THE PURIFICATION AND CHARACTERISATION OF A Z-PRO-PROLINAL INSENSITIVE Z-GLY-PRO-MCA DEGRADING PEPTIDASE FROM BOVINE SERUM

Thesis Submitted for the Degree of

Doctor of Philosophy

by

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In Loving Memory of my Grandparents

DECLARATION

I, hereby certify that the 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.

Signed Yvonne A. Birney **Date** 17th February 2000

Yvonne A. Birney

"He who knows not and knows not that he knows not-he is a fool,

ignore him

He who knows not and knows that he knows not-he can be taught,

teach him

He who knows and knows that he knows not all-he is wise,

follow him "

Anonymous

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ABSTRACT

*The study of a novel proline-specific peptidase from bovine serum is presented The enzyme readily cleaves Z-Gly-Pro-MCA liberating the fluorophore MCA thus allowing quantification of enzyme activity Unlike prolyl oligopeptidase (PO) which also hydrolyses this fluorogenic substrate, this peptidase is completely insensitive to Z-Pro-Prolinal and has been designated **Z**-Pro-Prolinal **I**nsensitive Z-Gly-Pro-MCA degrading **P**eptidase (**ZIP**)*

The two peptidases were successfully separated from each other by phenyl sepharose hydrophobic interaction chromatography and the subsequent purification focused on the isolation of ZIP from bovine serum In addition to phenyl sepharose, calcium phosphate cellulose and DEAE anion exchange chromatography were employed in the purification, with an overall enzyme yield of 33% and a purification factor of 4023 SDS PAGE and size-exclusion chromatography indicated a heterodimeric structure with a relative molecular mass of 180kDa

The enzyme remained stable at temperatures less than 50°C for up to an hour, while optimal activity was observed at 37°C ZIP was surprisingly stable over the pH range 2 5-10 0 Optimal activity was detected in the range pH 7 4-8 0 Isoelectric focusing revealed a pI value of 5 68 and the enzyme appears to be 33% glycosylated

Inhibition by AEBSF suggests the peptidase may be a serine protease and ZIP possibly contains a cysteine residue near the active site α_2M failed to inhibit activity suggesting an oligopeptidase specificity HPLC analysis revealed a broad substrate specificity for proline-containing peptides Kinetic analysis indicated that ZIP had a greater affinity for Z-Gly-Pro-MCA than PO with a K_M of 54μM deduced The oligopeptidase showed complete insensitivity to a number of PO-specific inhibitors, namely JTP-4819 and S-17092-1

ZIP exhibits similar biophysical characteristics to PO isolated from a number of sources However the peptidases do appear to be distinct and ZIP may represent a novel proline-specific serum peptidase, which may play a role in the degradation of oligopeptides

GENERAL ABBREVIATIONS

ψ	Indicates the peptide bond has been modified by the group in parenthesis immediately following
α_2M	α_2-macroglobulin
βNa	β-naphthylamide
$(NH_4)_2SO_4$	Ammonium sulphate
α-sAPP	Soluble amyloid precursor protein
2-NNAp	β-naphthylamide
Aβ	Amyloid β-protein
A_{280}	Absorbance at 280nm
AA	Amino acid
ACN	Acetone
AD	Alzheimer's disease
ADH	Alcohol dehydrogenase
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
APMSF	4-amidino-phenyl methane-sulphonyl fluoride
APP	Amyloid Precursor Protein
AVP	Arginine vasopressin
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
Bz	Benzoyl
Ca	Calcium
CALLA	Common acute lymphoblastic leukemia antigen
CAPS	3[cyclohexylamino]-1-propanesulphonic acid
Cd	Cadmium
CDTA	1,2-cyclo hexanediaminetetraaectic acid
Cl^-	Chloride ion
CMK	Chloromethylketone

CN	2-nitrile
Co	Cobalt
Conc	Concentration
CPC	Calcium cellulose phosphate
Cpp	*N*-[1-(RS)-carboxyl-3-phenylpropyl]
Cu	Copper
DEAE	Diethylaminoethyl
DFP	Diisopropyl fluorophosphate
DFP	Diisopropyl fluorophosphate
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNP	2,4-dinitrophenyl
DPP II	Dipeptidyl aminopeptidase II
DPP IV	Dipeptidyl aminopeptidase IV
DTNB	5,5-dithio-bis2-nitrobenzoic acid
DTT	Dithiothreitol
DXN	Dioxane
ed	Editors
EDTA	Ethylenediaminetetra acetic acid
EGTA	[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EH	Eadie-Hofstee
EtOH	Ethanol
Fmoc-	9-fluorenylmethyloxycarbonyl
FPLC	Fast protein liquid chromatography
GPL	Gly-Pro-Leu
H	Homogenate
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HEXXH	His-Glu-Xaa-Xaa-His

Hg	Mercury
HIC	Hydrophobic interactions chromatography
HIV-1	Human Immunodeficiency Virus 1
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HW	Hanes-Woolf
I	Iodine
IC_{50}	Concentration at which enzyme is 50% inhibited
IEF	Isoelectric focusing
IgE-pF	Immunoglobulin E potentiating factor
IgE-sF	Immunoglobulin E stimulating factor
JTP-4819	(S)-2-[[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-[phenylmethyl]-1-pyrrolidinecar-boxamide
K_2HPO_4	Potassium phosphate base
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate acid
K_i	Inhibition constant
KI	Potassium iodide
K_M	Michaelis constant
LB	Lineweaver-Burk
LHRH	Luteinising hormone-releasing hormone
Log	Logarithm
M W	Molecular weight
Mca	(7-methoxy-coumarin-4-yl)acetyl
MCA	7-amino-4-methyl coumarin
MCC	7-methoxycoumarin-3-carboxylyl
MeOH	Methanol
Meq	7-amino-4-methyl-2-quinoline
MES	2-[N-Morpholino]ethane-sulphonic acid
Mg	Magnesium

MM	Michaelis-Menten
Mn	Manganese
MOP	Mitochondrial oligopeptidase
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ND	Not determined
NEM	N-ethyl maleimide
Ni	Nickle
OH	Hydroxy
P	Pellet
P_n	Substrate subsite located n positions from the scissile bond on the N-terminal side
P_n'	Substrate subsite located n positions from the scissile bond on the C-terminal side
pAb	p-aminobenzoate
PAGE	Polyacrylamide gel electrophoresis
PAPI	Pyroglutamyl peptidase I
PBE	Polybuffer exchanger
PCMB	*p*-chloromercuribenzoate
PE	Perkin Elmer
PEG	Polyethylene glycol
pH	log of the reciprocal of the hydrogen ion concentration
pI	Isoelectric point
pK_a	Negative log of the equilibrium constant for an acid
PMSF	Phenylmethylsulphonylfluoride
PO	Prolyl oligopeptidase
PS	Phenyl sepharose
PVDF	Polyvinylidene difluoride
Pz	Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg

QF34	Mca-Gly-Gly –Phe-Leu-Arg-Arg-Ala-Lys(Dnp)
QF37	Mca-Gly-Gly –Phe-Ile-Arg-Arg-Ala-Lys(Dnp)NH_2
QFO1	Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys
QFO2	Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp)NH_2
R^2	Regression coefficient
RCO	Acyl
R_f	Relative mobility
RP	Reverse phase
Rpm	Revolutions per minute
S	Sulphur
S1	First supernatant
S2	Second supernatant
S9	Prolyl oligopeptidase family of serine proteases
SDS	Sodium dodecyl sulphate (Lauryl sulphate)
SE	Standard error
SEC	Size exclusion chromatography
SH	Thiol group
S_n	Enzyme subsite positioned n places from the active site on the N-terminal side
S_n'	Enzyme subsite positioned n places from the active site on the N-terminal side
SO_4	Sulphate
S-S	Disulphide Bridges
Suc-	Succinyl-
TEMED	N, N, N, N'-tetramethyl ethylenediamine
TFA	Trifluoroacetic acid
TLCK	Tosyl-L-lysylchloromethane
TOP	Thimet oligopeptidase
TRH	Thyrotropin-releasing hormone
Tris	Tris (hydroxymethyl) amino methane

UV	Ultraviolet
v/v	Volume per volume
V_e	Elution volume
V_o	Void volume
w r t	With respect to
Xaa	Any amino acid
Yaa	Any amino acid
Z-	*N*-benzyloxycarbonyl
Zaa	Any amino acid
ZIP	Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase
Zn	Zinc
ZnCl	Zinc chloride

UNITS

A	Amp
Da	Dalton
g	Gram
hr	Hour
k	Kilo
L	Litre
m	Metre
M	Molar
min	Minute
N	Normal
sec	Second

PREFIXES

c	centi ($1x10^{2}$)
m	milli ($1x10^{3}$)
μ	micro ($1x10^{6}$)
n	nano ($1x10^{9}$)
p	pico ($1x10^{12}$)

AMINO ACID ABBREVIATIONS

Ala	A	Alanine
Arg	R	Arginine
Asn	D	Asparagine
Asp	N	Aspartic acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylanaline
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

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1.0. INTRODUCTION

1 0 INTRODUCTION - OLIGOPEPTIDASES

1 1 BACKGROUND

The term oligopeptidase was once assigned to *any* peptide hydrolase, which appeared to act on oligopeptides yet showed no activity towards proteins However this vague terminology was soon disregarded on the discovery that many of these 'oligopeptidases' were in fact exopeptidases whose inactivity towards proteins was due to a low molar concentration of susceptible terminal groups available as substrate (Barrett and Rawlings, 1992) As recommended by the *Nomenclature Committee* (1992), an oligopeptidase is now defined as an endo acting oligopeptide hydrolase and no longer includes the exopeptidases

The earliest recognition of an oligopeptidase as a distinct sub-group of the endopeptidases was in 1979, by Camargo *et al*, with the discovery of two brain peptidases capable of hydrolysing the neuropeptide, bradykinin Further research led to the identification of the neurotensin-degrading ability of these oligopeptidases in rabbit brain (Camargo *et al*, 1983 and 1984) The enzymes then referred to as endo-oligopeptidase A and B are now widely recognised as thimet oligopeptidase and prolyl oligopeptidase respectively

Since the 1980's, there has been a surge of research into the proteinases, but little attention was given initially to their substrate-size limitations and hence the existence of the oligopeptidases as a distinct class of endopeptidases was not focused on This, coupled to the inconsistent naming of the same enzyme makes it difficult to identify the exact number of true oligopeptidases However, the recent classification, whether by catalytic type, reaction catalysed or evolutionary relationship (Barrett, 1994), and the trivial naming of the oligopeptidases, has made it somewhat easier to quantify, recognise and research them

1 2 CHARACTERISTICS

The oligopeptidases are a distinct sub-set of the endopeptidases, although they are not true proteinases due to their inability to hydrolyse peptide bonds within proteins They are confined to the degradation of oligopeptides or small polypeptide substrates, the size of which varies for individual enzymes within the class This strict substrate specificity is probably determined by their molecular structures though little structural information has been obtained and so the exact mechanism of action is not yet fully understood The ohgopeptidases are known to have few naturally occurring inhibitors Plasma is rich with inhibitors of various proteinases including α_2-macroglobulin (α_2M), which inhibits the majority of endopeptidases, irrespective of catalytic class (Barrett and Rawlings, 1992) Due to the large size of α_2M, oligopeptidases fail to cleave in the bait region, presumably for the same reason that they cannot hydrolyse proteins It is suggested that the endopeptidases cleave a peptide bond in a sensitive region of the macroglobulin, and that this results in a conformational change in the α_2M molecule that traps the enzyme irreversibly Access of substrates to the active site of the enzyme becomes sterically hindered, causing inhibition that is most pronounced with large substrate molecules (Barrett and Starkey, 1993)

The precise biological functions of ohgopeptidases are still unknown yet for some of the enzymes possible roles have been elucidated They are thought to perform important, specialized biological functions including the modification or destruction of peptide messenger molecules The fact that they lack natural inhibitors may also be of some biological significance

1 3 THE OLIGOPEPTIDASES

The exact number of oligopeptidases is difficult to ascertain due to the many different names given to the same enzyme and the ever-emerging new enzymes TABLE 1 1 highlights the most common ohgopeptidases studied and the ones which are going to be focused on in this review, while TABLE 1 2 lists other hydrolases which have been identified as oligopeptidases but have not been as extensively characterised

OLIGOPEPTIDASE	NUMBER	CATALYTIC TYPE	ALTERNATIVE NAMES
Prolyl Oligopeptidase	EC 3 4 21 26	Serine protease	Prolyl endopeptidase Proline endopeptidase Endo-oligopeptidase B Post-proline cleaving enzyme
Thimet Oligopeptidase	EC 3 4 25 15	Metalloprotease (thiol activated)	Pz peptidase Collagenase-like peptidase Endo-oligopeptidase A Soluble metalloendopeptidase Endopeptidase EC 24 15 Kinmase A EC 3 4 22 19
Neurolysin	EC 3 4 24 16	Metalloprotease	Oligopeptidase M EC 3 4 99 31 Thimet Peptidase II Microsomal metalloendopeptidase Mitochondrial oligopeptidase (MOP)
Neprilysin	EC 3 4 24 11	Metalloprotease	Neutral endopeptidase Enkephalinase Membrane-metalloendopeptidase A
Oligopeptidase B	EC 3 4 21 83	Serine Protease	Protease II

TABLE 1 1 THE PREDOMINANT OLIGOPEPTIDASES

OLIGOPEPTIDASE	NUMBER	CATALYTIC TYPE	ALTERNATIVE NAMES
Oligopeptidase A	EC 3 4 24 70	Metalloprotease	-
Oligopeptidase F	-	Metalloprotease	LEP I Oligopeptidase PepF Alkaline endopeptidase
Oligopeptidase MepB	-	Metalloprotease	
Oligopeptidase O	-	Metalloprotease	Neutral thermolysin-like oligopeptidase (NOP) Oligopeptidase PepO
Insulysin	EC 3 4 24 56	Metalloprotease	Insulinase Insulin-degrading peptidase
Oligopeptidase PepB	-	Metalloprotease	PepB
Pitrilysin	EC 3 4 99 44	Metalloprotease	Protease III Protease Pi
Protease IV	-	Unclassified	
Saccharolysin	EC 3 4 24 37	Metalloprotease	Oligopeptidase YscD

TABLE 1 2 THE EMERGING OLIGOPEPTIDASES

1 3 1 Prolyl Oligopeptidase

Prolyl oligopeptidase (EC 3 4 21 26) activity was first observed in 1971 in a study on human uterine homogenates and appeared to cause the release of leucylglycinamide from oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2), indicative of cleavage at the carboxy side of the proline residue (Walter *et al*, 1971) This enzyme was further purified and characterisation revealed a broader specificity than was initially suggested In fact this oxytocin-degrading enzyme was found to be a proline specific peptidase capable of hydrolysing the carboxy side of proline residues and was referred to as post-proline cleaving enzyme (Koida and Walter, 1976, Walter, 1976) Since its initial discovery, PO has been isolated from various sources and named according to the bioactive peptide acting as substrate For example, kinmase B isolated from rabbit brain (Oliveira *et al*, 1976) and TRH deamidase from rat brain (Rupnow *et al*, 1979) are now known to be the same enzyme Prolyl oligopeptidase is now the generally accepted term for this proline specific peptidase, as recommended in 1992 (Nomenclature Committee)

1 3 1 1 Assay

Following the discovery of PO, oxytocin and arginine-vasopressin were initially employed as natural and radiolabelled substrates for the enzyme (Walter *et al*, 1976) Koida and Walter (1976) reported the use of the Z-Gly-Pro-Leu-Gly as substrate, the ninhydrin assay being employed to detect the cleavage product Leu-Gly The development of spectrophotometric and fluorimetric substrates in the late 1970's, lead to the synthesis of a number of potential chromogens and fluorophores The basic structure of PO substrates focuses on N-blocked proline containing dipeptides linked to a molecule that is readily detected on cleavage The N-terminal blocking agents *N*-benzyloxycarbonyl- (Z-) and succinyl- (Suc-) are most commonly linked to Gly-Pro residues such is the case for Z-Gly-Pro-MCA The synthesis of this first fluorogenic substrate was described in 1979 by Yoshimoto *et al*, who reported a Michaelis constant (K_M) of 0 02nM for rat tissue PO The fluorophore MCA (7-amino-4-methyl-coumarin) can readily be detected at excitation and emission wavelengths of 370nm and 440nm respectively (Yoshimoto *et al*, 1979)

Various reports of novel fluorogenic synthetic substrates have been published since, and the superior sensitivity of fluorimetric over spectrophotometric assays is well-documented (Willard *et al*, 1988) Knisatschek *et al* (1980) reported a K_M value of 14μM for Z-Gly-Pro-2-NNAp, an alternative fluorogenic substrate Although PO assays incorporating 2-NNAp (β-naphthylamide) have been reported, MCA shows enhanced sensitivity over the naphthylamide derivative (Polgar, 1994) A ten-fold lower K_M (1 4μM) was determined by Noula *et al* (1997) using 7-amino-4-methyl-2-quinoline (Meq) as an alternative to MCA The enhanced affinity of PO was as a direct result of replacement of the P_2 positioned Gly with a cysteine-benzyl residue An added advantage of this fluorogenic substrate is the reduction in solvent concentration required for solubilisation Spectrophotometric assays have also been described using Z-Gly-Pro-4-nitroanilide (Polgar, 1992d) and nitrophenylesters (Walter and Yoshimoto, 1978, Polgar, 1992d) although these are generally less sensitive than their fluorescent counterparts To date, Z-Gly-Pro-MCA has been the most widely used, commercially available substrate employed for the detection of PO activity (Rennex *et al*, 1991, Goossens *et al*, 1995, Cunningham and O'Connor, 1998) It should be noted however, that the identification of a Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) by Cunningham and O'Connor (1997a) questions the classification of Z-Gly-Pro-MCA as a *specific* PO substrate

1 3 1 2 Purification

Prolyl oligopeptidase activity has been identified and subsequently purified from a wide range of mammalian and bacterial sources PO distribution studies in human and rabbit tissues (Orlowski *et al*, 1979, Kato *et al*, 1980) have revealed an ubiquitous distribution with consistently elevated levels in the brain, offering a wide range of source tissues Initially, tissue samples are homogenised using mechanical and centrifugal forces and this is usually followed by an ammonium sulphate fractionation with up to 80% saturation (Yoshimoto *et al*, 1981 and 1983, Moriyama and Sasaki, 1983, Kalwant and Porter, 1991) Diethylaminoethyl (DEAE) resins at pH 7 0 are widely employed as the first chromatographic separation step in PO purification schemes and the concept of re-chromatographing on the same resin is commonly practiced Yoshimoto *et al* (1983) employed the anion exchange resin twice in the purification of bovine brain PO, while four distinct DEAE cellulose steps were reported by Rosen *et al* (1991) for the purification of PO from human erythrocytes, resulting in a 64,000-fold purification Separation based on relative molecular size is commonly performed using a variety of size exclusion chromatography columns, ranging from Sephadex G-150 (Moriyama and Sasaki, 1983) to Sephacryl S-300 (Chevallier *et al*, 1992) Many PO purification schemes published incorporate hydroxyapatite (Yoshimoto *et al*, 1981, Kalwant and Porter, 1991, Rosen *et al*, 1991) and phenyl sepharose hydrophobic interaction (Goossens *et al*, 1995, Cunningham and O'Connor, 1998) chromatography resins Alternative purification methods include native polyacrylamide gel electrophoresis (Carvalho and Camargo, 1981) and multi-step fast protein liquid chromatography (Makinen *et al*, 1994) A membrane-bound form of PO was purified 1400-fold by size exclusion, calcium phosphate cellulose and hydrophobic interactions chromatography with a yield of 23% (O'Leary *et al*, 1996)

The effectiveness of these purification procedures is reflected in the overall purification factor achieved and the yield of biologically active peptidase PO purification factors ranging from 30 to 64,000 have been reported using the aforementioned procedures, with an average yield of 10%

1 3 1 3 Biochemical Characteristics

To date PO has been well characterised in terms of its biochemical and physiochemical properties It has been identified as a monomeric enzyme with a molecular weight ranging from 65 to 85kDa, the majority of reports indicating a molecular weight of 70 to 75kDa (Kato *et al*, 1980, Moriyama and Sasaki, 1983, Yoshimoto *et al*, 1987) Preliminary molecular weight determinations suggesting that PO was a dimer with native molecular weights ranging from 115 to 140kDa (Koida and Walter, 1976, Mizutam *et al*, 1984), have been disproved (Rennex *et al*, 1991) Ammo acid sequences have been deduced from cDNA clones of prolyl oligopeptidase and further verify a monomeric structure Both porcine brain and human T-cell PO revealed a 710 amino acid sequence and a deduced molecular weight of 80 75kDa (Rennex *et al*, 1991, Shirasawa *et al*, 1994) Bacterial PO has also been cloned and a molecular weight of 78 71kDa has been proposed for the 705 amino acid residue sequenced (Yoshimoto *et al*, 1992) In 1992, Chevallier *et al* verified this result and identified a 20 amino acid N-termmal signaling peptide (also reported by Kanatani *et al*, 1993), which when removed resulted in a molecular weight of 76 78kDa

In general, bacterial and mammalian PO have a neutral pH optimum with reported values between pH 6 5 and pH 8 3 depending on the source Isoelectric points of between 4 8 and 4 9 indicate the acidic nature of mammalian PO (Koida and Walter, 1975, Yoshimoto *et al*, 1983, Goossens *et al*, 1995), while bacterial PO isoelectric points are generally higher *Treponema denticola* PO was reported to have a pI of 6 5 (Makinen *et al*, 1994), while Yoshimoto *et al* (1980) reported a pI of 9 6 for PO isolated from *Flavobacterium meningosepticum* and a value of 5 2 for *Lyophyllum cinerascens* PO in 1988 Both forms have an optimum temperature of 37°C to 40°C (Yoshimoto *et al*, 1981, Kalwant and Porter, 1991, Makinen *et al*, 1994), with some reports of thermostability at 45°C (Koida and Walter, 1976, Yoshimoto *et al*, 1981)

1 3 1 4 Distribution

PO distribution is predominantly cytosolic in nature, the majority of studies reporting the purification or characterisation of soluble PO from bovine, human, lamb, mouse, porcine, rabbit or rat cytosol Camargo *et al* (1984) were the first to report a particulate form of this endopeptidase, reporting 10% of the total rabbit brain PO activity to be associated with the membrane fraction Particulate forms of PO have also been reported in the brush border of kidney (Sudo and Tanabe, 1985) and in neuroblastoma cells (Checler *et al*, 1986) O'Leary and O'Connor (1995) were first to report the complete purification and characterisation of a membrane-bound form of PO The particulate bovine brain enzyme displayed similar characteristics to cytosolic PO, however the synaptosomal membrane localisation may be of potential physiological significance in relation to neuropeptide cleavage (O'Leary *et al*, 1996)

As previously mentioned, PO is ubiquitously distributed throughout a variety of mammalian, plant and microbial sources, with high levels in the brain Localisation studies performed by Kato *et al*, 1980 and Orlowski *et al*, 1979, compared PO distribution in human and rabbit peripheral tissues respectively Highest levels of the peptidase in humans were observed in skeletal muscle, testes and kidney extracts while in rabbit peripheral tissues the small intestine, lung and spleen contributed to the highest PO activity Conversely in rabbit, skeletal muscle was the poorest source, followed by kidney and Camargo *et al* (1983) failed to detect the enzyme in rabbit spleen, lung or kidney The small intestine of rat exhibited poor PO levels, while activity in kidney and liver homogenates was substantial (Yoshimoto *et al*, 1979) These studies imply considerable species to species variation in PO activity in mammalian peripheral tissues

Similar species variation is not observed in mammalian brain Consistently high PO levels have been identified in brain homogenates of bovine, human, lamb, mouse, rabbit and rat species examined (Blumberg *et al*, 1980, Kato *et al*, 1980, Yoshimoto *et al*, 1981, Dresdner *et al*, 1982, Healy and Orlowski, 1992, Fukunari *et al*, 1994) However, localisation studies have revealed considerable species variation in the *regional* distribution of PO in the brain For example, PO activity was highest in the frontal cortex

of human brain (Kato *et al*, 1980), in the endohinal cortex and hippocampus of rabbit brain (Orlowski *et al*, 1979), but in the thalamus in bovine brain (Tate, 1981) This variation among specific regions within the brain may simply be species related (Kato *et al*, 1980), however the consistently high activity observed in the brain as a whole, indicates a possible functional role of PO in the brain

1 3 1 5 Catalytic Classification

PO has been classified as a serine protease, initially based on its inhibition by the active-site directed reagents, DFP and to a lesser extent by PMSF (Kalwant and Porter, 1991, Stone *et al*, 1992) Confirmation of its catalytic classification was achieved on identification of the active site by Rennex *et al* (1991) who successfully cloned PO from porcine brain Although PO is a serine protease, it differs from the established families of these enzymes in its substrate specificity, subcellular localisation and amino acid sequence Until recently serine proteases have been considered to consist of three groups, the trypsin, subtilisin and carboxypeptidase Y families (Neurath, 1989) The families have unrelated tertiary structures but possess similar catalytic sites and are believed to have evolved from a common origin (Polgar and Szabo, 1992)

When the deduced amino acid sequence of PO was reported, there was no apparent similarity between it and the other proteins in its class However in 1992, Polgar and Szabo (1992) detected a resemblance to DPP IV (EC 3 4 14 5), since both peptidases were proline specific and larger than classic serine proteases Rawlings *et al* (1991), while agreeing with Polgar and Szabo, claimed a clearer relationship between PO and acylaminoacyl-peptidase (EC 3 4 19 1) based on similar C-terminal residues It is now generally accepted that there is a fourth class of serine proteases, the prolyl oligopeptidase family, which includes, pig and *F meningosepticum* PO, human and rat DPP IV, pig and rat acylaminoacyl-peptidase, among others (Barrett and Rawlings, 1992)

Within each family of serine proteases, the primary structures of the enzymes are homologous and the tertiary structures similar, while enzymes from different families are not homologous Similarly the amino acids that surround the active site seryl residue are conserved in each family From TABLE 1 3 it is clear that the active-site sequence for PO showed no homology to those for the subtilisin, trypsin or carboxypeptidase families confirming its classification as a *new type of serine protease* (Rennex *et al*, 1991) DPP IV and acylaminoacyl-peptidase sequences from different sources are also shown

SERINE PROTEASE CLASS	ACTIVE SITE SEQUENCE *Residues 552-558*
Trypsin-like	Gly-Asn-**Ser**-Gly-Gly-Pro
Subtilisin-like	Gly-Thr-**Ser**-Met-Ala-Ser/Thr
Carboxypeptidase-like	Gly-Glu-**Ser**-Tyr-Ala-Gly
Prolyl oligopeptidase-like	Gly-Xaa-**Ser**-Yaa-Gly-Gly
Examples of PO-like family	
Pig PO	Gly-Gly-**Ser**-Asp-Gly-Gly
F meningosepticum PO	Gly-Arg-**Ser**-Asp-Gly-Gly
Pig acylaminoacyl-peptidase	Gly-Gly-**Ser**-His-Gly-Gly
Rat acylaminoacyl-peptidase	Gly-Gly-**Ser**-His-Gly-Gly
Rat DPP IV	Gly-Trp-**Ser**-Tyr-Gly-Gly
Human DPP IV	Gly-Trp-**Ser**-Tyr-Gly-Gly

TABLE 1 3 AMINO ACID SEQUENCES SURROUNDING THE ACTIVE SITE SER[554] RESIDUES OF SERINE PROTEASE FAMILIES

A second feature that distinguishes PO from the other families of serine proteases is the order of the catalytic triad The active site is believed to be located at the C-terminal end of the 710 amino acid sequence Barrett and Rawlings (1992) highlighted the sequence homology between PO family members at Asp^{529}, Ser^{554}, His^{680} and Asp^{641} residues, three of which represent the catalytic triad They concluded that Asp^{529} was located in a neutral, hydrophilic segment and was therefore most likely to be the catalytic aspartic acid In 1995, Goossens *et al*, in a study of the secondary structure of PO, indicated that Asp^{641} was in fact the third member of the catalytic triad Although the active sites of all serine protease in the SA, SB and SC clans (see Barrett *et al*, 1998 for classification) contain a catalytic triad of Ser, His and Asp residues (Rennex, 1991), the order for PO (Ser^{554}-His^{680}-Asp^{641}) appears to be unique in comparison to trypsin (His^{57}-Asp^{102}-Ser^{195}) and subtilisin (Asp^{32}-His^{64}-Ser^{221}) classes The linear arrangement of the active site residues of PO, differs from that of the established serine proteases and is in fact similar to the lipases and some carboxypeptidases (Polgar, 1992b) Goossens *et al* (1995) reported that the prolyl oligopeptidase is the first endopeptidase shown to adopt the α/β hydrolase fold structure, and the authors postulate that oligopeptidases are an evolutionary link between the exopeptidases and the endopeptidases

The prolyl oligopeptidase family (S9) is the most disparate of the serine peptidases (TABLE 1 4) Members include an endopeptidase that is confined to action on oligopeptides (EC 3 4 21 26), an exopeptidase that cleaves dipeptides from the N-terminal only when the terminal is free (EC 3 4 14 5) and an omega peptidase that preferentially cleaves N-terminal acetyl-aminoacyl residues from polypeptides (EC 3 4 19 1) (Rawlings *et al*, 1991) The mixture of thiol-dependent and particulate and cytosolic peptidases further emphasises the diversity of the members of the S9 family of serine proteases

PEPTIDASE	EC NO	PEPTIDASE TYPE	PROPERTIES
Acylaminoacyl peptidase	3 4 19 1	Exopeptidase- Omega peptidase	Cytosolic, thiol dependent Cleaves *N*-formyl or *N*-acetyl from the N-terminus of a peptide
Dipeptidyl peptidase IV	3 4 14 5	Dipeptidase	Particulate, not thiol dependent Releases N-terminal dipeptides, preferential for prolyl bonds
Dipeptidyl peptidase IV-like protein	-	Dipeptidase	Releases N-terminal dipeptides
Dipeptidyl aminopeptidase A	-	Aminopeptidase	Releases N-terminal dipeptides
Dipeptidyl aminopeptidase B	-	Aminopeptidase	Releases N-terminal dipeptides
Prolyl oligopeptidase	3 4 21 26	Endopeptidase- Oligopeptidase	Mainly cytosolic, thiol dependent Cleaves prolyl and some alanyl bonds
Oligopeptidase B	3 4 21 83	Endopeptidase- Oligopeptidase	Cytosolic Cleaves arginyl and lysyl bonds

TABLE 1 4 THE MEMBERS OF THE PROLYL OLIGOPEPTIDASE (S9) FAMILY OF SERINE PROTEASES

1 3 1 6 Mechanism of Action

The catalytic triad amino acids each play an important role in the catalysis of PO The histidine acts as a general acid-base catalyst activating the nucleophilic group The hydroxyl group of the serine acts as a nucleophile in the attack on the peptide bond while the aspartic acid stabilizes charged tetrahedral intermediates formed in the reaction (Fink, 1987, Rennex *et al* 1991) Although the mechanism of action of the serine proteases is still not completely understood, catalysis generally involves an acylation reaction in which the acyl (RCO-) moiety of the substrate is transferred to the enzyme's serine group This represents the first half of the catalytic reaction The second stage involves a reversal of this step i e deacylation, where the imidazole group of the histidine residue activates a water molecule by general-base catalysis ultimately leading to the formation of an acid product and enzyme (see Fink, 1987 for review of serine proteinase catalysis)

Polgar (1991) observed the remarkable sensitivity of PO to ionic strength and postulated the existence of two catalytically active forms while attempting to understand the catalytic mechanism of PO Usually the ionisation of the histidine residue in the active site of serine proteases governs the pH dependence of catalysis, exhibiting a pK_a of 7 0 However a second significant ionisation event in pH dependent kinetics was observed at pK_a 5 0 (Polgar, 1991) Examination of the two PO forms revealed that the rate determining step of the low pH form followed general acid/base catalysis, while that of the high pH form was due to a physical step The rate of PO catalysis was unaffected by the nature of the substrate leaving group Consequently it was concluded that the rate-limiting catalysis of PO is governed by a physical rather than a chemical step (Polgar, 1992c) In comparison, the rate-determining step in the catalysis of trypsin and subtilisin is a general acid/base catalysed chemical step, further substantiating the notion that PO is a member of a distinct family of serine proteases

X-ray crystallography has helped to elucidate the crystal structure of PO (Fulop *et al*, 1998), a breakthrough which has revealed and confirmed many interesting structural properties of the enzyme The catalytic (peptidase) domain consists of residues 1-72 and 428-710 As predicted by Goossens *et al*, 1995, this region of the domain exhibits a characteristic α/β hydrolase fold A second (β-propeller) domain was also identified between residues 73 to 427, connected to the catalytic domain by hydrophobic forces Its structure is based on a seven-fold repeat of four-stranded antiparallel β-sheets, which are twisted and radially arranged around a central tunnel It was also evident that the catalytic triad is covered by the central tunnel of this unusual β-propeller Unlike other β-propellers, the first and last blades of the PO propeller are not closed The resultant partial opening provides access via the tunnel to the active site This domain controls the size-limitation of PO substrates, by excluding large structured peptidases thus protecting large peptides and proteins from proteolysis within the cytosol The catalytic triad is hidden in the central cavity at the interface between the two domains The enzyme subsites, S_1 and S_2, have been located in the peptidase domain, suggesting that the truncated catalytic domain alone preserves enzyme specificity (Fulop *et al*, 1998)

The β-propeller is partially opened and the presence of a narrow hole at its bottom could be responsible for the conformational change allowing the substrate gain access to the active site It is also possible that the active site entrance could be widened by the movement of flexible side chains located on the propeller and this is believed to be the physical rate-limiting catalysis step hypothesised by Polgar (1992c)

1 3 1 7 Specificity

The ability of PO to hydrolyse a peptide is dictated by the presence of a particular residue (proline or alanine), the location of the residue and the length of the peptide Prolyl oligopeptidase is assumed to have five binding subsites (Walter *et al*, 1980) from S_3 to S_2' (see FIGURE 1 1 for terminology) The smallest peptide cleaved is an N-blocked tripeptide or a tetrapeptide with residues in positions P_3-P_1' As an oligopeptidase, PO has a substrate-size limitation and is therefore unable to act on proteins or peptides longer than 25 amino acids in length (Koida and Walter, 1976, Walter *et al*, 1980, Moriyama *et al*, 1988) This observation can be explained by the existence of the β-propeller domain described in section 1 3 1 6

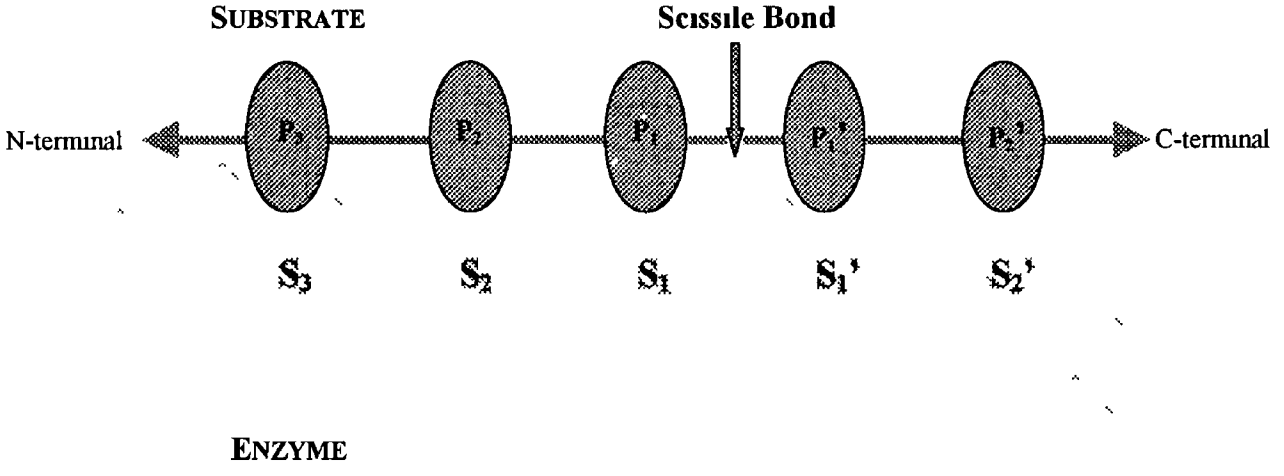


FIGURE 1 1 SCHEMATIC REPRESENTATION OF AN ENZYME-SUBSTRATE COMPLEX
Residues of the substrate and enzyme subsites are numbered according to their distance from the scissile bond and their location on the N- or C-terminal side of the cleavage site (Berger and Schechter, 1970, Nomenclature Committee, 1992)

PO is a proline specific peptidase, cleaving peptides with a proline in the P_1 position Cleavage will not occur if a free α-amine exists in the N-terminal sequence (P_3 position) or in the P_1' position Pro-Pro bonds are not hydrolysed (where a proline also occupies the P_1' site) nor are peptides with a proline residue at the P_2 site N-terminally blocked peptides are readily hydrolysed as long as the above criteria are observed Nomura (1986) investigated the importance of the residue in the substrate P_1 position and the corresponding effect on the subsite of the enzyme (S_1) A range of substrates was examined and it was observed that proline, alanine and N-methylalanine in the P_1 position yielded acceptable rates of bacterial PO catalysis This had been previously reported for mammalian PO, where Walter *et al* (1980) showed that the P_1 proline could be substituted for an alanine residue Although alanine and N-methyl derivatives of alanine and glycine are possible P_1 residues, the rate of hydrolysis is much slower, Nomura (1986) reporting this rate as only 16% the rate of the Pro bond cleavage It is generally accepted that the S_1 subunit of prolyl oligopeptidase is designed to specifically fit proline residues (FIGURE 1 2) but can tolerate residues carrying substituent groups, provided they do not exceed the size of proline's pyrrolidine (Walter *et al*, 1980, Nomura, 1986, Makinen *et al* , 1994, Kreig and Wolf, 1995)

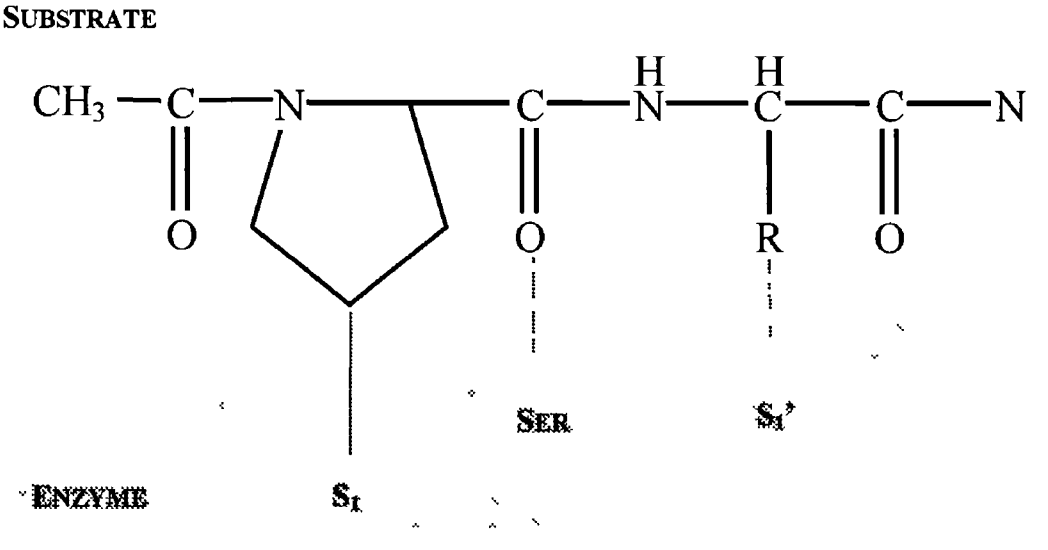


FIGURE 1.2. A SCHEMATIC MODEL OF THE ACTIVE SITE OF PO

The S_1 subsite is presumed to accommodate those residues with size and shape similar to proline (Nomura, 1986)

There is a preference for hydrophobic residues at the C-terminal of the scissile bond (P_1') with lower specificities for acidic and basic residues (Koida and Walter, 1976, Krieg and Wolf, 1995) This explains why PO exhibits such a high specificity for the hydrophobic MCA employed in fluorogenic substrate synthesis (Yoshimoto *et al*, 1979) Substrates incorporating phosphorylated serine residues in place of serine in the P_1' position were shown to induce an increase in the rate of hydrolysis Similarly substitution of aspartic acid with an asparagine residue in this position also increased the rate of catalysis (Momand and Clarke, 1987)

Prolyl oligopeptidase has a broad specificity for proline-containmg oligopeptides and its ability to cleave a number of bioactive peptides *in vitro* is well documented The first report of such cleavage was in 1976 when the enzyme was initially identified as an oxytocin-degrading peptidase in human uterus (Walter *et al*, 1976) Since then, many bioactive peptides were utilised as substrates including arginme-vasopressin, TRH and bradykinm TABLE 1 5 summarises the peptides reported to have been hydrolysed by PO and the identified cleavage sites Despite the volume of reports claiming PO hydrolysis *in vitro*, the precise role of the peptidase *in vivo* is still unclear, although research on specific inhibitors has helped in the elucidation of the role of PO in peptide turnover

PEPTIDE	AA	SEQUENCE AND CLEAVAGE SITE	REFERENCE
Angiotensin I	11	Asp-Arg-Arg-Val-Tyr-Ile-His-**Pro-Phe**-His-Leu	Moriyama *et al*, 1988
Angiotensin II	8	Asp-Arg-Val-Tyr-Ile-His-**Pro-Phe**	Moriyama *et al*, 1988
Angiotensin III	7	Arg-Val-Tyr-Ile-His-**Pro-Phe**	Moriyama *et al*, 1988
Arg-vasopressin	9	Cys-Tyr-Phe-Gln-Asn-Cys-**Pro-Arg**-Gly-NH_2	Walter *et al*, 1976
Bradykinin	9	Arg-Pro-**Pro-Gly**-Phe-Ser-**Pro-Phe**-Arg	Tate, 1981
LHRH	10	Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-**Pro-Gly**-NH_2	Wilk *et al*, 1979
Melanotropin	13	Ser-The-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-**Pro-Val**-NH_2	Tate, 1981
Neurotensin	13	Pyr-Leu-Tyr-Glu-Asn-Lys-**Pro-Arg**-Arg-**Pro-Tyr**-Ile-Leu	Camargo *et al*, 1984
Oxytocin	9	Cys-Tyr-Ile-Gln-Asn-Lys-**Pro-Leu**-Gly-NH_2	Walter *et al*, 1976
Substance P	11	Arg-Pro-Lys-**Pro-Gln**-Gln-Phe-Phe-Gly-Leu-Met	O'Leary *et al*, 1996
TRH	3	Pyr-His-**Pro-NH_2**	O'Leary *et al*, 1996
Tuftsin	4	The-Lys-**Pro-Arg**	Tate, 1981
Vasopressin	8	Cys-Tyr-Phe-Gln-Asn-**Pro-Arg**-Gly	O'Leary *et al*, 1996

TABLE 1 5 BIOACTIVE PEPTIDES HYDROLYSED BY PO

1 3 1 8 Inhibitors

Specific inhibitors are typically substrate analogues that possess a higher affinity for the active site thus binding reversibly or irreversibly to a region in the catalytic domain and preventing enzyme action Active site-directed inhibitors such as DFP and PMSF, exploit the primary sequence of a protein, however it is substrate analogue inhibitors, which play a role in identifying the physiological role of a peptidase Therefore a particular emphasis on the development of inhibitors that effectively inactivate prolyl oligopeptidase *in vivo* and can readily traverse the blood brain barrier has developed

The most suitable inhibitors were found to be ones in which the chain length corresponded to the three enzyme subsites, S_1, S_2 and S_3 In 1983, Wilk and Orlowski, reported the synthesis of a novel transition state aldehyde inhibitor, Z-Pro-Prolinal, which was shown to be remarkably potent against rabbit brain PO with an inhibition constant (K_i) of 14nM *In vivo* studies on the same inhibitor illustrated an 85% decrease in PO activity in various mouse tissues following intraperitoneal injection of 1 25mg/kg Z-Pro-Prolinal (Friedman *et al*, 1984) Further work on Z-Pro-Prolinal inhibition revealed that it was a competitive, slow-tight binding inhibitor which presumably binds to the S_1 subsite forming a hemiacetal with the active serine of the enzyme (Wilk and Orlowski, 1983, Bakker *et al*, 1990)

Since the initial research on Z-Pro-Prolinal, the synthesis of a large number of potential analogues has been reported Yokosawa *et al* (1984) substituted the (P_1) proline with a number of amino acid residues, of which valine proved to be the most potent (K_i 2 4nM) Other alterations include the conversion of prolinal to thioprolinal or thiazolidine and the substitution of proline with thioprohne The resultant inhibitors, Z-thioprolyl-thioprolinal and Z-thioprolyl-thiazolidine yielded K_i values of 0 01nM and 0 36nM respectively (Tsuru *et al*, 1988) The reported K_i for the latter inhibitor is comparable to that of Z-Pro-Prohnal yet the aldehyde group has been removed Previous studies showed the importance of the aldehyde group, believed to be responsible for the potency of Z-Pro-Prolinal (Friedman *et al*, 1984) Z-Pro-Prolinol and Z-Pro-Prolinal dimethylacetate did not exhibit the superior potency of Z-Pro-Prolinal, presumably due to the loss of the

aldehyde prolinal group Having said this, Z-Pro-Prolinal dimethylacetate can be converted to the aldehyde in the stomach and hence was investigated for its *in vivo* potency (Goossens *et al*, 1997)

Many other potent inhibitors which are variants of the N-blocked dipeptide chain have been developed (TABLE 1 5 lists some examples) The demonstration that nitriles could inhibit PO lead to the synthesis of the Fmoc derivatives by Li *et al* (1996) In addition to their potency (K_i of 5nM each), these inhibitors were also shown to be cell permeable, stable and capable of penetrating the blood-brain barrier Similarly Y-29794, a potent, competitive PO inhibitor was found to easily traverse this barrier (Nakajima *et al*, 1992) and Kato *et al* (1997) illustrated the prevention of amyloid-like deposits in the hippocampus of the mouse by oral administration of Y-29794 Z-Pro-3-fluoropyrrolidine, a derivative of Z-Pro-Prolinal, successfully inhibited PO activity *in vivo* Goossens *et al* (1997) reported a profound PO inhibition in the thymus, brain and testes of rats and claimed Z-Pro-3-fluoropyrrolidine showed no acute cell toxicity The potent PO inhibitors, JTP-4819, a pyrrolidinecar-boxamide derivative and S-17092-1, a perhydroindol carboxylic derivative have been devised for the treatment of Alzheimer's disease (AD) JTP-4819 reduces memory impairment by restoring TRH content, which occurs on inhibition of PO, known to hydrolyse TRH (Shinoda *et al*, 1996) Similarly S-17092-1 (Barelli *et al*, 1999), illustrates cell penetration while effectively inhibiting PO *in vivo* with a K_i of 1nM

Many inhibitors of bacterial origin also exhibit potent inhibition of prolyl oligopeptidase activity both *in vitro* and *in vivo* (see TABLE 1 6) Postatin, produced by *Streptomyces viridochromogenes* was first reported in 1991 (Aoyagi *et al*) as a potent inhibitor exhibiting an IC_{50} value of 0 03μg/mL Analogues containing postatins characteristic (S)-3-amino-2-oxovaleryl group increased IC_{50} values up to 5 8ng/mL (Tsuda *et al*, 1996) Kimura *et al* (1997a) reported the isolation of four bacterial PO inhibitors, propeptin, SNA-8073-B, staurosporine and enduracidin from *actinomycetes,* while quercetin and luteolin from caryophylli flocs yielded IC_{50} values of 0 17 and 0 19ppm respectively (Lee *et al*, 1998)

INHIBITOR	POTENCY	REFERENCE
Mammalian		
Fmoc-Ala-Pro-CN	K_i = 5nM	Li *et al*, 1996
Fmoc-Pro-Pro-CN	K_i = 5nM	Li *et al*, 1996
JTP-4819	IC_{50} = 0 83nM	Toide *et al*, 1995
ONO-1603	IC_{50} = 57nM	Toda *et al*, 1989
S-17092-1	K_i =1nM	Barelli *et al*, 1999
SUAM-1221	K_i = 190μM	Saito *et al*, 1991
Y-27924	IC_{50} = 0 95μM	Nakajima *et al*, 1992
Z-Indolinyl-Prolinal	K_i = 2 4nM	Bakker *et al*, 1991
Z-Pro-3-fluoropyrrolidine	K_i = 0 8nM	Goossens *et al*, 1997
Z-Pro-Prolinal	IC_{50} = 0 74nM	Wilk and Orlowski, 1983
Bacterial		
Eurystatin A	IC_{50} = 0 004μg/mL	Toda *et al*, 1992
Eurystatin B	IC_{50} = 0 002μg/mL	Toda *et al*, 1992
Postatin	IC_{50} = 0 03μg/mL	Aoyagi *et al*, 1991
Propeptin	K_i = 0 7μM	Kimura *et al*, 1997b
SNA-8073-B	K_i = 8μM	Kimura *et al*, 1997a
Staurosporine	K_i = 0 7μM	Kimura *et al*, 1990

TABLE 1 6 PO SPECIFIC INHIBITORS

1 3 1 9 Physiological Importance

Based on prolyl oligopeptidase's ability to hydrolyse a variety of proline-containing bioactive peptides and its ubiquitous distribution in tissues including the brain, it is believed that the enzyme must contribute to a number of biologically important functions (Makinen *et al*, 1994) Although much *in vitro* research has been carried out, the findings must also be correlated to *in vivo* studies To date, *in vivo* investigations carried out either compare enzyme activity levels in normal versus disease state environments (Maes *et al*, 1995) or determine physiological patterns based on PO inhibition (Shinoda *et al*, 1996)

Maes *et al* (1994) has performed extensive research with regard to comparative activity studies Lower serum PO levels were observed in patients suffering from major depression in comparison to control patients It was initially thought that the putative involvement of PO activity in the pathogenesis of major depression revolved around its role in the degradation of neuropeptides and hormones, however it is now known to be due to other factors A number of studies have suggested that PO may be involved in modification of T-cell function (Aoyagi *et al*, 1989) and in the pathogenetic mechanisms of inflammatory and autoimmune responses (Shoji *et al*, 1989) Depression is often accompanied by a number of these pathophysiological characteristics

Further research by Maes *et al* (1995) reported increased activity in the plasma of manic and schizophrenic patients compared with normal volunteers Higher serum PO levels were also measured in patients with post-traumatic related (Maes *et al*, 1999a) and physiological (Maes *et al*, 1998) stress 58 3% of alcohol-dependent patients showed a decrease in serum PO levels (Maes *et al*, 1999b) It was concluded that lower PO activity could contribute to the pathophysiology of major depression and alcohol dependence, while increased activity may be related to various stress conditions and psychotic conditions such as mania and schizophrenia

In contrast, abnormally low prolyl oligopeptidase activities have also been observed in neurodegenerative disorders Comparison of enzyme levels in brain tissues from normal and various neurodegenerating disease cases was performed by Mantle *et al* (1996) Data suggested a 65-70% reduction in PO activity in tissues from Parkinson's, Hungtington's and Alzheimer's diseases and in Lewy body dementia It was assumed that this reduction in activity was characteristic of a generalised process of neurodegeneration (Mantle *et al*, 1996) Yoshida *et al* (1996) observed similar reduced levels of serum PO activity in patients suffering from senile dementia of the Alzheimer type and even lower activities in vascular dementia cases Yoshida suggested that the brain of the dementia patients was affected by the metabolism of skeletal muscle, which subsequently caused a lowering of PO levels Conflicting data was observed in measurements of PO levels in AD brains Aoyagi *et al* (1990) reported enhanced peptidase activity while Ichai *et al* (1994), contradicted this data, claiming a substantial reduction of PO in the AD brain Lower than normal activity in AD brains was also reported in 1998 by Terwel *et al*

In addition to comparative activity studies, the effect of various inhibitors has been employed to aid in the elucidation of a possible physiological role of PO *In vitro,* substrates such as TRH, substance P and arginine vasopressin (AVP) are known to exert neuroprotective effects For example, AVP and TRH are known to improve the performance of animals in memory and learning tasks (De Wied, 1984, Griffiths, 1987) Therefore degradation of these peptides by peptidase activity is unfavourable and the inhibition of PO is targeted in an attempt to enhance neuroprotective effects Oral administration of Z-Pro-Prolinal successfully caused inhibition of the PO activity in rat brain and hence caused a significant increase in AVP levels in the brain (Miura *et al*, 1995) Similarly JTP-4819 enhanced memory processes in aged rats (Toide *et al*, 1995) and reversed an age-related decrease of substance P and TRH levels (Shinoda *et al*, 1995)

A second attribute of JTP-4819, is its apparent role in AD The deposition of amyloid in senile plaques in the brain is now widely accepted as the hallmark of AD, the major component being a 42 residue protein referred to as the amyloid β-protein (Aβ) (Ishiura, 1991) A number of peptidases have been implicated in the production of Aβ from its precursor APP (amyloid precursor protein) Ishiura *et al* (1990) identified prolyl oligopeptidase as a possible γ-secretase, cleaving APP between residues Ala^{713} and Thr^{714} therefore contributing to the generation of Aβ (Checler *et al*, 1995a) Based on this, a number of studies have investigated the use of potent PO inhibitors, such as JTP-4819 and S-17092-1 in preventing Aβ production Induced Aβ formation in a neuronal cell line was retarded upon addition of JTP-4819 (Shinoda *et al*, 1997) further implicating PO in the pathogenesis of Alzheimer's disease Despite these studies there are a few factors which question the ability of PO to degrade the APP and hence be involved in Aβ formation As previously discussed, PO is an oligopeptidase capable of cleaving peptides no greater than 25 amino acids in length, yet APP is composed of over 700 residues However, as discussed in section 4 8 10 it may be possible that APP is first cleaved by a β-secretase which produces an APP fragment short enough for PO to hydrolyse

In summary, prolyl oligopeptidase is a large cytosolic enzyme that belongs to a new class of serine proteases (S9) The presence of an unusual β-propeller sheet regulates proteolysis by limiting substrate size Although an exact function cannot be deduced, PO is believed to be a regulatory peptidase involved in the degradation of proline-containing neuropeptides and hormones It has been implicated in the pathogenesis of a number of neurodegenerative disorders, nevertheless the degree of involvement is not yet completely understood

1 3 2 Thimet Oligopeptidase

Thimet oligopeptidase (EC 3 4 24 15) is a thiol-dependent metalloprotease, which is widely distributed in animals and plants The history of thimet oligopeptidase (TOP) began in the 1960's with the design of the chromogenic substrate phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide) by Wunsch and Heidrich (1963) and the presence of an enzyme in mammalian tissues capable of cleaving this substrate at the Leu-Gly bond Initially, it was believed that this enzyme was mammalian collagenase, however it was soon shown that animal collagenase and Pz peptidase activity were separable by gel filtration and that the peptidase had no activity towards native collagen (Harper *et al*, 1970, Harris *et al*, 1972) The enzyme then became known as collagen-like peptidase (EC 3 4 99 31)

Camargo *et al* (1973) discovered a bradykinin enzyme from rabbit brain, endo-oligopeptidase A, which exhibited a clear thiol dependence It was believed to be a cysteine-type peptidase and was assigned EC 3 4 22 19 (Gomes *et al*, 1993) Meanwhile soluble metalloendopeptidase (endopeptidase 24 15) was being independently researched (Orlowski *et al*, 1983) Despite similarities between this metalloendopeptidase and endo-oligopeptidase A (substrate specificity and thiol dependence), it was assigned EC 3 4 24 15 not EC 3 4 22 19 In 1989, Barrett and Tisljar *et al*, independently suggested that all of these activities were in fact due to a single enzyme, which combined thiol and metal dependencies The enzyme became known as thimet oligopeptidase, EC 3 4 24 15, to account for this dual nature and also the unusual substrate-size dependence of the enzyme (Nomenclature Committee, 1992)

1 3 2 1 Assay

Pz-peptide was the first substrate used to identify thimet oligopeptidase activity (Wunsch and Heidrich 1963) This compound has the advantage that the chromophoric split product, Pz-Pro-Leu, is water insoluble and can be easily extracted into ethyl acetate and quantified spectrophotometrically This original assay was constantly modified and was popular until Juliano *et al* (1990) developed two quenched fluorescence substrates,

namely, Dnp(2,4-dinitrophenyl)-Pro-Leu-Gly-Pro-Trp-D-Lys (QF01) and 7-methoxycoumarin-3-carboxylyl (Mcc)-Pro-Leu-Gly-Pro-D-Lys(Dnp) (QF02) The fluorescent product formed on cleavage excites at 345nm and emits at 405nm (Tisljar *et al*, 1990) These compounds allow continuous monitoring of product formation (a major drawback with Pz-peptide) Continuous assays using substrates such as Suc-Gly-Pro-Leu-Gly-Pro-MCA and the aforementioned Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys and Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) are the most popular TOP assays due to the sensitivity of fluorimetric over spectrophotometric assays

An alternative discontinuous assay substrate to Pz-peptide is Bz-Gly-Ala-Ala-Phe-p-aminobenzoate (pAb) designed by Orlowski *et al* (1983) TOP readily cleaves the Gly-Ala bond, followed by cleavage of Phe-pAb by an added aminopeptidase The release of aminobenzoic acid can be quantified in a diazo-coupling procedure Although this assay is useful for purification purposes, it can produce complications in more specific assays due to the presence of the aminopeptidase (Barrett *et al*, 1995) Naturally occuring peptides e g bradykinin, neurotensin, dynorphin A(1-8) and dynorphin A(1-17) are often employed as TOP substrates It should be noted at this point that there are no specific assays for this thiol-dependent metalloprotease Neurolysin also cleaves dynorphin A(1-8) at the same bond as TOP, while serine proteases such as PO have been shown to cleave bradykmin and neurotensin (Tate, 1981, Camargo *et al*, 1984) Therefore the addition of peptidase inhibitors such as PMSF, Pro-Ile and phosphoramidon will inhibit serine proteases, neurolysin and neprilysin respectively

1 3 2 2 Distribution

TOP is widely distributed across species, including invertebrate animals and plants In higher animals it is present in most tissues, where it is mainly in the soluble fraction of the cytoplasm, not associated with any particular organelle A minor fraction (5-20%) is reported to be membrane-bound (Acker *et al*, 1987) Location is mainly intracellular, the peptidase rarely being secreted or presented on the plasma membrane (Barrett and Chen, 1998) TOP is highly active in testes, brain, pituitary and liver tissues Healy and Orlowski (1992) determined the immunocytochemical localisation of thimet

oligopeptidase in rat brain and peripheral tissues using a polyclonal antibody raised in rabbits Quantitation of enzyme levels suggested the activity of TOP in the kidney and other peripheral tissues (except testes) was only 20% of that identified in the brain, proposing a role for TOP in neuropeptide metabolism The existence of a particulate form of TOP was confirmed since subcellular fractionation of brain homogenates showed that 20% activity was associated with the membrane fraction (Healy and Orlowski, 1992)

1 3 2 3 Purification

The relative abundance of thimet oligopeptidase in rat testis, brain and chicken liver, make these sources ideal for purification purposes The first reported purification of TOP was by Aswamkumar and Radhakrishnan in 1972 from rat granuloma tissue, with a purification factor of 250 achieved In 1975 the same researchers reported an improved purification of TOP from monkey kidney (2500-fold purification)

To date the enzyme has been purified to homogeneity or near homogeneity from a number of sources including rat testis (Orlowski *et al*, 1989), rabbit brain (Carvalho and Camargo, 1981), rabbit heart (Tisljar *et al*, 1989), chicken embryo (Morales and Woessner, 1977) and chicken liver (Barrett and Browne, 1990) Purification from erythrocytes is worthwhile as purification factors of 40,000 have been reported (Dando *et al*, 1993) and contamination with plasma albumin is eliminated (Barrett *et al*, 1995) Recombinant rat TOP has successfully been produced (Pierotti *et al*, 1990) Chromatography resins commonly employed include size exclusion, phenyl sepharose, DEAE cellulose and hydroxyapatite, the latter two being of particular importance as they separate TOP from contaminating neurolysin (Checler *et al*, 1995b) Due to the thiol dependence of TOP, DTT (0 1mM) is usually added to all running buffers and substrates (Morales and Woessner, 1977) Similarly addition of ZnCl (0 1mM) in buffers markedly stabilises the peptidase thus increasing yields (Barrett *et al*, 1995)

1 3 2 4 Biochemical Characteristics

Thimet oligopeptidase has been identified as a monomeric enzyme with molecular weights determined to range from 73kDa to 75kDa depending on the source (Carvalho and Camargo, 1981, Camargo *et al*, 1983, Tisljar *et al*, 1989) cDNA cloning produced a 687 ammo acid sequence for TOP, from which a molecular mass of 78 3kDa was calculated (McKie *et al*, 1993) Isoelectric focusing by Carvalho and Camargo (1981) revealed an isoelectric point of 4 75 for TOP, which was verified two years later by the same researchers (Camargo *et al*, 1983) TOP exhibited an optimum pH in the 7 5 to 8 5 range (Morales and Woessner, 1977), with later reports of pH optima of 7 5 and 7 8 (Camargo *et al* 1983, Barrett and Chen, 1998) TOP does not contain disulphide bonds and there is no indication of any glycosylation based on the amino acid sequence (Barrett *et al*, 1995)

1 3 2 5 Catalytic Classification and Mechanism Action

The effects of site-directed inhibitors readily excluded TOP from the serine and aspartic types of endopeptidases yet it appeared to have characteristics of both cysteine and metallo-proteases (Barrett and Brown, 1990) A time-dependent decrease in activity was observed on incubation with EDTA and 1,10-phenanthrohne, while zinc, manganese, calcium, cobalt and cadmium metal ions restored activity after EDTA treatment (Barrett and Brown, 1990) It was these observations on chicken liver Pz peptidase that led Barrett to believe that it was in fact a metalloendopeptidase with thiol dependence, hence the name thimet was proposed and accepted

Thereafter, TOP has been classified as a member of the M3 family, the largest and most complex family of metalloproteases (Barrett *et al*, 1998a) All peptidases in the family (see TABLE 1 7) contain the His-Glu-Xaa-Xaa-His (HEXXH) motif and the mechanism of substrate binding and catalysis with respect to TOP has finally been elucidated (Cummins *et al*, 1999) As expected the active site of the peptidase exhibited the HEXXH motif, a common feature of zinc metalloenzymes His^{473}, Glu^{474} and His^{477} were confirmed by site-directed mutagenesis to be the residues essential for binding and catalysis of thimet oligopeptidase (Cummins *et al*, 1999)

It had been previously proposed that one of the Glu or His residues in the motif was also the ligand of the metal ion However Chen *et al* (1998) disproved this theory by mutagenesis claiming that neither of these amino acids close to the HEXXH motif were required for the binding of zinc Due to the fact that Glu^{497} is highly conserved in thermolysin and other metalloproteases it was then believed that this same residue was responsible for zinc binding in mammalian TOP Further characterisation by Cummins *et al* (1999) showed that a third putative metal ligand, presumed to coordinate directly to the active site zinc ion with the two histidine residues, was identified as Glu^{502} Activity towards synthetic and physiological substrates was substantially reduced on modification of any of the aforementioned residues, and the peptidase lost its ability to bind the active site directed inhibitor, *N*-[1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-paminobenzoate (Cpp-Ala-Ala-Phe-pAb), thus emphasising their importance in the catalysis of TOP

It is believed that TOP behaves in a similar manner to other metalloproteases e g carbonic anhydrase II (Lesburg *et al*, 1997) The histidine directly coordinates the active site zinc ion whereas the glutamate facilitates the acid/base catalysis by weakly co-ordinating to the zinc via a water molecule (Cummins *et al*, 1999) There are no indications of post-translational modifications in the biosynthesis of TOP or of a proteolytically activatable proenzyme (Barrett and Chen, 1998) Despite the results of Healy and Orlowski (1992), there is no apparent hydrophobic segment in the structure of TOP which would serve as a membrane spanning domain, questioning the existence of a particulate form of the metalloprotease (Pierotti *et al*,1990, Barrett and Chen, 1998)

PEPTIDASE	EC	TYPE	PROPERTIES
Thimet oligopeptidase	3 4 25 15	Oligopeptidase	Cytosolic, thiol dependent Cleaves when hydrophobic AA in P_1 and P_3' and Pro in P_2'
Neurolysin	3 4 24 16	Oligopeptidase	Cytosolic, little thiol dependence All substrates contain 17 or fewer AA
Saccharolysin	3 4 24 37	Oligopeptidase	Won't cleave peptides with free N-terminal, need hydrophobic AA in P_1 and P_3' and Pro in P_2'
Oligopeptidase MepB	-	Oligopeptidase	As for TOP
Oligopeptidase F	-	Oligopeptidase	Broad substrate specificity, cleaves peptides 7-23 AA long
Oligopeptidase PepB	-	Oligopeptidase	Cleaves small basic peptides, exact rules yet to be elucidated
Oligopeptidase A	3 4 24 70	Oligopeptidase	Hydrolyses N-blocked terminals containing at least four amino acids but not fewer than five AA
Peptidyl-dipeptidase Dcp	3 4 15 5	Dipeptidase	Cleaves penultimate bond in α-N-blocked tripeptidases, free tetra- and higher peptides
Mitochondrial intermediate peptidase	3 4 24 59	Endopeptidase	Involved in 2-step processing of mitochondrial protein precursors

TABLE 1.7 THE MEMBERS OF THE THIMET OLIGOPEPTIDASE (M3) FAMILY OF METALLOPROTEASES

1 3 2 6 Specificity

The substrate size specificity of human TOP was investigated using oligomers of (Gly-Pro-Leu)n, where n = 2, 3, 4 and 5 (Knight *et al*, 1995) Hydrolysis was most rapid with $(GPL)_3$ and slowest with $(GPL)_5$, indicating a preferentially binding of substrates composed of 9 residues, although the endopeptidase cleaves peptides that are between 6 and 17 amino acid residues in length (Barrett and Chen, 1998) This sharply defined upper limit of substrate size, which is also evident for prolyl oligopeptidase is responsible for the classification of TOP as an oligopeptidase Unfortunately, the precise rules governing TOP specificity remain unclear but general observations in specificity have been widely reported Primarily TOP cleaves on the carboxyl side of hydrophobic amino acids i e when the P_1 position (FIGURE 1 1) is occupied by a hydrophobic residue (Dando *et al*, 1993, Camargo *et al*, 1997) Isoleucine and valine, although hydrophobic in nature do not promote hydrolysis in TOP presumably due to their branched chain R-groups (Dando *et al*, 1993, Barrett *et al*, 1995)

A second prerequisite observed is that the presence of a hydrophobic or bulky ammo acid in the P_3' position and a proline residue at P_2 greatly increase the affinity of the substrate (or inhibitor) towards TOP (Dando *et al*, 1993) TOP has been shown to cleave a number of neuropeptides and hormones Dando *et al* (1993) extensively studied the specificity of human TOP, showing cleavage of oligopeptides like bradykinin, neurotensin, LHRH and dynorphin fragments TABLE 1 8 lists some of the reported naturally occurring peptide substrates of TOP It is clear from this table that the TOP hydrolysis site in a particular peptide is unpredictable, for example, TOP cleaves the Tyr-Ile bond in angiotensin II, yet fails to cleave the same bond in angiotensin I where hydrolysis of Pro-Phe bond is observed Knight *et al* (1995), observed that with isoleucine-containing peptides, TOP preferentially to cleaved these peptides near the C-terminal and this may explain why many of the scissile bond are three or four residues from the C-terminal (Barrett *et al*, 1995) In summary, despite the considerable peptide research that has been done, an exact description of TOP's substrate specificity has still not been formulated

PEPTIDE	AA	SEQUENCE AND CLEAVAGE SITES (**Bold** font)
Angiotensin I	11	Asp-Arg-Arg-Val-Tyr-Ile-His-**Pro-Phe**-His-Leu
Angiotensin II	8	Asp-Arg-Val-**Tyr-Ile**-His-Pro-Phe
Bradykinin	9	Arg-Pro-Pro-Gly-**Phe-Ser**-Pro-Phe-Arg
Dynorphin A(1-6)	6	Tyr-Gly-**Gly-Phe**-Leu-Arg
Dynorphin A(1-7)	7	Tyr-Gly-**Gly-Phe**-Leu-Arg-Arg
Dynorphin A(1-8)	8	Tyr-Gly-Gly-Phe-**Leu-Arg**-Arg-Ile
Dynorphin B	13	Tyr-Gly-Gly-Phe-**Leu-Arg**-Arg-Gln-**Phe-Lys**-Val-Val-Thr
LHRH	10	Pyr-**His-Trp**-Ser-**Tyr-Gly**-Leu-Arg-Pro-Gly-NH_2
Neurokinin A	10	His-Leu-Thr-**Asn-Ser**-Phe-**Val-Gly**-Leu-Met-NH_2
Neurotensin	13	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-**Arg-Arg**-Pro-Tyr-Ile-Leu
Substance P	11	Arg-Pro-Lys-**Pro-Gln**-Gln-**Phe-Phe-Gly**-Leu-Met

TABLE 1 8 BIOACTIVE PEPTIDES CLEAVED BY TOP (Dando *et al* , 1993)

1 3 2 7 Inhibitors

As a metalloprotease, TOP is sensitive to exposure to metal-chelating agents such as 1,10-phenanthroline and time dependently to EDTA (Barrett and Brown, 1990) Specific TOP inhibitors are rare, due its broad, unpredictable specificity In addition to this, neurolysin has an extremely similar specificity for TOP substrates and subsequently many inhibitors inactivate both metalloproteases Potent synthetic TOP inhibitors are generally analogues of the Orlowski substrate (Bz-Gly-Ala-Ala-Phe-pAb) in which the Bz-Gly group has been substituted with a Cpp group It is thought that the Cpp group binds to the zinc atom in the active site of the peptidase, with the 3-phenyl ring in the hydrophobic S_1 subsite (Knight and Barrett, 1991)

