10^{-7} \text{ M}^{-1}\text{s}^{-1}) / ((0.00017\text{M}) * (1.7 * 10^{-6}\text{M}))$$

$$k_{cat}/K_m \cong \underline{2768.2}$$

Figure 5.42: An example of a calculation to determine the k_{cat}/K_m values for the amidase activity of native porcine trypsin

Table 5.9: The esterase and amidase activity of native porcine and EG-trypsin in both aqueous and 30 % ACN/aqueous solution and the optimum values for enzyme and substrate concentration in these assays.

		[E] M	[S] M	k_{cat}/K_m ($s^{-1}M^{-1}$)
Native trypsin (aqueous)	Amidase	$4.23 \cdot 10^{-6}$	$1.7 \cdot 10^{-4}$	2768.2
	Esterase	$3.52 \cdot 10^{-7}$	$6.5 \cdot 10^{-5}$	$4.29 \cdot 10^4$
Native trypsin (30% ACN)	Amidase	$8.8 \cdot 10^{-7}$	$1.7 \cdot 10^{-4}$	157
	Esterase	$5.5 \cdot 10^{-8}$	$1.1 \cdot 10^{-6}$	$3.58 \cdot 10^5$
EG-trypsin (aqueous)	Amidase	$1.89 \cdot 10^{-6}$	$1.7 \cdot 10^{-4}$	263
	Esterase	$2.35 \cdot 10^{-7}$	$6.5 \cdot 10^{-5}$	$4.66 \cdot 10^4$
EG-trypsin (30% ACN)	Amidase	$8.8 \cdot 10^{-7}$	$9 \cdot 10^{-4}$	331
	Esterase	$6.2 \cdot 10^{-8}$	$6.5 \cdot 10^{-5}$	$1.4 \cdot 10^5$

Table 5.10: The esterase/amidase ratios of native porcine trypsin and EG-trypsin in aqueous and 30% aqueous/acetonitrile solutions.

	Esterase/amidase ratio
Native porcine trypsin, aqueous soln.	15.5
Native porcine trypsin, 30% ACN.	2280
Porcine EG-trypsin, aqueous soln.	177
Porcine EG-trypsin, 30% ACN.	423

5.2.2 Subtilisin Carlsberg kinetics

The amidase activity assay was optimised with respect to both enzyme and substrate concentration. The optimum enzyme concentration for amidase activity was found to be 6×10^{-10} M for native subtilisin Carlsberg and the optimum substrate concentration was calculated to be 5×10^{-4} M. Visual observation of the graph of absorbance versus time (seconds) showed that the optimum time for this reaction was 540 seconds. The optimum enzyme concentration for esterase activity was found to be 1.1×10^{-9} M and the optimum substrate concentration was 1.1×10^{-5} M. Visual observation of the graph showed that the optimum time for the reaction is 95 seconds (Table 5.11).

For the alcalase the optimum enzyme concentration was determined found to be 3×10^{-9} M for the amidase activity and 8×10^{-10} M for the esterase activity. The optimum substrate concentrations remained the same for both kinetic assays (Table 5.11).

The above assays were also used to determine the activity of these two enzymes in a 50% DMF/aqueous solution. The values obtained for the catalytic efficiency of the enzymes in this organic solvent/aqueous solution are shown in Table 5.11.

All of the above plots of absorbance versus time had $r^2 \geq 0.99$ over the period of the assay for both the amidase and esterase activity. Each assay was performed in triplicate and the average values obtained experimentally for all these assays are summarised in Table 5.11 and Table 5.12.

Table 5.11: Catalytic efficiency of both subtilisin Carlsberg and alcalase in both aqueous and 50%v/v aqueous/DMF solution and the optimum values for enzyme and substrate concentration in these assays.

		[E] in well (M)	[S] in well (M)	k_{cat}/K_m ($s^{-1}M^{-1}$)
Subtilisin Carlsberg (aqueous)	Amidase	$5.9 \cdot 10^{-10}$	$4.6 \cdot 10^{-4}$	$8.1 \cdot 10^5$
	Esterase	$1.2 \cdot 10^{-9}$	$1.2 \cdot 10^{-5}$	$2.3 \cdot 10^7$
Subtilisin Carlsberg (50% v/v DMF soln)	Amidase	$2.7 \cdot 10^{-9}$	$4.6 \cdot 10^{-4}$	$2.2 \cdot 10^5$
	Esterase	$1.3 \cdot 10^{-9}$	$1.2 \cdot 10^{-5}$	$2.5 \cdot 10^7$
Alcalase (aqueous)	Amidase	$3.1 \cdot 10^{-9}$	$4.6 \cdot 10^{-4}$	$1.1 \cdot 10^5$
	Esterase	$7.6 \cdot 10^{-10}$	$1.2 \cdot 10^{-5}$	$6.1 \cdot 10^7$
Alcalase (50% v/v DMF soln)	Amidase	$2.8 \cdot 10^{-9}$	$4.6 \cdot 10^{-4}$	$1.4 \cdot 10^5$
	Esterase	$1.3 \cdot 10^{-9}$	$1.2 \cdot 10^{-5}$	$6.1 \cdot 10^6$

Table 5.12: The esterase/amidase ratios of subtilisin Carlsberg and alcalase in aqueous and 50% aqueous/DMF solutions.

	Esterase/amidase ratio
Subtilisin Carlsberg, aqueous soln.	29
Subtilisin Carlsberg, 50% v/v DMF soln.	113
Alcalase, aqueous soln.	567
Alcalase, 50% v/v DMF soln.	43

5.3 Discussion:

At very low substrate concentrations, the combination of the substrate with the enzyme becomes rate limiting. That is, the binding of the molecule and the enzyme is a purely random event that occurs when the enzyme and molecule meet. If the substrate is in short supply this event will not occur frequently and the substrate binding therefore becomes the rate limiting effect. The Michaelis-Menten equation becomes $v \approx k_{cat}/K_m [E][S]$ and $[S] \ll K_m$. k_{cat} is defined as the turnover number of the enzyme and represents the number of times each enzyme molecule catalyses the reaction per unit of time. K_m is k_2+k_{-1}/k_1 , i.e. the dissociation constant of the ES complex (Figure 5.43).

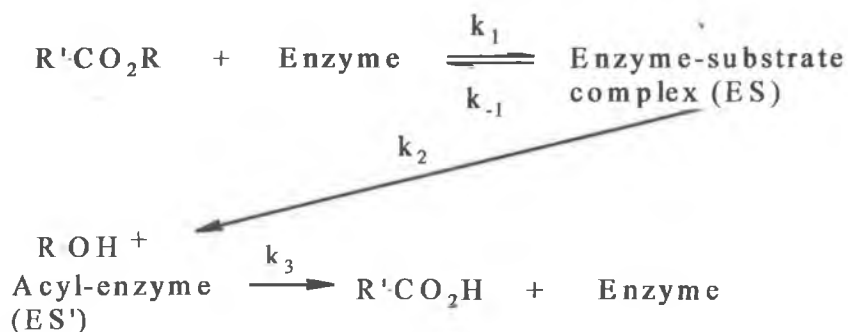


Figure 5.43: An overview of the kinetics involved in a serine proteinase reaction.

The mechanism for the hydrolysis of amide and ester substrates catalysed by serine proteases involves an acylenzyme intermediate, which is formed when the carboxyl portion of the substrate esterifies the hydroxyl group of the reactive serine (Figure 5.43).

The rate-determining step for the hydrolysis of ester substrates by these proteases is the breakdown of the acyl-enzyme intermediate i.e. deacylation (Guinn et al., 1991).

Assuming $k_3 \ll k_2$, then

$$K_m \approx k_1 k_3 / k_2$$

$$k_{\text{cat}} \approx k_3 \quad (\text{see Figure 5.43}).$$

Whereas, in the case of an amide substrate the rate-determining step is the acylation of the enzyme's active site Ser (Guinn et al., 1991).

Assuming $k_2 \ll k_3$, then

$$K_m \approx k_1$$

$$k_{\text{cat}} \approx k_2 \quad (\text{see Figure 5.43}).$$

The nucleophilic attack by the Ser in the active site requires the proper alignment between the enzyme and the bound substrate. Ester hydrolysis depends on the hydrolytic attack of water on the acyl-enzyme bond within the enzyme's active site. Therefore amidase activity should be more affected by active-site conformational changes than the esterase activity. This is important for peptide synthesis as high esterase activity is important for peptide bond formation and low amidase activity is needed to prevent the hydrolysis of the peptide bond formed. When the acyl-enzyme intermediate is formed it can take part in one of two reactions:

- (1) Hydrolysis – that is the breaking down of peptide bonds
- (2) Aminolysis – reaction of the acyl-enzyme with an amine nucleophile resulting in the formation of a peptide bond.

Therefore the higher the esterase/amidase ratio of an enzyme the more efficient it will be as a catalyst for peptide synthesis.

5.3.1 Trypsin:

The literature values for amidase activity of trypsin include Murphy (1996) who obtained a k_{cat}/K_m value of $960\text{s}^{-1}\text{M}^{-1}$ for bovine trypsin and Outzen et al., 1996 obtained a k_{cat}/K_m value of $1890\text{s}^{-1}\text{M}^{-1}$ for porcine (cationic) trypsin. The present value was comparable to these at $2768.2\text{s}^{-1}\text{M}^{-1}$ (Table 5.9). The literature value for esterase activity of bovine trypsin was $7.1 \times 10^5\text{s}^{-1}\text{mM}^{-1}$ (Murphy, 1996), while the present result showed a 60-fold higher value for esterase activity. The overall esterase/amidase ratio for the porcine trypsin was found to be 15.5 and for the bovine trypsin 0.96. These results agree with published literature reports that porcine trypsin is more stable than bovine trypsin to thermal denaturation (Walsh and Wilcox, 1970). It was also observed that porcine EG-trypsin increased its esterase/amidase ratio by 11-fold in an aqueous environment compared to a 3-fold decrease for bovine EG-trypsin under the same conditions (Murphy, 1996).

However for the native porcine trypsin in 30% acetonitrile/aqueous solution the amidase activity was determined to be $157\text{s}^{-1}\text{M}^{-1}$ and for the esterase activity the value was $3.58 \times 10^5\text{s}^{-1}\text{M}^{-1}$, which gave an esterase/amidase ratio of 2280. This was a 147-fold increase for the esterase/amidase ratio compared to that of the native trypsin in a totally aqueous environment. These results would indicate that the native porcine would be a useful catalyst for peptide synthesis, as was previously discussed the greater the esterase/amidase ratio the more efficient the enzyme will be for peptide synthesis (chapter 7). Guinn et al. (1991) also noted that water-miscible organic solvents gave decreased trypsin amidase activities with respect to esterase activities.

It should also be noted that the EG-trypsin in the organic/aqueous cosolvent showed a decreased esterase/amidase ratio compared with the native in the same cosolvent, a 5-fold decrease. The modified form had an increased affinity for the amide substrate and a decreased affinity for the ester substrate compared to that of the native. Amidase activity is more sensitive to active-site changes as proper alignment between the enzyme and the substrate is required for nucleophilic attack of the active-site serine on the substrate molecule bound in the active site (Guinn et al. 1991) and so it was surprising that it was the esterase activity that was adversely effected. The EG-trypsin would therefore not be as suitable a catalyst for peptide synthesis compared to the native in a 30% acetonitrile solution but it would be a better catalyst than the native if the reaction were taking place in an aqueous solution. This will be investigated further in chapter 7.

5.3.2 Subtilisin Carlsberg/Alcalase:

The amidase activity k_{cat}/K_m value established for subtilisin Carlsberg is $8.1 \cdot 10^5 \text{ s}^{-1} \text{ M}^{-1}$ and for the esterase activity $2.34 \cdot 10^7 \text{ s}^{-1} \text{ M}^{-1}$ in an aqueous solution. Chen et al. 1995 obtained a value of $41.7 \cdot 10^7 \text{ s}^{-1} \text{ M}^{-1}$ using the same ester substrate. The subtilisin Carlsberg was found to have an overall esterase/amidase ratio of 29. When Plettner et al. 1998 carried out the same experiments on subtilisin *Bacillus lentus* (SBL) they got an esterase/amidase ratio of 44, which compares quite well.

Alcalase showed a k_{cat}/K_m value of $1.1 \cdot 10^5 \text{ s}^{-1} \text{ M}^{-1}$ for its amidase activity and $6.1 \cdot 10^7 \text{ s}^{-1} \text{ M}^{-1}$ for its esterase value. Therefore, the esterase/amidase ratio was 567 for the alcalase in an aqueous solution. Very little kinetic work on alcalase has been published in the open scientific literature so it is difficult to compare these values. The amidase activity

for the alcalase is lower than the subtilisin Carlsberg (by ~ 14%) and the esterase activity is higher (by ~38%) so the esterase/amidase ratio is 13 fold greater for the alcalase compared to the subtilisin Carlsberg. This would indicate that the alcalase should be a better enzyme for peptide synthesis in an aqueous environment.

Kinetic studies were then carried out on the two enzymes in a 50% (v/v) DMF/aqueous solution. The C_{50} values for subtilisin Carlsberg and alcalase were 56% and 58% v/v DMF/aqueous solution respectively (section 4.2.1.3). While the two enzymes didn't demonstrate the greatest organotolerance in DMF (Table 4.6), it was picked for kinetic studies, as it is one of the most frequently used solvents to dissolve the starting materials for peptide synthesis (Chapter 7).

Subtilisin Carlsberg showed a k_{cat}/K_m value of $2.2 \cdot 10^5 \text{ s}^{-1}\text{M}^{-1}$ for its amidase activity and $2.5 \cdot 10^7 \text{ s}^{-1}\text{M}^{-1}$ for its esterase activity resulting in an esterase/amidase ratio of 113. This represented a 4-fold increase of the esterase/amidase ratio in the presence of DMF. Observations of the relative k_{cat}/K_m values (Table 5.11) for the enzyme in both an aqueous and 50 % DMF medium show that the esterase values show little change. However the amidase activity shows a 27% decrease in activity in the 50% (v/v) DMF/aqueous solution (Table 5.11), leading to a 4-fold rise in the esterase/amidase ratio. This would increase its effectiveness in terms of peptide synthesis (Chapter7). Lozano et al., 1996 investigated the effect of five aprotic solvents on the stability of another protease (α -chymotrypsin). They found that the more hydrophilic solvents (DMF and DMSO) showed a slight decrease in the deactivation rate constants (k_1 and k_2) at low solvent concentrations and so at these concentrations they helped protect against enzyme deactivation.

