Chemical Modification of Bovine Trypsin for use in Peptide Synthesis

By

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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.

Signed: Ann Murphy

Ann Murphy

Date: 2/8/1996

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ABBREVIATIONS

AA:	Amino acid
Å:	Angstrom
AA-NHS:	Acetic acid N-hydroxy-succinimide ester
Abs:	Absorbance
AMC:	7-Amino-4-methyl Courmarin
Arg:	Arginine
Asp:	Asparagine
BA:	N-Benzoyl-L-arginine
BAEE:	N-Benzoyl-L-arginine ethyl ester
$BALeuNH_2$:	N-Benzoyl-L-arginine Leucinamide
BAPNA:	N-Benzoyl-L-arginine p-nitroanilide
(Cbz)-Gly-Gly-Arg-AMC:	Benzyloxycarbonyl Glycine-Glycine-Arginine-7-Amino-4-methyl Courmarin
Cys:	Cysteine
D	Reversible unfolded protein
Da:	Dalton
DMF:	Dimethylformamide
DMSO:	Dimethylsulphoxide
DNA:	Deoxyribonucleic acid
DTNB:	5,5'-Dithiobis(2-nitrobenzioc acid)
ECS:	Equilibrium Controlled Synthesis
EDC:	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EG-NHS:	Ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester)
EGTA:	Ethylene glycol tetra acetic acid

GdnCl:	Guanidine hydrochloride
His:	Histidine
I:	Irreversibly inactivated Protein
K:	Equilibrium constant
k_{cat}:	Catalytic constant
KCS:	Kinetically Controlled Synthesis
kDA:	kilodaltons
K_m:	Michaelis Menton Constant
Lys:	Lysine
M:	Molar
mM:	millimolar
MES:	2-[N-Morpholino]ethanesulfonic acid
Mr:	Molecular weight
N:	Native state of protein
NHS:	N-hydroxysuccinimide
NPGB:	p-Nitrophenyl p'guanidinobenzoate
PEG:	Polyethylene glycol
Phe:	Phenylalanine
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
SA-NHS:	Suberic acid-bis(N-hydroxy-succinimide ester)
TA:	N-alpha-p-Toluenesulfonyl-L-arginine
$TALeuNH_2$:	N-alpha-p-Toluenesulfonyl-L-arginine Leucinamide
TAME:	N-alpha-p-Toluenesulfonyl-L-arginine methyl ester
TEMED:	N,N,N',N',-Tetramethyl ethylenediamine
TFA:	Trifluoroacetic acid
THF:	Tetrahydrofuran
TNBS:	2,4,6-Trinitrobenzenesulfonic acid
Tris:	Tris-(hydroxymethyl) methylamine (2-amino-(hydroxy-methyl)propane-1,3,-diol

Trp:	Tryptophan
Tyr:	Tyrosine
U:	Reversibly unfolded form of protein
UV/Vis.:	Ultra violet/Visible spectroscopy
v/v:	Volume per volume
V_{max}:	Maximum Velocity
w/v:	Weight per volume
Z-Lys-SBzl:	Thiolbenzyl benzyloxycarbonyl-L-lysinate

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Abstract

The first synthesis reaction achieved via reversal of the catalytic reaction of a hydrolytic enzyme was reported at the end of the last century. There has been a renewed interest in the use of proteases in peptide synthesis in the last twenty years. Proteases offer numerous advantages over conventional chemical methods, including reduction of racemization and side reactions. However, there are drawbacks associated with their use, including destabilization and loss of activity both at high temperatures and at high concentrations of organic solvents. Another major problem is autolysis which can occur when proteases are in solution. It is, therefore necessary to modify the enzyme so as to improve its stability.

The aim of this project was to chemically modify bovine trypsin in an attempt to enhance the stability of the enzyme and subsequently to use the stabilized forms in peptide synthesis. Initially, assay systems were developed and studies on native trypsin with respect to thermostability, organotolerance and stability to denaturants were undertaken. Chemical modification of bovine trypsin was carried out using carbodiimides and N-hydroxy-succinimide esters. Stabilized derivatives were produced using acetic acid N-Hydroxy-succinimide ester (AA-NHS) and ethylene glycol bis (succinic acid N-Hydroxy-succinimide ester) or EG-NHS for short. AA-NHS neutralizes the positive charge on lysine residues. Trypsin stabilized with EG-NHS a crosslinking reagent contained no dimers or higher order derivatives suggesting that intramolecular crosslinking had occurred. Approximately 8 out of 14 lysine residues per trypsin molecule were modified with AA-NHS and EG-NHS. The AA- and EG-NHS treated trypsins showed enhanced thermostability between 30 and 70°C compared with the native. T_{50} values for native, AA-NHS and EG-NHS trypsin were 46°C, 51°C and 59°C respectively. At 55°C, the AA- and EG-NHS modified trypsins' half-lives are 8.7 and 25 minutes versus 4.3 minutes for the native enzyme. Modified forms of trypsin exhibited decreased rates of autolysis. EG-NHS trypsin showed enhanced stability in aqueous mixtures of organic solvents, while AA-NHS trypsin showed enhanced stability at 65°C in aqueous mixtures of the following organic solvents: 1,4-dioxan, dimethylformamide, dimethylsulphoxide and acetonitrile.

Kinetic studies showed that both modified forms had decreased K_m and increased k_{cat} values compared with native trypsin for ester and amide substrates. The substrate specificity of modified trypsin was also enhanced. Catalytic activity of AA-trypsin in aqueous-organic mixtures was found to be higher than that of native in a range of organic solvents, while little or no difference between native and EG-trypsin was observed.

Each form of trypsin was used to prepare the dipeptide benzoyl arginyl leucinamide from derivatives of its constituent amino acids. The product yield increased with increasing organic solvent concentration and decreasing temperature. Maximum product yield was obtained in 95% acetonitrile at 4°C. The rate of peptide synthesis of modified trypsin was greater than native in 95% t-butanol and in 95% acetonitrile, with the highest rate being observed for EG-trypsin.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The ribosome is a vitally important organelle. It assembles proteins needed by the cell, using messenger and transfer RNA in the process called translation. In this way a genetic message, encoded in nuclear DNA and transcribed as messenger RNA, is converted into a polypeptide with a specific sequence of amino acids.

A second type of peptide synthesis occurs in nature. Some microorganisms can assemble certain peptides, notably antibiotics, without using the transcription/translation process. Bioactive peptides with linear and cyclic structures are produced NONribosomally by several bacteria and fungi with the aid of multienzymic complexes. Kleinkauf and von Dohren (1990) have reviewed this nonribosomal biosynthesis of peptide antibiotics. In many cases this is accomplished by an enzymatic scheme known as the "thiotemplate multienzymic mechanism". The reaction sequence leading to antibiotics such as actinomycin and gramicidin has been explored. Analysis of the primary structure of several peptide synthetases has revealed that they are organized in highly conserved and repeated functional domains. The aligned domains provide the template for peptide synthesis and their order determines the sequence of the peptide product. Numerous examples of biosynthesis of peptide antibiotics have been shown (Kleinkauf & von-Dohren, 1990; von-Dohren & Marahiel, 1990). Such antibiotics include penicillin in *Aspergillus nidulans* (van Liempt et al., 1989), bacilysin in *Bacillus subtilis* (Sakojoh et al, 1987) beta-lactams by the multienzyme Detla-(L-alpha-aminodipyl)-L-cysteinyl-D-valine-synthetase purified from *Aspergillus nidulans, Streptomyces clavuligerus* and *Acremonium chrysogenum* (Aharonowitz et al., 1993) and peptide antibiotic bialaphos (Thompson et al., 1990).

Scientists, however, can accomplish peptide synthesis *in vitro* in completely cell-free systems. The skills of organic chemistry allow synthesis of peptides from amino acid derivatives. Careful choice of reaction conditions can 'persuade' proteolytic enzymes to make, rather than break, peptide bonds. Both of these 'artificial' strategies are described here but the main emphasis is on the use of proteases for peptide

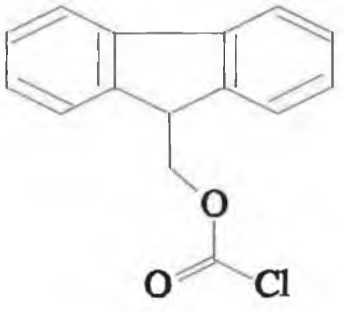
synthesis. Relevant aspects of enzyme technology, notably the use of organic solvents and methods of stabilization, are also discussed.

1.1 Chemical Peptide Synthesis

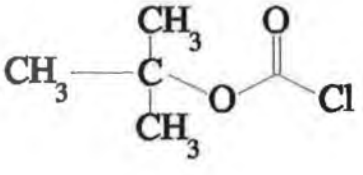
The common approach to peptide synthesis has been traditional chemical synthesis. This involves the joining of optically active amino acids in chains of known length with a predetermined sequence of amino acid residues. In chemical synthesis, it is essential to protect all functional groups except those actually involved in the formation of the peptide bond (Kent 1988; Jones, 1991). This includes alpha-amino and alpha-carboxyl groups as well as any reactive side chain groups. This protection must be reversible without damage to the assembled peptide chain. The most common types protect alpha-amino groups. The Boc (tertiary-Butyloxycarbonyl, or t-Boc) protecting group (Fig 1.1) is the most popular alpha-protecting group. The Boc group is completely stable to catalytic hydrogenolysis conditions (and reducing agents generally), but it is labile to acids. Basic and nucleophilic reagents have no effect at all on the Boc group, even on prolonged exposure. The Boc group is removed by trifluoroacetic acid (TFA) treatment at ambient temperature under anhydrous conditions, to preserve peptide bonds. Fmoc (9-Fluoroenylmethoxycarbonyl, Fig 1.1) protection is the most widely used base-labile alpha-protecting group in peptide synthesis. The Fmoc group is very stable to acidic reagents, but is cleaved rapidly under certain basic conditions. Deprotection with piperidine in dimethylformamide (DMF) is accomplished quickly at room temperature. In some cases one must protect the alpha-carboxy group, e.g. where the carboxy component is activated in the presence of the amino component. The usual method of carboxy protection is esterification (Jones, 1991). The parent carboxylic acid can be regenerated from the ester by acyl-oxygen or alkyl-oxygen fission. For peptides (and amino acids with the amine protected as a simple amide) racemization can occur, involving a five-membered intermediate called an "oxazolone", whenever the carboxylic acid OH group is replaced by a really good leaving group. The use of alkoxycarbonyl protected amino acids in

the coupling reactions results in almost no risk of racemization occurring (Bailey 1990).

Figure 1.1 Structures of some protecting groups commonly used in chemical peptide synthesis.



Fluorenylmethoxycarbonyl chloride
(Fmoc-Cl)



t-Butoxycarbonyl chloride
(tBoc-Cl)

There are three main types of chemical synthesis: solution phase synthesis, stepwise solid phase peptide synthesis and solid phase fragment condensation.

1.1.1 Solution phase synthesis

This involves classical wet organic chemistry. Its advantages include easy purification and characterization of intermediates after each peptide bond-forming step, based on ready discrimination between reactants and target product. However, flaws in this approach severely limit its practical use, notably the poor solubility of some protected peptides in organic solvents. This results from the tendency of protected peptide chains to form intermolecular aggregates rather than interact with organic solvents. Other serious problems include racemization of the activated C-terminal amino acid in peptides and the lack of highly resolving methods for the purification of protected peptide fragments. By incorporating an ionizable group into a (protected) peptide it is possible to selectively adsorb the intermediates of a lengthy synthesis to ion-exchange resins. Two such ionizable "handles" are *p*-dimethylaminoazobenzyl alcohol and 4-picolyl alcohol (Bodanszky 1993). Because the methods involved in this type of synthesis are quite sophisticated, high levels of training and experience are required.

1.1.2. Stepwise solid phase peptide synthesis (SSPPS)

This is based on the attachment of the C-terminal residue of the desired peptide to an insoluble polymer and the stepwise building of the peptide toward the amino end of the peptide. This avoids racemization by always adding a urethane-protected amino acid to the N-terminus of the growing chain. After the desired sequence has been achieved on the solid support, the peptide can be cleaved from the support and be liberated into solution. Techniques are simple, particularly the purification of resin bound intermediates by filtration and washing. However, this places much more stringent requirements on the chemistry because all resin-bound intermediates are carried over into the final product. The attachment of a protected peptide to a swollen resin support overcomes the solubility problem associated with solution phase

synthesis. Other problems associated with SSPPS include incomplete peptide bond formation, giving rise to peptides with one or more internal amino acids missing but with properties similiar to the target sequence. Other problems include side reactions and the use of toxic chemicals.

1.1.3 Solid phase fragment condensation

The target sequence is assembled by consecutive condensation on a solid support of protected fragments prepared by SSPPS. The peptide used as the acyl group in the condensation is usually chosen to have Gly or Pro as the C-terminal residue, which must be activated and is liable to become racemized. Purification of resin-bound intermediates is performed by filtration and washing. This approach overcomes the insolubility problem for the resin bound peptide intermediates. However, purification and characterization of protected fragments not bound to the resin is still difficult. This is particularly important because the fragments have been synthesized by SSPPS (see paragraph above).

1.2 Proteases and Peptide Synthesis

Proteases cleave peptide bonds between the amino acid residues of peptides and proteins. They are well-characterized enzymes of great importance in both life sciences and biotechnology. They are used in the laboratory in tissue culture and molecular biology. They are also useful industrially, in washing powders and detergents, in leather and hide processing, the food industry and so on. In all these applications proteases are used as cleavage and digestion agents in hydrolysis reactions. As catalysts, proteases must also catalyze the reverse reaction, i.e. the *formation* of a peptide bond resulting in oligopeptide *synthesis*. Many significant bioactive substances, such as hormones, antibiotics and neuroactive substances are oligopeptides. The artifical sweetener aspartame is a dipeptide (see next paragraph). Quite a number of peptides have been successfully synthesized using enzymatic methods: see Table 1.1

Table 1.1 Examples of Peptides Prepared by Enzymatic Synthesis

PEPTIDE	REMARKS	ENZYME(S)	REFERENCE
Aspartame	Precursor coupling	Th	Oyama et al. (1981)
Enkephalins	Kinetic synthesis	Ct,P	Kullmann (1984)
Oxytocin	Synthesis of fragments	Ct,P,Th	Cerovsky & Jost (1985)
Kytorphin	Scaled-up synthesis	Ct	Hermann et al. (1991)
Human insulin	Semisynthesis from pig insulin	C,Tr	Morihara et al. (1986)
Glyco - peptides	Preparation	S	Wong et al. (1993)

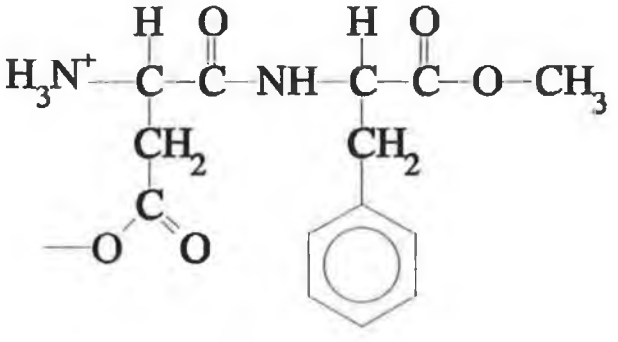
Key to enzymes: C, carboxypeptidase A; Ct, chymotrypsin; P, papain; S, subtilisin BPN'; Th, thermolysin; Tr, trypsin

Compared with chemical methods, enzymatic peptide formation proceeds stereospecifically without side reactions (except unwanted hydrolysis of peptide bonds pre-existing in the peptides to be condensed) and with minimal risk of racemization. It requires little (if any) side chain protection, especially when esters are used as acyl donors. Protease-catalyzed reactions are environmentally safe, whereas various chemical reactions produce ecologically unsafe by-products while forming peptide bonds. In addition, proteolytic reactions can be carried out in water/cosolvent mixtures, which provide enhanced substrate and product solubilities compared with the organic solvents commonly used in chemical synthesis. Finally, highly specific proteases can be used for fragment condensation. For a more detailed description see Gill et al. (1996).

One of the more prominent peptides to be synthesized by enzymatic means is the artificial sweetener aspartame, a dipeptide of aspartic acid and phenylalanine. The enzyme thermolysin was used to catalyze the coupling of two precursors, N-(benzyl-carbonyl)-L- aspartic acid and L-phenylalanine methyl ester, to form aspartame (Oyama et al., 1981) see Table 1.1 and Fig 1.2. Chaiken (1984) used stepwise synthesis from amino acid components to construct aspartame and other small peptides using enzymes. Other products synthesized using enzymatic peptide synthesis include antibiotics such as beta-lactams (Kasche & Haufler, 1984) and semisynthetic penicillins (Oyama et al., 1980). A superpotent analog of human somatoliberin was prepared by a two step enzymatic semisynthesis (Bongers et al., 1992). Here carboxypeptidase Y was used to amidate the C-terminus and V8 protease (from *S. aureus* endoprotease) to incorporate the N-terminal moiety. Widmer et al. (1984) combined the use of chemical and enzymatic methods for the synthesis of fragments of mouse epidermal growth factor. D- and unusual amino acid derivatives could be efficiently incorporated into peptides using enzymatic procedures (Wong et al., 1988). An example of this is the synthesis of protected enkephalin-releasing factors which were catalyzed by chymotrypsin. It was also found that the formation of LD dipeptides was virtually irreversible, while that of LL-dipeptides suffered from a secondary hydrolysis. Another area of development is

the preparation of glycopeptides (Wong et al., 1993). Subtilisin BPN' catalyzed the coupling of N- and O-glycosyl amino acids and glycopeptide fragments. Hermann et al. (1991) showed that enzymatic peptide synthesis could be scaled up for production of larger amounts of dipeptides. They successfully scaled up the production of kytorphin using the protease alpha-chymotrypsin.

Fig. 1.2 The dipeptide sweetner aspartame



Aspartame

Proteases can be classified into four groups depending on the structure of their active sites: serine, cysteine, metallo and aspartic proteases (see Table 1.2). The proteases can be further divided according to the specificity of their peptide cleavage. Therefore, one can select proteases for peptide synthesis on the basis of their specificity against amino acid residues on either side of the bond that is cleaved (the scissile bond).

1.2.1 Serine proteases

Serine proteases have a reactive serine residue in their active site which is included in the hallmark Ser-His-Asp catalytic triad. They also contain the backbone NH groups of Ser195 and Gly193 forming the so-called "oxyanion hole". An acylenzyme is formed during reaction as the carboxyl portion of the substrate esterifies the hydroxyl group of the serine. Reaction of these enzymes with diisopropylphosphorofluoridate (DFP) yields a phosphate ester resulting in complete inactivation. Cleavage of the substrate's terminal peptide bonds is inhibited by the charge on the amino N-terminus or carboxyl C-terminus, hence all members of the group are endopeptidases (Walsh & Wilcox, 1970). The serine proteases also exhibit strong esterolytic activity towards esters with structures analogous to the specific peptide substrate. In addition, hydrolytic activity extends to a variety of acyl compounds in which the acyl moiety is bonded to a good leaving group, such as p-nitrophenol or imidazole. Rapid acylation of the enzyme may be followed by very slow hydrolytic deacylation, depending on the pH of the solution and the nature of the acyl group (Fersht, 1984; Fink, 1987).

The specificities of members of this group range from the narrow specificity of the trypsins, directed toward the bond on the carboxyl side of arginine and lysine, to the rather broad specificity of the subtilisins which are active against bonds between a wide variety of amino acids. It should be noted, however, that specificity towards natural protein substrates may be influenced not only by the side chain next to the scissile peptide bond, but also by neighbouring residues in the sequence and the surrounding structure of the protein (Walsh & Wilcox, 1970).

1.2.2 Cysteine proteases

Cysteine proteases, also known as thiol proteases, depend on the presence of a thiol group for their catalytic activity (Brocklehurst, 1987). Like serine proteases, they are endopeptidases and form covalent intermediates. This transient thiol-ester intermediate forms as part of their catalytic mechanism. Many appear to have a thiol-imidazolyl system in their catalytic site. It has been suggested that the interactive system may be an ion pair where the thiolate anion is transiently acylated during catalysis. Proton transfer from the imidazolium ion assists the process. Further ionization may be required for catalytic competence. This appears to be a variable feature between members of the series.

Cysteine proteases are found in many species including plants, animals and bacteria. Most cysteine proteases have molecular weights in the range 20,000 to 35,000. Many are glycoproteins including bromelains, chymopapains, some ficins, calotropin FI and some of the cathepsins. Calpain and clostripain are large Mr 110,000 and Mr 55,000 respectively and are structurally quite different from papain. The cysteine proteases also vary considerably in substrate specificity and in reactivity.

1.2.3 Metalloproteases

Zinc is invariably the metal found in metalloproteases and participates in a variety of functions in metalloenzymes. It may participate directly in the catalytic process either in the binding step, the rate determining step, or both. It may regulate catalyic activity or help stabilize protein by affecting the the active site conformation structure (Auld, 1987).

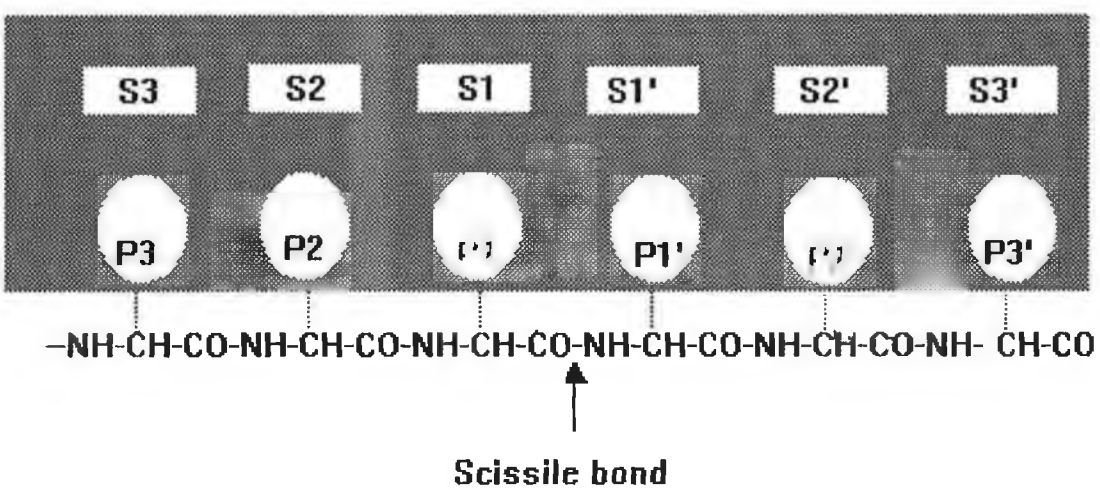
Metalloproteases can be endoproteases or exoproteases. The metallo-endoprotease thermolysin catalyzes enzymatic peptide synthesis in which a zinc atom behaves as a Lewis acid (an electron acceptor) and activates the peptide bond of interest. The enzyme has a distinct specificity for substrate with hydrophobic amino acid residues in P' (see Fig. 1.3) position being preferred. Studies on *P. aeruginosa* elastase have shown its potential use for enzymatic peptide synthesis (Pauchon, 1993). Like thermolysin it has a catalytically essential zinc atom and an active site histidine.

histidine. However unlike thermolysin it contains cysteines and has two disulphide bridges formed from it's 4 cysteines.

1.2.4 Aspartic proteases

Asparatic proteases, also described as carboxyl acid proteases, are mostly single-chain proteins with a molecular weight of 30-40 kD. The most important representative of this class is pepsin. Chymosin also belongs to this class and is responsible for a single specific cleavage of casein in cheese formation. In their active site, two carboxyl groups of aspartate residues engage in a general acid-base catalysis mechanism. With a few exceptions, the optimum pH for catalytic action of aspartic proteases is in the range 1.9-4.0. Two carboxyl groups with pK values of about 1.4 and 4.5 mediate the hydrolysis of peptide bonds in all cases (Fischer, 1987).

Fig 1.3 The Schecter and Berger nomenclature for binding of a peptide substrate to a peptidase (Schecter & Berger, 1967).



where the shaded area represents the protease, P_1 ... P_1' are the chains of six amino acids and S_1 ... S_1' are the corresponding subsites on the enzyme.

Table 1.2 Families of Proteases

FAMILY	EXAMPLES	ESSENTIAL RESIDUE	ACTIVE SITE COMPOSITION	C L E A V A G E MECHANISM
Serine	Trypsin, subtilisin	Serine	Ser-His-Asp catalytic triad	A c y l - e n z y m e intermediate
Cysteine	Papain, ficin	Cysteine	Cys-His charge transfer	Acyl-enzyme intermediate
Aspartic (acid)	Pepsin, chymosin	Aspartic acid	1 charged, 1 uncharged Asp	General acid-base
Metallo	Thermolysin	$[5]$ Zn^{2+}	Coordinated Zn^{2+}	Zn^{2+} acts as Lewis acid

For further details, see Creighton (1993). [5] Zinc ion is not an amino acid, of course, and hence is not a 'residue' but it is essential for metalloprotease function.

1.3 Strategies in Enzymatic Peptide Synthesis

There are two basic strategies for protease-mediated peptide bond formation, depending on the type of carboxyl component used. The carboxyl group not required in the reaction is protected (e.g by an amide group), while the carboxyl group taking part in the reaction can be free or activated depending on the strategy taken. In the first, equilibrium controlled synthesis (ECS, Scheme 1.1), the carboxyl terminus is free and formation of the peptide bond occurs as the reversal of peptide hydrolysis with thermodynamic control. In the second, kinetically controlled synthesis (KCS, Scheme 1.2), a chemically activated carboxyl component is used, which binds to the enzyme to form an alkyl-enzyme intermediate which reacts with either the nucleophile or water in a competing reaction. These two strategies are fundamentally different due to the energies required for the conversion of the substrates to the peptide products.

1.3.1 Equilibrium Controlled Synthesis

Protease-catalyzed reversal of peptide bond hydrolysis involves condensation of a carboxyl component with an unblocked nucleophilic alpha-amino function. Scheme 1.1 represents this synthetic approach in a simple way. The energy required for the transfer of a proton from the positively charged alpha-amino group to the negatively charged alpha-carboxyl group is the predominant barrier to peptide bond synthesis. The concentrations of the non-ionized forms in K_{ion}, control the concentration of the peptide product at equilibrium. Relatively high concentrations of the reactants are required to overcome the unfavourable equilibrium position. Some unusual techniques are needed to achieve an appreciable degree of synthesis. Use of reactants partially blocked at the terminal sites gives significantly higher concentrations of the reactive uncharged forms in the ionization equilibrium, in comparison with unprotected amino acids or peptides. Thus, measurable product formation will occur in the condensation reaction. In general, the pH optimum of the synthesis lies between the pK of the α-carboxyl group and that

of the α-amino group of the substrates (that is, normally between pH 6 and 7). Morihara (1987) has described some strategies used to increase peptide product formation in equilibrium controlled synthesis :

1. Precipitation Use of certain soluble carboxyl and amine components will lead to precipitation of the peptide products, causing the apparent equilibrium to shift towards synthesis since the product concentration in the reaction mixture will be decreased. The concentration of product is determined by the concentrations of the starting materials. Product formation is favoured by use of a reactant concentration yielding an equilibrium product concentration at which significant precipitation occurs. Large hydrophobic substituents used to block either the alpha-amino or alpha-carboxyl groups of the reactants make more insoluble products, but if the substituents are too hydrophobic, they may make the reactants insoluble. Lower concentrations of starting materials can be used for more insoluble products.

2. Use of biphasic systems Here the enzyme is localized in the aqueous phase of a two-phase aqueous/nonpolar organic solvent system. The enzyme is only subjected to the saturation concentration of the nonpolar organic solvent in water and is usually inhibited far less than by water-miscible solvents. There is the possibility of denaturation of the enzyme at the organic/aqueous interface. The water content is usually low (2-5%). When stirred vigorously, the reaction mixture can be regarded as an emulsion of an aqueous enzyme solution in a water-immiscible organic solvent. Starting materials dissolved in the organic phase will diffuse into the water and undergo enzyme-catalyzed peptide synthesis while the products will diffuse from water back into the organic phase. The system is suitable for the formation of water insoluble products. Generally, in solvents less polar than water, the effective pK_a of the $-COO^-$ may increase slightly while the effective pK_b of the $-NH_3^+$ may decrease slightly. Therefore, the portions of uncharged acid and amine forms increase and the equilibrium shifts towards synthesis at neutral pH. Extraction of products from the water into the organic phase drives the reaction towards product formation. Peptide synthesis in biphasic systems with product precipitation has advantages but is limited by the solubility of the substrate in nonpolar organic solvents.

3. Synthesis in the dissolved state Neither of the above schemes can be used in synthesis of water-soluble products (such as short peptides containing basic amino acid residues) or in semisynthesis of high molecular weight peptides and proteins. Reaction can be shifted towards synthesis by using any of the following :

(i) mass action - high concentration of one reactant

(ii) addition of a high concentration of water-miscible organic cosolvent.

(iii) the presence of a molecular trap. Sometimes the product formed can be removed from the equilibrium mixture by formation of a specific complex. This is termed a molecular trap and the idea can be used only in certain circumstances. The first and second factors, however, are generally applicable in synthesis.

Scheme 1.1 Equilibrium controlled peptide synthesis where K_{ion} represents the ionization equilibrium between the charged and uncharged forms of the peptides (or amino acids) to be coupled while K_{con} is the rate constant for formation of the peptide synthesis (see text for further details).

$$RCOO^- + H_3\overset{+}{N}R' \overset{K_{ion}}{\rightleftharpoons} RCOOH + H_2NR' \overset{K_{con}}{\rightleftharpoons} RCO\text{-}NHR' + H_2O$$

1.3.2 Kinetically Controlled Synthesis

In contrast to equilibrium-controlled synthesis, the target peptide product can be formed more rapidly than the thermodynamically-favoured hydrolysis product if the reaction is controlled kinetically. The product with the highest rate of appearance and lowest rate of disappearance will accumulate under kinetic control. The slower hydrolysis reaction becomes important only when the amount of product formed reaches a maximum. In equilibrium controlled synthesis any type of protease can be used but the kinetic approach is confined to proteases that form reactive acyl enzyme intermediates, i.e., serine and cysteine proteases.

The serine and cysteine proteases can catalyze acyl *transfer* from specific substrates to various nucleophiles via an acyl enzyme intermediate (Scheme 1.2). After the leaving group (H-X) departs, the resulting acyl-enzyme intermediate partitions between aminolysis (Scheme 1.2; rate constant k_4) and hydrolysis (rate constant k_3). This results in formation of the desired product RCO-NHR'. For maximum peptide synthesis, k_4 should be much greater than k_3. One can decrease the rate of hydrolysis (which occurs at the rate $k_3[H_2O]$) by reduction of the H_2O concentration (i.e., by increasing the concentration of organic solvents). There will then be less water available. Both aminolysis and hydrolysis are affected equally by factors affecting the rate of acyl-enzyme formation (equilibrium constant K_s and rate constant k_2). These include concentration of enzyme and of the acyl donor, the value of K_s, the rate of leaving group departure, etc. The partition ratio between aminolysis and hydrolysis, on the other hand, depends on the binding efficiency for the productive interaction of the amino component with the acyl-enzyme complex, the concentration of the nucleophile and other parameters.

One can conveniently use ester substrates as carboxyl components in serine and cysteine protease-catalyzed syntheses. Synthesis is rapid, allowing use of a much lower enzyme concentration than in equilibrium controlled synthesis. Also, reaction can be performed at alkaline pH where the rate of secondary product hydrolysis is small due

to the negligible peptidase activity at high pH. From a practical point of view, it is desirable to attain the highest aminolysis/hydrolysis ratio (k_4/k_3 - see Scheme 1.2) and hence the highest synthetic yields. For a comprehensive review of enzymatic peptide synthesis, see Jakubke (1987).

Scheme 1.2 Kinetically controlled peptide synthesis with H-E as serine or cysteine porteases, R-CO-X as carboxyl component and H_2N-R' as amino component. X = O-alk and R-CO-NH-R' = product peptide or substituted amide. K_s is the equilibrium constant for E-S complex formation while k_2 is the rate constant for formationof the acyl-enzyme intermediate. k_4 and k_3 are the rate constants for, respectively, synthesis of the peptide product and for hydrolysis of the acyl-enzyme intermediate.

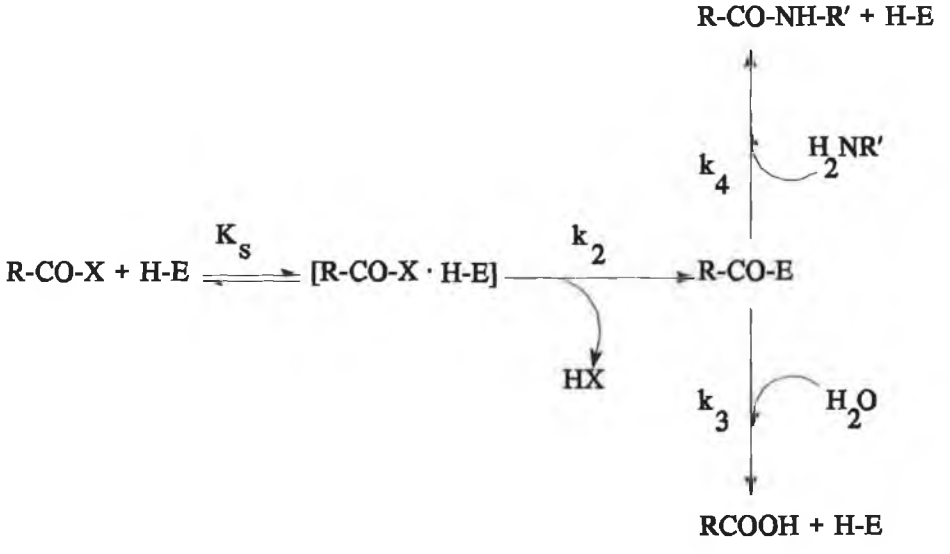


Table 1.3 Comparison of Equilibrium Controlled Synthesis (ECS) and Kinetically Controlled Synthesis (KCS)

PARAMETER	EQUILIBRIUM	KINETICALLY
Enzyme type	All proteases	Proteases forming acyl-enzyme intermediate
Reaction rate	Slow: days	Rapid: minutes
pH	Yield increases as pK_a difference between the $-NH_2$ and -COOH groups decreases	pH optimum lies above pK of P' amino group
Ionic strength	Yield decreases with increasing ionic strength	May influence nucleophile binding
Solvent composition	Influences activity: increase in apparent equilibrium with increase in organic solvent	Product yield increases as water content decreases

For a full account, see Jakubke (1987).

The use of organic cosolvents greatly increases synthetic yields in equilibrium controlled synthesis (and to some extent in kinetically controlled synthesis) but they can have serious adverse effects on enzyme activity and stability. These effects may be quite complex. Modification of the three dimensional structure of the enzyme may occur. The organic cosolvent may interact with the enzyme active centre and/or adsorb on recognition sites and so compete with substrates for the active site of the enzyme. There is a need for integrated study of the effects of the reaction medium composition on different parameters of industrial interest. Blanco et al., (1991) described the main parameters to be tested:

a. Time course of the synthetic reaction.

b. Solubility and stability of substrates and product.

c. Stability of enzyme and enzymatic derivative.

d. Synthetic yield

1.4 Enzyme Catalysis in Non-aqueous Solvents

Most enzymatic processes are carried out in water because it is generally accepted that organic solvents destabilize protein molecules to a greater or lesser extent. Many studies have been carried out on the use of enzymes in organic solvents and the effects of these solvents on enzyme conformation and function. By exchanging enzyme bound H_2O with T_2O (tritiated water), Gorman &Dordick (1992) showed that organic solvents strip water off enzymes. Polar solvents resulted in the highest degree of T_2O desorption (e.g. methanol desorbed 56-62% of the bound T_2O), while nonpolar solvents resulted in the lowest degree of desorption (e.g. hexane desorbed from 0.4% - 2% of the bound T_2O). This study showed that water stripping from an enzyme into a nonaqueous medium does occur and can be significant in polar solvents. A disadvantage resulting from these high concentrations is undoubtedly the decrease of solubility, stability and catalytic activity of the protease. Attempts to place enzymes in media other than pure water to improve peptide synthesis have been made (Barbas et al., 1988; Gaertner & Puigserver, 1990; Kise et al., 1990; Sakurai et al., 1990).

Many studies have been carried out on the use of enzymes in organic solvents and the effects of these solvents on enzyme conformation and function. Nearly anhydrous solvents, containing just a few percent water (and always less than its solubility limit) have been employed as media for enzymatic reactions. When carefully introduced into completely anhydrous solvents, enzymes often display remarkable novel properties (Klibanov, 1989). Proteases can be placed in media other than pure water to improve peptide synthesis. One noticeable effect of certain concentrations of water-miscible organic solvents on serine (chymotrypsin, subtilisin and trypsin) and cysteine (papain) proteases is their lack of amidase activity while their esterase activity remains significant. This prevents secondary hydrolysis of the formed product and makes enzymatic protocols especially useful for stepwise synthesis and fragment

coupling (Barbas et al., 1988). Guinn and colleagues found that solvent selectivity reduced the k_{cat} for amidase activity relative to that for esterase activity. The decline in k_{cat} for amidase activity began at solvent concentrations greater than 10% in a water-miscible organic solvent (9:1 mix of dimethylformamide and dimethylsulphoxide) mixture. This decrease correlated well with minor changes in the structure of the active site. In contrast, the esterase k_{cat} showed no significant decrease at any of the solvent concentrations studied (Guinn et al., 1991).

Catalytic activities of proteases in hydrophilic organic solvents have been markedly increased by immobilization or complexation with polysaccharides such as chitin or chitosan (Kise & Hayakawa, 1990) and immobilized enzymes demonstrated higher catalytic activities than free enzymes for amino acid esterification in many hydrophilic organic solvents (methanol and dimethylformamide were exceptions) (Kise & Hayakawa, 1991). Another approach to non-aqueous enzymology is to use biphasic mixtures. Blanco et al. (1989a) studied the effects of saturating concentrations of various immiscible organic solvents in aqueous solution on the activity and stability of chymotrypsin. The enzyme had been immobilized on agarose and was suspended in the organic phase. All showed the opposite trend for the influence of solvent polarity to that generally observed for biocatalysts. Their stabilized chymotrypsin-agarose derivatives were much more tolerant of these water-immiscible solvents. Studies on the stability performance of *Cynara cardunculus* L. acid protease in aqueous-organic biphasic systems revealed that presaturation of phases was found to increase enzyme stability in the cases of n-hexane and isooctane and to be an absolute requirement in the case of ethyl acetate (Barros et al., 1992)

Attempts have been made to develop a quantitative criterion for the selection of organic cosolvents to be used for enzymatic reactions. Log P values of water-miscible organic solvents (where P is the partition coefficient of a solvent in the water/octanol biphasic system) revealed a good correlation with their denaturing strength: the higher the log P value, the stronger the denaturing effect. However, the log P criterion applies only to solvents of the same functionality (e.g alcohols and polyols) (Mozhaev et al., 1989). When solvents of a different nature are included, the

correlation breaks down. Khmelnitsky and colleagues developed a thermodynamic model of protein denaturation by organic solvents in solution, which explained the molecular mechanism of the denaturation process and established a quantitative relationship between physicochemical properties of organic solvents and their denaturing strength. This relationship enabled construction of the DC (deactivation capacity) scale of organic solvents. The DC scale permits quantitative prediction of limiting concentrations of various organic solvents at which dissolved proteins will remain intact (Khmelmitsky et al., 1991).

One can also undertake peptide synthesis in reverse micelles. The solubilization of proteins in reverse micelles was found to be dependent on the method of protein addition. For the dry-addition method, the protein is not appreciably solubilized until the diameter of the reverse micelle is similar to that of the protein. For small micelles sizes, the injection method solubilizes more protein than the dry-addition method. In the injection method, protein solubilization is not strongly dependent on micelle size (Matzke et al. 1992). While exploring the enzymatic synthesis of Ac-Phe-Leu-NH_2 in reverse micelles, Jorba and co-workers found the cosurfactant concentration to be a critical parameter. One should try to achieve a balance between the cosurfactant's substrate-solubilizing properties and its tendency to yield transesterification by-product. High reaction rate values (k') were obtained by decreasing the cosurfactant concentration. In addition, product distribution P was affected by both structure and concentration of cosurfactants. Lower P values (and thus better peptide yields) were obtained by using less efficient nucleophiles. Extended incubation times led to slightly higher peptide yields due to the transesterification products' further reaction as acyl donors (Jorba et al., 1992).

Inada et al. (1986) showed that enzymes in organic solvents not only showed good solubility and high enzymatic activities but also catalyzed the formation of acid amide bonds. Klibanov (1989) has proposed certain rules to ensure that enzymes will be active in organic solvents:

1. The solvent should be hydrophobic and show very little affinity for water.
2. Enzymes to be used in organic solvents should be lyophilized from solutions with

pH values corresponding to the enzyme's optimum pH

3. The enzyme should be agitated vigorously or sonicated to ensure homogeneous dispersion in the organic solvent.

However enzyme enantioselectivity can be profoundly affected by the solvent (Sakurai et al. 1988). Fitzpatrick et al. (1992) used the x-ray crystal structure of subtilisin and interactive computer modeling to model the structures of the reactive enzyme complexes for the subtilisin-catalyzed transesterification between vinyl butyrate and S and R enantiomers of chiral secondary alcohols XCH(OH)Y. They showed that subtilisin's enantioselectivity depended markedly on the organic solvent used as the reaction medium. They successfully predicted changes in subtilisin's enantioselectivity as a function of the sizes of the X and Y substituents in the nucleophile and upon addition of certain inhibitors.

Organic cosolvents can maximize yields in equilibrium controlled synthesis (and to some extent in kinetically controlled synthesis) but can seriously compromise enzyme activity and stability. Blanco et al. studied the effects of reaction media compositions on the various parameters of peptide synthesis reactions. These included the synthetic reaction's time course, the solubility and stability of substrates and product, the catalytic stability of the enzyme (and derivatives) and the synthetic yield. They prepared benzoylarginine leucinamide using trypsin immobilized on agarose and estimated that synthetic productivities of up to 180 tons of dipeptide product per litre of catalyst per year were attainable (Blanco et al., 1991).