The most potent inhibitor of TOP was Cpp-Ala-Pro-Phe-pAb, which exhibited an inhibition constant of 1 3nM for human erythrocytes and 7 0nM for rat testis TOP (Dando *et al*, 1993) K_i values of 16nM and 40nM were obtained for the inhibition of human erythrocyte and rat recombinant TOP respectively using Cpp-Ala-Ala-Tyr-pAb (Barrett *et al*, 1995) Knight and Barrett (1991) investigated the effect of the p-aminobenzoate group on TOP inhibition Substitution of the pAb group for a hydroxy (OH) group to produce Cpp-Ala-Ala-Phe-OH, yielded a K_i of 200nM, five-fold poorer than with pAb (Knight and Barrett, 1991) It was believed that it was the negative charge on the pAb group that conferred potency and this was confirmed when K_i values drastically decreased to 7 6μM on removal of the C-terminal negative charge (Knight and Barrett, 1991)

N-[2R,4R)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarbonyl]-L-phenylalanine (SA898), an inhibitor designed by Ukai *et al* (1996), was shown to be a competitive inhibitor of rat TOP IC_{50} and K_i values of 23nM and 9 1nM respectively were obtained The inhibitory effect of SA898 on neprilysin activity was almost negligible The most specific TOP inhibitor to date is Z-(L,D)Phe Ψ $(PO_2\text{-}CH_2)$(L,D)Ala-Arg-Phe, which has a K_i of 0 16nM for TOP and one three orders of magnitude less for neurolysin (Jiracek *et al*, 1995) Activities of other zinc metalloproteases, such as neprilysin and angiotensin-converting enzyme were unaffected by this inhibitor

Substitution of the Phe residue with a methionine, was reported to yield a highly potent TOP inhibitor with a K_i of 70pM Jiracek *et al* (1995) concluded from these results that TOP has a preference for inhibitors containing basic residues such as arginine or lysine in the P_2' position, while neurolysin prefers a proline in this position Exploitation of this finding should allow a strategy for the development of potent TOP *specific* inhibitors

Natural occurring thimet ohgopeptidase inhibitors are few and the enzyme is not inhibited by α_2M, thus confirming its ohgopeptidase specificity Dynorphin A(1-13) has been shown to be a potent naturally occurring inhibitor of TOP activity, with reported K_i values of 11 2nM, 48nM and 26nM for human, rat and chicken TOP respectively (Dando *et al*, 1993)

1 3 2 9 Physiological Importance

Initially it was believed that TOP contributed to the metabolism of collagen based on its hydrolysis of Pz-peptide (Wunsch and Heidrich, 1963) However it is now readily accepted that thimet oligopeptidase is not involved in connective tissue matrix resorption (Barrett *et al*, 1995) *In vitro* studies have been shown TOP to hydrolyse many peptides e g enkephalin-containing peptides, LHRH, bradykinin and neurotensin (which posses important biological functions) Initial *in vivo* tests were not very accurate because the inhibitor used Cpp-Ala-Ala-Phe-pAb was also cleaved by neurolysin This second activity releases Cpp-Ala-Ala on cleavage which inhibits angiotensin-converting enzyme which indirectly supplied misleading results (Cardozo and Orlowski, 1993, Lew *et al*, 1996) Pierotti *et al* (1991) suggested that TOP contributes to hormonal balance by degrading LHRH, explaining its ubiquitous distribution in the testes The location of TOP, inside the cells, implicates a possible intracellular function, possibly participating in the intracellular catabolism of oligopeptides (Barrett *et al*, 1995)

Like prolyl oligopeptidase, TOP has been implicated in the pathogenesis of AD, based on its potential as a γ-secretase and subsequent generation of Aβ (McDermott *et al*, 1992) The ability of this oligopeptidase to cleave APP is unlikely, but as for PO, hydrolysis may occur following primary cleavage by β-secretase (FIGURE 4 7) However, a role of

TOP in AD seems unlikely since Brown *et al* (1996) indicated that TOP was not a γ-secretase, this was later verified by Chevallier *et al* (1997)

It was proposed that TOP in tandem with a multicatalytic proteinase is responsible for the cytoplasmic protein degradation pathway, producing small peptides (Orlowski, 1990) Indications that the enzyme may be involved in the *in vivo* metabolism of opioid peptides were also suggested by Orlowski *et al* (1988) TOP was found to the primary factor responsible for degradation of LHRH in brain and peripheral tissues, administration of Ccp-Ala-Ala-Phe-pAb increased the half-life of centrally and peripherally administered LHRH (Lasdun *et al*, 1989) In summary, TOP appears to have an unregulated biological activity, be fully active on synthesis, and show no evidence of pro-peptide presence Although an exact physiological function cannot be defined, the majority of studies suggest a role in the metabolism of some peptide hormones and bioactive neuropeptides In order for such claims to be substantiated TOP would have to exhibit an extracellular presence However a readily identifiable secretory signal sequence and a hydrophobic spanning domain would be expected for an extracellularly acting peptidase, features which are not present on the deduced TOP protein sequence Crack *et al* (1999) has recently associated thimet oligopeptidase with the extracellular surface of the cell plasma membrane, which is consistent with a putative role in neuropeptide metabolism acting at the external cell surface

1.3 3 Neurolysin

As previously discussed Wunsch and Heidrich (1963) discovered Pz-peptidase, an enzyme present in rat tissues capable of cleaving Pz-peptide A study by Tisljar and Barrett (1990) suggested that there were actually two forms of thimet ohgopetidase, the second a mitochondrial form (thimet peptidase II) which although similar to TOP was not identical Oligopeptidase M is predominantly a mitochondrial peptidase found in the liver of pig and rat (Serizawa *et al*, 1995) It is now accepted that neurolysin is an enzyme identical to oligopeptidase M (Barrett *et al*, 1995) and thimet peptidase II (Tisljar and Barrett, 1990) yet different to TOP, and is classified as a distinct metalloprotease (EC 3 4 24 16)

1 3 3 1 Assay

TOP substrates, such as Pz-peptide, QF01 and QF02, were initially used to detect neurolysin activity However due to a similar substrate specificity of TOP and neurolysin these peptides failed to distinguish the two peptidases Barelli and co-workers (1991) successfully distinguished the two peptidases using neurotensin as substrate Although TOP and neurolysin both hydrolyse this neuropeptide, the cleavage sites and hence cleavage products are distinct and so can be separated by HPLC analysis In general neurolysin cleaves neurotensin at the Pro^{10}-Tyr^{11} bond, while the Arg^{8}-Arg^{9} bond is hydrolysed by TOP (Millican *et al*, 1991) Analysis of neurotensin degradation by HPLC has the advantage of allowing the direct identification of the peptidase, but it is too laborious for repetitive biochemical assays Checler *et al* (1995b) describes a radiometric assay using tritiated neurotensin which can be applied for the identification of neurolysin and is superior over HPLC analysis in its speed and reproducibility

An alternative assay reported for the detection of neurolysin involves using the quenched fluorimetric substrate QF02 (Mcc-Pro-Leu-Gly-Pro-D-Lys-Dnp) and two inhibitors (Serizawa *et al*, 1995) Since PO, TOP and neurolysin all cleave QF02, the inhibitors are required to selectively assay for neurolysin activity Addition of Z-thioprolyl-thiazolidine inhibits PO while inclusion of the antibody R646/P successfully inhibits TOP by 100% This assay can also be employed for the selective detection of thimet

oligopeptidase by incorporating a neurolysin inhibiting antibody, K118 (Serizawa *et al*, 1995) Vincent *et al* (1996) also report the use of QF02 (referred to as QFS) for the detection of neurolysin by including Pro-Ile, a potent neurolysin inhibitor Neurolysin activity can be estimated by comparison of fluorescence recorded in the absence and presence of Pro-Ile Although no specific fluorimetric substrate is available for neurolysin detection, the inclusion of inhibitors into the assay mixture and HPLC analysis of neuropeptide degradation have enabled researchers to isolate and quantify the metalloprotease Unlike TOP, neurolysin does not exhibit a thiol dependence and so there is no need for DTT or 2-mercaptoethanol in assay solutions (Barrett *et al*, 1995)

1 3 3 2 Distribution

High concentrations of neurolysin have been isolated from the cytosol and membrane fractions of a variety of organs, particularly brain (Vincent *et al*, 1996) and kidney (Barelli *et al*, 1993) Checler *et al* (1995) identified the liver as the most abundant source, while Serizawa *et al* (1995) found high activity in rat spleen (see TABLE 1 9) It is thought to be primarily localised in the mitochondrial fraction and to a lesser extent in the cytoplasm, while no activity has been detected in microsomes or lysosomes (Barrett *et al*, 1995) It was neurolysin's mitochondrial localisation that eventually distinguished it from TOP activity (Tisljar and Barrett, 1990) More specifically neurolysin is localised on the inner membrane of the mitochondria (Rioli *et al*, 1998) Woulfe and co-workers (1992) attempted to elucidate the function of neurolysin in neurotensin hydrolysis by localisation of the peptidase in the brain Immunoradioautography revealed an ubiquitous distribution of neurolysin throughout the midbrain, many of the regions previously associated with high concentrations of neurotensin or abundant neurotensin receptors (Woulfe *et al*, 1992)

RAT TISSUE	NEUROLYSIN ACTIVITY (%) *Checler et al , 1995*	NEUROLYSIN ACTIVITY (%) *Serizawa et al , 1995*
Liver	100 0	80 5
Kidney	95 8	38 0
Bladder	80 3	ND
Testis	66 8	45 0
Spleen	49 4	100 0
Brain	26 9	40 5
Lung	23 8	32 0
Heart	14 8	13 0

TABLE 1 9 DISTRIBUTION OF NEUROLYSIN IN RAT ORGANS

Activities are expressed as percentages of the highest activity determined (100%) ND means not determined Both authors used the fluorimetric substrate QF02, Checler *et al* employed the inhibitor Pro-Ile, while Serizawa *et al* used immunoinhibitors to select for neurolysin activity

1 3 3 3 Purification

The first report of neurolysin purification in 1986 employing neurotensin as substrate was carried out on rat brain by Checler *et al* (1986) Following Triton X-100 solubilisation, DEAE anion exchange was performed yielding 15% active neurolysin with a purification factor of 541 A similar protocol was performed by Vincent *et al* (1996) with the addition of a hydroxyapatite chromatography step Although only 95-fold purification was attained, the inclusion of the extra resin served to separate neurolysin from contaminating peptidases including TOP and PO It was claimed that a MonoQ column served the same purpose and successfully distinguished TOP and neurolysin activities in HeLa cells (Krause *et al*, 1997) Barrett and Dando (1998) have raised a neurolysin monoclonal antibody available for academic purposes such as neurolysin purification

1 3 3 4 Biochemical Characteristics

Neurolysin in general has not been well characterised, in comparison to peptidases such as PO and TOP Many early studies on its characterisation were attributed to TOP, since they were believed to be the same enzyme A molecular weight of 70-75kDA has been estimated by SDS PAGE (Checler *et al*, 1986) and gel filtration chromatography on pig neurolysin revealed it to be a monomeric peptidase with a relative molecular mass of 80 76kDa (Barrett *et al*, 1995) Estimations of pI values all fall between pH 5 0 and 6 0 (Checler *et al*, 1986, Tisljar, 1993) On alignment of the amino acid sequences available for TOP and neurolysin, there appeared to be a 60% conservation between peptidases, which would explain the similar biochemical characteristics and specificities observed (Barrett *et al*, 1995)

1 3 3 5 Catalytic Classification and Mechanism of Action

The classification of neurolysin as a member of the thimet oligopeptidase family of metalloproteases (M3), is hardly surprising based on the similarities of the two enzymes Neurolysin unlike many members of the family has been identified as a zinc metalloprotease and like TOP contains the HEXXH motif Unlike TOP, neurolysin is not substantially activated by thiol agents such as DTT, however it is inhibited by the metal chelators EDTA and 1,10-phenanthroline (Tisljar, 1993) Both metallopeptidases contain 13-16 cysteine residues Three of these residues are conserved in TOP but are absent from neurolysin It is believed that the absence of one of these residues, most likely Cys^{246} or Cys^{253} is responsible for neurolysin's unique lack of thiol dependence (Barrett *et al*, 1995)

Amino acid sequences for neurolysin have been reported from pig and rat sources (Sugiura *et al*, 1992, Dauch *et al*, 1995) both studies independently deducing sequences of 704 amino acids Neurolysin appears to have 24 additional residues not present in TOP, which are situated at the N-terminus (Sugiura *et al*, 1992) It is likely that these residues may contain a mitochondrial targeting sequence (Glick *et al*, 1992) which evidently is not required by TOP Comparable to TOP the sequence for neurolysin

suggests no indication of a hydrophobic transmembrane region or glycosylation (Barrett *et al*, 1995)

1 3 3 6 Specificity

Neurolysin has been classified as an ohgopeptidase, hydrolysing peptides containing no greater than seventeen amino acids The enzyme's substrate specificity is common to TOP, both peptidases cleaving Pz-peptide, QF01, QF02 and a range of biologically active peptides Despite similar specificities, the oligopeptidases exhibit disparity in the bonds that they hydrolyse Neurolysin readily cleaves bradykinin and dynorphin A(1-8) at the same cleavage sites as TOP (TABLE 1 8), however TABLE 1 10 illustrates the contrasting cleavage sites for TOP and neurolysin on a number of peptides It is evident that neurolysin hydrolyses dynorphin A(1-17) at the Lys^{11}-Leu^{12} or Leu^{12}-Lys^{13} bond, however there is no hydrolysis by TOP of this peptide, which actually inhibits its activity (Dando *et al*, 1993)

PEPTIDE	AA	SEQUENCE AND CLEAVAGE SITES
Dynorphin A(1-17)	17	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-**Lys-Leu-Lys**-Trp-Asp-Asn-Gln
Neurotensin	13	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-*Arg-Arg*-**Pro-Tyr**-Ile-Leu
QF34	9	Mca-Gly-Gly-Phe-***Leu-Arg***-**Arg**-Ala-Lys(Dnp)
QF37	9	Mca-Gly-Gly-Phe-Ile-*Arg-Arg*-**Ala**-Lys(Dnp)NH_2

TABLE 1.10 BIOACTIVE PEPTIDES CLEAVED BY NEUROLYSIN (Serizawa *et al*, 1995)
Bold font indicates neurolysin hydrolysis site, while *italics* show the TOP cleavage site

1 3 3 7 Inhibitors

Inhibition of neurolysin by peptides known to potently inhibit TOP is not extraordinary due to their extremely similar specificities Barrett *et al* (1995) showed that although neurolysin is sensitive to Cpp-Ala-Ala-Tyr-pAb and its analogues, these inhibitors exert far less potency towards neurolysin than for TOP The most effective and specific neurolysin inhibitor is the dipeptide Pro-Ile, shown to inhibit bran neurolysin with an IC_{50} value of 90μM (Dauch *et al*, 1991) A second inhibitor of bacterial origin, referred to as phosphodiepryl 03 was shown to inhibit neurolysin yielding a K_i of 0 9nM TOP was also sensitive to this compound (K_i 7 5nM), however it had no detrimental affect on neprilysin, PO or angiotensin-converting enzyme activities (Checler *et al*, 1995b) In 1996, Jiracek *et al* reported the development of the first potent and selective inhibitor of neurolysin The need for a specific neurolysin inhibitor, to help elucidate its physiological role, was recognised and screening of phosphinic peptides was the approach taken Pro-L-Pheψ(PO_2CH_2)Gly-Pro was prepared based on the previous discovery that neurolysin had a preference for a proline in the P_2' position (Jiracek *et al*, 1995) The phosphinic peptide displayed a K_i value of 4nM for neurolysin and was 2000 times less potent on TOP activity

1 3 3 8 Physiological Importance

As with the majority of oligopeptidases it is difficult to assign an exact physiological function to neurolysin Its hydrolysis of neuropeptides has led to the belief that it is involved in neuropeptide, particularly neurotensin, metabolism However the bulk of neurolysin in most tissues is detected in the mitochondria and cytosol, an unfavourable location to mediate the degradation of an extracellular peptide such as neurotensin (Barrett and Dando, 1998) Therefore Barrett and Dando propose that neurolysin serves a function in the hydrolysis of intermediates in the turnover of cellular proteins

1 3 4 **Neprilysin**

Neprilysin (EC 3 4 24 11) is a metallopeptidase, which in most cases is bound to the plasma membrane It was first discovered in 1974 (Kerr and Kenny, 1974a) in the brush border membrane of porcine kidney and was known as neutral endopeptidase (EC 24 11) Microvillar structures of the kidney and intestine are enriched with neprilysin although it has also been isolated from many other tissues (see section 1 3 4 3) Neprilysin isolated in the brain became known as enkephalinase due to its ability to hydrolyse the Gly^3-Phe^4 amide bond of opioid enkephalins However the kidney neutral endopeptidase and enkephalinase were soon shown to have identical amino acid sequence (Malfroy *et al*, 1987), and are now both recognised as neprilysin

1 3 4 1 Assay

As for thimet oligopeptidase, there are no substrates specific for the determination of neprilysin activity with many aminopeptidases and chymotrypsin-like enzymes causing interference The most widely practiced assay for neprilysin detection uses synthetic chromogenic peptides such as Glu-Ala-Ala-Phe-4-MeO-2-Na Cleavage of the Ala-Phe bond by neprilysin, followed by the addition of an aminopeptidase, liberates 4-MeO-2-Na which can be spectrophotometrically or fluorimetrically monitored (see Chingwin and Hersh, 1995 for method) In general chymotrypsin-like enzymes compete for the substrate and so phosphoramidon is included in the assay mixture Phosphoramidon is a specific neprilysin inhibitor, conducting the assay in its presence and absence, coupled to the construction of a standard curve of free 4-MeO-2-Na, allows for the quantitation of neprilysin activity More sensitive radiometric and fluorimetric assay methods are often applied for neprilysin detection These assays involve the measurement of [tyrosyl-^{3}H]D-Ala-Gly hydrolysis and the release of internally quenched fluorimetric substrates respectively The most popular fluorogenic substrate used is Dansyl-D-Ala-Gly-Phe(NO_2)-Gly designed by Florentin *et al* (1983) which is readily cleaved at the Gly-Phe bond by neprilysin releasing the dansyl group flouophore (Chingwin and Hersh, 1995) [D-Ala^2, Leu^5]-enkephalin provides a convenient substrate for HPLC analysis (Turner, 1998)

1 3 4 2 Distribution

Neprilysin is an integral glycoprotein located in the plasma membrane of tissues in many diverse species In mammals kidney microvilli are a particularly rich source (Kerr and Kenny, 1974a, Malfroy *et al*, 1987) of the oligopeptidase followed by the epithelial cells of the intestine, lymph nodes and placenta (Danielsen *et al*, 1980) Brain is a common source (Wilk and Orlowski, 1979, Checler *et al*, 1984, Checler *et al*, 1986) however neprilysin activity is several hundred times greater in the kidney (Hardy and Orlowski, 1992) Significant levels of enzyme are localised to muscle cells in the stomach, colon and small intestine while lower activities have been identified in the adrenal glands, testis and lung (Chingwen and Hersh, 1995)

1 3 4 3 Purification

Because neprilysin is exclusively membrane bound, a detergent (e g Triton X-100) treatment is usually required prior to column chromatography Alternatively the use of papain-solubilised neprilysin has been reported which eliminates the need for detergent (Chingwen and Hersh, 1995) A range of purification protocols has been reported, using size exclusion (Kerr and Kenny, 1974), ion exchange (Relton et al, 1983), chromatofocusing and lectin affinity (Chingwen and Hersh, 1995) and hydrophobic interactions chromatography (Danielsen et al, 1980) techniques Purification of neprilysin has been much facilitated by the introduction of immunadsorbent columns harnessing monoclonal antibodies (Danielsen et al, 1980) Purification factors of 23,000 have been reported coupled to enzyme yields of 30% using immunadsorbent chromatography (Relton et al, 1983)

1 3 4 4 Biochemical Characteristics

Neprilysin is an integral membrane-bound zinc metalloprotease In most species it appears to exist as a monomer (Kerr and Kenny, 1974b, Gafford *et al* 1983, Checler *et al*, 1986), although the existence of dimeric subunits has also been reported (Fulcher and Kenny, 1983) Estimated molecular weights vary from 88kDa to 95kDa for the monomeric enzyme, with brain forms showing slightly lower molecular weights This has been attributed to the fact that kidney neprilysin is 15% glycosylated while the brain

enzyme contains only 13% carbohydrate (Relton *et al*, 1983) An isoelectric point between pH 5 0 and 5 3 was reported for porcine kidney neprilysin (Fulcher and Kenny, 1983), while Checler *et al* (1986) used two-dimensional SDS PAGE revealing a pI of 5 9-6 0 Neprilysin displays a broad neutral pH optimum range varying between pH 6 0 (Kerr and Kenny, 1974b) and pH 7 0 (Danielsen *et al*, 1980)

1 3 4 5 Catalytic Classification and Mechanism of Action

Neprilysin is a member of the MA clan of metalloproteases and is sub-classified in to the M13 family It is a zinc metalloprotease closely related to the bacterial enzyme thermolysin, although is over double its molecular weight (Barrett *et al*, 1998b) A histidine and an aspartic acid have been identified as catalytically essential residues in neprilysin and the zinc ligands have also been experimentally determined (Le Moual *et al*, 1993 and 1994) The ammo acid sequence of neprilysin has been elucidated from rat and human sources (Malfroy *et al*, 1987 and 1988 respectively) Neprilysin consists of 742 amino acids, with the presence of a single transmembrane spanning domain located close to the N-terminal (residues 28-50) which is believed to anchor the protease to membranes (Malfroy *et al*, 1987, Devault *et al*, 1987) Residues 1-27 represent the intracellular domain while the extracellular region (containing the active site) consists of the remaining amino acids (52-742) The C-terminal domain of neprilysin contains the classic zinc metalloprotease motif HEXXH, also seen in TOP and neurolysin The three zinc ligands have been identified as His^{583}, His^{587} and Glu^{646} (Turner, 1998)

1 3 4 6 Specificity

Although neprilysin can hydrolyse peptides up to forty amino acids in length, it is still classified as an oligopeptidase as substrate-size limitations do occur and there appear to be a lack of natural inhibitors of the peptidase Catalysis of small oligopeptides is favoured In general neprilysin cleaves peptides on the amino side of hydrophobic residues, unlike TOP The primary prerequisite for efficient catalysis is a substrate containing a bulky hydrophobic residue in the P_1' position (Turner, 1998) Amino acids with aliphatic side chains such as leucine are preferred to aromatic containing residues

e g phenylalanine (Connelly *et al*, 1985) A consensus sequence for efficient neprilysin activity Phe-Phe-Gly-Phe-Leu was proposed (Turner, 1998)

In vivo neprilysin is limited in the peptides that it cleaves Substance P appears to be the most efficiently cleaved (Matsas *et al*, 1984) and reports of bradykinin (Wilk and Orlowski, 1979) and neurotensin (Checler *et al*, 1983 and 1984) hydrolysis have also been reported Neprilysin once referred to as enkephalinase cleaves opioid enkephalins e g Leu- and Met-enkephalinase releasing intact enkephalin, a neurotransmitter involved in pain-related pathways See TABLE 1 11 for cleavage sites of bioactive peptides

PEPTIDE	AA	SEQUENCE AND CLEAVAGE SITES (-- **Bold** font)
[1]Angiotensin I	11	Asp-Arg-**Arg--Val-Tyr--Ile**-His-**Pro--Phe**-His-Leu
[1]Angiotensin II	8	Asp-**Arg--Val-Tyr--Ile**-His-Pro-Phe
[2]Bradykinin	9	Arg-Pro-**Pro--Gly**-Phe-Ser-**Pro--Phe**-Arg
[3]Cholecystokinin-8	8	Asp-Tyr-Met-**Gly--Trp**-Met-**Asp--Phe**-NH_2
[1]Leu-enkephalin	5	Tyr-Gly-**Gly--Phe**-Leu
[1]Met-enkephalin	5	Tyr-Gly-**Gly--Phe**-Met
[4]Neurotensin	13	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-**Pro--Tyr--Ile**-Leu
[5]Substance P	11	Arg-Pro-Lys-Pro-Gln-**Gln--Phe--Phe-Gly--Leu**-Met

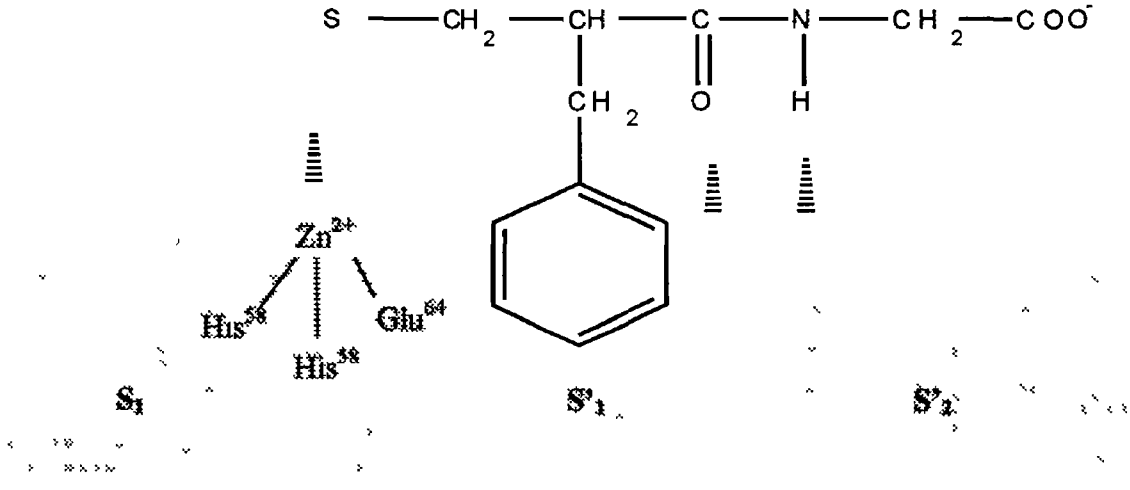
TABLE 1 11 BIOACTIVE PEPTIDES CLEAVED BY NEPRILYSIN

[1] Gafford *et al*, 1983, [2] Wilk and Orlowski, 1979, [3] Turner, 1998, [4] Checler *et al*, 1983, [5] Matsas *et al*, 1984

1 3 4 7 Inhibitors

Neprilysin is inhibited by zinc-chelating reagents such as EDTA (IC_{50} 30μM) and 1,10-phenanthrohne (IC_{50} 0 6mM) based on its absolute requirement for Zn^{2+} (Damelsen *et al*, 1980) Like TOP, neprilysin is also sensitive to the thiol reagents 2-mercaptoethanol and DTT (Fulcher and Kenny, 1983) Alternative neprilysin inhibitors include phosphoramidon, thiorphan and captopril Phosphoramidon an inhibitor of, and isolated from, thermolysin has been reported to yield an IC_{50} value of 10nM for neprilysin (Danielsen *et al*, 1980) Although not a specific inhibitor for neprilysin activity, use of concentrations in the μM region readily inhibit neprilysin yet show no effect on other enzymes in the M13 family, which require higher concentrations Thiorphan and captopril are of similar structure and exhibit inhibition constants of 4nM (Roques *et al*, 1995) and 4 7nM (Checler *et al*, 1983) respectively for neprilysin activity Roques *et al* (1995), generated a schematic illustrating the selective inhibition of neprilysin by thiorphan (FIGURE 1 3) A number of neprilysin specific inhibitors have been synthesised (see Roques *et al*, 1995) based on the general structure of zinc-peptidase inhibitors such as thiorphan

THIORPHAN



ENZYME

FIGURE 1 3 SELECTIVE INTERACTION BETWEEN NEPRILYSIN AND THIORPHAN (Roques *et al*, 1995)

1 3 4 8 Physiological Importance

The most conceivable physiological involvement of neprilysin is in pain and inflammation pathways (Turner, 1998) The generation of enkephalins (neurotransmitters known to relieve pain) emphasises this role and Wilkins (1993) demonstrated an increase in the amount of atrial natriuretic peptides in the circulation following administration of a neprilysin inhibitor It appears highly possible that neprilysin is involved in the metabolism of inflammatory peptides and highlights the potential of specific neprilysin inhibitors in contributing to pain relief

On cloning of the gene, Devault *et al* (1987) discovered an exact correlation between neprilysin and CALLA (common acute lymphoblastic leukemia antigen), a cell surface antigen Although the exact biological function of neprilysin in the immune system remains unclear, CALLA is expressed at various stages in lymphoblastic leukemia and hence can be employed as a marker for the disease (Anderson *et al*, 1984) In addition the CALLA/neprilysin antigen is also expressed in normal bone marrow, spleen and liver cells suggesting a role in metabolising some peptides in normal lymphoid differentiation (Le Bien and McCormack, 1989) Neprilysin may also serve physiological functions in bone metabolism (Howell *et al*, 1993)

1.3 5 Oligopeptidase B

Pacaud and Richaud (1975) first described the isolation of oligopeptidase B (EC 3 4 21 83) a trypsin like peptidase then referred to as protease II from *E coli* Its inability to hydrolyse high molecular weight proteins and the preferential specificity for **B**asic amino acids were the reason the peptidase was subsequently named oligopeptidase **B** A number of different bacterial and plant forms of oligopeptidase B have been isolated namely from, soybean seed (Nishikata, 1984), *Rhodococcus erythropolis* (Shannon *et al*, 1982), *Trypanosoma brucei* (Kornblatt *et al*, 1992) and *Trypanosoma cruzi* (Santana *et al*, 1992) Despite the wide distribution, physiochemical properties vary according to the source

1 3 5 1 Assay

Both a spectrophotometric and fluorimetric assay have been described for oligopeptidase B Benzoyl-Arg-β-naphthylamide (Bz-Arg-βNa) is readily hydroysed by *E coli* and *T brucei* oligopeptidases, releasing βNa which can be measured spectrophotometrically at 550nm (Tsuru and Yoshimoto, 1994) A similar substrate incorporating MCA in place of βNa is employed for more sensitive fluorimetric assays *T brucei* oligopeptidase B can also be fluorimetrically quantified using Z-Phe-Arg-MCA (Caler *et al*, 1998)

1 3 5 2 Distribution and Purification

Oligopeptidases B appear to be distributed widely in plant and prokaryotic cells The *T cruzi* form of the oligopeptidase is expressed in all life cycles of *T cruzi* and is often purified from epimastigotes, a rich source of the protozoan parasite (Burleigh and Andrews, 1998) The enzyme has been purified from *E coli* , *R. erythropolis*, *T brucei*, *Moraxella lacunanta* and soybean seed (Pacaud and Richaud, 1975, Shannon *et al*, 1982, Kornblatt *et al*, 1992, Yoshimoto *et al*, 1995, Nishikata, 1984 respectively) Ammonium sulphate fractionation, ion exchange chromatography and size exclusion chromatography have been described as successful purification tools (Tsuru and Yoshimoto 1994), purification factors from transformant cells ranging from 57 (Yoshimoto *et al*, 1995) to 90 (Kanatam *et al*, 1991)

1 3 5 3 Biochemical Characteristics

The biological properties of the oligopeptidases B vary greatly depending on the source of the enzyme TABLE 1 12 summarises the characteristics of a number of oligopeptidase B forms

SOURCE	MW *kDa*	PI	PH OPTIMUM	THERMAL STABILITY	SUBUNIT
[1]*E coli*	81 8	5 2	8 0	45°C	Monomer
[2]*T cruzi*	120	4 8-5 0	ND	ND	Dimer
[3]*T brucei*	80	5 1	8 2	ND	Monomer
[4]*M lacunata*	80	4 3	6 5	35°C	Monomer
[5]*R erythropolis*	82-90	5 1	7 0-7 2	37°C	Monomer
[6]Soybean seed	59	ND	8 5	ND	Monomer

TABLE 1 12. PHYSIOCHEMICAL PROPERTIES OF OLIGOPEPTIDASES B FROM A NUMBER OF SOURCES

[1]Kanatani *et al*, 1991, [2]Burleigh and Andrews, 1998, [3]Kornblatt *et al*, 1992, [4]Yoshimoto *et al*, 1995, [5]Shannon *et al*, 1982, [6]Nishikata, 1984

1.3.5.4 *Catalytic Classification and Mechanism of Action*

Oligopeptidases B catalyse the hydrolysis of peptides exclusively at the carboxyl side of basic amino acids such as lysine and arginine (Tsuru 1998) All oligopeptidases B are inhibited by the serine protease inhibitors, diisopropyl fluorophosphate (DFP), tosyl-L-lysylchloromethane (TLCK) and leupeptin, justifying their serine protease classification Although *E coli* oligopeptidase B is very similar to trypsin (except for its oligopeptidase specificity), the oligopeptidases B do not belong to the S1 family of trypsin peptidases In fact the oligopeptidases B show a high degree of homology with PO and have hence been classified as members of the S9 family of serine proteases The sequence of the *E coli* peptidase shows a 24-25% homology with PO (Tsuru, 1998), while less similarity (18-22%) is noted between prolyl oligopeptidase and *T cruzi* oligopeptidase B (Burleigh *et al*, 1997)

These members of the S9 family also exhibit the Gly-Xaa-**Ser**-Xaa-Gly-Gly consensus sequence common to the family (Barrett and Rawlings, 1992), where **Ser** is the active-site serine Using radiolabelled DFP, Ser^{532} was identified as the active site of *E coli* oligopeptidase B (Kanatani *et al*, 1991) The remaining members of the catalytic triad were deduced to be Asp^{617} and His^{652} (Tsuru, 1998) In distinction from that found with most serine endopeptidases, oligopeptidase B appears to have a physical rather than chemical rate-limiting step (Polgar, 1997), as is the case for prolyl oligopeptidase (Polgar, 1992c) Although the crystal structures of oligopeptidases B have not been determined, these serine protease oligopeptidases may possess a similar catalytic mechanism to any one of the other α/β fold hydrolases within the S9 family, possibly PO

1.3.5.5 Specificity and Physiological Importance

As the name suggests, oligopeptidases B readily cleave short peptides less than 6kDa and have a preference for basic amino acid residues in the P_1 position. Therefore, basic residues such as arginine and leucine are often incorporated into synthetic substrates, like Bz-Arg-βNa, Bz-Lys-βNa and Z-Phe-Arg-βNa. There are no reports of neuropeptide cleavage by the oligopeptidases B and so biological substrates and functions remain unidentified. A recent publication implicates *T. cruzi* oligopeptidase B in host cell invasion events. Caler *et al.* (1998) showed that the removal of the oligopeptidase B gene reduced host cell invasion, an essential step in the development of infection by intracellular pathogens such as *T. cruzi*. This infers a physiological role for oligopeptidase B from *T. cruzi* as a processing peptidase responsible for the signaling between pathogens and host cells.

1 3 6 Other Oligopeptidases

A number of other oligopeptidases exist most of which have not been well characterised The majority are prokaryotic metalloproteases belonging to the M3 family, however a few have yet to be classified TABLE 1 13 names such oligopeptidases and denotes an insight into their specificity and physiochemical characteristics

1 3 7 Z-Pro-Prolinal Insensitive Z-Gly-Pro-MCA Degrading Peptidase

Cunningham and O'Connor, (1997a) identified a novel proline-specific peptidase from bovine serum related to the oligopeptidase PO This enzyme is detected on hydrolysis of the fluorogenic substrate, Z-Gly-Pro-MCA and differentiates itself from PO in its insensitivity towards Z-Pro-Prolinal and other specific PO inhibitors In addition, the two peptidases can be separated by ion exchange and hydrophobic interactions chromatography This second peptidase has been referred to as ZIP, based on it being a Z-Pro-Prohnal insensitive peptidase and may well exhibit oligopeptidase specificity The aim of this project was to purify ZIP from bovine serum and subsequently characterise to determine biophysical properties, effects of a range of functional reagents and inhibitors and to elucidate its substrate specificity

OLIGOPEPTIDASE	MW *kDa*	PROPERTIES	INHIBITORS	PHYSIOLOGICAL FUNCTIONS
[1]Insulysin	Dimer	pI 5 9	1,10-phenanthroline,	Degradation of insulin and damaged
EC 3 4 24 56	110	pH 7 0-8 5	EDTA	proteins in cells
[2]Oligopeptidase MepB	Monomer	pI 5 6	1,10-phenanthroline,	Intracellular degradation of peptides
	82	pH 7 1	DTT EDTA, DFP,	
[3 4]Oligopeptidase A	Monomer	-	Zn^{2+}, Ni^{2+}, Cu^{2+}	Degradation of signal peptides
EC 3 4 24 70	77		EDTA	
[5]Oligopeptidase F	Monomer	pH 7 0-9 0	1,10-phenanthroline	Protein turnover in cheese making
	90	Temp 40°C	EDTA	
[6]Oligopeptidase O	Monomer	pH 6 0-6 5	EDTA	Cheese fermentation
	71 5	Temp 30-38°C	Cu^{2+}, Zn^{2+}	Degradation of casein derived peptides
		pI 4 62		
[7]Oligopeptidase PepB	Monomer	pI 4 9	-	Possibly peptide transport
	69 6			
[1]Pitrilysin	Monomer	pH 7 4	EDTA, DTT, bacitracin	Possibly turnover of abnormally folded
EC 3 4 24 55	105	pI 5 7	1,10-phenanthroline	proteins
[9]Protease IV	Tetramer	pH 7 2-7 6	DFP, PMSF, leupeptin,	Degradation of signal peptides
	268		antipain	
[10]Saccharolysin	Monomer	pH 5 5-7 9	Cpp-Ala-Ala-Phe-pAb,	Degradation of peptides resulting from
EC 3 4 24 37	81 8		EDTA, mercury	non-vacuolar proteolysis

Continued overleaf

OLIGOPEPTIDASE	CLAN FAMILY	TYPICAL SOURCE	SPECIFICITY AND SUBSTRATES
[1]Insulysin	ME M16	H sapiens, S cerevisiae,	Cleaves amino side of hydrophobic groups
EC 3 4 24 56		D melanogaster	Substrates insulin, glycogen, insulin B chain, Aβ
[2]Oligopeptidase MepB	MX M3	S typhimurium, E coli,	Cleaves N-terminal blocked peptides with at least 4 AA or 5
		H influenzae	AA if unblocked Substrates Ala5, AcAla4
[3 4]Oligopeptidase A	MX M3	L lactis, M genitalium,	Broad specificity for 7-23 residues
EC 3 4 24 70		M pneumoniae	Substrates bradykinin, Mcc-Pro-Leu-Gly-Pro-D-Lys
[5]Oligopeptidase F	MX M3	A fumigatus	Similar specificity as TOP Substrates Pz peptide, collagen
[6]Oligopeptidase O	MA M13	L lactis lactis,	Cleaves hydrophobic residue in P_1', hydrolyses peptides of 5-
		L lactis cremoris	30AA higher affinity for larger peptides
			Substrates oxidised insulin B chain α-, β- or κ- casein
[7]Oligopeptidase PepB	MX M3	S agalactiae	Hydrolyses small basic peptides, length and flexibility rather
			than AA are important Substrates bradykinin, neurotensin
[1]Pitrilysin	ME M16	E coli	Cleaves peptides less than 7kDa
			Substrates insulin B chain, insulin, substance P
[9]Protease IV	UX U7	C jejuni, E coli,	Hydrolyses N-or C- blocked peptides with hydrophobic
		H influenzae	residues in P_1 and P_1' positions, minimum of 6 AA
[10]Saccharolysin	MX M3	S cerevisiae	Hydrolyses N-terminally blocked peptides, hydrophobic
EC 3 4 24 37			residue in P_1 and P_3', proline in P_2' Substrates bradykinin

TABLE 1 13 SUMMARY OF OTHER OLIGOPEPTIDASES

[1]Roth, 1998a, [2] Ibrahim-Granet and d'Enfert, 1998, [3] Conlin and Miller, 1995, [4] Miller, 1998a, [5]Monnet, 1998, [6] Mierau and Kok, 1998, [7]Pritchard, 1998, [8] Roth, 1998b, [9]Miller, 1998b, [10] d'Enfert and Ibrahim-Granet, 1998

2.0. MATERIALS AND METHODS

2 1 MATERIALS

Sigma Chemical Company (Poole, Dorset, England)

α_2M

β-Galactosidase

1, 10-phenanthroline

1, 7-phenanthroline

2-mercaptoethanol

4, 7-phenanthroline

8-hydroxyquinoline

Acetic Acid

Acetone

ADH

AEBSF

Ala-MCA

Ammonium Persulphate

Angiotensin I, II & III

Antipain

APMSF

Aprotinin

Arg-MCA

Bacitracin

Benzamidine

Bisacrylamide

Blotting Paper

Blue Dextran

Boric Acid

Bovine Serum Albumin

Bradykinin

Brilliant Blue G Colloidal Stain

Cadmium Chloride

Cadmium Sulphate

Calcium Sulphate

CAPS Buffer

Carnitine

CDTA

Cellulose Type 50

Chymotrypsin

Cobalt Sulphate

Dithiothreitol

DTNB

EDTA

Egg Albumin

EGTA

Glycine

HRP

IEF Standards

Imidazole

Insulin B Chain

Iodine

Iodoacetate

Iodoacetic Acid

Leupeptin

LHRH

Magnesium Sulphate

Manganese Sulphate

MCA

Mercuric Sulphate

Myosin

Naphthol

N-Ethylmaleimide

Nickel Sulphate

Ninhydrin

Pepstatin A

PMSF

Ponceau S

Potassium Iodide

Potassium Phosphate (Dibasic)

Potassium Phosphate (Monobasic)

Pro-MCA

Puromycin

PVDF Membrane

SDS

Silver Stain High MW Standard Kit

Silver Stain Kit

Sodium Chloride

Sodium Phosphate

Sodium Sulphate

Substance P

Sulphuric Acid

TEMED

TFA

Thioglycolic Acid

Trifluoroacetic Acid

Tris

Trypsin Inhibitor from Soybean

Trypsinogen

Zinc Sulphate

BDH Chemicals Ltd (Poole, Dorset, England)

Acrylamide

Ammonia Solution

Biuret Reagent

Bromophenol Blue

Calcium Chloride

Citric Acid

Clinistix

Copper Sulphate

Dimethylformamide

Dimethylsulphoxide

Dioxane

Fehling's Solution A

Fehling's Solution B

Glacial Acetic Acid

Glycerol

Hydrochloric Acid

Methanol

Polyethylene Glycol 6000

Urea

Bachem Feinchemikalein AG (Bubendorf, Switzerland)

(Arg^{8}) Vasopressin

(Glu^{2})-TRH

(Phe^{2})-TRH

$A\beta_{1\ 28}$

$A\beta_{1\ 48}$

$A\beta_{25\ 35}$

Cyclo-(His-Pro)

Glu-Phe-MCA

Gly-Gly-Pro-Ala

Gly-Phe-Ala

Leu-MCA

Lys-Ala-Ala

Lys-Ala-MCA

Lys-MCA

N_{α}-benzoyl-Arg-MCA

Neurotensin

Pyr-His-Pro-MCA

Pyr-His-Gly

Z-Pro-Ala

Z-Pro-Gly

Gly-Pro-Ala

Gly-Pro-MCA

His-Pro

Leu-Gly

Z-Pro-Leu-Gly

Z-Pro-Pro

Z-Pro-Prolinal dimethylacetate

Merck Chemical Company (Frankfurt, Germany)

Ammonium Sulphate

Potassium Chloride

Sodium Hydrogen Phosphate

Sodium Hydroxide

Novex (San Diego, California, USA)

Anode Buffer

Cathode Buffer

Precast IEF Gels

Pharmacia Fine Chemical Company (Uppsala, Sweden)·

BioSep Sec 300

DEAE-Sepharose Fast Flow

Phenyl Sepharose CL-4B

Polybuffer 74

Polybuffer Exchanger 94

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

Z-Indolinyl-Prolinal

Biorad Laboratories (Hercules, California, USA).

Biogel HT Hydroxylapatite

Keypak Meats (Clonee Co Meath, Ireland):

Bovine Whole Blood

Pierce Chemical Company (Illinois, USA)

BCA Reagent

School of Biotechnology, Dublin City University

Ethanol

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

Fmoc-Ala-Pro-Nitrile

Fmoc-Pro-Pro-Nitrile

Z-Pro-Prolinal

Central Pharmaceutical Research Institute (Japan , Courtesy of Dr K Toide)·

JTP-4819

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

Postatin

NUI Galway (Courtesy of Dr. G O'Cuinn)

Ala-Arg

Ala-Gln

Ala-Pro

Leu-Gly

Lys-Ile

Lys-Tyr

Arg-Ala	Pro-Gly
Arg-Glu	Pro-Leu
Asp-Lys	Pro-Pro
Asp-Tyr	Pyr-Ala
Glu-Lys	Pyr-Gly
Glu-Val	Pyr-Phe
Gly-Pro	Pyr-Val
His-Pro	Z-Pro

IMPC du CNRS, (Valbonne, France, Courtesy of Dr F Checler)·

S170921	S19825

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

Boc-Glu ($NHBO_2$)-Pyr	Z-Phe-Ala-CMK
Ile-Pyrrolidide	Z-Phe-Pro-Methylketone
Ile-Thiazolidide	

2 2 FLUORESCENCE SPECTROMETRY USING 7-AMINO-4-METHYL-COUMARIN (MCA)

2 2 1 MCA Standard Curves

100μM 7-Amino-4-Methyl-Coumarin (MCA) stock solution was prepared containing 2%v/v dimethylsulphoxide (DMSO) in 100mM potassium phosphate, pH 7 4 All lower concentrations required were obtained using 100mM potassium phosphate, pH 7 4 containing 2%v/v DMSO, as diluant and stored at 4°C in the dark (for a maximum of three months) Standard curves were prepared in the MCA concentration ranges 0-10μM and 0-2 5μM as follows

- 100μL 100mM potassium phosphate, pH 7 4
- 400μL appropriate MCA concentration
- 1mL 1 5M acetic acid

Fluorescent intensities were measured using a Perkin Elmer LS-50 Luminescence Spectrophotometer at excitation and emission wavelengths of 370nm and 440nm respectively Emission slit widths were varied according to the extent of the fluorimetric intensities observed, while an excitation slit width of 10nm was maintained All solutions were assayed in triplicate and the mean fluorimetric intensity calculated

2 2 2 Inner Filter Effect

The inner filter effect (filtering) of enzyme samples was determined by combining 100μl of enzyme sample with 400μl MCA solution and 1mL 1 5M acetic acid Filtering of crude serum samples was monitored in the presence and absence of $5x10^{-4}$M Z-Pro-Prolinal or $2\ 5x10^{-4}$M JTP-4819 in 10%v/v methanol (MeOH) as appropriate These samples, prepared in triplicate, were fluorimetrically assayed as outlined in section 2 2 1

2.3 PROTEIN DETERMINATION

2 3 1 Biuret Assay

The Biuret assay was used to qualitatively monitor the elution of protein from a number of chromatography columns Dialysis was performed when necessary using 2 4mm pore visking tubing with a cut off of 12,000-14,000 Da (subsequent references to dialysis also utilised this tubing) to remove any interferents or contaminants such as incompatible buffers Fractions were dialysed against 100mM potassium phosphate, pH 7 4 overnight at 4°C and assayed on a 96-well plate as follows

- 50µL Post-column fraction
- 200µL Biuret Reagent
- Incubated for 30min at 37°C

The Biuret assay was also used to quantify protein in post-column chromatography enzyme pools containing between 2mg and 10mg protein per mL High protein content samples were diluted in 100mM potassium phosphate, pH 7 4 to achieve a concentration suitably determinable by the assay Bovine serum albumin (BSA) standards (0-10mg/mL) were prepared and 50µL of each was added in triplicate to 200µL Biuret reagent in a 96-well microtitre plate Absorbances were determined at 570nm using a Tecan Spectra Plate Reader and a standard curve was constructed Enzyme pools were assayed in parallel and the concentration of protein estimated using the standard curve

2 3 2 Standard BCA Assay

Post-column chromatography enzyme samples containing less than 2mg/mL protein were quantified using the standard bicinchoninic acid (BCA) assay protocol based on the method described by Smith *et al*, (1985) Samples were prepared as outlined in section 2 3 1 and BSA standards in the range 0mg/mL to 1 4mg/mL were included 50µL of sample or standard was assayed, in triplicate with 200µL BCA reagent at 37°C for 30min Absorbance of each sample was recorded at 570nm

The BCA reagent was also employed to follow the elution of protein from chromatography columns as described for the Biuret assay in section 2 3 1 using BCA reagent in place of Biuret reagent

2 3 3 Enhanced BCA Assay

The enhanced BCA assay was employed to determine protein concentrations in samples which could not be accurately quantified using the less sensitive standard BCA assay The protocol was performed as described in section 2 3 2 but at an incubation temperature of 60°C for 30min Samples were first dialysed against 100mM potassium phosphate, pH 7 4, and assayed in triplicate along with BSA standards in the range 0-100μg/mL

2.4. Enzyme Assays

2 4 1 Substrate Preparation

Prolyl oligopeptidase (PO) and Z-Pro-Prohnal insensitive Z-Gly-Pro-MCA degrading peptidase (ZIP) activities were determined using 0 1mM Z-Gly-Pro-MCA as a fluorimetric substrate, according to a modification of the original protocol of Yoshimoto *et al*, (1979) 10mM Z-Gly-Pro-MCA stock substrate solution was prepared in 100%v/v DMSO 100mM potassium phosphate, pH 7 4 at 37°C was slowly added to 300μL DMSO and 100μL stock substrate to a final volume of 10mL Substrate used for the determination of PO activity had 0 015g dithiothreitol (DTT) included, while 0 2922g sodium chloride (NaCl) was added to the substrate on determination of ZIP activity This resulted in a 'ZIP substrate' composed of 0 1mM Z-Gly-Pro-MCA in 4%v/v DMSO containing 500mM NaCl and a 'PO substrate' consisting of 0 1mM Z-Gly-Pro-MCA in 4%v/v DMSO containing 10mM DTT

2 4 2 Z-Gly-Pro-MCA Degrading Activities Assay

Prior to assay, both substrate and enzyme sample were pre-incubated at 37°C to allow them to reach thermal equilibrium 400μL 0 1mM substrate was added to 100μL enzyme sample in triplicate and incubated at 37°C for 60min Reactions were terminated by the addition of 1mL 1 5M acetic acid into each tube Negative controls consisted of 100μL enzyme sample to

which 1mL 1 5M acetic acid was added prior to substrate addition and 37°C incubation Liberated MCA was measured fluorimetrically as described in section 2 2 1 Fluorimetric intensities observed for each sample were converted to picomoles MCA released per min per mL using the appropriate filtered MCA standard curve as generated in section 2 2 2 Enzyme units were thus defined as picomoles of MCA released per min at 37°C (section 6 2)

2 4 3 Z-Pro-Prolinal Insensitive Z-Gly-Pro-MCA Degrading Activities Assay

The activity of ZIP was determined as described in section 2 4 2 in situations where it was certain that it had been separated from other Z-Gly-Pro-MCA degrading peptidases However, ZIP activity in crude bovine serum had to be differentiated from PO activity and so the following modification of the Z-Gly-Pro-MCA assay was necessary 100µL of enzyme sample was pre-incubated for 5min at 37°C with 20µL of $5x10^{-4}$M Z-Pro-Prolinal in 10%v/v MeOH prior to substrate addition Negative controls containing no Z-Pro-Prolinal but incorporating 20µL 10%v/v MeOH were included and determinations were carried out in triplicate MCA liberated from the substrate was determined fluorimetrically as described in section 2 2 1 and fluorimetric intensities were converted to enzyme units as outlined in section 6 2 , where MCA standard curves used had 20µL Z-Pro-Prolinal included Enzyme units were defined as picomoles MCA released per min at 37°C

2.4.4 Non-Quantitative Z-Gly-Pro-MCA Activities Assay

A non-quantitative fluorimetric microtitre plate assay was employed to facilitate the rapid identification of Z-Gly-Pro-MCA degrading activities in post-column chromatography fractions 200µL of 0 1mM Z-Gly-Pro-MCA in 4%v/v DMSO containing 500mM NaCl, at 37°C, was added to 100µL of sample in each well Post-phenyl sepharose fractions were assayed in the presence of 20µL $5x10^{-4}$M Z-Pro-Prolinal in 10%v/v MeOH and in its absence (where 20µL 100mM potassium phosphate, pH 7 4 containing 10%v/v MeOH was included in each well) The microtitre plate was incubated at 37°C for 30min MCA released was determined fluorimetrically as outlined in section 2 2 1 using the Perkin Elmer LS-50 Luminescence Spectrophotometer plate reader attachment

2 5 PURIFICATION OF BOVINE SERUM ZIP

All purification procedures were carried out in a 4°C cold room unless otherwise stated

2 5 1 Serum Preparation

Bovine whole blood was collected from a freshly slaughtered animal and transported to a 4°C cold room where the clot was allowed to shrink After 24hr, the remaining unclotted blood was decanted and centrifuged at 6000rpm for 60min at 4°C using a Beckman J2-MC centrifuge fitted with a JL-10 5 rotor The supernatant and loose cellular debris was decanted and re-centrifuged at 20,000rpm for 15min using a JL-20 rotor The serum (supernatant) was collected, pooled and stored in 20mL aliquots at -17°C

2 5 2 Phenyl Sepharose Hydrophobic Interaction Chromatography

A 30mL phenyl sepharose CL-4B hydrophobic interactions column (2 5cm x 7cm) was equilibrated with 100mL of 100mM potassium phosphate containing 200mM ammonium sulphate, pH 7 4 Crude serum was thawed at 37°C and solid ammonium sulphate ($(NH_4)_2SO_4$) was added to give a final concentration of 200mM $(NH_4)_2SO_4$ 20mL salted serum was applied to the equilibrated column followed by 100mL wash of 100mM potassium phosphate containing 200mM $(NH_4)_2SO_4$ The column was then washed with 100mL 100mM potassium phosphate containing 50mM ammonium sulphate, pH 7 4 Bound protein was eluted isocratically with 100mL distilled water Equilibration was carried out at 1mL/min, while loading, washing and elution steps were performed at 2mL/min 5mL fractions were collected throughout the purification step, which were assayed for ZIP following the microtitre plate assay protocol in section 2 4 4 Protein content in each fraction was determined spectrophotometrically using the Biuret assay as outlined in section 2 3 1 Fractions containing ZIP activity were combined to yield post-phenyl sepharose ZIP The phenyl sepharose resin was regenerated with 100mL wash EtOH at 0 5mL/min, 100mL distilled water wash at 2mL/min followed by equilibration as described previously

2 5 3 Calcium Phosphate Cellulose Chromatography

The calcium phosphate gel was prepared at room temperature 1L of 500mM sodium hydrogen phosphate was added (at a rate of 6mL/min) to 1 5L of constantly stirred 500mM calcium chloride Following a 15min agitation, 1 5mL concentrated ammonia solution was added and stirred for a further 10mm The precipitated gel was allowed to settle and the supernatant decanted and discarded 1L of distilled water was added and stirring continued for 5min Settling and decanting was performed and this procedure was repeated until the gel had been washed with 10L of distilled water The washed calcium phosphate gel was stored in 1L distilled water at 4°C 10g Sigma cellulose type 50 was soaked overnight in 200mL 500mM potassium phosphate containing 150mM potassium chloride, pH 6 8 The cellulose was washed eight times with distilled water and dried overnight at 70°C 2g washed and dried cellulose was dissolved in 20mL 20mM potassium phosphate, pH 7 5 and added to 24mL evenly suspended calcium phosphate gel This calcium phosphate cellulose was poured into a column (2 5cm x 3 0cm) and allowed to settle, yielding an 18ml packed resin

The column was equilibrated at 1mL/min with 10mM potassium phosphate, pH 7 4 The post-phenyl sepharose ZIP was applied to the column followed by a further 100mL wash with 10mM potassium phosphate, pH 7 4 100mL 500mM potassium phosphate, pH 7 4 was applied to elute bound protein Loading, washing and elution steps were carried out at 2mL/min and 5mL fractions were collected throughout the run The standard BCA assay was employed to determine protein in each fraction as described in section 2 3 2 Fractions containing ZIP activity (microtitre plate assay outlined in section 2 4 4) were pooled yielding post-calcium phosphate cellulose ZIP

2 5 4. Diethylaminoethyl Sepharose Anion Exchange Chromatography

100mL 50mM Tris/HCl, pH 8 0 was used to equilibrate a 30mL diethylaminoethyl (DEAE) sepharose anion exchange column (2 5cm x 6cm) at 1mL/min The post-calcium phosphate cellulose ZIP sample was dialysed overnight against 5L of Tris/HCl, pH 8 0 at 4°C The pH of the dialysate was checked prior to application onto the DEAE resin The column was washed with 100mL of equilibration buffer to remove unbound proteins ZIP was eluted with 100mL Tris/HCl containing 200mM NaCl, pH 8 0 The entire procedure was performed at a flow-rate of 1mL/min 5mL fractions were collected and assayed for ZIP activity using the microtitre plate procedure detailed in section 2 4 4 The column was regenerated with 2M NaCl at 1mL/min Removal of Tris buffer by dialysis against 100mM potassium phosphate, pH 7 4 was necessary prior to using the enhanced BCA assay (section 2 3 4) to determine protein content in collected fractions

Pooled post-DEAE fractions represented purified bovine ZIP, which was employed for all characterisation, assay development and localisation studies Dialysis was always performed at 4°C overnight to remove Tris buffer or salt prior to enzyme assays The dialysis buffer employed depended on the application, however 100mM potassium phosphate, pH 7 4 was most commonly used Therefore reference to *purified ZIP* indicates post-DEAE bovine ZIP which had been dialysed extensively against 100mM potassium phosphate, pH 7 4 The purified PO sample used in some of the assays had previously been purified in our laboratory from bovine brain, using fractionation, chromatofocusing, S-200 size-exclusion and phenyl sepharose hydrophobic interactions chromatography (unpublished data) It was extensively dialysed against 100mM potassium phosphate, pH 7 4 unless otherwise stated

2 6 PURITY DETERMINATION

2 6 1 Polyacrylamide Gel Electrophoresis

A discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) system based on the method of Laemmli, (1970) was employed to assess the effectiveness of the purification procedure and to estimate the relative molecular mass of ZIP (section 2 9 2) Electrophoresis was also used to determine the subunit structure of ZIP and the molecular weight of these subunits

2 6 1 1 Sample Preparation

Samples generated during the purification procedure, namely, crude serum, post-phenyl sepharose ZIP, post-calcium phosphate ZIP and post DEAE sepharose ZIP were electrophoresed using SDS PAGE Sample preparation involved an extensive dialysis (18hr) at 4°C against 2L 62 5mM Tris/HCl, pH 6 8, with buffer changes at 3, 6 and 12hr Dialysed samples were diluted with an equal volume of solubilisation buffer which consisted of 62 5mM Tris/HCl, pH6 8, 20%v/v glycerol, 8%w/v SDS, 10%v/v 2-mercaptoethanol and 0 01%w/v bromophenol blue Silver stain high molecular weight standards were also prepared These standards consisted of Myosin (205,000Da), β-Galactosidase (116,000Da), Phosphorylase B (97,000Da), Fructose-6-phosphate kinase (84,000Da), BSA (66,000Da), Glutamic dehydrogenase (55,000Da), Ovalbumin (45,000Da) and Glyceraldehyde-3-phosphate dehydrogenase (36,000Da) Samples in solubilisation buffer and markers were denatured on immersion in a boiling water bath for 90sec and immediately stored on ice prior to application onto the gel

2 6 1 2 Preparation of SDS Gel

TABLE 2 1 lists the stock solutions required for the production of an SDS gel All solutions were prepared in ultra-pure water and from these a 10%v/v resolving gel with a 3 75%v/v stacking gel was poured (see TABLE 2 2) 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 prepared samples and markers were loaded into the wells under the buffer Electrophoresis was carried out a constant current of 25mA per gel for approximately 3 5hr

2 6 1 3 Visualisation of Proteins

Polyacrylamide gels were stained based on the method of Heukeshoven and Dernick (1985) using a Sigma silver stain kit and by brilliant blue G colloidal stain kit from Sigma TABLES 2 3 and 2 4 outline the stages performed An image of each stained gel was captured using an Olympus digital camera coupled to Olympus C-2 OW95E software

SOLUTION	COMPOSITION
Acryl/bisacyrl solution	30%w/v acrylamide, 0 8%w/v bisacrylamide
Ammonium persulphate	1 5%w/v ammonium persulphate
Resolving gel buffer	3M Tris/HCl, pH 8 8
Running buffer	25mM Tris/HCl, 192mM glycine, 0 1%w/v SDS, pH 8 3
SDS	10%w/v SDS
Stacking gel buffer	500mM Tris/HCl, pH 6 8

TABLE 2 1 SDS PAGE BUFFER PREPARATION

SOLUTION	10%V/V RESOLVING GEL	3.75%V/V STACKING GEL
	Volume	*Volume*
Acryl/bisacyrl solution	10mL	2 5mL
Ammonium persulphate	1 5mL	1mL
Resolving gel buffer	3 75mL	-
SDS	300µL	200µL
Stacking gel buffer	-	5mL
TEMED	15µL	15µL
Ultra-pure water	14 45mL	11 3mL

TABLE 2.2. SDS PAGE GEL PREPARATION

STEP	REAGENT	VOLUME	TIME
Fixing	30%v/v ethanol in 10%v/v glacial acetic acid	3 x 300mL	2 x 10min 1 x overnight
Rinsing	Ultra-pure water	3 x 300mL	3 x 20min
Silver staining	Silver nitrate (kit)	300mL	30min
Rinsing	Ultra-pure water	300mL	10sec
Developing	Sodium carbonate formaldehyde (kit)	2 x 150mL	2 x 8min
Development stop	1%v/v glacial acetic acid	300mL	5min
Rinse	Ultra-pure water	300mL	10min

TABLE 2 3 SILVER STAINING PROCEDURE

STEP	REAGENT	VOLUME	TIME
Fixing	30%v/v EtOH 10%v/v glacial acetic acid	3 x 300mL	2 x 10min 1 x overnight
Rinsing	Ultra-pure water	3 x 300mL	3 x 10min
Staining	80%v/v brilliant blue 20%v/v MeOH	300mL	Overnight
Destaining	10%v/v acetic acid 25%v/v MeOH	300mL	60sec
Rinsing	25%v/v MeOH	3x300mL	2 x 10min 1 x overnight

TABLE 2 4 BRILLIANT BLUE G COLLOIDAL STAINING PROCEDURE

2 6 2 The Activity of ZIP Using Fluorimetric Substrates

A range of fluorimetric substrates was assayed in the presence of purified bovine serum ZIP to assess the enzymatic purity of the sample TABLE 2 5 lists the substrates used and the peptidases which they are most commonly used to detect Each substrate was prepared as a 10mM stock in 100%v/v MeOH 100mM potassium phosphate, pH 7 4 was heated to 37°C and added to 100µL each stock substrate and 300µL MeOH to a final volume of 10mL This yielded a final substrate concentration of 0 1mM in 4%v/v MeOH ZIP activity was also assayed in 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH, containing no NaCl, which acted as a control Purified ZIP was incubated with each substrate according to section 2 4 2 and fluorescent intensities were recorded

2 6 3. Purity Assessment using Reverse Phase HPLC

Fluorimetric substrates listed in TABLE 2 5 were again used to assess the purity of ZIP using HPLC analysis 100µL purified dialysed ZIP was incubated with 400µL 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 500mM NaCl at 37°C for 1hr The reaction was terminated by the addition of 25µL 5%v/v trifluoroacetic acid (TFA) Blanks were included in which TFA was added prior to substrate addition These samples were then analysed by ion-pair HPLC using a Varian Star system coupled to a fluorescent detector A Technicol Exsil 5 C_{18} analytical column (220mm x 4 6mm) and a Beckman Ultrasphere 4 6mm x 4 5mm guard column were employed The column was equilibrated at 1mL/min with 10mL solution of 79 9%v/v MeOH, 20%v/v distilled water containing 0 1%v/v TFA 20µL of sample was applied to the column followed by a 10min wash with equilibration buffer, at a flow rate of 1mL/min Column eluant was constantly monitored at excitation and emission wavelengths of 370nm and 440nm respectively with appropriate slit widths

SUBSTRATE	CLEAVED BY
Ala-MCA	Alanine aminopeptidase
Arg-MCA	Arginine aminopeptidase
Glu-Phe-MCA	Chymotrypsin
Gly-Pro-MCA	DPP IV
Leu-MCA	Leucine aminopeptidase
Lys-Ala-MCA	DPP II
Lys-MCA	Aminopeptidase B
Nα-benzoyl-Arg-MCA	Papain
Pyr-His-Pro-MCA	Pyroglutamyl aminopeptidase I
Pyr-MCA	Pyroglutamyl aminopeptidase II
Pro-MCA	Proline aminopeptidase
Z-Arg-MCA	Trypsin
	Papain
	Soya-bean trypsin-like enzyme
Z-Gly-Pro-MCA	Prolyl oligopeptidase
	ZIP
Z-Phe-Arg-MCA	Plasma kallikrein
	Cathepsin B
	Cathepsin L
	Papain
	Ohgopeptidase B

TABLE 2 5 FLUORIMETRIC SUBSTRATES TESTED

2 7 ASSAY DEVELOPMENT

2 7 1 MCA excitation and emission wavelengths

The excitation and emission wavelengths for MCA were tested using the luminescence spectrophotometer scan functions 1mL 5μM MCA in 0 2%v/v MeOH was placed in a glass cuvette and a pre-scan was performed The excitation wavelength obtained from the pre-scan was set and an emission scan was performed from 400-600nm at 5nm increments Similarly an excitation scan was performed between 250nm and 420nm using the previously determined optimum emission wavelength Optimal wavelengths were taken to be those at which fluorescence was at a maximum

2 7.2. Substrate Solvent Determination

10mM Z-Gly-Pro-MCA stock substrates were prepared in 100%v/v DMSO, dimethylformamide (DMF), dioxane (DXN), EtOH and MeOH These stock solutions were diluted with 100mM potassium phosphate, pH 7 4 to a final concentration of 0 1mM Z-Gly-Pro-MCA in 4%v/v solvent containing 500mM NaCl as described in section 2 4 1 Purified bovine serum ZIP was assayed in triplicate with suitable negative controls to determine the effect of these substrate solvents on enzyme activity

2 7 3 Solvent Concentration Determination

A range of substrates was prepared in order to assess the optimum methanol concentration for use in the Z-Gly-Pro-MCA degrading activities assay 0 1mM Z-Gly-Pro-MCA solutions containing 500mM NaCl in 2%, 3%, 4%, 5% and 6%v/v MeOH were prepared in 100mM potassium phosphate, pH 7 4 as described in section 2 4 1 Purified ZIP was assayed in triplicate using these substrates as outlined in section 2 4 2

2 7 4. Linearity of Enzyme Assays with Respect to Time

300µL purified ZIP and 1 2mL of 0 1mM Z-Gly-Pro-MCA containing 500mM NaCl in 4%v/v MeOH were pre-incubated separately at 37°C for 15min to allow each to reach thermal equilibrium A blank consisting of 1mL substrate and 300µL enzyme was used to auto-zero the fluorimeter Substrate and enzyme were combined in a pre-warmed cuvette and the fluorescence was continuously monitored for 60min at 37°C in an incubated cuvette holder Liberated MCA was measured at optimum excitation and emission wavelengths every 2sec and plots of fluorescent intensity versus time were prepared to determine the linearity of ZIP activity over time The experiment was also carried out using crude bovine serum in the presence and absence of Z-Pro-Prohnal 300µL crude serum was pre-incubated for 5min at 37°C with 20µL $5x10^{-4}$M Z-Pro-Prohnal prior to addition of substrate and continuous monitoring of liberated MCA Blanks were incorporated as before but 20µL of either inhibitor was included

2.7.5. Linearity of Enzyme Assays with Respect to Enzyme Concentration

A range of dilutions of purified ZIP was prepared in 100mM potassium phosphate, pH 7 4 Enzyme dilutions were assayed with 0 1mM Z-Gly-Pro-MCA containing 500mM NaCl in 4%v/v MeOH as described in section 2 4 2 Plots of fluorescent intensities versus enzyme concentration were prepared

2.7 6 Optimum Assay Temperature

Purified ZIP was assayed at 4°C, 20°C, 30°C, 32°C, 37°C, 39°C, 40°C, 50°C and 65°C by a modification of the assay described in section 2 4 2 Both the substrate and enzyme sample was pre-incubated at the appropriate temperature for 15min prior to the assay to ensure that thermal equilibrium had been reached

2 7 7 Effect of DTT on ZIP Activity

2mL aliquots of 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 500mM NaCl were prepared incorporating 0, 4, 8, 12 and 20mM dithiothreitol (DTT) Purified, dialysed ZIP was assayed in triplicate with this range of substrates as outlined in section 2 4 2

2 7 8 Effect of NaCl on ZIP Activity

2mL aliquots of 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH were prepared incorporating 0, 200, 300, 400, 500, 600, 700, 800, 900 and 1000mM NaCl Purified, dialysed ZIP was assayed with this range of substrates in triplicate as outlined in section 2 4 2

2 7 9 Prolyl Oligopeptidase Inhibitor Studies

In order to assay for ZIP activity alone in crude samples, it was necessary to find an inhibitor that was capable of inhibiting PO activity, yet effect no inhibition of ZIP Z-Pro-Prolinal was the potent prolyl oligopeptidase inhibitor initially employed, however due to depletion of the supply, an alternative inhibitor was required

2 7 9 1 Z-Pro-Prolinal Studies

A range of Z-Pro-Prohnal concentrations ($5x10^{-5}$ to $5x10^{-15}$M) was prepared in 10%v/v MeOH 20µL inhibitor was added to 100µL crude sample followed by the addition of 400µL 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 500mM NaCl Samples were incubated for 1hr at 37°C and the reaction terminated by the addition of 1 5M acetic acid Suitable controls and blanks were also included The effect of pre-incubation was also studied by incubating 20µL $5x10^{-4}$M Z-Pro-Prohnal with 100µL crude sample for 0, 5, 10, 15, 20, 25 and 30min Following this pre-incubation, samples were assayed at 37°C as outlined in section 2 4 2

