The Purification and Characterisation of a Prolyl Oligopeptidase from the Cytosolic Fraction of Bovine Whole Brain

Thesis Submitted for the Degree of Doctor of Philosophy by Oonagh Dowling B.Sc.

Supervised by Dr. Brendan O'Connor School of Biological Sciences Dublin City University

August 1998
Declaration

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

Signed: [Signature]  Date: 11/01/98
For my parents, Theresa and Lawrence, with love and thanks.

Hope

Hope is the thing with feathers
That perches in the soul,
And sings the tune without the words,
And never stops at all,

And sweetest in the gale is heard,
And sore must be the storm
That could abash the little bird
That kept so many warm.

I’ve heard it in the chillest land,
And on the strangest sea,
Yet, never in extremity,
It asked a crumb of me.

Emily Dickinson
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Last and by no means least, house-mates, confidantes, and dearest friends, Maria, Caroline and Deirdre I couldn’t imagine what the past four years would have been like without you and I’ll miss you
Abbreviations

The following abbreviations are used throughout this text.

ChAT  Choline acetyltransferase
ACTH  Adrenocorticotropic hormone
AD    Alzheimer's disease
ADNF-14 Activity-dependent neurotrophic factor-14
AEBSF 4-(2-Aminoethyl)-benzenesulfonylfluoride
APP   Amyloid A4 precursor protein
AVP   Arginine vasopressin
BCA   Bicinchoninic acid
Bisacryl Bisacrylamide
βNA   β-Naphthylamide
Boc   Butoxycarbonyl
BPP   Bradykinin-potentiating peptide
BSA   Bovine serum albumin
Bz    Benzyloxyl
CDTA  1,2-Cyclohexanediamine tetraacetic acid
CLIP  Corticotropin-like intermediate peptide
CN    2-Nitrile
Da    Daltons
DEAE  Diethylaminoethyl
DFP   Dusofluorophosphate
DMF   Dimethylformamide
DMSO  Dimethylsulphoxide
DPP II Dipetidyl amniopeptidase II
DPP IV Dipetidyl amniopeptidase IV
DTNB  5',5'-Ditho-(-2-nitrobenzoic acid)
DTT   Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
EGTA  [Ethylenbis(oxyethylenenitrilo)]tetraacetic acid
EH    Eadie-Hofstee
Fmoc  9-Fluorenylmethoxycarbonyl
HPLC  High performance liquid chromatography
HW    Hanes-Woolf
Ki    Inhibitor dissociation constant
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<tr>
<td>Km</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LB</td>
<td>Lineweaver-Burk</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption time of flight</td>
</tr>
<tr>
<td>MCA</td>
<td>7-Amino-4-methyl-coumarin</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-[(N-Morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>MM</td>
<td>Michaelis-Menten</td>
</tr>
<tr>
<td>M/Z</td>
<td>Mass/charge ratio</td>
</tr>
<tr>
<td>N.D.</td>
<td>Not determined</td>
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<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>ONp</td>
<td>p-Nitrophenyl ester</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBE</td>
<td>Polybuffer exchanger</td>
</tr>
<tr>
<td>PCMB</td>
<td>p-Chloromercuribenzoate</td>
</tr>
<tr>
<td>PDA</td>
<td>Photo diode array</td>
</tr>
<tr>
<td>PE</td>
<td>Prolyl endopeptidase/Proline endopeptidase</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pGlu</td>
<td>Pyroglutamic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>pNA</td>
<td>p-Nitroaniline</td>
</tr>
<tr>
<td>PO</td>
<td>Prolyl oligopeptidase</td>
</tr>
<tr>
<td>PPCE</td>
<td>Post-proline cleaving endopeptidase</td>
</tr>
<tr>
<td>PS</td>
<td>Paradoxical sleep</td>
</tr>
<tr>
<td>Pyrr</td>
<td>Pyrrolidine</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
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<tr>
<td>Rf</td>
<td>Relative mobility</td>
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<tr>
<td>S.D.</td>
<td>Standard deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>Standard error mean</td>
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<tr>
<td>SM</td>
<td>Sulphamethxazole</td>
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<tr>
<td>Suc</td>
<td>Succinyl</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-Tetramethyl ethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin releasing hormone</td>
</tr>
<tr>
<td>TRH-OH</td>
<td>Acid TRH</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)amino methane</td>
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Ve  Elution volume
Vo  Void volume
v/v  Volume per volume
w/v  Weight per volume
Xaa-  Any amino acid
Yaa-  Any amino acid
Z-  N-Benzylxycarbonyl-
ZIP  Z-Pro-proline insensitive Z-Gly-Pro-MCA hydrolysing peptidase
ZTTA  Z-Thiopro-thioprolinal
ZPP  Z-Pro-proline

Amino Acid Abbreviations

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

Cytosolic bovine brain prolyl oligopeptidase was purified from whole brain using ammonium sulfate precipitation and chromatography with DEAE sepharose, S200 gel filtration, chromatofocusing and phenyl sepharose. An overall recovery of 23% and purification factor of 253 was achieved.

The relative molecular mass of the brain PO, determined by gel filtration chromatography, was found to be 69.5 kDa. This was confirmed by SDS-PAGE. The purified enzyme was relatively unstable under assay conditions. However, the presence of 0.5% w/v BSA improved its stability and the assay linearity. Activity was also inhibited strongly by solvents with DMF being the most inhibitory. DMSO was found to be the optimal solvent in terms of enzyme activity and substrate solubility. Optimal enzyme activity was observed at 37°C with complete inactivation occurring at temperatures of 50°C or more. A pH optimum of 7.4 and a preference for phosphate buffer was found for the enzyme with complete inactivation of activity below pH 4.5 and above pH 10.

The brain PO was confirmed to be a serine protease based on its sensitivity to AEBSF. The enzyme was also inhibited strongly by some cysteine protease inhibitors such as NEM and DTNB and was activated by DTT. Sensitivity to these agents would suggest the presence of a cysteine residue in close proximity to the active site. Some divalent metal salts also exerted some inhibitory effects on activity with Hg²⁺, Cu²⁺, Cd²⁺, Co²⁺ and Ni²⁺ being the most potent.

Substrate specificity studies performed on the purified bovine brain, partially purified bovine serum and recombinant Flavobacterium meningosepticum PO activity revealed that this oligopeptidase could hydrolyse a range of proline containing peptides including TRH, LHRH, ADNF-14, substance P, neurotensin, CLIP, a 45 amino acid residue Gly-Pro-Ala polymer and the APP fragment 708-715. N-blocked proline containing dipeptides, including Z-Pro-Pro-OH were not hydrolysed by any of the enzyme. The smallest synthetic sequences hydrolysed were an N-blocked tripeptide and a tetrapeptide.

The brain, serum and bacterial activities hydrolysed Z-Gly-Pro-MCA, with Km values of 62.5, 14.6 and 38.5µM respectively. The TRH analog pGlu-His-Pro-MCA was also hydrolysed by all three activities with higher Km values of 99.8, 52.1 and 73.5µM for the brain serum and recombinant Flavobacterium meningosepticum enzyme respectively.

A number of proline-containing peptides were also found to competitively inhibit all three activities. Of these angiotensins I, II and III were the most potent. The TRH analogs, Glu²TRH and Phe³TRH exhibiting the lowest inhibitory potency.
Inhibition studies performed using a range of PO-specific inhibitors revealed α-ketobenzothiazole to be the most potent inhibitor of bovine brain PO with an IC50 value of 63pM. The classical PO inhibitor Z-Pro-proinal inhibited all three activities with IC50 values of 7-10nM. With regard to the majority of inhibitors tested, the brain, serum and bacterial enzymes were similar in their sensitivity. However, the brain and serum activities were approximately 4000 times less sensitive than the bacterial enzyme to inhibition by the N-blocked dipeptide analog, Z-Phe-Pro-methylketone. An investigation into the time course inhibition of brain and bacterial activities by Z-Phe-Ala-chloromethylketone found that while the bacterial enzyme was completely inhibited by $1 \times 10^{-5}$ M of this inhibitor after 60 minutes, the brain enzyme was completely insensitive to inhibition.
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Summary

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1. INTRODUCTION
1.1. **Proline**

Amongst the twenty amino acids coded for in protein synthesis proline occupies a unique position. A highly conserved residue, and the only mammalian imino acid, proline's unique cyclic structure influences not only the conformation of peptide chains but restricts their susceptibility to proteases (Yaron and Naider, 1993, MacArthur and Thornton, 1991)

1.1.1. **Structural Characteristics**

Amino acids are constructed around a single carbon atom (the $\alpha$ carbon) forming the centre of the tetrahedral array, the $\alpha$ carbon is bonded to an amino group (NH$_3$), a carboxyl group (COOH), a hydrogen atom (H) and a distinctive side chain or R-group (see figure 1 1a). Proline's uniqueness arises because the end of its side chain (CH$_2$CH$_2$CH$_2$) is covalently bonded to the $\alpha$-nitrogen resulting in a rigid cyclic structure (see figure 1 1b). Therefore, the presence of a proline residue in a peptide chain can confer important conformational and biologically significant characteristics on that peptide.

Proline's bulky ring structure limits the angle of rotation about the $\alpha$ carbon and nitrogen within a peptide bond, which is normally only reliant upon steric hindrance or electrostatic repulsion between R-groups of adjacent residues. As a result of this, proline normally introduces a fixed bend in a peptide chain changing its direction, a causative factor in the spherical or globular shape of proteins (MacArthur and Thornton, 1991).

As the amide proton is replaced by the CH$_2$ group, proline is unable to act as a hydrogen bond donor. This along with the bulkiness of the side chain, places restrictions on the residue preceding proline, disfavouring the $\alpha$ helix conformation. Inside an $\alpha$ helix, the possibility of making hydrogen bonds with the preceding turn is lost thus introducing a kink (helix breaker effect). It is not surprising therefore that proline is often found one or two residues after the end of an $\alpha$ helix (or $\beta$ sheet). There is also a tendency to find proline at the beginning of a helix. This can be explained both by the benefit of not needing a hydrogen bond partner and by the fact that proline's angle of rotation $\phi$ is permanently constrained to the angle typically found in a helix (Barlow and Thornton, 1988), (see figure 1 1 2).

Virtually all peptide bonds found in proteins are in the trans-configuration. However, proline residues have a relatively high probability of having the cis rather than the trans isomer of the preceding peptide bond, when compared to other amino acids (Brandts et al., 1975). The greater angle length of the X-pro peptide bond which results in redistribution of charge and lack of resonance stabilisation is caused by loss of the imide hydrogen (see figure 1 1 2 and 1 1 3). It has been suggested that proline may be actively involved in the regulation of transmembrane protein systems such as the sodium pump, by having cis/trans isomerization synchronous with ion translocation (Williams and Deber, 1991).
Figure 1.1.1a. General Amino Acid Structure. Carboxyl, amino and R group are attached to a chiral carbon (C').

Figure 1.1.1b. Structure of the Imino Acid, Proline. The bonded amino and R group create a cyclic structure.

Figure 1.1.2. Peptide bond structure
The α carbon of each amino acid are designated C' and C''. Angle of rotation ψ, ω and φ are indicated. In general the angle of rotation ω is normally fixed at 180° resulting in a fixed planar bond. However in proline the angle of rotation ω may be 180° (trans) or 0° (cis) and is not fixed (may rotate by -20° or +10°). The angle of rotation φ in a peptide bond involving proline is constrained, and it is this constraint that is responsible for introducing a fixed bend into peptide chains.
1.1.2. **Physiological Implications of Proline in Peptides**

A key physiological role played by proline is the protection of biologically active peptides from enzymatic degradation. As proline constitutes 5% of amino acid residue of total brain protein (Lajtha and Toth, 1974) and is indeed present in many neuropeptides/neurohormones and vasoactive peptides (VanHoof et al., 1995, Meintlein, 1988), (see table 1 1 1), proline-specific or selective peptidases should play an important role in the nervous system. The presence of proline may not only determine the properties of secondary structures necessary for a peptide's biological activity but may also hinder any nonspecific proteolytic degradation (Yaron and Naider, 1993)
<table>
<thead>
<tr>
<th>Protein/Peptide</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
</tr>
<tr>
<td>Substance P</td>
<td>Arg-Pro-Lys-Pro-Gln-Gly-Phe-Gly-Leu-Met</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Tyr-Pro-Ser-Lys-Pro-Asp-Asp-Pro-Gly-Ala-Pro-Ala</td>
</tr>
<tr>
<td>Pancreatic polypeptide (1-14)</td>
<td>Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro</td>
</tr>
<tr>
<td>Luteinizing hormone-releasing hormone</td>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂</td>
</tr>
<tr>
<td>Thyrotropin-releasing hormone</td>
<td>pGlu-His-Pro-NH₂</td>
</tr>
<tr>
<td>Gastrin releasing peptide (1-10)</td>
<td>Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-</td>
</tr>
<tr>
<td>Corticotropin-releasing hormone (1-10)</td>
<td>Ser-Glu-Glu-Pro-Pro-Ile-Ser-leu-Asp-Leu-</td>
</tr>
<tr>
<td>Calcitonin (20-32)</td>
<td>His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH₂</td>
</tr>
<tr>
<td>Bradykinnm-potentiating peptide</td>
<td>pGlu-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro</td>
</tr>
<tr>
<td>Tuftsin</td>
<td>Thr-Lys-Pro-Arg</td>
</tr>
<tr>
<td>Melanotropin</td>
<td>Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>pGlu-Leu-Tyr-Glu-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu</td>
</tr>
</tbody>
</table>

Table 1.1.1. Proline Containing Neuro- and Vasoactive Peptides in Human

1.1.3. Proline-Specific Peptidases

Given the unique structural characteristics of proline, it is not unexpected that the presence of proline in peptide bonds generally makes them resistant to hydrolysis by peptidases, even those of broad specificity (Yaron and Naider, 1993). However, there is now known to exist, a group of proline-specific peptidases, which have evolved to recognize the pyrrolidine ring of proline. The specificity of these peptidases is even further limited by both the size of the peptide and the position of the proline residue. For instance, Dipeptidyl aminopeptidase II can only act on peptides of three or four amino acid residues (Fukasawa et al., 1983). Aminopeptidase P requires proline to be situated at the N-terminal penultimate position, but carboxypeptidase P has a requirement for a C-terminal penultimate proline residue (Hedeager-Sorensen and Kenny, 1985; Yaron and Berger, 1970).
To date eight exopeptidases and one endopeptidase have been identified in mammals as being proline specific in their action (Cunningham and O'Connor, 1997a), (see table 1.1.2). It is the endopeptidase, prolyl oligopeptidase that will be the focus of this review.

