A Study of Two Proline Specific Peptidases from Bovine Serum

Thesis Submitted for the Degree of
Doctor of Philosophy
by

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Declaration

I hereby certify that this material, which I now submit for assessment on the program 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: Damian Cunningham Date: 24th Sept 1996
(Candidate)

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Rarely does one discover such strength and capacity to love so beautifully created - I have

Abbreviations

The following abbreviations are used throughout this text

Abu	Aminobutyric acid
Abz	2-Aminobenzoyl
ACE	Angiotensin converting enzyme
Acryl	Acrylamide
AEBSF	4-(2-Aminoethyl)-benzenesulfonylfluoride
AP	Ammonium persulphate
APP	Aminopeptidase P (EC 3 4 11 9)
arb	Arbitrary units
BCA	Bicinchoninic acid
Bisacryl	Bisacrylamide
βNA	β-Naphthylamide
Boc	Butoxycarbonyl
BSA	Bovine serum albumin
Bz	Benzyloxyl
CD26	Cluster differentiation antigen 26
cDNA	copy Deoxyribonucleic acid
CDTA	1,2-Cyclohexanediamine tetraacetic acid
CHO	Chinese hamster ovarian
CPC	Calcium phosphate cellulose
CPP	Carboxypeptidase P
Da	Daltons
DDR	Dipeptidyl peptidase IV deficient rats
DEAE	Diethylaminoethyl
DFP	Diisofluorophosphate
DLP	Direct linear plot
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
Dnp	Dinitrophenol
DPPII	Dipeptidyl peptidase II
DPPIV	Dipeptidyl peptidase IV
DTNB	5' 5'-Dithio-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EH	Eadie-Hofstee
ELISA	Enzyme linked immunosorbent assay
F.I	Fluorimetric intensity
Fmoc	9-Fluorenylmethoxycarbonyl
FPP	Fertility promoting peptide
gag	Group specific antigen gene
GRF	Growth hormone releasing factor
HIV	Human immunodeficiency virus
HIV1P	HIV-1-protease
HPLC	High performance liquid chromatography
HW	Hanes-Woolf
LB	Lineweaver-Burk
LHRH	Luteinizing hormone releasing hormone
MBP	Maltose binding protein
MCA	7-Amino-4-methyl-coumarin
MeOH	Methanol
MES	2-[N-Morpholino]ethane-sulphonic acid
MM	Michaelis-Menten
N.D	Not determined
n r.	Not reported
NAD	Nicotinamide adenine dinucleotide
NC	Nucleocapsid
NEM	N-Ethylmaleimide
NF-κ-β	Nuclear factor- κ-β
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PCMB	p-Chloromercuribenzoate

PCP	Prolyl carboxypeptidase	**SM**	Sulphamethoxazole
PDA	Photo diode array	**SP**	Sulphopropyl
PE	Prolyl endopeptidase	**Suc**	Succinyl
PEG	Polyethylene glycol	**TEMED**	N, N, N, N'-Tetramethyl ethylenediamine
PLD-D	Prolidase deficiency		
PMSF	Phenylmethylsulphonylfluoride	**TFA**	Trifluoroacetic acid
pNA	p-Nitroanilide	**Thi**	Thiazolidinecarboxylic acid
pol	Polymerase gene	**TRH**	Thyrotropin releasing hormone
Pyrr	Pyrrolidide	**TRH-OH**	Acid TRH (pGlu-His-Pro)
PYY	Peptide-YY	**v/v**	Volume per volume
Q	Quaternary anion	**w/w**	Weight per weight
R_f	Relative mobility	**Xaa-**	Any amino acid
RSH	Thiol reducing agent	**Yaa-**	Any amino acid
S D	Standard deviation	**Z-**	*N*-Benzyloxycarbonyl-
SDS	Sodium dodecyl sulphate	**ZIP**	Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase
SEC	Size exclusion chromatography		
SH	Sulphydryl	**ZPP**	Z-Pro-Prolinal

Amino acid abbreviations

Ala	Alanine	**Cys**	Cysteine	**His**	Histidine	**Met**	Methionine	**Thr**	Threonine
Arg	Arginine	**Gln**	Glutamine	**Ile**	Isoleucine	**Phe**	Phenylalanine	**Trp**	Tryptophan
Asn	Asparagine	**Glu**	Glutamate	**Leu**	Leucine	**Pro**	Proline	**Tyr**	Tyrosine
Asp	Aspartate	**Gly**	Glycine	**Lys**	Lysine	**Ser**	Serine	**Val**	Valine

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Abstract

Proline holds an important position among twenty naturally occurring amino acids, the building blocks of peptides and proteins It confers particular biological properties upon these physiologically important biomolecules due to its unique structural characteristics There has evolved a specialised group of enzymes that recognise this residue and can introduce peptide bond cleavage at either its carboxyl or amino terminus within a peptide chain The variety of these specialised peptidases cover practically all situations where a proline residue might occur in a substrate and their action can be of biological significance, leading to the inactivation or biotransformation of peptides and proteins

Two distinct proline specific peptidases were detected in bovine serum using the substrate Z-Gly-Pro-MCA, a reportedly specific fluorimetric substrate for prolyl endopeptidase (PE) One of these activities was inhibited by Z-Pro-Prolinal, a PE specific inhibitor, and was subsequently designated PE The second activity resisted inhibition by Z-Pro-Prolinal, even at high concentrations and increased preincubation times This activity was subsequently designated Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP)

Both PE and ZIP activities in bovine serum were successfully separated using SP-Sepharose cation exchange chromatography and were subsequently purified independently of each other

PE activity was partially purified, following its separation from ZIP, using Phenyl-Sepharose hydrophobic interaction, DEAE-Sepharose anion exchange and Sephacryl S-200 HR gel filtration chromatographies, with a final yield of 24% and a final purification factor of 30 achieved. The enzyme had a native molecular weight of 70,000 Da, as determined by gel filtration chromatography The subunit structure of the enzyme could not be determined by SDS PAGE due to the appearance of multiple bands following visualisation of the gel by silver staining

A pH optimum of 8 0, with a preference for phosphate buffer was determined for the partially purified PE. The enzyme was stable over a pH range of 5-9 Optimal activity was obtained from PE at a temperature of 37°C with little activity being detected above or below this temperature

The enzyme was inhibited by AEBSF indicating that the enzyme is a member of the serine protease family The enzyme was also inhibited by PCMB and activated by DTT, indicating the possible presence of an essential cysteine residue, close to the active site

PE hydrolysed the substrates Z-Gly-Pro-MCA and pGlu-His-Pro-MCA, with K_m values of 94μM and 62μM respectively The enzyme also cleaved a variety of proline containing bioactive peptides including LHRH, bradykinin, substance P and angiotensin II These peptides also competitively

inhibited PE activity towards Z-Gly-Pro-MCA PE demonstrated relatively high specificities towards bradykinin and angiotensin II with K_i values of 136μM and 113μM respectively

The enzyme was inhibited by a range of PE specific inhibitors with the highest inhibitory activity being observed for α-ketobenzothiazole (IC_{50} = 41 picomolar) and Z-Indolinyl Prolinal (IC_{50} = 45 picomolar)

Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) activity was purified, following its separation from PE, using Phenyl-Sepharose hydrophobic interaction , Calcium Phosphate Cellulose and Sephacryl S-200 HR gel filtration chromatographies, with a final yield of 14% and a final purification factor of 2250 achieved The enzyme had a native molecular weight of 185,000 Da, as determined by gel filtration chromatography The subunit structure of ZIP was determined to be tetrameric, based on the identification of a major band of 50,000Da by SDS PAGE following visualisation of the gel by silver staining

The enzyme exhibited a pH optimum of 8 5, and was stable over a pH range of 4-9 5 Optimal activity was obtained from ZIP at a temperature of 37°C- 40°C with significant activities being observed at 4°C and 20°C

The enzyme was inhibited by AEBSF indicating that it is probably a member of the serine protease family

ZIP hydrolysed the substrate Z-Gly-Pro-MCA, with a K_m value of 267μM The enzyme also cleaved proline containing bioactive peptides including LHRH, bradykinin and substance P These peptides also inhibited ZIP activity towards Z-Gly-Pro-MCA with bradykinin and LHRH demonstrating competitive K_i values of 2 5mM and 475μM respectively

The enzyme was inhibited by some of the PE specific inhibitors tested. Highest inhibitory activity was observed for α-ketobenzothiazole (IC_{50} = 15 picomolar)

Bovine serum PE activity, purified and characterised during this investigation, is similar to PE isolated from other sources with regard to its biophysical and biochemical characteristics, and its substrate specificity Its localisation in serum, and it activity towards proline containing bioactive peptides indicates that it may play and important physiological role in the metabolism of such peptides

ZIP activity may represent a novel proline specific peptidase localised in serum which might also play a role in the degradation of proline containing bioactive peptides

1. Introduction

1 1. Proline

More than two billion years ago, as part of the evolutionary development of protein and peptide structure, 20 discrete building blocks emerged as the optimal components of the biosynthetic chain Among these 20 α-amino acids a unique position is held by the imino acid proline Amino acids are constructed around a single carbon atom, the α-carbon Forming the centre of a tetrahedral array, the α-carbon is bound to an amino group (NH_3), a hydrogen atom, a carboxyl group (*COOH*) and a side chain R group (Figure 1 1 a) Proline differs from other amino acids in that its side chain R group (-CH_2-CH_2-CH_3) is bonded to both the amino group and α-carbon, resulting in a cyclic structure (Figure 1 1 b)

Chemical modification of the nitrogen atom in this manner, affects its basicity, and the overall polarity of the proline residue Furthermore, the cyclic nature of this amino acid places major constraints on structural aspects of the polypeptide backbone

1.2. Biological Significance of Proline

1.2.1. Structural Aspects

Due to its unique structural properties, important conformational attributes are observed when proline is introduced into a peptide sequence Its cyclic structure limits the angle of rotation about the α-carbon and nitrogen within a peptide bond, (ϕ, Figure 1 1 c), normally only affected by steric hindrance or electrostatic repulsion between the R groups of adjacent residues Consequentially, proline introduces a fixed bend into the peptide chain which has been found to be a potent repeated structure breaker It tends to change the direction of peptide chains, resulting in the spherical or globular shape of proteins This important structural event, a reverse turn or hairpin bend at the surface of proteins, is the most common occurrence of proline within proteins (Yaron and Naider, 1993, Anfinsen and Scheraga, 1975, Crawford *et al.*, 1973) A second consequence of prolines' cyclic nature is that it possesses no functional groups This prevents it from participating in hydrogen bonding or resonance stabilisation of a peptide bond Proline is, therefore, the only amino acid that is not compatible with α-helix or β-sheet secondary structures It *can* form a left handed helical structure when multiple proline residues occur sequentially in a protein This is not a common occurrence, but has been reported for residues 5 - 7 in bovine pancreas trypsin inhibitor A second helical structure that depends specifically on the structural attributes of proline is collagen, the main constituent of bone, tendons and supporting membranous tissue (Bornstein, 1974)

Steric hindrance and electrostatic repulsion effects, caused by interactions between the R groups of adjacent residues, energetically favour *trans* isomers of peptide bonds However, due to the close association of the R group of proline and the nitrogen atom of the peptide bond, *cis* isomers of proline and adjoining residues are common (Figure 1 1 d and 1 1 e) This allows rotation around the bond between the nitrogen and carbonyl groups to occur (ω), reducing the structural integrity conferred to

biosynthetic chains by the normally fixed planar peptide bond (Figure 1 1 c) The significance of prolines' ability to form *cis* peptide bonds is illustrated by the fact that only four examples of *cis* peptide bonds, not involving a proline residue, have been reliably reported in proteins Three in carboxypeptidase A and one in dihydrofolate reductase (Creighton, 1984, Rees, 1981, Kolaskar and Ramabrahman, 1982)

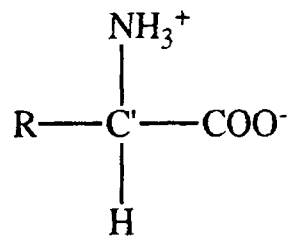
1 2.2 Physiological Aspects

A key physiological role played by proline is the protection of biologically active peptides against enzymatic degradation This is clearly evident in the regulation of post-translational modifications of peptide or protein precursors The biosynthesis and degradation of peptides, such as hormonal peptides and neurotransmitters, is recognised to be a highly ordered series of events Generation of an active polypeptide involves the action of an endopeptidase(s), which cleaves its precursor at a specific site(s), followed by proteolytic "trimming" by exopeptidases to reduce the polypeptide chain to its correct size The peptide chain must possess some structural element to prevent excessive hydrolysis and subsequent loss of biological activity Enzymatic modification of the peptide chain termini by acetylation, or, by the formation of a pyroglutamyl residue at the amino terminus and an amide at the carboxyl terminus, can regulate modification of peptides by exopeptidases However, this type of processing occurs after proteolytic trimming is complete Proline residues situated within polypeptide precursors act as structural elements limiting the susceptibility of the polypeptide chain to proteolysis They are also present at the modification site prior to enzymatic processing of the precursor This has been revealed by investigations into the specificity of exopeptidases involved in post translational modification of peptides and is confirmed by the observation that proline appears near the amino termini of many biologically active peptides (Persson *et al*, 1985, Bradbury *et al*, 1982, Yaron, 1987)

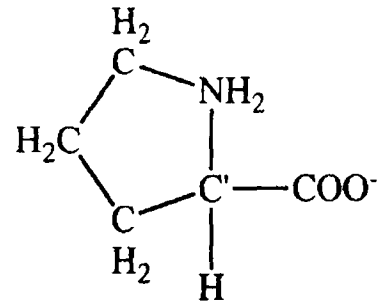
1.3. Proline Specific Peptidases

As previously discussed, the presence of a proline residue confers specific properties upon a polypeptide chain These structural characteristics give the molecule particular conformational properties and influence its interaction with other proteins The interaction between proline containing peptides and enzymes is no exception Restriction of the susceptibility of polypeptides to proteolytic cleavage conferred by proline, offers a mechanism of protection against degradation This is primarily true for the Xaa-Pro peptide bond, but is also evident for the Pro-Xaa bond These restrictions can also extend to amino acid residues not directly attached to the proline and are commonly associated with the isomeric configuration of the peptide chain Lin and Brandts, (1983), reported that the sequence -Lys-Phe-Pro was only hydrolysed at the Lys-Phe bond by trypsin when the Phe-Pro bond was in *trans* configuration They went on to use this method to determine the ratio of *trans-cis* proline isomerisation states in pentapeptide mixtures Because of the varied restrictions applied when dealing with proline containing peptides it is not surprising that nature saw fit to develop range of enzymes to deal specifically with this imino acid. The following pages will endeavour to discuss these enzymes, with specific reference to those responsible for the degradation of proline containing peptides

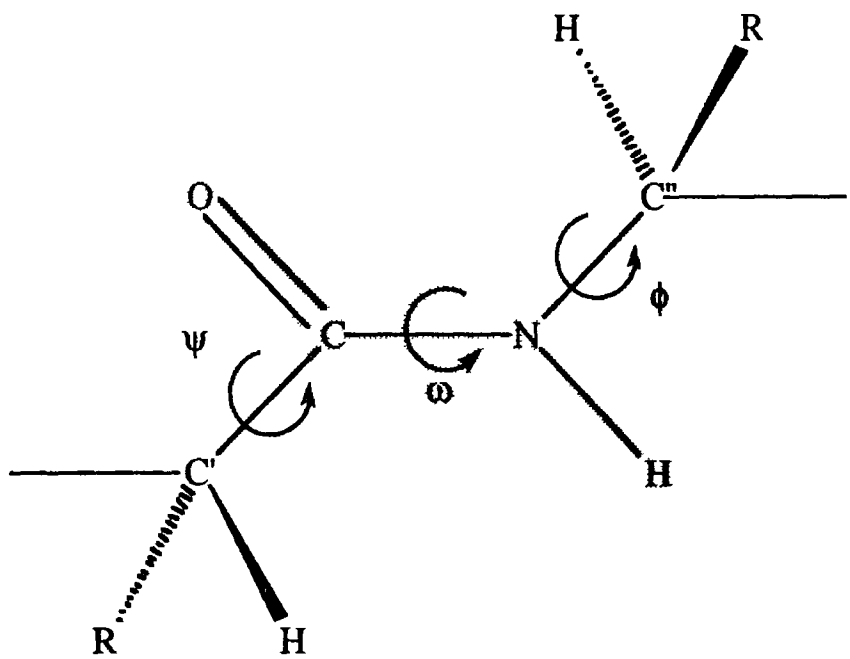
Figure 11



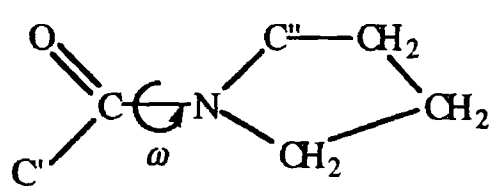
(a) Basic amino acid structure illustrating carboxyl, amino, and R groups attached to a chiral α-carbon (C')



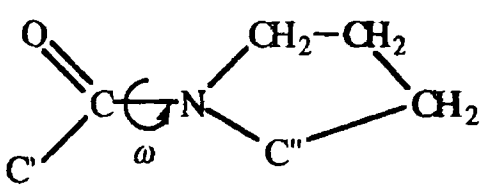
(b) Structure of proline, illustrating the bonded amino group and R group creating a cyclic structure The secondary nature of the nitrogen containing moiety makes proline an imino acid



(c) Structure of the peptide bond between two amino acids The α-carbon of each amino acid are designated C' and C" Angles of rotation ψ, ω and ϕ are indicated. The angle of rotation ω is normally fixed at 180° (trans configuration) resulting in the fixed planar bond" (shaded) In proline, the angle of rotation ω may be 180 or 0° (trans or cis) and is not fixed, but may rotate by -20° or +10° Furthermore, the angle of rotation ϕ in a peptide bond involving proline is constrained, and this constraint is responsible for introducing a fixed bend into peptide chains



(d) Peptide bond involving proline in the trans formation, i e ω = 180°



(e) Peptide bond involving proline in the cis formation, i.e ω = 0°

1 3 1. Prolyl Endopeptidase (EC 3 4 21 26)

Prolyl endopeptidase activity was first observed in homogenates of human uterus by Walter *et al*, (1976) They reported the release of leucylglycinamide from the C-terminus of oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) This activity was referred to as post proline cleaving enzyme by Koida and Walter later that year when they purified the activity from lamb kidney and determined that the enzyme cleaved peptide bonds on the carboxyl side of a proline residue A highly purified brain enzyme, which shared similar specificity to post-proline cleaving enzyme, was reported by Orlowski *et al*, (1978) The enzyme hydrolysed peptidyl prolyl-peptide and peptidyl prolylamino acid bonds, but was inactive towards substrates with an unsubstituted imino group of proline and was therefore designated prolyl endopeptidase In 1992, it was recommended that the enzyme be referred to as prolyl oligopeptidase, as it does not cleave proline bonds in proteins (Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, 1992)

1.3.1.1. Biophysical and Biochemical Aspects of Prolyl Endopeptidase

Early reports as to the molecular weight of prolyl endopeptidase (PE) indicated a dimeric subunit structure, with native molecular weights ranging from 115kDa to 140kDa (Mizutani *et al*, 1984, Koida and Walter, 1976) However, it is generally accepted that the molecular weight of PE ranges from 65kDa to 85kDa, with the majority of reports indicating a molecular weight of 70kDa to 75kDa. The enzyme is monomeric (Goossens *et al*, 1995, O'Leary and O'Connor, 1995, Kusuhara *et al*, 1993, Yokosawa *et al*, 1983) In 1990, Rennex *et al*, reported the cDNA cloning of porcine brain PE The deduced molecular weight, based on 710 amino acids sequenced, was 80,751Da. This has been confirmed recently for human T-cell PE (Shirasawa *et al*, 1994) PE from *Flavobacterium meningosepticum* has also been cloned and a molecular weight of 78,705Da was deduced for 705 amino acid residues sequenced (Yoshimoto *et al*, 1991) This result was also obtained by Chevallier *et al*, (1992), but these workers identified an N-terminal signalling peptide which, when removed, resulted in a protein consisting of 685 amino acids, the molecular weight of which was 76,784 Da The identification of an N-terminal signalling peptide was confirmed by Kanatani *et al*, (1993)

Mammalian PE has a pH optimum of 7 5 with a pI of 4 8-4 9 The bacterial enzyme has a similar pH optimum but its pI is higher at 5 5, with some reports of 6 5 Both forms have an optimum temperature of 35°C-40°C (Sharma and Ortwerth, 1994, Makinen *et al*, 1994, Goossens *et al*, 1995, Yoshimoto *et al*, 1995)

PE has been classified as a serine protease, based on its inhibition by DFP and to a lesser extent by PMSF Sequencing of the protein has confirmed this classification, but in tandem with biochemical characterisation studies, PE has been identified as a member of a distinct family of serine proteases

The known serine proteases have been classified into three distinct families that are named after the representative enzymes trypsin, subtilisin and carboxypeptidase Y (Neurath,1989, Breddam, 1986) Primary structures of each family differ though these structures are homologous within each family A consensus sequence for mammalian serine proteases was proposed by Brenner (1988), where the active site serine appears in the sequence Gly-Xaa-Ser-Xaa-Gly This consensus is adhered to by trypsin (Gly-Asp-Ser-Gly-Gly) but the carboxyl Gly is not present at the active sites of subtilisin (Gly-Thr-Ser-Met-Ala) or carboxypeptidase Y (Gly-Glu-Ser-Tyr-Ala) Rennex *et al*, 1990, identified the sequence surrounding the active site serine in porcine brain PE as Gly-Gly-Ser-Asn-Gly, which does not share homology with the 'classical' serine protease families It does, however, conform to Brenners' (1988) consensus sequence Bacterial PE active site sequences also share no homology with the classical serine proteases and are not in complete agreement with the mammalian sequence (Makinen *et al*, 1994, Chevallier *et al*, 1992, Yoshimoto *et al*, 1991)

A second feature setting PE and its family of serine proteases apart from classical forms, is the order in which members of the catalytic triad of residues occur Within the classical enzyme triads of Asp--His--Ser and His--Asp--Ser have been identified. PE, however, contains a catalytic triad sequence of Ser--Asp--His This evidence clearly distinguishes PE from classical serine proteases (Goossens *et al*, 1995, Vanhoof *et al*, 1995, Yoshimoto *et al*, 1995, Kanatani *et al*, 1993) The active site is located at the C-terminal end of the amino acid sequence, in a protease domain, the secondary structural organisation of which conforms to an α/β hydrolase fold (Goossens *et al*, 1995) PE is also widely reported to be inhibited by sulphydryl blocking agents such as PCMB This inhibition is thought to be due to the modification of a cysteine residue located near the active site (Goossens *et al*, 1995, Makinen *et al*, 1994)

László Polgar has carried out extensive work with regard to the catalytic mechanism of PE In 1991 he reported the existence of two pH dependent forms of the enzyme Serine proteases have a catalytically competent histidine residue, which facilitates both the formation and the decomposition of the intermediate acyl-enzyme and exhibits a pK_a of 7 0 The ionisation of this residue governs the pH dependence of catalysis However, Polgar observed a second significant ionisation event in pH dependent kinetic studies and concluded that a second, low pH (≈5 0) form of the enzyme existed Further investigations into kinetic deuterium isotopic effects on the two enzyme forms, revealed that the rate-determining step of the low pH form was general base/acid catalysis, while a physical step was rate limiting in catalysis performed by the high pH form. In 1992(b), he confirmed this hypothesis and suggested that the physical rate limiting step might be a conformational change, which he confirmed in 1993 He also reported that in contrast to classical serine proteases, hydrogen bond interaction between substrate residue P_2 and enzyme subsite S_2, made a greater contribution to catalysis than stabilisation of the oxyanion binding site

1 3.1.2 Substrate Specificity of Prolyl Endopeptidase

Before discussing the substrate specificity of PE, a brief explanation of the terminology used is warranted The standard nomenclature for referring to the manner in which various substrates bind to complementary sites on a peptidase was introduced by Schechter and Berger (1967) Residues of the substrate are denoted according to their distance from the scissile bond and their respective location on the C-terminal or N-terminal side of this bond, while the subsites on the enzyme to which these residues are bound are similarly designated (Figure 1 2)

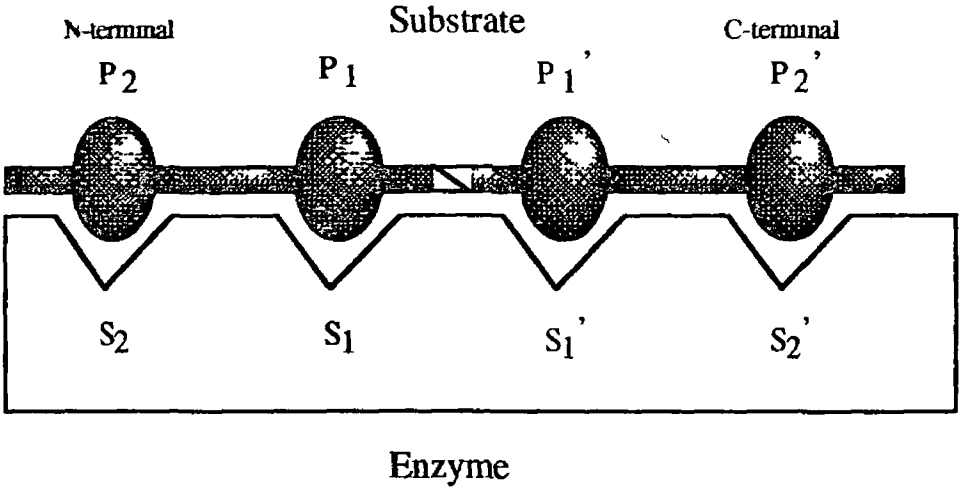


Figure 1.2. *Standard nomenclature for referring to the manner in which various substrates bind to complementary enzyme subsites (Schechter and Berger, 1967) Residues of the substrate are denoted according to their distance from the scissile bond and their respective location on the C-terminal or N-terminal side of the scissile bond (P_1, P_1'), while the subsites on the enzyme to which these residues are bound are similarly designated (S_1, S_1') The scissile bond is located between the P_1 and P_1' substrate residues.*

PE is an endopeptidase that cleaves a Pro-Xaa bond in a structure that consists of an acyl-Yaa-Pro-Xaa sequence Cleavage will not occur if a free α-amine exists in the N-terminal sequence Yaa-Pro-Xaa or Pro-Xaa. Pro-Pro bonds and N-blocked peptides of the sequence Z-Pro-Xaa are not cleaved. In peptides conforming to these restrictions, the P_1 proline can be replaced by alanine but significant losses in hydrolytic activity are observed (Walter *et al.*, 1980) The importance of a P_1 proline was investigated by Nomura (1986), using a substrate of the sequence Z-Gly-Xaa-Leu-Gly Where Xaa was replaced by proline, alanine, N-methylalanine or sarcosine, good substrates resulted Substitution of Xaa with aminobutyric acid, hydroxyproline, serine and glycine were not good substrates while substrates with N-methylvaline and N-methyleucine in the P_1 position were not cleaved Nomura concluded that the S_1 subsite was designed specifically to fit proline This subsite tolerated other residues carrying substituent groups, provided they did not exceed the size of prolines' pyrrolidine ring The requirement

of an imino acid in the P_1 position of a subsite was confirmed by Makinen *et al*, (1994) and Krieg and Wolf (1995) There is a preference for a hydrophobic residue at the C-terminal of the scissile bond (P_1') with lower specificities for basic and acidic residues respectively (Krieg and Wolf, 1995, Taylor *et al*, 1980, Koida and Walter, 1976) Phosphorylation of this residue leads to increased rates of cleavage (Rosen *et al*, 1991) The adjoining P_2 residue (located N-terminally with respect to the P_1 proline), is reported to be bound non-specifically (Krieg and Wolf, 1995) In all, five subsite binding pockets have been identified for PE (S_3-S_2') and the smallest peptide cleaved is a tetrapeptide with residues in positions P_3-P_1' The enzyme cannot cleave large proteins and is reported to hydrolyse a maximum substrate size of 3,000Da, thus its classification as an oligopeptidase The enzyme exhibits high stereo specificity for the two bonds spanning the P_2-P_1' substrate positions (Goossens *et al*, 1995, Makinen *et al*, 1994, Sattar *et al*, 1990, Yoshimoto *et al*, 1988) Proline containing bioactive peptides, conforming to the substrate specificity requirements of PE and subsequently hydrolysed by the enzyme are listed in Table 1 1

1 3.1 3. Assays for Prolyl Endopeptidase Activity

Following the enzymes discovery in 1976 by Walter *et al*, oxytocin and arginine-vasopressin were used in native or radiolabelled forms to detect PE activity Koida and Walter (1976), reported the use of Z-Gly-Pro-Leu-Gly as substrate for PE detection and quantitation, the product (Leu-Gly) being detected by ninhydrin determination (Walter, 1976)

In 1979, two reports of new synthetic substrates were published Yoshimoto *et al*, (1979), synthesised Z-Gly-Pro-MCA (7-amino-4-methyl-coumarin), a fluorimetric substrate, while Orlowski *et al*, (1979), synthesised Z-Gly-Pro-SM (sulphamethoxazole), a colorimetric substrate This basic structure of PE substrates (N-blocked dipeptide Gly-Pro- linked to a chromogenic or fluorogenic moiety) has changed little since Reports of assay development for PE using these substrates, have been published relatively recently (Goossens *et al*, 1992)

1.3.1.4. Inhibitors of Prolyl Endopeptidase

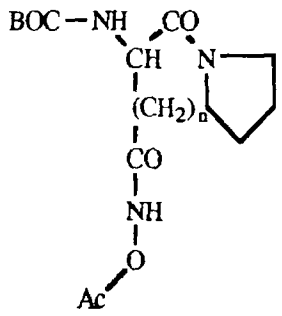
In 1983, Friedman *et al*, published the synthesis of Z-Pro-Prolinal, a specific inhibitor of PE which was described as being a transition state aldehyde inhibitor of this enzyme Since then, many modifications based on this inhibitor have been synthesised There has also been an increase in the number of non-peptide based or microbially sourced PE specific inhibitors A list of PE specific inhibitors is presented in Table 1 2 with structures presented in Figure 1 3

Peptide	Sequence	Reference
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-**Pro-Phe**-His-Leu	Moriyama *et al* , 1988
BPP	pGlu-Gly-Gly-Trp-**Pro-Arg**-Pro-Gly-**Pro-Glu**-Ile-Pro-Pro	Koida and Walter, 1976
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-**Pro-Phe**-Arg	Greene *et al* , 1982
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-**Pro-Gly**-NH_2	Mendez *et al* , 1990
Melanotropin	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-**Pro-Val**-NH_2	Tate *et al* , 1981
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-**Pro-Arg**-Arg-**Pro**-Tyr-Ile-Leu	Camargo *et al* , 1984
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-**Pro-Leu**-Gly-NH_2	Walter *et al* , 1976
Substance P	Arg-Pro-Lys-**Pro-Gln**-Gln-Phe-Phe-Gly-Leu-Met	Moriyama *et al* , 1988
TRH	pGlu-His-**Pro-NH_2**	O'Leary and O'Connor, 1995b
Tuftsin	Thr-Lys-**Pro-Arg**	Tate *et al* , 1980
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-**Pro-Arg**-Gly	Moriyama *et al* , 1988

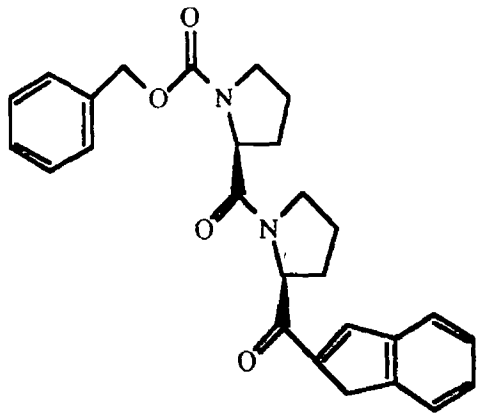
Table 1 1 *Bioactive peptides that are substrates for prolyl endopeptidase The scissile bonds are indicated in bold type TRH - Thyrotropin Releasing Hormone, LHRH - Leuteinising Hormone Releasing Hormone, BPP - Bradykinin potentiating peptide*

Inhibitor	Details	Reference
Poststatin	IC_{50} = 0 03μg/mL	Nagai *et al* , 1991
JTP-4819	IC_{50} = 0 83nM	Toide *et al* , 1995
Eurystatin A	IC_{50} = 0 004μg/mL	Kamei *et al* , 1992
Eurystatin B	IC_{50} = 0 002μg/mL	Kamei *et al* , 1992
Z-Thiopropyl-Thyoprolinal	K_1 = 0 01nM	Tsuru *et al* , 1988
Z-Pro-Prolinal	K_1 = 14nM	Wilk and Orlowski, 1983
α-Ketobenzothiazole	IC_{50} = 5nM	Tsutsumi *et al* , 1994
Z-Phe-Pro-Methylketone	K_1 = 1 8nM	Stemmetzer *et al* , 1993
Z-Cyclohexyl-Prolinal	K_1 = 3 0nM	Bakker *et al*, 1991
Z-Indolinyl-Prolinal	K_1 = 2 4nM	Bakker *et al*, 1991
Boc-Glu(NHO-Bz)-Pyrr	K_1 = 0 03μM	Demuth *et al* , 1993

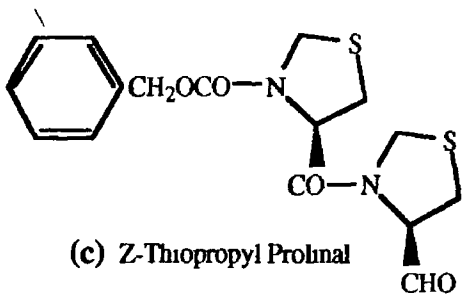
Table 1 2 *PE specific inhibitors Postatin and Eurystatin A and B are prolyl endopeptidase specific inhibitors isolated from fermentations of Streptomyces viridochromogenes MH535-30F3 and Streptomyces eurythermus R353-21 respectively Many of the inhibitors presented are derivatives of the original prolyl endopeptidase specific inhibitor, Z-Pro-Prolinal*



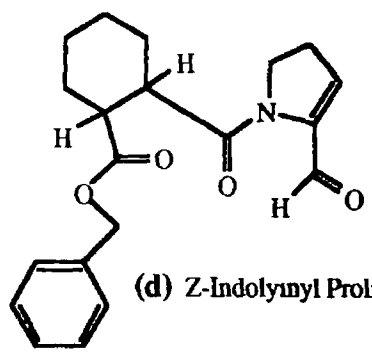
(a) BOC-Glu(NHO-Bz)-Pyr



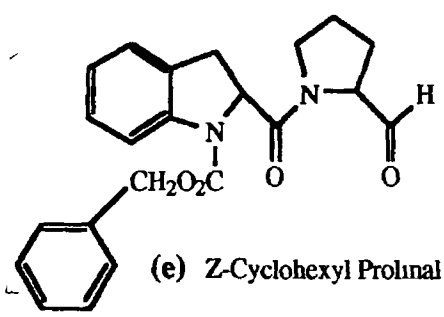
(b) α-ketobenzothiazole



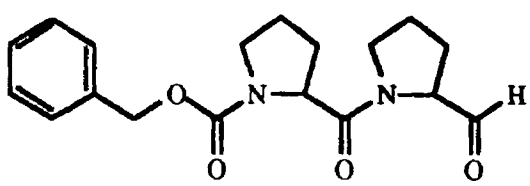
(c) Z-Thiopropyl Prolinal



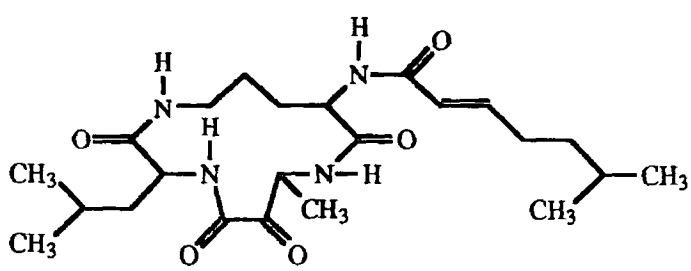
(d) Z-Indolyinyl Prolinal



(e) Z-Cyclohexyl Prolinal



(f) Z-Pro-Prolinal



(g) Eurystatin A

Figure 1 3 *Schematic representations of some prolyl endopeptidase specific inhibitors*

An endogenous inhibitor of PE activity was identified by Yoshimoto *et al*, (1982b) These workers purified the compound and reported it to have a molecular weight of 6 500Da It was distributed widely in both rat and porcine tissues with the highest levels detected in the pancreas Recent studies by Salers (1994), have suggested that this inhibitor is present in neonatal rats but that its levels decrease as the rat ages, explaining why PE activity in neonatal rats increases over the same period Ohmori *et al*, (1994), screened peptides from bovine brain homogenates for inhibitory activity against PE They isolated one octadecapeptide that demonstrated a K_i value of 8 6μM against PE activity obtained from *Flavobacterium meningosepticum*

1 3.1.5. Biological Relevance of Prolyl Endopeptidase

The wide distribution of PE and its activity towards may bioactive peptides (Table 1 1), implies that the enzyme may have important and/or specific biological functions However, despite the many reports involving *in vitro* studies concerning PE, it is only when these findings are confirmed *in vivo*, that the true function of PE will be elucidated For this reason, the *in vivo* implications of PE activity will be discussed.

In vivo studies involving PE can be divided broadly into two categories, those where abnormalities in the activity level of the enzyme are linked to a physiological or disease state (comparative activity studies) and those where the inhibition of the enzyme *in vivo* lead to changes in physiological parameters (cause and effect studies)

1.3.1.5.1. Comparative Activity Studies

By measuring the levels of PE activity in various tissues obtained from patients suffering from varied disease states, and comparing these activities to control levels, links between PE activity and its physiological role can be made In 1994, Maes *et al*, reported a significant decrease in serum PE levels obtained from patients suffering from melancholic, minor and simple major depressions The greatest reductions in enzyme activity were observed in serum from melancholically depressed subjects These workers indicated that peptides known to be involved in the pathophysiology of depression included arginine vasopressin, β-endorphin, TRH and LHRH all of which are natural peptide substrates for PE This group extended the study in 1995 to include manic and schizophrenic patients, confirming their earlier work and noting a significant increase in serum PE activity obtained from subjects suffering from the psychotic disorders They also suggested that PE may be involved in the activation of cell mediated immunity, autoimmune and inflammatory responses, which repeatedly occur in severe depression This link between PE and inflammatory or autoimmune syndromes has been made by other groups studying quite different physiological events

Shoji *et al*, (1989), reported depression of PE activity in inflamed skin, induced in hypersensitive guinea pigs with bovine-γ-globulin The depressed enzyme activity was caused by an endogenous PE inhibitor, generated by the inflammation

In a mouse model of systemic lupus erythematosus, PE activities were increased in the spleen of diseased subjects when compared to controls This increase was progressive with age and indicated an important role played by PE in the immunopathological disturbances associated with this syndrome (Aoyagi *et al*, 1985 and 1987) A further link between PE and immunological disturbances was made by Kanori *et al*, (1991), when they reported increased PE levels in synovial membrane preparations from patients suffering from rheumatoid arthritis

Abnormally high or low PE activities have also been linked to neurodegenerative disorders In studies evaluating the PE levels in postmortem brains, obtained from subjects who had suffered from Alzheimer's disease, significant increases in the levels of enzyme activity were observed (Aoyagi *et al*, 1990) Increases in PE activity were also observed in homogenates of spinal cord obtained from patients suffering from motor neuron disease (Falkous *et al*, 1995) However, in studies investigating the physiology of Parkinsonian and Huntington's Chorea patients, lowered PE activities were observed in the cerebrospinal fluid and basal ganglia respectively (Hagihara *et al*, 1987, Pittaway *et al*, 1984)

1.3.1.5.2. Cause and Effect Studies

The application of PE specific inhibitors has revealed further physiological possibilities for this enzyme

In 1995, Miura *et al*, reported that inhibition of PE activity in the brain of rats, through the oral administration of Z-Pro-Prolinal, significantly increased the levels of arginine vasopressin obtained in the septum area of the rat brain They deduced from this study that PE may contribute to the degradation of endogenous arginine vasopressin in the brain

Amnesia, induced in rats via administration of scopolamine, was reported to be protected against by eurystatins A and B, two PE specific inhibitors of microbial origin (Kamei *et al*, 1992) This confirmed an earlier report by Yoshimoto *et al*, (1987), whereby Z-Pro-Prolinal also protected rats from scopolamine induced amnesia. It is thought that the inhibition of PE prevents its activity towards Pyr-Asn-Cys-Cys-Pro-Arg, a metabolite of vasopressin which was deduced as having a direct involvement in memory and related processes (DeWied *et al*, 1984)

1.3.2. Dipeptidyl Peptidase IV (EC 3.4.14.5)

Dipeptidyl Peptidase IV was discovered by Hopsu-Havu and Glenner (1966) while studying enzymatic activities in rat liver They observed an activity capable of degrading Gly-Pro-β-naphthylamide and called this enzyme glycylprolyl-β-naphthylamidase Further substrate specificity studies carried out by McDonald *et al* (1971), led to its referral as dipeptidyl aminopeptidase IV The distribution of dipeptidyl peptidase IV seems to be ubiquitous, with activities purified from many mammalian, insect and bacterial sources (Table 1 3)

Source	Localisation	Reference
Flavobacterium meningosepticum	Soluble	Kabashima *et al.*, 1995
Porphyromonas gingivalis	Soluble	Gazi *et al*, 1995
Bovine kidney	Membrane	Brownlees *et al.*, 1992
Bovine uterus	Soluble	Liu and Hansen, 1995
Canine intestine	Membrane	Pemberton *et al*, 1995
Guinea-pig brain	Membrane	Smyth and O'Cuinn, 1994
Human lung	Membrane	Jackman *et al.*, 1995
Human lymphocytes	Membrane	Kurktschiev *et al*, 1993
Human oesophagus	Membrane	Christie *et al*, 1995
Human seminal plasma	Soluble	DeMeester *et al.*, 1996
Mouse lymphocyte	Membrane	Bernard *et al*, 1994
Porcine oesophagus	Membrane	Christie *et al*, 1995
Porcine seminal plasma	Soluble	Ohkubo *et al*, 1994
Rabbit intestine	Membrane	Bai, 1993
Rat brain	Soluble and Membrane	Alba *et al*, 1995

Table 1 3 *Distribution of Dipeptidyl peptidase IV (DPPIV) This table illustrates the ubiquitous nature of DPPIV among micro-organisms and various mammalian tissues DPPIV can be localised in the cytoplasm (soluble) but it is more common to find the enzyme membrane bound*

The enzyme has been characterised in both membrane and soluble forms and has also been identified as an important signalling molecule on T and β lymphocytes, where it is referred to as CD26

1 3 2 1 Biophysical And Biochemical Aspects of Dipeptidyl Peptidase IV

Dipeptidyl peptidase IV (DPPIV) is a glycoprotein Nine glycosylation sites have been identified within the DPPIV peptide sequence and sugars associated with these sites include sialic acids, mannose and galactose (Misumi *et al*, 1992, Marguet *et al*, 1992, Kyouden *et al*, 1992) The mammalian form is reported to be a dimer, with subunit molecular weights in the range of 100kDa-130kDa (Abbs and Kenny, 1983, Erikson *et al*, 1983, Puschel *et al*, 1982) There have been reports of 2 different molecular weight versions of DPPIV in human serum with monomeric values ranging from 105kDa to 175kDa and native values of 250kDa-550kDa (Duke-Cohan *et al*, 1996, Krepela *et al*, 1983) The structure of DPPIV has been deduced from its cDNA obtained from a variety of sources cDNA from *Flavobacterium meningosepticum*, human, murine and rat DPPIV code for 711, 766, 760 and 767 amino acids respectively The bacterial protein had a deduced subunit molecular weight of 80,626Da while the mouse and rat values were 87,500 and 88,107 Daltons respectively These values do not consider the added weight of glycosylation (Kabashima *et al*,.1995, Misumi et al, 1992, Tanaka et al, 1992, Marguet *et al*, 1992, Ogata *et al*, 1989) The gene coding for human DPPIV has been located on the long arm of chromosome 2 (2q24) (Darmoul *et al*, 1994, Abbott *et al*, 1994)

The pH optimum of DPPIV, though dependent to some degree on the substrate used, ranges from 8 0-9 0, with a general consensus of maximum activity obtained at pH 8 5 Similarly, pI values range from 3-5, with the majority of reports quoting pI figures of 4 7 Interestingly, conflicting reports as to the effect of neuraminidase, an enzyme that removes neuraminic acid from protein glycosylation sites, on the pI of DPPIV have been published, with workers observing both increased and decreased pI values for DPPIV following neuraminidase treatment (Liu and Hansen, 1995, DeMeester *et al*, 1992, Mineyama and Saito, 1991, Yoshimoto *et al*, 1982)

DPPIV has been classified mechanistically as a serine protease due to its inhibition by DFP and its resistance to inhibition by sulphydryl blocking agents and chelators (Ohkubo *et al*, 1994, Schon, 1993, Hama *et al*, 1982) Sequencing and cDNA studies have revealed more specific details regarding the active site of this protease The catalytic site is located at the C-terminal region of the polypeptide This has been described as the protease domain, which is attached to a non-catalytic structural domain (Loster *et al*, 1995, Polgar and Szabo, 1992) The active site catalytic triad has been identified as Ser624, Asp702 and His734 in murine DPPIV but the active site serine residue in the human enzyme is identified as Ser631 (David *et al*, 1993, Fujiwara *et al*, 1992) In humans, the consensus sequence for the active site serine residue is Gly-Xaa-Ser-Xaa-Gly This sequence is identified in DPPIV as Gly629-Trp-Ser-Tyr-Gly633 Substitution of serine or either of the glycine residues in this sequence and subsequent expression of the mutated protein results in the production of inactive enzyme (Fujiwara

et al, 1992, Ogata *et al*, 1992) The novel topology of the catalytic triad (Ser, Asp, His) residues and the lack in sequence homology between DPPIV and classical serine proteases (eg trypsin) indicates that this enzyme is a member of a recently identified subclass of serine proteases (David *et al*, 1993) Detailed thermodynamic analysis of the reaction mechanism of DPPIV has led Brandt *et al* (1996), to propose a novel mechanism for serine protease catalysis They suggest a stabilisation of the tetrahedral intermediate by oxazolidine ring formation with the P_2 - P_1 non-scissile bond and trans-cis isomerisation of this bond in the final steps of bond cleavage

1 3.2 2. Substrate Specificity of Dipeptidyl Peptidase IV

DPPIV cleaves dipeptides from substrates consisting of three or more amino acid residues or dipeptides linked to C-terminal chromogenic or fluorogenic compounds such as 2-naphthylamides or methylcoumarin amides (Yaron and Naider, 1993) Proline is the preferred residue at position P_1, but it may be substituted by alanine or hydroxyproline, resulting in lower activities against these substrates It should also be noted that the catalytic mechanism by which DPPIV cleaves dipeptides with P_1 alanine is different to the mechanism where P_1 is a proline residue (Hems *et al*, 1988) Bonds where both P_1 and P_1' residues are proline, are not cleaved and there is an absolute requirement for the P_1-P_1' bond to be in trans configuration (Puschel *et al*, 1992, Fischer *et al*., 1983)

Bovine growth hormone releasing factor (GRF) has been studied extensively with regards to DPPIV specificity The N-terminal sequence of this peptide, Tyr-Ala-Asp-, is cleaved quite efficiently by the enzyme However, substitution of the Tyr-Ala- moiety by His-Val- , protects GRF from DPPIV hydrolysis, retaining the peptides potency and indicating the importance of the P_1 residue (Campbell *et al*, 1994)

Bongers *et al* (1992), carried out extensive studies into the specificity of DPPIV for GRF and its analogues They observed no differences in the rates of hydrolysis obtained for GRF(1-44), GRF(1-29) and GRF(1-20) Lower rates of cleavage were observed for GRF(1-11) and GRF(1-3) indicating that substrate binding by the enzyme extends beyond the S_1' subsite Substitution of D configuration residues in positions P_2, P_1 or P_1' resulted in no hydrolytic activity, demonstrating DPPIV's strict requirement for L-residues in these positions Indeed, the requirement for L-residue configuration extends to P_4', indicating an S_4 subsite These workers also investigated the kinetic effects observed when alanine in the P_1 position was substituted The results obtained were as follows

Km Abu<Ala<Pro<Val<Ser<Gly<<Leu and kcat Abu>Pro>Ala>Ser>Gly=Val>>Leu

where Abu is α-aminobutyric acid The position of proline and alanine is particularly interesting As stated earlier, high hydrolytic activities are obtained when the P_1 position is occupied by proline However, DPPIV demonstrates greater specificity towards GRF with alanine in this position

Bongers *et al*, concluded that the ethyl side chain of α-aminobutyric acid was close to the optimal P_1 specificity of DPPIV which they described as being a hydrophobic residue, 0 25nm in length

Other substrates of interest are substance P and β-casomorphin The action of DPPIV on substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met), produces des[Arg^1-Pro^2] substance P which is subsequently hydrolysed to des[Arg^1-Pro^4] (Conlon and Sheehan, 1983) β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is cleaved sequentially at Pro^2 and Pro^6 (Kreil *et al*, 1983)

1.3 2.3. Assays for Dipeptidyl Peptidase IV Activity

Assays for the enzyme are based primarily on substrates whereby a chromophore or fluorophore is attached to the C-terminus of the dipeptide Gly-Pro- (Shibuyu-Saruta *et al*, 1995) An alternative assay for the enzyme was developed by Hoffman *et al* (1995), when capillary electrophoresis was used to analyse the fragments produced following the action of DPPIV on the N-terminal dodecapeptide of interleukin-2

1.3.2.4. Specific Inhibitors of Dipeptidyl Peptidase IV

Diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) are well known specific inhibitors of DPPIV activity and have been used extensively in the identification of this enzyme (Sedo and Revoltella, 1995, Umezawa *et al*, 1984)

Aminoacylpyrrolidine-2-nitriles have been recently developed to specifically inhibit DPPIV The carboxyl group of proline is replaced by a nitrile which is thought to interact with the active site serine, forming an imidate adduct. These compounds are synthesised easily, yet remain potent and stable DPPIV inhibitors (Li *et al*, 1995)

Some of the most effective DPPIV inhibitors are of the type Xaa-boroPro, where boroPro is an amino boronic acid analogue of proline In 1993, Gutheil and Bachovchin reported that the L-L-diastereomer of Pro-boroPro inhibited DPPIV with a K_i of 16 picomolar

1.3.2 5. Physiological Relevance of Dipeptidyl Peptidase IV

The ubiquitous distribution of DPPIV, implies that it may play an important role in different *in vivo* physiological processes It is likely that the localisation of the enzyme will influence the particular function is performs

1 3.2 5.1. Absorption Of Proline Containing Peptides.

The absorption or recycling of proline containing peptides is a vital process DPPIV's contribution to this process is twofold due to its brush border localisation in the small intestine and the kidney The functional role of DPPIV in the intestinal hydrolysis and assimilation of prolyl peptides was

investigated by Tirupathi *et al*, (1993), using rats which were genetically deficient in this enzyme When a reference diet, where proteins were normal in proline content, was fed to DPPIV deficient rats (DDR) and an appropriate control group, no difference in the growth rates of either group was observed When the protein source was changed to a proline rich protein, gliadin, the control groups maintained their weights, while the DDR group experienced significant weight loss Similarly, when Suzuki *et al*, (1993), fed high proline content diets to rats, the expression of intestinal brush border DPPIV increased dramatically, confirming the proteases role in the hydrolysis and assimilation of prolyl peptides and proline rich proteins Similar experiments carried out on DDR rats, but concentrating on the renal absorbtion of proline containing peptides, led workers to conclude positively with regards to the physiological role played by DPPIV (Miyamoto *et al*, 1987, Tirupathi *et al*, 1990)

1.3.2.5.2. Immunological Relevance

The role of DPPIV in the immune system became of interest when its presence on the surface of human peripheral lymphocytes as an ectoenzyme was demonstrated (Lojda, 1977, Sedo *et al*, 1989) It was soon concluded that a T-lymphocyte surface antigen, designated CD26, and a leukocyte activation marker (Tp103) were in fact DPPIV (Fox *et al*, 1984, Hegen *et al*, 1990) The enzyme is now known to be an activation marker on T-cells, natural killer cells and ß-cells (Buhling *et al*, 1995) The precise details on the mechanism and nature of its interaction with physiological substrates, and it subsequent activation of lymphocytes are unclear It is known that substrate or mitogenic interaction with CD26 can cause lymphocyte differentiation, increases in the rate of DNA synthesis and increased cytokine production It is also known that, although activation molecules need not be targeted directly to the active site of DPPIV, its enzymatic activity is required for signal potentiation (Dong *et al*, 1990, Kahne *et al*, 1995, Reinhold *et al*, 1994)

1.3.3. Dipeptidyl Peptidase II (EC 3.4.14.2)

Dipeptidyl peptidase II (DPPII) is, in may ways, similar to DPPIV, in that it catalyses the removal of N-terminal dipeptides from substrates Detailed biochemical and substrate specificity studies, however, distinguish this enzyme from DPPIV

The enzyme is primarily lysosomally located and is widely distributed It is a dimeric protein with reported molecular weights of between 80kDa and 130kDa for the native enzyme The protein is glycosylated (approximately 2% w/w) with mannose representing most of the attached sugar moieties Reported pH optima for DPPII range from 5 5 to 6 3, with a consensus among most reports that the pH optimum is 5 5, which is appropriate to its subcellular localisation The enzyme has a pI of 4 8-5 0, with no change being observed following treatment with neuraminidase, indicating that there are no sialic acids taking part in its glycosylation The enzyme has been classed as a serine protease, based on its inhibition by DFP and its resistance to sulphydryl blocking and chelating agents

DPPII removes N-terminal dipeptides from substrates of the general sequence Xaa-Pro-Yaa Substitution of the P_1 proline with alanine is accepted and results in comparable hydrolytic rates which is unusual among the proline specific enzymes discussed thus far Favoured residues in the P_2 position are basic and neutral amino acids, with 100 fold decreases in hydrolytic activity being observed with acidic residues at this position Another unusual aspect of the substrate specificity of DPPII is its ability to cleave Xaa-Pro-Pro bonds Tripeptides are the preferred chain length, with decreased activity towards tetrapeptides Substrates with more than four residues are not cleaved (Lynn, 1991 Mentlein and Struckhoff, 1989, Scott *et al*, 1988, Lampelo *et al*, 1987, Eisenhauer and McDonald, 1986, Fukusawa *et al*, 1983)

Lys-Ala-MCA was synthesised by Nagatsu *et al*, (1985), for the specific determination of DPPII activity in rat tissues This substrate and variations on the Lys-Ala theme are still used as the preferred substrate for DPPII activity determinations (Smyth and O'Cuinn, 1994)

DPPII is commonly used as a lysosomal marker enzyme It is thought to play a physiological role in peptide catabolism and post-translational modification, with suggestions that it plays an important role in the catabolic processes of cell differentiation (Roberts *et al*, 1990, Duve *et al*, 1995, Struckhoff, 1993) Significant increases in DPPII activity have been reported in the brain and cerebrospinal fluid of patients suffering from Parkinson's and Huntington's disease (Mantle *et al*, 1995, Hagihara *et al*, 1987b)

1.3.4. Aminopeptidase P (EC 3.4.11.9.)

Aminopeptidase P was first isolated from the soluble fraction of *Escherichia coli* by Yaron and Mlynar, (1968) These workers were studying proline containing polypeptides and the specificity of their enzymatic hydrolysis Using polymers of proline as substrates, several proteolytic enzymes were detected and isolated from bacteria. Aminopeptidase P was isolated and characterised during these investigations and was found to be responsible for the specific cleavage of N-terminal Xaa-Pro peptide bonds in both short and long peptides (Yaron and Berger, 1970) The enzyme is widely distributed among mammalian and microbial sources (Table 1 4)

1.3.4.1. Biophysical and Biochemical Characteristics of Aminopeptidase P

The molecular weight of Aminopeptidase P (APP) and its subunit structure are source dependent. Bacterial APP has been the best studied to date, and is reported to be multimeric with the molecular weight of the inactive monomer ranging from 50kDa to 60kDa. A cloned and sequenced APP from *E coli* had a monomeric molecular weight of 49,650 Da, deduced for 440 amino acid residues Subsequent expression of this gene revealed a native molecular weight of 200kDa, indicating a tetrameric subunit structure (Yoshimoto *et al*, 1988b and 1989) Oyama *et al*, (1989) obtained similar results when *E coli* APP was expressed in *Bacillus subtilis*

Source	Enzyme localisation	Reference
Bovine adrenal medulla	Soluble	Vanhoof *et al*, 1992b
Bovine lung	Membrane	Orawski *et al.*, 1987
Escherichia coli	Soluble	Yaron and Mlynar, 1968
Guinea pig serum	Soluble	Ryan *et al*, 1990
Human erythrocytes	Soluble	Sidorowicz *et al.*, 1984b
Human kidney	Membrane	Hooper and Turner, 1988
Human leukocytes	Soluble	Rusu and Yaron, 1992
Human lung	Membrane	Sidorowicz *et al.*, 1984
Human serum	Soluble	Holtzman *et al*, 1987
Lactococcus lactis	Soluble	Mars and Monnet, 1995
Rat brain	Soluble	Harbeck and Mentlein, 1991
Streptomyces lividans	Soluble	Butler *et al.*, 1993

Table 1 4 *Distribution of Aminopeptidase P This table is not an exhaustive list but rather a means to illustrate the ubiquitous nature of Aminopeptidase P among micro-organisms and mammals of different species*

Mammalian APP, however, varies with regard to its native molecular weight and subunit makeup Soluble forms of the enzyme, isolated from rat brain and human leukocytes, were dimers with a native molecular weight of 140kDa (Harbeck and Mentlein 1991, Rusu and Yaron, 1992) The rat brain soluble APP, however, was revealed to be trimeric when the native molecular weight was estimated by gel filtration chromatography in the absence of salt, indicating that its native molecular weight was dependent on salt concentration (Harbeck and Mentlein, 1991) Trimeric forms of soluble APP were also reported for guinea-pig serum and human platelets with native molecular weights of 220kDa (Ryan *et al*, 1992, Vanhoof *et al*, 1992) Similarly, a trimer of 280kDa was observed when membrane bound APP was isolated from porcine kidney by Hooper *et al*, (1990) APP from bovine lung was found to be tetrameric with a monomeric molecular weight of 95kDa. It is not surprising that the mammalian APP subunit molecular weight is considerably larger than that of the bacterial enzyme as recently, Vergas Romero *et al*, (1995), reported that the amino acid sequence of mammalian APP from porcine kidney consisted of 623 amino acids

Bacterial and, soluble and membrane mammalian APP, have been classified as metalloproteases based on their inhibition by metal chelators (Yoshimoto *et al*, 1989; Simmons and Orawski, 1992, Harbeck and Mentlein, 1991) This inhibition is caused by the removal of an essential metal atom from the active site of the enzyme Hooper *et al*, (1992), have recently identified the APP active site metal atom as zinc The metal atom provides an electrophilic "pull", co-ordinating the nucleophilic attack by water on the peptide bond It is co-ordinated tetrahedrally within the active site, being bound to two histidines residues A third attachment is to a water molecule which remains in the active site following substrate binding and may take part in the nucleophilic attack on the substrate A fourth interaction occurs between substrate and metal ion subsequent to binding (Dunn, 1989) The anchoring of the metal atom by two histidine residues in the active site is relevant as Lim and Turner, (1996), have recently identified two histidine residues in porcine kidney APP that are critical for enzyme activity Some confusion arises in the literature as to the effect of different divalent metal ions on APP activity Zinc is reported to be inhibitory towards APP from varied sources including porcine kidney, rat brain, human platelets and leukocytes (Harbeck and Mentlein, 1991, Hooper *et al*, 1990, Rusu and Yaron, 1992, Vanhoof *et al*, 1992) Zinc has also been reported as been activatory towards APP isolated from guinea-pig serum (Ryan *et al*, 1992) Divalent metal ions commonly associated with the activation of APP, or its reactivation following exposure to chelators, are Mn^{2+} and Co^{2+} (Rusu and Yaron, 1992, Hooper *et al*, 1990; Orawski and Simmons, 1995, Fleminger and Yaron, 1984) Only one report concerning human lung APP (Sidorowicz *et al*, 1984) showed no inhibition of the enzyme by chelators These workers did observe a decrease in APP activity but concluded that this decrease was due to the chelation of activating metal ions that were loosely associated with the enzyme

The inhibition of APP by thiol functional reagents has also been widely reported (Mars and Monnet, 1995, Ryan *et al*, 1992, Sodorowicz *et al*, 1984b) These results concur with the identification of four cysteine residues per subunit in the cloned *E coli* APP (Yoshimoto *et al*, 1989) The cysteine

residues might not be located in the active site, yet retain a functional significance The inhibition of APP by thiol reagents, such as DTT may be a competitive interaction between the reagent and substrate for the fourth metal atom co-ordination position (Campbell *et al*, 1988) Care must be taken when interpreting the inhibition of APP by metal ions or thiol functional reagents as these observed characteristics may be substrate dependent (Lloyd and Turner, 1995)

Optimal APP activity is obtained at pH 7 0 - pH 8 5 at an optimum temperature of 43°C (Rusu and Yaron, 1992, Orawski and Simmons, 1995) One interesting point, with regards to the biophysical and biochemical attributes of APP, was reported by Orawski and Simmons (1995) who observed that a membrane bound rat lung APP was inhibited by NaCl, complete inhibition being observed at 2M NaCl This may relate to the salt dependent nature of multimeric APP forms discussed earlier Considering that monomeric forms of the enzyme are reported to be inactive, the inhibition of APP activity by high salt might indicate that cooperativity between subunits is essential for substrate hydrolysis.