The alcalase showed amidase activity of $1.4 \cdot 10^5 \text{ s}^{-1}\text{M}^{-1}$ and the k_{cat}/K_m value for the esterase activity was found to be $6.1 \cdot 10^6 \text{ s}^{-1}\text{M}^{-1}$ in a 50% (v/v) DMF solution. This gave the enzyme an esterase/amidase ratio of 43 in 50% DMF solution. This is a 13-fold decrease in the esterase/amidase ratio in this environment compared to that of the aqueous. The amidase activity has stayed at approximately the same value whereas the esterase has had a 10-fold decrease in esterase activity. Khmelnitsky et al. (1988) stated that a maximal activity in organic solvent is achieved if the pH value of the starting aqueous enzyme solution corresponds to the pH optimum of its catalytic activity in water. The aqueous solution that was used in the kinetic studies was at pH 8.4 for both subtilisin Carlsberg and alcalase, which is in the optimum pH range for both enzymes. Shubhada and Sundaram (1995) have observed that the change in pH of the buffer caused by the addition of an organic solvent is due to:

- The nature of the buffer
- The nature of the solvent
- The concentration of the solvent

They determined that in the case of a 50% (v/v) DMF/0.01M sodium phosphate solution the pH would show an increase of 2. If the same happened in my solution this would leave the alcalase solution at pH 10. Alcalase demonstrates maximum activity at pH 8 – 9 and shows a decrease of approx. 10% in proteolytic activity at pH 10 (Figure 5.44 obtained from www.novozymes.com). Proteolytic activity is the catalytic efficiency of the enzyme on a large molecule as compared to esterase or amidase activity, which is measured against substrates like sAAPF-pNa. This may account for the change in catalytic activity of the enzyme. Subtilisin Carlsberg on the other hand maintains

stability in the pH range 7 –10 so this may be why there was no observable difference for its catalytic activity.

The alcalase in the aqueous solution showed the greatest catalytic efficiency (Table 5.12). This enzyme preparation is used in washing detergent formulations and so is designed to operate at a moderate alkalinity in an aqueous environment. These results would seem to indicate that the alcalase would be the most suitable for peptide synthesis in an aqueous system.

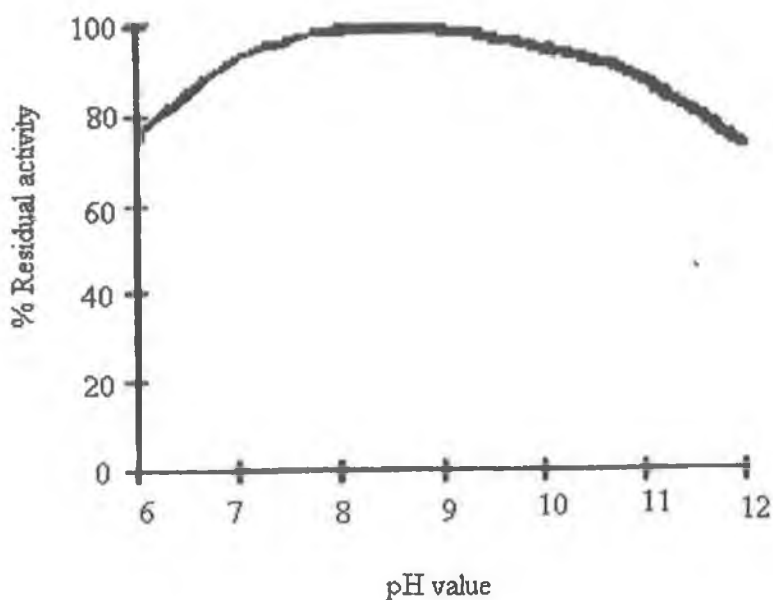


Figure 5.44: Activity of alcalase at different pH values (www.novozymes.com)

The performances of native and EG-modified trypsin, subtilisin Carlsberg and alcalase in peptide synthesis reactions will be explored in chapter 7.

CHAPTER 6

FORMYLATION of PORCINE TRYPSIN

6.1 Introduction

Trypsin demonstrates both amidase and esterase activity. Coletti-Previero et al. (1969) showed, by chemical modification of tryptophan residues in bovine trypsin to l-formyltryptophan, that the enzyme shows esterase activity but no amidase activity. This could be useful, as esterase activity is important in relation to peptide synthesis (Section 1.2.2). Coletti-Previero et al. (1969) formylated the tryptophan residues by using gaseous hydrochloric acid in anhydrous formic acid solution at room temperature.

A more convenient and less hazardous method was investigated, as gaseous HCl would have to be synthesised in the lab and great care would have to be taken when using it. Also, formic acid is very corrosive and extra safety measures would have to be implemented if it was to be used in the reaction solution. N-formylimidazole is very useful in many types of synthesis and it has been shown that it can be used for the N-formylation of amines to formamides. The tryptophan residues are a type of amine and l-formyl-tryptophan a type of formamide. Extensive investigation has been carried out in this area and there are many published reports on this particular type of reaction. For example, Villeneuve and Chan (1997) generated formyl chloride by the reaction of hexachloroacetone and triphenylphosphine at -78°C in dichloromethane. The formyl chloride could then have been reacted with imidazole to form N-formylimidazole. A disadvantage to this procedure would be maintaining the low temperatures required for the reaction. Kitagawa et al (1994) found that formyl chloride, which requires careful exclusion of moisture, could be produced *in situ* by the reaction of oxalyl chloride on formic acid. They then reacted this formyl chloride with imidazole to form N-formylimidazole in a "one-pot" reaction.

This experimental procedure was then taken and applied to the conversion of tryptophan (which is an amine) to l-formyltryptophan (a formamide) (Figure 6.45) in our laboratory. Investigations were carried out to determine the effects of this modification on the enzyme and to compare and contrast it with the native form. These experiments include; (i) Stability studies i.e. temperature profile, active site titration, (ii) Activity assays i.e. active site titration and (iii) Kinetic studies i.e. amidase and esterase assays.

As was previously described in Chapter 3 trypsin was crosslinked with the homobifunctional reagent EG-NHS. It was decided to formylate this EG-trypsin using the same procedure as for the native, to investigate if this would produce an even more stable enzyme and/or a better esterase/amidase ratio. The EG-trypsin has been shown to have increased stability and catalytic activity compared to the native enzyme (Chapter 3) and it was thought that a double modification might produce an even more efficient enzyme. Again investigations were carried out to determine the effects of this modification on the enzyme and to compare and contrast it with the native form and with the EG-trypsin.

It was also noted by Coletti-Previero et al. (1969) that the amidase activity could be restored after deformylation of the enzyme. This was achieved by keeping the enzyme at pH 9.5 using a pH stat for over an hour and measuring the change in the enzyme spectrophotometrically. This deformylation was conducted on the formylated trypsin obtained by the method described by Kitagawa et al. (1994).

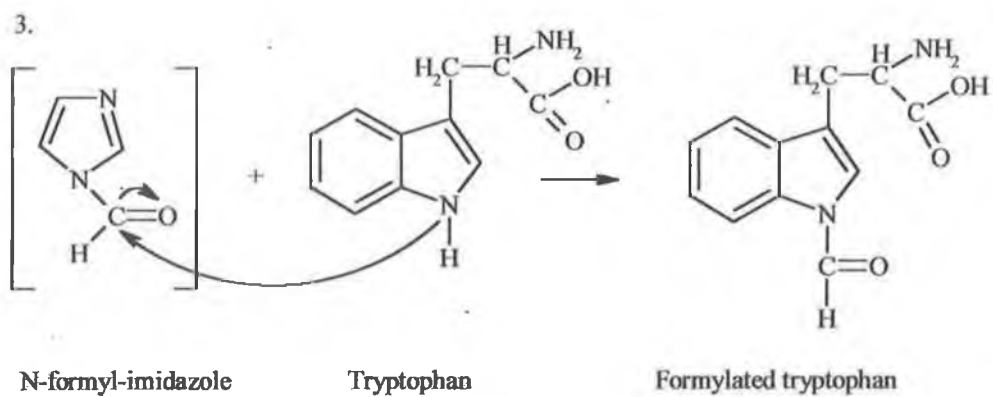
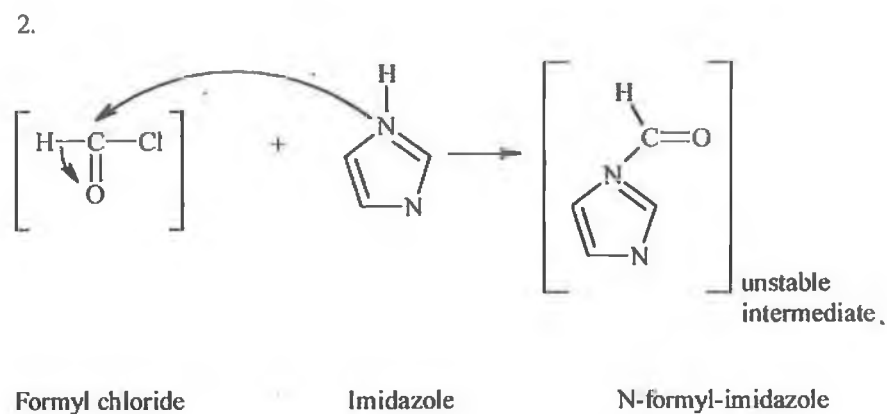
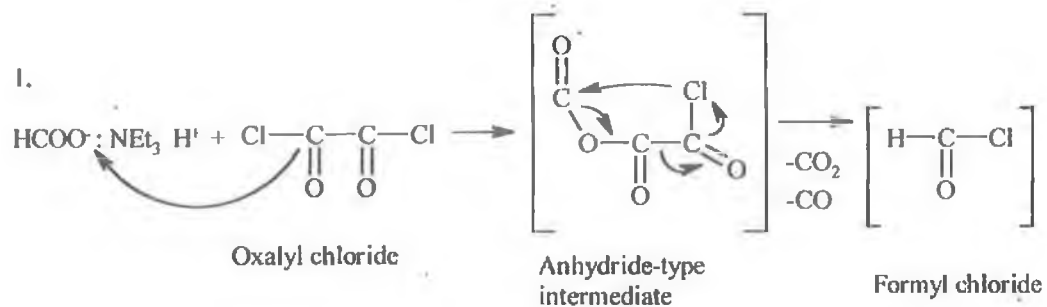


Figure 6.1: A schematic diagram showing the conversion of tryptophan to *l*-formyltryptophan.

6.2 Results

6.2.1 Formylation of native trypsin

6.2.1.1 UV-visible spectra to determine formylation

The UV-visible scan (Figure 6.46) clearly shows that the tryptophan residues have been converted to l-formyltryptophan as the indole ring of the tryptophan shows a peak at 280nm but when it is changed to an aldehyde the peak is much broader.

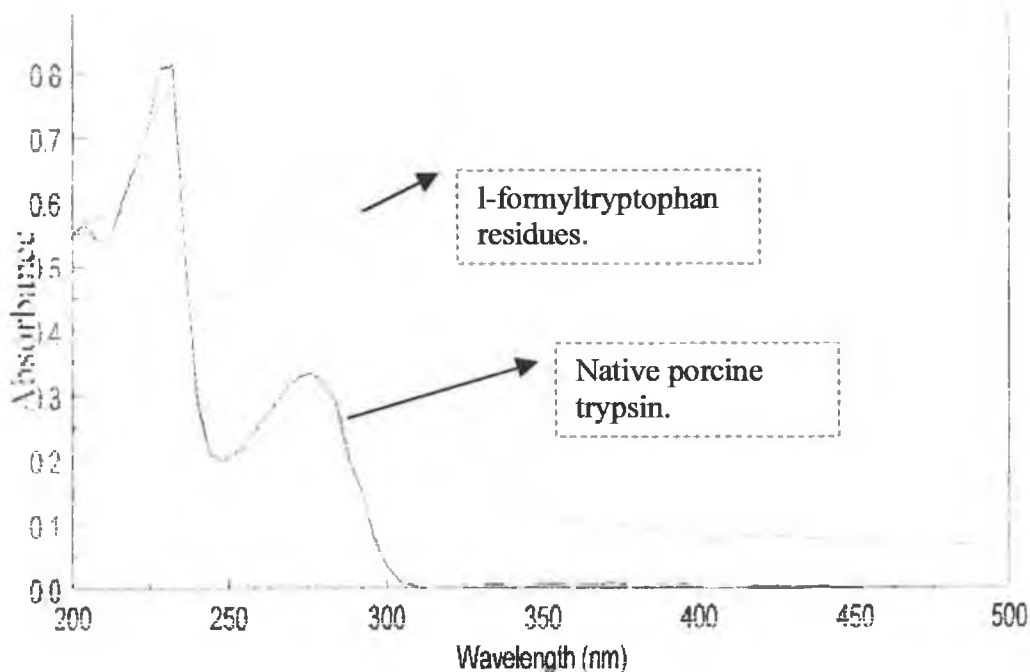


Figure 6.46: A UV/Visible scan of the formylation of the tryptophan residues in native porcine trypsin.