<table>
<thead>
<tr>
<th>Proline-Specific Peptidase</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl Oligopeptidase</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Dipeptidyl Peptidase IV</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Dipeptidyl Peptidase II</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Aminopeptidase P</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Prolidase</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Proline Iminopeptidase</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Prolinase</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Prolyl Carboxypeptidase</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Carboxypeptidase P</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1.1.2: Mammalian Proline-Specific Peptidases

Proline= ●, Amino acid= ○, ↓=Site of hydrolysis


1.2. The Discovery of Prolyl Oligopeptidase

Enzymatic hydrolysis of proline-containing peptides at either the carboxyl or amino terminus of a peptide has been known since the 1950s (Davis et al., 1957 and 1953). However the enzymatic degradation of peptidyl-prolyl-peptide bonds was not observed until much later. In 1971, in a study of
the products of uterine oxytocin degradation, an enzyme capable of cleaving the prolyl-leucyl bond of oxytocin resulting in the release of the dipeptide leucylglycinamide was identified and purified (Walter et al., 1971) Further studies on this oxytocin degrading enzyme found that this peptidase, rather than having a specificity for oxytocin was in fact specific for the amino acid residue, proline, and could mediate cleavage at the carboxyl side of this residue (Walter, 1976, Koida and Walter, 1976). This enzyme has since been termed post-proline cleaving endopeptidase (PPCE), prolyl or proline endopeptidase (PE), (Orlowski et al., 1979, Walter, 1976). Since its initial discovery, several peptidases isolated and named on the basis of their specificity for a particular bioactive peptide, were found to be identical to this proline-specific endopeptidase For instance, TRH deamidase isolated from rat brain (Rupnow et al., 1979) and bovine anterior pituitary (Knisatschek and Bauer, 1979), kininase B from rabbit brain, which was found to cleave bradykinin (Oliveira et al., 1976), and endo-oligopeptidase B, which hydrolysed bradykinin, angiotensin I and II, neurotensin and LHRH (Camargo et al., 1984, 1983, Greene et al., 1982), were all found to be identical to PPCE It is now accepted that the more correct term for PE/PPCE is prolyl oligopeptidase, a term recommended due to the substrate-size limitation of its specificity (Camargo et al., 1984, Barrett and Rawlings, 1992) The existence of a new family of serine-type peptidases related to prolyl oligopeptidase, known as the S9 or prolyl oligopeptidase family, is now recognized (Barrett and Rawlings, 1992)

1.3. Biochemical and Biophysical Characteristics of Prolyl Oligopeptidase

1.3.1. Molecular Weight

Preliminary studies on the molecular weight of PO reported that this peptidase had a dimeric structure with a molecular weight of 115-140kDa (Mizutani et al., 1984, Koida and Walter, 1976) However it is now known that mammalian, plant and microbial forms are monomeric with a molecular weight ranging from 62-77kDa (Kanatani et al., 1993, Yoshimoto et al., 1981), (see table 131) With the cDNA cloning of porcine brain PO, the reported amino acid sequence (710 amino acids) allowed the deduction of a molecular weight for the enzyme of 80.75kDa This was in conflict with the lower value obtain experimentally, in the same study, of 74.5kDa (Rennex et al., 1991) Another cDNA cloning study on the human T cell form revealed again a sequence of 710 amino acids with a deduced molecular weight of 80.75kDa (Shirasawa et al., 1994). Investigations into the amino acid sequence of the Flavobacterium meningosepticum enzyme revealed a complete amino acid sequence of 705 amino acids However this sequence was found to contain a signal peptide of 20 amino acids preceding the mature enzyme. It was confirmed that loss of this signal peptide leads to the mature form of PO with an estimated molecular mass of 76.782kDa, which correlated well with previously reported experimental values (Chevallier et al., 1992)
1.3.2. Isoelectric point, pH and Temperature Optima

In general mammalian and plant forms of PO have reported to be acidic with isoelectric points of between 4.5 and 4.9 (Goossens et al., 1995, Kalwant and Porter, 1991, Yoshimoto et al., 1983c) The *Flavobacterial meningosepticum* form of this peptidase however has a much higher PI of around 9.6 (Yoshimoto et al., 1980) This is indicative of a difference in the ionizable amino acid content which results in a predominantly basic protein. Another bacterial form of PO from *Aeromonas hydrophila* was found to have a PI of 5.5, which is again quite high, relative to mammalian forms (Kanatani et al., 1993), (see table 131)

Generally mammalian, bacterial and plant forms of PO have a broadly neutral pH optima with reported values ranging from 6.8 to 8.3 (see table 131) Optimal temperatures reported for activity are generally between 37 and 40°C (Kanatani et al., 1993, Yoshimoto et al., 1983c) There has been a report of a PO from *Pyrococcus furiosus* with a temperature optimum of between 85-90°C (Harwood et al., 1997)
## Table 1.3.1. Physical Characteristics of Prolyl Oligopeptidase from a Variety of Mammalian, Plant and Microbial Sources

<table>
<thead>
<tr>
<th>PO Source</th>
<th>Molecular Weight</th>
<th>Isoelectric Point</th>
<th>pH Optima</th>
<th>Temperature Optima(°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamb Kidney</td>
<td>115kDa*</td>
<td>4 8</td>
<td>7-5-8 0</td>
<td>47</td>
<td>Koida and Walter, 1976</td>
</tr>
<tr>
<td>Lamb Brain</td>
<td>74-77kDa</td>
<td>4 9</td>
<td>7 0</td>
<td>45</td>
<td>Yoshimoto et al., 1981</td>
</tr>
<tr>
<td>Bovine Brain</td>
<td>62-65kDa</td>
<td>4 8</td>
<td>7 0-7.5</td>
<td>40</td>
<td>Yoshimoto et al., 1983c</td>
</tr>
<tr>
<td>Bovine Anterior</td>
<td>76kDa</td>
<td>-</td>
<td>7 4-7 6</td>
<td>-</td>
<td>Knisatschek and Bauer, 1979</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Brain</td>
<td>66kDa</td>
<td>-</td>
<td>8 3</td>
<td>-</td>
<td>Orlowski et al., 1979</td>
</tr>
<tr>
<td>Human Brain</td>
<td>76 9kDa</td>
<td>4 75</td>
<td>6 8</td>
<td>-</td>
<td>Kalwant and Porter, 1991</td>
</tr>
<tr>
<td>Human Placenta</td>
<td>140kDa*</td>
<td>4 75</td>
<td>-</td>
<td>-</td>
<td>Mizutani et al, 1984</td>
</tr>
<tr>
<td>Human Lymphocytes</td>
<td>76kDa</td>
<td>4 8</td>
<td>-</td>
<td>-</td>
<td>Goossens et al, 1995</td>
</tr>
<tr>
<td>Human T Cells</td>
<td>80 75kDa**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Shirasawa et al, 1994</td>
</tr>
<tr>
<td>Porcine Brain</td>
<td>80 75kDa**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Rennex et al, 1991</td>
</tr>
<tr>
<td></td>
<td>74 5kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyophyllum cenerascens</td>
<td>76kDa</td>
<td>5 2</td>
<td>6 8</td>
<td>37</td>
<td>Yoshimoto et al, 1988</td>
</tr>
<tr>
<td>Daucus carota</td>
<td>75kDa</td>
<td>4 8</td>
<td>7 3</td>
<td>37</td>
<td>Yoshimoto et al, 1987a</td>
</tr>
<tr>
<td>F. meningosepticum</td>
<td>74kDa</td>
<td>9 6</td>
<td>7 0</td>
<td>40</td>
<td>Yoshimoto et al, 1980</td>
</tr>
<tr>
<td></td>
<td>78.78kDa**</td>
<td></td>
<td></td>
<td></td>
<td>Chevallier et al, 1992</td>
</tr>
<tr>
<td>Aeromonas hydrophilia</td>
<td>76 4kDa**</td>
<td>5 5</td>
<td>8 0</td>
<td>30</td>
<td>Kanatani et al, 1993</td>
</tr>
<tr>
<td>Pyrococcus furiosus</td>
<td>70kDa**</td>
<td></td>
<td></td>
<td>85-90</td>
<td>Harwood et al, 1997</td>
</tr>
</tbody>
</table>

- * molecular weight accounts for a dimeric structure
- ** molecular weight deduced from an amino acid sequence and includes the N-terminal signal peptide (see section 1 3 1)
1.3.3. Catalytic Classification of Prolyl Oligopeptidase

1.3.3.1. Sensitivity to Protease Inhibitors

One of PO’s distinguishing characteristics is its sensitivity to both serine and cysteine protease inhibitor reagents. PO has been classified as a serine peptidase based on its sensitivity to DFP, Z-Gly-Pro-CH₂Cl, Ac-Ala-Ala-Pro-CHN₂ and to a lesser extent PMSF (Stone et al., 1992; Kalwant and Porter 1991; Yoshimoto et al., 1987a; Orlowski et al., 1979, Yoshimoto et al., 1977). DFP is a reagent with a very high specificity for activated catalytically relevant serine residues and the stoichiometric inhibition of PO by this residue was definitive evidence for the presence of a catalytically competent active site serine. Mammalian and plant forms of the peptidase have also been found to be sensitive to cysteine protease inhibitors but at relatively high inhibitor enzyme ratios which is in contrast to the stoichiometric inhibition observed with DFP (Yoshimoto et al., 1977). It is plausible that bulky reagents could react with non-catalytically competent cysteine residues at or near the active site causing a level of steric hindrance which may interfere with catalytic activity. This hypothesis was confirmed in a series of experiments involving the cysteine protease inhibitors, NEM and the smaller molecule, iodoacetamide. When PO was treated with these reagents separately 85% and 50% inhibition was observed with NEM and iodoacetamide respectively. When PO, which had been treated with iodoacetamide, was exposed to NEM, the peptidase was not further inhibited. This would seem to indicate the presence of a cysteine residue that is close enough to the active site, to cause total exclusion of a substrate, when it is complexed to a bulky residue. A smaller reagent such as iodoacetamide would therefore be able to exert incomplete steric hindrance (Polgar, 1991). There does seem to be quite a variation in the sensitivity of PO to functional reagents depending on its source. PO from Flavobacterium meningosepticum and Aeromonas hydrophilia was found to be resistant to the cysteine protease inhibitor PCMB indicating that no cysteine residue was in close enough proximity to the active site so as to adversely influence catalytic activity (Kanatani et al., 1993; Yoshimoto et al., 1980).

1.3.3.2 Cloning and sequencing of Prolyl Oligopeptidase Gene

Confirmation of PO’s status as a serine protease was obtained through the eventual cloning of the PO gene and the deduction of its amino acid sequence. The amino acid sequence of PO from porcine brain was reported in 1991. As well as containing 16 half-cystinyl residues, an active site serine, confirmed on the basis of its inactivation by DFP was identified (Rennex et al., 1991). Essential catalytic residues have been identified as Ser554 and His680 by reaction with active site-directed reagents (Rennex et al., 1991; Stone et al., 1991). The amino acid sequence surrounding this active site serine of PO was found to be Gly-Gly-Ser-Asn-Gly-Gly, distinguishing it from other well characterised families of serine proteases (Shirasawa et al., 1994; Rennex et al., 1991). The amino acid residues that surround the active site serine are conserved in each family and are Gly-Asp-Ser-Gly-Gly for the
trypsin family, Gly-Thr-Ser-Met-Ala, for the subtilisin family and Gly-Glu-Ser-Tyr-Ala for the carboxypeptidase Y family (Barrett and Rawlings, 1992; Brenner, 1988). Therefore PO was thought to represent a new class of serine protease. The order of the triad residues in PO is also distinct from the other more commonly known serine protease families: His57-Asp102-Ser195 in chymotrypsin, Asp32-His64-Ser221 in subtilisin and Asp529-Ser554-His680 for PO (Barrett and Rawlings, 1992). PO has also now been cloned and sequenced from porcine and bovine brain, human T cells, Flavobacterium meningosepticum and Aeromonas hydrophilia all of which (their primary structures deduced from nucleotide sequences) show significant sequence homology to each other (Yoshimoto et al., 1997; Shirasawa et al., 1994; Kanatani et al., 1993, Chevallier et al., 1992; Yoshimoto et al., 1991; Rennex et al., 1991). This would suggest that the PO protein is highly conserved and is likely to play an important role in vivo.