1.3.4.2. Substrate Specificity of Aminopeptidase P

Although substrate specificity studies on mammalian APP are not as complete as those of the bacterial enzyme, both lead to similar conclusions APP is an exopeptidase which hydrolyses a variety of peptides ranging in size from dipeptides to proteins It cleaves between an N-terminal amino acid (P_1) and a penultimate proline (P_1') The N-terminal amino acid must have a free amino group, the penultimate residue must be proline and the scissile bond must be in the *trans* configuration (Lin and Brandts, 1979)

In 1983, Flemmger and Yaron reported the cleavage of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) by immobilised bacterial APP, with arginine and proline being released sequentially from the N-terminus. Reports of mammalian APP cleaving bradykinin followed (Sidorowicz *et al*, 1984, Ward *et al*, 1991, Hooper *et al*, 1992) These workers also observed the cleavage of dipeptides such as Phe-Pro, Arg-Pro and Ala-Pro Harbeck and Mentlein, (1991), reported that APP isolated from rat brain cytosol, selectively liberated all unblocked ultimate (P_1) residues from di-, tri- and oligopeptides with N-terminal Xaa-Pro- sequences, where Xaa (P_1) was preferentially a hydrophobic or basic residue Replacement of the penultimate residue (P_1') by alanine or any residue other than proline, led to decreased or no hydrolysis being observed

Bioactive peptides cleaved include bradykinin, substance P and melanostatin (Simmons and Orawski, 1992) Peptide-YY (PYY) cleavage by APP has also been reported (Medeiros and Turner, 1994) Yoshimoto *et al*, (1988c), identified a third subsite (S_2') in bacterial APP which preferentially bound aromatic amino acids at the P_2' position This preference was confirmed when Gly-Pro-pNA, Gly-Pro-pNA and Gly-Pro-MCA were observed as excellent substrates for the enzyme

In 1994, Yoshimoto *et al.*, carried out detailed substrate specificity studies on APP isolated from *E. coli*. These workers found that dipeptides were hydrolysed very slowly relative to longer substrates and a fourth enzyme subsite was identified (S_3'). S_1 and S_1' were highly stereospecific with preferential binding of hydrophobic residues at S_1. They also noted that bacterial APP could accommodate most amino acid residues in the S_2' subsite. This was in contrast to mammalian APP which could not accommodate bulky P_2' residues. The presence of four substrate binding sites for mammalian APP was confirmed by Orawski and Simmons, (1995), who also reported that the S_1, S_1' and the S_2' subsites had to be occupied for substrate hydrolysis to occur.

1.3.4.3. Assays for Aminopeptidase P Activity

The original assay procedures used to detect APP activity were developed by Yaron and Berger, (1970), and were based on the cleavage of polyproline. These substrates proved insufficient however, as they were neither sensitive nor specific, and were only hydrolysed slowly by purified mammalian APP (Rusu and Yaron, 1992; Harbeck and Mentlein, 1991). Specific substrates of the sequence Xaa-Pro-Pro were developed as they were resistant to dipeptidyl aminopeptidase and carboxypeptidase hydrolysis. Fleminger *et al.*, (1982), developed an intramolecularly quenched fluorimetric substrate, Lys-(Dnp)-Pro-Pro-Eda-Abz. The fluorophore, 2-aminobenzoyl (Abz), was quenched by the dinitrophenol (Dnp) moiety. Following the removal of Lys(Dnp) by APP, Abz could be detected fluorimetrically, resulting in a sensitive and specific assay for the enzyme.

Coupled enzyme assays have also been developed and used successfully for the detection of APP. These include colorimetric and fluorimetric substrates such as Gly-Gly-Pro-pNa (Lasch *et al.*, 1988) and Gly-Pro-MCA (Yoshimoto *et al.*, 1988), coupled with the enzymes dipeptidyl aminopeptidase IV or proline iminopeptidase respectively.

Radiolabelled assays are also in use, including radiolabelled bradykinin (^{3}H-Bradykinin) and a radiolabelled bradykinin fragment, (Arg-Pro-Pro-^{3}H)-benzylamide (Ryan *et al.*, 1992b).

1.3.4.4. Specific Inhibitors of Aminopeptidase P

Prechel *et al.*, (1995), designed the APP inhibitor, apstatin, based on an N-blocked tripeptide (Pro-Pro-Ala-NH_2). It was synthesised specifically to facilitate the inhibition of APP during investigations into the degradation of bradykinin, and was reported to have no inhibitory effect on angiotensin converting enzyme (ACE) or other known bradykinin degrading enzymes. It inhibited APP with a K_i of 2.6μM.

1.3.4.5. Physiological Relevance of Aminopeptidase P

A single report of a disease linked to human APP deficiency has been published by Blau *et al.* (1988). These workers discovered a new inborn error of metabolism, whereby a deficiency in APP led to the development of peptiduria.

However, the specificity of APP suggests that this enzyme may have a unique biological role Table 1 5 lists a number of bioactive polypeptides that contain N-terminal Xaa-Pro- bonds (Yaron and Naider, 1993) These include hormones, toxins and enzymes Because of the N-terminal Xaa-Pro bond, these peptides are ideal substrates for APP Their hydrolysis might lead to a loss or alteration in biological activity, or initiate a cascade of events leading to the eventual termination of biological activity and subsequent peptide/protein turnover For example, the action of APP on peptide-YY regulates the interconversion of this peptides' receptor selectivity (Medeiros and Turner, 1994)

It is also possible that APP plays an important role in the trimming of biosynthetic precursors during post-translational modification The specificity of APP makes it ideal for the final post-translational modification steps involving peptides containing an N-terminal proline

The high APP activity found in various blood components may also be of physiological significance Scharpe *et al* (1990) and Hendriks *et al*, (1991) reported that the majority of APP found in blood was located inside platelets, unlike other exopeptidases such as ACE and DAP IV These findings support the hypothesis that APP is involved in the regulation of cardiovascular and pulmonary functions Ryan *et al*, (1990) observed that APP can act *in vitro* on the antiarrhythmic peptide (Gly-Pro-Hyp-Gly-Ala-Gly) and substance P, peptides that are associated with cardiovascular physiology In 1995, Prechel *et al* reported that APP and ACE working together, fully accounted for the degradation of bradykinin in the rat pulmonary vascular bed Also, in 1995, Kitamura *et al* reported that blood pressure responses to administered bradykinin following treatment of rats with apstatin, were doubled They concluded that APP was an important *in vivo* kininase To combat the effects of APP on bradykinin activity, Ward *et al*, (1991), have developed bradykinin receptor agonists and antagonists resistant to APP attack B7644, an agonist of bradykinin, was found to be resistant to APP hydrolysis and more potent than the natural peptide

1.3.5. Prolidase (EC 3.4.13.19.)

Prolidase was first discovered by Bergmann and Fruton, (1937), while studying aminopeptidase and carboxypeptidase activities in porcine intestinal mucosal extracts It was subsequently purified and characterised from porcine kidney, and porcine and bovine intestine (Davis and Smith, 1957, Sjostrom *et al*, 1973, Yoshimoto *et al*, 1983)

Prolidase is closely related to aminopeptidase P in that it removes N-terminal amino acids from substrates with proline in the penultimate P_1' position yet it is manifestly dipeptide specific Like APP, the enzyme is widely distributed among mammalian and microbial sources (Table 1 6)

1 3.5 1 Biophysical and Biochemical Aspects of Prolidase

The molecular weight of prolidase, like the molecular weight of aminopeptidase P, is difficult to state definitively In previous reviews on proline specific peptidases, the native molecular weight of a

Polypeptide	N-terminal sequence
Basic trypsin inhibitor	Arg-Pro-Asp-Phe-Cys-
Bradykinin	Arg-Pro-Pro-Gly-Phe-
β-casomorphin	Tyr-Pro-Phe-Pro-Gly-
Cathepsin B	Leu-Pro-Ala-Ser-Phe-
Cathepsin D	Gly-Pro-Ile-Pro-Glu-
Cathepsin H	Gly-Pro-Tyr-Pro-Gly-
Cathepsin L	Leu-Pro-Asp-Ser-Val-
Eledoisin	pGlu-Pro-Ser-Lys-Asp-
Erythropoietin	Ala-Pro-Pro-Leu-Leu-
Factor XII	Ile-Pro-Pro-Trp-Glu-
Growth hormone	Phe-Pro-Ala-Met-Pro-
Interleukin 2	Ala-Pro-Thr-Ser-Ser-
Neurotensin 9-13	Arg-Pro-Tyr-Ile-Leu-
Papain	Ile-Pro-Glu-Tyr-Val-
Plasminogen	Glu-Pro-Leu-Asp-Asp-
Prolactin	Leu-Pro-Ile-Cys-Ser-
Streptavidin	Asp-Pro-Ser-Lys-Asp-
Substance P	Arg-Pro-Lys-Pro-Gln-
Thyrotropin	Phe-Pro-Asp-Gly-Glu-
Trypsinogen	Ala-Pro-Asp-Asp-Asp-

Table 1 5. *List of bioactive peptide sequences that contain proline in the N-terminal penultimate position These peptide sequences are ideal substrates for Aminopeptidase P (Yaron and Naider, 1993)*

Source	Reference
Bovine intestine	Yoshimoto *et al.*, 1983
Human erythrocytes	Ohhashi *et al.*, 1990
Human fibroblasts	Oono *et al*, 1990
Human kidney	Myara *et al.*, 1994
Human liver	Endo *et al.*, 1987
Human plasma	Cosson *et al.*, 1992
Human prostate	Masuda *et al.*, 1994
Lactobacillus delbruecku	Stucky *et al.*, 1995
Porcine intestine	Sjostrom *et al*, 1973
Porcine kidney	Mock and Liu, 1995
Rabbit kidney	Endre and Kuchel, 1985
Rat brain	Middlehurst *et al.*, 1989
Rat liver	Miech et al., 1988
Xanthomonas maltophilia	Suga *et al.*, 1995

Table 1.6 *Distribution of Prolidase This table is not an exhaustive list but rather a means to illustrate the ubiquitous nature of Prolidase among micro-organisms and various mammalian tissues Prolidase is localised in the cytoplasm (soluble) There have been no reports regarding a membrane form of the enzyme*

dimeric prolidase was reported as 110kDa (Yaron and Naider, 1993, Walter *et al*, 1980) Yaron and Naider based this value on the results of Endo *et al*, (1989) A combination of human prolidase gene cloning and Edman sequence determination allowed these workers to deduce a monomeric molecular weight of 54,305 Da, which agreed with an estimation of 56kDa for human erythrocyte prolidase and more recent results (Richter *et al*, 1989, Ohhashi *et al*, 1990, Myara *et al* 1994 Suga *et al*, 1995) However, in 1985, Butterworth and Priestman reported the separation of two dimeric forms of prolidase (I and II) from human tissues with native molecular weights of 112kDa and 185kDa. This was later confirmed by Oono *et al*, (1990) and Masuda *et al*, (1994)

With the exception of one report (Suga *et al*, 1995), the pI of mammalian and bacterial prolidase is 4 6 A pH optimum of 7 5 has been observed for all forms of the enzyme (Stucky *et al*, 1995, Myara *et al*, 1994, Oohashi *et al*, 1990)

Biochemical characterisations of prolidases in general indicate that they are Mn^{++} activated yet inhibited by sulphydryl group reagents, indicating that the enzyme is a cysteine or metallo-protease Prolidase type II however was reported to be inhibited by Mn^{++} ions (Butterworth and Priestman, 1984, Sjostrom and Noren, 1974, Masuda *et al*, 1994, Cosson *et al*, 1992) King *et al*, (1989), identified an active site Mn^{++} ion and concluded that prolidase is a metalloenzyme dimer whose subunits exhibited selective co-operative interaction pH dependent kinetic studies of prolidase activity towards picolinylprolines suggested an active site mechanism involving the participation of two acidic metal ions positioned adjacently within the active site (Mock and Green, 1990, Mock and Liu, 1995) A functional group within the active site having a pK_a of 6 6 has been widely reported This functional group is essential for catalytic activity and has been identified as a water molecule, rendered acidic through its co-ordination with the active site metal ion Productive chelation to this metal ion by the P_1 substrate residue was observed with subsequent displacement of the functional water molecule (Mock and Liu, 1995, Radzicka and Wolfenden, 1991, Mock *et al*, 1990) Active site arginine and aspartic acid functional residues have also been identified (Mock and Zhuang, 1991)

1.3 5 2 Substrate Specificity of Prolidase

Prolidase hydrolyses dipeptides in which the C-terminal residue is proline and the N-terminal residue is an amino acid with a free α-amino group Proline can be replaced by hydroxyproline (Hyp), resulting in significant losses in hydrolytic activity Tissue culture studies illustrated that an auxotrophic Chinese hamster ovary (CHO) cell line could utilise Gly-Pro as a proline source through the action of prolidase Inability to similarly utilise Gly-Hyp was observed, indicating prolidase's poor activity towards this substrate (Emmerson and Phang, 1993) When proline is replaced at the C-terminus by Thi, a proline analogue whereby a sulphur atom is substituted into the pyrrolidine ring, the dipeptide Gly-Thi was hydrolysed at 2 7 times the rate of Gly-Pro (Yaron and Naider, 1993) It was also discovered that Z-pipecolic acid exerted the same level of inhibition towards prolidase as Z-Proline, indicating that the proline binding S_1' subsite could bind a six-membered piperodine ring as well as the

five-membered pyrrolidine ring of proline. This led workers to conclude that the S_1' subsite was a hydrophobic cleft and could explain the observation that prolidase exhibited low rates of hydrolysis towards the substrates Leu-Ala and Ala-Leu (King *et al.*, 1989; Hui and Lajtha, 1980; Sjostrom *et al.*, 1973).

The requirement for a free α-amino group residue in the P_1 position has been questioned by some workers. Mock *et al.*, (1990), studied the specificity and pH dependence for acylproline catalysis by prolidase and found that at pH 6.6, significant hydrolysis of alkylthioacetyl prolines and haloacetyl prolines was evident.

Lin and Brandts, (1979b), concluded that prolidase had an absolute specificity for the substrate dipeptide bond to be in *trans* isomeric form. This was later confirmed using NMR analysis of Ala-Pro hydrolysis by the enzyme (King *et al.*, 1986).

1.3.5.3. Assays for Prolidase Activity

The prolidase assay is based on its hydrolysis of Gly-Pro, and methods used for product detection are primarily HPLC based with some reports of the use of isotachophoresis (Harada *et al.*, 1990; Mikasa *et al.*, 1985).

1.3.5.4. Specific Inhibitors of Prolidase

In 1989, King *et al.* reported the competitive inhibition of prolidase by N-blocked proline (Z-Proline - $K_i = 9.0 \times 10^{-5}M$) and Z-pipecolic acid. Mock and Green (1987) reported the use of trans-cyclopentane-1,2, dicarboxylic acid as a prolidase inhibitor. This compound is a substituted Xaa-Pro dipeptide, where the P_1 substrate position is replaced by dicarboxylic acid. A K_i value of $9.0 \times 10^{-8}M$ was reported for the interaction between this inhibitor and prolidase.

The most potent prolidase inhibitors reported to date are derivatives of phosphoenolpyruvate. Phosphoenolpyruvate is a metabolic intermediate which inhibits prolidase with a K_i of $8.0 \times 10^{-9}M$. A bromophosphoenolpyruvate derivative inhibited prolidase with a K_i value of $4.0 \times 10^{-9}M$ (Radzicka and Wolfenden, 1991).

1.3.5.5. Physiological Relevance of Prolidase

The primary biological function of prolidase is thought to be the metabolism of collagen degradation products and other Xaa-Pro dipeptides, with the subsequent recycling of proline. Prolidase deficiency (PLD-D) results in an inability to recycle proline from dipeptides and leads to abnormalities of the skin and other collagenous tissues, sometimes leading to mental retardation. Massive amounts of Xaa-Pro dipeptides are excreted by individuals suffering from PLD-D with Gly-Pro being the most prominent of these (Freij *et al.*, 1984).

PLD-D may be the result of biochemical alterations within prolidase rather than a marked reduction in the levels of the enzyme In 1984 and 1985, Butterworth and Priestman compared the characteristics of prolidase in cultured skin fibroblasts from healthy and PLD-D individuals Using Gly-Pro as substrate, there was a marked loss of prolidase activity in PLD-D cells which could not be restored by the addition of Mn^{++} However only slight reductions in PLD-D prolidase activity were observed when Phe-Pro, Ala-Pro and Leu-Pro were used as substrates Using these substrates, normal prolidase activity was stable during prolonged incubation with Mn^{++} while the PLD-D activity was progressively inactivated In 1986, Lombeck *et al*, reported that patients suffering from PLD-D were found to have significantly increased Mn^{++} levels in their blood cells, yet their arginase and prolidase activity levels were almost halved They concluded that an altered form of prolidase rendered the Mn^{++} ions inaccessible to the active site, preventing the enzymes activation This altered prolidase was also observed by Ohhashi *et al*, (1988), when prolidase from PLD-D serum could not be activated by Mn^{++}, but was activated by Co^{++}

The hereditary nature of PLD-D is classified as an autosomal recessive disorder (Boright *et al*, 1989) The structural organisation of the human prolidase gene was elucidated by Tanoue *et al*, (1990), and in PLD-D patients, several hundred base pair deletions were evident, including the whole of exon 14 A guanine to adenosine substitution at position 826 in exon 12, resulting in the replacement of an aspartic acid by asparagine in the polypeptide chain, was also observed The human prolidase gene and the arginine substituted PLD-D gene were expressed in mammalian cell cultures, and although both genes produced a prolidase polypeptide, the substituted protein was inactive It was also demonstrated that active prolidase could be recovered from PLD-D cells following transfection with the normal prolidase gene They concluded that gene replacement might be an appropriate therapeutic regime for individuals suffering from PLD-D It is also interesting to restate that an essential aspartic acid residue in the active site of prolidase was identified by Mock and Zhuang, (1991)

Although the deletion of exon 14 was confirmed by other groups, these mutations were not found in all patients tested, indicating that the molecular defects in PLD-D were heterogenous This was confirmed by Ledoux *et al*, (1994), when cDNA samples from five patients suffering from PLD-D were examined A wide variety of different genetic mutations were evident in all five subjects They concluded that PLD-D is caused by mutations in multiple prolidase alleles

1.3.6. HIV 1 Protease (EC 3.4.23.-)

HIV produces a small protease (HIV 1 protease) that specifically cleaves the polyprotein precursor in which structural proteins and enzymes of the virus are contained. This enzyme is becoming one of the most important proteins in medicine as it is absolutely required for the production of mature, infectious virions It is therefore the most attractive target for the development of anti-HIV therapeutic drugs

The relevance of the HIV 1 protease to this discussion is that it has a high specificity for Xaa-Pro sequences in the middle of a polypeptide chain As an endopeptidase that cleaves the Xaa-Pro tertiary amide it is a rarity among proteases

1 3.6 1. Biophysical and Biochemical Aspects of HIV 1 Protease

The HIV 1 protease (HIV1P) has been purified from virions isolated from HIV positive patients However, the development of recombinant and total chemical synthesis methodologies have led to the production and purification of large quantities of the protein Most published investigative work into the varied biochemical aspects of HIV1P has been carried out on these "artificial" forms of the enzyme

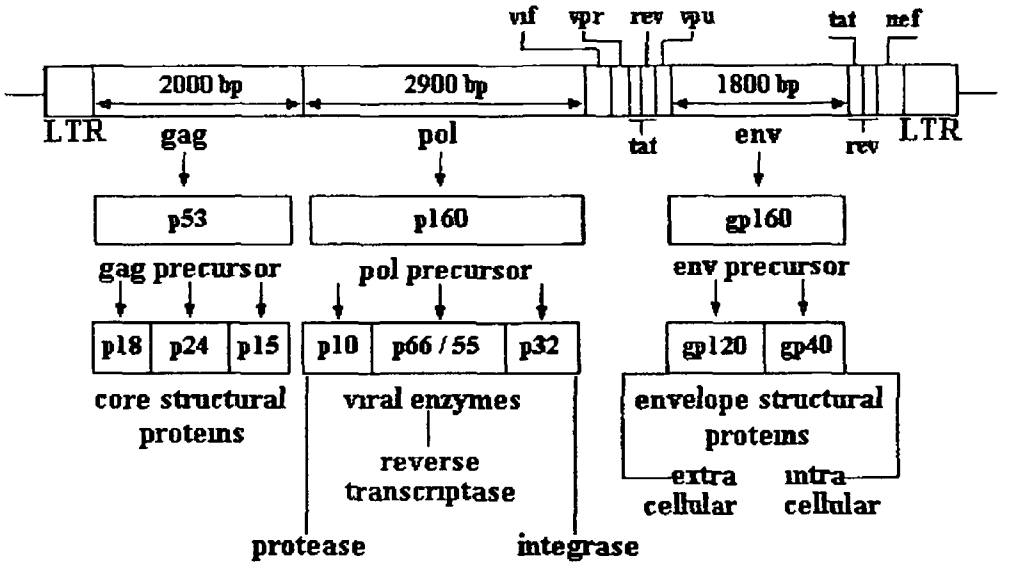
In type I human immunodeficiency virus (HIV-1), the capsid and nonstructural proteins are synthesised as a single polypeptide precursor The polypeptide is coded by two genes, *gag* and *pol* and includes the protease HIV1P (Figure 1 4 a) HIV1P autocatalytically excises itself from the precursor polypeptide by cleaving a Phe-Pro bond at its C- and N- termini at cleavage sites V and VI respectively (Figure 1 4 b) (Debouck *et al* , 1987, Graves *et al* , 1988)

A model was developed to examine the sequence of events leading to production of mature HIV1P, whereby, the protease polypeptide was flanked at its C- and N- termini by native sequences from the *gag-pol* precursor, the C- terminal sequence being fused to the maltose binding protein of *E. coli* (MBP) In its dimeric form, this polypeptide cleaved itself intramolecularly between the C- terminal flanking sequence and HIV1P, releasing a HIV1P + N- terminal flanking sequence as a short lived intermediate This intermediate possessed activity similar to that of the mature protease The dimeric intermediate intermolecularly removed the N-terminal flanking sequence producing the mature HIV1P (Wondrak *et al*, 1996, Louis *et al* , 1994, Boutelje *et al* , 1990)

Mature HIV1P is a dimer of 2 identical subunits, each with a molecular weight of 11 kDa and 99 amino acid residues Monomeric HIV1P is inactive (Strickler *et al*, 1989, Tomasselli *et al* , 1993) The rates of association of HIV1P from monomeric to dimeric form were found to be within the range commonly associated with protein-protein interaction ($4 \times 10^5 M^{-1}s^{-1}$), while the dissociation of the dimer was found to be strongly pH dependent and governed by a dissociation constant in the picomolar range The enzyme exhibits maximal activity between pH 4 0 and 6 0, with an observed optimum pH of 5 5 (Darke *et al* , 1994, Jordan *et al* , 1992, Menendez-Arias *et al*, 1992)

HIV1P is strongly inhibited by pepstatin and 1,2-epoxy-(3-(4-nitrophenoxy)propane) indicating that it is an aspartyl protease Indeed, its amino acid sequence of 99 residues has shared homology with other aspartyl proteases such as renin, pepsin and chymosin (Strakalaitis *et al* , 1991, Meek *et al* , 1989)

(a)



(b)

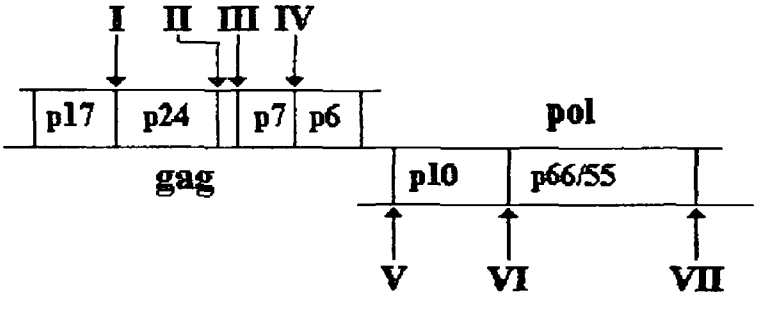


Figure 1 3 HIV-1 Genome, *(a) Schematic of the genome of HIV-1 LTR-long terminal repeats gag-group specific antigen gene encodes viral nucleopcapsid proteins p24 a nucleoid shell protein p7, p15, p17 and p55, pol-polymerase gene, encodes the viral enzyme, protease (p10), reverse transcriptase (p66/55, alpha and beta subunits) and integrase (p32), env-envelope gene, encodes the viral envelope glyocproteins gp120 and gp41 tat. encodes transactivator protein, rev encodes a regulator of expression of viral protein, vif associated with viral infectivity, vpu encodes viral protein U, vpr encode viral protein R, nef encodes a 'so-called' negative regulator protein (b) Schematic illustrating HIV1P cleavage sites (I-VII) on the gag and pol protein precursors*

An active site mechanism proposed by Hyland *et al* in 1991, was updated and detailed by Silva *et al*, 1996 Essentially, the substrate is asymmetrically bound to one active site aspartate residue Proton exchange between the aspartate and carbonyl group of the scissile bond with simultaneous hydration of the bond to introduce flexibility, facilitates optimised binding Further proton movement from the hydroxyl of the scissile bond to an aspartate residue with concerted proton exchange between the second aspartate and the amino group of the N-terminal product, introduces a bond break and regenerates the initial protonation state of the active site aspartates The active site aspartate residues have been identified at position 25 in each monomer Modification or substitution of these residues leads to inactivation of the protease (Lab *et al*, 1991 Grinde *et al*, 1992)

Two cysteine residues have been identified among the 99 amino acids of each monomer Although neither are required for enzyme activity, modification of Cys 67 leads to inactivation of the enzyme (Karlstrom *et al*, 1993)

The dimeric interface between identical subunits is composed of 4 well ordered β-strands from both the C- and N-termini of the 99 amino acid sequence with residues 86-94 having a helical formation Substitution of Asp-88 or Arg-87 with Lys lead to inactivation of HIV1P Further analysis of the protease domain surrounding these residues, revealed that their substitution affected the proteins' ability to form an active homo-dimeric complex (Wlodawer *et al*, 1989, Quenet *et al*, 1989, Louis *et al*, 1989)

In 1991, Hyland *et al* noted the non-specific activation of HIV1P in increasing ionic strength It was later revealed that at similar ionic strengths, different salts activated HIV1P activity with respect to their position in the Hofmeister series, a measurement of their salting out effect (Wondrak *et al*, 1991) Szeltner and Polgar (1996) confirmed that the increase in conformational stability and catalytic activity of HIV1P was due to stabilisation of the protease by preferential hydration

1 3 6.2. Substrate Specificity of HIV 1 Protease

The natural substrates for HIV1P are the seven cleavage sites that are involved in the maturation of the viral protein (Table 1 7), coded for by the *gag* and *pol* genes (Figure 1 4 b) Three of these cleavage sites are Xaa-Pro bonds and also form part of a consensus sequence, i e sequences that are also required recognition sites for other retroviral proteases (Pearl and Taylor, 1987) Sites V and VI are hydrolysed by the enzyme during its auto-excision from the polypeptide precursor

The substrate specificity of HIV1P is not only determined by the residues present at the scissile bond, but extends to many of the C- and N-terminal substrate residues flanking the cleavage site However, close scrutiny of the flanking amino acids presented in Table 1 7 , provides little insight into the subsite requirements of the enzyme

Cleavage site	HIV-1 sequence cleaved	Amino acid sequence of cleavage site
I	gag 124-138	-Ser-Gln-Ans-Tyr- - -Pro-Ile-Val-Gln-
II	gag 357-370	-Ala-Arg-Val-Leu- - -Ala-Glu-Ala-Met-
III	gag 370-383	-Ala-Thr-Ile-Met- - -Met-Gln-Arg-Gly-
IV	gag 440-453	-Pro-Gly-Asn-Phe- - -Leu-Gln-Ser-Arg-
V	pol 59-72	-Ser-Phe-Asn-Phe- - -Pro-Gln-Ile-Thr-
VI	pol 162-174	-Thr-Leu-Asn-Phe- - -Pro-Ile-Ser-Pro-
VII	pol 721-734	-Arg-Lys-Ile-Leu- - -Phe-Leu-Asp-Gly-

Table 1 7 *Identification of the positions within the gag-pol polypeptide precursor that represent cleavage sites for the viral protease, HIV1P It is of interest to note that excluding the Phe-Pro scissile bonds present in sites V and VI, all sites vary in their amino acid sequence The ability of HIV1P to cleave sites I-VII reveals little regarding the substrate specificity of this enzyme Sites V ands VI represent the amino acids that are cleaved by HIV1P during its auto-excision from the polypeptide precursor Scissile bond are represented by - - -*

A definitive picture of HIV1P specificity is difficult to portray and is complicated by the various models used for determining this specificity These include the enzymes' ability to cleave synthetic substrates, to autohydrolyse itself in native or substituted form and to auto-excise itself from polypeptide precursors where the equivalent V and VI site have been modified Because of these varied permutations and combinations, only the basic substrate specificity requirements of this enzyme will be discussed.

The specificity of the enzyme for the Tyr-Pro and Phe-Pro cleavage sites (I,V,VI, Table 1 7) are different with respect to the influence of flanking residues Single amino acid substitutions in these sites revealed that cleavage of Tyr-Pro (I) is severely inhibited by substitutions at the P_4, P_2, P_1 and P_2' positions The Phe-Pro site exhibits far greater tolerance to amino acid substitution at these positions (Partin *et al*, 1990) The P_1' residue of the Tyr-Pro cleavage site (Proline) could be substituted by hydrophobic amino acids such as Leu, Val and Phe, without greatly affecting the activity of HIV1P against this site (Kassel *et al.*, 1995) These results confirmed an earlier statistical analysis by Poorman *et al.* (1991), in which an extended viral substrate data base was used to predict that the highest stringency for particular amino acid residues were at P_2, P_1 and P_2' positions It is also interesting to note, however, that the subsites of the substrate binding pocket of HIV1P are capable of acting independently in their interaction with substrate amino acids (Cameron *et al.*, 1994)

1.3.6.3. Assays for HIV 1 Protease Activity

Various methods for the detection of HIV1P activity have been developed including ELISA, HPLC and fluorimetric substrate assays Two ELISA's have been developed that are based on the specificity of HIV1P for the p17/p24 cleavage site (I) They are both based on the reduced immunoreactivity of a fusion protein containing the p24 polypeptide following the action of HIV1P (Mansfield *et al*, 1993, Sarubbi *et al.*, 1991)

The p17/p24 cleavage site is also the focus of HPLC based assays A polypeptide containing the p17/p24 cleavage site (-Tyr-Pro-) is incubated with HIV1P samples and the cleavage products are separated and identified using HPLC (Louis *et al*, 1989, Cole *et al*, 1991)

A commercially available tetradecapeptide that includes the peptide sequence of angiotensin I plus N-terminally added Leu-Val-Tyr-Ser, is cleaved by HIV1P at the Leu-Leu bond, producing angiotensin I which can be subsequently quantified using HPLC or RIA methods (Sharma *et al.*, 1991, Wilkinson *et al*, 1993, Evans *et al.*, 1992, Hyland and Meet, 1991)

A fluorimetric substrate, N-α-b_2-Arg-Gly-Phe-Pro-MeO-βNa was developed by Tyagi and Carter, (1992) Cleavage at the Phe-Pro bond by HIV1P releases Pro-MeO-βNa which can be detected fluorimetrically

Inhibitor	Details	Reference
Resistomycin	Isolated from *Actinomycetes* cultures, IC_{50} = 21μM	Roggo *et al*, 1994
Spirodihydrobenzofuranlactams	Isolated from *Stachybotrys* sp, IC_{50} = 11μM	Roggo *et al*, 1996
A-98881	A synthesised azacyclic urea, K_i = 5pM	Sham *et al*, 1996
SB-206343	P_1'-P_2' inhibitor site bond is an isoteric acylimidazole ring, K_i = 0 6nM	Thompson *et al*, 1994
BILA-398	Synthetic competitive inhibitor of HIV1P, K_i = 0 5μM	Pargellis *et al*, 1994
RPI-856A	Isolated from *Streptmocyes* sp AL-322, K_i = 10nM	Asano *et al*, 1994
GR123976	A penicillin derivative, asymmetric inhibitor, IC_{50} = 2 3μM	Jhoti *et al*, 1994
Z-OVCA-NH-Bun	A peptide substrate derivative, K_i = 8nM	Sakurai *et al*, 1994

Table 1 8. *Specific inhibitors against HIV1P activity Due to the significant rise in the levels of interest expressed in HIV1P and its specific inhibitors, only those citations from 1994 onwards are included Inhibitors vary in type from modifications of peptide substrates for the protease, to novel compounds isolated from microbial cultures Much emphasis has been placed on the screening of microbial cultures for inhibitors of this protease*

1.3 6.4. Specific Inhibitors of HIV 1 Protease

It is understandable that, due to this enzymes function in HIV-1 infection, a proliferation of publications have arisen describing the synthesis and use of HIV1P specific inhibitors A brief list of recently published HIV1P specific inhibitors is presented in Table 1 8

1.3 6.5. Physiological Relevance of HIV 1 Protease

As stated earlier, HIV1P is absolutely required for the maturation of HIV-1 virions It is also thought to have some function in the assembly of virion particles Inhibitors of HIV1P have been shown to block the early steps of HIV-1 replication In H9 and HeLa CD4-LTR/β-gal cells infected with HIV-1, the protease was thought to be responsible for the cleavage of the nucleocapsid (NC) protein This cleavage may be required for the proper formation of a pre-integration complex and/or its transport to the cell nucleus (Nagy *et al*, 1994) However, apart from the role of this protease in the replication of new virions, interesting interactions between HIV1P and native cellular proteins have been identified NF-κ-β is a factor required by HIV-1 for transcription It is produced by native cells as a 105kDa precursor, located in the cytoplasm In its active form, it is translocated to the cell nucleus where it is involved in transcription Riviere *et al*, (1991) discovered that HIV1P can process the inactive precursor of NF-κ-β into its active form, suggesting that this function may also be part of HIV1P's role in virion replication

HIV1P can also cleave native cellular cytoskeleton proteins such as actin, spectrin and tropomyosin This weakening of the cytoskeleton of an infected cell may serve some purpose with regards to cell infection or virion expulsion (Shoeman *et al*, 1991, Adams *et al*, 1992) However, recent proposals suggest that elements within the cytoskeleton may play an important role in the regulation of large scale genetic regulation Therefore, the cleavage of cytoskeletal proteins by HIV1P may perturb the regulation of gene expression by these proteins and in turn, account for the increased incidence of cancer in HIV-1 infected patients (Shoeman *et al*, 1992)

1.3 7. Prolyl Carboxypeptidase (EC 3 4.16.2)

Prolyl carboxypeptidase (PCP) was first detected in the lysosomal fraction of porcine kidney by Young *et al*, (1968) and characterised by these workers as an enzyme capable of removing the C-terminal phenylalanine residue from angiotensin II Reports of the molecular weight of this enzyme vary greatly Human kidney PCP has a native molecular weight of 115kDa and is dimeric, while the porcine kidney enzyme is reported to have a native molecular weight of 210kDa (Walter *et al*, 1980) Recently, a tetrameric form of the enzyme was purified from *Xanthomonas maltophilia* with a native molecular weight of 330kDa (Suga *et al*, 1995b) Tan *et al*, (1993), cloned and sequenced human PCP The enzyme was found to consist of 451 amino acid residues with a calculated weight of 51,043Da. It was also identified as a glycoprotein with an estimated 12% carbohydrate (w/w) reported. The human enzyme has a pH optimum of 5 0-5 5, whereas, the bacterial PCP's pH optimum is 8 5

This enzyme is a serine protease, based on its inhibition by DFP (Suga *et al*, 1995) The enzyme cleaves C-terminal residues from peptides with proline being the preferred P_1 residue (Suga *et al*, 1995, Walter *et al*, 1980) Z-Pro-prolinal, the prolyl endopeptidase specific inhibitor, also inhibits PCP with a K_i of 2 6 x 10^{-7}M (Tan *et al*, 1993)

1 3.8 Carboxypeptidase P (EC 3.4.12-)

Carboxypeptidase P (CPP) was first discovered by Dehm and Nordwig, (1970), while investigating the cleavage of prolyl peptides by kidney peptidases The enzyme is a dimeric glycoprotein with a native molecular weight of 240kDa and a single, asparagine linked, mannose carbohydrate moiety being reported (Walter *et al*, 1980, Zieske *et al*, 1992) The pH optimum for CPP ranges from 6 0 to 7 8 Confusion persists over the mechanistic classification of this enzyme It is widely reported to be activated by Mn^{++} ions In 1985, Hedeager-Sorensen and Kenny identified one zinc atom present in each subunit of the dimer Erickson *et al*, (1989), reported subsequently that the enzyme was inhibited by chelators and suggested it to be a metalloprotease However, in 1992, Zieske *et al*, identified a serine residue that they determined to be necessary for catalytic activity The enzyme is responsible for the removal of C-terminal residues from peptides and proteins, with a preference for proline residues in the P_1 position Alanine and glycine substitutions of proline are also cleaved (Hedeager-Sorensen and Kenny, 1985) A general peptide and/or protein processing role has been suggested for CPP (Hoedemaeker *et al*, 1994) and it is therefore not surprising that the enzyme features regularly in the literature as part of peptide and/or protein sequencing methodologies (Thiede *et al*, 1995, Gray *et al*, 1994)

1.3.9. Summary

Proline holds an important position among twenty naturally occurring amino acids, the building blocks of peptides and proteins It confers particular biological properties upon these physiologically important biomolecules due to its unique structural characteristics There has evolved a specialised group of enzymes that recognise this residue and can introduce peptide bond cleavage at either its carboxyl or amino terminus within a peptide chain The variety of these specialised peptidases cover practically all situations where a proline residue might occur in a substrate and their action can be of biological significance, leading to the inactivation or biotransformation of peptides and proteins

The role played by prolme specific peptidases in physiological processes has been discussed, and, because of their unique role within these processes, their continued investigation is warranted.

Proline specific peptidases have been implicated in a variety of disease states based on (a), abnormalities in their levels or modes of action or (b), abnormalities associated specifically with their natural substrates In the latter case, therapeutic regimes often focus on the administration of the natural peptide to compensate for its deficiency or defect. Administration of such compounds often fails to alleviate symptoms or cure ailments due to their short lived efficacy, primarily due to

degradation by peptidases This has led to the development of peptide analogues as therapeutic agents, designed specifically to elicit the natural activity of the peptide yet resist enzymatic degradation

It is obvious that administration of such agents as part of a therapeutic regime will inevitably lead to their exposure to the degradative processes contained in serum To date, the physiochemical properties of proline specific peptidases in the blood/serum fraction has been poorly studied Although some work has been carried out on serum aminopeptidase P, prolidase and prolyl endopeptidase, the extent to which they have been studied varies from purification and detailed characterisation (e g aminopeptidase P and prolidase) to simply reporting the presence of the peptidase in serum

Therefore, more detailed studies are essential, in order to better understand the nature of these enzymes, and to identify links between them and their tissue counterparts The continued investigation into the biochemical characteristics and substrate specificity of serum proline specific peptidases is vital in overcoming problems associated with drug delivery

2. Materials & Methods

2 1 Materials

Sigma Chemical Company (Poole, Dorset, England)

2-Iodoacetamide
2 Mercaptoethanol
8-Hydroxyquinoline
1,10-Phenanthroline
Ala-MCA
Ammonium Persulphate
Angiotensin II
Aprotinin
Arg-MCA
Bacitracin
Benzamidine
Bisacrylamide
Blue Dextran
Bovine Serum Albumin
Bradykinin
Cadmium Sulphate
Calcium Sulphate
CDTA
Cellulose Type 50
Chymotrypsin Inhibitor
Cobalt Sulphate
Coomassie Brilliant Blue G
Dithiothreitol
DTNB
EDTA
EGTA
Glycine
Imidazole
Iodoacetate
Leupeptin
LHRH
Magnesium Sulphate
Manganese Sulphate
MCA
Mercuric Sulphate
MES
MW-GF-200 Marker Kit
N-Acetylimidazole
N-Decanoyl Co-A
N-Ethylmaleimide
Nickel Sulphate
PCMB
pGlu-His-Pro
Phenylmethylsulphonylfluoride
Potassium Phosphate (dibasic)
Potassium Phosphate (monobasic)
Proline
Pro-MCA
Puromycin
SDS
Sephadex G-25
Silver Stain High MW Standard Kit
Silver Stain Kit
Sodium Chloride
Substance P
TEMED
TRH-OH
Trizma Base
Trypsin Inhibitor from Soybean
Zinc Sulphate

Bachem Feinchemikalein AG (Bubendorf, Switzerland)

Alanine
Gly-Gly-Pro-Ala
Gly-Phe-Ala
Gly-Pro-MCA
Leu-Gly
Lys-Ala-MCA
pGlu-His-Pro-MCA
Thyroliberin
TRH-OH
Z-Gly-Pro-MCA
Z-Gly-Pro-Ala
Z-Pro-Ala
Z-Pro-Gly
Z-Pro-Leu-Gly
Z-Pro-Pro

BDH Chemicals Ltd (Poole, Dorset, England)

Acetone
Acrylamide
Ammonia Solution
Biuret Reagent
Bromophenol Blue
Calcium Chloride
Citric Acid
Copper Sulphate
Dimethylformamide
Dimethylsulphoxide
Dioxane
Glacial Acetic Acid
Glycerol
Hydrochloric Acid
Methanol
Polyethylene Glycol 6000
Urea
Zinc Chloride

Merck Chemical Company (Frankfurt, Germany)

Ammonium Sulphate
Potassium Chloride
Sodium Hydrogen Phosphate
Sodium Hydroxide

Pharmacia Fine Chemical Company (Uppsala, Sweden).

Activated Thiol Sepharose CL-4B
Blue Sepharose Fast Flow
DEAE-Sepharose Fast Flow
Phenyl Sepharose CL-4B
Q-Sepharose High Performance
Sephacryl S-200 HR
SP-Sepharose Fast Flow

Bio-Rad Laboratories (Hercules, California, USA)

Biogel HT Hydroxylapatite

Keypak Meats (Clonee, Co Meath, Ireland)

Bovine whole blood

Aldrich Chemical Company (Poole, Dorset, England)

1,7-Phenanthroline

4,7-Phenanthroline

Trifluoroacetic Acid

Calbiochem-Novabiochem (UK) Ltd (Nottingham, England)

AEBSF

Pepstatin

Pierce Chemical Company (Illinois, USA)·

BCA Reagent

Mount Sinai School of Medicine (New York, Courtesy of Dr S Wilk)

Fmoc-Pro-Pro-Nitrile

Z-Pro-Prolinal

University College Galway (Courtesy of Dr G O'Cuinn)

Gly-Ala-Phe

Gly-Pro-Ala

Lys-Ala-Ala

Pro-Gly

Z-Pro

Dublin City University (Courtesy of Dr J Dalton)

Boc-Val-Leu-Lys-MCA

Boc-Val-Pro-Arg-MCA

Glu-Phe-MCA

Gly-Arg-MCA

Suc-Ala-Phe-Lys-MCA

Suc-Gly-Pro-Leu-Gly-Pro-MCA

Z-Arg-MCA

Z-Arg-Arg-MCA

Z-Phe-Arg-MCA

Z-Phe-Val-Arg-MCA

University Rene Descartes (Paris, France, Courtesy of Prof B Roques)

Kelatorphan

Institute of Microbial Chemistry (Tokyo, Japan, Courtesy of Dr M Nagai)

Poststatin

Pfizer (Groton, CT, USA, Courtesy of Dr S Faraci)

Z-Indolinyl Prolinal

Z-Cyclohexyl Prolinal

Meiji Seika Kaisha, LTD (Yokohama, Japan, Courtesy of Dr S Tsutsumi)

α-Ketobenzothiazole

Z Pro-Prolinal

Hans-Knoell Institute of Natural Product Research (Germany, Courtesy of Prof H U Demuth)

Z-Phe-Pro-Methylketone

Boc-Glu(NHO-Bz)-Pyrr

Nagasaki University (Japan, Courtesy of Prof T Yoshimoto)

Z-Thiopropyl Thioprolinal

2 2 Fluorescence Spectrophotometry using 7-Amino-4-Methyl-Coumarin (MCA)

2 2 1. MCA Standard Curves

5mM MCA in 100% DMSO was diluted to 200µM MCA using 100mM potassium phosphate, pH 7 4, at 37 C This stock solution was stored at 4 C Lower MCA concentrations could be achieved using 100mM potassium phosphate, pH 7 4, as diluant Standard curves were prepared by combining 100µL 100mM potassium phosphate, pH 7 4, 400µL appropriate MCA concentration and 1mL 1 5M acetic acid Ranges of 0-2 5µM, 0-10µM and 0-20µM MCA were prepared in triplicate Fluorimetric analysis of these samples was achieved using a Perkin Elmer LS-50 Fluorescence Spectrophotometer at excitation and emission wavelengths of 370nm and 440nm respectively Excitation slit widths were maintained at 10nm while emission slit widths were adjusted to produce fluorimetric intensities appropriate for the range being analysed

2.2.2. Inner Filter Effect

The inner filter effect of enzyme samples was determined by combining 100µL enzyme sample, 400µL appropriate MCA solution and 1mL 1 5M acetic acid These samples, prepared in triplicate, were analysed fluorimetrically as described in section 2 2 1

2.3 Protein Determination

2.3 1 Absorbance at 280nm

The absorbance of proteins based on the λ_{max} of tryptophan residues at 280nm was used as a non-quantitative method of determining protein concentrations in post-column chromatography fractions A Shimadzu UV 160A Spectrophotometer was used to determine this absorbance

2.3.2 Biuret Assay

The Biuret assay was used to quantify protein concentrations in samples of approximately 2mg/mL protein or greater Samples were dialysed for 12 hours against 100mM potassium phosphate, pH 7 4, to remove possible interfering substances where necessary Samples with a protein concentration outside the limits of the Biuret assay (2-10mg/mL) were diluted with 100mM potassium phosphate, pH 7 4, to achieve a suitable protein concentration with respect to the assay limits 200µL Biuret reagent was added to 50µL sample in triplicate in a 96 well microplate and incubated for 30 minutes at 37°C BSA standard curves in the range 0-10mg/mL were included on each plate in triplicate The absorbance of each well at 560nm was determined using a Titertek Multiscan PLUS plate reader

2 3.3. Standard BCA Assay

The standard BCA assay protocol, based on the method of Smith *et al*, (1985), was used to quantify protein concentrations of samples that could not be determined accurately using the less sensitive

Biuret assay Samples were prepared as described for the Biuret assay 200µL BCA reagent was added to 50µL sample in triplicate in a 96 well microplate and incubated for 30 minutes at 37 C BSA standard curves in the range 0-1 4 mg/mL were included on each plate in triplicate Plates were read as described in section 2 3 2

2.3.4. Enhanced BCA Assay

The Enhanced BCA assay protocol was used to quantify protein concentrations of samples that could not be determined accurately by the less sensitive standard BCA assay The assay was performed as described in section 2 3 3 using an incubation temperature of 60°C The included BSA standard curves were in the range 0-100µg/mL and were prepared in triplicate on each plate

2 4 Serum Preparation

Whole blood was collected from a freshly killed bovine The whole blood was transported to a 4°C cold room and the clot allowed to shrink for 24 hours The remaining unclotted whole blood was then decanted and centrifuged at 6000 rpm (4100 x g) for 1 hour using a Beckman J2-MC refrigerated centrifuge fitted with a JA-21 rotor at 4°C The serum thus produced was divided into 20mL aliquots Aliquots were stored at -20°C

2.5. Enzyme Assays

2 5.1. Measurement of Z-Gly-Pro-MCA Degrading Activities

Z-Gly-Pro-MCA degrading activity was determined according to a modification of the original procedure of Yoshimoto *et al*, (1979) 10mM Z-Gly-Pro-MCA substrate stock in 100% DMSO was prepared 100mM potassium phosphate, pH 7 4, at 37°C, was added slowly to 300µL DMSO + 100µL substrate stock to a final volume of 10mL resulting in a final concentration of 0 1mM substrate, 4% DMSO (v/v) 400µL 0 1mM substrate was added to 100µL sample to be tested in triplicate and the reaction mixture was incubated for 1 hour at 37°C Both enzyme and substrate were preincubated at 37 C to allow them to reach thermal equilibrium The reaction was terminated by the addition of 1mL 1 5M acetic acid Negative controls were prepared by adding 1mL 1 5M acetic acid to enzyme sample prior to substrate addition and incubation at 37°C MCA liberated from the substrate was determined fluorimetrically as described in section 2 2 1 Fluorimetric intensities obtained for each sample were converted to picomoles (pmoles) MCA released per min per mL using standard curves described in section 2 2 1 Enzyme units were defined as pmoles MCA released per minute

2.5.2. Z-Pro-Prolinal Insensitive Activity

Z-Pro-Prolinal insensitive residual Z-Gly-Pro-MCA degrading activity in serum was determined as described in section 2 5 1 with the exception that 20µL of 10^{-4}M Z-Pro-Prolinal was added to each sample prior to substrate addition The sensitivity of residual Z-Gly-Pro-MCA degrading activity in serum to varied concentrations of Z-Pro-Prolinal was investigated by adding 20µL Z-Pro-Prolinal (that

varied in concentration from 10^{-9}M to 10^{-4}M to serum prior to substrate addition The effect of increased preincubation time with Z-Pro-Prolinal on residual Z-Gly-Pro-MCA degrading activity was also investigated by preincubating 100µL serum with 20µL 10^{-4}M Z-Pro-Prolinal for 0-30 minutes at 37 C, prior to substrate addition All determinations were carried out in triplicate with suitable negative controls

2.5 3 Microplate Assay

A non-quantitative microplate fluorimetric assay was developed to facilitate the rapid identification of Z-Gly-Pro-MCA degrading activities in post-column chromatography fractions 200µL 0 1mM substrate in 100mM potassium phosphate, pH 7 4, 4% (v/v) DMSO (+ 15mM DTT, 15mM EDTA for PE type activity) was added to 100µL sample in each well The plate was incubated for 30 minutes at 37 C MCA liberated from the substrate in each well was determined using the Perkin Elmer LS-50 Fluorescence Spectrophotometer with microplate reader accessory attached

2.6. Purification of Serum Z-Gly-Pro-MCA Degrading Activities

All procedures were carried out at 4°C unless otherwise stated

2.6 1. SP Sepharose Fast Flow Cation Exchange Chromatography

2 6.1.1. Separation of 2 Z-Gly-Pro-MCA Degrading Activities from Serum using SP Sepharose Fast Flow Cation Exchange Chromatography

A 20mL aliquot of serum was thawed at 37 C and dialysed against 4L 20mM MES, pH 4 5, for 12 hours The post-dialysis serum was centrifuged at 20,000rpm (48,500 x g) for 20 minutes using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor to remove post-dialysis precipitate A 25mL SP Sepharose column (2 5cm x 5cm) was equilibrated with 100mL 20mM MES, pH 5 5, at a flowrate of 1mL/min Dialysed serum supernatant was loaded followed by a 200mL wash with 20mM MES, pH 5 5 Bound protein was eluted isocratically with 100mL 100mM potassium phosphate, 1M ammonium sulphate, pH 7 4 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 2mL/min Fractions were assayed for Z-Gly-Pro-MCA degrading activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 3 1 Fractions containing Z-Gly-Pro-MCA degrading activity were combined to form the post-SP Sepharose PE and ZIP pools

2 6 1 2. Identification of PE Activity in Post-SP Sepharose Fractions

PE activity in the post SP Sepharose fractions could be distinguished from residual Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) activity by the addition of 20µL 10^{-5}M Z-Pro-Prolinal into each well prior to substrate addition

2 6.2. Phenyl Sepharose Hydrophobic Interaction Chromatography (PE)

The post-SP Sepharose PE pool was salted by addition of 50mL 200mM potassium phosphate, 2M ammonium sulphate, pH 7 4 The final volume was brought to 100mL with distilled water resulting in a final concentration of 100mM potassium phosphate, 1M ammonium sulphate

A 17mL Phenyl Sepharose column (2 5cm x 3 5cm) was equilibrated with 100mL 100mM potassium phosphate, 1M ammonium sulphate, pH 7 4, at a flowrate of 1mL/min The salted post-SP Sepharose pool was loaded followed by a 200mL wash with 100mM potassium phosphate, 800mM ammonium sulphate, pH 7 4 Bound protein was eluted with a 200mL linear gradient from 100mM potassium phosphate, 800mM ammonium sulphate, pH 7 4, to 100mM potassium phosphate, pH 7 4 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 2mL/min Fractions collected were assayed for PE activity using the microplate assay procedure described in section 2 5.3 Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 3 1 Fractions containing PE activity were combined to form the post-Phenyl Sepharose PE pool

2.6 3. DEAE Sepharose Fast Flow Anion Exchange Chromatography (PE)

The post-Phenyl Sepharose PE pool was dialysed against 2L 50mM Tris-HCl, pH 8 0, for 12 hours with buffer changes at 3, 6, and 9 hours The conductivity of the sample was measured before application onto DEAE Sepharose