6.2.1.2 Determination of optimum time for formylation

The UV-visible scans (Figure 6.47) show that the optimum time for the formylation experiment was 90 minutes, as up to this the indole ring of the trypsin remained

unchanged. After 90 minutes no further change was observed in the UV-visible spectra (data not shown)

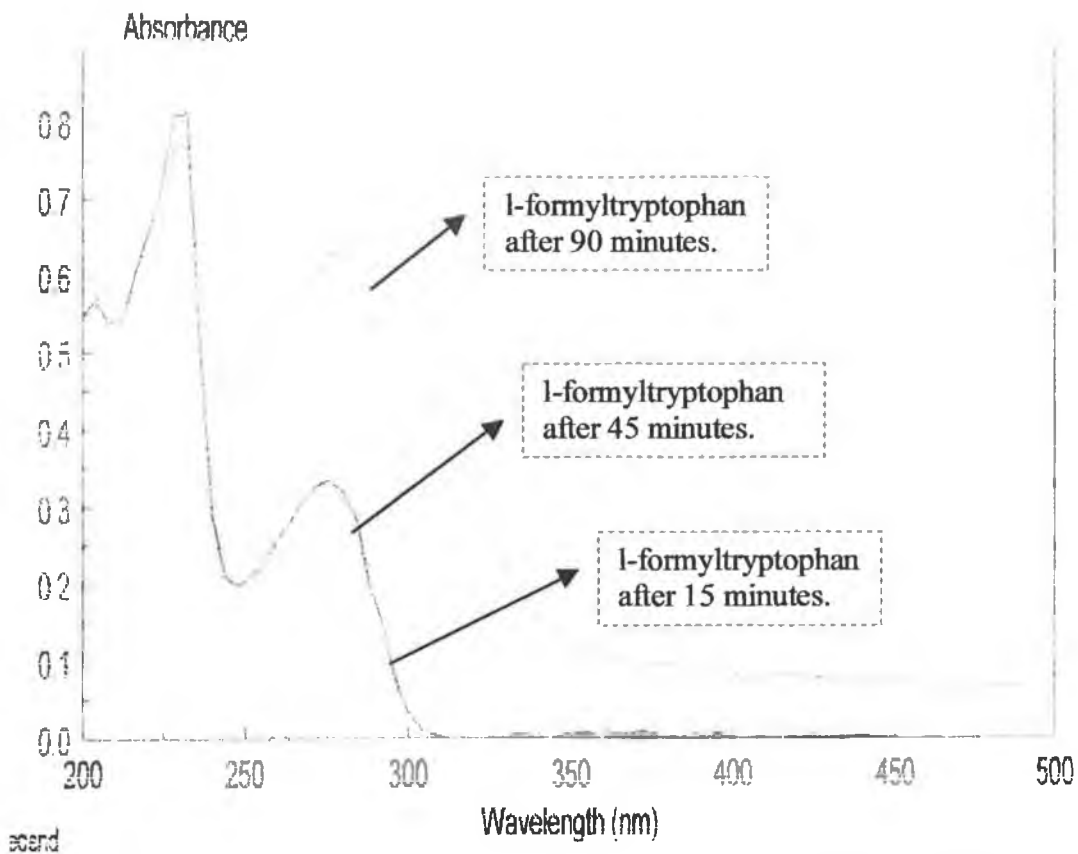


Figure 6.47: A UV-Vis scan of the change in the tryptophan residues in native porcine trypsin as compared with the l-formyltryptophan residues over time.

6.2.1.3 Temperature profile

The temperature profile shows that the formylated trypsin retained 100% esterase activity up to a temperature of 50 °C. The T_{50} value for formylated porcine trypsin was found to be 55 °C, whereas the T_{50} value for native porcine trypsin (Figure 6.48) was determined to be 45 °C, according to Section 2.3.4.1.

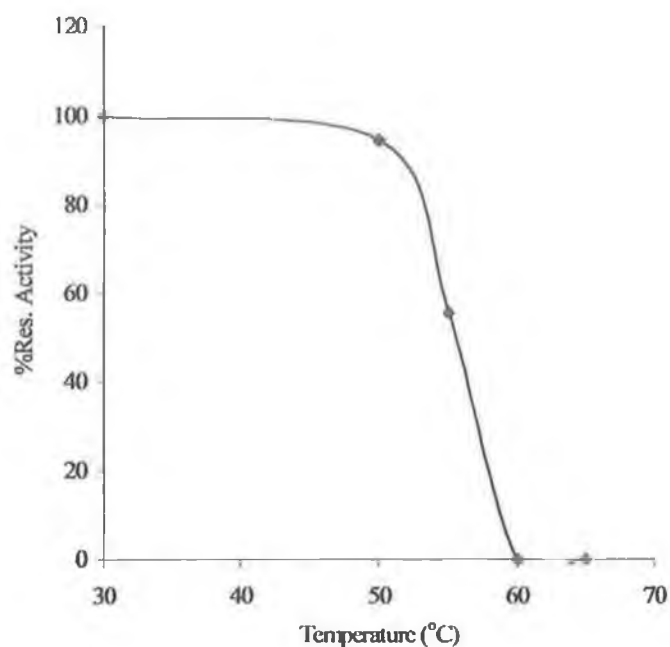


Figure 6.48: A temperature profile of the formylated trypsin.

6.2.1.4 Active site titration

An active site titration, using the method described in Section 2.3.1.3, showed that the formylated trypsin retained 75% of the activity of the native trypsin.

6.2.1.5 Amidase activity

The amidase activity of the formylated trypsin was investigated according to Section 2.3.4.1. No amidase activity was detected at this concentration, i.e. $0.225\text{mg}\cdot\text{ml}^{-1}$, and a further experiment with 10 times the concentration yielded no amidase activity either.

6.2.1.6 Esterase activity

The esterase activity was determined using the substrate Z-Lys-SBzl (Section 2.3.4.2). A linear graph for change in absorbance versus time was observed (Figure 6.49). The

graph was linear for up to 60 minutes whereas for native trypsin the graph (Figure 3.4) was linear only up to 20 seconds. The k_{cat}/K_m was found to be $1.7 \times 10^{-9} \text{ s}^{-1}\text{M}^{-1}$ for the ester substrate for the formylated trypsin.

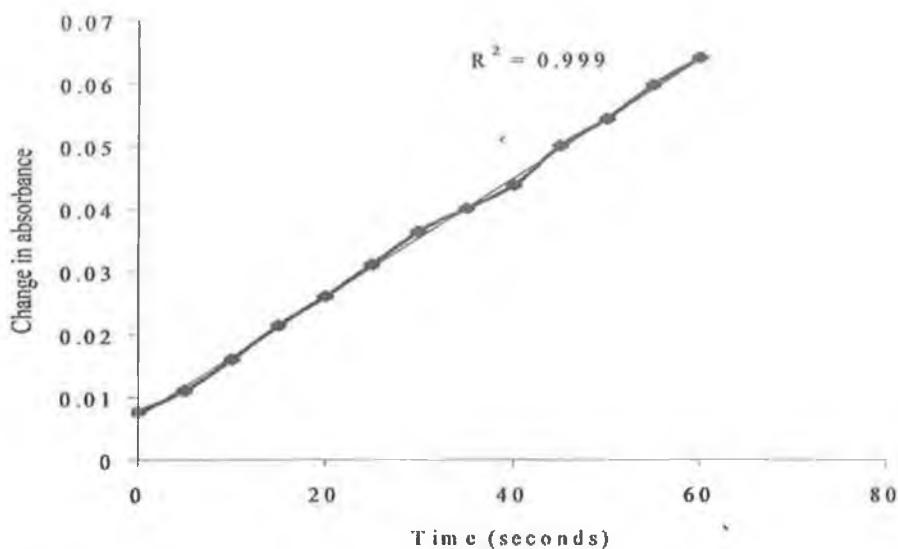


Figure 6.49: Change in absorbance versus time for formylated trypsin using Z-Lys-SBzl as the substrate.

6.2.2 Formylation of EG-trypsin

6.2.2.1 UV-visible spectra to determine formylation

A UV-visible scan (Figure 6.50) for formylated EG-trypsin revealed there was only a slight difference between it and the EG-trypsin. The time of the reaction used was 90 minutes in all cases as this produced optimum results.

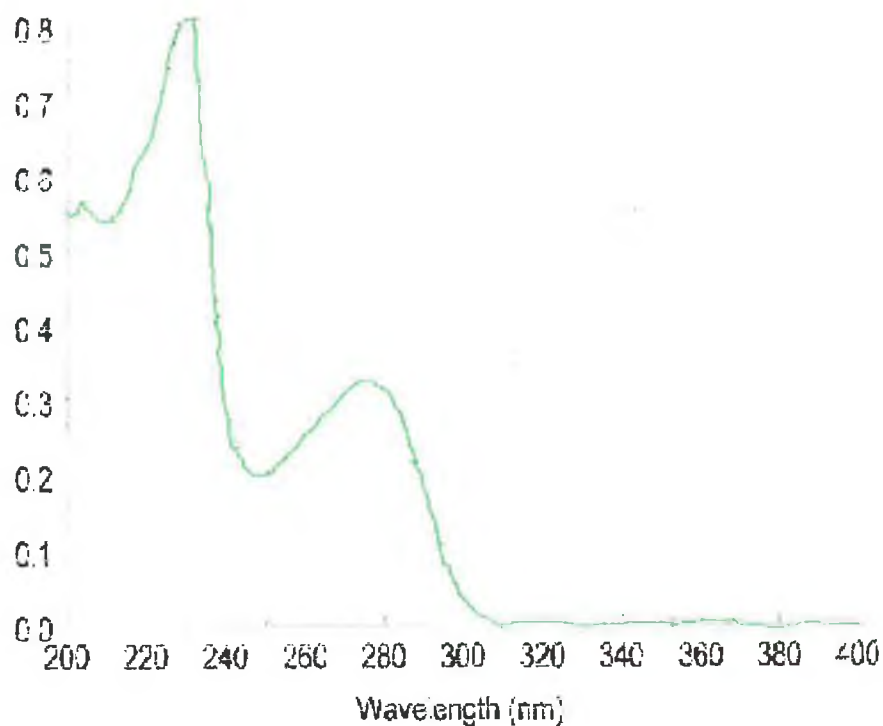


Figure 6.50: UV-visible scan of the formylated EG-trypsin.

6.2.2.2 Active site titration

The active site titration (Section 2.3.1.3) showed that the formylated EG-trypsin retained 82.5% of the activity of the native trypsin.

6.2.2.3 Temperature profile

The temperature profile (Figure 6.51) showed that the formylated EG-trypsin did not retain 100% of its esterase activity above 30°C. The T_{50} value was 45°C. This was the same as the native trypsin, but lower than the value for the formylated trypsin which was 55°C. So the formylation of the enzyme after EG treatment counteracts the stabilising effect of the EG treatment alone.

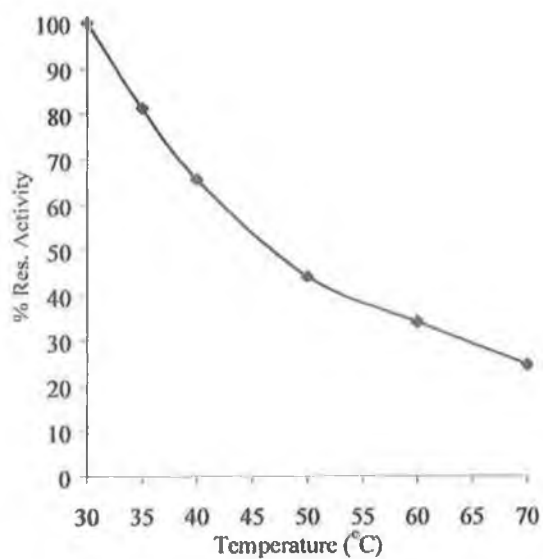


Figure 6.51: The temperature profile of formylated EG-trypsin.

6.2.2.4 Esterase activity

The esterase activity of the formylated EG-trypsin was found to be $4.73 \times 10^{-5} \text{ s}^{-1}\text{M}^{-1}$. The graph of the change in absorbance versus time (seconds) was linear for up to twenty seconds (Figure 6.52) which was similar to the graph obtained for the native trypsin (Figure 3.4).

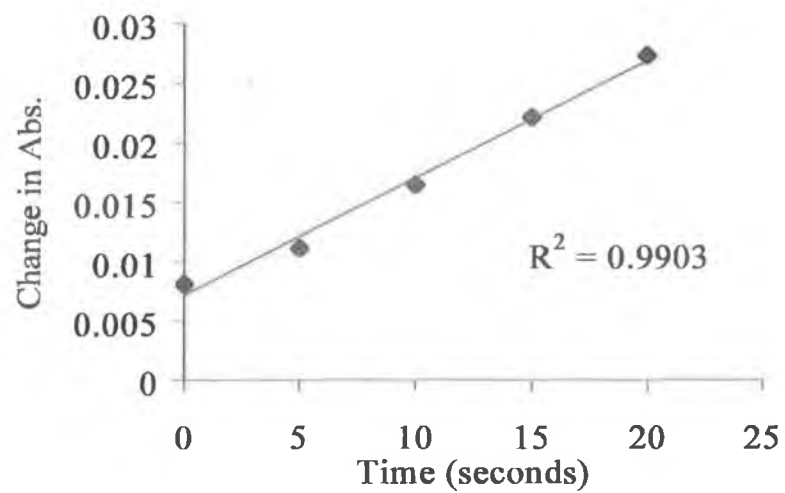


Figure 6.52: Change in absorbance versus time for the formylated EG-trypsin using Z-Lys-SBzl as the substrate.

6.3 Discussion

The UV-visible scan of the formylated trypsin shows no peak at 280nm whereas, for the same concentration, native trypsin does show a peak at this wavelength in 8M urea at pH 4.0. The indole ring of the tryptophan shows a peak at 280nm; when it is changed to an aldehyde, no peak is observed. The UV-visible scan closely resembles that published by Coletti-Previero et al. (1968).

The temperature profile would suggest that this type of modification makes the enzyme more thermostable as compared to native trypsin at the same concentration.