While there was initially no significant resemblance between the sequence as a whole or segments containing the catalytic residues to other known peptidases, it now appears that PO can be placed amongst a new evolutionary family of serine proteases, known as the S9 family (Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, 1992), (see table 1.3.1). The PO sequence shows moderate similarity to DPP IV and an even greater similarity to the acylaminoacyl-peptidases and protease II previously known as oligopeptidase B. The greatest similarities between the amino acid sequences of members of this prolyl oligopeptidase family are seen in the C-terminal third of the sequence which contains the catalytic triad (see figure 1.3.1). The consensus sequence GXSXGGZZ (X is any amino acid and Z is a hydrophobic amino acid) may be useful in identifying other members of this family. It was only with the discovery of this family of related peptidases that the Asp residue of the catalytic triad was deduced to be Asp529. Two aspartic acid residues were found to be conserved between family members, Asp529 and Asp642 (or Asp641 in slightly shifted alignment). While the environment surrounding the Asp642 varied, the Asp529 residue was found in a uniformly neutral to mildly hydrophilic segment in all members of the family allowing the conclusion that Asp529 is most likely a member of the catalytic triad (Barrett and Rawlings, 1992; Rawlings et al., 1991). While members of the S9 family of serine peptidases display some sequence homology, with respect to catalytic activity, specificity and subcellular distribution they are one of the most disparate families of peptidases recognized to date. For instance PO’s action is confined to oligopeptides (Barrett and Rawlings, 1992), DPP IV, an exopeptidase, cleaves dipeptides from the N-termini of polypeptides, only when the N-terminus is free (McDonald et al., 1971), and acyl-aminoacylpeptidase, an omega peptidase preferentially cleaves N-terminal acetyl-aminoacyl residues from polypeptides (acyl-aminoacyl-peptidase), (Mitta et al., 1989). Protease II an oligopeptidase cleaves peptide bonds, specifically lysine and arginine residues at the carboxyl end of amino acids (Kanatani et al., 1991). PO and acyl-aminoacyl peptidase are soluble/cytosolic (Mitta et al., 1989; Torres et al., 1986) but DPP IV is heavily glycosylated and membrane associated (Misumi et al., 1992). DPP IV and PO show specificity for prolyl bonds but no such specificity has been
observed for acyl-aminoacyl peptidase (Mitta et al., 1989, Koida and Walter, 1976, McDonald et al., 1971)

Structural similarities have also been noted between lipases and members of the prolyl oligopeptidase family, more specifically similarities between the short segments comprising the catalytic triad residues. These similarities include sequence homology, topology and the not entirely open active site. This similarity has provided a possible explanation for the physical rate-limiting catalytic step likely to be a conformational change. In the case of lipases the catalytic triad is covered by a surface loop or flap and the repositioning of this flap is necessary to render the catalytic site accessible to the substrate. This flap may be conserved in the peptidases of the prolyl oligopeptidase, S9 family (Polgar, 1992c)

1 NCFDDFQCAA
2 NCFDDFQCAA
3 NVFDIFIAAG
4 NTNFDYLDAC
5 QDVKDVQFAV
6 QDVKDVQFAV
7 LEVEDQIEAAA
8 FEVEDQISAA
9 YEARDQISAA

* Location of catalytically competent triad residues
1 Human prolyl oligopeptidase (Shirasawa et al., 1994)
2 Pig prolyl oligopeptidase (Rennex et al., 1991)
3 Flavobacterium meningosepticum prolyl oligopeptidase (Chevallier et al., 1992)
4 Escherichia coli protease II (Kanatan et al., 1991)
5 Pig acylaminoacyl-peptidase (Mitta et al., 1989)
6 Rat acylaminoacyl-peptidase (Kobayashi et al., 1989).
7 Rat dipeptidyl peptidase IV (Ogata et al., 1989)
8 Human dipeptidyl peptidase IV (Abbot et al., 1994).
9 Yeast dipeptidyl amnuopeptidase B (Roberts et al., 1989)
<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Peptidase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfamily A.</td>
<td>Oligopeptidase B/Protease II</td>
<td>Kanatani et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Prolyl Oligopeptidase</td>
<td>Shirasawa et al., 1994</td>
</tr>
<tr>
<td>Subfamily B.</td>
<td>Dipeptidyl aminopeptidase A</td>
<td>Anna-Arriola and Herskowitz, 1994</td>
</tr>
<tr>
<td></td>
<td>Dipeptidyl aminopeptidase B</td>
<td>Roberts et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Dipeptidyl peptidase IV</td>
<td>Abbot et al., 1994</td>
</tr>
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<td></td>
<td>Dipeptidyl peptidase V</td>
<td>Beauvais et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Fibroblast activation protein subunit</td>
<td>Goldstein et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Seprase</td>
<td>Goldstein et al., 1997</td>
</tr>
<tr>
<td>Subfamily C</td>
<td>Acyl-aminoacyl peptidase</td>
<td>Kobayashi et al., 1989</td>
</tr>
</tbody>
</table>

Table 1.3.1.  The S9 or Prolyl Oligopeptidase Family of Serine Proteases

*This family of peptidases contains deeply divergent groups and is therefore divided in subfamilies (Rawlings and Barrett, 1993).

1.3.3.3. Prolyl Oligopeptidase Catalytic Mechanism Studies

Investigations into the mechanistic features of PO catabolism have revealed further distinctions from the extensively characterised serine protease, trypsin/chymotrypsin and subtilisin, families. Until the confirmation of PO's status as a serine peptidase, it was assumed that serine proteases shared the same simple pH rate profile controlled by a single ionizing group (histidine) of pKa 7 (Polgar, 1989; Blow, 1969). Briefly a classical serine protease (chymotrypsin) catalytic reaction has been predicted to proceed as follows: **Acylation:** The nucleophilic hydroxyl group of the catalytic serine attacks the substrate carbonyl group and this reaction is stabilised by general base catalysis, a contribution by the catalytic histidine side chain, which removes the proton from the attacking oxygen. **Deacylation:** The resulting tetrahedral intermediate (covalent enzyme-substrate interaction) then decomposes by general acid catalysis afforded by the protonated histidine. This results in the return of free enzyme (see figure 1.3.2). It is the catalytically competent histidine residue that facilitates both the formation and decomposition of the intermediate acyl-enzyme and it is the ionization of this residue that determines the pH dependence of catalysis (Blow, 1969). These general base/acid reactions are said to be rate limiting in the case of the chymotrypsin/subtilisin families and ionic strength has little or no effect on these proteases. In contrast PO displays a double sigmoidal pH/rate profile indicative of two
enzyme forms existing between pH 5 and 9, and its catalysis is sensitive to ionic strength (Polgar, 1992a, Polgar 1992b)

One of the methods routinely used in the studies on the mechanism of serine protease reactions is the investigation of kinetic deuterium isotopic effects. It was discovered that the acylation/deacylation steps for a chymotrypsin catalysed reaction, proceeded slower in deuterium oxide by a factor of around 2-3, indicating that base/acid catalysis i.e. a chemical step, is rate limiting for the overall reaction (Polgar, 1992b). Similar experiments involving PO showed that while the low pH form displayed a significant deuterium isotope effect, the high, physiologically competent pH form showed none. This would indicate an isotopically silent step at physiological pH (Polgar, 1991). This precluded the possibility of a rate limiting conformational change in PO catalysis. Further evidence involved an investigation of the effects of substrates with different chemical leaving groups on PO reaction rates. A substrate with a better leaving group is known to react with a peptidase at a higher rate provided the rate limiting step is chemical. This was proved to be the case for chymotrypsin with nitrophenyl ester substrates displaying a much higher reactivity relative to amide substrate derivatives. In the case of PO there appeared to be no significant difference between rate constants using various derivatives of the substrate, Z-Gly-Pro-Leaving Group, on PO catalysis which would indicate a rate-limiting physical rather than chemical step (Polgar 1992b). Thus some conformational change in PO can be associated with the catalytic process.

Investigations on another member of the prolyl oligopeptidase family of serine peptidases, oligopeptidase B (formerly known as protease II), isolated from E Coli, (Pacaud and Richaud, 1975), have found that this protease has mechanistic features similar to PO. While this peptidase is trypsin-like in its hydrolysis of oligopeptides at the carboxy-side of paired basic residues, no kinetic deuterium isotopic effect was observed for this peptidase indicative of a rate-limiting conformational change (Polgar, 1997).
Figure 1.3.2. *Chemical Mechanism for Chymotrypsin-mediated Cleavage:*

Asp residue pulls a proton from the neighbouring His, which in turn pulls a proton from the nearby Ser, forming an anion. This form of Ser is more nucleophilic enabling it to attack the carbonyl of the appropriate amide bond. Mechanisms in which protons are transferred during catalysis are examples of acid/base catalysis. Following the attack by serine and formation of the transition state, a portion of the peptide containing the new amino terminus is released. A second portion of the peptide remains bound to serine via an ester linkage, which is hydrolysed to form the second half of the new peptide with a new carboxy-terminus. Protons are then transferred to regenerate chymotrypsin in its original form, completing the cycle (Menger and Brock, 1968)

1.4. **Prolyl Oligopeptidase Specificity**

1.4.1. *Specificity for Proline Residues*

Prolyl oligopeptidase's ability to hydrolyse peptides is dictated not only by the presence of a particular residue (proline) but also the position of that residue and the size of the peptide. While PO was originally identified as an oxytocin degrading enzyme (Walter et al., 1971), it was soon discovered that rather than having a unique specificity for oxytocin, this peptidase had a specificity for Pro-Xaa
bonds. An exception to this was found to be an inability to act on the Pro-Pro bond (Walter, 1976, Koida and Walter, 1976). The rate of cleavage of the Pro-Xaa bond was found to be fastest when Xaa was a hydrophobic residue. However, the catalytic rate decreased progressively when Xaa was replaced with basic and acidic residues (Koida and Walter, 1976). An example of this selectivity for P1' residues (see figure 14.1.), was observed in the hydrolysis of bradykinin potentiating peptide. PO hydrolyses BPP, in vitro, at the Pro-Phe, Pro-Gly and Pro-Glu bonds. However, hydrolysis of the Pro-Phe bond occurs more rapidly than the Pro-Gly, while the Pro-Glu bond is very slowly hydrolysed.

This action on BPP highlights PO's preference for hydrophobic residues. BPP also possesses a Pro-Pro bond which was not hydrolysed (Koida and Walter, 1976). PO was found to be unable to cleave a Pro-Xaa bond when the proline residue was at the amino terminus of a peptide, but could hydrolyse the bonds when Xaa was the carboxyl terminal residue. The introduction of a blocking group at the amino terminus to give Z-Pro-Xaa sequence did not result in hydrolysis. Replacement of P1 or P2 residues with D-amino acids was found to result in substrates that were resistant to hydrolysis, but replacement of the P3 residue had minor effects. In contrast, D-amino acid substitution in the P1' and P2' positions decreased catalytic activity, but replacement of the P3' residue elicited no change. From these observations, it has been suggested that PO has an extended binding site region of three subsites on the amino side of the scissile bond and two on the carboxyl side (see figure 14.1.). (Walter and Yoshimoto, 1978). PO also has an ability to act on alanyl bonds (Ala-Xaa) but at a rate 1/100-1/1000 that of Pro-Xaa bonds. In contrast to this peptidases inability to hydrolyse oligoproline, it was found to act on oligoalanine residues (Walter and Yoshimoto, 1978). Replacement of the P1 residue with N-methylalanine and sarcosine resulted in relatively good substrates. It can be concluded from these studies that the S1 subsite of PO was designed specifically to fit proline and only accommodated residues that did not exceed proline's pyrrolidine ring (Nomura et al., 1986). With respect to the P1' residue, it was found that the phosphorylation of a serine residue in this position increased the rate of hydrolysis (Rosen et al., 1991), and the replacement of an Asp residue with Asu (a result of spontaneous rearrangement of Asp and Asn residues) also resulted in a ten-fold increase in hydrolysis. This could suggest a possible role for PO in the disposal of non-functional proteins from a cell (Momand and Clarke, 1987).
Figure 1.4.1. Substrate and Complementary Prolyl Oligopeptidase Subsite

Prolyl oligopeptidase has 3 subsites for the amino acid residues at the amino side of the scissile bond (P1, P2, and P3) and two at the carboxyl side (P1' and P2'). The complementary enzyme subsites are also illustrated (S4-S2'). Diagram is an illustration of the standard nomenclature used to refer to substrates and their complementary enzyme subsites (Schecter and Berger, 1967)

1.4.2. Substrate- Size Limitation

As well as its selectivity for proline residues PO also displays a certain selectivity in the size of the peptides it will act on. Preliminary studies have shown that while this peptidase was capable of cleaving a wide variety of proline containing peptides, it had a tendency to cleave low molecular weight peptides much faster than larger molecules, the smallest peptide hydrolysed being a tetrapeptide. PO was unable to act on larger proteins such as albumin, collagen, myoglobin, insulin or glucagon and in general could not act on proteins/peptides greater than 25 amino acid residues long (Moriyama et al., 1988, Walter et al., 1980, Koda and Walter, 1976). PO was first termed an endo-oligopeptidase based on its inability to hydrolyse Gly-Gly-Arg-bradykinin bound to succmylated polylysme or Affigel 10 (Camargo et al., 1979). An oligopeptidase or endo-acting oligopeptide hydrolase is a peptidase limited to the hydrolysis of peptide bonds in smaller polypeptides and is unable to act on proteins. It was only recently that the oligopeptidases received a separate classification distinguishing them from endopeptidases (Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, 1992). Some of the known oligopeptidases known to date are listed in Table 141. The mechanism of the substrate-size limitation of oligopeptidases is not well understood but must in some way be intrinsically linked to.
the molecular structure of these peptidases and the high degree of conformational freedom of oligopeptides. Possibly the binding sites of these peptidases could be analogous to receptor binding sites. Barrett likened the action of oligopeptidases on their peptide substrates to a receptor interacting with a specific biologically active ligand, for instance the receptor mediated biological activity mediated by bradykinin is not shown by kinogen (Barrett and Rawlings, 1992).