A 20mL DEAE Sepharose column (2 5cm x 4cm) was equilibrated with 100mL 50mM Tris-HCl, pH 8 0, at a flowrate of 1mL/min The dialysed post-Phenyl Sepharose PE pool was loaded followed by a 200mL wash with 50mM Tris-HCl, pH 8 0 Bound protein was eluted with a 200mL linear gradient from 50mM Tris-HCl, pH 8 0, to 50mM Tris-HCl, 100mM NaCl, pH 8 0 A further 100mL wash with 50mM Tris-HCl, 100mM NaCl, pH 8 0, was applied to the column to complete elution 5mL fractions were collected throughout the run Loading and washing steps were carried out at a flowrate of 2mL/min Elution was carried out at a flowrate of 1mL/min Fractions collected were assayed for PE activity using the microplate assay procedure described in section 2.5.3 Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 3 1 Fractions containing PE activity were combined to form the post-DEAE Sepharose PE pool

2.6 4 Sephacryl S-200 HR Sepharose Gel Filtration Chromatography (PE)

The post-DEAE Sepharose PE pool was concentrated via reverse osmosis using polyethylene glycol Glycerol was added to the concentrated sample to a final concentration of 10% (v/v)

A 220mL Sephacryl S-200 Sepharose column (2 5cm x 45cm) was equilibrated with 400mL 100mM potassium phosphate, 200mM KCl, pH 7 4, at a flowrate of 1mL/min The concentrated sample was loaded under the buffer head and the column was washed with 300mL equilibration buffer at a flowrate of 1mL/min 5mL fractions were collected and assayed for PE activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using the BCA assay described in section 2 3 3 Fractions containing PE activity were pooled and stored on ice

2.6 5. Phenyl Sepharose Hydrophobic Interaction Chromatography (ZIP)

A 10mL Phenyl Sepharose column (2 5cm x 5cm) was equilibrated with 100mL 100mM potassium phosphate, 1M ammonium sulphate, pH 7 4, at a flowrate of 1mL/min The post-SP Sepharose ZIP pool was loaded followed by a 200mL wash with 10mM potassium phosphate, 50mM ammonium sulphate, pH 7 4 The bound protein was eluted isocratically with 100mL distilled water 5 mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 2mL/min Fractions collected were assayed for ZIP activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 3 1 Fractions containing ZIP activity were combined to form the post-Phenyl Sepharose ZIP pool

2.6 6. Calcium Phosphate Cellulose Chromatography (ZIP)

Calcium Phosphate Cellulose was prepared according to the method of Tiselius *et al*, (1956) with the exception that the cellulose used was SigmaCell 50

A 7mL Calcium Phosphate Cellulose column (1 5cm x 4cm) was equilibrated with 50mL 10mM potassium phosphate, pH 7 4, at a flowrate of 0 6mL/min The post-Phenyl Sepharose ZIP pool was loaded followed by a 50mL wash with 10mM potassium phosphate, pH 7 4 The bound protein was eluted with a 100mL linear gradient from 10mM potassium phosphate, pH 7 4, to 500mM potassium phosphate, pH 7 4 6mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 0 6mL/min Fractions collected were assayed for ZIP activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using the BCA assay described in section 2 3 3 Fractions containing ZIP activity were combined to form the post-Calcium Phosphate Cellulose ZIP pool

2 6 7 Sephacryl S-200 HR Sepharose Gel Filtration Chromatography (ZIP)

The post-Calcium Phosphate Cellulose ZIP pool was concentrated via reverse osmosis using polyethylene glycol Glycerol was added to the concentrated sample to a final concentration of 10% (v/v)

A 220ml Sephacryl S-200 HR Sepharose column (2 5cm x 45cm) was equilibrated with 400mL 100mM potassium phosphate, 200mM KCl, pH 7 4, at a flowrate of 1mL/min The concentrated sample was loaded under the buffer head and the column was washed with 150mL equilibration buffer at a flowrate of 1mL/min 5mL fractions were collected throughout the run Fractions collected were assayed for ZIP activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using the enhanced BCA assay described in section 2 3 4 Fractions containing ZIP activity were pooled and stored on ice

2.6 8. Alternative Chromatographic Regimes Used to Further Purify PE

The following chromatographic media were used to develop further steps as part of the PE purification

2.6.8.1. Q Sepharose Fast Flow Anion Exchange Chromatography

Post-Phenyl Sepharose PE activity was produced as outlined in section 2 6.2. and dialysed as outlined in section 2 6 3 A 20mL Q Sepharose column (2 5cm x 4cm) was equilibrated with 100mL 20mM Tris-HCl, pH 8 0, at a flowrate of 1mL/min The dialysed post-Phenyl Sepharose PE pool was loaded followed by a 200mL wash with 20mM Tris-HCl, pH 8 0 Bound protein was eluted with a 100mL linear gradient from 20mM Tris-HCl, pH 8 0, to 20mM Tris-HCl, 500mM NaCl, pH 8 0 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 2mL/min Fractions collected were assayed for PE activity using the microplate assay procedure described in section 2 5 3 Protein determinations for each fraction were achieved using the Biuret assay as described in section 2 3 2

2 6 8.2. Calcium Phosphate Cellulose Chromatography

The Calcium Phosphate Cellulose column outlined in section 2 6 6 was used. Post-Phenyl Sepharose PE activity was dialysed against 2L 10mM potassium phosphate, pH 7 4, for 12 hours with buffer changes at 3, 6 and 9 hours The Calcium Phosphate Cellulose column was equilibrated as outlined in section 2 6 6 at a flowrate of 0 5mL/min The dialysed PE pool was loaded followed by a 50mL wash with 10mM potassium phosphate, pH 7 4 The bound protein was eluted with a 100mL linear gradient from 10mM potassium phosphate, pH 7 4, to 500mM potassium phosphate, pH 7 4 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 0 5mL/min Fractions collected were assayed for PE activity using the microplate assay procedure

described in section 2 5 3 Protein determinations for each fraction were achieved using the Biuret assay as described in section 2 3 2

2.6 8 3. Biogel HT Hydroxylapatite Chromatography

Post-Phenyl Sepharose PE activity was dialysed as outlined in section 2 6 8 2 A 10mL Biogel HT Hydroxylapatite column (2 5cm x 2cm) was equilibrated with 100mL 10mM potassium phosphate, pH 7 4, at 0 5mL/min The dialysed PE activity was loaded followed by a 100mL wash with 10mM potassium phosphate, pH 7 4 Bound protein was eluted with a 200mL linear gradient from 10mM potassium phosphate, pH 7 4, to 500mM potassium phosphate, pH 7 4 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at a flowrate of 0.5mL/min Fractions collected were assayed for PE activity using the microplate assay procedure as outlined in section 2 5 3 Protein determinations for each fraction were achieved using the Biuret assay as described in section 2 3 2

2.6.8.4. Blue Sepharose Fast Flow Chromatography

Post-Phenyl Sepharose PE pool was dialysed against 2L 20mM potassium phosphate, pH 7 4, for 12 hours with buffer changes at 3, 6 and 9 hours. A 40mL Blue Sepharose column (2 5cm x 8cm) was equilibrated with 150mL 20mM potassium phosphate, pH 7 4, at a flowrate of 1mL/min The dialysed PE activity was loaded followed by a 100mL wash with 20mM potassium phosphate, pH 7 4 Bound protein was eluted with a 150mL linear gradient from 20mM potassium phosphate, pH 7 4, to 20mM potassium phosphate, 2M NaCl, pH 7 4 Loading, washing and elution steps were carried out at a flowrate of 1mL/min 5mL fractions were collected throughout the run. Fractions collected were assayed for PE activity using the microplate assay procedure as outlined in section 2 5 3 Protein determinations for each fraction were achieved using the Biuret assay as described in section 2 3 2

2.6 8 5. Activated Thiol Sepharose 4B Chromatography

PE activity was eluted from the Phenyl Sepharose column as described in section 2 6 2 with the exception that 5mM DTT was incorporated into both elution buffers DTT removal from the enzyme sample was an important step before application onto the Activated Thiol Sepharose column This was achieved by two different methods

2.6 8.5.1. Removal of DTT by Dialysis

The post-Phenyl Sepharose PE pool, containing 5mM DTT, was dialysed for 6 hours against 1L 20mM potassium phosphate, pH 7 4, with buffer changes at 2 and 4 hours

2 6.8.5 2. Removal of DTT using Sephadex G-25 Chromatography

The post-Phenyl Sepharose PE pool, containing 5mM DTT was concentrated via reverse osmosis using polyethylene glycol Following concentration, glycerol was added to a final concentration of 10% (v/v) A 25mL Sephadex G-25 column (1.5cm x 15cm) was equilibrated with 100mL 20mM

potassium phosphate, pH 7 4 at a flowrate of 0 5mL/min The concentrated PE pool was loaded under the buffer head and the column was washed with 150mL 20mM potassium phosphate, pH 7 4, at a flowrate of 0 5mL/min 5mL fractions were collected and assayed for PE activity using the microplate assay procedure outlined in section 2 5 3 Protein determinations for each fraction were achieved using the Biuret assay as described in section 2 3 2 DTT elution from the column could also be determined due to its interference with the Biuret assay Fractions containing PE activity were pooled for application onto Activated Thiol Sepharose

2 6.8.5.3. Application of PE onto Activated Thiol Sepharose

A 5mL Activated Thiol Sepharose column (1 5cm x 3cm) was equilibrated with 50mL 20mM potassium phosphate, pH 7 4, at a flowrate of 0 5mL/min The PE activity, having had the DTT removed by dialysis or de-salting using Sephadex G-25, was loaded followed by a 25mL wash with 20mM potassium phosphate, pH 7 4 Flow was stopped for 30 minutes to allow protein to bind to the column A further wash of 25mL 20mM potassium phosphate, pH 7 4, was followed by isocratic elution of bound protein using 50mL 20mM potassium phosphate, 5mM DTT, pH 7 4 5mL fractions were collected throughout the run Loading, washing and elution steps were carried out at 0.25mL/min Fractions collected were assayed for PE activity using the microplate assay procedure as outlined in section 2 5 3 Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 3 1

2.7. Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was used to determine the success of the purification of both enzymes It was also used to determine the subunit structure of both enzymes and the molecular weight of these subunits A non-native, discontinuous SDS PAGE system based on the method of Laemmli, (1970) was used.

2.7.1. Sample Preparation

Samples to be electrophoresed were dialysed extensively over a 24 hour period against 2L 62 5mM Tris-HCl, pH 6 8, at room temperature with buffer changes at 6 and 12 hours Dialysed samples were then diluted with an equal volume of sample solubilisation buffer which consisted of 62 5mM Tris-HCl, pH 6 8, 20% v/v glycerol, 8% w/v SDS, 10% v/v 2-mercaptoethanol and 0 01% w/v bromophenol blue A molecular marker cocktail, containing six known molecular weight markers, was prepared from a Sigma MW-SDS-200 marker kit. This included carbonic anhydrase (29,000Da), ovalbumin (45,000Da), BSA (66,000Da), phosphorylase B (97,400Da), ß-galactosidase (116,000Da) and myosin (205,000Da) For gels that were subjected to Silver Staining, a specific Silver Stain Molecular Weight Marker kit was obtained from Sigma. This kit contained no myosin or ovalbumin marker, but did contain a Fumarase marker (48,500) Samples and markers thus prepared were incubated in a boiling water bath for 2 minutes prior to application onto the gel.

2 7 2 Preparation of SDS Gels

The following stock solutions were prepared in deionised water

Resolving Gel Buffer	3M Tris-HCl, pH 8 8
Stacking Gel Buffer	0 5M Tris-HCl, pH 6 8
Acryl / Bis-Acryl Stock	30% w/v acrylamide, 0 8% bisacrylamide
Ammonium Persulphate	1 5% w/v AP freshly prepared
SDS	10% w/v
Running Buffer	0 025M Tris-HCl, 0 192M Glycine, 0 1% SDS, pH 8 3

Table 2 1 outlines how these stock solutions were combined to produce a 10% resolving gel overlayed with a 3 75% stacking gel Gels were prepared in an ATTO vertical electrophoresis system (160mm x 160mm x 1mm) The system was filled with running buffer and 20µL of the appropriately prepared samples were loaded into the wells under the buffer The gels were then electrophoresed using a current of 25mA per gel for approximately 3 hours

2.7.3. Visualising Proteins in Polyacrylamide Gels

2.7.3.1 Staining Polyacrylamide Gels with Coomassie Brilliant Blue

Following electrophoresis as outlined in section 2 7 2 , gels were fixed for 30 minutes in a solution of 40% v/v methanol, 7% v/v glacial acetic acid Gels were then stained for 1 hour in a solution of 0 1% w/v Coomassie Brilliant Blue G, 25% v/v methanol and 5% v/v acetic acid Following staining, the gels were destained for 12-24 hours in a solution of 25% v/v methanol, 10% v/v glacial acetic acid

2.7.3.2. Silver Staining Polyacrylamide Gels

Polyacrylamide gels were stained with silver using a Silver Staining kit obtained from Sigma. The kit operates according to the method of Heukeshoven and Dernick, (1985) Table 2 2 outlines the steps involved

2 7.4. Recording and Storage of Polyacrylamide Gel Images

Digital images of gels were obtained using a UVP ImageStore 7500 This incorporated a UVP White/UV Transilluminator/Camera unit driven by ImageStore 7500 software Images acquired were either stored digitally on disk, or printed using a Sony Videographics Printer UP-860 CE

Solution	10% Resolving gel (mL)	3 75% Stacking gel (mL)
Acryl / Bisacryl Stock	10	2 5
Resolving Gel Buffer	3 75	-
Stacking Gel Buffer	5	-
SDS	0 3	0 2
Water	14 45	11 3
Ammonium Persulphate	1 5	1
TEMED	0 015	0 015

Table 2 1 *Volumes required for SDS PAGE resolving and stacking gels All solutions with the exception of TEMED were prepared in deionised water (refer to section 2 7 2) Quantities indicated sufficient to cast 2 x (160mm x 160mm x 1mm) gels*

Step	Solvent Reagent	Duration
Fixing	30% v/v Ethanol, 10% v/v Glacial Acetic Acid	60 minutes
Rinsing	Deionised Water	30 minutes
Silver Staining	Silver Nitrate	30 minutes
Rinsing	Deionised Water	20 seconds
Developing	Sodium Carbonate, Formaldehyde	30 minutes
Development Stop	1% Glacial Acetic Acid	5 minutes
Rinsing	Deionised Water	30 minutes
Reducing	Sodium Thiosulphate, Sodium Carbonate	30 seconds
Rinsing	Deionised Water	24 hours

Table 2 2 *Steps involved in Silver Staining polyacrylamide gels Solutions used for Silver Staining, Developing and Reducing were supplied as part of Sigma Silver Staining Kit No AG-25 Durations of each step were appropriate for a 1mm thick polyacrylamide gel*

2 8. Assay Development for Purified Serum Z-Gly-Pro-MCA Degrading Activities

2.8.1. Substrate Solvent Determination

10mM Z-Gly-Pro-MCA stocks were prepared in 100% DMSO, DMF and dioxane Purified enzyme (PE or ZIP) was assayed in triplicate with suitable negative controls using 0 1mM Z-Gly-Pro-MCA (+15mM DTT, 15mM EDTA in the case of purified PE), prepared from each stock as described in section 2 5 1 , to determine the effect of different substrate solvents on enzyme activity

2.8.2 Solvent Concentration Determination

0 1mM Z-Gly-Pro-MCA in 2%, 4%, 6%, 8% and 10% dioxane (v/v) were prepared in 100mM potassium phosphate, pH 7 4 Purified enzyme (PE or ZIP) was assayed in triplicate with these substrates as described in section 2 5 1 to determine the effect of solvent concentration on enzyme activity Suitable negative controls were prepared

2.8.3. Linearity of Enzyme Assays with respect to Time

300µL purified enzyme (PE or ZIP) and 1 2mL 0 1mM Z-Gly-Pro-MCA were preincubated separately at 37°C to allow each to attain thermal equilibrium The liberation of MCA from the reaction of substrate and enzyme combined was monitored continuously over a 2 hour period in an incubated cuvette holder to determine the linearity of enzyme activity over time The experiment was repeated for serum in the presence and absence of 20µL 10^{-4}M Z-Pro-Prolinal

2 8.4 Linearity of Enzyme Assays with respect to Enzyme Concentration

A range of purified enzyme concentrations (PE or ZIP) were prepared using 100mM potassium phosphate, pH 7 4, as diluant These samples were assayed in triplicate as described in section 2 5 1 Suitable negative controls were prepared The experiment was repeated on purified ZIP activity as described here, with the exception that 200mM KCl was incorporated into substrate and diluant. The linearity of observed ZIP activity with respect to enzyme concentration was also repeated on ZIP activity that had been dialysed extensively against 100mM potassium phosphate, pH 7 4, to remove post-gel filtration KCl.

2.8.5. DTT Activation of Purified Z-Gly-Pro-MCA Degrading Activities

0 1mM Z-Gly-Pro-MCA was prepared in a range of DTT concentrations from 0 to 25mM Purified enzyme activity (PE or ZIP) was assayed in triplicate with these substrates as described in section 2 5 1 Suitable negative controls were prepared This experiment determined the DTT concentration at which purified enzyme was maximally activated under assay conditions where DTT was present in substrate only

2 8 6. Salt Activation of Purified Z-Gly-Pro-MCA Degrading Activities

0 1mM Z-Gly-Pro-MCA was prepared in a range of NaCl concentrations from 0 to 1M Purified enzyme activity (PE or ZIP) was assayed with these substrates in triplicate as described in section 2 5 1 Suitable negative controls were prepared This experiment determined the NaCl concentration at which the purified enzyme was maximally activated under assay conditions where NaCl was present in substrate only

2.8.7. Optimised Assay for Z-Gly-Pro-MCA Degrading Activities

The optimised assay used to determine PE and ZIP activities, based on the results from the assay development experiments, was as described in section 2 5 1 with the following modifications 10mM stock substrate was prepared in 100% dioxane and the final concentration of dioxane in 0 1mM Z-Gly-Pro-MCA prepared in 100mM potassium phosphate, pH 7 4, was 2% (v/v) PE activities were assayed with substrate incorporating 15mM DTT, 15mM EDTA while ZIP activities were assayed with substrate incorporating 500mM NaCl unless the presence of these agents interfered with a particular study It should be noted that the optimised assays for each enzyme were used to evaluate the success of each purification protocol

2 9. Characterisation of Purified Z-Gly-Pro-MCA Degrading Activities

2.9.1. Relative Molecular Mass Determination

The relative molecular masses of purified PE and purified ZIP were determined by Gel Filtration Chromatography, Size Exclusion Chromatography using HPLC and SDS Polyacrylamide Electrophoresis

2.9.1 1. Sephacryl S-200 HR Gel Filtration Chromatography

The S-200 gel filtration column, similar to the column previously mentioned in sections 2 6 4 and 2 6 7 , was calibrated for relative molecular mass determination

2 9.1 1.1. Void Volume Determination

The S-200 column was equilibrated with 300mL 100mM potassium phosphate, pH 7 4, 200mM KCl at 1mL/min 1mL of a 2mg/mL dextran blue solution, containing 10% v/v glycerol, was loaded onto the S-200 column under the buffer head The column was washed with equilibration buffer at 1mL/min and 2mL fractions were collected Dextran blue elution was determined by monitoring the absorbance at 620nm in fractions collected The void volume (V_0) of the column was calculated to be the volume at which the absorbance at 620nm reached a maximum.

2 9 1.1.2. Calibration of the S-200 Column with Molecular Mass Standards

The S-200 column was equilibrated as outlined in section 2 9 1 1 1 1mL of each standard at a concentration of 2mg/mL, containing 10% v/v glycerol, were loaded onto the S-200 column under the buffer head The column was washed with equilibration buffer at 1mL/min and 2mL fractions were collected The elution volume (V_e) of each standard was monitored by assaying the fractions for protein using the BCA assay described in section 2 3 3 Cytochrome C elution was monitored at its λ_{max} of 405nm Standards applied to the column included cytochrome C (12,400Da), carbonic anhydrase (29,000Da), BSA (66,000Da), alcohol dehydrogenase (150,000Da) and ß-amylase (200,000Da) A plot of Log molecular mass versus V_e/V_o of each standard, was prepared, and a calibration curve for the column was thus constructed.

2 9.1 1.3. Estimation of Relative Molecular Mass of Purified Enzymes

The S-200 column was equilibrated as outlined in section 2 9 1 1 1 1mL of each enzyme, containing 10% v/v glycerol, was loaded onto the S-200 column under the buffer head The column was washed with equilibration buffer at 1mL/min and 2mL fractions were collected Elution of each enzyme activity was monitored using the microplate assay described in section 2 5 3 The elution volume for each enzyme (V_e) was analysed using the calibration curve produced in section 2 9 1 1.2 to determine a relative molecular mass

2 9.1.2. Biosep SEC-3000 High Performance Size Exclusion Chromatography

A Beckman System Gold HPLC system was used to carry out high pressure size exclusion chromatography The system was fitted with a Beckman Autosampler for sample injection, a Beckman Programmable Solvent Module for solvent delivery, and a Beckman Photo Diode Array (PDA) detection system The column used was a Phenomenex Biosep SEC-3000 (7 8mm x 300mm)

2.9.1.2.1. Calibration of the Biosep SEC-3000 Column with Molecular Mass Standards.

The Biosep SEC-3000 column was equilibrated with 50mL 100mM potassium phosphate, pH 7 4, at a flowrate of 0 5mL/min The standards used to calibrate the S-200 column in section 2 9 1 1 2 were also used to calibrate the Biosep SEC-3000 column with the exception that apoferritin (443,000Da) was included. 5µL injections of 2mg/mL of each standard were applied and eluted from the column with 15mL equilibration buffer at a flowrate of 0 5mL/min. The elution of each standard was monitored at 214nm, 280nm and by continuous scanning of the column eluant using the PDA. Peak detection and retention time determination for each standard was achieved using Beckman System Gold peak integration software The elution volume (V_e) was calculated as the retention time

(minutes) of each standard multiplied by the flowrate (0 5mL/min) A plot of Log molecular mass versus V_e of each standard, was prepared, and a calibration curve for the column was thus constructed

2 9 1 2 2 Estimation of Relative Molecular Mass of Purified Enzymes

Purified PE and ZIP samples were concentrated 10 fold via reverse osmosis as outlined in section 2 6 4 and 2 6 7 respectively 5µL injections of concentrated enzyme were applied and eluted from the column with 15mL equilibration buffer at a flowrate of 0 5mL/min while 0 2min fractions were collected The fractions collected were assayed for Z-Gly-Pro-MCA degrading activity as outlined in section 2 8 7 and the elution volume (determined as outlined in section 2 9 1 2 1) was analysed using the calibration curve produced in section 2 9 1 2 1 The relative molecular mass of each enzyme was thus determined

2.9.1.3. SDS Polyacrylamide Gel Electrophoresis

The SDS electrophoresis of enzyme samples and molecular weight markers has been described in section 2 7 The distance migrated by each of the standards, purified enzyme bands and bromophenol blue dye front was determined by measuring the distance from the stacker-reslover interface to the appropriate band/dye front. The relative mobility (R_f) of the molecular weight standards and the purified protein samples were calculated by dividing the distance migrated by each standard or enzyme sample, by the distance migrated by the bromophenol blue dye front. A plot of Log molecular mass of each standard versus the R_f calculated for each standard was prepared and a calibration curve for the gel was thus constructed This calibration curve was then used to determine the molecular weight of the purified enzyme samples based on their respective R_f values

2.9 2. Temperature Effects on Purified Enzymes

The effect of assaying purified PE and purified ZIP at various temperatures was determined Purified enzymes were premcubated at 4°C, 20°C, 37°C, 45°C, 60°C and 80 C for 10 minutes to achieve thermal equilibrium after which enzyme activities were determined in triplicate as described in section 2 8 7 at each temperature Activities were determined over assay times of 15, 30 and 45 minutes Substrate was preincubated at each temperature to achieve thermal equilibrium and suitable negative controls were prepared

2.9.3. pH Effects on Purified Enzymes

2.9.3 1. pH Activity Profiles

20mL purified PE and purified ZIP were dialysed for 12 hours against 4L distilled water at 4°C 0 1mM Z-Gly-Pro-MCA was prepared in a range of 20mM buffer systems at various pH values Enzyme activities were determined in triplicate as outlined in section 2 8 7 using substrate prepared over a pH range of 2.5 to 10 5 Suitable negative controls were prepared The buffer systems used

were citric acid/potassium phosphate basic species (pH 2 5-7 5), potassium phosphate (pH 6 0-8 0), Tris/HCl (pH 7 0-9 0) and glycine/NaOH (pH 8 5-10 5)

2 9 3 2. pH Inactivation Profiles

50µL purified enzyme samples prepared as described in section 2 9 3 1 were preincubated with 50µL 40mM buffer at various pH for 15 minutes at 37°C The buffer systems used are described in the preceding section 2 9 3 1 Enzyme activities were determined in triplicate as described in section 2 8 7 using substrate prepared in 100mM potassium phosphate, pH 7 4 Suitable negative controls were prepared

2.9.4. Effect of Functional Reagents on Purified Enzyme Activities

The effect of various functional reagents on purified PE and ZIP activities were investigated The reagents tested and details on stock preparations of these reagents are listed in Table 2.3 50µL of each reagent to be tested were incubated with 50µl of purified enzyme for 15 minutes at 37°C prior to substrate addition Samples were assayed as outlined in section 2 8 7 , with the exception that neither DTT nor NaCl were included in the substrate used Each reagent was tested in triplicate with suitable negative controls Fluorimetric standard curves were prepared for each reagent to account for any inner filter effect produced (filtered standard curves) Where functional reagents were prepared in acetone or 0 1M NaOH, suitable positive controls were also prepared

2.9.5. Effect of Divalent Metal Salts on Purified Enzyme Activities

The effect of various divalent metal salts on purified PE and ZIP activities were investigated The metal salts used were $NiSO_4$, $MnSO_4$, $ZnSO_4$, $CdSO_4$, $CoSO_4$, $HgSO_4$, $CuSO_4$, $CaSO_4$ and $MgSO_4$ Each salt was used at a concentration of 1mM 50µL of each metal salt to be tested were incubated with 50µl of purified enzyme for 15 minutes at 37°C prior to substrate addition Samples were assayed as outlined in section 2 8 7 , with the exception that neither DTT nor NaCl were included in the substrate used. Each metal salt was tested in triplicate with suitable negative controls Fluorimetric standard curves were prepared for each reagent to account for any inner filter effect produced

Functional reagent	Concentration	Preparation
DTT	20mM	Dissolved in buffer [a]
DTNB	20mM	Dissolved in buffer with heating [b]
Iodoacetamide	20mM	Dissolved in buffer [a]
Iodoacetate	20mM	Dissolved in basic phosphate buffer [c]
NEM	20mM	Dissolved in buffer [a]
Leupeptin	2mM	Dissolved in buffer [a]
2-Mercaptoethanol	20mM	Dissolved in buffer [h]
PCMB	10mM	Dissolved in 10% v/v 0 1M NaOH [d]
EDTA	20mM	Dissolved in buffer [a]
CDTA	20mM	Dissolved in buffer [a]
EGTA	20mM	Dissolved in buffer [a]
Imidazole	20mM	Dissolved in buffer [a]
8-Hydroxyquinoline	20mM	Dissolved in 5% v/v acetone with heating [e]
1,10 Phenanthroline	20mM	Dissolved in 5% v/v acetone [f]
1,7 Phenanthroline	20mM	Dissolved in 5% v/v acetone [f]
4,7 Phenanthroline	20mM	Dissolved in 5% v/v acetone with heating [e]
PMSF	2mM	Dissolved in 5% v/v acetone [g]
AEBSF	20mM	Dissolved in buffer [a]
Puromycin	2mM	Dissolved in buffer [a]
N-Acetylimidazole	20mM	Dissolved in buffer [a]
Benzamidine	20mM	Dissolved in buffer [a]
Trypsin inhibitor	2mg/mL	Dissolved in buffer [a]
Chymotrypsin inhibitor	1mg/mL	Dissolved in buffer [a]
Pepstatin	1mg/mL	Dissolved in 5% v/v acetone [f]
Aprotinin	1mg/mL	Dissolved in buffer [a]
Bacitracin	2mg/mL	Dissolved in buffer [a]
N-Decanoyl Co A	2mg/mL	Dissolved in buffer [a]

Table 2 3 *Preparation of functional reagent stock solutions [a]100mM potassium phosphate, pH 7 4, at 37°C [b]100mM potassium phosphate, pH 7 4, with subsequent heating in a boiling water bath. [c]100mM potassium phosphate basic species at 37°C to aid solubility and to maintain a final pH of 7 0 [d]Dissolved initially in 0 1M NaOH followed by dilution with appropriate 100mM basic or acidic potassium phosphate species at 37°C to maintain pH at 7 0 [e]Dissolved initially in 100% acetone followed by dilution with 100mM potassium phosphate, pH 7 4, with subsequent heating in a boiling water bath. [f]Dissolved initially in 100% acetone with subsequent dilution with 100mM potassium phosphate, pH 7 4 [g]As for [f] with the exception that preparation was carried out 10 minutes prior to preincubation [h]20µL 2-mercaptoethanol diluted with 10mL 100mM potassium phosphate pH 7 4*

2.9.6. Substrate Specificity Studies on PE and ZIP

The substrate specificity of purified PE and purified ZIP activities was determined using Reverse Phase HPLC , fluorimetric and kinetic analysis methodologies

2.9 6 1 Substrate Specificity Determination Using Reverse Phase HPLC

A Beckman Gold HPLC system, as previously described in section 2 9 1 2 , was used The column used was a Beckman Ultrasphere C-8 (Octyl) Reverse Phase Column (4 6mm x 250mm) which was fitted with a Beckman Ultrasphere Reverse Phase Guard Column (4 6mm x 45mm),

2.9 6.1 1 Preparation of Stock Substrates and Standard Fragments

All stock substrates and standard fragments used were prepared in a similar manner They were initially dissolved in 0 5mL 100% MeOH (filtered and degassed), followed by dilution to 10mL with 100mM potassium phosphate, pH 7 4 (filtered and degassed) Table 2 4 lists the substrates and standard fragments that were used

2.9.6.1.2. Reaction of Substrates and Purified Enzyme Activities

200µL of each substrate (1mM) was added to 50µL purified enzyme activity (PE or ZIP) The reaction between substrate and enzyme was allowed to continue for 24 hours at 37°C Suitable buffer, enzyme, substrate and fragment controls were prepared Substrate specificity studies on PE were performed in the presence and absence of 15mM DTT, 15mM EDTA , and in the presence and absence of Z-Pro-Prolinal

2.9.6.1.3. Reverse Phase HPLC of Samples

Following the completion of the enzyme-substrate reactions, and preparation of suitable controls, samples were transferred to HPLC vials and positioned in the Beckman Autosampler Running buffers for the reverse phase chromatography were **A 100% MeOH + 0.2% TFA (v/v)** and **B Ultrapure water + 0 2% TFA (v/v)** Both buffers were filtered and degassed prior to use The reverse phase column was equilibrated with 10mL 15% buffer A / 85% buffer B at a flowrate of 1mL/min 20µL injections of each sample to be analysed were applied followed immediately by a 10mL linear gradient from 15% buffer A / 85% buffer B to 70% buffer A / 30% buffer B Following a 5mL wash at 70% buffer A / 30% buffer B and a subsequent 5mL wash with 100% buffer A to clean the column, the column was re-equilibrated prior to application of the next sample Eluant from the column was monitored using the Beckman PDA detector previously described in section 2 9 1 2 Wavelengths used were 214nm, 280nm and continuous scanning from 190nm to 590nm at 4nm intervals Scans were performed twice every second

Substrate / Fragment	Solubility in 100% MeOH *	Solubility in buffer**	Heating needed†	Stock conc (mM)††
LHRH	Yes	Yes	No	1 0
TRH	Yes	Yes	No	2 0
TRH-OH	Yes	Yes	No	3 5
Bradykinin	Yes	Yes	No	0 35
Substance P	Yes	Yes	No	0 15
Angiotensin II	No	Yes	No	0 20
Pro-Gly	No	Yes	No	7 0
Pro	No	Yes	No	14 0
Gly	No	Yes	No	56 0
Z-Pro-Gly	Yes	Yes	No	6 5
Gly-Pro-Ala	No	Yes	No	3 5
Gly-Pro	Yes	Yes	No	6 0
Ala	No	Yes	No	15 0
Z-Gly-Pro-Ala	Yes	Yes	No	5 5
Gly-Gly-Pro-Ala	No	Yes	No	6 5
Gly-Ala-Phe	No	No	Yes 80°C	5 0
Lys-Ala-Ala	No	Yes	No	8 0
Gly-Phe-Ala	No	Yes	No	4 5
Z-Pro-Pro	No	Yes	No	5 5
Z-Pro-Ala	Yes	Yes	No	5 0
Z-Pro-Leu-Gly	Yes	Yes	No	4 5
Leu-Gly	Yes	Yes	No	5 5
Z-Pro	Yes	Yes	No	6 0

Table 2 4 *Preparation of stock substrates and fragments for substrate specificty studies using reverse phase HPLC * Indicates whether substrate / fragment was solubilised in initial 0 5mL 100% MeOH ** Indicates whether (a) substrate / fragment that failed to dissolve in MeOH was finally dissolved upon addition of 100mM potassium phosphate, pH 7 4, or (b) substrate / fragment that was dissolved in MeOH remained in solution following addition of buffer † Indicates whether heating was needed to dissolve substrate / fragment †† 1mM stock substrate / fragment was the intended final concentration however some substrates were in short supply and had to be made up at lower concentrations Those substrates that were made up to a final concentration greater than 1mM were diluted appropriately prior to use in substrate specificity studies Those peptides listed in bold type were also used in kinetic analysis studies as described in section 2 9 6 3 3*

2 9 6 2. Substrate Specificity Determination Using Fluorimetric Substrates

Various substrates, to which MCA was attached, were used for this study

2 9.6 2 1 Preparation of Stock Substrates and Standard Fragments

All stock substrates used were prepared in a similar manner They were initially dissolved in 100% dioxane followed by dilution with 100mM potassium phosphate, pH 7 4, at 37°C so that the final concentration of each substrate stock was 1mM in a final dioxane concentration of 20% (v/v) Table 2 5 lists the substrates that were used

2 9.6.2.2. Reaction of Substrates and Purified Enzyme Activities

The activity of purified PE and ZIP activities against the various substrates was determined in a similar manner to that described previously in section 2 8 7 Each substrate stock, prepared as outlined in section 2 9 6 2 1 , was diluted 10 fold with 100mM potassium phosphate, pH 7 4, (+15mM DTT for PE activity determination or +500mM NaCl for ZIP activity determination) to a final concentration of 0 1mM The final dioxane concentration present in each substrate was 2% (v/v) 400µL of each substrate to be tested was added to 100µL of purified PE or ZIP activity The reaction mixture was then incubated for 1 hour at 37°C followed by the addition of 1mL 1 5M acetic acid to terminate the assay Substrates were tested in triplicate and suitable negative controls were prepared MCA liberated from each substrate was determined fluorimetrically as outlined in section 2 2 1

2 9.7. Substrate Specificity Studies on PE and ZIP Based on Kinetic Analysis

Kinetic analyses of PE and ZIP activities against Z-Gly-Pro-MCA and pGlu-His-Pro-MCA, coupled with the effects of proline containing peptides on the kinetic parameters obtained, were investigated.

2 9.7.1. Determination of K_m for Z-Gly-Pro-MCA (PE and ZIP)

The Michaelis-Menten Constant (K_m) of purified enzymes (PE or ZIP) for the substrate Z-Gly-Pro-MCA was determined Stock substrates for PE and ZIP studies were 0 2mM Z-Gly-Pro-MCA, 5% dioxane (v/v), 15mM DTT, 15mM EDTA and 0 5mM Z-Gly-Pro-MCA, 10% dioxane (v/v), 500mM NaCl respectively A range of substrate concentrations from 0-100% stock substrate were prepared using an appropriate diluant. Enzyme activities were determined in triplicate as outlined in section 2 8 7 Suitable negative controls were prepared The results were subjected to Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and Direct Linear Plot analysis to determine K_m (Michaelis and Menten, 1913, Lineweaver and Burk, 1934, Hofstee *et al*, 1959, Hanes, 1932, Eisenthal and Cornish-Bowden, 1974)

Substrate	Typical activity detected	Reference
Ala-MCA	Alanine Aminopeptidase	Mantle *et al.* ,(1983)
Arg-MCA	Arginine Aminopeptidase	Barrett *et al.* ,(1981)
Z-Arg-MCA	Trypsin	Nishikata *et al.* ,(1985)
Pro-MCA	Proline Aminopeptidase	Yoshimoto *et al.* ,(1983)
pGlu-MCA	PAP Type I	Cummins and O'Connor(1996)
Glu-Phe-MCA	Chymotrypsin	Zimmerman *et al.* ,(1977)
Gly-Arg-MCA	DPP I	Chan *et al.* ,(1985)
Gly-Pro-MCA	DPP IV	Checler *et al.* ,(1985)
Lys-Ala-MCA	DPP II	Nagatsu *et al.* ,(1985)
Z-Arg-Arg-MCA	Cathepsin B	Hiwasa *et al.* ,(1987)
Z-Phe-Arg-MCA	Plasma Kallikrein	Barrett *et al.* ,(1981)
Z-Gly-Pro-MCA	Prolyl Endopeptidase	Yoshimoto *et al.* ,(1979)
pGlu-His-Pro-MCA *	PAP type II	O'Leary and O'Connor ,(1995)
Boc-Val-Leu-Lys-MCA	Plasmin	Kato *et al.* ,(1980)
Boc-Val-Pro-Arg-MCA	Thrombin	Kawabata *et al.* ,(1988)
Suc-Ala-Phe-Lys-MCA	Plasmin	Pierzchala *et al.* ,(1979)
Z-Phe-Val-Arg-MCA	Trypsin	Somorin *et al.* ,(1978)
Suc-Gly-Pro-Leu-Gly-Pro-MCA	Collegenase	Shoji *et al.* ,(1989)

Table 2 5. *Fluorimetric substrates used to determine the substrate specificity of purified PE and ZIP activities The enzymes which are normally associated with activity against these substrates are also listed.* * *This substrate can also be used to detect Prolyl Endopeptidase activity PAP - Pyroglutamyl aminopeptidase, DPP - Dipeptidyl Peptidase*

2 9 7 2. Determination of K_m for pGlu-His-Pro-MCA (PE)

The Michaelis-Menten Constant (K_m) of purified PE for the substrate pGlu-His-Pro-MCA was determined 0 5mM pGlu-His-Pro-MCA, 15mM DTT, 15mM EDTA was prepared in 100mM potassium phosphate, pH 7 4 A range of substrate concentrations from 0-100% of this stock substrate was prepared in 100mM potassium phosphate, pH 7 4, 15mM DTT 15mM EDTA Enzyme activities towards pGlu-His-Pro-MCA were determined in triplicate in a similar manner to that described in section 2 8 7 Suitable negative controls were prepared The results were subjected to Michaelis-Menten, Lineweaver-Burk, Eadie Hofstee, Hanes-Woolf and Direct Linear Plot analysis to determine K_m

2.9 7.3. Determination of K_i Values for Proline Containing Peptides (PE and ZIP)

The effects of proline containing peptides on the K_m values obtained for PE and ZIP activities towards the substrate Z-Gly-Pro-MCA were investigated The peptides used are listed in Table 2 4 in bold type Stock substrates for PE and ZIP studies were 0 2mM Z-Gly-Pro-MCA, 5% dioxane (v/v), 15mM DTT, 15mM EDTA and 0 5mM Z-Gly-Pro-MCA, 10% dioxane (v/v), 500mM NaCl respectively A range of substrate concentrations from 0-100% stock substrate were prepared using an appropriate diluant. A fixed concentration of each proline containing peptide to be tested was incorporated into the substrate ranges Where possible the concentration of each peptide in the substrate was maintained at 1mM Enzyme activities were determined in triplicate as described in section 2 8 7 Suitable negative controls were prepared The results were subjected to Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and Direct Linear Plot analysis to determine K_m and apparent K_m values The relationships between the K_m and apparent K_m values thus obtained, and the nature of the inhibition observed, were investigated using Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf analyses K_i values were thus determined

2.9.8. Effect of PE Specific Inhibitors on Purified PE and ZIP Activities

The effect of various PE and proline specific peptidase inhibitors was investigated Table 2 6 lists these inhibitors and details of their preparation and use Stock substrates for PE and ZIP studies were 0 2mM Z-Gly-Pro-MCA, 4% dioxane (v/v), 30mM DTT, 30mM EDTA and 0 2mM Z-Gly-Pro-MCA, 4% dioxane (v/v), 500mM NaCl respectively 1mL of each inhibitor at various concentrations was added to 1mL of substrate prior to enzyme addition Thus the final concentration of substrate used was 0 1mM, 2% dioxane (v/v) Enzyme activities against these substrates were determined in triplicate as outlined in section 2 8 7 Suitable negative and positive controls were prepared

Inhibitor	Reference	Preparation	Concentration range used (M)
Fmoc-Pro-Pro-Nitrile	Dr Sherwin Wilk (PC)	20% (v/v) Dioxane [a]	2.6×10^{-15} - 2.6×10^{-6}
Z-Thiopropyl-Thyoprolinal	Tsuru *et al* (1988)	50% (v/v) Dioxane [a]	1.9×10^{-14} - 1.9×10^{-5}
Z-Pro-Prolinal	Wilk and Orlowski (1983)	20% (v/v) Dioxane [a]	2.8×10^{-12} - 2.8×10^{-3}
α-Ketobenzothiazole	Tsutsumi *et al.* (1994)	50% (v/v) Dioxane [a]	6.9×10^{-15} - 6.9×10^{-6}
Poststatin	Nagai *et al.* (1991)	20% (v/v) Dioxane [a]	8.1×10^{-13} - 8.1×10^{-4}
Z-Phe-Pro-Methylketone	Steinmetzer *et al* (1993)	Buffer [b]	1.0×10^{-15} - 1.0×10^{-6}
Z-Cyclohexyl-Prolinal	Bakker *et al* (1991)	50% (v/v) Dioxane [a]	2.0×10^{-15} - 2.0×10^{-6}
Z-Indolinyl-Prolinal	Bakker *et al.* (1991)	50% (v/v) Dioxane [a]	1.1×10^{-13} - 1.1×10^{-4}
Boc-Glu(NHO-Bz)-Pyrr	Demuth *et al* (1993)	20% (v/v) Dioxane [a]	8.6×10^{-13} - 8.6×10^{-4}
Kelatorphan	Barelli *et al* (1993)	Buffer [b]	1.0×10^{-10} - 1.0×10^{-2}

Table 2.6 *PE specific inhibitors used to characterise purified PE and ZIP activities as described in section 2.9.7* [a] *Inhibitors were initially dissolved in 100% dioxane to which 100mM potassium phosphate, pH 7.4, at 37°C was added resulting in the final dioxane concentration presented.* [b] *100mM potassium phosphate, pH 7.4, at 37°C (PC) - Personal Communication*

3. Results

Note Error bars on all graphs represent the standard deviation values determined for triplicate observations

3 1. MCA Standard Curves and the Inner Filter Effect

MCA standard curves were prepared as outlined in section 2 2 1 Plots of fluorimetric intensity observed versus MCA concentration are presented in Figures 3 1 1 , 3 1 2 , and 3 1 3 These plots also include standard curves prepared as described in section 2.2.2 to demonstrate the inner filter effect observed when using serum as the enzyme sample Table 3 1 lists the slopes obtained from each curve It should be noted that the expression of MCA concentration on the X-axis of Figures 3 1 1 , 3 1 2 and 3 1 3 represent the concentration contained in 400µL used to construct the curve (refer to section 2 2 1)

3.2. Protein Standard Curves

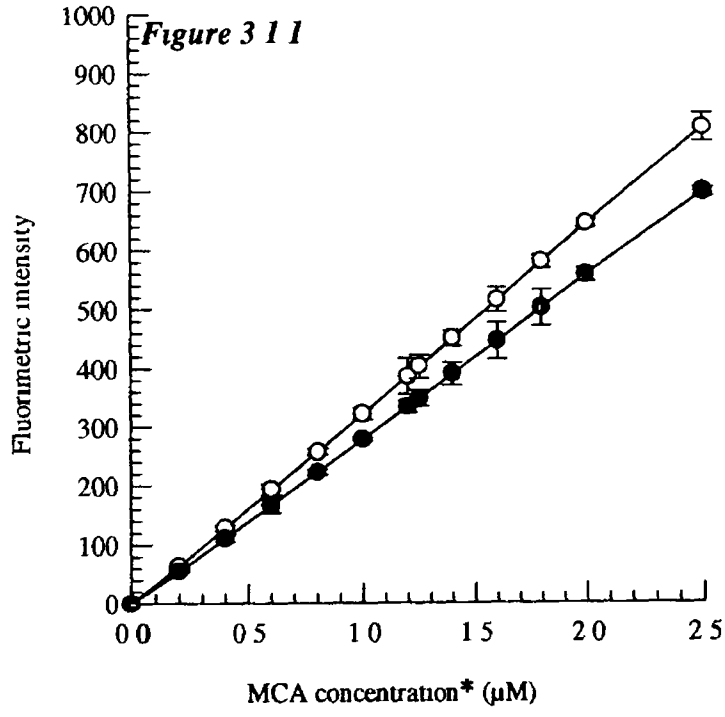
Protein standard curves were prepared using BSA as outlined in section 2 3 2 , 2 3 3 and 2 3 4 Plots of Absorbance at 560nm versus BSA concentration are presented in Figures 3 2 1 , 3 2 2 and 3 2 3 for the Biuret , Standard BCA and Enhanced BCA assays respectively

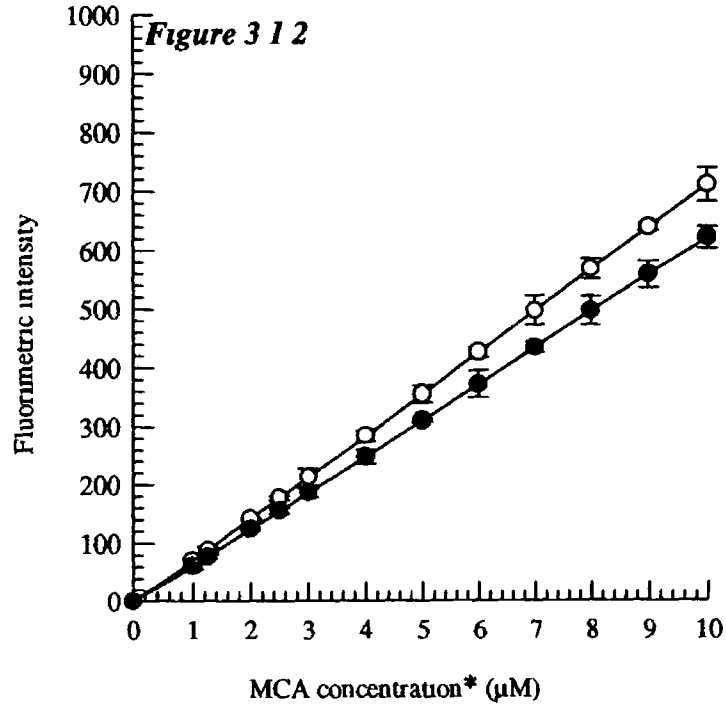
3.3. Serum Preparation

Serum was prepared as described in section 2 4 From 9L whole blood collected, 2L unclotted blood was removed following 24 hours at 4°C and 1 6L serum were produced following centrifugation

Curve	Slope * (No filter effect)	Slope including filter effect
0 - 2 5 μM (Figure 3 1 1)	321 07 F I./μM MCA	277 61 F I /μM MCA
0 - 10 μM (Figure 3 1 2)	70 85 F I /μM MCA	61 91 F I /μM MCA
0 - 20 μM (Figure 3 1 3)	11 16 F I /μM MCA	9 85 F I /μM MCA

Table 3.1 *Slopes obtained from MCA standard curves * F I = Fluorimetric Intensities MCA standard curves were constructed as outlined in section 2 2 1 This table clearly indicates Inner Filter effect observed when serum is incorporated into the MCA standard curves with slopes reduced by an average of approximately 12 5%*





Figures 3 1 1 and 3 1 2 MCA Standard Curves *Plots of fluorimetric intensity versus MCA concentration demonstrating the inner filter effect 100µL buffer (o-o) or 100µL serum (•-•) were combined with 400µL MCA and 1mL 1 5M acetic acid before being analysed fluorimetrically as outlined in section 2 2 1 Emission slit widths were set to 10nm and 5nm for Figures 3 1 1 and 3 1 2 respectively * MCA concentrations represented as described in section 3 1*

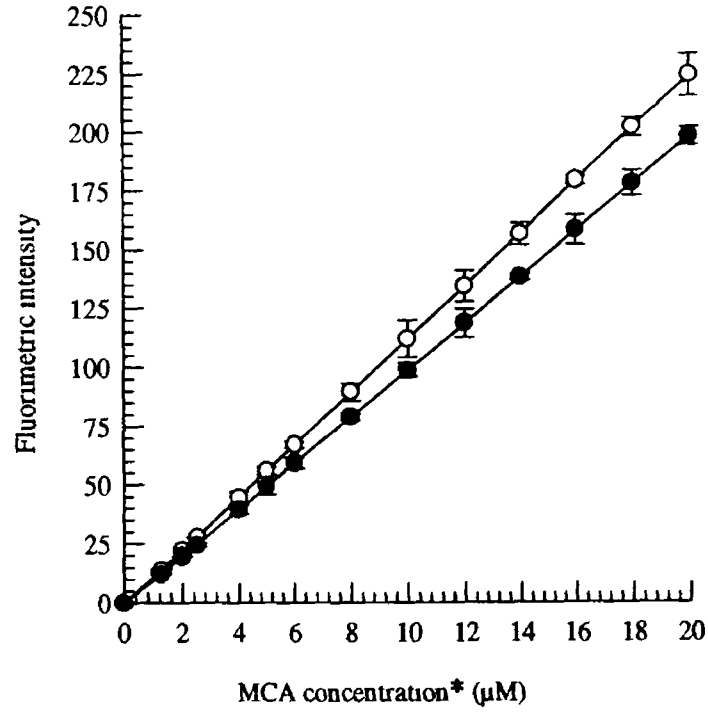


Figure 3 1 3 MCA Standard Curve *Plot of fluorimetric intensity versus MCA concentration demonstrating the inner filter effect of serum (•-•) compared to that of buffer (o-o) Curve constructed as outlined in section 2 2 1 and 2 2 2 Emission slit width set to 2 5nm. * MCA concentrations represented as described in section 3 1*

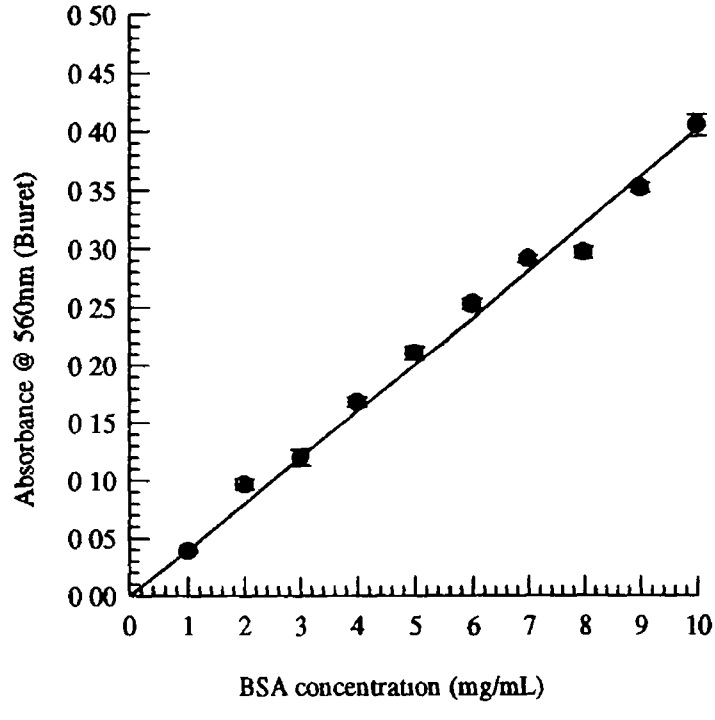
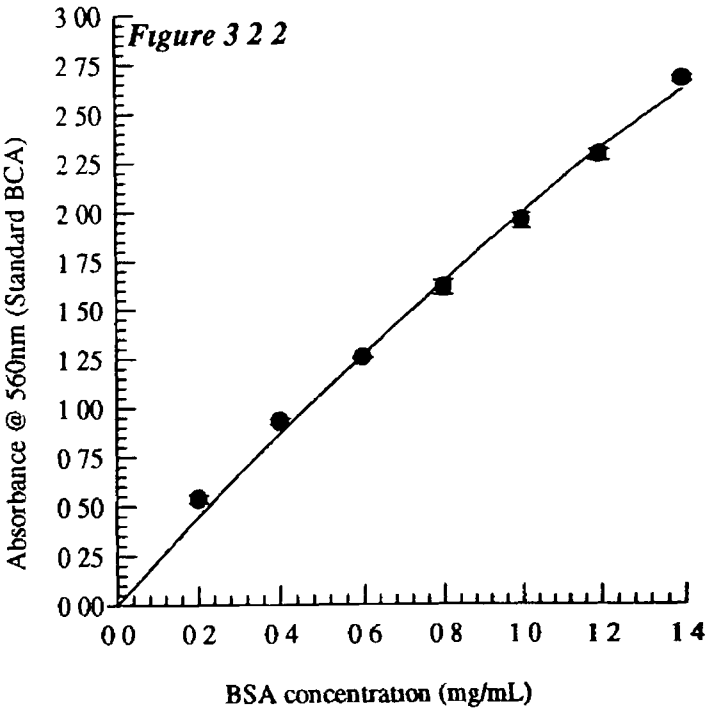
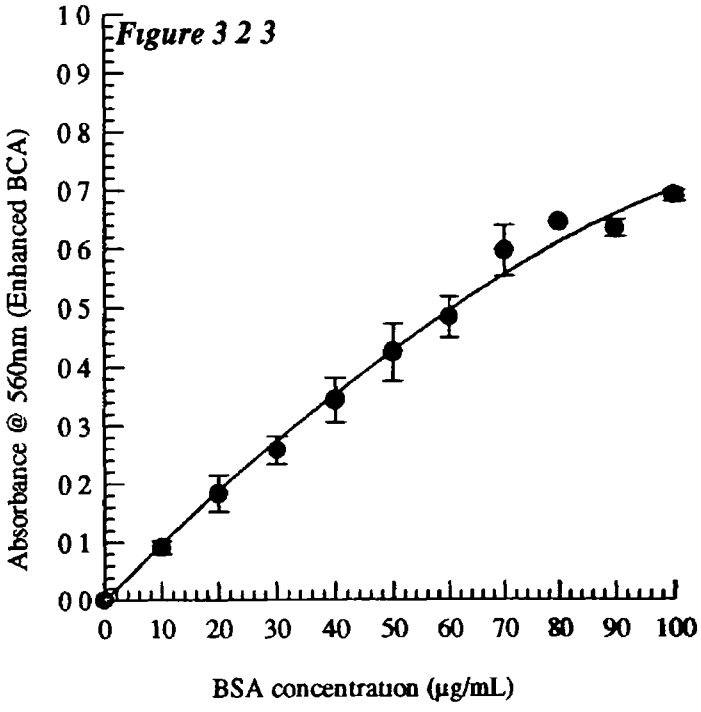


Figure 3.2.1. BSA Standard Curve *Plot of absorbance at 560nm versus BSA concentration obtained using the Biuret assay Curve constructed as outlined in section 2 3 2 Absorbances at 560nm determined using a Titertek Multiscan PLUS plate reader*





Figures 3 2 2 and 3 2 3 BSA Standard Curves *Plots of absorbance at 560nm versus BSA concentration obtained using the Standard BCA and Enhanced BCA assays respectively 50µL BSA was combined with 200µL BCA reagent in a 96 well plate, which was incubated for 30 minutes at 37°C or 60°C for the Standard BCA or Enhanced BCA assays respectively as outlined in sections 2 3 3 and 2 3 4 Absorbances at 560nm were determined using a Titertek Multiscan PLUS plate reader*

3 4. Measurement of Z-Gly-Pro-MCA Degrading Activity in Serum

Serum Z-Gly-Pro-MCA degrading activities were determined as outlined in section 2 5 1 Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading activity (ZIP) in serum was determined as described in section 2 5 2 Figure 3 4 1 demonstrates the presence of Prolyl Endopeptidase (PE) and ZIP in bovine serum Figure 3 4 2 and Figure 3 4 3 confirm that Z-Gly-Pro-MCA degrading activity in serum could not be further inhibited by increasing Z-Pro-Prolinal concentration, or by increasing the preincubation time of serum with Z-Pro-Prolinal at 37°C prior to substrate addition, respectively It should be noted that the Z-Pro-Prolinal concentrations expressed on the X-axis of Figure 3 4 2 represent the inhibitor concentration present in the 20µL added to serum prior to substrate addition (refer to section 2 5 2)

3 5 Conversion of Fluorimetric Intensities to Enzyme Units

Conversion of fluorimetric intensities to enzyme units, defined as pmoles MCA released per minute, were accomplished using the following formula.

$$\text{Enzyme Units} = \frac{\text{F.I.}}{\text{SLOPE}} \times 6\,67$$

where F.I is the fluorimetric intensity observed, SLOPE is the slope of an appropriate standard curve and 6 67 is a factor that considers how the standard curves were constructed (refer to section 2 2 1) The conversion from µM to pmoles and from hours to minutes is also considered Using this formula and multiplying by a factor of 10 to convert to Units per mL, the PE and ZIP activities presented in Figure 3 4 1 are 342 Enzyme Units per mL and 222 Enzyme Units per mL respectively