The esterase activity showed a k_{cat}/K_m value of $1.7 \times 10^{-9} \text{ s}^{-1}\text{M}^{-1}$, this is 10^{-10} times smaller than the k_{cat}/K_m value for the native porcine trypsin obtained in Section 4.2.1. There was no detectable amidase activity. The loss of this activity is thought to be due to the fact that the polar formyl group causes a decrease in the hydrophobic interactions inside the trypsin molecule. The esterase activity appears to be unaffected by these decreased hydrophobic interactions in the trypsin molecule. This modification would not increase the ability of the enzyme to be used in peptide synthesis. Even though the esterase activity is present it is demonstrating a very low k_{cat}/K_m value i.e. $1.7 \times 10^{-9} \text{ s}^{-1} \text{M}^{-1}$ and the greater the esterase/amidase ratio the better in regards to peptide synthesis (Kasche, 1989).

The UV-visible scan of the formylated EG-trypsin showed no change to the tryptophan residues, as there was still a peak at 250nm but it isn't as broad as the corresponding peak for the formylated trypsin. The UV-visible scan of the EG-trypsin was the same as that for the formylated EG-trypsin and for the native, showing that there was no change in the tryptophan residues of the trypsin.

The temperature profile showed that the formylated EG-trypsin was no more stable than the native trypsin. Therefore, there would be no advantage to this type of double modification. There was esterase activity present but no amidase activity, suggesting that some sort of reaction took place.

The EG-trypsin is a crosslinked form of trypsin. As can be seen from Figure 6.9, some of the lysine residues lie very close to the tryptophan residues and the crosslinkers may have caused a type of hindrance to the formylation experiment. The porcine trypsin has been cross-linked with EG-NHS, which has a maximum molecular linkage length of 16Å (Ji, 1983) between the lysine residues (Figure 6.53) before the formylation experiment was attempted.

The deformylation experiment was not successful. It was thought that perhaps the use of the organic solvent dichloromethane irreversibly changed the enzyme and so the l-formyltryptophan residues couldn't be converted to tryptophan residues as described by Coletti-Previero et al (1969).

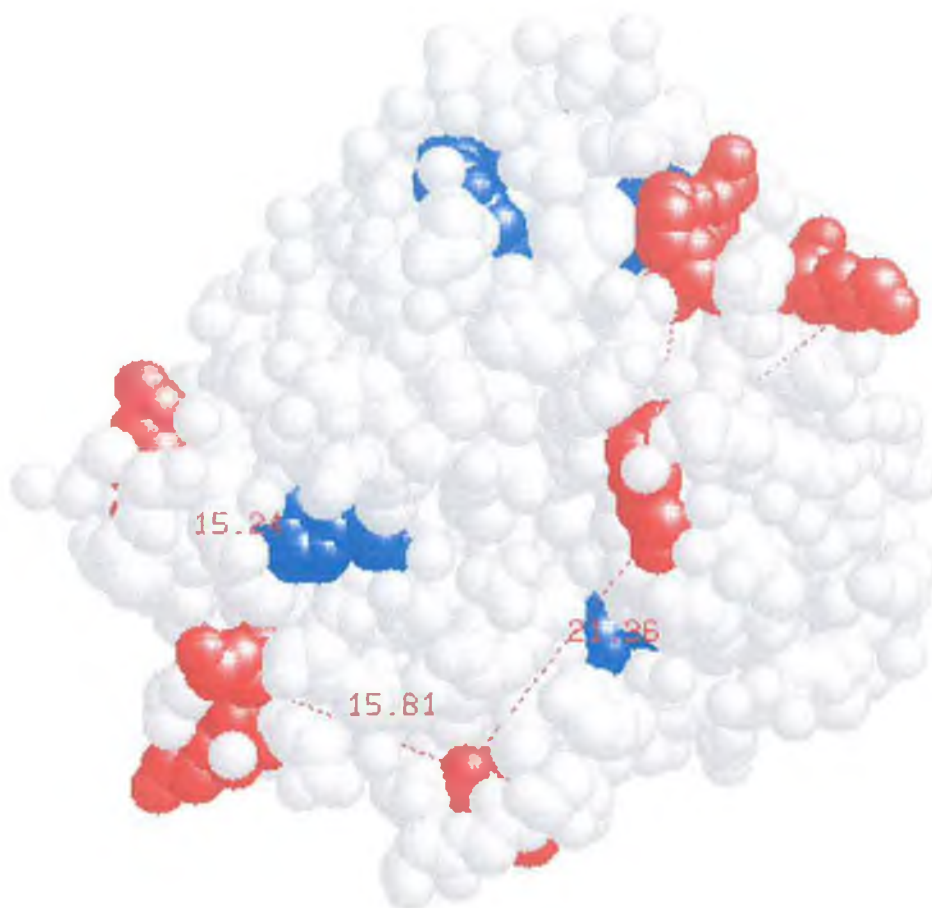


Figure 6.53: The distance between some of the lysine (red) nearest to the tryptophan residues (blue) in a native porcine trypsin molecule (Bernstein et al., 1977 and www.umass.edu/microbio/rasmol).

CHAPTER 7

PEPTIDE SYNTHESIS

7.1 Introduction.

Proteases belong to a group of enzymes whose catalytic function is to hydrolyse (break down) the peptide bonds of proteins. However, as already discussed in section 1.4.2, they can be manipulated to form peptide bonds under certain reaction conditions. Some of the advantages of using this type of peptide synthesis compared to a chemical approach are: a need for side-chain protection is minimal, no racemization occurs in solution, only mild reaction conditions are required for synthesis, high regioselectivity and as there are less hazardous by-products this method is also better for the environment.

There are also a few disadvantages associated with this type of peptide synthesis. In particular, proteases are prone to thermoinactivation and autolysis. A way of reducing these effects so that the enzyme will be a more efficient peptide synthesis catalyst is to chemically modify it. Chemical modification by crosslinking with EG-NHS has been previously discussed for trypsin (Chapter 3) and subtilisin Carlsberg and alcalase (Chapter 4) and has shown to produce enzymes more resistant to denaturing effects such as high temperatures. In some cases, the catalytic efficiency of the enzyme was improved due to the EG-modification (Chapter 5).

As previously discussed, (Section 1.4.2) there are two types of enzymatic peptide synthesis: thermodynamically controlled synthesis (TCS) and kinetically controlled synthesis (KCS). The kinetic approach was chosen here, as it requires lower volumes of enzymes and has a shorter reaction time compared to the TCS approach.

Initially, synthesis of the dipeptide Bz-Arg-Leu-NH₂ was carried out using porcine trypsin, as this peptide had been successfully synthesised previously with bovine trypsin

(Murphy, 1996). Leucinamide was used as the nucleophile and Bz-Arg-OEt as the acyl donor. Murphy (1996) had determined that the optimum conditions for this synthesis were at 4°C in aqueous acetonitrile, which gave a yield of 59% after 72 hours, so these were the conditions used with the porcine trypsin. The aqueous acetonitrile solution is an ideal reaction environment for peptide synthesis, as the water allows the enzyme to retain its essential bound water and the acetonitrile encourages the reaction to go in the direction of aminolysis rather than hydrolysis of the acyl-enzyme intermediate, thereby allowing peptide synthesis rather than hydrolysis of the intermediate (Figure 1.4).

This reaction was also carried out using EG-trypsin. EG-trypsin demonstrates an increased esterase/amidase ratio compared to native porcine trypsin in both an aqueous and acetonitrile solution (Table 5.10) and so it was thought that it might also be a more efficient catalyst for peptide synthesis.

Klein et al. (1991) synthesised the tetrapeptide Z-Val-Trp-Gly-Gly using subtilisin Carlsberg and Chen et al. (1991) obtained the product Cbz-Tyr-Gly-Gly-OH using alcalase. Both these methods were combined to synthesise the tripeptide Z-Tyr-Gly-Gly-NH₂ and the catalytic efficiencies of subtilisin Carlsberg and alcalase were compared.

The C₅₀ values for subtilisin Carlsberg and alcalase were 56% and 58% v/v DMF/aqueous solution respectively (Section 4.2.1.3) and the subtilisin Carlsberg in particular demonstrates high catalytic efficiency (k_{cat}/K_m) in a 50% v/v DMF solution (4-fold higher esterase/amidase ratio) compared to in an aqueous solution (Table 5.12). So, synthesis of the tripeptide was also carried out in the 50% v/v DMF solution to observe the difference in the rate of the reaction.

7.2 Results:

7.2.1 Synthesis of Bz-Arg-Leu-NH₂ using porcine trypsin:

7.2.1.1 Effect of time on the reaction conditions.

The enzymatic peptide synthesis of Bz-Arg-Leu-NH₂ was carried out as described in Section 2.3.5.1.1. As can be seen from Figure 7.54, all of the BAEE, the acyl donor in this reaction, was consumed after fifty minutes. So, it was decided that the optimum time for this reaction would be sixty minutes to ensure that the entire acyl donor was consumed.

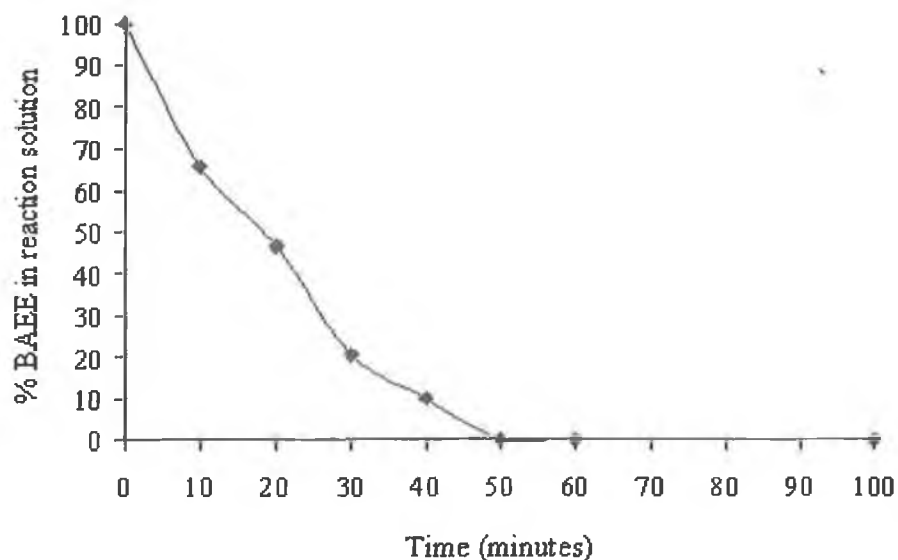


Figure 7.54: Hydrolysis of BAEE during the synthesis of the peptide Bz-Arg-Leu-NH₂.

7.2.1.2 A comparison of native and EG-trypsin in aqueous solution

The reaction was carried out as described in Section 2.3.5.1.1. The actual concentration of the peptide couldn't be determined, as there was no standard peptide available for calibration curves as described by Blanco et al. (1991). Therefore, a graph of the increasing absorbance of the peptide product at 230nm was plotted against time (Figure 7.55). By visual observation of the graph it can be seen that use of EG-trypsin increased the rate of peptide synthesis by approximately 3-fold compared to the native in an aqueous solution.

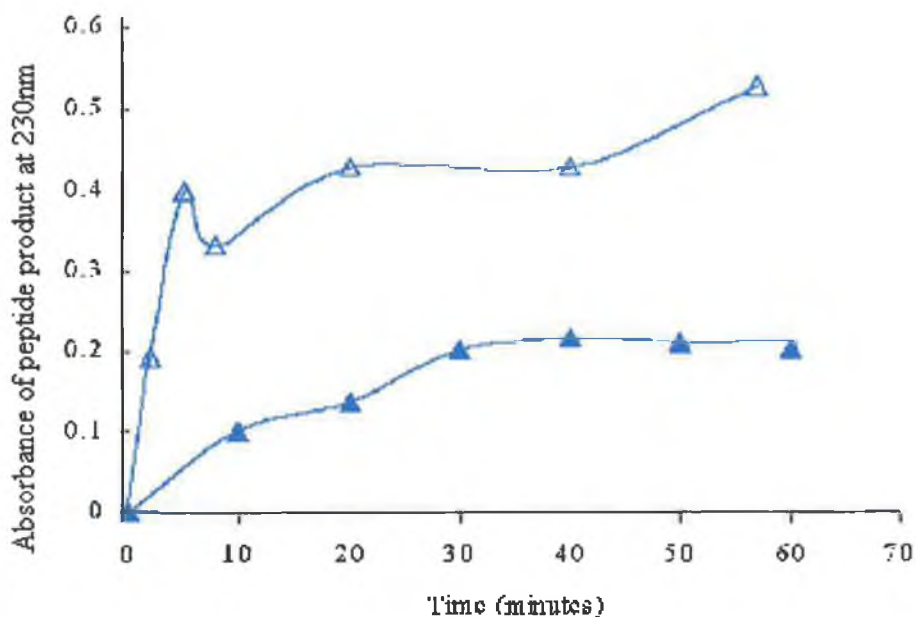


Figure 7.55: Change in absorbance at 230nm of the peptide product versus time with

▲ native trypsin and ▲ EG-trypsin.

7.2.1.3 A comparison of native and EG-trypsin in 30% acetonitrile solution.

The peptide synthesis reaction was carried out as described in Section 2.3.5.1.2 in 30% v/v acetonitrile and the increasing absorbance of the product versus time was plotted (Figure 7.56). The native enzyme had the higher peptide synthesis activity in 30% v/v acetonitrile compared to an aqueous solution. This was to be expected, as native trypsin demonstrated a dramatic increase in esterase/amidase activity in 30% v/v acetonitrile (Table 5.10). EG-trypsin retained approximately the same rate of synthesis in 30% v/v acetonitrile (Figure 7.56) as in aqueous solution.