<table>
<thead>
<tr>
<th>Peptidase</th>
<th>*Peptidase Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl Oligopeptidase</td>
<td>Serine</td>
<td>Moriyama et al., 1988</td>
</tr>
<tr>
<td>Thimet Oligopeptidase</td>
<td>Thimet</td>
<td>Barrett and Brown, 1990</td>
</tr>
<tr>
<td>Neprilysin</td>
<td>Thimet</td>
<td>Danielsen et al., 1980</td>
</tr>
<tr>
<td>Pitrilysin</td>
<td>Metallo</td>
<td>Anastasi et al., 1993</td>
</tr>
<tr>
<td>Neurolysin</td>
<td>Thimet</td>
<td>Serzawa et al., 1995</td>
</tr>
<tr>
<td>Oligopeptidase M</td>
<td>Metallo</td>
<td>Krause et al., 1997</td>
</tr>
<tr>
<td>Streptococcal PepB oligopeptidase</td>
<td>Thimet</td>
<td>Lin et al., 1996</td>
</tr>
<tr>
<td>Lactococcus lactus PepF oligopeptidase</td>
<td>Metallo</td>
<td>Monnet et al., 1994</td>
</tr>
<tr>
<td>Lactococcus cremoris endopeptidase I</td>
<td>Metallo</td>
<td>Yan et al., 1987</td>
</tr>
<tr>
<td>Lactococcus cremoris endopeptidase II</td>
<td>Metallo</td>
<td>Yan et al., 1987</td>
</tr>
<tr>
<td>E. coli Protease II (Oligopeptidase B)</td>
<td>Serine</td>
<td>Paucaud et al., 1975</td>
</tr>
</tbody>
</table>

Table 1.4.1. Oligopeptidases

*With the exception of prolyl oligopeptidase and E. coli protease II, the known oligopeptidases are metallo/thiol proteases.

1.4.3. Prolyl Oligopeptidase Hydrolysis of Neuro/Vasoactive peptides

In *vivo* PO has been found to hydrolyse a wide variety of proline containing neuroactive peptides including TRH, LHRH bradykinin and neurotensin. Table 1.4.2 illustrates a range of neuro- and vasoactive peptides hydrolysed *in vitro* by PO and their sites of hydrolysis.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu</td>
<td>Moriyama et al., 1988</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>Tate, 1981</td>
</tr>
<tr>
<td>BPP</td>
<td>pGlu-Gly-Gly-Trp-Pro-Arg-Arg-Gly-Pro-Glu-Ile-Pro-Pro</td>
<td>Koida and Walter, 1976</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td>Tate, 1981</td>
</tr>
<tr>
<td>LHRH</td>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td>Mendez et al., 1990</td>
</tr>
<tr>
<td>TRH</td>
<td>pGlu-His-Pro-NH₂</td>
<td>Tate, 1981</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>pGlu-Leu-Tyr-Glu-Asn-Cys-Pro-Leu-Gly-NH₂</td>
<td>Tate, 1981</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Cys-Tyr-Ile-Gln-Asp-Cys-Pro-Leu-Gly-NH₂</td>
<td>Walter, 1976</td>
</tr>
<tr>
<td>Substance P</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met</td>
<td>Moriyama et al., 1988</td>
</tr>
<tr>
<td>AVP</td>
<td>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly</td>
<td>Walter, 1976</td>
</tr>
<tr>
<td>Melanotropin</td>
<td>Ser-Tyr-Ser-Met-Glu-Asn-Lys-Pro-Leu-Gly-NH₂</td>
<td>Tate, 1981</td>
</tr>
<tr>
<td>Tuftsin</td>
<td>Thr-Lys-Pro-Arg</td>
<td>Tate, 1981</td>
</tr>
</tbody>
</table>

Table 1.4.2. Physiological Neuro-/Vasoactive peptides Hydrolysed by Prolyl Oligopeptidase in vitro.
Scissile bonds are indicated in bold.

It is still unclear however, what precise role this peptidase plays in the turnover of these peptides in vivo but the recent development of potent and specific PO inhibitors has contributed to numerous studies attempting to elucidate the role of PO in neuropeptide turnover.

In the case of TRH degradation in vivo several studies have shown that PO had in fact no influence on physiological TRH levels. Several studies using Z-Pro-prolinal have shown that in vitro and in vivo treatment with this inhibitor did not alter TRH levels. There have been similar observations made in the case of LHRH (Salers et al., 1992; Mendez et al., 1990; Friedman and Wilk, 1989). Ontogenic studies revealed a poor correlation between TRH, TRH-OH and PO activity (Salers et al., 1992). In conflict with the conclusion, drawn from these results, that PO had no involvement in the degradation of TRH in vivo, was the report that the potent PO inhibitor, Y29794, potentiated the effect of TRH on release of acetylcholine in rat hippocampus (Nakajima et al., 1992). PO has also been implicated in the biotransformation of angiotensin I and II to form angiotensin 1-7, a peptide that mimicks the vasopressin-releasing and cardiovascular effects of angiotensin II (Schiavone et al., 1988). The production of angiotensin 1-7 in canine brain homogenates was found to be inhibited by Z-Pro-prolinal (Welches et al., 1991). The portal plasma of sheep was discovered to contain a significantly higher level of angiotensin 1-7, than in jugular plasma. It has been speculated that a high level of PO, found in the median eminence, could contribute to the metabolism of angiotensin I delivered by
arterial blood (Lawrence et al., 1992). Administration of the PO specific inhibitor, JTP-4819 to aged rats was found to increase substance P immunoreactivity. However there was found to be no significant change in AVP immunoreactivity, suggesting that it had no involvement in the hydrolysis of this peptide (Toide et al., 1995)

1.4.4. Prolyl Oligopeptidase Specific Assays

Since POs initial discovery in human uterine extracts, radiometric, spectrophotometric and spectrofluorometric assays have been developed and used in this peptidase’s detection. Following its discovery, known substrates of PO oxytocin and arginine-vasopressin were used in native or radiolabelled forms to detect PO activity (Walter et al., 1971, Walter, 1976). Today the vast majority of PO assays are based on the synthetic substrate N-blocked-Gly-Pro linked to a colourmetric or fluorimetric label. In 1979 Yoshimoto et al., reported on the synthesis and use of Z-Gly-Pro-MCA (7-amino-4-methylcoumarin) a fluorescent substrate which had a Km of 20µM for PO purified from lamb kidney, while Orlowski et al., synthesised Z-Gly-Pro-SM (sulphamethoxazole) a colourmetric substrate. This basic structure (N-blocked-Gly-Pro-colourmetric/fluorimetric label) has changed little over time even though the solvents needed to solubilise these substrates were known to adversely effect POs activity (Knisatschek et al., 1980). In an investigation, that examined a range of fluorimetric substrates, it was reported that glycine was in fact a poor choice of amino acid for the P2 position as PO could accommodate bulky residues and had a preference for positively charged groups (Noula et al., 1997). This confirms the suggestion that there are one or more negative charges at or near the active site which are responsible for electrostatic attraction or repulsion between PO and charged substrates (Polgar, 1992a). Other high affinity substrates developed by Noula et al., include Z-Lys-Pro-NH-Meq, Z-Lys(Boc)-Pro-NH-Meq and Z-Glu-Pro-NH-Meq with Km values of 2.1, 2.9 and 5.0µM respectively for the porcine kidney enzyme. Another potential reason why further substrate development studies are required, was the discovery of a second distinctive Z-Gly-Pro-MCA hydrolysing activity in bovine serum (Cunningham and O’Connor, 1997b). As this peptidase is insensitive to Z-Pro-prolinal (hence its name Z-Pro-prolinal insensitive peptidase or ZIP), this specific PO inhibitor can be incorporated into the assay as a method of distinguishing these two activities. It is however imperative that further substrate specificity studies on this new peptidase be completed in order to develop specific, high affinity substrates for both peptidases.
1.5. Specific Inhibitors of Prolyl Oligopeptidase

It is still not clear, what physiological role, if any is played by PO in the metabolism of neuropeptides. In order to investigate the potential function of PO, the availability of powerful and selective inhibitors for this peptidase, for use *in vitro* and *in vivo* is a prerequisite. There has been a particular emphasis on the development of inhibitors that effectively inhibit the enzyme *in vivo*, and can readily traverse the blood brain barrier where they produce long lasting inhibition of brain PO. Inhibitors of PO to date are described in section 1.5.1. to 1.5.3. (Table 1.5.1. lists some of these inhibitors and their Ki and/or IC50 values. Figures 1.5.1. and 1.5.2. illustrates some of the structures of these inhibitors).

1.5.1. Synthetic Inhibitors

Peptide aldehydes are known to be potent inhibitors of some proteolytic enzymes. For example N-blocked argininals strongly inhibit cysteine proteases and trypsin. N-blocked lysinal derivatives have been shown to be specific for lysyl endopeptidases and leupeptin, a transition state aldehyde, has also been shown to inhibit trypsin (Vinitsky *et al.*, 1992). In 1983 Wilk and Orlowski reported on the synthesis of N-benzylxycarbonyl-L-prolyl-L-prolinal (Z-Pro-prolinal) (1). This N-blocked peptidyl aldehyde was reported to be a non-competitive, transition state inhibitor with a reported Ki of 14nM for the rabbit brain enzyme. This inhibitor was found to be quite potent *in vivo* and its lipophilic nature allowed it to readily traverse the blood brain barrier to the brain where it seems to be very slowly eliminated. Both the alcohol(2) and acid derivatives of Z-Pro-prolinal were found to be 3000 times less inhibitory than the aldehyde (Friedman *et al.*, 1984, Wilk and Orlowski, 1983). In 1990 it was proposed that Z-Pro-prolinal was in fact a competitive slow binding inhibitor of mouse and human brain PO with Ki values of 0.35nm and 0.5nm respectively. PO a serine protease should be capable of a nucleophilic attack on the aldehyde carbonyl group resulting in a hemiacetal adduct. The absence of any apparent slow tight binding in the case of Z-Pro-nrolinal (2) highlights the importance of the aldehyde group in this slow-tight binding inhibition (Bakker *et al.*, 1990). Since the initial synthesis of Z-Pro-prolinal, a large number of potentially potent ZPP analogs have been investigated as possible PO inhibitors. In 1984 Yokosawa *et al* substituted the P2 prolyl residue with a range of amino acids. Of these Z-Val-prolinal was found to be the most potent with a reported Ki of 2.4nM for PO derived from sperm of the ascidian, *H. roretzi*. It has also been reported that replacement of the L-prolyl residue with a thiopropyl residue and the conversion of the prolinol group to thioprolinal, increased inhibitory potency with a reported IC50 and Ki of 0.035nm and 0.01nm respectively using bovine brain PO (Z-Thioprop-thioprolinal (3)). When the thioprolinal was replaced with a thiazolidine group this inhibitor remained very potent with slightly lower IC50 and Ki values of 0.14nM and 0.36nM respectively (Z-Thioprop-Thiazolidine(4)), (Tsuru *et al.*, 1988). This is interesting as this compound lacks an aldehyde group but is yet comparable to Z-Pro-prolinal in its inhibitory activity.
In studies using Z-pro-prokmal and derivatives it was found that a main chain length of the inhibitor corresponding to 3 subsites, S1, S2 and S3 is most suitable for inhibitory activity. This is a good reflection of the fact that PO is very active towards low molecular weight peptides but inert towards high molecular mass peptides. For a potent inhibitor specific for PO, a restricted chain length in the main chain interacting with the S1, S2, S3 subsites of the enzyme was critical, with a hydrophobic pocket in the S3 subsite of the enzyme which seemed to interact hydrophobically with a certain N-blocked group of the inhibitor, such as the benzylxoycarbonyl group. How the introduction of sulphur atom affects the enzyme-inhibitor interaction is unknown, but somehow it either induces steric fitness in the enzyme-inhibitor interaction or it introduces an electron attracting or donating force which results in a firm interaction between the enzyme and the inhibitor (Tsuru et al., 1988).

Prodrugs based on Z-Pro-prokmal and its derivatives have also been synthesised. Because the aldehyde group of this inhibitor is very reactive, it was disguised with an acetal group which could be converted in the stomach to the aldehyde moiety. Interestingly these acetals were also potent in their inhibition of PO in vitro with a reported IC50 for Z-Pro-prokmal dimethyl acetate of 0.13 mM (Goossens et al., 1997). Many other potent PO inhibitors many of which are variations of the N-blocked dipeptide chain have been developed. The most potent of these include SUAM 1221 (5), a phenyl butanoyl prolyl-pyrrolidine derivative with a reported IC50 of 190 nM and a derivative of this, (replacement of proline to thioproline) which had an IC50 67 nM (6), (Saito et al. 1991). The conversion of the pyrrolidine group of Z-Pro-pyrrolidine, to a fluoropyrrolidine to give Z-Pro-fluoropyrrolidine, resulted in a compound with a Ki of 0.8 nM (Goossens et al., 1997).

Fmoc-aminoacylpyrrolidine-2-nitriles have also been found to possess potent inhibitory activity against PO. Both Fmoc-Ala-Pro-PyrCN and Fmoc-Pro-PyrCN had Ki values of 5 nM for PO and were found to be very stable, cell permeable and crossed the blood brain barrier (Li et al., 1996).

Natural substrates of PO, oxytocin and vasopressin, are already inhibitors of this peptidase. These peptide hormones have intramolecular disulphide bridges between cysteine residues and it has been suggested that a thiol-disulphide exchange between the substrate and a thiol group in or near the active site PO could be a mechanism for inhibition. Inhibitors mimicking these natural substrates, which incorporated an NH-O-acyl moiety (N-peptidyl-O-acyl hydroxylamines are known inhibitors of thiol enzymes (Bromme et al., 1989)) have been prepared, the most potent of them being Boc-Glu(NH-O-Bz)Pyr (9), (Demuth et al., 1993).

A wide variety of dipeptidyl-α keto heterocycles (10), both one-ring and two-ring types, have also proved themselves to be potent PO inhibitors. It was suggested that the potency of these compounds was dependent on the presence of an sp² nitrogen atom which is able to form a hydrogen bond at a β-position from the ketone moiety (Tsutsumi et al., 1994).

A reversible and competitive non-peptide PO inhibitor Y-29794, was reported by Nakajima et al., in 1992. This inhibitor was found to be potent (Ki=0.95 nM) highly specific and easily penetrated the blood-brain barrier.
Pentidyl diazomethanes and peptidyl chloromethanes have also been reported to have inhibitory activity against PO. These agents have contributed tremendously in the study of the active site of PO, confirming its status as a serine protease. PO's position as a serine protease was clarified by its inhibition with DFP and Z-Gly-Pro-CH₂Cl (Yoshimoto et al., 1977) Active site directed chloromethanes are thought to alkylate active site histidines of serine proteases and also cysteine residues in various enzymes. The competitive PO inhibitor Ac-Ala-Ala-Ala-CH₂Cl was found, out of 23 histidine residues, to selectively alkylate His-680, providing evidence that this residue is indeed the catalytic histidine. This agent also alkylated some PO cysteine residues (Stone et al., 1991) Modification of at least one of these cysteine residues may be at least partly responsible for inactivation of the enzyme. In this respect PO could be similar to certain members of the subtilisin serine protease family that are inactivated by modification of a non-essential cysteine residue within the active site.