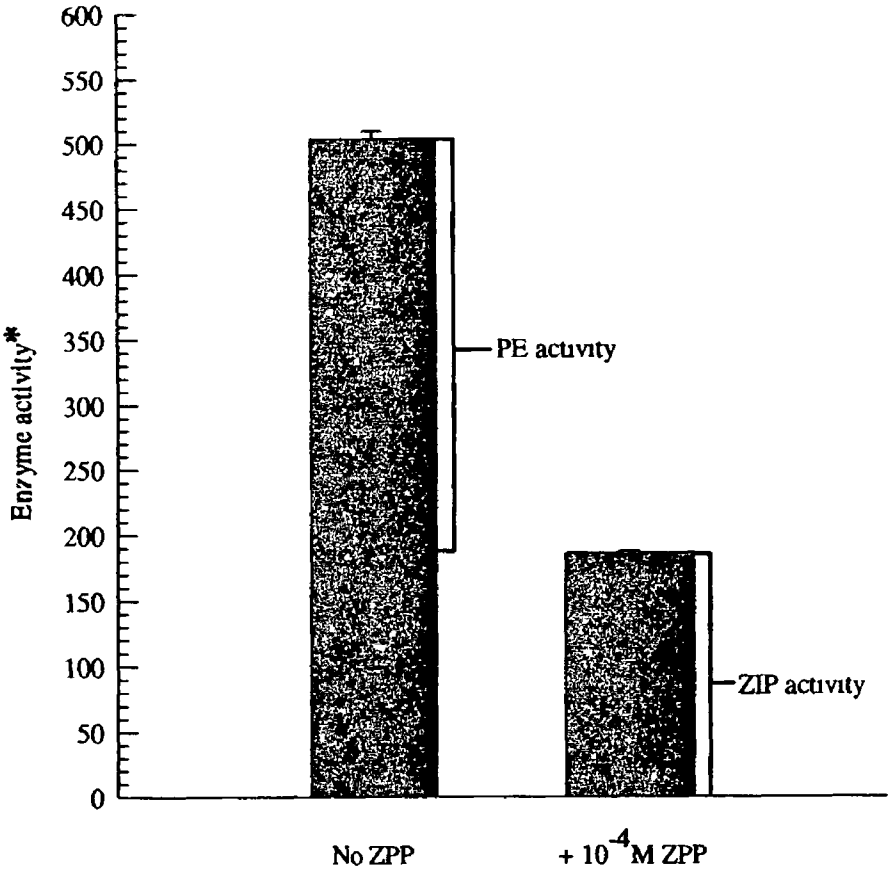
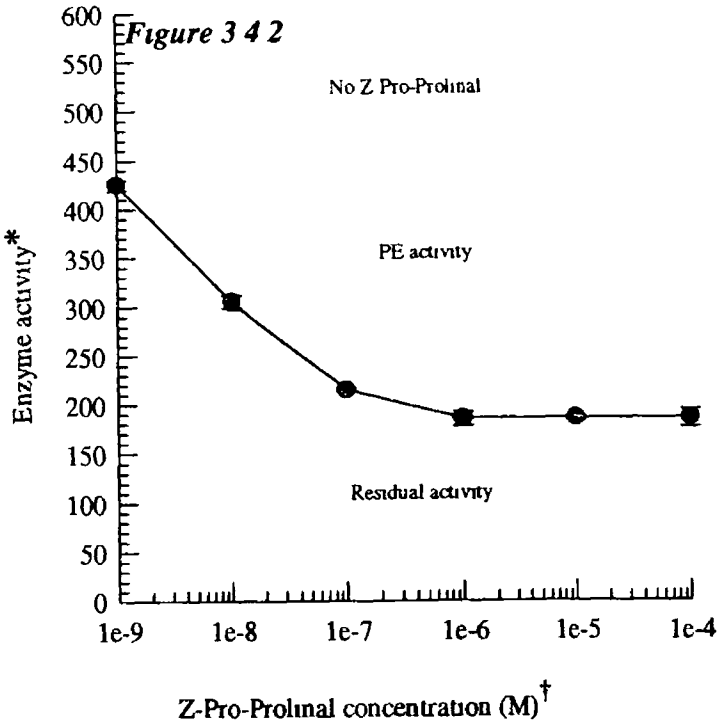
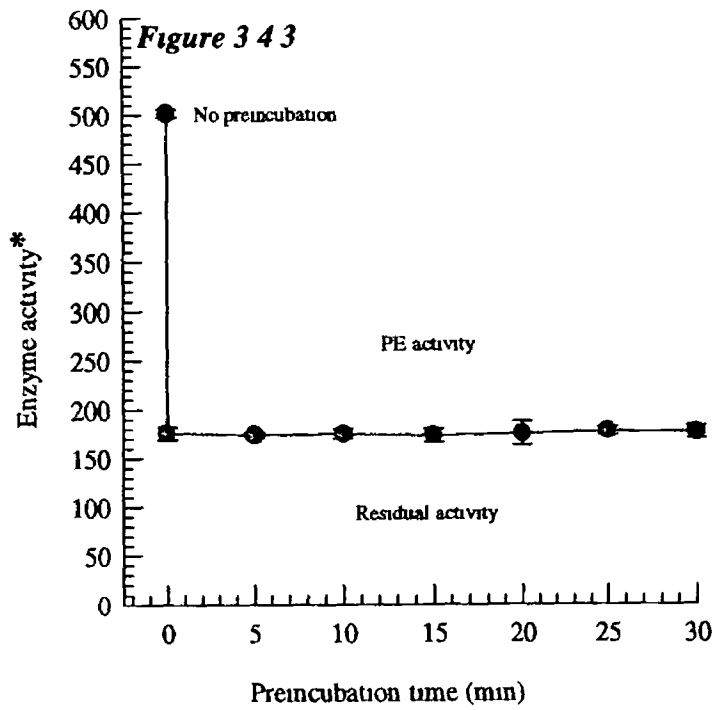


Figure 3.4 1. Demonstration of 2 distinct Z-Gly-Pro-MCA degrading activities in bovine serum *Plot of bovine serum enzyme activity against Z-Gly-Pro-MCA in the presence and absence of Z-Pro-Prolinal (ZPP) PE activity is represented by the disappearance of Z-Gly-Pro-MCA degrading activity in the presence of Z-Pro-Prolinal ZIP activity is represented by the residual activity against Z-Gly-Pro-MCA in the presence of Z-Pro-Prolinal. Activities determined as outlined in sections 2 5 1 and 2 5 2 * Enzyme activity expressed as fluorimetric intensities*





Figures 3 4.2 and 3 4 3 Resistance of residual Z-Gly-Pro-MCA degrading activity in bovine serum to Z-Pro-Prolinal inhibition *Plots of bovine serum enzyme activity against Z-Gly-Pro-MCA versus Z-Pro-Prolinal concentration (Figure 3 4 2) or increased preincubation time with Z-Pro-Prolinal (Figure 3 4 3) Plots demonstrate that residual Z-Gly-Pro-MCA degrading activity in serum is resistant to Z-Pro-Prolinal inhibition regardless of inhibitor concentration or preincubation time used PE activity in serum is completely inactivated by Z-Pro-Prolinal concentrations of 10^{-6}M or greater * Enzyme activity expressed as fluorimetric intensities † Z-Pro-Prolinal concentrations in Figure 3 4 2 are as described in section 3 4*

3 6. Purification of Z-Gly-Pro-MCA Degrading Activities from Bovine Serum

3 6 1 SP Sepharose Fast Flow Cation Exchange Chromatography

Following dialysis of 20mL bovine serum, post-dialysis precipitate was removed by centrifugation as outlined in section 2 6 1 1 producing 21 5mL clear supernatant, pH 5 5 85% PE and 73% ZIP activities were recovered following dialysis and centrifugation 1mL of the dialysed supernatant was retained for enzyme activity and protein determinations while the remainder was applied to an SP Sepharose column as described in section 2 6 1 1 Two activity peaks were detected from this column The first peak eluted during the wash and a second peak was eluted following the application of an isocratic salt wash to the column as illustrated in Figure 3 6 1 1 These activities are distinguished according to their sensitivity towards Z-Pro-Prolinal which is illustrated in Figure 3 6 1 2 Fractions 4-12 and 51-56 were combined as the post-SP Sepharose PE (41 5mL) and ZIP (27 5mL) pools respectively 1mL of each pool was retained for enzyme activity and protein determinations

3 6.2 Phenyl Sepharose Hydrophobic Interaction Chromatography (PE)

The post-SP Sepharose PE pool was salted and applied to the Phenyl Sepharose column as outlined in section 2 6 2 PE activity was eluted from the column by application of a linear decreasing salt gradient (Figure 3 6 2) Fractions 74-91 were combined as the post-Phenyl Sepharose PE pool (84mL) 1mL of this pool was retained for enzyme activity and protein determinations

3 6 3. DEAE Sepharose Fast Flow Anion Exchange Chromatography (PE)

The post-Phenyl Sepharose PE pool was dialysed as outlined in section 2 6 3 Following dialysis the volume of the pool had increased to 110mL and its conductivity had reached 3 98 millisiemens (mS) The conductivity of the DEAE Sepharose equilibration buffer was recorded as 3 84mS The pool was applied to the column as described in section 2 6 3 PE was eluted from the column with an linear increasing salt gradient and final salt wash as illustrated in Figure 3 6 3 Fractions 94-111 were combined as the post-DEAE Sepharose PE pool (82mL) 1mL of this pool was retained for enzyme activity and protein determinations

3 6.4. Sephacryl S-200 HR Sepharose Gel Filtration Chromatography (PE)

The post-DEAE Sepharose PE pool was concentrated to 2mL via reverse osmosis 200µL glycerol were added to the concentrated enzyme which was loaded and eluted from the S-200 column as outlined in section 2 6 4 86mL were washed through the column before fractions were collected Figure 3 6 4 illustrates the elution profile of PE from the column Fractions 11-23 were pooled (61mL) and stored on ice 1mL of this pool was retained for enzyme activity and protein determinations The effectiveness of the PE purification strategy is presented in Table 3 2

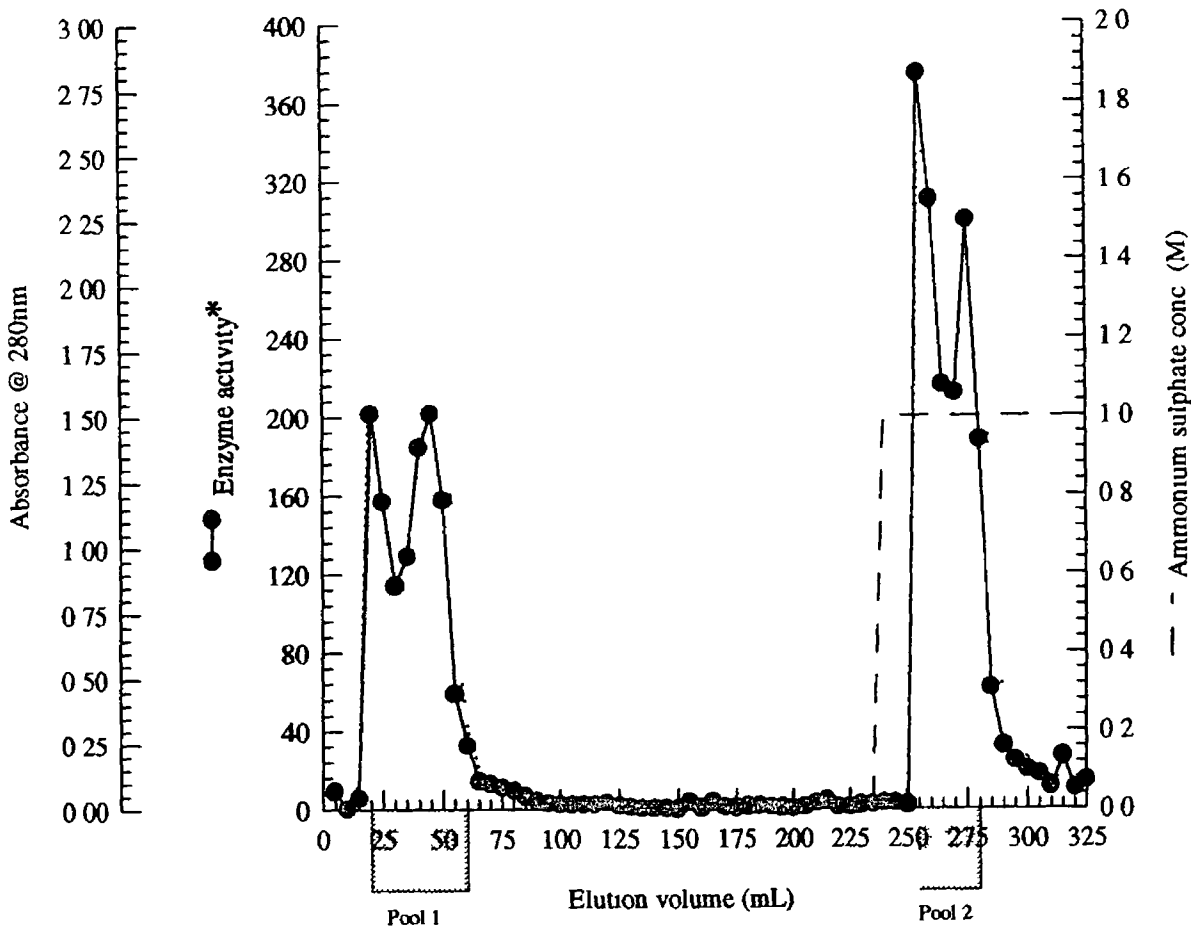


Figure 3 6 1.1. Elution of PE and ZIP activities from SP Sepharose cation exchange chromatography *Plot of absorbance at 280nm ( ), Z-Gly-Pro-MCA degrading activity (●-●) and ammonium sulphate concentration (——) versus elution volume Plot illustrates run through and bound Z-Gly-Pro-MCA degrading activities in bovine serum being separated by cation exchange chromatography Pool 1 (fractions 4 - 12, 41 5mL) represents PE activity Pool 2 (fractions 51 - 56, 27 5mL) represents ZIP activity SP Sepharose chromatography performed as described in section 2 6 1 1* * *Enzyme activity expressed as fluorimetric intensities*

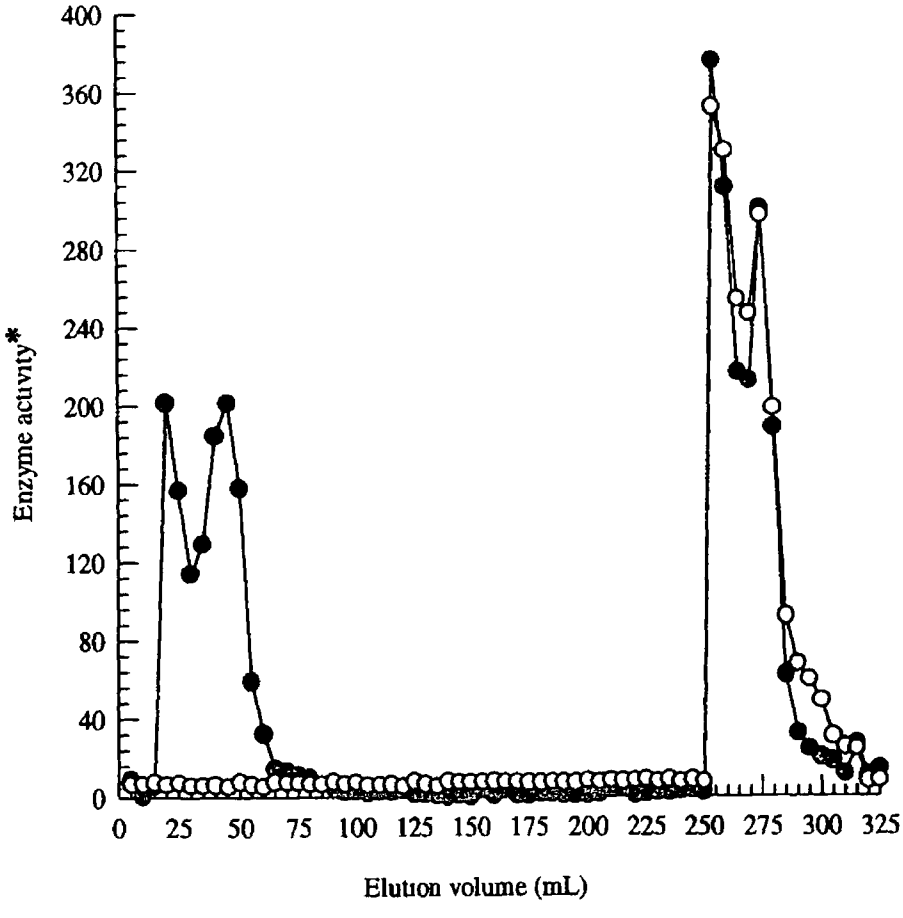


Figure 3 6 1 2 Sensitivity of post-SP Sepharose fractions to Z-Pro-Prolinal inhibition *Plot of enzyme activity versus elution volume Plot illustrates Z-Gly-Pro-MCA degrading activity in fractions assayed in the presence (o-o) and absence (●-●) of Z-Pro-Prolinal Run through activity (≈20 - 60mL) was totally inhibited by Z-Pro-Prolinal while bound activity (≈250 - 280mL) was totally insensitive to Z-Pro-Prolinal inhibition Fractions assayed as outlined in sections 2 6 1 1 and 2 6 1 2 * Enzyme activity expressed as fluorimetric intensities*

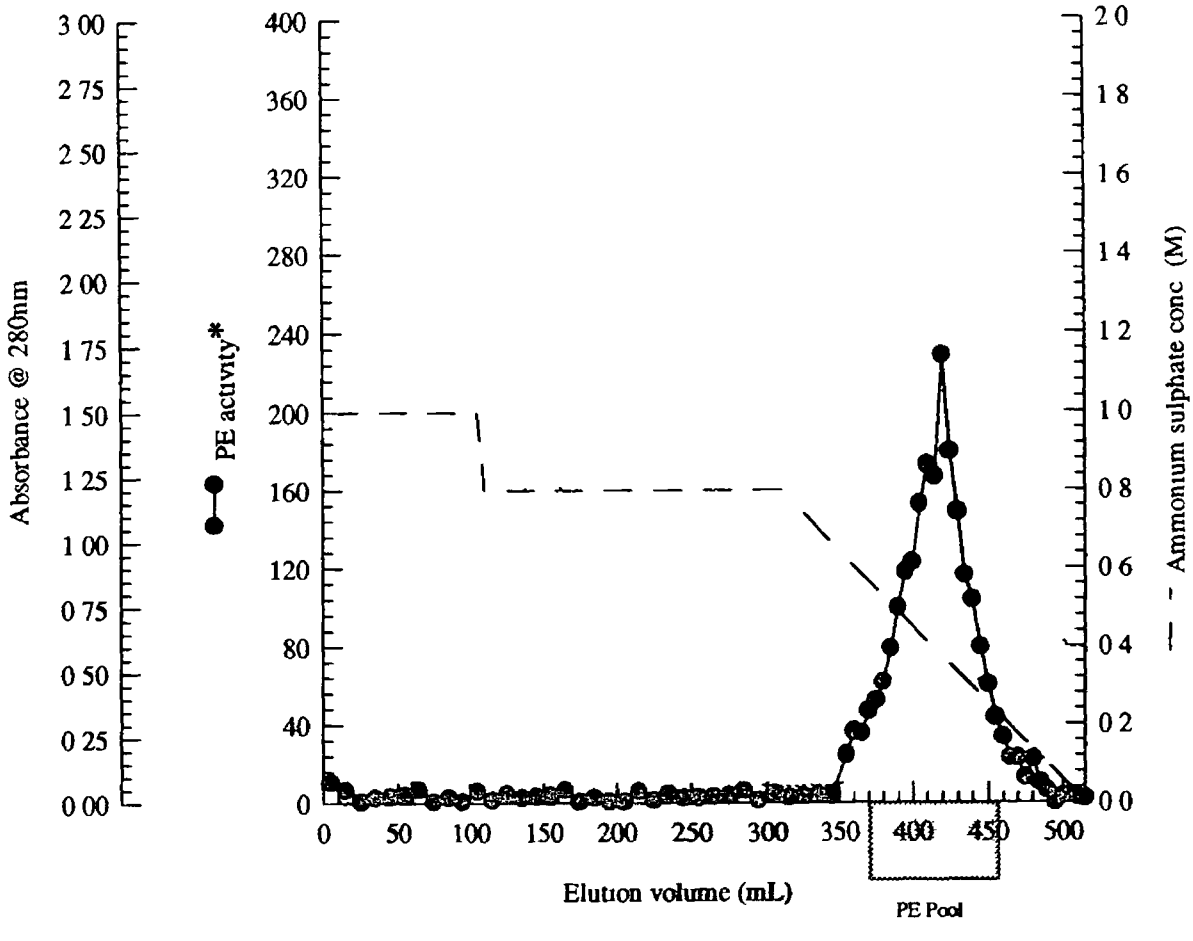


Figure 3 6 2 Elution of PE activity from Phenyl Sepharose hydrophobic interaction chromatography *Plot of absorbance at 280nm ( ), PE activity (●-●) and ammonium sulphate concentration (— —) versus elution volume Bound PE activity was eluted using a linear decreasing ammonium sulphate gradient Fractions 74 - 91 were combined to form the post-Phenyl Sepharose PE pool (84mL) Phenyl Sepharose chromatography was performed as described in section 2 6 2* * *Enzyme activity expressed as fluorimetric intensities*

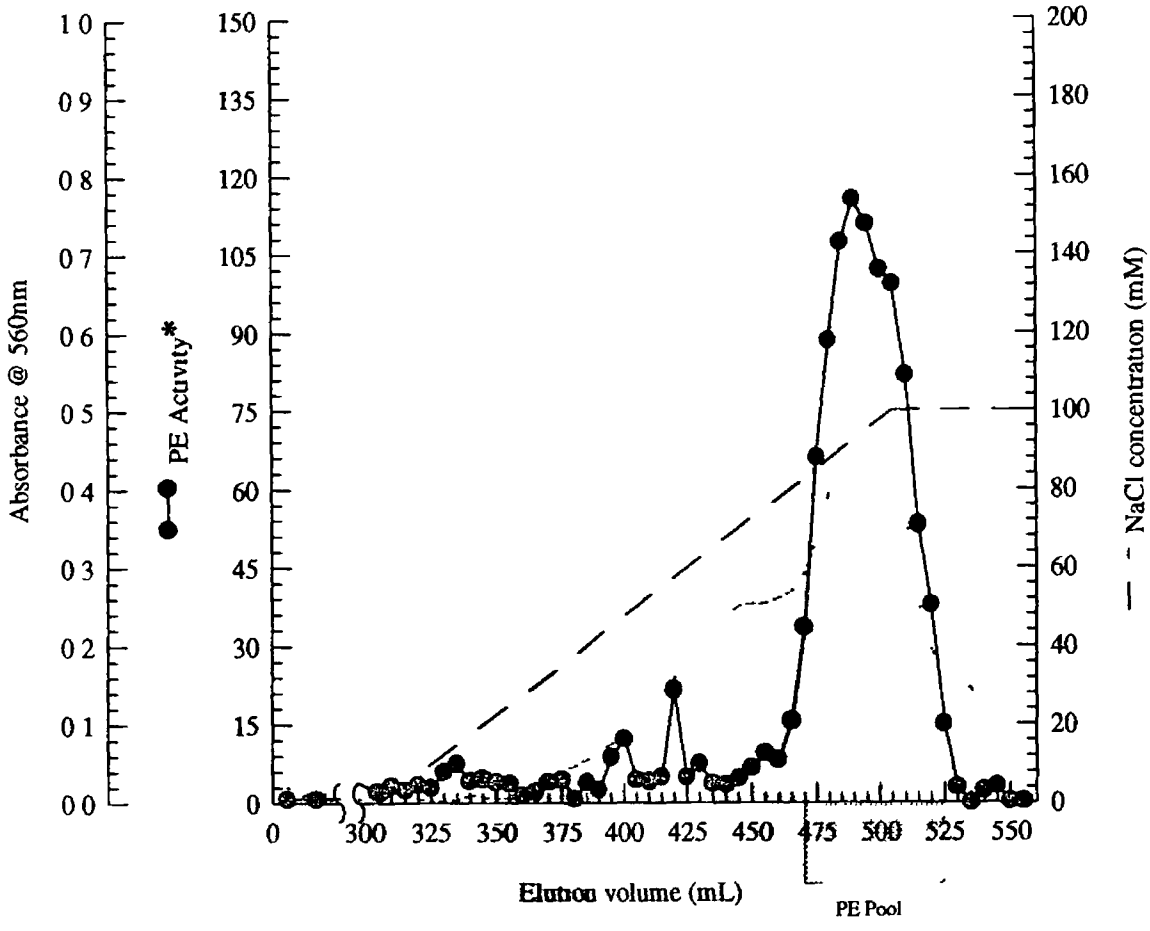


Figure 3 6 3 Elution of PE from DEAE Sepharose anion exchange chromatography *Plot of absorbance at 280nm ( ), PE activity (●-●) and NaCl concentration (——) versus elution volume Bound PE activity was eluted using a linear increasing NaCl gradient Fractions 94 - 111 were combined to form the post-DEAE Sepharose PE pool (82mL) DEAE Sepharose chromatography was performed as described in section 2 6 3 * Enzyme activity expressed as fluorimetric intensities*

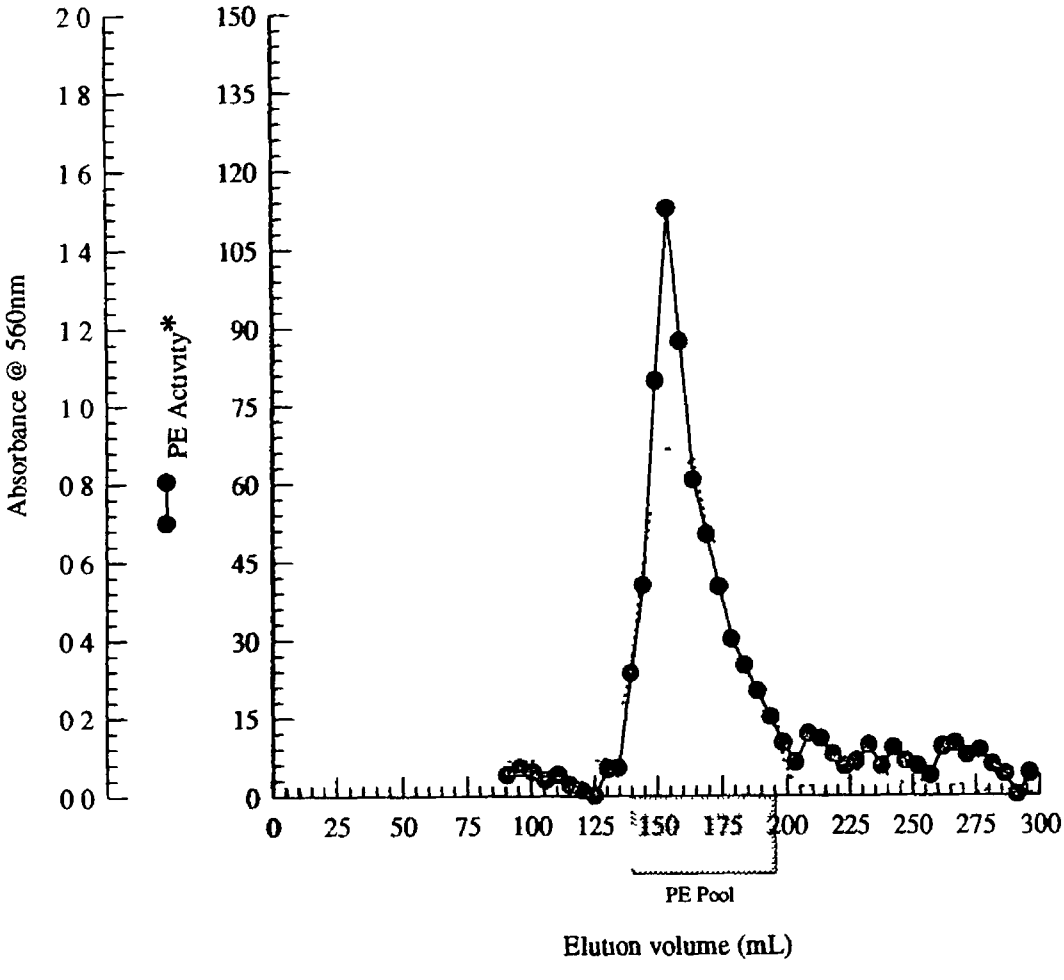


Figure 3 6.4. Elution of PE from Sephacryl S-200 HR Sepharose gel filtration chromatography. *Plot of absorbance at 560nm ( ) and PE activity (●–●) versus elution volume Protein was determined using the Standard BCA assay as described in section 2 3 3 Fractions 11 - 23 were combined to form the post-Sephacryl S-200 pool (61mL) and were stored on ice for characterisation studies Sephacryl S-200 HR Sepharose chromatography was performed as outlined in section 2 6.4 * Enzyme activity expressed as fluorimetric intensities*

Purification stage	Total protein (mg)	Total activity (Units)*	Specific activity (Units/mg)	Purification factor	Recovery (%)
Serum	1,420	10,228	7 20	1 00	100
Dialysis pH 5 5	1,084	8,716	8 04	1 12	85
SP Sepharose	422	4,994	11 83	1 64	49
Phenyl Sepharose	31	4,243	137 21	19 05	41
DEAE Sepharose	12	2,420	205 88	28 59	24
Sephacryl S-200 HR	11	1,197	104 29	14 48	12

Table 3 2 *Purification of prolyl endopeptidase from bovine serum. * Units are expressed as pmoles MCA released per minute Pools representing each purification stage were assayed for PE activity as outlined in section 2 8 7, with 15mM DTT, 15mM EDTA incorporated into substrate*

3 6.5 Further Purification of ZIP Using Phenyl Sepharose Hydrophobic Interaction Chromatography (ZIP)

The post-SP Sepharose ZIP pool was applied to the Phenyl Sepharose column as described in section 2 6 5 ZIP activity was eluted with an isocratic distilled water wash as illustrated in Figure 3 6 5 Fractions 53-56 were combined as the post-Phenyl Sepharose ZIP pool (18mL) 1mL of this pool was retained for enzyme activity and protein determinations

3 6.6 Calcium Phosphate Cellulose Chromatography (ZIP)

The post-Phenyl Sepharose ZIP pool was further purified using a Calcium Phosphate Cellulose column as described in section 2 6 6 The enzyme was eluted from the column following application of an linear increasing phosphate gradient as illustrated in Figure 3 6 6 Fractions 20-24 were combined as the post-Calcium Phosphate Cellulose pool (29mL) 1mL of this pool was retained for enzyme activity and protein determinations

3 6.7. Sephacryl S-200 HR Sepharose Gel Filtration Chromatography (ZIP)

The post-Calcium Phosphate Cellulose ZIP pool was concentrated via reverse osmosis to 2mL 200µL glycerol were added to the concentrated enzyme which was loaded and eluted from the S-200 column as described in section 2 6 7 and illustrated in Figure 3 6 7 85mL were washed through the column before fractions were collected. Fractions 7-12 were pooled (28 5mL) and stored on ice 1mL of this pool was retained for enzyme activity and protein determinations The effectiveness of the ZIP purification strategy is presented in Table 3 3

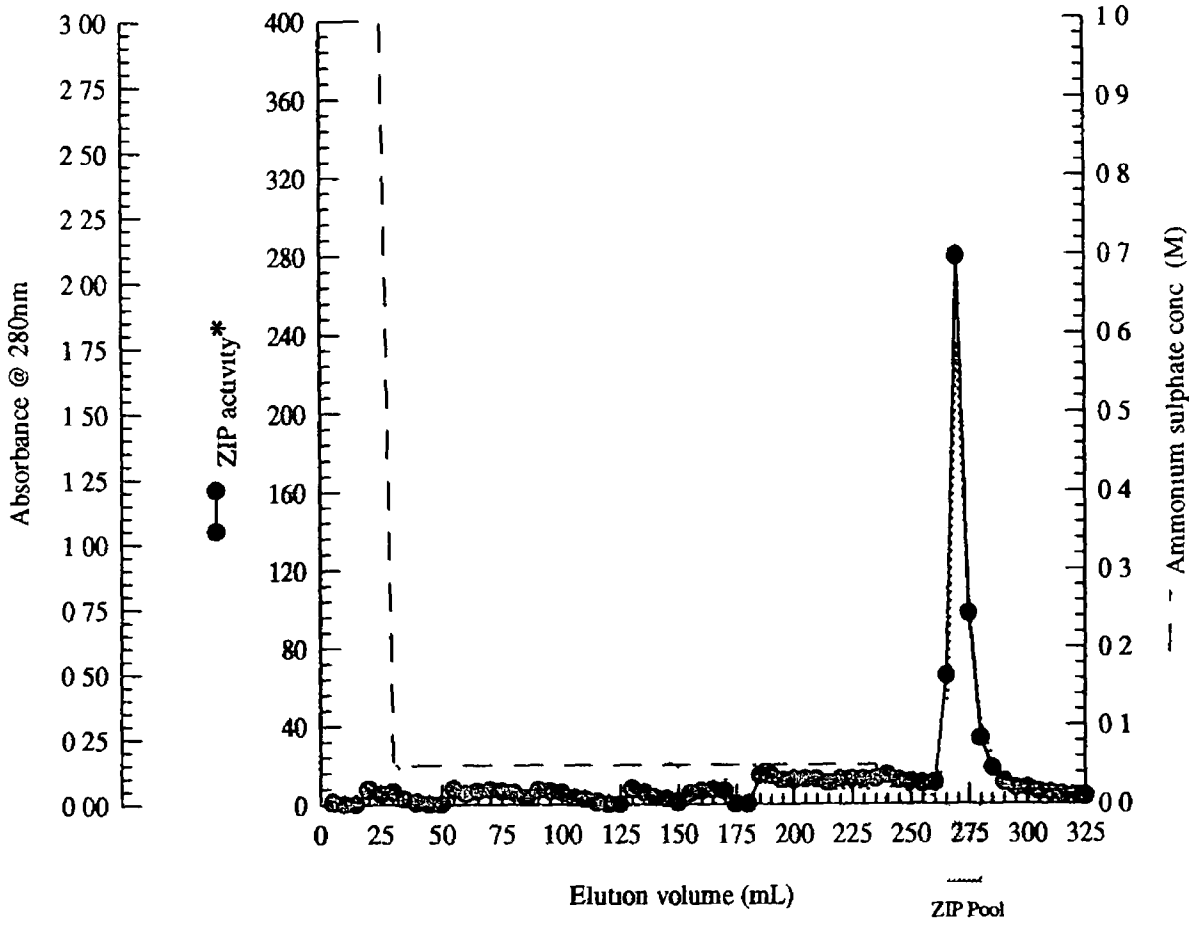


Figure 3 6 5 Elution of ZIP from Phenyl Sepharose hydrophobic interaction chromatography *Plots of absorbance at 280nm ( ), ZIP activity (●-●) and ammonium sulphate concentration (— —) versus elution volume Bound ZIP activity was eluted isocratically with a distilled water wash Factions 53 - 56 were combined to form the post-Phenyl Sepharose ZIP pool (18mL) Phenyl Sepharose chromatography was performed as described in section 2 6 5 * Enzyme activity expressed as fluorimetric intensities*

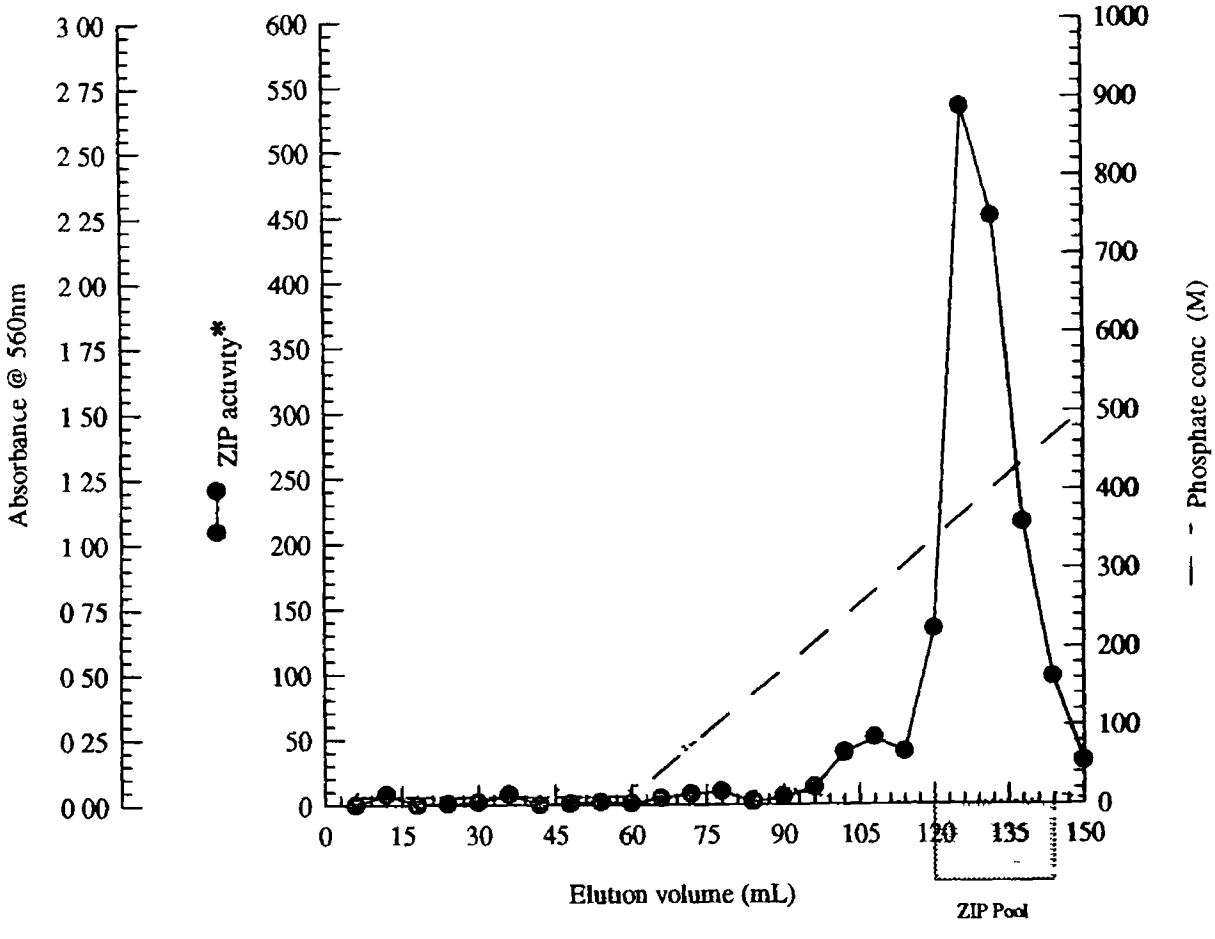


Figure 3 6 6 Elution of ZIP from Calcium Phosphate Cellulose chromatography

*Plots of absorbance at 560nm ( ), ZIP activity (●-●) and potassium phosphate concentration (— —) versus elution volume Bound ZIP activity was eluted with a linear increasing phosphate gradient Protein determined using the Standard BCA assay as described in section 2 3 3 Fractions 20 - 24 were combined to form the post-Calcium Phosphate Cellulose ZIP pool (29mL) Calcium Phosphate Cellulose Chromatography was performed as described in section 2 6 6 * Enzyme activity expressed as fluorimetric intensities*

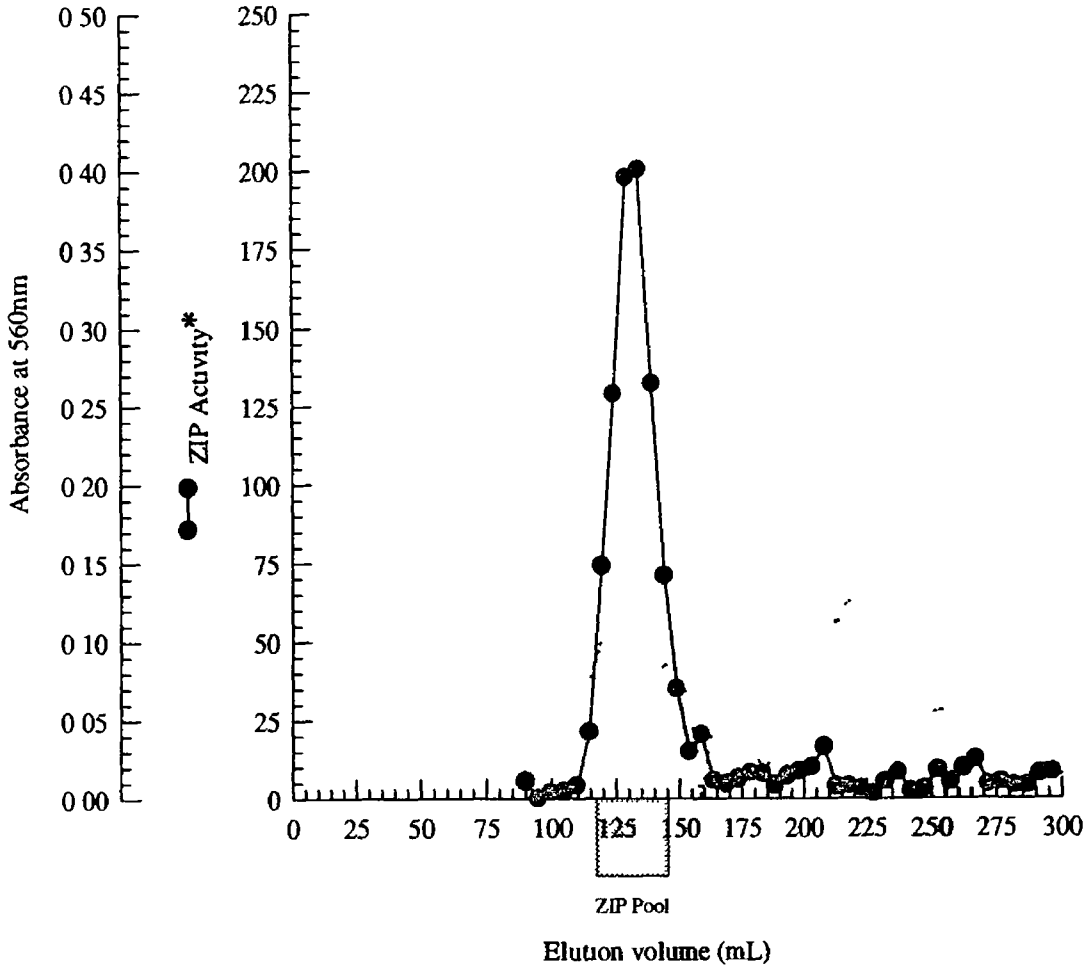


Figure 3.6 7 Elution of ZIP from Sephacryl S-200 HR Sepharose gel filtration chromatography *Plots of absorbance at 560nm ( ) and ZIP activity (●-●) versus elution volume Protein determined using the Enhanced BCA assay as described in section 2 3 4 Fractions 7 - 12 were combined to form the post Sephacryl S-200 HR Sepharose ZIP pool and were stored on ice for characterisation studies Sephacryl S-200 HR Sepharose chromatography was performed as described in section 2 6 7 * Enzyme activity expressed as fluorimetric intensities.*

Purification stage	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification factor	Recovery (%)
Serum	1,420	5,412	3 81	1 00	100
Dialysis pH 5 5	1,084	3,950	3 64	0 96	73
SP Sepharose	579	3,852	6 66	1 75	71
Phenyl Sepharose	16	2,132	129 44	33 96	39
Calcium Phosphate Cellulose	0 1	1,257	11,811	3,098	23
Sephacryl S-200 HR	0 09	757	8,601	2,256	14

Table 3 3 *Purification of ZIP from bovine serum* * *Units are expressed as pmoles MCA released per minute Pools representing each purification stage were assayed for ZIP activity as outlined in section 2 8 7, with 500mM NaCl incorporated into substrate*

3 6 8. Alternative Chromatographic Regimes Used to Further Purify PE

3 6 8 1. Q Sepharose Fast Flow Anion Exchange Chromatography

Post-Phenyl Sepharose PE activity was dialysed, applied and eluted from Q Sepharose as outlined in section 2 6 8 1 Figure 3 6 8 1 illustrates the elution of PE activity following application of a linear increasing salt gradient. This Figure also illustrates the failure of Q Sepharose to resolve the PE activity from contaminating protein

3 6.8.2 Calcium Phosphate Cellulose Chromatography

Figure 3 6 8 2 illustrates attempts made to bind PE activity to Calcium Phosphate Cellulose under conditions described in section 2 6 8 2

3 6.8.3. Biogel HT Hydroxylapatite Chromatography

Post-Phenyl Sepharose PE activity was prepared and applied to Biogel HT Hydroxylapatite as outlined in section 2 6 8 3 Figure 3 6 8 3 illustrates that under these conditions it was possible to bind PE activity However, elution of the PE activity by applying an increasing phosphate gradient failed to resolve PE from contaminating protein

3.6.8.4. Blue Sepharose Fast Flow Chromatography

Figure 3 6 8 4 demonstrates the failure of Blue Sepharose to bind PE activity under the conditions outlined in section 2 6 8 4

3.6.8 5. Activated Thiol Sepharose Chromatography

DTT was removed from post-Phenyl Sepharose PE by dialysis as described in section 2 6 8 5 1 Removal of DTT from the enzyme sample was also achieved by de-salting using a Sephadex G-25 column as outlined in section 2 6 8 5 2 Figure 3 6 8.5 1 illustrates the separation of PE activity from DTT in post-Sephadex G-25 fractions Failure to bind PE activity to Activated Thiol Sepharose is illustrated in Figure 3 6 8 5.2

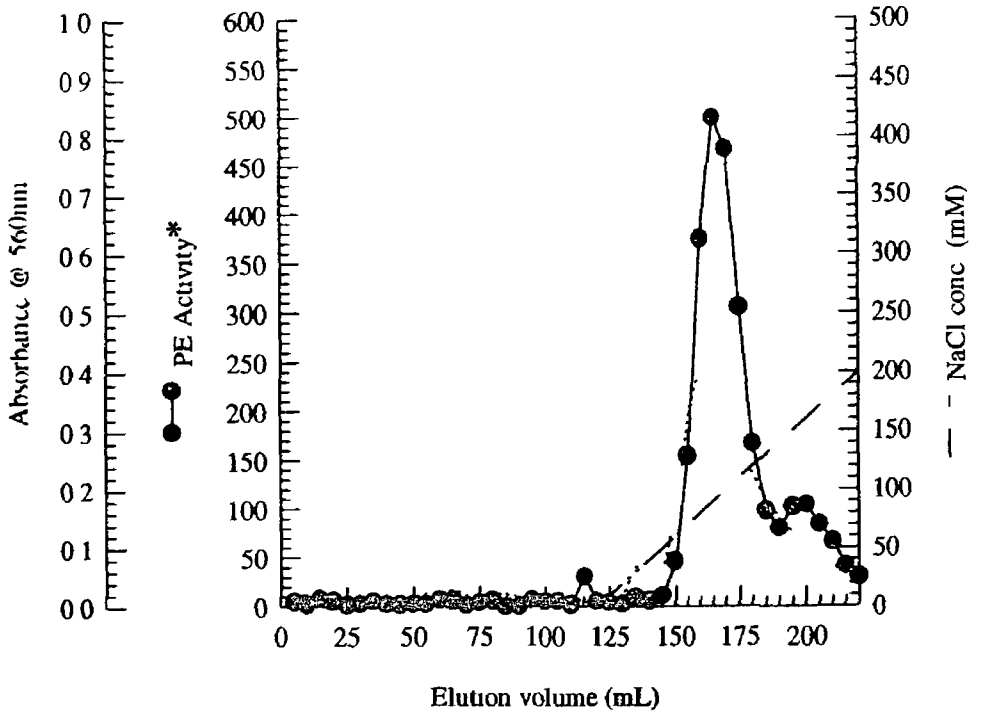


Figure 3 6 8 1 Elution of PE from Q Sepharose *Illustration of failure to resolve PE activity (●-●) from protein ( ) using a linear increasing NaCl gradient (—·—) Q Sepharose chromatography performed as described in section 2 6 8 1 * Enzyme activity expressed as fluorimetric intensities*

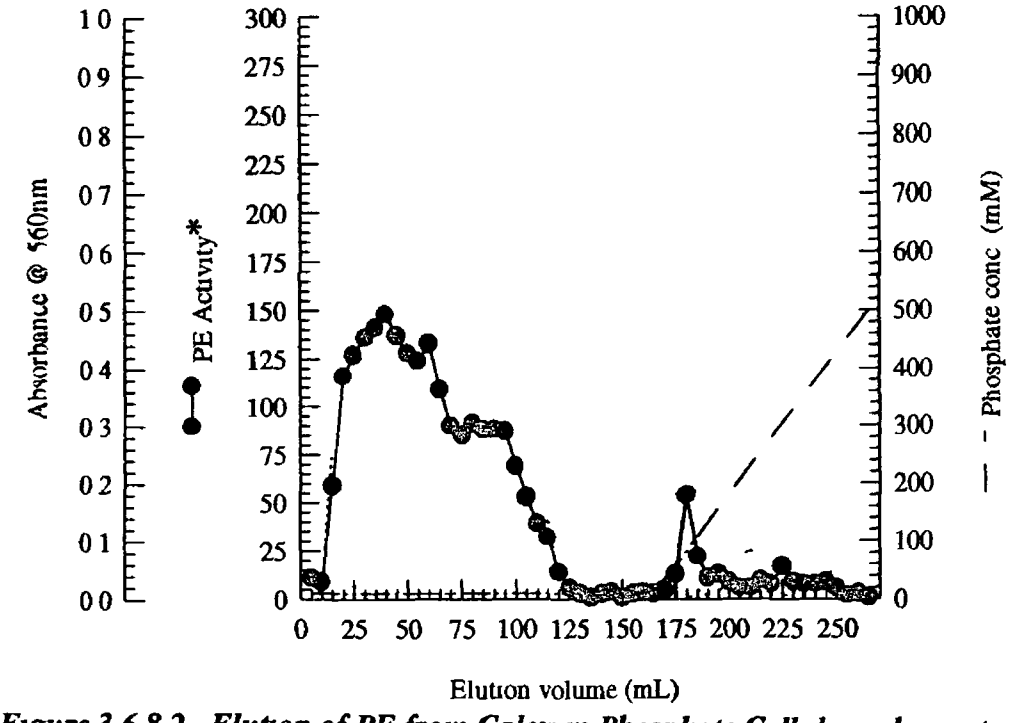


Figure 3 6 8 2 Elution of PE from Calcium Phosphate Cellulose chromatography *Illustration of failure to bind PE activity (●-●) to Calcium Phosphate Cellulose Calcium Phosphate Cellulose chromatography performed as described in section 2 6 8 2 * Enzyme activity expressed as fluorimetric intensities ( ) Protein concentration, (—·—) Phosphate concentration.*

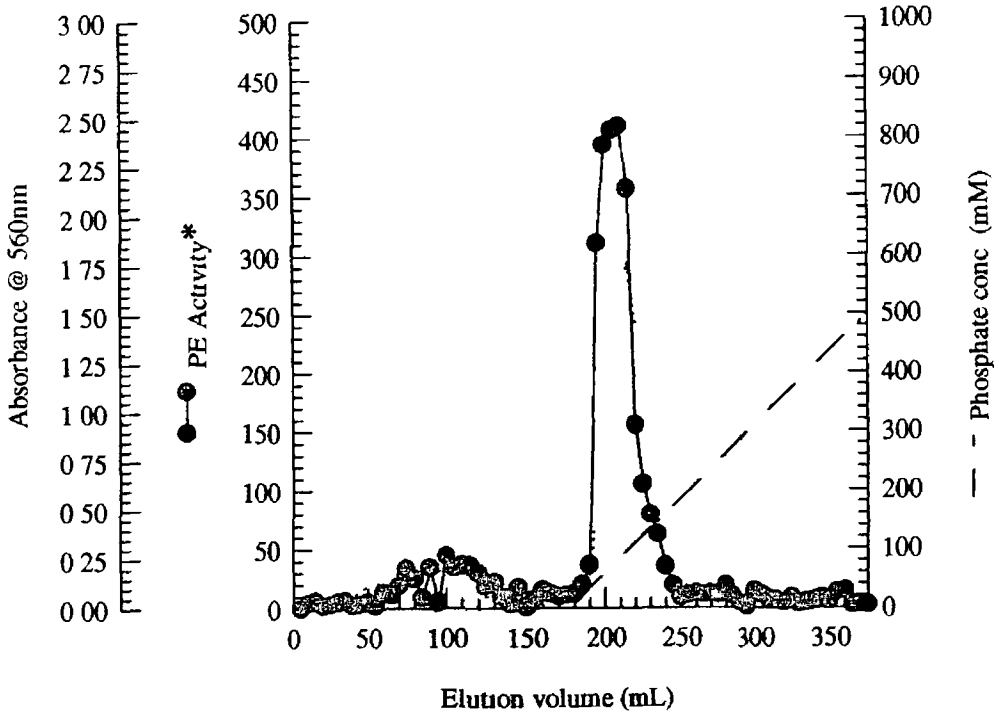


Figure 3 6 8 3 Elution of PE from Hydroxylapatite *Illustration of failure to resolve PE activity (●-●) from protein ( ) using a linear increasing potassium phosphate gradient (— —) Hydroxylapatite chromatography performed as described in section 2 6 8 3 * Enzyme activity expressed as fluorimetric intensities*

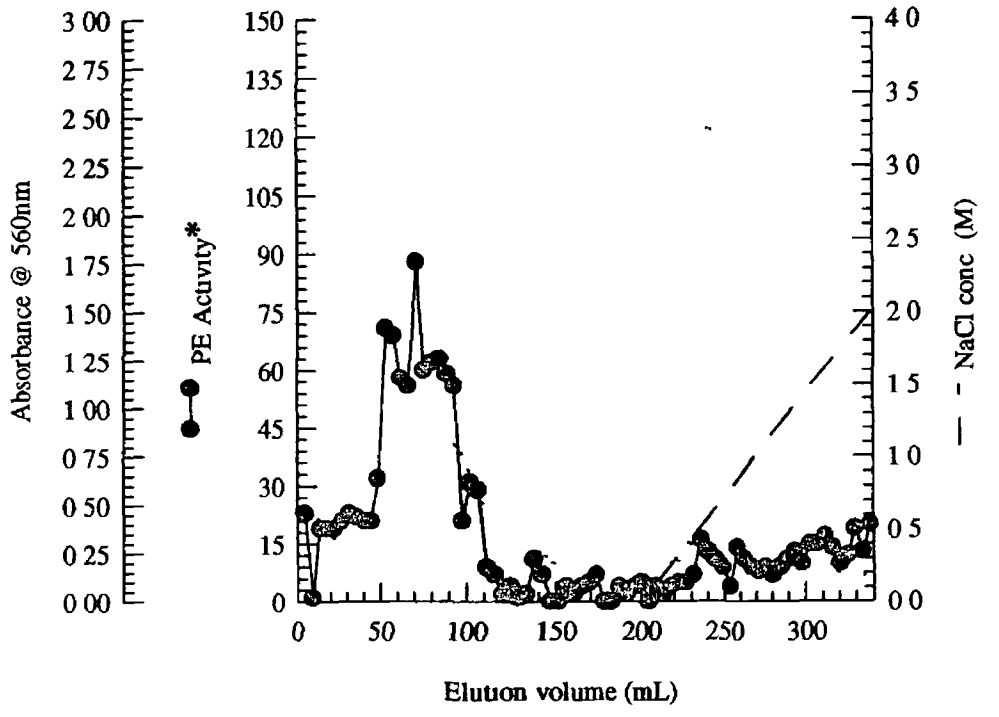


Figure 3 6 8 4 Elution of PE from Blue Sepharose *Resolution of PE (●-●) from protein ( ) was achieved with an increasing NaCl gradient (—·—), however PE activity was severely affected Blue Sepharose chromatography was performed as described in section 2 6 8 4 * Enzyme activity expressed as fluorimetric intensities*

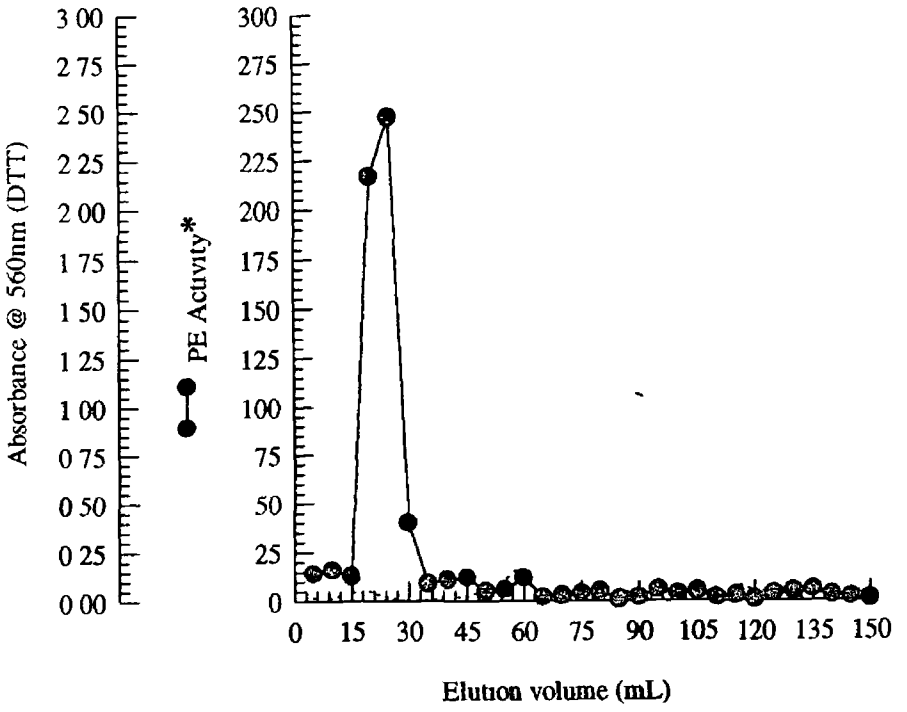


Figure 3 6 8 5 1 Elution of PE and DTT from Sephadex G-25 *Illustration of the removal of DTT ( ) from PE (●-●) prior to Activated Thiol Sepharose chromatography Sephadex G-25 chromatography performed as described in section 2 6 8 5 2 DTT determined according to its interference with the Biuret assay as described in section 2 3 2 * Enzyme activity expressed as fluorimetric intensities*

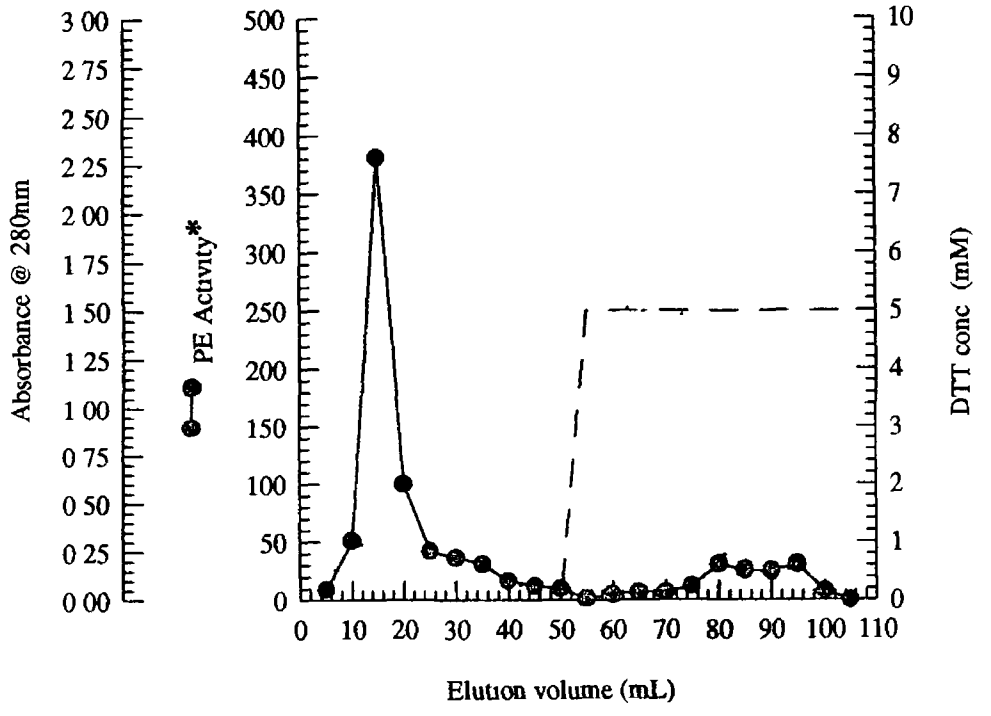


Figure 3 6 8 5 2 Elution of PE from Activated Thiol Sepharose *Illustration of failure to bind PE activity (●-●) Activated Thiol Sepharose Chromatography was performed as described in section 2 6 8 5 3 * Enzyme activity expressed as fluorimetric intensities (— —) DTT concentration, ( ) Absorbance at 280nm*

3.7. SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed as outlined in section 2.7. to determine the effectiveness of the purification protocols used. Post S-200 PE and Post S-200 ZIP samples were prepared as outlined in section 2.7.1. and subjected to electrophoresis on 1mm x 160mm x 160mm gels consisting of a 10% resolving gel overlayed with a 3.75% stacking gel. Following eletrophoresis, gels were stained with Coomasie Brilliant Blue and Silver Stain. No bands were visualised with the Coomassie Brilliant Blue stain. The silver stained gel image was recorded digitally as outlined in section 2.7.4. Figure 3.7.1. represents the silver stained gel images for ZIP and PE . Molecular weight markers applied to the gels are also presented. The appearance of multiple bands on the silver stained gel image for PE prevented positive identification of this protein on the gel.