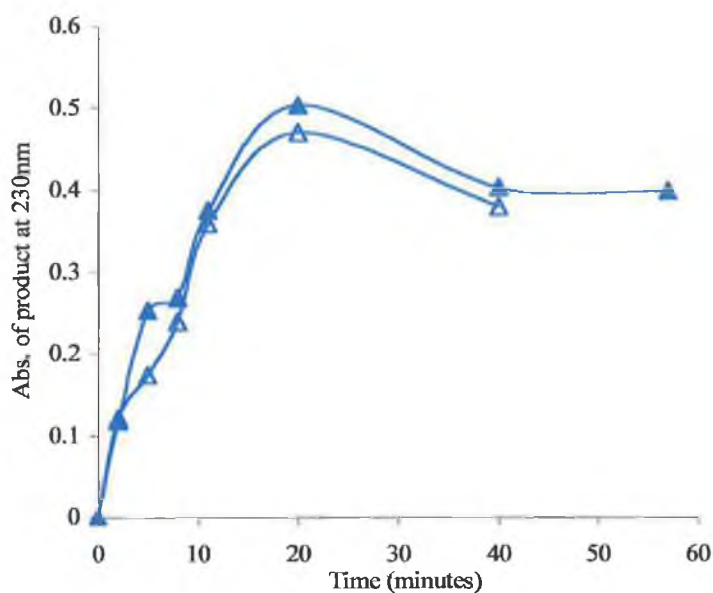


Figure 7.56: Absorbance of the peptide product versus time with ▲ native trypsin and △ EG-trypsin.

7.2.2 Synthesis of Z-Tyr-Gly-Gly-NH₂:

7.2.2.1. Enzyme concentration

Reaction solutions containing subtilisin Carlsberg and alcalase concentrations of 1mg/ml and 2mg/ml were incubated at 4°C as described in Section 2.3.5.2.2. Both concentrations produced the same yield of tripeptide product so it was decided to use the lower concentration to conserve enzyme. A calibration curve was constructed using stock solutions and so the molar concentration of the product could be determined.

7.2.2.2 Effect of temperature on reaction

Using the method described in section 2.3.5.2.3, reaction solutions were incubated at 25°C (Figure 7.57), 37°C (Figure 7.58) and 4°C (Figure 7.59). The greatest yield was observed by the native subtilisin Carlsberg at 25°C, where 1.6mmol of tripeptide product was synthesised. At 37°C, the yield was slightly lower at 1.4mmol product; at this temperature, native alcalase was the more efficient enzyme.

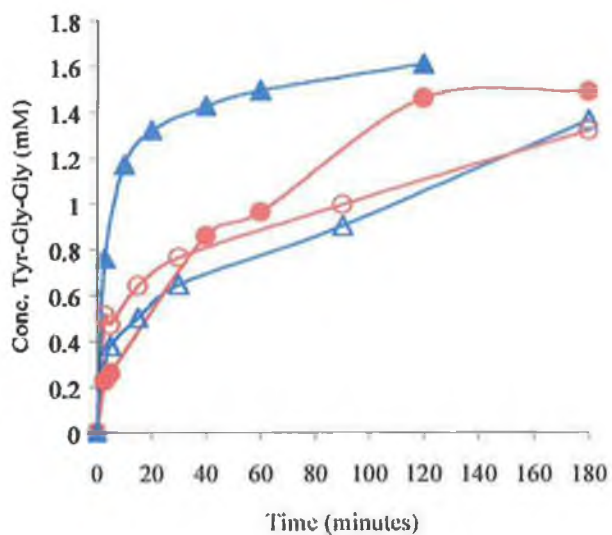


Figure 7.57: Synthesis of the tripeptide Z-Tyr-Gly-Gly at 25°C where ▲ is the native subtilisin Carlsberg, △ is the EG-subtilisin Carlsberg, ● is the native alcalase and ○ is the EG-alcalase.

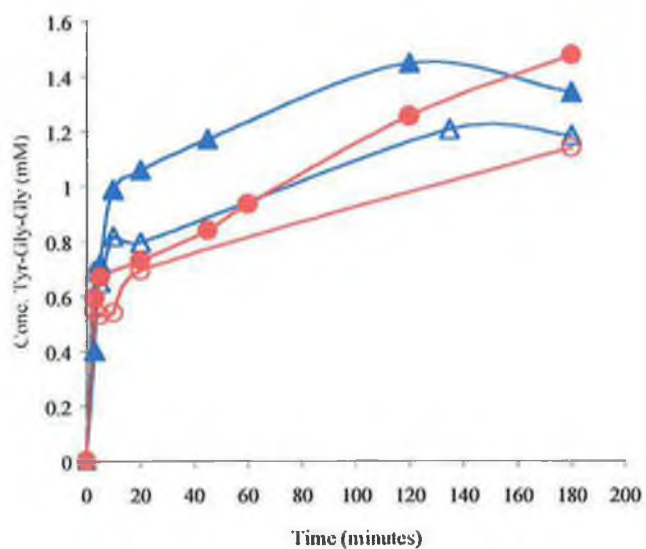


Figure 7.58: Synthesis of the tripeptide Z-Tyr-Gly-Gly at 37°C where ▲ is native subtilisin Carlsberg, △ is EG-subtilisin Carlsberg, ● is the native alcalase and ○ is the EG-alcalase.

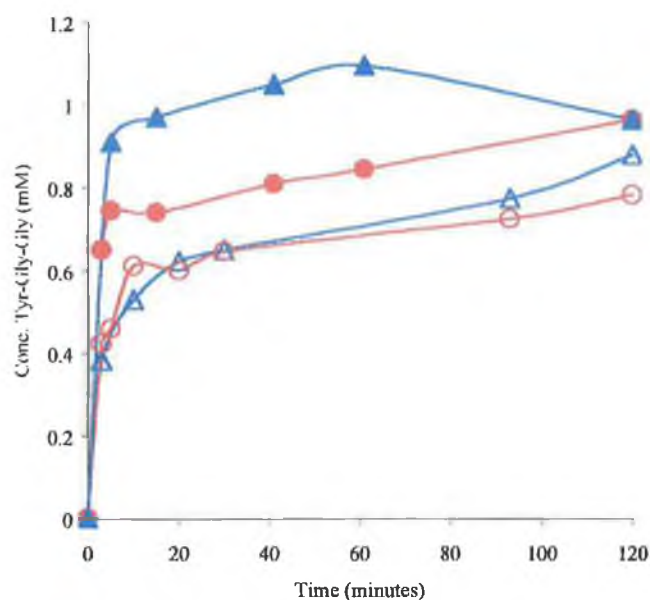


Figure 7.59: Synthesis of the tripeptide Z-Tyr-Gly-Gly at 4°C where ▲ is the native subtilisin Carlsberg, △ is the EG-subtilisin Carlsberg, ● is the native alcalase and ○ is the EG-alcalase.

The lowest yield obtained was at 4°C, where only 1mmol of Z-Tyr-Gly-Gly was formed using native subtilisin Carlsberg. At all temperatures the EG-enzymes gave lower yields of tripeptides compared with the native forms in an aqueous solution. The lowest yields obtained with both EG-alcalase and EG-subtilisin Carlsberg was at 4°C, where they both synthesised only 0.8mmol of peptide product.

7.2.2.3 Effect of DMF on the synthesis of Z-Tyr-Gly-Gly

The reaction was carried out as described in Section 2.3.5.2.4. EG-subtilisin Carlsberg demonstrated the greatest tripeptide yield synthesising 1.12mmol of product over 60 minutes in 50% v/v DMF solution (Figure 7.60) compared to native subtilisin Carlsberg

(which gave a yield of 0.8mmol) in an aqueous solution. This reaction was carried out at 4°C and comparing this to the native subtilisin Carlsberg at the same temperature in an aqueous solution slightly more peptide had been synthesised using the 50% v/v DMF solution. The native alcalase also showed slightly enhanced synthesis in the 50% v/v DMF solution compared to the product yield obtained in the aqueous solution. This was unexpected as the esterase/amidase ratio for the alcalase in the 50% v/v DMF solution was 13-fold lower than the corresponding value in an aqueous solution which should suggest that the enzyme would not be as efficient a catalyst for peptide synthesis in this environment (Table 5.12). As was previously discussed in section 5.3.2, the reason for the decreased esterase/amidase ratio may be due to the fact that the pH of the reaction solution was changed by the addition of the DMF. However when the peptide synthesis reaction was carried out triethylamine was added to ensure the conservation of the pH and so this reaction medium may have increased the catalytic efficiency of the alcalase and subtilisin Carlsberg.

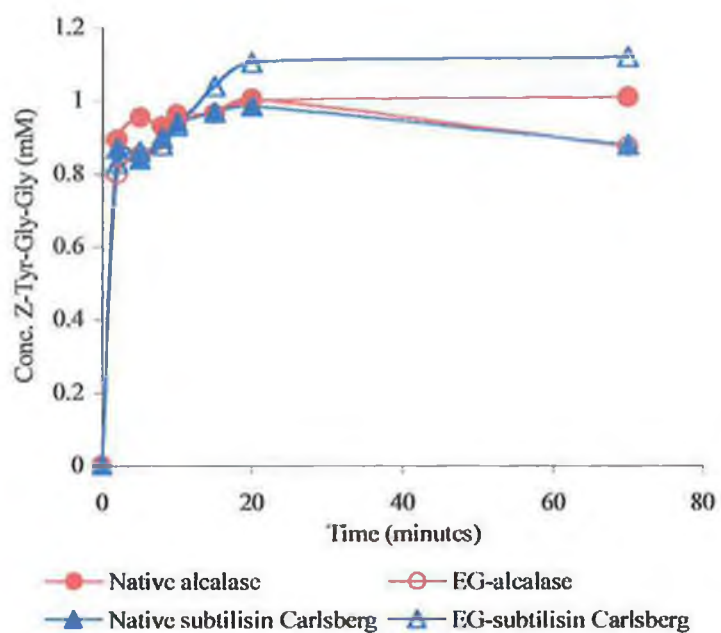


Figure 7.60: Synthesis of the tripeptide Z-Tyr-Gly-Gly in 50% v/v DMF where \blacktriangle is the native subtilisin Carlsberg, \triangle is the EG-subtilisin Carlsberg, \bullet is the native alcalase and \circ is the EG-alcalase.

7.3 Discussion:

The optimum time for the synthesis of the dipeptide, Bz-Arg-Leu-NH₂, was determined to be sixty minutes. This was the time at which the entire amount of the acyl donor i.e. BAEE had been consumed. The leucinamide, which is the nucleophilic acyl acceptor, was used in two-fold molar excess and so it was the BAEE, which determined the maximum amount of peptide that could be synthesised. In this reaction system the BAEE could have undergone two different reaction pathways: an unwanted hydrolysis reaction or conversion to the required peptide product (Figure 7.61).

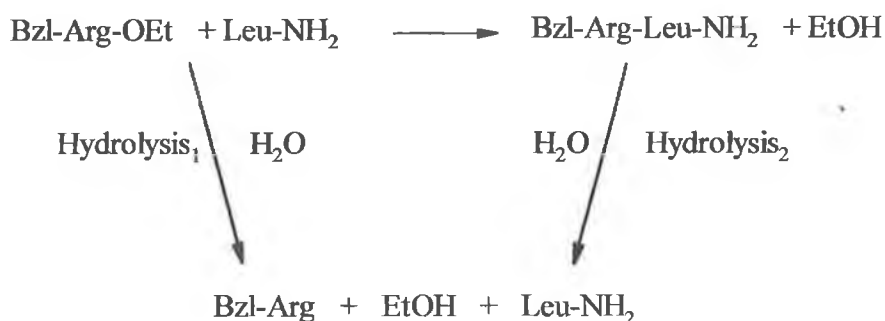


Figure 7.61: A schematic diagram of the dipeptide synthesis of Bz-Arg-Leu-NH₂ using trypsin (Blanco et al., 1991).

This synthesis reaction was carried with both native and EG-trypsin in an aqueous solution to investigate the effect of EG-NHS crosslinking on the pig enzyme's ability to catalyse peptide synthesis (Figure 7.55). EG-trypsin demonstrated an absorbance value for the dipeptide product that was approximately 3-fold higher than that obtained by the native enzyme under the same reaction conditions. This was to be expected, as the EG-trypsin demonstrated a 11-fold increase in esterase/amidase ratio compared to the native

in an aqueous solution (Table 5.10). As already discussed in Section 5.3, synthesis of a peptide by a serine protease increases with increasing esterase/amidase activity, as the acyl-enzyme intermediate is more likely to undergo aminolysis rather than hydrolysis. Some of the advantages of using non-aqueous solutions for enzymatic peptide synthesis are: (i) the reaction proceeds in the direction of peptide synthesis and undesirable hydrolytic side reactions occur to a lesser extent; (ii) many of the starting materials used in peptide synthesis show increased solubility in organic solvents; (iii) many enzymes demonstrate enhanced thermostabilities in low water media; (iv) there is the reduced risk of microbial contamination. Hydrophobic solvents are not normally used in this type of synthesis, as they don't dissolve many of the amino acid derivatives used as starting materials. Hydrophilic solvents such as acetonitrile are generally used but care must be taken as, even though these solvents have good solvating properties, they may have a negative effect on enzyme activity and stability. Solvent effects are enzyme-specific, so it is difficult to predict how a given enzyme will react to a specific solvent (Batra and Gupta, 1994).

The synthesis of the dipeptide Bz-Arg-Leu-NH₂ was then repeated using the native and EG-trypsin in a 30% acetonitrile solution. The native trypsin's ability to catalyse the peptide was increased in this organic/aqueous solution as the absorbance value of the product had increased 2.5-fold. The EG-trypsin had a slight increase in its catalytic efficiency also (Figure 7.56). Butler (1979) stated that an aqueous-organic solvent mixture may resemble the enzyme's natural microenvironment more closely than a purely aqueous system and so the enzyme might become a more efficient catalyst in this type of environment. Gupta et al. (1997) state that enzyme activity increases initially as

the organic solvent is added, generally going beyond the activity of the enzyme in a purely aqueous system and reaching a maximum around 20% (v/v) organic cosolvent concentration. Above this concentration, the activity starts to decrease and at higher concentrations the enzyme will become totally inactive. This is what has been observed here: the enzyme is displaying maximum activity when there is a small amount of acetonitrile present in the reaction solution.