Peptidyl diazomethanes have been useful in confirming PO's status as a serine protease. It was initially thought that peptidyl diazomethanes were reagents that specifically inhibited cysteine proteases by alkylation of the active site cysteine residues (Shaw, 1990). Some serine proteases however are now known (members of the subtilisin family) to be inhibited by these diazomethane derivatives through the formation of a covalent bond with the active site histidine (Ermer et al., 1990) Peptidyl diazomethanes such as Ac-Ala-Ala-Pro-CHN₂ have been found to be reversible slow tight binders of PO with the formation of a covalent complex between the active site serine and the diazomethane (Stone et al., 1992).

1.5.2. Inhibitors of Bacterial Origin.
A number of compounds which are bacterial in origin were found to possess quite potent PO inhibitory activity (see table 1.5.1) These include bacitracin, staurosporine(11), (Kimura et al., 1990), postatin (13), (Tsuda et al., 1996), curvatains A and B (12), (Toda et al., 1992), propeptin (Kimura et al., 1997a), purpurogallin (Inamori et al., 1997), lipohexin (Henze et al 1997) and SNA-8073B (Kimura et al., 1997b), a stereosomer of the antibiotic fujinamycin B. A variety of derivatives of postatin have been chemically synthesised in an attempt to obtain a greater inhibitory potency and selectivity. Two postatin analogs containing its characteristic (S)-3-amino-2-oxovaleryl moiety were found to have IC₅₀s of 5.8 ng/mL and 8.2 ng/mL (in contrast to postatin's 0.030 µg/mL) and unlike postatin had no inhibitory effect on cathepsin B activity (Tsuda et al., 1996).

1.5.3. Endogenous Inhibitors
Since POs initial discovery, a number of naturally occurring inhibitors have been purified from a variety of mammalian tissues including porcine pancreas (Yoshimoto et al., 1982), neonatal rat
pancreatic islet cell extracts (Salers, 1994), rat brain (Soeda et al., 1985) and bovine brain (Ohmori et al., 1994). Distribution studies performed using porcine and rat tissues found this inhibitor to be uniformly distributed with highest levels in the pancreas (Yoshimoto et al., 1982). The molecular weight of inhibitors purified from various tissues ranged from 6.5 to 7.0 kDa. Amino acid analysis on the purified inhibitor from bovine brain revealed it to be identical to segment 38-55 of glial fibrillary acidic protein and had a Ki of 8.6 μM (Ohmori et al., 1994, Maruyama et al., 1996). It is known that some protease inhibitors released from glial cells (neurins) are involved in neurite growth regulation. This coupled with the observation that staurosporine, another PO inhibitor, was found to stimulate neurite growth in a rat cell line suggests a possible role for PO in cell growth (Kimura et al., 1990). This will be discussed further in section 1.7.1.

Other endogenous inhibitors of PO from rat liver cytosol include coenzyme A, its acyl derivatives and acyl carnitine (Yamakawa et al., 1990).
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$</th>
<th>$IC_{50}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Pro-prolinal (1)</td>
<td>0.5 nm/14 nm*</td>
<td></td>
<td>*Wilk &amp; Orlowski, 1983</td>
</tr>
<tr>
<td>Z-Pro-prolinal (2)</td>
<td>10$^9$ nM</td>
<td></td>
<td>Bakker et al, 1990</td>
</tr>
<tr>
<td>Z-Pro-prolinal dimethylacetate</td>
<td>0.13 μM</td>
<td></td>
<td>Bakker et al, 1990</td>
</tr>
<tr>
<td>Z-Thiopro-thioproline (3)</td>
<td>0.01 nm</td>
<td>0.35 nm</td>
<td>Tsuru et al, 1988</td>
</tr>
<tr>
<td>Z-Thiopro-thiazolidine (4)</td>
<td>0.36 nm</td>
<td>0.14 nm</td>
<td>Tsuru et al, 1988</td>
</tr>
<tr>
<td>SUAM 1221 (5)</td>
<td>190 nm</td>
<td></td>
<td>Sato et al, 1991</td>
</tr>
<tr>
<td>Fmoc-Ala-Pyr-CN</td>
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</tr>
<tr>
<td>Fmoc-Pro-Pyr-CN</td>
<td>5 nM</td>
<td></td>
<td>Lin et al, 1996</td>
</tr>
<tr>
<td>ONO 1603</td>
<td>57 nm</td>
<td></td>
<td>Toda et al, 1989</td>
</tr>
<tr>
<td>Y-29794</td>
<td>0.95 nM</td>
<td></td>
<td>Yoshimoto et al, 1992</td>
</tr>
<tr>
<td>Boc-Glu(NHO-Bz-Pyr) (9)</td>
<td>0.03 μM</td>
<td></td>
<td>Demuth et al, 1993</td>
</tr>
<tr>
<td>Z-Phe-Pro-methylketone</td>
<td>1.8 nM</td>
<td></td>
<td>Steinmetzer et al, 1993</td>
</tr>
<tr>
<td>Z-Cyclohexyl-prolinal (7)</td>
<td>3.0 nM</td>
<td></td>
<td>Bakker et al, 1991</td>
</tr>
<tr>
<td>Z-Indoliny1-prolinal (8)</td>
<td>2.4 nM</td>
<td></td>
<td>Bakker et al, 1991</td>
</tr>
<tr>
<td>JTP-4819</td>
<td>0.83 nm</td>
<td></td>
<td>Toide et al, 1995</td>
</tr>
<tr>
<td>α-Ketobenzothiazole (10)</td>
<td>5.0 nM</td>
<td></td>
<td>Tsutsumi et al, 1994</td>
</tr>
<tr>
<td>Postatin (13)</td>
<td>0.030 μg/mL</td>
<td></td>
<td>Nagai et al, 1990</td>
</tr>
<tr>
<td>Staurosporine (11)</td>
<td>0.70 μM</td>
<td>77 μM</td>
<td>Kimura et al, 1990</td>
</tr>
<tr>
<td>Eurystatin A (12)</td>
<td>0.004 μg/mL</td>
<td></td>
<td>Kamei et al, 1992</td>
</tr>
<tr>
<td>Eurystatin B (12)</td>
<td>0.002 μg/mL</td>
<td></td>
<td>Kamei et al, 1992</td>
</tr>
<tr>
<td>Propeptin</td>
<td>0.70 μM</td>
<td></td>
<td>Kimura et al, 1997a</td>
</tr>
<tr>
<td>Purpurogallin</td>
<td>1.6 x 10$^{-5}$</td>
<td>8 μM</td>
<td>Inamori et al, 1997</td>
</tr>
</tbody>
</table>

Table 1.5.1. PO Specific Inhibitors

Numbers in brackets refer to figures 15.1 and 15.2
Figure 1.5.1. Prolyl Oligopeptidase Specific Synthetic Inhibitors
Figure 1.5.2. Prolyl Oligopeptidase Inhibitors of Bacterial Origin.
1.6. Distribution

Since its discovery in human uterine homogenates (Walter et al., 1971), prolyl oligopeptidase has been identified in a wide variety of organisms; mammalian, plant and microbial Distribution studies performed in human (Kato et al., 1980), rat (Yoshimoto et al., 1979), rabbit (Orlowski et al., 1979) and mouse tissue sources (Dauch et al., 1993) have revealed a ubiquitous tissue distribution with uniformly high levels in the brain. Significant differences in peripheral tissues levels reflect a substantive species to species variation (see table 16.1). With respect to peripheral organs, highest levels of PO in humans were observed in skeletal muscle, testis and kidney, with low levels in aorta, heart tissue and serum (Kato et al., 1980). In contrast, rabbit levels were lowest in skeletal muscle and highest in the intestine, lung and spleen (Orlowski et al., 1979). In rat, highest PO activity was found in kidney and liver while the pancreas and small intestine had little or no activity (Yoshimoto et al., 1979).

Consistently high levels were detected in the brain of all species examined, with some variation in regionalization of activity (see table 16.2). Cortical levels in all species examined were high. In human brain, the frontal cortex had highest levels with the thalamus showing lowest levels. It is important to note that the thalamus levels were less than 10-fold lower than cortical levels, not as great a difference in comparison to the trend in levels in peripheral tissues (Kato et al., 1980). In rabbit, brain, highest levels were found in the endorhinal cortex, hippocampus and striatum, with the frontal and parietal cortex also having relatively high levels. Lowest levels were observed in rabbit medulla and pons (Orlowski et al., 1979). In bovine brain, high PO levels were detected in the caudate nucleus and thalamus with low levels in the anterior pituitary (Tate, 1981).

Such uniform tissue distribution of a peptidase activity is highly supportive of a role in peptide metabolism in a particular organism. This coupled with PO’s unique specificity for internal proline bonds makes it an ideal candidate for further study.

The majority of subcellular localization studies performed to date on PO have found it to be primarily cytosolic in nature in a variety of sources including rabbit, rat and bovine brain, hamster hypothalamus and murine macrophages, PO having been either co-localized with LDH and ChAT, two cytoplasmic marker enzymes or having stayed in soluble fraction under conditions that would sediment membrane fraction, lysosomes and other subcellular granules (Torres et al., 1986, Greene and Shaw, 1983, Dresdner et al., 1982, Tate, 1981). In a Sarcophagia peregrina embryonic cell line, PO was found to be entirely localized in the nucleus using immunofluorescent staining (Ohtsuki et al., 1997). In a follow up study on mouse Swiss 3T3 cells, activity was detected in the cytoplasm and nuclei (Ishmo et al., 1998). There have been reports however of a particulate form of this peptidase (Camargo et al., 1984, Dalmaz et al., 1986) and in 1996, O'Leary et al., reported on the purification of a particulate prolyl endopeptidase type peptidase from the synaptosomal membranes of bovine brain. As well as its membrane location, this PO type activity displayed some unique characteristics, such as an insensitivity to serine protease inhibitors and sensitivity to the metal chelator 1,10-
phenanthroline. Because of POs cytosolic location its role in neuropeptide metabolism and physiological significance is obscured. It is generally assumed that the action of neuropeptides is catalysed by membrane bound ectoenzymes and very little is known about the internalisation of these peptides for degradation. The brain cytosol is a rich source of peptide degrading enzymes. Resident exo- and oligopeptidases are thought likely to participate in the terminal stages of intracellular protein degradation. However their physiological significance and their role in neuropeptide metabolism is unknown. There is the possibility that cytosolic peptidases may control the intracellular generation of biologically active peptides. They may function to degrade peptides delivered to the cell by receptor mediated endocytosis or possibly degrade peptides which have been released intracellularly from damaged vesicles. Alternatively they may control levels of peptides targeted for the nucleus for signalling. Whatever the role of this cytosolic prolyl oligopeptidase, its ability to act on a number of physiologically relevant proline-containing neuropeptides, warrants that this peptidase be subject to further study.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Human¹</th>
<th>Rabbit²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal Muscle</td>
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<td>1</td>
</tr>
<tr>
<td>Testis</td>
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<td></td>
</tr>
<tr>
<td>Kidney</td>
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</tr>
<tr>
<td>Submandibular Gland</td>
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</tr>
<tr>
<td>Pancreas</td>
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<td>2.04</td>
</tr>
<tr>
<td>Liver</td>
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<td>2.9</td>
</tr>
<tr>
<td>Tonsil Gland</td>
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<tr>
<td>Thyroid</td>
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<td></td>
</tr>
<tr>
<td>Stomach</td>
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<td></td>
</tr>
<tr>
<td>Small Intestine</td>
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<tr>
<td>Urinary Bladder</td>
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<td></td>
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<tr>
<td>Thymus</td>
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<td></td>
</tr>
<tr>
<td>Adrenal Gland</td>
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</tr>
<tr>
<td>Lung</td>
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<td>3.75</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.15</td>
<td>3.08</td>
</tr>
<tr>
<td>Diaphragm</td>
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<td>2.42</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.12</td>
<td>2.42</td>
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<tr>
<td>Heart</td>
<td>0.09</td>
<td>2.29</td>
</tr>
<tr>
<td>Serum</td>
<td>0.0001</td>
<td></td>
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</tbody>
</table>

Table 1.6.1. **Comparison of PO Distribution in Human and Rabbit Peripheral Organs.**
Activities expressed as a factor of levels in skeletal muscle ¹ Assayed with Suc-Gly-Pro-MCA, Kato et al., 1980, ² Assayed with Z-Gly-Pro-SM, Orlowska et al., 1979
Table 1.6.2. Distribution of Prolyl Oligopeptidase in Human Brain

Activities expressed as a factor of levels in frontal cortex

<table>
<thead>
<tr>
<th>Human Brain Region</th>
<th>Activity</th>
<th>Rabbit Brain Region</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>1</td>
<td>Frontal cortex</td>
<td>1</td>
</tr>
<tr>
<td>Nucleus caudatus</td>
<td>0.46</td>
<td>Parietal cortex</td>
<td>0.94</td>
</tr>
<tr>
<td>Pallidum interior</td>
<td>0.19</td>
<td>Entorhinal cortex</td>
<td>1.3</td>
</tr>
<tr>
<td>Pallidum exterior</td>
<td>0.20</td>
<td>Occipital cortex</td>
<td>0.77</td>
</tr>
<tr>
<td>Thalamus anteriores</td>
<td>0.008</td>
<td>Stratum</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>Hippocampus</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>Cerebellum</td>
<td>0.84</td>
</tr>
<tr>
<td>Pulvinar</td>
<td>0.26</td>
<td>Midbrain</td>
<td>0.65</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.19</td>
<td>Medulla</td>
<td>0.51</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>0.26</td>
<td>Pons</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypothalamus</td>
<td>0.86</td>
</tr>
</tbody>
</table>

1 Assayed with Suc-Gly-pro-MCA, Kato et al., 1980; 2 Assayed with Z-Gly-Pro-SM, Orlowski et al., 1979

1.7. Physiological Relevance of Prolyl Oligopeptidase

Because of its ubiquitous distribution and ability to act on a large number of biologically relevant peptides (Wilk, 1983, Kato et al., 1980), it is generally believed that prolyl oligopeptidase could contribute to a number of important biological functions. Studies involving the physiological relevance of prolyl oligopeptidase in vivo usually involve an observation of abnormal levels of activity pertinent to some particular disease state or the induction of some physiological change after the administration of specific prolyl oligopeptidase inhibitors.