1.5 Stabilization of Proteases

Even though peptide synthesis has certain advantages over chemical methods, biocatalysts are extremely vulnerable to impairment of activity and especially to the effects of temperature and solvents. The activity of a protease requires proper folding, to ensure that structural and functional integrity of the active domain are retained. Proteases catalyzing segment condensation may also split other bonds already formed. There is also the risk of autolysis of the enzyme occuring while in solution. Therefore,

the enzyme must be altered in some way so as to enhance its stability without losing activity. Common approaches to enzymes stability include chemical modifications, coupling, crosslinking and immobilization of the enzyme. Protein engineering is increasingly being employed in the stabilization of proteases.

The three-dimensional structure of the enzyme is in a functional active form in a native enzyme. However, this three-dimensional structure (and hence the integrity of the enzyme) can be irreversibly disrupted by a range of forces both physical and chemical. Physical forces include heating, freezing and irradiation, chemical forces include oxidation, reduction, ionic strength, extremes of pH, effects due to chemicals, solvents, and metal ions and biological forces such as enzyme modification and degradation. Thermal inactivation is the most important mode of enzyme inactivation. In industrial applications elevated temperatures and the use of chemicals and organic solvents are frequently involved. Proteins would not encounter such forces in vivo and when such situations arise problems with stability may arise. Enzymes can however, be stabilized by a variety of procedures to prevent loss of activity.

Before discussing the various options available for the stabilization of proteins it is first necessary to explain the terms "denaturation" and "stabilization". Gianfreda & Scarfi (1991), regarded the native structure of a protein as the conformation exhibited by proteins within the cellular environment or by isolated proteins at their maximum biological activity. Protein denaturation is a process involving a major or minor change of this three-dimensional native structure, without altering the amino acid sequence (Tanford, 1968; Lapanjie, 1978). Enzyme inactivation results from a change of the protein structure that compromises the right arrangement of the active site (Klibanov 1983). Using these terms stabilization of protein molecules means preventing this change and preserving the native structure of proteins.

Mozhaev (1993) described the reversible and irreversible inactivation of enzymes. Reversible unfolding is a two state transition, where N is folded native and U is unfolded denatured:

$$N <-> U$$

Reversible unfolding can be constrained by either immobilization or by adding new

stabilizing interactions, e.g. by protein engineering.

Irreversible inactivation is thought to be a multi-step process. The first step is the reversible (unfolding) followed by irreversible processes:

$$N <-> D -> I$$

where N, D and I are the native, reversibly unfolded and irreversibly inactivated forms of the protein, respectively. Klibanov (1983) suggested that irreversible inactivation was a result of covalent changes giving rise to a chemically altered enzyme and noncovalent changes causing aggregated or incorrectly folded enzyme. Based on these observations Mozhaev (1993) suggests that methods such as covalent modification of amino acid residues or 'medium engineering' (by the addition of low-molecular-weight compounds) could be effective against irreversible (incorrect) unfolding, while the most effective approach for preventing chemical deterioration is genetic modification of the protein sequence.

1.5.1 Chemical modification

A serious drawback associated with protease-catalyzed synthesis of peptides (and semisynthesis of larger peptides via segment coupling) is the potential loss of product due to the hydrolysis of sensitive peptide bonds by the protease. Chemical modification can alter an enzyme so that it exhibits esterase activity but little or no amidase activity. Nakatsuka et al. (1987) prepared thiolsubtilisin (a chemical derivative of subtilisin in which a thiol group replaces the serine hydroxyl in the active site). This acted poorly as a protease but proved to be a promising catalyst in the synthesis of small and larger peptides. The enzyme became a rudimentary peptide ligase by virtue of the thiol-acyl intermediate's preference to react with an alpha-amine rather than to hydrolyze (Nakatsuka et al., 1987). Similarly, conversion of subtilisin's active site serine (Ser 221) into selenocysteine yielded an acyl transferase with selectivity properties suitable for use as a peptide ligase (Wu & Hilvert, 1989). Methylated subtilisin also lost most of its amidase activity but its esterase activity was still significant and useful for peptide synthesis (Zhong et al., 1991). Chemical modification can also be used to stabilize proteases, i.e., to increase their resistance to inactivation under conditions used

for peptide synthesis. Some recent approaches to chemical stabilization of soluble (as distinct from immobilized) enzymes have been reviewed (Ó Fágáin, 1995).

1.5.2 Crosslinking

Crosslinking of proteins is often employed to increase the stability of proteins. Crosslinking reagents are usually bifunctional reagents, i.e., reagents with two reactive groups, that can be used to introduce both inter- and intra-molecular bridges in proteins (Han et al., 1984). The introduction of intramolecular crosslinks can be used to stabilize the tertiary structure of an enzyme. Bifunctional crosslinking reagents are either homobifunctional, in which reactive groups are the same, or heterobifunctional, in which the reactive groups are different. Homobifunctional reagents include imidoesters and bismaleimides, that are amino- and thiol- group specific respectively. Heterobifunctional reagents include derivatives of N-hydroxysuccinimide that are specific for both amino and thiol groups. For a more detailed description of homo- and hetero-bifunctional crosslinkingreagents see Ji, 1983; Means & Feeney, (1990); Ó Fágáin & O'Kennedy, (1991); Mozhaev et al., (1990) and Wong & Wong, (1992).

Bifunctional reagents crosslink different parts of the enzyme molecule. The active conformation of the enzyme should be maintained in any crosslinking procedure. In effect, "braces" are placed across the molecule preventing unfolding (Ó Fágáin et al., 1988). It is known that unfolding is an essential step in protein denaturation (Torchilin et al., 1978) and this may be prevented by crosslinking. Crosslinking strenthens the compact structure of the molecule, preventing denaturation by heat and other forces. Enhancing stability of an enzyme by treatment with a bifunctional reagent largely depends on the length of the bifunctional molecule, and hence the distance between the molecular centres (Torchilin et al., 1978, and 1983). It is possible that a crosslinking reagent may form molecular bridges in one type of enzyme molecule and not in another. When crosslinking occurs, then the target enzyme has molecular distances that suit the crosslinking reagent used.

Crosslinking reagents that have enhanced the stability of proteases include glutaraldehyde, bisimidoesters, adipimidates, dianhydrides, suberimidates and

succinimide esters. The half-life of α-Chymotrypsin crosslinked with dianhydrides and N-hydroxysuccinimide esters of carbonic acid increased from 1 hour to 22.2 hours at 50°C and their pH optima shifted to a higher pH value, while isoelectric points shifted to the acid region (Tafertshofer et al. 1989). Gleich et al. (1992) found that trypsin crosslinked with reagents such as N-hydroxysuccinimide esters (NHS) of dicarboxylic acids, dianhydrides and bisimidoesters showed enhanced stability while retaining sufficiently high activity. Autolysis and thermal denaturation were also reduced. Crosslinking *Trichodermas koningii* extracellular alkaline protease with glutaraldehyde insolubilised the protease and also increased its stability (Manonmani & Joseph 1993). Rajput and coworkers crosslinked trypsin using glutaraldehyde and bisimidoesters (Rajput et al. 1987) and later with dimethyladipimidate (Rajput et al. 1988). Trypsin crosslinked with glutaraldehyde was only marginally more stable towards autolysis compared with native trypsin and retained 90% of amidase activity. Trypsin crosslinked with dimethylsuberimidate (DMS) and dimethyladipimate showed lower esterase and caseinolytic activity but decreased autolysis. Likewise, trypsin crosslinked with dextran-dialdehydes showed increased stability towards autolysis, pH and temperature (Kobayashi and Takatsu 1994). Treating *Pseudomonas aeruginosa* elastase with dimethyl suberimidate increased its thermostability and seemed to intramolecularly crosslink the enzyme (Besson et al. 1995). Both St. Clair & Navia (1992) and Ampon & Whitaker (1993), chemically crosslinked enzyme crystals which managed to retain their catalytic activity in harsh conditions including high temperature, pH extremes and organic solvents. Thermolysin crystals crosslinked with glutaraldehyde showed remarkable temperature, pH and solvent stability compared with native thermolysin. Crosslinked enzyme crystals (CLC's) showed enhanced resistance to proteolysis. This is thought to be due to the exclusion of exogenous protease from the small solvent channels defined by the crystal lattice.

1.5.3 Hydrophilization

Hydrophilization involves preferential modification of $-NH_2$ groups on the protein surface by alkylation or acylation to achieve an artificial hydrophilization of the

surface area of the protein globule. Generally, an enhanced hydrophilization of the surface layer of protein globules (transformation of protein -NH_2 groups into -$NHCH_2COOH$ groups or analogues of carboxyl derivatives) results in increased enzyme stability. This effect can be explained by a decrease of nonpolar clusters located on the protein surface, contact of which with water is thought to destabilize the enzyme (Gianfreda & Scarfi, 1991).

Hydrophilization of proteases has been showed to protect them from thermoinactivation. Mozhaev et al.(1988) hydrophilized trypsin by transforming surface tyrosine residues to aminotyrosine via nitration followed by reduction with sodium dithionite. In this way the -OH group of the tyrosine was replaced by an -NH_2 group. Stabilization of up to 100 times greater than the native was realized and was found to be exclusively dependent on the number of aminotyrosines introduced, the stabilizing effect increasing with the number of aminotyrosine residues in trypsin. The same report described alteration of chymotrypsin's surface by treatment with anhydrides or chloroanhydrides of aromatic carboxylic acids. This allowed a variable number of carboxylic groups (up to 5) to be added to each Lys residue altered. Acylation of the enzyme in this way resulted in a more hydrophilic protein (as measured by partitioning in an aqueous biphasic system) with greatly enhanced thermostability (300 times more stable than native chymotrypsin). A 100 - 1,000 fold increase in stability against thermoinactivation was observed in α-Chymotrypsin acylated with carboxylic acid anhydrides or reductively alkylated with aliphatic aldehydes. There is a direct relationship between the degree of hydrophilization and protein stability, to a limiting value. Above this value further increases in hydrophilicity did not increase thermostability. For both native and the low-temperature form of the modified enzyme, inactivation was due to incorrect refolding. Hence, the hydrophilization stabilizes the unfolded high temperature conformation by eliminating the incorrect refolding (Mozhaev et al. 1992).

Inactivation of proteases in organic solvents has been reduced by modifying the enzymes with hydrophilic reagents. The reductive alkylation of trypsin with acetaldehyde, propionaldehyde, octaldehyde and benzaldehyde, resulted in about 5- to

6- fold increase in esterification activities in DMF compared with the native (unmodified) enzyme at temperatures between 20 and 60°C (Ampon et al., 1991). Khmelnitsky and co workers postulated that hydrophilized enzymes had the ability to keep their hydration shell, thereby enhancing their stability in organic solvents. They showed that chymotrypsin modified with pyromellitic dianhydride endured much higher concentrations of organic solvents without loss of catalytic activity as comparred to native enzyme (Khmelnitsky et al., 1991b).

1.5.4 Protein coupling to polyethylene glycol (PEG) and polysaccharides

A particular type of chemical modification which can sometimes stabilize enzymes is the covalent attachment of large molecular weight polyhydroxy entities such as polyethylene glycol (PEG) or carbohydrates to proteins. PEG coupling has been used to dissolve enzymes in non-aqueous solvents opening up a new area of enzyme chemistry. Covalent attachment of PEG to a protein can result in minimal loss of activity and help stabilize the structure. PEG-modified subtilisin showed enhanced stability towards temperature and pH (Yang et al., 1996). However, the main purposes of PEG coupling have been to dissolve enzymes in non-aqueous solvents and to use them for novel reactions (see 'Enzyme catalysis in non-aqueous solvents'). Enzymes modified with PEG have both hydrophilic and hyrophobic properties. PEG modified chymotrypsin retained activity and solubility in aqueous and organic systems. It also catalyzed amide bond formation in organic systems (Inada et al., 1986). Likewise, PEG-modified thermolysin catalyzed peptide synthesis in organic solvents such as benzene (Ferjancic et al., 1988). Sakurai and colleagues modified trypsin, chymotrypsin, papain, thermolysin and pepsin with PEG. Each PEG-protease became soluble and active in organic solvents and also catalyzed peptide bond formation with its respective substrate specificity. Using five kinds of PEG-protease, various kinds of tripeptides were synthesized by a solid phase system in methanol or dimethylformamide (Sakurai et al., 1990). Inada et al. (1986) described the use of enzymes (including chymotrypsin) modified with PEG which had both hydrophobic and hydrophilic properties. Direct modification of chymotrypsin caused complete loss

chymotrypsinogen was modified and then activated using trypsin. The modified chymotrypsin retained 57% of its esterase activity in aqueous systems and was soluble in organic solvents such as benzene, toluene and chlorinated hydrocarbons. It also catalyzed the formation of amide bonds in benzene at 37°C. Zalipsky and co-workers have described a new means for covalent attachment of PEG to proteins. They converted methoxypolyethylene glycol (molecular weight 5,000) to a reactive succinimidyl carbonate form (SC-PEG). SC-PEG was sufficiently reactive to produce extensively modified proteins under mild conditions within 30 min, showing the highest reactivity around pH 9.3. Their PEG-trypsin conjugates had no proteolytic activity, well preserved esterolytic activity and enhanced activity toward chromogenic p-nitroanilide substrates (Zalipsky et al., 1992).

Polyethylene glycol modified proteases have been successfully used in synthesis rather than hydrolysis reactions in organic solvents (Takahashi et al., 1984; Matsushima et al. 1984). PEG-modified chymotrypsin catalyzed peptide bond formation in benzene. Ferjancic et al. (1988) observed that substrate specificity of PEG-modified thermolysin changed in organic solvents where hydrophilic as well as acidic amino acids were better carboxyl group donors than hydrophobic residues.

Like PEG, carbohydrate moieties have been coupled to proteases to enhance their stability. Marshall (1975) coupled a number of enzymes, including trypsin, to cyanogen bromide-activated dextran via the ε-amino groups on their lysine residues. All the enzyme derivatives were more thermostable and the rate of autolysis in the trypsin derivative decreased. Carbohydrate protease conjugates (CPC's) acquired higher stability due to increased aldehyde content in aqueous solution. CPC were capable of synthesizing peptides in acetonitrile (Wartchow et al. 1995).

1.5.5 Protein engineering

This allows alteration of an enzyme's amino acid sequence (and, therefore, of its structural and catalytic properties) by manipulation of its coding DNA sequence. One can improve enzyme stability and function in environments somewhat different from the optimal "in vivo" conditions. However, a detailed knowledge of the structure of the

target protease is needed. Not only must the exact amino acid sequence be known but also the spatial conformation of the protein molecule.

The substrate specificities of proteases have been altered by using protein engineering. Craik et al. studied the relationship between structure and function of trypsin. They substituted alanines for the glycine residues at positions 216 and 226 in the substrate binding cavity. The resulting three variants (two single mutants and a double mutant) showed enhanced substrate specificity, but decreased catalytic rates, relative to wild type trypsin (Craik et al., 1985). By substituting the aspartic acid residue at the base of the binding pocket with α-lysine residue all catalytic activity was lost; however, an inherent but low chymotrypsin-like activity was observed. It appears that the positively charged $^{+}NH_3$ group on the lysine side chain is directed outside the substrate binding pocket. The resulting hydrophobic cavity may explain the substrate specificity of the mutant enzyme (Graf et al., 1987). The effects of substituting specific amino acids in trypsin that are not critical determinants for catalysis and substrate specificity were also studied (Craik et al., 1990). Hedstrom and colleagues converted trypsin to a chymotrypsin-like protease. They replaced the S1 binding site (see Fig. 1.3 in Section 1.2) of trypsin with the analogous residues for chymotrypsin and exchanged the chymotrypsin surface loops 185 through 188 and 221 through 225 for the analogous trypsin loops. This mutant enzyme was equilivant to chymotrypsin in its catalytic rate, but its substrate binding is impaired (Hedstrom et al., 1992).

Subtilisin has been designed for efficient ligation of peptide bonds in aqueous solution. Nakatsuka et al. (1987) showed that conversion of Ser221 to cysteine resulted in the a rudimentary peptide ligase by virtue of the thiol-acyl intermediate's preference to react with an α-amine rather than to hydrolyze. Although sluggish, the enzyme's ligase activity could be dramatically enhanced and peptidase activity suppressed by making more room in the active site for the larger catalytic thiol. This was done by compressing the α-helix (Abrahmsen et al., 1991). Bonneau et al. reduced subtilisin BPN's rate of amide hydrolysis without affecting its esterolysis. They achieved this by converting the wild type Met222 to phenylalanine. The Met222→Phe mutant seems to be an excellent candidate for preparative scale peptide synthesis applications (Bonneau

et al., 1991). The peptide ligase activity of endopeptidase from *Flavobacterium meningosepticum* in aqueous solution was increased by substituting Cys in place of the catalytic Ser556 (Kreig & Wolf, 1995).

Proteases must function in organic solvents if they are to be used in peptide synthesis. It has been proven possible to stabilize proteases in organic solvents using site directed mutagenesis (Zhong et al., 1991; Chen et al., 1991; Martinez & Arnold, 1991; Martinez et al., 1992 and Economou et al., 1992a) and random mutagenesis (Economou et al., 1992a & b; Chen & Arnold, 1991 & 1993). Arnold (1993) improved the thermal and conformation stability in organic solvents of cytochrome-C and subtilisin-E by incorporating simple metal-chelating sites. A metal ion inert to ligand exchange (Ru^{2+}) stabilized proteins by crosslink formation in denatured and folded states. Xu et al. (1992) attempted to redesign the active site of subtilisin BPN' for optimal function in non-aqueous media. Polar mutation was studied; however, variants showed lower catalytic activity.

Likewise, protein engineering of protease has enhanced their thermostability. Bryan et al. (1986a & b) developed substilisin variants with enhanced resistance to thermal inactivation. They looked at engineering disulphide bonds and modifying calcium binding sites known to be critical for stabilization and found two variants that showed significantly more stability than that of the wild type. Also, by introducing mutations "in vitro" to random sites within the cloned subtilisin gene, the stability was greatly enhanced: in one case 50 times greater stability than the wild type was observed (Bryan et al., 1987). Based on computer graphics, Grandi et al. (1990) predicted changes that should enhance activity by comparing *Bacillus subtilis* neutral protease (BSNP) to thermolysin. They inserted a 10 amino acid external loop, which coordinates 1 calcium ion in thermolysin, into BSNP in an attempt to increase thermostability. However, no effect on thermostability was observed. When Gly189 was replaced by alanine the stability of BSNP was increased.

1.5.6 Immobilization

By definition, an immobilized enzyme is a protein physically localized in a certain

region of space or converted from a water-soluble, mobile state to a water insoluble immobile one (Gianfreda & Scarfi, 1991). Immobilized enzymes may very closely simulate the state of enzymatic proteins within the intracellular microenvironment of living cells and so they can provide a good system to study and solve some basic problems in enzymology. Because the enzyme is immobilized it is therefore recoverable and hence reusable.

Proteases can be immobilized without loss of function and the potential of immobilized proteolytic enzymes for peptide synthesis has been demonstrated (Blanco et al., 1989a & 1991; Jakubke & Konnacke, 1987; Nilsson & Mosbach, 1984; Alvaro et al., 1991; Kise & Hayakawa, 1991). It is thought that multipoint enzyme-support attachment may exert an important stabilizing effect on the insolubilized enzyme derivative. These "more rigid" enzyme molecules must be more resistant to conformational changes induced by heat and organic solvents than the corresponding unmodified enzyme molecule (Klibanov, 1983). It is therefore not surprising that proteases have been immobilized to a range of different types of supports.

Thermoinactivation studies by Martinek and coworkers on native and immobilized trypsin and chymotrypsin found that the immobilized enzymes had increased thermostability (Martinek et al., 1975; 1977a & b). The thermostabilities of trypsin and chymotrypsin attached with multipoint linkages to the lattice of polymeric acrylamide or sodium methacrylate gel were found to be several orders of magnitude higher than that of the respective native enzymes, with high catalytic activity being retained (Martinek et al., 1977b). They also looked at chymotrypsin and trypsin mechanically entrapped into polymethacrylate and polyacrylamide gels and found an increase in the rigidity (and hence the thermostability) of the enzyme (Martinek et al. 1977a).

Enzymes covalently incorporated in polyacrylamide gel were found to be hundreds of times more stable against irreversible thermoinactivation than native enzymes. The reversible conformational changes which also lower enzyme activity at elevated temperatures were completely suppressed following immobilization. It was concluded that the copolymerized enzyme preparations had high operational stability

at elevated temperatures (Mozhaev et al., 1983). Likewise, Reddy et al. (1986) immobilized trypsin onto alginic acid poly(glycidyl methacrylate) graft copolymer. Both the pH and thermal stabilities were greater than those of soluble enzyme.

Blanco and co workers found that trypsin immobilized by multipoint attachment in the presence of its competitive inhibitor benzamidine were more active but less stable than derivatives prepared without benzamidine (Blanco & Guisan, 1988). They also tried one-point attachment of enzymes and found it to be reversible. However, immobilization became apparently irreversible when it occurred through a two-point or greater multipoint attachment. It was thus assumed that enzyme molecules become irreversibly attached to the support through their areas containing the highest densities of amine groups. They looked at the effects of a number of variables on the trypsin (amine)-agarose (aldehyde) interactions processes: surface density of aldehyde groups, pH, contact time and temperature (Blanco et al., 1989b). The most stable derivative was 5,000-fold more stable than the native trypsin. Seven lysine residues per trypsin molecule had reacted with the activated support. They also developed different activity/stability tests to evaluate the possibilities of using fully dispersed chymotrypsin derivatives as industrial catalysts in biphasic systems (Blanco et al. 1992). They concluded that fully dispersed immobilized stabilized derivatives seemed to be an interesting alternative to native chymotrypsin for the development of chymotrypsin catalyzed industrial biphasic processes.

Other novel approaches to stabilization of proteases by immobilization include the preparation of soluble-insoluble immobilized enzyme (Fujimura et al., 1987). Using methacrylic acid-methacrylate copolymer (MPM-06), a papain derivative was insoluble in aqueous solution when below pH 4.8 and soluble above pH 5.8. It was found to be more stable against water-miscible organic solvents than native proteases. Furthermore, various proteases could be immobilized by this method with high activity. Cohenford et al. (1986) crosslinked bovine trypsin to human serum albumin (HSA) with glutaraldehyde to form soluble and insoluble copolymers. Both soluble and insoluble trypsin-HSA polymers showed enhanced resistance to heat inactivation. The trypsin-HSA polymers also showed shifts in pH optima, an increase in activation

energy and a broadening of their pH stability profiles. Couturier et al. (1988) used covalently immobilized proteases on polystrene and nylon as a tool for the study of chromatin structure. Tyagi and coworkers developed a method of reversible immobilization for trypsin. The surface charges on the enzyme were modified by pyromellitic dianhydride and the trypsin derivative bound to DEAE-cellulose. The immobilization is reversed by ion-exchange (Tyagi et al., 1994).

Studies on the immobilization of enzymes onto Perflex (a new fluorocarbon based technology) show that significant loss of enzymatic activity may occur upon immobilization of certain enzymes due to the hydrophobic character of the support. This appears to be due to a large conformational change of the protein ("inversion"). Pretreatment of the Perflex support with fluorosurfant improved the retention activity for sensitive enzymes (Boivin et al., 1991).

Proteases used in peptide synthesis have also been immobilized to a number of different supports. Thermolysin, immobilized using various methods, synthesized N-(benzyloxycarbonyl)-L-aspartyl-phenyalanine methyl ester, the precursor of the synthetic sweetner aspartame (Oyama et al., 1981). α-Chymotrypsin immobilized to tresylchloride-activated agarose for peptide synthesis in aqueous-organic solvent mixtures was found capable of distinguishing between L- and D- configurations of the acceptor amino acid. It was also noted that multiple points of attachment had a favourable effect on the stability of the enzyme (Nilsson and Mosbach 1984). Fadanavis & Lusi (1991) showed that α-chymotrypsin entrapped within a polyacrylamide gel crosslinked with N,N'-methylenebisacrylamide, catalyzed peptide synthesis using water soluble substrate in reverse micellar suspension. Proteases immobilized to porous chitosan beads showed higher catalytic activities than free enzymes for amino acid esterification in many hydrophilic organic solvents (methanol and dimethylformamide were exceptions). The immobilized enzymes were highly stable in organic solutions and could easily be separated from the reaction mixtures (Kise & Hayakawa, 1991). Chymotrypsin deposited on Celite™ catalyzed peptide synthesis reactions in organic solvents. The substrate specificity in the reaction was due to the combined effects of the substrate specificity of the enzyme and the influence of

the solvents (Clapes et al., 1992). Blanco and coworkers found that chymotrypsin immobilized by multipoint attachments to crosslinked agarose catalyzed peptide synthesis in the presence of water-immiscible solvents over long periods, and enzyme activity was retained at the end (Blanco et al., 1989). Later, they found that trypsin-agarose derivatives greatly broadened the range of usable conditions in both kinetically controlled (KCS) and equilibrium controlled (ECS) syntheses (upto 1M ammonium sulfate (KCS) or 90% organic cosolvents (ECS) could be tolerated). Under these conditions, the derivative retained more than 95% of activity after 2 months (Blanco et al., 1991). One-point and multipoint covalently attached chymotrypsin and trypsin derivatives were used to synthesize dipeptides (Arg-Leu and Tyr-Arg). Because of the stability of the enzyme derivatives, drastic experimental conditions (high concentrations of organic solvents and ammonium sulfate) could be applied (Alvaro et al., 1991).

1.6 Novel approaches to Enzymatic Peptide Synthesis

Enzymatic peptide synthesis has been carried out in many different organic systems and using a range of modified forms of proteases. Others novel approaches to enzyme catalyzed peptide synthesis have been explored. These include low temperatures and the use of unprotected amino acids, inverse substrates and salt hydrates. It has been observed that amounts of the desired peptide product increase with decreasing temperature as the rate of hydrolysis is depressed (Chen et al., 1994). It has also been observed that unprotected amino acids (as acyl acceptors) can successfully be used in enzymatic peptide synthesis. Tougu et al. (1993) found that the efficiency of amino acids as acceptors in the formation of the coresponding dipeptide (H-Try-X-OH and Mal-Tyr-X-OH) depended on the substrate used in chymotrypsin - catalyzed synthesis. These amino acids were not found to act as acyl acceptors in liquid water. Schuster et al (1993) used chymotrypsin to form dipeptides from unprotected amino acids in frozen solution. It is thought that an increase in the concentration of the reactants in an unfrozen liquid phase as a result of freezing increased the aminolysis reaction compared to the hydrolysis reaction.

Khmelnitsky and coworkers found that the presence of nonbuffer salts dramatically enhanced activity of lyophilized enzymes. They suggested that salt-induced activation of enzyme activity in organic solvents was due to a protective effect afforded by the salt matrix against deactivation by direct contact with the organic solvent (Khmelnitsky et al., 1994). Kuhl et al. (1992) exploited this phenomenom by performing enzyme-catalyzed peptide synthesis in the presence of salt hydrates. Thermolysin synthesized N-protected dipeptides and tripeptides in hexane in the presence of $Na_2SO_4.10H_2O$. Cerovsky went one step further by enzymatically synthesizing peptides in solvent-free systems in the presence of $Na_2CO_3.10H_2O$. The salt hydrate provides the alkaline conditions required for enzymatically-catalyzed peptide synthesis and the necessary water source for the enzyme (Cerovsky, 1992).

Enzymatic peptide synthesis using "inverse substrates" has also been explored as a possible means of increasing peptide yield (Schellenberger et al., 1991a & b). In an inverse substrate for trypsin, the cationic centre is included in the leaving group instead of being in the acyl moiety (P1 position) (Tanizawa et al., 1977). Using Benzloxycarbonyl-L-alanine p-guanidinophenyl ester as an acyl donor, trypsin catalyzed the synthesis of peptide bonds that cannot be split by this enzyme. This approach would prevent hydrolysis of the product.

A wide range of protease are capable of catalyzing peptide synthesis in heterogeneous "eutectic" mixtures of substrates (Gill & Vulfson, 1994). A "eutectic" can be described as a mixture of two or more substances which displays a minimum value for the melting temperature, at a defined composition in the corresponding phase diagram (for a more detailed description see Murrell & Boucher, 1982). The minimum melting point (the eutectic temperature) is significantly lower than the melting points of the pure constituents reflecting their interaction at a molecular level. As a result of molecular interaction at this minimum melting point, the eutectic temperature is significantly lower than the melting points of the pure constituents. When two solid amino acid derivatives are mixed, they form liquid or semiliquid eutectics. Under these conditions, proteases were capable of catalyzing peptide bond formation when derivatives of amino acids were mixed. Furthermore, the addition of small amounts of

solvents, known as adjuvants, promoted the formation of eutectics (Gill & Vulfson 1993, Lopez et al., 1994). Another approach to enzymatic peptide synthesis involves the use of starting materials and products in the form of suspended solids. The low aqueous solubilities resulting from many established protecting groups may not be a barrier to peptide synthesis. Solid-to-solid conversion is independent of the solvent used, at a fixed water activity, and proceeds until the excess solid of at least one reactant has been completely consumed (Halling et al., 1995).

1.7 Potential of trypsin as a tool for peptide synthesis

Trypsin is a serine protease that hydrolyses peptides, amides, esters etc. at bonds involving the carboxyl group of L-arginine and L-lysine. It is a proteolytic enzyme, whose inactive precursor trypsinogen is formed in the exocrine cells of the pancreas and is secreted into lumen of the intestine. The proenzyme is converted to the active enzyme by enterokinase and also autocatalytically by the action of the trypsin formed. Bovine trypsin is a single-chain structure of 229 amino acid residues, crosslinked by six disulphide bridges. The amount of active trypsin in solution can be determined either from the rate of catalysis of a specific substrate or by direct titration of its active site (Rick, 1974).

Trypsin was chosen because it is a well characterized enzyme with numerous assay systems. Trypsin from beef pancreas was among the first proteolytic enzymes isolated in pure form in amounts sufficient for exact chemical and enzymological studies. Its applications in cell culture, molecular biology and immunology are well documented. Trypsin is also used to activate precursor proteins produced in recombinant expression systems, such as glucagon-like peptide precursor (Rasmussen et al., 1995). Other applications include the use of trypsin in biosensors to detect peptides (Taguchi et al., 1990) and in waste-water treatment to inhibit the growth (or to enhance the destruction) of slime-causing filamentous bacteria (Nalco-Chem, 1994). It has also been used to treat paper containing polyamide resin (Mead, 1994) and to produce soluble collagen which can be used in paper making (Fink & Brody, 1996;

Battle-Mem. Inst. Ohio, 1994). In the food industry, trypsin is used to degrade milk proteins to produce a substance that has no antigenic activity for use in infant formula food (Teagasc, 1993; Nestle, 1993).

Trypsin has successfully been used in the synthesis of biologically active peptides including a Hepatitis B antigen and also in semisynthesis and modification of proteins such as human insulin analogue (for more details see Gill et al., 1996). Model system dipeptide synthesis have previously been carried out by Blanco et al. (1991) and Cervosky (1990). It is the aim of this project to chemical modify this well characterized enzyme using a range of chemical reagents. Any of these modified trypsin that exhibit enhanced stability will be used in peptide synthesis.

1.8 Conclusion

Enzymatic peptide synthesis is an area of rapid growth and development. Enzymatic peptide synthesis offers numerous advantages over conventional methods including prevention of racemization, reduced use of toxic solvents, reduced side-chain functional protection and possible reuse of the catalyst. There are, however, a number of drawbacks associated with the use of enzymes including denaturation and inactivation at high solvent concentrations and autolysis of the protein molecule. Substrate specificity also poses some problems: whereas a high specificity drastically reduces application, proteases with broad substrate specificity have the advantage of a more universal use, but this is always coupled with the potential of unspecific proteolytic cleavages in the starting substrates and the end product. However, new data and information along with improved techniques for enzyme stabilization (including immobilization, chemical modification and protein engineering) are instrumental in overcoming some of these limitations.

It is not expected that protease-catalyzed peptide synthesis will replace chemical peptide synthesis but chemical and enzymatic synthesis should be combined in such a way as to allow the individually most effective variant to be preferred. The continued development of enzyme technology will contribute toward the goal of steadily

increasing the potential of enzymatic peptide synthesis.

CHAPTER 2

MATERIALS and METHODS

2.1 MATERIALS

Sigma Chemical Company,

Poole, Dorset, England.

Bovine pancreatic trypsin (EC 3.4.21.4) type III, benzoyl DL -arginine *p*-nitroanilide (DL-BAPNA), benzoyl-L-arginine *p*-nitroanilide (L-BAPNA), L-Leucinamide HCl, acetic acid N-hydroxy-succinimide ester (AA-NHS), Ethylene Glycol-bis(succinic acid N-hydroxysuccinimide ester) (EG-NHS), Suberic Acid bis(N-hydroxysuccinimide ester) (SA-NHS), Tris-(hydroxymethyl) aminomethane (Tris), Borax, 2-(N-Morpholino) ethanesulfonic acid (MES), sodium bicarbonate, sodium hydroxide, calcium chloride, sodium chloride, 2,4,6-trinitrobenzenesulfonic acid (TNBS), urea, guanidine hydrochloride, sodium dodecylsulfate (SDS), benzamidine, glycine, 1,7-diaminoheptane, 1,8-diaminooctane, 1,10-diaminodecane, 1,12-diaminododecane, adipic acid, sebacic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-N'-methylene-bis-Acrylamide, Nα-p-Toluenesulfonyl-L-arginine methyl ester (TAME), Nα-p-Toluenesulfonyl-L-arginine (TA), N-benzoyl-L-arginine (BA), N-benzoyl-L-arginine ethyl ester (BAEE), p-Nitrophenyl p'-guanidinobenzoate HCl (NPGB), thiobenzoyl benzoyloxycarbonyl-L-lysinate (Z-Lys-SBzl), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), Coomassie Brilliant blue R-250, Nile Red, bromophenol blue sodium salt, ammonium persulphate, N,N,N',N'-tetramethyl ethylenediamine (TEMED), Sephadex G-25™ and molecular weight markers: Albumin, Bovine serum; Albumin, Egg (ovalbumin); Glyceraldehyde-3-phosphate dehydrogenase; Carbonic anhydrase; Trypsinogen, Bovine pancreas; Trypsin inhibitor, soybean and α - Lactalbumin, Bovine milk.

Labscan Ltd.,

Dublin, Ireland.

Acetone, acetonitrile, dimethylformamide (DMF), dimethylsulphoxide (DMSO), 1,4-dioxan, methanol and tetrahydrofuran (THF).

BDH Chemicals Ltd.,

Poole, Dorset, England.

Acetic acid, ethylamine, t-butanol, trifluoroacetic acid, hydrochloric acid, potassium chloride, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium dihydrogen orthophosphate dihydrate, disodium hydrogen phosphate dihydrate, triethylamine.

Bio-Rad Ltd.,

Munich, Germany.

Coomassie blue Bio-Rad protein assay.

Bachem Feinchemikalien AG.,

Bubendorf, Switzerland.

Benzyloxycarbonyl Glycine-Glycine-Arginine-7-Amino-4-methyl Courmarin ((Cbz)-Gly-Gly-Arg-AMC).

Riedel-de-Haen AG.,

Germany.

2-Mercaptoethanol.

Queen's University Belfast,

U.K.

Amyloidbeta-protein(H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH):Ac-Gly-Ser-Asn-Lys-OMe (activated ester), Gly-Ala-Ile-Ile-Gly-Leu-Met-NH_2 and Benzoyl arginine-leucinamide (BzlArgLeuNH_2) were kindly donated by Dr. Brian Walker ,Division of Biochemistry and Dr. Pat Harriott, Biosyn Ltd..

2.2 Measurement of Enzyme Activity

2.2.1 Amidase Activity

The amidase activity of trypsin was determined based on the method described by Erlanger et al. (1961). Substrate stock solution (1mM) was prepared by dissolving 43.5mg of DL-BAPNA in 1ml DMSO. The solution was brought to 100ml with 0.05M Tris buffer pH 8.2 containing 0.02M $CaCl_2$. Enzyme stock solution (0.45mg ml^{-1}) was prepared by dissolving 4.5mg trypsin in 10ml 0.001M HCl. Water (0.45ml) was added to 2.5ml of substrate stock solution, and the mixture was allowed to equilibrate in a waterbath at 30°C for 5 minutes. At time zero, 0.05ml of enzyme stock solution (9-45μg. trypsin) was added, and the reaction was allowed to run for 15 minutes. The reaction was terminated by the addition of 0.5ml. of 30% acetic acid. The absorbance was measured at 410nm on a Shimadzu UV-160A spectrophotometer. A control containing no enzyme was also set up.

2.2.2 Microassay

A microassay was developed based on the above method. 200μl of 1.67 mM BAPNA (29mg dissolved in 1ml DMSO added to 39ml of 0.1M Tris-HCl, pH 8.2 containing 20mM $CaCl_2$) was equilibrated in a 30°C waterbath for 5 minutes, 50μl of sample was added and incubated in waterbath at 30°C for 15 mins. The reaction was allowed to run for 15 minutes and was then terminated with 50μl of 30% acetic acid. The absorbance was read at 405nm on an ICN flow Multiskan plus MK11 Titretek reader. 50μl of each sample was placed in 4 different wells and the mean calculated. A blank containing substrate and enzyme buffer was included in the 8 wells of lane 1; its mean was calculated and subtracted from other values automatically.

2.2.3 Esterase Activity

The esterase activity of trypsin was determined using TAME as described by Rick (1974). 2.6ml of 48mM Tris buffer, pH 8.1 containing 11.5mM $CaCl_2$ was added to 0.3ml of 10mM TAME stock in H_2O and equilibrated at 30°C for 5 minutes. 0.1ml of

approximately 0.3mg ml^{-1} enzyme solution was added to the mixture and the absorbance read at 247nm over 5 minutes using a Unicam UV/Vis spectrophotometer UV2. The temperature was kept constant at 30°C during the assay using a Tempette Junior TE - 85 circulating waterbath attached to the spectrophotometer. Reference cells containing reaction mixture with no enzyme were used as blanks at each concentration. Esterase activity was determined using $\Delta A\ min^{-1}$ values for each sample. All assays were carried out in triplicate and their average calculated.

2.3 Chemical Modification

2.3.1 Reaction with carbodiimides

2.3.1.1 Activation of carboxyl groups on trypsin

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), a water soluble carbodiimide, was used to activate carboxyl groups on trypsin. EDC was prepared at a concentration of 15mM to give a 10 fold excess over available carboxyl groups on trypsin. 15 mM EDC was added to a reaction mixture containing 1mg ml^{-1} trypsin and 30mM benzamidine in aqueous solution (1mM HCl/NaCl, pH4.5). Solutions were mixed and reaction was carried out at room temperature for 2 hours, after which time the pH was adjusted to >7 by the addition of concentrated NaOH. Crosslinking with diamine was carried out as described in Section 2.3.1.2.

2.3.1.2 Crosslinking with Diamines

The following diamines were used to crosslink activated trypsin: 1,7-diaminoheptane, 1,8-diaminooctane, 1,10-diaminodecane and 1,12-diaminododecane. Ethylamine (a monoamine incapable of forming a crosslink) was included as a control. The concentration of monoamine and diamine equalled that of the carbodiimide used, i.e. a trypsin sample treated with 15mM carbodimmide (as described in Section 2.3.1.1) was mixed with 15mM diamine. Because of the poor solubility of 1,12-diaminododecane, it was necessary to dissolve it first in 0.5ml DMSO. The

crosslinking reactions proceeded for 15 minutes at room temperature after which time the samples were dialyzed against 1mM HCl for 20 hours at 4°C.

2.3.1.3 Crosslinking with dicarboxylic acids

The dicarboxylic acids were first reacted with EDC by mixing 2mM EDC with 0.5mM dicarboxylic acid in aqueous solution (1mM HCl/NaCl, pH4.5). The total volume of the reaction mixture was 5ml. Reaction proceeded for 1.5 hours at room temperature, then the pH was increased to 8.2 and the enzyme solution was added to a final trypsin concentration of 1mg ml^{-1}. The mixture was incubated for an additional 1.5 hours and the reaction then stopped by dialysis in 1mM HCl for 20 hours at 4°C. The dicarboxylic acid was used sebacic acid. Acetic acid (a monocarboxylic acid incapable of forming a crosslink) was included as a control.

2.3.2 Reaction of trypsin with monofunctional succinimide (AA-NHS)

Reaction of trypsin with AA-NHS was carried in aqueous solution (3mM KH_2PO_4, 0.1M KCl, pH 8.2 containing 30 mM benzamidine) at room temperature for 20 min with a working concentration of 2mg ml^{-1} of both enzyme and AA-NHS. Reaction mixture contained 20mg of trypsin in 10ml of buffer and 0.5ml of DMSO containing 20mg AA-NHS. The reaction was terminated with an equal volume of 0.1M Tris-HCl pH7. The sample was dialyzed in 3mM KH_2PO_4, 0.1MKCl, pH 8.2, for 20 min at 4°C. Alternatively, the reaction was terminated by removing excess reagent by Sephadex™ G-25 gel filtration using the method described in Section 2.4.

2.3.3 Crosslinking of trypsin with Homo-bifunctional Succinimides (EG-NHS and SA-NHS)

Crosslinking of trypsin with the succinimides was carried in aqueous solution (3mM KH_2PO_4, 0.1M KCl, pH 8.2 containing 3mM benzamidine) at room temperature for 20 minutes with a working concentration of 2mg ml^{-1} of both enzyme and succinimide. Reaction mixture contained 5mg of trypsin in 2.375ml of buffer and 0.125ml DMSO containing 5mg succinimide. The reaction was terminated by removing excess reagent

by G-25 gel filtration using the method described in Section 2.4.