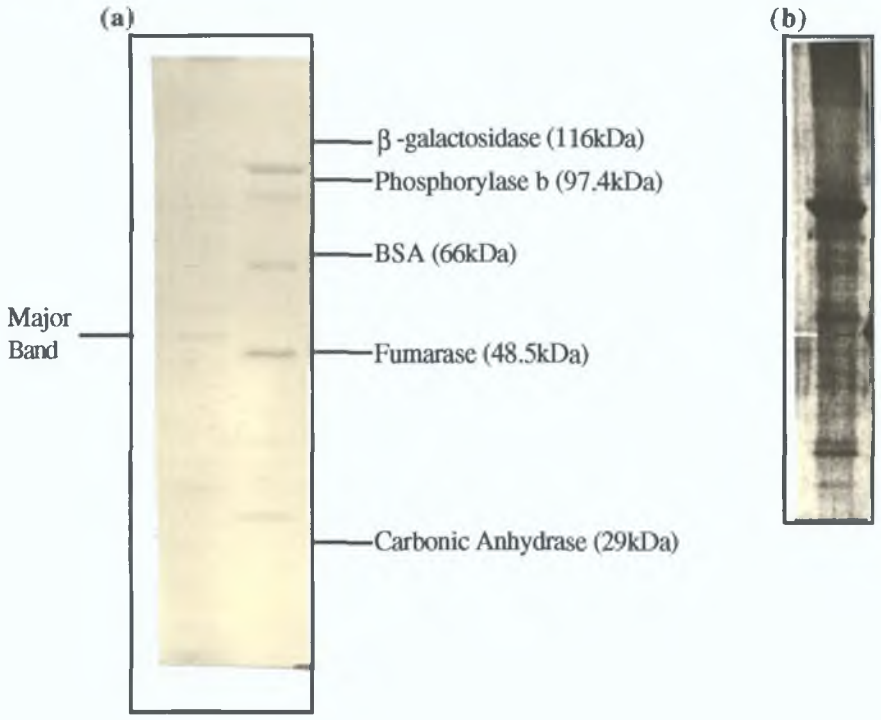


Figure 3.7.1. Polyacrylamide Gel Electrophoresis Results. *SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as outlined in section 2.7. with the silver stained image digitally recorded as described in section 2.7.4.* ***(a)*** *Image illustrates a major band obtained when purified ZIP was subjected to electrophoresis with subsequent silver staining. The position of this band relative to the positions of the included molecular weight markers indicates that it has a molecular weight of approximately 50,000 Da.* ***(b)*** *Image illustrates the appearance of multiple bands when partially purified PE was subjected to elctrophoresis with subsequent silver staining. The major band corresponds with BSA (66,000) and indicates that this protein is the major contaminant present in the PE preparation. The subunit make up of PE could not be determined due to the multiple band obtained.*

3 8 Assay Development for Purified Z-Gly-Pro-MCA Degrading Activities

3 8 1. Determination of Suitable Solvent for Substrate Solubilisation

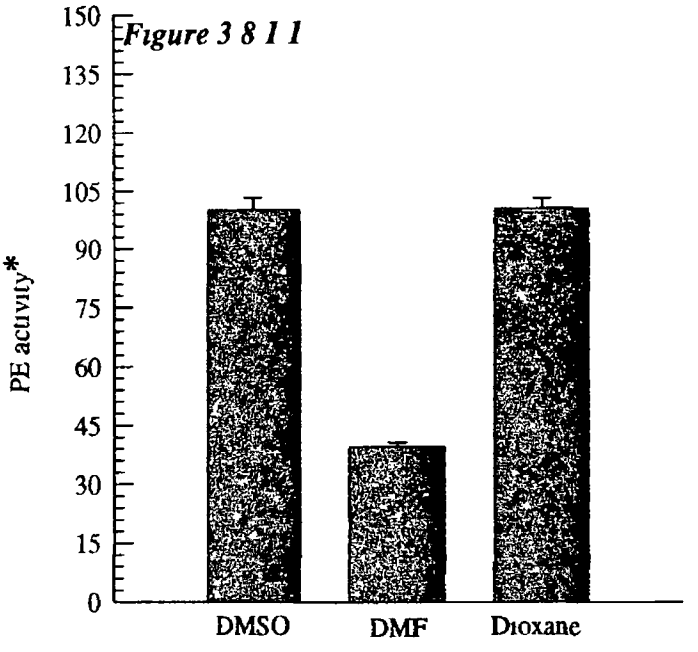
Stock substrates were prepared as outlined in section 2 8 1 in DMSO, DMF and dioxane. The effects of using these solvents in substrate preparation are illustrated in Figures 3 8 1 1 and 3.8 1.2. for PE and ZIP assays respectively Dioxane was chosen as the most suitable solvent for subsequent substrate solubilisation

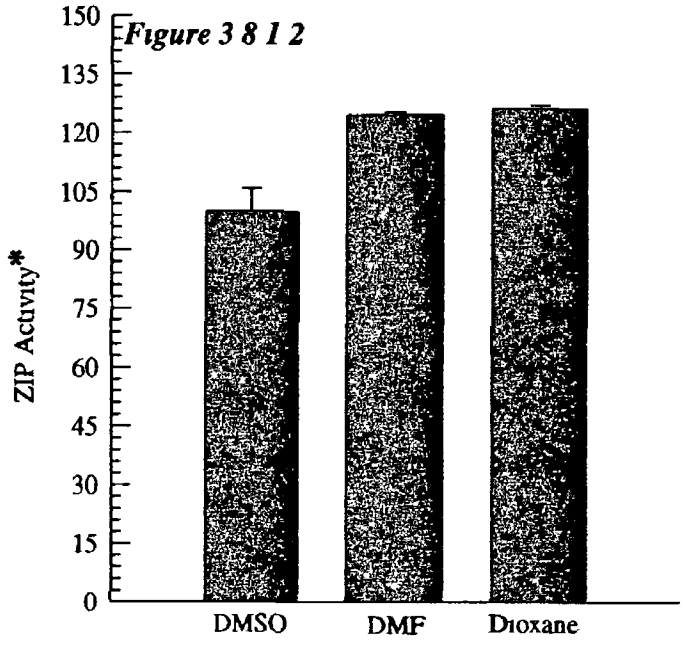
3.8 2 Effect of Dioxane Concentration on Purified Enzymes

Figures 3 8 2 1 and 3 8 2 2 illustrate the effect of increasing dioxane concentration in substrate preparations on purified PE and ZIP activities respectively 2% (v/v) dioxane was chosen as the most suitable final dioxane concentration in subsequent substrate preparations for both purified PE and purified ZIP

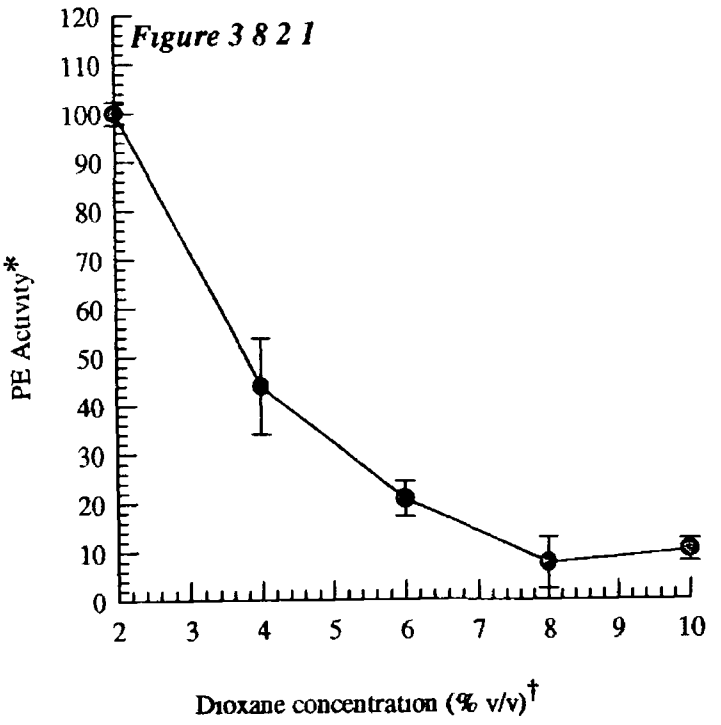
3 8 3. Linearity of Enzyme Assays with Respect to Time

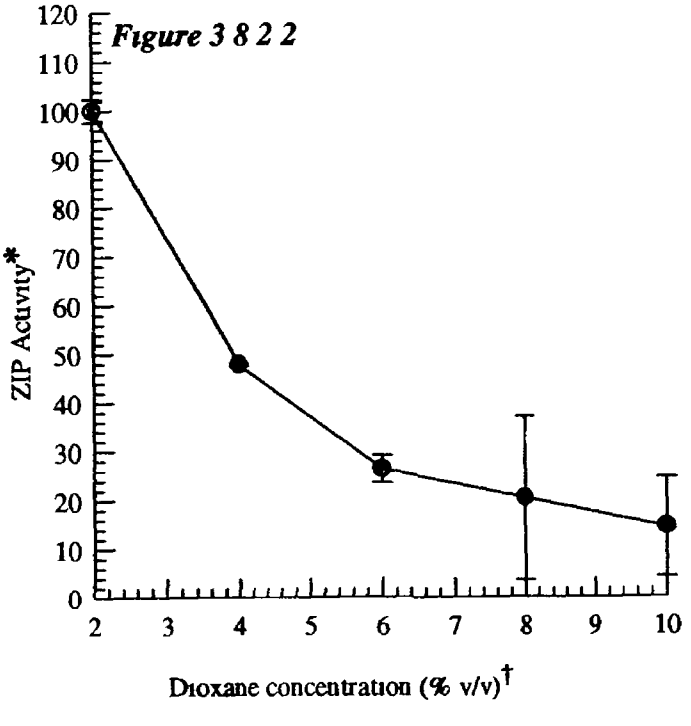
Linearity of PE and ZIP assays was determined as outlined in section 2 8 3 Purified PE activity was linear over a 2 hour assay period in the presence and absence of DTT (Figure 3 8.3 1.). Purified ZIP activity was also linear over this period as illustrated by Figure 3 8 3.2 Assays for both PE and ZIP activities working in tandem in serum (Figure 3 8 3 3) and ZIP activity working alone in serum (Figure 3 8 3 4) were linear over a 1 hour period at 37°C



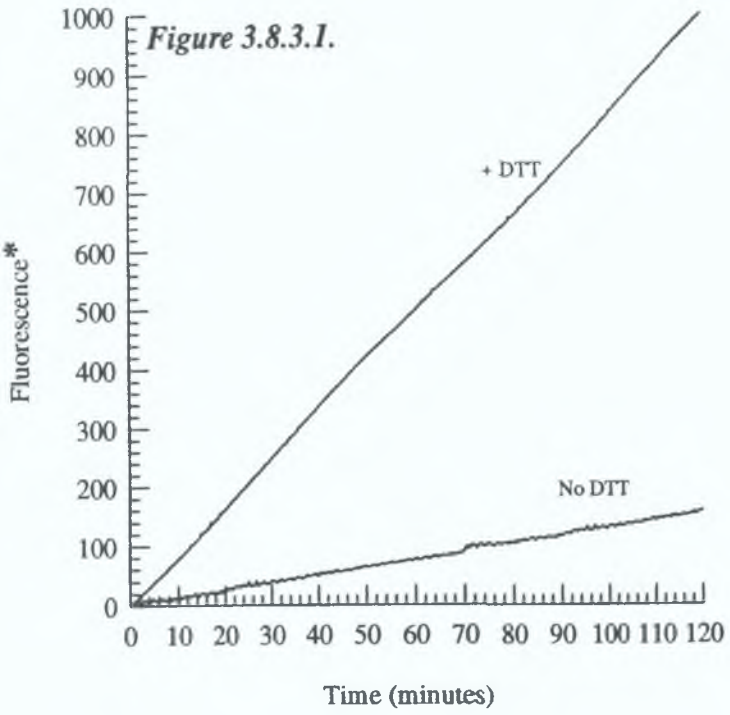


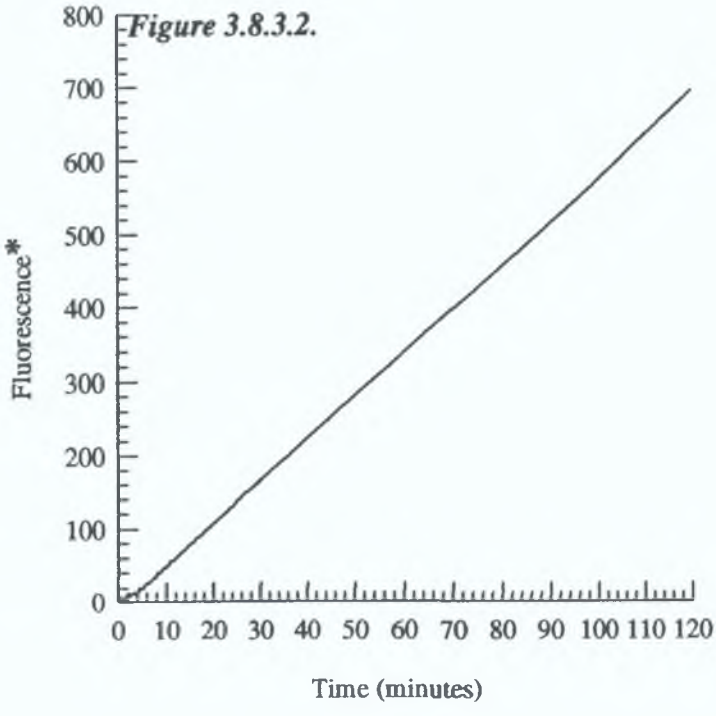
Figures 3 8 1 1 and 3 8 1 2 Effect of substrate solubilisation solvent on purified enzyme activities *Plots of enzyme activity versus solvent choice 10mM Z-Gly-Pro-MCA stocks were prepared in 100% DMSO, DMF or dioxane as outlined in section 2 8 1 Purified PE (Figure 3 8 1 1) and purified ZIP (Figure 3 8 1 2) were assayed with 0.1mM Z-Gly-Pro-MCA prepared from these stocks as outlined in section 2 5 1 Figure 3 8 1 1 illustrates that DMF was not a suitable substrate solvent for determining PE activity Figure 3 8 1 2 illustrates that DMSO was not a suitable substrate solvent for determining ZIP activity Dioxane, therefore, was chosen as the single most suitable substrate solvent for determining PE and ZIP activities * Enzyme activities expressed as % activity obtained in 4% (v/v) DMSO*



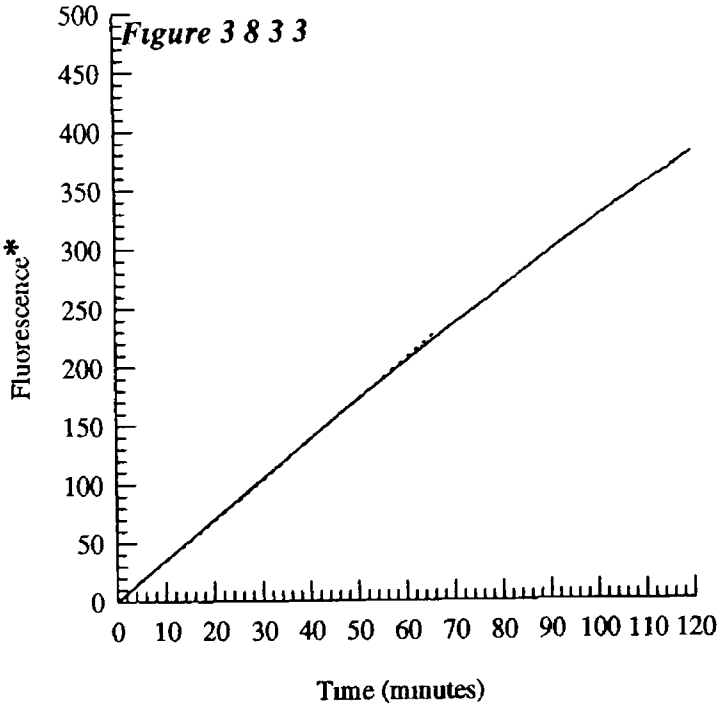


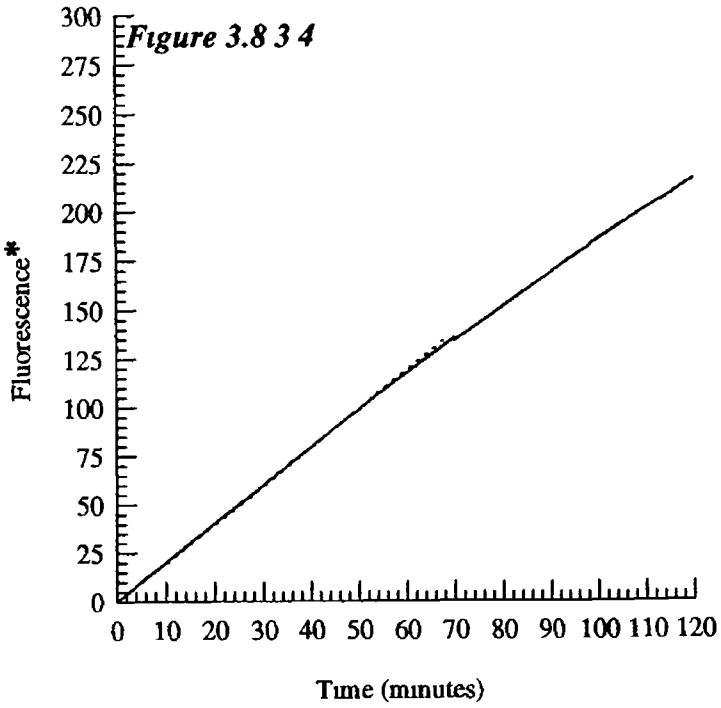
Figures 3 8 2 1 and 3 8 2.2 Effect of final dioxane concentration in substrate on purified PE and ZIP activities. *Plots of enzyme activity versus dioxane concentration for purified PE (Figure 3 8 2 1) and purified ZIP (Figure 3 8 2 2) Investigation performed as described in section 2 8 2 Figures 3 8 2 1 and 3 8 2 2 illustrate that assays with substrate containing a dioxane concentration of 2% (v/v) produce maximum PE and ZIP activities respectively Increasing dioxane concentration to 4% (v/v) reduces apparent sensitivity of both PE and ZIP assays by greater than 50% 2% (v/v) dioxane, therefore, was chosen as the most suitable solvent concentration in substrate used to determine PE and ZIP activities * Enzyme activity expressed as % of activity obtained at a solvent concentration of 2% (v/v) † Dioxane concentration expressed as final solvent concentration in substrate (v/v)*





Figures 3.8.3.1. and 3.8.3.2. Linearity of purified PE and ZIP assays with respect to time. *Plots of enzyme activity versus assay time for PE (Figure 3.8.3.1.) and ZIP (Figure 3.8.3.2.). Assays performed as described in section 2.8.3. where enzyme activity was monitored continuously over a 2 hour period in an incubated cuvette at 37°C. Plots demonstrate that both the PE and ZIP assays were linear over a period of 2 hours, assuring the linearity of 1 hour discontinuous assay for both enzymes. Figure 3.8.3.1. illustrates linearity of PE assay in the presence and absence of DTT.* * *Enzyme activity expressed as fluorimetric intensities.*





Figures 3 8 3 3 and 3.8 3 4 Linearity of Z-Gly-Pro-MCA degradation in bovine serum. *Plots of Z-Gly-Pro-MCA degradation versus assay time in the presence (Figure 3 8 3 4) and absence (Figure 3 8 3 3) of Z-Pro-Prolinal Investigation performed as outlined in section 2 8 3 Plots demonstrate that Z-Gly-Pro-MCA degradation in bovine serum is linear for up to 1 hour for PE and ZIP activities working in tandem (Figure 3 8 3 3) and ZIP activity working alone (Figure 3 8 3 4), assuring the linearity of a 1 hour discontinuous assay under similar conditions (—) observed velocity ( ) initial velocity * Enzyme activity expressed as fluorimetric intensities*

3 8 4 Linearity of Assays with Respect to Enzyme Concentration

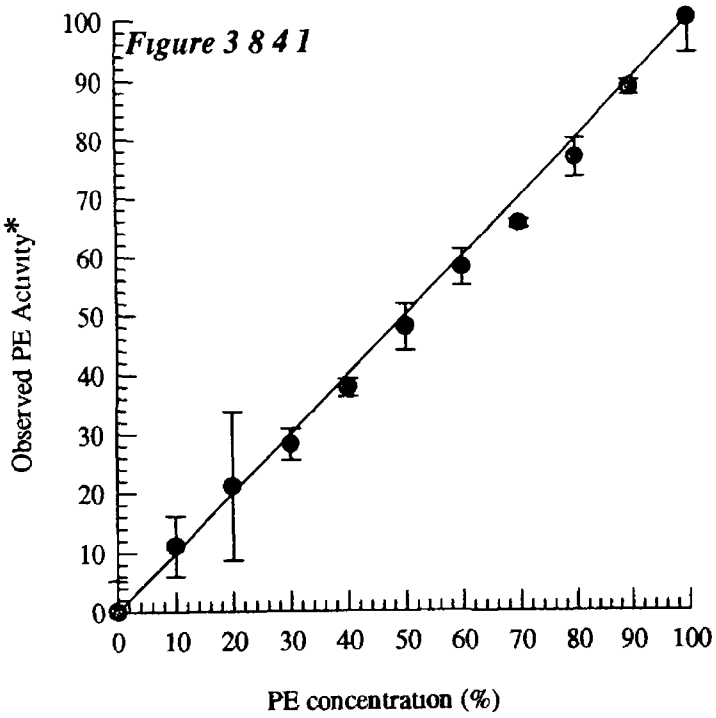
Linearity of PE and ZIP assays with respect to enzyme concentration was determined as outlined in section 2 8 4 Figure 3 8 4 1 illustrates that the purified PE assay was linear with respect to enzyme concentration Figure 3 8 4 2 demonstrates that the purified ZIP assay was not linear with respect to enzyme concentration under the conditions described in section 2 8 4 Figure 3 8 4 3 illustrates the linear reaction velocity of a 50% ZIP sample monitored over 1 hour as outlined in section 2 8 3 Inclusion of 200mM KCl in substrate and diluant produced a linear assay response to enzyme concentration (Figure 3 8 4 4) Following removal of post-gel filtration KCl by dialysis as outlined in section 2 8 4 , ZIP produced a linear assay response to enzyme concentration (Figure 3 8 4 5) where no KCl was present in substrate or diluant

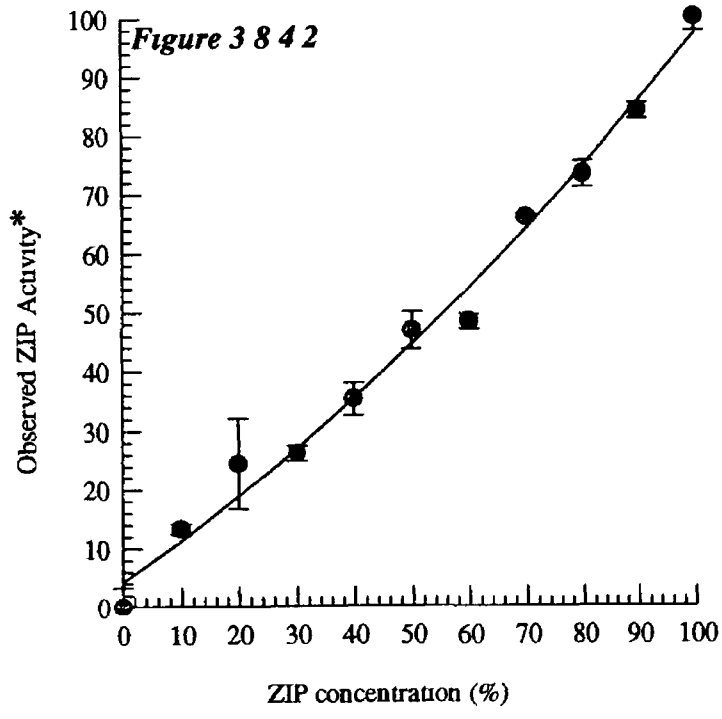
3.8.5. DTT Activation of Purified Enzyme Activities

Figure 3 8 5 illustrates that maximal PE activity is obtained when at least 12mM DTT is included in the substrate ZIP showed a 30% decrease in observed activity at a DTT concentration of 20mM

3 8 6. Salt Activation of Purified Enzyme Activities

ZIP demonstrated enhanced activity in the presence of increasing concentrations of NaCl This enhanced activity was maximal at a salt concentration of approximately 500mM NaCl as illustrated in Figure 3 8 6 while PE demonstrated a 25% decrease in activity at 800mM NaCl





Figures 3 8 4 1 and 3 8 4 2. Linearity of purified enzyme assays with respect to enzyme concentration *Plots of observed enzyme activity versus purified PE (Figure 3 8 4 1) or purified ZIP (Figure 3 8 4 2) concentration Investigation performed as described in section 2 8 4 Plots demonstrate that while the PE assay* **was** *linear with respect to enzyme concentration, the ZIP assay* **was not** *Data from a plot of enzyme activity with respect to ZIP concentration in Figure 3 4 8 2 fitted a second order regression curve better than it did a first order linear regression.* * *Enzyme activity expressed as % of activity obtained from undiluted enzyme*

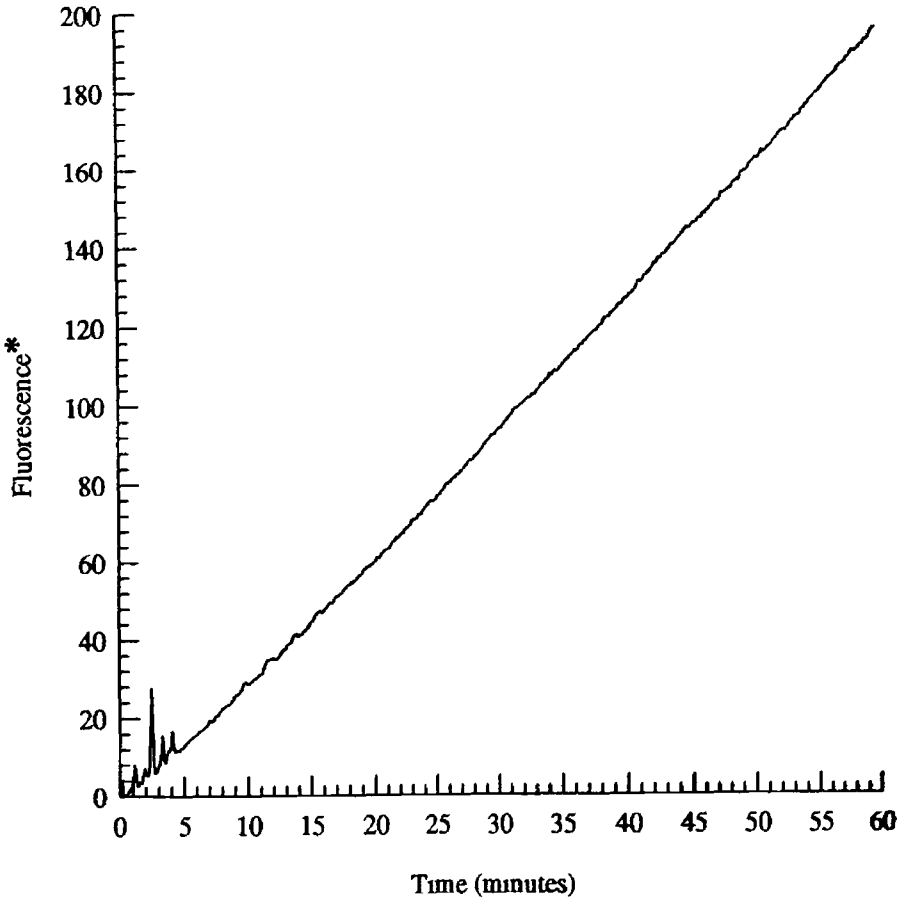


Figure 3.8 4 3 Linearity of a 50% ZIP concentration assay with respect to time

Plot of enzyme activity versus time Assay performed as outlined in section 2 8 3 with ZIP activity being monitored continuously in an incubated cuvette Plot illustrates that dilution of ZIP activity prior to assay does not affect the stability of the enzyme under assay conditions over a period of 1 hour Therefore, the lack of linearity with respect to ZIP concentration observed in Figure 3 8 4.2. is <u>not</u> *related to any instability of the enzyme when in diluted form.* * *Enzyme activity expressed as fluorimetric intensities*

Figure 3.8.4.4.

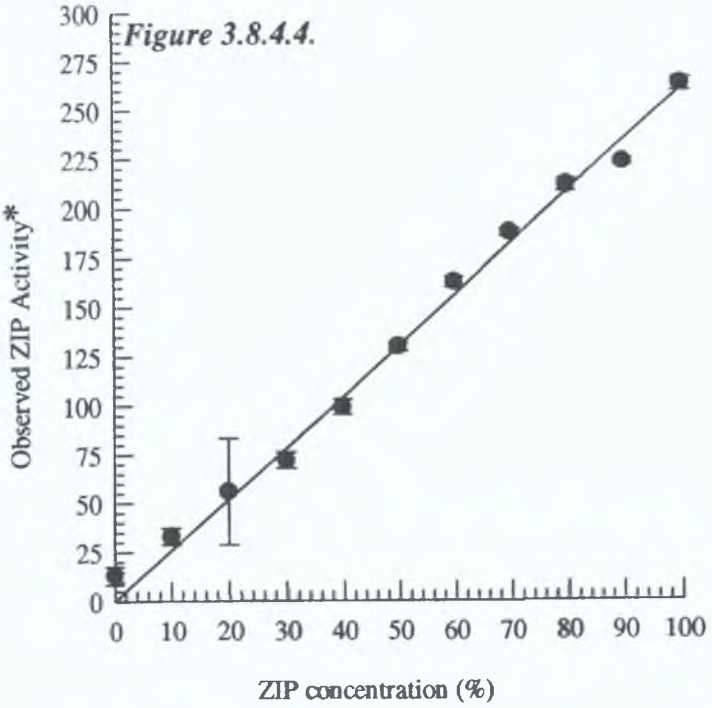
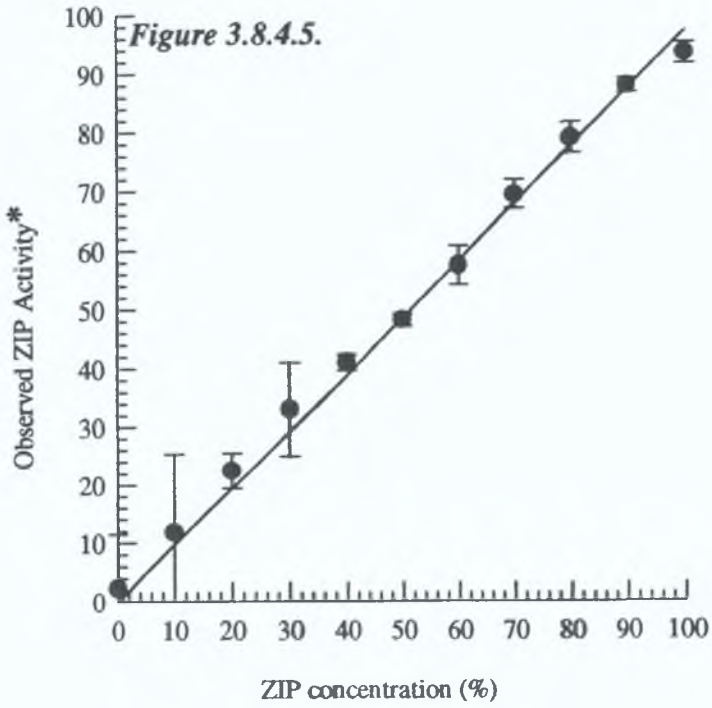
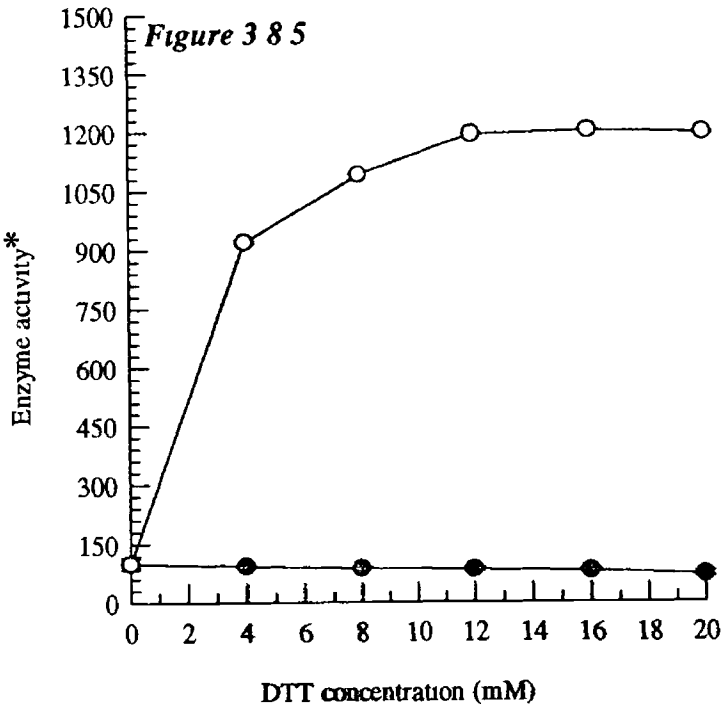
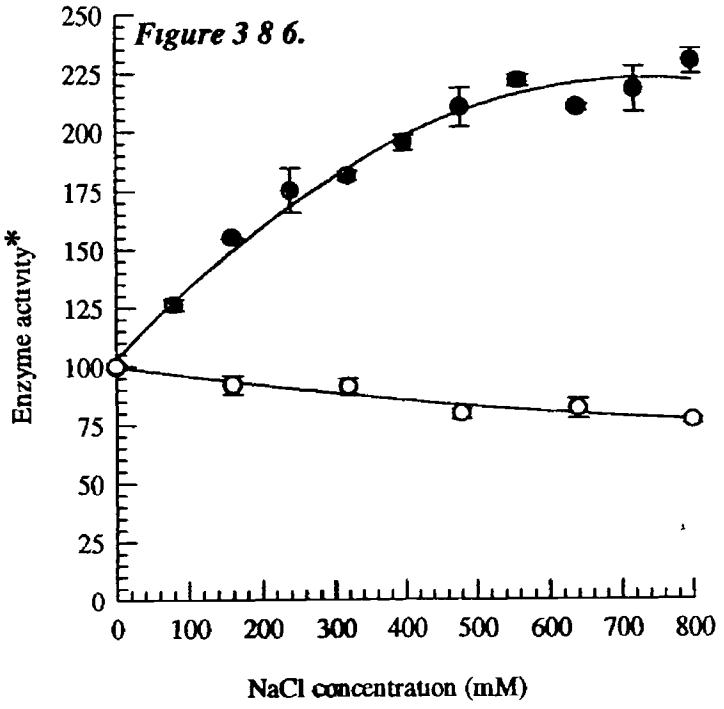


Figure 3.8.4.5.



Figures 3.8.4.4. and 3.8.4.5. Linearity of purified ZIP assays with respect to ZIP concentration : The effects of KCl. *Plots of observed ZIP activity versus ZIP concentration. In Figure 3.8.4.4. ZIP activity was diluted and assayed in buffer containing 200mM KCl while in Figure 3.8.4.5. ZIP activity, with post gel filtration KCl removed by dialysis, was diluted and assayed in buffer containing no KCl. Investigation performed as described in section 2.8.4. Both plots are linear with respect to ZIP concentration. A salt enhanced activity was elucidated for purified ZIP with enzyme activities being increased by a factor of 2.75 in the presence of 200mM KCl. Post gel filtration KCl was therefore responsible for non-linearity of Figure 3.8.4.2.* * *Enzyme activity expressed as % activity obtained from undiluted dialysed enzyme.*





Figures 3 8 5 and 3 8 6 Effect of DTT and NaCl concentrations on purified PE and ZIP activities *Plots of PE (o-o) and ZIP (•-•) activities versus DTT concentration (Figure 3 8 5) and NaCl concentration (Figure 3 8.6) respectively Figure 3 8 5 illustrates the DTT enhanced activity of purified PE while ZIP activity is independent of DTT concentration Figure 3 8 6 illustrates the salt enhanced activity of ZIP while PE activity is inhibited by 25% at 800mM NaCl Maximum enzyme activities were obtained when 12mM DTT or 500mM NaCl are incorporated into substrate used to assay PE or ZIP activity respectively * Enzyme activity expressed as % activity of enzyme assayed in the absence of DTT or NaCl*

3 9 Characterisation of Purified PE and ZIP Activities

3 9 1 Relative Molecular Mass Determination

3 9 1.1 Sephacryl S-200 HR Gel Filtration Chromatography

A Sephacryl S-200 gel filtration column was calibrated as outlined in section 2 9 1 1 producing a calibration curve for the column which is illustrated in Figure 3 9 1 1 Also included on this plot of Log molecular mass versus V_e/V_O are the positions of ZIP and PE, calculated from the calibration curve equation based on their respective elution volumes The calibration curve obtained was

$$\text{Log(molecular mass)} = -2\ 82(V_e/V_O) + 9\ 1$$

where V_e and V_O are elution volume and void volume (101 5mL) respectively It was deduced from this equation that the molecular weights of PE and ZIP are 69,700 Daltons and 184,200 Daltons respectively

3.9.1.2. Biosep SEC-3000 High Performance Size Exclusion Liquid Chromatography

A Biosep SEC-3000 column was calibrated as outlined in section 2 9 1 2.2 producing a calibration curve for the column which is illustrated in Figure 3 9 1 2 Also included on this plot of Log molecular mass versus V_e are the positions of ZIP and PE , calculated from the calibration curve equation based on their respective elution volumes The calibration curve obtained was

$$\text{Log(molecular mass)} = -\ 0\ 27(V_e) + 9\ 67$$

It was deduced from this equation that the molecular weights of PE and ZIP are 54,500 Daltons and 191,000 Daltons respectively

3.9.1.3. SDS Polyacrylamide Gel Electrophoresis

Non-native SDS gels were prepared as outlined in section 2 7 A calibration curve based on the relative mobility of the molecular weight standards (R_f) was produced as described in section 2 9 1 3 and is presented in Figure 3 9 1 3 Also included on this plot of Log molecular weight versus R_f are the positions of PE and ZIP calculated from the gel calibration curve equation, based on their respective R_f values. The calibration curve obtained was

$$\text{Log(molecular mass)} = -1\ 0(R_f) + 5\ 2$$

It was deduced from this equation that the molecular weight of ZIP was 50,000 Daltons and that the enzyme was tetrameric The deduced molecular weight of PE could not be determined by this method because multiple bands were visualised on the gel

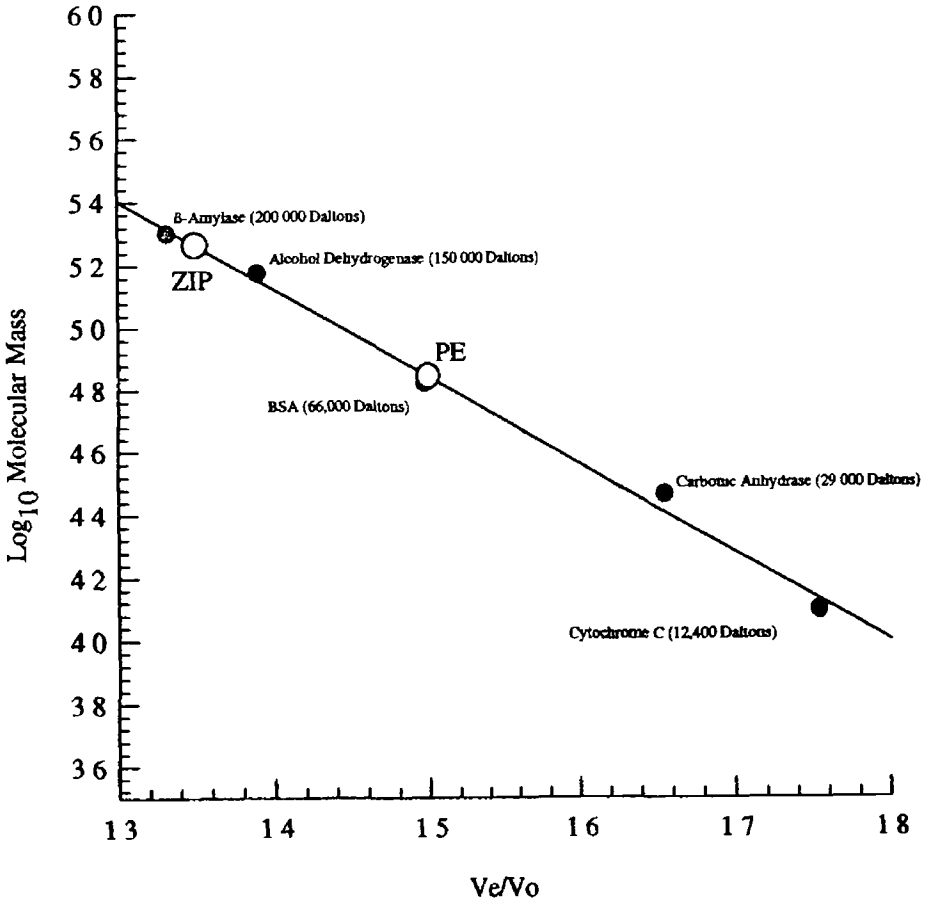


Figure 3 9 1.1 Sephacryl S-200 HR Sepharose molecular mass calibration curve

Plot of Log molecular mass versus V_e/V_O where V_e is the elution volume of a molecular mass standard (●-●) or enzyme activity (O) and V_O is the void volume of the column used (101 5mL) V_e and V_O were determined as described in sections 2 9 1 1 1 and 2 9 1 1 2 Linear regression analysis of the data obtained from the molecular mass standards produced the following calibration equation

$$Log(molecular\ mass) = -2\ 82(V_e/V_O) + 9\ 1$$

From this equation the molecular mass of PE and ZIP were calculated to be 69,700 Daltons and 184,200 Daltons respectively The positions of PE and ZIP are indicated on the calibration curve

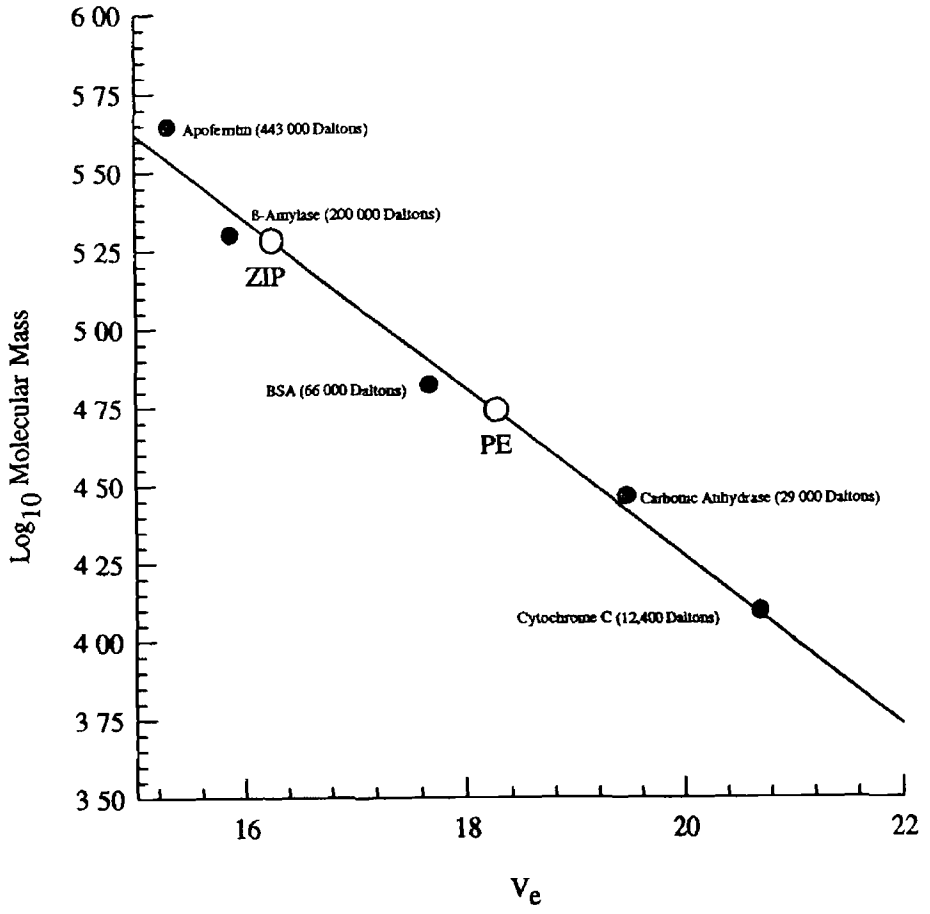


Figure 3 9 1 2 Biosep SEC-3000 molecular mass calibration curve *Plot of Log molecular mass versus V_e where V_e is the elution volume of a molecular mass standard (●-●) or enzyme activity (O) V_e was determined as described in section 2 9 1 2 1 Linear regression analysis of the data obtained from the molecular mass standards produced the following calibration equation*

$$Log(molecular\ mass) = -0.27(V_e) + 9.67$$

From this equation the molecular mass of PE and ZIP were calculated to be 54,500 Daltons and 191,000 Daltons respectively The positions of PE and ZIP are indicated on the calibration curve

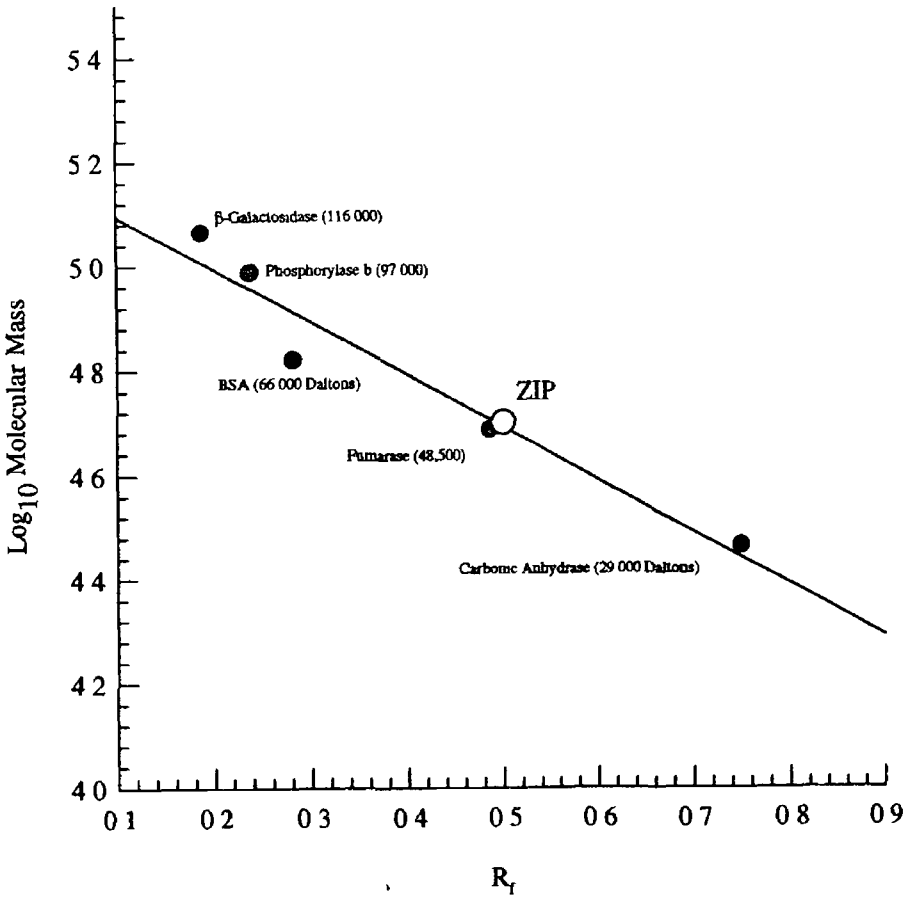


Figure 3 9 1 3 SDS PAGE molecular mass calibration curve *Plot of Log molecular mass versus Rf Rf values were determined as described in section 2 9 1 3 Linear regression analysis of the data obtained from the molecular mass standards (●-●) produced the following calibration equation*

$$Log(molecular\ mass) = -1\ 0(R_f) + 5\ 2$$

From this equation the molecular mass of ZIP was calculated to be 50,000 Daltons The molecular weight of PE was not determined The position of ZIP is indicated on the calibration curve (O)

3 9 2 Assay Temperature effects on Purified Enzyme Activities

The effects of assay temperature on the purified enzymes was investigated as outlined in section 2 9 2 Figure 3 9 2 1 demonstrates that PE has a narrow assay temperature profile with an optimum assay temperature of 37°C ZIP activity demonstrates a broad assay temperature profile as depicted in Figure 3 9 2 2 with an optimum assay temperature of 37°C - 45°C

3 9.3 pH Effects on Purified Enzyme Activities

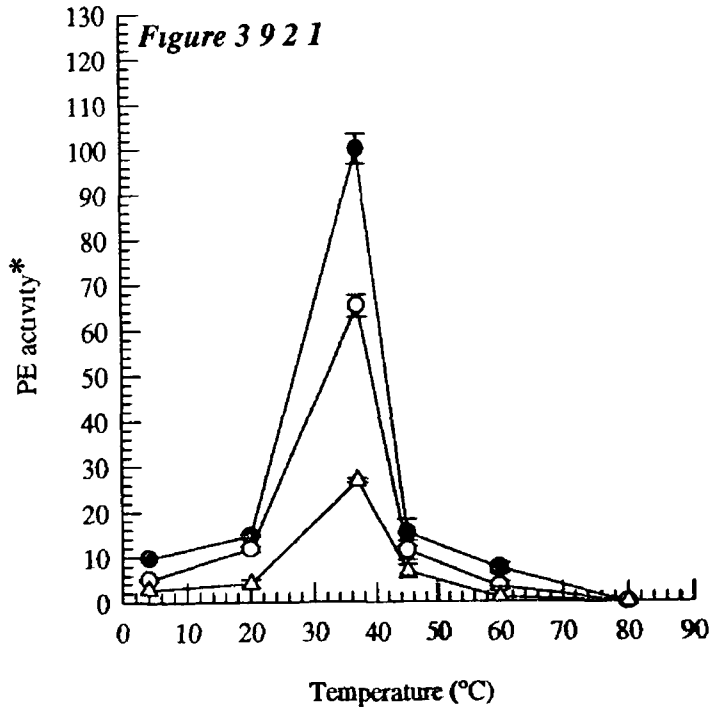
The effects of varied pH on purified enzyme activities were investigated as outlined in section 2 9.3 1 and 2 9 3 2 Figure 3 9 3 1 illustrates that purified PE exhibits a broad pH activity profile with an optimum pH, with respect to activity, observed at pH 8 0 The enzyme demonstrated a preference for a potassium phosphate buffer system Figure 3 9 3 2 illustrates the inactivation of purified PE at pH outside the pH range 5 0 to 9 0 Purified ZIP also exhibits a broad pH activity profile (Figure 3 9 3 3) with an optimum pH, with respect to activity, of 8 5, and no preference towards a particular buffer system Figure 3 9 3 4 demonstrates that inactivation of ZIP occurs at a pH range outside 4 0 to 9.5

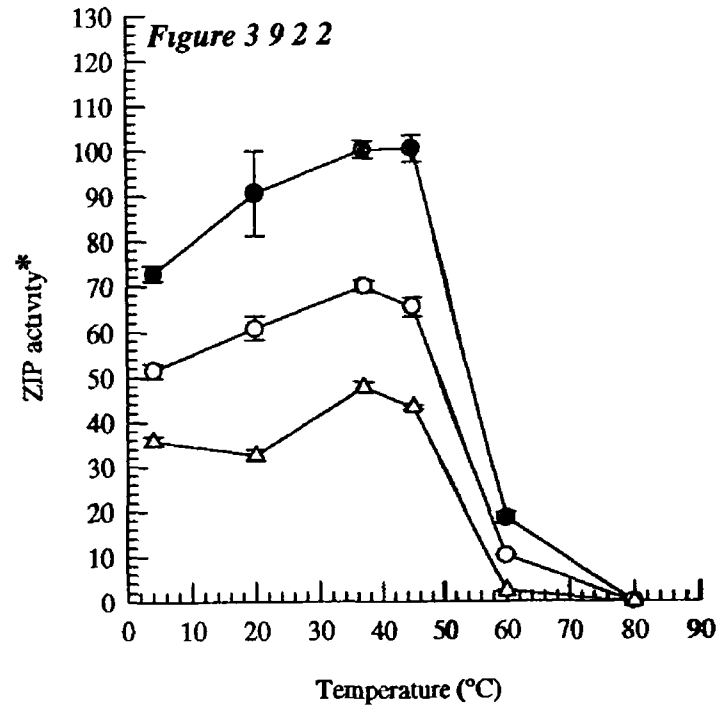
3 9.4. Effect of Functional Reagents on Purified Enzyme Activities