2 7 9 2 Alternative Inhibitors

TABLE 2 6 lists the other inhibitors whose potency towards PO was examined A stock concentration of each inhibitor was prepared in a minimum volume of an appropriate solvent (DXN, DMSO or MeOH) From these stock solutions, a range of dilutions was prepared using 100mM potassium phosphate, pH 7 4 incorporating the appropriate solvent and maintaining its concentration Each inhibitor was tested to assess PO and ZIP sensitivity towards them The reaction was performed as outlined in section 2 4 3 , where 20µL each inhibitor concentration was added to 100µL serum and incubated with 0 1mM Z-Gly-Pro-MCA for 60min at 37°C Acetic acid was used to terminate the reaction and appropriate

blanks and controls were incorporated The effect of pre-incubating serum with 2 5x10 ^{4}M JTP-4819 for 0, 5, 10, 15, 20, 25 and 30min prior to substrate addition was also examined

INHIBITOR	STOCK CONCENTRATION *M*	PREPARATION
Z-Pro-Prolinal Dimethylacetate	5x10 ^{5}	10%v/v DXN
Fmoc-Pro-Pro-CN	2 6x10 ^{5}	1%v/v DMSO
Fmoc-Ala-Pro-CN	5x10 ^{4}	1%v/v DMSO
Z-Indolinyl-Prohnal	1x10^{-4}	13%v/v DXN
Postatin	1x10^{-4}	10%v/v DXN
JTP-4819	1x10 ^{2}	10%v/v MeOH

TABLE 2 6 PREPARATION OF ALTERNATIVE INHIBITORS

2 7.10 Optimised ZIP Assay

Based on the results observed in the assay development experiments, the ZIP and PO activities assay was modified slightly Substrate was prepared as a 10mM Z-Gly-Pro-MCA stock solution as described in section 2 4 1 except that 100% MeOH was used in place of DMSO This stock was subsequently employed to prepare 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH by combination of 100µL stock substrate solution and 300µL MeOH, made to a final volume of 10mL with 100mM potassium phosphate, pH 7 4 at 37°C 500mM NaCl or 10mM DTT was incorporated as described in section 2 4 1 for the detection of ZIP and PO activities respectively These substrate solutions were then employed in the Z-Gly-Pro-MCA degrading activities assays described in sections 2 4 2 and 2 4 3 Subsequent MCA solutions were prepared as outlined in section 2 2 1 except that the solubilisation solvent was changed from DMSO to MeOH

Initially ZIP and PO activities in crude samples were differentiated by the inclusion of $5x10^{-4}$M Z-Pro-Prolinal (sections 2 4 3 and 2 4 4), however, as explained in section 2 7 9, an alternative inhibitor was required and based on the results, JTP-4819 was selected as the substitute PO inhibitor Therefore, experiments to differentiate ZIP and PO activities in crude samples subsequent to the assay development section, incorporated $2.5x10^{-4}$M JTP-4819 in place of $5x10^{-4}$M Z-Pro-Prolinal but were otherwise performed as described in section 2 4 3 The linearity of the Z-Gly-Pro-MCA degrading activity assay with respect to time was reassessed as outlined in section 2 7 4 but including 20µL $2.5x10^{-4}$M JTP-4819 These modifications to the assay were applied throughout the characterisation protocols following

2 8 LOCALISATION STUDIES

2 8 1 Tissue Preparation

Liver, lung, spleen, kidney and brain samples were obtained from a freshly sacrificed bovine and transported to the laboratory on ice Each organ was divided into approximately 25g portions which were stored at -17°C if not being used immediately A 25g piece of each tissue was suspended in 100mL 100mM potassium phosphate buffer, pH 7 4 These were

homogenised at full speed for 15sec using a Sorvall Omni Mixer, followed by 15sec intermission to minimise heating and foaming effects Homogenisation was continued until the tissue in question was visually disrupted The homogenate (H) was then collected and centrifuged at 15,000rpm for 45min at 4°C in a Sorvall RC-SB refrigerated centrifuge fitted with an SS-34 rotor The supernatant was decanted and retained (S_1) The pellet was resuspended in 25mL ice-cold 100mM potassium phosphate buffer, pH 7 4 and re-centrifuged at 15,00rpm for 45min The supernatant was again decanted (S_2) The homogenate, pellet and two supernatants obtained for each tissue sample were assayed for total protein, PO and ZIP activity as described below

2 8 2. Identification of Z-Gly-Pro-MCA Degrading Activities

The substrate preparation and assays used to detect the presence of Z-Gly-Pro-MCA degrading activities in the tissue samples was as described in sections 2 4 1, 2 4 2 and 2 4 3 incorporating the modifications described in section 2 7 9 The inclusion of 2 5x10^{-4}M JTP-4819 in 10%v/v MeOH allowed for the identification of ZIP alone The total Z-Gly-Pro-MCA activity present was obtained in the absence of the potent inhibitor and PO activity was taken to be the residual non-ZIP activity Due to the high-colour content of the samples, the effect of filtering was investigated for the pellet, homogenate and supernatants of each tissue type MCA standards were prepared (in 4%v/v MeOH) in the range 0-8μM and the extent of filtering was determined as outlined in sections 2 2 1 and 2 2 2 Enzyme levels were quantified using the slopes of the filtered standard curves and the fluorescence intensities observed as outlined in section 6 2

2 8 3 Protein Determination

The concentration of protein present in each sample was measured using the biuret assay as outlined in section 2 3 1 and specific activities of ZIP and PO were calculated for each tissue (section 6 2)

2 9 CHARACTERISATION

2.9 1 Determination of N-terminal Protein Sequence

The N-terminal amino acid sequence of ZIP was determined commercially at the University of Durham, England, by Dr John Gilroy It was required that the enzyme be electroblotted onto a stained polyvinylidene diflouride (PVDF) membrane and this was achieved as follows

2 9 1 1 SDS Polyacrylamide Gel ELectrophoresis

An SDS PAGE was pre-cast as outlined in section 2 6 1 2 and stored overnight at 4°C to prevent N-terminal blocking by free amines 1mL post DEAE sepharose ZIP was dialysed against 1L 62 5mM Tris/HCl, pH 6 8 at 4°C overnight and then diluted in an equal volume of solubilisation buffer (see section 2 6 1 1) Denaturation was initiated by boiling for 90sec Molecular weight markers listed in section 2 6 1 1 were again employed for this PAGE and were prepared in the same manner

The gel was pre-run at 50V for 30min with 200µM thioglycolic acid in the upper reservoir to provide a scavenger for free radicals The chamber was emptied and rinsed with reservoir buffer and further electrophoresis was carried out at a constant current of 25mA for 3hr The gel was not stained but was prepared for electroblotting

2 9 1 2 PVDF Electroblotting

1L 10X CAPS buffer was prepared by dissolving 23 13g CAPS in 900mL ultra-pure water and adjusting the pH to 11 0 using 2M sodium hydroxide (NaOH) The volume was then adjusted to 1L Electroblotting buffer (1X CAPS in 10%v/v MeOH) was prepared and stored at 4°C The gel was removed from the electrophoresis chamber and equilibrated in electroblotting buffer for 1hr Meanwhile the PVDF membrane was dipped in MeOH for 10sec and also equilibrated for 1hr in electroblotting buffer

A transblotting sandwich was constructed using 8 layers of pre-soaked filter paper (Sigma electrophoresis grade blotting paper) cut to the exact size of the SDS gel The PVDF membrane was gently placed on top of the filter paper followed by the gel Eight more layers of wet filter paper was added to complete the sandwich A sterile pipette was rolled over the sandwich surface to expel air bubbles and the filter paper was extensively moistened Electroblotting was carried out at a constant current of 76 8mA (0 8mA x area of gel in cm^2) for 1hr 10min Removal of the membrane was followed by a rapid membrane wash in ultra-pure water The blot was then saturated in 100% MeOH for 10sec and stained in Ponceau S (Sigma) for 3min Destaining in 1%v/v acetic acid was repeated three times and the blot was extensively rinsed in ultra-pure water The blot was allowed to completely air dry and the corresponding ZIP band was identified using the molecular weight markers and marked with a ball point pen The blot was wrapped in cling-film, stored and transported at -17°C for sequencing

2.9 2 Relative Molecular Mass Determination

The relative molecular mass of the purified bovine ZIP was determined using polyacrylamide gel electrophoresis and size exclusion chromatography

2 9 2 1 SDS Polyacrylamide Gel Electrophoresis

The SDS PAGE of samples and standards has previously been outlined in section 2 6 1 The gel obtained in the assessment of enzyme purity was also employed to estimate the relative molecular mass of the purified enzyme and to determine the number of sub-units present A standard curve representing the log of molecular weight versus the relative mobility (R_f) was constructed for each of the molecular weight markers electrophoresed (see TABLE 3 3) The R_f is defined as the distance migrated by the standard or sample divided by the distance migrated by the bromophenol blue dye front The relative molecular weight of ZIP was determined by calculating its R_f value and using the calibration curve to estimate the log of the molecular weight

2 9 2 2 Size Exclusion Chromatography

The relative molecular mass of ZIP was estimated using three size-exclusion chromatography columns namely HiPrep Sephacryl S-200, Sephacryl S-200 and Biosep SEC 3000 For all methods a flow rate of 1mL/min was maintained TABLE 2 7 lists the molecular weight standards and the concentrations at which they were used All standards were prepared in 100mM potassium phosphate, pH 7 4 and samples and standards analysed using S-200 resins had 20%v/v glycerol added prior to application on to the column Blue dextran at a concentration of 2mg/mL was used to calculate the void volume (V_o) for each of the columns Each standard was individually applied and its elution volume (V_e) determined A graph of V_e/V_o versus log of molecular weight was constructed for each of the resins (TABLE 2 7 lists which standards were employed for each resin) ZIP samples were concentrated five-fold using PEG 6000 prior to analysis and V_e/V_o determined for the purified enzyme Using the calibration curves constructed, the molecular weight of ZIP was estimated by each of the three methods The elution of enzyme activity from each column was monitored using the microtitre plate assay as described in section 2 4 4

2 9 2 2 1 S-200 Sephacryl HR Size Exclusion Chromatography

A 200mL Sephacryl S-200 HR size exclusion column (2 5cm x 40cm) was poured and equilibrated with 300mL 100mM potassium phosphate containing 150mM NaCl, pH 7 4 at 4°C 2mL of each standard or blue dextran were applied directly to the top of the resin, under the buffer head using a syringe The column was washed with equilibration buffer until the protein of interest had eluted 2 5mL fractions were collected and the absorbance of each measured using the biuret assay as outlined in section 2 3 1 A calibration graph of V_e/V_o versus log of molecular weight was constructed for the six standards and the molecular weight of ZIP was estimated using the equation of this line

STANDARD	M W *kDa*	SOURCE	CONCENTRATION *mg/mL*
Myosin [1 2 3]	205	Bovine muscle	4 6
ADH [1,2 3]	150	Baker's yeast	2 0
β-Galactosidase [2 3]	116	*E coli*	2 0
BSA [1 2 3]	66	Bovine serum	4 0
Ovalbumin [1 2]	45	Ova	2 0
Horseradish peroxidase [1,3]	44	Horseradish root	2 0
Trypsinogen [2 3]	25	Bovine pancreas	2 0
Chymotrypsin [1]	24	Bovine pancreas	2 0

TABLE 2 7 MOLECULAR WEIGHT MARKERS USED IN SIZE-EXCLUSION CHROMATOGRAPHY

[1] Standards employed for section 2 9 2 2 1

[2] Standards employed for section 2 9 2 2 2

[3] Standards employed for section 2 9 2 2 3

2 9 2 2 2 FPLC Size Exclusion Chromatography

A BioRad BioLogic Chromatography System was used to perform fast protein liquid chromatography (FPLC) The system consisted of a HR Workstation with two biocompatible dual piston pump-heads, fixed wavelength (214nm and 280nm) UV detector and conductivity detector A model 2128 fraction collector was attached to the detector outlet The size exclusion column employed was a Pharmacia HiPrep Sephacryl S-200 (16mm x 60mm) Equilibration was performed at 1mL/min using 300mL 100mM potassium phosphate containing 150mM NaCl, pH 7 4 1mL standard or purified ZIP was injected followed by a 200mL wash 100mM potassium phosphate containing 150mM NaCl, pH 7 4 at 1mL/min The elution of each sample was continuously monitored using the UV detector at 280nm ZIP elution was determined using Z-Gly-Pro-MCA as described in section 2 4 4 A graph of V_e/V_o versus log of molecular weight was generated and the molecular weight of ZIP determined

2 9 2 2 3 HPLC Size Exclusion Chromatography

A Shimadzu LC-9A liquid chromatography system coupled to a Shimadzu SPD-6AV UV-VIS Spectrophotometric detector was used to carry out high performance size exclusion chromatography A Phenomonex BioSep SEC-3000 column (7 8mm x 300mm) was fitted to the system and equilibrated with 100mM potassium phosphate, pH 7 4 20µL sample was applied using a micro-syringe and eluted from the column with 30mL equilibration buffer at 1mL/min The elution of each standard and concentrated ZIP sample was monitored by continuous scanning of the column eluant at 280nm Retention time determinations were made using LDC Analytical thermochrom software (version 3 86) from which a plot of V_e/V_o versus log of molecular weight was constructed for this system

2 9.3. Thermostability Studies

2 9 3 1 Stability at Various Temperatures over Time

The effect of pre-incubation on ZIP activity was determined at various temperatures over a range of time points Purified enzyme was pre-incubated at 20, 30, 40, 50, 60 and 70°C 500µL aliquots were removed after 0, 10, 20, 30, 40 and 50min and then assayed as outlined in section 2 7 10

2 9 3 2 Overnight Stability

The stability of ZIP at 37°C overnight was assessed prior to the substrate specificity studies by HPLC analysis Purified ZIP was incubated at 37°C for 14hr Enzyme aliquots of 500µL were taken at time 0, 1, 2, 4, 6, 8, 10, 12 and 14hr and ZIP activity measured as described in section 2 7 10

2.9 4. pH Effects

2 9 4 1 pH Inactivation

5mL purified bovine ZIP was dialysed for 7hr against 4L distilled water at 4°C with water changes at 1 and 3hr 50µL dialysate was pre-incubated at 37°C with 50µL of each of the buffers listed in TABLE 2 8 The concentration of each buffer was 50mM and the pH increments were 0 5 pH units The effect of pH on the inactivation of ZIP was determined by assaying these samples with 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 500mM NaCl, as outlined in section 2 7 10

pH Range	Buffer	pH Adjuster
2 0-6 0	Citric acid	50mM potassium phosphate dibasic (K_2HPO_4)
6 0-8 0	Potassium phosphate	50mM potassium phosphate monobasic (KH_2PO_4)
8 5-9 0	Tris	0 1M HCl
9 5-10 5	Glycine	0 1M NaOH

TABLE 2 8 BUFFER SYSTEMS USED IN pH INACTIVATION AND ACTIVATION OF ZIP

2 9 4 2 pH Activation

The activation of ZIP at a range of pH values was determined in a manner similar to that outlined in section 2 9 4 1 50µL dialysed enzyme was pre-incubated for 15min with 50µL buffer (TABLE 2 8) at 37°C Z-Gly-Pro-MCA was prepared in a range of 50mM buffer systems at various pH values 400µL of each substrate was added to the appropriate sample buffer pH and ZIP activity was determined as detailed in section 2 7 10

2 9 5 Isoelectric Point Determination

2 9 5 1 Chromatofocusing Chromatography

A 20mL polybuffer exchanger (PBE 94) column was poured (1 5cm x 10cm) and equilibrated with 300mL 50mM imidazole/HCl pH 7 8 at 1mL/min Polybuffer 74 was diluted 1 8 in distilled water and the pH adjusted to 4 5 using HCl 15mL of this was applied to the column at 1mL/min, immediately after equilibration Post DEAE sepharose ZIP was dialysed overnight against 5L 50mM imidazole/HCl pH 7 8 at 4°C Post-dialysis enzyme was applied to the top of the chromatofocusmg resin followed by 300mL polybuffer, pH 4 5 wash again at 1mL/min 5mL fractions were collected throughout and assayed for ZIP activity using the microtitre plate assay as described in section 2 4 4 using the substrate preparation method described in section 2 7 10 The pH of each fraction was also measured and a plot of both fluorescent intensity and pH versus elution volume was constructed

2 9 5 2 Isoelectric Focusing

A vertical isoelectric focusing (IEF) gel was employed to determine the isoelectric point (pI) of ZIP Pre-cast gel, cathode and anode buffers were purchased from Novex Post DEAE sepharose ZIP was extensively dialysed (18hr) against 1L 100mM potassium phosphate, pH 7 4 at 4°C to remove contaminants and interferents with buffer changes after 3 and 6hr Dialysed samples were diluted with an equal volume of sample buffer (TABLE 2 9)

Isoelectric focusing markers were purchased from Sigma (TABLE 2 10) and reconstituted as directed Gels were prepared in an ATTO vertical electrophoresis system (160mm x 160mm x 1mm) The bottom of the chamber was filled with 1X anode running buffer, the gels placed in position and the upper chamber filled with cathode buffer 20μL of the prepared samples and markers were loaded into the wells under cathode buffer Electrophoresis was carried out at 100V for 60min, 200V for 60min and 500V for 30min, the current running from 5mA–6mA/gel Staining was performed as described in TABLE 2 4 using brilliant blue G colloidal stain and the image was captured using a UVP white /UV transluminator camera unit driven by ImageStore 7500 software A standard curve representing the pI versus the R_f was constructed for each of the IEF markers electrophoresed The R_f represents the distance migrated by the standard or sample divided by the distance to the anoidic lip of the gel The pI of ZIP was estimated using the calibration curve by calculating its R_f value from the stained gel

SOLUTION	COMPOSITION
Anode buffer (50X)	4 7%w/v phosphoric acid
Cathode buffer (10X)	3 5%w/v arginine, 2 9%w/v lysine
Sample buffer	20%v/v 10X cathode buffer 30%v/v glycerol

TABLE 2 9 IEF BUFFERS EMPLOYED FOR PI DETERMINATION OF ZIP

MARKER	SOURCE	PI
Amyloglucosidase	*Aspergillus niger*	3 6
Trypsin Inhibitor	Soybean	4 6
β-Lactoglobulin A	Bovine Milk	5 1
Carbonic Anhydrase II	Bovine Erythrocytes	5 9
Carbonic Anhydrase I	Human Erythrocytes	6 6
Myoglobin	Horse Heart	6 8-7 2
Lentil Lectin	*Lens culinaris*	8 2-8 8
Trypsinogen	Bovine Pancreas	9 3

TABLE 2 10 IEF MARKERS EMPLOYED FOR PI DETERMINATION OF ZIP

2 9 6 Catalytic Classification

Stock solutions of a range of functional reagents were prepared, as presented in TABLE 2 11 , to aid in the catalytic classification of ZIP All stock solutions were prepared in 50mM KH_2PO_4 or K_2HPO_4, or both, ensuring a final pH of 7 4 Water insoluble reagents were first dissolved in a minimum volume of acetone and then the volume and pH adjusted using the phosphate species Lower concentrations of each functional reagent were achieved using 50mM KH_2PO_4 or K_2HPO_4, pH 7 4 ± acetone as appropriate (TABLE 2 11) 25mL post-DEAE sepharose ZIP was dialysed against 4L of 50mM potassium phosphate pH 7 4 overnight at 4°C 50µL dialysed enzyme was pre-incubated for 15min at 37°C with an equal volume of each functional reagent under investigation Positive controls were included where the functional reagent was replaced with 50mM phosphate buffer, pH 7 4 containing the appropriate %v/v acetone as the corresponding reagent ZIP activity was determined fluorimetrically in triplicate as detailed in section 2 7 10 The fluorescent filtering effect of these functional reagents was assessed by the construction of unfiltered and filtered MCA standard curves as described in sections 2 2 1 , 2 2 2 and 2 7 10

CLASS	REAGENT	STOCK CONC [1]	PREPARATION
Serine protease inhibitors	AEBSF	45mM	Buffer
	APMSF	20mM	5%v/v acetone
	Aprotinin	2 5mg/mL	Buffer
	Benzamidine	20mM	Buffer
	Leupeptin	2mM	Buffer
	PMSF	5mg/mL	Buffer
Metallo-protease inhibitors	1,10-phenanthroline[3]	40mM	10%v/v acetone
	1,7-phenanthroline[3]	30mM	10%v/v acetone
	4,7-phenanthroline[3]	40mM	10%v/v acetone
	8-hydroxyquinoline[3]	20mM	5%v/v acetone
	CDTA[2]	20mM	Buffer
	EDTA	40mM	Buffer
	EGTA[2]	20mM	Buffer
	Imidazole	20mM	Buffer
Cysteine protease inhibitors	DTNB	20mM	Buffer
	Iodoacetamide	20mM	Buffer
	Iodoacetic acid	40mM	Buffer
	NEM	20mM	Buffer
Cysteine protease activators	2-mercaptoethanol	2%v/v	Buffer
	DTT	20mM	Buffer

TABLE 2 11 REAGENTS USED IN THE CATALYTIC CLASSIFICATION OF ZIP

Buffer is 50mM KH_2PO_4 or K_2HPO_4, or both, ensuring a final composition of 50mM potassium phosphate, pH of 7 4

[1] Concentration of the functional reagent before added to the enzyme (i e 2X)

[2] Samples had to be heated at 37°C to achieve dissolution

[3] Samples had to be maintained at 50°C until diluted

2.9.7 Effect of Other Functional Reagents

The effect of a range of other functional reagents on ZIP activity was also tested. As in section 2.9.6, all stock solutions were prepared in 50mM monobasic or dibasic anhydrous potassium phosphate or both with a final pH of 7.4. Addition of minimum volume acetone aided the dissolution of water-insoluble compounds and as before 50mM potassium phosphate pH 7.4 ± acetone was used as diluant. 6mL of purified ZIP was dialysed against 2L of 50mM potassium phosphate pH 7.4 overnight at 4°C. 50µL dialysed enzyme was pre-incubated for 15min at 37°C with an equal volume of each functional reagent to be tested. Positive controls and blanks were included and ZIP activity was determined fluorimetrically in triplicate as detailed in section 2.7.10. The effect of fluorescent filtering by these functional reagents was assessed as outlined in section 2.2.2.

REAGENT	INHIBITS	STOCK	PREPARATION
Antipain	Papain, Trypsin	5mg/mL	Buffer
Bacitracin	Protein proteases	2mg/mL	Buffer
Carnitine	PO	20mM	Buffer
Pepstatin A	Pepsin, Renin HIV-I protease	2mg/mL	5%v/v acetone
Puromycin	Protein proteases	2mg/mL	Buffer
Trypsin Inhibitor	Trypsin	2mg/mL	Buffer

TABLE 2.12. PREPARATION OF OTHER FUNCTIONAL REAGENTS TESTED

Buffer is 50mM KH_2PO_4 or K_2HPO_4, or both, ensuring a final composition of 50mM potassium phosphate, pH of 7.4. Pepstatin A was initially dissolved in 100%v/v acetone and adjusted to stock concentration by addition of 50mM potassium phosphate buffer.

2.9 8 Effect of Metal Ions on ZIP Activity

The effects of metal salts on ZIP activity was assessed using Ca, Cd, Co, Cu, Mg, Mn, Hg, Na, Ni, Zn sulphates 2mM stock solutions were prepared in either 50mM Tris or 50mM potassium phosphate buffer as outlined in TABLE 2 13 The pH was adjusted to 7 4 using 100mM HCl or 50mM KH_2PO_4 Post anion exchange ZIP was dialysed against 4L 50mM Tris or 50mM potassium phosphate buffer as appropriate for 5hr at 4°C 50µL metal salt and 50µL dialysed enzyme was pre-incubated in triplicate at 37°C for 15min Negative controls had 1mL 1 5M acetic acid added prior to incubation while positive controls consisted of 50µL preparation buffer in place of the metal salt Following pre-incubation, substrate containing no sodium chloride and prepared in either 50mM Tris/HCl or 50mM potassium phosphate buffer, pH 7 4 was added and assayed as described in sections 2 4 2

METAL	CONCENTRATION	PREPARATION AND DIALYSIS BUFFER
	mM	*50mM*
$CaSO_4$	2 0	Tris/HCl
$CdSO_4$	2 0	Tris/HCl
$CoSO_4$	2 0	Potassium phosphate[1]
$CuSO_4$	2 0	Potassium phosphate
$HgSO_4$	2 0	Tris/HCl[2]
$MnSO_4$	2 0	Potassium phosphate[3]
$MgSO_4$	2 0	Tris/HCl
$NaSO_4$	2 0	Tris/HCl
$NiSO_4$	2 0	Tris/HCl
$ZnSO_4$	2 0	Tris/HCl

TABLE 2 13 PREPARATION OF METAL SALTS TESTED ON ZIP ACTIVITY

[1] Heating at 37°C required

[2] Initially dissolved in 1M HCl then diluted with Tris, final pH 7 4

[3] Readily falls out of solution

2 9.9. Carbohydrate Analysis

A number of carbohydrate tests were performed to detect the presence of glycan moieties in purified ZIP

2 9 9 1 Fehling's Test for Reducing Sugars

Equal volumes of Fehling's solutions A and B were added 2mL of this Fehling's working solution were incubated with 1mL dialysed ZIP at 100°C for 15min The assay was performed in triplicate and a control was included where the enzyme had been replaced by 100mM potassium phosphate, pH 7 4 A brick-red precipitate denoted the presence of reducing sugars

2 9 9 2 Molisch, Glucose and Starch Tests

1 drop of Mohsch's reagent (10%w/v naphthol in 95%v/v ethanol) was added to 1mL dialysed ZIP, followed by the addition of 2mL concentrated sulphuric acid The presence of a violet colour at the junction of the two liquids denoted a positive test Clinistix reagent strips were used to detect the presence/absence of glucose in ZIP Three drops of I_2/KI solution was added to 1mL dialysed ZIP to test for starch A blue-black colour was indicative of the presence of starch

2 9 9 3 Glycoprotein Carbohydrate Estimation

A Pierce Glycoprotein Carbohydrate Estimation Kit was purchased to determine whether ZIP was a glycoprotein and subsequently to deduce the percentage of carbohydrate present 10mM sodium meta-periodate was prepared using the sodium meta-periodate and glycoprotein assay buffer provided Glycoprotein detection reagent was supplied and a 5%w/v solution was prepared in 1N NaOH 1mL glycoprotein assay buffer was used to reconstitute each vial of standard or negative control 1mL post DEAE sepharose ZIP was dialysed against 3L 100mM sodium phosphate containing 150mM NaCl, pH 7 2 at 4°C overnight The protein content of the dialysate was measured using the enhanced BCA assay (section 2 3 3) and ZIP was concentrated using PEG 6000 until the protein concentration was at 0 25mg/mL 50µL sample, standard or control was placed in the well of a microtitre plate in triplicate 25µL 10mM sodium meta-periodate was added to each well and the plate was mixed for 30seconds

and incubated at room temperature for 10min 150µL glycoprotein detection reagent was then added to the wells followed by plate shaking and room temperature incubation for 60min A dark purple colour indicated the presence of carbohydrates The absorbance of each well was recorded at 550nm using a Tecan Spectra Plate Reader A calibration curve of absorbance at 550nm versus total carbohydrate content was constructed for the standard and the percentage of carbohydrate in ZIP was estimated using the equation of the line

2 9 10 Oligopeptidase Determination

Classification of ZIP as an oligopeptidase was attempted using high performance liquid chromatography and fluorescence analysis α_2-macroglobulin (α_2M), BSA and insulin B-chain were each prepared at a concentration of 1mg/mL in 100mM potassium phosphate, pH 7 4, the latter containing 10%v/v MeOH

2 9 10 1 Reverse Phase HPLC Analysis

Purified ZIP and PO from bovine serum and brain respectively were dialysed against 100mM potassium phosphate, pH 7 4 overnight at 4°C 400µL α_2M, BSA or insulin B-chain was incubated with 50µL dialysed ZIP or PO at 37°C for 24hr Negative controls consisting of buffer in place of the enzymes and blanks were also included The assay was terminated by the addition of 25µL 0 5%v/v TFA The resulting solutions were analysed by ion-pair HPLC (Varian Star model, coupled to a UV-VIS detector) A Technicol Exsil 5 C_{18} analytical column (220mm x 4 6mm) coupled to a Beckman Ultrasphere 4 6mm x 4 5mm guard column, was employed The columns were equilibrated for 10min with 70%v/v buffer A, 30%v/v buffer B (TABLE 2 14 for buffer compositions) at a flow-rate of 1mL/min 20µL of each post-incubation sample, control and blank were applied at 1mL/min followed by a 4mL wash of 30%v/v buffer A, 70%v/v buffer B Elution was achieved by a 6mL linear gradient of increasing MeOH (30%v/v buffer A, 70%v/v buffer B to 0%v/v buffer A, 100%v/v buffer B), again at 1mL/min The absorbance of the eluant was continuously monitored at 214nm A 4mL wash of 100%v/v buffer B, succeeded by a 10mL equilibration was carried out between each injection

COMPONENT	BUFFER A	BUFFER B
	%v/v	%v/v
Ultra-pure Water	94 85	20 00
HPLC-grade MeOH	5 00	79 90
TFA	0 15	0 10

TABLE 2 14. BUFFER COMPOSITION USED IN HPLC ANALYSIS

Buffers were filtered through a 0 45µm hydrophilic polypropylene membrane filter and degassed prior to use

2 9 10 2 Fluorimetric Analysis

A range of dilutions of α_2M were prepared in 100mM potassium phosphate, pH 7 4 50µL purified ZIP which had been dialysed against phosphate buffer was added to 50µL of each α_2M dilution These samples were pre-incubated for 15min at 37°C prior to substrate addition and assaying as detailed in sections 2 4 2 and 2 7 10

2 9.11 Substrate Specificity

2 9 11 1 Ion-Pair Reverse Phase HPLC Analysis

HPLC analysis was carried out to determine the substrate specificity of ZIP towards a number of synthetic and naturally occurring proline-containing bioactive peptides A Varian Star Chromatographic System was employed with a Technicol Exsil 5 C_{18} analytical column (220mm x 4 6mm) and a Beckman Ultrasphere guard column (4 6mm x 4 5mm) TABLES 2 15 and 2 16 list the prohne-containing peptides tested and the buffers required for their preparation 100mM potassium phosphate, pH 7 4 was always used as diluant and in the cases of water-insoluble peptides, the substance was initially dissolved in a minimum volume solvent followed by phosphate buffer addition 400µL of each prohne-containing peptide was incubated with 50µL purified, dialysed ZIP or PO at 37°C for 24hr Negative controls (no enzyme) and blanks (no peptide) were also included 25µL 0 5%v/v TFA was added to

terminate the reaction after 24hr 20µL of each post-incubation sample, control and blank were applied at 1mL/min to the column which had been equilibrated with 30%v/v buffer A, 70%v/v buffer B (see TABLE 2 14 for buffer composition) A 4mL wash of 30%v/v buffer A, 70%v/v buffer B was performed followed by a 6mL linear gradient elution from 30%v/v buffer A, 70%v/v buffer B to 0%v/v buffer A, 100%v/v buffer B at 1mL/min A further 5mL wash of 100%v/v buffer B was required to elute certain peptides from the C_{18} column The column was equilibrated between injections The absorbance of the eluant was continuously monitored at 214nm

2 9 11 2 Cadmium Ninhydrin Assay

The cadmium ninhydrin assay is based on a modification of the method described by Doi *et al*, (1981) (see FIGURE 4 8 for reaction mechanism) This assay was performed in an attempt to determine whether or not ZIP could cleave a number of dipeptides 5mL purified ZIP and PO were dialysed against 2L 100mM borate buffer, pH 7 4 for 4hr at 4°C Borate was chosen in preference to phosphate to minimise interference effects A range of dipeptides was prepared in 100mM borate buffer, pH 7 4, as outlined in TABLE 2 17 10µL of each peptide was incubated with 50µL ZIP or PO and 40µL 100mM borate pH 7 4 at 37°C for 1hr The assay was terminated by the addition of 700µL of cadmium ninhydrin reagent and 100µL distilled water The cadmium ninhydrin reagent was prepared by dissolving 0 96g ninhydrin in 96mL ethanol and adding 12mL 100% acetic acid To this, 1 2g of cadmium chloride which had been dissolved in 1 2mL distilled water was added The samples were incubated at 84°C for 5min and the absorbance read at 505nm Blanks were included which consisted of the cadmium ninhydrin reagent being added and the samples being heated to 84°C for 5min prior to the 37°C incubation Negative controls were also included where 50µL of borate was added in place of enzyme

SYNTHETIC PEPTIDES	CONCENTRATION *mM*	PREPARATION
Cyclo-(His-Pro)	5 0	Buffer
Gly-Ala-Phe	1 3	Buffer
Gly-Gly-Pro-Ala	2 0	10%v/v MeOH
Gly-Phe-Ala	5 0	Buffer
Gly-Pro-Ala	5 0	10%v/v MeOH
Lys-Ala-Ala	5 0	Buffer
Pyr-His-Gly	5 0	Buffer
Pyr-His-Pro	5 0	Buffer
Z-Gly-Pro-Ala	1 3	10%v/v MeOH
Z-Pro-Ala	1 3	10%v/v MeOH
Z-Pro-Gly	1 3	10%v/v MeOH
Z-Pro-Leu-Gly	1 3	10%v/v MeOH
Z-Pro-Pro	1 3	Buffer

TABLE 2.15 PREPARATION OF HPLC SYNTHETIC PROLINE-CONTAINING PEPTIDES

Buffer refers to 100mM potassium phosphate, pH 7 4 which was also used as the diluant for water insoluble peptides

PEPTIDE	CONCENTRATION *mM*	BUFFER
$A\beta_{1\ 28}$	0 15	50%v/v acetic acid
$A\beta_{1-43}$	0 045	Buffer
$A\beta_{25-35}$	0 2	0 4%v/v TFA
Angiotensin I	2 0	Buffer*
Angiotensin II	2 0	Buffer*
Angiotensin III	2 0	Buffer*
(Arg^8)- Vasopressin	1 0	Buffer
Bradykinin	2 0	Buffer
(Glu^2)-TRH	1 0	10%v/v MeOH
LHRH	2 0	10%v/v MeOH
Neurotensin	2 0	10%v/v MeOH
(Phe^2)-TRH	2 0	10%v/v MeOH
Substance P	1 5	10%v/v 1 5M Acetic Acid*
TRH	1 3	10%v/v MeOH
TRH-Gly	2 0	10%v/v MeOH

TABLE 2 16. PREPARATION OF HPLC BIOACTIVE PROLINE-CONTAINING PEPTIDES

* Samples had to be sonicated in ultrasonic water bath to achieve complete dissolution Buffer refers to 100mM potassium phosphate, pH 7 4 which was always used as diluant

DIPEPTIDES	CONCENTRATION *mM*	PREPARATION
Ala-Arg	5 0	Buffer
Ala-Gln	5 0	Buffer
Ala-Pro	5 0	Buffer
Arg-Ala	5 0	Buffer
Arg-Glu	5 0	Buffer
Asp-Lys	5 0	Buffer
Asp-Tyr	5 0	20%v/v MeOH
Glu-Lys	5 0	Buffer
Glu-Val	5 0	Buffer
Gly-Pro	5 0	Buffer
His-Pro	5 0	Buffer
Leu-Gly	5 0	Buffer
Lys-Ile	5 0	Buffer
Lys-Tyr	5 0	Buffer
Pro-Gly	5 0	Buffer
Pro-Leu	5 0	40%v/v MeOH
Pro-Pro	5 0	Buffer
Pyr-Ala	5 0	Buffer
Pyr-Gly	5 0	Buffer
Pyr-Phe	5 0	Buffer
Pyr-Val	5 0	Buffer
Z-Pro	5 0	20%v/v MeOH

TABLE 2 17 PREPARATION OF DIPEPTIDES USED IN THE CADMIUM NINHYDRIN ASSAY

Buffer refers to 100mM borate, pH 7 4 which was also used as the diluant for water insoluble peptides

2 9 11 3 Kinetic Analysis

2 9 11 3 1 K_M determinations for Z-Gly-Pro-MCA

Stock solutions of 0 5mM Z-Gly-Pro-MCA in 15%v/v MeOH containing either 500mM NaCl or 10mM DTT were prepared 2 5mL 10mM Z-Gly-Pro-MCA in 100%v/v MeOH (section 2 7 10), 5 625mL MeOH and 1 461g NaCl or 0 077g DTT were made up to a final volume of 50mL with 100mM potassium phosphate, pH 7 4 pre-warmed to 37°C Using these solutions a range of dilutions were prepared in 100mM potassium phosphate buffer, pH 7 4 containing 500mM NaCl or 10mM DTT Purified ZIP and PO were assayed with each concentration of the appropriate substrate in triplicate as outlined in sections 2 4 2 and 2 7 10 Various kinetic models were applied to the data and the Michaelis constant (K_M) for ZIP and PO were estimated using Z-Gly-Pro-MCA (section 6 4 1)

2 9 11 3 2 K_i Determinations for Proline-containing Peptides

The effect of a variety of proline-containing peptides on ZIP and PO activities for Z-Gly-Pro-MCA was determined The peptides tested and their preparation is outlined in TABLE 2 18 A 500μM stock solution of Z-Gly-Pro-MCA in 10%v/v MeOH was prepared containing either 10mM DTT or 500mM NaCl for PO or ZIP respectively A range of dilutions were prepared from 0-500μM using 100mM potassium phosphate, pH 7 4 containing 10mM DTT or 500mM NaCl as appropriate as diluant The MeOH concentration was maintained at 10%v/v 1mL of peptide (TABLE 2 18) was added to an equal volume of each substrate dilution ZIP and PO activities were assayed in triplicate using these mixtures (sections 2 4 2 and 2 7 10) The data was applied to a number of kinetic models and the inhibition constant (K_i) and type of inhibition observed were determined as outlined in sections 6 4 2 and 6 4 3 respectively

PEPTIDE	STOCK CONC *mM*	PREPARATION	ASSAY CONC *mM*
$A\beta_{1\,43}$	0 09	Buffer	0 06
Angiotensin I	0 10	Buffer*	0 07
Angiotensin II	0 10	Buffer*	0 07
Angiotensin III	0 10	Buffer*	0 07
(Arg^8)-Vasopressin	0 02	Buffer	0 01
Bradykinin	0 20	10%v/v MeOH	0 13
(Glu^2)-TRH	0 02	10%v/v MeOH	0 01
LHRH	0 40	10%v/v MeOH	0 27
(Phe^2)-TRH	0 40	10%v/v MeOH	0 27
Substance P	0 10	10%v/v 1 5M acetic acid	0 07
TRH	0 10	10%v/v MeOH	0 07
TRH-Gly	0 10	10%v/v MeOH	0 07
Gly-Gly-Pro-Ala	1 00	Buffer	0 67
Z-Gly-Pro-Ala	0 13	10%v/v MeOH	0 09

TABLE 2 18 PREPARATION OF PEPTIDES FOR K_i DETERMINATIONS

Buffer refers to 100mM potassium phosphate, pH 7 4

* Samples had to be sonicated in ultrasonic water bath to achieve complete dissolution

2 9 12 Inhibitor Studies

The potency of a range of peptidase-specific inhibitors towards ZIP and purified PO was assessed TABLE 2 19 outlines the preparation and stock concentration of the inhibitors under investigation Stock solutions were diluted in 100mM potassium phosphate, pH 7 4 (± solvent as appropriate) or distilled water to produce a range of serial dilutions of each inhibitor 1mL of each dilution was added to 1mL of double strength substrate, resulting in a final substrate concentration of 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 500mM NaCl for ZIP and 0 1mM Z-Gly-Pro-MCA in 4%v/v MeOH containing 10mM DTT for PO 100µL of purified ZIP or PO was incubated in triplicate with 400µL of appropriate substrate for 1hr at 37°C Suitable blanks and controls were included Due to a shortage of PO, only four inhibitor concentrations were tested for this enzyme The IC_{50} for each inhibitor were determined for PO and ZIP as explained in section 6 5

INHIBITOR	STOCK CONCENTRATION *M*	PREPARATION
Boc-Glu-($NHOB_2$)-Pyr	8.6×10^{-4}	10%v/v DXN
Fmoc-Ala-Pro-CN	1×10^{-3}	10%v/v MeOH
Fmoc-Pro-Pro-CN	2×10^{-4}	10%v/v MeOH
Ile-Pyrrolidide *	1×10^{-3}	10%v/v MeOH
Ile-Thiazolidide *	1×10^{-3}	10%v/v MeOH
JTP-4819	2.5×10^{-2}	10%v/v MeOH
Postatin	8×10^{-4}	20%v/v DXN
S-17092-1	1×10^{-5}	Distilled water
S-19825	1×10^{-5}	Distilled water
Z-Phe-Ala-CMK	5×10^{-5}	10%v/v acetone
Z-Phe-Pro-Methylketone	1×10^{-3}	Potassium phosphate
Z-Pro-Prolinal	5×10^{-5}	10%v/v MeOH
Z-Pro-Prolinal dimethylacetate	1×10^{-3}	10%v/v MeOH

TABLE 2 19. PREPARATION OF SPECIFIC INHIBITORS

All inhibitors listed are PO-specific except for * which are DPP IV inhibitors 100mM potassium phosphate, pH 7 4 was used as diluant in the preparation of each stock solution, except for S-17092-1 and S-19825, which were prepared in 100%, distilled water

3.0. RESULTS

3 0 RESULTS

The majority of upcoming graphs illustrate data points that represent the average of triplicate fluorescence or absorbance readings, minus the blank measurement All error bars represent the standard error of the mean of these triplicate values (see section 6 1)

3 1. MCA STANDARD CURVES AND THE INNER FILTER EFFECT

MCA standard curves were prepared as outlined in section 2 2 1 Plots of fluorescent intensity versus MCA concentration were constructed for both MCA ranges and the slope of the lines calculated FIGURES 3 1 1 and 3 1 2 represent typical MCA standard curves at emission slit widths of 5nm and 10nm respectively Demonstration of the inner filter effect was also performed as described in section 2 2 2 to assess the effect on fluorescence of including crude serum or post column fractions in the assay mixture FIGURES 3 1 3 and 3 1 4 are plots of fluorescence versus MCA concentration for crude serum and the post phenyl sepharose pool Graphs for post calcium phosphate cellulose and post DEAE are not shown since the inner filter effect was not observed for these samples TABLE 3 1 lists the slopes calculated for each curve and the degree of filtering observed for enzyme samples

SAMPLE	R^2	SLOPE	FILTERING %
10nm, 5nm			
Buffer	0 998	68 82	0 0
Serum	0 999	58 99	14 3
Serum + 5x10 ^{4}M Z-Pro-Prolinal	0 995	59 03	14 2
Serum + 2 5x10 ^{4}M JTP-4819	0 997	59 07	14 2
Post-phenyl sepharose	0 999	65 99	4 1
10nm, 10nm			
Buffer	0 998	296 26	0 0
Post-calcium phosphate cellulose	0 997	295 81	0 2
Post DEAE	0 999	296 00	0 0

TABLE 3 1 SLOPES OF FILTERED STANDARD CURVES

FIGURE 3 1 1

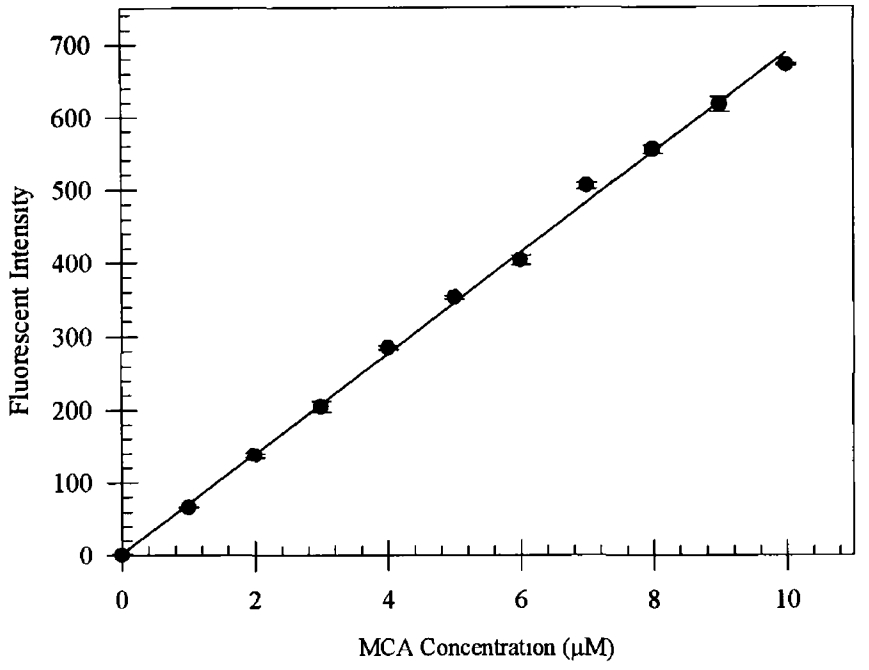
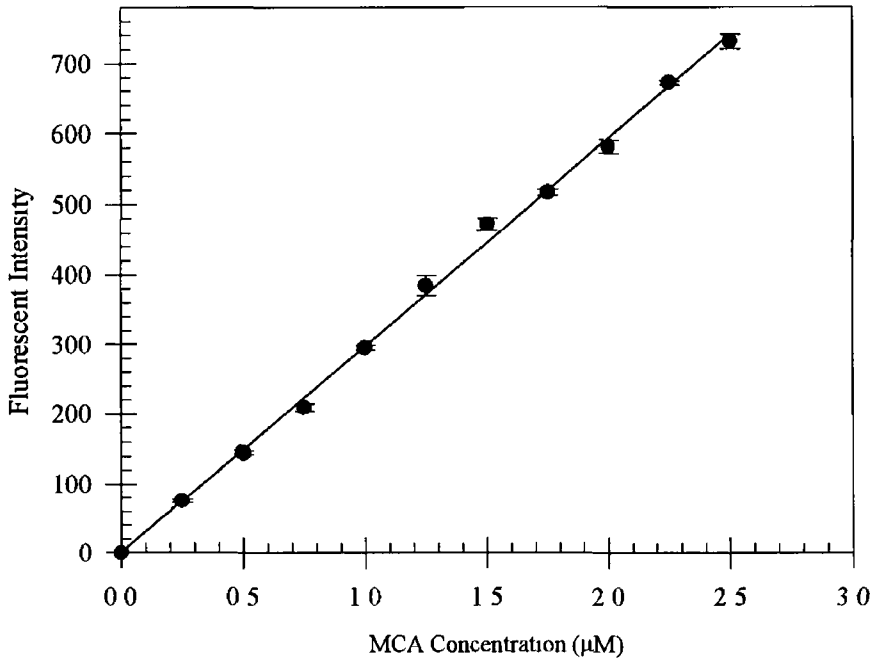


FIGURE 3 1 2.



FIGURES 3 1 1 and 3 1 2 MCA STANDARD CURVES

Plot of fluorescent intensity versus MCA concentration Excitation slit width was maintained at 10nm, while emission slit widths were 5nm and 10nm for FIGURES 3 1 1 and 3 1 2 respectively

FIGURE 3 1 3

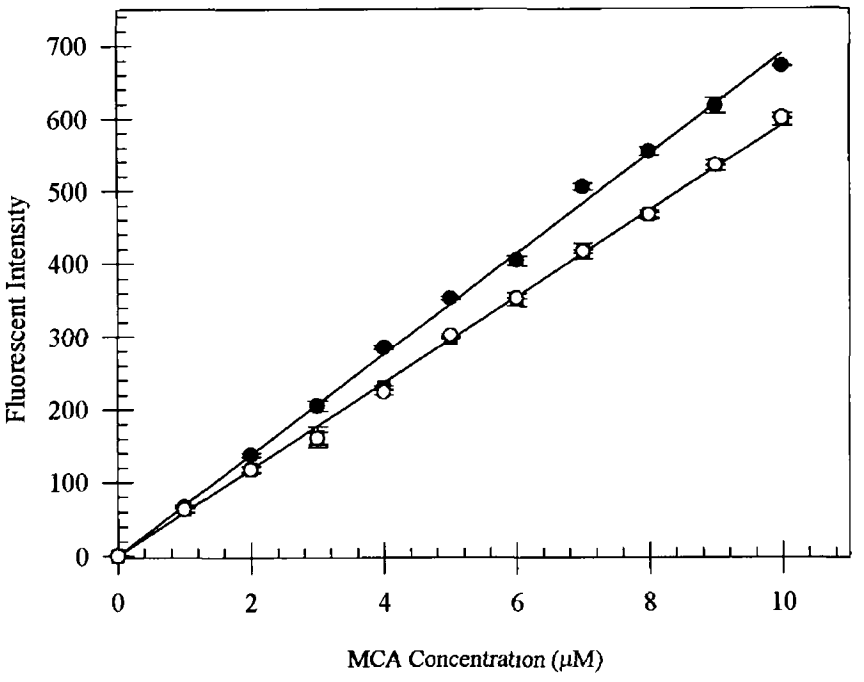
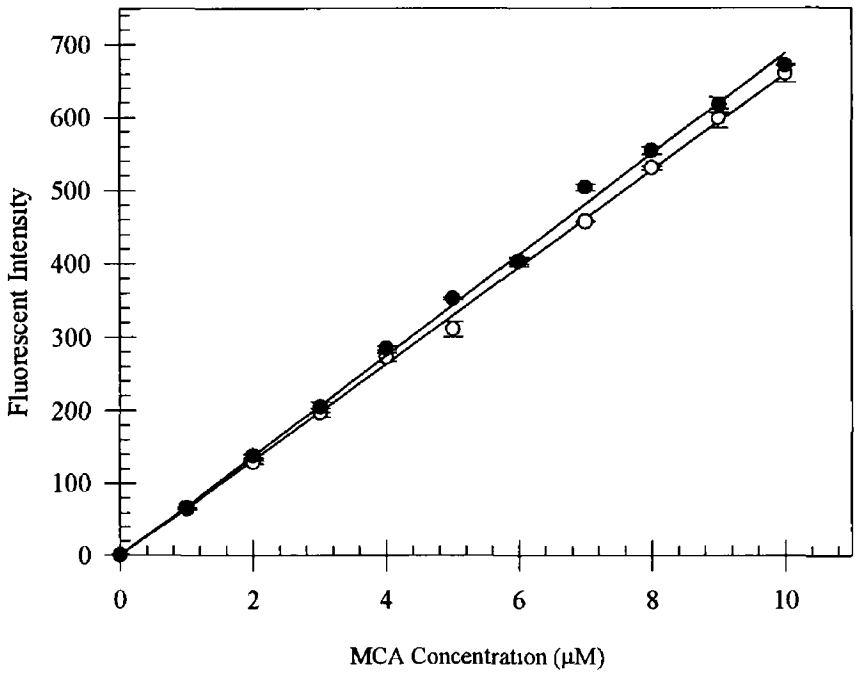


FIGURE 3.1.4.



FIGURES 3 1 3 AND 3.1 4 FILTERED MCA STANDARD CURVES

Plot of fluorescent intensity versus MCA concentration FIGURE 3 1 3 illustrates the filter effect for serum (•-•) and serum + Z-Pro-Prolinal (▪-▪) in comparison to unfiltered slope for buffer (o-o) FIGURE 3 1 4 shows the filter effect for post CPC ZIP (•-•) versus unfiltered slope (o-o)

3 2 PROTEIN DETERMINATION

BSA standard curves were prepared according to the methods given in section 2 3 Plots of protein absorbance 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 MEASUREMENT OF Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN SERUM

Serum Z-Gly-Pro-MCA degrading activities were determined as outlined in sections 2 4 2 and 2 4 3 Bovine serum was found to have 2459units of Z-Gly-Pro-MCA degrading activity, which was equally attributable to PO (1232 units) and ZIP (1227units) activities A unit of enzyme activity is defined as one picomole of MCA released per min at 37°C FIGURE 3 3 1 illustrates the presence of both PO and ZIP in crude bovine serum, differentiated by the addition of Z-Pro-Prohnal or JTP-4819

FIGURE 3.2 1

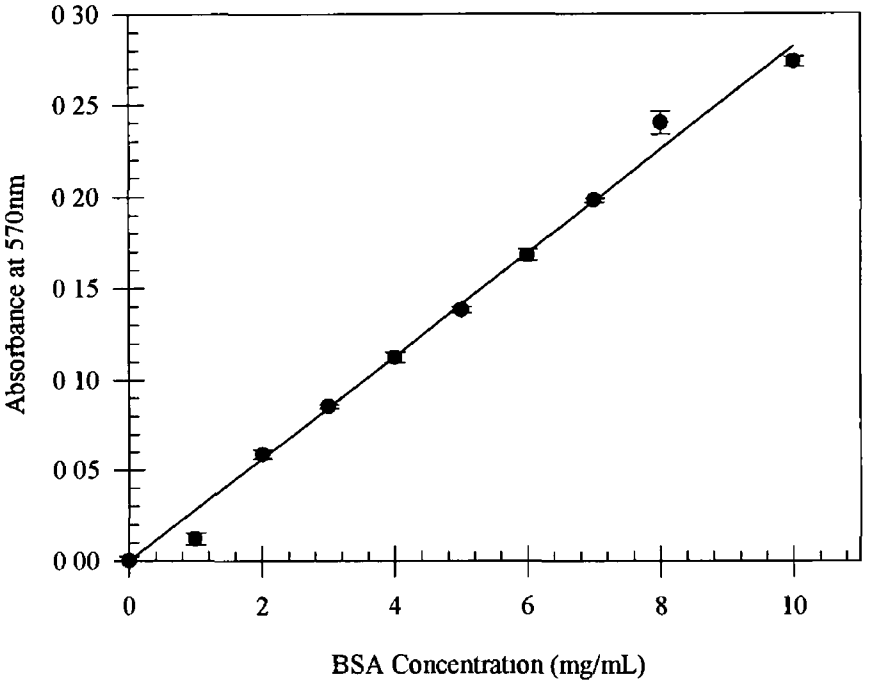


FIGURE 3 2 1 BSA STANDARD CURVE

Plot of Absorbance at 570nm observed using the Biuret Assay versus BSA concentration

FIGURE 3 2 2

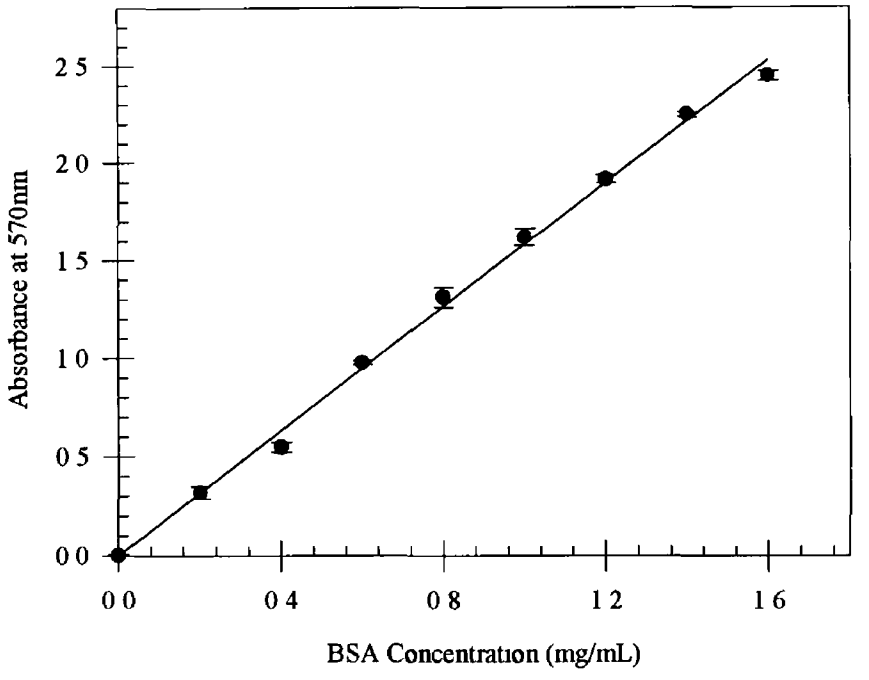
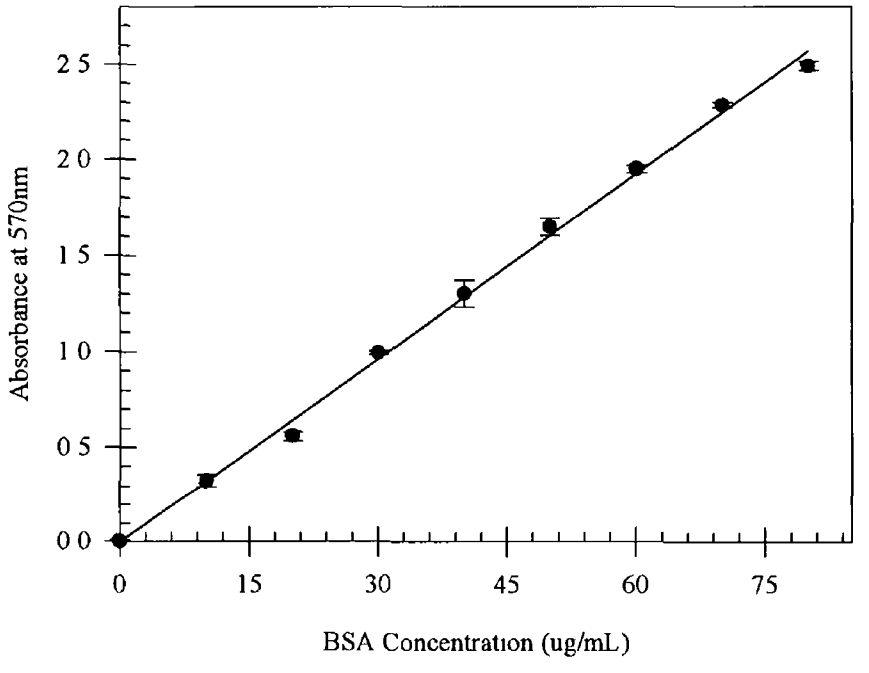


FIGURE 3.2 3



FIGURES 3 2 2. AND 3 2 3 BSA STANDARD CURVES

Plots of absorbance at 570nm versus BSA concentration FIGURE 3 2 2 was constructed using the BCA Assay, while FIGURE 3 2 3 illustrates the use of the enhanced BCA Assay

FIGURE 3 3 1

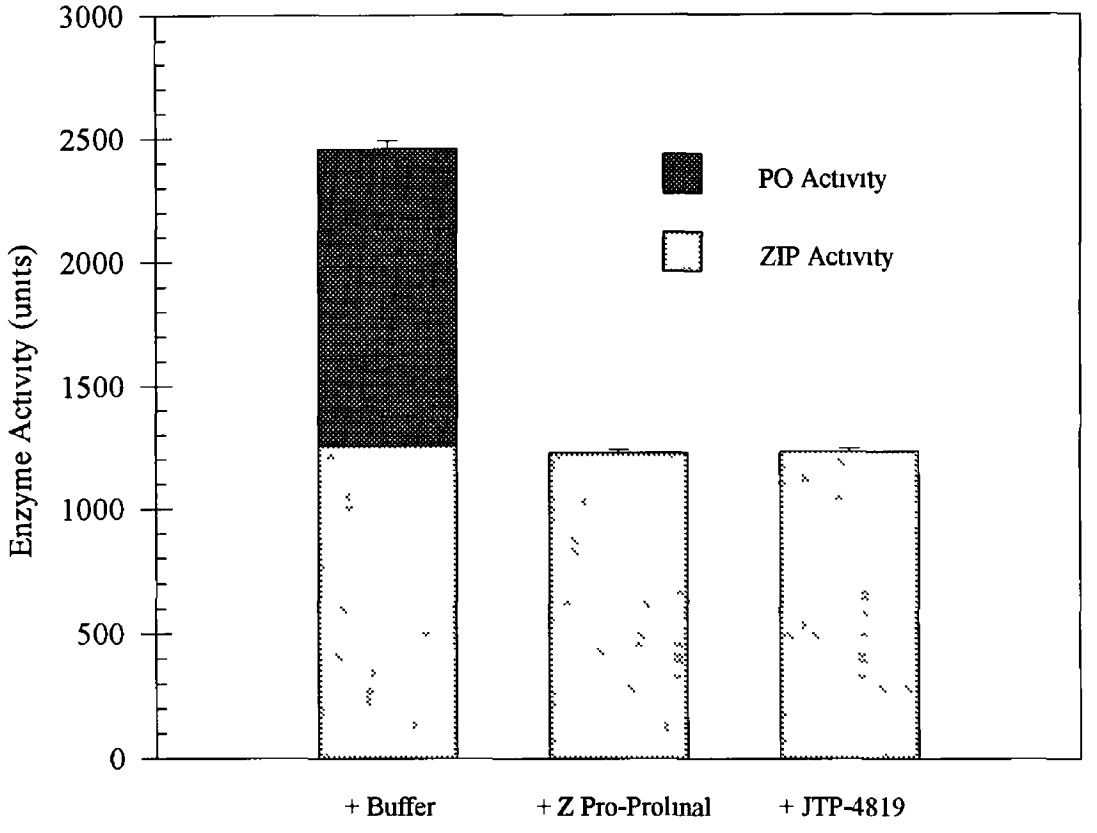


FIGURE 3.3 1 Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN BOVINE SERUM

Plot showing the presence of two distinct enzymes capable of cleaving Z-Gly-Pro-MCA ZIP is represented by the residual activity □ towards the substrate in the presence of either Z-Pro-Prolinal or JTP-4819 PO is inhibited by both Z-Pro-Prolinal and JTP-4819 and so its activity is represented by the disappearance of Z-Gly-Pro-MCA degrading activity ■ in the presence of these inhibitors

3.4 PURIFICATION OF BOVINE SERUM ZIP

3 4 1 Serum Preparation

9L fresh bovine whole blood was collected which resulted in the formation of 1 8L unclotted blood after 4°C storage for 24hr Centrifugation yielded 1 3L of serum

3 4 2 Phenyl Sepharose Hydrophobic Interaction Chromatography

The crude serum containing 200mM $(NH_4)_2SO_4$ was applied to a phenyl sepharose hydrophobic interaction chromatography column as outlined in section 2 5 2 Two activity peaks were observed in the eluant from this column as shown in FIGURE 3 4 2 1 , the first during the 100mL 100mM potassium phosphate wash containing 200mM ammonium sulphate, pH 7 4 and the second eluting with distilled water These activities are distinguished according to their sensitivity towards Z-Pro-Prolinal which is evident from FIGURE 3 4 2 2 Fractions 54-57 were combined to form a 20mL post-phenyl sepharose ZIP sample 2mL of this pool were retained for activity and protein determinations

3 4 3 Calcium Phosphate Cellulose Chromatography

The post-phenyl sepharose ZIP pool was further purified using calcium phosphate cellulose as outlined in section 2 5 3 The Z-Gly-Pro-MCA degrading peptidase bound to the column and eluted following application of an increasing phosphate gradient FIGURE 3 4 3 illustrates the observed elution profile from the column Fractions 48-50 were pooled yielding 15mL of post-calcium phosphate cellulose pool 2mL of this pool were retained for activity and protein determinations

3 4 4 DEAE Sepharose Anion-Exchange Chromatography

Dialysed post-calcium phosphate cellulose pool was applied to a DEAE sepharose column as described in section 2 5 4 ZIP activity was detected in the bound fractions from this anion-exchange column The elution profile may be seen in FIGURE 3 4 4 A 15mL post-DEAE sepharose pool was formed after combination of fractions 33-35 inclusive 2mL were retained for estimation of enzyme recovery and protein determinations An average yield of 145µg ZIP was obtained and stored at -17°C until required

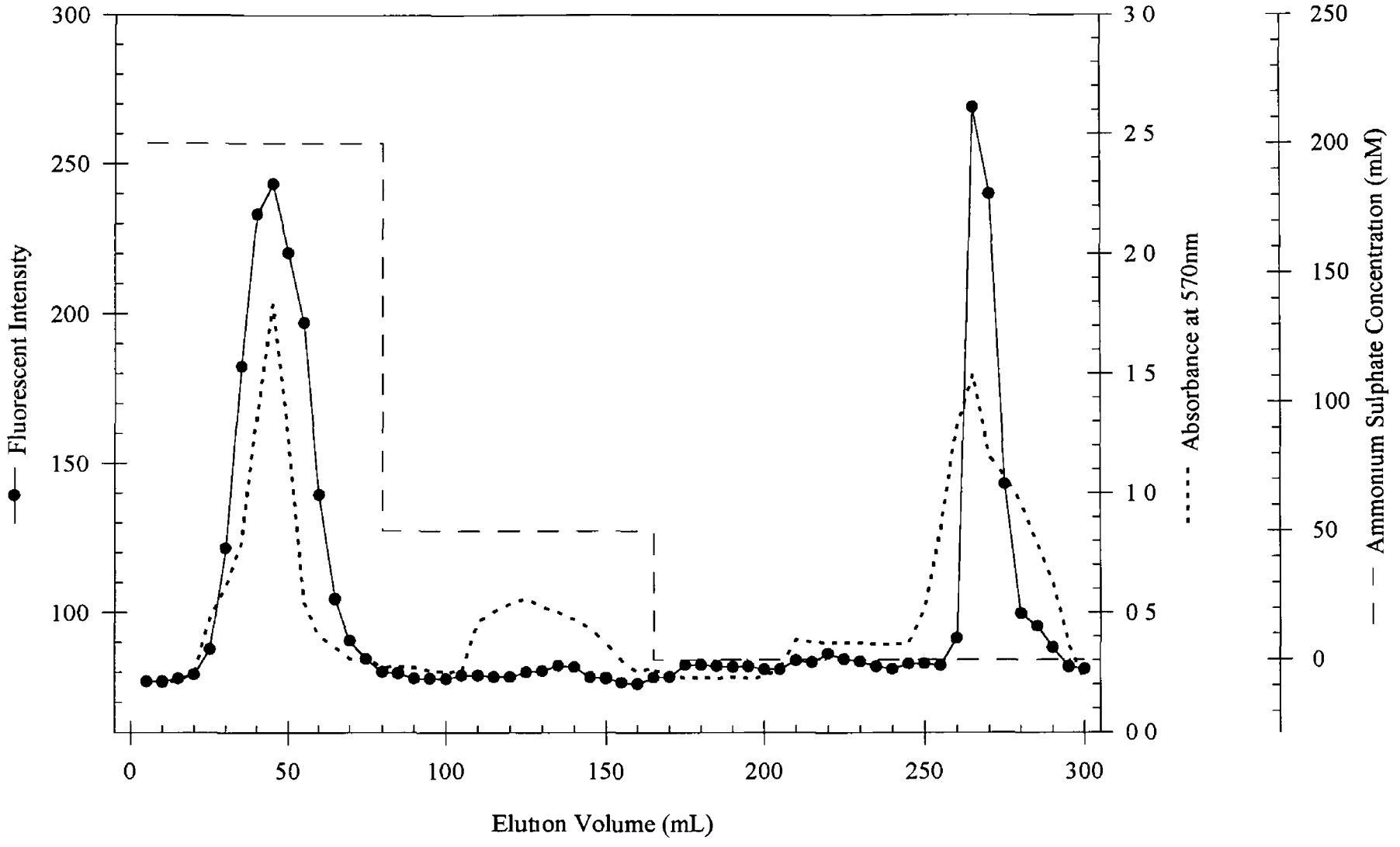


FIGURE 3 4 2 1. PHENYL SEPHAROSE ELUTION PROFILE

Plot of fluorescent intensity (•-•), protein (- - -) and ammonium sulphate concentration (— - - —) versus elution volume from phenyl sepharose resin Elution shows the presence of two distinct Z-Gly-Pro-MCA degrading activities in bovine serum which are separable by hydrophobic interactions chromatography

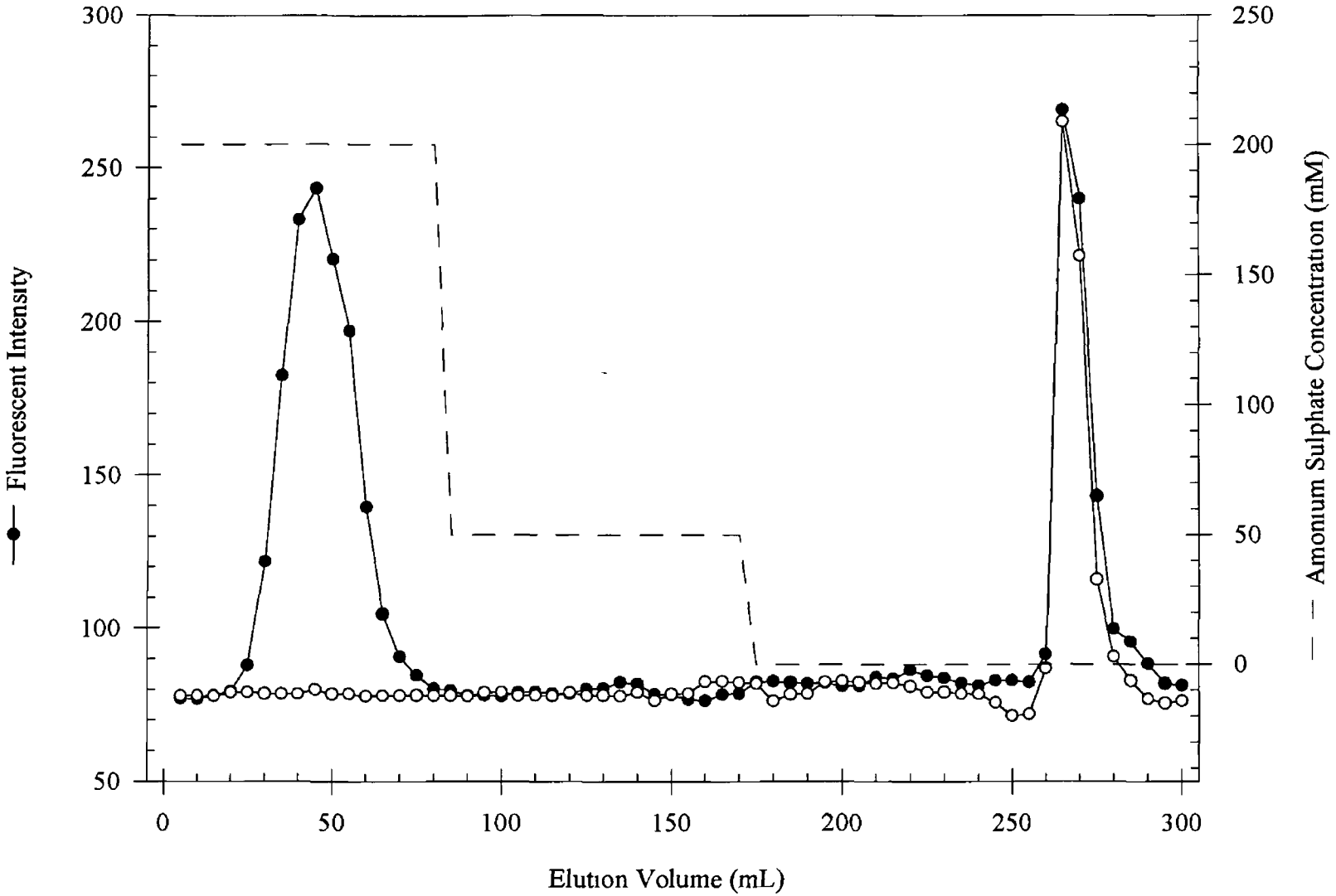


FIGURE 3 4 2 2 PHENYL SEPHAROSE ELUTION PROFILE

Plot shows the differentiation of the two distinct Z-Gly-Pro-MCA degrading activities (●-●) by the inclusion of Z-Pro-Prolinal (o-o) ZIP activity, in fractions 54-57 (270-285mL) binds to the column and is insensitive to Z-Pro-Prolinal, while PO (fractions 6-13 (30-65mL)) runs through and is completely inhibited by Z-Pro-Prolinal