1.7.1. Prolyl Oligopeptidase Ontogenic Studies-Evidence for a Possible Role in Cell Growth

Since the first ontogenic studies were completed on prolyl oligopeptidase evidence has accumulated implicating this peptidase in a possible role in cell growth and proliferation. Various ontogenic studies performed in relation to PO in a variety of mammalian tissues have revealed a common pattern in its expression during mammalian development and suggest that it is a
developmentally regulated peptidase. Corresponding to the first couple of weeks of life, a period of cell growth and development, is a dramatic increase in PO levels. This is followed by a slow decline to normal adult levels. In rat brain, lung, kidney and liver and mouse brain, PO levels increased dramatically during the first two weeks of life followed by a gradual decrease to adulthood levels (Dauch et al., 1993, Fuse et al., 1990). A similar trend was noted in a separate study on rat pancreas when it was found that PO levels increased during the first 2-3 weeks of life before dropping to adult levels (Salers et al., 1992). In prenatal investigations performed using primary cultures of rat cerebral cortex, PO activity was seen to augment dramatically during the first week of growth, corresponding to a period of rapid neuronal differentiation and development. After the second week of growth, levels dropped to adult levels (Szappanos et al., 1994). In an investigation of regenerating rat liver cells an increased PO activity was noted corresponding to the early stage of regeneration. Accompanied by this increased activity was a decrease in an endogenous PO inhibitor. Treatment of cells with Z-Gly-Pro-CH$_2$ resulted in inactivation of intracellular PO which was accompanied by a suppression of the regeneration process (Yamakawa et al., 1994). Using cultured embryonic cells and imaginal discs of Sacrophaga peregrina (flesh fly) it was found that the specific PO inhibitor ZTTA inhibited DNA synthesis and thus cell/disc differentiation and proliferation. Furthermore, PO was found to be exclusively located in the nucleus suggesting an endogenous nuclear substrate (Ohtsuki et al., 1997 and 1994). In a follow up study using mouse Swiss 3T3 cells findings regarding ZTTA mediated inhibition of cell proliferation were confirmed (Ishno et al., 1998).

Perhaps the most striking case of cell growth and tissue proliferation is the growth of cancer cells. It is now widely believed that peptidases do have an important role to play in tumour development and progression (Gottesman, 1990), and studies have established a link between elevated PO levels and cancerous tumours. Increased PO levels have to date been found in lung tumours, prostate, breast and sigmoid malignances (Goossens et al., 1996a, Sedo et al., 1991). Further evidence connecting PO to cancer PO was found to be localised on chromosome 6q22 (Goossens et al., 1996b). It is a well established fact that a region on chromosome 6 containing this locus is susceptible to rearrangements resulting in loss of tumour suppressor genes (Trent and Ziegler, 1992), which could by some mechanism lead to elevated PO activity.

What is evident from all of the investigations on PO levels during development and cell growth, is the possibility that the peptidase may be intrinsically linked to cell growth, development and specifically DNA synthesis. To further clarify PO's role in cell growth, possible endogenous substrates and specifically nuclear substrates of this peptidase must be identified.
1.7.2. Involvement of Prolyl Oligopeptidase In The Pathogenesis of Neurological and Psychiatric Disorders.

Over the last three decades over forty small peptides have been identified in the mammalian central nervous system (Krieger, 1984; Donlon, 1995). As the distribution, neurochemical, electrophysiological and pharmacological effects of this group of neuromodulators have been studied it has become increasingly evident that these peptide containing neural circuits may be pathologically altered in neuropsychiatric disorders such as major depression, Alzheimer's disease and schizophrenia (Nemeroff and Bissette, 1985). Perturbations in certain peptidase activities could be linked to abnormal levels of these bioactive peptides and in turn lead to neuroendocrine and neurochemical disturbances.

To date a lot of interest has been generated in prolyl oligopeptidases possible role in certain neurological and psychiatric disorders because of its distribution in the brain (Tate, 1981; Kato et al., 1980), and its broad specificity of action on proline-containing bioactive peptides in vitro (Wilk, 1983; Kato et al., 1980; Koida and Walter, 1976).

1.7.2.1. Prolyl Oligopeptidase and Major Depression

Major depression is characterized by numerous neuroendocrine and immunological disturbances that theoretically could be linked to prolyl oligopeptidase activity. The following are just some of the neuroendocrine/immunological hallmarks of major depression.

- Increased concentrations of certain neuropeptides such as TRH (Kirkegaard et al., 1979), and Substance P (Rimon et al., 1984), in cerebrospinal fluid.
- Hypothalamic-Pituitary-Adrenal axis hyperactivity leading to increased secretion of certain neuropeptides such as arginine vasopressin, corticotropin releasing hormone, β-endorphin and adrenocorticotrophic hormone (ACTH), (Maes et al., 1993a, 1991; VonBardeleben et al., 1985).
- Systemic immune activation characterised by T cell activation, B cell proliferation and increased levels of phagocytic cells in peripheral blood (Maes, et al., 1995a, 1994, 1993a).
- An increased production of inflammatory cytokines such as interleukin-1β, interleukin-6 and interferon γ which may underlie both immune activation and an acute phase response (Maes et al., 1993a).
- Moderate autoimmune response (Maes et al., 1993b, Gastpar and Muller, 1981).

A possible role for PO in the pathophysiology of major depression stems from not only its ability to act on neuropeptides such as TRH and substance P in vitro but also suggestions of its possible involvement in the pathogenic mechanisms of inflammatory and autoimmune responses (Aoyagi et
I al, 1984, Shoji et al 1989) and its possible involvement in the modification of T cell function. Several studies have already shown that bidirectional communication exists between the immune and neuroendocrine systems due to shared signal molecules. A number of neuropeptide receptors have been identified on cells of the immune system and the functions of these cells can be affected by neuropeptides. For instance, substance P receptors have been found on lymphocytes and are believed to regulate the functions of these cells (Li et al., 1998). These possible characteristics of PO in vivo prompted studies on a possible link between prolyl oligopeptidase and depression. In 1994, decreased PO levels were observed in serum of major depressed patients in comparison to normal controls (Maes et al., 1994). In fact, PO activity was found to be inversely related to the severity of depression. Further studies attempting to establish a possible role for prolyl oligopeptidase in the pathophysiology of major depression established that treatment of major depressed patients with antidepressants resulted in a significant increase in PO activity. Furthermore, one of these antidepressants, fluoxetine, was found, in vitro, to enhance PO isolated from human platelets (Maes et al., 1995b).

It is still uncertain if diminished PO activity in major depression is involved in the neuroendocrine and immune pathophysiology of this disorder or whether prolyl oligopeptidase levels are simply a marker for this condition. However, the possibility of POs involvement in the modulation of neuroactive peptides in vivo, its role in immune response generation and the significant enhancement of its activity following antidepressant therapy warrants further investigations into the role of PO in major depression.

### 1.7.2.2. Prolyl Oligopeptidase and Schizophrenia

Studies on neuropeptide concentrations in patients with schizophrenia have found significant alterations in levels of these neurally active peptides in comparison to normal controls. Of these neuropeptides, possible PO substrates in vivo, substance P, neurotensin, TRH, β-endorphin, and arginine vasopressin were all found to be significantly altered (Nemeroff et al., 1987). It has been speculated that disorders in peptide metabolizing enzymes may be present in schizophrenia (Wiegant et al., 1988, Beckmann et al., 1984). Low molecular weight hyperpeptiduria is a characteristic in schizophrenia and may be associated with diminished peptidase activity (Reichelt et al., 1981). It has also been shown that certain antipsychotic drugs can induce the activity of some peptidases in vivo, therefore increasing peptide metabolism (Traficante and Turnbull, 1982).

An increased level PO activity was observed in serum samples from schizophrenic patients in comparison to normal controls (Maes et al., 1995b). While this might seem to contradict the observation of low molecular weight hyperpeptiduria in schizophrenia, it has been speculated that a selective reduction of some peptidases together with an increase in the activity of other peptidases such as PO may be a characteristic of this disorder. In addition, it was found that treatment with the
antipsychotic drug valproate reduced PO activity (Maes et al., 1995b). Further evidence for a possible role for PO in schizophrenia includes observed decreases in cerebrospinal fluid arginine vasopressin and neurotensin and postmortem brain TRH. However there has been conflicting reports of an increase in neurotensin and substance P in postmortem brain of schizophrenic patients (Nemeroff et al., 1987).

The evidence for a possible role for PO in psychotic disorders such as schizophrenia and mania is still unclear. However the results suggest that PO could be used as a marker in these conditions.

1.7.2.3. Prolyl Oligopeptidase and Neurodegenerative Disorders

Many studies have been completed on the possible role of PO in neurodegenerative disorders such as Huntington’s, Parkinson’s and Alzheimer’s disease. In 1984 Pittaway et al observed a decreased PO activity in post-mortem brain of subjects with Huntington’s disease. At the time it was speculated that decreased PO activity could be reflective of neuronal cell damage particularly with the evidence that loss of PO activity took place where degeneration was most pronounced (basal ganglia, frontal cortex and substantia nigra). In a complementary study (Hagihara and Nagatsu., 1987) a decrease in PO activity was observed in CSF fluid of Parkinsonian patients. Extensive work has been done in the past decade on the possible role of PO in the pathogenesis of Alzheimer’s disease. In 1990 Gutroy found that the highly potent and PO specific inhibitor, Z-Pro-prolinal induced neuronal degeneration in primary rat hippocampus neurons in vivo, specifically causing an accumulation of phosphorylated neurofilaments. This observation coupled with evidence that PO has a preference for substrates with a phosphoserine in the P1 position and thus may act on phosphorylated peptides in vivo (Rosen et al., 1991), implies a possible contribution by PO to the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease. Several studies involving the measurement of PO levels in post-mortem brain, serum and CSF from AD subjects are in agreement with this data, all reporting a decrease in PO level (Mantle et al., 1996; Yoshida et al., 1996). The question still remains however as to whether PO levels are simply reflective of neuronal cell damage or whether PO can be implicated in the disease process through its degradation of neuropeptides in vivo or in its possible role in cell stress response. It seems unlikely however that PO is simply a “marker” for cell damage as it was observed that PO activity uniformly decreased in both grey and white matter of post-mortem brains of AD subjects (Mantle et al., 1996). Results of studies completed on serum however must be treated with caution due to the discovery of a second Z-Gly-Pro-MCA hydrolysing, Z-Pro-prolinal insensitive peptidase (Cunningham and O’Connor, 1997b).

Other studies on the link between PO and Alzheimer’s disease have attempted to establish a connection between PO activity and the generation of amyloid A4 protein. Amyloid A4 protein has been found in the deposits in senile plaques characteristic of Alzheimer’s disease. This 42 amino acid peptide has a tendency to self aggregate and induce neuronal apoptosis (Yamaguchi et al., 1988;
Amyloid A4 is derived from a larger transmembrane amyloid precursor protein (Haass et al., 1992). It is thought proteolytic processing of this precursor protein at residues 672 and 714 could lead to the formation of the pathogenic amyloid A4 protein (Checler et al., 1995). Using a model synthetic substrate, Suc-Ile-Ala-MCA, which is homologous to the C-terminal portion of the A4 peptide, a peptidase identified as prolyl oligopeptidase was isolated from rat brain (Ishiura et al., 1990). Additional evidence involved the effect of PO specific inhibitors on amyloid A4 protein in cell culture. The specific and potent PO inhibitor ONO-1603 was found to have neuroprotective effects on rat cerebellar granule cells as well as increasing levels of muscarinic cholinergic neurons which are thought to induce normal secretion and processing of amyloid precursor protein (secreted APP is thought to have neuroprotective and neurotrophic functions). It is conceivable that induction of muscarinic cholinergic neurons could normalise the processing and secretion of APP and suppress the production of neurotoxic amyloid A4 peptides (Katsube et al., 1996). Another inhibitor study using the highly specific PO inhibitor, JTP-4819, found that cleavage of the model substrate Suc-Ile-Ala-MCA was inhibited on treatment of N-6108-15 neuronal cell line with this inhibitor. Induced amyloid A4 formation in these cultures was retarded upon addition of this inhibitor to cultures (Shinoda et al., 1997).

Another study that reinforces the link between PO and amyloidogenesis in the brain found that amyloid A4-like immunoreactivity in hippocampus of senesence accelerated mouse matched PO-like immunoreactivity (Funkunari et al., 1994).

While all the evidence accumulated appears to suggest that PO plays some role in the neuropathogenesis of Alzheimer’s disease, specifically through its role in the processing of amyloid precursor protein current evidence suggests that this peptidase is an oligopeptidase incapable of cleaving peptides greater than 25 amino acid residues. There is also the question of PE’s cytosolic location. It is possible that internalised APP could be initially processed by an unknown “B-secretase” at the N-terminal portion of amyloid A4 protein. This fragment could then be acted upon by PE to give the final neurotoxic, amyloidogenic amyloid A4 peptide (Ishiura et al., 1990).