2.4 Sephadex™ G-25 Gel Filtration

The method was based on that of Helmerhorst and Stokes (1980). Ten grams of Sephadex™ G-25 was swollen in 100ml of 3mM KH_2PO_4, 0.1MKCl, pH 8.2. This was stirred continuously for 2 hours, then 20ml of the slurry was poured continuously (by means of an adaptor) into a 10ml column. The column was then capped and stored until required. Alternatively, a prepacked Sigma G-25 column was equilibrated using 3mM KH_2PO_4, 0.1M KCl, pH 8.2. Sample (1.5ml) was applied to a G-25 Sephadex™ column which was centrifuged at 1,800 rpm for 3 minutes in a bench centrifuge.

2.5 Active site titration

Determination of the number of active sites was carried out using *p*-nitrophenyl-*p*'guanidinobenozoate (NPGB) as described by Walsh (1970) but changed slightly. Thirty microlitres of 0.01M NPGB (3.37mg in 1ml DMF) was added to 3ml of sample in 0.1M phosphate buffer, pH 7.0 and absorbance read at 410nm on a Unicam UV2 UV/Vis spectrphotometer after 15 minutes. A control containing 3ml of buffer was also included. All determinations were carried out in triplicate and their mean calculated.

2.6 Amino group estimation

The method of Rajput et al. (1987) using TNBS for the estimation of free amino groups in trypsin was followed. 1.5ml of sample was mixed with 0.5ml $NaHCO_3$ (8% w/v) pH 8.5 containing 20mM benzamidine and 1ml of TNBS (0.1% w/v). The reaction mixture was incubated for 2 hours at 40°C after which time 1ml SDS (10% w/v) and 0.5ml 1N HCl were added. The absorbance was read at 340nm on a Unicam UV2 UV/Vis spectrophotometer. N-α-Acetyl-L-Lysine in the range 0.1mM - 1mM was used for a standard curve. A control containing buffer instead of sample was also included. All readings were carried out in triplicate and their means determined.

2.7 Trypsin autolysis

The rate of autolysis was measured using Coomassie Blue dye as described by Bickerstaff & Zhou (1993). A volume of 30-50ml containing approximately 0.2mg ml-1 enzyme solution in 0.1M Tris-HCl pH 8.2 was incubated in a waterbath at 50°C. At regular intervals, aliquots of 2.6ml were taken, cooled quickly to room temperature on ice and 0.4ml of Coomassie blue added. The sample was mixed and after 5 minutes the absorbance was read at 595nm on a Unicam UV2 UV/Vis spectrophotometer. A control of buffer and Comassie Blue was included at each time reading. All readings were carried out in triplicate and the means calculated.

2.8 Thermoinactivation

A solution of approximately 0.05mg ml^{-1} trypsin in aqueous solution (3mM KH_2PO_4, 0.1M KCl pH 8.2) was incubated in a waterbath at 55°C for both native and modified trypsin. At intervals, aliquots were taken, cooled rapidly on ice and the residual activity of trypsin assayed as described in Section 2.2.2. Activity (% value) was calculated from the initial activity of the sample. Autolysis under these conditions was found to be negligible.

2.9 Temperature profile

1ml volumes of approximately 0.05mg ml^{-1} of trypsin in aqueous solution (3mM KH_2PO_4, 0.1M KCl pH 8.2) were incubated for 10 minutes over a range of temperatures between 30°C and 75°C. The samples were cooled rapidly on ice for 1 minute, residual activity was assayed as described in Section 2.2.2 and compared to that of sample incubated at 30°C.

2.10 Enzyme stability in organic solvents

Approximately 0.05mg ml^{-1} trypsin in an aqueous (3mM KH_2PO_4, 0.1M KCl pH 8.2)/organic mixture in the range 0-90% (v/v) solvent was incubated at 30°C for 1 hour. The residual activity of the trypsin was assayed as described in Section 2.2.2 and compared with that of controls containing no organic solvent. Blanks containing no

enzyme were included for each solvent concentration.

2.11 Thermostability of enzyme in organic solvents

To show the combined effects of organic solvents and elevated temperature, the procedure described as in Section 2.8 was followed but the samples were instead incubated at 65°C for 1 hour. The activity (% value) was calculated from activity of samples containing no solvent and incubated at 30°C for 1 hour.

2.12 Enzyme stability in denaturing agents

Approximately 0.05mg ml^{-1} trypsin in aqueous buffer containing guanidine HCl (in the range 0-1M) or urea (in the range 0-12.5M) were incubated at 20°C for one hour after which time the residual trypsin activity was assayed as described in Section 2.2.2. Controls containing aqueous buffer only were used for comparisons with the samples.

2.13 UV/Visible Spectral analysis

Spectra of native and modified forms of trypsin, in 3mM KH_2PO_4, + 0.1M KCl, pH8.0, were determined using the spectrum mode on a Shimadzu recording spectrophotometer. Protein concentration of samples was approximately 0.1mg ml^{-1}. Absorbances were read over the wavelength range 200nm to 600nm. The effect of urea and guanidine hydrochloride on the conformation of native trypsin was investigated.

2.14 SDS-Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out based on the method of Laemmli (1970). This procedure was used to assess the homogeneity of modified trypsin and also to determine if intermolecular crosslinking had occurred in the EG-NHS trypsin. This procedure was also used for autolysis studies. 10%, 12% and 15% resolving gel and a 3% stacking gel were used.

2.14.1 Solution Preparation

Table 2.1 gives details on the preparation of gels using the following solutions:

Acrylamide/bisacrylamide solution

Acrylamide - 29.2g

Bis-acrylamide - 0.8g

made up to 100mls in distilled water

Tris HCl pH 8.8

Tris buffer - 22.6g

made up to 100ml distilled water and pH adjusted to 8.8, (1.87M).

Tris HCl pH 6.8

Tris buffer - 6.06g

made up to 100ml with distilled water and pH adjusted to 6.8 (0.5M).

Ammonium persulpate (10% w/v) solution

Ammonium persulpate - 0.1g

made up to 1ml with distilled water fresh each day.

10% (w/v) SDS solution

SDS - 0.1g

made up to 1ml with distilled water.

Running buffer

Tris - 3.028g (0.025M)

Glycine - 14.4g (0.192M)

SDS - 1g (0.1% w/v)

made up to 1 Litre with distilled water and the pH adjusted to 8.3.

Solubilizing buffer

Tris - 0.969g

Glycerol - 10ml

Bromophenol blue - 10ml

Mercaptoethanol - 5ml

SDS - 2g (2% w/v)

made up to 100ml with distilled water.

Staining solution

Coomassie Brilliant Blue - 2.5g

Methanol - 500ml

Acetic acid - 100ml

made up to 1 Litre with distilled water and filtered through Whatman paper No. 1.

Destaining solution

Methanol - 250ml

Acetic acid - 70 ml

made up to 1 Litre with distilled water.

2.14.2 Sample preparation

Samples (1mg ml^{-1}) and molecular weight markers for electrophoresis were mixed 1:1 with solubilization buffer and boiled for 2 minutes. 10µl - 20µl of each sample was then loaded onto a prepared minigel under running buffer. Molecular weight markers in the range of 14,200 - 66,000 Daltons were used. A constant current of 20-25mA per gel was used with a running time of approximately 3 hours (or until dye runs off end of gel). Electrophoresis equipment used was a Consort minigel system with Consort E443 power pack.

Molecular weight markers (Sigma);.

Albumin, Bovine serum	66,000

Albumin, Egg (ovalbumin)	45,000
Glyceraldehyde-3-phosphate dehydrogenase	36,000
Carbonic anhydrase	29,000
Trypsinogen, Bovine pancreas	24,000
Trypsin inhibitor, soybean	20,100
α - Lactalbumin, Bovine milk	14,200

Table 2.1 Details of preparation of gels for SDS-polyacrylamide gel electrophoresis

TYPE OF GEL	RESOLVING (10%)	RESOLVING (12%)	STACKING (3%)
STOCK SOLUTION	VOLUME (ml)	VOLUME (ml)	VOLUME (ml)
10% SDS	9.0	7.0	7.8
Acryl/bisacryl	10.0	12.0	1.0
Tris HCl pH 8.8	11.2	11.2	---
Tris HCl pH 6.8	---	---	1.25
Ammonium persulphate (10% w/v)	0.15	0.15	0.075
TEMED*	0.05	0.05	0.05

*TEMED acts as a catalyst for gel polymerization so it is added last. The commercial solution was used as supplied.

Gels were placed in staining solution for 15-20 minutes, then placed in destaining solution, which was changed periodically until clear backgrounds were obtained.

2.15 pH profile

0.1M Buffer solutions, containing 20mM $CaCl_2$, of pH 4.5 to 6, 7 to 9, and 8 to 10 were made with acetic acid-sodium acetate buffer, Tris-HCl buffer, and glycine-NaOH buffer respectively. Enzyme solution of approximately 0.05mg ml^{-1} was diluted in buffer of each pH. Substrate solutions were likewise prepared using buffer of each pH and activities were measured as described for Tris pH 8.2 in Section 2.2.2. The activity was calculated (%) relative to the maximum activity of each sample.

2.16 Hydrophobic Probes

The method of Sackett and Wolff (1987) using Nile Red as a hyrophobic probe was followed with some slight modifications. 0.1M MES buffer, pH6.9, containing 1mM EGTA and 1mM $MgCl_2$ was prepared. A stock solution of 250mM Nile Red in DMSO was prepared. Enzyme solution (approximately 2.0 $mgml^{-1}$) in 3mM KH_2PO_4, + 0.1M KCl, pH8.0, was placed in a waterbath held at the temperature required for thermoinactivation. At regular intervals 0.4ml aliquots were removed added to 0.8ml of MES buffer and plunged in ice for 1 minute. Four microlitres of stock Nile Red (1 micromole) was added and mixed. Samples were allowed to stand for 15 minutes before being read. Fluorescence was performed on a Perkin Elmer LS50 Luminescence Spectrometer. The sample was excited at 550nm and the emission scan was monitored from 560nm to 700nm. Excitation and emission slit widths of 5nm were used.

2.17 Kinetics of Trypsin and Derivatives

2.17.1 Amidase kinetics using l-BAPNA

The amidase kinetics of native and modified forms of trypsin were determined using L-BAPNA based on the method of Erlanger (1961). 4.8mM stock solution of L-

BAPNA was prepared by dissolving 42mg of L-BAPNA in 1ml of DMSO and adjusting the total volume to 20ml with 0.1M tris pH 8.2, containing 20mM $CaCl_2$. An enzyme stock solution of 225µg ml-1 was prepared in 3mM KH_2PO_4 pH 8.2, containing 0.1M KCl. 0.93ml of substrate was added to the cuvette and allowed to equilibrate at 30°C for 5 minutes. 0.067ml of enzyme was added and the reaction was monitored at 410nm using a Unicam UV2 UV/Vis spectrophotometer for 3 minutes. The temperature was maintained at 30°C during the assay by a Tempette Junior TE - 85 circulating waterbath attached to the spectrophotometer. Reference cells containing reaction mixture with no enzyme were used as blanks for each concentration of substrate. $\Delta A\ min^{-1}$ values were recorded for each sample. All samples were assayed in triplicate and their average calculated. The K_m and k_{cat} values for both native and modified trypsin were determined using the Enzfitter programme (Biosoft, Cambridge, U.K) and also using the direct linear plot within Sigma Plot.

2.17.2 Amidase Kinetics using (Cbz)-Gly-Gly-Arg-AMC

The amidase kinetics of the tripeptide (Cbz)-Gly-Gly-Arg-AMC were determined based on the method of O'Donnell-Tormey & Quigley (1983). 4mM stock solution of substrate was prepared by adding 25mg to 0.5ml DMSO. The volume was adjusted to 10ml by the addition of 9.5ml 0.1M Tris pH 8.2, containing 20mM $CaCl_2$. 0.125ml of substrate was added to a test tube and allowed to equilibrate at 30°C in a waterbath for 5 minutes. 10µl of 5µg ml^{-1} solution of enzyme was added to the test tube and the reaction allowed to proceed at 30°C. The reaction was terminated after 5 minutes by the addition of 0.125ml of 30% (v/v) acetic acid. The sample was diluted to 5ml using distilled water. The release of 7-amino-4-methylcoumarin (AMC) from the fluorescent substrate was measured in a Perkin Elmer LS50 Luminescence Spectrometer at an excitation wavelength of 370nm and an emission wavelength of 440nm with slitwidths of 5nm. All samples were assayed in triplicate and their average calculated. The K_m and k_{cat} for both native and modified trypsin were determined using the Enzfitter programme (Biosoft, Cambridge, U.K) and also using the direct linear plot within Sigma Plot.

2.17.3 Esterase activity using Z-Lys-SBzl

The esterase kinetics of native and modified trypsin were determined using thiobenzoyl benzoyloxycarbonyl-L-lysinate (Z-Lys-SBzl) by the method of Coleman and Green (1981). Stock solutions of 110 mM DTNB in 50mM Na_2HPO_4, 20mM Z-Lys-SBzl in H_2O and 200mM Na phosphate plus 200mM NaCl (Pi-NaCl), pH 7.5 were prepared. 1 part each of DTNB and Z-Lys-SBzl solutions were added to 100 parts Pi-NaCl. 950µl of this solution was added to a cuvette and allowed to equilibrate for 5 minutes at 30°C. 50µl of enzyme solution was added to the cuvette. The reaction was monitored at 412nm using a Unicam UV2 UV/Vis spectrophotometer for 3 minutes. Temperature was maintained at 30°C by a Tempette Junior TE - 85 circulating waterbath attached to the spectrophotometer. Reference cells containing reaction mixture without enzyme were used as blanks for each concentration of substrate. ΔA min^{-1} values were recorded for each sample. All samples were assayed in triplicate and their average calculated. The K_m and k_{cat} values for both native and modified trypsin were determined using Enzfitter and Direct linear plot.

2.18 Catalytic activity in organic solvents

TAME substrate was used to determine the esterase catalytic activity in the presence of various concentrations of organic solvents. The method used was that described in Section 2.2.3 with some modifications. 0.26ml of 0.46M Tris pH 8.1, containing 0.115M $CaCl_2$ was added to 0.3ml of 10mM TAME stock solution. The mixture was made up to 2.9ml by addition of H_2O and organic solvent to give the required concentrations. 0.1ml of approximately 0.3mg ml^{-1} enzyme solution was added to the reaction mixture and the absorbance was read at 247nm over 3 minutes. Reference cells without enzyme were included at each solvent concentration. DMF absorbed strongly at this wavelength and was not used for analysis.

2.19 Enzymatic peptide Synthesis

2.19.1 Model dipeptide synthesis using BAEE and Leucinamide

The enzymatic synthesis of Bz-Arg-Leu-NH_2 using BAEE and leucinamide was carried out based on the methods described by Cerovsky (1990) and Blanco et al. (1991). A stock reaction mixture of 33.5mg $LeuNH_2$ (0.2M) and 34.5mg BAEE (0.1M) in 950µl of 0.1M borate buffer pH 9.0 was prepared. 190µl of reaction mixture was removed and equilibrated for 10 minutes at 4°C. 10µl of approximately 1mg ml^{-1} enzyme was added to the reaction mixture and incubated at 4°C. At regular intervals 10µl aliquots were removed, added to 0.5ml of 50% (v/v) MeOH in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC. HPLC was preformed on a Beckman System Gold apparatus equipped with a System Gold Autosampler 507, System Gold Diode Array Detector Module 168 and Beckman C_8 column (0.46mm x 25cm); flow rate 1ml min^{-1}, detection at 204 and 230nm, mobile phase methanol with 0.05% aqueous trifluoroacetic acid (50/50 by volume). 20 microlitre was injected onto the column using a autosampler. Both sustrates and products were identified and quantified by comparisons with standards of each.

2.19.2 Effect of enzyme concentration on rate of peptide synthesis

Reaction mixture was prepared as described in Section 2.19.1. 190µl of reaction mixture was equilibrated 10 minutes at 4°C. 10µl of the following stock enzyme concentrations was added to the reaction mixture: 0.05, 0.1 or 1.0 mg ml^{-1}. At regular intervals 10µl 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 as described in Section 2.19.1.

2.19.3 Effect of Leucinamide concentration on rate of peptide synthesis

Reaction mixtures were prepared with 0.1M BAEE and the following concentrations of leucinamide : 0.1, 0.2 or 0.4 M. 10 microlitre of approximately 1mg ml-1 enzyme was added to the reaction mixture. At regular intervals 10µl aliquots were removed,

added to 0.5ml of 50% (v/v) MeOH in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC as described in Section 2.19.1.

2.19.4 Effect of temperature on rate of peptide synthesis

Reaction mixture was prepared as described in Section 2.19.1. 190µl of reaction mixture was equilibrated at the following temperatures for 10 minutes: 4, 25 and 37°C. 10µl of approximately 1mg ml^{-1} enzyme was added to the reaction mixture and incubated at the required temperature. At regular intervals 10µl aliquots were removed, added to 0.5ml of 50% (v/v) MeOH in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC as described in Section 2.19.1.

2.19.5 Effect of organic solvents on Peptide synthesis

The effects of organic solvents on peptide synthesis were examined. A stock reaction mixture of 33.5mg $LeuNH_2$ and 34.5mg BAEE in 950µl aqueous/organic solvent mixture and 45µl of triethylamine (to ensure that the rection mixture was alkaline) was prepared. This mixture (199µl) was removed and equilibrated for 10 minutes at 4°C. 10µl of enzyme solution was added and the entire reaction mixture incubated at 4°C. 10µl aliquots were removed at regular intervals, added to 0.5ml of 50% (v/v) MeOH in aqueous solutionn containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC as described in Section 2.19.1. Controls containing 10µl of aqueous solution in the reaction mixture (but no enzyme) were included for each concentration of solvent.

2.19.6 Effect of salt hydrate on peptide synthesis

Reaction mixture was prepared containing 0.1M BAEE, 0.2M $LeuNH_2$ and 45µl triethylamine in 95% DMF. The following concentrations of salt hydrate ($Na_2CO_3.10H_2O$) were included in the mixture: 0.01, 0.1 and 0.5M. This mixture (199µl) was removed and equilibrated for 10 minutes at 4°C. 10µl of enzyme solution was added and the entire reaction mixture incubated at 4°C. 10µl aliquots were removed at regular intervals, added to 0.5ml of 50% (v/v) MeOH in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC as described in Section

2.19.1. Controls without $Na_2CO_3.10H_2O$ were included.

2.19.7 Effect of a sugar alcohol (xylitol) on peptide synthesis

Reaction mixture was prepared as described in Section 2.19.1 containing the following concentrations of xylitol: 0.01, 0.1 and 0.5M. This mixture (199µl) was removed and equilibrated for 10 minutes at 4°C. 10µl of enzyme solution was added and the entire reaction mixture incubated at 4°C. 10µl aliquots were removed at regular intervals, added to 0.5ml of 50% (v/v) MeOH in aqueous solution containing 1% (v/v) trifluoroacetic acid and analyzed by HPLC as described in Section 2.19.1. Controls without xylitol were included.

2.19.8 Model dipeptide synthesis using TAME and leucinamide

19mg TAME, 17mg $LeuNH_2$ and 45µl of triethylamine were added to 950µl of acetonitrile. To 199µl of this mixture was added 10µl of enzyme solution and the full reaction mixture was incubated at 4°C. At regular intervals 10µl aliquots were removed, added to 0.5ml of 50% (v/v) aqueous MeOH containing 1% (v/v) trifluoroacetic acid and then analyzed on HPLC. 37mg TAME (0.1M), 33.5mg $LeuNH_2$ (0.2M), were added to 950µl of 0.1M borate buffer, pH 9.0 /acetonitrile. 190µl was removed and equilibrated for 10 minutes at 4°C. 10µl of enzyme solution was added and the reaction mixture was incubated at 4°C. At regular interval 10µl was removed and added to 0.5ml of 50% aqueous MeOH containing 1% trifluoroacetic acid and analyzed on HPLC. HPLC was performed on Beckman System Gold equipped with a System Gold Autosampler 507, System Gold Diode Array Detector Module 168 and Beckman C_8 column (0.46mm x 25cm); flow rate 1ml min^{-1}, detection at 204 and 230nm, mobile phase methanol with 0.05% aqueous trifluoroacetic acid (40/60 by volume). 10µl was injected onto the column using a autosampler. Substrates and product (TA) were identified and quantified by comparisons with standards. No standard for $TALeuNH_2$ was available and therefore it was quantified indirectly by calculating the amount of TA and TAME in the samples and hence the amount of $TALeuNH_2$ formed.

2.20 Oligopeptide synthesis

Stock solutions were prepared as follows: 11mg Ac-Gly-Ser-Asn-Lys-OMe was dissolved in 200µl of H_2O, 11mg Gly-Ala-Ile-Ile-Gly-Leu-Met-NH_2 was dissolved in 200µl DMSO. 300µl of 0.1M borate buffer, pH 9.0 and were added to a reaction mixture containing 50µl of each of the sustrates 10µl EG-trypsin (1mg ml^{-1}). At regular interval 10µl was removed and added to 0.5ml of 50% aqueous acetonitrile containing 1% trifluoroacetic acid and analyzed on HPLC. HPLC was performed on Beckman System Gold equipped with a System Gold Autosampler 507, System Gold Diode Array Detector Module 168 and Beckman C_8 column (0.46mm x 25cm); flow rate 1ml min^{-1}, detection at 210nm, mobile phase acetonitrile with 0.05% aqueous trifluoroacetic acid (35/65 by volume).

CHAPTER 3

STUDIES on NATIVE TRYPSIN

3.1 Introduction

Trypsin is a proteolytic enzyme that hydrolyzes bonds in proteins and peptides involving the carboxyl group of lysine or arginine. Amides and esters of these amino acids are also hydrolyzed. The activity of trypsin can be measured using denatured proteins such as haemoglobin or casein as substrates. The amides or esters of arginine and lysine are also suitable substrates, e.g. N-p-toluene-sulfonyl-L-arginine methyl ester, N-benzoyl-DL-arginine-naphthylamide, N-benzoyl-DL-arginine-p-nitroanilide.

N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) when hydrolyzed by trypsin releases *p*-nitroaniline. This can be estimated colorimetrically, making it an ideal substrate to determine the amidase activity of trypsin. A change in colour of the reaction mixture from colourless to yellow indicates trypsin amidase activity. The intensity of the yellow colour is proportional to the concentration of enzyme present. Absorbance values are directly correlated to enzyme amidase activity. The assay used here is based on the method described by Erlanger et al (1961). This method requires large quantities of reagent and is time consuming when working with large numbers of samples. A scaled-down version of the assay using a microplate reader was developed, allowing large numbers of samples to be analyzed simultaneously. The esterase activity of trypsin can be determined using N-p-toluene-sulfonyl-L-arginine methyl ester (TAME). TAME when hydrolyzed releases N-p-toluene-sulfonyl-L-arginine (TA). The absorption of TAME at 247nm is much weaker than that of TA, so hydrolysis of the ester can be followed spectrophotometrically.

Studies on a number of different parameters of native trypsin were necessary prior to the commencement of modifications. The first of these was the thermal stability. Mozhaev et al (1993) described a two-step model of irreversible thermoinactivation that involves preliminary reversible unfolding and an irreversible step. He suggested that reversible unfolding is best prevented by covalent immobilization, while irreversible unfolding was effectively prevented by covalent modification of amino acid residues or by the addition of low-molecular weight compounds. An enzyme denatures at extreme temperatures due to unfolding of the

protein backbone. At high temperatures, non-covalent interactions that maintain the native enzyme structure at moderate temperatures (30-40°C) are lost. Activity is lost if the heating is prolonged, as unfolding disrupts the active or functional site to an extent that the whole process is irreversible. Irreversible enzyme inactivation is the process responsible for the gradual loss of enzyme activity with time at high temperatures. Chemical modifications and crosslinking can stabilize an enzyme against irreversible thermoinactivation.

The pattern of thermal denaturation and the inherent stability of the native enzyme must be established prior to any of these modification experiments. Once these properties of the native enzyme have been established, any changes in the thermal denaturation pattern of the native enzyme following modification can be monitored using suitable systems. Thermostability (N->I, where N is native and I is irreversibly inactivated forms of protein) can be assessed by measuring residual activity of an enzyme that has been incubated at a particular temperature for a certain length of time (Mozhaev et al, 1988; Torchilin et al, 1983). Various other methods for measuring thermostability are available such as: measurement of the melting temperature Tm, measuring of the protein free (Gibbs) energy values and estimation of the temperature of maximum stability T_s. These methods refer to conformational or folding stability (N->U). The retention of catalytic activity is an important stability index if modified trypsins are to be used for peptide synthesis or other applications. Measurement of residual activity at a particular temperature was chosen as it is easy to perform, is relatively rapid and does not demand the use of expensive equipment. Various incubation temperatures were studied to ascertain a temperature at which the enzyme inactivation rate could be conveniently measured. One particular temperature (55°C) was chosen and trypsin's activity loss with time was studied to determine the reaction order of its activity loss.

A temperature profile of the native trypsin incubated for a certain length of time over a range of temperatures was also determined. Residual activity of trypsin was measured at each temperature after a uniform time and the apparent melting temperature T_{50} calculated.

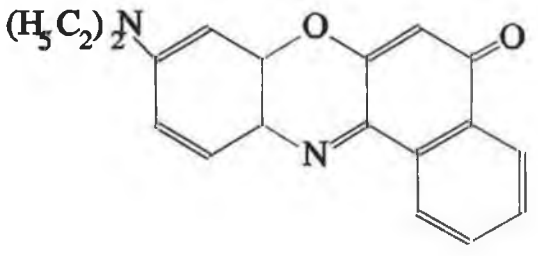
Nonpolar groups are usually found buried in the interior of the molecule. When protein unfolding occurs these hydrophobic groups are exposed. A method sensitive to change in polarity of the protein was required to confirm that unfolding was occurring. A simple method to detect changes in the structural integrity involves the use of fluorescent hydrophobic probes (Semisotnov et al., 1991; Sackett & Wolff, 1987). These probes are based on a much stronger affinity of the probe to the unfolded state (due to the absence of rigid packing of hydrophobic clusters in the unfolded polypeptide). A folded protein has buried hydrophobic clusters and less affinity for a probe molecule.

Nile red is an uncharged phenoxazone dye whose fluorescence is strongly influenced by the polarity of its environment (see Fig. 3.1). Nile red was found to be a useful probe of hydrophobic sites on many proteins. Unlike other probes which are limited by their ultraviolet or near uv excitation maxima, Nile red has a visible excitation wavelength, removing problems of absorption interference from proteins or cofactors. The significant Stokes shifts observed in emission wavelength allow the detection of small changes in protein structure.

An assessment of the stability of native trypsin to denaturing agents was also undertaken. The primary structure of a protein determines its native structure only under suitable environmental conditions. Addition of chaotropic agents leads to denaturation and is commonly accompanied by deactivation and dissociation of assembed structures, as well as solubilization of aggregates (Jaenicke & Rudolph, 1993). The degree of denaturation depends on the enzyme, as co-factors and disulphides bridges may strongly influence the stability so that extreme conditions may be required for unfolding. Usually high concentrations of GdnCl or urea are sufficient to denature the polypeptide chains.

It was decided to look at the denaturing effects of both guanidinium hydrochloride (GdnCl) and urea on trypsin. The effects of these reagents can be

Fig. 3.1 Chemical structure of the dye Nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one).



assessed by measuring residual activity of trypsin that has been incubated in the presence of these denaturants for a given length of time. Various concentrations of denaturants were studied so that the range where catalytic activity is lost could be established. UV-visible spectral analysis of the sample was also performed to see if any structural change in the native trypsin could be observed.

The effect of a range of organic solvents on trypsin was investigated. Several properties of organic solvents can affect enzymes including hydrophobicity, polarity and solvophobicity (the ability of a solvent to dissolve in water). The lower activities of enzymes in polar organic solvents, as compared with nonpolar solvents is due to the immediate loss in enzymic hydration that takes place when the enzymes are placed in polar organic solvents due to the fact that polar organic solvents strip water off an enzyme almost immediately (Gorman & Dordick, 1992).

One of the most common parameters used in correlating enzyme activity with solvent properties is hydrophobicity, conveniently represented as log P, where P, is the

measure of partitioning of a given solvent between 1-octanol and water. Mozhaev et al. (1989) evaluated the denaturing strength of different organic cosolvents and correlated these values with their physiochemical characteristics. They found that cosolvents with lower log P value reduced the overall water content of the reaction system without significant loss of enzyme activity. However this criterion is valid only when a series of organic cosolvents of the same functionality (e.g. alcohols) is used. Later Khmelnitsky et al. (1991) established a quantitative relationship between the physiochemical properties of organic solvents and their denaturing strength. As a result they were able to construct the denaturation capacity (DC) scale of organic solvents. This scale can be used to predict quantitatively the limiting concentrations of various organic solvents at which dissolved proteins still retain their native properties.

A number of organic solvents varying in their log P values were chosen (see Table 3.1). Their effects on trypsin was assessed by measuring the residual activity of trypsin incubated in varying concentrations of these solvents for a given length of time.

3.2 Results

3.2.1 Trypsin Amidase Assay Optimization

The assay used is based on the method described by Erlanger et al. (1961) and described in Section 2.2.1. The assay was optimized with respect to enzyme and substrate concentration and time. As shown in Fig. 3.2, the rate of hydrolysis of DL-BAPNA at a concentration of 0.83mM is directly proportional to trypsin concentration over at least five fold range up to 7.5μg enzyme ml^{-1}.

Trypsin microassay was performed as described in Section 2.2.2. A range of trypsin concentrations from 25μg ml^{-1}-200μg ml^{-1} (actual concentration in reaction mixture was 4μg ml^{-1}-34μg ml^{-1}) was assayed (see Fig. 3.3). The effect of substrate volume was also investigated: 200μl was found to be the optimum.

Fig. 3.2 **Concentration (ug ml^{-1}) versus absorbance at 410 nm for bovine trypsin at $30^{o}C$**

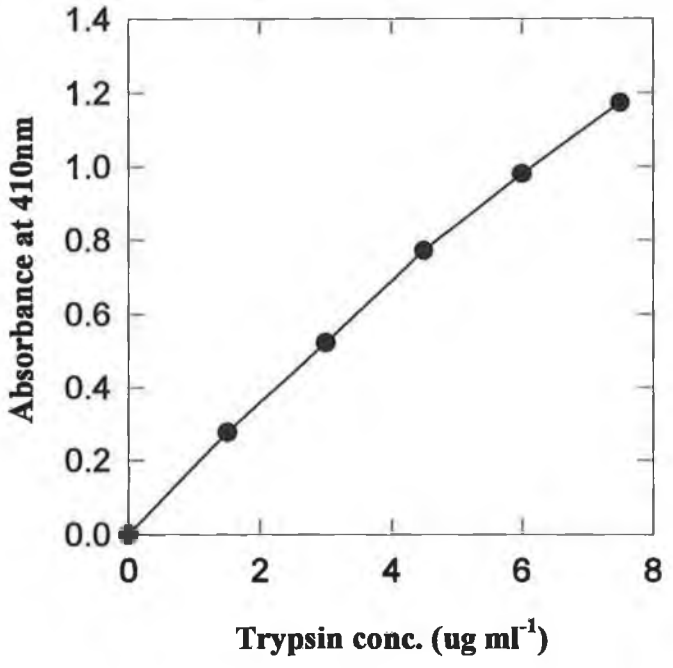
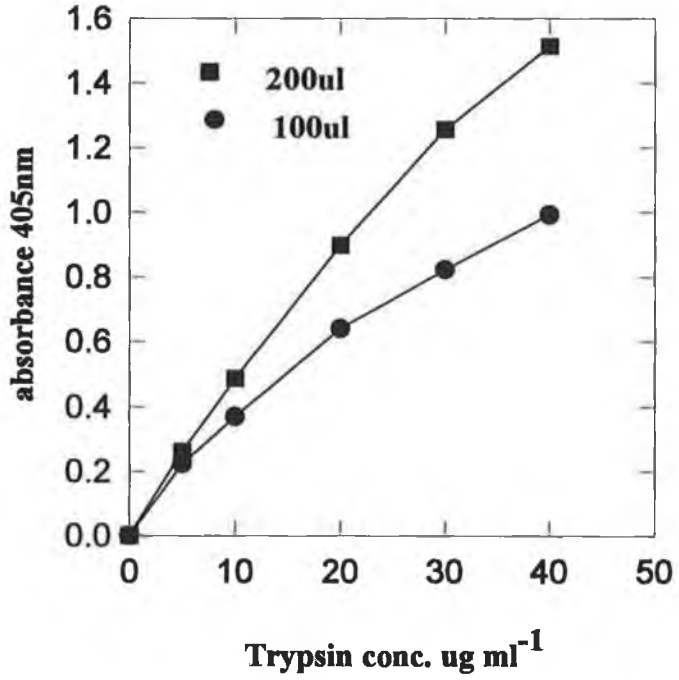


Fig. 3.3 **Concentration versus absorbance at 405nm for trypsin mircroassay at 1.67 mM BAPNA**



3.2.2 Trypsin esterase assay optimization

The esterase activity of trypsin was determined as described by Rick (1974) (see Section 2.2.3). The assay was found to be linear over the enzyme concentration range of 0 - 0.01mg ml^{-1} (see Fig. 3.4). The pH optimum of the reaction is around pH 8.1 and the optimum substrate concentration is 1mM; calcium ions at a final concentration of 10mM are added to stabilize the enzyme (Rick, 1974)

3.2.3 Thermal Stability of Trypsin

Thermoinactivation of trypsin at various temperatures was performed as described in Section 2.8. Initially the enzyme solution was prepared in 0.001M HCl and then thermoinactivated. The enzyme retained activity in this solution even after one hour's incubation at very high temperatures (see Fig. 3.5). When the enzyme was changed to 3mM KH_2PO_4, 0.1MKCl, pH8.2 the trypsin was more readily inactivated. Thermoinactivation curves for native trypsin incubated at 55°C and 65°C are shown in Fig. 3.6. These curves are plotted as % residual amidase activity vs. time. At 65°C most of the enzyme activity is lost after 10 minutes, while for 55°C an even decay rate is observed for incubated enzyme sample over time.

The kinetics of thermoinactivation of trypsin were studied at 55°C. The results of % residual activity vs. time plots were analyzed using the computer programme, Enzfitter. Data was fitted to first order exponential equation and visual observation of the graphic fit shows that trypsin thermal inactivation obeys first-order rate equations. The standard deviation of the first-order rate constant, k, and the intercept were very low, typically 5%.

3.2.3.1 Polarity-Sensitive fluorescent probe of hydrophobic protein surfaces

Reaction of trypsin with Nile Red was performed as described in Section 2.16. Heat treatment resulted in a progressive change in trypsin which shifted the emission of the dye to 630nm. A range of temperatures was used and it was observed that the higher temperatures gave the highest emission readings and the greatest shift to 630nm (see Fig. 3.7).

Fig. 3.4 Trypsin concentration versus absorbance at 247nm for bovine trypsin

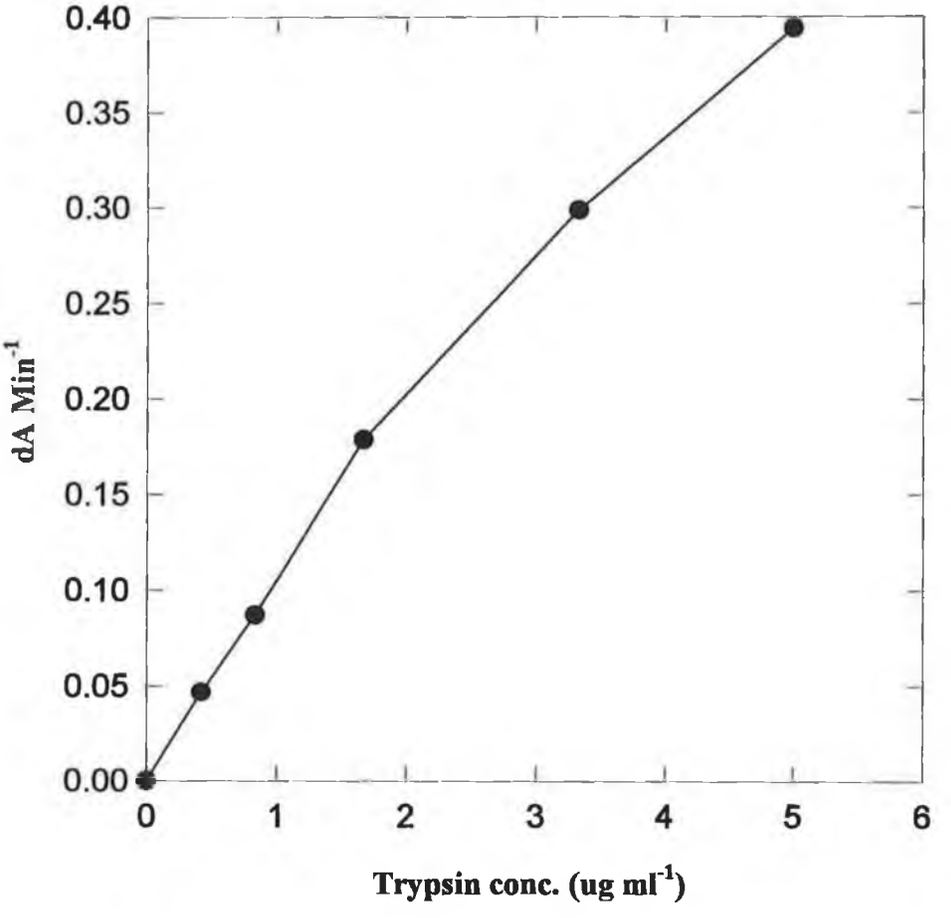


Fig. 3.5 **Thermostability studies on native trypsin in 1mM HCl at 65^{0}C over time**

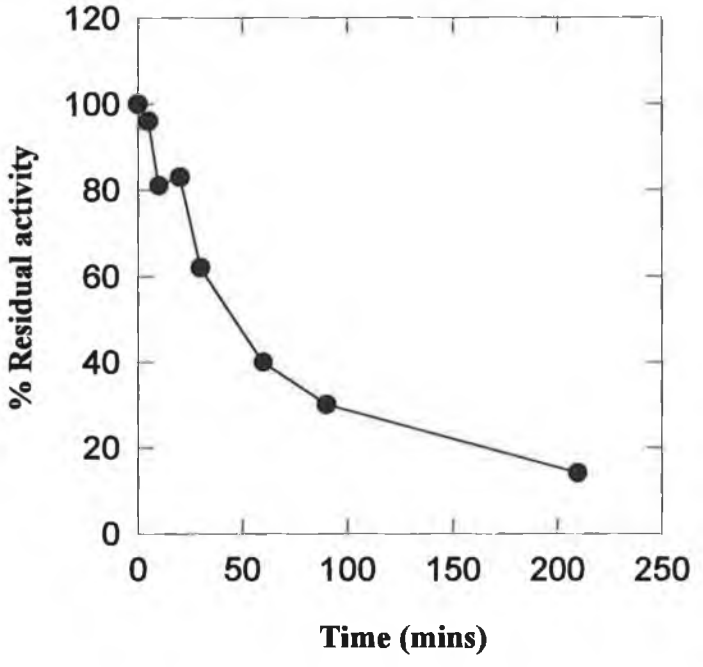


Fig. 3.6 **Thermostability studies on native trypsin at pH 8.2 at 55^{0}C and 65^{0}C over time**

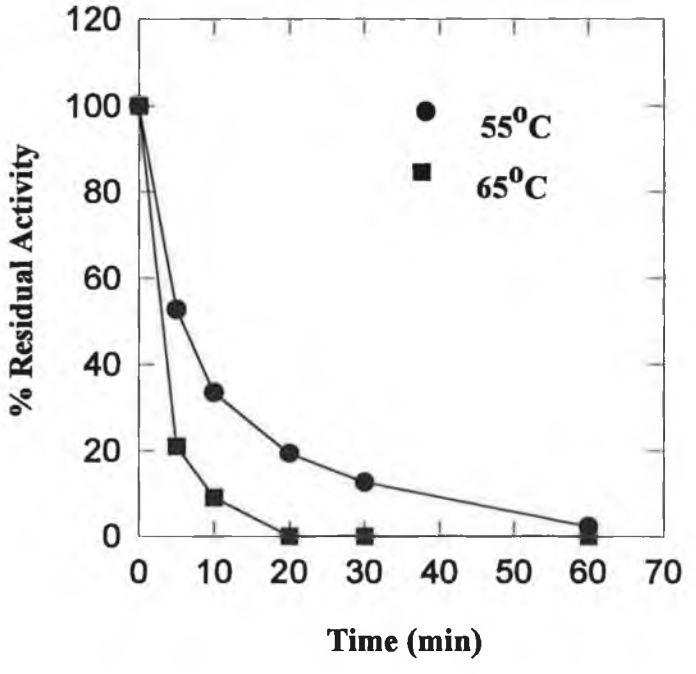
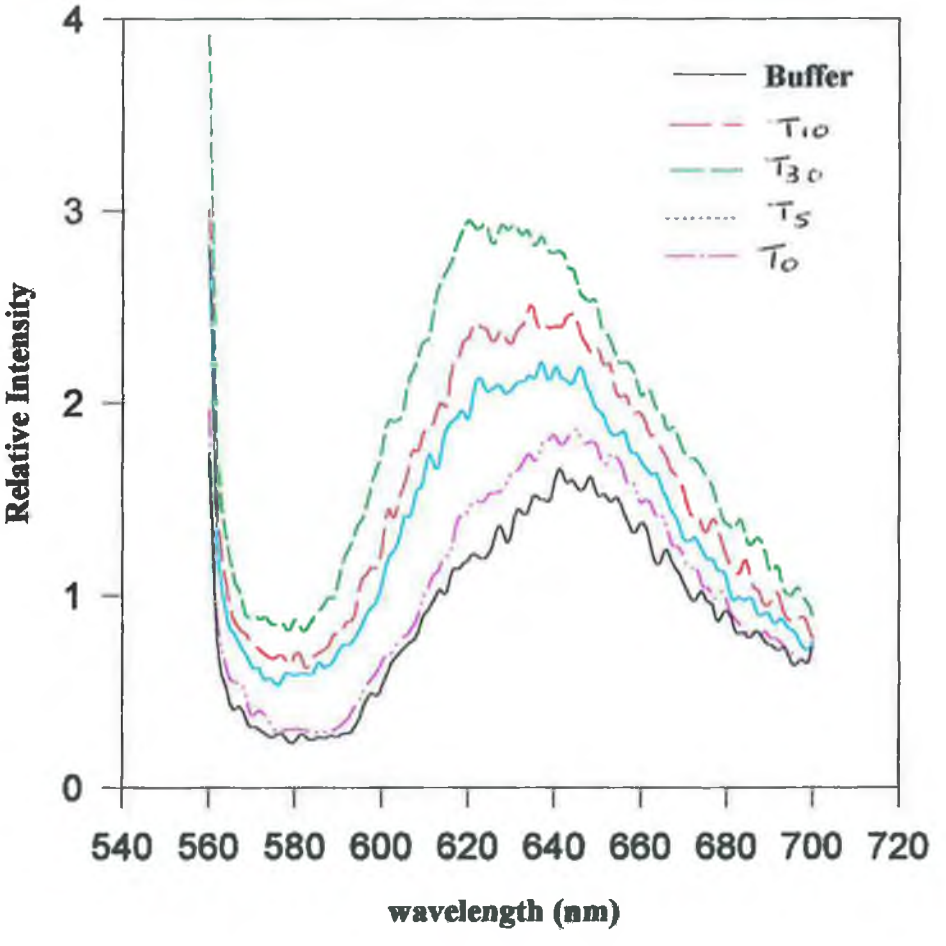


Fig. 3.7 **Fluorescence of Nile Red in the presence of heat treated native trypsin at 85^{0}C at different time intervals**



3.2.4 Organotolerance

Organotolerance of native trypsin was determined as described in Section 2.10 for the following organic solvents: acetone, acetonitrile, DMF, DMSO, 1,4-dioxan, methanol and THF. Graphs of % residual activity vs. concentration of solvent were plotted for each solvent. In general the graphes were sigmoid. Percent residual activity was calculated as a percentage of native trypsin's activity in aqueous solution containing no solvent. The amidase activity was enhanced at lower concentrations of solvent. The concentration at which half the amidase activity remained (C_{50}) was calculated for each solvent (see Table 3.1). It was found that THF was the most potent denaturing solvent and this corresponded with its also having the highest log *P* value, i.e, it was the most hydrophobic.