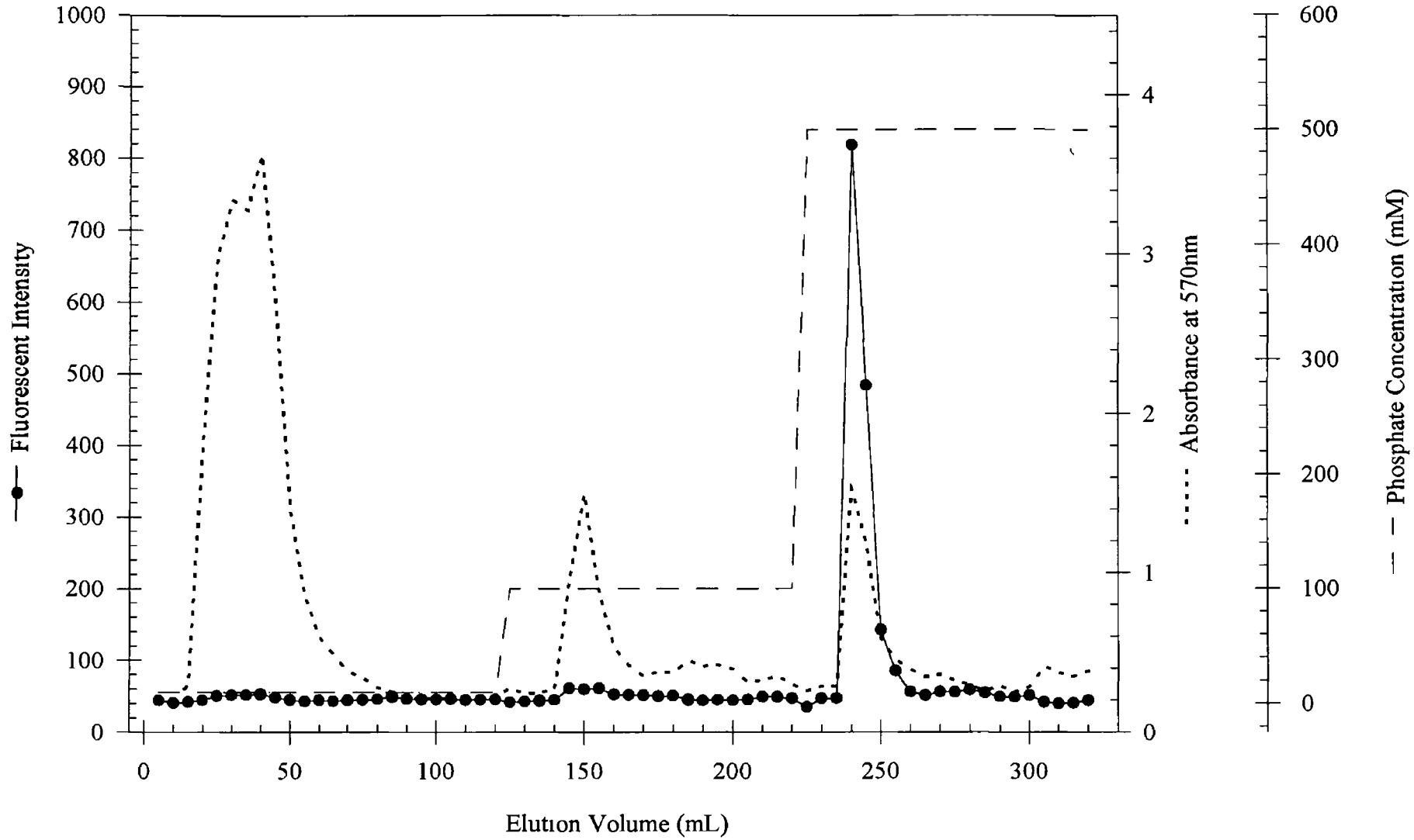


FIGURE 3 4 3 CALCIUM PHOSPHATE CELLULOSE CHROMATOGRAPHY ELUTION PROFILE

Plot of Z-Gly-Pro-MCA degrading activity (●-●), protein (- - -) and potassium phosphate concentration (— - - —) versus elution volume Bound ZIP activity was eluted using an increasing linear gradient of phosphate Fractions 48-50 were pooled to form post calcium phosphate cellulose ZIP sample

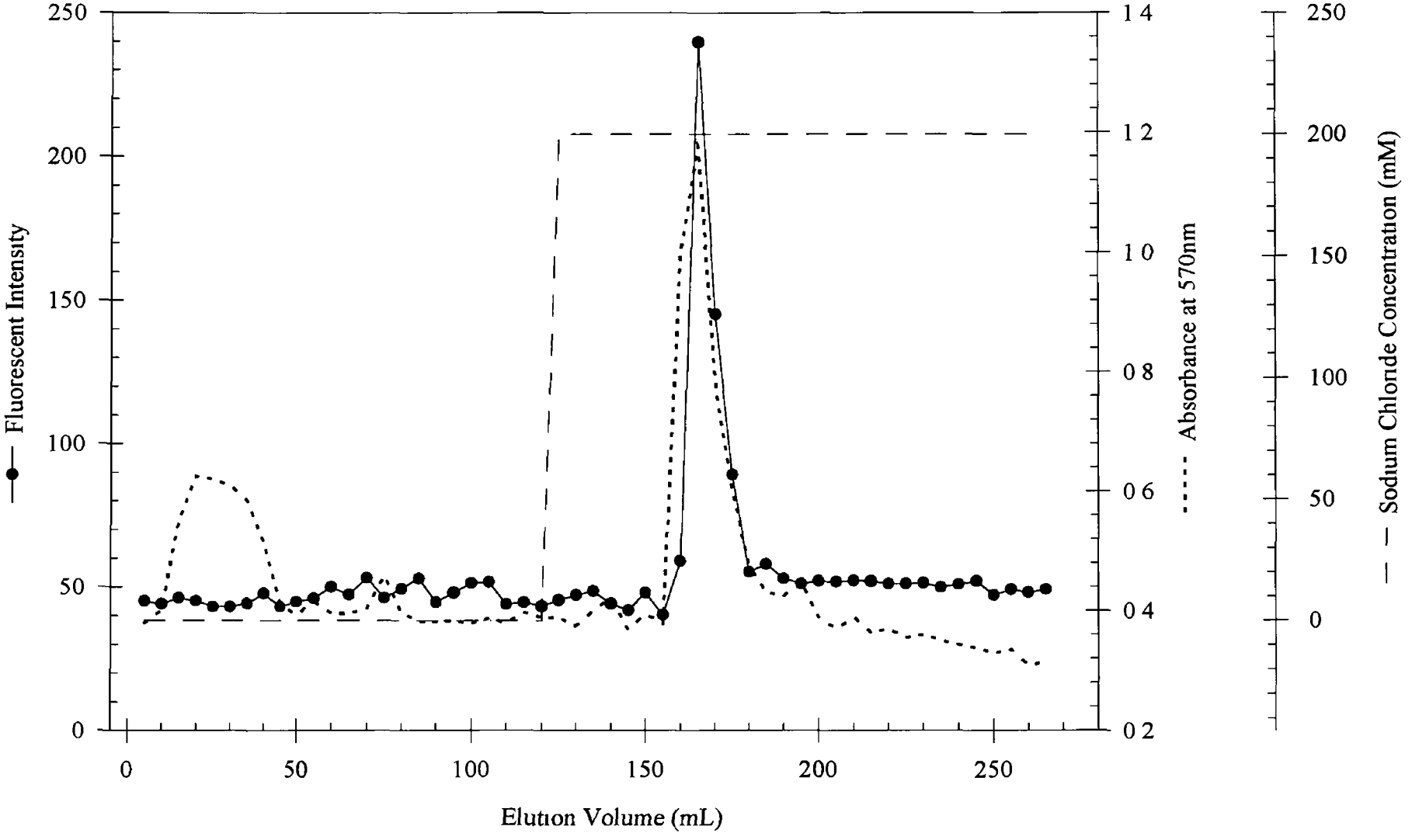


FIGURE 3 4 4 DEAE SEPHAROSE ELUTION PROFILE

Plot of Z-Gly-Pro-MCA degrading activity (●-●), protein (- - -) and NaCl concentration (— -- —) versus elution volume Bound ZIP activity was eluted isocratically using sodium chloride Fractions 33-35 were combined to form post DEAE sepharose ZIP pool

A purification table was constructed to assess the overall effectiveness of the purification protocol (TABLE 3 2) Calculations were performed as highlighted in section 6 3

PURIFICATION STAGE	TOTAL ACTIVITY *unit*	TOTAL PROTEIN *mg*	SPECIFIC ACTIVITY *unit/mg*	PURIFICATION FACTOR	YIELD %
Serum	5 29	1743	0 003	1 0	100
Post PS	4 5	23 94	0 188	63	85
Post CPC	3 74	0 764	4 895	1632	71
Post DEAE	1 75	0 145	12 07	**4023**	**33**

TABLE 3 2 PURIFICATION TABLE FOR BOVINE ZIP

3 5. PURITY DETERMINATION

3 5 1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed as described in section 2 6 1 FIGURES 3 5 1 and 3 5 2 represent images of the silver and brilliant blue stained gels respectively, showing molecular weight markers and the various post-column ZIP pools TABLE 3 3 summarises the labels used to identify each of the markers on the gels and the relative molecular weight of each

3 5 2 The Activity of ZIP Using Fluorimetric Substrates

The presence of contaminating peptidase activities in the purified ZIP pool was determined by the detection of the hydrolysis of a number of fluorimetric substrates These substrates were prepared and tested as outlined in section 2 6 2 TABLE 3 4 illustrates that no cleavage of these substrates was detected in the presence of purified ZIP activity

SUBSTRATE	HYDROLYSIS	SUBSTRATE	HYDROLYSIS
Ala-MCA	No	N_{α}-benzoyl-Arg-MCA	No
Arg-MCA	No	Pyr-His-Pro-MCA	No
Glu-Phe-MCA	No	Pyr-MCA	No
Gly-Pro-MCA	No	Pro-MCA	No
Leu-MCA	No	Z-Arg-MCA	No
Lys-Ala-MCA	No	Z-Gly-Pro-MCA	Yes
Lys-MCA	No	Z-Phe-Arg-MCA	No

TABLE 3 4 PURITY ASSESSMENT USING FLUORIMETRY

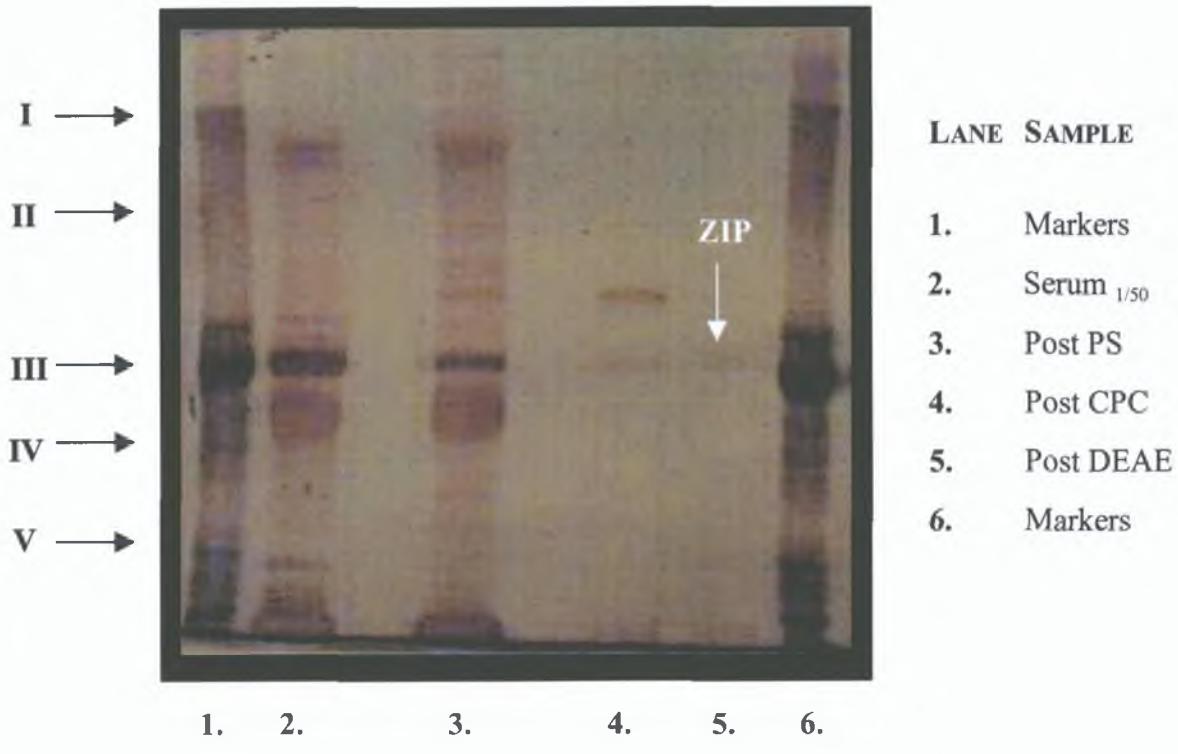


FIGURE 3.5.1. SILVER STAINED SDS POLYACRYLAMIDE GEL

LABEL	Marker	MOLECULAR WEIGHT *kDa*
I	Myosin	205
II	β-Galactosidase	116
III	BSA	66
IV	Fumarase	48.5
V	Carbonic anhydrase	29

TABLE 3.3. MOLECULAR WEIGHT MARKERS USED IN SDS PAGE

LANE	SAMPLE
1.	Markers
2.	Serum $_{1/50}$
3.	Post PS
4.	Post CPC
5.	Post DEAE
6.	Markers

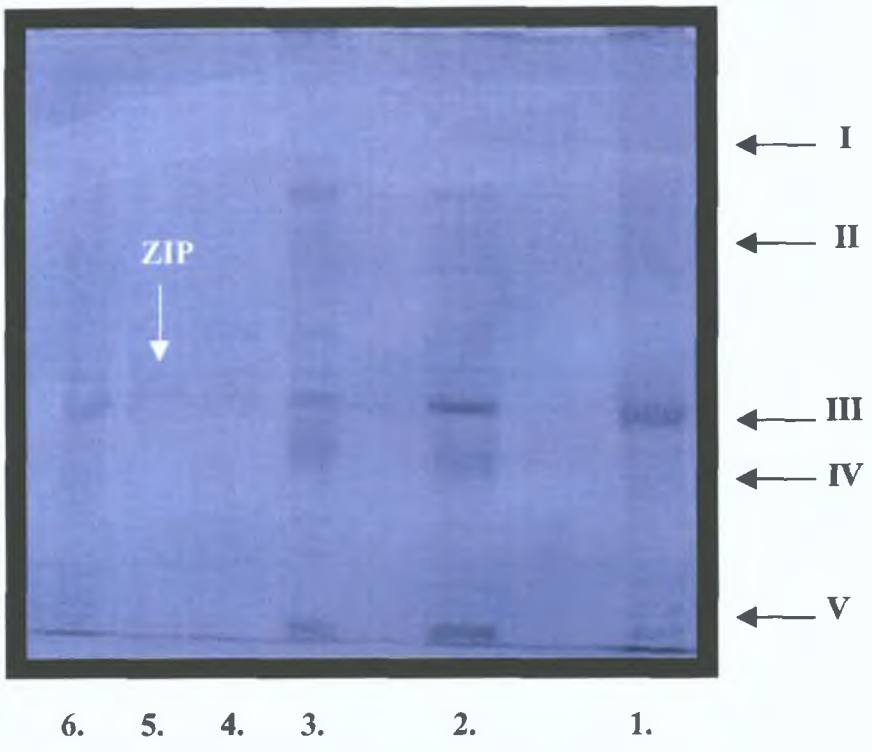


FIGURE 3.5.2. BRILLIANT BLUE STAINED SDS POLYACRYLAMIDE GEL

LABEL	Marker	MOLECULAR WEIGHT *kDa*
I	Myosin	205
II	β-Galactosidase	116
III	BSA	66
IV	Fumarase	48.5
V	Carbonic anhydrase	29

TABLE 3.3. MOLECULAR WEIGHT MARKERS USED IN SDS PAGE

3 5.3. Purity Assessment Using Reverse Phase HPLC

The cleavage of fluorimetric substrates was also investigated using HPLC analysis as an alternative means of elucidating the purity of the enzyme Fluorimetric substrates (TABLE 2 4) were prepared and analysed as described in section 2 6 3 Liberated MCA from these substrates was identified based on the knowledge that MCA has a retention time of 3 1min TABLE 3 5 summarises the results obtained, while FIGURES 3 5 3 1 , 3 5 3 2 and 3 5 3 3 illustrate the lack of hydrolysis of Pyr-His-Pro-MCA, Glu-Phe-MCA and Lys-Ala-MCA respectively by ZIP FIGURE 3 5 3 4 demonstrates the cleavage of Z-Gly-Pro-MCA by purified ZIP

SUBSTRATE	HYDROLYSIS	SUBSTRATE	HYDROLYSIS
Ala-MCA	No	N_{α}-benzoyl-Arg-MCA	No
Arg-MCA	No	Pyr-His-Pro-MCA	No
Glu-Phe-MCA	No	Pyr-MCA	No
Gly-Pro-MCA	No	Pro-MCA	No
Leu-MCA	No	Z-Arg-MCA	No
Lys-Ala-MCA	No	Z-Gly-Pro-MCA	Yes
Lys-MCA	No	Z-Phe-Arg-MCA	No

TABLE 3 5. PURITY ASSESSMENT USING HPLC

FIGURE 3 5 3 1

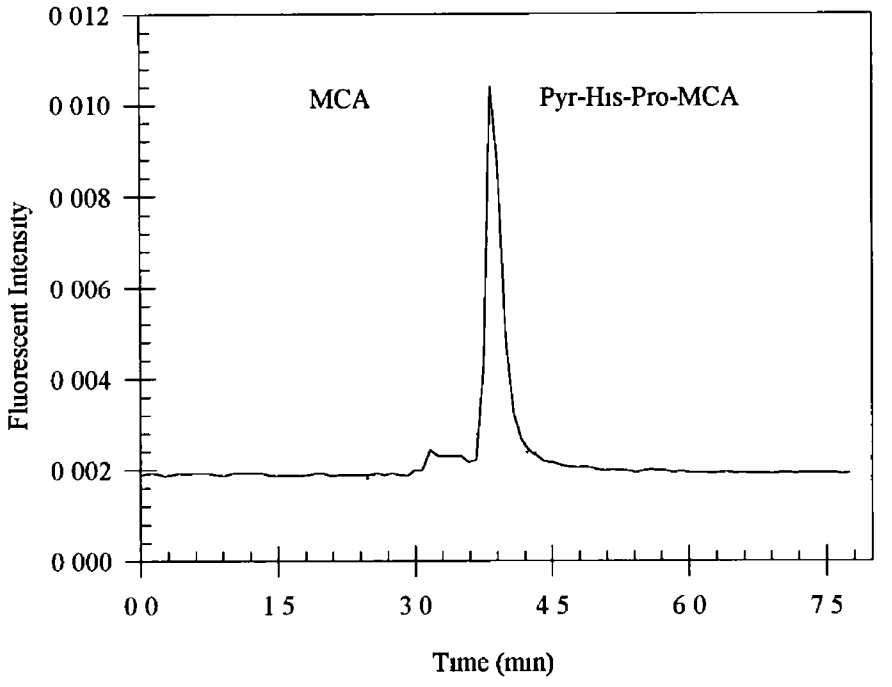
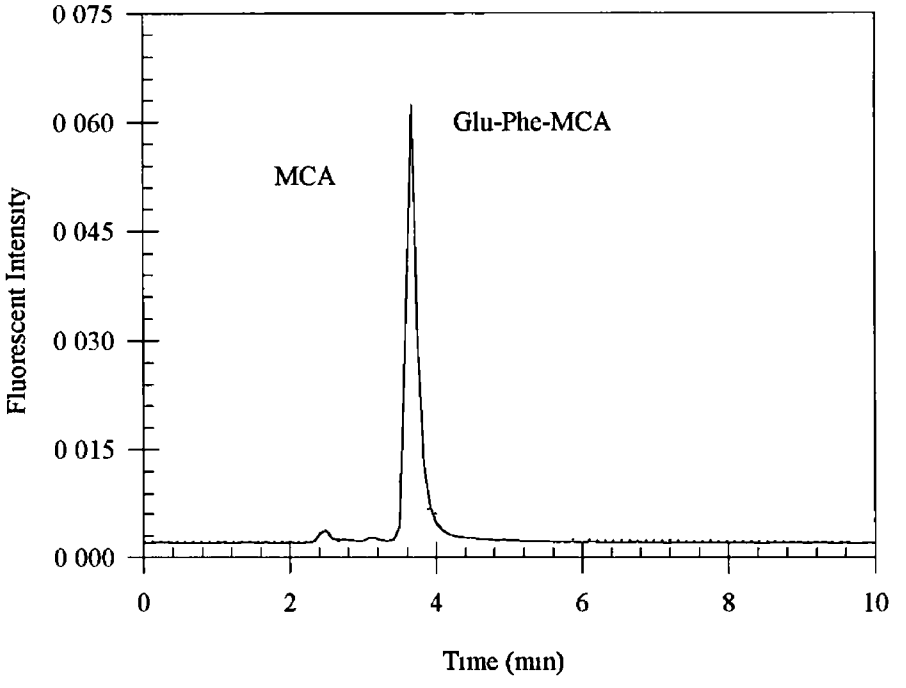


FIGURE 3 5.3 2



FIGURES 3 5 3 1 AND 3 5 3 2 PURITY ASSESSMENT USING HPLC

Plots of fluorescent intensity versus time for ZIP incubated with substrate (___) and free MCA (- - -) FIGURE 3 5 3 1 represents the chromatograms for ZIP incubated with Pyr-His-Pro-MCA, while FIGURE 3 5 3 2 illustrates the chromatograms obtained for ZIP incubated with Glu-Phe-MCA

FIGURE 3.5.3.3.

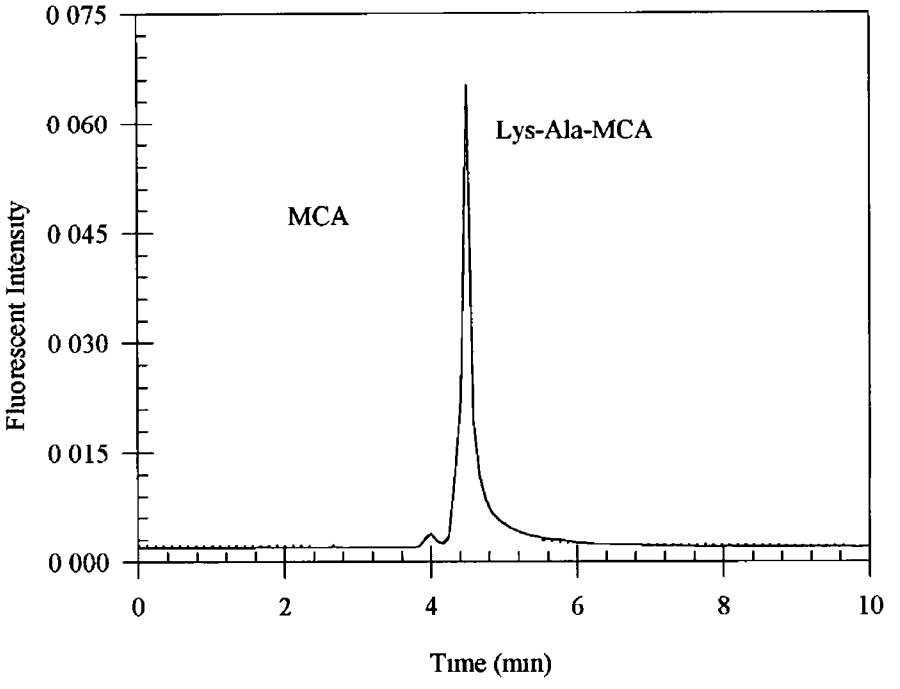
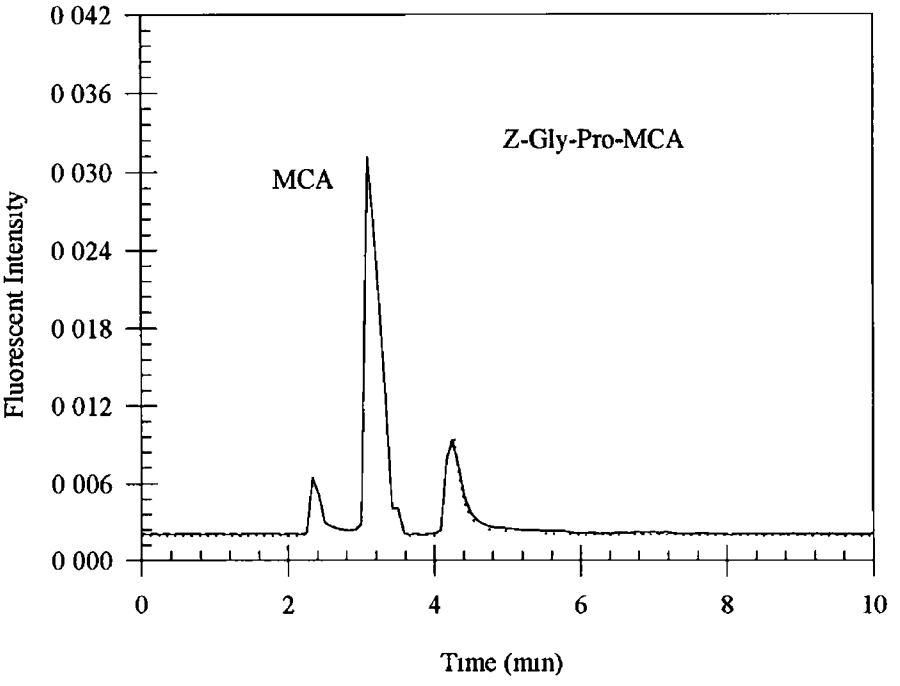


FIGURE 3 5 3 4



FIGURES 3 5 3.3. AND 3 5 3 4 PURITY ASSESSMENT USING HPLC

Plots of fluorescent intensity versus time FIGURE 3 5 3 3 represents the chromatograms of Lys-Ala-MCA when incubated with ZIP (___) and free MCA (- - -) FIGURE 3 5 3 4 illustrates the chromatograms of free Z-Gly-Pro-MCA (- - -) and that of ZIP incubated with Z-Gly-Pro-MCA (___) The liberated MCA peak is also shown

3 6 ASSAY DEVELOPMENT

3 6 1 Optimum MCA Excitation and Emission Wavelengths

Scans of fluorescence versus wavelength were performed on free MCA as outlined in section 2 7 1 in order to determine the optimum excitation and emission wavelengths for this compound FIGURES 3 6 1 1 and 3 6 1 2 show that the optimum excitation and emission wavelengths for MCA are 370nm and 440nm respectively

3 6 2 Substrate Solvent Determination

Stock substrates were prepared as outlined in section 2 7 2 in DMSO, DMF, DXN, EtOH and MeOH The effects of using these solvents on ZIP activity is illustrated in FIGURE 3 6 2 Methanol was chosen to be the most suitable solvent and was thus used for subsequent substrate solubilisation

3 6 3 Solvent Concentration Determination

FIGURE 3 6 3 shows the effect of increasing methanol concentration in substrate preparations on purified ZIP Although 2% and 3%v/v MeOH yielded higher activities of ZIP, the solubility of the substrate at these low concentrations was problematic, triplicates were poor and standard errors increased with decreasing methanol content Therefore 4%v/v MeOH was chosen as the most suitable final solvent concentration in the substrate for the assaying of ZIP

FIGURE 3.6 1 1.

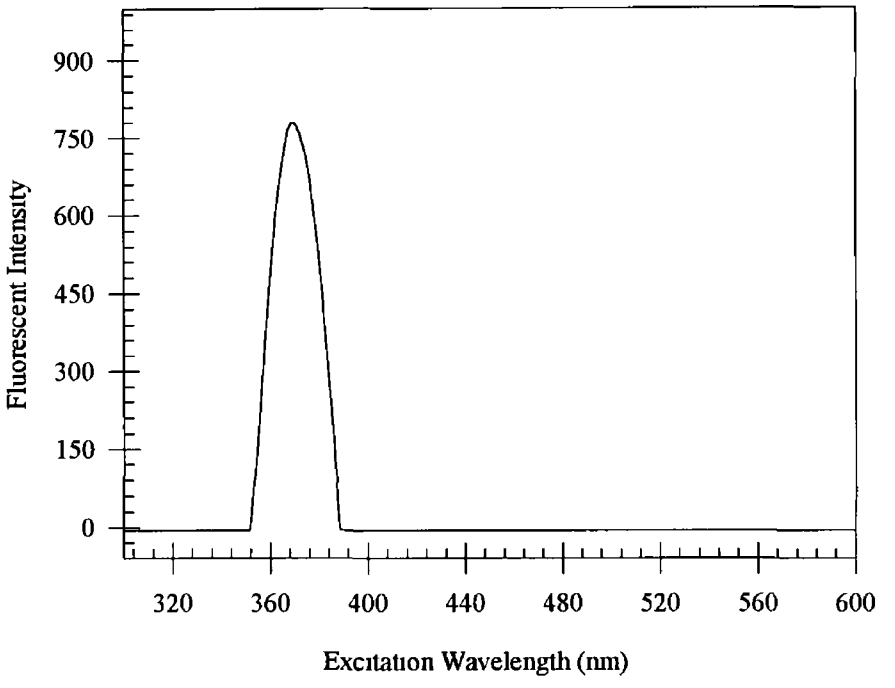
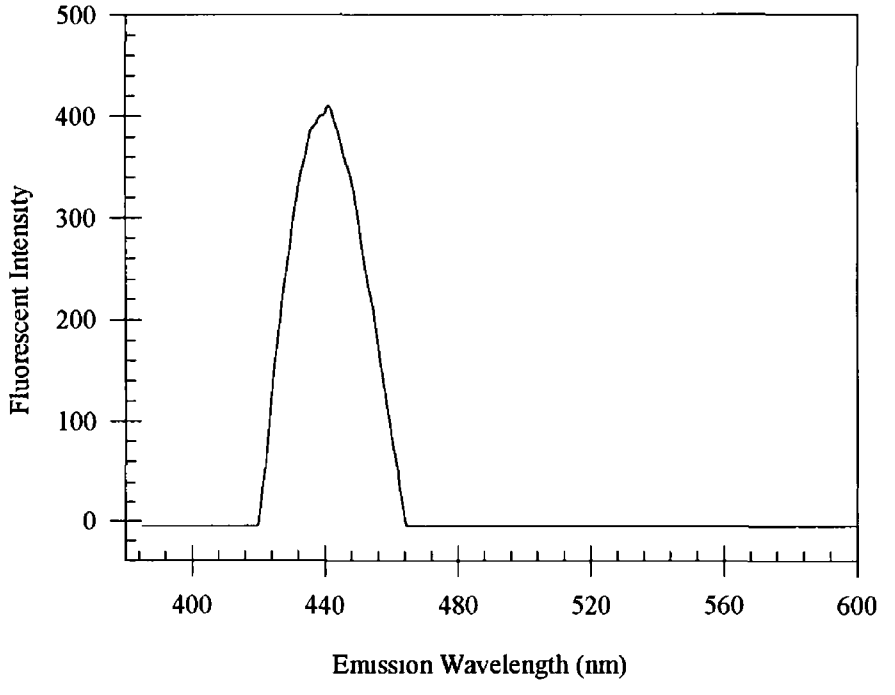


FIGURE 3 6 1 2.



FIGURES 3.6 1.1. AND 3 6.1.2. MCA EXCITATION AND EMISSION SCANS.

Plot of fluorescent intensity versus wavelength for free MCA in solution FIGURE 3 6 1 1 illustrates an optimum excitation wavelength of 370nm for MCA while FIGURE 3 6 1 2 shows an emission wavelength of 440nm as optimal

FIGURE 3 6.2

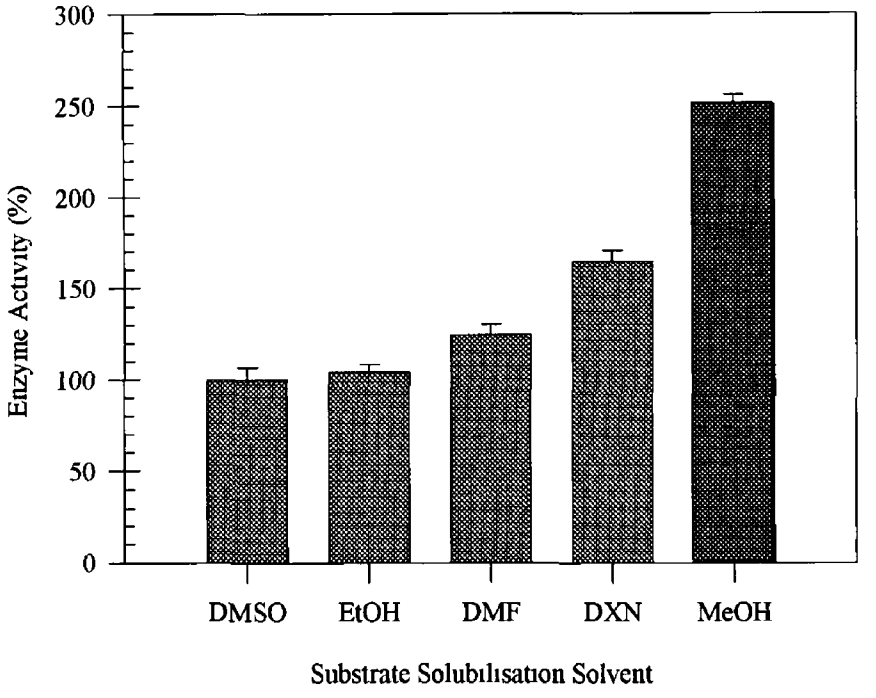
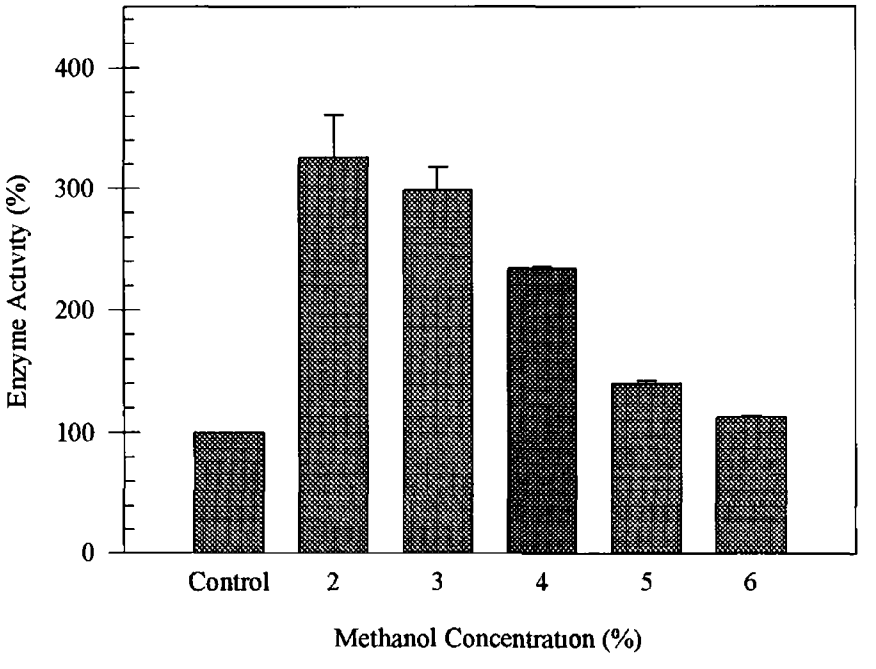


FIGURE 3 6 3



FIGURES 3 6 2 AND 3.6 3 OPTIMAL SUBSTRATE SOLVENT AND SOLVENT CONCENTRATION

Plot of enzyme activity versus substrate solubilisation solvent (FIGURE 3 6 2) FIGURE 3 6 3 illustrates a plot of enzyme activity versus methanol concentration Enzyme activities are expressed as a percentage of the fluorescence observed using substrate solubilised in 4% DMSO

3 6 4 Linearity of Enzyme Assays with Respect to Time

The linearity of the Z-Gly-Pro-MCA degrading activity assay as a function of time was examined as described in section 2 7 4 FIGURE 3 6 4 1 illustrates that ZIP activity in post DEAE purified sample is linear over a period of 60 minutes (R^2 0 9997) Similarly, total Z-Gly-Pro-MCA activity in crude serum is linear (R^2 0 9999) over the same time period (FIGURE 3 6 4 2) The activity of ZIP in serum was assessed by the inclusion of Z-Pro-Prohnal, or JTP-4819 FIGURES 3 6 4 3 and 3 6 4 4 illustrate the linearity of ZIP in crude serum with respect to time using Z-Pro-Prolinal and JTP-4819, yielding regression coefficients of 0 9999 and 0 9989 respectively Continuous assays of post phenyl sepharose and post calcium phosphate cellulose ZIP also yielded linear progress curves with respect to time

3 6 5 Linearity of Enzyme Assays with Respect to Enzyme Concentration

Linearity of the Z-Gly-Pro-MCA degrading activity assays with respect to enzyme concentration was determined as outlined in section 2 7 5 FIGURE 3 6 5 indicates that the purified ZIP assay is linear (R^2 0 9980) with respect to enzyme concentration

3 6.6. Optimum Assay Temperature

The effect of incubating purified ZIP at different temperatures was investigated according to section 2 7 6 The optimal temperature for the assay of bovine serum ZIP was determined to be 37°C as FIGURE 3 6 6 illustrates

3 6 7 Effect of DTT on ZIP Activity

The incorporation of DTT into Z-Gly-Pro-MCA was carried out as detailed in section 2 7 7 FIGURE 3 6 7 illustrates the effect on purified ZIP activity of the inclusion of DTT into the substrate DTT inhibited ZIP activity with over 35% inhibition observed at 20mM DTT

3.6 8. Effect of NaCl on ZIP Activity

The inclusion of NaCl in the substrate resulted in proportional enhancement of purified ZIP activity, up to a concentration of 400mM NaCl at which point the activity of ZIP began to plateau FIGURE 3 6 8 shows the pattern observed

FIGURE 3 6.4 1

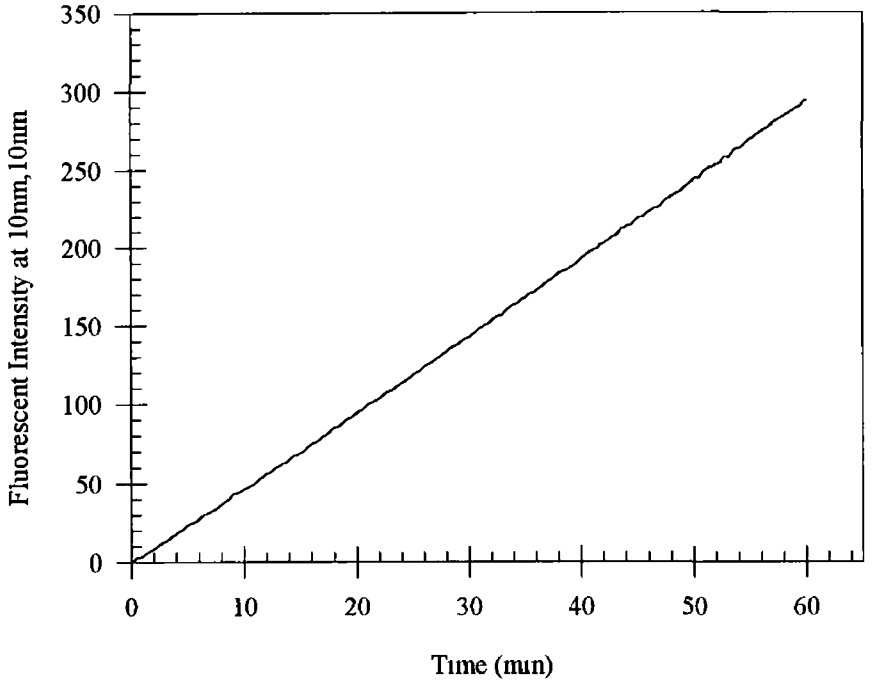
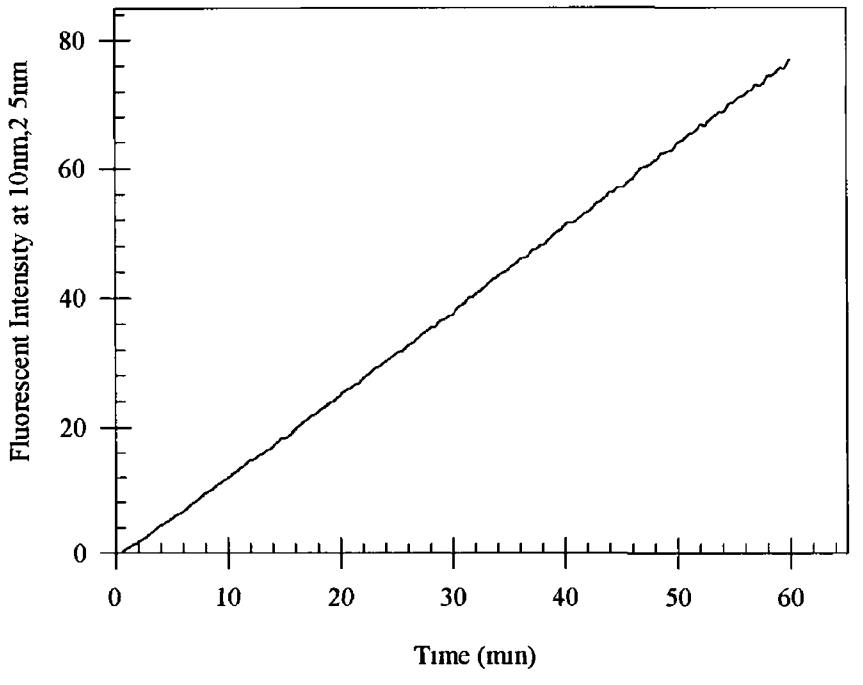


FIGURE 3 6 4 2



FIGURES 3 6 4.1 AND 3 6 4 2 LINEARITY WITH RESPECT TO TIME

Plots of fluorescent intensity versus time FIGURE 3 6 4 1 shows the linearity of the assay using purified ZIP, while FIGURE 3 6 4 2 illustrates linearity using crude serum

FIGURE 3 6 4 3.

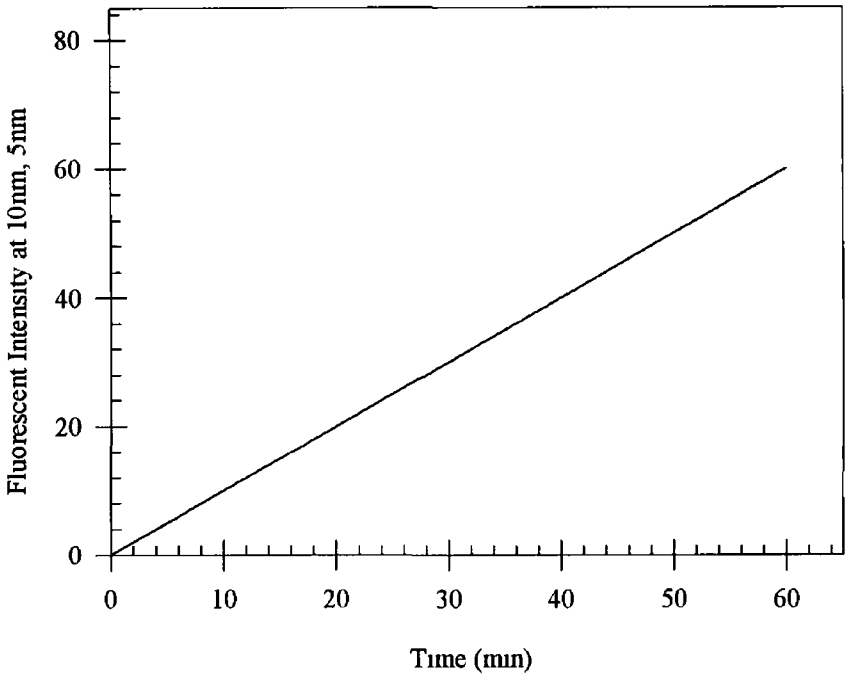
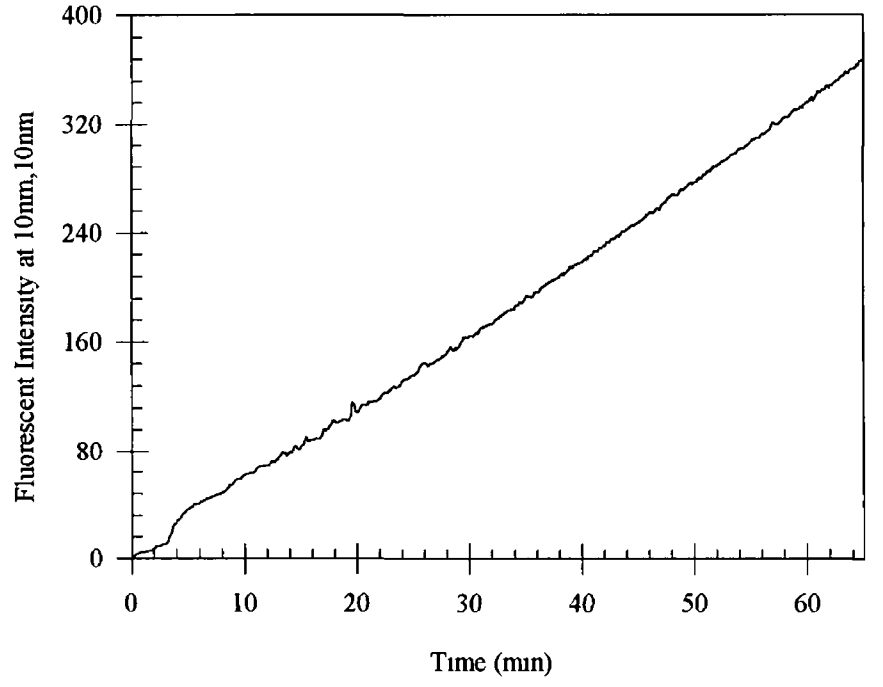


FIGURE 3 6 4 4



FIGURES 3 6 4 3 AND 3 6 4 4 LINEARITY WITH RESPECT TO TIME

Plots of fluorescent intensity versus time for crude serum FIGURE 3 6 4 3 illustrates maintenance of linearity on the inclusion of Z-Pro-Prohnal and FIGURE 3 6 4 4 shows that JTP-4819 does not effect the linearity of the enzyme assay with respect to time

FIGURE 3 6 5.

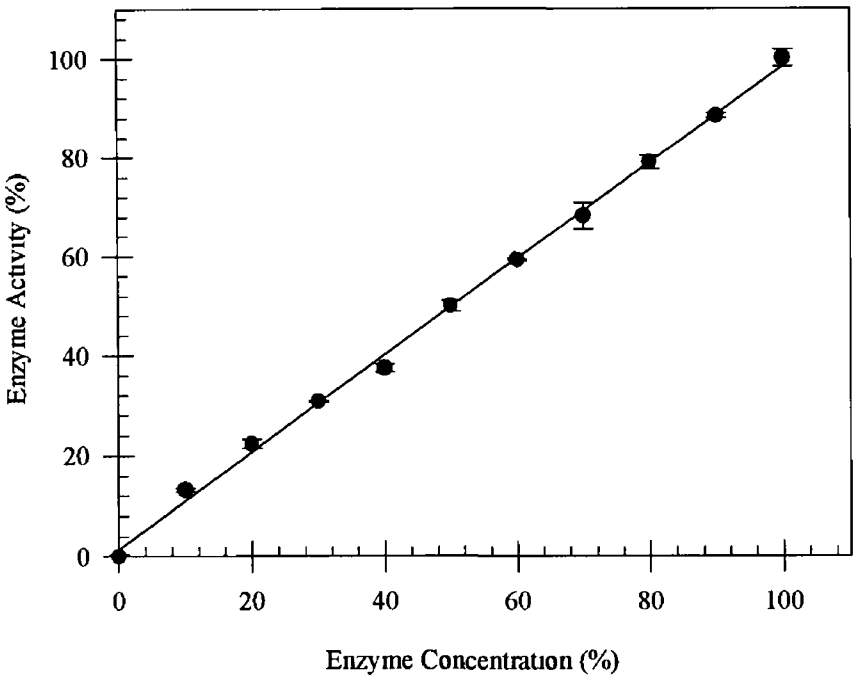


FIGURE 3 6.5 LINEARITY WITH RESPECT TO ENZYME CONCENTRATION

Plot of fluorescent intensity versus enzyme concentration (100% enzyme represents 50μg/mL)

FIGURE 3 6 6

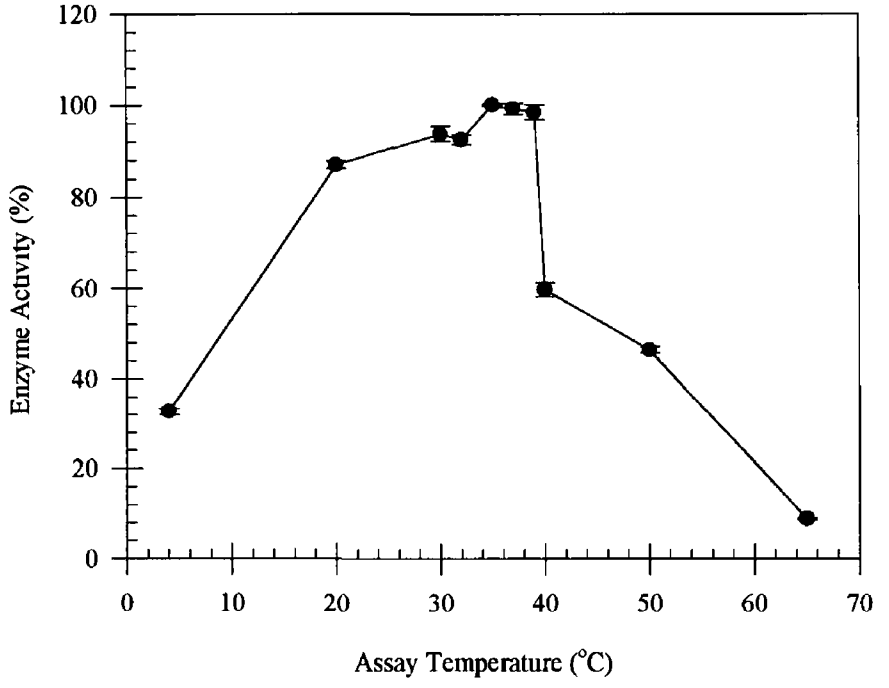


FIGURE 3.6.6 OPTIMUM ASSAY TEMPERATURE

Plot of enzyme activity versus temperature for purified ZIP Enzyme activity is expressed as a percentage of the fluorescence observed at 37°C

FIGURE 3 6 7

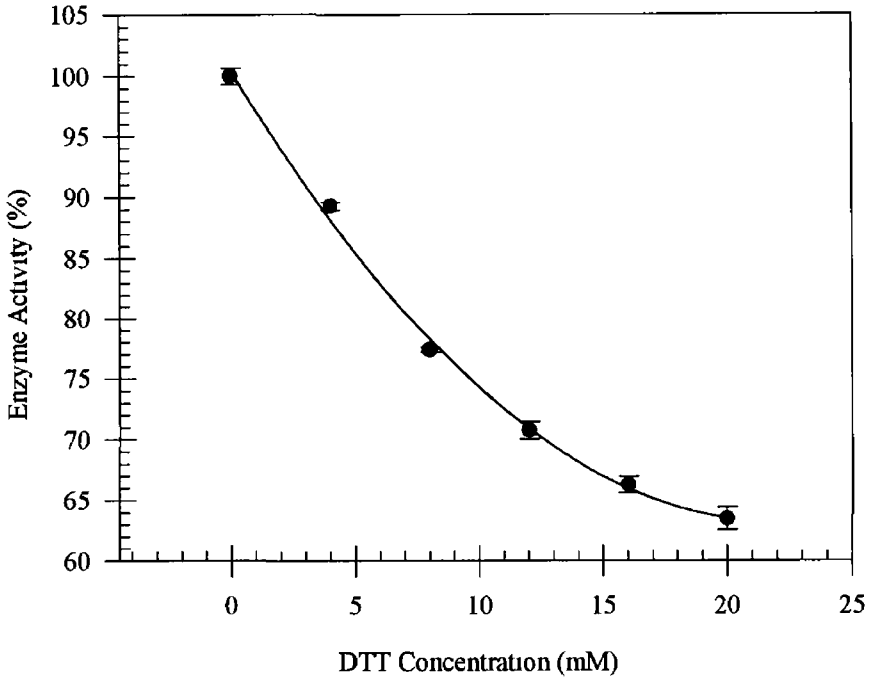
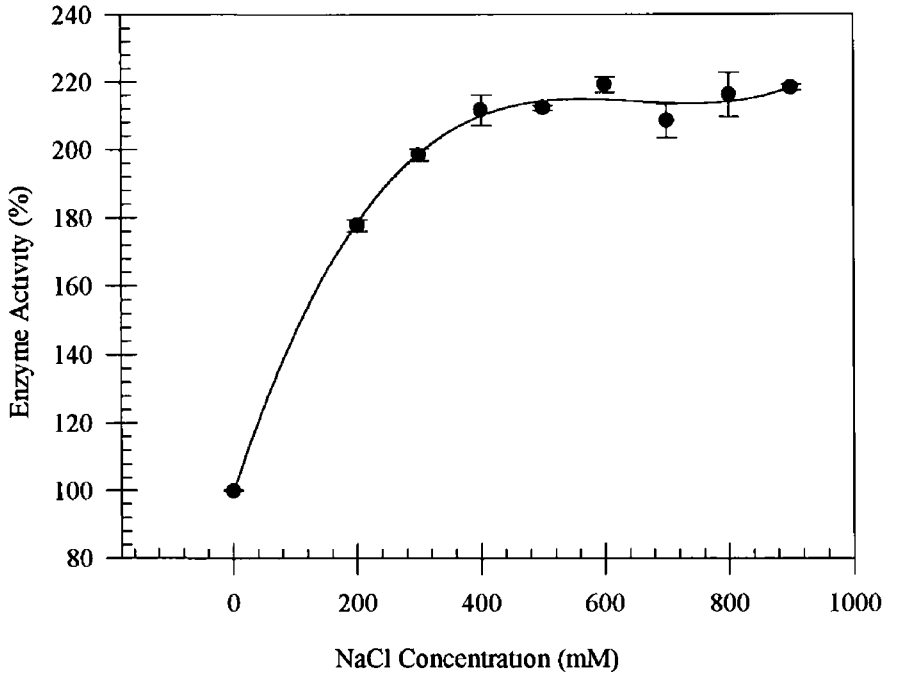


FIGURE 3 6 8



FIGURES 3.6 7 AND 3.6.8. EFFECT OF DTT AND NACL ON ZIP ACTIVITY

Plots of enzyme activity versus DTT (FIGURE 3 6 7) or NaCl (FIGURE 3 6 8) concentration Enzyme activities are expressed as a percentage of fluorescence recorded with neither DTT nor NaCl

3 6 9 Prolyl Oligopeptidase Inhibitor Studies

Initially Z-Pro-Prolinal was employed as the potent PO inhibitor in crude samples to differentiate the two distinct Z-Gly-Pro-MCA degrading activities FIGURE 3 6 9 1 shows the inhibition profile observed on the incubation of PO with increasing concentrations of Z-Pro-Prohnal while FIGURE 3 6 9 8 verifies that PO cannot be further inhibited by increased pre-incubation

On depletion of this source a number of inhibitors were tested as outlined in section 2 7 9 2 in an attempt to find one which could completely inhibit PO yet be ineffective on ZIP activity This was necessary to allow for the detection of ZIP alone in crude samples containing both Z-Gly-Pro-MCA degrading activities FIGURES 3 6 9 2, 3 6 9 3, 3 6 9 4, 3 6 9 5 , 3 6 9 6 and 3 6 9 7 show the inhibition of Z-Gly-Pro-MCA degrading activities in crude serum by Z-Pro-Prohnal dimethylacetate, Fmoc-Pro-Pro-CN, Fmoc-Ala-Pro-CN, Z-Indohnyl-Prohnal, postatin and JTP-4819 respectively FIGURE 3 6 9 9 shows the effect of pre-incubating serum with JTP-4819

FIGURE 3 6 9 1

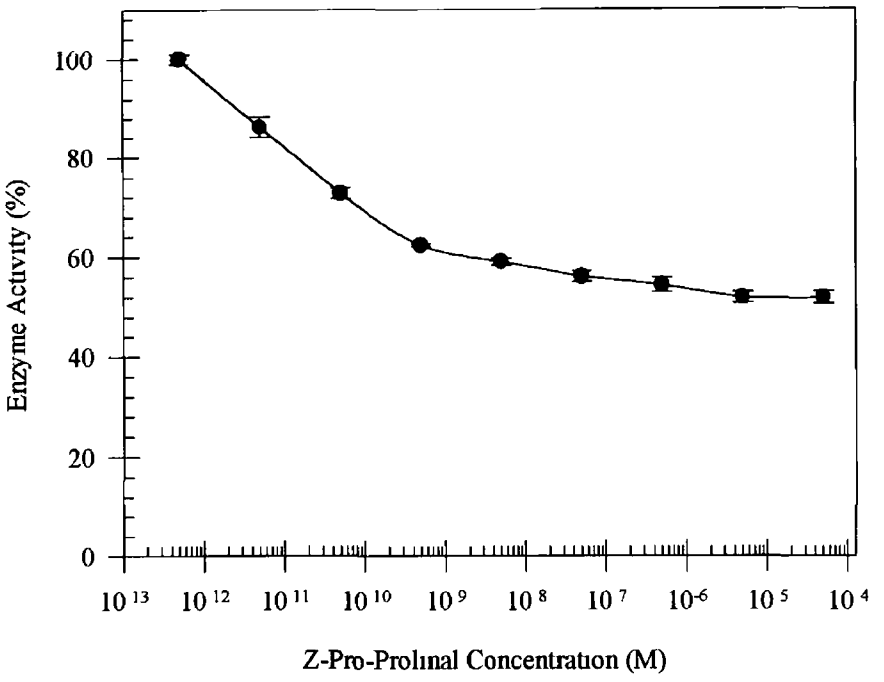
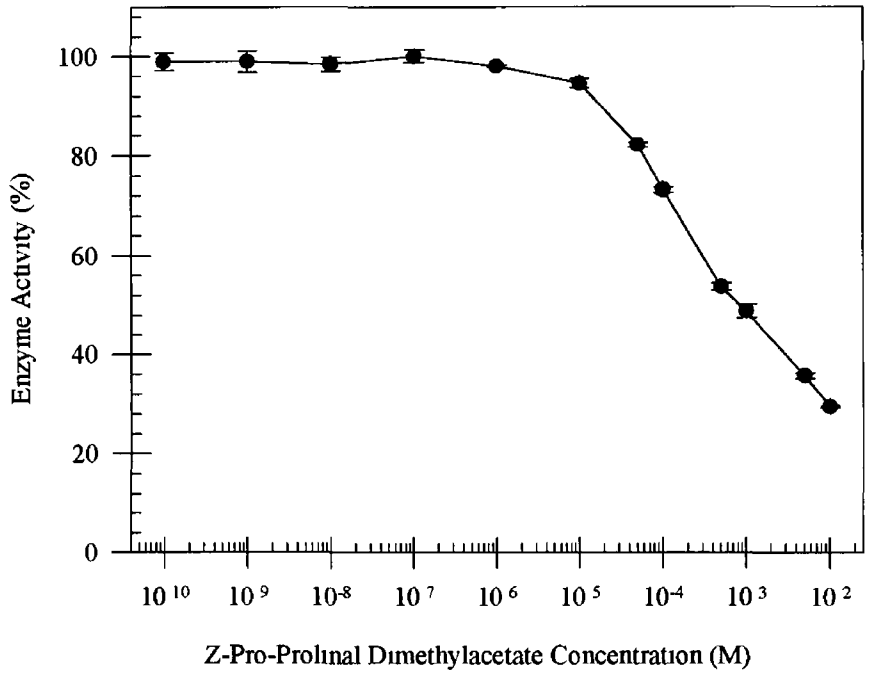


FIGURE 3 6 9 2



FIGURES 3 6 9 1 AND 3 6 9 2. EFFECT OF Z-PRO-PROLINAL AND Z-PRO-PROLINAL DIMETHYLACETATE ON Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN SERUM

Semi log plots of enzyme activity versus specific inhibitor concentration Enzyme activity is expressed as a percentage of uninhibited enzyme (100%)

FIGURE 3 6 9 3

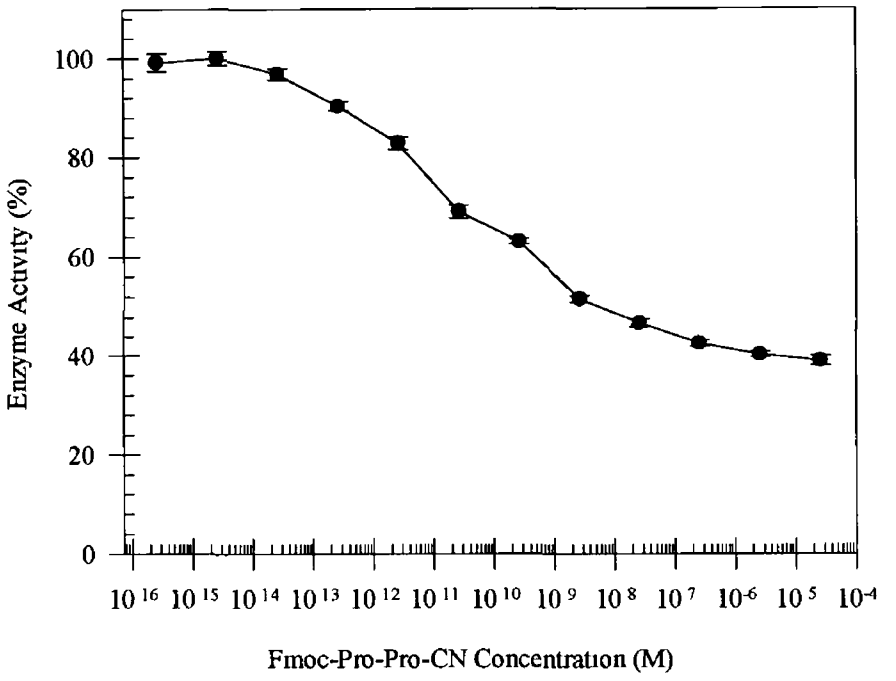
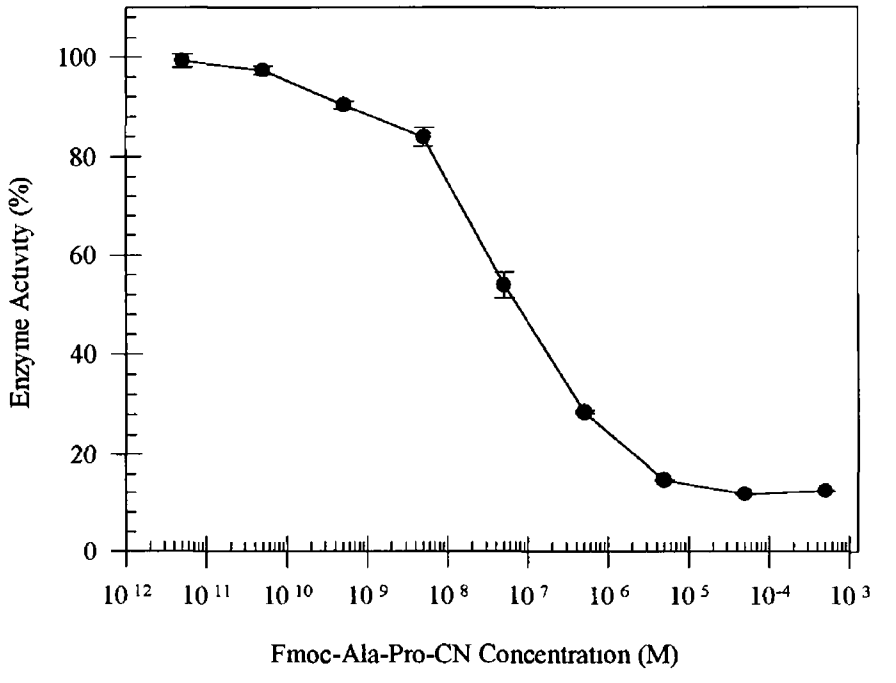


FIGURE 3.6.9.4.



FIGURES 3 6 9 3 AND 3 6 9 4. EFFECT OF FMOC-PRO-PRO -CN AND FMOC-ALA-PRO-CN ON Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN SERUM

Semi log plots of enzyme activity (expressed as a percentage of uninhibited activity) versus specific inhibitor concentration

FIGURE 3.6 9 5

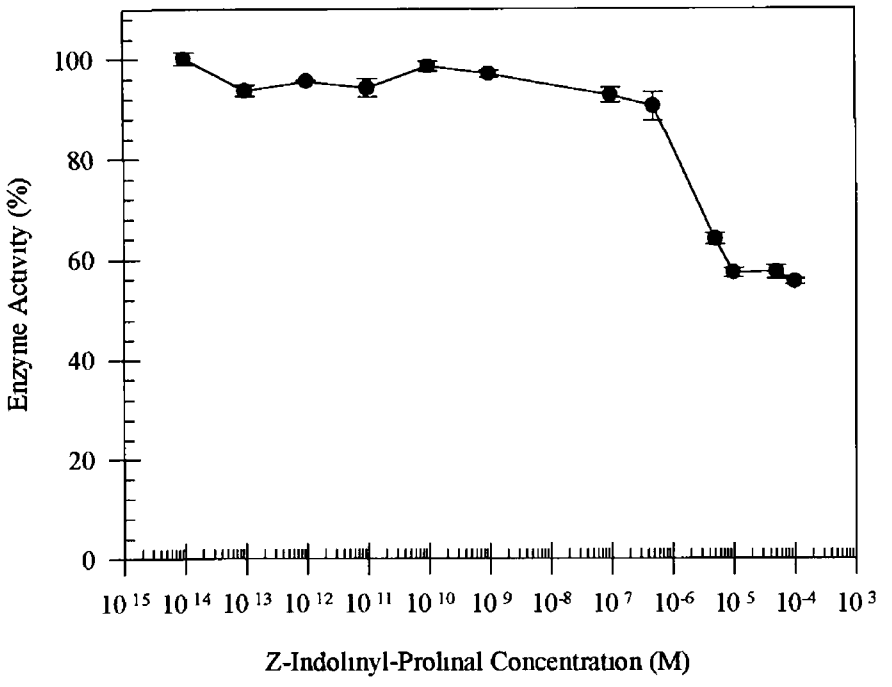
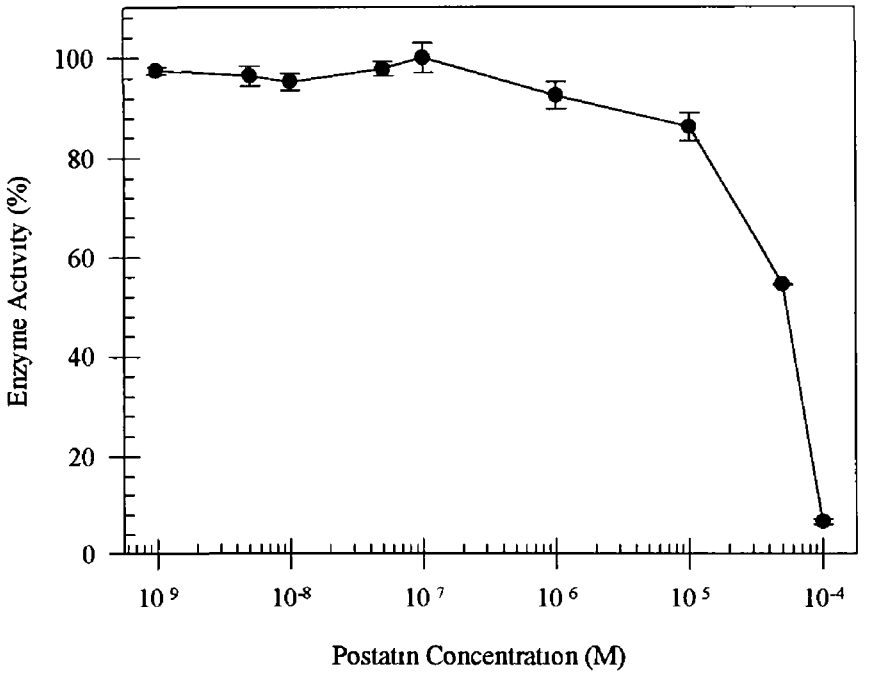


FIGURE 3 6 9 6



FIGURES 3 6 9 5. AND 3 6 9 6 EFFECT OF Z-INDOLINYL-PROLINAL AND POSTATIN ON Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN SERUM.