1.7.2. Prolyl Oligopeptidase and Cerebral Ischemia, Memory and Learning

Ever since the development of potent and specific PO inhibitors, several studies have established a possible link between PO and cerebral ischemia effects, memory and learning. A number of PO substrates in vitro, including TRH, Substance P and AVP are known to exert a neuroprotective effect through their ability to potentiate ACh release, thus improving a cholinergic imbalance, and have been shown to improve the performance of animals in memory and learning tasks (Tanaka et al., 1996; Shibata et al., 1992; Griffiths, 1987; DeWied, 1984; Schlesinger et al., 1983). TRH has also been known to prevent excessive release of the neurotoxic glutamate, a characteristic of ischemic episodes (Renaud et al., 1979).
Several specific PO inhibitors have been found to prevent the induction of scopolamine or lesioning induced ischemia, including Z-Pro-prolinal, Z-Val-prolinal, Z-Gly-Pro-CH₂Cl and ZTTA (Shishido et al., 1996, Yoshimoto et al., 1987b). The inhibitor JTP-4819 has been found to enhance the acquisition and retrieval of memory processes, and enhance ACh release in aged rats (Toide et al., 1995). This inhibitor also reversed an age-related decrease of cortical and hippocampal substance P and TRH levels, possibly through the prevention of their inactivation (Shinoda et al., 1995)

It is evident from all of the studies on PO's possible involvement in neuropsychiatric and neurodegenerative disorders, whether its role is simply as a "marker" for cell injury or whether it is directly involved, that further studies are needed to finally clarify its function in the brain.
2. MATERIALS AND METHODS
2.1. Materials

*Sigma Chemical Company (Poole, Dorset, England):*

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

*Bachem Feinchemikalien AG (Bubendorf, Switzerland):*

- Activity-Dependent Neurotrophic Factor-14
- Bradykxin
- (Glu²)-TRH
- Gly-Gly-Pro-Ala
- Substance P
- TRH
- TRH-OH
- Z-Gly-Pro-MCA
(Gly-Pro-Ala)n Polymer
Gly-Pro-MCA
Leu-Gly
LHRH
Lys-Ala-MCA
(Phe)-TRH
pGlu-His-Pro-MCA

Z-Gly-Pro-Ala
Z-Pro-Ala
Z-Pro-Gly
Z-Pro-Leu-Gly
Z-Pro-Pro
Z-Pro-prolinal-dimethylacetate

**BDH Chemicals Ltd. (Poole, Dorset, England):**

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<tr>
<td>Copper Sulphate</td>
<td>Urea</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>Zinc Chloride</td>
</tr>
</tbody>
</table>

**Merck Chemical Company (Frankfurt, Germany):**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulphate</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>Sodium Hydrogen Phosphate</td>
<td></td>
</tr>
</tbody>
</table>

**Pharmacia Fine Chemical Company (Uppsala, Sweden):**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated Thiol Sepharose CL-4B</td>
<td>Phenyl Sepharose CL-4B</td>
</tr>
<tr>
<td>Blue Sepharose Fast Flow</td>
<td>Polybuffer Exchanger 94</td>
</tr>
<tr>
<td>Chelating Sepharose Fast Flow</td>
<td>Polybuffer 74</td>
</tr>
<tr>
<td>DEAE-Sepharose Fast Flow</td>
<td>Sephacryl S200</td>
</tr>
</tbody>
</table>

**Bio-Rad Laboratories (Hercules, California, USA):**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biogel HT Hydroxylapatate</td>
<td></td>
</tr>
<tr>
<td>Bio-Rad Protein Assay Dye Reagent Concentrate</td>
<td></td>
</tr>
</tbody>
</table>
Kevpak Meats (Clonee, Co. Meath, Ireland):
Bovine whole brain
Bovine whole blood

Aldrich Chemical Company (Poole, Dorset, England):
1, 7-Phenanthroline
4, 7-Phenanthroline
Trifluoroacetic acid

Calbiochem-Novabiochem (UK) Ltd. (Nottingham, England):
AEBSF
Angiotensin I
Angiotensin II

Pierce Chemical Company (Illinois, USA):
BCA Reagent

Mount Sinai School of Medicine (New York, Courtesy of Dr. S. Wilk):
Fmoc-Ala-Pro-Nitriole
Fmoc-Pro-Pro-Nitriole
Z-Pro-Proinal

University College Galway (Courtesy of Dr. G. O’Cuinn):
Gly-Ala-Phe
Gly-Pro-Ala

University Rene Descartes (Paris, France, Courtesy of Prof. B. Roques):
Kelatorphan
Institute of Microbial Chemistry (Tokyo, Japan, Courtesy of Dr. M. Nagai):

Postatin

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

Z-Indoliny1 Prolinal

Z-Cyclohexyl Prolinal

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

α-Ketobenzothiazole

Z-Pro-Prolinal

Hans-Knoell Institute of Natural Product Research (Halle, Germany, Courtesy of Dr. H.U. Demuth):

Amyloid Precursor Protein 37-44 Z-Phe-Ala-Chloromethylketone

Boc-Glu(NHO-Bz)-Pyr

ACTH 18-35

Z-Phe-Pro-Methylketone

Recombinant Flavobacterium meningosepticum PO

Nagasaki University (Japan, Courtesy of Prof. T. Yoshimoto):

Z-Thiopropyl Prolinal
2.2. Fluorescence Spectrophotometry using 7-Amino-4-Methyl-Coumarin (MCA)

2.2.1. MCA Standard Curves
250μM stock MCA solution in 8% DMSO was prepared in 100mM potassium phosphate pH 7.4 at 37°C. This stock solution was stored at 4°C for up to one month. Lower MCA concentrations were prepared using 100mM potassium phosphate pH 7.4 as diluent. MCA standard curves were prepared by combining 100μL of 100mM potassium phosphate pH 7.4, 400μL of appropriate MCA concentration and 1mL of 1.5M acetic acid. Standard curves between the ranges 0-10μM and 0-100μM MCA were prepared in triplicate. Fluorimetric analysis of these samples was achieved using a Perkin Elmer LS50 Fluorescence Spectrophotometer at excitation and emission wavelengths of 370nm and 440nm respectively. Excitation slit widths were maintained at 10nm while emission slit widths were adjusted to produce fluorimetric intensities appropriate for the range being analysed.

2.2.2. Inner Filter Effect
The inner filter or 'quenching' effect of enzyme samples was determined by combining 100μL of enzyme sample, 400μL of appropriate MCA dilution and 1mL 1.5M acetic acid. These samples were prepared in triplicate and assayed as outlined in section 2.2.1.

2.3. Protein Determination

2.3.1. Absorbance at 280nm
The absorbance of proteins based on the λ max of tryptophan residues at 280nm was used as a qualitative method of determining protein levels in post-column chromatography fractions during purification procedures. A Shimadzu UV 160A Spectrophotometer was used to determine this absorbance.

2.3.2. Standard BCA Assay
The standard BCA protocol was used to quantify protein concentrations of samples between the range of 0-2mg/mL. Samples were dialysed for 12 hours against 10mM potassium phosphate pH 7.4 to remove any interfering substances. Samples with a protein concentration above 2mg/mL were diluted to a suitable protein concentration with 10mM potassium phosphate pH 7.4. 200μL of BCA reagent was added to 10μL of sample in triplicate in 96 well microplate and incubated for 30min at 37°C. 0-
2mg/mL BSA standard curves were prepared in triplicate. The absorbance of each well was determined at 560nm using a Titertek Multiscan PLUS plate reader.

2.3.3. **Enhanced BCA Assay**

The enhanced BCA assay protocol was used to quantify protein concentrations of samples that could not be determined by the less sensitive standard BCA assay protocol. This assay was performed as described in section 2.3.2 using a sample/standard volume of 20μL and an incubation temperature of 60°C. 0-500μg/mL BSA standard curves were prepared in triplicate.

2.3.4. **Biorad Protein Microassay**

The Biorad protein microassay was used to quantify protein concentrations that could not be determined by the less sensitive enhanced BCA assay protocol. Samples were dialysed for 12 hours against 10mM potassium phosphate pH 7.4 to remove any interfering substances. 200μL of Biorad reagent was added to 800μL of sample in triplicate and incubated for 5 minutes at room temperature. BSA standard curves in the range 0-25μg/mL were also prepared in triplicate. The absorbance of each sample was determined at 595nm using a Shimadzu UV 160A Spectrophotometer.

2.4. **Enzyme Assays**

2.4.1. **Quantitative Measurement of Prolyl Oligopeptidase Activity**

Z-Gly-Pro-MCA degrading activity was determined according to a modification of the original procedure of Yoshimoto et al., (1979). 10mM Z-Gly-Pro-MCA substrate stock was prepared in 100% DMSO. 100mM potassium phosphate with 5mM DTT and 0.5mM EDTA, pH 7.4 at 37°C was added slowly to 600μL DMSO and 200μL substrate stock to a final volume of 10mL. This gave a final substrate concentration of 200μM in 8% DMSO (v/v). 400μL of substrate solution was added to 100μL of sample in triplicate. This reaction mixture was incubated for 30 minutes at 37°C. Both samples and substrate were preincubated at 37°C to allow them to reach thermal equilibrium. The reaction was terminated by the addition of 1mL of 15M acetic acid. Negative controls were prepared by the addition of 1mL of acetic acid to the sample prior to the addition of substrate. These controls were also incubated at 37°C for 30 minutes. MCA released from the substrate was determined fluorimetrically as described in section 2.2.1. Fluorimetric intensities obtained for each sample were converted to picomoles MCA released per min per mL using standard curves prepared as outlined in section 2.2.1. Enzyme units were defined as pmoles of MCA released per min at 37°C.
2.4.2. Quantitative Measurement of Z-Pro-Prolinal-Insensitive Z-Gly-Pro-MCA Hydrolysing Activity

Z-Gly-Pro-MCA substrate was prepared as outlined in section 2.4.1. 400μL of substrate solution was added to 80μL of sample which had been preincubated for 15 minutes at 37°C with 20μL of 5x10^{-4}M Z-Pro-prolinal (10% v/v methanol). This reaction mixture was terminated by the addition of 1mL of 1.5M acetic acid. Negative controls were prepared as in section 2.4.1 incorporating Z-Pro-prolinal. MCA released was determined fluorometrically as described in section 2.2.1. Fluorimetric intensities obtained for each sample were converted to picomoles MCA released per min per mL using standard curves (incorporating Z-Pro-prolinal), prepared as outlined in section 2.2.1. Enzyme units were defined as picomoles MCA released per min at 37°C.

2.4.3. Non-Quantitative Microplate Procedure for Measurement of Prolyl Oligopeptidase Activity

This assay was developed to assist in the identification of post column chromatography PO containing fractions. Substrate was prepared as outlined in section 2.4.1. 100μL of substrate solution was added to 100μL of sample in each well and the microplate was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 100μL of 1.5M acetic acid and MCA liberated was measured using a Perkin Elmer LS50 Fluorescence Spectrophotometer with a plate reader attachment.

2.4.4. Non-Quantitative Microplate Procedure for Measurement of Z-Pro-Prolinal Insensitive Z-Gly-Pro-MCA Hydrolysing Activity

As outlined in section 2.4.3, except 100μL of substrate was added to 20μL of 5x10^{-4}M Z-Pro-prolinal and 100μL of sample.

2.5. Partial Purification of Prolyl Oligopeptidase from Bovine Serum

2.5.1. Bovine serum production

Bovine whole blood was collected from a freshly slaughtered animal. This was then stored at 4°C for 24 hours to allow clot formation. The remaining unclotted whole blood was decanted and centrifuged at 6000rpm (4000g) for 1 hour at 4°C using a Beckman J2-MC centrifuge fitted with a pre-cooled JA21 rotor. The supernatant/serum was removed, pooled and divided into 20mL aliquots for storage at -17°C.

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2.5.2. Phenyl Sepharose CL-4B Hydrophobic Interactions Chromatography I

A 40mL Phenyl Sepharose CL-4B hydrophobic interactions column (2.5x6cm) was equilibrated with 200mL (5 bed volumes) of 200mM ammonium sulphate in 100mM potassium phosphate, 5mM DTT, 0.5mM EDTA, pH 7.4. Solid ammonium sulphate was dissolved in 20mL of bovine serum to give a final concentration of 200mM. This sample was applied to the column which was then washed with 150 mL of equilibration buffer. Bound protein was eluted with a distilled water wash. 5mL fractions were collected throughout the procedure at a flow rate of 1mL/min. The entire procedure was carried out at 4°C. Fractions were assayed for PO and ZIP activity using the fluorimetric assays as outlined in sections 2.4.3 and 2.4.4, respectively. Protein levels in each fraction were determined by measuring their absorbance at 280nm. Fractions containing PO activity were pooled and enzyme activity and protein levels were quantified using the fluorimetric assay as outlined in section 2.4.1 and the standard BCA assay as outlined in section 2.3.2. Samples were dialysed against 10mM potassium phosphate buffer pH 7.4, (section 2.3.2) prior to protein analysis.

2.5.3. Phenyl Sepharose CL-4B Hydrophobic Interactions Chromatography II

A 20mL Phenyl Sepharose CL-4B hydrophobic interactions column (2.5x3cm) was equilibrated with 100mL of 1M ammonium sulphate in 100mM potassium phosphate, 5mM DTT, 0.5mM EDTA, pH 7.4. Solid ammonium sulphate was dissolved in the post phenyl sepharose pool I (pH was maintained at 7.4 using 1M NaOH), to give a final concentration of 1M. This sample was centrifuged for 30 minutes at 15,000rpm at 4°C using a Beckman J2-MC centrifuge fitted with a pre-cooled JA20 rotor. The resulting supernatant was applied to the column which was then washed with 100mL of equilibration buffer. Bound protein was eluted with a 100mL linear gradient of 1-OM ammonium sulphate, 100-10mM potassium phosphate and 0-15% v/v glycerol, pH 7.4. The column was then washed with 60mL of 10mM potassium phosphate with 15% (v/v) glycerol, 5mM DTT, 0.5mM EDTA, pH 7.4. Fractions were collected, assayed and pooled as outlined in section 2.5.2.