The effects of various functional reagents on purified PE and ZIP activities were determined as outlined in section 2 9 4 These results are presented in Tables 3 4 and 3 5 for PE and ZIP activities respectively Tables 3 4 and 3 5 present residual PE and ZIP activities following modification with functional reagent for 15 minutes at 37°C (section 2 9 4) when compared to unmodified enzyme (100%) PE activity was inhibited by PCMB (5mM and 0.5mM), 1,7 Phenanthroline (10mM) and AEBSF (10mM) by 50% or more Its activity was significantly enhanced by DTT (10mM and 1mM) and N-Acetylimidazole (10mM) ZIP activity was inhibited by PCMB (5mM), 1,7 Phenanthroline (10mM) and 4,7 Phenanthroline (10mM) by 50% or more Its activity was enhanced significantly by 8-Hydroxyquinoline (10mM), Trypsin Inhibitor preparation (1mg/mL) and Aprotinin (500µg/mL)

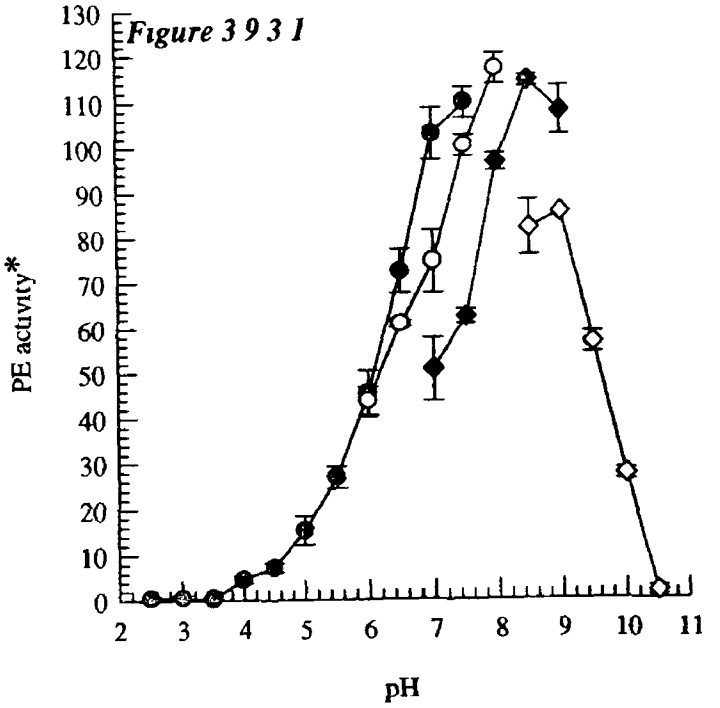
3 9.5. Effect of Divalent Metal Salts on Purified Enzyme Activities

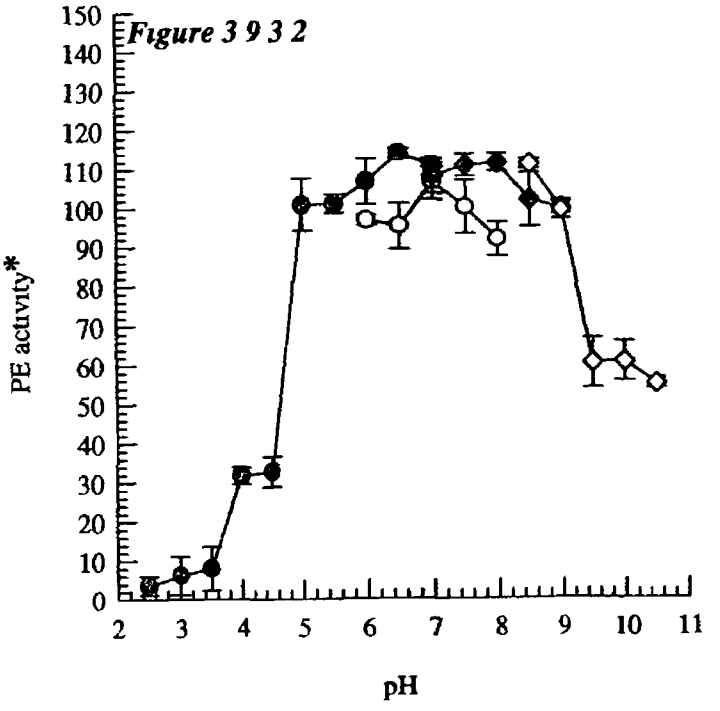
The effects of various divalent metal salts on purified PE and ZIP activities were determined as outlined in section 2 9 5 The results are presented in Table 3 6 where residual PE and ZIP activities are expressed as a percentage of unmodified enzyme activity (100%) Both PE and ZIP activities were inhibited by greater than 80% by $HgSO_4$



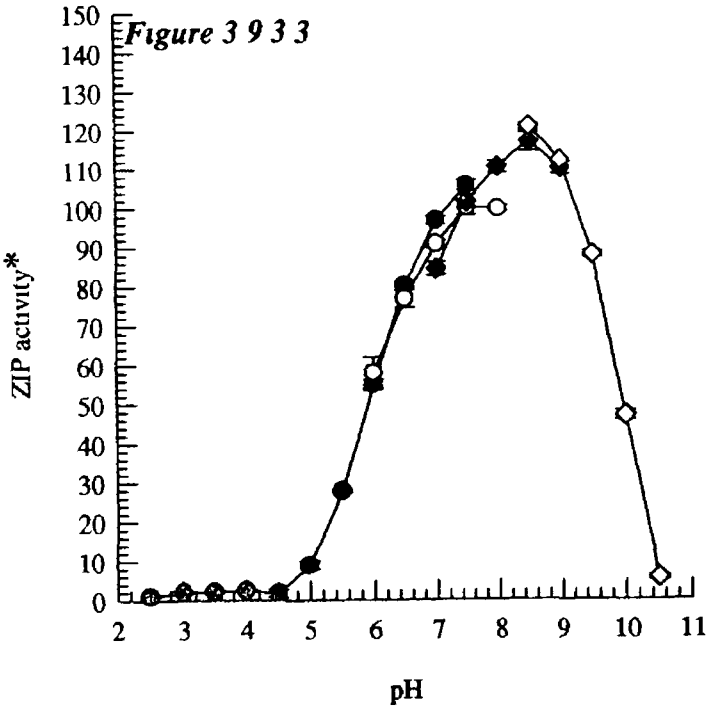


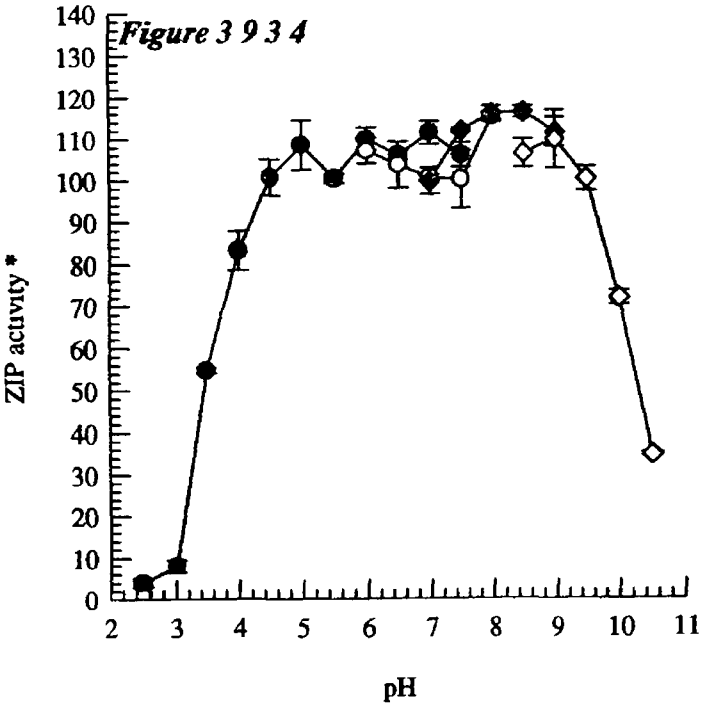
Figures 3 9 2 1 and 3 9 2 2 Effect of assay temperature on purified enzyme activities. *Plots of enzyme activity versus assay temperature for purified PE (Figure 3 9 2 1) and purified ZIP (Figure 3 9 2 2) Investigation performed as described in section 2 9 2 Enzymes were assayed over periods of 15 minutes (Δ-Δ), 30 minutes (o-o) and 45 minutes (●-●) Plots illustrate the comparison between the narrow temperature range of PE and the broad temperature range of ZIP PE was optimally active at 37°C while ZIP was optimally active at 37°C to 45°C. * Enzyme activity expressed as % activity obtained at 37°C*





Figures 3 9 3 1 and 3 9.3 2 Effect of pH on PE activity. *Plots of PE activity versus pH Investigations performed as described in section 2 9 3 1 and 2 9 3 2 Buffer systems used were citrate / phosphate (●-●), phosphate (o-o), Tris / HCl (♦-♦) and glycine / NaOH (◇-◇) Figure 3 9 2 1 represents the pH activity profile of PE indicating that the enzyme has a broad pH range with an optimum pH of 8 0 PE also demonstrates selectivity towards different buffers with optimum activity obtained in potassium phosphate Figure 3 9 2 2 represents the pH inactivation profile of PE illustrating the instability of PE outside the pH range 5 - 9 * Enzyme activity expressed as % of activity obtained in potassium phosphate, pH 7 4*





Figures 3 9 3 3 and 3 9.3.4 Effect of pH on ZIP activity *Plots of ZIP activity versus pH Investigations performed as described in section 2 9 3 1 and 2 9 3 2 Buffer systems used were citrate / phosphate (●-●), phosphate (o-o), Tris / HCl (♦-♦) and glycine / NaOH (◇-◇) Figure 3 9 2 1 represents the pH activity profile of ZIP indicating that the enzyme has a broad pH range with an optimum pH of 8 5 ZIP does not demonstrate selectivity towards different buffer systems Figure 3 9 3 4 represents the pH inactivation profile of ZIP illustrating the instability of ZIP outside the pH range 4 - 9 5 * Enzyme activity expressed as % of activity obtained in potassium phosphate, pH 7 4*

	Residual PE Activity † (%)				
Functional reagent	0 1mM	0 5mM	1mM	5mM	10mM
DTT		-	110*	-	1210*
DTNB	-	-	94	-	86
Iodoacetamide	-	-	81*	-	80*
Iodoacetate	-	-	92	-	88*
NEM	-	-	92*	-	60*
Leupeptin	88	-	85*	-	-
2-Mercaptoethanol	-	-	100	-	103*
PCMB	-	50	-	33*	-
EDTA	-	-	98	-	84
CDTA	-	-	95	-	62*
EGTA	-	-	93	-	86
Imidazole	-	-	81*	-	72*
8-Hydroxyquinoline	-	-	80*	-	80*
1,10 Phenanthroline	-	-	96	-	51*
1,7 Phenanthroline	-	-	100	-	40*
4,7 Phenanthroline	-	-	75*	-	51*
PMSF	84	-	77*	-	-
AEBSF	-	-	59*	-	20*
Puromycin	113*	-	84	-	-
N-Acetylimidazole	-	-	106	-	159*
Benzamidine	-	-	94	-	108

	50µg/mL	100µg/mL	500µg/mL	1mg/mL
Trypsin Inhibitor	-	74	-	67
Chymotrypsin Inhibitor	80	-	75	-
Pepstatin	-	-	98	-
Aprotinin	84	-	75	-
Bacitracin	-	81	-	73*
N-Decanoyl Co A	-	-	-	62*

Table 3 4 *Effect of functional reagents on purified PE* † *Residual activity is expressed as percentage activity remaining following exposure to functional reagent when compared to unmodified enzyme (100%) Enzyme was preincubated with each functional reagent for 15 minutes at 37°C prior to substrate addition.* * *P < 0 05 (data analysed statistically using the Paired Student t-Test)*

	Residual ZIP Activity † (%)				
Functional reagent	0 1mM	0 5mM	1mM	5mM	10mM
DTT	-	-	103	-	107
DTNB	-	-	98	-	97
Iodoacetamide	-	-	93	-	93*
Iodoacetate	-	-	84*	-	69*
NEM	-	-	102	-	108
Leupeptin	113	-	110	-	-
2-Mercaptoethanol	-	-	110	-	90*
PCMB	-	64*	-	44*	-
EDTA	-	-	97	-	86*
CDTA	-	-	96	-	92
EGTA	-	-	92	-	82
Imidazole	-	-	90	-	86
8-Hydroxyquinoline	-	-	96	-	138*
1,10 Phenanthroline	-	-	95	-	114
1,7 Phenanthroline	-	-	91	-	30*
4,7 Phenanthroline	-	-	79*	-	22*
PMSF	94	-	55*	-	-
AEBSF	-	-	91*	-	16*
Puromycin	92	-	107	-	-
N-Acetylimidazole	-	-	98	-	91
Benzamidine	-	-	93	-	95

	50µg/mL	100µg/mL	500µg/mL	1mg/mL
Trypsin Inhibitor	-	112*	-	131*
Chymotrypsin Inhibitor	112	-	115	-
Pepstatin	-	-	111	-
Aprotinin	111*	-	125*	-
Bacitracin	-	91*	-	76*
N-Decanoyl Co A	-	-	-	79*

Table 3 5 *Effect of functional reagents on purified ZIP † Residual activity is expressed as percentage activity remaining following exposure to functional reagent when compared to unmodified enzyme (100%) Enzyme was preincubated with each functional reagent for 15 minutes at 37°C prior to substrate addition. * $P < 0.05$ (data analysed statistically using the Paired Student t-Test)*

Metal salt	Concentration†	PE Residual Activity††	ZIP Residual Activity††
$NiSO_4$	1mM	83*	69*
$MnSO_4$	1mM	87*	71*
$ZnSO_4$	1mM	78*	59
$CdSO_4$	1mM	94*	80*
$CoSO_4$	1mM	83	71*
$HgSO_4$	1mM	5*	12*
$CuSO_4$	1mM	85*	71*
$CaSO_4$	1mM	83*	94
$MgSO_4$	1mM	86*	77*

Table 3 6 *Effect of divalent metal salts on purified PE and ZIP † Concentration of metal solution used. Actual concentration during preincubation was 500μM and during assay was 100μM †† Residual activity is expressed as percentage activity remaining following exposure to divalent metal salt when compared to unmodified enzyme (100%) Enzyme was preincubated with each divalent metal salt for 15 minutes at 37°C prior to substrate addition * $P < 0.05$ (data analysed statistically using the Paired Student t-Test)*

3 9 6 Substrate Specificity Studies on Purified PE and ZIP

Substrate specificity studies on purified PE and ZIP were performed as described in section 2 9 6 using Reverse Phase HPLC and fluorimetric substrate studies

3.9.6.1. Substrate Specificity Studies Using Reverse Phase HPLC

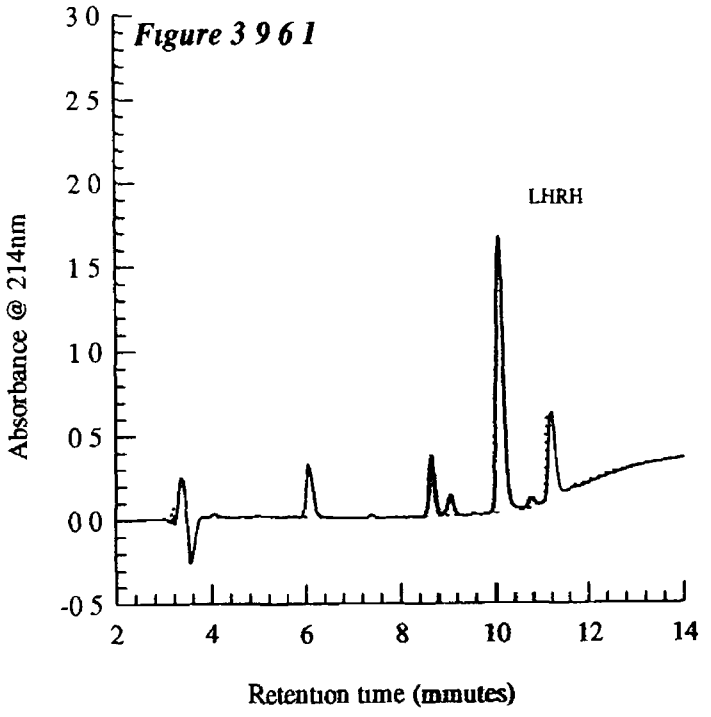
Reverse Phase HPLC was used to determine whether purified PE or ZIP activities produced cleavage products from various peptides listed in Table 2 4 Reactions between the purified enzymes and peptides under investigation were performed as outlined in section 2 9 6 1 2 and samples were analysed by Reverse Phase HPLC as outlined in section 2 9 6 1 3 Table 3 7 illustrates what peptides were and were not cleaved by purified PE and ZIP activities under conditions outlined in section 2 9 6 1 2 Figures 3 9 6 1 to 3 9 6 9 represent the spectrophotometric data obtained from the PDA detector (section 2 9 1 2) Plots of absorbance at 214nm versus retention time for LHRH, TRH, Bradykinin, Substance P and Angiotensin II are presented Cleavage fragments produced by purified PE and ZIP activities on these peptides are also represented Both PE and ZIP activities produced cleavage fragments from LHRH, Bradykinin and Substance P The cleavage product produced by the action of ZIP towards LHRH (Figure 3 9 6.2 and insert) coincided with the major cleavage product detected following the degradation of LHRH by purified PE activity (Figure 3 9 6 1) This was also the case with regards to the cleavage of Bradykinin by PE and ZIP activities (Figures 3 9 6 5 and 3 9 6 6) No cleavage of TRH by PE was detected as illustrated by the failure to detect TRH-OH in Figure 3 9 6 4 There was no similarity between the cleavage products detected for PE and ZIP activities towards Substance P

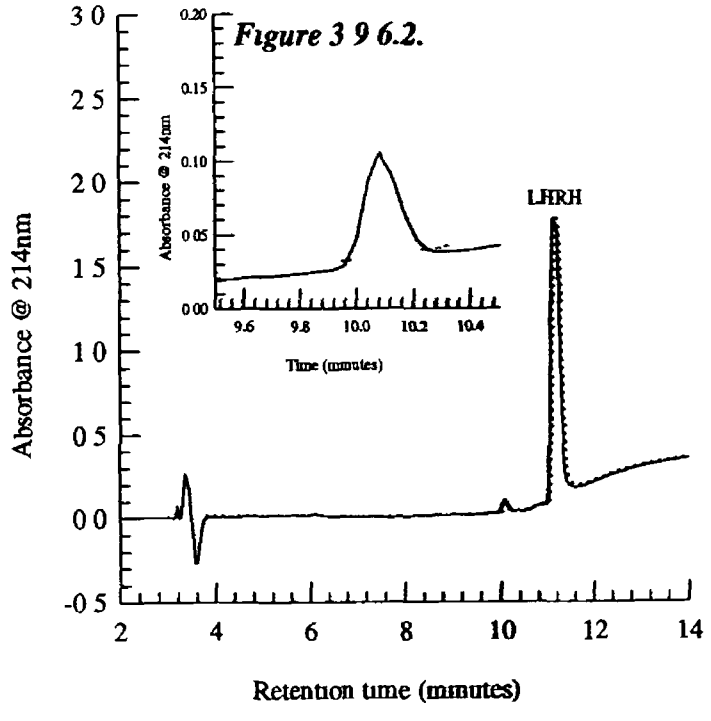
3.9.6.2 Fluorimetric Substrate Specificity Studies

Cleavage of fluorimetric substrates by purified enzyme activities was also investigated as a means of elucidating the substrate specificity of PE and ZIP activities Fluorimetric substrates (Table 2 5) were prepared and reacted with purified PE and ZIP activities as outlined in section 2 9 6 2 MCA released from these substrates was determined as described in section 2 9 6 2.2 Table 3 8 presents the results obtained from this investigation Purified enzyme activities towards the various substrates are expressed as a percentage of their activity towards Z-Gly-Pro-MCA PE demonstrated a high relative activity against pGlu-His-Pro-MCA, a fluorimetric TRH analogue ZIP demonstrated a high relative activity against the proline aminopeptidase substrate, Pro-MCA

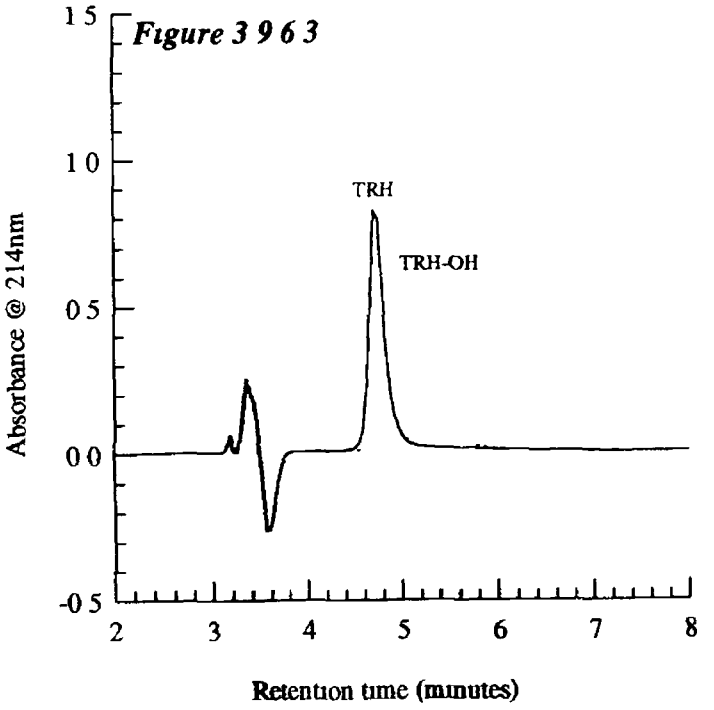
Peptide	Cleavage detected following reaction with purified enzyme activity	
	PE activity	ZIP activity
LHRH	Yes	Yes
TRH	No	No
Bradykinin	Yes	Yes
Substance P	Yes	Yes
Angiotensin II	Yes	No
Pro-Gly	No	No
Z-Pro-Gly	No	No
Gly-Pro-Ala	No	No
Gly-Pro	N D	N.D
Z-Gly-Pro-Ala	No	No
Gly-Gly-Pro-Ala	No	No
Gly-Ala-Phe	Yes	No
Lys-Ala-Ala	No	No
Gly-Phe-Ala	No	No
Z-Pro-Pro	No	No
Z-Pro-Ala	No	No
Z-Pro-Leu-Gly	No	No
Z-Pro	No	No

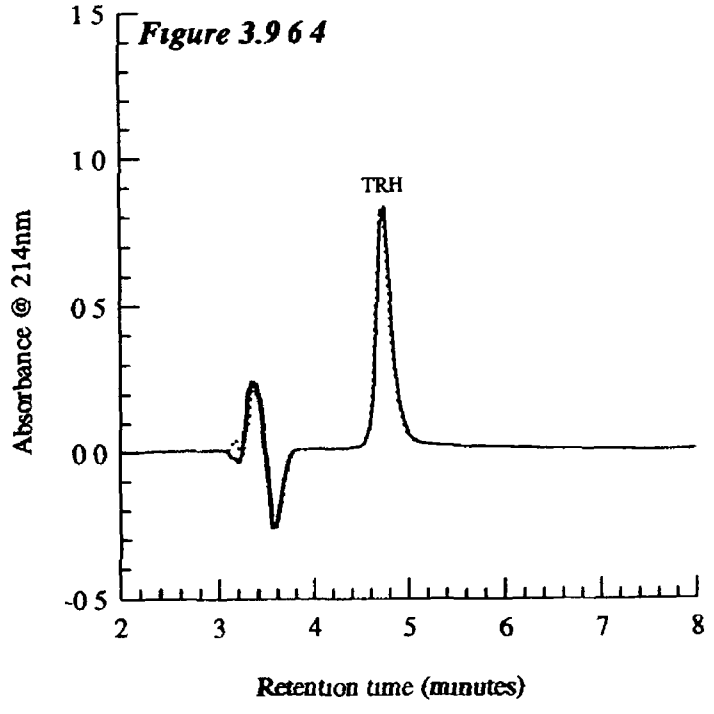
Table 3 7 *Results from substrate specificity studies using Reverse Phase HPLC Investigation was performed as described in section 2 9 6 1 Table indicates whether cleavage products were detected following incubation of peptide with purified PE or ZIP activities as outlined in section 2.9 6 1 2 N D - Not determined. No result could be presented for this peptide as it was not detected using the method described in section 2 9 6 1 3*



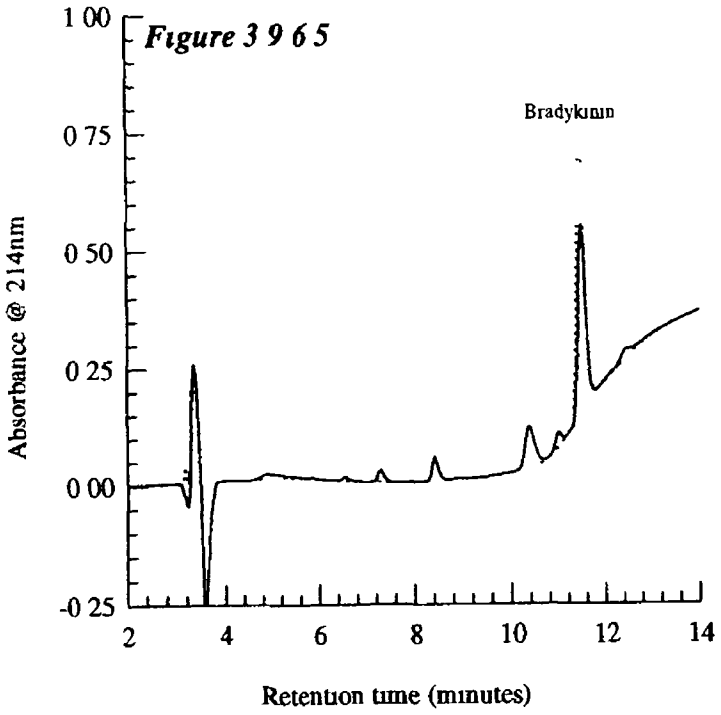


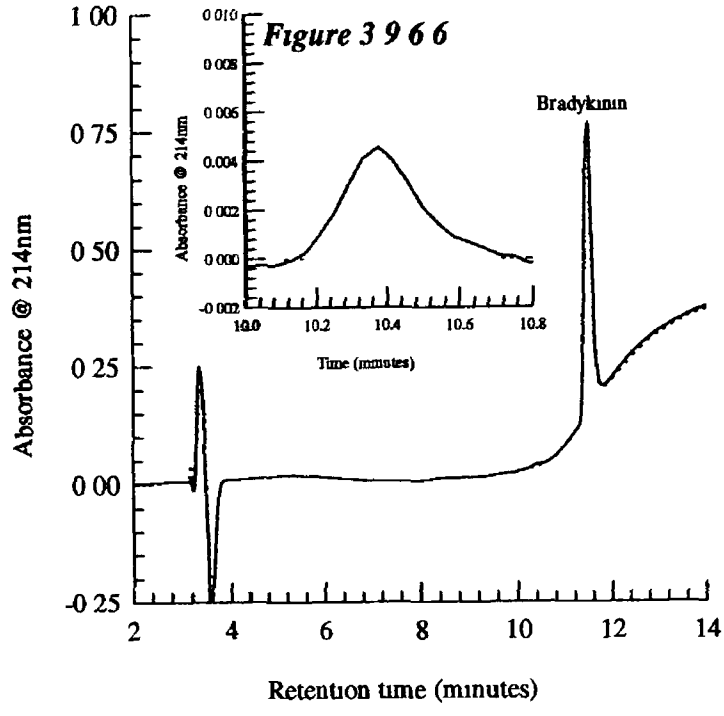
Figures 3 9 6.1 and 3 9 6 2 Cleavage of LHRH by PE and ZIP activities respectively *Plots of absorbance at 214nm versus retention time Substrate specificity studies using Reverse Phase HPLC were carried out as outlined in section 2 9 6 1 Plots present data obtained from LHRH control ( ) and LHRH following incubation with purified PE and ZIP activities (——) The major cleavage product detected in Figure 3 9 6 1 coincides with the cleavage product detected in Figure 3 9 6 2 (see insert) This indicates that PE and ZIP may share a common cleavage site on LHRH*



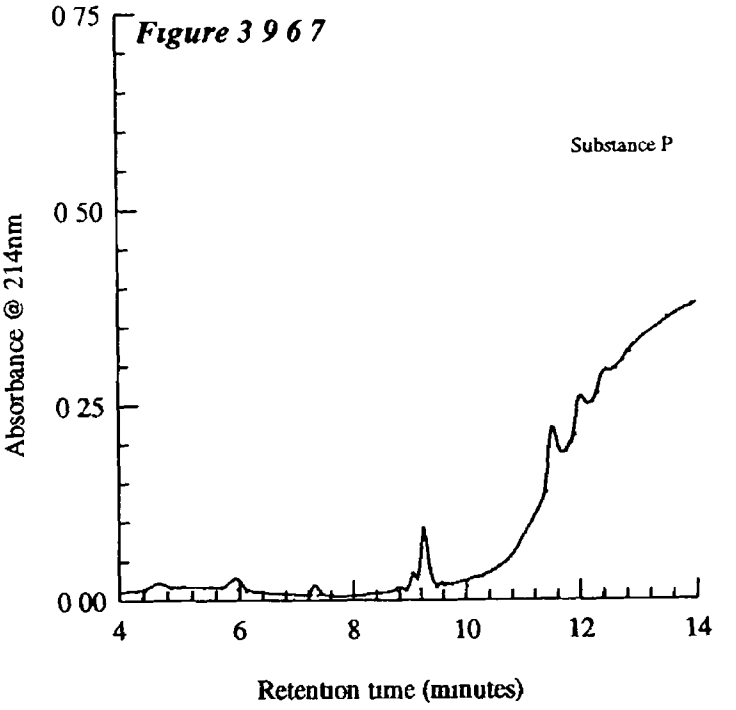


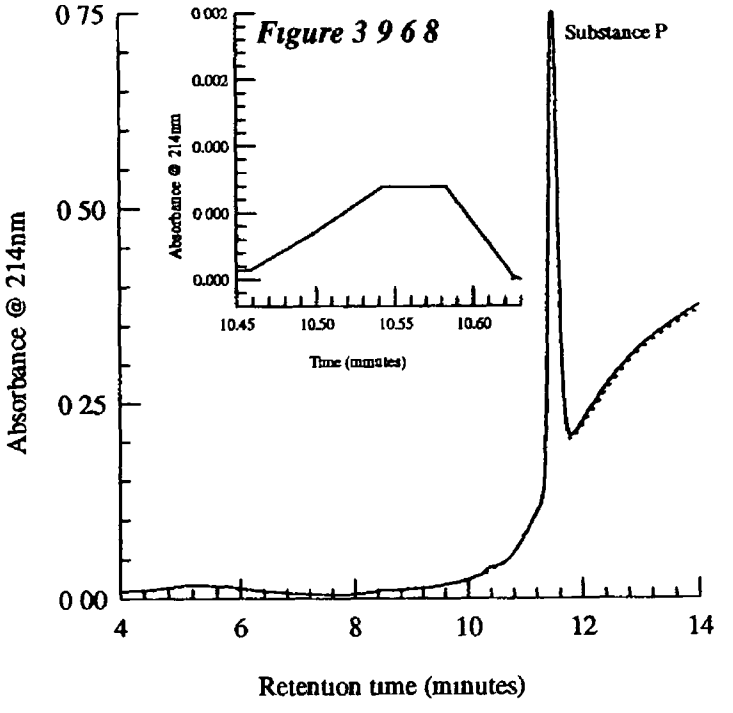
Figures 3 9 6 3 and 3 9.6.4. Failure to Detect TRH Cleavage by PE *Plots of absorbance at 214nm versus retention time. Substrate specificity studies using Reverse Phase HPLC were carried out as outlined in section 2 9 6 1 In Figure 3 9 6 3 the detection of TRH (——) and TRH-OH ( ) as they elute from the Reverse Phase HPLC column is illustrated TRH and TRH-OH are well resolved by the method described in section 2 9 6 1 3 and are easily distinguishable Figure 3 9 6 4 illustrates that following the reaction of TRH with PE, no TRH-OH is detected.*





Figures 3 9.6 5 and 3 9.6.6 Cleavage of Bradykinin by PE and ZIP activities respectively. *Plots of absorbance at 214nm versus retention time Substrate specificity studies using Reverse Phase HPLC were carried out as outlined in section 2 9 6 1 Plots present data obtained from Bradykinin control (-) and Bradykinin following incubation with purified PE and ZIP activities (——) The major cleavage product detected in Figure 3 9 6 1 coincides with the cleavage product detected in Figure 3 9 6.2 (see insert) This indicates that PE and ZIP may share a common cleavage site on Bradykinin.*





Figures 3.9 6 7 and 3 9 6 8. Cleavage of Substance P by PE and ZIP activities respectively *Plots of absorbance at 214nm versus retention time Substrate specificity studies using Reverse Phase HPLC were carried out as outlined in section 2 9 6 1 Plots present data obtained from Substance P control ( ) and Substance P following incubation with purified PE and ZIP activities (——) A minor cleavage product was detected following the action of ZIP toward Substance P (Figure 3 9 6 8 insert) but this does not correspond with any cleavage products detected following the action of PE on Substance P (Figure 3 9 6 7)*

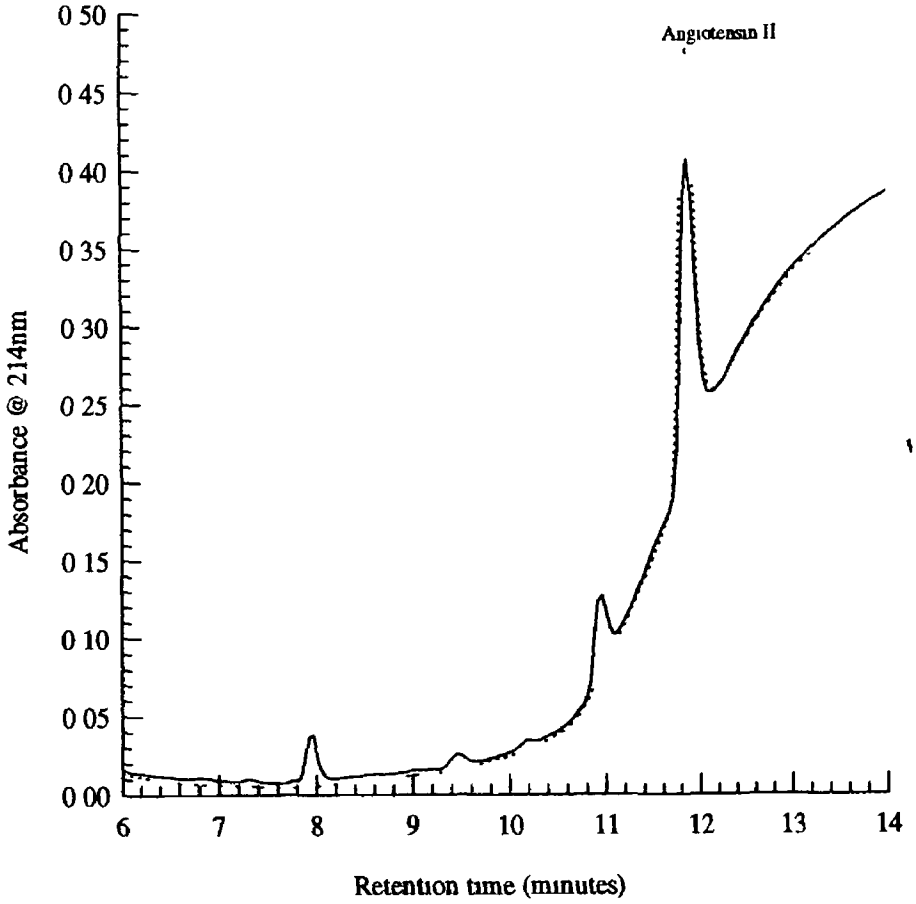


Figure 3 9 6 9 Cleavage of Angiotensin II by PE *Plot of absorbance at 214nm versus retention time Substrate specificity studies using Reverse Phase HPLC were carried out as outlined in section 2 9 6 1 Plot illustrates the elution of an Angiotensin II control ( ) and the elution of Angiotensin II and it cleavage products produced by the action of purified PE (——)*

Substrate	Relative PE activity (%)† (± s d)	Relative ZIP activity (%)† (± s d)
Z-Gly-Pro-MCA	**100 (±0 8)**	**100 (±3 3)**
Ala-MCA	3 0 (±5 1)	3 0 (±1 7)
Arg-MCA	4 0 (±1 2)	2 0 (±2 9)
Z-Arg-MCA	1 0 (±6.2)	11 0 (±6 1)
Pro-MCA	6 0 (±4 3)	**38 0 (±1.5)**
pGlu-MCA	1 0 (±4 6)	0 0 (±2 6)
Glu-Phe-MCA	0 0 (±4 1)	0 0 (±2 2)
Gly-Arg-MCA	1 0 (±1.2)	0 0 (±1 0)
Gly-Pro-MCA	2 0 (±5 2)	1 0 (±2 6)
Lys-Ala-MCA	0 0 (±0 5)	1 0 (±1 2)
Z-Arg-Arg-MCA	1 0 (±1 4)	7 0 (±2 5)
Z-Phe-Arg-MCA	2 0 (±8 0)	4 0 (±2 8)
pGlu-His-Pro-MCA	**63.0 (±4 6)**	4 0 (±2 1)
Boc-Val-Leu-Lys-MCA	1 0 (±1 7)	0 0 (±7 5)
Boc-Val-Pro-Arg-MCA	8 0 (±1 1)	3 0 (±0 8)
Suc-Ala-Phe-Lys-MCA	0 0 (±6 1)	0 0 (±7 3)
Z-Phe-Val-Arg-MCA	0 0 (±5 6)	1 0 (±3 5)
Suc-Gly-Pro-Leu-Gly-Pro-MCA	**10 0 (±0.5)**	1 0 (±11 8)

Table 3 8 *Cleavage of fluorimetric substrates by purified PE and ZIP activities † Activities are presented as a percentage of PE and ZIP activities against Z-Gly-Pro-MCA Standard deviations are presented in brackets Purified PE activity was highly active against pGlu-His-Pro-MCA, a fluorimetric analogue of TRH whereas ZIP activity was active against Pro-MCA, the proline aminopeptidase substrate Substrates cleaved by 10% or greater with respect to the cleavage of Z-Gly-Pro-MCA (100%) are presented in bold type*

3.9.7. Substrate Specificity Studies on PE and ZIP Based on Kinetic Analysis

Kinetic analysis of the interactions of PE and ZIP with various substrates was performed as outlined in section 2 9 7

3.9 7.1. Determination of K_m for Z-Gly-Pro-MCA and purified PE and ZIP Activities

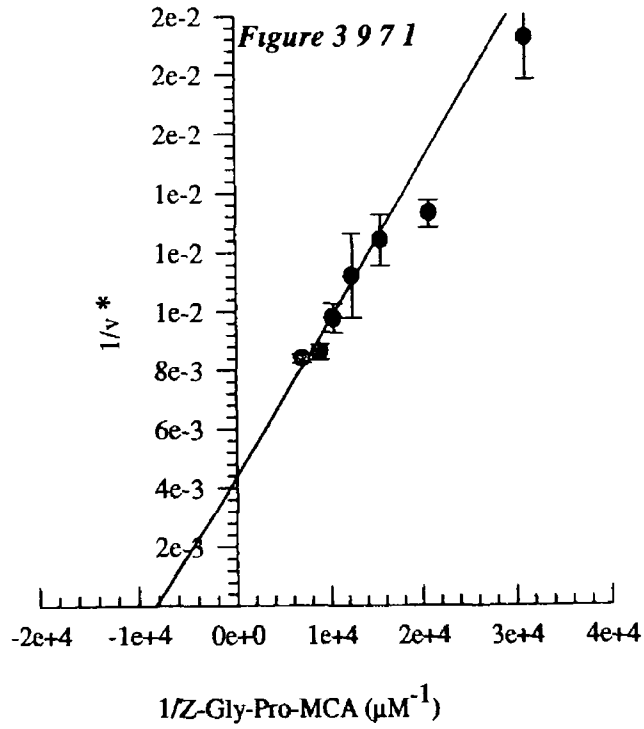
A K_m value was determined for the reaction between Z-Gly-Pro-MCA and both purified enzyme activities as outlined in section 2 9 7 1 Figures 3 9 7 1 and 3 9 7 2 present the data obtained from this study as Lineweaver-Burk plots The K_m values were determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and Direct Linear Plot analyses and the results are presented in Table 3 9 Direct Linear Plot analysis is statistically the more reliable of the five analysis methods used and accordingly K_m values of 94μM and 267μM were obtained for PE and ZIP activities respectively This indicates that the interaction between Z-Gly-Pro-MCA and PE is of a more specific nature than that of ZIP and the same substrate

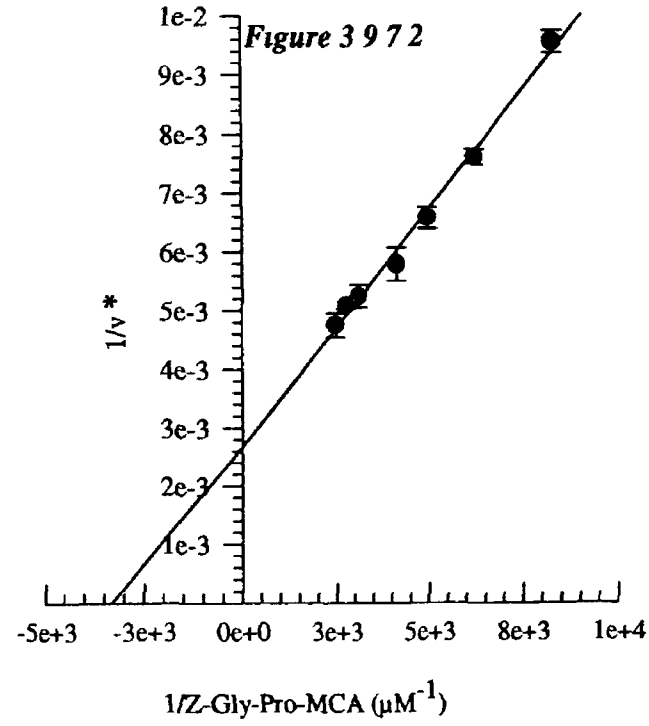
3.9.7.2. Determination of K_m for pGlu-His-Pro-MCA and purified PE

A K_m value was determined for the reaction between pGlu-His-Pro-MCA and PE as outlined in section 2 9 7 2 Figure 3 9 7 3 presents the data obtained from this study as a Lineweaver-Burk plot. The K_m value was determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and Direct Linear Plot analyses and the results are presented in Table 3 9 A K_m value of 69μM for the interaction of PE and pGlu-His-Pro-MCA was returned following Direct Linear Plot analysis.

3.9.7.3. Determination of K_i Values for Proline Containing Peptides

The effect of proline containing peptides on the interaction between PE and ZIP and the substrate Z-Gly-Pro-MCA was investigated as outlined in section 2 9 3 Data obtained was analysed using the five analytical techniques previously mentioned The nature of inhibition observed when proline containing peptides were introduced into assays between purified enzyme activities and the substrate Z-Gly-Pro-MCA was determined using Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots Figures 3 9 7 4 to 3 9 7 8 are Lineweaver-Burk plots illustrating the competitive or non-competitive nature of PE and ZIP inhibition by selected proline containing peptides The K_i values for the interactions studied are presented in Table 3 10 PE was competitively inhibited by LHRH, TRH, Bradykinin, Angiotensin II and Z-Gly-Pro-Ala with K_i values of 523μM, 680μM, 136μM, 113μM and 894μM respectively ZIP was competitively inhibited by LHRH, Bradykinin and Z-Gly-Pro-Ala with K_i values of 475μM, 2497μM and 722μM respectively





Figures 3 9 7 1 and 3 9 7.2 Kinetic analysis of PE and ZIP activities respectively

*Lineweaver-Burk reciprocal plots of reaction velocity versus substrate concentration Data was obtained according to the methods described in section 2 9 7 1 K_m values are represented by the negative intercept on the X-axis Results obtained following kinetic analysis are presented in Table 3 9 K_m values obtained for PE and ZIP interaction with Z-Gly-Pro-MCA were 94μM and 267μM respectively * Velocities are expressed as fluorimetric intensities*

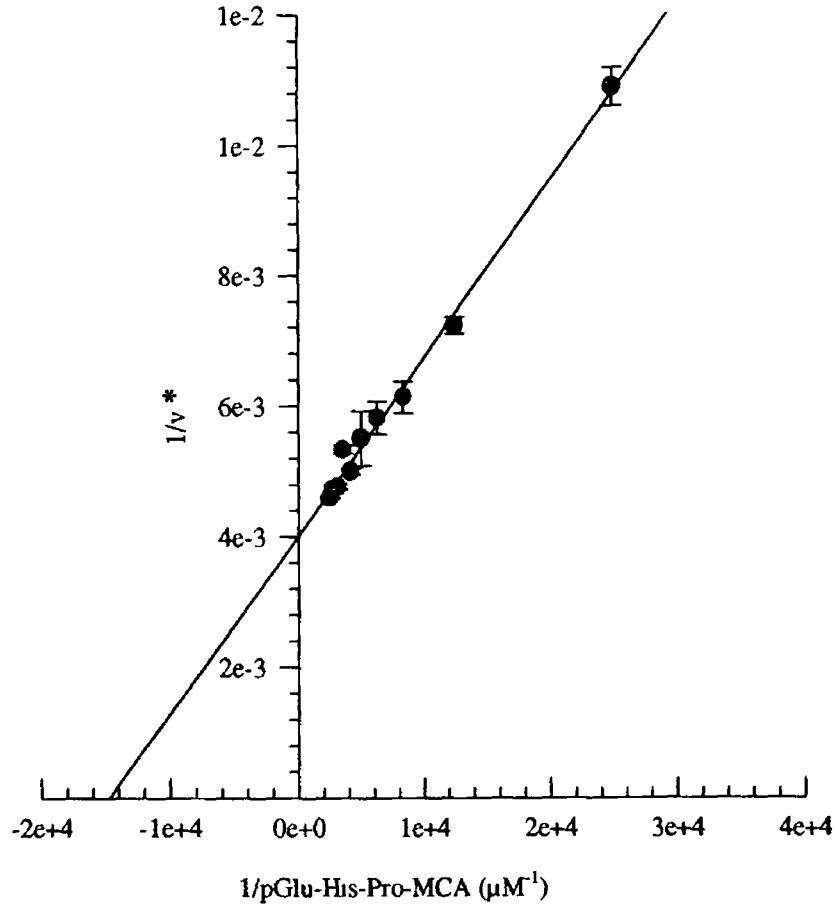
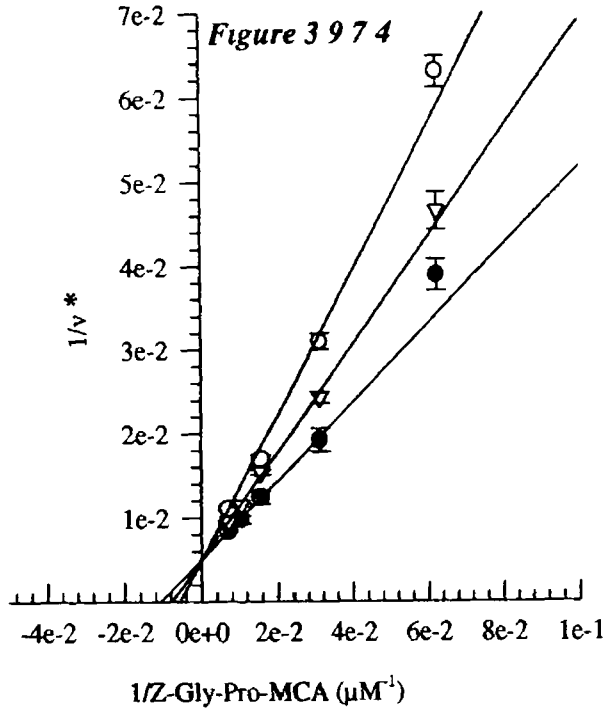


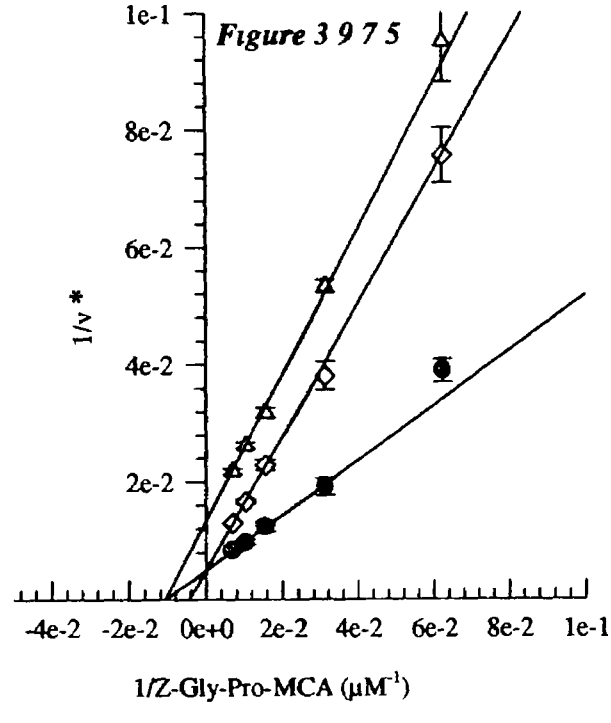
Figure 3 9 7 3 Kinetic analysis of PE activity towards pGlu-His-Pro-MCA

*Lineweaver-Burk reciprocal plot of reaction velocity versus substrate concentration Data was obtained according to the methods described in section 2 9 7 2 K_m values are represented by the negative intercept on the X-axis Results obtained following kinetic analysis are presented in Table 3 9 The K_m value obtained for PE interaction with pGlu-His-Pro-MCA was 69μM * Velocities are expressed in fluorimetric intensities*

Kinetic study	K_m obtained following data analysis (μM)				
	Michaelis-Menten	Lineweaver-Burk	Eadie-Hofstee	Hanes-Woolf	Direct Linear Plot
PE + Z-Gly-Pro-MCA	88	151	95	101	94
ZIP + Z-Gly-Pro-MCA	320	300	271	266	267
PE + pGlu-His-Pro-MCA	66	67	66	70	69

Table 3 9 *Results obtained from kinetic determinations carried out as described in sections 2 7 1 , 2 7 2 and 2 7 3 respectively Table illustrates K_m determinations for PE and ZIP activities using Z-Gly-Pro-MCA as substrate and K_m determinations for PE activity using pGlu-His-Pro-MCA (fluorimetric TRH analogue) as substrate Data from each experiment was analysed using the multiple kinetic models presented PE shows a markedly higher affinity for Z-Gly-Pro-MCA than ZIP PE demonstrates a slightly speificty for pGlu-His-Pro-MCA than for Z-Gly-Pro-MCA*





Figures 3 9 7 4 and 3 9 7 5 Kinetic analysis of the effect of proline containing peptides on PE activity towards Z-Gly-Pro-MCA *Lineweaver-Burk reciprocal plots of reaction velocity versus substrate concentration. Investigation into the effect of proline containing peptides on the interactions of PE activity towards Z-Gly-Pro-MCA were carried out as described in section 2 9 7 3 Figure 3 9 7 4 illustrates the competitive inhibition of PE by LHRH (o-o) and TRH (∇-∇) when assayed with Z-Gly-Pro-MCA (●-●) Figure 3 9 7 5 illustrates the competitive and non-competitive inhibition of PE by Bradykinin (◇-◇) and TRH-OH (Δ-Δ) respectively K_i values are presented in Table 3 10 * Reaction velocities are expressed as fluorimetric intensities*

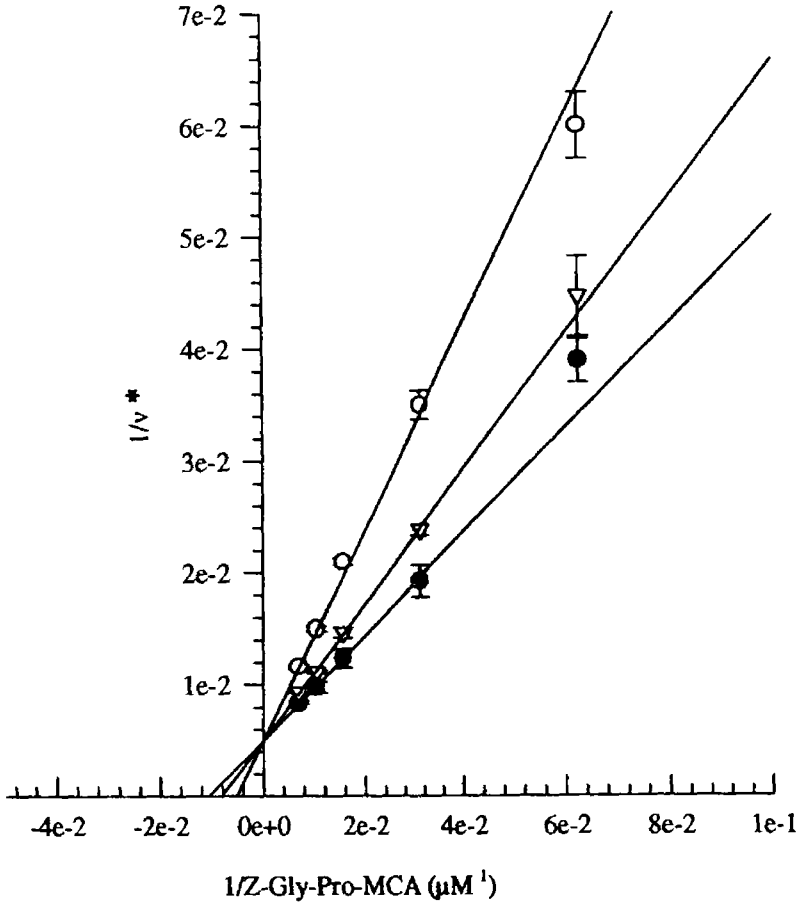
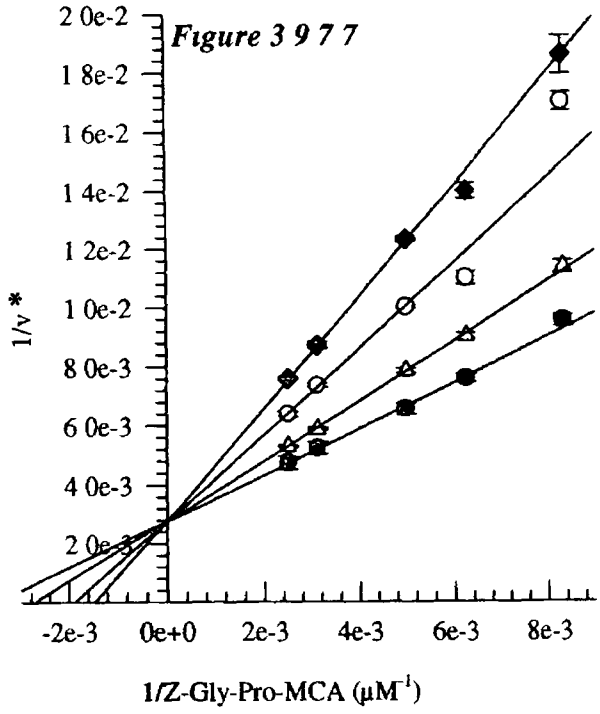
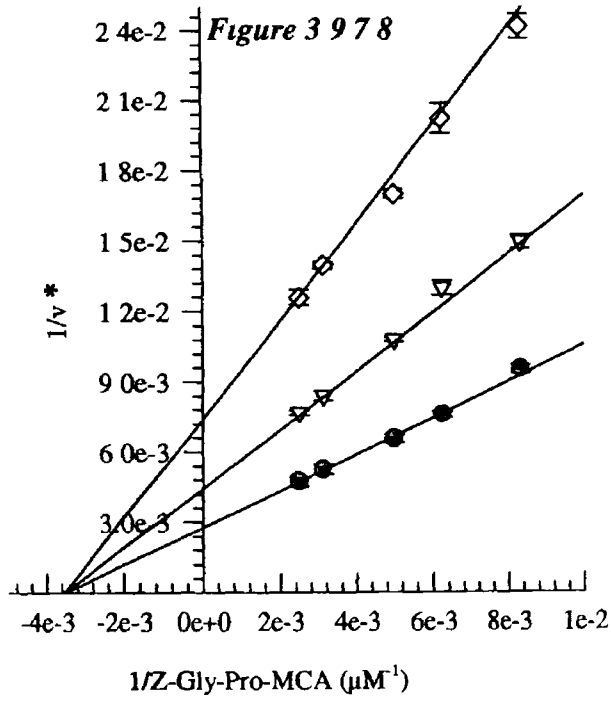


Figure 3 9 7 6 Kinetic analysis of the effect of Angiotensin II and Z-Gly-Pro-Ala on PE activity towards Z-Gly-Pro-MCA *Lineweaver-Burk reciprocal plot of reaction velocity versus substrate concentration. Investigation into the effect of proline containing peptides on the interactions of PE activity towards Z-Gly-Pro-MCA were carried out as described in section 2 9 7 3 Figure 3 9 7 6 illustrates the competitive inhibition of PE by Angiotensin II (o-o) and Z-Gly-Pro-Ala (∇-∇) when assayed with Z-Gly-Pro-MCA (●-●) K_i values are presented in Table 3 10 * Reaction velocities are expressed as fluorimetric intensities*