Semi log plots of enzyme activity versus specific inhibitor concentration Enzyme activity is expressed as a percentage of uninhibited activity (100%)

FIGURE 3.6 9 7

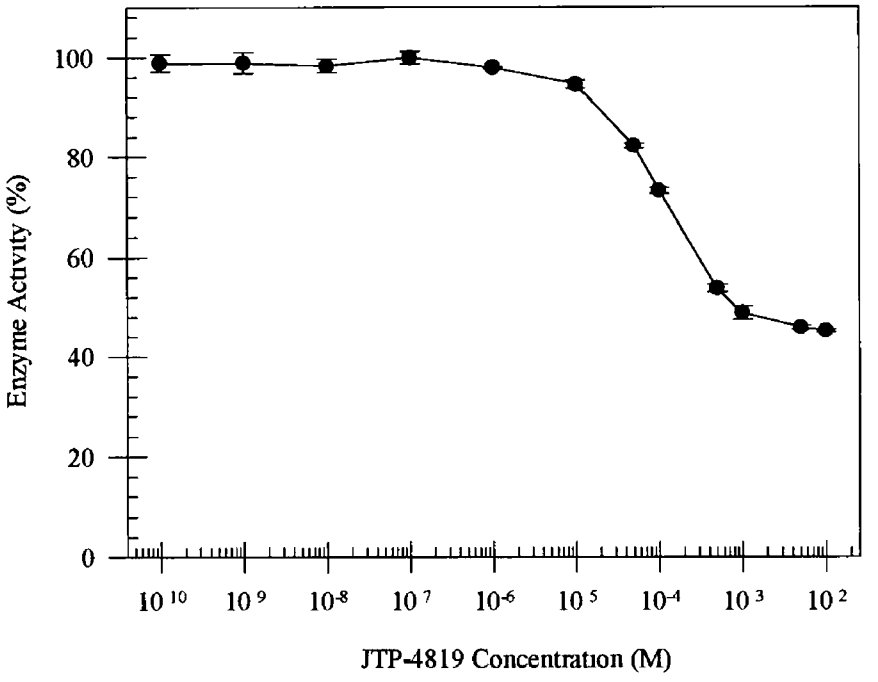


FIGURE 3 6 9 7. EFFECT OF JTP-4819 ON Z-GLY-PRO-MCA DEGRADING ACTIVITY IN SERUM.

Semi log plot of enzyme activity versus JTP-4819 Concentration Enzyme activity is expressed as a percentage of uninhibited enzyme (100% activity)

FIGURE 3 6.9.8.

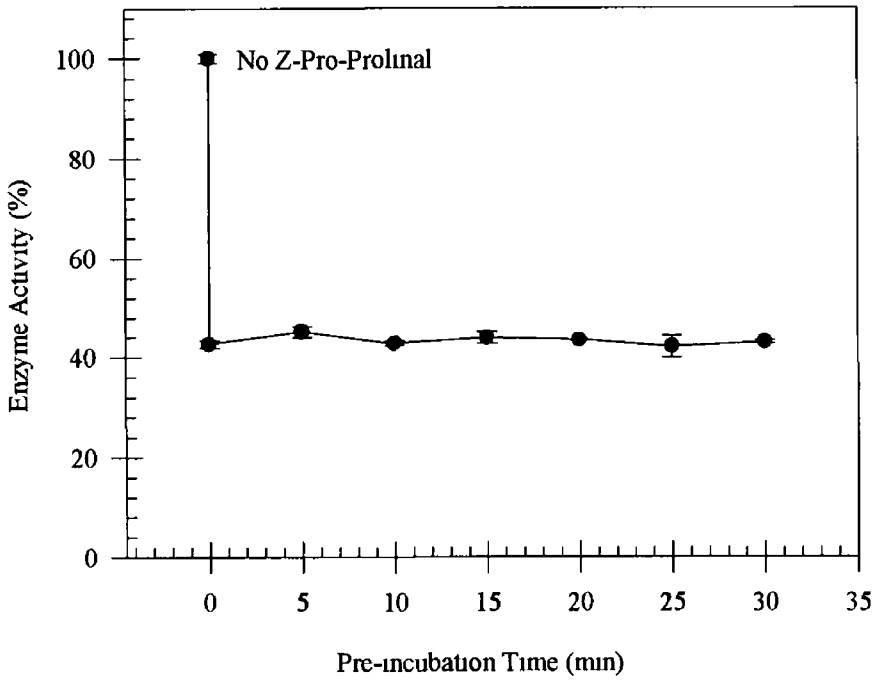
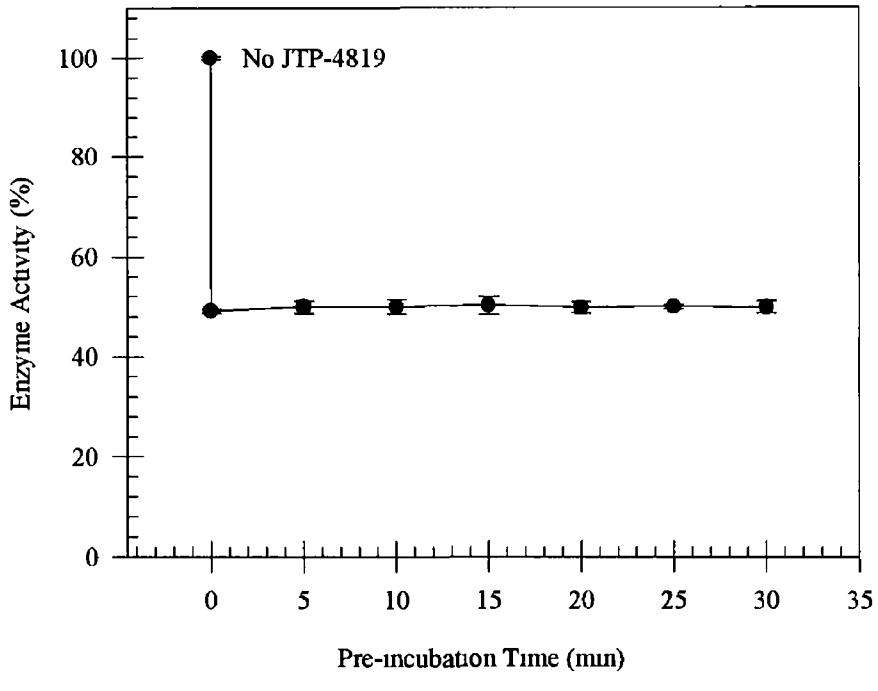


FIGURE 3.6 9.9



FIGURES 3.6 9.8. AND 3 6 9 9. EFFECT OF PRE-INCUBATION WITH Z-PRO-PROLINAL OR JTP-4819 ON Z-GLY-PRO-MCA DEGRADING ACTIVITIES IN SERUM

Plots of enzyme activity versus pre-incubation time FIGURE 3 6 9 8 illustrates the effect of pre-incubating serum with Z-Pro-Prolinal over time, while FIGURE 3 6 9 9 shows the effect of pre-incubation with JTP-4819 over time Enzyme activity is expressed as a percentage of activity observed using no inhibitor

3 7 LOCALISATION STUDIES

Bovine tissue samples were obtained and homogenised as outlined in section 2 8 1 The resulting homogenate, pellet and two supernatants were assayed for both Z-Gly-Pro-MCA degrading activities using JTP-4819 as a potent inhibitor of PO activity (section 2 8 2) The effect of filtering was examined and FIGURES 3 7 1 to 3 7 6 represent the filtered standard curves for each fraction from brain, kidney, liver, lung, spleen and serum samples respectively TABLE 3 6 lists the degree of filtering estimated from each filtered standard curve FIGURE 3 7 7 shows the linearity of the Biuret standard curve used to estimate total protein content in the samples Significant PO and ZIP activities were not detected in the pellets or in the second supernatants of any of the tissues TABLE 3 7 summarises the units of enzyme activity, the total protein and the specific activity in each of the supernatant (S1) samples obtained for ZIP and PO (see section 6 2 for calculations) TABLE 3 8 lists the percentage of the total Z-Gly-Pro-MCA degrading activity due to each peptidase FIGURES 3 7 8 and 3 7 9 graphically illustrate specific activities in the supernatant (S1) of each tissue for ZIP and PO respectively

BOVINE TISSUE	**DEGREE OF FILTERING OBSERVED %**			
	Homogenate (H)	*Pellet (P)*	*Supernatant (S1)*	*Supernatant (S2)*
Brain	56	40	23	15
Kidney	55	22	31	16
Liver	52	23	45	23
Lung	45	23	41	16
Spleen	83	43	60	16
Serum	-	-	17	-

TABLE 3 6 FILTERING EFFECTS OF BOVINE TISSUES

Degree of filtering is expressed as a percentage of the slope of the unfiltered standard curve constructed using 100mM potassium phosphate buffer

FIGURE 3.7.1.

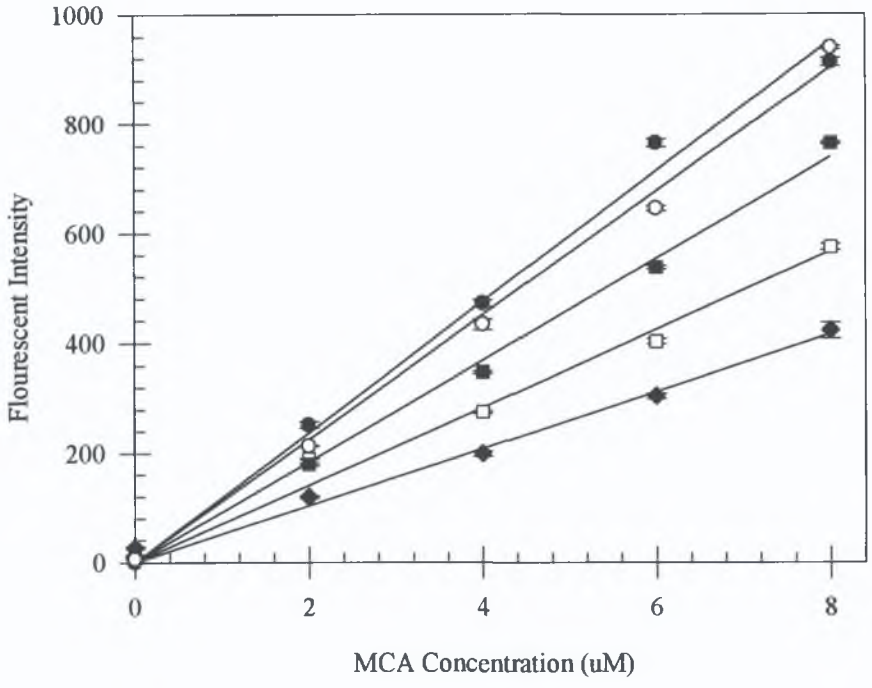
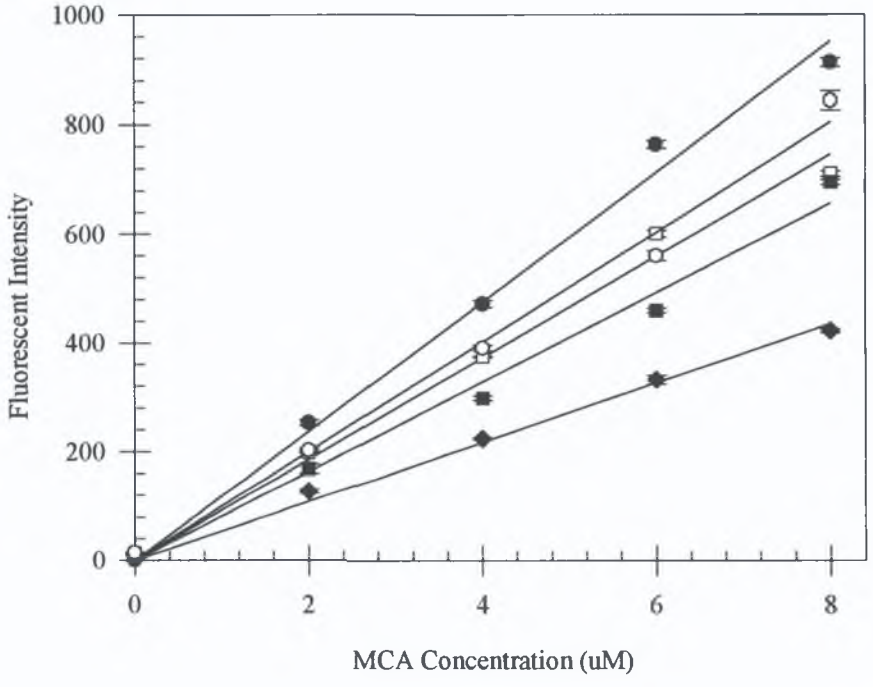


FIGURE 3.7.2.



FIGURES 3.7.1. AND 3.7.2. FILTERED MCA STANDARD CURVES

Plots of fluorescent intensity versus MCA concentration for various samples; unfiltered (●), S_1 (■), S_2 (○), pellet (□) and homogenate (◆). FIGURE 3.7.1. represents filtering effects of these samples in brain tissue, while FIGURE 3.7.2. in kidney tissue.

FIGURE 3 7 3.

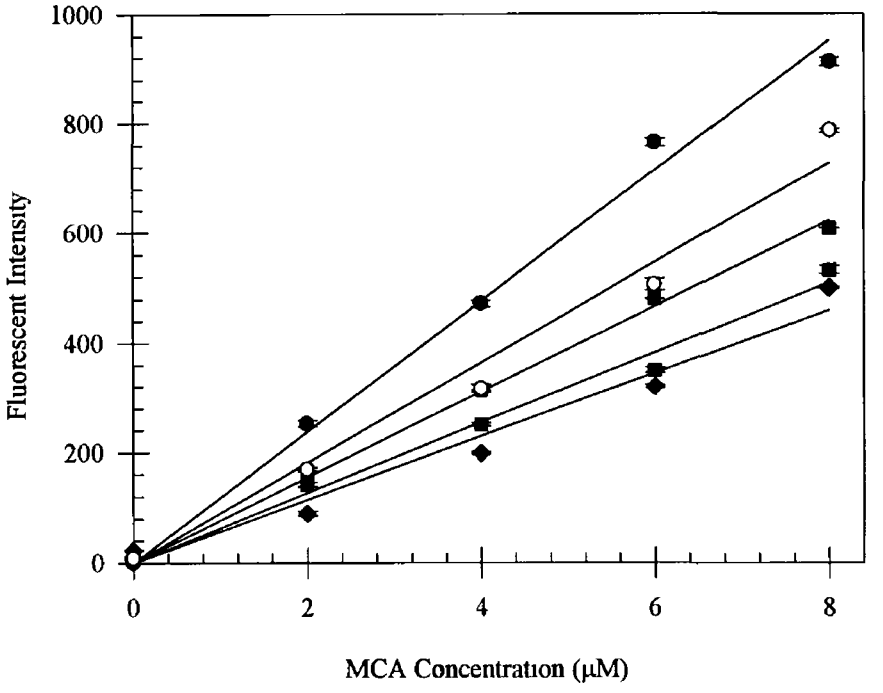
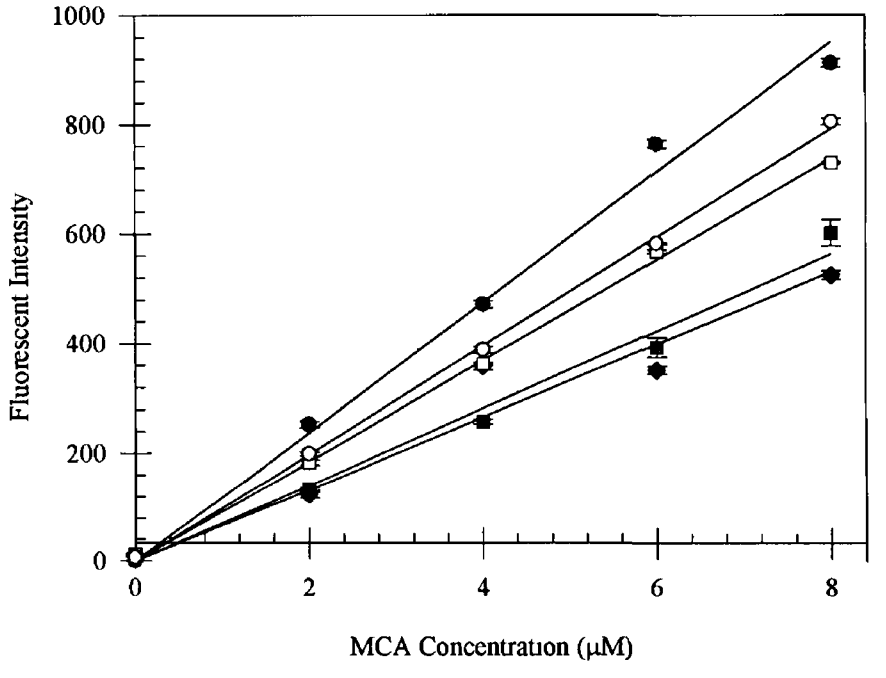


FIGURE 3 7 4



FIGURES 3 7 3 AND 3 7 4 FILTERED MCA STANDARD CURVES

Plots of fluorescent intensity versus MCA concentration for various samples, unfiltered (●), S2 (○), S1 (■), pellet (□) and homogenate (◆) FIGURE 3 7 3 represents filtering effects of these samples in liver tissue, while FIGURE 3 7 4 in lung tissue

FIGURE 3.7.5.

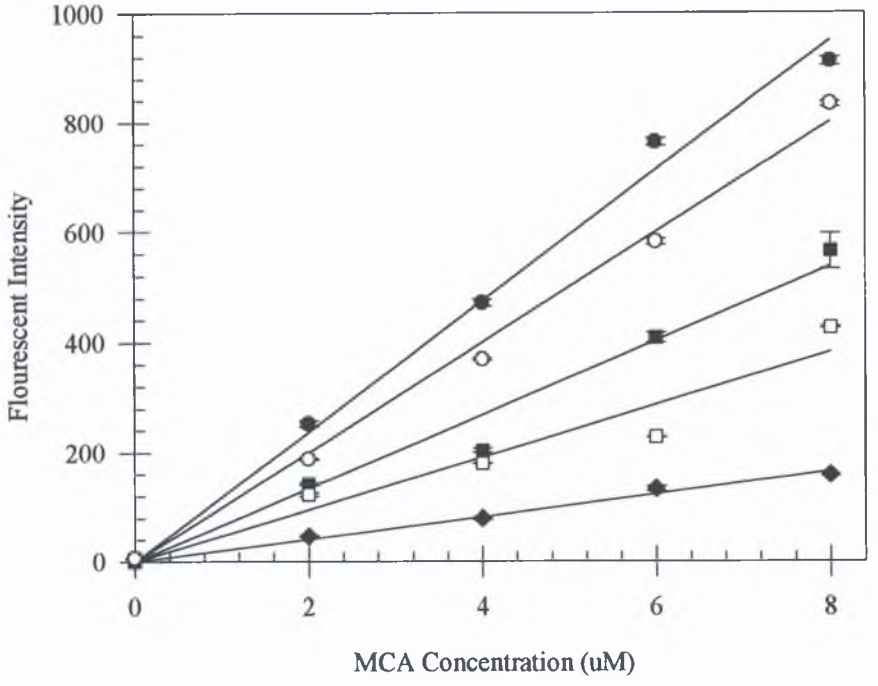
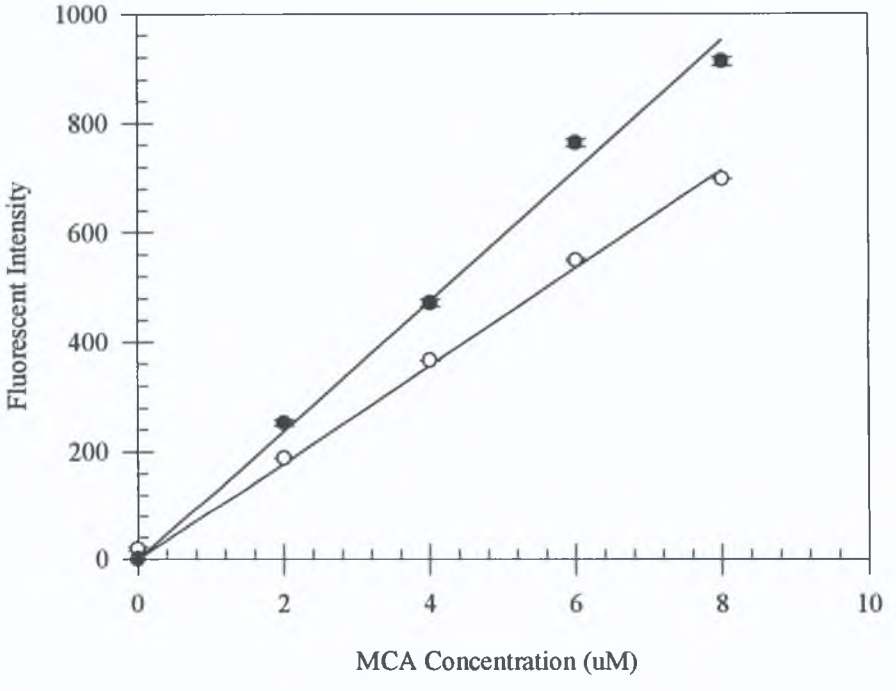


FIGURE 3.7.6.



FIGURES 3.7.5. AND 3.7.6. FILTERED MCA STANDARD CURVES

Plots of fluorescent intensity versus MCA concentration for various samples; unfiltered (●), S2 (○), S1 (■), pellet (□) and homogenate (◆). FIGURE 3.7.5. represents filtering effects of these samples in spleen tissue, while FIGURE 3.7.6. represents serum.

FIGURE 3 7.7

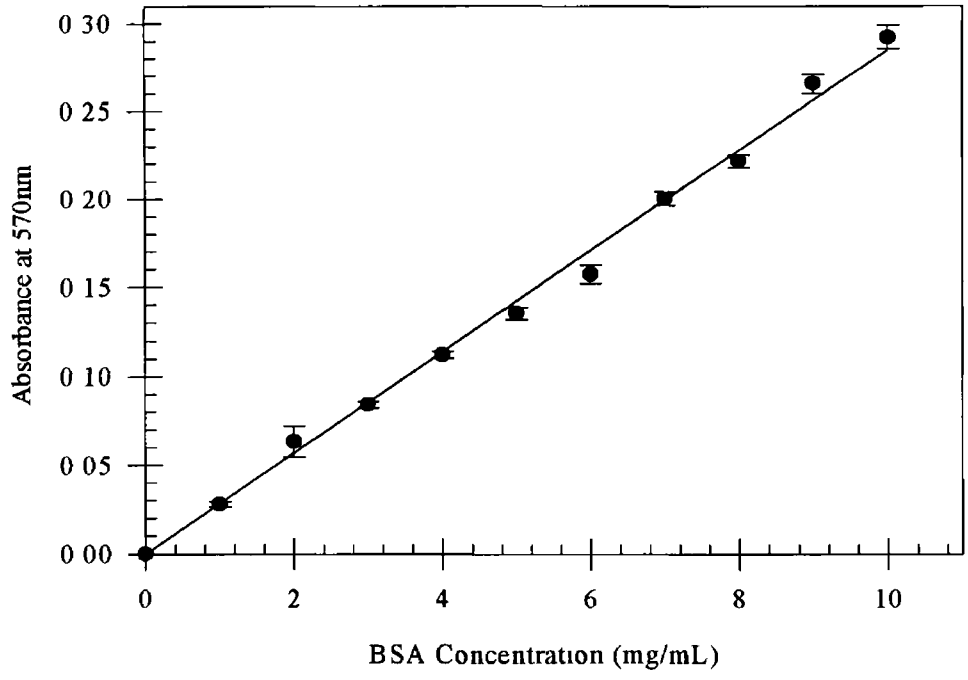


FIGURE 3.7 7 BSA STANDARD CURVE

Plot of absorbance at 570nm versus BSA concentration Biuret Assay standard curve employed for the estimation of protein content in tissue samples

TISSUE	TOTAL ACTIVITY	TOTAL PROTEIN	SPECIFIC ACTIVITY
S1 samples	*unit*	*mg*	*unit/mg*
ZIP			
Brain	37 3	179	0 209
Kidney	172 0	455	0 379
Liver	118 9	663	0 179
Lung	50 5	405	0 125
Spleen	44 3	486	0 091
Serum	122 7	660	0 186
PO			
Brain	565 3	179	3 158
Kidney	1071 8	455	2 356
Liver	1601 4	663	2 415
Lung	469 8	405	1 161
Spleen	846 0	486	1 741
Serum	123 7	660	0 187

TABLE 3 7 ZIP AND PO ACTIVITY, PROTEIN AND SPECIFIC ACTIVITY IN BOVINE EXTRACTS

FIGURE 3.7 8

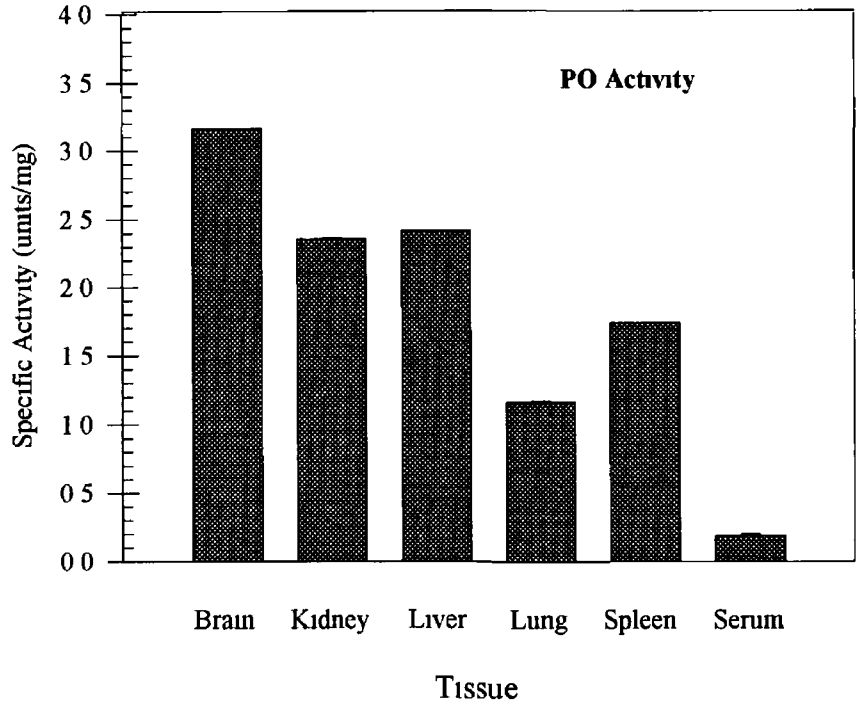
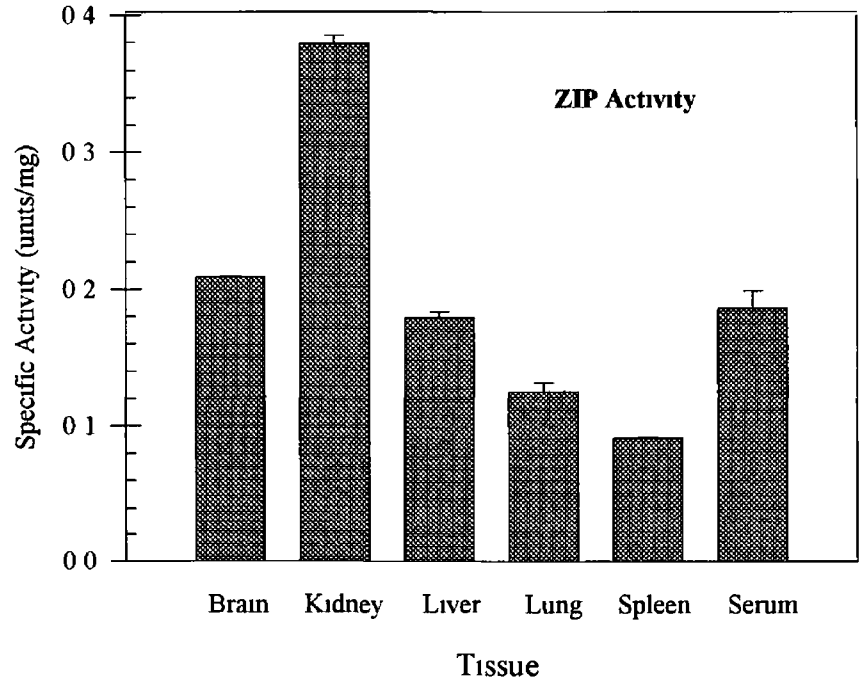


FIGURE 3 7.9.



FIGURES 3 7 8 AND 3 7 9 PO AND ZIP LEVELS IN THE S_1 FRACTION OF VARIOUS BOVINE TISSUES

Plots of specific activity versus organ tissue Specific activity is a ratio of total protein to total enzyme activity FIGURE 3 7 8 shows PO levels and FIGURE 3 7 9 illustrates ZIP levels found in the range of organs

TISSUE	ZIP ACTIVITY	PO ACTIVITY
S1 samples	*%*	*%*
Brain	6	94
Kidney	14	86
Liver	7	93
Lung	10	90
Spleen	5	95
Serum	50	50

TABLE 3 8 LEVELS OF ZIP AND PO ACTIVITIES IN VARIOUS BOVINE SAMPLES

Activities are expressed as a percentage of the total Z-Gly-Pro-MCA degrading activity (100%)

3 8 CHARACTERISATION

3 8 1 N-terminal Sequence Determination

SDS PAGE was performed as detailed in section 2 9 1 1 followed by electroblotting on to a PVDF membrane (section 2 9 1 2) The Ponceau S-stained blot was sent to Dr John S Gilroy, Department of Biological Sciences, University of Durham, South Rd, Durham DHI 3LE to be sequenced Despite many attempts, the N-terminal sequence of ZIP could not be successfully determined An initial attempt at sequencing by Dr Brian Dunbar (Protein Sequencing Department, University of Dundee, Scotland) revealed the presence of two equimolar components with the following sequence following a run of six cycles on the sequencer

CYCLE	1	2	3	4	5	6
RESIDUE	ND	Glu/Pro	Asn/Gly	Asn/Glu	Gln/Arg	Asn/Gln

3 8 2 Relative Molecular Mass Determination

3 8 2 1 SDS PAGE

A non-native SDS gel was prepared as outlined in section 2 6 1 and employed to estimate the relative molecular weight of the purified ZIP activity The distance migrated by the bromophenol blue dye front was calculated to be 66mm and 69mm for the silver and colloidal blue stained gels respectively A plot of log of molecular weight versus relative mobility (R_f) was constructed for the five standards as described in section 2 9 2 1 FIGURES 3 8 2 1 1 and 3 8 2 1 2 show the calibration curves obtained for the gels when stained with silver stain and brilliant blue respectively From the equations of the lines (TABLE 3 9) the molecular weight of ZIP was determined to be 87,810Da (silver stained gel) and 95,820Da (blue stain) It was deduced from this that ZIP is a dimer

FIGURE 3 8.2.1 1

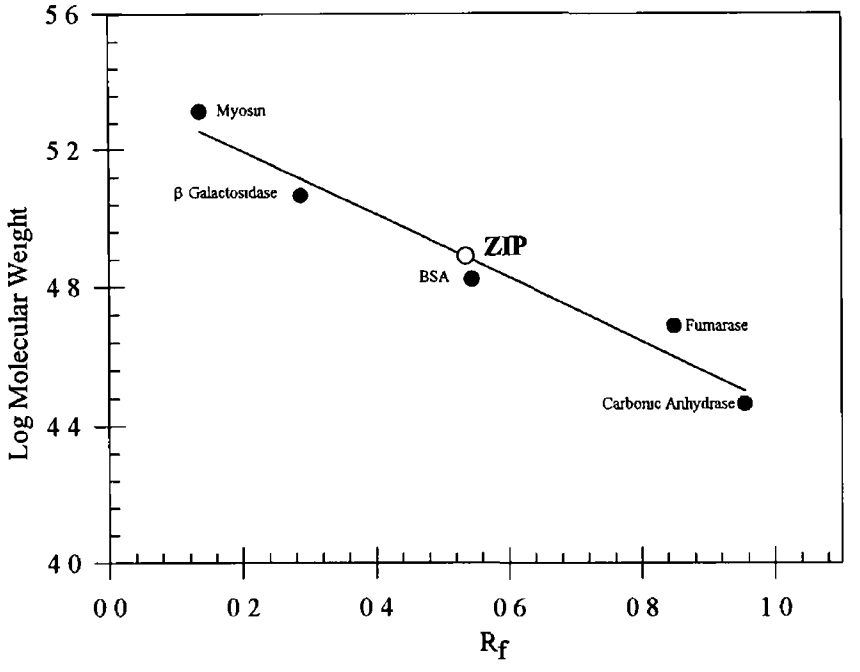
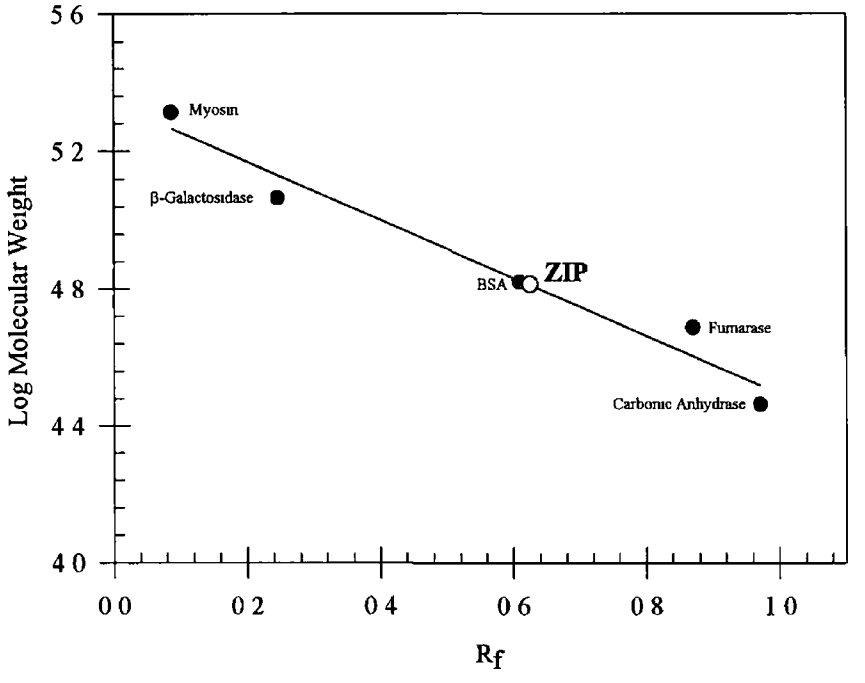


FIGURE 3.8.2.1 2



FIGURES 3 8 2 1 1 AND 3 8 2 1 2 SDS PAGE STANDARD CURVES FOR MOLECULAR WEIGHT DETERMINATION

Plots of log molecular weight versus R_f value, where the R_f value is the distance migrated by the standard (•) or sample (o) divided by the distance migrated by the bromophenol dye front FIGURE 3 8 2 1 1 illustrates the standard curve constructed from the silver stained gel, while FIGURE 3 8 2 1 2 shows that of the colloidal blue stained gel

3 8 2 2 Size Exclusion Chromatography

The relative molecular mass of purified ZIP was estimated using three different size-exclusion chromatography resins, namely sephacryl S-200, HiPrep sephacryl S-200 fast-performance and a BioSep SEC-3000 HPLC column Each of these columns was calibrated with a range of standards as outlined in sections 2 9 2 2 1 - 2 9 2 2 3 The void volume of the Sephacryl S-200 was 81mL, the HiPrep Sephacryl S-200 was 36mL while that of the BioSep SEC-3000 was 5 69mL This allowed for the production of a calibration graph for each resin representing the log molecular weight versus V_e/V_o as described in section 2 9 2 2 These graphs (FIGURES 3 8 2 2 1 , 3 8 2 2 2 and 3 8 2 2 3) were then used to estimate the molecular weight of purified ZIP TABLE 3 9 shows the equation of the lines obtained and the deduced molecular weights obtained using PAGE and size exclusion chromatography

METHOD	EQUATION OF LINE	R^2	MW *Da*
SDS PAGE (silver stain)	$Log\,MW = -0\,92\,(R_f) + 2\,38$	0 958	175,620*
SDS PAGE (blue stain)	$Log\,MW = -0\,84\,(R_f) + 2\,34$	0 962	191,640*
Sephacryl S-200	$Log\,MW = -0\,6(V_e/V_o) + 5\,91$	0 999	194,922
HiPrep sephacryl S-200	$Log\,MW = -0\,95(V_e/V_o) + 6\,32$	0 977	172,288
BioSep SEC-3000	$Log\,MW = -0\,41(V_e/V_o) + 6\,03$	0 976	164,369

TABLE 3.9. MOLECULAR WEIGHTS DETERMINED

* These molecular weights are estimated based on the SDS PAGE indication that ZIP is a hetero-dimer

The average molecular weight was determined to be **179,768** ± 11,670Da

FIGURE 3.8.2 2 1

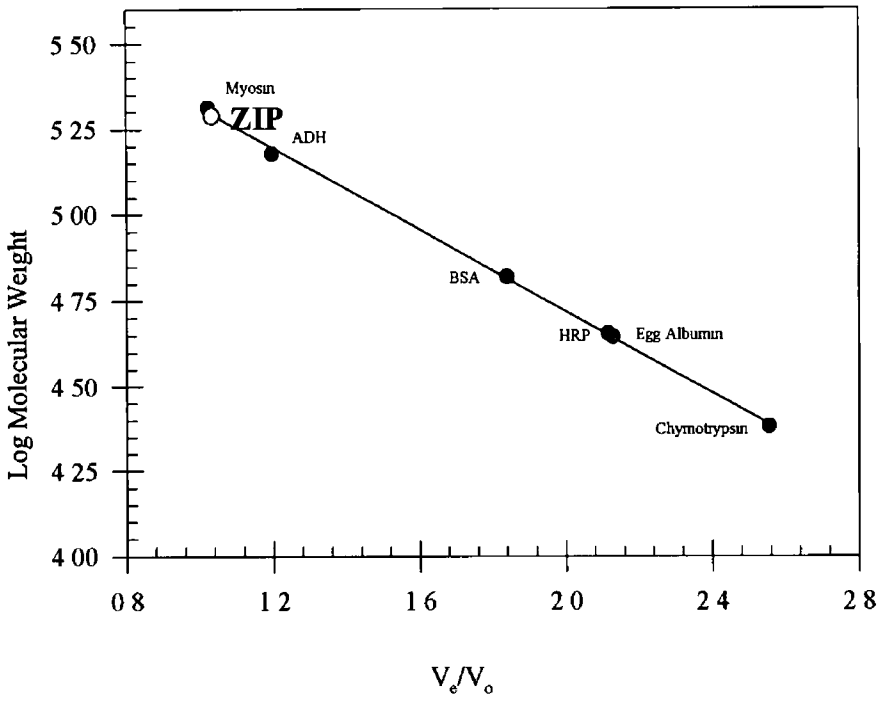
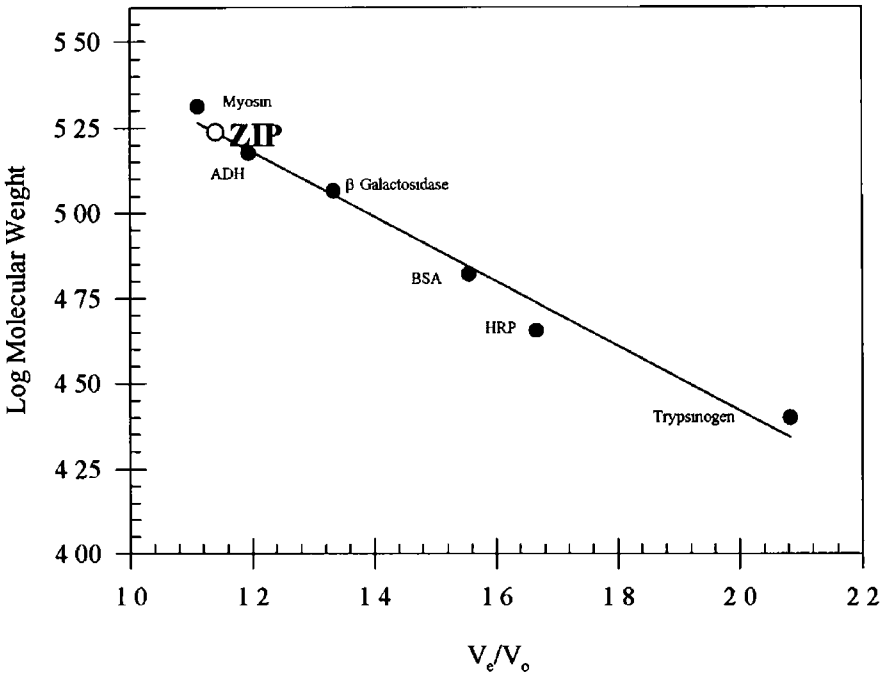


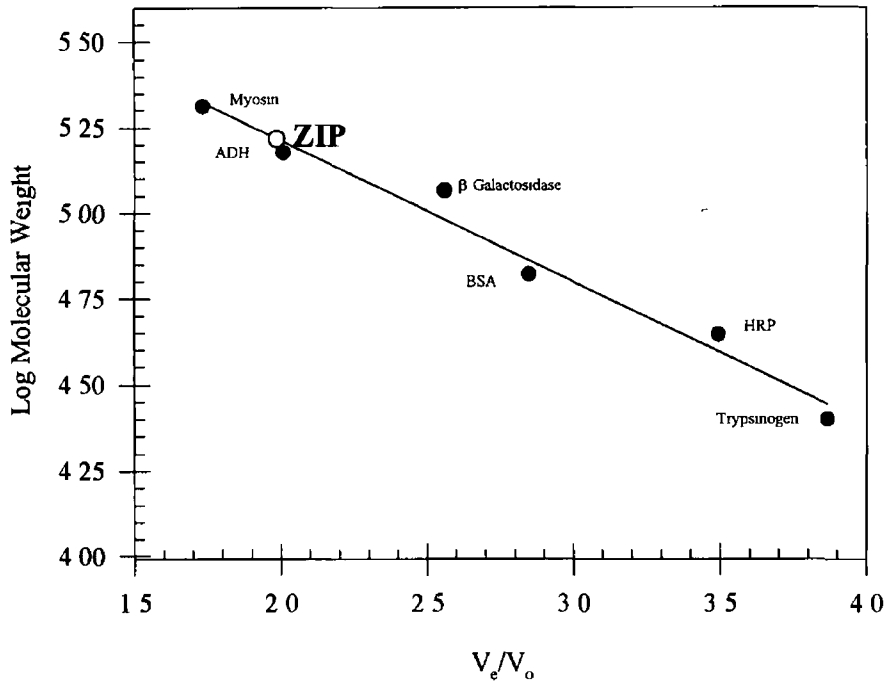
FIGURE 3 8.2.2 2



FIGURES 3 8 2 2.1 AND 3.8.2 2 2. SIZE EXCLUSION CHROMATOGRAPHY STANDARD CURVES FOR MOLECULAR WEIGHT DETERMINATION

Plots of log molecular weight versus V_e/V_o, where V_e is the elution volume of the standard (•) or sample (o) and V_o is the void volume FIGURE 3 8 2 2 1 illustrates the standard curve constructed using Sephacryl S-200, while FIGURE 3 8 2 2 2 shows that of a HiPrep Sepahcryl S-200 resin

FIGURE 3.8.2.2 3



FIGURES 3 8 2 2 3 SIZE EXCLUSION CHROMATOGRAPHY STANDARD CURVE FOR MOLECULAR WEIGHT DETERMINATION

Plot of log molecular weight versus V_e/V_o, where V_e is the elution volume of the standard (•) or sample (o) and V_o is the void volume of the column (5 69mL) A HPLC BioSep SEC-3000 column was employed

3 8 3 Thermostability Studies

3 8 3 1 Stability at Various Temperatures over Time

The effect of pre-incubating ZIP at a range of temperatures for different times prior to assaying at 37°C was investigated as described in section 2 9 3 1 FIGURE 3 8 3 1 illustrates that ZIP activity is completely inhibited by pre-incubation at 60°C, however 70% enzyme activity remains after 55min pre-incubation at 50°C ZIP is unaffected by pre-incubation at temperatures less than 50°C

3 8 3 2 Overnight Stability

The stability of ZIP at 37°C over 24hr was examined as outlined in section 2 9 3 2 Enzyme activity remained relatively stable over this time frame, with a maximum loss of 30% activity after 4hr However ZIP activity was restored following further incubation for 24hr FIGURE 3 8 3 2 illustrates the activity profile at 37°C overnight for purified ZIP

3 8 4 pH Effects

The effects of pH on ZIP activity were monitored in two ways Primarily, ZIP was incubated at a range of pH values and then assayed for activity as described in section 2 9 4 1 This inactivation of ZIP is graphically illustrated in FIGURE 3 8 4 1 The bell-shape curve illustrates inhibition of enzyme activity at pH 2 0 and pH 10 5 Above pH 2 0, ZIP is surprisingly active with 70% activity being observed, similarly at pH 10 0 ZIP is insensitive to pH inactivation between pH 4 0 and pH 8 0

The activation of ZIP by pH was also assessed as described in section 2 9 4 2 where the enzyme was incubated with substrates prepared in a range of pH values FIGURE 3 8 4 2 illustrates this pH activation profile, suggesting activity is optimum between pH 7 4 and pH 8 0 The enzyme exhibits a slight preference towards a potassium phosphate buffering system

FIGURE 3.8 3 1

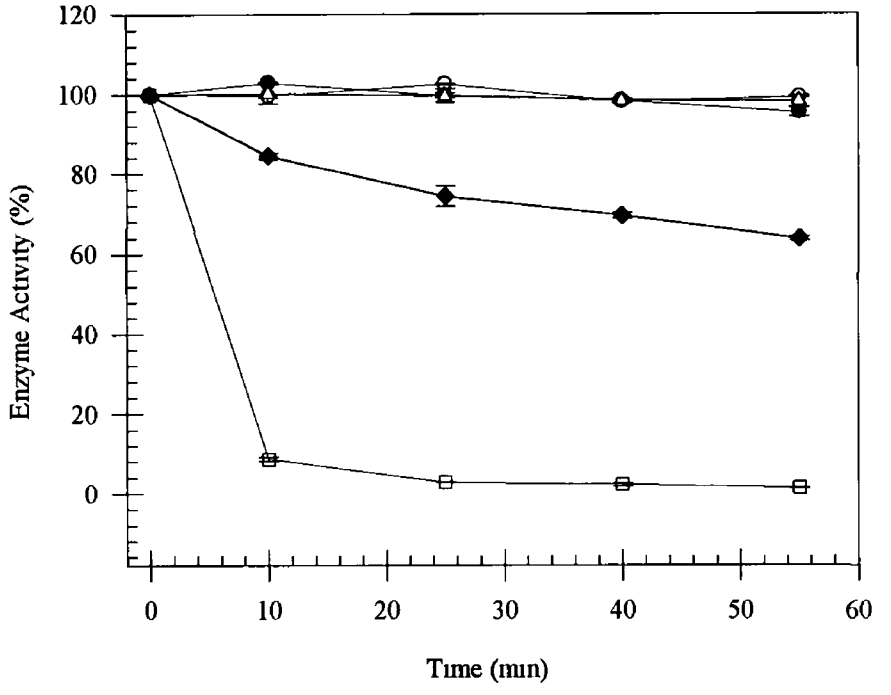
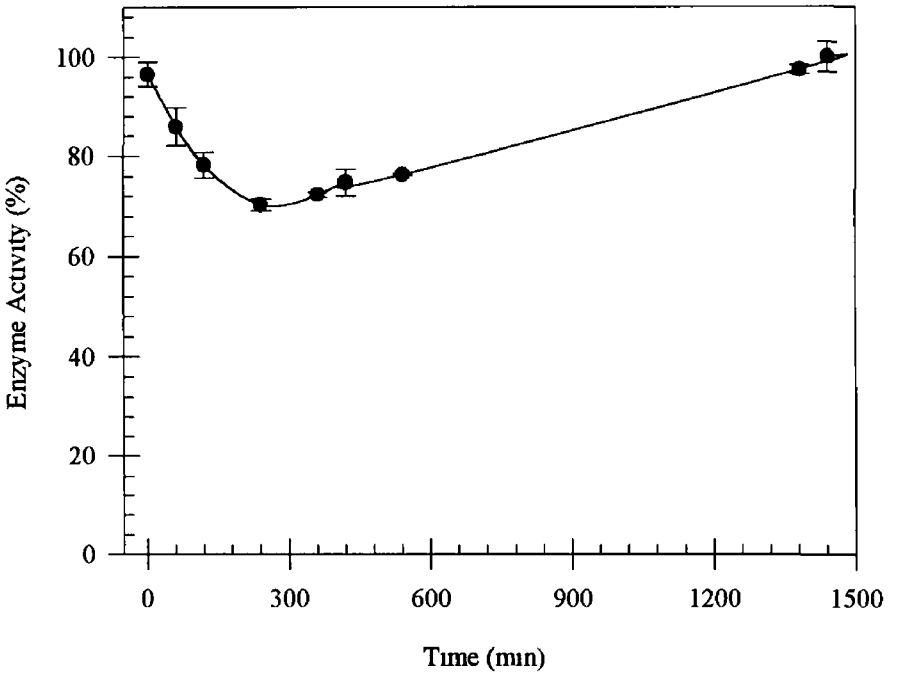


FIGURE 3 8.3.2.



FIGURES 3.8 3 1 AND 3.8 3 2 THERMOSTABILITY STUDIES

Plots of enzyme activity versus time at various temperatures FIGURE 3 8 3 1 shows the stability of ZIP at 20°C (o), 37°C (•), 40°C (△), 50°C (◆) and 60°C (□) over time Enzyme activity is expressed as a percentage of unheated enzyme FIGURE 3 8 3 2 illustrates the effect of a 24 hour pre-incubation on ZIP activity

FIGURE 3 8 4 1.

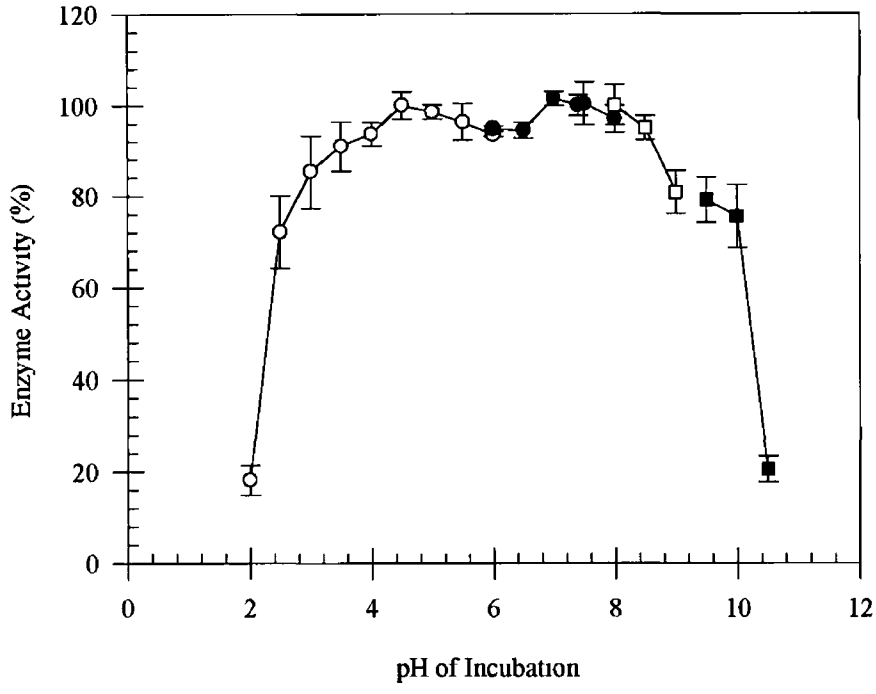
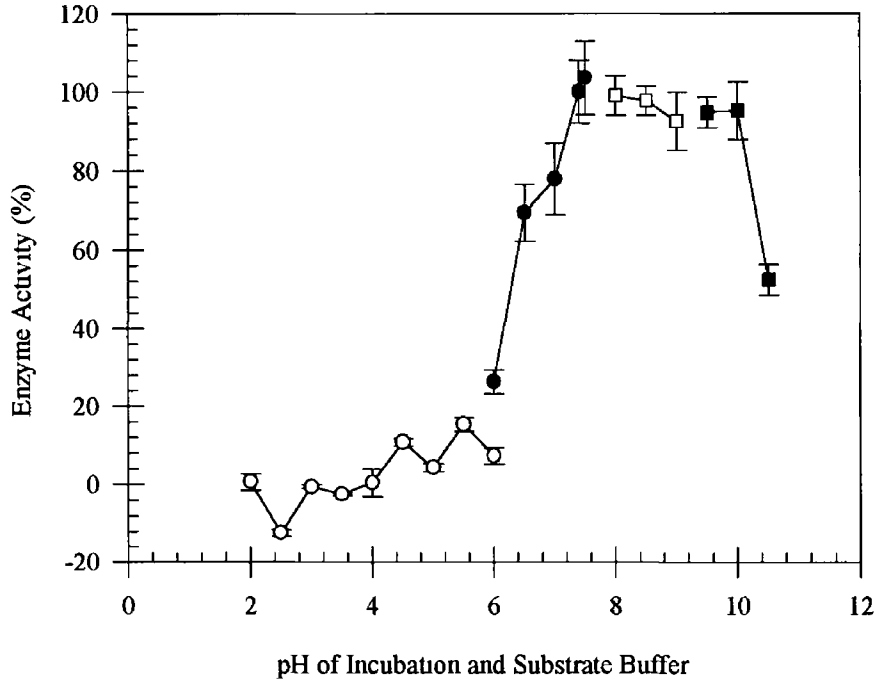


FIGURE 3 8 4 2



FIGURES 3 8 4 1 AND 3 8 4.2 pH EFFECTS ON ZIP

Plots of enzyme activity versus pH Buffers tested were citrate (o), phosphate (•), Tris (□) and glycine (■) FIGURE 3 8 4 1 represents the pH inactivity profile of ZIP while FIGURE 3 8 4 2 corresponds to the pH activation of ZIP Enzyme activity is expressed as a percentage of activity obtained at pH7 4 which is represented as 100%

3 8 5 Isoelectric Point Determination

3 8 5 1 Chromatofocusing

A PBE 94 chromatofocusing resin was employed in an attempt to determine the isoelectric point of ZIP as described in section 2 9 5 1 The elution profile obtained from this column can be seen in FIGURE 3 8 5 1 ZIP activity was eluted in a decreasing linear pH gradient and activity was detected in fractions 78-83 inclusive corresponding to a pH of between 5 5 and 5 8 The pH of this pool was measured to be 5 7, which was taken to be the pI of bovine ZIP

3 8 5 2 Isoelectric Focusing

A vertical IEF acrylamide gel was run as outlined in section 2 9 5 2 and stained with brilliant blue G colloidal FIGURE 3 8 5 2 represents the stained gel image showing the pI markers and purified ZIP The relative mobility (R_f) of each standard and ZIP was calculated as described in section 2 9 5 2 The distance to the anoidic edge of the plate was 7 1cm A plot of R_f versus pI was constructed (FIGURE 3 8 5 3) and the pI of ZIP was estimated using the following equation of the line

$$pI = -4\ 47\ x\ R_f + 7\ 62$$

The pI of ZIP was calculated to be **5 68**

FIGURE 3 8 5 1

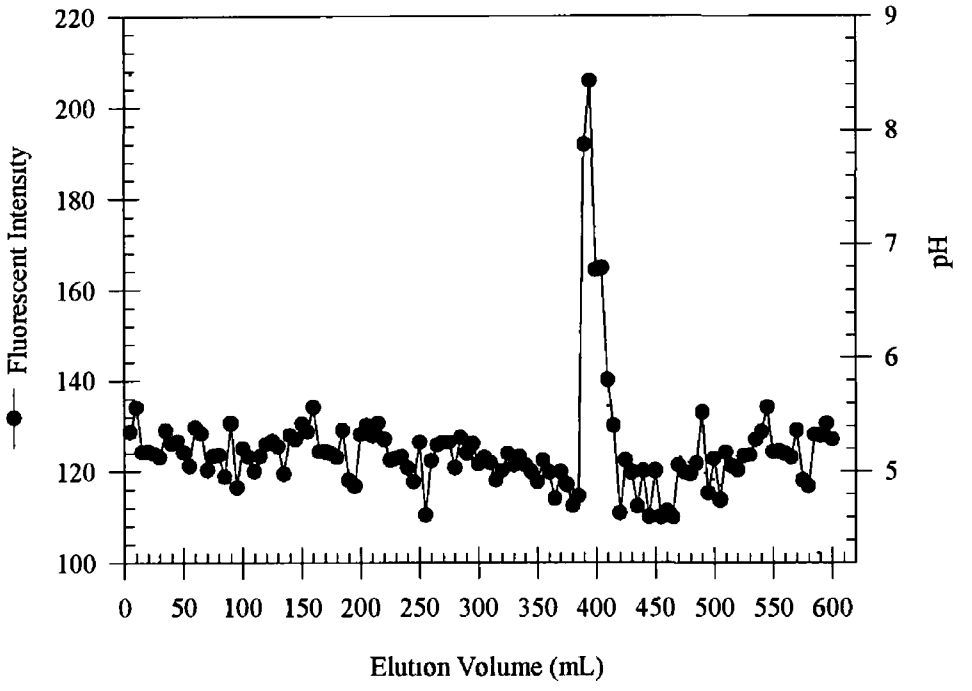
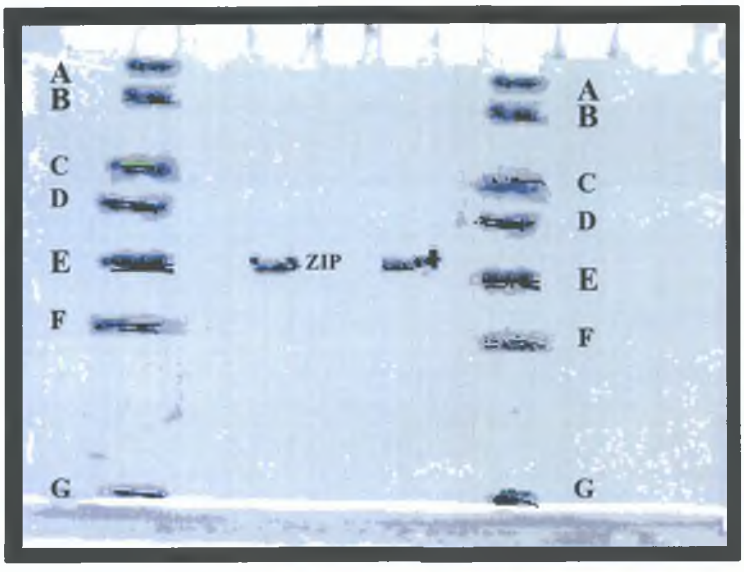


FIGURE 3 8 5.1. CHROMATOFOCUSING ELUTION PROFILE

Plot of fluorescent intensity (●-●) and pH (- - -) versus elution volume

FIGURE 3.8.5.2.



A Lentil Lectin

B Myoglobin

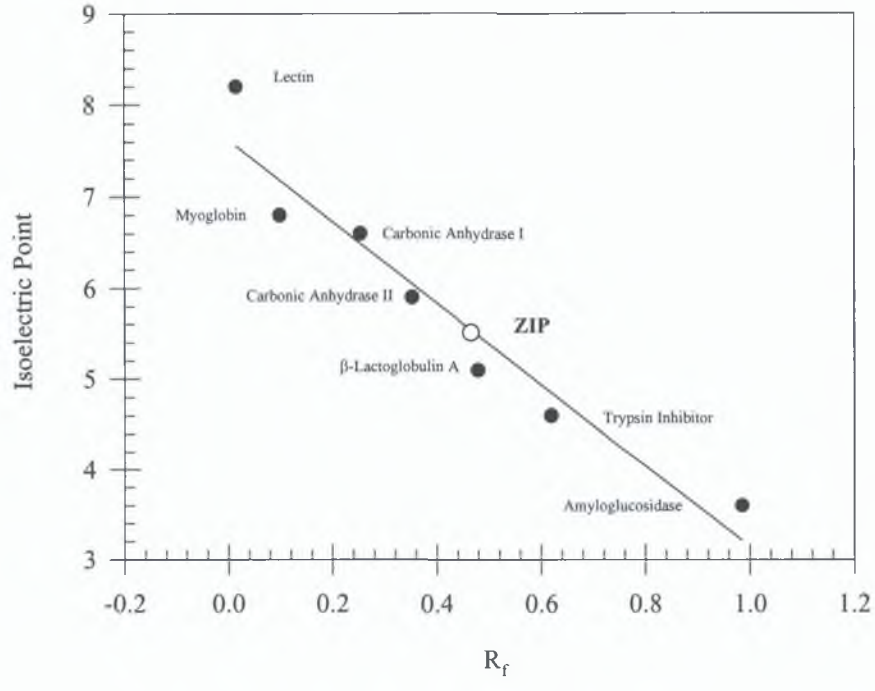
C Carbonic Anhydrase I

D Carbonic Anhydrase II

E β-Lactoglobulin A

F Trypsin Inhibitor

FIGURE 3.8.5.3.



FIGURES 3.8.5.2. AND 3.8.5.3. ISOELECTRIC FOCUSING

FIGURE 3.8.5.2. represents a copy of the isoelectric focusing gel produced. The distances migrated by each standard or sample was calculated and divided by the length of the plate to yield a R_f value. These values for standards (●) and samples (○) are plotted versus pI in FIGURE 3.8.5.3.

3 8 6 Catalytic Classification

The effect of various functional reagents on ZIP activity was investigated as outlined in section 2 9 6 TABLES 3 10, 3 11, 3 12 and 3 13 outline residual ZIP activity following incubation with a range of serine protease inhibitors, metallo-protease inhibitors, cysteine protease inhibitors and cysteine protease activators respectively Residual enzyme activity is expressed as a percentage of positive control activity where no functional reagent was added (represented as 100% activity) The concentration listed is the concentration of functional reagent in the pre-incubation mixture (100µL) These results suggest that ZIP is inhibited by the serine protease inhibitors, AEBSF (58% inhibition at 22 5mM) and aprotinin, (35% inhibition at 1 25mg/mL) 1,7-phenanthroline inhibited ZIP by 77% at 30mM, while 20mM 4,7-phenanthroline caused 90% inhibition The cysteine protease inhibitors, DTNB and NEM inhibited activity by 60% and 47% respectively at concentrations of 10mM

SERINE PROTEASE INHIBITOR	CONCENTRATION	RESIDUAL ZIP ACTIVITY	STANDARD ERROR
		%	%
AEBSF	22 50mM	42 49	0 28
	11 25mM	50 06	4 26
	4 50mM	64 66	9 94
	2 25mM	67 18	0 06
APMSF	10 00mM	95 74	9 78
	5 00mM	93 75	2 97
	2 50mM	92 20	3 49
	1 25mM	90 65	1 05
Aprotinin	1 25mg/mL	66 56	3 77
	0 63mg/mL	68 27	0 23
	0 25mg/mL	73 12	1 13
	0 13mg/mL	79 30	8 65
Benzamidine	10 00mM	90 27	1 20
	5 00mM	90 41	2 87
	1 00mM	98 68	5 42
Leupeptin	1 00mM	97 52	2 01
	0 50mM	93 76	2 25
	0 26mM	98 13	3 57
PMSF	2 50mg/mL	95 47	2 27
	1 25mg/mL	97 15	1 58
	0 50mg/mL	99 34	3 67
	0 25mg/mL	106 94	3 37

TABLE 3.10 EFFECTS OF SERINE PROTEASE INHIBITORS

METALLO-PROTEASE INHIBITOR	CONCENTRATION *mM*	RESIDUAL ACTIVITY %	STANDARD ERROR %
1,10-phenanthroline	20 00	96 73	5 24
	10 00	100 94	7 75
	5 00	100 79	0 42
	1 00	102 87	1 67
1,7-phenanthroline	30 00	23 32	2 22
	7 50	55 09	5 38
	3 75	76 38	7 79
	0 75	76 65	0 47
4,7-phenanthroline	20 00	9 56	1 90
	10 00	10 43	0 28
	5 00	28 30	1 57
	1 00	98 67	4 41
8-hydroxyquinoline	10 00	91 57	2 29
	5 00	96 77	6 12
	2 50	98 64	2 31
CDTA	10 00	69 64	4 65
	5 00	80 51	3 88
	1 00	85 91	1 85
EDTA	20 00	86 04	2 91
	10 00	86 37	0 26
	5 00	88 94	0 16
	1 00	95 99	0 44
EGTA	10 00	92 82	1 82
	5 00	96 13	4 14
	1 00	94 73	2 65
Imidazole	10 00	85 20	2 93
	5 00	106 01	7 99
	1 00	93 27	0 10

TABLE 3 11 EFFECTS OF METALLO-PROTEASE INHIBITORS

CYSTEINE PROTEASE INHIBITOR	CONCENTRATION *mM*	RESIDUAL ZIP ACTIVITY %	STANDARD ERROR %
DTNB	10 0	39 26	5 89
	5 00	58 56	4 84
	1 00	79 82	7 53
NEM	10 00	53 22	2 60
	5 00	72 82	2 50
	1 00	77 66	3 20
Iodoacetamide	10 00	90 31	2 65
	5 00	85 63	2 71
	1 00	88 94	1 58

TABLE 3.12 EFFECTS OF CYSTEINE PROTEASE INHIBITORS

CYSTEINE PROTEASE ACTIVATOR	CONCENTRATION *mM*	RESIDUAL ZIP ACTIVITY %	STANDARD ERROR %
2-mercaptoethanol	10 00	78 56	2 22
	5 00	74 69	2 46
	1 00	79 08	4 11
DTT	10 00	95 61	1 05
	5 00	97 27	1 41
	1 00	79 29	2 94

TABLE 3 13 EFFECTS OF CYSTEINE PROTEASE ACTIVATORS

3 8 7 Effect of Other Functional Reagents

The effect of a range of other functional reagents on ZIP activity was also examined as outlined in section 2 9 7 The residual activities and standard error of the means detected following incubation with these reagents are listed in TABLE 3 14 2 5mg/mL antipain, 1mg/mL bacitracin, 1mg/mL pepstatin A and 1mg/mL puromycin inhibited ZIP by 55%, 41%, 42% and 41% respectively Carnitine and trypsin inhibitor had no effect on enzyme activity

FUNCTIONAL REAGENT	CONCENTRATION	RESIDUAL ZIP ACTIVITY %	STANDARD ERROR %
Antipain	2 50mg/mL	45 70	4 37
	1 25mg/mL	54 13	0 74
	0 50mg/mL	58 33	3 56
Bacitracin	1 00mg/mL	58 58	2 48
	0 50mg/mL	69 27	0 89
	0 10mg/mL	73 55	1 24
Carnitine	10 00mM	96 27	8 53
	5 00mM	85 81	0 55
	1 00mM	78 21	4 97
Pepstatin A	1 00mg/mL	57 94	1 70
	0 50mg/mL	79 22	3 72
	0 20mg/mL	72 81	1 15
Puromycin	1 00mg/mL	59 18	4 26
	0 50mg/mL	66 25	1 13
	0 20mg/mL	68 30	1 29
Trypsin Inhibitor	1 00mg/mL	131 58	21 08
	0 50mg/mL	101 98	5 05
	0 10mg/mL	94 12	5 71

TABLE 3 14 EFFECT OF OTHER FUNCTIONAL REAGENTS

3.8 8 Effect of Metal Ions on ZIP Activity

The effect of metal salts on purified ZIP activity was investigated by incorporating the metal salts into the fluorimetric assay as outlined in section 2 9 8 The results are presented in TABLE 3 15 $HgSO_4$ had the most detrimental effect on activity, with a loss of 66% activity, followed by zinc, manganese, cadmium, sodium and calcium sulphates

METAL SULPHATE	ZIP RESIDUAL ACTIVITY	STANDARD ERROR
	%	%
Control	*100 00*	*8 5x10 ^{4}*
Cobalt	99 57	4 0x10^{-4}
Copper	97 53	5 1x10 ^{4}
Nickle	92 86	6 9x10^{-4}
Magnesium	92 66	7 6x10 ^{4}
Calcium	70 38	5 5x10 ^{4}
Sodium	69 72	5 6x10^{-4}
Cadmium	63 57	6 9x10^{-4}
Manganese	61 10	6 6x10 ^{4}
Zinc	56 60	6 2x10^{-4}
Mercury	44 29	2 1x10^{-4}

TABLE 3 15 EFFECT OF METAL IONS ON ZIP ACTIVITY

3 8 9 Carbohydrate Analysis

3 8 9 1 Fehling's Test for Reducing Sugars

The Fehling's test for reducing sugars was performed as outlined in section 2 9 9 1 Following 15min incubation at 100°C, the formation of a brick red precipitate was observed, indicating the presence of reducing sugars in the enzyme sample

3 8 9 2 Molisch, Glucose and Starch Test

Purified ZIP was tested for carbohydrates using the Molisch test and was also tested for glucose and for starch as outlined in section 2 9 9 2 The addition of Mohsch's reagent to ZIP followed by sulphuric acid addition produced a violet colour at the junction of the two liquids, indicating a positive result ZIP tested negative for both glucose and starch

3 8 9 3 Glycoprotein Carbohydrate Estimation

The classification of ZIP as a glycoprotein was determined using a Pierce glycoprotein estimation kit as described in section 2 9 9 3 FIGURE 3 8 9 shows the standard curve constructed on plotting total carbohydrate content versus absorbance at 550nm for each of the standards The absorbance of ZIP was measured and the total carbohydrate content estimated using the following equation of the line

$$\textit{Total carbohydrate content} = 4\ 06 \times \textit{absorbance at } 550nm + 0\ 43$$

ZIP was determined to be a glycoprotein according to this kit with **33%** total carbohydrate content

FIGURE 3 8 9.