The tripeptide Z-Tyr-Gly-Gly-NH₂ was synthesised from the acyl donor Z-Tyr-OMe and the nucleophilic component Gly-Gly-NH₂ using both subtilisin Carlsberg and alcalase (Section 2.3.5.2.1). Klein et al. (2000) had catalysed the peptide bond formation between Z-Val-Trp-OMe and various nucleophilic components using subtilisin Carlsberg. They used many amino acid derivatives as nucleophiles and they discovered that subtilisin Carlsberg had a strong preference for the glycine residue in the P₁' position. They discovered that Gly-NH₂ gave a yield of 78%, Gly-Gly-NH₂ a yield of 90% and Gly-Gly-Gly-NH₂ a yield of 73% when they were used as the acyl acceptors. This is why Gly-Gly-NH₂ was chosen as the nucleophile in the present experiments.

The peptide synthesis reaction was then carried out at 4°C, 25°C and 37°C to investigate the effects of temperature. It has been widely published in literature that the lower the reaction temperature used, the higher the yield of peptide product (Jönsson et al., 1996). After five minutes, the greatest yield of peptide was obtained at 4°C using native subtilisin Carlsberg: 0.9mmol compared to 0.7mmol for the same enzyme solution at 37°C. However, as the time progressed the optimum yields for both native and modified forms of the alcalase and subtilisin Carlsberg was obtained at 25°C (Figure 7.57). This was also the temperature that Klein et al. (2000) had chosen for their peptide synthesis

reaction. It has been published in literature that alcalase is stable and active at ambient or higher temperatures (Chen and Wang, 1992). This may be why it behaves slightly differently to the other proteases studied.

Klein et al. (2000) stated that they had used a 50% v/v DMF solution in their peptide synthesis reaction, as it was necessary to dissolve the Z-Tyr-OMe. It is essential to optimise the use of organic solvents in the synthesis of a peptide using an enzyme-catalyzed approach, as the solvents affect the enzyme stability, solubility of the substrate and the yield of the peptide product (Zhou et al., 2003). Subtilisin Carlsberg also demonstrates a 4-fold increase in its esterase/amidase ratio when used in a 50% v/v DMF solution compared to a purely aqueous solution (Table 5.12). Alcalase showed a decrease in esterase/amidase activity when placed in 50% v/v DMF, so it was decided to investigate the effect of 50% v/v DMF on the rate of synthesis of the tripeptide Z-Tyr-Gly-Gly-NH₂. EG-subtilisin Carlsberg and native alcalase gave a slight increase in the yield of the tripeptide in the DMF solution compared to an aqueous solution. EG-alcalase and native subtilisin Carlsberg displayed equivalent peptide synthesis abilities in the 50% DMF solution compared to the reaction in an aqueous solution.

CHAPTER 8

CONCLUSION

8 Conclusion:

The activity and stability of porcine trypsin were compared with those of bovine trypsin, which had been studied previously by Murphy (1996). The T_{50} value for the porcine trypsin was found to be 45°C while the bovine trypsin was less thermo-stable, with a T_{50} value of 38°C. The $t_{1/2}$ value at 55°C obtained for native bovine trypsin was found to be 4 minutes compared to 110 minutes for porcine trypsin.

Two types of chemical modification were carried out on porcine trypsin. The first was crosslinking of the enzyme with the homobifunctional reagent EG-NHS. This modification was notably successful, as it increased the enzyme's stability and activity. The EG-trypsin demonstrated increased thermostability: it retained 100% amidase activity for 150 minutes at 55°C, whereas the native was completely inactive after this period of time. EG-trypsin also demonstrated increased proteolytic activity using casein, being 1.5-fold more active than the native. This modified form of trypsin showed an 11-fold increase in its esterase/amidase ratio compared to the native in an aqueous solution. This change in the enzyme's catalytic efficiency suggested that the modified enzyme might be useful for peptide synthesis. The second type of modification attempted on the porcine trypsin was the formylation of the tryptophan residues of the enzyme. Coletti-Previero et al. (1969) had stated that this caused the removal of the amidase activity while retaining the enzymes esterase activity. A more modern, convenient and less hazardous method was investigated and an increase of 10°C in the T_{50} value (up to 55°C) of the enzyme was observed, which was unexpected and had not been reported previously. The formylated trypsin did lose its amidase activity completely (as predicted) and retained its esterase activity up to 50°C, but overall activity levels were

much lower. Accordingly, it was decided that the esterase activity retained by the formylated enzyme would not be sufficient to catalyse peptide synthesis. Synthesis of the dipeptide Bz-Arg-Leu-NH₂ was achieved using both native and EG-trypsin. EG-trypsin increased the rate of peptide synthesis by approximately 3-fold compared to the native in an aqueous solution.

Trypsin, a mammalian serine protease, was then compared in terms of stability and activity to subtilisin Carlsberg, a bacterial serine protease, and alcalase, a commercially produced enzyme. Subtilisin Carlsberg demonstrated a T₅₀ value of 68°C and the alcalase a value of 72°C. The t_{1/2} value for alcalase was found to be 4.6 minutes at 75°C and that for subtilisin Carlsberg 3.9 minutes at the same temperature.

These enzymes were then modified using the EG-NHS reagent that had been successfully used on trypsin. The modification increased the thermostability of the two enzymes at 65°C: EG-alcalase retained 100% amidase activity for 100 minutes, while native became inactive after 85 minutes; EG-subtilisin Carlsberg retained 50% amidase activity up to 65 minutes while the native was inactive after 25 minutes. It was decided to investigate the effect of this modification on alcalase and subtilisin Carlsberg in terms of their ability to catalyse peptide synthesis. The synthesis of the tripeptide Z-Tyr-Gly-Gly-NH₂ was successful using both the native and modified forms of the alcalase and subtilisin Carlsberg. The optimum conditions were found to be 25°C and an aqueous environment.

The original aims of the project have been met as the three enzymes were crosslinked with EG-NHS and then used to successfully synthesis various peptides. As these

modified forms demonstrated enhanced activity and stability they could be useful in a variety of biocatalytic applications and in the synthesis of other peptides.

BIBLIOGRAPHY

BIBLIOGRAPHY

- Anderson, A.J., Fomsgaard, J., Thorebek, P. and Aasmul-Olsen, S. (1991) "Current enzymatic possibilities in the technology of peptide synthesis". *Chimicaoggi* **9**,3, 17-23.
- Arnold, F.H. (1990) "Engineering enzymes for non-aqueous solvents". *TIBTECH* **8**, 244-249.
- Bahadur, A. and Bahadur, P. (1985) "Trypsin Immobilization on anionic polymeric latex". *Indian Journal of Biochemistry and Biophysics* **22**, 107-110.
- Bakhtiar, S., Andersson, M.M., Gessesse, A., Mattiasson, B. and Hatti-Kaul, R. (2003) "Stability characteristics of a calcium-independent alkaline protease from *Nesterenkonia* sp.". *Enzyme and Microbial Technology* **6252**, 1-7.
- Batra, R. and Gupta, M.N. (1994) "Enhancement of enzyme activity in aqueous-organic solvent mixtures". *Biotechnology Letters* **16**, 10, 1059-1064.
- Bender, M.L., Begué-Cantón, M.L., Blakeley, R.I., Brubacher, L.J., Feder, J., Gunter, C.R., Kézdy, F.J., Killheffer Jr., J.V., Marshall, T.H., Miller, C.G., Roeske, R.W. and Stoops, J.K. (1966) "The determination of the concentration of hydrolytic enzyme solutions: α -Chymotrypsin, Trypsin, Papain, Elastase, Subtilisin and Acetylcholinesterase". *Journal of the American Chemical Society* **88**, 24, 5890-5913.
- Bernstein, F.C., Koetzle, T.F., Williams, G.J., Meyer Jr., E.E., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) "The Protein Data Bank: a computer-based archival file for macromolecular structures". *Journal of Molecular Biology* **112**, 535.
- Beutel, S., Klein, K., Knobbe, G., Königfeld, P., Petersen, K., Ulber, R. and Scheper, T. (2002) "Controlled enzymatic removal of damaging casein layers on medieval wall paintings". *Biotechnology and Bioengineering*, **80**, 1, 13-21.
- Bickerstaff, G.F. and Zhou, H. (1993) "Protease activity and autodigestion (autolysis) assays using coomassie blue dye binding". *Analytical Biochemistry* **210**, 155-158.
- Binggen, R., Jinzhu, D., Yaohui, Z., Liansheng, C., Yisheng, N., Guohua, T. and Longxiang, Z. (1980) "Active products of porcine trypsin after autolysis". *SCIENTIA SINICA* **11**, 1453-1460.
- Blanco, R.M., Alvaro, G. and Guisán, J.M. (1991) "Enzyme reaction engineering: design of peptide synthesis by stabilised trypsin". *Enzyme Microbial Technology* **13**, 573-583.
- Bradford, M.M. (1976) "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding". *Analytical Biochemistry* **72**, 248-254.

Briand, L., Chobert, J.-M., Gantier, R., Declerck, N., Tran, V., Léonil, J., Mollé, D. and Haertlé, T. (1999) "Impact of the lysine-188 and aspartic acid-189 inversion on activity of trypsin". *FEBS Letters* **442**, 43-47.

Briand, L., Chobert, J.-M., Tauzin, J., Declerck, N., Léonil, J., Mollé, D., Tran, V. and Haertlé, T. (1997) "Regulation of trypsin activity by Cu^{2+} chelation of the substrate binding site". *Protein Engineering* **10**, 5, 551-560.

Briedigkeit, L. and Frömmel, C. (1989) "Calcium ion binding by thermitase". *FEBS Letters* **253**, 1, 2, 83-87.

Broun, G.B. (1977) "Chemically aggregated enzymes" in *Methods in enzymology* (Mosbach, K., Ed.) Academic Press, New York. **44**, 263-269.

Bryan, P.N. (2000) "Protein engineering of subtilisin". *Biochimica et Biophysica Acta* **1543**, 203-222.

Bryjak, J. and Kolarz, B.N. (1998) "Immobilisation of trypsin on acrylic copolymers". *Process Biochemistry* **33**, 4, 409-417.

Butkus, E.P., Zaslavsky, B.Y., Mestechkina, N.M. and Martinek, K. (1988) "Protein stabilization via hydrophilization-covalent modification of trypsin and α -chymotrypsin". *European Journal of Biochemistry* **173**, 147-154.

Butler, L.G. (1979) "Enzymes in non-aqueous solvents". *Enzyme and Microbial Technology* **1**, 4, 253-259.

Carrea, G. and Riva, S. (2000) "Properties and synthetic applications of enzymes in organic solvents". *Angew. Chem. Int. Ed.* **39**, 2226-2254.

Carrea, G., Ottolina, G. and Riva, S. (1995) "Role of solvents in the control of enzyme selectivity in organic media". *TIBTECH* **13**, 63-70.

Castro, G.R. (1999) "Enzymatic activities of proteases dissolved in organic solvents". *Enzyme and Microbial Technology* **25**, 689-694.

Čeřovský, V. (1990) "Free trypsin-catalyzed peptide synthesis in acetonitrile with low water content". *Biotechnology Letters* **12**, 12, 899-904.

Chen, S.-T. and Wang, K.-T. (1992) "Peptide synthesis in organic solvents catalyzed by an industrial alkaline protease "Alcalase"". *Journal of the Chinese Chemical Society* **39**, 683-691.

Chen, S.-T., Chen, S.-Y., Hsiao, S.-C. and Wang, K.-T. (1991) "Application of industrial protease "Alcalase" in peptide synthesis". *Biomed. Biochim. Acta* **50**, 181-186.

Chen, S.-T., Chen, S.-Y., Kao, C.-L. and Wang, K.-T. (1994) "Proline as nucleophile in kinetically controlled peptide synthesis catalyzed by alcalase in 2-methyl-2-propanol". *Bioorganic and Medicinal Chemistry Letters* **4**, 3, 443-448.

Chen, S.-T., Chen, S.-Y., Tu, C.-C., Chiou, S.-H. and Wang, K.-T. (1995) "Physiochemical properties of alkaline serine proteases in alcohol". *Journal of Protein Chemistry* **14**, 4, 205-215.

Chen, S.-T., Tseng, P.-H., Yu, H.-M., Wu, C.-Y., Hsiao, K.-F., Wu, S.-H. and Wang, K.-T. (1997) "The studies of microwave effects on the chemical reactions". *Journal of the Chinese Chemical Society* **44**, 169-182.

Chen, Y.-C., Zhang, X.-Z., Zheng, K., Chen, S.-M., Wang, Q.-C. and Wu, X.-X. (1998) "Protease-catalysed synthesis of precursor dipeptides of RGD with reverse micelles". *Enzyme and Microbial Technology* **23**, 243-248.

Chobert, J.-M., Briand, L., Tran, V. and Haertlé, T. (1998) "How the substitution of K188 of trypsin binding site by aromatic amino acids can influence the processing of β -casein". *Biochemical and Biophysical Research Communications* **246**, 847-858.

Coleman, P.L. and Green, G.D.J. (1981) "A coupled photometric assay for plasminogen activator". *Methods in Enzymology* **80**, 408-414.

Coletti-Previero, M.-A., Previero, A. and Zuckerkandl, E. (1969) "Separation of the proteolytic and esterase activities of trypsin by reversible structural modifications". *Journal of Molecular Biology* **39**, 493-501.

Colombo, G. and Carrea, G. (2002) "Modeling enzyme reactivity in organic solvents and water through computer simulations". *Journal of Biotechnology* **96**, 23-33.

Craik, C.S., Largman, C., Fletcher, T., Roczniak, S., Barr, P.J., Fletterick, R. and Rutter, W.J. (1985) "Redesigning trypsin: alteration of substrate specificity". *Science* **228**, 291-297.

Dallas Johnson, K., Clark, A. and Marshall, S. (2002) "A functional comparison of ovine and porcine trypsins". *Comparative Biochemistry and Physiology Part B* **131**, 423-431.

Davis, B.G., Khumtaveeporn, K., Bott, R.R. and Jones, J.B. (1999) "Altering the specificity of subtilisin *Bacillus lentus* through the introduction of positive charge at single amino acid sites". *Bioorganic and Medicinal Chemistry* **7**, 2303-2311.