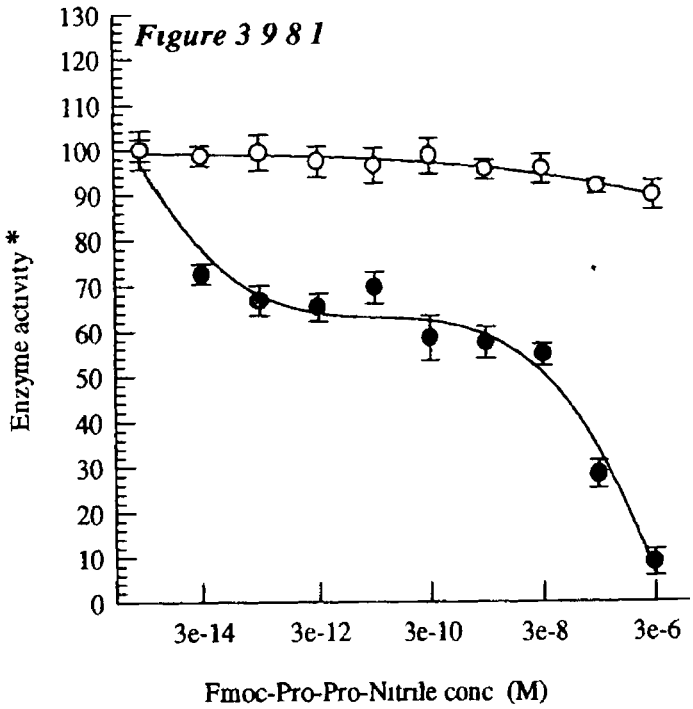
Figures 3 9 7 7 and 3 9 7 8 Kinetic analysis of the effect of proline containing peptides on ZIP activity towards Z-Gly-Pro-MCA *Lineweaver-Burk reciprocal plots of reaction velocity versus substrate concentration Investigation into the effect of proline containing peptides on the interactions of ZIP activity towards Z-Gly-Pro-MCA were carried out as described in section 2 9 7 3 Figure 3 9 7 7 illustrates the competitive inhibition of ZIP by LHRH (o-o), Bradykinin (♦-♦) and Z-Gly-Pro-Ala (Δ-Δ) when assayed with Z-Gly-Pro-MCA (●-●) Figure 3 9 7 8. illustrates the non-competitive inhibition of ZIP by TRH (∇-∇) and TRH-OH (o-o) K_i values are presented in Table 3 10 * Reaction velocities are expressed as fluorimetric intensities*

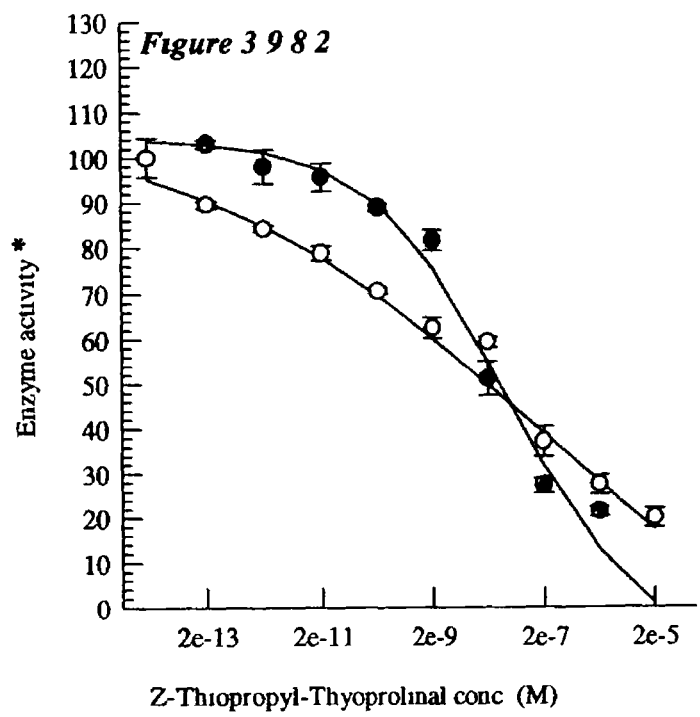
		Inhibition observed										
		K_I (μM) determined for PE					K_I (μM) determined for ZIP					
Peptide	Conc (μM) †	Inhibition type	MM	LB	EH	HW	**DLP**	Inhibition type	MM	LB	EH	HW	**DLP**
LHRH	400	Competitive	460	762	504	512	**523**	Competitive	460	498	478	458	**475**
TRH	200	Competitive	529	-	815	794	**680**	Non-competitive	317	281	306	315	**316**
TRH-OH	800	Non-competitive	508	365	490	480	**412**	Non-competitive	460	416	446	457	**465**
Bradykinin	175	Competitive	150	282	191	180	**136**	Competitive	3525	1930	2853	3648	**2497**
Substance P	75	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-
Angiotensin II	100	Competitive	125	235	153	149	**113**	Mixed	-	-	-	-	-
Z-Pro-Gly	800	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-
Z-Gly-Pro-Ala	200	Competitive	726	-	977	1073	**894**	Competitive	674	896	642	608	**722**
Z-Pro-Pro	800	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-
Z-Pro-Ala	800	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-
Z-Pro-Leu-Gly	800	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-
Z-Pro	800	Mixed	-	-	-	-	-	Mixed	-	-	-	-	-

Table 3 10 *K_I values obtained for proline containing peptides when determined for both PE and ZIP activities as outlined in section 2 9 7 3 K_I values were determined using the apparent K_m and apparent V_{max} parameters obtained using the following data analysis MM- Michaelis-Menten, LB - Lineweaver-Burk I H - Eadie-Hofstee, HW - Hanes-Woolf and DLP - Direct Linear Plot The figures obtained form Direct Linear Plot analysis have been highlighted as they are statistically the most reliable "-" = values not determined. † Concentration of proline containing peptide under assay conditions*

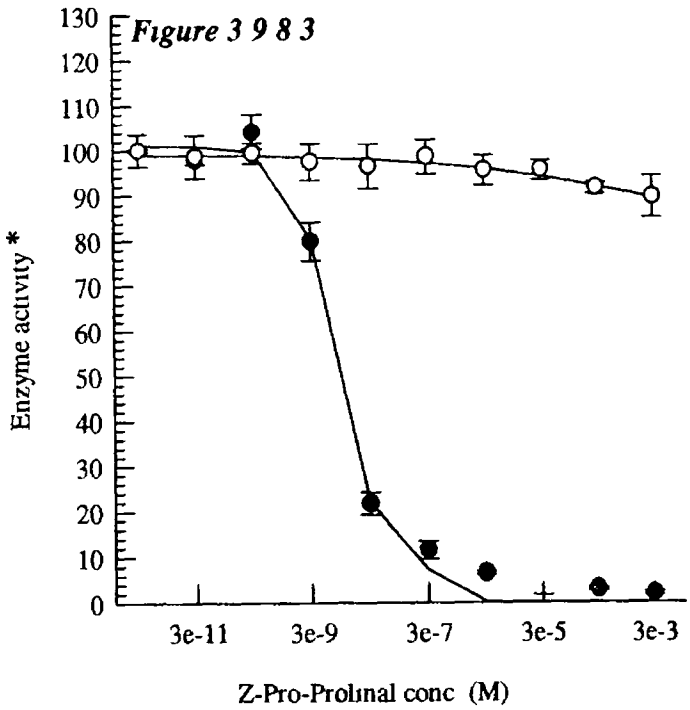
3.9 8 Effect of PE Specific Inhibitors on Purified PE and ZIP Activities

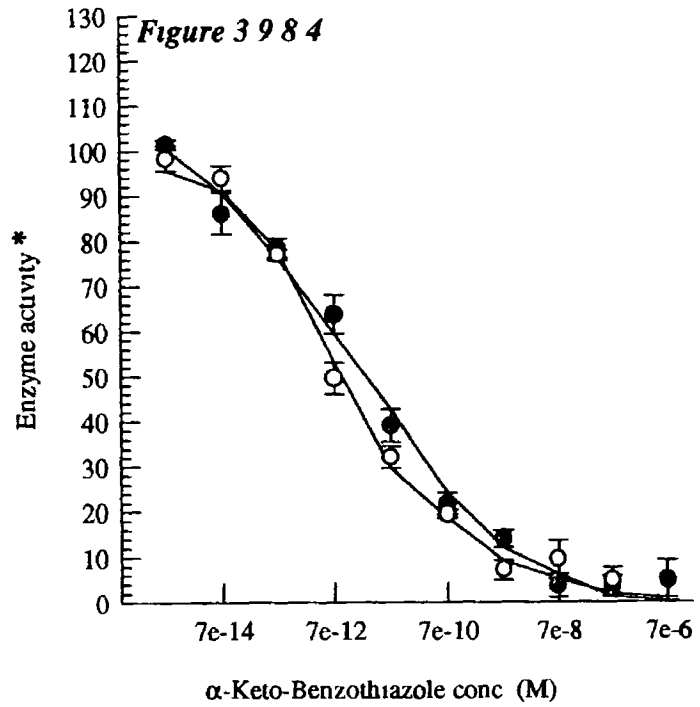
The effect of various PE and proline specific peptidase inhibitors was investigated as described in section 2 9 8 Figures 3 9 8 1 to 3 9 8 10 illustrate the effect that these inhibitors exerted on purified PE and ZIP activities Table 3 11 presents the IC_{50} values determined for each inhibitor with respect to PE and ZIP activity PE and ZIP activities were unaffected by Kelatorphan up to a concentration of 10mM PE was strongly inhibited by α-Ketobenzothiazole and Z-Indolinyl Prolinal with IC_{50} values of 4.1×10^{-11}M and 4.5×10^{-11}M respectively ZIP activity was strongly inhibited by α-Ketobenzothiazole with an observed IC_{50} value of 1.5×10^{-11}M



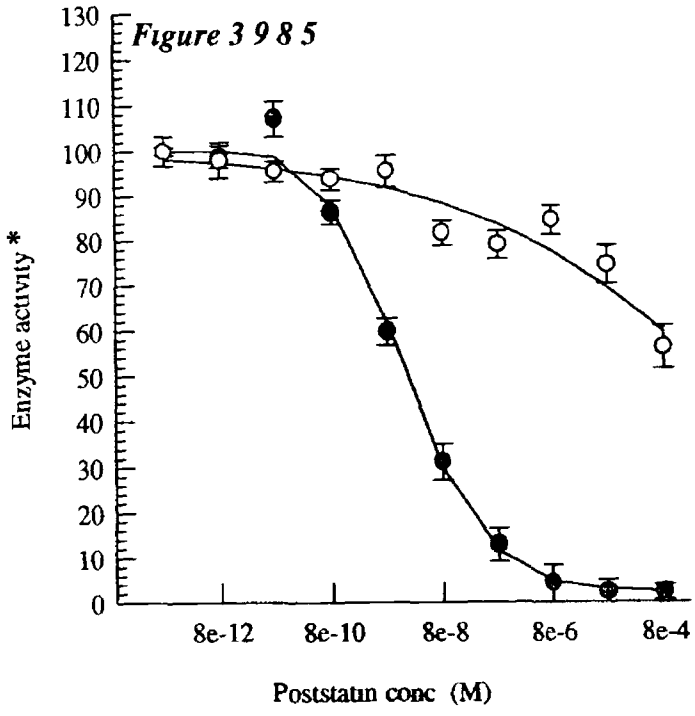


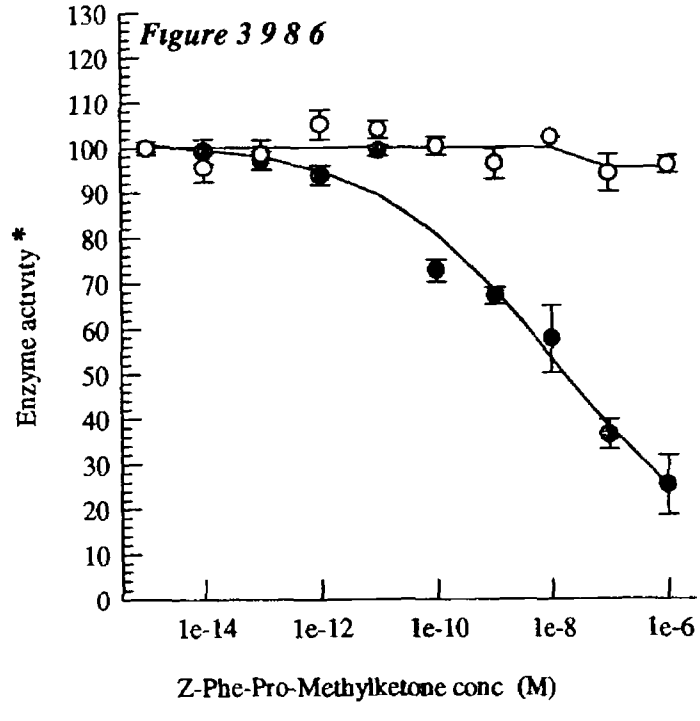
Figures 3 9 8 1 and 3 9 8.2 The inhibition of PE and ZIP by Fmoc-Pro-Pro-Nitrile and Z-Thiopropyl-Thyoprolinal *Semi log plots of residual PE (●-●) and ZIP (o-o) activity versus specific inhibitor concentration Investigations into the effect of proline specific peptidase inhibitors were performed as outlined in section 2 9 8 Figure 3 9 8 1 illustrates that Fmoc-Pro-Pro-Nitrile exhibits no significant inhibitory activity against ZIP but effectively inhibits PE activity Figure 3 9 8 2 shows that PE and ZIP are inhibited in a similar manner by Z-Thiopropyl-Thyoprolinal IC_{50} values determined for these inhibitors against PE and ZIP activity are presented in Table 3 11 * Enzyme activity expressed as % of uninhibited enzyme (100%)*



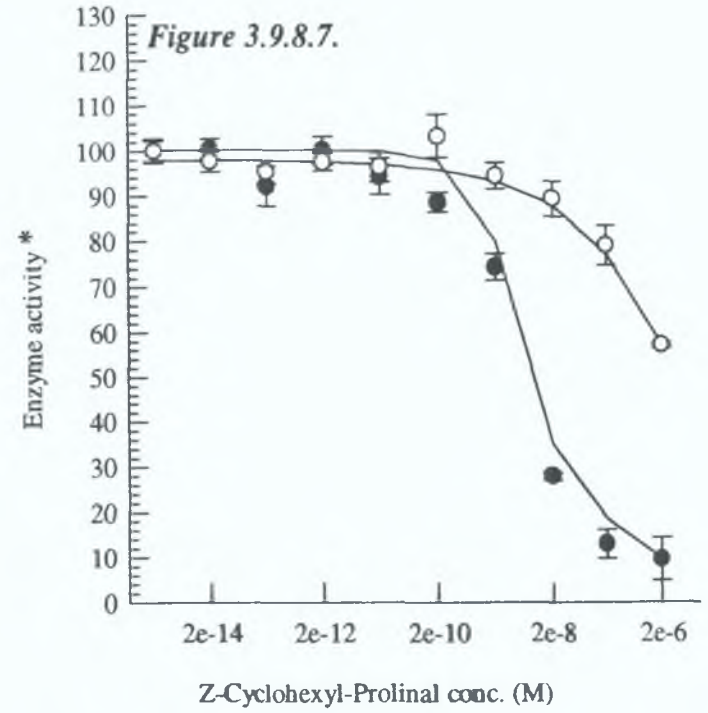


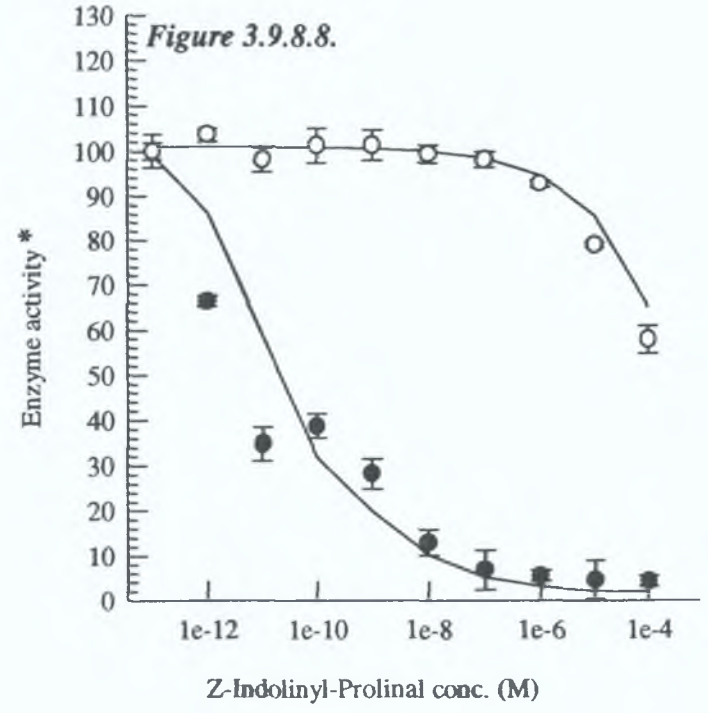
Figures 3 9 8 3 and 3 9 8 4 The inhibition of PE and ZIP by Z-Pro-Prolinal and α-Ketobenzothiazole *Semi log plots of residual PE (●-●) and ZIP (o-o) activity versus specific inhibitor concentration Investigations into the effect of proline specific peptidase inhibitors were performed as outlined in section 2 9 8 Figure 3 9 8 3 illustrates that Z-Pro-Prolinal exhibits no significant inhibitory activity against ZIP but is effective against PE activity Figure 3 9 8 4 shows that PE and ZIP are inhibited in a similar manner by α-Ketobenzothiazole IC_{50} values determined for these inhibitors against PE and ZIP activity are presented in Table 3 11 * Enzyme activity expressed as % of uninhibited enzyme (100%)*



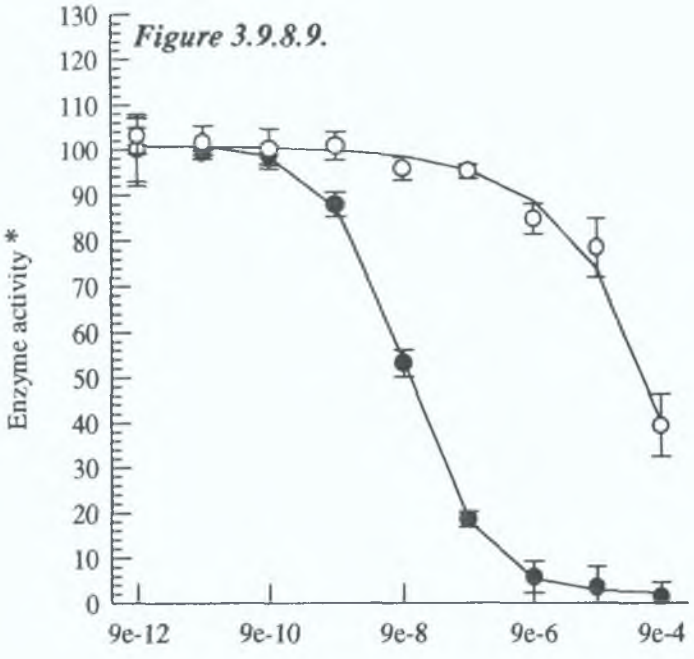


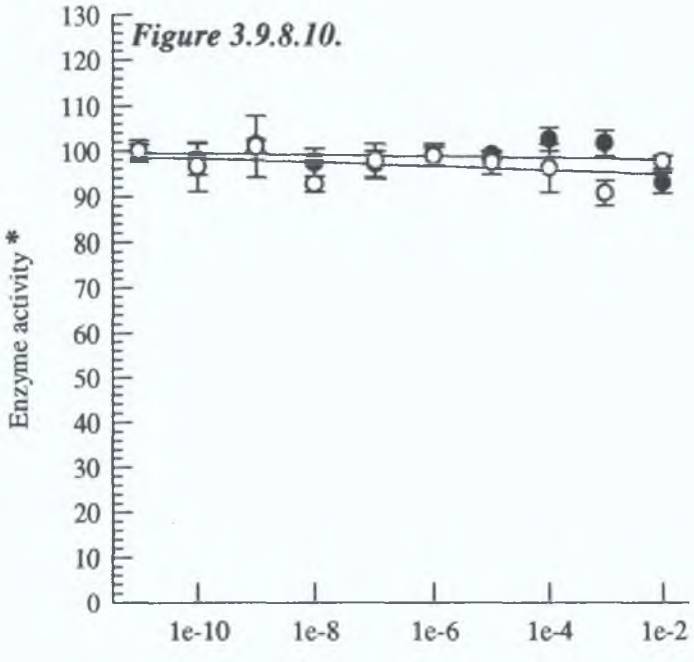
Figures 3 9 8 5 and 3 9 8 6 The inhibition of PE and ZIP by Poststatin and Z-Phe-Pro-Methylketone *Semi log plots of residual PE (●-●) and ZIP (o-o) activity versus specific inhibitor concentration Investigations into the effect of proline specific peptidase inhibitors were performed as outlined in section 2 9 8 Figure 3 9 8 5 illustrates that Poststatin exhibits some inhibitory activity against ZIP but is a more effective inhibitor of PE activity Figure 3 9 8 6 shows that ZIP is not inhibited by Z-Phe-Pro-Methylketone which is an effective inhibitor of PE activity IC_{50} values determined for these inhibitors against PE and ZIP activity are presented in Table 3 11 * Enzyme activity expressed as % of uninhibited enzyme (100%)*





Figures 3.9.8.7. and 3.9.8.8. The inhibition of PE and ZIP by Z-Cyclohexyl-Prolinal and Z-Indolinyl-Prolinal *Semi log plots of residual PE (●-●) and ZIP (o-o) activity versus specific inhibitor concentration. Investigations into the effect of proline specific peptidase inhibitors were performed as outlined in section 2.9.8. Figure 3.9.8.7. illustrates that Z-Cyclohexyl-Prolinal exhibits some inhibitory activity against ZIP but is a more effective inhibitor of PE activity. Figure 3.9.8.8. shows that ZIP is weakly inhibited by Z-Indolinyl-Prolinal in comparison to this inhibitors effect on PE activity. IC_{50} values determined for these inhibitors against PE and ZIP activity are presented in Table 3.11. * Enzyme activity expressed as % of uninhibited enzyme (100%)*





Figures 3.9.8.9. and 3.9.8.10. The inhibition of PE and ZIP by Boc-Glu(NHO-Bz)-Pyrr and Kelatorphan. *Semi log plots of residual PE (●-●) and ZIP (o-o) activity versus specific inhibitor concentration. Investigations into the effect of proline specific peptidase inhibitors were performed as outlined in section 2.9.8. Figure 3.9.8.9. illustrates that Boc-Glu(NHO-Bz)-Pyrr exhibits inhibitory activity against ZIP and PE activity. Figure 3.9.8.10. shows that Kelatorphan has no inhibitory effect on either PE or ZIP activities. IC_{50} values determined for these inhibitors against PE and ZIP activity are presented in Table 3.11. * Enzyme activity expressed as % of uninhibited enzyme (100%)*

Specific inhibitor	IC_{50} value (M)	
	PE	ZIP
Fmoc-Pro-Pro-Nitrile	3.203×10^{-8}	ND
Z-Thiopropyl-Thyoprolinal	5.472×10^{-8}	1.882×10^{-8}
Z-Pro-Prolinal	1.597×10^{-8}	ND
α-Ketobenzothiazole	4.163×10^{-11}	1.505×10^{-11}
Poststatin	3.509×10^{-8}	$>1.0 \times 10^{-3}$
Z-Phe-Pro-Methylketone	3.099×10^{-8}	ND
Z-Cyclohexyl-Prolinal	1.450×10^{-8}	$>2.0 \times 10^{-6}$
Z-Indolinyl-Prolinal	4.540×10^{-11}	$>1.0 \times 10^{-4}$
Boc-Glu(NHO-Bz)-Pyrr	1.656×10^{-7}	6.351×10^{-4}
Kelatorphan	ND	ND

Table 3.11. *IC_{50} values determined for proline specific peptidase inhibitors on PE and ZIP activity. Investigations into the effect of specific inhibitors against PE and ZIP activity were performed as outlined in section 2.9.8. Results are expressed in M units where IC_{50} is the concentration (M) of inhibitor needed to inhibit PE or ZIP acitvity by 50% under the conditions described in section 2.9.8.*

4. Discussion

4 1 Fluorimetry using 7-Amino-4-Methyl-Coumarin (MCA).

The primary focus of this work centred around the study of two Z-Gly-Pro-MCA degrading activities from bovine serum This internally quenched substrate, so called because the attachment of MCA to the N-blocked dipeptide compromises its fluorescent activity, was first synthesised and applied to prolyl endopeptidase (PE) detection by Yoshimoto *et al*, (1979) Peptide bond cleavage on the carboxyl side of proline releases free MCA, which, under constant exposure to electromagnetic radiation at a wavelength of 370nm (excitation), leads to the emission of electromagnetic radiation at a wavelength of 440nm Fluorimetric assays of this type provide greater sensitivity than their colorimetric counterparts In fact Yoshimoto *et al*, (1979) reported a 100 fold increase in sensitivity when using Z-Gly-Pro-MCA, as opposed to a colorimetric substrate, Z-Gly-Pro-ß-napthylamide The use of fluorimetric substrate assays also provide a safer and less labour intensive procedure than radiolabelled substrate assays

4 1.1. The Inner Filter Effect

Because fluorimetric assays rely upon the excitation of a fluorophore by electromagnetic radiation at a specific wavelength, and the subsequent detection of emitted electromagnetic radiation at a higher specific wavelength, their sensitivity is often compromised by the presence of molecules within the reaction mixture that also absorb at these wavelengths This phenomenon is known as the inner filter effect. In the case of MCA based fluorimetric assays, molecules within the reaction mixture that absorb at 370nm will reduce the number of MCA molecules that are excited, leading to a decrease in detected fluorescence Also, molecules within the reaction mixture that absorb at 440nm will prevent the detection of emitted radiation from excited MCA molecules In crude biological samples, this effect can be quite significant, and lead to inaccurate determination of free MCA concentration within a particular reaction mixture

To overcome this problem, MCA standard curves were constructed in a manner designed to replicate assay conditions Specifically, enzyme sample was incorporated into the make up of each standard curve (section 2 2 2) The effect of this on standard curves is clearly demonstrated in Figures 3 1 1, 3 1 2 and 3 1 3 and in Table 3 1 where serum was incorporated as the enzyme sample The "filtered" standard curves demonstrated a reduced slope which is indicative of reduced sensitivity

It was also observed that the percentage reduction in slope varies in a manner dependent on emission slit width As the emission slit width was increased the percentage reduction in slope also increased The reduction in slopes for Figure 3 1 1 and Figure 3 1 3 were 13 5% and 11 7% respectively with emission slit width settings of 10nm and 2 5nm respectively Expanding the emission slit width merely broadens the bandwidth over which the fluorimeter integrates the light emitted from a particular sample With an emission wavelength of 440nm and an emission slit width of 2 5nm, the fluorimeter includes light radiated from 438 75nm to 441 25nm as part of the integration calculations for that

sample If the emission slit width is expanded to 10nm the fluorimeter then includes light radiated from 435nm to 445nm as part of its integration calculations It is obvious, therefore, that increasing the emission slit width will not only increase sensitivity, but also increase the likelihood of inner filter effect interference

One of the more interesting features of the inner filter phenomenon is the effect produced when a fluorimetric assay is used to monitor a protein purification process Starting material for such a process would generally constitute a crude, highly coloured, protein preparation, and as such, is more likely to exhibit the inner filter phenomenon than a purified sample Measurement of the initial activity in the starting material, without due consideration for the inner filter effect, will reveal lower activities than are actually present in the starting material Assuming that in a single purification step, all activity is recovered, and all contaminants responsible for the inner filter effect are removed, the yield from this step will be considerably higher than 100%, due to the erroneous initial activity determination. It was realised therefore, that as part of this work, the preparation of "filtered" standard curves was a useful method in ensuring the accuracy of results obtained from fluorimetric assays

A problem arose, however, based on the need to produce "filtered" standard curves, and the practicalities of preparing such curves In order to construct one "filtered" standard curve, like those presented in Figures 3 1 1 , 3 1 2 or 3 1 3 , 100µL serum were incorporated as part of each MCA concentration in triplicate and almost 4mL serum were required in total It was unacceptable that 4mL of any enzyme sample were used merely to produce "filtered" standard curve A compromise was therefore reached with the production of a two point standard curve It was observed from Figures 3 1 1 - 3 1 3 that the inner filter effect, although reducing the slopes of individual standard curves, did not affect their linearity On the basis of this observation a "filtered" curve prepared using only two points in triplicate, namely a zero point (standard curve origin) and either a 2µM or a 10µM MCA point, was used routinely These curves reduced the amount of enzyme sample needed to quantify the inner filter effect from 4mL to 600µL

4 2 Serum Preparation

Serum preparation from whole blood was a simple procedure involving clotting, subsequent clot shrinkage, followed by centrifugation Z-Gly-Pro-MCA degrading activities in serum produced in this manner remained stable for up to six months when stored at -20 C, by which time the serum batch was normally exhausted Some batch to batch variation was observed with regard to levels of Z-Gly-Pro-MCA degrading activities, but these variations did not pose specific problems due to the minimal variations observed and the fact that these variations did not affect the efficiency of the purification protocols

4.3. Measurement of Z-Gly-Pro-MCA Degrading Activity in Bovine Serum

PE activity in serum was measured according to a modification of the original procedure as described in section 2 5 1 and the results are presented in Figure 3 4 1 Z-Gly-Pro-MCA was first synthesised by Yoshimoto *et al*, (1979), and in that report the susceptibility of Z-Gly-Pro-MCA to non-specific degradation was examined These workers reported that the substrate was not degraded by high concentrations of trypsin, α-chymotrypsin, elastase, thrombin, urokinase, leucine aminopeptidase, carboxypeptidase A or post-proline dipeptidyl aminopeptidase Therefore, Z-Gly-Pro-MCA represented a specific substrate for the detection of PE The synthesis and use of Z-Pro-Prolinal was first reported by Wilk *et al*, (1983) It was described as a tetrahedral transition state intermediate analogue of the PE enzyme-substrate complex At high concentrations, Z-Pro-Prolinal failed to inhibit trypsin, chymotrypsin, post-proline dipeptidyl aminopeptidase, cation-sensitive neutral endopeptidase or membrane-bound metalloendopeptidase (enkephalinase) These workers thus concluded that Z-Pro-Prolinal was a selective inhibitor of PE The use, therefore, of the specific PE substrate Z-Gly-Pro-MCA, coupled with the specific PE inhibitor, Z-Pro-Prolinal, has formed the basis of a definitive assay for PE activity over the last decade In this light, the observation that residual PE activity against Z-Gly-Pro-MCA (40%) was detected in bovine serum in the presence of Z-Pro-Prolinal was unexpected (Figure 3 4 1) Increased Z-Pro-Prolinal concentrations (Figure 3 4 2.) and increased preincubation times (Figure 3 4 3) failed to reduce this residual PE activity The possibility that there may have been a second Z-Gly-Pro-MCA degrading activity in bovine serum was not considered a likely possibility at this point. Instead, it was thought that incomplete inhibition of PE activity in serum could be explained by either, (a), the high levels of protein in serum interfering in some way with the enzyme-inhibitor interaction, or (b), the susceptibility of Z-Pro-Prolinal to degradation by aldehyde and alcohol dehydrogenases (Friedman *et al*, 1984) may have effectively reduced the concentration of intact Z-Pro-Prolinal available to inhibit the enzyme It was decided, therefore, to continue with efforts to purify PE from bovine serum

4.4. Purification of Z-Gly-Pro-MCA Degrading Activities from Bovine Serum

4.4.1 Ion Exchange Chromatography

Initial attempts to purify PE from bovine serum focused on the use of ion exchange resins Both anion and cation exchange resins were considered Figure 3 6 1 1 is representative of the protein and activity profiles that were obtained repeatedly from both cation and anion exchange columns under various conditions This type of profile, activity binding to the column with a significant amount of activity also running through the column (unbound), is normally associated with a binding capacity problem which can be related to an insufficiently large column, overloading the column with too much enzyme sample, or non-ideal binding and running conditions Increasing the size of the various ion exchange columns tested, followed by a substantial reduction in the amount of enzyme sample applied to the

column, failed to improve the activity binding profile Column configurations and binding conditions were also optimised, but failed, without any apparent explanation, to prevent a relentless run through of enzyme activity The observation that similar Z-Gly-Pro-MCA degrading activity profiles (bound activity and run through activity) were obtained whether the column used was an anion or cation exchange resin, was also inexplicable The presence of two distinct Z-Gly-Pro-MCA degrading activities in bovine serum now seemed a likely possibility

Confirmation of this was forthcoming when, fractions obtained from an SP Sepharose cation exchange column (section 2 6 1 1), were assayed in the presence and absence of Z-Pro-Prolinal The resulting profiles presented in Figure 3 6 1 2 confirmed that there were indeed two Z-Gly-Pro-MCA degrading activities in bovine serum, a PE activity that was totally inhibited by Z-Pro-Prolinal, and a Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase, henceforth designated as ZIP This realisation subsequently explained why it was not possible to totally inhibit Z-Gly-Pro-MCA degrading activity in bovine serum (Figure 3 4 1) Attention now focused on the purification of both of these activities

The SP Sepharose column proved most useful in separating PE and ZIP activities, allowing these enzymes to be further purified independently of one another The applied protein was separated roughly into two equal parts, the run through pool containing PE, and the bound pool containing ZIP activity Attempts to resolve bound ZIP activity further from bound protein, using gradient elution techniques, failed to improve activity-protein resolution and the simpler isocratic elution with high salt was retained The recovery of applied PE activity was relatively low (approx 57%) and this was thought to be caused by the long term exposure of this activity to low pH The recovery of ZIP activity from this column was practically 100%

One final element of the SP Sepharose profile depicted in Figure 3 6 1 1 should be discussed, the apparent "split" or "shouldered" PE and ZIP activity peaks Responsibility for this lay with the non-quantitative microplate assay used to determine activities in post-column fractions, described in section 2 5 3 , and the inner filter effect, which has already been discussed. Figure 4 1 illustrates the light paths taken by excited and emitted light through samples that are being fluorimetrically analysed in a cuvette or in one well on a 96 well plate A hypothetical fluorophore is placed at a distance farthest from the excitation source and the emission detector In the case of the cuvette, the total distance travelled through the sample by excitation and emission radiation is 1 4cm, while in the case of the well, total distance travelled by radiation through the sample is 2cm. In a cuvette based assay the final sample concentration (% v/v) is approximately 6 5%, while in the well assay this percentage is approximately 35% The increased exposure of excitation and emission radiation to the enzyme sample, coupled with the increased concentration of enzyme sample in the well, amplifies the inner filter effect of the assay mixture

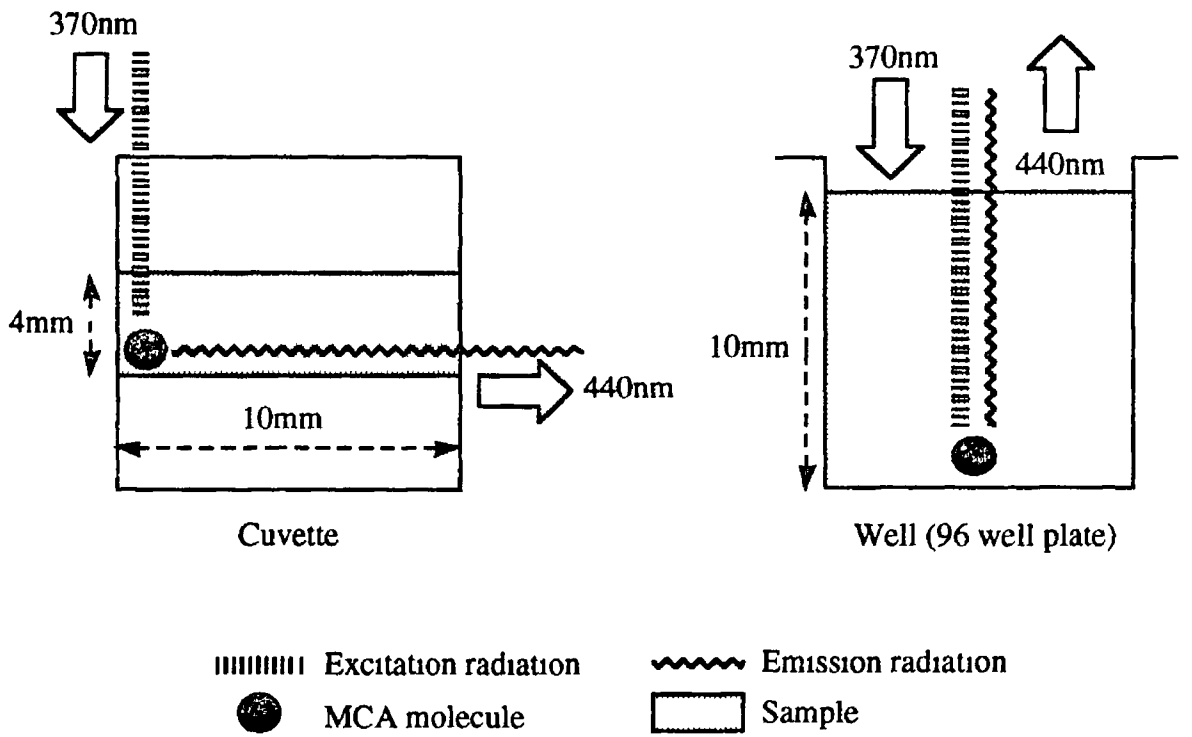


Figure 4 1 *Light paths travelled through a glass cuvette and an individual well on a 96 well plate demonstrating the increased exposure of emission and excitation electromagnetic radiation to sample when fluorimetric assays are performed in a 96 well plate as opposed to a cuvette*

This amplification of the inner filter effect was so severe in fractions towards the centre of the run through and bound protein peaks of the SP Sepharose column, that the fluorimetric activity in these fractions seemed to decrease, producing "split" or "shouldered" peaks

4 4 2. Further Purification of PE

The post-SP Sepharose PE pool was salted and applied to a Phenyl Sepharose hydrophobic interaction column Elution of PE activity from the column with a decreasing salt gradient produced very good resolution between enzyme and contaminating protein, as illustrated in Figure 3 6 2 Recovery of applied enzyme activity was 84% and, coupled with a purification factor of 12 for the column, this step proved to be the most successful part of the entire PE purification

The post-Phenyl Sepharose PE pool was applied to a DEAE Sepharose anion exchange column and eluted from that column with an increasing salt gradient, as described in section 2 6 3 , and illustrated in Figure 3 6 3 Some resolution of protein was obtained and 60% of the applied activity was recovered However, the overall purification factor for the column was only 1 5

The purification strategy for PE is presented in Table 3.2 The greatest purification factor obtained was 29 with an overall yield of 24% This provides some insight into the difficulties encountered during the development of the procedure Whether the final aim of a purification is high yield or high levels of purity, successful steps must resolve the protein of interest from contaminating proteins without serious detrimental effects on the activity or integrity of the protein of interest Sections 2 6 8 and 3 6 8 document some of the alternative methods attempted to further purify PE following the successful Phenyl Sepharose step Single protocols for each regime attempted are presented in sections 2 6 8 and 3 6 8 but many different protocols were attempted using each of the resins mentioned The failure of these alternative procedures to further purify PE could be broadly divided into two categories, (a) failure to resolve protein or (b), failure to maintain PE activity

Failure to resolve PE activity from contaminating protein proved to be a difficult problem to overcome in order to further purify PE activity following the Phenyl Sepharose hydrophobic interaction step It was possible to bind PE activity to Q Sepharose (Figure 3 6 8 1) and Hydroxylapatite (Figure 3 6 8 3) columns under conditions outlined in sections 2 6 8 1 and 2 6 8 3 respectively Although no reference to the use of Q Sepharose anion exchange chromatography could be found in the relevant PE literature, Hydroxylapatite has been used by many groups successfully Table 4 1 lists workers that have used Hydroxylapatite as an integral part of successful PE purifications However in the case of bovine serum PE, under no circumstances was it possible to resolve protein and enzyme activities Combinations of low flow rates and shallow phosphate gradients failed to make Hydroxylapatite a useful part of the bovine serum PE purification strategy

Author	Source	Purification Factor Obtained (Fold)
Koida *et al*, (1976)	Lamb kidney	6
Knisatschek *et al*, (1980)	Lamb kidney and pituitary	n r
Yoshimoto *et al*, (1980)	*Flavobacterium*	3
Moriyama *et al*, (1988)	Porcine muscle	4
Sattar *et al*, (1990)	*Agaricus bisporous*	2
Kalwant *et al*, (1991)	Human brain	9
Ohtsuki *et al*, (1994)	Flesh fly	10

Table 4 1 *Use of Hydroxylapatite in prolyl endopeptidase purification These workers published Hydroxylapatite purification steps as successful parts of PE purification strategies However, Hydroxylapatite failed to bind bovine serum PE and could not be used for its purification n r - Not Reported*

Q Sepharose, being an anion exchanger, was expected to work in a similar manner to DEAE Sepharose, but again, no resolution between protein and activity was forthcoming The resolution obtained by using the DEAE Sepharose failed to equal some resolutions obtained by other workers using this technique (Table 4 2), with the purification factor achieved using this column being a mere 1 5 fold

There is one possible factor that may be responsible for this lack of resolution Although many of the workers presented in Table 4 2 , used animal tissues as their sources, no purification of PE from serum has been reported It is obvious that the greatest single source of protein contaminant in bovine serum is bovine serum albumin. With a pI of 5 0, and considering that PE has a similar pI (Wilk, 1983), it would be very difficult to separate these proteins on the basis of charge using an anion exchange resin

Other techniques used simply failed to bind PE activity Calcium Phosphate Cellulose (Figure 3 6 8 2), which can be used under similar operating conditions to Hydroxylapatite, failed repeatedly to bind PE activity, and in fact, this column failed to bind any of the post-Phenyl Sepharose PE pool protein Browne and O'Cuinn, (1983), used Calcium Phosphate Cellulose as part of their purification of PE from guinea pig brain, but are the only workers in the literature to have done so, or to have reported this technique as a successful purification step It is very likely that the Calcium Phosphate Cellulose produced in our laboratory had different chromatographic properties to that used by Browne and O'Cuinn in 1983 This is a common problem associated with the use of this "home-made" resin.

Assuming that BSA was a major contaminant, it was hoped that Blue Sepharose, a resin used regularly for binding albumins as well as NAD^+ and $NADP^+$ linked enzymes, might help in the purification of PE The resin not only failed to bind PE activity, but fractions collected in the wash from the column repeatedly displayed high losses of PE activity (Figure 3 6 8 4) No reports of the use of Blue Sepharose to purify PE activity were found in the literature

One other chromatographic technique was investigated thoroughly as a possible part of the PE purification strategy, Activated Thiol Sepharose affinity chromatography Figure 4 2 demonstrates how Activated Thiol Sepharose may be used to bind proteins The protein sample must be reduced with a suitable reducing agent, converting all disulphide bonds to thiols The reducing agent, in this case DTT, must then be removed quickly, as it will displace the capping group, and prevent protein binding to the column, in the same way that high salt will prevent protein binding to an ion exchange resin However, in the absence of DTT, thiols will revert slowly to disulphides, which will not bind the resin It can be a difficult process, but previously it has been used with success in our laboratory (Cummins and O'Connor, 1996)

Author	Source	Purification Factor Obtained (Fold)
Koida *et al*, (1976)	Lamb kidney	25
Orlowski *et al*, (1979)	Rabbit brain	5
Yoshimoto *et al*, (1981)	Lamb brain	34
Browne *et al* (1983)	Guinea-pig brain	8
Moriyama *et al*, (1988)	Porcine muscle	42
Kalwant *et al*, (1991)	Human brain	3
Kusuhara *et al*, (1993)	Rat skin	3

Table 4 2 *Use of DEAE anion exchange in prolyl endopeptidase purification. These workers published DEAE anion exchange purification steps as successful parts of PE purification strategies DEAE anion exchange chromatography was also used as part of the bovine serum PE purification strategy, however the efficiency of this step was hindered by the presence of BSA which has a pI equal to that reported for PE*

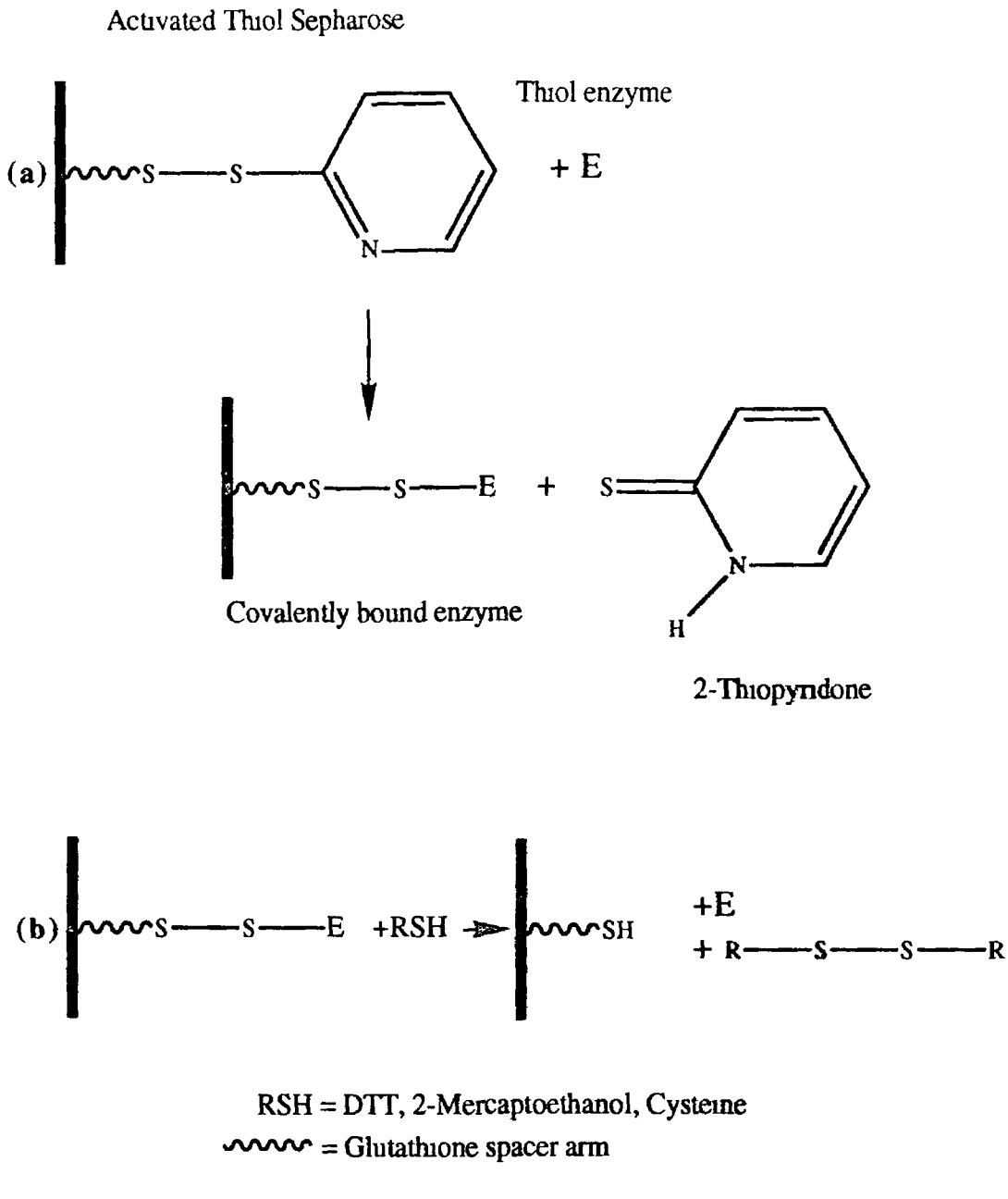


Figure 4 2 *Protein purification strategy using Activated Thiol Sepharose (ATS) (a) Protein in reduced form is applied to ATS resin that is capped with 2-Thiopyridone linked to Sepharose via a glutathione spacer arm. The protein binds covalently via a disulphide bond to the resin, displacing the capping group (2-Thiopyridone) (b) Application of a reducing agent (RSH) such as DTT, reduces the disulphide bond, eluting the bound protein from the resin*

Unfortunately, having optimised the removal of DTT using Sephadex G-25 as a de-salting column, neither PE nor great amounts of contaminating protein bound to the column as illustrated in Figure 3 6 8 5 2 This plot may be somewhat misleading in that the A280nm protein profile seems to suggest that quite alot of protein was bound to the column, and under these conditions, the column could have been used successfully as a purification step However the A280nm peak bound to the resin is not representative of protein Rather, it illustrates the elution of the capping group, 2-Thiopyridone, from the column following the application of DTT 2-Thiopyridone absorbs maximally at 343nm but interferes significantly with A280nm determinations Subsequent analysis of protein in fractions obtained from the column indicated that 95% of the applied protein was recovered in the run through peak

4 4.3 Further Purification of ZIP

The separation of the bovine serum Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP), from bovine serum PE has already been discussed (Section 4 4 1)

The second step used in the purification of ZIP was, as for PE, a Phenyl Sepharose hydrophobic interaction column This column, when run as described in section 2 6 5 , produced excellent resolution between ZIP activity and contaminating protein (Figure 3 6 5) The isocratic elution of ZIP activity with distilled water could not be improved upon using gradient elution techniques Indeed, gradient elution merely broadened the eluting ZIP peak without any resolution of protein Recovery of applied activity (55%) coupled with a purification factor of 19 for the column, made this step a good bulk protein separation step (Table 3 3)

The post-Phenyl Sepharose ZIP pool could be loaded directly onto a Calcium Phosphate Cellulose Column This resin was produced in the lab as outlined in section 2 6 6 As a "home-made" resin, it proved to be a very successful purification tool Used as outlined in section 2 6 6 , it produced excellent resolution between contaminating protein and ZIP activity (Figure 3 6 6) 59% of the applied ZIP activity was recovered, and, coupled with a purification factor of almost 100 fold for the column, this step proved invaluable in the overall ZIP purification strategy (Table 3 3)

As a final step, the post-Calcium Phosphate Cellulose ZIP pool was concentrated and applied to a Sephacryl S-200 HR Sepharose gel filtration column The elution profile presented in Figure 3 6 7 , shows that further resolution of activity from protein was achieved

The purification strategy for ZIP, presented in Table 3 3., illustrates the success of the ZIP purification It would seem that application of the post-Calcium Phosphate Cellulose ZIP onto the S-200 gel filtration column was unwarranted, as the overall purification factor dropped after this column However, this drop can be explained by a loss in yield rather than a failure to further purify the

enzyme As Figure 3 15 illustrates, purification of ZIP was achieved by the gel filtration step On this basis the gel filtration step was retained as part of the ZIP purification

4 5 Z-Gly-Pro-MCA Degradation Assay Development

Having purified both enzyme activities, it was decided to re-evaluate the assay procedure described in section 2 5 1 This assay was a modification of the procedure of Yoshimoto *et al*, (1979) In fact it was a *modification* of a modification of the original procedure

Yoshimoto *et al*, (1979), first synthesised Z-Gly-Pro-MCA for PE detection Their assay procedure involved the addition of 50µL 0 5mM Z-Gly-Pro-MCA in 100% dioxane to 3mL 100mM phosphate buffer, pH 7 0, followed by the addition of 50µL enzyme solution Thus, the final substrate concentration was 0 08mM Z-Gly-Pro-MCA and the final solvent concentration was 0 16% (v/v) In 1983, Browne and O'Cuinn modified the original procedure adding 10µL enzyme sample to 490µL 0 2mM Z-Gly-Pro-MCA in 100mM potassium phosphate, pH 7 4, 2mM DTT, 2mM EDTA, in a final concentration of 5% (v/v) DMF O'Leary and O'Connor, (1995), made a further modification of this procedure O'Leary developed the assay in this laboratory as part of her work here In this procedure, 400µL 0 1mM Z-Gly-Pro-MCA in 100mM potassium phosphate, pH 7 4, with a final DMSO concentration of 2% (v/v) was added to 100µL enzyme sample As part of the work presented here, problems arose with the preparation of 0 1mM Z-Gly-Pro-MCA in a final DMSO concentration of 2% (v/v) At this final DMSO solvent concentration, substrate solubility was quite poor, resulting in an unreliable protocol for substrate preparation Initially this problem was overcome by preparing substrate in a final DMSO concentration of 4% (v/v), which proved to be more reliable, and this is the method described in section 2.5 1 To make substrate preparation even more reliable and reproducible a further change in the methodology was made Substrate had been prepared by dissolving the required amount of Z-Gly-Pro-MCA in 1mL DMSO, bringing the volume finally to 50mL with 100mM potassium phosphate buffer at 37°C This not only had the potential for inaccuracies in weighing out the small amounts of substrate needed but also forced the user to make up substrate batches of at least 50mL to confidently produce a 0 1mM substrate solution, when far less substrate might actually be needed The preparation of 10mM Z-Gly-Pro-MCA stock in 100% DMSO, and subsequent preparation of 10mL batches of 0 1mM substrate from this stock as described in section 2 5 1 led to an easier and more flexible procedure for substrate preparation

The decision to re-evaluate the assay procedure described in section 2 5 1 was primarily due to the discovery of the ZIP activity The assay systems described thus far were designed to detect and quantify PE activity It was unclear whether they would be sufficient for accurate and reproducible determinations of ZIP activity

4 5 1. Determination of Suitable Solvent for Substrate Solubilisation

To re-evaluate the assay the basics were first considered What was the most suitable solvent to be used for stock substrate preparation, and what final concentration of this solvent would provide optimum solubility, with due consideration to enzyme activity ? The determination of what was the most suitable solvent for stock substrate preparation was investigated as described in section 2 8 1 , and the results are presented in section 3 8 1 and Figures 3 8 1 1 and 3 8 1 2 In Figure 3 8 1 1 , although no discernible difference may be made between the activity of PE when assayed with substrates prepared in DMSO or dioxane, DMF adversely affected the activity of the enzyme, reducing the sensitivity of the assay by 60% when compared to the other solvents

The same results presented for ZIP in Figure 3 8 1 2 demonstrated that 20% reduced sensitivity was observed when DMSO was used as the substrate solvent. Substrate solubility in all three solvents at a final concentration of 4% (v/v) was satisfactory On the basis that dioxane produced the lowest deleterious effects on assay sensitivity for PE and ZIP, it was chosen as the most suitable solvent to use for substrate solubilisation

4.5.2. Effect of Dioxane Concentration on Purified Enzymes

Having decided that dioxane was the best choice of solvent in which to prepare 10mM substrate stock, the optimum final concentration of solvent in 0 1mM buffered substrate was investigated as outlined in section 2 8 2 Section 3 8 2 and Figures 3 8 2 1 and 3 8 2 2 illustrate the results obtained as part of this investigation It was observed that for both PE and ZIP activities, a final solvent concentration of 2% (v/v) dioxane in substrate produced optimal results Increasing this concentration to 4% (v/v) reduced the sensitivity of both assays by greater that 50% Substrate solubility at a final concentration of 2% (v/v) dioxane was attained easily in a reproducible manner Therefore the preparation of 10mM stock substrate in 100% dioxane, with the use of 0 1mM substrate with a final dioxane concentration of 2% (v/v) was accepted as being the optimum substrate make up for both PE and ZIP activities

Once satisfied with substrate make up, the two most important considerations for developing a quantitative and accurate assay are linearity of the assay with respect to (a) time and (b) enzyme concentration

4 5.3. Linearity of Enzyme Assays with Respect to Time

Figure 4 3 represents the typical progression of an enzyme-substrate reaction velocity curve The change in reaction velocity over time may be due to substrate depletion, instability of the enzyme under assay conditions, or inhibition of the enzyme by product formed Using a continuous assay system, this decrease in reaction velocity over time may be observed but does not cause any specific problems, provided the decrease in reaction velocity is caused by substrate depletion A tangent to the reaction curve, drawn through the origin, represents the initial rate of reaction, which may be used in

quantitative activity measurements However, in a discontinuous assay system, where activity measurements are only determined at one discrete point along the reaction curve, deviation from linearity is not detected, and may lead to inaccurate activity estimates A hypothetical reaction curve is presented in Figure 4 3 At 25 minutes, determination of the amount of product formed using either a continuous, (represented by initial velocity measurements) or discontinuous assay leads to identical results, i e 20 units However, at 100 minutes, 45 units are observed when determining the amount of product formed using a discontinuous assay, while the initial velocity determination (the true activity measurement) is 80 units

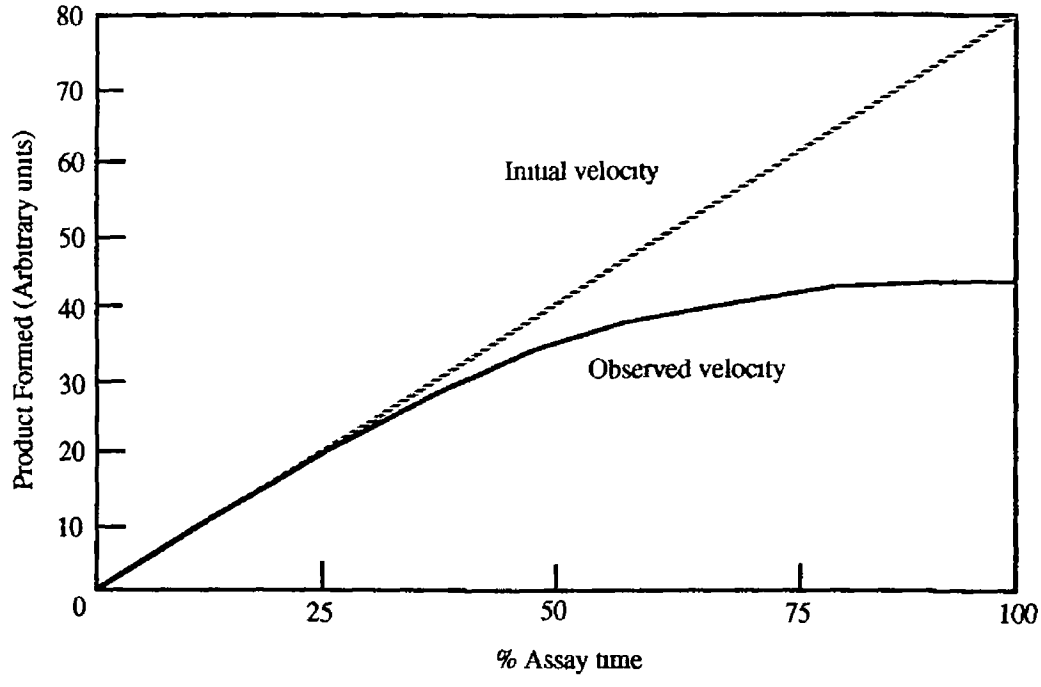


Figure 4 3. Progress of an enzyme-substrate reaction velocity curve *Plot of product formed versus assay time Deviation of observed velocity from initial velocity due to substrate depletion, enzyme instability or inhibitor production over the assay period.*

In order to overcome this difficulty with discontinuous assays, the progression of the reaction curve in a linear fashion, over the assay period must be confirmed Sections 2 8 3 , 3 8 3 , and Figures 3 8 3 1 - 3 8 3 4 outline how this linearity was confirmed and what results were obtained From Figures 3 8 3 1 and 3 8 3 2 it is apparent that Z-Gly-Pro-MCA degradation by purified PE and ZIP was linear with respect to time for up to two hours Given that the assay described in section 2 5 1 is a one hour discontinuous assay, the linearity of this assay was assured for purified PE and ZIP activity measurements However, Figures 3 8 3 3 and 3 8 3 4 demonstrate that a loss in linearity was apparent over the two hour period when Z-Gly-Pro-MCA degradation by PE and ZIP activities in serum, and ZIP activity alone in serum, were studied respectively Because the curvature was only observed in the crude serum samples, it was reasonable to assume that this loss in linearity was due to enzyme

depletion caused by proteolysis This curvature did not, however, affect the reaction curve over the first sixty minutes, and therefore a one hour discontinuous assay was still valid

4 5 4. Linearity of Assays with Respect to Enzyme Concentration

A quantitative assay system determining the levels of activity in given enzyme sample should be linear with respect to enzyme concentration This aspect of the Z-Gly-Pro-MCA assay was investigated as outlined in section 2 8 4 , and the results obtained are presented in section 3 8 4 and Figures 3 8 4 1 - 3 8 4 5 The PE assay was found to be linear with respect to enzyme concentration as illustrated in Figure 3 8 4 1 It was immediately apparent however, that the ZIP assay posed problems in this regard Figure 3 8 4 2 illustrates that activities observed from dilutions of purified ZIP with phosphate buffer were not consistent with ZIP concentration, and in fact the data fitted a second order linear regression analysis quite well The linearity of a 50% purified ZIP sample with respect to time over the assay period was confirmed in Figure 3 8 4 3 confirming that the lack of linearity seen in Figure 3 8 4 2 was not due to instability of the enzyme under assay conditions, caused by dilution effects While pondering these results in an attempt to understand why the purified ZIP assay would not behave linearly with respect to enzyme concentration, it was noted that apart from the varying concentration of ZIP produced by diluting with phosphate buffer, one other factor was being varied, the concentration of salt in the enzyme sample The purified ZIP and PE used for these studies were post-Sephacryl S-200 HR Sepharose gel filtration samples, and as such, contained 200mM KCl as part of the gel filtration running buffer Dilution of ZIP activity with phosphate would produce a pattern of varied salt concentrations for each ZIP concentration Repeating the experiment with the inclusion of 200mM KCl in diluant and substrate, produced a linear response to enzyme concentration (Figure 3 8 4 4) When the experiment was again repeated, with post-gel filtration KCl first removed by dialysis, and with no KCl included in either diluant or substrate, linearity of the purified ZIP with respect to enzyme concentration was again apparent (Figure 3 8 4 5) It was also noted that enzyme activity in Figure 3 8 4 4 , when expressed as a percentage of ZIP activity with no salt included, was approximately 2 75 times that of the dialysed ZIP sample These results not only helped validate the purified PE and ZIP assays, but also revealed a hitherto unobserved enhancement of ZIP activity in the presence of salt.