Table 3.1 Threshold concentrations of organic solvents for trypsin

Solvent	log *P*[a]	C_{50}
Tetrahydrofuran	0.46	45%
1,4-Dioxane	-0.27	55%
Acetonitrile	-0.34	60%
Dimethylformamide	-1.01	65%
Dimethyl sulphoxide	-1.35	65%
Acetone	-0.24	66%
Methanol	-0.74	70%

[a] Values taken from Khmelniysky et al. (1991)

3.2.5 Effects of denaturing agents on trypsin

Denaturation of trypsin at various concentrations of GdnCl was performed as described in Section 2.12. Denaturation curves for native trypsin incubated at room emperature for 5 minutes and 1 hour are shown in Fig. 3.8a. The effects of GdnCl can be seen almost instantaneously with very little difference observed between the two incubation periods used. The optimum range was found to be between 0-2M GdnCl. The competitive inhibitor 30mM benzamidine was found to protect the trypsin slightly against the effects of GdnCl.

Spectral analysis of trypsin in varying concentrations of GdnCl (as described in Section 2.13) showed as decrease in absorbance 200nm - 300nm with increasing concentrations of GdnCl (see Fig. 3.8b).

Denaturation of trypsin at various concentrations of urea was performed as described in Section 2.12. Denaturation curves for native trypsin incubated at room temperature for 5 minutes are shown in Fig. 3.9a. Trypsin was not completely inactivated even at saturating concentrations of urea. Little or no difference was observed between the two incubation times. The inclusion of 30mM benzamidine had no effect on trypsin's stability.

Spectral analysis (as described in Section 2.13) showed that there was a change in the profile of trypsin after exposure to urea. A decrease in absorbance from 200nm - 300nm was observed in trypsin in the presence of increasing concentrations of urea see Fig. 3.9b

Fig. 3.8a **Effect of guanidine HCl on native trypsin**

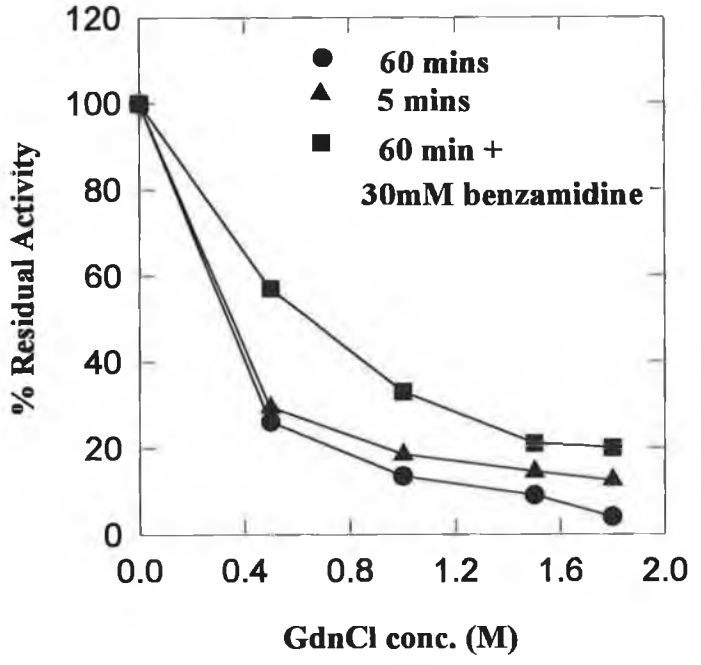


Fig. 3.8b **Effect of Guanidine Chloride on**

on the structure of native trypsin

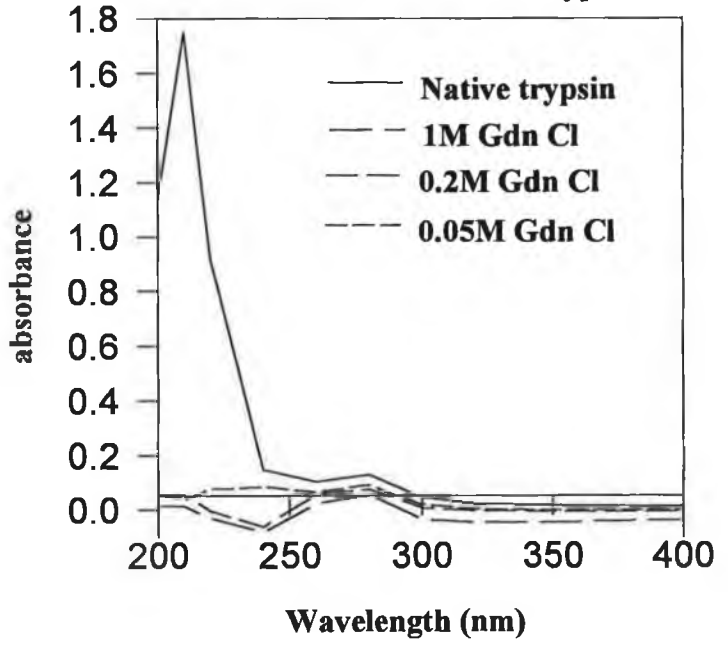


Fig. 3.9a **Effect of Urea on native trypsin**

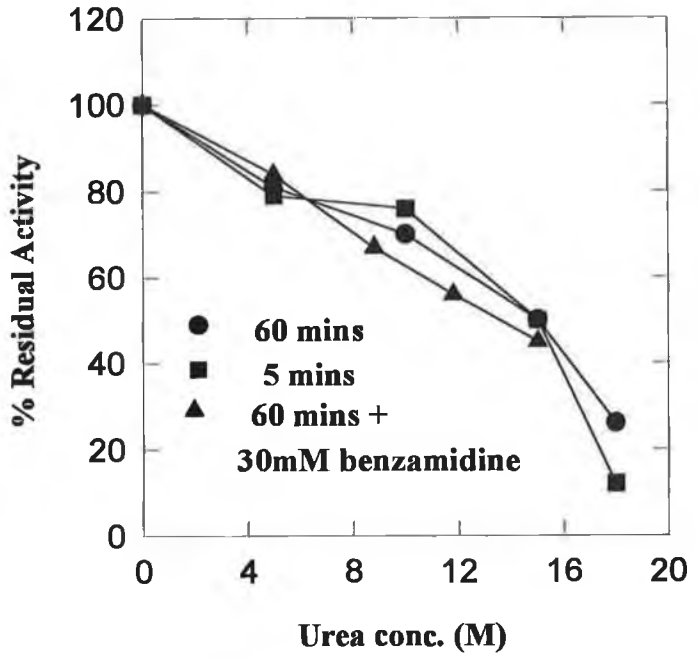
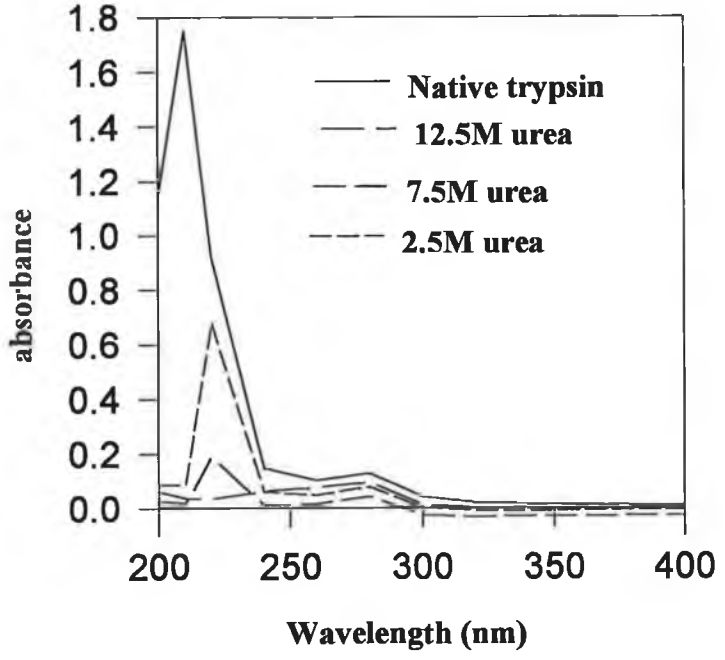


Fig. 3.9b **Effect of urea on the structure of native trypsin**



3.3 Discussion

The BAPNA assay used to determine trypsin's amidase activity based on the method of Erlanger et al (1961) who optimized conditions with respect to enzyme concentration, substrate concentration, time, pH and temperature. The optimal activity for hydrolysis of the arginine derivative occurs near pH 8.1 and a temperature of 30°C.

This method required large quantities of reagent and was time consuming when working with large numbers of samples. It was therefore decided to use a scaled-down version of the assay, allowing use of a microplate reader. The following volumes were chosen after various trials: 50 microlitres enzyme sample, 200 microlitres substrate solution and 50 microlitres of 30% acetic acid. The trypsin BAPNA assay can be terminated by addition of acetic acid. Because no filter for 410nm was available it was decided to use the 405nm filter for the assay. p-Nitroaniline (the product of hydrolysis by trypsin) absorbs between 280-500nm while BAPNA absorbs up to 400nm. Above 400nm there is no substrate interference in the absorption readings of the product. A range of trypsin concentrations from 25μg ml^{-1} -200μg ml^{-1} (actual concentration in reaction mixture was 5μg ml^{-1}-40μg ml^{-1}) were assayed. Because trypsin is a proteolytic enzyme, autolysis can occur solution; and hence 0.001M HCl and later 3mM KH_2PO_4, 0.1MKCl, pH8.2) was used to prepare enzyme stock solutions which were kept on ice. It was also found best to prepare the enzyme solution just before use to ensure minimal autolysis.

The esterase activity of trypsin was determined as described by Rick (1974), with optimum conditions of: pH 8.1, temperature of 30°C, 1mM TAME and a calcium ion concentration of 10mM. The optimum enzyme concentration was found to be approximately 0.005 mg ml^{-1}.

pH was found to influence the rate of thermoinactivation of trypsin. At pH 8.2 trypsin was completely inactivated after 20 minutes at 65°C, while at pH 3 it retained 80% activity after 20 minutes at this temperature. This suggests that pH plays an important part in thermostability. Walsh et al (1970) confirmed this by showing that below pH 8 elevated temperatures resulted in reversible denaturation, while for pH >8

elevated temperatures induced irreversible denaturation. Therefore all thermoinactivation experiments were carried out at pH >8. At 65°C most of the enzyme activity is lost after 10 minutes, while at 55°C an even decay rate is observed for incubated enzyme sample over time. 55°C was chosen as the temperature at which to study enzyme activity loss with time. The enzyme inactivates at a conveniently measurable rate at 55°C and so it is ideal for studying thermal stability of modified derivatives. Thermoinactivation data fitted well to first order exponential equation. From the derived first-order rate constant, k, the enzyme's half-life can be determined at the same temperature. The Arrhenius equation can be used to determine activation energies from first-order kinetics data.

An enzyme denatures at an elevated temperature due to unfolding of the protein backbone. At high temperatures, non-covalent interactions (that usually maintain the native enzyme structure at moderate temperature) are lost. Non-native, non-covalent bonds are acquired by the enzyme at high temperatures. These irregular noncovalent interactions are unstable and remain during the heating process (Mozhaev & Martinek, 1982). Activity is lost if the heating is prolonged as unfolding disrupts the active, or functional, site to an extent that is irreversible. Irreversible enzyme thermoinactivation is the process that is responsible for the gradual loss of enzyme activity with time at an elevated temperature.

To determine if unfolding of the trypsin polypeptide was in fact occurring a hydrophobic probe was employed. As the enzyme unfolds more hydrophobic groups (found in the interior of the molecule) are exposed. The Nile Red associates with these groups, resulting in an increase in intensity and also shifting the emission wavelength of the dye to about 630nm. As expected heat-treated trypsin caused a significant Stokes shift in emission wavelength. This suggests that heat treatment caused trypsin to unfold and hence resulted in a loss of activity as observed with the amidase activity. Prolonged heating and higher temperatures increase these shifts.

Trypsin was found to denatured by GdnCl almost instantaneously with very little difference observed in activity observed after prolonged incubation. The optimum range was found to be between 0-2M GdnCl. The degree of inactivation increased as

the denaturant concentration increased. The competitive inhibitor benzamidine was found to protect the trypsin slightly against the effects of GdnCl. It has been shown that the inhibitor protects the enzyme from distortive conformational changes induced either by heat or multipoint covalent attachment to agarose(aldehyde) gels (Blanco et al., 1988). It is thought that the inhibitor strongly adsorbs to the native trypsin and hence protects this native structure from conformational changes. This is probably the case here where it protects the native trypsin against the denaturing effects of GdnCl. From spectral analysis it observed that there was change in the spectrum of native trypsin when exposed to GdnCl. This confirms that the GdnCl causes structural changes in native trypsin.

Higher concentrations of urea (0-15M) were necessary for unfolding as it is not as potent a denaturant as GdnCl (Jaenicke & Rudolph, 1993). It was found that trypsin was not completely inactivated by urea even at saturation concentrations. As in the case of GdnCl prolonged incubation has little effect on the activity indicating that the denaturing effect of urea seemed instantaneous. Unlike GdnCl, the inclusion of benzamidine with urea had no effect on the stability of trypsin. Spectral analysis showed that urea also caused a change in the profile of trypsin, indicating that urea (like GdnCl) causes structural changes in native trypsin.

In most of the solvents tested the amidase activity was enhanced at lower concentrations, being most pronounced in acetone where the activity was enhanced by as much as 140%. The concentration at which half the amidase activity remained (C_{50}) was calculated for each solvent (see Table 3.1). It was found that THF was the most potent denaturing solvent and this corresponded with its also having the highest log *P* value, i.e, it was the most hydrophobic and the highest denaturing capacity (DC). This correlated well with the findings of Mozhaev et al (1989). The first five solvents in the table are quite similar in chemical properties in that they are chemically inert and therefore tend not to react with the enzyme. THF is the least polar has the poorest solvating ability of the group while both DMF and DMSO are very polar (Morrison and Boyd, 1987). Both acetone and methanol are more chemically active, forming charged molecules in solution. These solvents would therefore have the ability to

interact chemically with the enzyme. Hence the solvent profile of these solvents would be more complex compared with the other five.

As will be discussed in Chapter 4, the aim of chemical modification experiments is to stabilize trypsin against denaturing and inactivating influences. Enhanced stability will be seen in increased resistance of the modified protein towards these factors, relative to the unmodified form.

CHAPTER 4

CHEMICAL MODIFICATION of TRYPSIN

4.1 Introduction

4.1.1 Crosslinking with Carbodiimides

Carbodiimides are bivalent coupling reagents with the general formula R-N=C=N-R' (Ji, 1983). The R and R' groups may be aliphatic or aromatic, like or unlike. The nature of these R groups determines the solubility of the carbodiimide. Carbodiimides can react with several functional groups, such as carboxyl and amino groups, under mild conditions of neutral pH. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is a heterobifunctional crosslinker. Use of EDC allows the formation of an amide bond between an amine and a carboxylic acid group. Two types of modification were attempted involving EDC. The first involved the activation of carboxylic groups on trypsin with EDC and then the introduction of diamines to form crosslinks between the activated carboxyl groups. The second procedure involved the use of diacids as described by Torchilin et al (1983). Diacids when activated with EDC can form amide bonds with lysine amino groups of the protein. The structure of EDC is shown in Fig. 4.1a. The reaction of EDC with carboxyl groups and the subsequent reaction of the activated carboxyl group is shown in Fig. 4.1b

4.1.1.1 Diamine Modification

A diamine is a bifunctional amine molecule of a particular length. The diamines used in this section were diaminoheptane, diaminooctane and diaminodecane. Diamines form amide linkages when used in conjunction with a water soluble carbodiimide. The carboxylic groups on the protein can be activated using EDC and the diamines can be introduced into the activated protein mixture to form crosslinks between the activated carboxyl groups on the protein.

4.1.1.2 Diacids

A diacid is a bifunctional carboxylic acid molecule of a particular length. The diacid used was sebacic acid. Diacids when activated with EDC can form amide bonds with lysine amino groups of the protein globule.

Fig. 4.1a Structure of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide

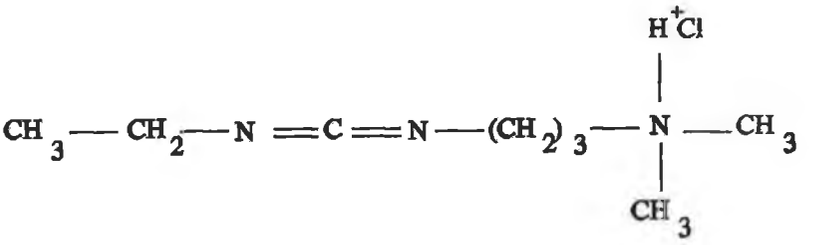
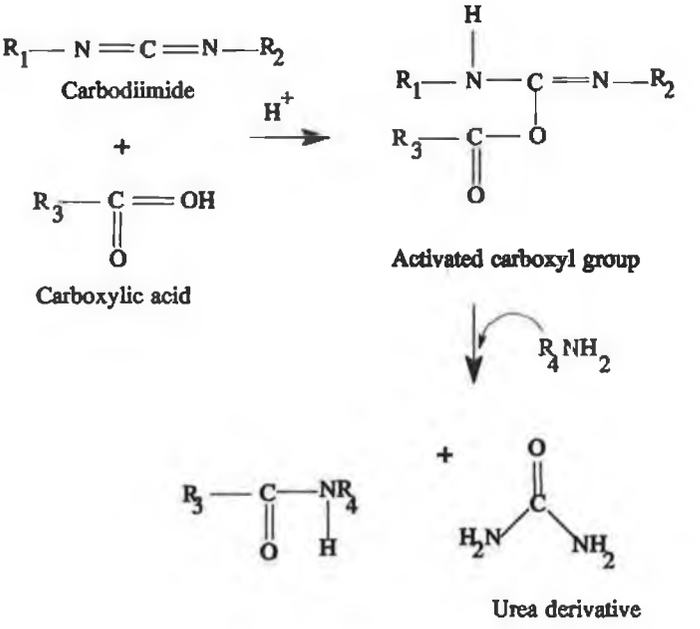


Fig. 4.1b Carbodiimide reaction Scheme.

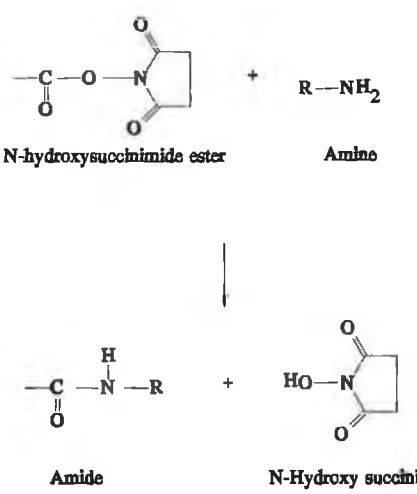


4.1.2 Modification with succinimides

NHS esters are popular reagents because of their mild and speedy reaction conditions and also their high chemical reactivity and specificity. NHS esters are not readily soluble in aqueous buffer (Ji, 1983) and must be dissolved initially in a minimal amount of an organic solvent, such as dimethylsulphoxide. The NHS ester in organic solvent forms an emulsion which will allow the reaction to occur.

The reaction proceeds most efficiently at pH 7.0-8.0. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The NHS ester reaction in aqueous solution consists of two competing reactions. One is the reaction with the primary amine and the other is hydrolysis of the NHS ester. Hydrolysis inactivates the NHS ester and so decreases efficiency of the reaction. Hydrolysis is favoured in dilute protein solutions and acylation is favoured in more concentrated protein solutions. The rapid reaction ensures that the most accessible protein amino groups are attacked within 10-20 minutes. A 10 fold molar excess of an NHS ester is usually sufficient to acylate amino groups. NHS esters react over a wide range of temperatures (4-25°C). The reaction buffer should be free of extraneous amines such as Tris or glycine (Ji, 1983).

Fig. 4.2 NHS Ester Reaction Scheme

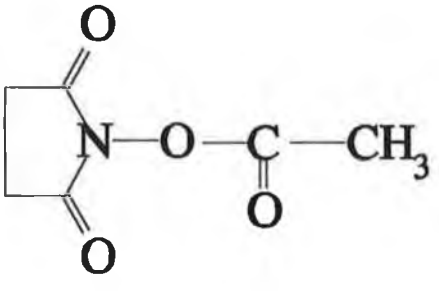


4.1.2.1 Chemical modification with Succinimide

Acetic acid N-hydroxy-succinimide ester is a monofunctional reagent, i.e., it has a single reactive group and cannot form a crosslink. The structure of this ester is given in Fig. 4.3. AA-NHS reacts with primary amine functions, amine groups on lysine residue or available N-terminus amines. The reaction involves the nucleophilic attack of an amine on the ester carbonyl of an N-hydrosuccinimide ester to form an amide, with the release of the N-hydroxy-succinimide so the positive charge on the original amino group is lost (Ji, 1983).

There are several reasons to choose this modification of trypsin. It has been previously shown that crosslinking with bifunctional N-hydroxysuccinimide esters diminished autolysis and thermal denaturation (Gleich et al., 1992). However, no studies were carried out on a one-point modification using the monofunctional AA-NHS. Trypsin has many lysine residues that are not involved in the catalytic site and, because amine groups are polar, they are usually exposed to the medium on the protein surface.

Fig. 4.3 Structure of AA-NHS



4.1.2.2 Crosslinking with succinimides

Bis-N-hydroxysuccinimide esters are homobifunctional crosslinking reagents, i.e. they have two reactive groups, one at either end of the molecule. A series of esters of succinimides of various chain length are available. The N-hydroxysuccinimide (NHS) esters used in this section were suberic acid (SA) ester of NHS and ethylene glycol succinic acid (EG) ester of NHS. The structures of these two esters are shown in Fig. 4.4a and Fig. 4.4b, respectively.

The maximum molecular linkage length of SA-NHS is 11Å (Ji 1983) and that of EG-NHS is 16Å. These esters are derivatives of suberic acid and ethylene glycol respectively (Han et al, 1984). Each reactive end of these molecules is amino specific. As with AA-NHS, they react preferentially with primary amino functions and the same conditions were used.

Crosslinking strengthens the compact structure of the molecule, preventing denaturation. Disulphide bonds can significantly stabilize the native structure of enzymes (Perry & Wetzel 1984, Matsumura et al. 1989). Like disulphide bonds, chemical crosslinking reagents have successfully been used to stabilize proteins (Ji 1983, Wong & Wong 1992). Proteases have been stabilized by a wide range of reagents. Crosslinking was performed using the NHS esters mentioned above. It has been previously shown that point modification with AA-NHS increased thermostability and diminished autolysis (Murphy & O'Fágáin, 1996). To compare the effects of crosslinking and point modification of trypsin, trypsin was reacted with the above mentioned succinimides. The stabilized NHS derivatives of trypsin were characterized. In this way, one can distinguish between the effects of crosslinking lysines and the covalent modification of lysine residues using charge-neutralizing succinimides (as described previously in Section 4.1.2.1).

Fig. 4.4a Structure of suberic acid (SA) ester of NHS

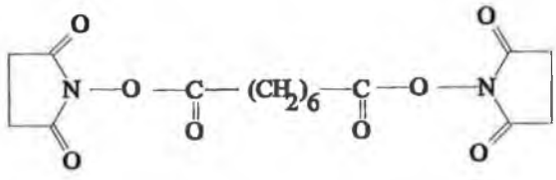
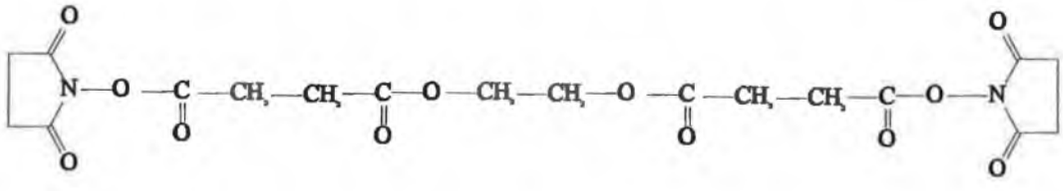


Fig. 4.4b Structure of Ethylene glycol-bis(succinic acid N-hydroxy-succinimide ester)



4.2 Results

4.2.1 Crosslinking with Carbodiimides

4.2.1.1 Diamine Modification

Modification of carbodiimide-activated trypsin with diamines was performed as described in Section 2.3.1.2. Various lengths of diamine were used which reacted in an amidination reaction with activated carboxyl groups on trypsin. A monoamine (ethylamine) was also included as a control to account for the effects on stability of a one-point noncrosslinking modification of the carboxyl groups.

Amidase activity was reduced compared with that of the native trypsin for all the diamines and the monoamine used. The number of active sites (performed as described in Section 2.5) also decreased upon modification (see Table 4.1).

4.2.1.1.1 Effect of Temperature on Diamine crosslinked Trypsin

Thermoinactivation of native and modified trypsin was performed as described in Section 2.8. All the modified forms showed very slight enhanced thermostability compared with native (see Fig 4.5). The monoamine control showed similiar thermostability as the diamine derivatives.

4.2.1.1.2 Stability in Organic Solvents

Organotolerance of native and modified trypsin was performed as described in Section 2.10 using the solvents 1,4-dioxan and DMF. Modified forms of trypsin was found to be less tolerant of both solvents than was native (see Fig 4.6). The monoamine displayed similiar properties to the native in both solvents.

Fig. 4.5 **Thermostability studies on native and diamine crosslinked trypsin at 55°C**

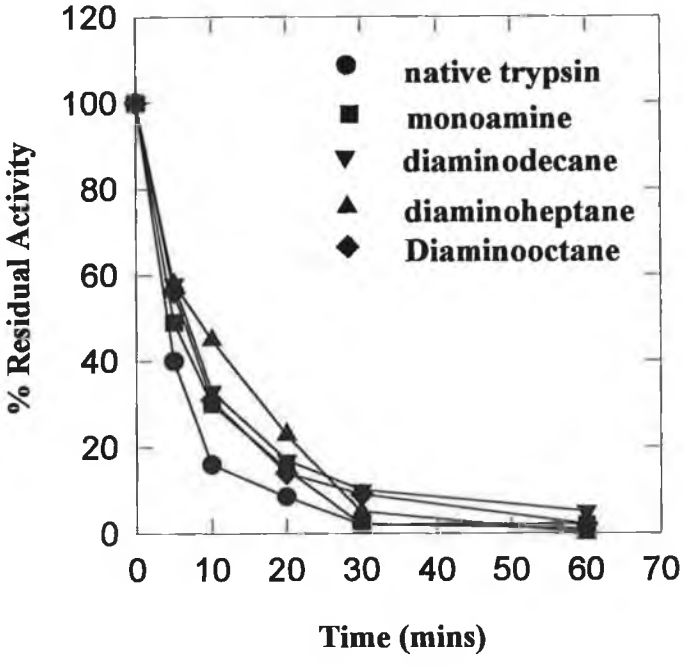


Fig. 4.6 **Organotolerance studies on native and diamine crosslinked trypsin in DMF**

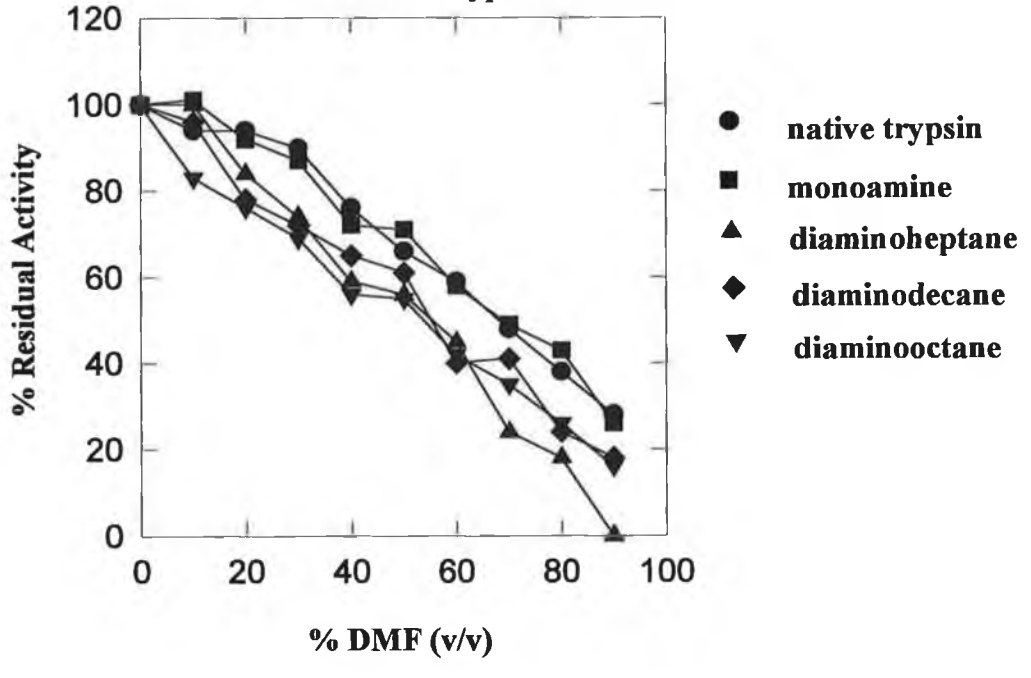


Table 4.1 Effects of various lengths of diamine crosslinks on trypsin activity

	Amidase activity (%)	Active Site Titration (%)
Native trypsin	100%	100%
Ethylamine (mono)	68%	78%
Diaminoheptane	73%	75%
Diaminooctane	65%	74%

4.2.1.2 Diacids

Modification of trypsin with carbodiimide-activated diacids was performed as described in Section 3.3.1.3. using the diacid sebacic acid. A monoacid, (acetic acid) was included as a control. The retained amidase activity of the modified forms was high, but slightly reduced compared with native trypsin (see Table 4.2). The number of active sites was also slightly reduced.

4.2.1.2.1 Effect of Temperature on Diacids crosslinked Trypsin

Thermoinactivation of native and modified trypsin was performed as described in Section 2.8. The thermoinactivation of native and modified trypsin is shown in Fig 4.7. Modified forms of trypsin (including the monoacid control) showed no enhanced thermostability when compared with native.

4.2.1.2.2 Stability in Organic Solvents

Organotolerance of native and modified trypsin was performed as described in Section 2.10. using the solvents 1,4-dioxan and DMF. The organotolerance of trypsin did not appear to be enhanced by the modification. For both solvents tested no enhanced stability was observed (see Fig 4.8). The monoacid exhibited similar properties to those of the diacid.

Fig. 4.7 **Thermostability studies on native and diacid crosslinked trypsin at 55^{0}C**

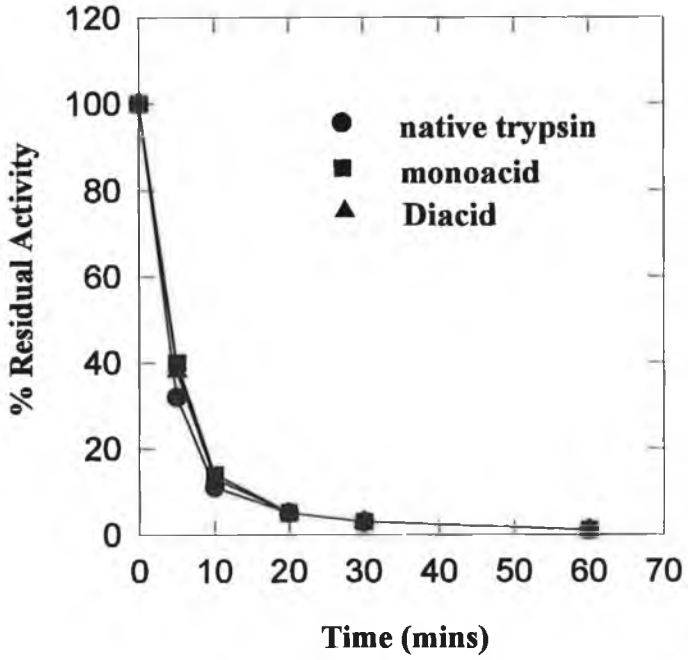


Fig. 4.8 **Organotolerance studies on native and diacid crosslinked trypsin in 1,4-Dioxan**

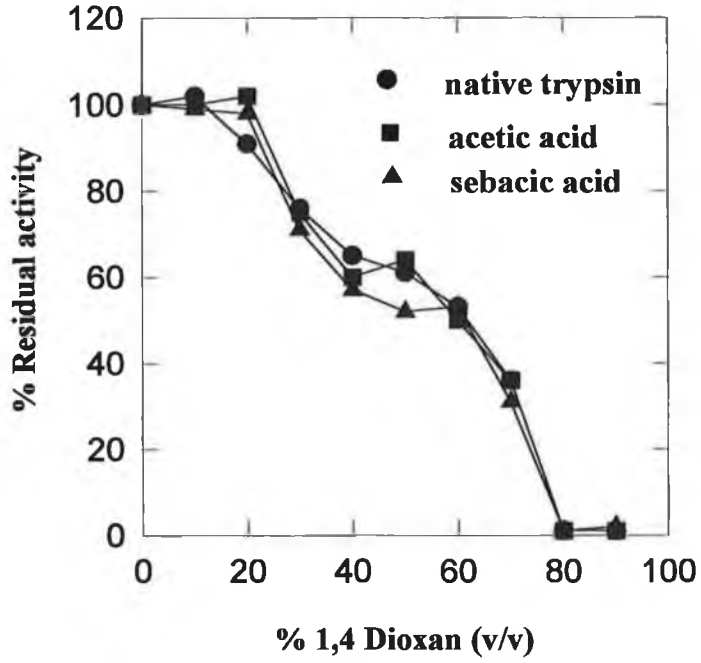


Table 4.2 Effects of various diacid crosslinks on trypsin activity

	Amidase activity (%)	Active Site Titration (%)
Control	100%	100%
Acetic acid	88%	91%
Sebacic acid	89%	88%

4.2.2 Modification with succinimides

4.2.2.1 Modification Using Acetic acid N-hydroxy-succinimide ester

4.2.2.1.1 Optimizaton of Reaction

The modification reaction was optimized with respect to succinimide concentration. The following concentrations of AA-NHS were included in the reaction mixture: 0.4, 2.0 and 10.0mg ml^{-1}. The amidase activity of all derivatives was enhanced compared with the native. The percentage of lysine residues modified was similar for the two higher concentrations of AA-NHS (approximately 60%) while at 0.4mg ml^{-1} less lysine residues were modified approximately 40%). At all AA-NHS concentrations tested, the modified trypsins were more thermostable than native (see Fig 4.9). The highest thermostability was observed at the two higher concentrations. The active site titration showed that, at all AA-NHS concentrations, the number of active sites available was the same as in the native. A concentration of 2mg ml^{-1} AA-NHS in the reaction mixture gave maximum modification, resulting in enhanced thermostability and activity. Higher concentrations had no adverse effects on the enzyme; however, no further increase in the number of lysine residues modified nor in stability was observed. Other reaction conditions (pH, reaction time and temperature) had previously been optimized by Miland et al. (1996) for horseradish peroxidase. These conditions were successfully applied to the trypsin modification reaction.

Fig. 4.9 **Studies on the effects of succinimide concentration on the thermostability of trypsin at 55°C**

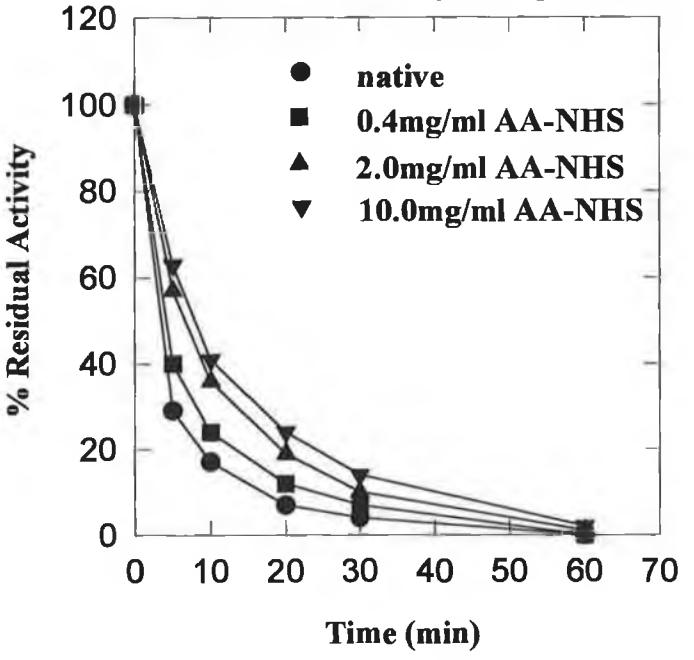


Table 4.3 Comparison of activity native and AA-trypsin

	Native trypsin (%)	AA-NHS trypsin (%)
Active Site Titration (NPGB)	100	92 ± 10
Amidase Activity (BAPNA)	100	132 ± 5
Esterase Activity (TAME)	100	88 ± 6
Lysine residues unmodified (TNBS)	100	40 ± 2

4.2.2.1.2 Characterization of AA-trypsin

Modification of the lysine residues of trypsin with AA-NHS is accompanied by an increase in the enzyme amidase activity (1.3 times the native). This may be due to the neutralization of the positive charge of surface lysine residues by the succinimide (Ji, 1983). The esterase activity of modified trypsin was slightly lower (90%) than the native. From the free amino group determination using TNBS it was found that approximately 40% of the lysine residues remained unmodified, suggesting that 8 lysine residues per trypsin molecule were modified. This result was no different in the presence and absence of 30mM benzamidine. Active site titration of trypsin with NPGB showed that the modified trypsin's activity was slightly lower than that of the native (approximately 90% compared with that of the native). The presence of benzamidine again seemed to have little or no effect on the modification

4.2.2.1.3 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as described in Section 2.14. The stained gel showed bands in similiar positions for all the samples (see Fig 4.10). This indicates that no aggregation or artifacts occurred as a result of the modification procedure and that the enzyme mixture was homogenous.

4.2.2.1.4 Autolysis of AA-Trypsin

The rates of autolysis of native and AA-NHS modified trypsin were studied as described in Section 2.7. Trypsin cleaves proteins and peptides at the carboxylic side of the basic amino acids arginine and lysine. Therefore, modifying the lysine residues should provide some protection against digestion. As expected, the AA-NHS trypsin was more resistant to autolysis than native trypsin. The absorbance of AA-NHS trypsin slightly increased initially but then remained constant throughout the experiment, showing that autolysis was not occurring. The initial increase could be due to the formation of artifacts or to slight aggregation. In contrast, native trypsin showed a steady decrease in absorbance dropping to 60% after 2 hrs (see Fig 4.11).

Fig. 410 SDS-PAGE of native and AA-trypsin.

Modified and native trypsin (20 μg per well in both cases) each migrated as a single band. The molecular weight markers on the right were (from top) bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa) and soybean trypsin inhibitor (20.1 kDa). the wells between the samples and molecular weight markers were left empty.