Dawson, M.T. (1996) "Gene technology" (Dawson, M.T., Powell, R. and Gannon, F. eds.) Bios Scientific Publishers, Oxford UK.

DeSantis, G. and Jones, J.B. (1999a) "Probing the altered specificity and catalytic properties of mutant subtilisin chemically modified at position S156C and S166C in the S1 pocket". *Bioorganic and Medicinal Chemistry* **7**, 1381-1387.

DeSantis, G. and Jones, J.B. (1999b) "Chemical modification of enzymes for enhanced functionality". *Current Opinion in Biotechnology* **10**, 324-330.

Dickman, M. and Jones, J.B. (2000) "Covalent modification of subtilisin *Bacillus lentus* Cysteine mutants with enantiomerically pure chiral auxiliaries causes remarkable changes in activity". *Bioorganic and Medicinal Chemistry* **8**, 1957-1968.

Elsner, C., Grahn, S., Bauer, S., Ullmann, D., Kurth, T. and Jakubke, H.-D. (2000) "Effects of chemical modification of lysine residues in trypsin". *Journal of Molecular Catalysis B: Enzymatic* **8**, 4-6, 193-200.

Erbeldinger, M., Mesiano, A.J. and Russell, A.J. (2000) "Enzymatic catalysis of formation of Z-aspartame in ionic liquid - An alternative to enzymatic catalysis in organic solvents". *Biotechnology Progress* **16**, 6, 1129-1131.

Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) "The preparation and properties of two new chromogenic substrates of trypsin". *Archives of Biochemistry and Biophysics* **95**, 271-278.

Fernández, M., Fragoso, A., Cao, R., Baños, M. and Villalonga, R. (2002) "Chemical conjugation of trypsin with monoamine derivatives of cyclodextrins catalytic and stability properties". *Enzyme and Microbial Technology* **31**, 543-548.

Ferreira, L., Ramos, M.A., Dordick, J.S. and Gil, M.H. (2003) "Influence of different silica derivatives in the immobilization and stabilization of a *Bacillus licheniformis* protease (Subtilisin Carlsberg)". *Journal of Molecular Catalysis B: Enzymatic* **21**, 189-199.

Fitzpatrick, P.A., Ringe, D. and Klibanov, A.M. (1992) "Computer-assisted modeling of subtilisin enantioselectivity in organic solvents". *Biotechnology and Bioengineering* **40**, 735-742.

Fitzpatrick, P.A., Steinmetz, A.C.U., Ringe, D. and Klibanov, A.M. (1993) "Enzyme crystal structure in a neat organic solvent". *Proceedings of the National Academy of Science* **90**, 8653-8657.

Fukui, S. and Tanaka, A. (1985) "Enzymatic reactions in organic solvents". *Endeavour, New Series* **9**, 1, 10-17.

Getun, I.V., Filippova, I.Y., Lysogorskaya, E.N. and Oksenoit, E.S. (2001) "SDS-subtilisin catalyzed synthesis of tetra-peptides containing multifunctional amino acid residues in ethanol". *Journal of Molecular Catalysis B: Enzymatic* **15**, 105-110.

Getun, I.V., Filippova, I.Y., Lysogorskaya, E.N., Oksenoit, E.S., Anisimova, V.V., Kolobanova, S.V., Bacheva, A.V. and Stepanov, V.M. (1997) "SDS-subtilisin complex efficiently catalyses synthesis of peptides in ethanol and 2-propanol". *Bioorganic and Medicinal Chemistry Letters* **7**, 20, 2691-2696.

Gill, I., López-Fandiño, R., Jorba, X. and Vulfson, E.N. (1996) "Biologically active peptides and enzymatic approaches to their production". *Enzyme and Microbial Technology* **18**, 162-183.

- Gleich, M., Talsky, G. and Spannagl, R. (1992) "Stabilization of trypsin by modification with bifunctional reagents". *DECHEMA Biotechnology Conferences* **5**, 121-124.
- Gorman, L.A.S. and Dordick, J.S. (1992) "Organic solvents strip water off enzymes". *Biotechnology and Bioengineering* **39**, 392-397.
- Graf, L., Craik, C.S., Patthy, A., Rocznai, S., Fletterick, R.J. and Rutter, W.J. (1987) "Selective alteration of substrate specificity by replacement of aspartic acid-189 with lysine in the binding pocket of trypsin". *Biochemistry* **26**, 2616-2623.
- Guinn, R.M., Blanch, H.W. and Clark, D.S. (1991) "Effect of a water-miscible organic solvent on the kinetic and structural properties of trypsin". *Enzyme Microbial Technology* **13**, 320-326.
- Guo, Y. and Clark, D.S. (2001) "Activation of enzymes for nonaqueous biocatalysis by denaturing concentrations of urea". *Biochimica et Biophysica Acta* **1546**, 406-411.
- Gupta, M.N., Batra, R., Tyagi, R. and Sharma, A. (1997) "Polarity Index: The guiding solvent parameter for enzyme stability in aqueous-organic cosolvent mixtures". *Biotechnology Progress* **13**, 284-288.
- Halfon, S. and Craik, C.S. (1998) "Trypsin" in "Handbook of proteolytic enzymes" (Barrett, A.J., Rawlings, N.D. and Woessner, J.F. eds.), p. 12-21. Academic Press Inc.
- Häring, D. and Schreier, P. (1999) "Cross-linked enzyme crystals". *Current Opinion in Chemical Biology* **3**, 35-38.
- He, Z., Zhang, Z. and He, M. (2000) "Kinetic study of thermal inactivation for native and methoxypolyethylene glycol modified trypsin". *Process Biochemistry* **35**, 1235-1240.
- Helmerhost, E. and Stokes, G.B. (1980) "Microcentrifuge Desalting: A rapid quantitative method for desalting small amounts of protein". *Analytical Biochemistry*, **104**, 130-135.
- Hilvert, D. (1991) "Extending the chemistry of enzymes and abzymes". *TIBTECH* **9**, 11-17.
- Hirata, J., Ariese, F., Gooijer, C. and Irth, H. (2003) "Continuous-flow protease assay based on fluorescence resonance energy transfer". *Analytica Chimica Acta* **478**, 1-10.
- Huang, X.L., Catignani, G.L. and Swaisgood, H.E. (1997) "Comparison of the properties of trypsin immobilized on 2 CeliteTM derivatives". *Journal of Biotechnology* **53**, 21-27.
- Inada, Y., Furukawa, M., Sasaki, H., Kodera, Y., Hiroto, M., Nishimura, H. and Matsushima, A. (1995) "Biomedical and biotechnology applications of PEG- and PM-modified proteins". *TIBTECH* **13**, 86-91.

Ito, Y., Fujii, H. and Imanishi, Y. (1993) "Catalytic peptide synthesis by trypsin modified with polystyrene in chloroform". *Biotechnology Progress* **9**, 128-130.

Ji, T.H. (1983) "Bifunctional reagents" in "Methods in Enzymology" vol. **91** (Hirs, C.H.W. and Timasheff, S.N. eds.), Academic Press, New York, p. 580-609.

Jiang, H., Zou, H., Wang, H., Ni, J., Zhang, Q. and Zhang, Y. (2000) "On-line characterization of the activity and reaction kinetics of immobilized enzyme by high-performance frontal analysis". *Journal of Chromatography A* **903**, 77-84.

Jönsson, Å., Wehtje, E., Adlercreutz, P. and Mattiasson, B. (1996) "Temperature effects on protease catalysed acyl transfer reactions in organic media". *Journal of Molecular Catalysis B: Enzymatic* **2**, 43-51.

Kallow, W., Pavela-Vrancic, M., Dieckmann R. and von Döhren, H. (2002) "Nonribosomal peptide synthetases-evidence for a second ATP-binding site". *Biochimica et Biophysica Acta* **1601**, 93-99.

Kasche, V. (1989) "Proteases in peptide synthesis" in "Proteolytic enzymes a practical approach", Chapter 7, 125-143.

Katchalski-Katzir, E. (1993) "Immobilized enzymes - learning from past successes and failures". *TIBTECH* **11**, 471-478.

Kawashiro, K., Sugahara, H., Sugiyama, S. and Hayashi, H. (1997) "Effect of organic solvents on enantioselectivity of protease catalysis". *Biotechnology and Bioengineering* **53**, 26-31.

Ke, T. and Klivanov, A.M. (1999) "Markedly enhancing enzymatic enantioselectivity in organic solvents by forming substrate salts". *Journal of American Chemical Society* **121**, 3334-3340.

Khan, S. S. and Siddiqi, A.M. (1985) "Studies on chemically aggregated pepsin using glutaraldehyde". *Biotechnology and Bioengineering* **27**, 415-419.

Khare, S.K., Vaidya, S. and Gupta, M.N. (1991) "Entrapment of proteins by aggregation within sephadex beads". *Applied Biochemistry and Biotechnology* **27**, 205-216.

Khmelnitsky, Y.L., Levashov, A.V., Klyachko, N.L. and Martinek, K. (1988) "Engineering biocatalytic systems in organic media with low water content". *Enzyme Microbial Technology* **10**, 710-724.

Khmelnitsky, Y.L., Mozhaev, V.V., Belova, A.B., Sergeeva, M.V. and Martinek, K. (1991) "Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis". *European Journal of Biochemistry* **198**, 31-41.

- Khumtaveeporn, K., DeSantis, G. and Bryan Jones, J. (1999) "Expanded structural and stereospecificity in peptide synthesis with chemically modified mutants of subtilisin". *Tetrahedron: Asymmetry* **10**, 2563-2572.
- Kitagawa, T., Ito, J. and Tsutsui, C. (1994) "Improved procedure for N-formylation of amines to formamides using formic acid, oxalyl chloride and imidazole". *Chem. Pharm. Bull.* **42**, 9, 1931-1934.
- Klein, J.U., Prykhodzka, A. and Čerovský, V. (2000) "The applicability of subtilisin Carlsberg in peptide synthesis". *Journal of Peptide Science* **6**, 541-549.
- Kleinkauf, H. and von-Döhren, H. (1990) "Nonribosomal biosynthesis of peptide antibiotics". *European Journal of Biochemistry* **192**, 1-15.
- Klibanov, A.M. (2001) "Improving enzymes by using them in organic solvents". *Nature* **409**, 241-246.
- Kobayashi, M. and Takaatsu, K. (1994) "Cross-linked stabilization of trypsin with dextran-dialdehyde". *Bioscience Biotechnology and Biochemistry* **58**, 2, 275-278.
- Krishna, S.H. (2002) "Developments and trends in enzyme catalysis in nonconventional media". *Biotechnology Advances* **20**, 239-267.
- Kwon, O.H., Imanishi, Y. and Ito, Y. (1999) "Catalytic activity and conformation of chemically modified subtilisin Carlsberg in organic media". *Biotechnology and Bioengineering* **66**, 4, 265-270.
- Liu, G., Zhang, S.-D., Xia, S.-Q. and Ding, Z.-K. (2000) "Solid-phase synthesis of muramyl dipeptide (MDP) derivatives using a multipin method". *Bioorganic and Medicinal Chemistry Letters* **10**, 1361-1363.
- Lozano, P., de Diego, T. and Iborra J.L. (1996) "Influence of water-Miscible aprotic solvents on α -chymotrypsin stability". *Biotechnology Progress* **12**, 488-493.
- Malmsten, M. and Larsson, A. (2000) "Immobilization of trypsin on porous glycidyl methacrylate beads: effects of polymer hydrophilization". *Colloids and Surfaces B: Biointerfaces* **18**, 277-284.
- Melik-Nubarov, N.S., Mozhaev, V.V., Šikšnis, S. and Martinek, K. (1987) "Protein stabilization via hydrophilization: Stabilization of α -chymotrypsin by reductive alkylation with glyoxylic acid". *Biotechnology Letters* **9**, 10, 725-730.
- Miyazawa, T., Kayoko, T., Ensatsu, E., Yanagihara, R. and Yamada, T. (1998) "Remarkable effects of donor esters on the α -Chymotrypsin catalysed couplings of inherently poor amino acid substrates". *Tetrahedron Letters* **39**, 997-1000.
- Morato, A.F., Carreira, R.G., Junqueira, R.G. and Silvestre, M.P.C. (2000) "Optimization of casein hydrolysis for obtaining high contents of small peptides: use of subtilisin and trypsin". *Journal of Food Composition and Analysis* **13**, 843-857.

Mozhaev, V.V. (1993) "Mechanism-based strategies for protein thermostabilization". *TIBTECH* **11**, 88-95.

Mozhaev, V.V., Šikšnis, V.A., Torchilin, V.P., and Martinek, K. (1983) "Operational stability of copolymerized enzymes at elevated temperatures". *Biotechnology and Bioengineering* **25**, 1937-1945.

Mozhaev, V.V., Šikšnis, V.A., Melik-Nubarov, N.S., Galkantaite, N.Z., Denis, G.J., Butkus, E.P., Zaslavsky, B.Y., Mestechkina, N.M. and Martinek, K. (1988) "Protein stabilization via hydrophilization-covalent modification of trypsin and α -chymotrypsin". *European Journal of Biochemistry* **25**, 1937-1945.

Murphy, A. (1996) "Chemical modification of bovine trypsin for use in peptide synthesis". Ph.D. thesis, Dublin City University.

Murphy, A. and Ó' Fágáin, C. (1996a) "Bypassing the ribosome: peptide synthesis without translation". *Essays in Biochemistry* **31**, 61-75.

Murphy, A. and Ó' Fágáin, C. (1996b) "Stability characteristics of chemically-modified soluble trypsin". *Journal of Biotechnology* **49**, 163-171.

Murphy, A. and Ó' Fágáin, C. (1997) "Dipeptide synthesis using acetylated trypsin derivative". *Biotechnology Techniques* **11**, 13-16.

Murphy, A. and Ó' Fágáin, C. (1998) "Chemically stabilised trypsin used in dipeptide synthesis". *Biotechnology and Bioengineering* **58**, 4, 365-373.