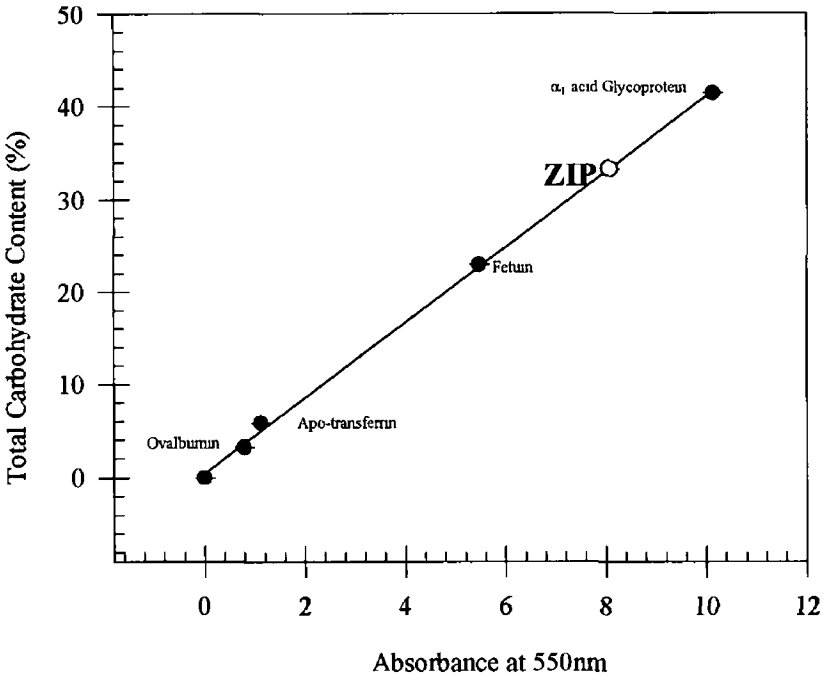


FIGURE 3.8 9 GLYCOPROTEIN CARBOHYDRATE ESTIMATION

Plot of total carbohydrate content versus absorbance at 550nm for various glycoproteins (●) and ZIP (O)

3 8 10 Oligopeptidase Determination

3 8 10 1 Reverse Phase HPLC Analysis

Ion-pair HPLC was performed as outlined in section 2 9 10 1 on three peptides, namely α_2M, BSA and insulin B-chain, to determine whether or not ZIP was an oligopeptidase FIGURES 3 8 10 1 , 3 8 10 2 and 3 8 10 3 are the chromatograms illustrating the failure of ZIP to cleave α_2M, BSA and insulin B chain respectively PO, a known oligopeptidase, also failed to cleave any of the tested peptides (results not shown)

3 8 10 2 Fluorimetric Analysis

Purified ZIP and PO were incubated with a range of α_2M concentrations as described in section 2 9 10 2 FIGURES 3 8 10 4 and 3 8 10 5 highlights the inhibition profiles observed It is clearly evident that neither PO nor ZIP was inhibited by α_2M, although a slight activation of PO was evident with increasing concentrations of the macroglobulin

3 8 11 Substrate Specificity

3 8 11 1 Ion-Pair Reverse Phase HPLC

The ability of purified ZIP and PO activities to hydrolyse a range of synthetic and bioactive peptides was investigated using ion-pair HPLC as outlined in section 2 9 11 1 TABLE 3 16 lists the peptides which were tested and whether or not cleavage was detected The cleavage of Angiotensin I, Angiotensin II, LHRH and TRH by both PO and ZIP can be seen in the chromatograms of absorbance versus retention time in FIGURES 3 8 11 1 to 3 8 11 8 respectively Cleavage products are highlighted

FIGURE 3 8 10 1.

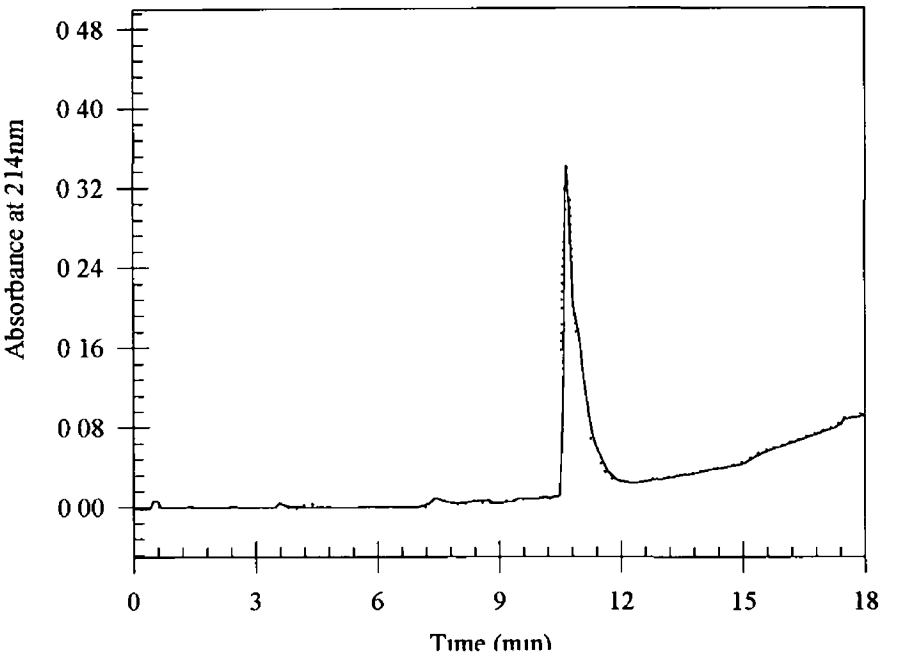
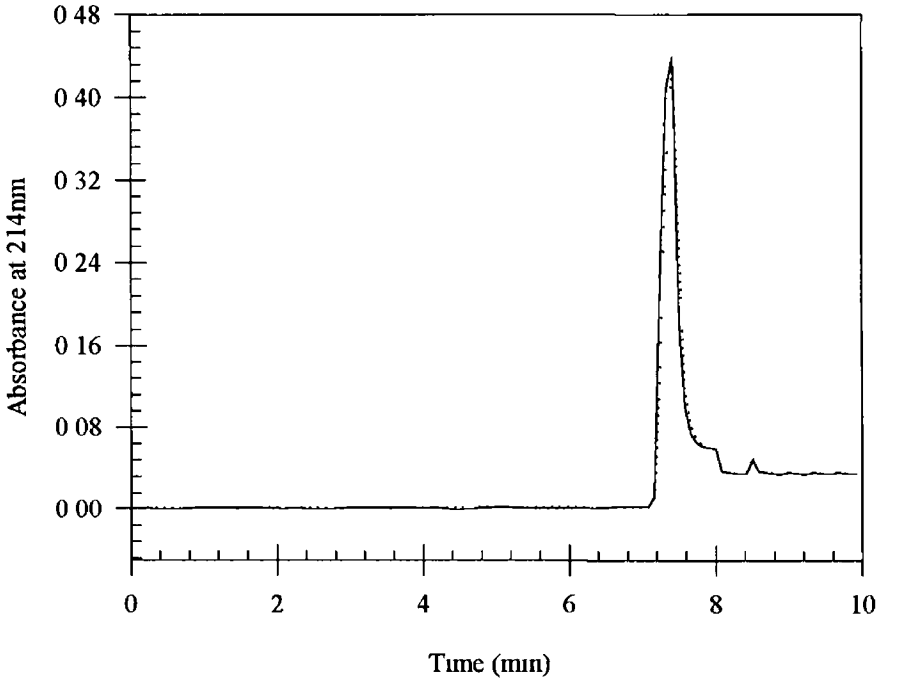


FIGURE 3.8.10 2



FIGURES 3 8 10 1. AND 3 8.10.2. OLIGOPEPTIDASE DETERMINATION

Plots of absorbance at 214nm versus time for peptide (- -) and peptide ZIP incubation (___)

FIGURE 3 8 10 1 illustrates the failure of ZIP to cleave α2-macroglobulin while FIGURE 3 8 10 2 shows that ZIP also fails to cleave bovine serum albumin

FIGURE 3 8 10 3

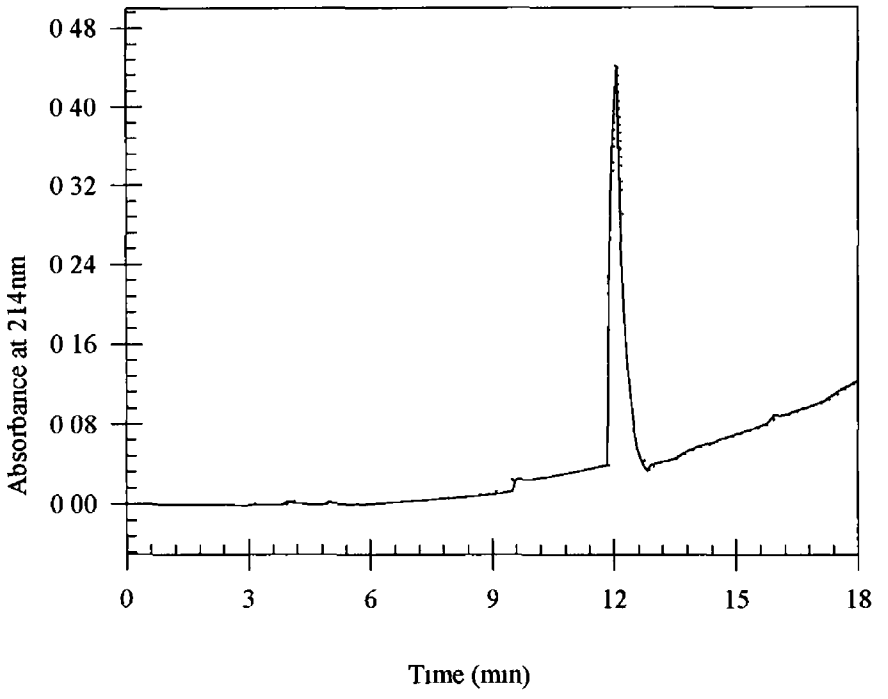


FIGURE 3 8 10 3 OLIGOPEPTIDASE DETERMINATION

Plot of absorbance at 214nm versus time for insulin B chain (- - -) and ZIP incubated with insulin B chain (____)

FIGURE 3 8 10 4

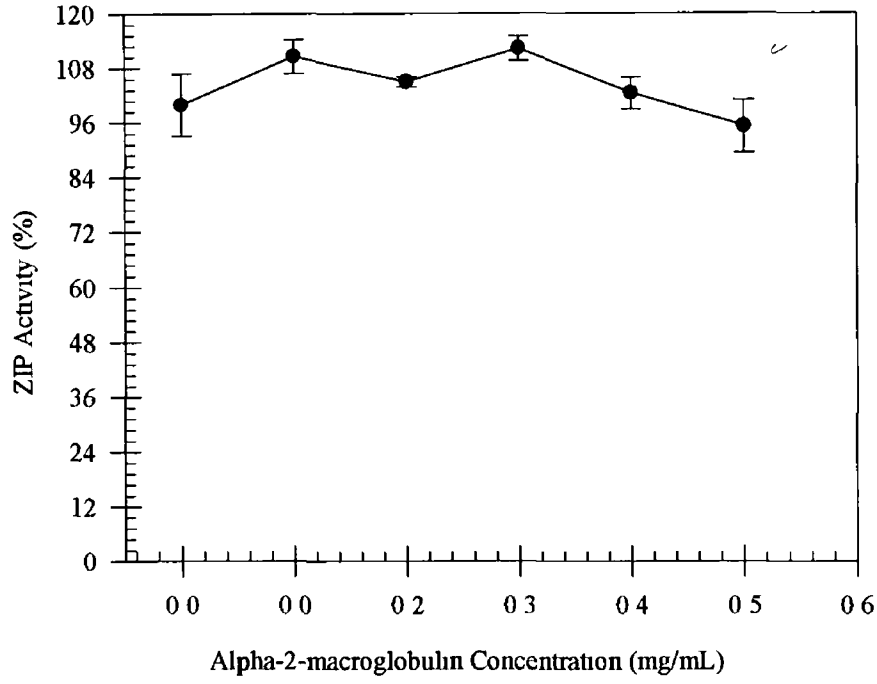
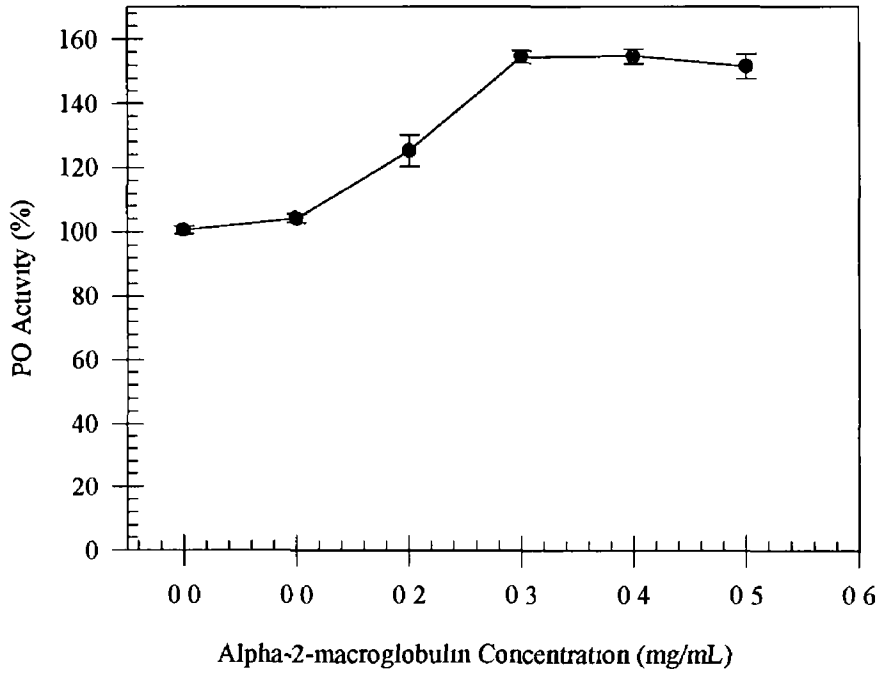


FIGURE 3 8 10 5



FIGURES 3 8.10.4. AND 3 8 10 5 α_2-MACROGLOBULIN TEST FOR OLIGOPEPTIDASES

Plot of enzyme activity versus α_2M concentration for ZIP (FIGURE 3 8 10 4) and PO (FIGURE 3 8 10 5)

PEPTIDE	HYDROLYSIS BY PO	HYDROLYSIS BY ZIP
$A\beta_{1\ 28}$	No	No
$A\beta_{1\ 43}$	No	No
$A\beta_{25\text{-}35}$	No	No
Angiotensin I	Yes	Yes
Angiotensin II	Yes	Yes
Angiotensin III	Yes	Yes
(Arg^8)-Vasopressin	No	No
Bradykinin	Yes	No
(Glu^2)-TRH	Yes	No
LHRH	Yes	Yes
Neurotensin	No	No
(Phe^2)-TRH	Yes	No
Substance P	No	No
TRH	Yes	Yes
TRH-Gly	Yes	No
Cyclo (His-Pro)	No	No
Gly-Ala-Phe	No	No
Gly-Gly-Pro-Ala	No	No
Gly-Phe-Ala	No	No
Gly-Pro-Ala	No	No
Lys-Ala-Ala	No	No
Pyr-His-Gly	No	No
Pyr-His-Pro	No	No
Z-Gly-Pro-Ala	Yes	Yes
Z-Pro-Ala	No	No
Z-Pro-Gly	No	No
Z-Pro-Leu-Gly	No	No
Z-Pro-Pro	No	No

TABLE 3 16. ION-PAIR HPLC SUBSTRATE SPECIFICITY ANALYSIS

FIGURE 3 8 11 1

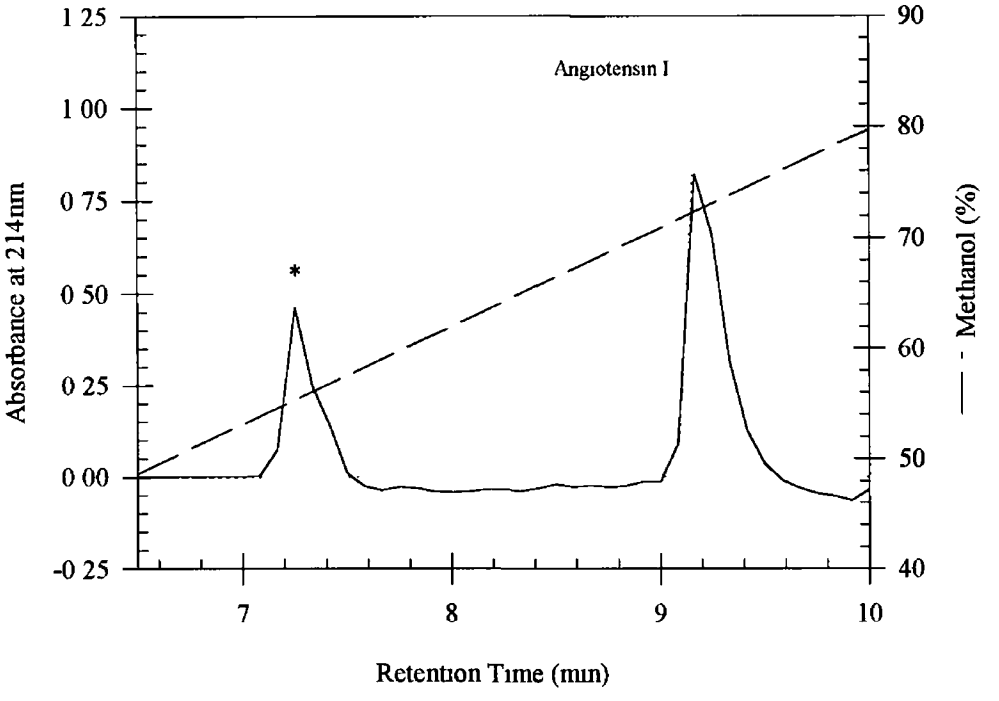
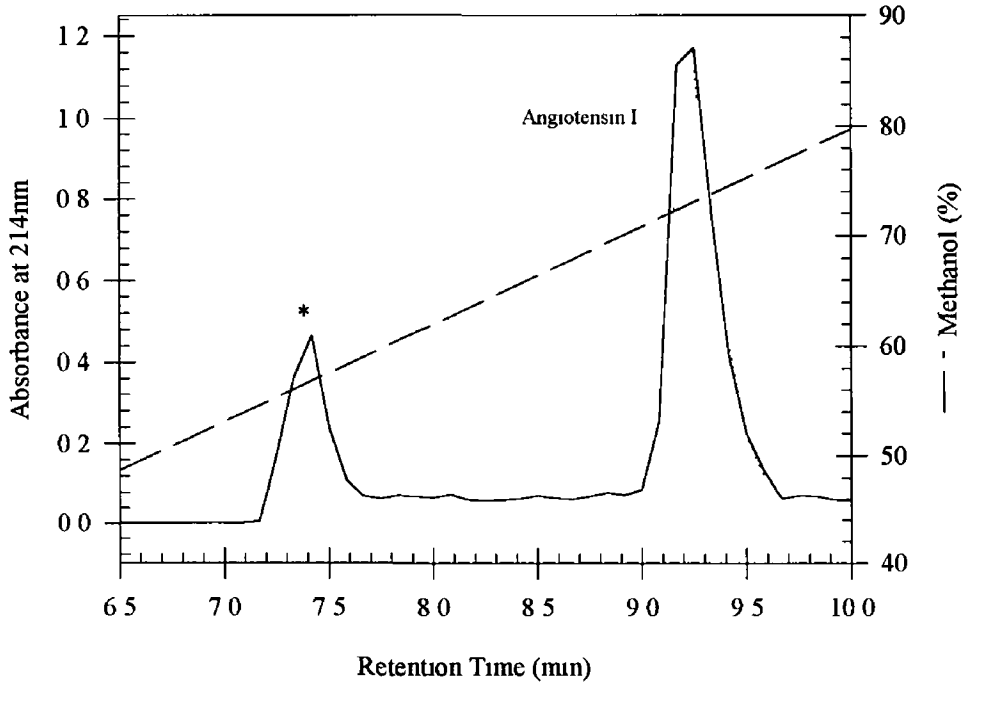


FIGURE 3 8 11 2



FIGURES 3 8 11 1 AND 3 8 11 2 ION-PAIR HPLC SUBSTRATE SPECIFICITY STUDIES

Plots of absorbance at 214nm versus time for Angiotensin I (- - -) and enzyme incubated with Angiotensin I (____) FIGURE 3 8 11 1 illustrates the cleavage of Angiotensin I by PO while FIGURE 3 8 11 2 represents Angiotensin I cleavage by ZIP Cleavage products are denoted *

FIGURE 3.8.11 3

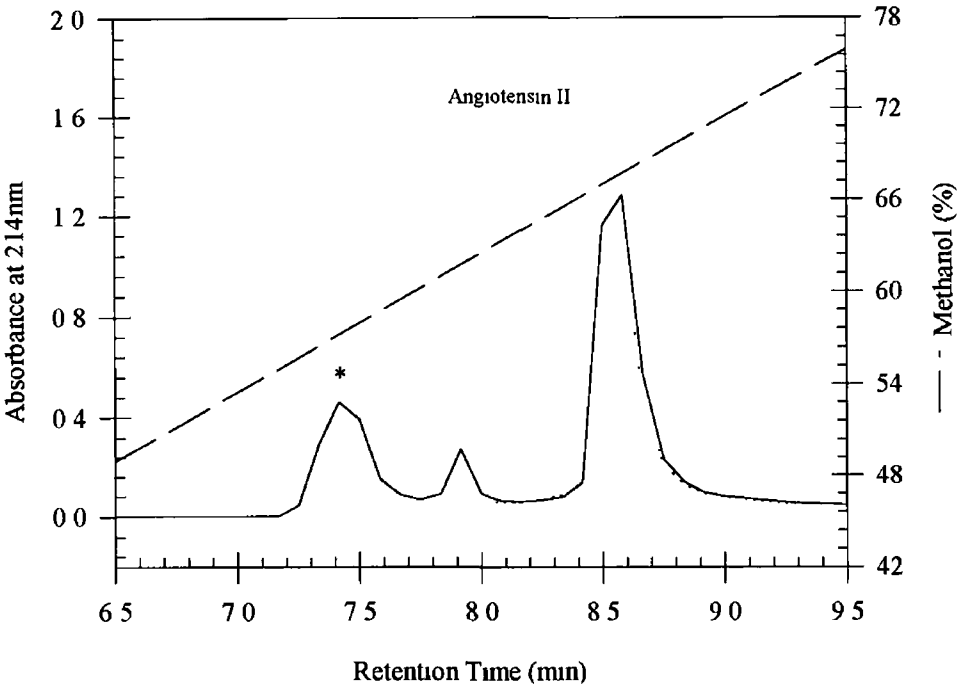
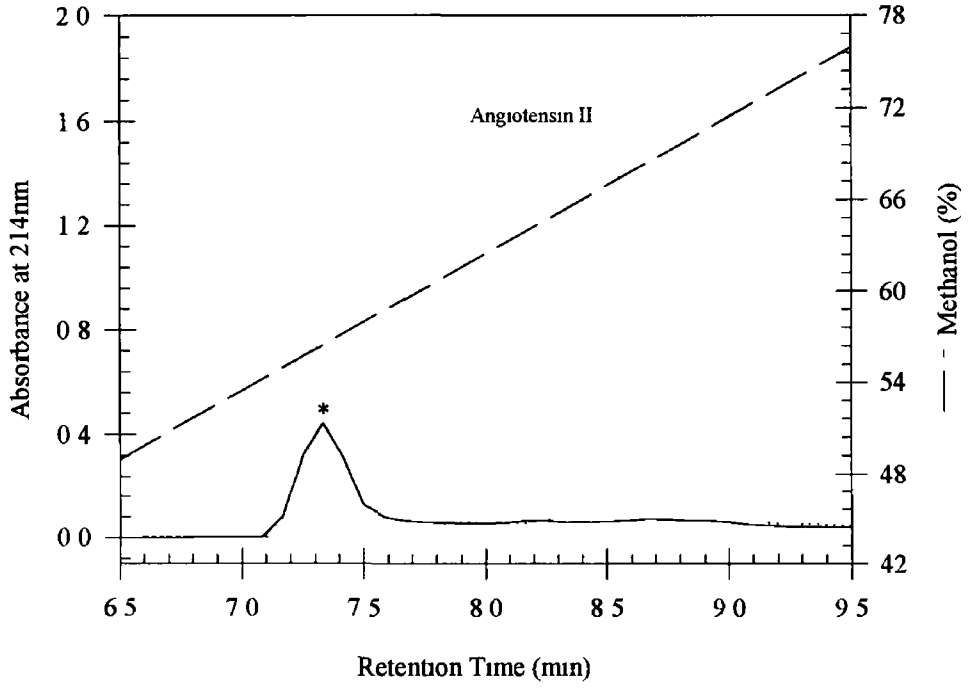


FIGURE 3 8.11 4



FIGURES 3 8.11.3. AND 3.8.11.4. ION-PAIR HPLC SUBSTRATE SPECIFICITY STUDIES

Plots of absorbance at 214nm versus time for Angiotensin II (- - -) and enzyme incubated with Angiotensin II (____) FIGURE 3 8 11 3 shows Angiotensin II cleavage by PO while FIGURE 3 8 11 4 illustrates the cleavage of Angiotensin II by ZIP Cleavage products are denoted *

FIGURE 3 8 11 5.

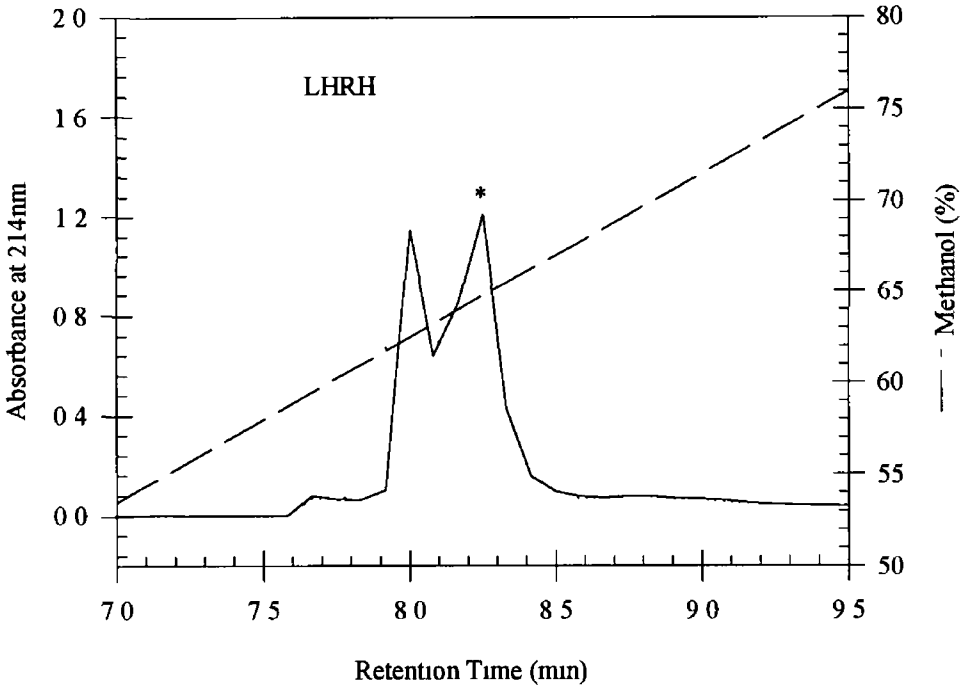
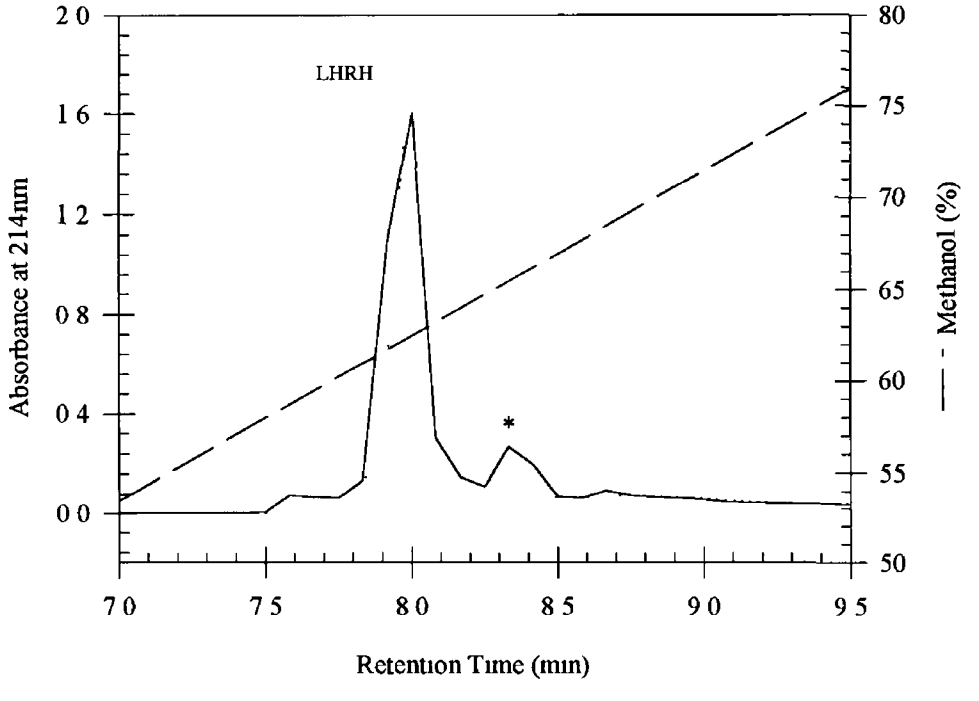


FIGURE 3 8 11 6



FIGURES 3.8.11.5. AND 3.8.11.6. ION-PAIR HPLC SUBSTRATE SPECIFICITY STUDIES

Plots of absorbance at 214nm versus time for LHRH ( ) and enzyme incubated with LHRH (__) FIGURE 3 8 11 5 illustrates cleavage by PO while FIGURE 3 8 11 6 represents LHRH cleavage by ZIP Cleavage products are denoted *

FIGURE 3.8.11.7.

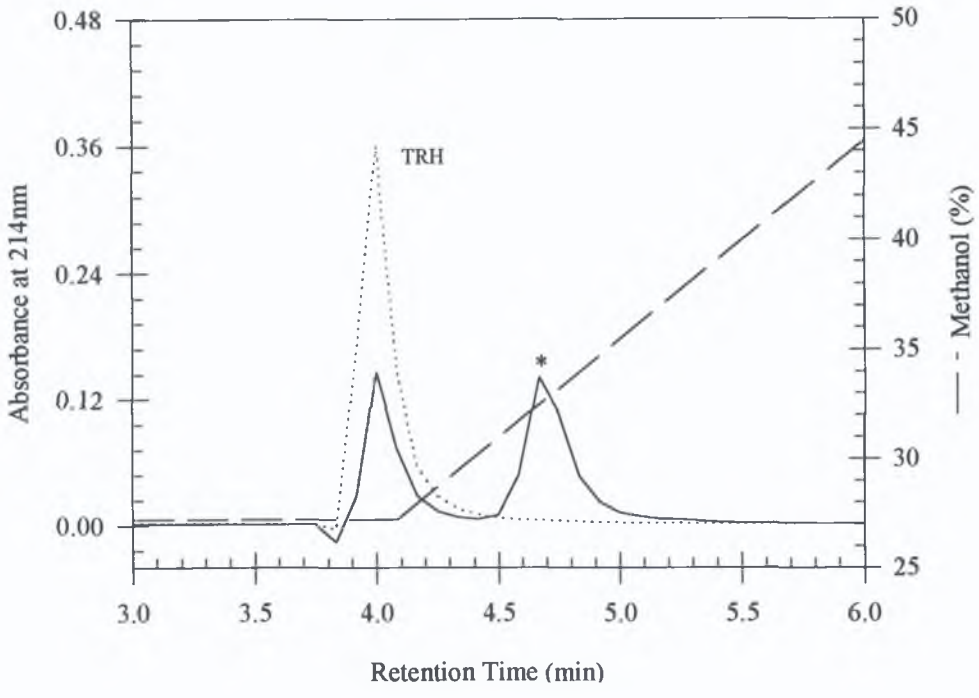
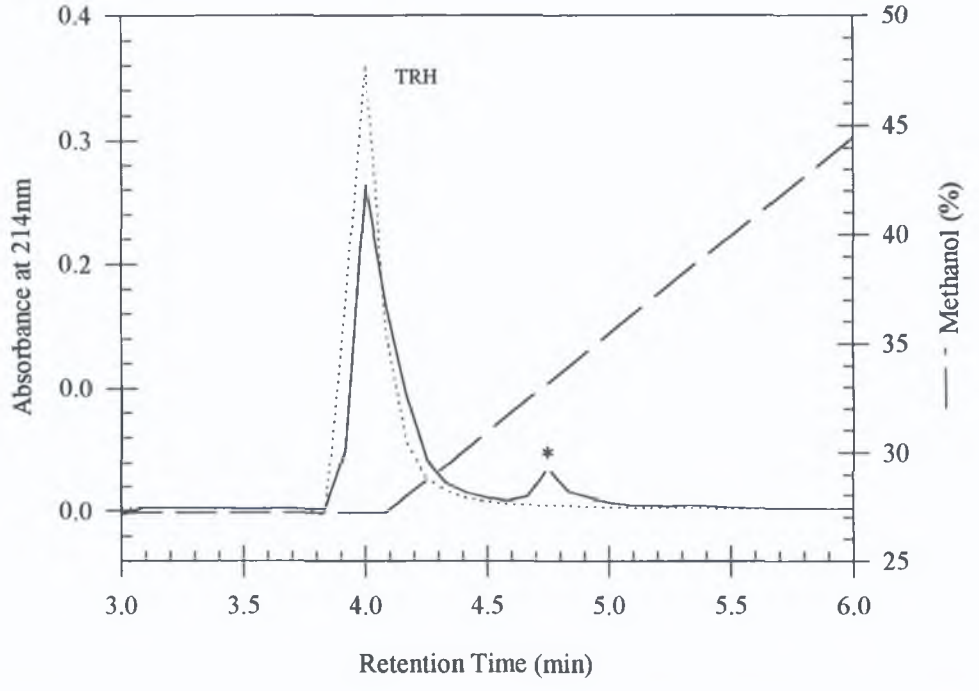


FIGURE 3.8.11.8.



FIGURES 3.8.11.9. AND 3.8.11.10. ION-PAIR HPLC SUBSTRATE SPECIFICITY STUDIES

Plots of absorbance at 214nm versus time for TRH (...) and enzyme incubated with TRH (___). FIGURE 3.8.11.7. illustrates TRH cleavage by PO while FIGURE 3.8.11.8. represents the cleavage of TRH by ZIP. Cleavage products are denoted *.

3 8 11 2 Cadmium Ninhydrin Assay

The ability of ZIP to cleave a number of dipeptides was examined using the cadmium-ninhydrin assay as outlined in section 2 9 11 2 Spectrophotmetric analysis at 504nm of samples and blanks allowed for the detection of cleavage products, recognisable as an increase in absorbance The affinity of ZIP towards these dipeptides could be quantified on construction of free amino acid standard curves, however as TABLE 3 17 illustrates, no cleavage was detected

SUBSTRATE	HYDROLYSIS	SUBSTRATE	HYDROLYSIS
Ala-Arg	No	Leu-Gly	No
Ala-Gln	No	Lys-Ile	No
Ala-Pro	No	Lys-Tyr	No
Arg-Ala	No	Pro-Gly	No
Arg-Glu	No	Pro-Leu	No
Asp-Lys	No	Pro-Pro	No
Asp-Tyr	No	Pyr-Ala	No
Glu-Lys	No	Pyr-Gly	No
Glu-Val	No	Pyr-Phe	No
Gly-Pro	No	Pyr-Val	No
His-Pro	No	Z-Pro	No

TABLE 3 17 CADMIUM-NINHYDRIN ASSAY FOR DIPEPTIDES

3 8 11 3 Kinetic Analysis

3 8 11 3 1 K_M Determinations for Z-Gly-Pro-MCA

The K_M values for the reactions between Z-Gly-Pro-MCA and ZIP and between Z-Gly-Pro-MCA and PO were determined experimentally as described in section 2 9 11 3 1 K_M values were determined using Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf analysis as described in section 6 4 1 TABLE 3 18 lists the K_M values deduced using these models FIGURES 3 8 11 9 to 3 8 11 12 illustrate Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf plots for ZIP assayed using Z-Gly-Pro-MCA The same plots for PO assayed with Z-Gly-Pro-MCA are shown in FIGURES 3 8 11 13 to 3 8 11 16

MODEL	K_M FOR ZIP	V_{MAX}	K_M FOR PO	V_{MAX}
	μM	*Fluorescent units*	*μM*	*Fluorescent units*
Michaelis-Menten (MM)	50	36	80	450
Lineweaver-Burk (LB)	65	45	108	376
Eadie-Hofstee (EH)	57	37	102	562
Hanes-Woolf (HW)	41	ND	106	ND
Average	*54*	*39*	*99*	*463*

TABLE 3.18. KINETIC ANALYSIS OF ZIP AND PO USING Z-GLY-PRO-MCA

3 8 11 3 2 K_i Determinations for Proline-containing Peptides

The effect of the inclusion of various proline-containing peptides on the kinetic interaction between enzyme activities and Z-Gly-Pro-MCA was determined as described in section 2 9 11 3 2 A K_M value was determined for both ZIP and PO using the kinetic models described in section 3 8 11 3 1 K_{app} values were graphically deduced for each of the proline containing peptides and subsequently a K_i value was estimated for each of the peptides tested (see appendix, section 6 4 2) The nature of the inhibition was determined as outlined in section 6 4 3 The K_i values for the ZIP and PO interactions studied are presented in TABLES 3 19 and 3 20 respectively FIGURES 3 8 11 17 and

FIGURE 3.8 11 9

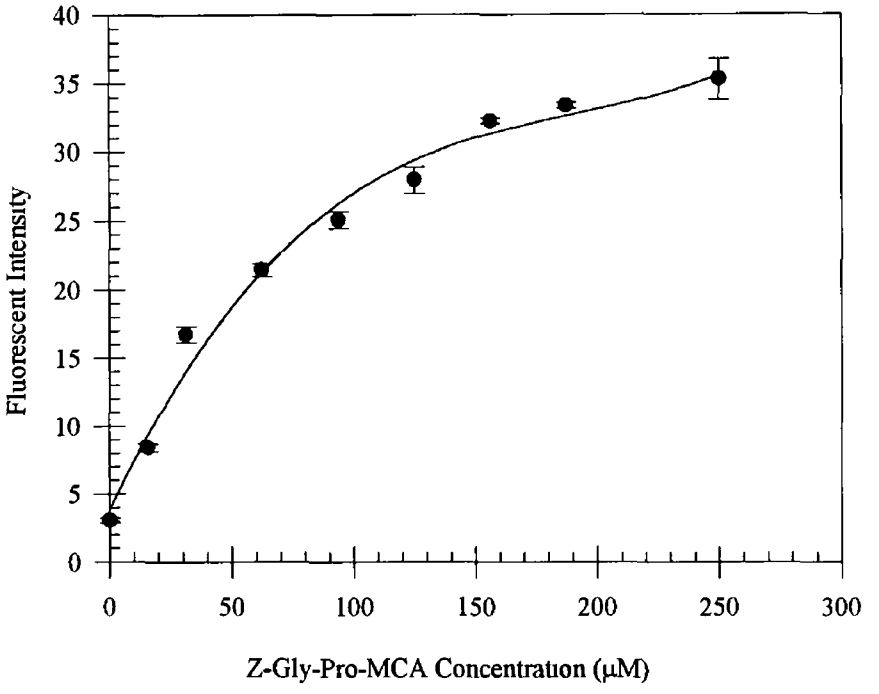
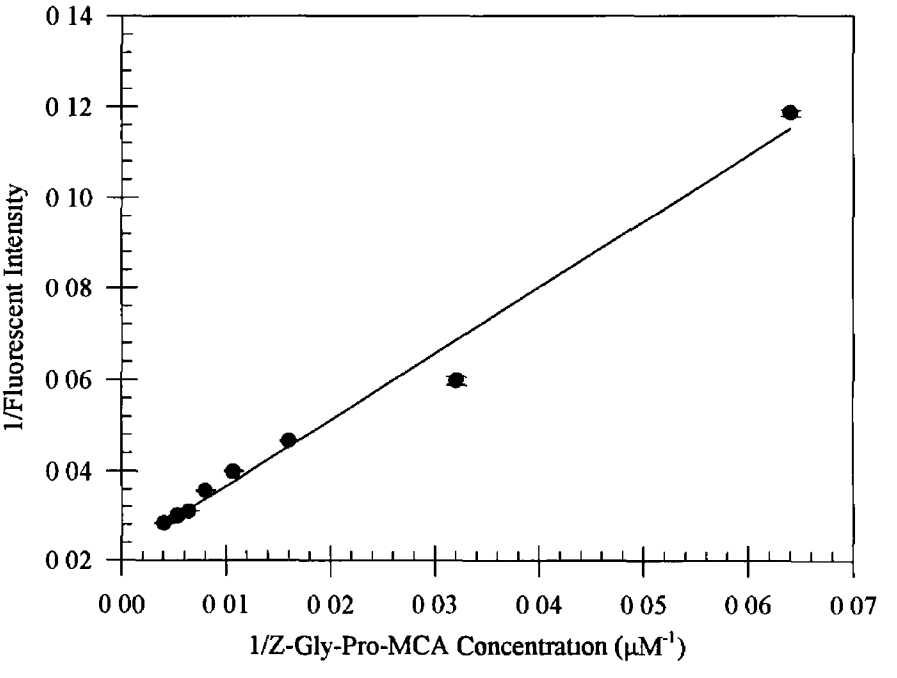


FIGURE 3 8 11 10.



FIGURES 3 8.11.9. AND 3 8 11 10 K_M DETERMINATION FOR ZIP USING Z-GLY-PRO-MCA

FIGURE 3 8 11 9 represents the Michaelis-Menten plot (fluorescent intensity versus Z-Gly-Pro-MCA concentration) for ZIP FIGURE 3 8 11 10 illustrates a plot of 1/fluorescent intensity versus 1/Z-Gly-Pro-MCA concentration (Lineweaver-Burk plot) for ZIP

FIGURE 3.8 11 11

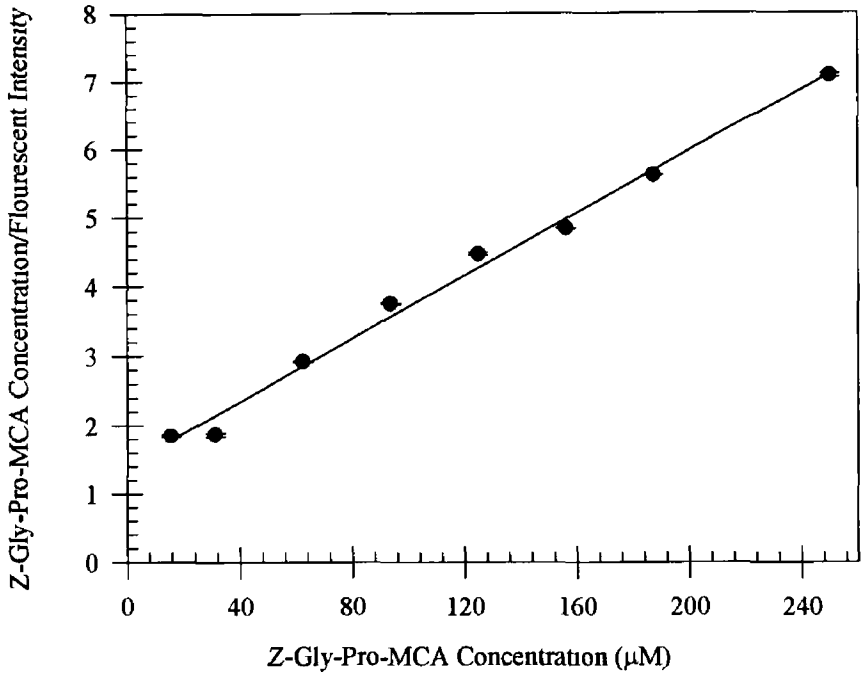
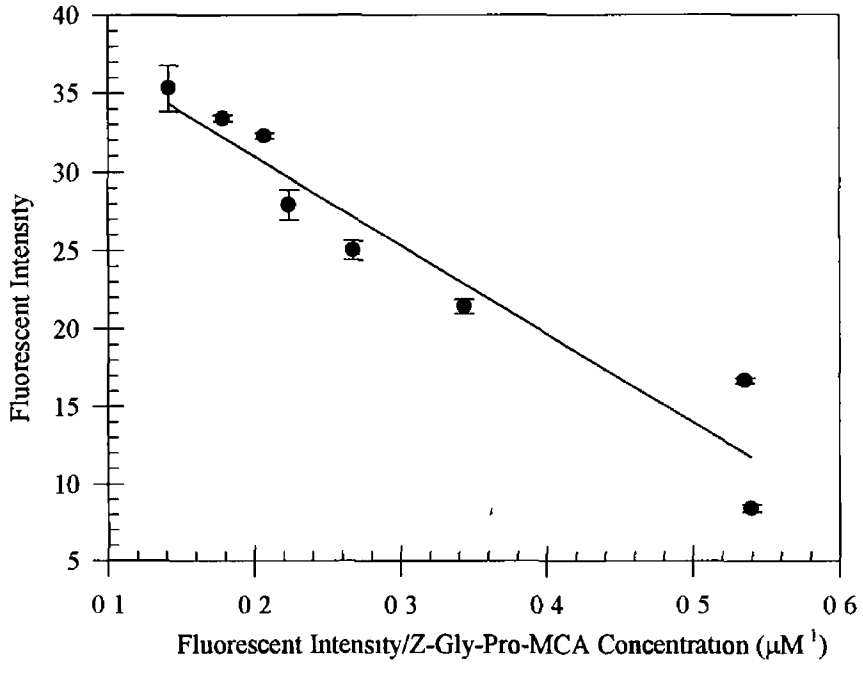


FIGURE 3.8 11 12



FIGURES 3.8.11.11 AND 3 8 11.12 K_M DETERMINATIONS FOR ZIP USING Z-GLY-PRO-MCA

FIGURE 3 8 11 11 is a plot of Z-Gly-Pro-MCA concentration/fluorescent intensity versus Z-Gly-Pro-MCA concentration (Hanes-Woolf plot) for ZIP, while FIGURE 3 8 11 12 is a plot of fluorescent intensity versus fluorescent intensity/Z-Gly-Pro-MCA concentration (Eadie-Hofstee plot)

FIGURE 3 8.11.13

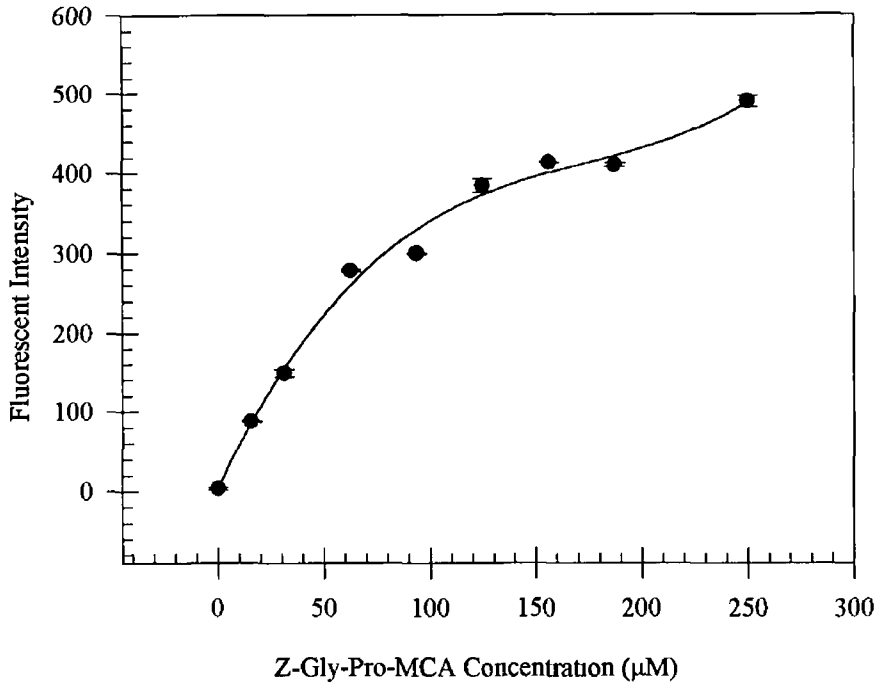
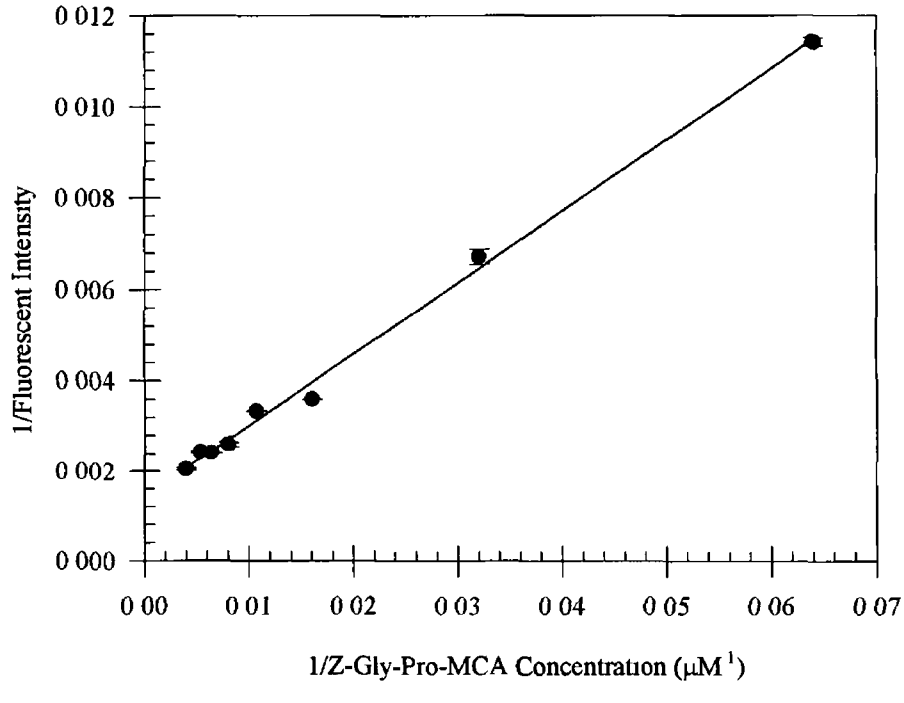


FIGURE 3 8 11.14



FIGURES 3 8 11.13. AND 3 8 11 14 K_M DETERMINATIONS FOR PO USING Z-GLY-PRO-MCA

FIGURE 3 8 11 13 represents the Michaelis-Menten plot of fluorescent intensity versus substrate concentration FIGURE 3 8 11 14 shows the double reciprocal Lineweaver-Burk plot (1/fluorescent intensity versus 1/Z-Gly-Pro-MCA concentration)

FIGURE 3 8.11 15

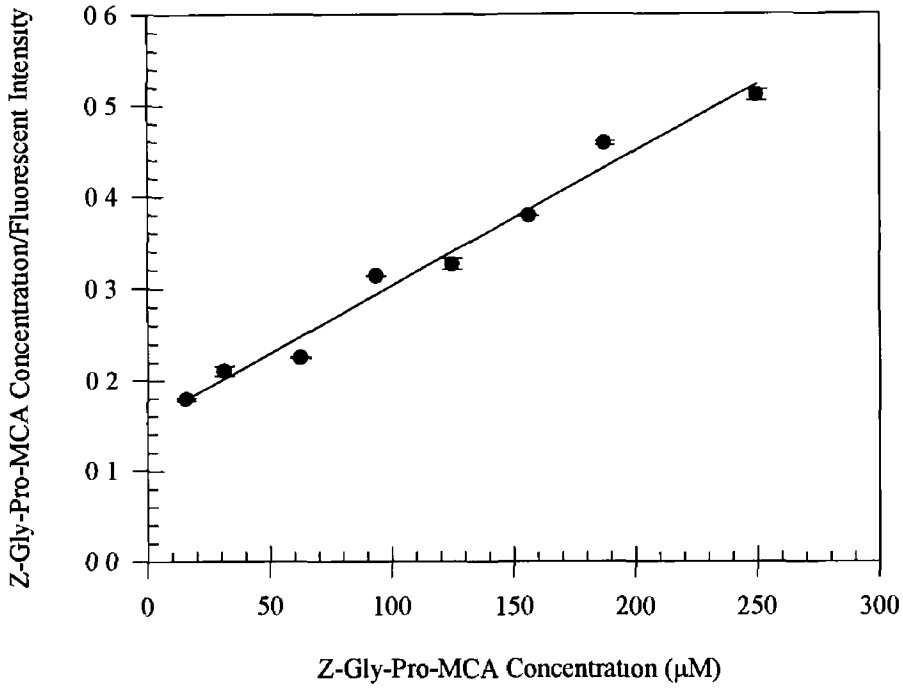
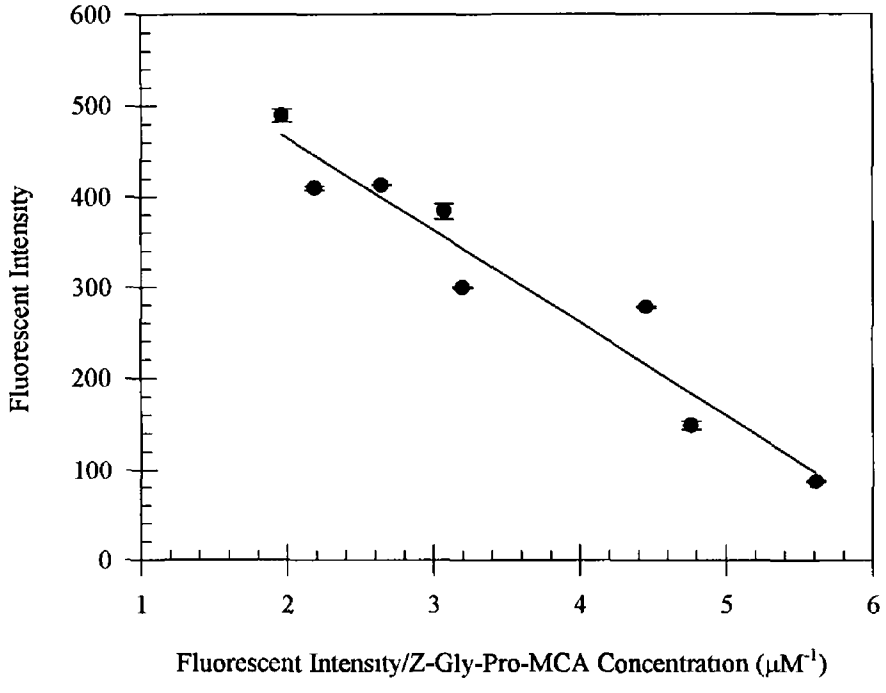


FIGURE 3 8 11 16



FIGURES 3 8 11 15. AND 3 8 11.16 K_M DETERMINATIONS FOR PO USING Z-GLY-PRO-MCA

FIGURE 3 8 11 15 illustrates the Hanes-Woolf plot of substrate concentration/fluorescent intensity versus substrate concentration FIGURE 3 8 11 16 represents a plot of fluorescent intensity versus fluorescent intensity/Z-Gly-Pro-MCA concentration (Eadie-Hofstee plot)

3 8 11 18 illustrate the competitive inhibition of ZIP by angiotensin I and uncompetitive inhibition by LHRH respectively Competitive inhibition of PO by angiotensin I and angiotensin II and that of bradykinin and Z-Gly-Pro-Ala are represented in FIGURES 3 8 11 19 and 3 8 11 20 respectively FIGURE 3 8 11 21 shows uncompetitive inhibition of purified PO by substance P

PEPTIDE	INHIBITION TYPE	K_i FOR ZIP μM			
Model		**MM**	**LB**	**EH**	**HW**
$A\beta_{1\ 43}$	Mixed	-	-	-	-
Angiotensin I	Competitive	90	24	73	37
Angiotensin II	Mixed	-	-	-	-
Angiotensin III	Mixed	-	-	-	-
(Arg^8)-Vasopressin	Mixed	-	-	-	-
Bradykinin	Mixed	-	-	-	-
(Glu^2)-TRH	Mixed	-	-	-	-
LHRH	Uncompetitive	100	45	15	56
(Phe^2)-TRH	Mixed	-	-	-	-
Substance P	Mixed	-	-	-	-
TRH	Mixed	-	-	-	-
TRH-Gly	Mixed	-	-	-	-
Gly-Gly-Pro-Ala	Mixed	-	-	-	-
Z-Gly-Pro-Ala	Mixed	-	-	-	-

TABLE 3 19. KINETIC ANALYSIS OF ZIP IN THE PRESENCE OF PROLINE CONTAINING PEPTIDE INHIBITORS

- means not calculated Assay concentrations of inhibitors as given in TABLE 2 18 Models used MM=Michaelis-Menten, LB=Lineweaver-Burk, EH=Eadie Hofstee, HW=Hanes Woolf (see section 6 4 for calculations)

PEPTIDE	INHIBITION TYPE	K_i FOR PO μM			
Model		**MM**	**LB**	**EH**	**HW**
$A\beta_{1\ 43}$	Mixed	-	-	-	-
Angiotensin I	Competitive	75	16	26	27
Angiotensin II	Competitive	150	19	31	35
Angiotensin III	Mixed	-	-	-	-
(Arg^8)-Vasopressin	Mixed	-	-	-	-
Bradykinin	Competitive	275	133	0 78	707
(Glu^2)-TRH	Mixed	-	-	-	-
LHRH	Mixed	-	-	-	-
(Phe^2)-TRH	Mixed	-	-	-	-
Substance P	Uncompetitive	177	161	192	165
TRH	Mixed	-	-	-	-
TRH-Gly	Mixed	-	-	-	-
Gly-Gly-Pro-Ala	Mixed	-	-	-	-
Z-Gly-Pro-Ala	Competitive	476	171	167	154

TABLE 3 20 KINETIC ANALYSIS OF PO IN THE PRESENCE OF PROLINE CONTAINING PEPTIDE INHIBITORS

- means not calculated Assay concentrations of inhibitors were as given in TABLE 2 18 Models used MM=Michaelis-Menten, LB=Lineweaver-Burk, EH=Eadie Hofstee, HW=Hanes Woolf (see section 6 4 for calculations)

FIGURE 3.8.11.17.

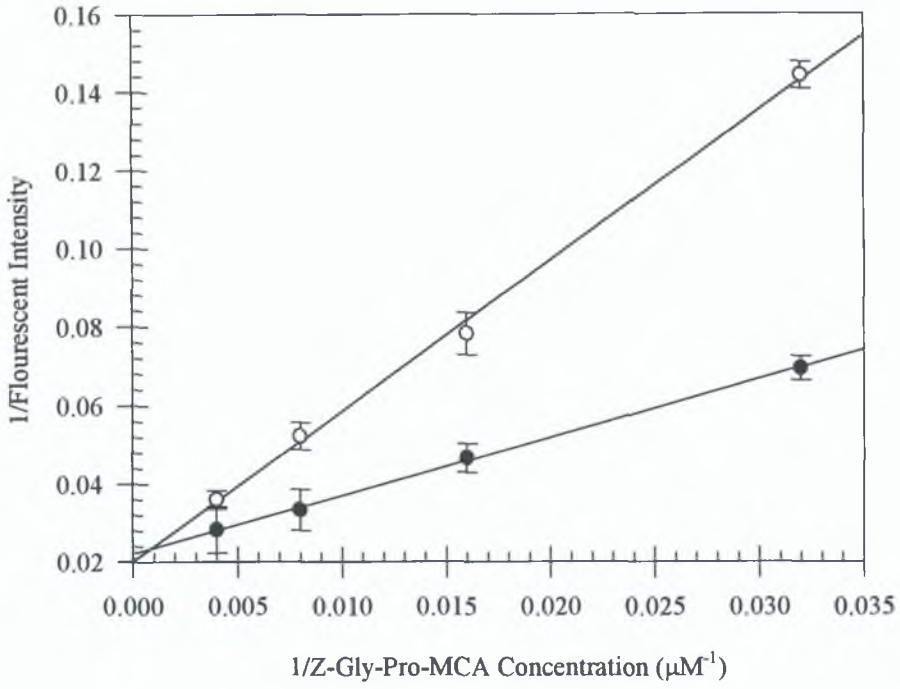
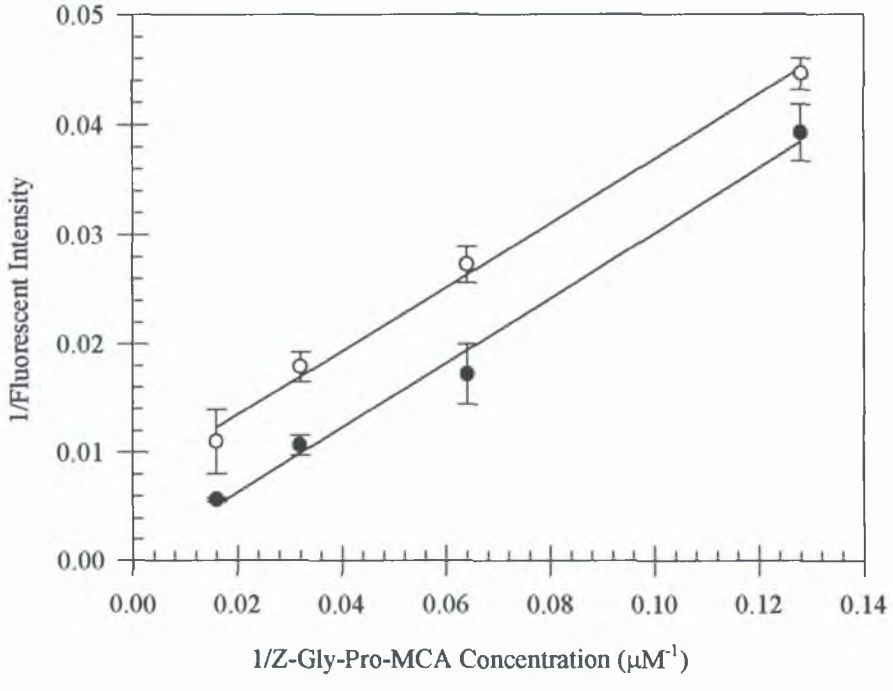


FIGURE 3.8.11.18.



FIGURES 3.8.11.17. AND 3.8.11.18. INHIBITION OF ZIP BY PROLINE-CONTAINING PEPTIDES

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Z-Gly-Pro-MCA concentration for proline containing peptides (o-o) when assayed with Z-Gly-Pro-MCA (•-•). FIGURE 3.8.11.17. illustrates the competitive inhibition of ZIP by angiotensin I, while FIGURE 3.8.11.18. shows the uncompetitive inhibition of ZIP activity by LHRH.

FIGURE 3 8 11 19.

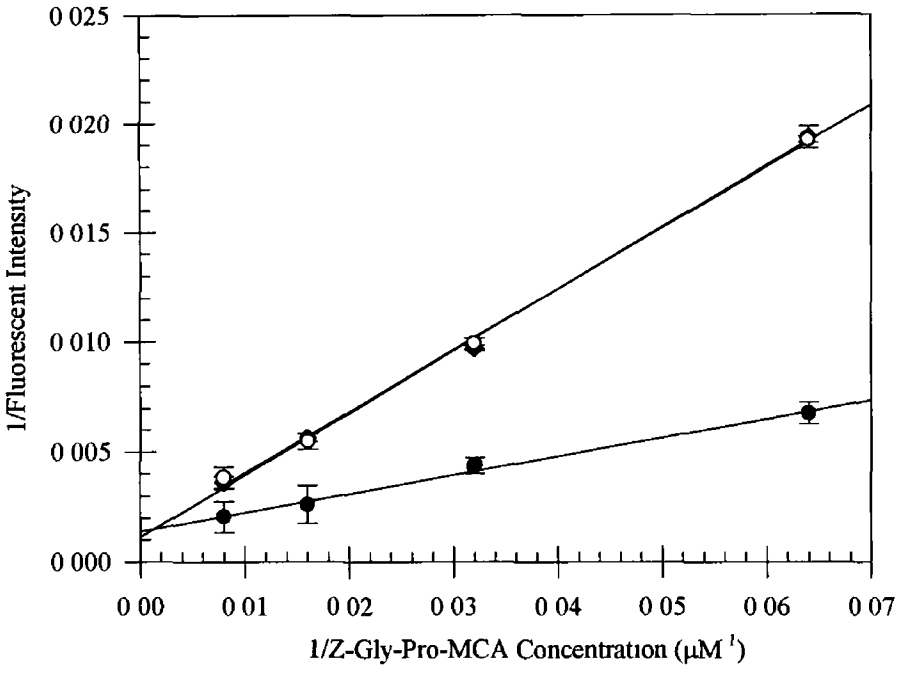
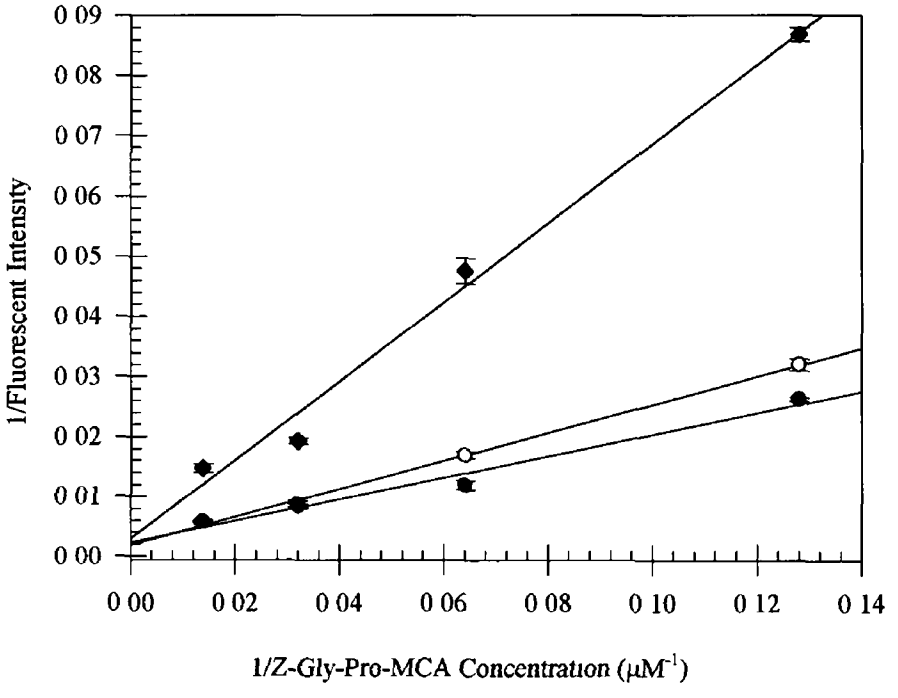


FIGURE 3 8 11 20.



FIGURES 3 8 11 19 AND 3 8 11 20 INHIBITION OF PO BY PROLINE-CONTAINING PEPTIDES

Lineweaver-Burk reciprocal plots of fluorescent intensity versus Z-Gly-Pro-MCA concentration FIGURE 3 8 11 19 illustrates the competitive inhibition of PO by angiotensin I (♦-♦) and angiotensin II (o-o) when assayed with Z-Gly-Pro-MCA (•-•) FIGURE 3 8 11 20 shows the competitive inhibition of PO by bradykinin (♦-♦) and Z-Gly-Pro-Ala (o-o) when assayed with Z-Gly-Pro-MCA (•-•)

FIGURE 3 8 11.21

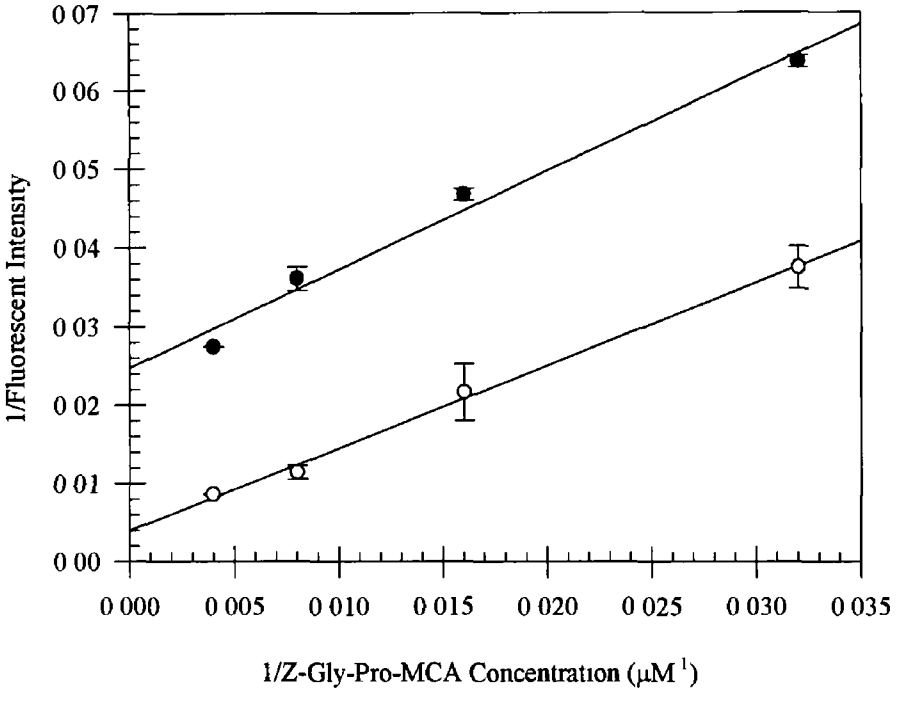


FIGURE 3 8 11 21. INHIBITION OF PO BY PROLINE-CONTAINING PEPTIDES

Lineweaver-Burk reciprocal plot of fluorescent intensity versus Z-Gly-Pro-MCA concentration, illustrating the uncompetitive inhibition of PO by substance P (o-o) when assayed with Z-Gly-Pro-MCA (•-•)

3 8 12 Inhibitor Studies

The effect of specific proline containing peptidase inhibitors on ZIP and PO activities was determined (see section 2 9 12 1) Plots of enzyme activity versus inhibitor concentration were constructed and are shown in FIGURES 3 8 12 1 - 3 8 12 12 TABLE 3 21 summarises the IC_{50} values determined for ZIP and PO as described in section 6 5

INHIBITOR	IC_{50} FOR ZIP	IC_{50} FOR PO
	M	*M*
Boc-Glu-($NHOB_2$)-Pyr	$5x10^{-10}$	$1x10^{-5}$
Fmoc-Ala-Pro-CN	ND	$1x10^{-12}$
Fmoc-Pro-Pro-CN	ND	$5 x10^{-7}$
Ile-Pyrrolidide	ND	ND
Ile-Thiazolidide	ND	$1x10^{-8}$
JTP-4819	ND	$2.5x10^{-6}$
Postatin	ND	$5x10^{-7}$
S-17092-1	ND	$1x10^{-8}$
S-19825	ND	$1x10^{-9}$
Z-Phe-Ala-CMK	$1x10^{-4}$	ND
Z-Phe-Pro-Methylketone	ND	$1x10^{-7}$
Z-Pro-Prolinal	ND	$5x10^{-7}$
Z-Pro-Prolinal dimethylacetate	$1x10^{-4}$	$1x10^{-5}$

TABLE 3 21 EFFECT OF SPECIFIC INHIBITORS ON ZIP AND PO ACTIVITIES

ND implies IC_{50} value could not be determined Concentrations as given in TABLE 2 19

FIGURE 3 8.12 1.