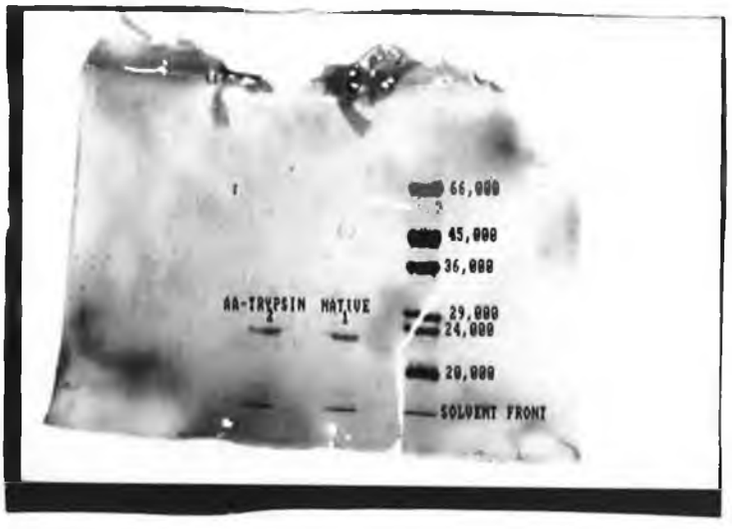
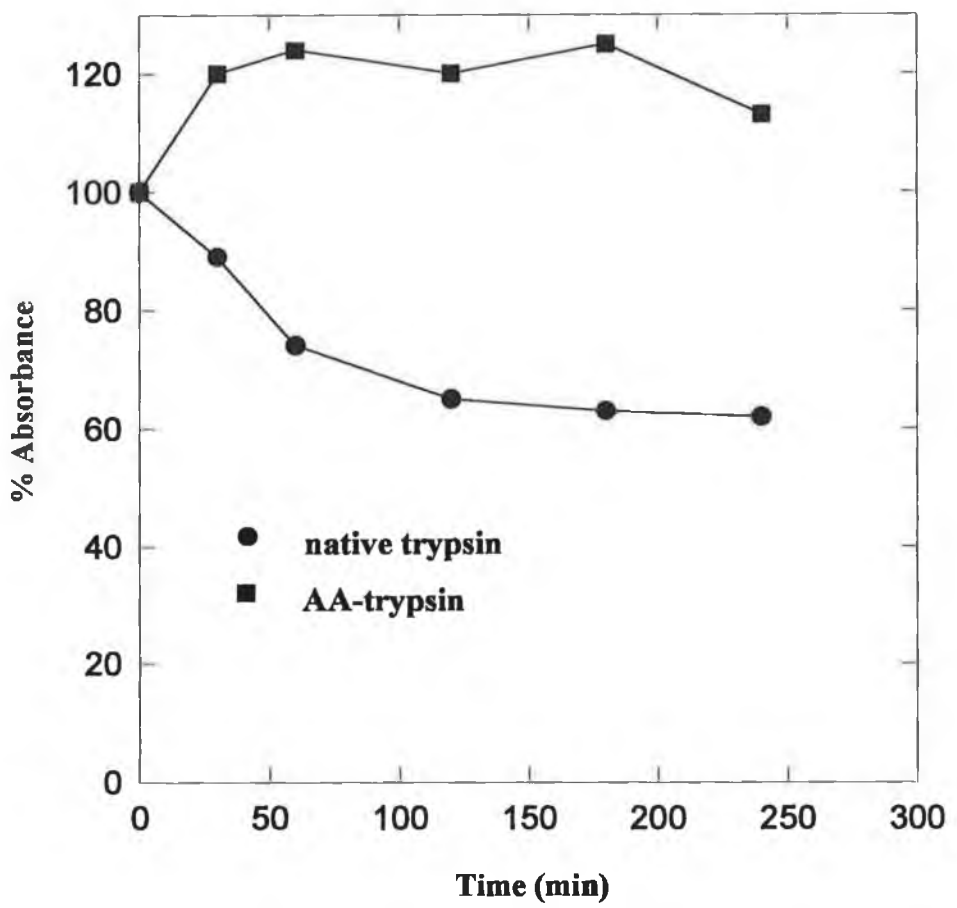


Fig. 4.11 **Autolysis of native and AA-trypsin at 50°C**



The absorbance is shown in percentage relative to initial absorbance of sample

4.2.2.1.5 Effects of temperature on AA-trypsin

Thermostability of AA-trypsin was compared with that of native enzyme as described in Section 2.8. Modified trypsin was more thermostable than the native (see Fig. 4.12). The half-life of the modified at 55 °C was double that of the native: 8.7 min ($k = 0.08$ min^{-1}) for the AA-trypsin compared with 4.3 min ($k = 0.16$ min^{-1}) for the native trypsin. The increased resistance of AA-trypsin to temperature is most likely due to the change in surface charges which may affect the folding and unfolding of the molecule. A number of different buffering systems have been previously tested to ascertain conditions of minimal autolysis. Under the conditions described for thermoinactivation, autolysis was negligible.

Temperature profiles of native and modified trypsin were determined as described in Section 2.9. The residual activity of modified trypsin was higher compared with the native over the temperature range 30 °C to 75 °C (see Fig 4.13). Modified trypsin retained 100% activity up to 50°C while the native trypsin lost some activity at 40 °C. T_{50} values for native and AA-trypsin were 46 °C and 51 °C respectively.

Reaction of AA-trypsin with Nile Red was performed as described in Section 2.16. Heat treatment resulted in a progressive change in trypsin which shifted the emission of the dye to 630nm. A range of temperatures was used and it was observed that the higher temperature gave the highest emmission readings and the greatest shift to 630nm (see Fig. 4.14).

4.2.2.1.6 Stability of AA-trypsin in organic solvents

Organotolerance was determined as described in Section 2.10. The stability of both native and AA-trypsin were compared in the following solvents: acetone, acetonitrile, DMF, DMSO, 1,4-dioxan, methanol and THF. After incubation for one hour at 30 °C, the modified enzyme showed no enhanced stability towards any of the solvents tested. However, when the samples were incubated at 65 °C for 1 hour all solvents except THF showed protecting effects against thermoinactivation (see Fig 4.15a, b & c). For methanol the AA-NHS, trypsin showed enhanced stability at lower solvent concentrations while the native trypsin was more thermostable at higher concentrations

Fig. 4.12 **Thermostability studies on native and AA-trypsin at 55^{0}C over time**

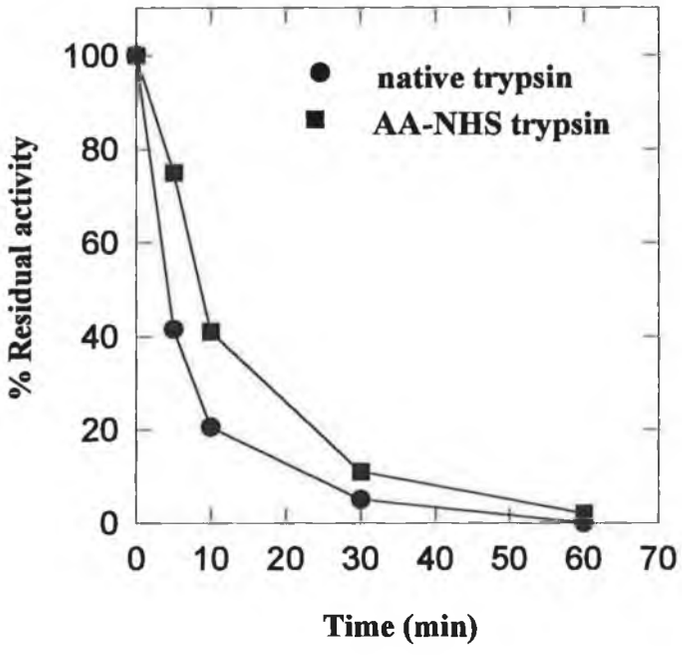


Fig. 4.13 **Temperature profile of native and AA-NHS modified trypsin**

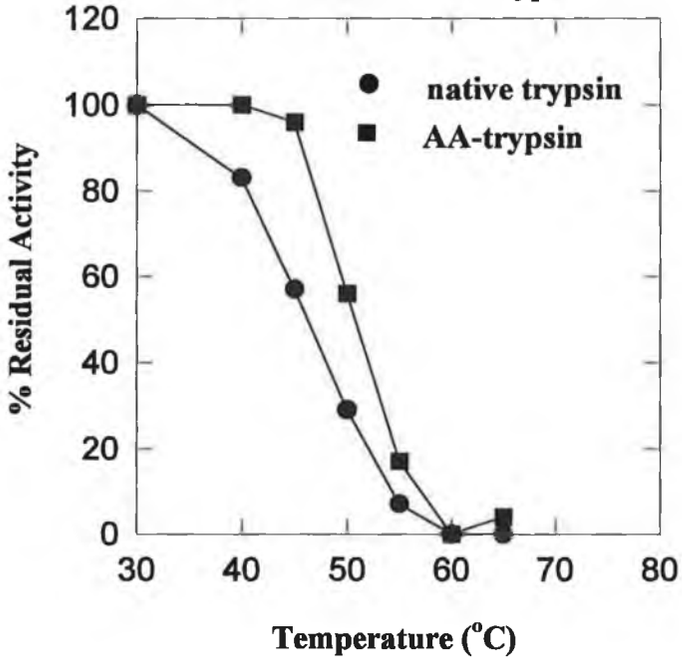
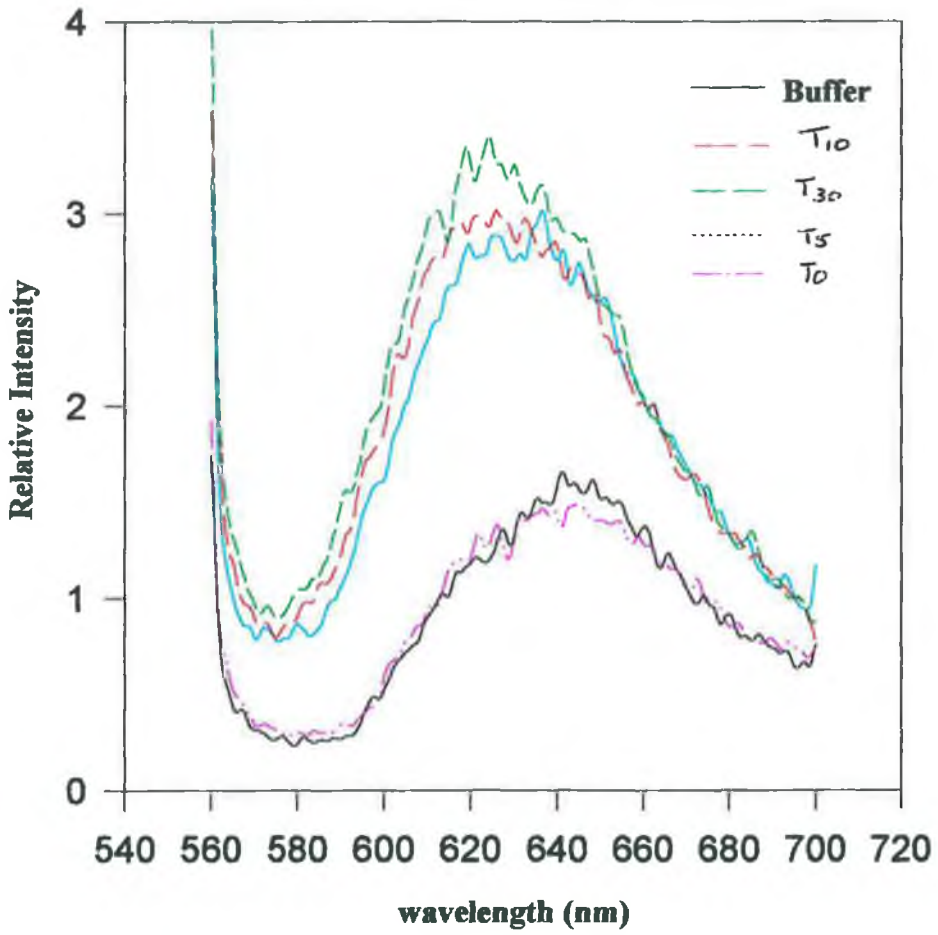


Fig. 4.14 Fluorescence of Nile Red in the presence of heat treated AA-trypsin at 85^{0}C at different time intervals



(see Fig 4.15d). THF afforded little protection against thermoinactivation: little or no activity was retained over the concentration range 0 - 90 % THF by either the native or AA-trypsin. (Note that activity was compared with a control incubated at 30°C in each case, so that the combined effect of both temperature and organic solvent could be examined).

4.2.2.1.7 Effects of pH on AA-trypsin

pH profiles were determined as described in Section 2.15. The activities of native and AA-trypsin were compared over a range of pH values. The modified trypsin retained slightly higher activity in alkaline conditions compared with native trypsin (see Fig 4.16). Both native and modified forms of trypsin exhibited similar activities in acid and neutral pH ranges.

4.2.2.1.8 Effects of Denaturants on AA-trypsin

The denaturing effects of urea and guanidine hydrochloride on both the native and modified trypsin were investigated as described in Section 2.12. Modified trypsin showed no increase in stability compared with trypsin in either denaturant.

4.2.2.2 Crosslinking with Succinimides

4.2.2.2.1 Optimization of reaction

Modification of trypsin with crosslinking succinimides was performed as descrided in Section 2.3.2. Activity of native and modified trypsin was determined for two types of bis-succinimides: EG-NHS and SA-NHS. Modification with SA-NHS (the shorter of the two) decreased the amidase activity of trypsin (38%), while EG-NHS enhanced trypsin's amidase activity (203%), see Table 4.4. EG-NHS trypsin showed increased activity over the concentration range tested while SA-NHS trypsin exhibited decreased activity (see Table 4.4).

Because of the low activity of SA-trypsin and the poor solubility of SA-NHS it was decided not to use this reagent further. The EG-NHS was, however, used to

Fig. 4.15a **Effects of DMF on native and AA-trypsin at 65°C**

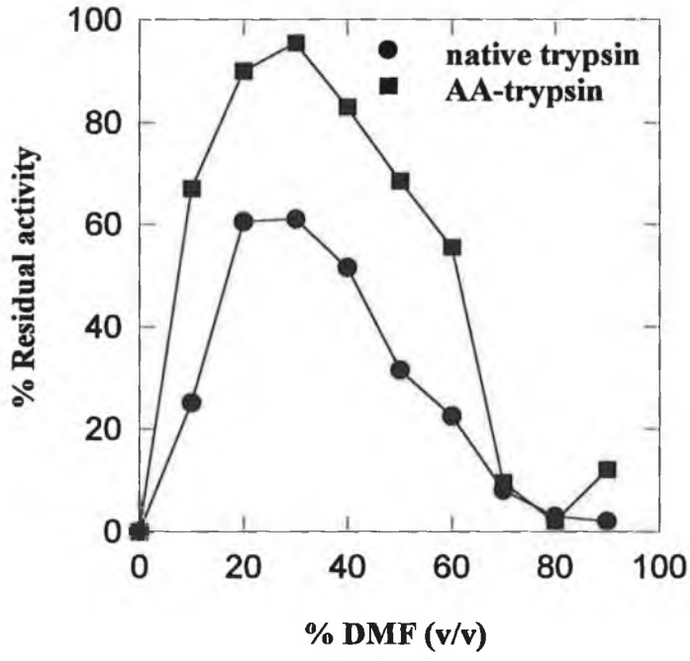


Fig. 4.15b **Effect of 1,4-dioxan on native and AA-trypsin at 65°C**

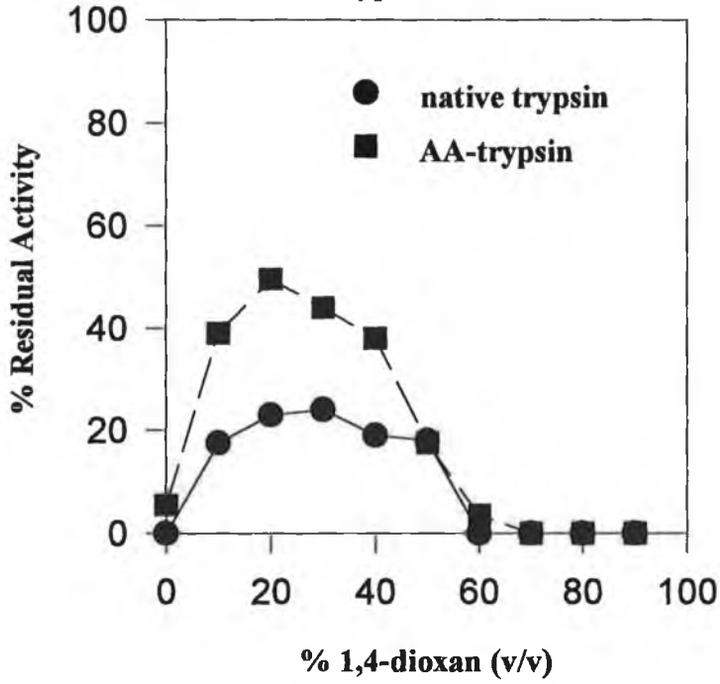


Fig. 4.15c **Effects of Acetonitrile on native and AA-trypsin at 65°C**

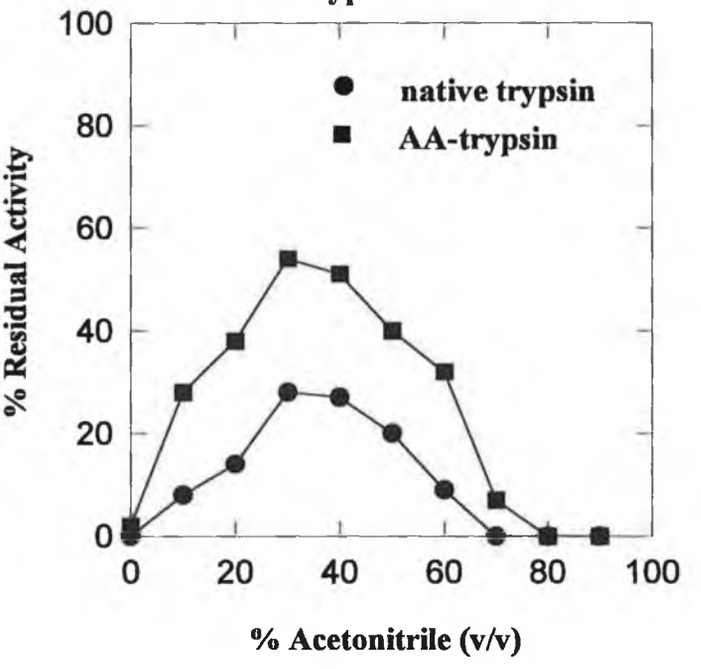


Fig. 4.15d **Effects of Methanol on native and AA-trypsin at 65°C**

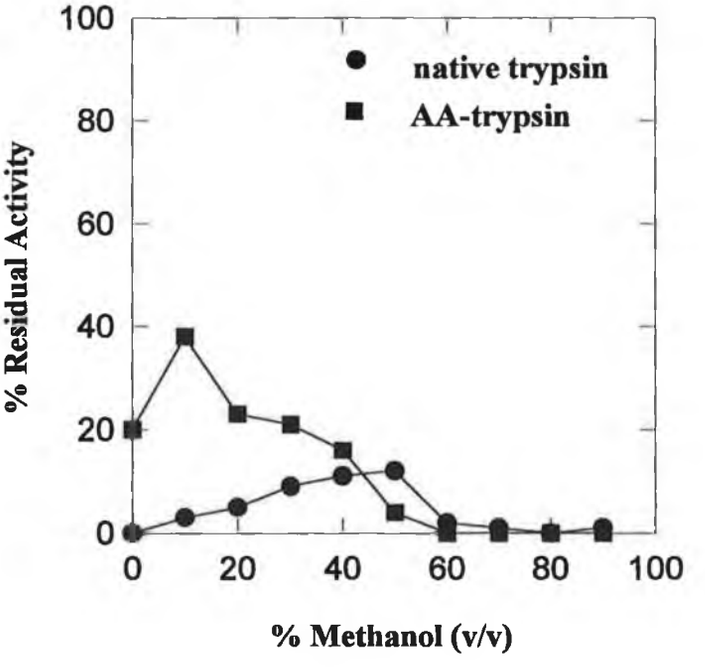
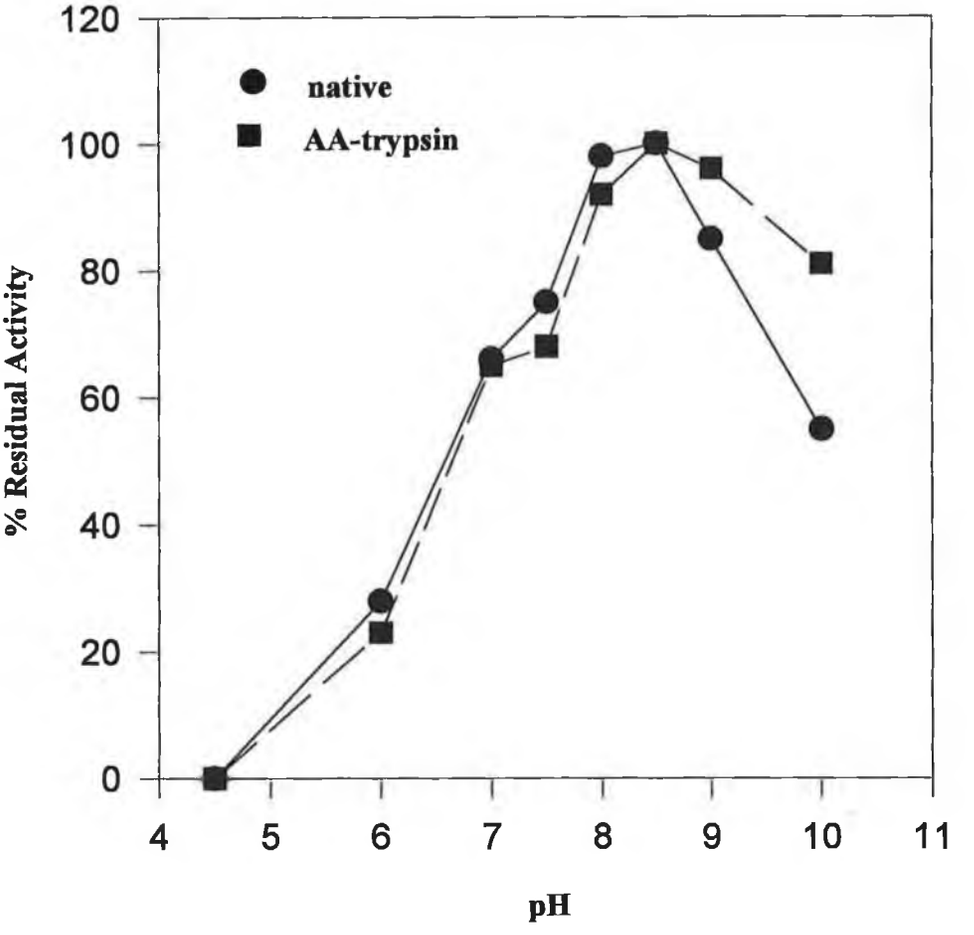


Fig. 4.16 **Effects of pH on the amidase activity of native and AA-trypsin**



The buffer solutions, (containing 20 mM CaCl2) of pH 4.5-6, 7-9, 8-10 were made with sodium acetate, Tris-HCl and glycine-NaOH buffer (I = 0.1 M), respectively. The activity is shown as percentage of the maximum activity of each sample.

modify trypsin and further characterization studies of EG-Trypsin performed. The reaction was optimized with respect to EG-NHS concentration. The following concentrations of succinimide were included in the reaction mixture: 0.4, 2.0 and 10.0mg ml^{-1} and the derivatives formed were analyzed. It was found that the amidase activity of all derivatives was enhanced compared with that of the native (Table 4.4). 2.0mg ml^{-1} gave the highest activity (203%). The percentage of lysine residues modified was highest for 10.0mg ml^{-1}. All three derivatives were more thermostable than the native: see Fig 4.17. The active site titration showed that an equal number of active sites was present in the native and all modified forms. 2.0mg ml^{-1} was the optimal concentration, as it exhibited the highest activity and had enhanced thermostability. 10mg ml^{-1} was slightly more thermostable but had lower amidase activity. All other reactions conditions had previously been modified as described by Miland et al (1996).

4.2.2.2.2 Characterization of EG-trypsin

The modified trypsin showed enhanced amidase activity (200%), but slightly lower esterase activity (90%) compared with the native (see Table 4.5). The number of active sites was the same for both native and modified forms. From the free amino group determination using TNBS, it was found that approximately 40% of the lysine residues remained unaltered suggesting that 8 lysine residues per trypsin molecule were modified.

4.2.2.2.3 SDS-polyacrylamide gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as described in Section 2.14. The modified trypsin did not show any higher molecular weight species as compared with the native molecule (see Fig 4.18). Thus no intermolecular crosslinking had occurred and the product was free of dimeric or higher molecular weight aggregates of trypsin.

Table 4.4 The effect of concentration of EG- and SA- succinimides on the activity of trypsin.

Succinimide conc. (mg/ml)	% Activity	
	EG-NHS	SA-NHS
0.2	115 ± 5	46 ± 2
0.4	186 ± 4	n.d
2.0	203 ± 4	38 ± 1
10.0	131 ± 5	n.d

Fig. 4.17 **Studies on the effects of EG-NHS concentration on the thermostability of trypsin at 55°C**

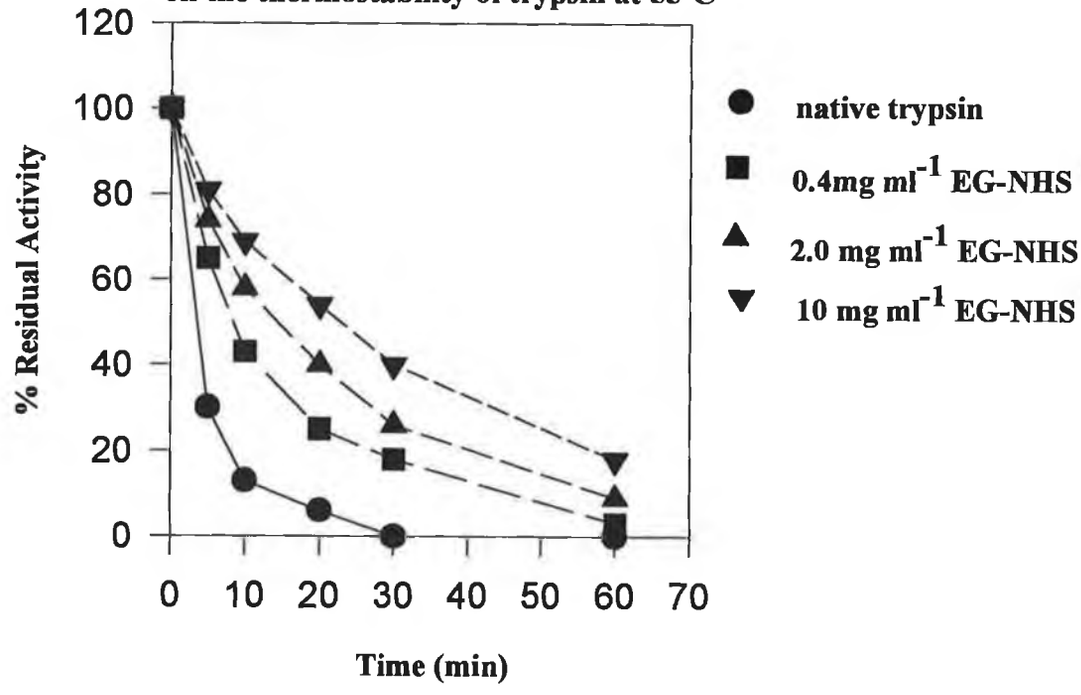


Fig. 4.18 SDS-PAGE of native and EG-trypsin.

Modified and native trypsin (20 μg per well in both cases) each migrated as a single band. The molecular weight markers on the right were (from top) bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa) and soybean trypsin inhibitor (20.1 kDa). the wells between the samples and molecular weight markers were left empty.

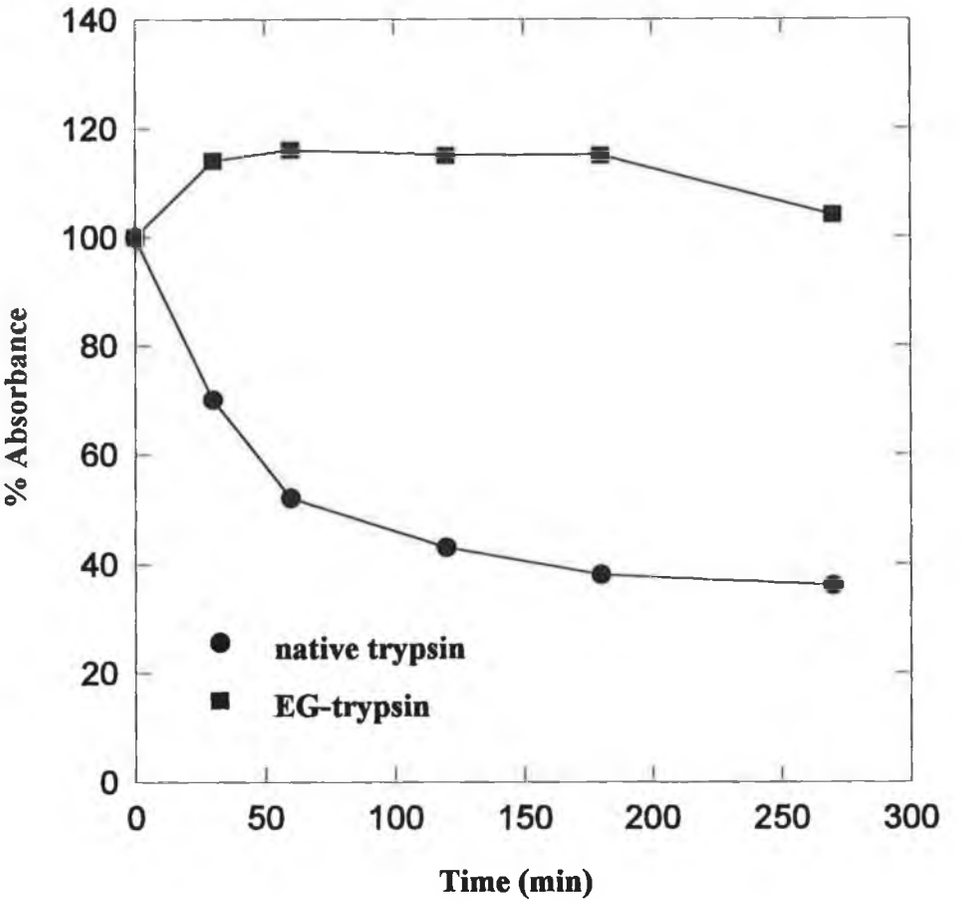
Table 4.5 Comparison of activity of native and EG-trypsin for a range of substrates

	Native trypsin (%)	EG-NHS trypsin(%)
Active Site Titration (NPGB)	100	110 ± 5
Amidase Activity (BAPNA)	100	203 ± 4
Esterase Activity (TAME)	100	90 ± 1
Lysine residues unmodified (TNBS)	100	45 ± 4

4.2.2.2.4 Autolysis of EG-trypsin

The rates of autolysis of native and modified trypsins were determined as described in Section 2.7. The modified trypsin showed decreased to autolysis compared with the native (see Fig 4.19.). The absorbance of the native trypsin had dropped to 40% of initial value after 5 hours while the modified trypsin remained stable. This enhanced stability was probably a combination of lysine residue modification (making then no longer a target for cleavage) and the actual crosslinks themselves: these would hold fragments together and also act as steric hindrances to proteolytic attack.

Fig. 4.19 **Autolysis of native and EG-trypsin at 50°C**



The absorbance is shown in percentages relative to initial absorbance of sample

4.2.2.2.5 Effects of temperature on EG-trypsin

Thermostability of EG-trypsin was compared with native trypsin as described in Section 2.8. EG-NHS modified trypsin was more thermostable than the native (see Fig 4.20). The half-life of modified trypsin at 55°C was 25 mins compared with 5 mins for native trypsin. The residual activity of EG-trypsin was compared with that of the native over the temperature range 30°C to 75°C as described in Section 2.9. The modified trypsin retained 100% activity up to 50°C while the native began losing activity at 40°C. T_{50} values for native and EG-trypsin were 46°C and 59°C (see Fig 4.21)

Reaction of trypsin with Nile Red was performed as described in Section 2.16. Heat treatment resulted in a progressive change in trypsin which shifted the emission of the dye to 630nm. A range of temperatures was used and it was observed that the higher temperature gave the highest emission readings and the greatest shift to 630nm (see Fig. 4.22).

4.2.2.2.6 Stability of EG-trypsin in organic solvents

Organotolerance was determined as described in Section 2.10. Organotolerance of native and EG-trypsin were compared in acetonitrile, methanol, dimethylformamide, and tetrahydrofuran. In all solvents the EG-trypsin showed enhanced organotolerance (see Fig 4.23a & b). The C_{50} values of EG-trypsin were higher than those of native for all solvents tested (see Table 4.6). Both enzymes in water-miscible organic solvent systems have suppressed activity at higher concentrations.

4.2.2.2.7 Effects of pH on EG-trypsin

pH profiles were determined as described in Section 2.15. The activities of modified and native trypsin were compared over a range of pH values. The pH profiles for both types of trypsin were similiar: see Fig 4.24. No difference in the amidase activity's pH optimum was observed between the modified trypsin and native trypsin.

Fig. 4.20 Thermostability studies on native and EG-trypsin at 55^{o}C over time

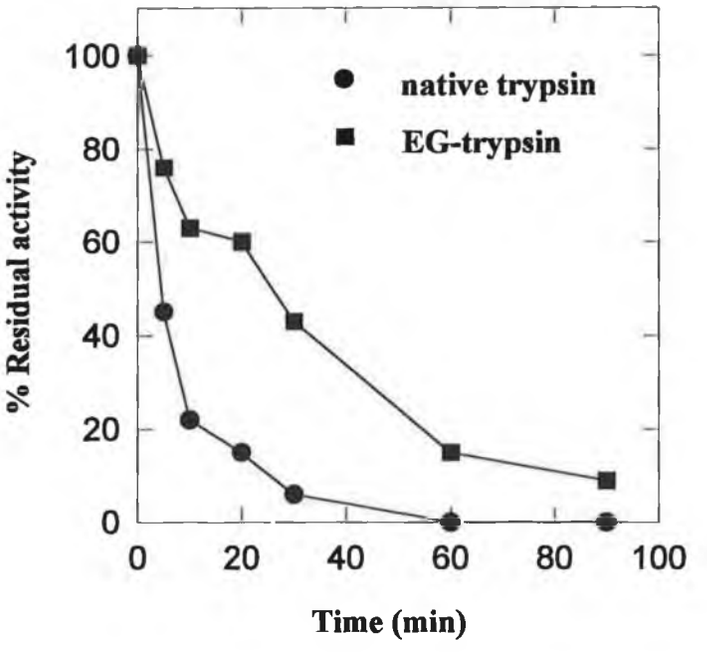


Fig. 4.21 Temperature profile of native and EG-trypsin

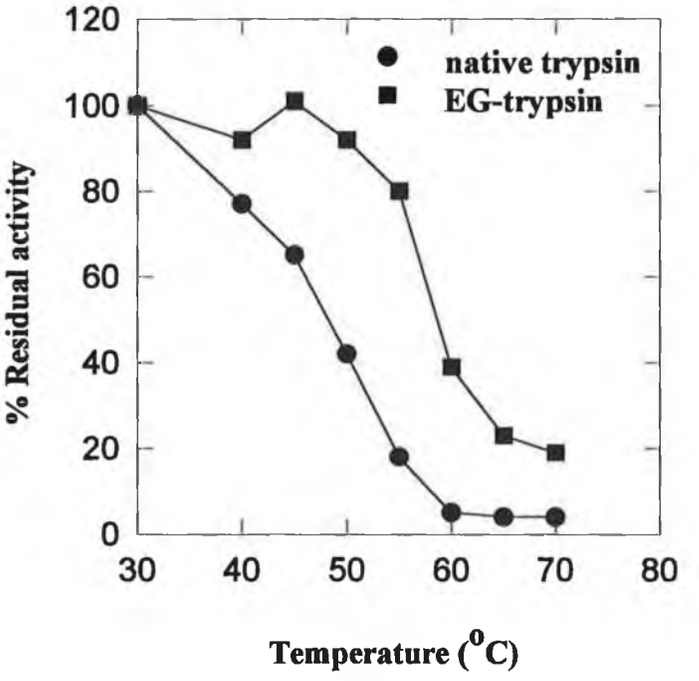


Fig. 4.22 Fluorescence of Nile Red in the presence of heat treated EG-trypsin at 85^{0}C at different time intervals

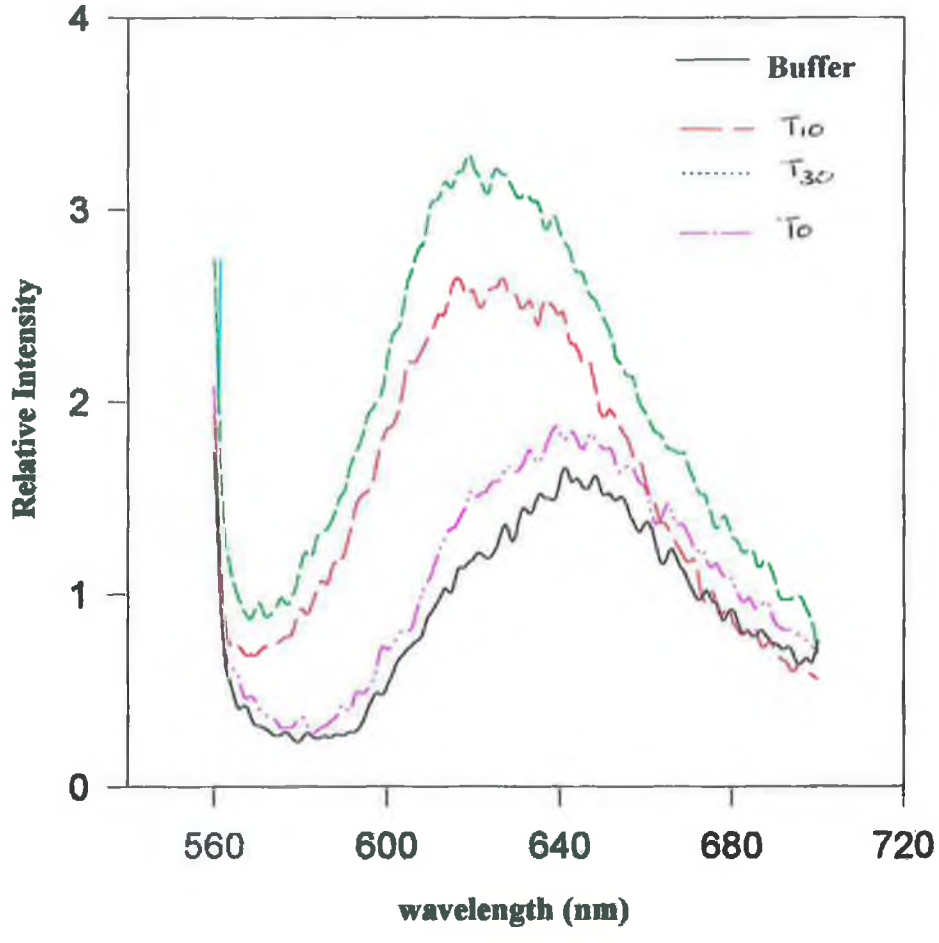


Fig. 4.23a **Effect of Acetonitrile on native and EG-trypsin**

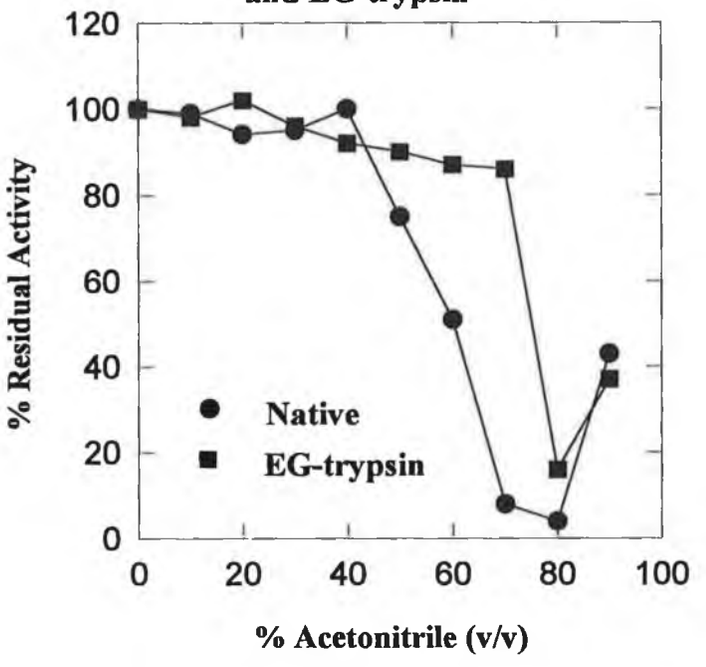
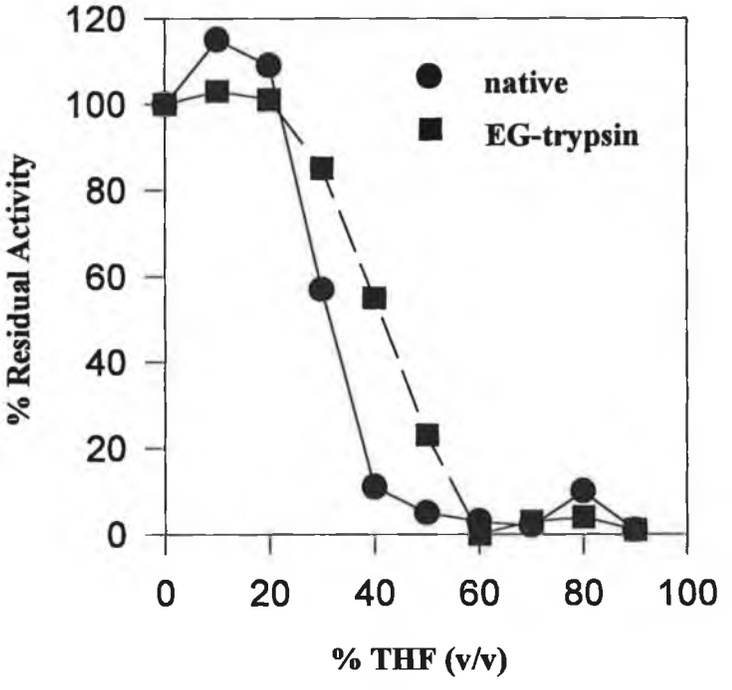


Fig. 4.23b **Effect of THF on native and EG-trypsin**



4.2.2.2.8 Effects of Denaturants

The denaturing effects of urea and guanidine hydrochloride on both the native and modified trypsin were investigated as described in Section 2.12. Modified trypsin was found to be more tolerant of urea, retaining higher activity compared with native trypsin over the concentration range tested (see Fig 4.25). Again this is likely due to the conformational rigidity introduced via crosslinkage. No difference between the native and EG-trypsin was observed for guanidine hydrochloride .