Nilsson, K. and Mosbach, K. (1984) "Peptide synthesis in aqueous-organic solvent mixtures with α -chymotrypsin immobilized to tresyl chloride-activated agarose". *Biotechnology and Bioengineering* **26**, 1146-1154.

Nurok, D., Kleyle, R.M., Muhoberac, B.B., Frost, M.C., Hajdu, P., Robertson, D.H., Kamat, S.V. and Russell, A.J. (1999) "Study of enzyme-catalysed reactions in organic solvents using multiple linear regression". *Journal of Molecular Catalysis B: Enzymatic* **7**, 273-282.

Ó' Fágáin, C. (1997) "Stabilizing protein function". p.4-6, Springer, Berlin.

Ogino, H. and Ishikawa, H. (2001) "Enzymes which are stable in the presence of organic solvents". *Journal of Bioscience and Bioengineering* **91**, 2, 109-116.

Outzen, H., Berglund, G.I., Smalås, A.O. and Willassen, N.P. (1996) "Temperature and pH sensitivity of trypsin from atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin". *Comparative Biochemistry and Physiology* **115B**, 1, 33-45.

Perischetti, R.A., St. Clair, N.L., Griffith, J.P., Navia, M.A. and Margolin, A.L. (1995) "Cross-linked enzyme crystals (CLECs) of thermolysin in the synthesis of peptides". *Journal of the American Chemical Society* **117**, 2732-2737.

- Perona, J.J., Tsu, C.A., McGrath, M.E., Craik, C.S. and Fletterick, R.J. (1993) "Relocating a negative charge in the binding pocket of trypsin". *Journal of Molecular Biology* **230**, 934-949.
- Philipp, M. and Bender, M.L. (1983) "Kinetics of subtilisin and thiolsubtilisin". *Molecular and Cellular Biochemistry* **51**, 5-32.
- Plettner, E., Khumtaveeporn, K., Shang, X. and Bryan Jones, J. (1998) "A combinatorial approach to chemical modification of Subtilisin *Bacillus lentus*". *Bioorganic and Medicinal Chemistry Letters* **8**, 2291-2296.
- Plou, F.J. and Ballesteros, A. (1994) "Acylation of subtilisin with long fatty acyl residues affects its activity and thermostability in aqueous medium". *FEBS Letters* **339**, 200-204.
- Pouvreau, L., Chobert, J.-M., Briand, L., Quillien, L., Tran, V., Guéguen, J. and Haertlé, T. (1998) "Effect of pea and bovine trypsin inhibitors on wild-type and modified trypsins". *FEBS Letters* **423**, 167-172.
- Rajput, Y.S. and Gupta, M.N. (1987a) "A conjugate of trypsin and chymotrypsin". *Applied Biochemistry and Biotechnology* **16**, 201-210.
- Rajput, Y.S. and Gupta, M.N. (1987b) "Reaction of trypsin with some crosslinking reagents". *Enzyme Microb. Technol.* **9**, 161-163.
- Rajput, Y.S. and Gupta, M.N. (1988) "Coaggregate of trypsin and Chymotrypsin". *Biotechnology and Bioengineering* **31**, 220-223.
- Reddy, C.R., Reddy, C.R., Raghunath, K. and Joseph, K.T. (1986) "Immobilization of trypsin on alginic acid-poly (glycidyl methacrylate) graft copolymer". *Biotechnology and Bioengineering* **28**, 609-612.
- Rick, W. (1974) "Trypsin" in "Methods of enzymatic analysis" (Bergmeyer, H.U. ed), 1013-1024. Academic Press Inc.
- Rosell, C.M., Vaidya, A.M. and Halling, P.J. (1995) "Prediction of denaturing tendency of organic solvents in mixtures with water by measurement of naphthalene solubility". *Biochimica et Biophysica Acta* **1252**, 158-164.
- Ru, M.T., Dordick, J.S., Reimer, J.A. and Clark, D.S. (1999) "Optimizing the Salt-Induced Activation of Enzymes in Organic Solvents: Effects of Lyophilization Time and Water Content". *Biotechnology and Bioengineering* **63**, 2, 233-241.
- Ru, M.T., Wu, K.C., Lindsay, J. P., Dordick, J.S., Reimer, J.A. and Clark, D.S. (2001) "Towards more active Biocatalysts in Organic Media: Increasing the Activity of Salt-Activated Enzymes". *Biotechnology and Bioengineering* **75**, 2, 187-196.
- Sakurai, K., Kashimoto, K., Kodera, Y. and Inada, Y. (1990) "Solid phase synthesis of peptides with polyethylene glycol-modified protease in organic solvents". *Biotechnology Letters* **12**, 9, 685-688.

Sakurai, T., Margolin, A.L., Russell, A.J. and Klibanov, A.M. (1988) "Control of enzyme enantioselectivity by the reaction medium". *Journal of American Chemical Society* **110**, 7236-7237.

Schellenberger, V., Jakubke, H.-D., Zapevalova, N.P. and Mitin, Y.V. (1991) "Protease-catalyzed peptide synthesis using inverse substrates: The influence of reaction conditions on the trypsin acyl transfer efficiency". *Biotechnology and Bioengineering* **38**, 104-108.

Schmitke, J.L., Stern, L.J. and Klibanov, A.M. (1998) "Comparison of x-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water". *Proceedings of the National Academy of Science* **95**, 12918-12923.

Schulze, B. and Klibanov, A.M. (1991) "Inactivation and stabilization of subtilisins in neat organic solvents". *Biotechnology and Bioengineering* **38**, 1001-1006.

Sears, P. and Wong, C.-H. (1996) "Engineering enzymes for bioorganic synthesis: peptide bond formation". *Biotechnology Progress* **12**, 423-433.

Sears, P., Witte, K. and Wong, C.-H. (1999) "The effect of counterion, water concentration and stirring on the stability of subtilisin BPN' in organic solvents". *Journal of Molecular Catalysis B: Enzymatic* **6**, 297-304.

Sekizaki, H., Itoh, K., Toyota, E. and Tanizawa, K. (1996) "Trypsin-catalysed peptide synthesis with various p-Guanidinophenyl esters as acyl donors". *Chem. Pharm. Bull.* **44**, 8, 1585-1587.

Sekizaki, H., Itoh, K., Toyota, E. and Tanizawa, K. (1997) "Enzymatic coupling of α,α -Dialkyl amino acids using inverse substrates as acyl donors". *Tetrahedron Letters* **38**, 1777-1780.

Sewald, N. and Jakubke, H.-D. (2002) "Peptides: Chemistry and Biology". WILEY-VCH.

Shubhada, S. and Sundaram, P.V. (1995) "The role of pH change caused by the addition of water-miscible organic solvents in the destabilization of an enzyme". *Enzyme and Microbial Technology* **17**, 330-335.

Simon, L.M., László, K., Vértési, A., Bagi, K. and Szajáni, B. (1998) "Stability of hydrolytic enzymes in water-organic solvent systems". *Journal of Molecular Catalysis B: Enzymatic* **4**, 41-45.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) "Measurement of protein using bicinchoninic acid". *Analytical Biochemistry* **150**, 76-85.

So, J.-E., Shin, J.-S. and Kim, B.-G. (2000) "Protease-catalysed tripeptide (RGD) synthesis". *Enzyme and Microbial Technology* **26**, 108-114.

Sowdhamini, R. and Balaram, P. (1993) "Protein structure and stability" in "Thermostability of enzymes"

St. Clair, N.L. and Navia, M.A. (1992) "Cross-linked enzyme crystals as robust biocatalysis". *Journal of the American Chemical Society* **114**, 7314-7316.

Svendsen, I. and Breddam, K. (1992) "Isolation and amino acid sequence of a glutamic acid specific endopeptidase from *Bacillus licheniformis*". *European Journal of Biochemistry* **204**, 165-171.

Sweadner, K.J. (1991) "Trypsin inhibitor paradoxically stabilizes trypsin activity in sodium dodecyl sulfate, facilitating proteolytic fingerprinting". *Analytical Biochemistry* **194**, 130-135.

Tafertshofer, G. and Talsky, G. (1989) "Intra- and intermolecular crosslinking of enzymes by dianhydrides and N-hydroxysuccinimide esters". *DECHEMA Biotechnology Conferences* **3**, 171-174.

Tommasi, R.A., Nantermet, P.G., Shapiro, M.J., Chin, J., Brill, W., K.-D. and Ang, K. (1998) "Novel method for the loading of nucleophiles on multipin crowns". *Tetrahedron Letters* **39**, 5477-5480.

Tyagi, R. and Gupta, M.N. (1993) "Chemical modification and chemical crosslinking for enhancing thermostability of enzymes" in "Thermostability of enzymes" (Gupta, M.N. Ed.) p.146-160, Springer-Verlag.

Tyagi, R. and Gupta, M.N. (1998) "Stability of enzymes in water/organic co-solvent mixtures". *Biotechnology Techniques* **12**, 7, 569-570.

Tyagi, R., Batra, R. and Gupta, M.N. (1999) "Amorphous enzyme aggregates: stability toward heat and aqueous-organic cosolvent mixtures". *Enzyme and Microbial Technology* **24**, 348-354.

Tyagi, R., Singh, D.K. and Gupta, M.N. (1994) "Reversible immobilization of chemically modified trypsin on DEAE-cellulose". *Biotechnol. Appl. Biochem.* **20**, 93-99.

Ulijn, R.V., Baragaña, B., Halling, P.J. and Flitsch, S.L. (2002) "Protease-Catalyzed Synthesis on Solid Support". *J. Am. Chem. Soc.*, **124**, 37, 10988-10989.

Van Rantwijk, F., Lau, R. M. and Sheldon, R.A. (2003) "Biocatalytic transformations in ionic liquids". *Trends in Biotechnology* **21**, 3, 131-138.

Van Unen, D.-J., Engbersen, J.F.J. and Reinhoudt, D.N. (2001) "Sol-gel immobilization of serine proteases for application in organic solvents". *Biotechnology and Bioengineering* **75**, 2, 155-158.

Van Unen, D.-J., Sakodinskaya, I.K., Engbersen, J.F.J. and Reinhoudt, D.V. (1998) "Crown ether activation of cross-linked subtilisin Carlsberg crystals in organic solvents". *J. Chem. Soc., Perkin Trans.* **1**, 3341-3343.

Venkatesh, R. and Sundaram, P.V. (1993) "Stabilization of trypsin by crosslinking". *Protein Engineering* **6**, 118.

Venkatesh, R. and Sundaram, P.V. (1998) "Modulation of stability properties of bovine trypsin after *in vitro* structural changes with a variety of chemical modifiers". *Protein Engineering* **11**, 8, 691-698.

Veronese, F.M. (2001) "Peptide and protein PEGylation: a review of problems and solutions". *Biomaterials* **22**, 405-417.

Villeneuve, G.B. and Chan, T.H. (1997) "A rapid, mild and acid-free procedure for the preparation of acyl chlorides including formyl chloride". *Tetrahedron Letters* **38**, 37, 6489-6492.

Walsh, K.A. and Wilcox, P.E. (1970) in "Methods in Enzymology" vol. **19** (Perlmann, G.E. & Lorand, L. eds) p.31-41, Academic Press, New York, San Francisco & London.

Walsh, K.A. (1970) "Trypsinogen and trypsins of various species" in "Methods in Enzymology" vol. **XIX**, (Perlmann, G.E. and Lorand, L. eds) p.41-63, Academic Press, New York.

Wang, P., Sergeeva, M. V., Lim, L. and Dordick, J.S. (1997) "Biocatalytic plastics as active and stable materials for biotransformations". *Nature Biotechnology* **15**, 789-793.

Wilken, J. and Kent, S.B.H. (1998) "Chemical protein synthesis". *Current Opinion in Biotechnology* **9**, 412-426.

Wong, C.-H. (1992) "Engineering enzymes for chemoenzymatic synthesis". *TIBTECH* **10**, 378-381.

Yang, Z., Domach, M., Auger, R., Yang, F.X. and Russell, A.J. (1996) "Polyethylene glycol-induced stabilization of subtilisin". *Enzyme and Microbial Technology* **18**, 82-89.

Yasui, M., Shiroya, T., Fujimoto, K. and Kawaguchi, H. (1997) "Activity of enzymes immobilized on microspheres with thermosensitive hairs". *Colloids and Surfaces B: Biointerfaces* **8**, 311-319.

Zaks, A. and Klivanov, A.M. (1988) "Enzymatic catalysis in nonaqueous solvents". *The Journal of Biological Chemistry* **263**, 7, 3194-3201.

Zalipsky, S., Seltzer, R. and Menon-Rudolph, S. (1992) "Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins". *Biotechnology and applied biochemistry* **15**, 100-114.

Zhang, L.-Q., Zhang, Y.-D., Xu, L., Li, X.-L., Yang, X.-C., Xu, G.-L., Wu, X.-X., Gao, H.-Y., Du, W.-B., Zhang, X.-T. and Zhang, X.-Z. (2001) "Lipase-catalysed synthesis of RGD diamide in aqueous water-miscible organic solvents". *Enzyme and Microbial Technology* **29**, 129-135.

Zhong, Z., Liu, J.L.-C., Dinterman, L.M., Finkelman, M.A.J., Mueller, W.T., Rollence, M.L., Whitlow, M. and Wong, C.-H. (1991a) "Engineering subtilisin for reaction in Dimethylformamide". *J. Am. Chem. Soc.* **113**, 683-684.

Zhong, Z., Bibbs, J.A., Yuan, W. and Wong, C.-H. (1991b) "Active-site-directed modification of subtilisin". *Journal of American Chemical Society* **113**, 2259-2263.

Zhou, Y.-Y., Yang, T., Wang, N., Xu, L., Huang, Y.-B., Wu, X.-X., Yang, X.-C. and Zhang, X.-Z. (2003) "Chemo-enzymatic synthesis of tripeptide RGD in organic solvents". *Enzyme and Microbial Technology* **33**, 55-61.