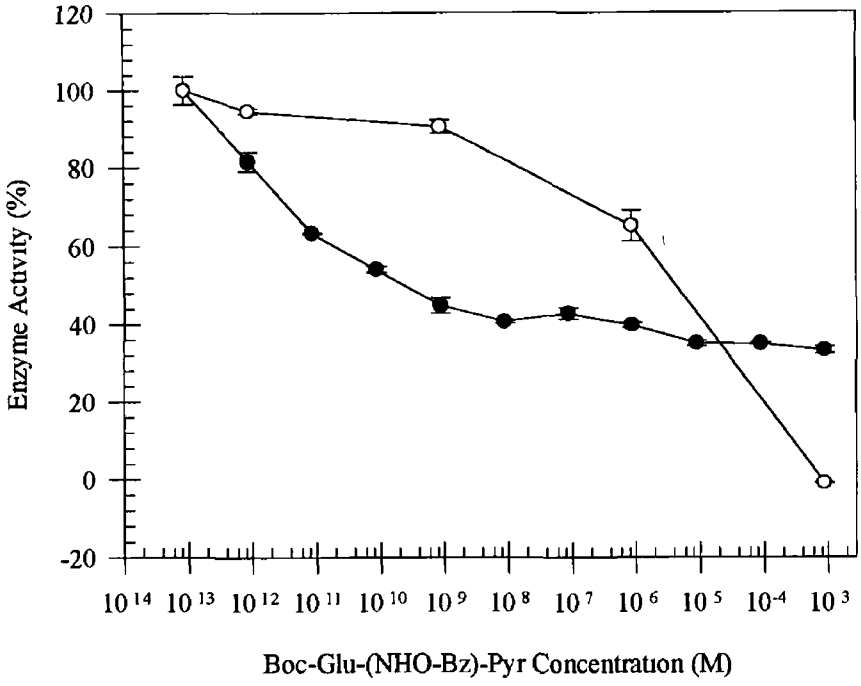
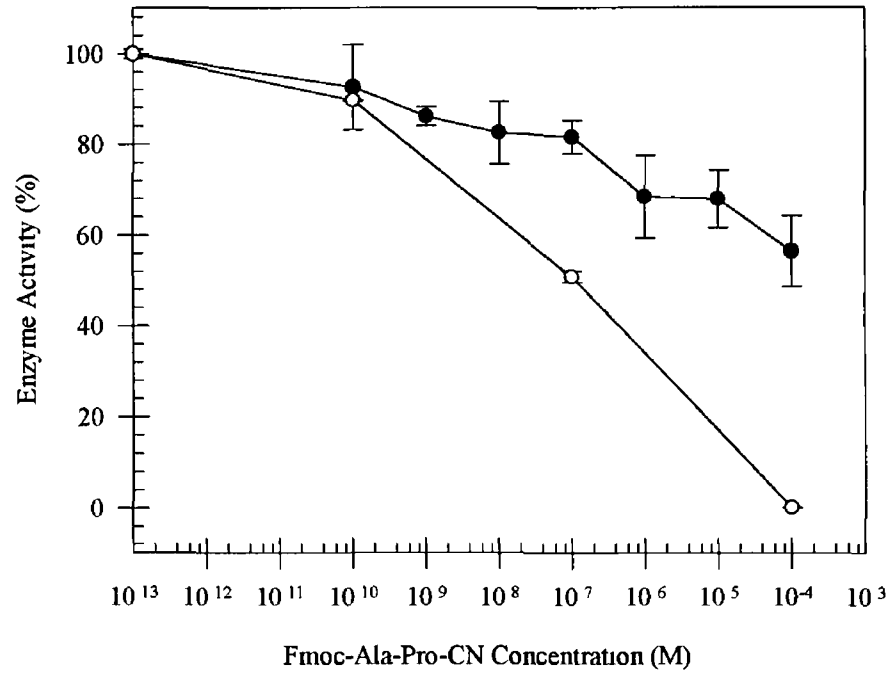


FIGURE 3 8 12 2



FIGURES 3 8 12 1 AND 3 8.12 2. INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus specific inhibitor concentration FIGURE 3 8 12 1 illustrates the effect of Boc-Glu(NHO-Bz)-Pyr on enzyme activities while FIGURE 3 8 12 2 represents the enzyme inhibition profiles observed using Fmoc-Ala-Pro-CN Enzyme activities are expressed as percentages of uninhibited enzyme

FIGURE 3.8 12 3.

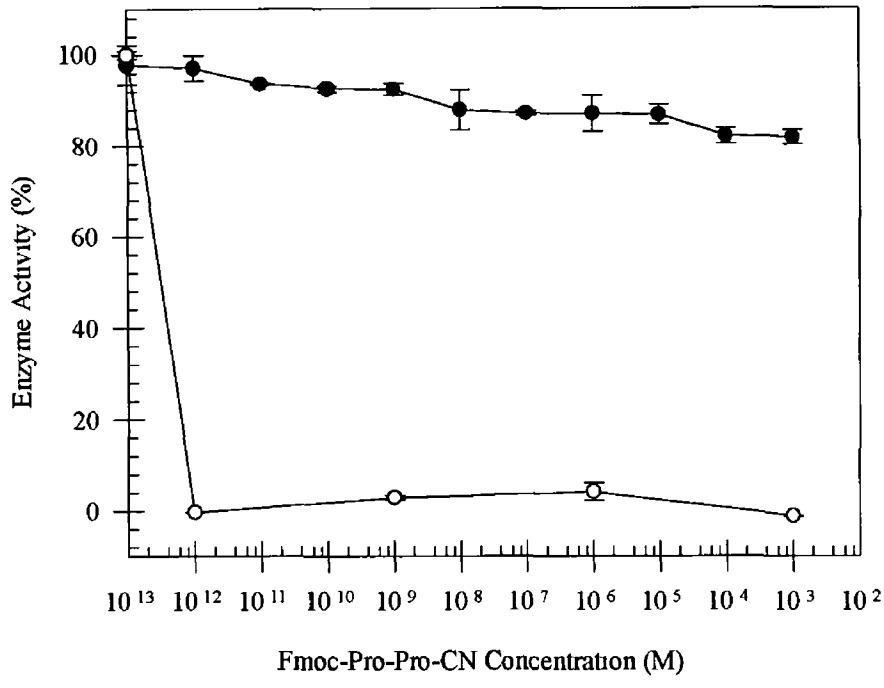
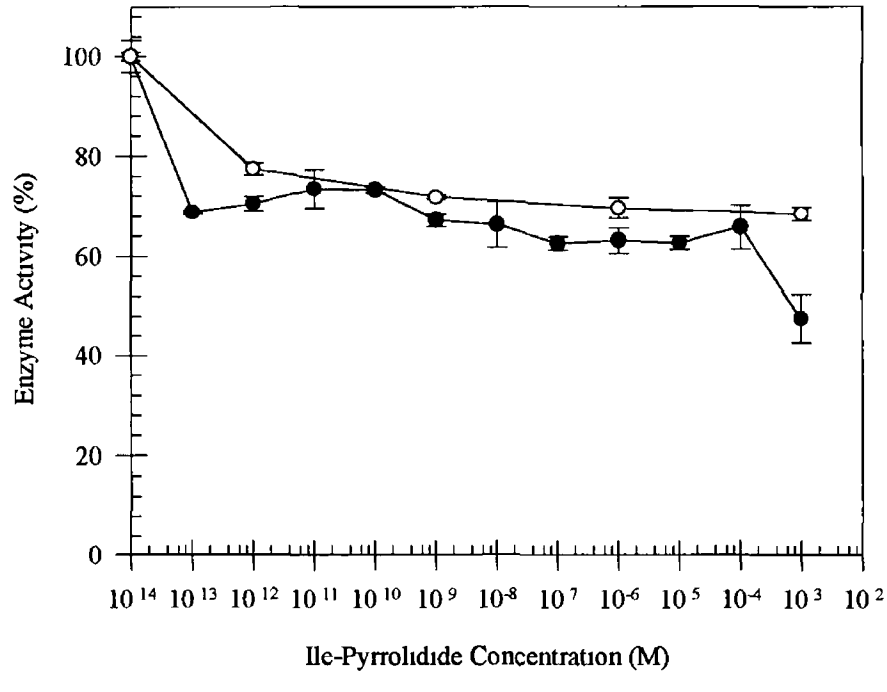


FIGURE 3.8 12 4



FIGURES 3 8 12 3 AND 3 8 12 4 INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus specific inhibitor concentration FIGURE 3 8 12 3 illustrates the effect of Fmoc-Pro-Pro-CN on enzyme activities while FIGURE 3 8 12 4 represents the effect of Ile-Pyrrolidide Enzyme activities are expressed as a percentage of uninhibited enzyme activity

FIGURE 3.8.12.5.

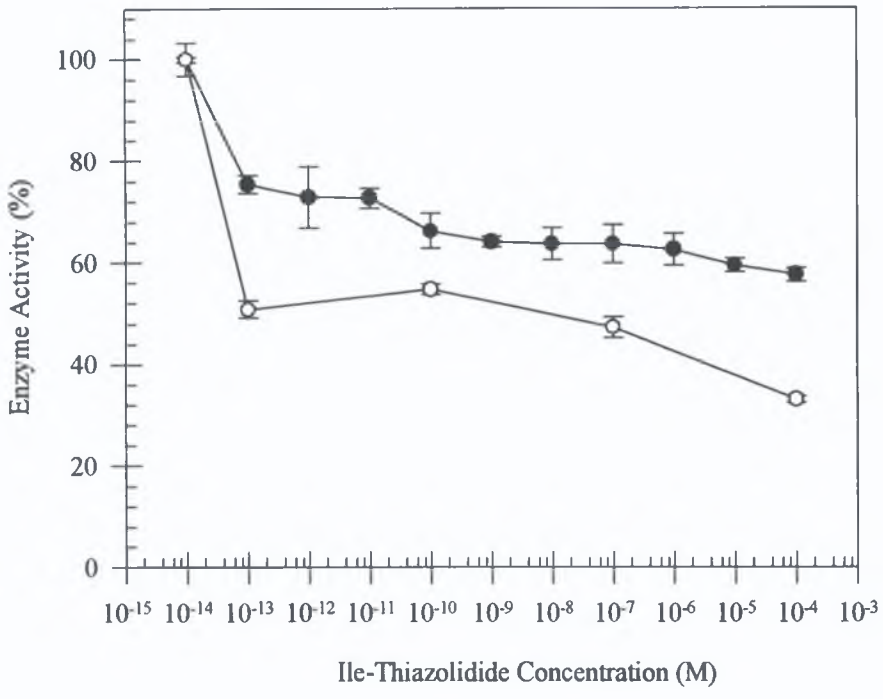
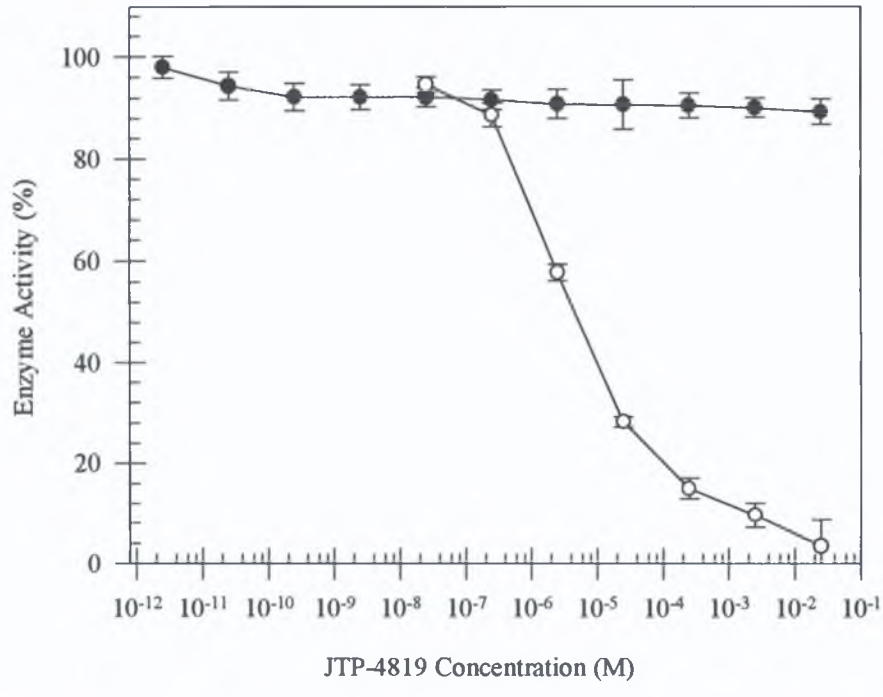


FIGURE 3.8.12.6.



FIGURES 3.8.12.5. AND 3.8.12.6. INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus inhibitor concentration. FIGURE 3.8.12.5. represents the effect of Ile-Thiazolidide on activity while FIGURE 3.8.12.6. illustrates the effect of JTP-4819 on enzyme activity. Enzyme activities are expressed as a percentage of uninhibited enzyme activity.

FIGURE 3 8 12 7

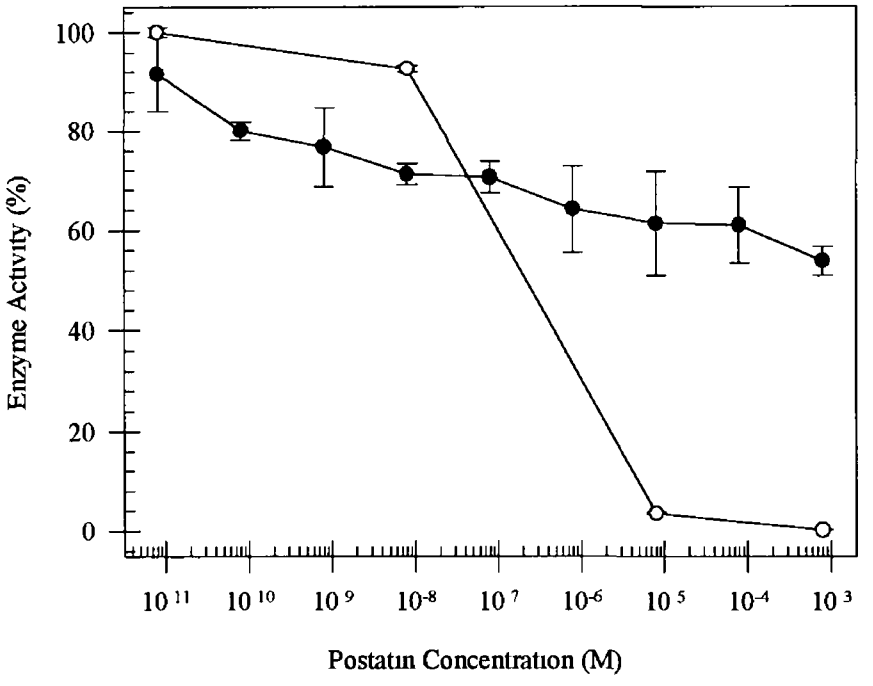
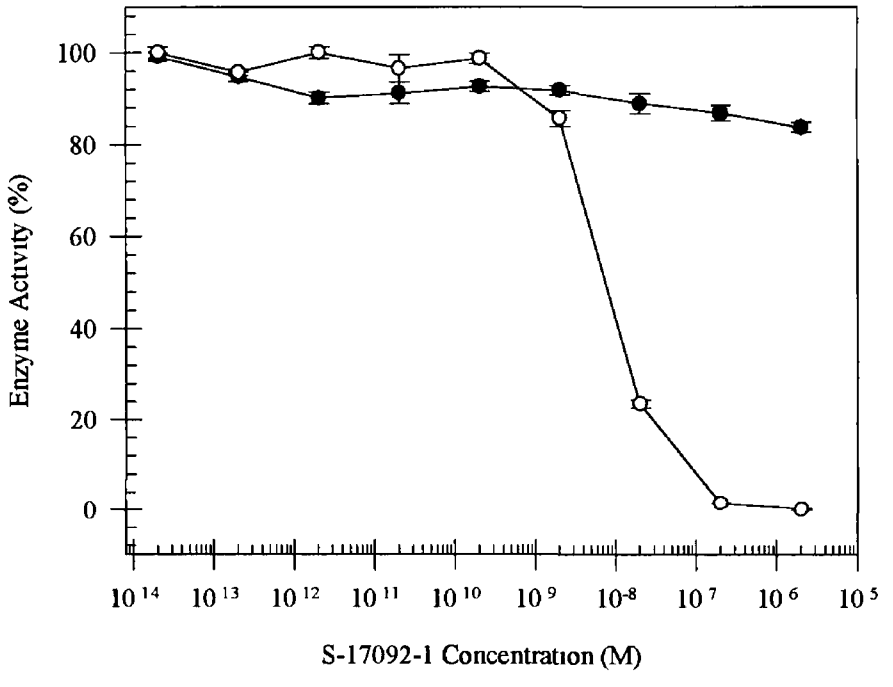


FIGURE 3 8 12 8



FIGURES 3 8 12 7 AND 3.8 12 8 INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus specific inhibitor concentration FIGURE 3 8 12 7 illustrates the effect of postatm on activity and FIGURE 3 8 12 6 illustrates the effect of S-17092-1 on enzyme activity Enzyme activities are expressed as percentages of uninhibited enzyme

FIGURE 3 8 12.9.

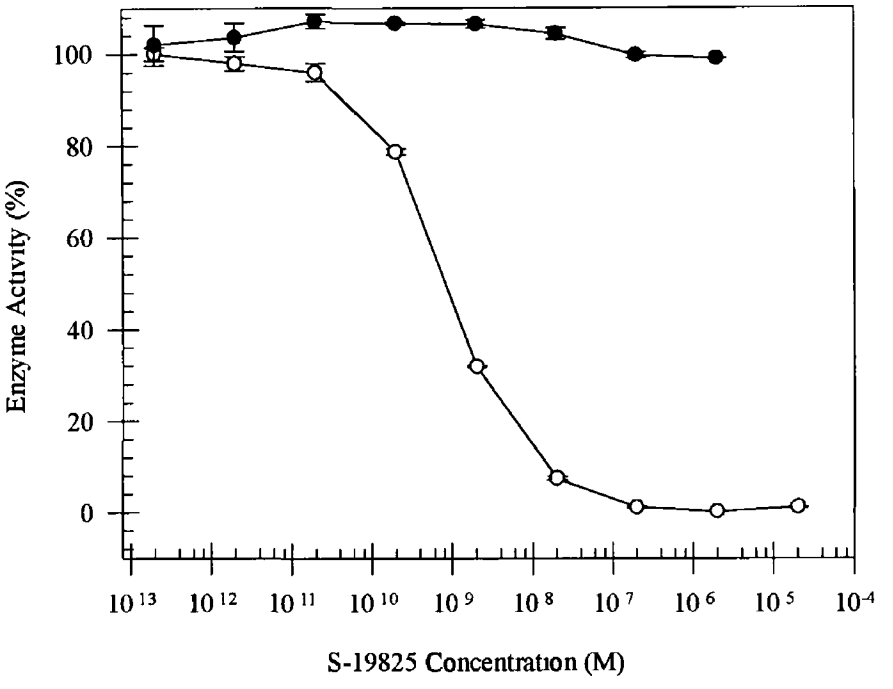
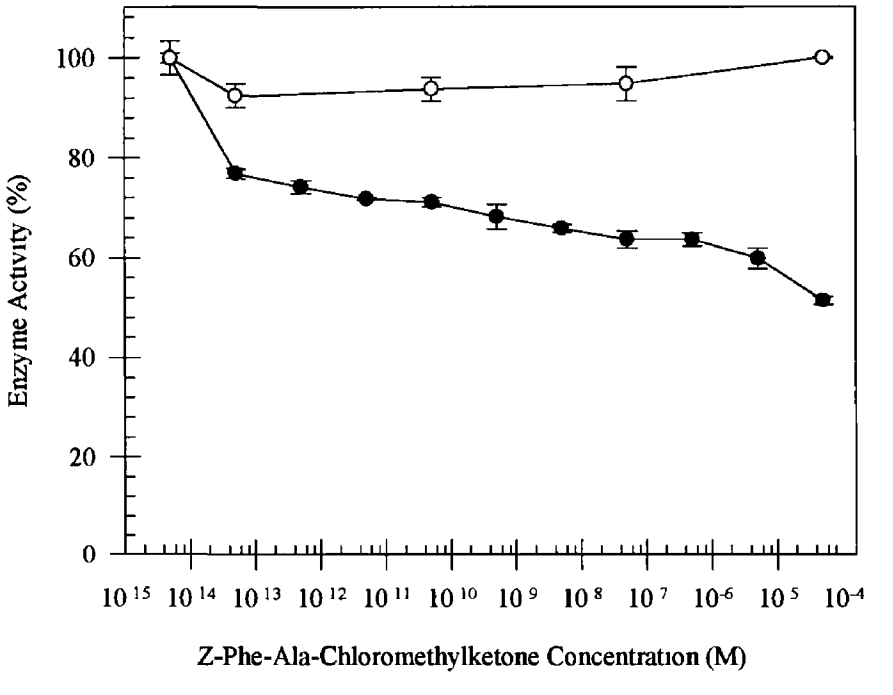


FIGURE 3 8.12.10



FIGURES 3 8 12 9 AND 3 8.12 10 INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus inhibitor concentration FIGURE 3 8 12 9 shows the effect of S-19825 on activities while FIGURE 3 8 12 10 illustrates the effect of Z-Phe-Ala-Chloromethylketone on enzyme activities Activities are expressed as a percentage of uninhibited enzyme

FIGURE 3 8 12.11

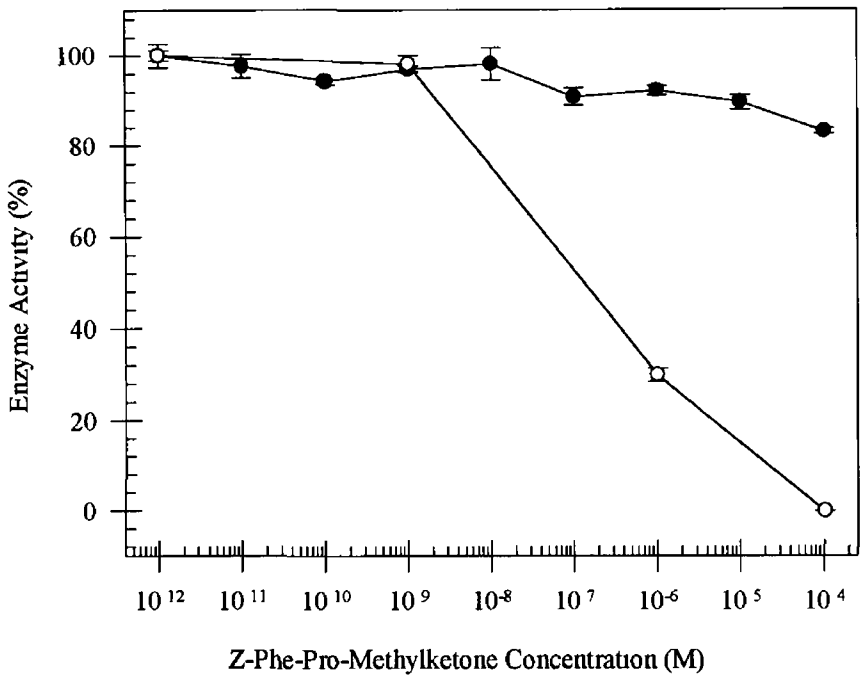
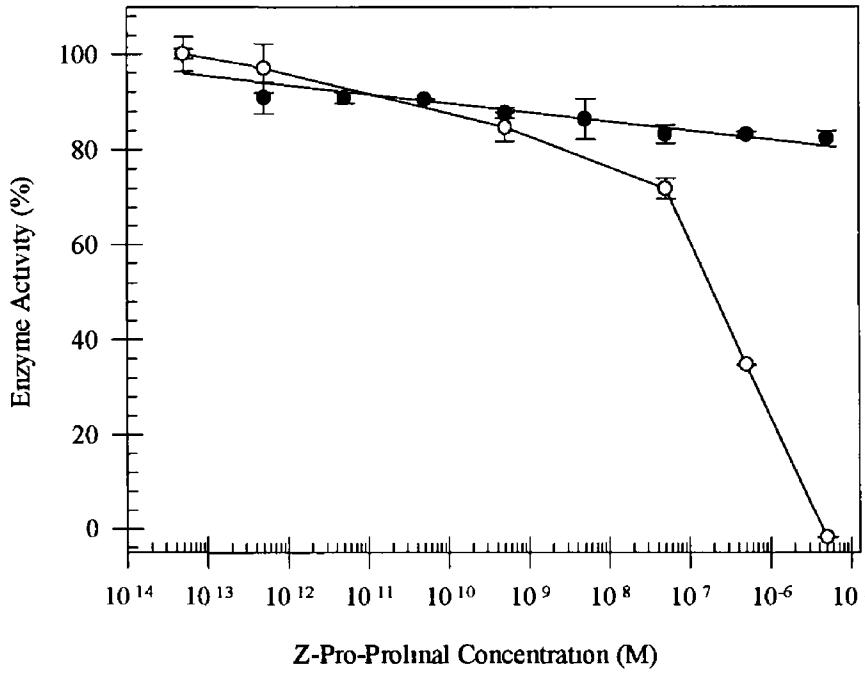


FIGURE 3 8 12.12



FIGURES 3 8 12 11. AND 3 8 12 12 INHIBITOR STUDIES

Semi log plots of ZIP (•-•) or PO (o-o) activity versus inhibitor concentration FIGURE 3 8 12 11 represents the effect of Z-Phe-Pro-Methylketone on enzyme levels while FIGURE 3 8 12 12 illustrates the effect of Z-Pro-Prolinal on enzyme activities Enzyme activities are expressed as percentages of uninhibited enzyme

FIGURE 3 8.12 13

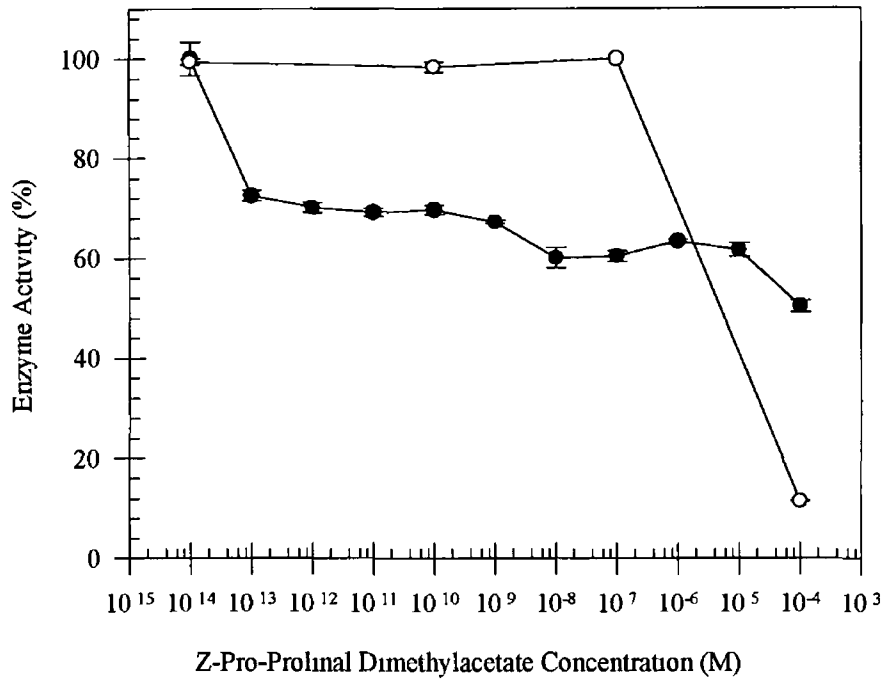


FIGURE 3.8.12 13 INHIBITOR STUDIES

Semi log plots of ZIP (•-•) and PO (o-o) activity versus inhibitor concentration FIGURE 3 8 12 13 represents the effect of Z-Pro-Prolinal dimethylacetate on enzyme activities Enzyme activities are expressed as a percentage of uninhibited enzyme

4.0. DISCUSSION

4 0 DISCUSSION

4 1 FLUORESCENCE SPECTROMETRY USING 7-AMINO-4-METHYL-COUMARIN

This work focused on the study of two specific peptidases, namely ZIP and PO from bovine serum and brain respectively A method capable of detecting low-levels of these enzyme activities was essential Walter *et al*, (1971) employed a radiometric assay using oxytocin for the detection of PO, however the superiority of the fluorimetric assay has resulted in its preferential use Generally, fluorescence spectroscopy offers increased safety, sensitivity, and specificity over colorimetric, spectrophotometric and radiometric assays The high sensitivity results from the wavelength difference between the exciting and fluorescence radiation, resulting in minimal background, while the high specificity is due to the dependence on both an excitation and emission spectra (Willard *et al*, 1988)

Z-Gly-Pro-MCA was first synthesised and applied in the detection of PO by Yoshimoto *et al*, in 1979, who determined a K_M of 0 02mM and increased sensitivity over selected proline derivatives This externally quenched fluorimetric substrate allows for peptide cleavage on the carboxyl side of the proline residue thus liberating the fluorophore, MCA (FIGURE 4 1) Excitation to a higher energy state occurs at 370nm due to constant absorption of electromagnetic radiation by MCA By the nature of fluorescence, this is followed by the return of the molecule to a lower state and the subsequent reemission of radiation at 440nm The sensitivity of fluorimetric assays may be improved by expansion of the emission slit width, which consequently broadens the bandwidth over which light is integrated For instance, at an emission wavelength of 440nm and a slit width of 5nm, the fluorimeter integrates light radiated from 437 5nm to 442 5nm, while if the slit width is increased to 10nm, light radiated from 435nm to 445nm is integrated Therefore, for samples of particularly low enzyme activity and hence low fluorescence, adaptation of the emission slit width ensures improved sensitivity

N-benzyloxycarbonyl-Gly-Pro-7-amino-4-methyl-coumarin (Z-Gly-Pro-MCA)

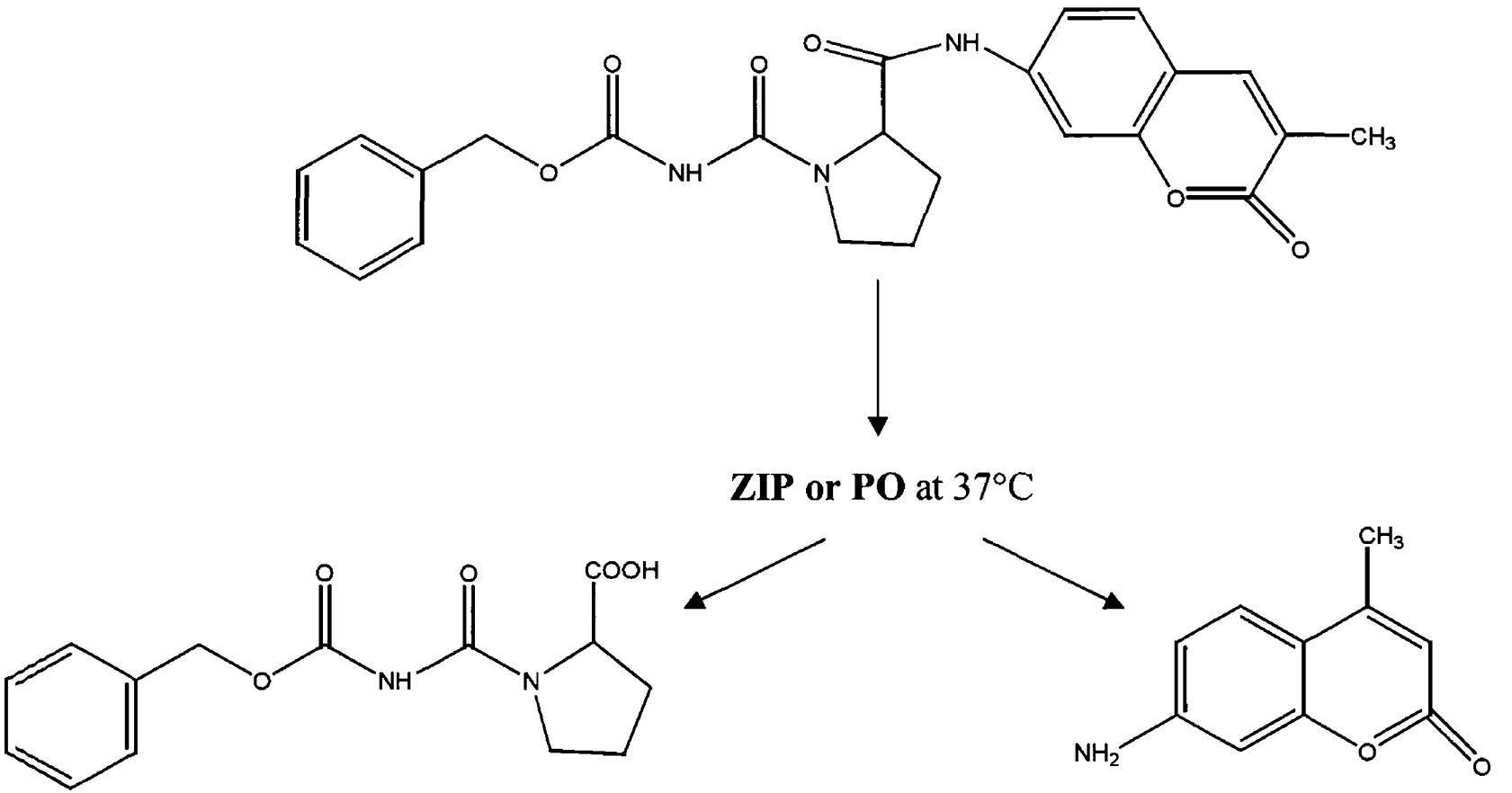


FIGURE 4 1 Z-GLY-PRO-MCA ASSAY MECHANISM

A major disadvantage of fluorimetric assays is the occurrence of two phenomena, namely quenching and the inner filter effect Both effects lead to an apparent reduction in fluorescent intensity and hence can be problematic where quantitation of fluorescence is important Quenching involves the removal of energy from a molecule in the excited state by another molecule, the most common form (collisional impurity quenching) is due to the collision of molecules This type of quenching may be attributed to the presence of heavy metals or dissolved oxygen in samples (Willard *et al*, 1988) The inner filter effect is a result of an overlap of the excitation or emission band of the fluorescing sample with the absorption band of a contaminating compound

4.1 1 MCA Standard Curves and the Inner Filter Effect

Liberated MCA by peptide cleavage was quantified by reference to standard curves as described in section 2 2 1 FIGURES 3 1 1 and 3 1 2 clearly exhibit the linear relationship between MCA concentration and fluorescence at excitation and emission wavelengths of 370nm and 440nm respectively However, the presence of contaminants in samples, particularly those which excite and emit at the same wavelengths as the fluorophore (the inner filter effect), compromise assay sensitivity and hence hinder accurate quantitation of liberated MCA This becomes problematic in the cases of crude biological samples such as serum and tissue extracts and thus appropriate compensation is required

Section 2 2 2 describes a protocol involving the incorporation of biological samples into the MCA standard curves This allows for the difference in fluorescence to be measured between free MCA and MCA in the presence of samples FIGURES 3 1 3 and 3 1 4 clearly demonstrate the effect of filtering on fluorescence The observed decrease in fluorescence when serum is incorporated into the assay is characteristic of the inner filter effect This decrease is reflected in a reduced slope, which is indicative of the reduction in sensitivity Expansion of the emission slit width would consequently broaden the bandwidth, thus improving sensitivity, however this would undoubtedly result in a corresponding increase in the inner filter effect Bovine serum was responsible for the highest degree of filtering, with 14% filtering observed at excitation and emission slit

widths of 10nm and 5nm respectively Post column chromatography fractions, namely, post phenyl sepharose and post calcium phosphate cellulose samples were responsible for 4% and 0 2% filtering respectively Purified ZIP did not exhibit the inner filter effect

These results raise two interesting issues Firstly, they suggest that crude, highly coloured, high protein-content samples, such as serum, are more likely to exhibit the filter effect than a purified sample Subsequently, this emphasises the importance of considering the effect of filtering on experiments, particularly in the assessment of a purification procedure Starting material for such a process would generally constitute a crude sample, highly susceptible to the inner filter effect Failure to consider filtering would reveal a lower activity in the crude starting material than actually present, which could in turn could lead to inaccuracies in the estimation of the overall effectiveness of the purification procedure Therefore, it was evident that the inclusion of filtered MCA standard curves was paramount if reliable, accurate results were to be obtained from fluorimetric assays

Preparation of a filtered MCA standard curve, consisting of ten MCA concentrations each in triplicate, required 3mL of enzyme sample Obviously, this is a considerable volume just to establish the effect of filtering It was observed that although a decrease in the slope occurred on inclusion of a biological sample, there was no apparent deviation from linearity (see FIGURES 3 1 3 and 3 1 4) Therefore, in order to limit the amount of sample required, filtered standard curves were prepared using only three MCA concentrations This method still yielded an accurate slope and hence the effect of filtering could be determined while minimal enzyme volume was required

In addition to biological samples such as serum and post column fractions, many other compounds contributed to the inner filter effect Therefore, for all characterisation studies carried out, the inner filter effect of buffers, functional reagents, inhibitors and metal salts was always considered This was achieved by construction of a three-point MCA filtered curve prior to calculation of the exact enzyme activity or specific activity

4 2 PROTEIN DETERMINATION

The fluorimetric assay described previously was essential for the determination of enzyme activity in a particular sample, however, the determination of the total protein concentration was equally important This was particularly true in relation to the purification of ZIP, where calculation of the specific activity relied heavily on accurate assessment of the protein content in a sample Many techniques for protein determination have evolved, each of which have advantages and limitations (Dunn, 1989) The use of ultraviolet spectrometry, most commonly the measurement of absorbance at 280nm, provides an inexpensive, rapid and non-destructive method of protein determination The assay exploits the ability of the aromatic amino acids, tyrosine and tryptophan to absorb at 280nm The method requires 0 5 to 5 0mg protein and unfortunately is inaccurate unless the extinction coefficient of the protein (dependant on precise amino acid composition) is known or the protein is relatively pure This technique is also very sensitive to interference from nucleic acids, nucleotides and haem-contaimng compounds which absorb at 280nm and can be very abundant in crude biological samples (Stevens, 1992)

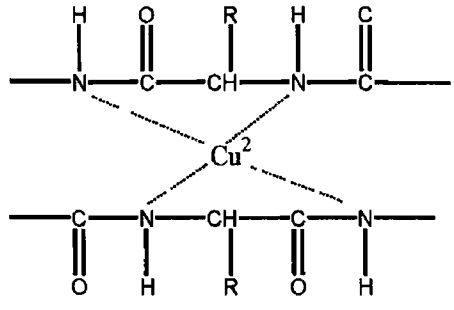


FIGURE 4 2. BASIS OF THE BIURET ASSAY

Copper ions (Cu^{2+}) readily complex with adjacent peptide bonds yielding a purple colour formation with an absorbance maximum at 562nm

A method unaffected by the amino acid composition of the protein and less prone to interference is the Biuret assay for protein determination Under alkaline conditions copper ions readily complex adjacent peptide bonds, producing a purple colour with an absorption between 540 and 590nm (maximum of 562nm) (FIGURE 4 2) This assay proved ideal for the determination of total protein concentration in relatively crude samples (containing 2-10mg/mL protein) as described in section 2 3 1 The linear relationship observed between protein concentration and absorbance at 570nm (FIGURE 3 2 1) allowed for the estimation of total protein in enzyme samples or fractions The major advantage of the Biuret assay over the UV spectrophotometric procedure was the reduction in interference effects The Biuret assay was performed in a microtitre plate, requiring only 50µL sample thus minimising the sample volume required

Samples containing less than 2mg/mL protein were assayed using the bicinchoninic acid (BCA) assay first developed in 1985 (Smith *et al*,) This assay, although closely related to the Lowry procedure (Lowry *et al*, 1951), is generally more tolerant to the presence of interferents and hence was chosen for the determination of total protein in purified samples The Pierce BCA assay used combines the biuret reduction of Cu^{2+} by protein to Cu^{1+} with the highly sensitive and selective colorimetric detection of the cuprous cation by a unique reagent containing bicinchoninic acid (FIGURE 4 3) Alteration of the standard BCA assay temperature (section 2 3 2) allows for the accurate estimation of protein as low as 100µg/mL (section 2 3 3) As with the Biuret assay, 50µL sample is sufficient for the analysis and protein content can be graphically estimated (FIGURES 3 2 2 and 3 2 3)

TABLE 4 1 summarises the common protein assays available, the limitations and advantages of different procedures

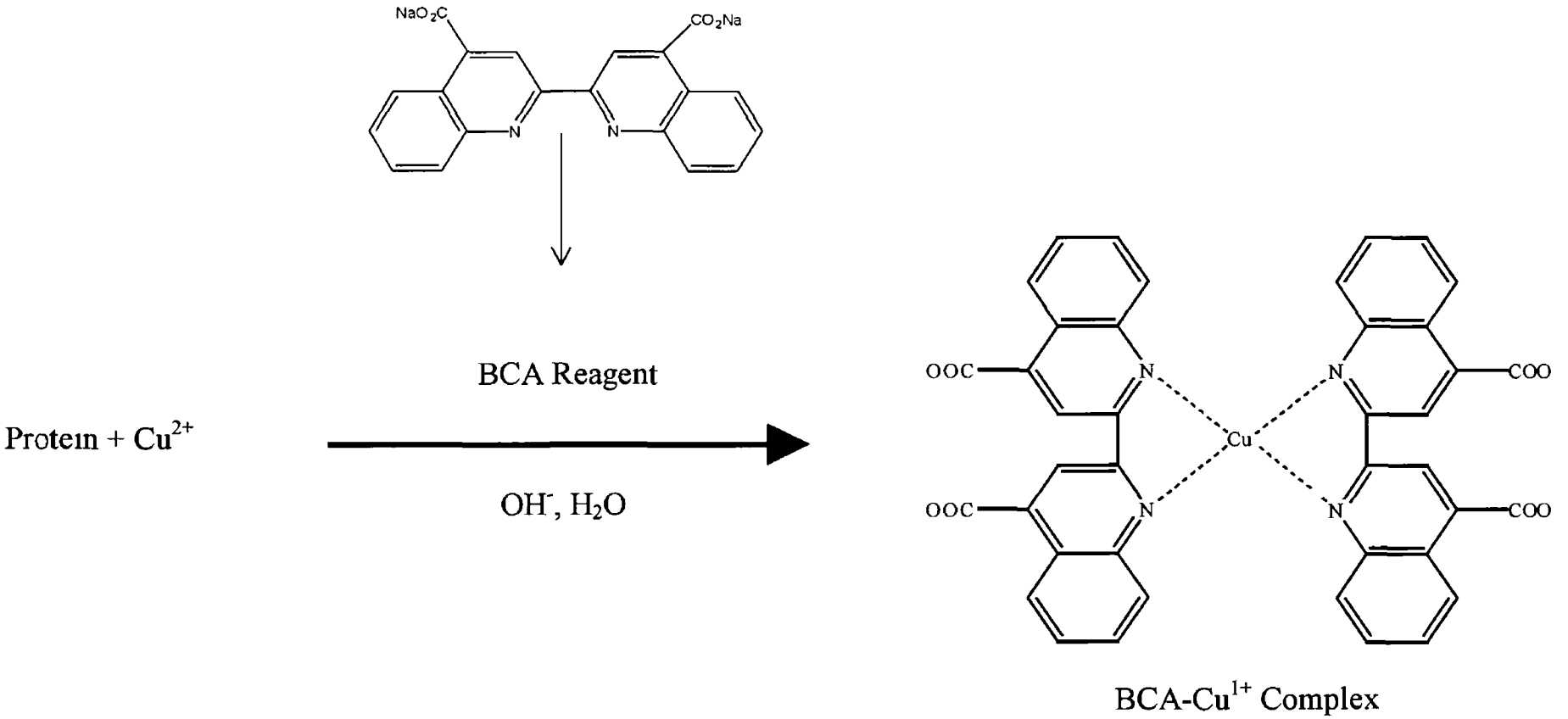


FIGURE 4 3 BICINCHONINIC ACID (BCA) ASSAY MECHANISM

Biuret reaction of protein with Cu^{2+} results in the formation of Cu^{1+} These ions subsequently react with protein in the presence of BCA to produce a purple coloured BCA-Cu^{1+} complex with a maximum absorbance at 562nm

METHOD AND DETECTION RANGE	INTERFERENTS	COMMENTS
A_{280} 0 5-5mg/mL	Nucleic acids, nucleotides, haem-containmg compounds, sulphydryl groups	Relatively insensitive, affected by amino acid composition, prone to interferents[f], inaccurate unless protein is pure Rapid and non destructive
Biuret 2-10mg/mL	Bile pigments, ammonia Sucrose*, Tris*, glycerol*	Low sensitivity Relatively few interferents, unaffected by amino acid composition, simple to perform, stable reagent
Lowry 0 1-1mg/mL	Amino acids, sulphydryl reagents, salts, detergents, drugs, sugars, lipids, nucleic acids	pH dependent reaction, prone to interferents, standard curve is non-linear at high concentrations, unstable reagents, complicated assay Sensitive
BCA 0 02-1 2mg/mL	Reducing agents Sugars*, protease inhibitors*	Extremely sensitive, particularly the enhanced assay, very few interferents

TABLE 4 1 COMPARISON OF A SELECTION OF PROTEIN DETERMINATION ASSAYS

* The presence of these interferents may be compensated for by the inclusion of an appropriate blank

[f]Interference can be minimised by measuring absorbance at 260nm and applying a correction formula (Layne, 1957)

4.3 ENZYME ASSAYS

The activities of the Z-Gly-Pro-MCA degrading peptidases studied in this work were determined by measurement of MCA released As described in section 4 1 enzyme assays were fluorimetric, monitoring the liberation of MCA from the substrate, leaving the *N*-blocked dipeptide Z-Gly-Pro The linear relationship between fluorescence and MCA concentration allowed for the subsequent quantification of enzyme activity (section 6 2) Yoshimoto *et al*, (1979) classified Z-Gly-Pro-MCA as a specific substrate for PO detection based on its resistance to degradation by 0 1-0 5mg/mL α-chymotrypsin, carboxypeptidase A, elastase, leucine aminopeptidase, post-proline dipeptidyl aminopeptidase, thrombin, trypsin and urokinase

However, the discovery of a second Z-Gly-Pro-MCA hydrolysing activity in this laboratory (Cunningham and O'Connor, 1997a) questioned this specificity Cunningham identified **ZIP**, a **Z**-Pro-Prolinal **I**nsensitive **Z**-Gly-Pro-MCA degrading **P**eptidase Z-Pro-Prolinal (FIGURE 4 4) was shown to be a potent PO inhibitor with a reported K_i of 14nM (Wilk and Orlowski, 1983) It is a transition state analogue, which forms a tetrahedral complex with prolyl ohgopeptidase at its catalytic serine site irreversibly inhibiting enzyme activity (Kahyaoglu *et al*, 1997)

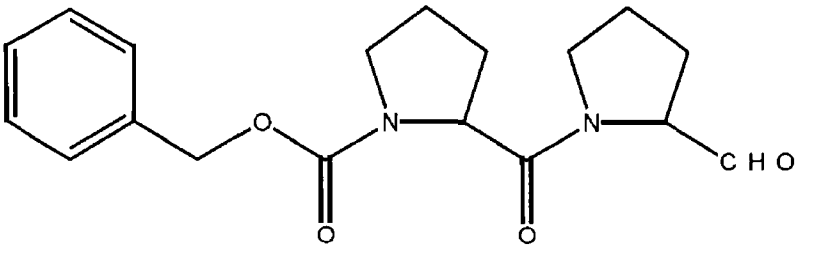


FIGURE 4 4 STRUCTURE OF Z-PRO-PROLINAL

The identification of this second activity induced the development of the selective PO assay (Cunningham and O'Connor, 1997a) Inclusion of Z-Pro-Prolinal in the fluorimetric assay allows for the differentiation of both activities The assay described in section 2 4 2 was employed for the detection of total Z-Gly-Pro-MCA hydrolysing activity, while the protocol outlined in section 2 4 3 was for the determination of ZIP activity alone It should be noted at this stage that samples shown to be free of PO activity (e g post column pools) were assayed according to section 2 4 2

These assays were therefore ideal, proving specific for both PO and ZIP activities, highly sensitive, quantifiable, rapid and simple and therefore met the requirements of any enzyme assay Coupled to this, was the fact that both described assays were cuvette based The magnitude and characteristics of inner filter effects strongly relies on the sample-cell geometry The use of a cuvette-based assay ensured perpendicular geometry between the sample and the incident beam In this case the fluorescence emitted is collected along an axis at right angles to the excitation beam Because of its freedom from the effects of scattered and transmitted excitation, this is the preferential geometric orientation for fluorimetric assays (Lloyd, 1981)

The third fluorimetric assay described in this work (section 2 4 4) again depended on Z-Gly-Pro-MCA as substrate and the enzymatic liberation of the fluorophore MCA This microtitre plate assay was developed to enable rapid identification of enzyme activities in post column chromatography fractions Although not of the preferred perpendicular geometrical orientation, this assay proved invaluable in the initial stages of purification This assay employed front surface geometry (FIGURE 4 5) where the fluorescence is collected and measured through the illuminated surface This arrangement increases inner filter effects and light scattering while the fibre optical cable in the plate-reader attachment itself was responsible for a sharp reduction in assay sensitivity Despite these factors, the microtitre plate assay was ideal for non-quantitative enzyme detection, using less sample, substrate and acid volumes than the cuvette form of the assay and taking only a fraction of the time

Using these assays the levels of Z-Gly-Pro-MCA hydrolysing activities in bovine serum were determined FIGURE 3 3 1 clearly illustrates the presence of two distinct activities, PO and ZIP in a ratio of 1 1 The level of ZIP activity determined here was slightly higher than previously reported (Cunningham and O'Connor, 1997a), where only 40% of the total Z-Gly-Pro-MCA degrading activity was attributed to ZIP This may be due to batch to batch variations in bovine whole blood or serum samples Similar ratios between PO and ZIP levels in human serum were observed, where 47 8% of Z-Gly-Pro-MCA degrading activity was credited to the Z-Pro-Prohnal insensitive peptidase

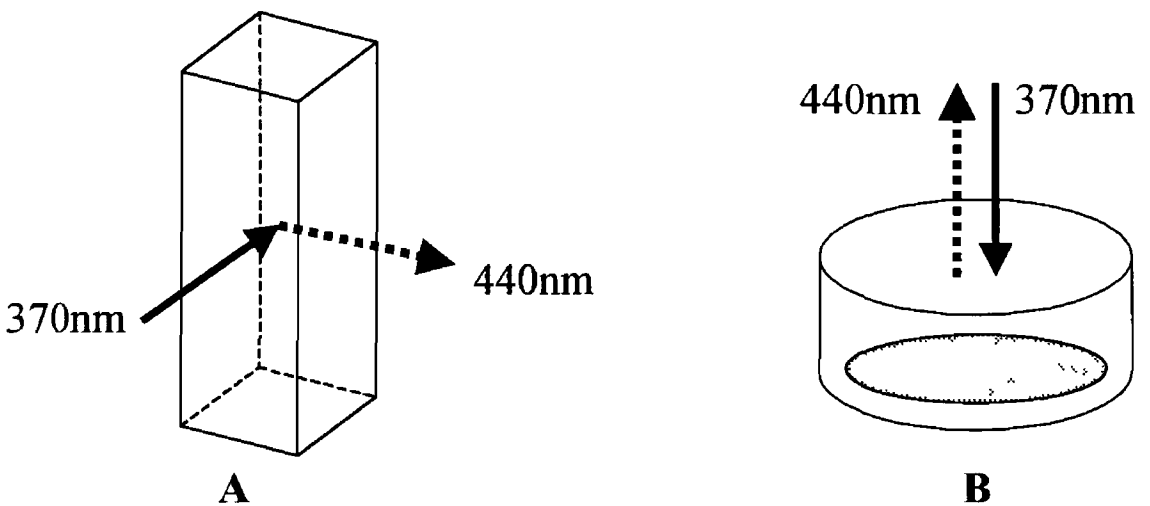


FIGURE 4 5 SAMPLE-CELL GEOMETRY

MCA (▨) absorbs electromagnetic radiation at 370nm (⟶) and emits fluorescence at 440nm (⟶)

A Cuvette-based assay - Fluorescence is emitted along an axis at 90° to the excitation beam - Perpendicular geometry

B Microtitre plate assay - Fluorescence is emitted along the same axis as the excitation beam - Front surface geometry

4 4 PURIFICATION OF BOVINE SERUM ZIP

Protein purification involves the isolation of a single protein from a mixture that may only constitute 1% of the species of interest It is achieved by the exploitation of known physical, chemical and/or biological characteristics of the target protein Prior to any purification protocol, there are a number of factors which must be considered The most important of these is the final purpose of the purified protein This in turn will dictate the amount required and the acceptable purity and whether or not maintenance of biological activity is necessary This research revolved around the characterisation of ZIP, a relatively novel proline-specific peptidase and therefore called for the production of large volume of high-purity enzyme Obviously, the most important factor to be considered in this particular application was the conservation of biological activity ZIP proved to be a fairly labile enzyme, which hampered purification procedures and limited the range of techniques that could be applied Coupled to this was the fact that the enzyme had not been well characterised and so limited understanding on its biochemical properties was available Despite this a highly effective purification procedure was established

4 4 1. Serum Preparation

A large volume of purified enzyme was required for optimisation of the purification protocol, necessitating a correspondingly large amount of starting material Therefore, a reliable, commercially available, inexpensive source that was high in ZIP content was essential Bovine serum fulfilled these requirements and was thus selected as an ideal source for the purification of ZIP Serum was prepared as outlined in section 2 5 1 by centrifugation of the non-clotted portion of bovine whole blood Storage at -17°C had no effect on the stability of the Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA degrading activity, however PO activity began to diminish after one month storage Fortunately, this did not cause problems as it was maintenance of the biological activity of ZIP which was imperative An alternative method of serum preparation was assessed, which would eliminate the 24hr shrinkage step This involved the collection of whole blood which had the anti-coagulant, heparin added Although the yield of serum was similar to that achieved using the clotting method, the behaviour of the ZIP on the initial

chromatography column was compromised Because of this and a difficulty in effective heparin removal, the initial method of serum preparation was preferred

4 4 2 Phenyl Sepharose Hydrophobic Interactions Chromatography

Unacceptable yields of active ZIP following ammonium sulphate precipitation lead to the idea of applying crude serum directly to a chromatography column Phenyl sepharose is classified as a high capacity resin, well able to withstand protein concentrations of 20mg protein/mL resin (Roe, 1989) Therefore, the hydrophobic interaction resin was selected as the first column in the purification procedure The number, size and distribution of hydrophobic regions is different for each protein and it is this characteristic that is exploited in hydrophobic interactions chromatography (HIC)

The addition of salt to crude serum and running buffers prior to column use was essential since ionic strength controls hydrophobicity (Roe, 1989) Solid $(NH_4)_2SO_4$, to a final concentration of 200mM, was added to serum samples as outlined in section 2 5 2 This method of salting avoided unnecessary dilution and dialysis, which were shown to substantially decrease biological activity FIGURE 3 4 2 1 illustrates the elution profile obtained from the HIC resin, clearly showing the presence of two distinct Z-Gly-Pro-MCA degrading activity peaks Based on the knowledge of the existence of ZIP, it was assumed that one peak could be attributed to PO activity while the other reflected the presence of ZIP in bovine serum This was readily shown by incubation of the column fractions with Z-Pro-Prolinal, the potent PO inhibitor As expected, PO was completely inhibited (FIGURE 3 4 2 2) as illustrated by the disappearance of the first peak Z-Pro-Prolinal had no effect on the second Z-Gly-Pro-MCA hydrolysing activity, which was hence deduced to be ZIP

Considering the purification of ZIP alone, this column successfully separated the peptidase from the bulk of contaminating protein Three protein peaks were observed (FIGURE 3 4 2 1) The first is evident in the column run-through (fractions 5-15), the second loosely bound to the column and was eluted on reduction of ammonium sulphate

concentration The third protein peak bound tightly to the HIC resin and co-eluted with ZIP in the complete absence of salt

The effectiveness of this resin in the purification of ZIP is reflected in the values obtained for the specific activity and enzyme yield, presented in TABLE 3 2 A 63-fold purification factor is due to the successful reduction in total protein (1743mg to 23 94mg) coupled to the minimal loss (15%) of ZIP activity Phenyl sepharose therefore served a dual purpose, primarily as a method of partially purifying ZIP but also as a tool for separating the two Z-Gly-Pro-MCA hydrolysing activities Because ZIP and PO both cleave the same substrate it was essential that they be separated at an early stage in the purification

4 4 3 Calcium Phosphate Cellulose Chromatography

The second step used in the purification of ZIP was a calcium phosphate cellulose column This resin was produced in the laboratory according to a modification of the methods described by Barranger (1976) and Donlon and Kaufman (1980) Although, an elaborate procedure, the production of a home-made resin considerably reduced expense and concerns of batch to batch variation were minimised by bulk production The precise mechanism of action of calcium phosphate cellulose (CPC) chromatography is unknown, limiting its popularity as a routinely used purification tool However, it is generally accepted that CPC crystals have two absorption sites on their surface, phosphate and calcium, and that these are responsible for the binding of basic and acidic groups of proteins respectively (Bernardi and Kawasaki, 1968, Bernardi *et al*, 1972) Due to the fact that the modes of protein adsorption and desorption are different from the majority of other techniques, CPC may be a successful purification step where other procedures have failed This indeed proved to be the case in the purification of ZIP

The elution profile generated using CPC is presented in FIGURE 3 4 3 illustrating excellent resolution between enzyme and contaminating protein ZIP readily bound to the resin and was isocratically eluted with increasing phosphate concentration The second wash at 100mM phosphate (section 2 5 3) was warranted for two reasons Primarily it successfully removed a portion of contaminating protein, but more

importantly it was responsible for the separation of ZIP from a number of contaminating peptidases First-time analysis of post CPC ZIP indicated the presence of a number of peptidases, namely Z-Phe-Arg-MCA, Lys-Ala-MCA and Gly-Pro-MCA hydrolysing enzymes Attempts at size-exclusion chromatography were successful in the removal of the Lys-Ala-MCA and Z-Phe-Arg-MCA degrading peptidases, however almost total loss of ZIP activity made this method unacceptable Replacement of the CPC linear phosphate gradient with two isocratic washes successfully separated the contaminating peptidases from ZIP activity

An 86% recovery of applied peptidase activity, coupled to a mass reduction (97%) in protein, resulted in a remarkable purification factor of 1632 (TABLE 3 2) Therefore this CPC resin, although often unpredictable, proved invaluable in the overall purification of ZIP from bovine serum

4.4 4 Diethylaminoethyl Sepharose Ion Exchange Chromatography

Diethylaminoethyl (DEAE) sepharose is a weak anion exchanger, frequently employed in the purification of enzymes with isoelectric points (pI) below 7 0 Despite the fact that the pI of ZIP was unknown at this stage, it was expected to fall between pH 5 0 and pH 7 0 (similar to other mammalian peptidases) Since anion exchange uses buffers at a pH above rather than below the pI, it was selected over cation exchange due to the preferential stability of ZIP at neutral-slightly alkaline pH values Ion exchange provides medium resolution and capacity and therefore is ideal when the volume and protein content of the extract have been reduced (Doonan, 1996), as was the case for ZIP

ZIP was dialysed and applied to a DEAE sepharose resin as outlined in section 2 5 4 FIGURE 3 4 4 shows the elution of peptidase activity and protein observed ZIP bound to the resin at pH 8 0 (indicative of pI below this) and its isocratic elution could not be improved using a linear chloride gradient, which actually resulted in peak tailing A number of parameters were investigated in an attempt to improve this elution profile, including pH, salt concentration, resin volume and sample volume, however, this procedure appeared to be optimal for the purification of ZIP using DEAE sepharose

TABLE 3 2 illustrates the effectiveness of the resin as a final clean-up step in the purification of ZIP The 38% loss of biological activity observed is excellent for such a late stage in a purification procedure and a substantial reduction in protein (0 76mg to 0 15mg) was also achieved

In summary, the purification of ZIP proved difficult and time consuming, however appropriate precautions and considerations were taken throughout A temperature of 4°C was constantly maintained, dialysis and concentration steps were avoided where possible, neutral buffers were used and the different stages was carried out consecutively to minimise enzyme degradation An overall purification factor of 4023 and a conservation of 33% Z-Pro-Prolinal insensitive Z-Gly-Pro-MCA hydrolysing activity proved it to be a worthwhile, successful endeavour

4 5 PURITY DETERMINATION

The concept of purity in relation to proteins is not entirely straightforward A pure protein would imply that the purified sample contains, in addition to water and buffer ions, only one population of molecules all with identical covalent and three-dimensional structures This is an unattainable and often unnecessary goal (Doonan, 1996) For the purpose of this work, purity was assumed if the sample was shown not to contain any species which would interfere with the experiments for which ZIP was intended This was achieved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and fluorimetry-based analysis

FIGURES 3 5 1 and 3 5 2 represent images of silver and brilliant blue stained polyacrylamide gels respectively Multiple bands were visualised following electrophoresis of crude serum and post-phenyl sepharose samples (lanes 2 and 3), however as the purification progressed there was an evidential reduction in the number of bands present Post DEAE sepharose ZIP is represented by a faint single band corresponding to a molecular weight of 88kDa for the silver stained gel and 96kDa for the blue stained gel (lanes 5) The discrepancy in molecular masses determined is not related to the staining procedure but rather to experimental error and is less than 5%

Although the band is relatively faint, this deduced molecular weight for ZIP was confirmed by size-exclusion chromatography (section 2 9 2 2)

A number of fluorimetric assays were performed to detect the presence of contaminating peptidases in the post DEAE sepharose ZIP pool The substrates employed are listed in TABLE 2 5 and the presence of the appropriate peptidase was determined by a measure of liberated MCA Two detection methods were employed The PE luminescence spectrophotometer was used to measure possible release of MCA as described in section 2 6 2 The second detection technique involved HPLC analysis on a C_{18} column, followed by eluant monitoring on a fluorescence detection system (section 2 6 3) No cleavage of any substrate other than Z-Gly-Pro-MCA was detected for either method (TABLES 3 4 , 3 5 and FIGURES 3 5 3 1 -3 5 3 3) The absence of these peptidases, particularly PO, DPP II and DPP IV, was essential since their presence would ultimately interfere with future experiments Based on SDS PAGE and fluorimetric analysis, post DEAE sepharose ZIP was deemed to be acceptably pure for further applications

4 6 ASSAY DEVELOPMENT

Using the purified ZIP sample, a number of experiments were carried out to assess the effectiveness of the Z-Gly-Pro-MCA assays in an attempt to enhance peptidase detection levels

4 6 1 MCA Excitation and Emission Wavelengths

The use of the fluorophore MCA in the production of fluorimetric peptide substrates is well documented (Kanaoka *et al*, 1977, Kato *et al*, 1978, Fujiwara and Tsuru, 1978, Yoshimoto *et al*, 1979, Blackmon *et al*, 1992) However, there does appear to be slight variations in the reported excitation and emission wavelengths at which it fluoresces The most frequently employed excitation wavelength is 380nm (Kanaoka *et al*, 1977, Kato *et al*, 1978, Nishikata *et al*, 1986, Bakker *et al*, 1990, Blackmon *et al*, 1992), while Fujiwara and Tsuru (1978), and Yoshimoto *et al* (1979) report an optimal excitation wavelength of 370nm The emission wavelength of the fluorophore also seems to be in question, ranging between 440nm and 460nm by the above researchers It must be noted though that none of these studies make any mention of the slit widths at which the fluorescence was measured Optimum wavelengths were tested as described in section 2 7 1 and 370nm and 440nm were concluded to represent the optimum excitation and emission wavelengths of MCA respectively (FIGURES 3 6 1 1 and 3 6 1 2) However, if the excitation slit width of 10nm employed is considered, MCA excites between 365 and 375nm Similarly, the emission wavelength broadens to anywhere between 435 and 445nm when read at slit widths of 2 5, 5 0 or 10 0nm Considering this, these results obtained are in keeping with those reported in the literature

4 6 2 Substrate Solvent Studies

The substrate employed for the detection of ZIP and PO activities, Z-Gly-Pro-MCA, is water insoluble, so the addition of a non-aqueous organic solvent was essential for solubilisation The majority of enzymes, excluding lipases, esterases, dehydrogenases, which function in natural hydrophobic environments (Dordick, 1989), did not evolve to function in non-aqueous environments and so the use of organic solvents is never favoured Having said this, it has been hypothesised that although water remains

essential for catalysis, an enzyme molecule in aqueous solution becomes fully hydrated when surrounded by a few layers of water (Gorman and Dordick, 1992) Therefore, bulk water is not crucial for catalytic activity and replacement of the remaining water with an organic solvent should be possible without adversely affecting the enzyme (Klibanov, 1986)

A non-aqueous solvent was sought which proved optimal for both substrate solubility and enzyme activity Z-Gly-Pro-MCA was prepared in a range of organic solvents and assayed with purified ZIP as described in section 2 7 2 FIGURE 3 6 2 compares the effect on ZIP activity of EtOH, DMF, DXN and MeOH as substrate solvent to that of DMSO, the previously favoured solvent (section 2 4 1) All solvents yielded identical solubility patterns when used at 4%v/v It is clearly evident that there was a 2 5-fold increase in ZIP activity when MeOH was used in comparison to DMSO This, coupled to its effective solubilisation properties, resulted in MeOH being selected as the optimal solvent for Z-Gly-Pro-MCA preparation

Generally short chain aliphatics cause a lower degree of denaturation than solvents such as butanol (Harris, 1989), and this may explain why ZIP activity was least affected by the presence of MeOH in the substrate Organic solvents effect both the denaturation and catalytic activity of enzymes, but not necessarily in a parallel manner Aliphatic solvents (EtOH and MeOH) show only moderate effect on activity denaturation and loss of catalysis DMF on the other hand, is a strong denaturant which also reduces catalytic activity, while dioxane does not induce denaturation but does diminish catalytic power This may explain why DXN and DMF resulted in lower peptidase activities than MeOH (Harris, 1989) Similar results have been cited in the literature Ulbrich-Hofmann and Selisko (1993) observed that DMF, DXN and DMSO showed greater inactivating influences against glucoamylase and invertase activity than MeOH They also suggested that the pH of a buffer and MeOH mixture was more likely to remain at the pH value of the added buffer component (in this case pH 7 4), than in mixtures composed of DXN, DMSO, DMF and acetonitrile This may offer another explanation as to why optimal enzyme activity was observed using MeOH as the substrate solvent

Once MeOH had been selected as the optimal solvent for substrate preparation, the concentration at which it was most effective had then to be determined Experiments were carried out according to section 2 7 3 in which the concentration of MeOH used in Z-Gly-Pro-MCA preparation was varied As expected, enzyme activity decreased with increasing concentrations of solvent (FIGURE 3 6 3) This graph reiterates the results obtained in FIGURE 3 6 2 , in that methanol is less inhibitory on ZIP activity than DMSO even at 6%v/v More importantly though, this result illustrates that 2%v/v MeOH is optimal for substrate preparation, yielding over three times the activity observed with 4%v/v DMSO Z-Gly-Pro-MCA was therefore prepared in 2%v/v MeOH, however a difficulty arose with respect to solubility The substrate solution constantly precipitated unless heated to at least 45°C Related to this was a marked increase in the standard error observed between triphcate fluorescent readings For these reasons, high ZIP activity due to low concentrations of solvent (2%v/v and 3%v/v) was compromised for acceptable substrate solubility and accurate triplicate values As outlined in section 2 7 10 , the substrate preparation described in section 2 4 1 was modified to incorporate 4%v/v MeOH in place of 4%v/v DMSO

4 6 3 Linearity Studies

A typical progress curve for an enzyme-catalysed reaction is presented in FIGURE 4 6 The time course is initially linear but the rate of product formation starts to decline after prolonged incubation This departure from linearity may be due to a number of factors, most frequently, substrate depletion, enzyme or substrate instability, products inhibiting the reaction, or a change in assay conditions It can be deduced from this that enzyme activity should only be quantified at a point where these factors are known not to be an issue This point is known as the initial velocity of the reaction and can be calculated by drawing a tangent through the origin to the linear part of the progression curve as shown in FIGURE 4 6 Using a continuous assay system, this decrease in reaction velocity with respect to time is evident and generally due to substrate depletion