Table 4.6 Comparison of C_{50} values for native and EG-trypsin in a range of solvents

Solvent	**Native** C_{50} **(%)**	**EG-trypsin** C_{50} **(%)**
Acetonitrile	39 ± 0	60 ± 2
Methanol	63 ± 3	73 ± 3
Dimethylformamide	63 ± 2	67 ± 2
Tetrahydrofuran	35 ± 4	49 ± 9
1,4-Dioxan	46 ± 1	54 ± 3

Fig. 4.24 **Effect of pH on the amidase activity of native anf EG-trypsin**

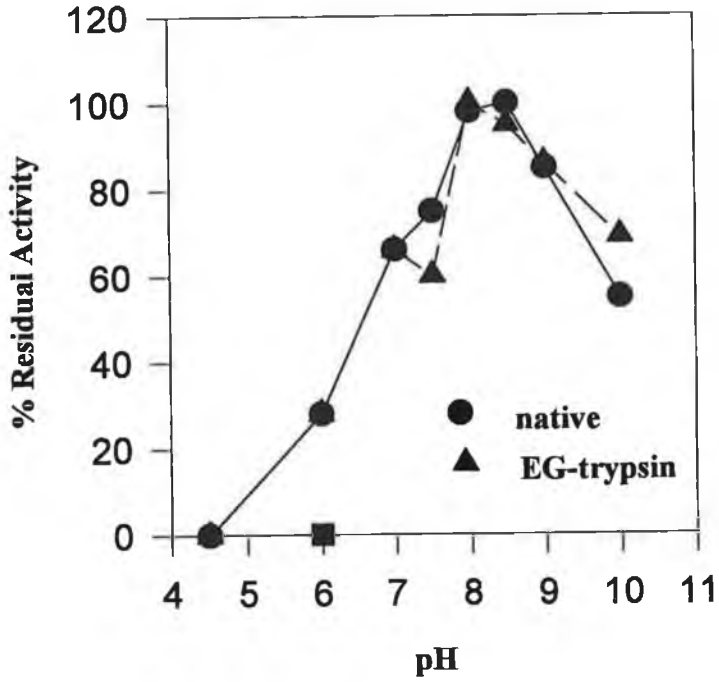
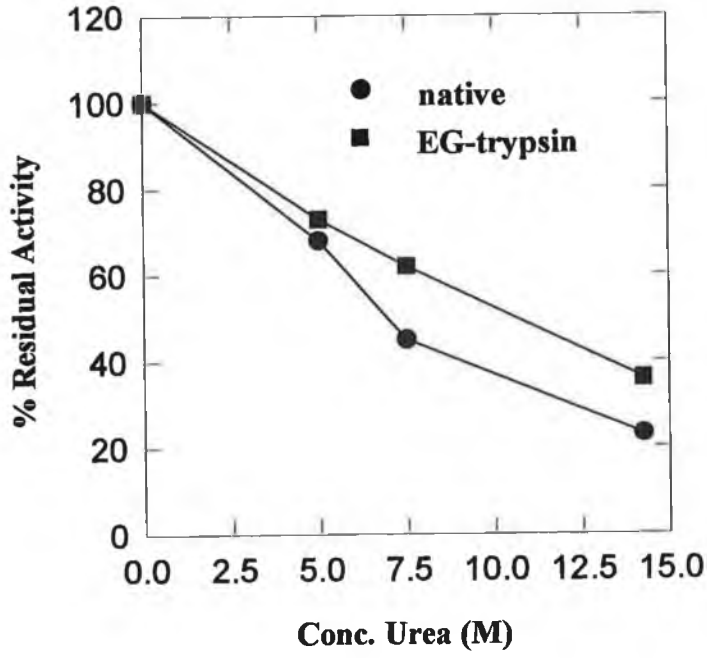


Fig. 4.25 **Effect of urea on native and EG-trypsin**



4.3 DISCUSSION

4.3.1 Modification using Carbodimides

Crosslinking has been used extensively to stabilize proteases in adverse environments (see Section 1.5.2). Because of the chemical nature of EDC (see Fig 4.1a) it is possible to modify both the carboxyl and amine groups on trypsin. The advantages of using carbodiimide amidation coupling procedures as a chemical modification technique are that the reactions are simple to perform and proceed under very mild conditions.

4.3.1.1 Diamine Modification

Diamines ($NH_2(CH_2)_nNH_2$) serve as nucleophiles to carbodiimide-activated trypsin. Amide bonds are formed between an activated carboxyl group and an amino group of the trypsin via the introduced amine. The activation of the carboxyl groups is carried out at pH <7 (approximately pH 4.5). It is necessary to adjust to pH >7 before adding the diamine. Various lengths of diamine (n=7,8 & 10) were used in amidination reactions with activated carboxyl groups on trypsin.

A trypsin concentration of 1mg ml^{-1} was used and a 10-fold excess of EDC over available COO^- groups was used. The carboxylic groups on trypsin consist of 14 glutamic acids and 22 aspartic acids (Walsh & Wilcox, 1970). A concentration of 15mM EDC, therefore, gives a 10-fold excess over available carboxyl groups. The concentration of diamine used was the same as that of EDC, i.e., 15mM. A monoamine was also included as a control to account for the effects on stability of a one-point, non-crosslinking modification of the carboxyl groups.

Modified derivatives of trypsin retained relatively high activity. For all the diamines and the monoamine used, it was found that the amidase activity was approximately 70% of native trypsin (see Table 4.1). Similiar results were obtained from the active site titration. The active site titration is less sensitive than the amidase assay but has the inherent advantage of providing an absolute standardization of the concentration of active enzyme. It therefore seems that these modifications have a slight adverse effects on the enzyme. Perfetti et al (1976) found that modification of

the carboxyl and tyrosyl residues of papain with EDC lead to irreversible inactivation. This inactivation might be due, at least in part, to a reaction of some kind between the carbodiimide and the imidazole moiety of His-159, a known constituent of the active site of papain.

The thermoinactivation of native and modified trypsin is shown in Fig 4.5. All the modified forms showed a very slight enhanced thermostability compared with the native. This limited degree of enhanced thermostability could be due to decreased conformational flexibility of the enzyme. However, since the monoamine control also exhibits this stability, it is more probably due to modification of trypsin's carboxyl groups than to crosslinks. Organotolerance of the enzyme did not appear to have been enhanced by the modifications. The diamines instead exhibited a slight decrease in stability for both the solvents tested, Dioxan and DMF (Fig 4.6). The monoamine displayed similiar properties to the native for both solvents. The length of the crosslinking diamine had no effect on the stability for the range of compounds tested. Torchilin et al. (1978) looked at the effect of different lengths of crosslinkers on the stability of chymotrypsin. The crosslinking of the carbodiimide activated chymotrypsin with tetramethylenediamine (n=4) resulted in the greatest thermostability of chymotrypsin. This seems to be due to the fact that this very agent has the length fitting the distance between the carboxy groups of the protein molecule, facilitating the formation of a greater number of intramolecular cross-linkages than with other diamines. Stabilization of the enzyme also occured with other diamines: n=2, 5 and 6. The number of crosslinkages could be increased by succinylating chymotrypsin, resulting in even greater thermostability. In the case of succinyl-alpha-chymotrypsin the optimal diamine was ethylenediamine (n=2). The stabilization observed in diamine-treated chymotrypsin is a result of intramolecular crosslinkage and is not due simply to chemical modification: modification of chymotrypsin with a monoamine slightly reduced the thermostability. It is probable that the slight thermostability changes seen in modified forms of trypsin was just a result of chemical modification of the carboxyl groups, rather than being due to crosslinks, as the monoamine control also exhibited the same stability. It is conceivable that shorter diamine might be better suited than

that chosen for the formation of crosslinks in trypsin. Pretreatment of trypsin, by succinylation etc., to increase the number of carboxyl groups available might also increase the number of crosslinks formed.

The presence or absence of crosslinks between the carboxyl groups on trypsin is speculative. Because no significant increase in stability was observed as a result of this modification no further characterization of these derivatives was undertaken.

4.3.1.2 Diacid Modification

The diacids (HOOC-$(CH_2)_n$-COOH) were first reacted with EDC to activate the carboxyl groups, as described by Torchilin et al (1983). A 4-fold excess of of EDC over diacid was used, i.e., 2mM EDC to 0.5mm diacid. This reaction was carried out at a pH >7. 1 mg ml^{-1} trypsin was added to the activated carboxyl group after adjusting the pH <7 to ensure reaction of the amine group. There are 14 lysine residues on trypsin which are available to form amide bonds with the activated carboxyl groups. Again, a monoacid, (acetic acid) was included as a control to account for the effects of one-point modification on the enzyme.

Both the monoacid and diacid retained high, but slightly reduced (about 90%) amidase activity when compared with the native trypsin. The same was also observed from the active site titration, suggesting that the modification again had a slight adverse effect on the activity of trypsin. The thermoinactivation of native and modified trypsin is shown in Fig. 4.3. The modified enzyme showed no enhanced thermostability when compared with the native.The organotolerance of trypsin did not appear to be enhanced by the modification. For the two solvents tested, dioxan and DMF, no enhanced stability was observed. The monoacid exhibited similar properties to those of the diacid indicating that if any crosslinks were actually formed they did not offer the conformational stability associated with crosslinkage.

Torchilin et al. (1983) found that glyceraldehyde-3-phosphate dehydrogenase could be stabilized against deactivation by using different diacids. The stabilization is due to a decrease in intermolecular (considering the enzyme as a functional oligomer) protein mobility. The degree of stability again was related to the length of the diacid

with succinic and glutaric acids (n=2 & 3 respectively) giving the greatest thermostability. As no difference between native and modified trypsin was observed, it is likely that no crosslinks formed. As with diamine modification, different length diacids may be more suitable for the formation of crosslinks.

Whether the activated diacid actually reacted with the lysine residues is speculative. The number of lysine residues modified could be determined using TNBS. Because no enhanced stabilty was observed in the derivatives no further characterizations were persued.

4.3.2 Modification using N-hydroxysuccinimides

NHS esters are popular reagents for modifying proteins because of their mild and speedy reaction conditions and their high chemical reactivity and specificity. One drawback is the fact that NHS esters are not readily soluble in aqueous buffers (Ji, 1983). The NHS esters must be dissolved initially in a minimal amount of organic solvent, such as DMSO or DMF. The effects of charge neutralization through chemical modification of lysine residues and the effect of crosslinking using NHS esters were investigated.

4.3.2.1 Modification with AA-NHS

There have been previous reports of trypsin stabilization by crosslinking agents (Rajput & Gupta, 1987 & 1988) including bis-succinimide esters (Gleich et al., 1992). It was wished to observe the effects of a non-crosslinking covalent modification of trypsin's lysine residues to distinguish between the effects of charge-retaining imidates and charge-neutralizing succinimides (Ji, 1983). Important differences between the stabilizing abilities of these two classes of compound have been noted for horseradish peroxidase (Ryan et al., 1994). Among proteolytic enzymes, notable stability gains resulted for chymotrypsin (Melik-Nubarov et al., 1987) and trypsin (Mozhaev et al., 1988) following non-crosslinking chemical modification of surface groups.

Trypsin cleaves proteins and peptides at the carboxylic side of the basic amino acids arginine and lysine. Modification of lysine residues provides some protection

against digestion. As expected, the AA-trypsin was more resistant to autolysis than native trypsin. The absorbance of AA-trypsin increased slightly initially but then remained constant throughout the experiment, showing that autolysis was not occurring. The initial increase could be due to the formation of artifacts or to slight aggregation. In contrast, native trypsin showed a steady decrease in absorbance to 60 % of the initial value, indicating autolysis.

The half-life of the AA-NHS derivative at 55 °C was double that of the native trypsin (8 min and 4 min respectively). From the temperature profiles (Fig 4.13), the AA-trypsin retained 100 % activity up to 50 °C while the native lost some of its activity at 40 °C. T_{50} value for the AA-NHS derivative was 5 °C higher than that of the native (51 °C and 46 °C respectively). The use of the hydrophobic Nile Red confirmed that unfolding was still occurring in the AA-trypsin. It is probable that the point modification offers the enzyme some protection against incorrect refolding rather than preventing unfolding of the native structure. The two-fold thermostabilization is modest compared with those achieved by Mozhaev et al. (1988) who gained a >100 fold increase in stability at 56.5 °C by hydrophilization of a hydrophobic patch on the surface of the trypsin molecule. Gaertner and Puigserver (1992) conjugated polyethylene glycols (PEGs) of varying molecular masses to trypsin amino groups. Although the inactivation at 60 °C was polyphasic (so no comparison of half lives could be attempted), $PEG_{5,000}$-modified trypsin retained approximately 70 % initial activity after 2 h. while native trypsin lost 50 % activity within approximately 6 minutes. The present two-fold stabilization at 55 °C is nonetheless significant since it has resulted from a very small and simple chemical change. Approximately 8 lysine residues per trypsin molecule were modified. The AA-NHS reaction leads to an apparent increase in amidase (BAPNA) activity to 1.3 times that of the native trypsin, while the esterase activity was sligthly lower (88%) compared with native.

Tolerance towards organic solvents (acetone, acetonitrile, DMF, DMSO, 1,4-dioxan, THF and methanol) was not enhanced in AA-trypsin. However, when the AA-trypsin was subjected to the combined effects of elevated temperature (65 °C) and organic solvents, it showed enhanced stability. Methanol was the least protecting

solvent. This could be related to the fact that it is protic, i.e. it solvates cations through unshared electron pairs and it solvates anions through hydrogen bonding. Methanol, like water, has an -OH group but it is less polar. The other solvents tested are aprotic. Aprotic solvents do not hydrogen bond to anions and they dissolve ionic compounds chiefly through solvation of cations (Morrison & Boyd, 1987). Of this group, THF afforded little or no protection against thermoinactivation. It is probable that THF at lower concentrations inactivates the enzyme due to its high denaturation capacity (DC), as described by Khmelnitsky et al. (1991). The other solvents, which protect the enzyme against thermoinactivation at lower concentrations, have lower DC values. Volkin et al (1991) showed that some enzymes are extremely thermostable in anhydrous organic solvents. Enzymes have been shown to lose their bound water when suspended in organic solvents, with polar solvents resulting in the highest degree of desorption (Gorman and Dordick, 1992). Polar cosolvents can solvate a partially dehydrated protein, replacing the displaced bound water. Only above a critical polar solvent concentration does this process lead to denaturation (unfolding) (Khmelnitsky et al., 1991). At concentrations below their critical denaturing concentrations, they protect the enzyme against thermoinactivation by removing some of the water. However, at higher concentrations the solvent itself causes inactivation.

From the pH profile (Fig 4.15) it can be seen that the pH optimum for the amidase activity of modified trypsin is shifted slightly towards alkaline conditions. The modified trypsin showed higher relative activity at high pH values compared with the native. This correlates well with the findings of Labouesse & Gervais (1967), who found that pH for esterase activity of acetylated trypsin shifted to a more alkaline pH as compared with trypsin.

No differences were observed between the effects of denaturants urea and guanidine HCl on native and modified trypsin. Since the AA-NHS modification affects only the surface of trypsin without forming any crosslinks, one would not expect any increased resistance to these conformation-disruptive denaturants.

Labouesse & Gervais (1967) described the preparation and properties of an acetylated trypsin ('acetyl trypsin'). Use of acetic anhydride at pH 6.7 resulted in

complete acetylation of the epilson-amino groups of lysine and acetylation of exposed tyrosyl residues of trypsin. A comparison of native and acetyl trypsin (Sigma) by Gleich et al. (1992) showed no enhanced stability at 50 °C for acetyl trypsin, unlike our AA-NHS trypsin which shows decreased autolysis at this temperature and increased thermal stability at 55 °C. [Cupo and colleagues (1982) have also reported that chymotrypsinogen acetylated via acetic anhydride was less stable than the native zymogen at neutral or mildly alkaline pH values.] The present modification using acetic acid N-hydroxy-succinimide ester was carried out at pH 8.2. Unlike acetic anhydride, AA-NHS is specific for lysine residues. Only 60 % of these are modified, leaving some lysines free for other purposes such as immobilization. A detailed description of stability towards temperature, organic solvents and autolysis has been given.

The treatment of trypsin with a non-crosslinking succinimide ester (AA-NHS) leads to improved thermostability in aqueous buffer and in solvents acetone, acetonitrile, DMF, DMSO, 1,4-dioxan and methanol. Autolysis is much reduced but resistance to the denaturing solutes urea and guanidine HCl is unaffected. These findings are relevant to the use of trypsin in organic milieux (see Sakurai et al., 1990) and for protein engineering of enzymes for non-aqueous systems (Arnold, 1990).

4.3.2.2 Crosslinking of Trypsin

Modification of the lysine residues on trypsin using AA-NHS enhances thermostability in aqueous and organic solvents, and reduces autolysis. However, stability towards organic solvents was unaltered at room temperature. The effect of crosslinking the lysine residues of trypsin using succinimide esters was also examined. This was to distinguish between the effects of crosslinking lysines and their covalent modification with charge neutralizing succinimides as described previously (see Section 4.3.2.1).

Crosslinking strengthens the compact structure of the molecule, preventing denaturation. Disulphide bonds can significantly stabilize the native structure of enzymes (Perry & Wetzel, 1984; Matsumura et al., 1989). Like disulphide bonds, chemical crosslinking reagents have successfully been used to stabilize proteins (Ji

1983; Wong & Wong 1992). Proteases have been stabilized by a wide range of reagents.

Bis-N-hydroxysuccinimide esters are homobifunctional crosslinking reagents, i.e., they have two reactive groups, one at either end of the molecule. Two types of bis-N-hydroxysuccinimide esters were used to crosslink trypsin: suberic acid (SA) ester of NHS and ethylene glycol-bis(succinic acid) (EG) ester of NHS. The maximum molecular linkage length of SA-NHS is 11A (Ji 1983) and that of EG-NHS is 16.1A (see Fig 4.4a & b). The difference in the size of crosslinkers used has a drastic effect on the activity of trypsin. Modification with SA-NHS decreased the activity to 38%, while EG-NHS increased the activity to 203%. Torchilin et al. (1978) found that the length of carbon chain in the intramolecular crosslinks influenced the degree of thermostability of alpha-chymotrypsin. Similarly the degree of stability for intramolecularly crosslinked glyceraldehyde-3-phosphate dehydrogenase was dependent on the length of the bifunctional used (Torchilin et al., 1983).

Modification of trypsin's lysine residues with EG-NHS is accompanied by an apparent increase in activity by 200%. Approximately 8 lysine residues per trypsin molecule were modified. The rate of autolysis was reduced by the introduction of crosslinks. It has been shown previously that crosslinking of trypsin results in enhanced stability towards autolysis (Gleich et al., 1992; Rajput and Gupta, 1987 & 1988) by increasing the rigidity of the enzyme. The observed stability towards autolysis is due to the combined effects of crosslinking and modification of lysine residues as observed in covalently modified AA-trypsin (Murphy and O'Fagain, 1996).

EG-trypsin showed increased resistance to elevated temperature. At 55°C the half-life of EG-trypsin was 5 times greater than native trypsin's. From the temperature profile (Fig 4.20) T_{50} value for EG-NHS was 10°C higher than native (58°C and 48°C respectively). Mozhaev (1993) outlines a two step model of irreversible thermoinactivation that involves preliminary reversible unfolding and an irreversible step. The use of hydrophobic Nile Red confirmed that unfolding was still occurring in the EG-trypsin. It is probable that the modification offers the enzyme some protection against incorrect refolding rather than preventing unfolding of the native structure as

was observed with AA-trypsin.

Chemical crosslinking of the protein with bifunctional reagents is generally thought to stabilizes it with against reversible unfolding. The introduction of a disulphide bond into lysozyme enhanced its thermostability (Perry & Wetzel, 1984). White and Olsen (1987) suggested that their thermostable crosslinked hemoglobin could be explained by Le Chatelier's principle, since crosslinking prevents dissociation of the beta-subunits. Gleich et al. (1992) showed that trypsin crosslinked with N-hydroxysuccinimide esters of dicarboxylic acids, dianhydrides and bisimidoesters had enhanced thermostability. From my results the half-life of EG-trypsin was calculated to be 5 times more stable than native trypsin at 55°C. This stability is a result of structural rigidity of the enzyme as a result of intramolecular bonds, preventing reversible unfolding of the molecule.

Tolerance of organic solvents (acetonitrile, DMF, THF and methanol) was enhanced in EG-trypsin. The C_{50} values of EG-trypsin were higher than the native enzyme's for all solvents tested (see Table 4.6). Enzymes in water-miscible organic solvent systems have suppressed activity at higher concentrations. The hydration state of the enzyme is distorted by the replacement of water in the protein surface layer by solvent, causing loss of conformation. Gorman and Dordick (1992) showed that enzymes lose their bound water when suspended in organic solvents, with the highest degree of desorption resulting from polar solvents. The stabilization observed in the EG-trypsin is a result of the conformational rigidity introduced by the intramolecular crosslinks and the prevention of the stripping of water from the protein surface or as a result of neutralizing positive charges with succinimides. St Clair and Navia (1992) showed that chemically crosslinked crystals of thermolysin (T-CLEC's) had enhanced stability in organic solvents. The operational stability of T-CLEC's was demonstrated by synthesis of the chiral precursors of the artificial sweetener aspartame in water-immiscible organic solvents and in mixtures of water and miscible organic solvents (Perichetti et al., 1995).

EG-trypsin was found to be more tolerant of urea compared with the native. Again this is likely due to the conformational rigidity introduced via crosslinkage. No

difference between the native and EG-trypsin was observed for the denaturant guanidine hydrochloride. No difference in the pH profile of amidase activity was observed between modified and native trypsin.

Gleich et al. (1992) croslinked trypsin with N-hydroxysuccinimide esters of dicarboxylic acids, anhydrids and bisimidoesters. Crosslinked derivatives showed diminished autolysis and thermal denaturation. Modified forms exhibited decreased activity towards the substrates L-BAEE and L-BAPNA. No studies on the effect of solvents and denaturants on these derivatives were performed. The EG-trypsin, described here showed enhanced activity, decreased autolysis and thermal denaturation and also, enhanced stability towards the effects of solvents and the denaturant urea.

CHAPTER 5

KINETICS of AMIDASE and ESTERASE ACTIVITIES of NATIVE and MODIFIED TRYPSIN

5.1 Introduction

The kinetics of modified and native trypsin were studied to determine the effects of the modifications on trypsin-catalyzed aminolysis and hydrolysis and to compare the respective K_m and k_{cat} values. The Lineweaver-Burk double reciprocal plot is commonly used to calculate the K_m and V_{max}. However, from a statistical viewpoint, this method gives large errors when determining the values of K_m and V_{max}, due to large distortions of tiny errors in the raw data when the reciprocals are taken. The only graphic method sufficiently reliable for determining the kinetic data directly from a plot is the direct linear plot (Tipton, 1992). The direct linear plot plots each observation as a straight line and the values of K_m and V_{max} appear as a point rather than as the slope and intercept of a line.

Three substrates were studied as described in Section 2.17: two amides, (L-BAPNA and (Cbz)-Gly-Gly-Arg-AMC) and an ester (Z-Lys-SBzl). BAPNA was chosen because it is a very common substrate for laboratory assays of trypsin. (Cbz)-Gly-Gly-Arg-AMC (a tripeptide) was chosen to determine the effect of modification with succinimides on the kinetics of a somewhat larger substrate. Z-Lys-Sbzl was chosen in preference to TAME and BAEE because, in both cases, the substrate and product absorb at the same wavelength. Therefore, spectrophotometric detection is difficult and usually a pH stat is the preferred method of detection. Z-Lys-Sbzl binds with DTNB to give a colorimetric product that is easily detected and was found suitable for kinetic determination (Coleman & Green, 1981).

Previously, the effect of organic solvents on the structural integrity of modified and native trypsin was examined. The enzyme was placed in an aqueous/miscible organic solvent system for one hour, after which amidase activity was determined. It is also necessary to look at the effect organic solvents have on the catalyic activity by including the solvents in the assay mixture. Because the esterase activity of trypsin influences its usefulness in peptide synthesis, the effect of miscible organic solvents on esterase catalytic activity was examined. It has been reported that trypsin's esterase

activity is enhanced in the presence of certain organic solvents while the amidase activity is suppressed (Barbas et al., 1988; Guinn et al., 1991). The catalytic activity was determined as described in Section 2.18 for the native and modified forms of trypsin using the following solvents: acetonitrile, methanol, 1,4-dioxan and THF. These solvents were chosen as they did not absorb at the wavelength used in the assay, DMF and acetone were excluded for this reason.

5.2 Results

5.2.1 Kinetic studies using BAPNA

The amidase kinetics of native and modified trypsin using L-BAPNA were determined as described in Section 2.17.1. The kinetics of L-BAPNA hydrolysis was found to obey normal Michaelis-Menten kinetics for native and modified trypsin. K_m and V_{max} values were calculated for each form of trypsin using Enzfitter and also using the direct linear plot; the results are summarized in Table 5.1a & b. All Lineweaver-Burk and Eadie-Hofstee plots (not shown) were linear when inspected visually on the screen. Using the data collected from the directlinear plot, V_{max} was converted from $\Delta A\ min^{-1}$ to Ms^{-1} using an extinction coefficient of 8,800 $M^{-1}\ cm^{-1}$ (Erlanger, 1961). k_{cat} values were calculated in turn using these V_{max} values (see Table 5.1). The K_m of native corresponded to the value of 2.2 mM reported by Sears and Clark (1993). The K_m values of trypsin for L-BAPNA decreased upon modification, with the EG-trypsin showing the greater decrease of the two. The k_{cat} values increased 2-fold for both modified trypsins. The ratios of k_{cat}/K_m values for AA-trypsin and EG-trypsin were more than 2- and 3- fold, greater respectively, than that of native trypsin.

Table 5.1a Comparison of K_m values as calculated using Enzfitter and Direct Linear Plot for the amide substrate L-BAPNA

	K_m [a] (mM)	K_m [b] (mM)
Native	2.2 ± 0.3	2.34
	2.4 ± 0.5	2.62
AA-trypsin	2.1 ± 0.2	2.01
	2.2 ± 0.4	1.88
EG-trypsin	1.5 ± 0.2	1.55
	0.8 ± 0.1	1.07

[a] K_m values as calculated using Enzfitter sofware

[b] K_m values as calculated using Direct Linear Plot

Table 5.1b Kinetic parameters for native and modified trypsin for the amide substrate L-BAPNA calculated using the direct-linear plot

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Native	2.34	2.5	1069
	2.62	2.21	843
AA-trypsin	2.01	4.71	2343
	1.88	4.48	2381
EG-trypsin	1.55	5.47	3530
	1.07	4.07	3803

5.2.2 Kinetics studies using (Cbz)-Gly-Gly-Arg-AMC

The amidase kinetics of native and modified trypsin using (Cbz)-Gly-Gly-Arg-AMC were determined as described in Section 2.17.2. The kinetics of hydrolysis of the tripeptide (Cbz)-Gly-Gly-Arg-AMC were also found to obey normal Michaelis-Menten kinetics for native and modified trypsin. All Lineweaver-Burk and Eadie-Hofstee plots (not shown) were linear. k_{cat} values were calculated from V_{max} values that were converted to Ms^{-1} using a standard curve of AMC fluorescence. K_m and k_{cat} values of native and modified trypsin were compared (see Table 5.2). The K_m values for AA- and EG-trypsin decreased to 78% and 53% that of native trypsin respectively. k_{cat} values for modified forms of trypsin increased. The ratios of k_{cat}/K_m values of AA- and EG-trypsin were 2.7 and 2.9 times greater than the native trypsin.

Table 5.2 Kinetic parameters for native and modified trypsin for the amide substrate (Cbz)-Gly-Gly-Arg-AMC calculated using the direct-linear plot

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Native	0.49	0.95	1930
	0.46	0.9	1942
AA-trypsin	0.22	1.27	4885
	0.26	1.23	5668
EG-trypsin	0.25	1.39	5560
	0.25	1.33	5320

5.2.3 Kinetics studies using Z-Lys-Sbzl

The esterase kinetics of native and modified trypsin using Z-Lys-Sbzl were determined as described in Section 2.17.3. These obeyed normal Michaelis-Menten kinetics for native and modified trypsins. Km and k_{cat} values for form of trypsin, calculated using the direct linear plot, are shown in Tables 5.3. All Lineweaver-Burk and Eadie-Hofstee plots were linear as visualized on screen. Using the data collected from the direct linear plot, V_{max} was converted from $\Delta A\ min^{-1}$ to Ms^{-1} using an extinction coefficient of 14,150 $M^{-1}\ cm^{-1}$ (Coleman and Green, 1981). The K_m values for EG-trypsin decreased, while AA-trypsin's K_m was approximately the same as the native enzyme. k_{cat} of AA-trypsin showed an increase while that of EG-trypsin decreased. The ratio of k_{cat}/K_m values increased in both modified forms of trypsin.

Table 5.3 Kinetic parameters for native and modified trypsin for the ester substrate Z-Lys-Sbzl calculated using the direct-linear plot

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Native	0.043	27	6.2×10^5
	0.039	31	8.0×10^5
AA-trypsin	0.042	46	11.1×10^5
	0.033	43	13.1×10^5
EG-trypsin	0.018	22	12.4×10^5
	0.035	24	7.1×10^5

5.2.4 Catalytic activity of trypsin in various aqueous/organic solvent systems.

The esterase catalytic activities of native and modified trypsins using TAME as substrate were compared in acetonitrile, 1,4-dioxan, methanol and THF as described in Section 2.18. For all forms of trypsin, lower concentrations of solvents enhanced esterase activity (see Fig 5.1a & b) and even at high concentrations of solvents, esterase activity was retained. AA-trypsin showed higher activity than native for the solvents tested, while little or no difference was observed between EG-trypsin and native. The C_{50} values (where C_{50} refers to concentration (% v/v) at which the enzyme has 50% of its aqueous catalytic activity) for native and modified trypsin were determined for the solvents tested (see Table 5.4). The C_{50} for AA-trypsin was higher than that of both native and EG-trypsin, while no significant difference in values was observed between native and EG-trypsin.

Table 5.4 Comparison of the C_{50} values of native and modified trypsin in a range of miscible organic solvents using TAME as substrate.

SOLVENT	NATIVE TRYPSIN C_{50} (% solvent)	AA-TRYPSIN C_{50} (% solvent)	EG-TRYPSIN C_{50} (% solvent)
Acetonitrile	69 $\pm$ 4	75 $\pm$ 7	64 $\pm$ 6
1,4-Dioxan	52 $\pm$ 2	59 $\pm$ 2	52 $\pm$ 1
Methanol	63 $\pm$ 2	66 $\pm$ 2	62 $\pm$ 2
THF	71 $\pm$ 3	75 $\pm$ 1	71 $\pm$ 1

Fig 5.1a **Effect of acetonitrile on esterase activity of native and modified trypsin**

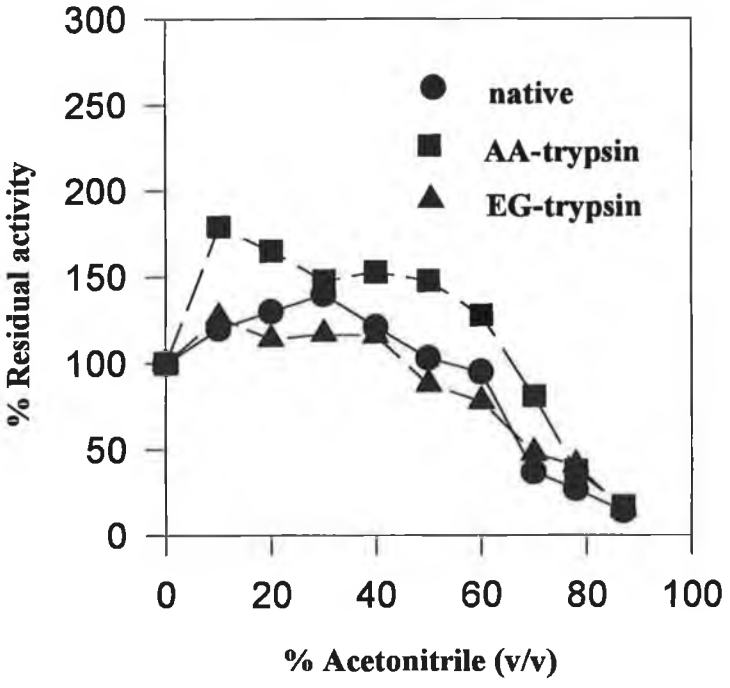
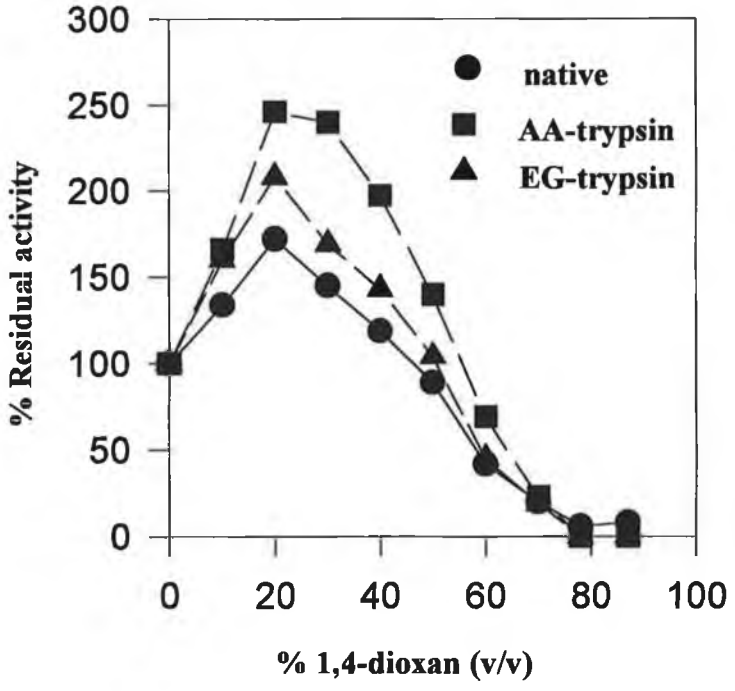


Fig. 5.1b **Effect of 1,4-dioxan on esterase activity on native and modified trypsin**



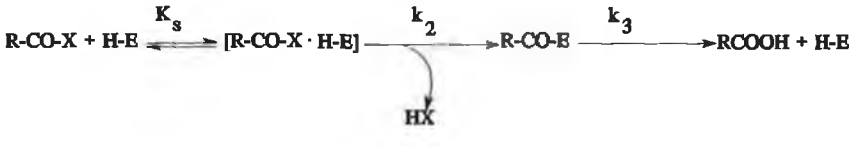
5.3 Discussion

K_m values for native and modified trypsin for each substrate were calculated using Enzfitter and the direct linear plot. The two methods gave siimilar results (see Table 5.1a for L-BAPNA results). The tabulated kinetic parameters show that there is generally a significant decrease in K_m upon modification for both amide substrates. For the ester substrate a similiar decrease in K_m was observed. k_{cat} values for both AA and EG-trypsin were double that of native trypsin for L-BAPNA, while for the tripeptide these values were 1.3-fold and 1.4-fold greater than native for AA- and EG-trypsin respectively. For the ester substrate, the k_{cat} values for AA-trypsin were again double those of the native, but no difference in k_{cat} was observed for EG-trypsin. For both amide and ester substrates, the ratio of k_{cat}/K_m was higher for modified forms of trypsin. For L-BAPNA hydrolysis by AA- and EG-trypsin, this ratio was 2.5 and 3.5 times greater respectively than that of native trypsin. For the tripeptide, this ratio was 2.7 and 2.8 times greater than native's for AA- and EG- trypsin, respectively. The ratio for the ester substrate was double for AA-trypsin and for EG-trypsin the k_{cat}/K_m was 1.4 times greater than the native's. The k_{cat}/K_m values reflect the specific activity for the substrates, showing that the modified forms of trypsin had increased affinity for the substrates.

As previously mentioned, modification of trypsin with succinimides results in the positive charge on the lysine residue being neutralized. As the "scissile residues" (lysine and arginine) binding to the active site of trypsin are positively charged these would be repelled by positive charges in the vicinity of the active site. It is therefore conceivable that neutralization of positive charges on some of the lysine residues of trypsin would enhance binding of the substrate to the active site. Enhanced activities of trypsin toward simple substrates have previously been observed as a consequence of chemical modification of the lysine residues. Crosslinking of trypsin with albumin using glutaraldehyde (which reacts with the amine group of lysine residues) resulted in a decreased K_m for the amide substrate BAPNA (Cohenford et al., 1986). (Note,

however, that glutaraldehyde is not very specific and can react with other residues other than lysine). This was accounted for by the attraction of the positively charged substrate to the negatively charged human serum albumin surrounding the enzyme and to the lowered lysine content on trypsin after glutaraldehyde treatment. Likewise, attachment of polyethylene glycol (PEG) via the lysine residues on trypsin resulted in a decreased K_m and an increased k_{cat} (Gaertner & Puigserver, 1992; Zalipsky et al., 1992). Non-enzymatic glycation of trypsin, lysine residues using the Maillard reaction resulted in decreased k_{cat} and K_m values (Kato et al., 1993). Crosslinking of trypsin's lysines with dextran-dialdehyde, however, resulted in an increased K_m for the ester substrate substrate BAEE. Immobilization of trypsin was found to increase the K_m values for a number of ester and amide substrates, while k_{cat} decreased: the ratio of k_{cat}/K_m decreased significantly, indicating that the immobilization decreased the affinity of trypsin for the substrates BAEE, TAME and BAPNA (Sears & Clarke, 1993). An increase in K_m is common in immobilized enzymes (Serban & Banateanu, 1984; Yan et al., 1988; Tyagi et al., 1994).

Scheme 5.1 Mechanism of hydrolysis of amides and esters catalyzed by serine proteases



where R-CO-X are the amides or esters, E is the serine protease and R-CO-E is an acylenzyme intermediate.

The currently accepted mechanism for the hydrolysis of amides and esters catalyzed by serine proteases is by the acylenzyme mechanism (see Scheme 5.1). The enzyme and substrate form a tetrahedral intermediate when the hydroxyl of the catalytic serine residue in the active site attacks the substrate. The intermediate then collapses to give

the acylenzyme (RO-CO-E in Scheme 5.1), releasing the amine or alcohol (H-X in Scheme 5.1). This acylenzyme then hydrolyzes to form the enzyme-product complex. For ester substrates, the acylenzyme intermediate accumulates ($k_2 > k_3$), while for amide substrates accumulation does not occur ($k_2 < k_3$) (Fersht, 1984).

As described by Guinn et al. (1991) for trypsin, the breakdown of the acyl-enzyme intermediate is the rate-determining step for the hydrolysis of ester substrates. Assuming $k_3 << k_2$, then

$$K_m = K_s k_3/k_2$$

$$k_{cat} = k_3 \qquad \text{(see Scheme 5.1)}$$

For the hydrolysis of amide substrates the rate-determining step is acylation of the enzyme, $k_2 << k_3$. Thus,

$$K_m = K_s$$

$$k_{cat} = k_2 \qquad \text{(see Scheme 5.1)}$$

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, the rate-determining chemical step in amide hydrolysis. Ester hydrolysis may be less dependent on the surrounding structure of the active site, as it is governed by hydrolytic attack of water on the scissile bond of the acyl-enzyme intermediate. Amidase activity is therefore expected to be more sensitive to active-site conformational changes than esterase activity and is more strongly affected by immobilization (Sears & Clarke, 1993). Immobilization of an enzyme commonly results in an increase in the apparent K_m as a consequence of diffusional limitations. Partial blockage of the active site by the support might be expected to impede the binding of the substrate to the active site of the enzyme. This increases the dissociation constant, K_s, for the enzyme-substrate complex. This effect would lead to an increase in K_m as for amidase activity $K_m = K_s$ while for esterase activity, $K_m = K_s k_3/k_2$ (see above and Scheme 5.1).

The greater sensitivity of amidase activity to active-site changes (compared with esterase activity), could possibly account for the more pronounced increases in k_{cat} and specific activity observed for amide hydrolysis in the modified forms of trypsin. On

the other hand, esterase activity, is less dependent on the active site surroundings and the enhancement in k_{cat} and k_{cat}/K_m is not as great. This is particularly true of the EG-trypsin.

For the amide substrates EG-trypsin showed the greater k_{cat} and k_{cat}/K_m values, For the ester substrate the k_{cat} was slightly lower than that of the native and k_{cat}/K_m was 1.4 times that of the native. AA-trypsin showed significant increases in k_{cat} and k_{cat}/K_m for both types of substrate. This chemical modification is therefore, more advantageous than immobilization, which leads to an increased K_m and lower k_{cat} as a result of impeding the binding of substrate to the enzyme (Sears & Clark, 1993).

The final part of the kinetic studies of modified trypsins involved the effect of solvents on their esterase activity. A range of water-miscible solvents was included in the reaction mixture and the esterase activity measured. Enhanced activity was observed at lower concentrations of solvents for both the native and modified forms of trypsin. These findings correlate with those of Barbas et al. (1988) and Guinn et al. (1991). Addition of organic solvents at low concentrations is thought to accelerate deacylation slightly. As mentioned earlier, the rate limiting step of ester hydrolysis is deacylation of the acyl-enzyme intermediate and hence $k_{cat} = k_3$ (the deacylation constant: see Scheme 5.1). The addition of low concentrations of organic solvents increases k_{cat} thereby appearing to accelerate deacylation.

These modified forms of trypsin will be used in peptide synthesis. As high concentrations of solvents are used to suppress hydrolysis in peptide synthesis, it is necessary that the enzyme retains esterase activity in these conditions. AA-trypsin showed higher activity compared with native in the solvents tested while EG-trypsin differed very little from native. C_{50} values for the different forms showed that AA-trypsin had higher values compared with both EG and native trypsin.