It was apparent from results obtained for the assay linearity with respect to enzyme concentration investigation (Figures 3 8 4 4 and 3 8 4 5) that the sensitivity of the ZIP assay could be increased by incorporating NaCl into the substrate It was also apparent from results obtained for the assay linearity with respect to time investigation (Figure 3 8 3 1) that the sensitivity of the PE assay could be increased by incorporating DTT into the substrate In order to produce the most sensitive assay for each enzyme, investigations were performed as outlined in section 2 8 5 and 2 8 6 to determine the effect on observed PE and ZIP activities in the presence of increasing concentrations of DTT and NaCl and the results are presented in Figures 3 8 5 and 3 8 6 respectively

4 5 5 DTT Activation of Purified Enzyme Activities

Figure 3 8 5 illustrates the effect of increasing DTT concentration on PE and ZIP activities ZIP activity demonstrated no increased activity in the presence of DTT In fact, at a DTT concentration of 20mM, the observed ZIP activity was decreased by 30% PE however demonstrated a significant increase in observed activity in the presence of low concentrations of DTT At a DTT concentration of 12mM observed PE activity increased by 12 fold At DTT concentrations of greater than 12mM no deleterious effects on observed PE activity were detected indicating that PE could be assayed in the presence of excess DTT (greater than 12mM) without affecting the sensitivity of the assay This increased PE activity in the presence of DTT agrees with the literature on PE where DTT is normally included as part of substrate preparations used to detect this enzyme The extent to which this purified bovine serum PE activity is enhanced by low levels of DTT is quite different however to that previously reported Orlowski *et al*, (1979) reported a 40% increase in observed activity from PE that had been purified from rabbit brain at a DTT concentration of 1mM From Figure 3 8 5 it is clear that at a DTT concentration of 1mM the observed activity of PE is enhanced by approximately 300% The precise nature of the DTT activation of PE is not well understood Reducing agents are normally associated as being necessary components of assay systems designed to detect cysteine protease activities The mechanistic class assigned to PE by this work and by the literature will be discussed in detail later It is generally accepted, however, that PE is a serine protease with a cysteine residue located near the active site In the case of cysteine proteases, the presence of reducing agents such as DTT, activates the active site cysteine residue, increasing its ability to initiate a nucleophillic attack upon a bound substrate In the case of PE, it is possible that activation of the cysteine residue by reducing agents may play some role in the binding of substrate, or stabilising the covalent tetrahedral intermediate formed during the catalysis of peptide bond scission

4 5.6. Salt Activation of Purified Enzyme Activities

Figure 3 8 6 illustrates the effect of increasing NaCl concentration on purified PE and ZIP activities PE exhibited a decreased observed activity in the presence of high concentrations of NaCl. At 1M NaCl the decrease in observed PE activity was approximately 25% This result is in stark contrast to that obtained by Polgar, (1991) where PE activity, purified from pig muscle, was enhanced in a linear fashion up to 0 3-0 5M NaCl at pH 8 0 Polgar also showed that purified porcine muscle PE demonstrated a double sigmoidal pH profile and concluded that two different catalytic forms of the enzyme existed at pH 6 0 and at pH 8 0 due to the fact that two pK_a values of 5 37 and 6 16 were determined for the enzyme This qualified his salt enhanced PE activity results which only occurred at pH 8 0 At pH 6 0 no enhanced activity was detected and slight inhibition of PE at low salt concentrations were evident It is difficult to reconcile Polgars' observations and those presented here because the method used (section 2 8 6) determined the effect of NaCl on PE activity at a pH of 7 4 This pH is above both pK_a values determined by Polgar and therefore PE would be in the same ionisation state at pH 7 4 or at pH 8 0 Orlowski *et al*, (1979) reported no effect on purified rabbit brain PE activity when NaCl was included in the substrate

ZIP activity was enhanced in the presence of NaCl with maximum observed activity being obtained at 500mM NaCl The increase in activity detected was approximately two fold (Figure 3 8 3 5) Increased activity in the presence of salts is commonly attributed to increased stability of a protein molecule in the presence of such salts Certain salts such as NaCl and ammonium sulphate can reduce the solubility of hydrophobic groups on a protein molecule by increasing the ionic strength of the solution In addition, these salts enhance the formation of water clusters around the protein which causes a loss of the total free energy of the system (Volkin and Klibanov, 1990) The salt itself is excluded from the water shell thus formed around the protein This is also known as preferential hydration (Timasheff and Arakawa, 1990) and causes the protein to become more compact and therefore more stable Increased stability, however, does not necessarily account for enhanced activity During the discussion on assay linearity with respect to time it was obvious that ZIP activity towards the substrate Z-Gly-Pro-MCA was stable over the assay period employed Likewise a 50% ZIP sample was stable for a similar period Polgar, (1995), examined the salt activation of PE with regards to stability effects and concluded that NaCl actually de-stabilises PE via a mechanism opposing preferential hydration He suggests that NaCl penetrates the zone of preferential hydration and binds to the enzyme at specific sites The binding of NaCl will weaken possible electrostatic forces that may stabilise the protein conformation. Polgar also suggests that the reduced water shell surrounding PE in the presence of NaCl can account for increased catalytic activity It must be remembered that this is Polgars' account for the activation of PE by NaCl, a result that is not consistent with this study His conclusions are presented here as a possible mechanism for the enhanced activity of ZIP in the presence of NaCl At NaCl concentrations greater than 500mM NaCl no deleterious effects on ZIP activity were observed This indicates (as was the case with PE and DTT) that ZIP could be assayed in the presence of excess NaCl (>500mM) without affecting the sensitivity of the assay It should be noted however that at NaCl concentrations approaching 1M in 100mM potassium phosphate, pH 7 4, some substrate solubility problems were observed

Having determined the optimal conditions for a sensitive and accurate fluorimetric assay for PE and ZIP using the substrate Z-Gly-Pro-MCA, a finalised protocol for such an assay was formulated, and is presented in section 2 8 7

One final question was left unanswered with regard to the salt enhanced activity of ZIP It was apparent, for ZIP, that consideration of salt in the design of linearity with respect to enzyme concentration experiments was necessary, but was salt dependence alone responsible for the second order data presented in Figure 3 8 4 2 ? Looking closely at Figure 3 8 6 , it is apparent that over a 0 - 200mM salt range the increase in ZIP activity is linearly enhanced by 0 - 50% Assuming that diluted ZIP would respond in a similar fashion Table 4 3 was constructed to estimate activity that would be observed when a ZIP sample containing 200mM salt was diluted with buffer containing no salt. The table was constructed as follows If ZIP (+200mM salt) was diluted with buffer (no salt) in a ratio of 1 1 then the final enzyme concentration would be 50% of the undiluted ZIP and the final salt

[ZIP] following dilution (%)	Activity expected (%)	[Salt] following dilution (mM)*	Assumed enhancement (%)**	Expected activity + enhancement (%)†
0	0	0	0	0
25	25	50	12 5	28 125
50	50	100	25	62 5
75	75	150	37 5	103 125
100	100	200	50	150

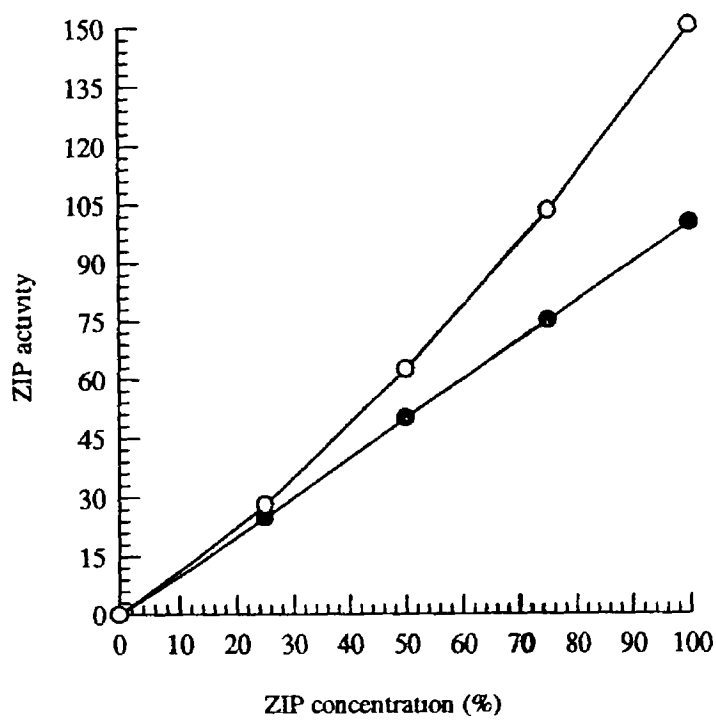


Table 4 3 and Figure 4 4. *ZIP activities expected when enzyme containing 200mM salt is diluted with buffer containing no salt* * *Salt concentration based on 200mM salt present in undiluted enzyme sample* ** *Assumed on the basis that 0 - 200mM salt produces a linear activity increase of 0 - 50% (Figure 3 8 6)* † *Expected activity plus expected activity x (assumed enhancement) % The data obtained is presented graphically in Figure 4 4 , a plot of ZIP activity versus ZIP concentration, clearly illustrating a second order curve (●-●) expected ZIP activity (o-o) ZIP activity determined according to assumed salt dependence*

concentration would be 100mM Therefore the activity expected from the diluted sample would be 50% of the undiluted ZIP activity plus the enhanced activity obtained from ZIP in 100mM salt It is assumed that because 0 - 200mM salt produces a linear increase in ZIP activity of 0 - 50% (Figure 3 8 6) then 100mM salt will produce an activity increase of 25% Thus the actual activity observed will be 50% of the undiluted ZIP plus a 25% enhancement, which leads to a final activity of 62 5% of the undiluted enzyme If this logic is applied to a range of dilutions, as is presented in Table 4 3, and the data obtained is plotted (Figure 4 4), a second order curve is produced This model therefore confirms that salt present in the post gel filtration ZIP was responsible for the data obtained in Figure 3 8 4 2

4.6. Characterisation of Purified PE and ZIP Activites

4 6.1. Relative Molecular Mass Determination

The molecular weight of both PE and ZIP were determined by SEC-HPLC, Gel Filtration Chromatography and SDS PAGE as outlined in section 2 9 1 The results of these investigations are presented in section 3 9 1 and Figures 3 9 1 1 - 3 9 1 3 The native molecular weight of PE was estimated to be 69,700Da and 54,500Da by gel filtration chromatography (Figure 3 9 1 1) and SEC-HPLC (Figure 3 9 1 2) respectively Multiple bands visualised following SDS-PAGE of PE, meant that the subunit structure or molecular weight of PE could not be reliably determined by this method. The molecular weights obtained agree well with the previously published molecular weights determined for PE from various sources (Table 4 4) In all cases but one, PE was monomeric One report suggested that PE had a molecular weight of 115,000Da and that the enzyme was dimeric (Koida *et al*, 1976) but this result was later corrected (Yoshimoto *et al*, 1977) The native molecular weight of ZIP was estimated to be 184,200 and 191,000 by gel filtration chromatography (Figure 3 9 1 1) and SEC-HPLC (Figure 3 9 1.2.) respectively Two bands were visualised following SDS PAGE using Silver Stain (Figure 3 7.2), the major of the two having a molecular weight of 50kDa (Figure 3 9 1 3) This indicates that ZIP exists as a tetramer of four subunits of equal molecular weight. The discrepancy between figures obtained from gel filtration chromatography on S-200 Sepharose and SEC-HPLC are difficult to reconcile, though these types of methods for determining molecular weight are accurate to approximately 10% (Welling and Welling-Wester, 1989) The basis upon which this value is obtained is not explained by Welling, but one insight into the use of gel filtration methods for molecular weight determination is discussed. As with all quantitative methods, the determination of a final result is based on the measurement of a single parameter In the case of gel filtration methods this parameter is volume (mL) The calibration curve is constructed on the basis of volumes at which the molecular mass standards elute from a column and the determination of the molecular weight of any unknown molecule is determined based on its elution volume from a column The calibration curve equations for the S-200 HR Sepharose and Biosep SEC-3000 columns are presented in sections 3 9 1 1 and 3 9 1 2 respectively Closer examination of these equations reveal that 1mL of eluant collected from these columns represents a molecular weight range of 4,000Da for

the S-200 HR Sepharose column and 30,000Da for the Biosep SEC-3000 column Considering these facts coupled with the fraction sizes collected as part of each method, the error in S-200 HR Sepharose and Biosep SEC 3000 gel filtration estimation of molecular weights could be as large as 8,000Da and 3 000Da respectively However, careful and precise column calibration and determination of elution volumes means that gel filtration chromatography as a method for determining molecular weights is generally accepted as a reliable procedure

4 6.2 Assay Temperature Effects on Purified PE and ZIP

The effects of assay temperature on the purified enzyme activities were investigated as outlined in section 2 9 2 and the results are presented in Figures 3 9 2 1 and 3 9 2 2 It should be noted that the method outlined in section 2 9.2 included the preincubation of enzyme and substrate for 10 minutes at each temperature tested prior to substrate addition This protocol, although allowing for the possibility of heat denaturation to occur before the assay was initiated, was necessary to avoid results inconsistent with the actual ability of enzyme to tolerate and catalyse substrate hydrolysis at the temperatures under investigation Failure to preincubate the enzyme and substrate, resulting in a lack of thermal equilibrium, would lead to observed results that were representative of the rate of thermal equilibration and the rate of thermal inactivation, particularly at the higher temperatures which would have proven difficult, if not impossible, to interpret correctly The limitations of this experimental design lie in the fact that results obtained relate specifically to the method employed and may not be extended to form further hypotheses on the thermal stability of the enzymes under investigation This method does, however, provide useful information into the difference between the purified PE and ZIP activities In Figure 3 9.2 1 the narrow temperature optimum of PE is illustrated This profile is centred narrowly about 37°C It is obvious, therefore, that at the two lower temperatures of 4°C and 20°C that there was not enough free energy within the system to allow catalysis to occur The lack of free energy in the system caused by lower temperatures can theoretically affect the binding of enzyme to substrate, the catalysis of the reaction itself, the dissociation of enzyme and product(s) following catalysis, or combinations of all three of these factors The lack of activity at the lower temperatures is not stability related as PE was purified at 4°C and stored on ice prior to characterisation experiments No cold-labile characteristics were observed for PE At temperatures of 45°C and greater, it is probable that PE underwent heat denaturation leading to low or non-existent activities being observed.

ZIP demonstrated a quite different temperature profile, with an optimum assay temperature of 37 C - 45°C Inactivation does occur, however, at temperatures greater than 45°C The most interesting observation was the activity of ZIP at the lower temperatures At 4°C, purified ZIP hydrolysed substrate at a rate equivalent to 70% of the rate of hydrolysis at 37°C Although no further investigations were performed into this aspect of ZIP activity against Z-Gly-Pro-MCA, it is apparent that lower amounts of free energy are required by the enzyme to hydrolyse Z-Gly-Pro-MCA than those required for PE to catalyse the same reaction. Again, this lower energy requirement for the hydrolysis

of Z-Gly-Pro-MCA by ZIP could be related to substrate binding, catalysis of the reaction or release of the products formed

4 6 3 pH Effects on Purified Enzymes

Investigations into the effects of pH on the activity and stability of purified PE and ZIP were performed as described in section 2 9 3 1 and 2 9 3 2 with results being presented in Figures 3 9 3 1 - 3 9 3 4 Two distinct investigations were conducted on each purified enzyme The first, as described in section 2 9 3 1 , determined the pH and buffer system at which optimal enzyme activity could be observed The second study, conducted as outlined in section 2 9 3 2 , demonstrated the pH range over which the enzyme remained stable, retaining its ability to hydrolyse substrate This study and the results obtained from it were important for the design and implementation of further characterisation work, which may have involved inadvertent pH changes For example, in the functional reagent studies that will be discussed, some compounds used were only slightly soluble in neutral buffers and had to be prepared in highly basic or acidic solutions Preincubation of these compounds with purified enzyme activity may have resulted in observed inhibition that was caused solely, or in part, by the pH of the solution being tested and not the functional reagent itself The study outlined in section 2 9 3 2 set the pH limits that could be successfully used in characterising PE and ZIP activities

Figures 3 9 3 1 and 3 9 3 2 illustrate the pH optimum and pH inactivation profiles for PE respectively The criteria upon which the optimum pH for PE activity was determined was based on maximum activity observed Thus it was determined that the pH optimum of PE was pH 8 0 in a potassium phosphate buffer system On closer examination of the data presented in Figure 3 9 3 1 however, it was noted that in a Tris-HCl buffer system, the pH at which maximum PE activity was obtained was pH 8 5 Because potassium phosphate buffer could not be used at pH 8 5, the possibility exists that the pH optimum for PE is actually 8 5, but that the enzyme retains a preference for phosphate buffer Following the discussions where it was concluded that PE activity was not enhanced by the presence of NaCl, it is unlikely that this preference for phosphate buffer is related to the ionic strength of the buffer systems used In Figure 3 9 3 2 , the pH inactivation profile of PE is presented From this data it was determined that PE was inactivated outside the pH range 5 0 - 9 0 It was also observed that greater than 50% PE activity could be detected over the pH range 5 0 - 10 0 These results are only relevant and reliable under the conditions used to perform the study, i e preincubation at each pH for 15 minutes at 37°C and cannot be extended to longer times or different temperatures The results obtained from the investigation into pH effects on PE activity agree well with previously published information (Table 4 4)

Source	Molecular weight	Subunit structure	pH Optimum	pH Stability Range	Optimum Temperature (°C)	Reference
Agaricus bisporous	78,000	-	7 5	5 0 - 9 0	37	Sattar *et al* (1990)
Flavobacterium	74,000	Mono	7 0	5 0 - 9 0	40	Yoshimoto *et al* (1980)
E coli	75,000	Mono	9 0	-	-	Sommer (1993)
Shakashimeji	76,000	Mono	6 8	5 75 - 7 4	37	Yoshimoto *et al* (1988)
Rat brain	70,000	Mono	7 0 - 8 0	-	-	Rupnow *et al* (1979)
Rat brain	70,000	-	7 5 - 8 0	-	-	Andrews (1980)
Bovine brain	75,000	Mono	7 5	5 0 - 9 0	40	Yoshimoto *et al* (1983)
Bovine pituitary	76,000	-	7 4	6 0 - 8 2	40	Knisatschek *et al* (1979)
Lamb brain	77,000	Mono	7 0	5 5 - 9 0	45	Yoshimoto *et al* (1981)
Lamb kidney	115,000	Di	7 5 - 8 0	-	-	Koida *et al* (1976)
Porcine liver	72,000	Mono	6 5	6 0 - 7 0	-	Moriyama and Sasaki (1983)
Rat muscle	69,000	-	7 0 - 7 5	5 0 - 9 0	-	Daly *et al* (1985)
Rat skin	70,000	Mono	5 8	-	40	Kusuhara *et al* (1993)
Felsh fly	84,000	Mono	7 5	-	-	Ohtsuki *et al* (1994)
Bovine serum	**70,000**	-	**8 0**	**5 0 - 9 0**	**37**	**This Investigation**

Table 4 4 *Molecular weight and subunit make up of PE from various sources The molecular weights obtained for bovine serum PE agree well with the data presented here The dimeric lamb kidney PE was later corrected by Yoshimoto et al (1977) Result for bovine serum (in bold type) were obtained as part of this investigation*

The pH optimum and pH inactivation profiles for ZIP are presented in Figures 3 9 3 3 and 3 9 3 4 respectively The pH at which optimum ZIP activity was observed was 8 5, but in contrast to PE, no buffer preference was exhibited by ZIP activity The pH inactivation profile produced for ZIP indicates that ZIP activity was stable over a pH rage of 4 5 - 10 5 with greater than 50% of the observed activity being retained over the pH range of 3 5 - 10 0 Once more, it should be noted that these figures relate only to the method used to investigate these pH effects

4 6.4. Effects of Functional Reagents on Purified PE and ZIP Activities

The interaction between enzymes and various functional reagents can be used to determined the mechanistic class of the enzyme There are four mechanistic classes, serine, cysteine, metallo- and aspartic To understand how functional reagent studies help to identify the mechanistic class of a particular enzyme, a brief discussion on enzyme catalysis and the subtle differences between the catalytic activities of each mechanistic class is warranted This discussion will focus on the catalysis of peptide bond scission by proteases and peptidases

Peptide bond scission involves the following steps , (a) nucleophilic attack on the slightly electrophilic carbonyl carbon atom, (b) base catalysis to remove the proton from the attacking nucleophile, (c) electrophilic assistance which influences the carbonyl oxygen and (d) acid catalysis to facilitate the leaving of an amine The difference between the mechanistic classes of proteases lies, not in the steps themselves but, in the variety of groups in the active site that perform each of these functions Figure 4 5 illustrates these steps with regard to a serine protease

In the case of serine proteases, oxygen from the hydroxyl side chain of serine initiates the nucleophilic attack, while a neighbouring histidine residue stabilises this interaction A covalent enzyme substrate interaction producing a tetrahedral ester intermediate results Cysteine proteases also form a covalent intermediate The side chain sulphur of a cysteine residue is the attacking nucleophile, with a neighbouring histidine again stabilising the attack The metallo- and aspartic proteases do not initiate the nucleophilic attack upon a peptide bond using an active site functional group, and therefore do not form covalent intermediates Instead, the metal atom or pair of aspartic acid residues present in the active sites of these proteases co-ordinate the nucleophilic attack on the peptide bond by water molecules So it is clear that although there are common steps involved in the catalysis of peptide bond scission, each mechanistic class performs these steps in distinct ways It is this specific difference between the proteases that can be utilised to determine the mechanistic class to which each protease belongs via functional reagent studies (Dunn, 1989)

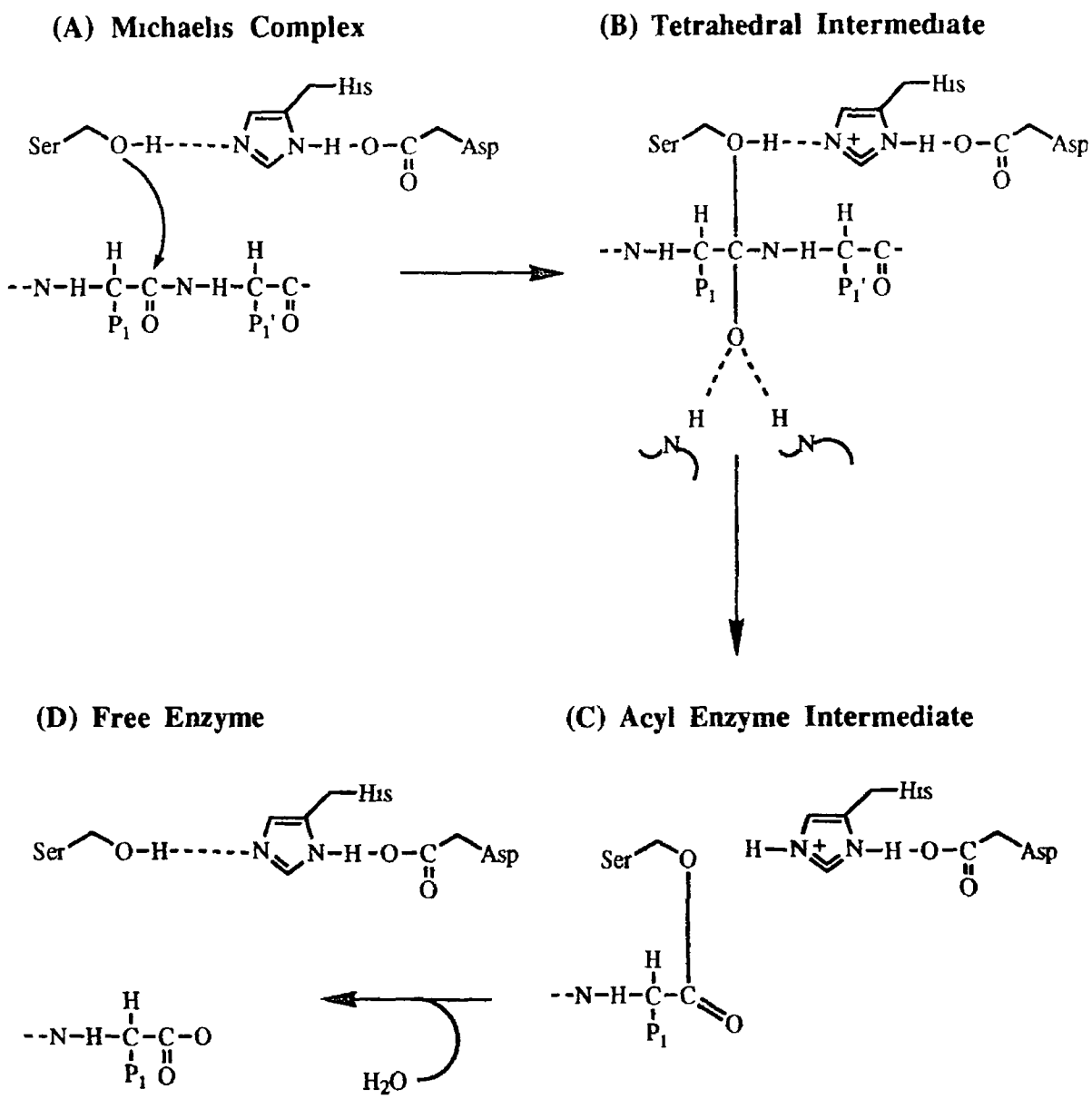


Figure 4 5 Schematic representation of the main steps involved in peptide bond scission *The figure illustrates the mechanism of serine protease catalysis (A) Following substrate binding and formation of the Michaelis Complex, oxygen, located on the side chain or serine, undergoes nucleophilic attack on the carbonyl carbon of the substrate peptide bond. (B) A tetrahedral intermediate is formed The intermediate is stabilised by the removal of a proton from the attacking oxygen by histidine and by electrophilic assistance offered by hydrogen atoms exposed to the enzyme substrate complex. (C) Formation of the Acyl Enzyme Intermediate results in the leaving of the P_1' amine (D) Release of the bound P_1 product via acid catalysis using water, results in return of free enzyme*

4 6 4 1. Functional Reagent Studies on PE.

Investigations were carried out as described in section 2 9 4 and the results illustrating the effect of functional reagents on purified PE, activity are presented in Table 3 4 Those results that are significant with regards to the mechanistic classification of PE are as follows

4.6.4 1.1 Enhanced PE Activity in the Presence of DTT

The activation of purified PE by increasing concentrations of DTT in substrate have been discussed (section 4 5) In Table 3 5 PE demonstrated a 10 fold increase in observed activity in the presence of 10mM DTT This DTT enhanced activity has being reported previously and the values presented in Table 3 5 agree well with published results (Kato *et al*, 1980, Orlowski *et al*, 1979, Walter, 1976) This DTT enhanced activity is normally indicative of a cysteine protease for reasons which were discussed previously An interesting point, with regards to the values presented in Table 3 4 for DTT activation of PE activity, is that at low DTT concentrations (1mM), the activation of DTT is significantly lower than that obtained when DTT was supplied to the assay mixture by substrate alone (Figure 3 8 5) This seems to suggest that the preincubation of enzyme with DTT leads to less enhanced activity than reduction of the substrate with DTT prior to assay initiation

4.6 4.1.2. Inhibition of PE by Cysteine Protease Inhibitors

Purified PE was inhibited by iodoacetamide (10mM), NEM (10mM) and PCMB (5mM) by 20%, 40% and 70% respectively Of these three compounds PCMB is the most effective and specific cysteine protease inhibitor However, all three compounds may interact with cysteine residues that are not located in the active site of an enzyme (Andrews *et al*, 1980) The inhibition of PE by cysteine protease inhibitors has been widely reported, the most effective inhibition being observed with PCMB (Daly *et al*, 1985, Mizuitani *et al*, 1984, Rosen *et al*, 1991, Taylor and Dixon, 1976) Andrews *et al*, (1980), having determined that PE was a serine protease, proposed that the inhibition of PE by cysteine protease inhibitors was the result of the interaction of these compounds with a non-catalytic cysteine residue located near the active site This has been previously reported for other serine proteases (Bai and Hayashi, 1979) The results presented here agree well with results obtained by Polgar, (1991) He related the size of the compounds interacting with the cysteine residue near the active site of PE with their ability to inhibit the enzymes activity Smaller thiol reagents, such as iodoacetamide, were less likely to exert stearic effects on the binding of substrate to the active site and therefore were less likely to significantly inhibit the enzyme Larger thiol reagents such as NEM, were of sufficient size to interfere with the binding of substrate to enzyme and therefore exerted a stronger inhibitory effect. This increase of inhibitory activity with relationship to thiol reagent size was consistent with the highest levels of inhibition being exerted by PCMB

4.6.4.1.3. Inhibition of Purified PE by Serine Protease Inhibitors

Purified PE was significantly inhibited by AEBSF (80% inhibition at 10mM) and to a lesser extent by PMSF AEBSF is a very specific serine protease inhibitor and its inhibition of PE is indicative that PE

is a serine protease PE has been confirmed as a serine protease by many authors (Kusuhara *et al*, 1993, Strohmeier *et al*, 1994, Yoshimoto *et al*, 1983), however no reports of the use of AEBSF have been published AEBSF was chosen as it represented a non-toxic but extremely potent alternative to DFP

4 6 4 1 4 Inhibition of PE by Phenanthrolines

PE was inhibited by 10mM 1,10-, 4,7- and 1,7-phenanthroline by 50%, 50% and 60% respectively The inhibition of proteases by the chelator, 1,10-phenanthroline, is normally indicative of a metallo-protease However, EDTA and 8-hydroxyquinoline did not inhibit PE activity significantly This, coupled with the fact that 1,7- and 4,7-phenanthroline are not chelators, indicates that the inhibition of PE by phenathrolines was not due to metal chelation, but rather, it was due to some structural similarity between these compounds Czekay and Bauer, (1993) reported that pyroglutamyl aminopeptidase type II (PAP II) purified from rat brain was a metallo-protease, but was also inhibited by 4,7- and 1,7-phenanthroline They proposed that the inhibition of this enzyme by the phenanthrolines was probably due to the non-specific hydrophobic interaction of the enzyme with the aromatic structures shared by these compounds They supported this observation with the fact that PAP II interacted strongly with phenyl sepharose hydrophobic interaction chromatography media. This explanation, therefore, could be applied to the effect of the phenanthrolines towards PE activity, as it also interacts strongly with phenyl sepharose (Figure 3 6 2)

4 6.4.2. Functional Reagent Studies on ZIP

The results illustrating the effect of functional reagents on purified ZIP activity are presented in Table 3 5 and the investigations were carried out as described in section 2 9 4 Those results that are significant with regards to the mechanistic classification of ZIP are as follows

4 6.4 2.1. Inhibition of ZIP by Cysteine Protease Inhibitors

Purified ZIP was inhibited by PCMB (5mM) and NEM (10mM) by 55% and 30% respectively Unlike PE, there was no significant inhibition obtained with the other cysteine protease inhibitors Of these two compounds PCMB is the most effective However, as with PE, these two compounds may interact with cysteine residues that are not located in the active site of an enzyme and ZIP may not be a cysteine protease This is supported by the fact that ZIP activity was not enhanced by the presence of reducing agents such as DTT or 2-mercaptoethanol (Table 3.5) The steric effect discussed earlier with regards to the inhibition of PE by PCMB and NEM may also apply to ZIP

4 6.4.2.2. Inhibition of Purified ZIP by Serine Protease Inhibitors

Purified ZIP was significantly inhibited by AEBSF (85% inhibition at 10mM) and to a lesser extent by PMSF (45% inhibition at 1mM) AEBSF has already been discussed as a specific non-toxic inhibitor of serine proteases Its inhibition of ZIP is therefore indicative that ZIP is a serine protease

4 6 4.2 3 Inhibition of ZIP by Phenanthrolines

Like PE, ZIP was inhibited by 10mM 1,7- and 4,7-phenanthroline by 70% and 80% respectively This inhibition is probably best explained by the same process whereby the phenanthrolines inhibited PE activity (Czekay and Bauer, 1993) The supporting argument made by these authors that the interaction of an enzyme with the aromatic structures of the phenanthroline compounds was hydrophobic in nature, is also supported by the interaction of ZIP with phenyl sepharose (Figure 3 6 5) It is interesting to note that ZIP interacted with phenyl sepharose in a stronger manner than PE, demonstrated by the need to use distilled water to elute the enzyme from the column The increased capacity of ZIP for hydrophobic interaction may be responsible for the greater inhibition demonstrated by ZIP when exposed to the phenanthrolines

4.6 5. The Effect of Divalent Metal Salts on Purified PE and ZIP Activities

The effect of divalent metal salts on purified PE and ZIP activities was determined as described in section 2 9 5 and the results are presented in Table 3 6 Of significance is the fact that both PE and ZIP activities were inhibited strongly by $HgSO_4$ (approximately 90% inhibition observed for both enzymes) Inactivation by heavy metal poisoning is well documented (Vallee and Ulmer, 1972) Heavy metal cations such as mercury (Hg^{2+}) are known to react with protein sulphydryl groups, converting them to mercaptides, as well as histidine and tryptophan residues In addition, disulphide bonds can be hydrolytically degraded by the catalytic action of mercury (Volkin and Klibanov, 1990) This supports the evidence presented thus far, that interaction with cysteine residues or with sulphydryl groups of both PE and ZIP leads to an inhibition of both enzymes The inhibition of PE by Hg^{2+} is well documented in the literature (Yoshimoto *et al*, 1981, Strohmeier *et al.*, 1994, Sattar *et al*, 1990, Mizutani *et al.*, 1984) Likewise the inhibition presented for Zn^{2+} is also in agreement with previous reports (Yoshimoto *et al.*, 1988, Kato *et al*, 1980; Kalwant and Porter, 1991) However, these authors also reported significant inhibition of PE by Cu^{2+} and Ni^{2+} which was not observed in this work ZIP activity, although strongly inhibited by only Hg^{2+}, demonstrated a greater sensitivity to inhibition by divalent metal salts that PE (Table 3 6)

4 6.6. Substrate Specificity Studies on Purified PE and ZIP

Substrate specificity studies were carried out as outlined in sections 2 9 6 and 2 9 7 and the results are presented in section 3.9.6 Three methods of investigation were employed to determine the substrate specificity of both PE and ZIP Reverse Phase HPLC focused on detecting cleavage products produced when various peptides were incubated with PE or ZIP Fluorimetric substrates were used to determine the ability of both enzymes in releasing MCA from the carboxyl terminus of a variety of peptides Kinetic analysis of the effect of proline containing peptides on the release of MCA from Z-Gly-Pro-MCA by purified PE and ZIP activities was also investigated The relevance of results obtained from these investigations will be discussed with regards to PE and ZIP substrate specificity

4.6 6 1. Substrate Specificity Studies on Purified PE

Table 3 8 presents the results obtained when PE was incubated with various fluorimetric substrates to determine its ability to release free MCA from these peptides This not only helped to elucidate the substrate specificity of PE but also indicated whether the purified PE preparation may have contained secondary peptidase contaminants PE exhibited activity against the fluorimetric TRH analogue, pGlu-His-Pro-MCA (TRH-MCA) and Suc-Gly-Pro-Leu-Gly-Pro-MCA The release of MCA from TRH-MCA poses interesting questions with regards to the specificity of PE towards TRH and its analogues PE cleaved this substrate at approximately 60% the rate at which it cleaved Z-Gly-Pro-MCA (Table 3 8), yet the cleavage of the natural peptide, pGlu-His-Pro-NH_2, was not detected using Reverse Phase HPLC as illustrated in Figures 3 9 6 3 and 3 9 6 4 The product expected following the deamidation of TRH by PE is TRH-OH Figure 3 9 6 3 illustrated clearly that the HPLC method employed could distinguish easily between TRH and TRH-OH It is likely that the failure to detect TRH-OH following incubation of TRH with PE is not because TRH was not cleaved by PE, but that the HPLC method was not sensitive enough to detect any TRH-OH that was produced The K_m determined for PE activity towards TRH-MCA was 69μM (Table 3 9), however the competitive action of TRH on PE activity towards Z-Gly-Pro-MCA produced a K_i of 680μM (Table 3 10), illustrating a 10 fold decrease in specificity This K_i value suggests that PE might not play an important physiological role with regard to TRH The results presented in Table 3 8 indicate that there is no major contaminating peptidase activity in the purified PE preparation as no significant cleavage of substrates other than TRH-MCA and Suc-Gly-Pro-Leu-Gly-Pro-MCA was observed Reverse Phase HPLC investigations showed that PE hydrolysed the bioactive peptides LHRH (Figure 3 9 6 1), Bradykinin (Figure 3 9 6 5), Substance P (Figure 3 9 6 7) and Angiotensin II (Figure 3 9 6 9) The cleavage of these substrates by PE has been previously reported (Bai, 1994, Barelli *et al.*, 1989, Blumberg *et al* , 1980; Chappell *et al.*, 1990, Emson and Williams, 1983) Results obtained from kinetic analysis of the interaction of PE towards these peptides are presented in Table 3 10 PE exhibits a low specificity towards LHRH (K_i of 523μM) similar to the figure obtained for TRH However a higher specificity towards Bradykinin (K_i of 136μM) and Angiotensin II (K_i of 113μM) was observed These specificities approximate to the specificity of PE towards its fluorimetric substrate, Z-Gly-Pro-MCA (K_m of 94μM)

4 6 6 2. Substrate Specificity Studies on Purified ZIP

ZIP exhibited significant activity against the fluorimetric substrate Pro-MCA, the substrate commonly used to detect proline aminopeptidase activity However, ZIP activity against Z-Gly-Pro-MCA cannot be explained by the action of an aminopeptidase activity and therefore the cleavage of Pro-MCA by purified ZIP preparation could represent a contaminating peptidase activity Reverse Phase HPLC investigations demonstrated that ZIP cleaved LHRH, Bradykinin and Substance P The cleavage products obtained from the action of ZIP towards LHRH and Bradykinin indicate that PE and ZIP may share a common cleavage site on each bioactive peptide In both cases, the fragment obtained following incubation of LHRH and Bradykinin with ZIP, corresponded to the major fragment observed when these substrates were incubated with purified PE (Figures 3 9 6 1 , 3 9 6 2 , 3 9 6 5 and 3 9 6 6)

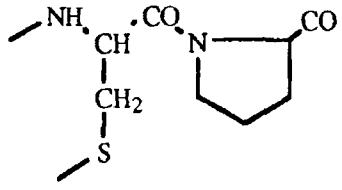
Kinetic analysis of these interactions (Table 3 10) indicate that ZIP exhibits a similar specificity towards LHRH as does PE (K_i of 475μM) but its specificity towards Bradykinin is approximately 20 fold lower (K_i of 2497μM)

4 6 7 The Effects of Proline Specific Peptidase Inhibitors on Purified PE and ZIP Activities

The investigation into the effect of proline specific inhibitors was carried out as outlined in section 2 9 8 and the results are presented in section 3 9 8 Having first discovered ZIP activity in bovine serum and identified its resistance to Z-Pro-Prolinal inhibition (Figures 3 4 2 and 3 4 3) other PE and proline specific peptidase inhibitors were sought from various international groups This list of inhibitors is presented in Table 2 6 The investigation carried out using these inhibitors was simple in nature and was implemented solely to determine the similarities or differences obtained when either ZIP or PE activities were determined in the presence of these compounds IC_{50} values were calculated to illustrate the differences in sensitivity of both enzymes to inhibition by these compounds, and are presented in Table 3 11 All compounds with the exception of kelatorphan inhibited PE activity The least effective of these was Boc-Glu(NHO-Bz)-Pyrr with an IC_{50} value of $1\ 6x10^{-7}M$ This inhibitor was synthesised by Demuth *et al* , (1993) as part of a series of amino dicarboxylic acid pyrrolidide inhibitors designed to inhibit proline specific peptidases such as PE and DAP IV Figure 4 6 (a) shows the structural similarity between the inhibitor and the Cys-Pro sequence of residues found in Oxytocin and Vasopressin, both natural substrates of PE The most effective inhibitors based on their very low IC_{50} values were α-Ketobenzothiazole and Z-Indolinyl Prolinal The former was obtained from Tsutsumi *et al* , (1994) and its structure is presented in Figure 4 6 (b) It was synthesised as part of a series of inhibitors produced to inhibit PE activity specifically Tsutsumi *et al.* reported an IC_{50} value of $5\ 0x10^{-9}M$ for the action of this inhibitor against PE activity, in comparison to the value presented in Table 3 11 of $4x10^{-11}M$ Bovine serum PE was therefore 100 times more sensitive to the inhibitory action of α-Ketobenzothiazole than that of the partially purified porcine kidney PE used by Tsutsumi *et al* Z-Indolinyl-Prolinal, supplied by Dr Steven Faraci (Bakker *et al*, 1991) demonstrated an IC_{50} value of $5\ 0x10^{-11}M$ against PE activity The structure of this compound is illustrated in Fig 4 6 (c) Bakker *et al* described this compound as a slow tight binding inhibitor of PE activity

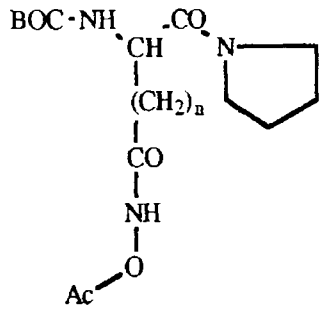
ZIP activity was resistant to inhibition by Z-Pro-Prolinal, Foc-Pro-Pro-Nitrile, Kelatorphan and Z-Phe-Pro-Methylketone With the exception of kelatorphan, all of these inhibitors are derivatives of Z-Pro-Prolinal ZIP was inhibited to some extent by Z-Indolinyl-Prolinal and Boc-Glu-(NHO-Bz)-Pyrr with IC_{50} values of approximately $1\ 0x10^{-4}M$ Also of interest was that Z-Thiopropyl-Thioprolinal, a derivative of Z-Pro-Prolinal (Tsuru *et al.*, 1988) was a potent inhibitor of ZIP activity ($IC_{50} = 1\ 8x10^{-8}M$) The most potent inhibitory activity observed against ZIP was that of α-Ketobenzothiazole, which was also the most potent PE inhibitor tested The IC_{50} values observed for α-Ketobenzothiazole inhibition of PE and ZIP were comparable at $4\ 1x10^{-11}M$ and $1\ 5x10^{-11}M$ respectively

Oxytocin, Vasopressin

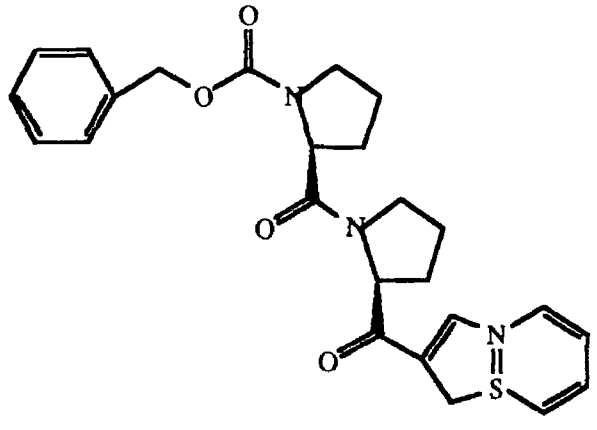


Cys-Pro

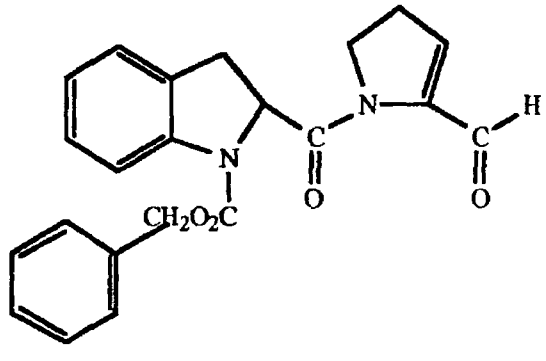
Boc-Glu(NHO-Bz)-Pyrr



(a)



(b) α-Ketobenzothiazole



(c) Z-Indolinyl-Prolinal

Figure 4 6. *(a) Structure of BOC-Glu(NHO-Bz)-Pyrr and its relationship to the Cys-Pro bond found in oxytocin and vasopressin, natural substrates for PE (b) Structure of the PE inhibitor, α-Ketobenzothiazole This inhibitor was the most potent inhibitor tested for both PE and ZIP activities (c) Structure of Z-Indolinyl-Prolinal, a very potent inhibitor of PE activity*

4 7. General Summary

Two distinct proline specific peptidases were detected in bovine serum using the substrate Z-Gly-Pro-MCA, a reportedly specific fluorimetric substrate for prolyl endopeptidase (PE) One of these activities was inhibited by Z-Pro-Prolinal, a PE specific inhibitor, and was subsequently designated PE The second activity resisted inhibition by Z-Pro-Prolinal, even at high concentrations and increased preincubation times This activity was subsequently designated Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP)

Both PE and ZIP activities in bovine serum were successfully separated using SP-Sepharose cation exchange chromatography and were subsequently purified independently of each other

4.7.1. PE - Summary and Conclusions

PE activity was partially purified, following its separation from ZIP, using Phenyl-Sepharose hydrophobic interaction, DEAE-Sepharose anion exchange and Sephacryl S-200 HR gel filtration chromatographies, with a final yield of 24% and a final purification factor of 30 achieved The enzyme had a native molecular weight of 70,000 Da, as determined by gel filtration chromatography The subunit structure of the enzyme could not be determined by SDS PAGE due to the appearance of multiple bands following visualisation of the gel by silver staining

A pH optimum of 8 0, with a preference for phosphate buffer was determined for the partially purified PE. The enzyme was stable over a pH range of 5-9 Optimal activity was obtained from PE at a temperature of 37°C with little activity being detected above or below this temperature

PE activity was inhibited by AEBSF indicating that the enzyme may be a member of the serine protease family The enzyme was also inhibited by PCMB and activated by DTT, indicating the possible presence of an essential cysteine residue close to the active site

PE hydrolysed the substrates Z-Gly-Pro-MCA and pGlu-His-Pro-MCA, with K_m values of 94μM and 62μM respectively The enzyme also cleaved a variety of proline containing bioactive peptides including LHRH, bradykinin, substance P and angiotensin II These peptides also competitively inhibited PE activity towards Z-Gly-Pro-MCA PE demonstrated relatively high specificities towards bradykinin and angiotensin II with K_I values of 136μM and 113μM respectively

The enzyme was inhibited by a range of PE specific inhibitors with the highest inhibitory activity being observed for α-ketobenzothiazole (IC_{50} = 41picomolar) and Z-Indolinyl Prolinal (IC_{50} = 45 picomolar)

In conclusion, bovine serum PE activity, purified and characterised during this investigation, has revealed itself to be similar to PE activities isolated from other sources with regard to its biophysical

and biochemical characteristics, and its substrate specificity However, based on its poorly investigated activity towards proline containing bioactive peptides, this serum form of PE may play an important physiological role in the metabolism of such peptides

4 7.2 ZIP - Summary and Conclusions

Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) activity was purified, following its separation from PE, using Phenyl-Sepharose hydrophobic interaction , Calcium Phosphate Cellulose and Sephacryl S-200 HR gel filtration chromatographies, with a final yield of 14% and a final purification factor of 2250 achieved The enzyme had a native molecular weight of 185,000 Da, as determined by gel filtration chromatography The subunit structure of ZIP was determined to be tetrameric based on the identification of a major band of 50,000Da by SDS PAGE following visualisation of the gel by silver staining

The enzyme exhibited a pH optimum of 8 5, and was stable over a pH range of 4-9.5 Optimal activity was obtained from ZIP at a temperature of 37°C- 40 C with significant activities being observed at 4°C and 20°C

The enzyme was inhibited by AEBSF indicating that it is probably a member of the serine protease family

ZIP hydrolysed the substrate Z-Gly-Pro-MCA with a K_m value of 267μM being determined. The enzyme also cleaved a variety of proline containing bioactive peptides including LHRH, bradykinin and substance P These peptides also inhibited ZIP activity towards Z-Gly-Pro-MCA with bradykinin and LHRH demonstrating competitive K_I values of 2 5mM and 475μM respectively

The enzyme was inhibited by some of the PE specific inhibitors tested Highest inhibitory activity was observed for α-ketobenzothiazole (IC_{50} = 15 picomolar)

The investigation of ZIP activity in bovine serum leads to the following conclusions.

(1) The fluorimetric assay used to assess PE activity in serum, using the substrate Z-Gly-Pro-MCA, is not valid unless the activity observed can be completely inhibited by Z-Pro-Prolinal.

(2) ZIP activity, isolated and characterised from bovine serum, is proline specific based on its inhibition by PE specific inhibitors, particularly those that are based on a proline dipeptide structure, and its ability to release free MCA from the fluorimetric substrate, Z-Gly-Pro-MCA.

(3) The biophysical and biochemical characteristics, determined for ZIP during this investigation, are dissimilar to those of any individual proline specific peptidase, previously characterised.

(4) The ability of ZIP to release free MCA from the substrate Z-Gly-Pro-MCA indicates that it might be classified as either an endopeptidase or carboxypeptidase activity However, the enzymes ability to cleave LHRH, bradykinin and substance P indicate that it is a proline specific endopeptidase To date, the only reported proline specific endopeptidase activity matching these substrate specificity requirements has been prolyl endopeptidase (PE) This investigation has determined that ZIP activity in bovine serum is distinct from PE

Therefore, ZIP activity may represent a novel proline specific endopeptidase localised in serum and may, like PE, play an important physiological role in the metabolism of proline containing bioactive peptides

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Appendices

A 1. Kinetic Analysis

Data obtained from relevant kinetic investigations were subjected to analysis based on Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and Direct Linear Plot models

A.1.1 Michaelis-Menten Analysis

The rate equation for the Michaelis-Menten model of enzyme catalysed reaction is as follows

$$V_0 = V_{max}[S] / (K_m + [S])$$

where V_0 = initial velocity, V_{max} = maximal velocity, [S] = substrate concentration and K_m is the Michaelis-Menten rate constant.

A.1.2. Lineweaver-Burk Analysis

A double reciprocal transformation of the Michaelis-Menten rate equation leads to the following

$$1/V_0 = K_m/V_{max}[S] + 1/V_{max}$$

A plot of $1/V_0$ versus 1/[S] produces a straight line The slope of this line represents K_m/V_{max}, the Y-axis intercept represents $1/V_{max}$ and the X-axis intercept represents $-1/K_m$

A.1.3. Eadie-Hofstee Analysis

This rate equation represents a derivation of the Michaelis-Menten rate equation and is as follows.

$$V_0/[S] = V_{max}/K_m - V_0/K_m$$

A plot of $V_0[S]$ verus V_0 produces a straight line The slope of the line represents $-1/K_m$, the Y-axis intercept represents V_{max}/K_m and the X-axis intercept represents V_{max}

A 1 4. Hanes-Woolf Analysis

This rate equation also represents a derivation of the Michaelis-Menten rate equation and is as follows.

$$[S]/V_0 = K_m/V_{max} + [S]/V_{max}$$

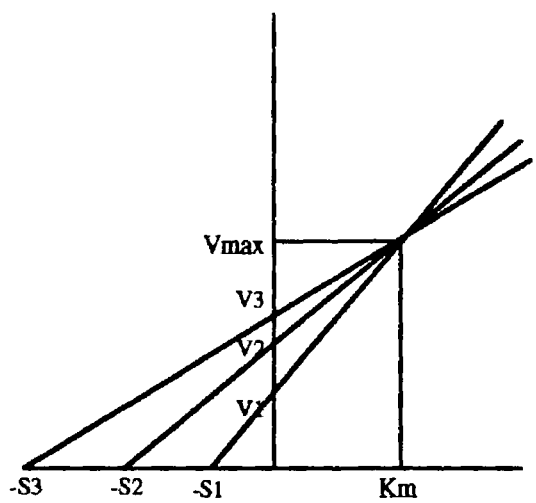
Plotting $[S]/V_0$ versus [S] produces a straight line The slope of the line represents $1/V_{max}$, the Y-axis intercept represents K_m/V_{max} and the X-axis intercept represents $-K_m$

Data obtained from kinetic studies was entered into a SigmaPlot worksheet. The data was transformed to produce the relevant X and Y axis cordinates applicable to each of the four previously mentioned

models Curve fitting protocols, based on the four models, were created and run to determine Km values for the data.

A 1 5. Direct Linear Plot Analysis

This method is based on plotting substrate concentration and initial velocity values as lines in in parameter space rather than as points in observation space Where as in the previously mentioned models, substrate concentration and initial velocity values were used to place a point in observation space at particular X, Y coordinates, in this model a line is constructed by joining a point at a distance of -[S] from the origin on the X-axis to a point at a distance V_O from the origin on the Y-axis and extending that line into positive X and Y space Plotting multiple lines using multiple substrate concentration and initial velocity combinations will lead ideally to a unique intersection point, whose X and Y coordinates represent K_m and V_{max} respectively



In practise, many intersection points are obtained and the K_m and V_{max} parameters are represented by the median values Beacuse the median value of the list of possible determinations is used, the Direct Linear Plot is less sensitive to the effects of outliers, making it statistically better than the previous models A simple BASIC program was created to take data obtained from kinetic investigations and calculate the intesection point of lines created as described, and to determine the median values

A.2. Statistical Analysis

Standard deviations were used to express error bar amplitudes on all plots The mathematics used to produce the standard deviation (s d) from triplicate determinations were as follows.

$$\text{Mean} = (a + b + c)/3$$
$$\text{Variance} = (a^2 + b^2 + c^2)/3 - \text{Mean}^2$$
$$\text{s d} = \text{Variance}^{1/2}$$

Where a, b, and c represent the three individual determinations