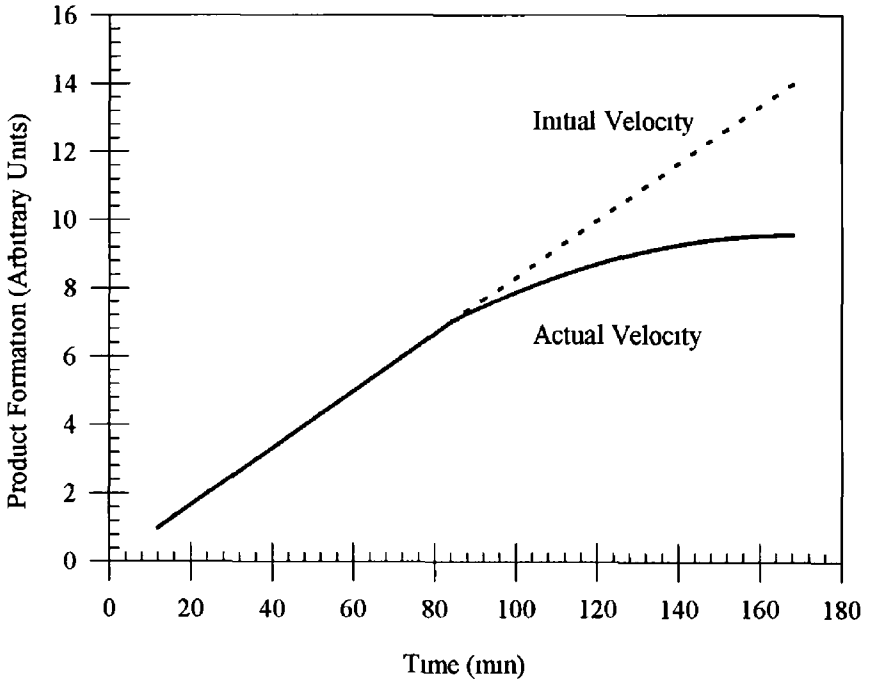


FIGURE 4 6. TYPICAL PROGRESS CURVE OF AN ENZYME-CATALYSED REACTION

Plot of product formation versus time

However in a discontinuous assay, activity measurements are determined at one discrete point along the reaction progression curve, and deviation from linearity may not be readily detected Quantification of ZIP activity using a discontinuous assay system cannot be relied upon unless the linearity of the assay has previously been assured using a continuous assay system

Because quantification of ZIP activity in a number of fractions was required for the purification table construction, the linearity with respect to time of crude serum (in the presence and absence of PO inhibitors) and post column chromatography samples was deemed necessary FIGURES 3 6 4 1 and 3 6 4 2 represent the progress curves obtained for serum in the absence of inhibitor and purified ZIP respectively It is clearly evident that the degradation of Z-Gly-Pro-MCA was linear over the 60min time frame examined Identical linear progress curves were generated for post phenyl sepharose and post calcium phosphate cellulose enzyme pools (results not shown) The inclusion of Z-Pro-Prolinal and JTP-4819 in the continuous assay system was necessary to ensure that these compounds were not responsible for any deviation from linearity A slight downward curvature of the line may have been expected on the inclusion of inhibitors as illustrated for trypsin by Dixon and Webb, (1979) However, this was not the case and FIGURES 3 6 4 3 and 3 6 4 4 clearly indicate that linearity was maintained The fact that the serum was pre-incubated for 5min with each inhibitor prior to the execution of the assay implies that the pre-incubation totally inhibited PO and that the linear velocity observed is due solely to ZIP This was further verified in section 3 6 9 where prolonged incubation failed to alter the level of ZIP activity detected from that measured after 5min

Another parameter that was important to consider in the quantification of ZIP, was the linearity of the assay with respect to enzyme concentration FIGURE 3 6 5 illustrates that fluorescent intensity increased linearly with increasing concentrations of enzyme thus allowing quantification of enzyme levels based on a fluorescent reading (section 6 3) Therefore both the Z-Gly-Pro-MCA degrading activities assay and the Z-Pro-Prohnal insensitive Z-Gly-Pro-MCA degrading activities assay were linear with respect to time over the 60min assay time frame and with respect to enzyme concentration

4.6 4 Optimum Assay Temperature

The effect of assay temperature on purified ZIP activity was investigated as outlined in section 2 7 6 Samples were allowed to reach thermal equilibrium by pre-incubation for 15mm at the appropriate temperature prior to assaying This ensured that the observed activity genuinely reflected the activity of the peptidase at the temperature under investigation Optimal activity was observed when the Z-Gly-Pro-MCA degrading activity assay was performed at 37°C as FIGURE 3 6 6 illustrates The rapid decline in fluorescent intensities observed in assays performed at greater than 37°C is most probably due to instability of the Z-Gly-Pro-MCA rather than inactivation of ZIP This was verified by the large background fluorescence recorded for the blanks at these temperatures and the fact that ZIP remained active when incubated at 40°C and then assayed at 37°C (see FIGURE 3 8 3 1) This result is not surprising considering the mammalian origin of this enzyme and that optimal physiological temperature is 37°C Yoshimoto *et al*, 1987 and 1988, reported an identical optimal assay temperature for the detection of PO from *D carota* and *L cinerascens* respectively using Z-Gly-Pro-MCA This assay temperature has previously been shown to be optimal for the detection of bovine serum PO (Cunningham and O'Connor, 1998) Cited assay temperatures for PO range from 37°C to 47°C depending on the source (TABLE 4 2)

4 6 5 Effect of DTT and NaCl on ZIP Activity

Purified ZIP was incubated with a range of dithiothreitol (DTT) concentrations in an attempt to study the effects of this thiol-reducing agent on its Z-Gly-Pro-MCA degrading ability FIGURE 3 6 7 illustrates the pattern observed Increasing concentrations of DTT caused a rapid decline in ZIP activity when compared to a peptidase sample containing no DTT (100% activity) Inclusion of 5mM DTT in the substrate resulted in a loss of over 10% activity, while 35% inhibition was observed in the presence of 20mM DTT

This result is quite interesting as it illustrates a distinct difference between the two Z-Gly-Pro-MCA degrading activities present in bovine serum Numerous reports have shown PO to be substantially activated by DTT (Walter, 1976, Orlowski *et al*, 1979,

Source	Assay temp ℃	MW *kDa*	Thermal Stability ℃	Optimum pH	pI	Reference
Bovine brain	40	62-65	ND	7-7 5	4 8	Yoshimoto *et al*, 1983
Bovine serum	37	69 7	37	8 0	ND	Cunningham and O'Connor,1998
Daucus carota	37	75	ND	7 3	4 8	Yoshimoto *et al*, 1987
Flavobacterium meningosepticum	40	74	ND	7 0	9 6	Yoshimoto *et al*, 1980
Human lymphocytes	ND	76	ND	ND	4 8	Goossens *et al*, 1995
Lamb brain	45	74-77	40	7 0	4 9	Yoshimoto *et al*, 1981
Lamb kidney	47	74-77	45	7 7	4 8	Koida and Walter, 1976
Lyophyllum cinerascens	37	76	ND	6 8	5 2	Yoshimoto *et al*, 1988
Porcine liver	37	72-74	ND	6 5	4 9	Moriyama and Sasaki, 1983
Rabbit brain	ND	66	ND	8 3	ND	Orlowski *et al*, 1979
Rat brain	37	70	ND	5 8-6 5	ND	Kato *et al*, 1980
Treponema denticola	ND	75-77	37	6 5	6 5	Makinen *et al*, 1994

Table 4 2. Summary of PO Physiochemical Properties

ND means not determined

Kalwant and Porter, 1991) Cunningham and O'Connor, 1998, reported a twelve-fold increase in PO activity in the presence of 10mM DTT It is generally accepted that this marked increase is due to the reduction of disulphide bridges (S-S) to thiol groups (SH +SH) by DTT This suggests the existence of a cysteine residue, situated near the active site, which when reduced increases the ability of PO to initiate a nucleophilic attack upon a bound substrate (Polgar, 1991) The results obtained suggest that ZIP, although not activated by DTT, may have a cysteine residue near its active site (see also section 4 8 6) which in the presence of thiol-reducing agents somehow contributes to the inactivation of enzyme activity

Salt exerts effects on proteins that can be denaturing or stabilising depending on the nature and concentration of the salt employed SO_4^{2} and Cl ions are classified as protein stabilisers, reducing the solubility of hydrophobic groups on the protein molecule by increasing the ionic strength of the solution (Volkin and Klibanov, 1990) Salts such as sodium chloride (NaCl) and ammonium sulphate ($(NH_4)_2SO_4$) therefore enhance water clusters around proteins inducing stability by compacting proteins This phenomenon is known as preferential hydration (Timasheff and Arakawa, 1989) The effect of NaCl was tested on purified ZIP as described in section 2 7 8 FIGURE 3 6 8 represents the salt enhancement of enzyme activity As the concentration of NaCl was increased there was a noticeable 220% ZIP activity in comparison to the control (100%) Unfortunately, this increase could not be extended by further increasing the salt concentration, evident by the graphical plateau, and most probably due to poor substrate solubilisation at high NaCl concentrations Based on these observations, 10mM DTT was included in substrates for the determination of PO, but since the thiol reducing agent had detrimental effects on ZIP activity it was omitted from the ZIP substrate Similarly, 500mM NaCl was incorporated into substrate preparations for the detection of ZIP activity Due to conflicting reports on the effect of salt on PO activity (Orlowski, 1979 and Polgar, 1991), NaCl was omitted from the PO substrate

4 6 6 Prolyl Oligopeptidase Inhibitor Studies

The original ZIP-specific assay was defined by Cunningham and O'Connor (1997a), involving the incorporation of the prolyl ohgopeptidase inhibitor, Z-Pro-Prolinal into the assay mixture as described in section 2 4 3 FIGURE 3 6 9 1 represents a typical inhibition curve of the inhibitor in crude serum Z-Pro-Prolinal appears to inhibit Z-Gly-Pro-MCA degrading activity by approximately 50%, however even at increased concentrations there is negligible further inhibition The residual Z-Gly-Pro-MCA hydrolysing activity has been attributed to ZIP as previously discussed The effect of extending the pre-incubation period was also examined, however no further inhibition of the Z-Pro-Prolinal insensitive activity could be achieved (FIGURE 3 6 9 8) These profiles are in keeping with the findings of Cunningham and O'Connor (1997a)

Unfortunately stocks of Z-Pro-Prolinal became unavailable and unsuccessful attempts to synthesise the aldehyde led to the search for an alternative inhibitor, which could exert identical behavioural characteristics to Z-Pro-Prolinal A number of specific PO inhibitors, listed in TABLE 2 6, were purchased or kindly donated (section 2 1) The effect of each inhibitor on Z-Gly-Pro-MCA degrading activities in serum was assessed and the results are illustrated in FIGURES 3 6 9 2 - 3 6 9 7 Fmoc-Ala-Pro-CN and postatin were deemed unsuitable as they both inhibited the total Z-Gly-Pro-MCA degrading activities in serum and therefore could not distinguish between PO and ZIP The commercially available Z-Pro-Prolinal dimethylacetate successfully inhibited almost 40% of the total Z-Gly-Pro-MCA hydrolysing activity, although it was difficult to establish if ZIP activity was affected A defined plateau was not observed and higher concentrations could not be examined as this would have required inhibitor preparation in 100%v/v solvent

Fmoc-Pro-Pro-CN, Z-Indolinyl-Prolinal and JTP-4819 however showed potential as specific PO inhibitors each illustrating the presence of an inhibitor-insensitive residual activity (see FIGURES 3 6 9 3, 3 6 9 5 and 3 6 9 7) JTP-4819 was selected as the *Z-Pro-Prolinal replacement* primarily because there was an ample supply and also because,

similar to Z-Pro-Prolinal, no further inhibition was observed on increased pre-incubation (FIGURE 3 6 9 9)

Accumulation of the assay development results allowed for the development of an optimal Z-Gly-Pro-MCA degrading activities assay (section 2 7 10) The sensitivity and suitability of the assay was assured and the linearity studies allowed for accurate quantification of enzyme concentrations The incorporation of 4%v/v MeOH and 500mM NaCl in the substrate solution, resulted in a 5-fold increase in ZIP activity

4 7 LOCALISATION STUDIES

The presence of PO and ZIP activities in a number of bovine tissues was determined as outlined in section 2 8 The purpose of this study was to ascertain the tissue of origin of the two Z-Gly-Pro-MCA degrading peptidases and/or to establish the optimum source for future study of either enzyme Extraction of soluble proteins from the animal tissues is relatively straightforward due to the weakness of the cell membranes and the absence of a cell wall A combination of mechanical and centrifugal forces were employed in the extraction of Z-Gly-Pro-MCA hydrolysing activities from each tissue as outlined in section 2 8 1 Lung tissue proved the most difficult to homogenise, due to its tough cellular composition and so was semi-thawed prior to homogenisation

Z-Gly-Pro-MCA degrading activities were measured in the tissues and serum as described in section 2 8 2 As discussed in section 4 1 1 the inner filter effect becomes a problem in coloured samples of high protein content, therefore filtered MCA standard curves were prepared for each extract The degree of filtering did not vary greatly between the same fraction of individual tissues (with the exception of spleen extracts) as is evident in TABLE 3 6 Considering this effect, fluorescent intensities were converted into enzyme units (one unit of enzyme activity is defined as the amount of enzyme which releases 1picomole MCA per minute at 37°C) as outlined in section 6 3 Following quantification of ZIP and PO activities, and total protein determination (Biuret assay, section 2 3 1), specific activities were estimated for ZIP and PO (TABLES 3 7 and 3 8 respectively) For all samples, the bulk peptidase activity was contained in the S1

fraction and so the discussion will focus on specific activities calculated in these supernatants

FIGURE 3 7 8 clearly shows that PO activity was most abundant in bovine brain, liver and kidney extracts with specific activities of 3 2, 2 4 and 2 4unit/mg respectively Spleen was also an acceptable source of PO while the low levels of activity in serum correlate to the results of Kato *et al* (1980) who observed a similar pattern in humans PO distribution studies performed in rat (Yoshimoto *et al*, 1979), human (Kato *et al*, 1980), rabbit (Orlowski *et al*, 1979) and mouse (Dauch *et al*, 1993) tissues have revealed an ubiquitous distribution with uniformly high levels in the brain The results presented are in agreement It is highly possible that kidney or liver may be the site of origin of bovine PO since significant species-to-species variation has been observed in such peripheral tissues Although liver is rich in PO activity, it is not an ideal tissue source Clarified homogenates are difficult to obtain due to the presence of nucleic acids and large quantities of fats It also contains much larger proportions of soluble protein than kidney or brain, which increases subsequent purification problems (Doonan, 1996) Therefore, brain or kidney would appear to be the most practical PO sources and are in fact most commonly employed in the extraction of prolyl oligopeptidase (TABLE 4 3)

FIGURE 3 7 9 clearly displays positive identification of ZIP activity in bovine kidney, brain, serum, liver, lung and spleen As previously mentioned, this ubiquitous distribution has also been reported for PO In general, ZIP specific activity levels were considerably lower than those for PO in the same tissue extracts, with the exception of serum which revealed a 1 1 ratio of ZIP PO TABLE 3 8 lists the percentages of total Z-Gly-Pro-MCA degrading activity attributed to each peptidase Although FIGURE 3 7 9 illustrates the highest ZIP activity to be present in bovine kidney, TABLE 3 8 shows that this activity only represents 14% of the total Z-Gly-Pro-MCA hydrolysing activities in the organ Despite the low levels, ZIP was identified in all tissues, its presence in the brain being of particular importance Previous attempts in our laboratory failed to detect ZIP in bovine brain yet these results suggest activity, albeit only 6 2% of the total activity However further studies, such as subcellular fractionation of the brain, would be

required before the presence of brain ZIP could be confidently claimed Observation of the actual enzyme activity values (TABLE 3 7) shows that kidney and liver contained comparable levels of ZIP to serum and could therefore serve as acceptable alternative sources for ZIP purification, although extraction procedures are more difficult and time-consuming

SOURCES	REFERENCE
Brain	
Bovine	Blumberg *et al.*, 1980, Hersh, 1981, Yoshimoto *et al.*, 1983, O'Leary and O'Connor, 1995.
Human	Kato *et al.*, 1980, Kalwant and Porter, 1991.
Lamb	Yoshimoto *et al.*, 1981.
Mouse	Fukunari *et al.*, 1994.
Rabbit	Carvalho and Camargo, 1981, De Camargo *et al.*, 1982, Dresdner *et al.*, 1982.
Rat	Kato *et al.*, 1980, Healy and Orlowski, 1992.
Kidney	
Porcine	Dehm and Nordwig, 1970, Hauzer *et al.*, 1984, Odya *et al.*, 1987.
Liver	
Porcine	Moriyama and Sasaki, 1983.
Rat	Yamakawa *et al.*, 1994, Matsubara *et al.*, 1998.
Other	
Rat heart	Cicilini *et al.*, 1994.
Human uterus	Walter *et al.*, 1971
Porcine pancreas	Yoshimoto *et al.*, 1982.

TABLE 4.3. COMMON MAMMALIAN SOURCES OF PO ACTIVITY

4 8 CHARACTERISATION

TABLE 4 2 summarises biochemical characteristics of PO reported and will be referred to throughout this section

4 8 1 Determination of N-terminal Sequence

As explained in section 3 8 1 unfortunately the N-terminal sequence of ZIP was not determined using the method described in section 2 9 1 1 Although it was possible that the N-terminal was blocked, this was unlikely since a previous attempt at sequencing discovered no blocking group present (see section 3 8 1) In addition to this, the method employed involved pre-casting of the gel coupled to pre-electrophoresis in thioglycolic acid, two procedures known to reduce the occurrence of N-terminal blocking by amines during electrophoresis The most plausible reason for the unsuccessful sequencing is due to the large molecular weight of ZIP, as the larger the volume of amino acid residues present the more difficult it is to sequence It was recommended that ZIP be digested using a protease such as V8 protease which cleaves at glutamic acid residues, resulting in smaller fragments which may then be sequenced and aligned (method employed by Malfroy *et al*, 1987 for the sequencing of rat neprilysin) This is currently being investigated and should allow the N-terminal sequencing of this novel proline-specific peptidase to be determined

The sequence obtained for ZIP, shown in section 3 8 1 was actually obtained following an acetone precipitation of the purified enzyme The presence of two equimolar components was initially believed to be due to the presence of a contaminant however, it was more likely that the acetone had a detrimental effect on ZIP activity, and hence the electroblotting method was attempted Irrespective of the dual sequence obtained, it is worth noting that no sequence homology between it and PO is evident in the first six residues The N-terminal amino acid sequence (residues 1-50) of prolyl oligopeptidase from porcine brain is {Met-Leu-Ser-Phe-Gln-Tyr-Pro-Asn-Val-Tyr-Arg-Asn-Glu-Thr-Ala-Ile-Gln-Asn-Tyr-His-Gly-His-Lys-Val-Cys-Asn-Pro-Tyr-Ala-Trp-Leu-Glu-Asn-Pro-Asn-Ser-Glu-Gln-Thr-Lys-Ala-Phe-Val-Glu-Ala-Gln-Asp-Lys-Ile-Thr} as deduced by Rennex *et al* (1991)

4 8 2 Relative Molecular Mass Determination

The anionic detergent sodium dodecyl sulphate (SDS) readily binds to proteins in a ratio of 1 4 1, effectively masking the intrinsic charge of the polypeptide chains (Dunn, 1989) The net charge per unit mass becomes approximately constant, therefore separation of SDS-treated proteins is effectively based on molecular weight alone SDS polyacrylamide gel electrophoresis (PAGE) was employed in the estimation of the molecular mass of purified ZIP as outlined in section 2 9 2 1 A 10%v/v gel was prepared since the molecular weight was expected to be in the range 15-200kDa FIGURES 3 5 1 and 3 5 2 represent images of the silver and blue stained gels respectively Measurement of the molecular weight marker R_f values allowed for the construction of standard curves (FIGURES 3 8 2 1 1 and 3 8 2 1 2) Using these, molecular masses of ZIP were determined to be 87,810Da (silver stain) and 95,820Da (blue stain) The interaction of SDS with proteins causes the irreversible denaturation of the 3-dimensional structure and proteins are thus broken down into individual polypeptide chains (monomers) Therefore SDS-PAGE yields no insight into the native molecular mass of a protein and so a second method for the determination of molecular mass is regularly performed

Size-exclusion chromatography (SEC) is frequently employed as a non-denaturing technique for molecular mass estimation Separation is based on the hydrodynamic volume of a protein or simply its size, larger proteins eluting faster than smaller ones that interact with the pores of the column Three SEC columns were run as described in section 2 9 2 2, namely a low-pressure sephacryl S-200, a medium pressure HiPrep sephacryl and a high pressure BioSep SEC-3000 FIGURES 3 8 2 1 3 to 3 8 2 1 4 represent the standard curves of log molecular weight versus elution volume/void volume for each resin Molecular masses of 194,922, 172,288 and 164,369Da were estimated from these graphs for S-200, HiPrep and SEC-3000 sephacryl resins respectively The 2 4% discrepancy between these values is lower than the frequently reported 10% inaccuracy for SEC (Welling and Welling-Wester, 1989) Based on these results, it was assumed that the estimated 88kDa and 96kDa SDS PAGE bands corresponded to the mass of a subunit of the peptidase It was deduced, due to the presence of a single band

following PAGE analysis that ZIP exists as a dimer of two subunits of equal molecular weight Combination of SDS PAGE and SEC for the determination of molecular weight, compensated for the inadequacies of each technique and so an average molecular weight of 179,768Da (TABLE 3 9) can be confidently postulated for ZIP Conversely, PO is generally accepted to be a monomer, with a molecular weight in the range 62-77kDa depending on the source (TABLE 4 2) This provides yet another biochemical dissimilarity between the two Z-Gly-Pro-MCA degrading peptidases

4 8 3 Thermostability Studies

The inactivation profile of ZIP following pre-incubation at various temperatures for different times is presented in FIGURE 3 8 3 1 Naturally, stability of this mammalian enzyme would be expected at temperatures up to 37°C, and this was clearly the case However, ZIP activity was less than 40% inhibited after incubation at 50°C for 1hour Relatively high thermostability can be attributed to the presence of *protecting* proteins, which increase interactions between water molecules thus strengthening hydrophobic interactions (explaining why BSA is often added to purified enzyme samples, Chang and Mahony, 1994) Progression of a purification procedure causes the protein of interest to become more heat-labile, due to the removal of such stabilising proteins Although ZIP was highly pure, there was no requirement for protein addition because of its excellent thermostability profile ZIP activity could be maintained at 4°C for up to a week or at room temperature for 3hr Reports of similar thermostability profiles have not been reported for PO and it appears that the peptidase is quite susceptible to temperatures above 40°C (TABLE 4 2)

The substrate specificity studies performed by HPLC analysis on purified ZIP required an overnight incubation at 37°C and so the stability of ZIP over this time frame was examined FIGURE 3 8 3 2 illustrates the interesting profile observed It is evident that the activity decreased with prolonged incubation showing a maximum inactivation of 30% after 4hr However, Z-Gly-Pro-MCA degrading activity measured after this time began to exhibit a steady increase in activity, 100% catalytic activity being restored after 24hr incubation One possible explanation for this unusual profile can be attributed to the

nature of protein denaturation and subsequent unfolding Although rare, an enzyme can unfold to an intermediate state that does not possess any activity and then further unfold to exhibit some activity (Sadana, 1993) Further work into this unusual observation would have to be performed to obtain a greater understanding of the mechanism of ZIP at 37°C Irrespective of the mechanism by which this phenomena occurred, this assay showed the stability of ZIP at 37°C overnight and hence the HPLC procedure could be performed without concern over the thermal degradation of the peptidase

4 8 4 pH Effects

Two distinct investigations into the effects of pH on ZIP stability and activity were performed Section 2 9 4 1 describes how the pH range over which the Z-Gly-Pro-MCA degrading peptidase remained stable was examined This was achieved by pre-incubation at a range of pH values followed by assaying with substrate prepared in buffer at pH 7 4 Citrate, phosphate, Tris and glycine buffer systems (pK_a values 4 76, 7 20, 8 06 and 9 78 respectively, Blanchard, 1984) were chosen to reflect a range of pK_a values, since buffering capacity is maximal at the pK_a FIGURE 3 8 4 1 represents the bell-shaped inactivation curve constructed for ZIP It is surprising how stable ZIP activity appeared to be with respect to pH, inactivation being observed only below pH 2 0 or above pH 10 5 In fact, 70% ZIP activity was detected at pH 2 5 and at pH 10 0 and over 80% at pH 3 0 This broad pH stability range implies that ZIP is little affected by the pH of the incubation buffer over 15min On closer examination of the graph it is clear that maximum Z-Gly-Pro-MCA degrading activity occurred at pH 7 5 in a phosphate buffer system

The second study served to determine the buffer system and substrate buffer pH at which ZIP activity was optimal Pre-incubation of purified enzyme with a range of buffers was conducted followed by assaying in a range of substrates each prepared at a different pH value as detailed in section 2 9 4 2 It was deduced from FIGURE 3 8 4 2 that >90% ZIP activity was observed between pH values of 7 4 (control-100%) and pH 10 0 in phosphate, Tris and glycine buffer species Optimal activity was observed using Z-Gly-Pro-MCA prepared in phosphate buffer at pH 7 0

Despite the availability of zwitteronic *Good buffers* (Good *et al*, 1966), such as HEPES and MES, inorganic buffers including phosphate are less expensive for protein purification where large buffer volumes are required Phosphate buffer was selected over Tris where possible due to Tris' ineffectiveness below pH 8 0, its highly temperature dependent pK_a and its interference with a number of protein assays due to primary amine presence (Blanchard, 1984) Inactivation and activation pH ranges determined for ZIP are very similar to those reported for PO from numerous sources (TABLE 4 2) On comparison with an identical source, bovine serum PO was optimally active at pH 8 0-8 5, demonstrated a preference for phosphate buffer and remained stable over a pH range of 5 0-9 0 (Cunningham and O'Connor, 1998)

4 8 5. Isoelectric Point Determination

Chromatofocusing was employed in the determination of the isoelectric point (pI) of ZIP It differs from ion exchange in that the anion exchange matrix, pre-adjusted to a given pH, has distinct buffering properties that allows the creation of a pH gradient on the column rather than outside it Chromatofocusing can be employed as an isoelectric focusing or purification technique Section 2 9 5 1 outlines the conditions at which a PBE 94 resin was run Binding of ZIP was achieved at the initial pH of 7 8 and eluted during a pH gradient between pH 5 5 and 5 8 (FIGURE 3 8 5 1) This implies that in this range ZIP had no net charge and therefore is a good indication of its pI

Chromatofocusing could have been applied in the purification protocol, but the low working pH range was not optimal for ZIP activity, although it did not cause a high degree of inactivation (section 4 8 4) DEAE sepharose proved to be a more successful anion exchanger in comparison, yielding higher activity and greater reduction in total protein Coupled to this, polybuffer can be difficult to remove, requiring a further step such as ammonium sulphate precipitation, size-exclusion or affinity chromatography Despite this, chromatofocusing has successfully been reported in various PO purifications (Browne and O'Cuinn, 1983, Rennex *et al*, 1991)

In order to confirm the pI deduced using chromatofocusing, vertical isoelectric focusing (IEF) was performed which is based on the following theory In the presence of an electric field and pH gradient, charged proteins migrate towards the anode or cathode until they reach their isoelectric point at which they have no charge and hence cannot further migrate Vertical IEF systems are becoming more common due to their simplicity and there is no need for a separate IEF system as PAGE electrophoresis equipment can be employed The relatively large pore size of agarose made it an ideal matrix for the large MW ZIP FIGURES 3 8 5 2 and 3 8 5 3 represent an image of the blue stained gel and the standard curve constructed as described in section 2 9 5 2 respectively The isoelectric point of ZIP was estimated to be 5 68 using this technique This value correlates with the chromatographic estimate of 5 5-5 8 and due to the superior resolving capabilities of IEF, the pI of ZIP was concluded to be 5 68 TABLE 4 2 highlights similar isoelectric points estimated for PO

4 8 6 Catalytic Classification and Effect of Other Functional Reagents

Sensitivity to specific active-site-directed inhibitors coupled to ammo acid sequence analysis is frequently investigated to determine the catalytic class of an enzyme In this study a number of serine, metallo- and cysteine protease inhibitors were incubated with purified ZIP to establish inhibition effects Cysteine protease activators and some non-specific inhibitors were also examined as detailed in sections 2 9 6 and 2 9 7

Inhibition of ZIP by classic serine protease inhibitors, 4-(2-aminoethyl)-benzenesulphonyl-fluoride (AEBSF) and aprotinin was observed to be 58% and 35% respectively (TABLE 3 10) AEBSF was employed as a safer, more stable, water-soluble alternative to di-isopropyl fluorophosphate (DFP) However, APMSF (4-amidino-phenyl methane-sulphonyl fluoride), a reportedly specific, irreversible serine protease inhibitor had no effect on ZIP activity Similarly the reversible cysteine and serine protease inhibitors, phenylmethyl-sulphonyl fluoride (PMSF) and leupeptin, failed to inhibit the Z-Gly-Pro-MCA degrading peptidase

PO has been classified as a serine protease based on its sensitivity towards inhibitors such as DFP and PSMF (Moriyama and Sasaki, 1983, Rennex *et al*, 1991) A number of researchers have reported contrasting results, Yoshimoto *et al*, (1983) for example, reported complete inhibition of PO by DFP yet none on incubation with PMSF, and still classified PO as a serine protease Similarly Goossens *et al*, (1995) demonstrated PO's sensitivity towards DFP and PMSF yet it exhibited resistance towards aprotinin It can be concluded from this that peptidases are often classified as serine proteases despite not exhibiting sensitivity towards *all* of the active-site-targeted serine protease inhibitors Based on results of a similar pattern and the identical substrate specificity of PO and ZIP, it is highly possible that ZIP too is of the serine protease catalytic family, although it is Z-Pro-Prolinal insensitive

Of the metallo-protease inhibitors examined (TABLE 2 11), ZIP showed no sensitivity towards the chelators, EDTA, EGTA, 8-hydroxyquinoline, imidazole and 1,10-phenanthroline The most substantial ZIP inhibitions noted were 90% by 4,7-phenanthrohne and 77% by 1,7-phenanthrohne This sensitivity may justify classification of ZIP as a metallo-protease, however similar inhibition by such non-chelating phenanthrolines has been reported in the cases of non-metallo-proteases One possible explanation put forward by Czekay and Bauer (1993) for pyroglutamyl aminopeptidase II, is that non-specific hydrophobic interactions occur between the aromatic ring structure on the phenanthrohnes and some hydrophobic region of the enzyme The presence of a hydrophobic pocket on ZIP has previously been illustrated by its ability to strongly bind to a HIC resin (FIGURE 3 4 2 1) Similar inhibition data for these phenanthrolines was acquired for bovine serum PO (classified a serine protease) which also readily interacts with phenyl sepharose resins (Cunningham and O'Connor, 1998)

It has been speculated that PO possesses a non-catalytically effective cysteine residue near its active serine site (Polgar, 1991) This explains the reported sensitivity towards classic sulphydryl inhibitors, such as PCMB (*p*-chloromercuribenzoate) by various researchers (Daly *et al*, 1985, Rosen *et al*, 1991, Cunningham and O'Connor, 1998) A

range of cysteine protease inhibitors and activators were incubated with purified ZIP to establish the presence or absence of a similar cysteine residue TABLES 3 12 and 3 13 summarise the results obtained Purified ZIP was inhibited by 10mM 5,5-dithio-bis2-nitrobenzoic acid (DTNB), and 10mM N-ethyl maleimide (NEM) by 60% and 47% respectively ZIP could be a cysteine protease or at least house a cysteine residue near its active site, since these compounds have been reported to interact with non-active site residues (Andrews *et al*, 1980) This theory, may also explain why ZIP was inactivated in the presence of thiol-reducing agents, DTT (when incorporated into the substrate buffer) and 2-mercaptoethanol A second hypothesis is that the ZIP cysteine residue, unlike that of PO, is in the reduced form (SH) and so is not activated by the presence of DTT If this were the case, what would explain the observed inhibition by DTNB and NEM? Polgar (1991) surmised a relationship between the size of the thiol reagent and the extent of enzyme inhibition The larger the reagent to interact with the cysteine residue, the greater the steric effects causing the active site to become less accessible to the substrate This may clarify why DTNB inhibited ZIP to a greater extent than NEM and iodoacetamide (decreasing size), and further proposes the presence of a cysteine residue in close proximity to the active serine site

The effect of a number of non-specific functional reagents was also explored 55% inhibition was observed on incubation of ZIP and 2 5mg/mL antipain, while 1mg/mL bacitracin, puromycin and pepstatin A, each inhibited ZIP by 41% These results verify the non-specificity of these functional reagents, which probably share a common inhibition mechanism which is not specific for any one type of peptidase No inhibition by carnitine or trypsin inhibitor was observed because these functional reagents are relatively more specific (TABLE 2 12)

No firm conclusions can be drawn based on these results It is highly probable that ZIP is a serine protease that contains a cysteine residue near its active site but for some reason is not activated in the presence of DTT Further analysis e g ammo acid sequence analysis needs to be performed on this novel peptidase to elucidate its exact catalytic classification

4.8 7 Effect of Metal Ions on ZIP Activity

The effect of metal ions on ZIP activity was examined as detailed in section 2 9 8 Dissolution of some of the metal salts in phosphate buffer proved difficult, and so for these salts, Tris/HCl buffer species was employed (TABLE 2 13) All salts had the counterion sulphate and controls in both buffer systems were included, so that effects observed were wholly due to the presence of the metal ion TABLE 3 15 illustrates that the ZIP was not affected significantly by cobalt, copper, nickle or magnesium ions Considerable inhibition of 30%, 31% and 37% was reported for Ca^{2+}, Na^{+} and Cd^{2+} respectively ZIP showed the greatest sensitivity towards zinc and mercury, with 56% and 44% activity remaining following incubation Inactivation by heavy metals is well documented (Vallee and Ulmer, 1972, Falkous *et al*, 1995) Heavy metal cations such as mercury are known to react with histidine and tryptophan residues (Volkin and Klibanov, 1990) and dithiols Therefore, it is not surprising that ZIP was substantially inactivated by $HgSO_4$ Inhibition of PO by zinc and mercury ions is well documented in the literature (Kalwant and Porter, 1991, Strohmeier *et al*, 1994) These results verify that ZIP is not a metallo-protease and may offer support for the presence of a non-catalytically active cysteine residue, since mercury has also been reported to hydrolytically degrade disulphide bridges (Volkin and Klibanov, 1990)

4 8 8 Carbohydrate Analysis

Since the majority of extracellular proteins of higher animals are glycosylated, it was decided to carry out a preliminary investigation into the detection of carbohydrate (oligosaccharide) moieties in purified ZIP The Molisch test for general carbohydrates was performed as outlined in section 2 9 9 2 Although a positive violet result was obtained, this test is non-specific for glycan moieties and so a more conclusive experiment had to be attempted ZIP tested positive for reducing sugars using the more specific Fehling's test as outlined in section 2 9 9 1 Most mono- and disaccharides are classified as reducing sugars based on the presence of potentially free aldehyde or ketone groups Detection of glucose and starch in the purified ZIP sample supplied negative results The data indicates that purified ZIP does contain carbohydrate residues, probably reducing sugar residues, and is a glycoprotein A Pierce glycoprotein estimation kit was

employed to investigate this possibility and to subsequently estimate the extent of glycosylation FIGURE 3 8 9 illustrates the standard curve constructed from which it was estimated that ZIP was indeed a glycoprotein, 33% of its total weight represented by a carbohydrate component To date it does not appear that PO is glycosylated

Glycosylation is regarded as an extension of protein biosynthesis and is one of the major metabolic activities in the lumen of the endoplasmic reticulum Oligosaccharide side chains most commonly attach to asparagine residues on the backbone of the protein in sequences Asn-X-Ser/Thr where X≠Pro (termed *N*-linked oligosaccharides) and there may be more than one attachment site (Einspahr, 1989) In contrast the *O*-glycosidic bond presents a wide variety of linkages including serine and threonine linkages The oligosaccharide moiety can have a significant effect on the physiochemical properties of the protein although the purpose of the carbohydrate is not always clear Glycoproteins display a greater increased resistance to denaturation factors, depending on the degree of glycosylation, including thermal stability and solubility It is believed that the carbohydrate moiety attached to the protein molecule affects its hydration, therefore enhancing stability (Cabral and Kennedy, 1993)

Different molecules of a glycoprotein may contain different sequences and locations of oligosaccharides (Voet *et al*, 1999) and recent studies suggest that some glycoproteins may have the same protein core, yet different biological activity, determined by their respective oligosaccharide moieties This has been shown to be the case with two IgE factors, one of which stimulates IgE production (IgE-pF) while the other (IgE-sF) inhibits the synthesis of IgE by lymphocytes (Goochee and Monica, 1990) It may be possible that PO and ZIP share a common backbone structure and differ by way of the degree of glycosylation After glycoprotein formation, modification of the core polysaccharide can occur, such as the removal of a mannose group (McGilvery, 1979) and such a phenomena can result in the formation of glycoproteins of identical origin which differ slightly in terms of the final glycosylation If this does prove to be the case with ZIP and PO, it may explain some of the dissimilarities observed in the physiochemical characterisation of the peptidases The presence of carbohydrate moieties would obviously affect the relative

molecular mass, which may explain why ZIP appears heavier than PO Glycosylation of ZIP may also explain differences in binding affinities to HIC and CPC resins and may even be responsible for the resistance of ZIP towards Z-Pro-Prolinal and DTT Since glycosylation is tissue and cell type specific within a given species, it may be possible that ZIP is a glycosylated form of PO

4.8 9 Oligopeptidase Determination

PO has been classified as an oligopeptidase (Camargo *et al*, 1979, Barrett and Rawlings, 1992) based on the substrate-size limitation of its specificity In general, short peptides or positively charged peptides have been found to be efficient PO substrates while longer peptides are less favourable Polgar (1992a) proposed a hypothesis of a tunnel-like binding site with a negatively charged active site, allowing the enzyme to exclude large, negatively charged peptides from the active site and hence classify PO as an oligopeptidase Considering this, the possibility that ZIP may too be an oligopeptidase was investigated as outlined in section 2 9 10 The cleavage of α_2-macroglobulin (α_2M), BSA and insulin B chain by purified ZIP was analysed using ion-pair reverse phase HPLC FIGURES 3 8 10 1 -3 8 10 3 represent the chromatograms showing the failure of ZIP to cleave all three peptides This data implies that ZIP is an oligopeptidase since it did not interact with α_2M (FIGURE 3 8 10 1) a characteristic of all oligopeptidases (Barrett and Rawlings, 1992) Secondly there was no hydrolysis of BSA (66kDa) or the proline-containing insulin B chain {Phe-Val-Asn-Gln-His-Leu-Cys[SO_3H]-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys[SO_3H]-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala} presumably due to the size of these peptides Oligopeptidases can only cleave small oligopeptides usually less than 20 amino acid residues

The effect of α_2M on ZIP and PO activities was fluorimetrically assessed as detailed in section 2 9 10 2 FIGURES 3 8 10 4 and 3 8 10 5 clearly show that this plasma serine protease inhibitor had no inhibitory effect on Z-Gly-Pro-MCA degrading activities up to 0 5mg/mL The failure of PO and ZIP to cleave the bait region of the macroglobulin, which is sensitive to the action of the great majority of endopeptidases of all catalytic

types (Barrett and Starkey, 1973), is a clear demonstration of the oligopeptidase specificity of PO and ZIP

4.8.10 Substrate Specificity

The substrate specificity of PO has been well documented from various sources and the peptidase has been classified as a proline specific oligopeptidase (Cunmgham and O'Connor, 1997b) Bovine brain PO which had been previously purified in our laboratory was analysed with bovine serum ZIP to allow comparison of their substrate specificities HPLC, colorimetric and kinetic analyses were carried out to gain an insight into the specificity of ZIP

The excellent resolving power of reverse phase high performance liquid chromatography (RP HPLC) has resulted in it becoming the predominant HPLC technique for peptide separation In this study ion-pair RP HPLC was used to detect cleavage products following incubation of peptides with purified enzyme (PO or ZIP) A silica based analytical column containing octadecyl ligands (C_{18}) was employed as the non-polar stationary phase The polar mobile phase of water and MeOH contained the hydrophobic ion-pairing *reagent* trifluoroacetic acid (TFA) In the pH range dictated by the stability of the C_{18} column, basic amino acids and the terminal amino group are fully ionised It has been suggested that TFA ion pairs with basic charges and this interaction tends to increase the affinity of the peptide for the column (Bennett, 1991) Thus, ion-pair RP HPLC was chosen as the optimum analytical tool for this substrate specificity investigation

A range of synthetic peptides was examined (TABLE 2 15) and the results are presented in TABLE 3 16 ZIP and PO had matching specificities and both peptidases failed to cleave any tri- or N-blocked di-peptides, such as Gly-Pro-Ala or Z-Pro-Gly It is well documented that PO fails to cleave any peptides less than four residues in length or of three residues with a blocked N-terminus (Walter and Yoshimoto, 1978) Identical cleavage products were detected following the hydrolysis of Z-Gly-Pro-Ala by ZIP and PO This result was hardly surprising since both enzymes readily cleave Z-Gly-Pro-

MCA One interesting result though, was that no hydrolysis was detected for the proline-containing tetrapeptide, Gly-Gly-Pro-Ala by either ohgopeptidase Based on the size of this peptide, these results are not in keeping with the current understanding of PO specificity, and it may be possible that the enzyme levels were not sufficient to mediate detectable hydrolysis

TABLE 4 4 outlines the amino acid sequences of the bioactive peptides investigated as described in section 2 9 11 1 Disappointedly, the peptides that were hydrolysed by ZIP were also cleaved by PO (TABLE 3 16) and so the two peptidases appear to have identical substrate specificities There were a number of peptides which were only hydrolysed by PO, namely, bradykinin, (Glu^2)-TRH, (Phe^2)-TRH and TRH-Gly, however this was most probably due to low enzyme concentration rather than differences in specificity characteristics ZIP and PO cleaved all three of the angiotensin peptides tested presumably at the **Pro-Phe** bond (TABLE 4 4) It is evident from the chromatograms in FIGURES 3 8 11 1 , 3 8 11 2 , 3 8 11 3 and 3 8 11 4 that ZIP and PO share a common cleavage site for all angiotensin I and II, based on the identification of a similar cleavage products Angiotensin III (chromatogram not shown) exhibited a similar chromatogram to angiotensin I and II and again it was clear that both peptidases readily hydrolysed the bioactive peptide at the same scissile bond The cleavage of angiotensin I, II and III has been previously reported for porcine brain PO (Moriyama *et al* , 1988) LHRH and TRH were both hydrolysed by PO and ZIP at the carboxyl side of the proline residue while PO was found to also cleave the TRH analogues, (Glu^2)-TRH, (Phe^2)-TRH and TRH-Gly Although cleavage of these bioactive peptides by PO is well documented (Tate, 1981, Mendez *et al* , 1990), the precise role this peptidase plays in the turnover of these peptides *in vivo* remains unclear

Amyloid β-protein (Aβ), a 4kDa protein subunit present in the neuritic plaques, neurofibrillary tangles and cerebrovascular deposits in Alzheimer's disease (AD) patients is the hallmark of the disease Although the specific biochemical events leading to the deposition of amyloid are not known, Aβ is believed to be involved in the pathogenesis of AD (Tanzi *et al* , 1988) Aβ is derived from the amyloid precursor protein (APP)

which is known to be metabolised by at least two major pathways that are mutually exclusive and involve distinct proteolytic activities (Almkvist *et al*, 1997) FIGURE 4 7 shows the proposed major pathways involving an enzyme activity known as α-secretase that produces a soluble derivative of APP (α-sAPP) An alternative metabolic pathway involves at least two proteolytic activities known as β- and γ-secretase that collectively generate Aβ PO has been implicated in this latter pathway (Ishiura *et al*, 1990) and is believed to act as a γ-secretase cleaving the Ala_{42}-Thr_{43} bond of $A\beta_{1\text{-}43}$

For this reason, the cleavage of $A\beta_{1\text{-}43}$ by PO and ZIP was investigated by RP HPLC analysis Fragments $A\beta_{1\text{-}28}$ and $A\beta_{25\text{-}35}$ were also examined since studies have shown these to exhibit identical antigenic, structural, neurotrophic and neurotoxic effects as the Aβ peptide (Kang *et al*, 1987, Yanker *et al*, 1990) The presence of alanine residues (marked in *italic* in TABLE 4 4) on these peptides serve as potential cleavage sites for PO, which also has the ability to hydrolyse alanyl bonds (Walter and Yoshimoto, 1978) HPLC chromatograms obtained reveal that neither PO nor ZIP appeared to hydrolyse any of the Aβ peptides This was disappointing, though not that surprising for a number of reasons Firstly, the molar concentration of the peptides tested was very low (TABLE 2 16) and the level of enzyme activity may not have been sufficient to induce a detectable cleavage product Although prolyl oligopeptidase does have an affinity for alanyl bonds, the rate of hydrolysis is orders of magnitude lower than that for prolyl bonds (Walter and Yoshimoto, 1978) Finally, PO and ZIP may be incapable of cleaving a 43 amino acid peptide ($A\beta_{1\text{-}43}$) based on their classification as oligopeptidases

Despite the identification of PO as a γ-secretase, the ability of PO to cleave the Ala_{714}-Thr_{715} of APP to produce $A\beta_{1\text{-}42}$, is impossible due to the length of the APP peptide (742 residues) However, if the β-secretase cleavage was to occur first, PO may then be able to act as a γ-secretase on the remaining 27 residue peptide (FIGURE 4 7) This hypothesis has been proposed by Yamaguchi *et al*, (1988) yet the ability of PO or ZIP to hydrolyse a 27 amino acid residue peptide remains highly debatable Despite the controversy, PO has also been implicated in AD due to its ability to cleave neuropeptides such as arginine-

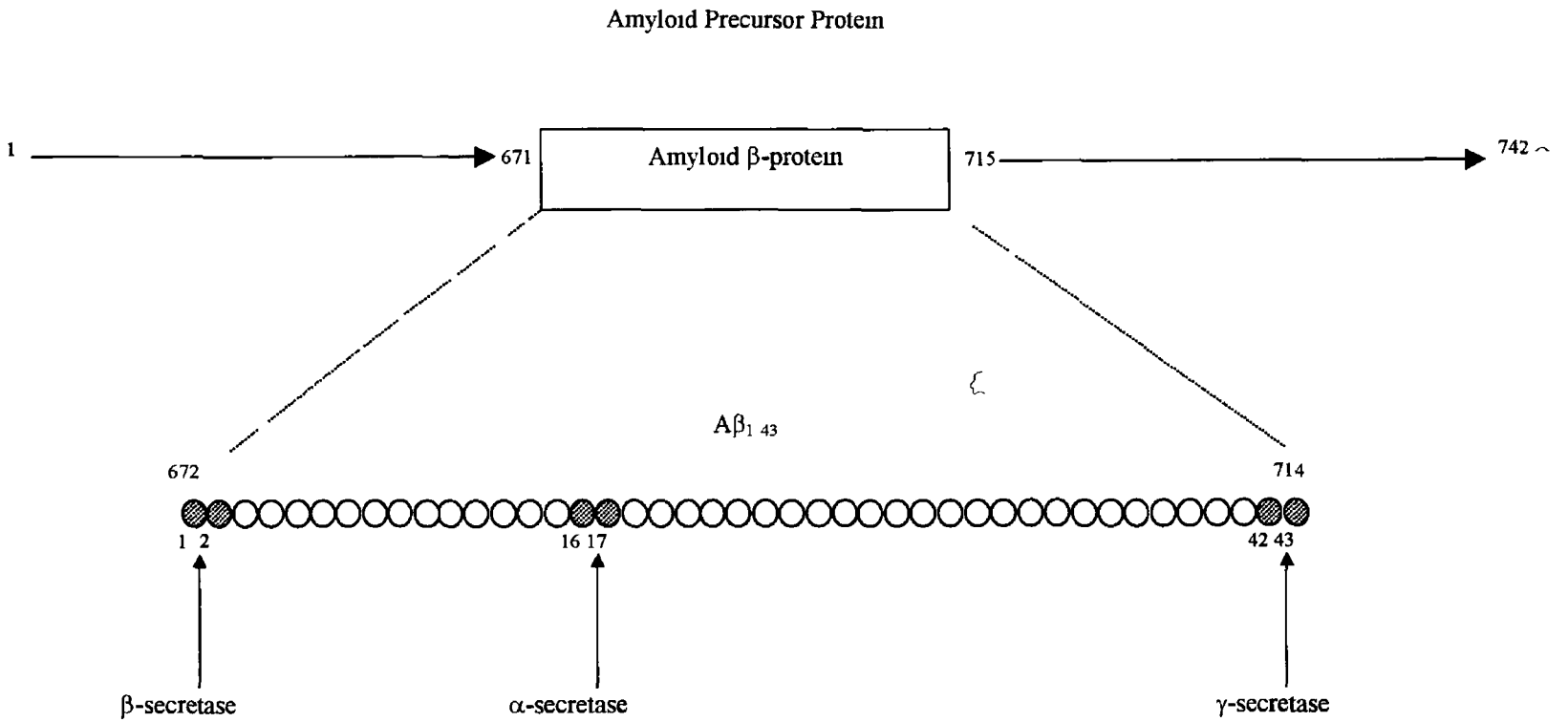
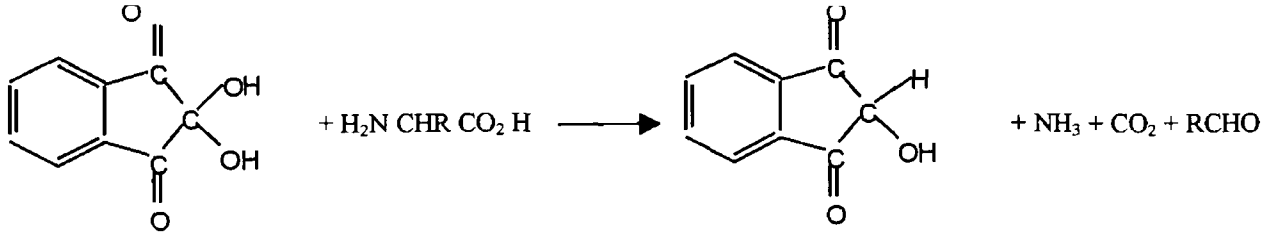
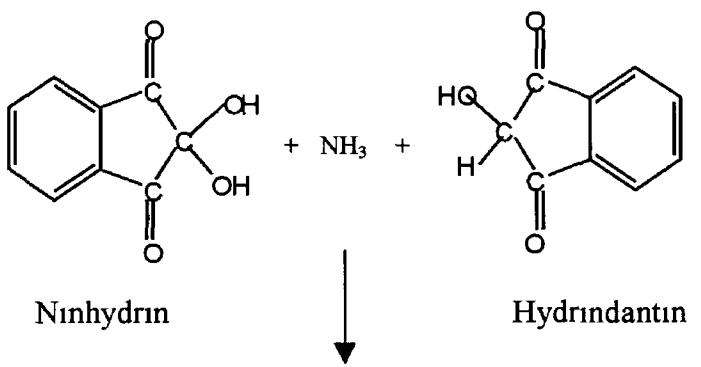


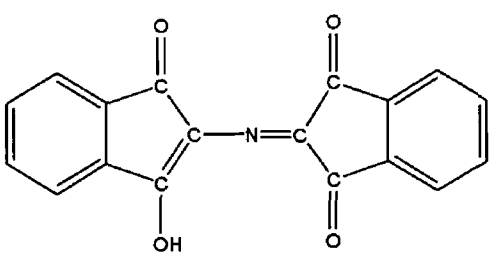
FIGURE 4.7. PROPOSED MECHANISM OF AMYLOID PRECURSOR HYDROLYSIS RESULTING IN Aβ1-43 PEPTIDE FORMATION

vasopressin, substance P, angiotensin II and TRH, which have suggested roles in memory and learning (Schlesinger *et al*, 1983, Griffiths, 1987) In view of the various potent biological effects elicited by neuropeptides, an inhibitor of the enzyme that regulates their metabolism may have the potential to be a therapeutic agent for AD (see section 4 8 11 for discussion on prohne-specific inhibitors)

As previously discussed, the substrate specificity of PO favours hydrolysis of tetra- or N-blocked tri-peptides and there have been no reports of dipeptide cleavage HPLC analysis indicated similar specificities between ZIP and PO and the cadmium ninhydrin assay was performed to investigate the substrate specificity of ZIP with respect to dipeptides The method employed was a modification of that of Doi *et al* (1981) and TABLE 3 17 highlights that ZIP did not cleave any of the dipeptides tested The colorimetric cadmium ninhydrin assay is based on the interaction of ninhydrin with an amino group as outlined in FIGURE 4 8, producing a purple complex which absorbs at 570nm in the absence of cadmium and at 505nm in its presence This coupled to the HPLC data suggests that the substrate specificity of PO and ZIP are extremely alike, both requiring at least four amino acid residues and a Pro in the P_1 position for hydrolysis







Ruheman's Purple

FIGURE 4 8 BASIS OF THE CADMIUM NINHYDRIN COLORIMETRIC ASSAY

Michaelis constants (K_M) were determined for purified ZIP and PO activities as described in section 2.9.11.3.1 using Z-Gly-Pro-MCA. Four kinetic models were applied to the data, namely the Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf models. Section 6.3 details how each model utilised the data to produce a K_M value for the two enzymes. TABLE 3.17 presents the calculated constants for ZIP and PO. It is obvious that both enzymes had a high affinity for Z-Gly-Pro-MCA, since constants in the μM region were obtained. However, PO appeared to have a lower affinity for the N-blocked substrate showing an average K_M of 99μM in comparison to 54μM for ZIP. This K_M determined for PO is higher than that originally reported (20μM) by Yoshimoto *et al* (1979). Yet, it is comparable to that of Cunningham and O'Connor (1998) for bovine serum PO and for membrane-bound brain PO (O'Leary *et al*, 1996).

PEPTIDE	AA	SEQUENCE AND PROPOSED CLEAVAGE SITE
$A\beta_{1\ 28}$	28	Asp-*Ala-Glu*-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-*Ala-Glu*-Asp-Val-Gly-Ser-Asn-Lys
$A\beta_{1\text{-}43}$	43	Asp-*Ala-Glu*-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-*Ala-Glu*-Asp-Val-Gly-Ser-Asn-Lys-Gly-*Ala-Ile*-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-*Ala-Thr*
$A\beta_{25\ 35}$	11	Gly-Ser-Asn-Lys-Gly-*Ala-Ile*-Ile-Gly-Leu-Met
Angiotensin I	11	Asp-Arg-Arg-Val-Tyr-Ile-His-**Pro-Phe**-His-Leu
Angiotensin II	8	Asp-Arg-Val-Tyr-Ile-His-**Pro-Phe**
Angiotensin III	7	Arg-Val-Tyr-Ile-His-**Pro-Phe**
Arg^8 Vasopressin	9	Cys-Tyr-Phe-Gln-Asn-Cys-**Pro-Arg**-Gly-NH_2
Bradykinin	9	Arg-Pro-**Pro-Gly**-Phe-Ser-**Pro-Phe**-Arg
(Glu^2)-TRH	3	Pyr-Glu-**Pro-NH_2**
LHRH	10	Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-**Pro-Gly**-NH_2
Neurotensin	13	Pyr-Leu-Tyr-Glu-Asn-Lys-**Pro-Arg**-Arg-**Pro-Tyr**-Ile-Leu
(Phe^2)-TRH	3	Pyr-Phe-**Pro-NH_2**
Substance P	11	Arg-Pro-Lys-**Pro-Gln**-Gln-Phe-Phe-Gly-Leu-Met
TRH	3	Pyr-His-**Pro-NH_2**
TRH-Gly	4	Pyr-His-**Pro-Gly**

TABLE 4.4. SEQUENCE OF BIOACTIVE PEPTIDES EMPLOYED FOR SUBSTRATE SPECIFICITY STUDIES

Scissile cleavage sites are marked in **bold** font, while *italic* residues indicate possible cleavage sites

The effect of a range of prolme-containing peptides on the K_M values determined for ZIP and PO using Z-Gly-Pro-MCA was examined as outlined in section 2 9 11 3 2 Angiotensin I was found to be a competitive inhibitor of ZIP with a K_i of 90μM as determined by the Michaelis-Menten plot (see TABLE 3 19 for results and section 6 4 2 for calculations) Values obtained from the Michaelis-Menten plot were taken as the most accurate representation of K_i values since the model does not involve reciprocals which can conceal a poor fit (Henderson, 1992) ZIP was uncompetitively inhibited by LHRH with a K_i of 100μM These results suggest that ZIP has a greater specificity for Z-Gly-Pro-MCA than for either of these two bioactive peptides K_i values for the other prolme-containing peptides could not be determined since the exact type of inhibition could not be identified (see section 6 3 3) PO was competitively inhibited by angiotensin I and II, bradykinin and Z-Gly-Pro-Ala with Michaelis-Menten K_i values of 75, 150, 275 and 476μM respectively These results agree with those of Moriyama *et al* (1988) who also found angiotensin I and II to be the most potent competitive inhibitors of PO among a number of bioactive peptides Previous reports in the literature though have claimed PO to be competitively inhibited by TRH, LHRH and bradykmin (Cunningham and O'Connor, 1998) although in this experiment, the exact mode of inhibition could not be determined for these peptides The poor specificity of PO towards Z-Gly-Pro-Ala (476μM) is surprising considering a K_M value of 99μM was observed for Z-Gly-Pro-MCA This may be due to a preference for a large hydrophobic group such as MCA in the P_1' subsite

In summary, these studies verify the broad proline-specific substrate specificity of prolyl oligopeptidase and suggest a similar specificity pattern for ZIP

4 8.11 Inhibitor Studies

The specificity of PO described by Polgar (1992a) in relation to its exclusion of negatively charged peptides from the active site, has also been observed with PO specific inhibitors (Kolosko *et al*, 1994) Therefore, the design of PO specific inhibitors has centred on mimicking the structure of short positively charged peptides (e g Z-Pro-Prolinal) A number of proline specific peptidase inhibitors were incubated with purified ZIP to determine its sensitivity towards them, purified PO was also assayed for comparison purposes TABLE 2 19 lists the inhibitors studied, most of which were kindly donated due to commercial unavailability Fmoc-Pro-Pro-CN, JTP-4819, S-17092-1, S-19825, Z-Phe-Pro-Methylketone and Z-Pro-Prolinal had little or no inhibitory effect on purified ZIP activity Conversely, PO was extremely sensitive to these inhibitors with IC_{50} values listed in TABLE 3 21 This specific inhibition of PO has been previously reported in the literature for these inhibitors (TABLE 4 5) The insensitivity of ZIP towards Z-Pro-Prolinal has been previously reported (Cunningham and O'Connor, 1997a), however this investigation highlights a number of alternative inhibitors exhibiting a similar profile Fmoc-Pro-Pro-CN (Li *et al*, 1996) and Z-Phe-Pro-Methylketone (Steinmetzer *et al*, 1993) are Z-Pro-Prolinal derivatives which may explain the similarity of the PO IC_{50} values obtained for these inhibitors in comparison to that for Z-Pro-Prolinal (TABLE 3 21) Fmoc-Ala-Pro-CN (Li *et al*, 1996), was the most potent PO inhibitor tested with an IC_{50} of $1x10^{-12}M$, however ZIP was 60% inhibited at $1x10^{-4}M$

JTP-4819 (FIGURE 4 9) is a potent inhibitor of PO activity exhibiting a high specificity for the target enzyme, with no effect on other proline-related enzymes (Toide *et al*, 1995) The inhibitor is currently being considered as a potential agent for the treatment of AD The pharmacological actions of JTP-4819 include its inhibitory effects on PO activity which in turn may prevent neuropeptide degradation in the brain, and its enhancement of memory impairment in rats (Shinoda *et al*, 1996, Toide *et al*, 1998) JTP-4819 proved to be a relatively potent PO inhibitor yielding an IC_{50} value of $2.5x10^{-6}M$, yet more importantly ZIP remained completely resistant to inhibition This result is in parallel to those discussed in section 4 6 6 where JTP-4819 was selected as the

optimum inhibitor for the detection of ZIP alone in crude samples Using JTP-4819, Shinoda *et al* (1997) have implicated PO in the production of the APP fragment $A\beta_{1-42}$ believed to be the hallmark of AD as previously discussed (section 4 8 10) The relevance of this is that if PO and ZIP possess a common substrate specificity as results of the HPLC analyses suggested, it is highly probable that if PO is responsible for Aβ production then so too is ZIP However, it should be considered that ZIP is not inhibited by JTP-4819, and so the total Z-Gly-Pro-MCA degrading activity is not being inactivated by this novel inhibitor Therefore, use of JTP-4819 to minimise neuropeptide cleavage and eliminate Aβ production would prove futile This is of particular relevance in light of the findings outlined in section 4 7 in which ZIP activity was detected in the brain

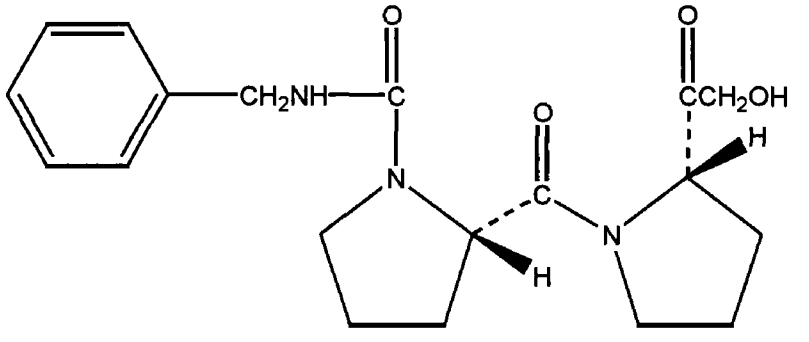


FIGURE 4.9 STRUCTURE OF JTP-4819

S-17902-1 and S-19825 were donated by Dr F Checler The perhydroindol carboxylic derivatives proved to be excellent PO inhibitors yielding IC_{50} values of $1x10^{-8}M$ and $1x10^{-9}M$ respectively (TABLE 3 21) These inhibitors also proved to be PO-specific and did not affect the activity of ZIP (FIGURES 3 8 12 8 and 3 8 12 9) A similar specificity of S-17092-1 has been reported by Barelli *et al* (1999), who noted a resistance by aminopeptidases B and M, DPP IV and angiotensin-converting enzyme, among others, towards the inhibitor Like JTP-4819, S-17092-1 is also being investigated as a potential tool in the determination of the contribution of PO to AD (Barelli *et al*, 1999) and so the observed insensitivity of ZIP to it, should not be overlooked

Commercially available Z-Pro-Prolinal dimethylacetate and Boc-Glu-($NHOB_2$)-Pyr, yielded the poorest IC_{50} values ($1x10^{-5}M$ each) for PO, and also inhibited ZIP activity (FIGURES 3 8 12 1 and 3 8 12 13) Goossens *et al* (1997) reported an IC_{50} of 0 13μM ($1\ 3x10^{-7}M$) for Z-Pro-Prolmal dimethylacetate, however a value of $1x10^{-5}M$ was obtained in this study Boc-Glu-($NHOB_2$)-Pyr was synthesised by Demuth *et al* (1993) as the most potent proline specific peptidase inhibitor from a range of aminodicarboxylic acid pyrrolidides (TABLE 4 5) An interesting profile was that of Z-Phe-Ala-Chloromethlyketone which appeared to exert a greater inhibitory effect on ZIP than on PO (FIGURE 3 8 12 10) Postatin, an inhibitor of bacterial origin was adequate in its inhibition of PO (IC_{50} $5x10^{-7}M$) in comparison to $3\ 5x10^{-8}M$ observed for PO from the same source by Cunningham and O'Connor (1998) Ile-Thiazohdide and Ile-Pyrrolidide were designed as DPP IV specific inhibitors (Pederson *et al*, 1998, Pauly *et al*, 1999) with reported K_i values of 130nM and 250nM respectively However in this study, it is clear that Ile-Thiazolidide was also a potent inhibitor of PO activity yielding an improved IC_{50} value ($1x10^{-8}M$) in comparison to DPP IV ($1\ 3x10^{-7}M$) This observed inhibitory action of Ile-Thiazolidide for both PO and DPP IV is likely to be due the specificity of both peptidases for a proline residue at the scissile bond (Cunningham and O'Connor, 1997b)

INHIBITOR	K_I	IC_{50}
Boc-Glu-($NHOB_2$)-Pyr	0 03μM	ND
Fmoc-Ala-Pro-CN	5nM	ND
Fmoc-Pro-Pro-CN	5nm	ND
JTP-4819	ND	0 83nM
Postatin	ND	0 03μg/mL
S-17092-1	1nm	ND
Z-Phe-Ala-Chloromethlyketone	ND	ND
Z-Phe-Pro-Methylketone	1 8nM	ND
Z-Pro-Prolinal	ND	0 74nM
Z-Pro-Prolinal dimethylacetate	ND	0 13μM

TABLE 4.5. SPECIFIC INHIBITORS OF PO

ND means not determined

REFERENCE

Demuth *et al* , 1993

Li *et al* , 1996

Li *et al* , 1996

Toide *et al* , 1995

Aoyagi *et al* , 1991

Barelli *et al* , 1999

Stone *et al* , 1991

Steinmetzer *et al* , 1993

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6.0. APPENDICES

6 0 APPENDICES

6 1 ERROR BARS

Error bars on all graphs represent the standard error of the mean of triplicate fluorescence or absorbance values The standard error (SE) of mean is defined as

$$SE = \sigma/\sqrt{n}$$

Where

σ is the standard deviation i e $\sqrt{\text{variance}}$ (variance is $(a^2 + b^2 + c^2)/3$, where a, b and c are the triplicate values)

n is the number of repeat values measured (three in these studies)

6 2 ENZYME QUANTITATION

One unit of enzyme activity is defined as the amount of enzyme which releases 1picomole MCA per minute at 37°C

Derivation

Let MCA released $= X\mu M$

$= X\mu moles/Litre$

(assay uses 400µL MCA, 100µL enzyme)

$= \frac{X \times 10^4 \times 0.4}{60}$ µmoles/min/100µL

$= \frac{X \times 10^4 \times 0.4 \times 10^{+6}}{60}$ picomoles/min/100µL

$= \underline{X \times 40}$ picomoles/minute/100µL
60

$= \underline{X \times 400}$ picomoles/minute/1000µL
60

$= \underline{X \times 400}$ picomoles/minute/mL
60

$= \underline{X}$ picomoles/min/mL
0 15

But.

1unit of activity is defined as amount of enzyme which releases 1picomole MCA/min,

Therefore

$= \underline{X}$ units/ml
0 15

And

MCA released (X) is also defined as fluorescent intensity observed/slope of the appropriate MCA standard curve, so

Unit/mL = Fluorescent Intensity
Slope of filtered standard curve x 0 15

Therefore

Unit of activity = Flourescent Intensity
Slope of filtered standard curve x 0.15

6 3. PURIFICATION TABLE CALCULATIONS

Total activity (unit). Units of enzyme activity, calculated from fluorescent intensity as described in section 6 2

Total protein (mg) mg/mL protein estimated from appropriate BSA standard curve (using Biuret or BCA assays) x volume of sample in mL

Specific Activity (unit/mg) Total activity/total protein

Purification factor Specific activity of sample/specific activity of starting sample (serum)

Yield (%) (Total activity of sample/total activity of starting sample (serum)) x 100

6 4. KINETIC ANALYSIS

6 4 1 K_M Determinations

The Michaelis constant (K_M) is defined as the dissociation equilibrium constant for the enzyme-substrate complex It may be measured by the analysis of initial velocities at a series of substrate concentrations and one concentration of enzyme The data points can then be fitted to a number of models as outlined below

Michaelis-Menten – Plot of fluorescent intensity versus substrate concentration
K_M is the substrate concentration at half the maximum fluorescent intensity

Lineweaver-Burk - Plot of 1/fluorescent intensity versus 1/substrate concentration
The intercept of the line at the x-axis is given as $-1/K_M$

Eadie-Hofstee – Plot of fluorescent intensity versus fluorescent intensity/substrate concentration
The slope is given as $-K_M$

Hanes-Woolf - Plot of substrate concentration/fluorescent intensity versus substrate concentration
The intercept at the x-axis is given as $-K_M$

6 4 2 K_i Determinations

K_i, the inhibition constant, is defined as the constant of dissociation of inhibitor from a reversibly bound enzyme-inhibitor complex

$$K_i = \frac{K_M \times I}{K_{app} - K_M}$$

Where

K_i is the inhibition constant

K_M is the Michaelis constant with no inhibitor present

I is the inhibitor concentration

K_{app} is the apparent Michaelis constant and is taken to be the 'new K_M' observed when a reversible inhibitor is incorporated into the assay K_{app} is measured as for K_M (described in section 6 4 1)

6 4 3 Types of Reversible Inhibition

Reversible inhibition involves noncovalent forces that bind inhibitors to enzymes The following are the common forms of reversible inhibition

E- enzyme

I-inhibitor

S-substrate

P-product

Competitive inhibition involves binding of the enzyme to the inhibitor forming an enzyme-inhibitor complex The inhibitor competes with the substrate for the enzyme active site

$$E + S \leftrightarrow ES \leftrightarrow E + P$$

$$+$$

$$I$$

$$\updownarrow K_i$$

$$EI$$

With competitive inhibition, the slope of the Lineweaver-Burk plot increases as does the K_M Competitive inhibition is recognisable when plots intersect at a common point of intersection on the positive y-axis

Uncompetitive inhibition occurs when the inhibitor binds to the enzyme-substrate complex, binding to a site other than the substrate binding site

$$\begin{array}{ccccccc} E & + & S & \leftrightarrow & ES & \rightarrow & E + P \\ & & & & + & & \\ & & & & I & & \\ & & & & \updownarrow K_i & & \\ & & & & ESI & & \end{array}$$

This type of inhibition is identified by the presence of parallel lines in the Lineweaver-Burk plot K_M decreases but slope values remain the same

Non-competitive inhibition involves the random binding of inhibitor to either enzyme or enzyme-substrate complex

$$\begin{array}{ccccccc} E & + & S & \leftrightarrow & ES & \leftrightarrow & E + P \\ + & & & & + & & \\ I & & & & I & & \\ \updownarrow K_i & & & & \updownarrow K_i & & \\ EI & + & S & \leftrightarrow & ESI & & \end{array}$$

Non-competitive inhibition may be identified by lines intersecting on the negative portion of the y-axis K_M remains the same but V_{max} decreases

Mixed inhibition occurs when inhibitor binds unequally to enzyme or enzyme-substrate complex V_{max} decreases and K_M may increase or decrease Non-competitive-uncompetitive mixed inhibition is evident when the type of inhibition lies between the two types and is identified by lines intersecting below the negative portion of the y-axis Competitive-non-competitive inhibition is recognisable by lines intersecting above the negative portion of the y-axis

6.5 IC_{50} Determinations

The IC_{50} is defined as the concentration at which an enzyme is half inhibited It can be calculated graphically from a plot of enzyme activity versus inhibitor concentration, by locating 50% activity on the y-axis and calculating the corresponding concentration on the x-axis