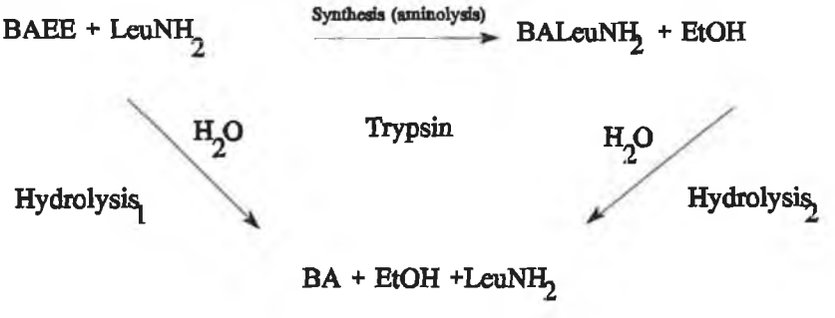
CHAPTER 6

PEPTIDE SYNTHESIS

6.1 Introduction

Proteases are very useful tools in the area of peptide synthesis, with numerous advantages over conventional chemical methods. There are, however, a number of disadvantages associated with their use as described earlier (see Section 1.2 for more details). By modifying trypsin with succinimides, some of these problems, such as autolysis and thermoinactivation, have been largely overcome. It is necessary to see if these modified forms of trypsin can synthesize peptides and, if so, are they more efficient compared with native trypsin. A model peptide synthesis system was set up. As previously mentioned (Section 1.3), there are two types of enzymatic peptide synthesis: Kinetically controlled synthesis and Equilibrium controlled synthesis. The model system of the kinetic approach, making use of ester substrates, was preferred, owing to its lower enzyme requirements and shorter reaction times. The process adopted can be summarized as follows:

Scheme 6.1 Model of kinetically controlled dipeptide synthesis using trypsin



Initially dipeptide synthesis of benzoyl argininyl leucinamide using BAEE and leucinamide was carried out. Temperature, leucinamide concentration and enzyme concentration were varied in this study. The effect of time was also examined. Dipeptide synthesis using TAME was also carried out to examine the effects of different protecting and leaving groups on peptide synthesis. Peptide synthesis was carried out based on the methods of Cerovsky et al. (1990) and Blanco et al. (1991), as described in Section 2.18.

Synthesis in water-free organic solvents would guarantee prevention of hydrolysis of substrate esters and formed products. As the amount of water bound to the enzyme surface is small a monolayer cannot form around the enzyme, therefore, trace amounts of water are necessary to obtain catalytic activity of proteolytic enzymes. There are other advantages in employing enzymes in organic solvents as opposed to aqueous solvents, such as increased solubility of nonpolar substrates. However, enzyme molecules may be distorted or competitively inhibited (through specific interactions) by organic solvents, which could lead to changes in reaction kinetics and substrate specificity. The distortion or partial unfolding of peptide chains may also change stereospecificity in enzymes.

Water/water-miscible systems have many advantages over other systems such as biphasic systems. Amino acid derivatives which are often poorly soluble in nonpolar solvents dissolve readily in water-miscible organic solvents. The concentration of substrates and product can be easily controlled, since the reaction solutions are monophasic and no separate aqueous phase is present. This prevents excessive concentration of substrates and products around enzymes; in two-phase systems, inhibition or inactivation of enzymes may occur due to excessive partitioning of substrates or products into an aqueous phase of small volume. Diffusional resistance across the water/organic interface is eliminated in monophasic systems leading to an increased overall reaction rate. Complexity in kinetic analysis and the need to determinate the true substrate specificity of enzymes are also eliminated.

Modified forms of trypsin were more tolerant of high concentrations of organic solvents than native trypsin (Section 4.2.2). It is hoped that the modified trypsin will

have enhanced synthetic ability and as a result be a more efficient catalyst. The following solvents were chosen for the synthetic system: acetonitrile, DMF and t-butanol.

Previously it has been observed that salts dramatically enhance activity of enzymes in organic solvents (Kuhl et al., 1992; Cerovsky, 1992; Khmelnitsky et al., 1994). Khmelnitsky et al. (1994) proposed that activation of subtilisin in a salt matrix was due to the protective effect afforded by the salt against deactivation by direct contact with the organic solvent. Previously it was observed that, in 95% DMF, no peptide synthesis activity occurred. This could be a result of denaturation of the enzyme by the solvent as suggested by Cervosky (1990). It was hoped that the inclusion of high concentrations of a salt hydrate or a sugar alcohol might offer some protecting effect against this denaturation. A reaction mixture containing 95% DMF including either salt hydrate or sugar was set up and monitored.

Finally it is hoped that these modified forms of trypsin are able to bind segments of an oligopeptide chain together. One of the disadvantages associated with chemical peptide synthesis is the low recovery of long-chain peptide products; the longer the chain, the smaller the recovery. Enymatic peptide synthesis helps to overcome this problem by joining two oligopeptides fragments together.

Recently there has been alot of interest in an oligopeptide found in the brain of Alzheimer's. This oligopeptide, amyloid beta-protein (H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH) is deposited in the brains of Alzheimer's patients with disease. A portion of the amyloid beta-Protein (amino acids 25-35) mediates both the neurotropic and neurotoxic effects and is homologous to the tachykinin neuropeptide family (Yanker et al., 1990). Two segments of the chain : Ac-Gly-Ser-Asn-Lys-OMe (activated ester) and Gly-Ala-Ile-Ile-Gly-Leu-Met-NH_2 (amide) were prepared. Trypsin should be able to bind these two segments together due to its lysine specificity.

6.2 Results

6.2.1 Optimization of reaction conditions

6.2.1.1 HPLC conditions

From the spectra obtained from the photodiode array (PDA) detector and the spectrophotometer, the optimum wavelengths for analysis were found to be 204nm and 230nm. Initially a mobile phase of 50% methanol / 50% H_2O containing 0.05% TFA gave good resolution between the substrates and the products for Bzl-Arg-LeuNH_2 synthesis. For dipeptide synthsesis using TAME as substrate, a mobile phase of 40% methanol / 60% H_2O containing 0.05% TFA was used. An injection volume of 20 microlitre was used for analysis.

6.2.1.2 Enzyme concentration

Reaction mixtures containing the following concentrations of native enzyme: 0.05, 0.1 and 1mg ml^{-1}, in 0.1M $CaCl_2$ were incubated at 37°C as described in Section 2.19.2. The highest enzyme concentration gave the fastest rate of peptide synthesis, while after one hour the product yield for the 0.1mg ml^{-1} concentration was similiar. It was decided to work at a concentration of 1mg ml^{-1} to ensure that the concentration of enzyme was not limiting.

6.2.1.3 Effect of concentration of leucinamide on reaction

The concentration of leucinamide in the reaction was varied: 0.1, 0.2 and 0.4M leucinamide, as described in Section 2.19.3. The reaction mixtures were incubated at 37°C and aliquots removed at regular intervals and analyzed. A concentration of 0.2 M gave the highest rate of peptide synthesis (see Fig 6.2). This concentration was used in subsequent reaction mixtures.

Fig. 6.1 **Effect of native trypsin concentration on the rate of peptide synthesis**

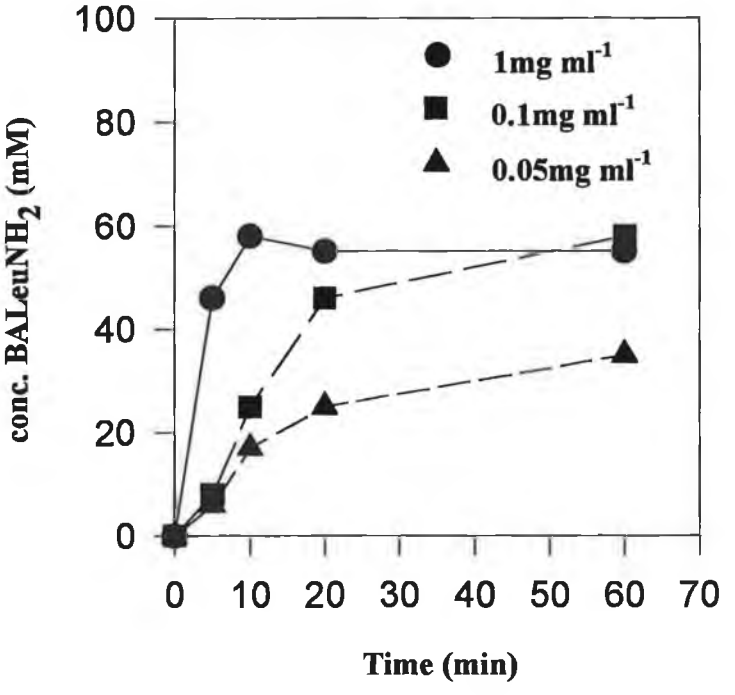
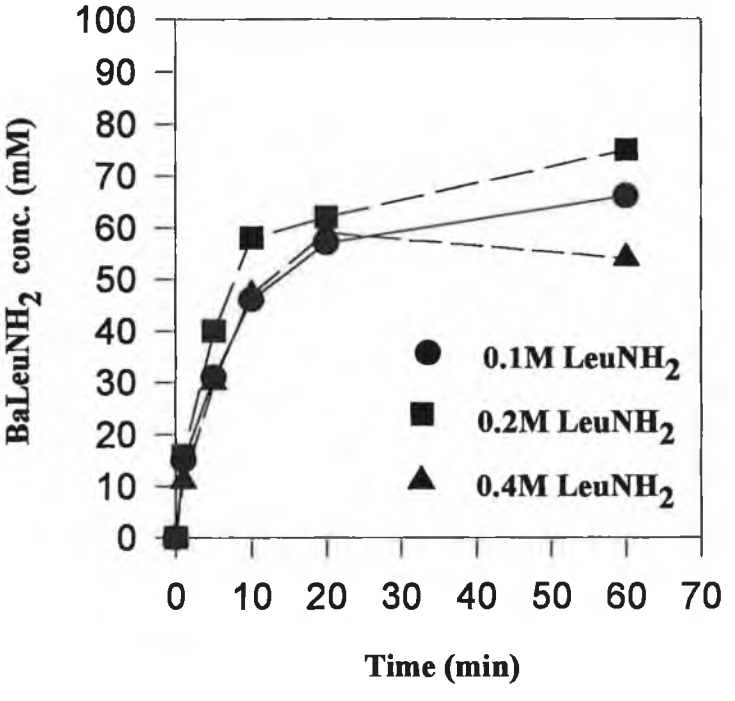


Fig. 6.2 **Effect of $LeuNH_2$ concentration on the rate of peptide synthesis for native trypsin**



6.2.1.4 Effect of temperature on reaction

To show the effect of temperature on the rate of peptide synthesis, reaction mixtures were incubated at the following temperatures: 4, 25 and 37°C as described in Section 2.19.4. The rate of peptide synthesis was highest for 25°C (see Fig 6.3). However, it was noted on prolonged incubation that the product formed was hydrolysed to the greatest extent at 37°C, while at 4°C very little (if any) hydrolysis occurred. Although the reaction rate was slowest at 4°C, hydrolysis of Bzl-Arg-LeuNH_2 was minimal, so that maximal product yield was observed at this temperature. All subsequent reactions were incubated at 4°C.

6.2.2 A comparison of native and modified trypsin

6.2.2.1 Effect of acetonitrile on enzymatic peptide synthesis

The effects of acetonitrile were examined as described in Section 2.18.4. As the concentration of acetonitrile increased in the reaction mixture, substrate hydrolysis decreased and secondary product hydrolysis of the product also decreased (see Fig 6.4). These values were taken after 1 day, the low value for 95% acetonitrile is due to a slower rate of synthesis. After 6 days maximum product yield was obtained. For lower concentrations hydrolysis had occurred after 6 days. This was observed with both native and modified forms of trypsin. For the lower concentrations of acetonitrile, little or no difference between the native and modified forms of trypsin was observed. The rate of synthesis decreased as the concentration of organic solvent increased. At 95% acetonitrile the rate of hydrolysis was very low and both modified forms of trypsin showed enhanced synthetic rate compared with native. EG-trypsin showed the highest rate of synthesis (see Fig 6.5). After one day, EG-trypsin had utilized all the BAEE while for native not all the BAEE had been utilized after 3 days. In 95% acetonitrile the ratio of synthesis to hydrolysis was approximately 9:1, with the modified forms trypsin converting 90% of the sustrate to Bzl-Arg-LeuNH_2 (see Table 6.1). For the substrate TAME similiar results were obtained, with the rate both of hydrolysis and of synthesis decreasing as the % acetonitrile increased

Fig. 6.3 **Effect of temperature on the rate of peptide synthesis for native trypsin**

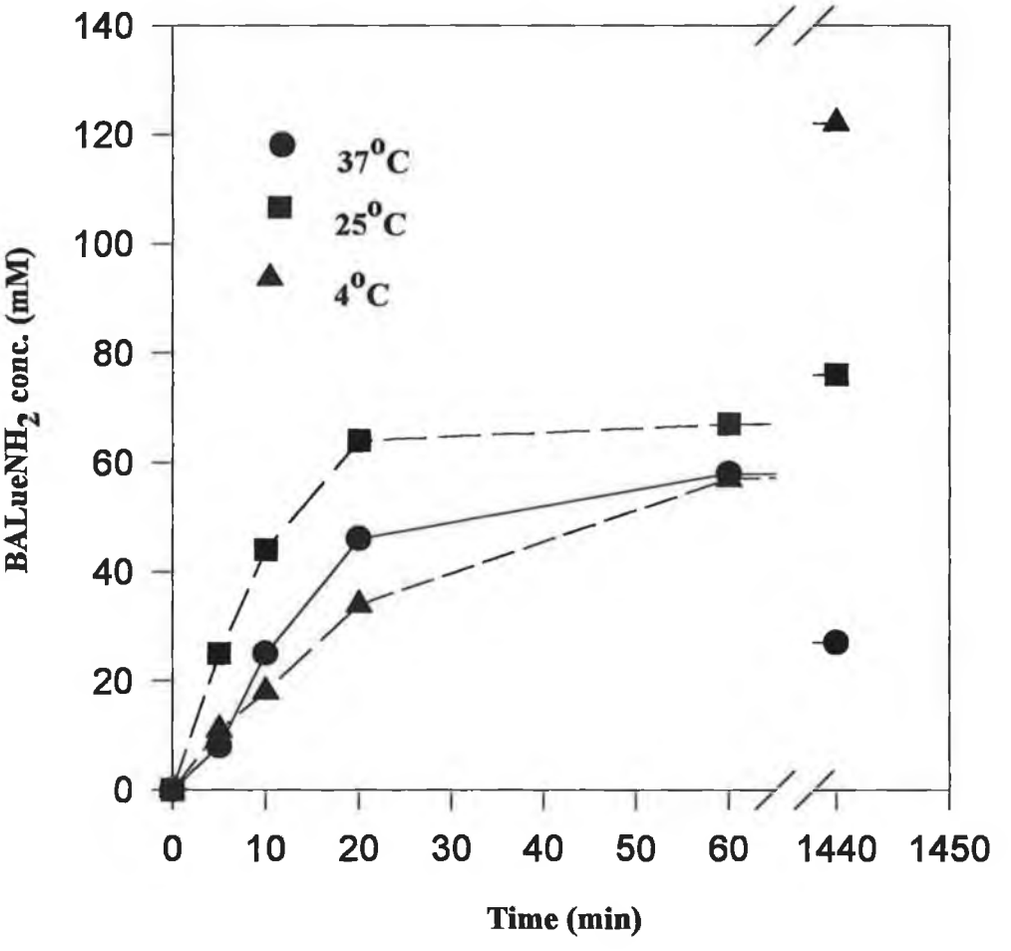
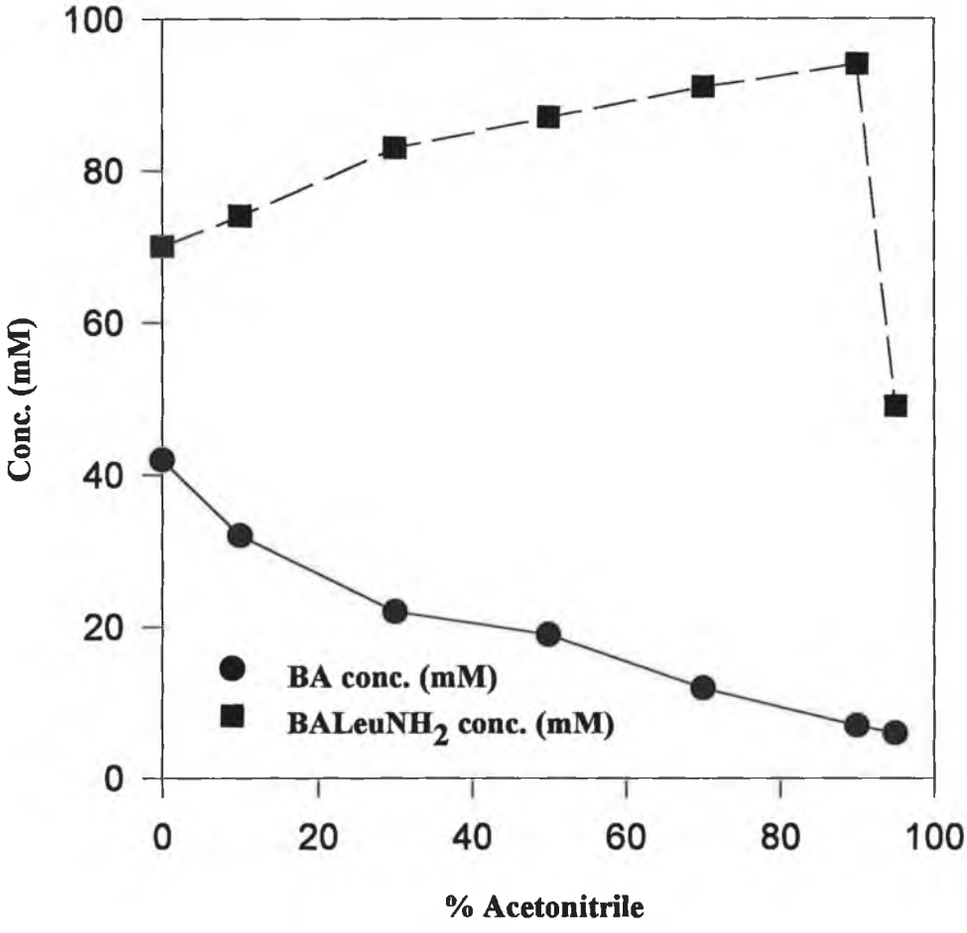


Fig. 6.4 Effect of acetonitrile on the formation of BA (hydrolysis) and $BALeuNH_2$ (aminolysis) for native trypsin



* Values of BA and $BALeuNH_2$ were taken after 1 day.

Fig. 6.5 **Rate of peptide synthesis by native and modified trypsin in 95% acetonitrile (v/v)**

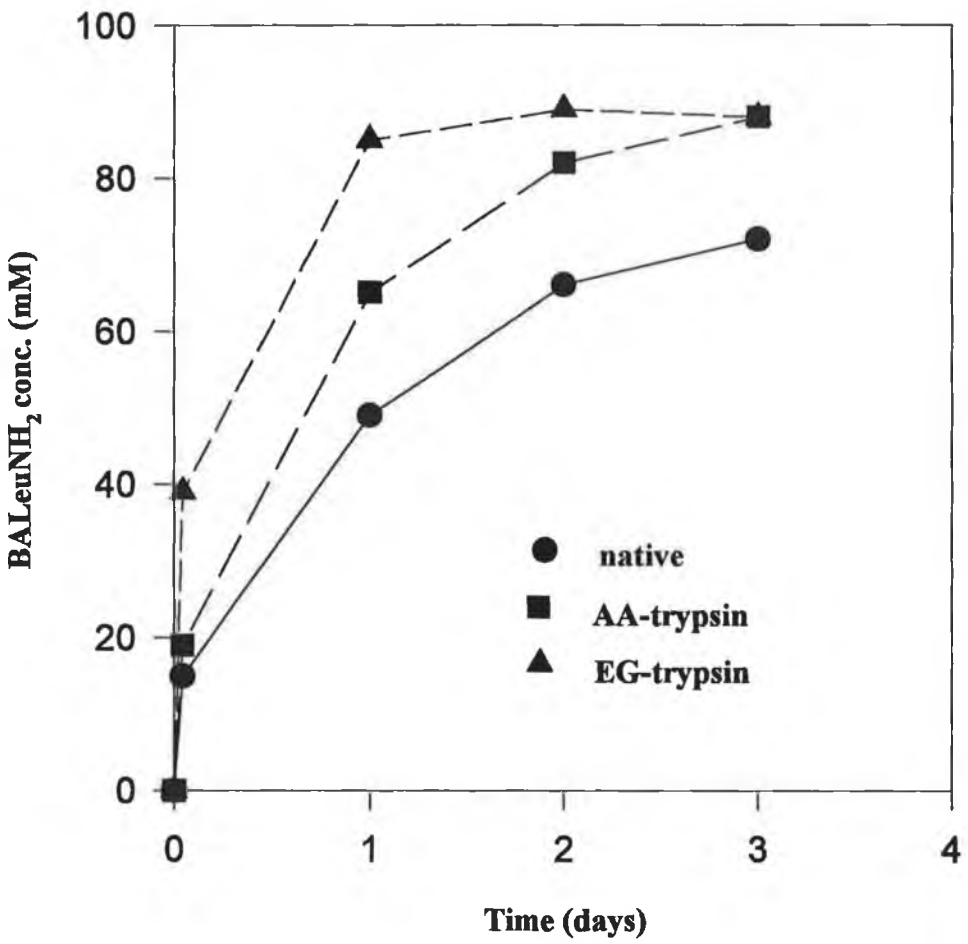


Table 6.1 Comparison of rate of hydrolysis (Bzl-Arg) and aminolysis (Bzl-Arg-$LeuNH_2$) of native and modified trypsin after 72 hours in 95% (v/v) solvent solution.

SOLVENT	NATIVE TRYPSIN		AA-TRYPSIN		EG-TRYPSIN	
	% Bzl-Arg	% Bzl-Arg-LeuNH$_2$	% Bzl-Arg	% Bzl-Arg LeuNH$_2$	% Bzl-Arg	% Bzl-Arg LeuNH$_2$
Acetonitrile[b]	8 ± 2	59 ± 9	9 ± 1	90 ± 1	11 ± 1	88 ± 1
t-Butanol[c]	24 ± 1	62 ± 3	26 ± 2	62 ± 3	30 ± 9	65 ± 3
DMF[d]	0	0	0	0	0	0

[a] Initial concentration of BAEE was 0.1M.

[b] BAEE was not completely utilized by native trypsin after 3 days in 95% acetonitrile (v/v).

[c] BAEE was not completely utilized by native or modified forms of trypsin after 3 days in 95% t-butanol (v/v).

[d] No peptide synthesis was observed in 95% DMF.

6.2.2.2 Effect of other solvents on Enzymatic peptide synthesis

Enzymatic peptide synthesis was carried out in systems containing DMF as described in Section 2.18.4. In 50% DMF, modified forms of trypsin had a slightly higher reaction rate compared with native. Maximum product yield was achieved after 20 minutes, after which time hydrolysis of the product was observed. After 24 hours product concentration had dropped to 60% Bzl-Arg-LeuNH$_2$ for the modified trypsins and to 70% Bzl-Arg-LeuNH$_2$ for native. A high concentration of DMF was therefore required to prevent hydrolysis. In 95% DMF, no enzymatic peptide synthesis by either native or modified trypsin was observed.

In systems containing 95% t-butanol, both native and modified forms of trypsin were capable of peptide synthesis. Again, the modified forms showed enhanced synthetic rates compared with native trypsin (see Fig 6.6). After one day EG-trypsin reached maximum product yield, while native had not reached maximum product yield after 6 days. Over 60% of the substrate was converted to Bzl-Arg-LeuNH$_2$ and the remainder to Bzl-Arg (the hydrolysis product), see Table 6.1.

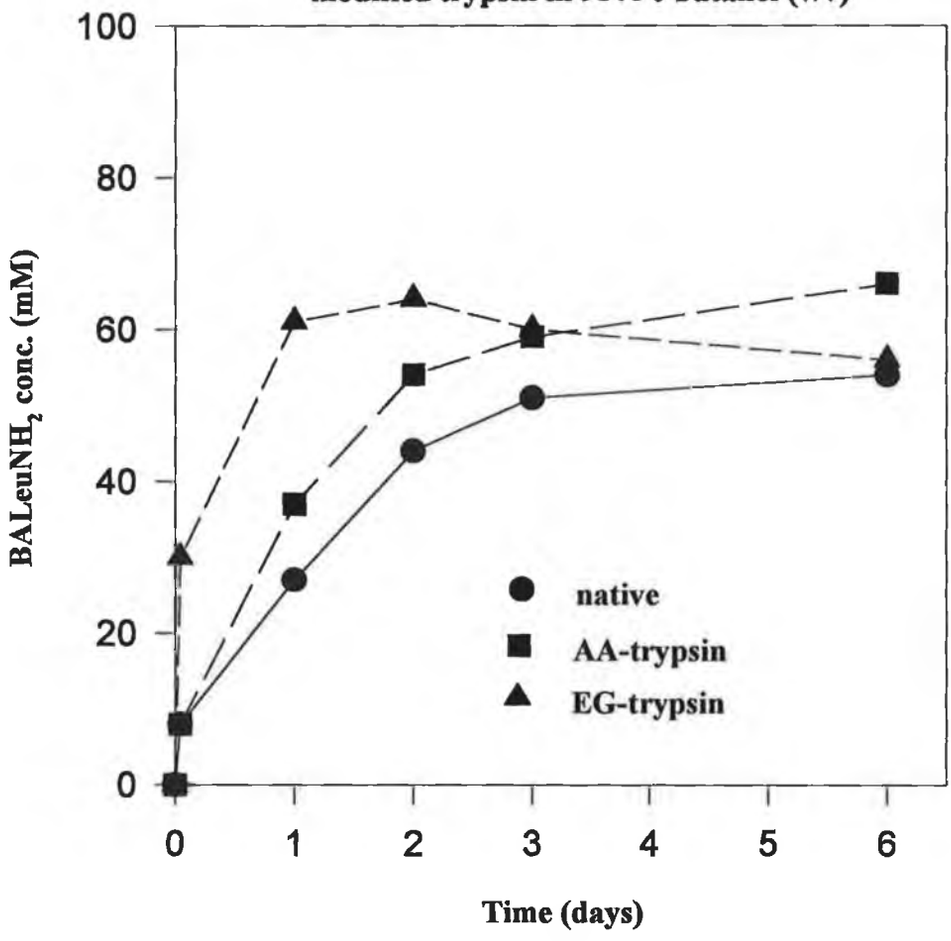
6.2.3 Effect of salt hydrates on peptide synthesis

The following concentrations of $Na_2CO_3.10H_2O$ were included in the reaction mixture containing 95% DMF: 0.01, 0.1 and 0.5M as described in Section 2.18.5. Samples were removed after 30 minutes, 1 and 24 hours and analyzed. In no case was peptide synthesis observed in the presence of the salt hydrate.

6.2.4 Effect of a sugar-alcohol (xylitol) on peptide synthesis

Similarly, concentrations of 0.01, 0.1 and 0.5M of the sugar-alcohol xylitol were included in the reaction mixture containing 95% DMF as described in Section 2.18.6. Again no peptide synthesis was observed in the presence of any of the concentrations of Xylitol used.

Fig 6.6 **Rate of peptide synthesis by native and modified trypsin in 95% t-butanol (v/v)**



6.2.5 Synthesis of Amyloid beta-Protein (25-35) by modified trypsin

Synthesis of Amyloid beta-Protein (25-35) was performed as described in Section 2.19. From the spectra obtained from the photodiode array (PDA) detector and the spectrophotometer the optimum wavelength for analysis was found to be 210nm. No peptide synthesis was observed under the conditions described even after prolonged incubation.

6.3 DISCUSSION

As expected, trypsin-catalyzed synthetic yields increased with decreasing temperature. Similarly, chymotrypsin-catalyzed synthesis of X-Phe-Leu-NH_2 had higher reaction yields at low temperatures (Calvet, 1992). The rate of hydrolysis of the acyl-enzyme complex (hydrolysis$_1$ or Bzl-Arg formation) and peptide hydrolysis (hydrolysis$_2$; see Scheme 6.1) increased with temperature much more than did the rate of peptide synthesis. Blanco et al. (1991) suggested that the absorption constant of the nucleophile to the enzyme/complex decreases with increasing temperature: at higher temperatures, the concentration of trypsin molecules containing leucinamide in their active centres is much lower than the at lower temperatures. This results in synthetic rates that do not increase in proportion with increasing temperature. Schuster et al. (1993) showed that chymotrypsin was capable of peptide synthesis using unprotected amino acids as acyl acceptors in ice. The freezing is thought to have increased the concentration of the reactants in a frozen liquid phase which is in equilibrium with the ice crystals. Other factors such as reduced water activity, altered dielectric constant and the subzero temperatures may have influenced the relative improvement of the aminolysis reaction compared to hydrolysis. Tougu et al. (1993) also showed to be chymotrypsin capable of peptide synthsis in frozen solutions using free amino acids as nucleophiles. Although frozen conditions have not been used here, it can be seen that lower temperature favours aminolysis over hydrolysis.

Leucinamide concentration also had an effect on the reaction rate. Having an excess of leucinamide over BAEE would be expected to favour peptide formation by the law of mass action. A two-fold molar excess of acyl acceptor (leucinamide) over the acyl donor (BAEE) gave the highest rate of aminolysis. At higher concentrations no further increase in aminolysis was seen.

Inagaki et al. (1994) suggested that the effects of organic solvents on the microenvironment of the enzyme largely depends on the molecular structure of the solvents. They found a linear correlation among the same category of organic solvents, but not between categories, for thermolysin-catalyzed peptide synthesis in various groups of organic solvents. With organic solvents with high log P values, the enzyme activity increased as the organic solvent content increased to a critical concentration, but further increases in the organic solvent content decreased the enzymatic activity. Organic solvents with low log P values were found to inhibit the enzymatic reaction even by the initial addition of small quantities of organic solvents. Here the effect of organic solvents on peptide synthesis was investigated using the solvents acetonitrile, t-butanol and DMF. Peptide synthesis was carried out in varying concentrations of acetonitrile. Acetonitrile was chosen firstly because of the high stability of both forms of modified trypsin in its presence. Secondly, acetonitrile has successfully been used in peptide synthesis. Cerovsky (1990) showed that trypsin was capable of peptide synthesis in acetonitrile with low water content (5% v/v). He observed that at lower water concentration hydrolysis of BAEE (hydrolysis$_1$) and secondary peptide hydrolysis (hydrolysis$_2$; see Scheme 6.1), were suppressed. He also observed that the reaction was slower. These findings correlate well with the effects of varying acetonitrile concentrations on the rate of peptide synthesis by native and modified trypsin. The ratio of aminolysis to hydrolysis increased as the concentration of acetonitrile increased, resulting in the highest accumulation of peptide at 95% acetonitrile. At this high concentration of solvent, both forms of modified trypsin showed higher rates of peptide synthesis than did native trypsin. EG-trypsin had the highest activity, suggesting that the "crosslinked" form of the enzyme was the most tolerant of acetonitrile, while AA-trypsin also showed enhanced activity. 95% acetonitrile in the

reaction mixture gave highest yields and lowest hydrolysis compared with other concentrations (see Table 6.1).

Peptide synthesis in 95% t-butanol, an alcohol, showed both modified trypsins had higher reaction rates compared with native trypsin. Like acetonitrile, this solvent was found suitable for peptide synthesis with product hydrolysis suppressed. DMF was chosen as it is more polar than acetonitrile. Previously no peptide synthesis was observed at high concentrations of DMF (Cerovsky, 1990). This could be the result of inactivation of the enzyme by DMF. Modification with succinimides has been shown to increase trypsin's tolerance of organic solvents (Section 4.2.2). Peptide synthesis was performed in 50% and 95% DMF. In 50% DMF, 80% of BAEE was converted to product after 20 minutes. No peptide product was formed in 95% DMF. It was concluded that the high concentration of DMF inactivated both modified and native trypsins. From organotolerance studies (see Sections 4.2.2.1.7 and 4.2.2.2.6) no amidase activity was observed at 95% DMF for native, AA- and EG-trypsin. These findings agree with those of Cerovsky (1990) who observed little or no synthesis by trypsin in 95% DMF. Calvet et al. (1992) found higher reaction yields at low DMF concentrations in peptide synthesis catalyzed by chymotrypsin. Gololobov et al. (1994) found that DMF had different effects on different enzymes. With chymotrypsin, reaction rates decreased with increasing DMF content, while for subtilisin 72 the rate of substrate consumption increased with increasing DMF content.

The present results for trypsin-catalyzed peptide synthesis correlate with the effects of solvents on chymotrypsin-catalyzed peptide synthesis (Nagashima et al., 1992). They found that acetonitrile, t-butanol and DMF gave the yields of 80, 74 and 0% after 24 hours in approximately 95% solvent systems. Similarly for trypsin-catalyzed peptide synthesis, the highest yields were recorded for acetonitrile, while for DMF no product was formed (see Table 6.1).

No peptide synthesis occurred at 95% DMF probably due to the inactivation of the trypsin by DMF. By including salt hydrates or sugar-alcohol it was hoped that the trypsin would be stabilized and able to synthesize peptides. Xylitol (sugar) and $Na_2CO_3.10H_2O$ (salt hydrate) were mixed with trypsin and included in the reaction

mixture containing 95% DMF. These additives had no effect on peptide synthesis as no product was formed. It was concluded that no stabilization of trypsin had occurred as a result of their inclusion in the reaction mixture.

The final objective in dipeptide synthesis was to examine the effect of different protecting groups. The substrate TAME was used instead of BAEE and the rate of peptide synthesis determined in buffer solution and in 50% acetonitrile in aqueous solution. No analysis of peptide syntheses was attempted at 95% acetonitrile because of the insolubility of TAME. For each form of trypsin, little or no difference in the rate of synthesis was observed. As with BAEE, increasing the concentration of solvent decreased the reaction rate, but also reduced hydrolysis allowing the accumulation of product. Little or no difference was observed between this substrate and BAEE showing that there is little practical distinction between the tosyl and benzoyl protecting groups and between the methyl and ethyl leaving groups.

The last part of the project involved the synthesis of an oligopeptide fragment to show the potential use of modified trypsin in such situations. Because EG-trypsin was found to be more efficient than either AA- or native trypsin at peptide synthesis in high solvent concentrations this form was used for oligopeptide synthesis (fragment coupling). The oligopeptide chosen was Amyloid beta-Protein (25-35). Both the oligopeptide and the the substrate fragments were specially synthesized. The n-terminal glycine of the tetrapeptide fragment had an acetyl group attached and the lysine residue was activated using a methyl ester group. The second acyl acceptor fragment had a amide group attached to the C-terminal methionine group. The activated ester tetrapeptide contained a charged amino acid group and was therefore readily soluble in H_2O. The amide fragment, on the other hand, had no charged or polar R-groups present and therefore DMSO was the solvent chosen to dissolve this fragment. Because of the high volume of DMSO present in the reaction mixture it was decided to carry out the reaction at room temperature (as DMSO solidifies at 18.5°C) and not 4°C as was optimal for dipeptide synthesis. Standards of the substrates and product were analyzed on HPLC and the optimal resolution conditions were determined. A mobile phase of 35% acetonitrile / 65% H_2O containing 0.05% TFA and a wavelength of

210nm was selected. No peptide bond formed between the two fragments. This could be due to modified enzyme's inability to form peptide bonds between the two fragments. EG-trypsin was capable of dipeptide synthesis between arginine and leucinamide residues, although no dipeptide synthesis between lysine and glycine residues was undertaken. However, it is more likely that the reaction conditions were non-ideal for peptide bond formation. The concentrations of substrates were very low compared with other examples of oligopeptide synthesis. It is also possible that the presence of DMSO (needed to dissolve one of the substrate peptides) could have inhibited the reaction. Because of the limiting quantities of substrate available optimization of reaction conditions was not possible. It is conceivable, that under suitable reaction conditions, that EG-trypsin could synthesize a peptide bond between these two fragments.

Proteases have successfully been used for the racemization-free condensation of chemically synthesized oligopeptide fragments (see Gill et al., 1996, for a more detailed review). Human insulin has been synthesized by enzymic semisynthesis involving the condensation of two fragments using trypsin (Hansicke et al., 1988). V8 protease catalyzed the condensation of fragments in a superpotent analog of human growth hormone-releasing factor (Bongers et al., 1992). Modified proteases likewise have catalyzed condensation reactions between oligopeptide fragments. Nakatsuka et al. (1987), found that the semisynthetic enzyme thiolsubtilisin (where the the catalytic -OH is replaced with -SH in the active site) was capable of peptide segment couplings such as [Leu]^5 enkephalin amide and a fragment of ribonuclease T_2. Carbohydrate-protease conjugates (CPC) of chymotrypsin, papain and thermolysin synthesized oligopeptides containing 2-8 amino acids in an acetonitrile solvent system (Wartchow et al., 1995).

CHAPTER 7

CONCLUSION

7 Conclusion

Initially, this project explored the baseline stability properties of native bovine trypsin. The microassay developed allowed rapid determination of large quantities of sample, was time saving and minimized use of reagents. 55°C prove to be the best temperature for thermoinactivation studies in later modification experiments. At 55°C there is an even, first order loss of enzyme activity over time. Guanidine hydrochloride was a more potent denaturant of native trypsin than urea. Concentrations of 0-2M guanidine hydrochloride and 0-15M urea were used for denaturation studies of modified derivatives. Organotolerance studies revealed that little or no loss in activity occurred at lower concentrations of the solvents, in most cases the activity was actually enhanced. A dramatic decrease in activity occurred at a critical solvent concentration. This concentration varied with solvent, with THF being the most potent solvent tested.

Modification experiments had mixed effects. EDC and diamines or diacids had little or no effect on stability. Slightly enhanced thermostability was observed for both the diamines and the monoamine control. This suggests that one-point modification of trypsin carboxyl groups was responsible for this enhanced stability rather than the actual formation of crosslinks. No enhanced stability was observed towards the organic solvents tested.

Modification of trypsin with succinimides, however, yields catalytically active derivatives (AA-trypsin and EG-trypsin) with altered and/or crosslinked amino groups. The succinimide modifications yields notable benefits. Both AA- and EG-trypsin showed increased resistance to heat and to a range of water-miscible organic solvents. They also show improved esterase/amidase ratios. These stability gains and alterations to modified trypsins' catalytic properties suggested that they could be particularly useful for peptide synthesis. Successful synthesis of the the dipeptide N-benzoyl-L-Arginine Leucinamide was achieved with the native and modified trypsins. By tailoring the reaction conditions to exploit the "improved" properties of the modified trypsins, significant increases in product yield could be obtained.

The original research objectives of stabilizing trypsin and using it for peptide synthesis have, to a large extent, been achieved. It is hoped that the present data will be of interest and use to scientists working in the field of protein stability, enzyme catalysis and enzymatic peptide synthesis. It has been shown that trypsin was successfully stabilized, using succinimides. The modification is simple and proceeds under mild reaction conditions. The modified forms of trypsin were applied to peptide synthesis and were more efficient at dipeptide synthesis. These modified trypsins have potential roles in a number of different areas. Because only 8 out of the 14 lysine residues were modified, it could be possible to immobilize the modified forms of the trypsin via the remaining lysine by single point attachment as described by Blanco et al. (1992). Because of the enhanced activity and stability of the modified forms of trypsin they could be applied to the area of waste-water treatment, food technology and biosensors. These modified forms might also be more efficient in animal cell cultures for the trypsinization of cells etc..

BIBLIOGRAPHY

Bibliography

Abrahmsen, C., Tom, J., Burnier, J., Butcher, K.A., Kossiakoff, A. & Wells, J.A. (1991) "Engineering subtilisin and its substrates for efficient ligation of peptide bonds in aqueous solution." Biochemistry, **30**, 4151-4159.

Aharonwitz, Y., Bergmeyer, J., Contoral, J.M., Cohen, G., Demain, A.L., Fink, U., Kinghorn, J., Kleinkauf, H., MacCabe, A., Palissa, H., Pfeifer, E., Schwecke, T., van Liempt, H., von Dohren, H., Wolfe, S. & Zhang, J. (1993) "S-(l-alpha-Aminoxlipyl)-L-Cysteinyl-D-Valine Synthetase the multienzyme Integrating the four primary reactions in beta-Lactam Biosynthesis, as a model peptide synthesis". Biotechnology, **11**, 807-810.

Alvaro, C., Blanco, R.M., Bastida, A., Cuesta, C., Fernandez-Lafuente, R. & Guisan., J.M (1991). "Industrial design of peptides synthesis catalyzed by immobilised and stablised enzyme derivative". Med. Fac. landbouuw. Rijksuniv gent., **56**, 1777-1779.

Ampon, K., Salleh, A.B., Teoh, A., Yunus, W.M.Z., Razak, C.N.A. & Basri, M. (1991) "Sugar esterification catalysed by alkylated trypsin in dimethylformamide". Biotechology Letters, **13**, 25-30

Ampon, K., Basri, M., Razak, C.N.A., Yunus, W.M.Z. & Salleh, A.B. (1992) The effect of attachment of hydrophobic modifiers on the catalytic activities of lipase and trypsin. Biocatalysis in non-conventional media, **8**, 331-338.

Ampon, K. & Whitaker, J.R., (1993) "Bioconjugated enzymes for special applications." Abstr. Pap. Am. Chem. Soc., **206** Meet., pt. 1, AGFD60.

Andersen, A.J., Fomsgaard, J., Thorbek, P. & Aasmul-Olsen, A. (1991) Current enzymatic possibilities in the technology of peptide synthesis II. Process development in enzymatic peptide synthesis. Chimicaoggi **4**, 17-23.

Arnold, F.H, (1990) "Engineering enzymes for non-aqueous solvents". Trends in Biotechology, **8**, 244-249.

Arnold, F.H., (1993) "Engineering enzymes for non-natural environments: Improved biocatalysts for the chemical and biotechnology industries". Protein Engineering, **6**, 59.

Auld, D.S. (1987) in "Enzyme Mechanism" (Page M.I. & Williams, A. eds) p.240-258, The Royal Society of Chemistry, London.

Bailey, P.D., (1990) in "An introduction to peptide chemistry" . J.Wiley & Sons, Chichester.

Barbas, C.F., Matos, M., Blairwest, J.R. & Wong, C.-H. (1988) "A search for peptide ligase: cosolvent-mediated converion of proteases to esterases for irreversible synthesis of peptides". J. Am. Chem. Soc., **110**, 5162-5166.

Barros, M.T., Carvalho, M.G.V., Garcia, F.A.P. & Pires, E.M.V. (1992) "Stability performance of *Cynara cardunculus* L. Acid protease in aqueous-organic biphasic systems". Biotech. Letts., **14**, 179-184.

Battle-Mem. Inst. Ohio (1994) Patent "Production of high molecular weight highly soluble collagen". Patent no. US 5316942.

Besson, C., Favin-Bonvin, G., Ó Fágáin, C. & Wallach, J. (1995) "Chemical derivatives of *Pseudomonas Aeruginosa* elastase showing increasing stability." Enzyme Microb. Technol., **17**, 877-881.

Bickerstaff, G.F. and Zhou, H. (1993) "Protease activity and autodigestion assays using Coomasie Blue dye binding". Anal. Biochem., **210**, 155-158.

Blanco, R.M. & Guisan, J.M. (1988) "Protecting effect of competitive inhibitors during very intense insolubilizing enzyme-activated support multipoint attachments: trypsin (amine)-agarose (aldehyde) system". Enzyme Microb. Technol., **10**, 227-232.

Blanco, R.M, Guisan, J.M. & Halling, P.J., (1989a) "Agarose-chymotrypsin as a catalyst for peptide and amino acid ester synthesis in organic media". Biotechnol. Letters, **11**, 811-816.

Blanco, R.M., Calvete, J.J. & Guisan, J.M. (1989b) "Immobilisation-stabilisation of enzymes: variables that control the intensity of the trypsin (amino)-agarose (aldehyde) multipaint attachments". Enzyme Microb. Technol., **11**, 353-359.

Blanco, R.M. & Guisan, J.M. (1989c) "Stabilisation of enzymes by multipoint covalent attachment to agarose-aldehyde gels. Borohyride reduction of trypsin-agarose derivatives." Enzyme Microb. Technol., **11**, 360-366.

Blanco, R.M., Alvaro, G. & Guisan, J.M. (1991) "Enzyme reaction engineering: design of peptide synthesis by stabilised trypsin". Enzyme Microb. Technol., **13**, 573-583.

Blanco, R.M., Halling, P.J., Bastida, A., Cuesta, C. & Guisan, J.M. (1992) "Effect of Immiscible organic solvents on activity/stability of native chymotrypsin and immobilised-stabilised derivatives". Biotech. Bioeng., **39**, 75-84.

Bodanszky, M. in "Principles of Peptide Synthesis". Springer-Verlag Berlin.

Bohlen, P., Stein, S., Darman, W. & Udenfriend, S. (1973) Fluorometric Assay of proteins in the nanogram range. Arch. Biochem. & Biophysics, **55**, 213-220.

Boivin, P., Kobos, R.K., Papa, S.L. & Scouten, W.H. (1991) "Immobilisation of perfluoroalkylated Enzymes in a Biologically active state onto perflex support". Biotech. Appl. Biochem., **14**, 155-169.

Bongers, J., Lambros, T., Mushtaq Ahmad, W.L., Campbell, R.M., Felix, A.M. & Heimer, E.P. (1992) "Enzymatic semisynthesis of a Superpotent analogy of Human Growth Hormone-releasing Factor". J. Med. Chem. **35**, 3934-3941.

Bonneau, P.R., Graycar, T.P., Estell, D.A. & Jones, J.B. (1991) "Alteration of the specificity of subtilisin BPN by site-directed mutagenesis in its S1 and S1' binding sites". J. Am. Chem. Soc., **113**, 1026-1030.

Brocklehurst, K. (1987) in "Enzyme Mechanism" (Page, M.I. & Williams, A. eds), p.140-158, The Royal Society of Chemistry, London.

Bryan, P., Pantoliano, M., Rollence, M., Wood, J., Gilliland, G. & Finzel, B., (1986a) "Protein Engineering of Subtilisin; Proteases of enhanced stability". J. Cell. Biochem. Suppl. **10A**, 271.

Bryan, P., Pantoliano, M., Rollence, M., Wood, J., Gilliland, G., Finzel, B., Ladner, R., & Poulos, T. (1986b) "Protein Engineering of Subtilisin; Proteases of enhanced stability". Fed. proc. Fed. Am. Soc. exp. Biol. **45**, 1519.

Bryan, P., Pantoliano, M., Rollence, M., Wood, J., Howard, A. & Ladner, B. (1987) "Protein engineering of subtilisin: proteases of enhanced stabilities". J. Cell. Biochem., Suppl. **11C**, 223.

Calvet, S., Clapes, P., Vigo, J.P., Xano, N., Jarba, X., Mas, R.M., Torres, J.L. & Valencia, G. (1992) "Application of Empirical Design Methodologies to the study of the influence of reaction conditions and n-alpha-protecting group structureon the enzymatic x-Phe-Leu-NH2 Dipeptide synthesis in buffer/DMF solvent systems". Biotech. Bioeng., **39**, 539-549.

Cerovsky, V. & Jost, K. (1985) "Enzymatically catalyzed synthesis of oxytocin fragments 1-6 and 7-9 using alpha-chymotrypsin, thermolysin and papain". Coll. Czech. Chem. Commun., **50**, 2775-2782.

Cerovsky, V. (1990) "Free trypsin-catalysed peptide synthesis in acetonitride with low water content". Biotech. Letters, **12**, 899-904.

Cerovsky, V. (1992) "Protease-catalysed peptide synthesis in solvent-free system". Biotechnol. Tech., **6**, 155-160.

Cerovsky, V. & Jakubke, H.D. (1994) "Acyl transfer reactions catalysed by native and modified alpha-Chymotrypsin in acetonitrite with low water content." Enzyme Microb. Technol., **16**, 596-601.

Chaiken, I.M. (1984) "Using proteases to synthesise biologically active peptides and proteins". Abstr. Pap. Am. Chem. Soc. **188**, Meet MBTD48.

Chen, K., Robinson, A.C, Van-Dam, M.E., Martinez, P., Economou, C. & Arnold, F.H. (1991) "Enzyme engineering for nonaqueous solvents. II. Additive effects of mutations on the stability and activity of subtilisin-E in polar organic media". Biotechnol. Prod., **1**, 125-129.

Chen, K. & Arnold, F.H. (1991) "Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance activity of subtilisin in polar organic media". Biotechnology, **9**, 1073-1077.

Chen, K. & Arnold, F.H. (1993) "Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in DMF". Pro. Natl. Acad. Sci. USA, **90**, 5618-22.

Chen, S.-T., Chen, S.-Y., Kau, C.L. & Wang K.-T. (1994) "Improved yield by cryoeffect in Kinetically Controlled Peptide Synthesis". Biotech. Letters. **16**, 1075-1080.

Clapés, P., Valencia, G. &Aldercreutz, P. (1992) "Influence of solvent and water activity on kinetically controlled peptide synthesis". Enzyme Microb. Technol., **14**, 575-580.

Cohenford, R., Santoro, P.F., Urbanowski, J.C. & Dain, J.A. (1986) "Effect of immobilisation on the physical and kenetic properties of soluable and insoluable Trypsin-Albumin polymer". Biotech. Bioeng., **18**, 736-40.

Coleman, P.L. & Green, G.D.J. (1981) "A coupled Photometric Assay for Plasminogen Activator". in "Methods in Enzymology." **80**, (edited by Laszlo Lorand) p.408-414. Academic Press.

Coleman, P.L. & Green, G.D.J. (1981) "A sensitive coupled assay for Plasminogen activator using a thiol ester substrate for plasmin". Ann. N.Y. Acad. Sci., **370**, 617-626.

Couturier, R., Hacques, M.F., Ville, A. & Marion, C. (1988) "Covalently immobilised proteases on polystrene and nylon spheres: A tool for the study of chromatin structure". Mol. Cell. Biol. (Life Sci. Adv.), **7**, 107-115.

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

Craik, C.S., Evnin, L., Fletterick, R., Higaki, J., McGrath, M., Tsu, C., Vasquez, J. & Wilke, M. (1990) "Redesigning proteins to understand their structure and function". Abstr. Pap. Am. Chem. Soc., **199** Meet., pt.1 Biol25.

Creighton, T.E. (1993) in "Proteins: structures and Molecular Properties", 2nd ed., p.417-433. W.H. Freeman, New York.

Cupo, P., El-Deiry, W.S., Whitney, P.L. & Awad, W.M. (1982) "Stability of acetylated and superguanidinated chymotrypsinogens". Arch. Biochem. Biophys., **216**, 600-604.

Economou, C., Chen, K. & Arnold, F.H. (1992a) "Subtilisin engineered for organic solvents: characterization of the variants and applications in synthesis". Abstr. Pap. Am. Chem. Soc., **203**, Meet. pt.1, BIOT43.

Economou, C., Chen, K. & Arnold, F.H. (1992b) "Random Mutagenesis to enhance the activity of subtilisin in organic solvents: Charactarisation of Q1030 substilisin E". Biotech. Bioeng., **39**, 658-662.

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

Fadanavis, N.W. & Lusi P.L. (1991) "Immobilised enzymes in reverse micelles: studies with gel-entrapped trypsin and alpha-chymotrypsin in AOT reverse micelles". Biotech. Bioeng., **33**, 1277-84.

Ferjancic, A., Puigserver, A. & Gaertner, H. (1988) "Unusual specificity of polyethylene glycol-modified thermolysin in peptide sythesis catalysed in organic solvents". Biotech. Letters., **10**, 101-106.

Fersht, A. (1984) in "Enzyme Structure and Mechanism", Freeman, W.H & Company Reading & San Francisco.

Fersht, A.R., Leatherbarrow, R.J. & Weals, T.N.C. (1986) Quantitative analysis of structure-activity relationships. Nature, 322, 284-286.

Fink, A.L. (1987) in "Enzyme Mechanism" (Page, M.I. & Williams A. eds) p.159-77, The Royal Society of Chemistry, London.

Fink, D.J. & Brody, R.S. Patent (1996) "Producing a solution of soluble collagen". Patent no. US 5460967.

Fischer, G. (1987) in "Enzyme Mechanism" (Page, M.I. & Williams, A. eds) p.229-239, the Royal Society of Chemistry, London.

Fischer, A., Bommarius, A.S., Drauz, K. & Wandrez, C. (1994) "A Novel approach to enzymatic synthesis using highly solubilizing N-alpha. protecting groups of amino acids". Biocatalysis, **8**, 289-307.

Fitzpatrick, P.A., Ringe, D. & Klibanov, A.M. (1992) "Computer-assisted Modeling of subtilisin enantioselectivity in organic solvents". Biotech. Bioeng., **40**, 735-42.

Fujimura, M., Mori, T. & Tosa, T. (1987) "Preparation and properties of soluble-insoluble Immobilised proteases". Biotech. Bioeng., **19**, 747-52.

Fukui, S. & Tanaka, A. (1985) Enzymatic reaction in organic solvents. Endeavour New Series, 9, 10-17.

Gaertner, H., & Puigserver, A. (1989) "Kinetic and speciaficity of serine proteases in peptide synthesis catalyzed in organic solvents". Eur. J. Biochem., **181**, 207-213.

Gaertner, H.F. & Puigserver, A.J. (1990) "Protein-protein interaction in low water organic media". Biotech. Bioeng., **36**, 601-607.

Gaertner, H., & Puigserver, A. (1992) "Increased activity and stability of poly (ethylene glycol)-modified trypsin". Enzyme Microb. Technol., **14**, 150-155.

Gianfreda, L. and Scarfi, M.R. (1991) "Enzyme stabilization: state of the art". Mol. Cell. Biochem. **100**, 97-128.

Gill, I. & Vulfson, E.N. (1993) "Enzymatic synthesis odf short peptides in heterogenous mixtures of substrates". J. Am. Chem. Soc., **115**, 3348-3349.

Gill, I. & Vulfson, E.N. (1994) "Enzymic catalysis in heterogeneous Eutectic mixtures of substrates". Tibtech., **121**, 118-122.

Gill, I., Lopez-Fandino, R., Jorba, X. & Vulfson, E.N. (1996) "Biologically active peptides and enzymatic approaches to their production." Enzyme Microb. Technol., **18**, 162-183.

Gleich, M., Talsky, G. & Spannagl, R. (1992) "Stabilization of trypsin by modification with bifunctional reagents". Dechema-Biotechnol. Conf., **5(A)**, 121-124.

Gololobov, Y.M., Stepanov, V.M., Voyushimo, T.L., Morozova, I.R. & Aldercreutz, P. (1994) "Side reactions in enzymatic peptide synthesis in organic media: Effects of enzyme solvent and substrate concentrations". Enz. Microb. Technol., **16**, 522-528.

Gorman, L.S. and Dordick, J.S. (1992) "Organic solvents strip water off enzymes". Biotech. Bioeng. **39**, 392-397.

Graf, L., Craik, C.S., Pattly, A., Roczniak, S., Fletterick, R.J. & 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.

Grandi, G., Toma, S., Margarit, I., Campagnoli, S., Gianna, R., Bellini, A.V., Zamai, M. & Carrera, P. (1990) "The *Bacillus subtilis* neutral protease: a model case for site-directed mutagenesis and protein engineering". Chim. oggi, **8**, 7-13.

Guinn, R.M., Blanch, H.W. & Clark, D.S. (1991) "Effect of a water-misible organic solvent on the kinetic and structural properties of trypsin". Enzyme Microb. Technol., **13**, 320-26.

Guisan, J.M. (1988) Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme Microb. Tech., **10**, 375-382.

Halling, P.J., Eichcorn, U., Kuhl, P. & Jakubke, H.-D. (1995) "Thermodynamic of solid-to-solid conversion and application to enzymic peptide synthesis". Enzyme Microb. Tech., **17**, 601-606.

Han, K.K., Richard, C. & Delacourte, A. (1984) "Chemical crosslinks of proteins by using bifunctional reagents." Int. J. Biochem., **16**, 129-145.

Hansicke, A., Beyermann, M., Oehlke, J., Mitlay, E., Kertscher, U., Bespalova, D., Tito, M.I., Bienert, M., Kaufman, K.D., & Niedrich, H. (1988) "Synthesis of [3H-TYRB26]-Human Insulin by Enzymic semisynthesis". Collection Czechoslovk Coumun, **53**, 2936-2943.

Hedstrom, L., Szilagyi, L. & Rutter, W.J. (1992) "Converting Trypsin to chymotrypsin: The role of Surface Loops". Science, **255**, 1249-1253.

Helmerhorst, E. & Stokes, G.B. (1980) "Microcentrifuge Desalting: A rapid Quantitative method for desalting small amounts of protein". Anal. Biochem., **104**, 130-135.

Hermann, G., Schwarz, A., Wandrey, C., Kula, M.R., Knaup, G. & Drauz, K.H. (1991) "Scale-up of enzymatic peptide synthesis in an enzyme membrane reactor". Biotechnol. Appl. Biochem. **13**, 346-353.

Inada, Y., Takahashi, K., Yoshimoto, T., Ayima, A., Matsushima, A. & Saito, Y. (1986) "Application of PEG-modified enzymes in biotechnological processes: organic solvent-soluble enzymes". Tibtech., 7, 190-94.

Inagaki, T., Tadasa, K. & Kayahara, H. (1994) "Modification effects of organic solvents on microenvironment of the enzyme in thermolysin-catalyzed peptide synthesis of N-(Benzyloxycarbonyl)-L-phenylalany-L-phenylalanine Methyl Ester". Biosci. Biotechnol. Biochem., **58**, 439-442.

Jaenicke, R. & Rudolph, R. (1989) in "Protein Structure - A Practical Approach" (Creighton, T.E. ed), p.191-223, IRL Press, Oxford, .

Jaenicke, R. & Rudolph, R. (1993) in "Proteins: structures and Molecular Properties", 2nd ed. (Creighton, T.E ed.) , W.H. Freeman, New York.

Jakubke, H.D. (1987) "Enzymatic Peptide Synthesis", Chapter 3 in "The Peptides", vol.**9**, p.103-165. Academic Press, New York.

Jakubke, H.D. & Konnecke, A. (1987) "Peptide Synthesis using immobilized proteases" in "Methods in Enzymology" vol. **136**, Chapter 18, (Mosbach, K. ed.) p.178-189. Academic Press, New York,

Jennes, L. & Stumpf, W. (1983) in Methods in Enzymology (Conn, P.M. ed), Academic Press, New York. p.448-453. Academic Press, New York,

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.

Jones, J. (1991) in "The Chemical Synthesis of Peptides" (Rowlinson, J.S., Holpern, J., Green, M.L.H. & Mukaiyama, T. eds) Clarendon Press, Oxford.

Jones, J. (1992) in "Amino Acid and Peptide Synthesis" (Davies, S.E ed) Oxford University Press, Oxford, New York & Toyko.

Jorba, X., Clapes, P., Xaus, N., Calvet, S., Torres, J.L., Valencia, G. & Mata, J. (1992) "Optimization and Kinetic Studies of the Enzymatic Synthesis of Ac-Phe-Leu-NH2 in reversed micelles". Enzyme Microb. Technol., **14**, 117-124.

Kasche, V. & Haufler, U. (1984) "Enzyme catalyzed semisynthesis of condensation products kinetic vs. equilibrium control". Eur. Congr. Biotech. Meet., **1**, 425-430.

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

Kato, Y., Matsuka, T. & Nakomura, R. (1993) "Improvement of physiochemical and enzymatic properties of bovine trypsin by non-enzymatic glycation". Biosci. Biotech., **57**, 1-5.

Kent, S. (1988) "Chemical Synthesis of Peptides and Proteins". Ann. Rev. Biochem., **57**, 957-989.

Khmelmitsky, Y.L., Levashov, A.V., Klyachko, N.L. & Martinek, K. (1988) "Engineering biocatalytic systems in organic media with low water content". Enzyme Microb. Technol., **10**, 710-724.

Khmelmitsky, Y.L., Belova, A.B., Levashos, A.V. & Mozhaev, U.V. (1991) "Relationship between surface hydrophilicity of a protein and its stability against denaturation by organic solvents". Febs., **284**, 267-269.

Khmelmitsky, Y.L., Mozhaev, V.V., Belova, A.B., Sergeeva, M.V. & Martinek, K. (1991) "Denaturation capacity: a new quantitative criterion for selection of orgaanic solvents as reaction media in biocatalysis". Eur. J. Biochem., **198**, 31-41.

Khmelmitsky, Y.L., Welch, S.H., Clark, D.S. & Dordick, J.S. (1994) "Salts dramatically enhance activity of enzymes suspended in organic solvents". J. Am. Chem. Soc., **116**, 2647-2648.

Kise, H., Hayakawa, A. & Noritomi, H. (1990) "Protease-catalyzed synthetic reactions and immobilization activation of the enzymes in hydrophilic organic solvents". J. Biotech., **14**, 239-254.

Kise, H. & Hayakawa, A. (1991) "Immobilization of proteases to porous chitosan beads and their catalysis for ester and peptide synthesis in organic solvents". Enzyme Microb. Technol., **13**, 584-589.

Kleinkauf, H. & von-Doehren, H. (1982) "A survey of enzymatic biosynthesis of peptide antibiotics". Trends Antibiot. Res., Proc. Conf. Toyko, **6**, 220-232.

Kleinkauf, H. & von-Dohren, H. (1990) "Nonribosomal biosynthesis of peptide antibiotics". Eur. J. Biochem., **192**, 1-15.

Klibanov, A.M. & Mozhaev, V.V. (1978) "The mechanism of irreversible thermoinactivation of enzymes and possibilities for reactivation of "irreversibly" inactivated enzymes". Biochem. Biophys. Res. Comm. **83**, 1012-1017.

Klibanov, A.M. (1983) "Stabilization of enzymes against thermal inactivation". Adv. in Appl. Microbiol., **29**, 1-23.

Klibanov, A.M. (1989) "Enzymatic catalysis in anhydrous organic solvents". TIBS, **14**, 41-44.

Kobayashi, M. & Takatsu, K. (1994) "Cross-linked Stabilization of Trypsin with Dextran-dialdehyde". Biosci. Biotech. Biochem., **58**, 275-278.

Krieg, F. & Wolf, N. (1995) "Enzymatic peptide synthesis by the recombinant proline-specific endopeptidase from *Flavobacterium meningosepticum* and its mutationally altered Cys-556 variant." Appl. Microbiol. Biotechnol., **42**, 844-852.

Kuhl, P., Eichorn, U. & Jakubke, H-D. (1992) "Thermolysin- and chymotrypsin-catalyzed peptide synthesis in the presence of salt hydrates". Prog. Biotechnol., **8**, 513-518.

Kullmann, W. (1984) "Kinetics of chymotrypsin- and papain-catalyzed synthesis of [leucine]enkephalin and [methionine]enkephalin". Biochem. J., **220**, 405-416.

Labouesse, J. & Gervais, M. (1967) "Preparation of chemically defined N-Acetylated Trypsin". E. J. Biochem., **2**, 215-223.

Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of Bacteriophage T4". Nature, **227**, 680-685.

Lapanjiie, S. (1978) in "Physicochemical aspects of proteins denaturation." J. Wiley & Sons, New York.

Lopez-Fandino, R., Gill, I. & Vulfson, E.N. (1994) "Enzymatic catalysis in heterogenous mixtures of substrates: The role of the liquid phase and the effects of *adjuvants*". Biotechnol. Bioeng., **43**, 1016-1023.

Lozano, P., Iborra, J.L., Manjon, A. & Combes, D. (1992) "One-step synthesis of Gly-Gly-PheNH2 from N-unprotected amino acid derivatives by papain in one-phase liquid media". Biotech. Letters, **14**, 933-936.

Maekawa, K. & Liener, I.E. (1960) The reaction of S-Methylglucosylisothiourea with Histidine. Archives of Biochem & Biophysics 91, 101-107.

Manonmani, H.k. & Joseph, R., (1993) "Preparation and properties of insolubilized proteinase of *Trichoderma Koningii*." Process-biochem., **28**, 325-329.

Margolin, A.L., Tai, D.F. & Klibanov, A. (1987) I"ncorporation of D-amino acids into peptides via enzymatic condensation in organic solvents". J. Am. Chem. Soc., **109**, 7885-7887.

Marshall, J.J. & Rabinowitz, M.L. (1975) "Enzyme stabilization by covalent attachement of carbohydrate". Arch. Biochem. & Biophys., **167**, 777-779.

Martinek, K., Goldmacher, V.S., Klibanov, A.M. & Berezin, I.V. (1975) "Denaturing agents (urea, acrylamide) protect enzymes against irreversible thermoinactivation: a study with native and immobilized alpha-chymotrypsin and trypsin". Febs Letts. **51**, 152-155.

Martinek, K., Klibanov, A.M., Goldmacher, V.S. & Berezin, I.V. (1977a) "The principles of enzyme stabilization (1) Increase in thermostability of enzymes covalently bound to a complementary surface of a polymer support in a multipoint fashion". Biochimia et Biophysica Acta, **485**, 1-12.

Martinek, K., Klibanov, A.M., Goldmacher, V.S., Tchernysheva, A.V., Mozhaev, V.V., Berezin, I.V. & Glotov, B.O. (1977b) "The principles of enzyme stabilization. II Increase in the thermostability of enzymes as a result of multipoint noncovalent interaction with a polymeric support". Biochimia. et Biophysica Acta, **485**, 13-28.

Martinez, P. & Arnold, F.H. (1991) "Surface charge substitutions increase the stability of alpha-lytic protease in organic solvents". J. Am. Chem. Soc., **113**, 6336-6337.

Martinez, P., Van-Dam, M.E., Robinson, A.C., Chen, K. & Arnold, F.H. (1992) "Stabilization of Subtilisin E in organic solvents by Site directed mutagenesis". Biotech. & Bioeng., **39**, 141-147.

Matsumura, M., Signor, G. & Mathews, B.W. (1989) "Substantial increase of protein stability by multiple disulphide bonds". Nature, **342**, 291-293.

Matsushima, A., Okada, M., Inada, Y. (1984) "Chymotrypsin modified with polyethylene glycol catalyzes peptide synthesis reactions in benzene."FEBS letts., **178**, 275-277.

Matzke, S.F., Creagh, A.L., Haynes, C.A., Prausnitz, J.M. & Blanch, H.W. (1992) "Mechanism of protein solubilization in reverse micelles". Biotech. & Bioeng., **40**, 91-102.

Mead. (1994) Patent "A method for treating paper or paperboard containing polyamide wet strength resin". Patent no. US 5330619.

Means, G.E. and Feeney, R.E. (1990) "Chemical modification of proteins: History and applications". Bioconjugate chem. **1**, 2-12.

Melik-Nubarov, N.S., Mozhaev, V.V., Šikšnis, S. and Martinek, K. (1987) "Protein stabilization via hydrophilization: stabilization of alpha-chymotrypsin by reductive alkylation with glyoxylic acid". Biotech. Letts. **9**, 725-730.

Miland, E.; Smyth, M.R. & Ó Fágáin, C. (1996) "Increased thermal and solvent tolerance of acetylated Horseradish peroxidase". Enzyme Microb. Technol. (in press)

Morihara, K., Ueno, Y. & Sakina, K. (1986) "Influence of temperature on the enzymic semisynthesis of human insulin by couplingand transpeptidationmethods". Biochem. J., **240**, 803-810.

Morihara, K. (1987) "Using proteases in peptide synthesis". Trends Biotech. **5**, 164-170.

Morrison, R.T. & Boyd, R.N. (1987) "Role of the solvent: secondary bonding" in "Organic Chemistry" 5th ed. chapter 6, p.223-247. Allyn & Bacan Inc.

Mozhaev, V.V., & Martinek, K. (1982) "Inactivation and reactivation of proteins (enzymes)." Enzyme Microb. Technol., **4**, 299-309.

Mozhaev, V.V., Siksnis, V.A., Torchilin, V.P. & Martinek, K. (1983) "Operational Stability of copolymerized enzymes at elevated temperatures". Biotech. Bioeng., **25**, 1937-1945.

Mozhaev, V.V., Siksnis, V.A, Melik-Nubarov, N.S., Galkantarte, N.Z., Denis, G.J., Butkus, E.P., Zalslavsky, B.Y., Mestechkina, N.M. & Martinek, K. (1988) "Protein stabilization via hydrophilization-covalent modification of trypsin and alpha-chymotrypsin". Eur. J. Biochem., **173**, 147-154.

Mozhaev, V.V., Khmelnitsky, Y.L., Sergeeva, M.V., Belova, A.B., Khyacko, Y.L., Levashov, A.V., & Martinek, K. (1989) "Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures:- alpha-chymotrypsin and laecasa in mixed water/alcohol,water/glycol and water/formanide solvents". Eur. J. Biochem., **184**, 597-602.

Mozhaev, V.V., Melik-Nubarov, N.S., Siksnis, V. & Martinek, K., (1990) "Strategy for stabilizating enzymes Part 2. Increasing enzyme stability by selective chemical modification". Biocatalysis, **3**, 189-196.

Mozhaev, V.V., Sergeeva, M.V., Belova, A.B. & Khmelnitsky, Y.L. (1990) "Multipoint attachements to a support protects enzyme from inactivation by organic solvents: alpha-chymotrypsin in aqueous solutions of alcohols and diols". Biotech. & Bioeng., **35**, 653-659.

Mozhaev, V.V., Melik-Nubarov, N.S. & Levitsky (1992) "High Stability to irreversible inactivation at elevated temperatures of enzymes covalently modified by hydrophilic reagents alpha-Chymotrypsin". Biotech. Bioeng., **40**, 650-662.

Mozhaev, V.V (1993) "Mechanism based strategies for protein thermostabilization". Trends Biotech., **11**, 88-94.

Murphy, A. & Ó Fágáin, C. (1996) "Stability characterization of chemically-modified soluble trypsin". J. Biotechnology, (in press).

Murrell, J.N. & Boucher, E.A. (1982) in "Properties of liquids and solutions." Wiley.

Nagashima, T., Watanabe, A. & Kise, H. (1992) "Peptide synthesis by proteases in organic solvents: Medium effect on substrate specificity". Enzyme Microb. Technol., **14**, 842-847.

Nakatsuka, T., Sasaki, T. & Kaiser E.T. (1987) "Peptide segment coupling catalyzed by the semisynthetic enzyme thiolsubtilisin". J. Am. Chem. Soc., **109**, 3808-3809.

Nalco-Chem (1994) Patent "Filamentous bacterium removal using trypsin, endoprotease, chymotrypsin and a biocide." Patent no. US 5324432.

Nestle (1993) Patent "Two-stage continuous enzymatic protein hydrolysis". Patent no. EP-566877.

Nilsson, K. & Mosbach, K. (1984) "Peptide Synthesis in aqueous-organic solvent mixtures with alpha-chymotrypsin, immobilized to tresyl chloride-activated agarase". Biotech. Bioeng., **16**, 1146-1154.

O'Donnell-Tormey, J. & Quigley, J.P. (1983) "Detection and parcial charactarization of a chymostatin-sensitive endopeptidase in transformed fibroblasts." Proc. Natl. Acad. Sci. USA, **88**, 344-348.

Ó Fágáin, C., Sheehan, H., O'Kennedy, R. & Kilty, C. (1988) "Maintenance of enzyme structure. Possible methods for enhancing stability." Process Biochem., **23**, 166-171.

Ó Fágáin, C. & O'Kennedy, R., (1991) "Functionally-stabilised proteins - a review". Biotech. Adv., **9**, 351-409.

Ó Fágáin, C. (1995) "Understanding and increasing protein stability". Biochim. Biophys. Acta, **1252**, 1-14.

Owens/Illinois glass container (PATENT) (1987) Protein modification to provide enzyme activity. US PAT No. 4716116.

Oyama, K., Nishimura, S., Nonaka, Y., Kihara, K-I. & Hashimoto, T. (1981) "Synthesis of an aspartame precursor by immobilized thermolysin in an organic solvent". J. Org. Chem., **46**, 5241-5242.

Pauchon, V., Besson, C., Saulnier, J. & Wallach, J. (1993) "Peptide Synthesis catalyzed by *Pseudomonas aeruginosa* elastase". Biotechnol. Appl. Biochem., **17**, 217-221.

Perjetti, R.B., Anderson, C.D. & Hall, P.L. (1976) "The chemical modification of Papain with 1-Ethyl-3-(-3-dimethylaminopropyl)carbodimide)". Biochem., **15**, 8, 1735-1743.

Perry, L.J. & Wetzel, R. (1984) "Disulfide Bond engineered into T4 lysozyme: Stabilization of the protein toward Thermal Inactivation. Science," **226**, 555-557.

Perichetti, R.A., St. Clair, N.L., Griffith, J.P., Havin, M.A. & Margolin, A.L. (1995) "Cross-linked enzyme crystals (CLECs) of Thermolysin in the synthesis of peptides". J. Am. Chem. Soc., **117**, 2732-2737.

Rajput, Y.S. and Gupta, M.N. (1987) "Reaction of trypsin with some crosslinking reagents". Enzyme Microb. Technol., **9**, 161-163.

Rajput, Y.S. & Gupta, M.N., (1988) "Reaction of Trypsin with dimethyl adipimidate:purification and charactarization of a trypsin derivative with decreased autolysis". Enzyme Microb. Technol., **10**, 143-150.

Rasmussen, J.S., Thim, L. & Bjorn, S.E. (1995) Patent "Production of glucagon-like peptide-1 (7-36)". Patent no. WO 9517510.

Reddy, C.R., Reddy, C.R., Raghunalx, K. & Joseph, K.T. (1986) "Immobilization of Trypsin on alginic acid-poly(glycidyl methacrylate) Graft polymer". Biotech & Bioeng., **28**, 609-612.

Rick, W. (1974) "Trypsin" in "Methods of enzymatic analysis" (Bergmeyer, H.U. ed.), p. 1013-1024. Academic Press Inc.

Ryan, O., Smyth, M.R. and Ó Fágáin, C. (1994) "Thermostabilized chemical derivatives of horseradish peroxidase". Enzyme Microb. Technol. **16**, 501-505.

Sackett, D.L. & Wolff, J. (1987) "Nile Red as a polarity-sensitive fluorescent probe of hydrophobic protein surfaces". Analytical biochemistry, **167**, 228-234.

Sakajoh, M., Solomon, N.A. & Demain, A.L. (1987) "Cell-free synthesis of the dipeptide antibiotic bacilysin". J. Industrial Micribiology, **2**, 201-208.

Sakurai, T., Margolin, A.L., Russell, A.-J. & Klibanov, A.M. (1988) "Control of enzyme enantioselectivity by the reaction medium". J. Am. Chem. Soc., **110**, 7236-7237.

Sakurai, K., Kashmitoto, K., Kodera, Y. & Inada, Y. (1990) "Solid phase synthesis of peptides with polyethylene glycol modified protease in organic solvents". Biotech. Letters, **12**, 685-688.

Schecter, I. & Berger, A. (1967) Biochim. Biophys. Res. Commun., **27**, 157.

Schellenberger, V., Schellenberger, U. & Jakubke, H-D. (1991a) "Protease-catalyzed peptide synthesis using inverse Substrates: The synthesis of Pro-Xaa-Bonds by trypsin". Biotech. Bioeng., **38**, 319-321.

Schellenberger, V. & Jakubke, H-D. (1991b) "Protease-Catalyzed peptide synthesis using inverse substrates: The influence of reaction conditions on the trypsin acyl transfer efficiency". Biotech. & Bioeng., **38**, 104-108.

Schuster, M., Ullman, G., Ullman, U. & Jakubke, H-D. (1993) "Chymotrypsin-catalyzed peptide synthesis in ice: use of unprotected amino acids as acyl acceptors". Tetrahedron Letters, **34**, 5701-5702.

Sears, P.S. & Clark, D.S. (1993) "Comparison of soluble and immobilized trypsin kinetics: Implications for peptide synthesis". Biotech. & Bioeng., **42**, 118-124.

Semisotnov, G.V., Rodionova, N.A., Razgalgaev, O.L., Uversky, V.N., Gripas, A.F. & Gilmanshin R.J. (1991) "Study of the `molten globule' intermediate state in protein folding by a hydrophobic flourescent probe". Biopolymers, **31**, 119-128.

Serban, M. & Banateanu, D. (1984) "New amylopectin derivative. I Preparation and some catalytical properties of immobilized trypsin on CNBr activated cross-linked amylopectin". Rev. roum: Biochim., **21**, 219-225.

St. Clair, N.L. & Navia, M.A. (1992) "Cross-linked Enzyme Crystals as robust biocatalysts". J. Am. Chem. Soc., **114**, 7314-7316.

Tafertshofer, G. and Talsky, G. (1989) "Intra- and intermolecular crosslinking of enzymes by dianhydrides and N-hydroxysuccinimide esters". Dechema-Biotechnol. Conf. **3(A)**, 171-174.

Taguchi, H.; Ishihara, N.; Okumura, K. & Shimabayashi, Y. (1990) "Biosensor for peptide determination constructed by immobilizing proteolytic enzymes on coated-wire electrodes". Anal. Chim. Acta, **1**, 13-17.

Takahashi, K., Nishimura, H., Yoshimoto, T., Okada, M., Ajima, A. & Matsushima, A. (1984) "Biotech. Letts., **6**, 765-770.

Talsky, G., Tafertshofer, G. and Gleich, M. (1990) "Influence of intra- and intermolecular crosslinking on activity, stability and kinetic of enzymes". Dechema-Biotechnol. Conf. **4(A)** 135-138.

Tanford, C. (1968) "Protein denaturation." Advance in Proteins Chemistry, **28**, 121-282.

Tanizawa, K., Kasaba, Y. & Kanaoka, Y. (1977) "*Inverse Substrates* for Trypsin. Efficient enzymatic hydrolysis of certain esters with a cationic centre in the leaving group". J. Am. Cmem. Soc., **99**, 4485-4488.

Teagasc (1993) Patent "Hyperallergenic whey protein hydrolyzate production". Patent no. WO9304593.

Thompson, C., Holt, T., Riabaud, A., Laurent, C., Chang, C. & Myers, P. (1990) "Regulation and synthesis of the peptide antibiotic Bialaphos in *Streptomyces Hygroscopicus*". GIM-90, **1**, 1393-1402.

Tipton, K.F. (1992) in "Enzyme assays A practical Approach" (Eisenthal, R. & Danson, M.J. eds.) Chapter 1, p.1-58.

Torchilin, V.P., Maksimenko, A.V., Smirnov, V.N, Berezin, I.V., Klibanov, A.M. & Martinek, K. (1978) "The principles of enzyme stabilization III. The effects of the length of intra-molecular cross-linkages on the thermostabilityof enzymes." Biochim. Biophys. Acta, **522**, 277-283.

Torchilin, V.P., Trubetsky, V.S., Omel'Yanenko, V.G. & Martinek, K. (1983) "Stabilization of subunit enzyme by intersubunit crosslinking with bifunctional reagents: studies with glyceraldchyde-3-phosphate dehydrogenase". J. of Mol. Catalysis, **19**, 291-301.

Tougu, V., Meos, H., Hato, M., Aaviksaar, A. & Jakubke, H-D. (1993) "Peptide synthesis by chymotrypsin in frozen solution, Free amino acids as nucleophiles". Febs Lett., **329**, 40-42.

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

Van-Liempt, H., Dohren, H.V. & Klienkauf, H. (1989) "δ-(L-alpha-Aminoadipyl)-L-cysteinyl-D-valine Synthetase from *Aspergillus nidulans*". J. Biol. Chem., **264**, 3680-3684.

Varley, P.G. & Pain, R.H. (1991) Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinase from yeast and Thermus thermophilus. J. Mol. Biol., 220, 531-538.

Vazquez-Duhalt, R., Fedorak, P.M. & Westlake, D.W. (1992) Role of enzyme hydrophobicity in biocatalysis in organic solvents. Enzyme Microb. Technol., 14, 837-841.

Venkatesh, R. & Sundaram, P.V. (1993) Stabilization of trypsin by crosslinking. Protein Eng., 6, suppl., 118.

Volkin, D.B., Staubli, A., Langer, R. & Klibanov, A.M. (1991) "Enzyme thermoinactivation in anhydrous organic solvents". Biotech. Bioeng. **37**, 843-853.

Von-Dohren, H. & Marahiel, M.A. (1990) "Antibiotics of Bacillus". Gim 90, **2**, 691-702.

Wartchow, C.A., Wang, P., Bednarski, M.D. & Callstrom, M.R. (1995) "Carbohydrate protease conjugates: stabilized proteases for peptide synthesis". J. Org. Chem., **60**, 2216-2226.

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,.

Walsh, K.A. & 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.

White, F.L. & Olsen, K.L. (1987) "Effects of crosslinking on the thermal stability of Hemoglobin. 1. The use of Bis(3.5-dibromosalicyl) fumarate". Archives of Biochemistry & Biophysics, **258**, 51-57.

Widmer, F., Baynes, S., Houen, G., Moss, B.A., Rigby, R.B., Whitaker, R.G. & Johansen, J.T. (1984) in "Peptides" (Ragnarsson, U., ed) p.193-200, Almquist & Wiksall Stockholm.

Wong, C.-H., Matos, J.R., West, J.B. & Barbas, C.F. (1988) "17. Practical enzymatics approaches to unusual peptides". Dev. Ind. Microbiol. **29**, 171-177.

Wong, C.H., Chen, S.T., Hennen, W.J., Bibbs, J.A., Wong, Y.F., Lieu, J.L-C., Pantolieno, M.W., Whitlow, M. & Bryan, P.N. (1990) "Enzymes in Organic Synthesis: Use of Subtilisin and a highly stable mutant derived from multiple site-specific mutations". J. Am. Chem. Soc., **112**, 945-953.

Wong, C.-H., (1992) "Engineering enzymes for chemoenzymatic synthesis. Part 2 modifying proteases for peptide synthesis". Trends Biotechnol. **10**, 378-381.

Wong, C.-H., Schuster, M., Wang, P. & Sears, P. (1993) "Enzymatic synthesis of N- and O-linked glycopeptides". J. Am. Chem. Soc **115**, 5813-5901.

Wong, S.S. & Wong, L.-J. G. (1992) "Chemical crosslinking and the stabilization of proteins and enzymes". Enzyme Microb. Technol., **14**, 860-874.

Wu, Z.P. & Hillvert, D. (1989) "Conversion of a protease into an acyl transferase: Selenolsubtilisin". J. Am. Chem Soc., **111**, 4513-4514

Xu, Z.-F., Focht, K. & Dordick, J.S. (1992) "Engineering Subtilisin for use in organic solvents". Ann. N.Y. Acad. Sci., **672**, 94-99.

Yan, C., Kitano, H. & Ise, N. (1988) "Functional properties of proteins immobilized on albumin microspheres". Biotech. & Applied Biochem., **10**, 13-20.

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

Yanker, B.A., Duffy, L.K. & Kischner, D.A. (1990) "Neurotrophic and neurotoxic effects of amyloid Beta Proteins Reversal by Tachykinin Neuropeptides". Science, **250**, 279-282.

Zaks, A. & Klibanov, A.M. (1985) Enzyme-catalyzed processes in organic solvents. Proc. Natl. Acad. Sci. USA, 82, 3192-3196.

Zaks, A. & Klibanov, A.M. (1986) "Substrate specificity of enzymes in organic solvents versus water is reversed". J. Am. Chem. Soc., **108**, 2767-2768.

Zalipsky, S., Seltzer, R. & Menon-Rudolph, S. (1992) "Evaluation of a new reagent for covalent attachement of polyethylene glycol to proteins". Biotech. Appl. Biochem., **15**, 100-114.

Zhong, Z., Bibbs, J.A., Yuan, W. & Wong, C.H. (1991) "Active-site directed modification of subtilisin", J. Am. Chem. Soc., **113**, 2259-2263.

APPENDIX

Appendix

List of Publications

1. Stability characterization of chemically-modified soluble trypsin.
   Ann Murphy and Ciaráin Ó Fágáin
   The Journal of Biotechnology (1996) in press

2. Bypassing the ribsome: Peptide synthesis without translation.
   Ann Murphy and Ciaráin Ó Fágáin
   Essays in Biochemistry (1996) in press