2.6. Purification of Prolyl Oligopeptidase from Bovine Brain

All steps were performed at 4°C unless otherwise stated.

2.6.1. Bovine Brain Preparation

Bovine whole brain was obtained from a freshly slaughtered animal. This brain was divided into 50g slices for storage at -80°C. A 50g brain slice was defrosted and homogenised, using a Sorvall Omni Mixer in 200mLs of ice-cold 100mM potassium phosphate buffer with 5mM DTT, 0.5mM EDTA, pH 7.4. The resulting homogenate was centrifuged for 45 minutes at 15,000rpm using a Beckman J2-MC
refrigerated centrifuge fitted with a JA-20 rotor. The supernatant (S1) was retained. The remaining pellets were resuspended in 100mLs ice-cold distilled water (osmotic shock step to remove any occluded prolyl oligopeptidase) and centrifuged as before. The supernatant (S2) was combined with the first supernatant (S1). The combined supernatants were then ultracentrifuged at 38,000rpm (100,000g) using a Beckman L8-M Ultracentrifuge fitted with a 70Ti rotor. The resulting supernatants were pooled and divided into 40mL aliquots for storage at -80°C

2.6.2. **Ammonium Sulphate Precipitation**
Solid ammonium sulphate was added to 40mLs of post ultracentrifugation aliquot with constant stirring to give 45% saturation (pH was adjusted with 1M NaOH). After 1 hour of constant stirring this aliquot was centrifuged for 45 minutes at 15,000rpm (see section 2.6.1). The resulting supernatants (S1) were pooled and solid ammonium sulphate was added as before to give 75% saturation. After 1 hour of constant stirring the aliquot was centrifuged as before. The pellets (P2) were resuspended in a total volume of 5mL Tris-HCl with 5mM DTT, 0.5mM EDTA, pH 8.0 at 4°C.

2.6.3. **DEAE Sepharose Fast Flow Anion Exchange Chromatography**
The post ammonium sulphate PO aliquot was dialysed for 12 hours against 3L of 50mM Tris-HCl buffer with 5mM DTT and 0.5mM EDTA pH 8.0 at 4°C. A 20mL DEAE sepharose column (2.5x3cm) was equilibrated with 100mL of 50mM Tris/HCl with 5mM DTT, 0.5mM EDTA pH 8.0. The dialysed sample was applied to the column which was washed with 100mLs of equilibration buffer. Bound activity was eluted with a 100mL linear 0-350mM NaCl gradient in Tris-HCl buffer, 5mM DTT, 0.5mM EDTA pH 8.0 followed by a 60mL 350mM NaCl wash in 50mM Tris-HCl, 5mM DTT, 0.5mM EDTA pH 8.0. 5mL fractions were collected and assayed for PO activity using the qualitative microplate assay procedure as outlined in section 2.4.1. Protein determinations for each fraction were made qualitatively by measuring their absorbance at 280nm. Protein levels in post DEAE pool were determined quantitatively using the BCA standard microplate assay protocol as outlined in section 2.3.2 after overnight dialysis against 5L of 25mM potassium phosphate pH 7.4. All equilibration, loading, washing and elution steps were carried out at 1mL/min.

2.6.4. **Sephacryl S-200 Sepharose Gel Filtration Chromatography**
Post DEAE sepharose pool was concentrated to 2mL by reverse osmosis using polyethylene glycol. Glycerol was added to reverse dialysed sample to give a 10% v/v final concentration. A 230mL Sephacryl S200 column (2.5cmx47cm) was equilibrated with 300mL of 25mM imidazole-HCl with
5mM DTT and 0.5mM EDTA, pH 6.3. The reverse dialysed sample was applied, using a syringe to the top of the resin and the column was washed with 300mL of equilibration buffer. 5mL fractions were collected and assayed for PO activity using the microplate procedure as outlined in section 2.4.3. Samples of fractions were dialysed overnight against 10mM potassium phosphate buffer, pH 7.4 for protein determinations using the BCA enhanced microplate assay as outlined in section 2.3. Samples of fractions were dialysed against 10mM potassium phosphate buffer, pH 7.4 and pooled. Equilibration and elution steps were carried out at 1mL/min.

2.6.5. PBE94 Chromatofocusing
Post sephacryl S200 HR pool was dialysed for 3 hours against 2L of 25mM imidazole/HCl with 5mM DTT, 0.5mM EDTA, pH 6.3. Buffer was changed after 1 and 2 hours. A 35mL PBE 94 column (1.5cm x 20cm) was equilibrated with 1L of 25mM imidazole/HCl with 5mM DTT, 0.5mM EDTA, pH 6.3, followed by a 20mL wash with 1:8 dilution of polybuffer 74, with 5mM DTT, 0.5mM EDTA, pH 4.5. The buffer head of the column was allowed to fall to just above the top of the resin and dialysed post sephacryl S200 HR pool was applied. The column was then washed with 250mL of 1:8 diluted polybuffer 74, 5mM DTT 0.5mM EDTA, pH 4.5. 5mL fractions were collected and assayed for PO activity using the microplate procedure as outlined in section 2.4.3. Samples of fractions were dialysed against 10mM potassium phosphate buffer, pH 7.4 and protein was determined using the Biorad microassay procedure as outlined in section 2.3. PO containing fractions were pooled. Equilibration, loading and elution steps were carried out at 1mL/min.

2.6.6. Phenyl Sepharose CL-4B Hydrophobic Interactions Chromatography
A 20mL phenyl sepharose CL-4B Hydrophobic Interactions column (2.5x3cm) was equilibrated with 100mL of 50mM potassium phosphate buffer with 5mM DTT, 0.5mM EDTA and 1M ammonium sulphate, pH 7.4. Solid ammonium sulphate was added to the post chromatofocusing pool to bring it to a final concentration of 1M. Sample was applied to column which was then washed with 50mL of equilibration buffer. Bound activity was eluted with a 50mL linear gradient of 1-0M ammonium sulphate, 50-10mM potassium phosphate, 0-15% v/v glycerol gradient, followed by a 60mL wash of 10mM potassium phosphate buffer with 5mM DTT, 0.5mM EDTA and 15% v/v glycerol, pH 7.4. 5mL fractions were collected and assayed for PO activity using the microplate procedure as outlined in section 2.3.3. Samples of fractions were dialysed against 10mM potassium phosphate buffer, pH 7.4 for protein measurements using the Biorad microassay procedure as outlined in section 2.3.4. PO containing fractions were pooled and stored with 0.5% w/v protease-free BSA at -80°C until further use. Equilibration step was carried out at 2mL/min while loading, washing and elution steps were carried out at 1mL/min.
2.7. Determination of Purity of Purified Bovine Brain Prolyl Oligopeptidase

2.7.1. Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was used to determine the purity of the post phenyl sepharose PO pool. Electrophoresis was also used to determine the molecular weight of this enzyme.

2.7.1.1. Sample Preparation

Samples generated over the duration of the bovine brain PO purification (crude brain homogenate to post phenyl sepharose pool) were used in electrophoresis. These samples were dialysed for 24 hours against 1L of 62.5mM Tris/HCl, pH 6.8 with buffer changes at 3, 6 and 12 hours. Dialysed samples were then diluted with an equal volume of solubilisation buffer (62.5mM Tris/HCl, pH 6.8, 20% v/v glycerol, 8% w/v SDS, 10% v/v 2-mercaptoethanol and 0.01% w/v bromophenol blue). Silverstain high molecular weight standards were also prepared. These standards consisted of Carbonic Anhydrase (29,000Da), Fumarase (48,500Da), Bovine Serum Albumin (66,000Da), Phosphorylase B (97,000Da) and β-Galactosidase (116,000Da). Both samples and markers were boiled for 60 seconds and kept on ice until use.

2.7.1.2. Preparation of SDS gels

10% resolving gel and 3.75% stacking gel were prepared according to Table 2.7.1 using the following stock solutions which were prepared in deionised water.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolving Gel Buffer</td>
<td>3M Tris/HCl, pH 8.8</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>0.5M Tris/HCl, pH 6.8</td>
</tr>
<tr>
<td>Acryl/Bisacryl Stock</td>
<td>30% w/v Acrylamide, 0.8% Bisacryl</td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>1.5% Ammonium Persulphate</td>
</tr>
<tr>
<td>SDS</td>
<td>10% w/v SDS</td>
</tr>
<tr>
<td>Running Buffer</td>
<td>0.025M Tris/HCl, 0.192M Glycine, 0.1% SDS, pH 8.3</td>
</tr>
</tbody>
</table>

A 160mmx160mmx1mm 10% stacking gel overlayed with a 3.75% stacking gel was cast in an Atto vertical electrophoresis system. 20μL of sample or standard was loaded into the wells and gels were electrophoresed for 4 hours at a constant current of 25mA/gel.
Solution | Volume Required for | Volume Required for |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% Resolving Gel</td>
<td>3.75% Stacking Gel</td>
</tr>
<tr>
<td>Acryl/Bisacryl Stock</td>
<td>10mL</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Resolving Gel Buffer</td>
<td>3.75mL</td>
<td>-</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>-</td>
<td>5mL</td>
</tr>
<tr>
<td>SDS</td>
<td>300μL</td>
<td>200μL</td>
</tr>
<tr>
<td>Deionised Water</td>
<td>14.45mL</td>
<td>11.3mL</td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>1.5mL</td>
<td>1mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>15μL</td>
<td>15μL</td>
</tr>
</tbody>
</table>

Table 2.7.1. **SDS Gel Electrophoresis Gel Preparation**

2.7.1.3. Visualising Proteins in Polyacrylamide Gel-Silver Staining

Polyacrylamide gels were stained using a Sigma (AG-25) Silver Stain kit, which was based on the method of Heukeshoven and Dernick (1985) Table 2.7.2 outlines the steps involved. It should be noted that a number of steps in the original silver staining procedure including a reducing step after development were found to be unnecessary. An image of the stained gel was obtained using a UVP ImageStore 7500 with a UVP white/UV translumminator camera unit driven by ImageStore 7500 software.

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Volume</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>30% v/v Ethanol, 10% v/v Glacial Acetic Acid</td>
<td>3x300mL</td>
<td>3x20 min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Deionised water</td>
<td>3x300mL</td>
<td>3x10 min</td>
</tr>
<tr>
<td>Silver Staining</td>
<td>Silver Nitrate</td>
<td>300mL</td>
<td>30 min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Deionised Water</td>
<td>300mL</td>
<td>10 sec</td>
</tr>
<tr>
<td>Developing</td>
<td>Sodium carbonate/Formaldehyde</td>
<td>150mLx2</td>
<td>8mmx2</td>
</tr>
<tr>
<td>Development Stop</td>
<td>1% v/v Glacial Acetic Acid</td>
<td>300mL</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7.2. **Silver Staining Procedure**
2.7.2. **Fluorimetric Assays**

Purified bovine brain PO activity was assayed for various peptidase activities as outlined in section 2.4.1, replacing 200 μM Z-Gly-Pro-MCA substrate with various quenched fluorimetric substrates. These substrates, with details of their preparation are listed in Table 2.7.3. All assays were performed in triplicate and suitable negative controls were included.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity Detected</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-MCA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Alanine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Arg-MCA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Arginine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Arg-MCA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Dipeptidyl Aminopeptidase I</td>
<td>Buffer</td>
</tr>
<tr>
<td>Gly-Pro-MCA&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Dipeptidyl Aminopeptidase IV</td>
<td>Buffer</td>
</tr>
<tr>
<td>Leu-MCA&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Leucine Aminopeptidase</td>
<td>Buffer</td>
</tr>
<tr>
<td>Lys-Ala-MCA&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Dipeptidyl Aminopeptidase II</td>
<td>Buffer</td>
</tr>
<tr>
<td>pGlu-MCA&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Pyroglutamyl Aminopeptidase I</td>
<td>4% v/v DMSO</td>
</tr>
<tr>
<td>*pGlu-His-Pro-MCA&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Pyroglutamyl Aminopeptidase II</td>
<td>4% v/v DMSO</td>
</tr>
<tr>
<td>Z-Arg-MCA&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Trypsin</td>
<td>8% v/v DMSO</td>
</tr>
<tr>
<td>*Z-Gly-Pro-MCA&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Z-Pro-Prolinal Insensitive Prolyl</td>
<td>8% v/v DMSO</td>
</tr>
</tbody>
</table>

Table 2.7.3. **Fluorimetric substrates used to determine the presence or absence of contaminating peptidase activity.**

The buffer used in all preparations was 100 mM potassium phosphate, pH 7.4. This was heated to 37°C prior to dissolution of substrate. Where required, DMSO was used to dissolve substrate prior to addition of buffer.

* Assay was performed in the presence and absence of Z-Pro-prolinal, which was incorporated in order to inhibit PO activity and thus eliminate the possibility of interference (see section 2.4.2.)

1 Mantle *et al.*, (1983)
3 Chan *et al.*, (1985)
4 Checler *et al.*, (1985)
5 Kagga *et al.*, (1998)
6 Nagatsu *et al.*, (1985)
7 Cummins and O’Connor (1996)
9 Nishikata *et al.*, (1985)
10 Cunningham and O’Connor (1998)
2.8. Purified Prolyl Oligopeptidase Assay Development

2.8.1. Solvent Effects on Purified Prolyl Oligopeptidase Activity and Assay Sensitivity
The effect of different concentrations of various solvents on PO activity, and substrate solubility in these solvents was investigated. 10mM stock of Z-Gly-Pro-MCA was prepared in 100% DMSO, DMF, dioxane, methanol and ethanol. This stock substrate solution was diluted with 100mM potassium phosphate buffer with 5mM DTT and 0.5mM EDTA, pH 7.4 and appropriate solvent to give a final concentration of 200µM substrate with 5-10% v/v solvent. Purified PO was assayed in triplicate with these substrates as outlined in section 2.4.1.

2.8.2. Linearity of Purified Prolyl Oligopeptidase Activity Assay with Respect to Time

2.8.2.1. Discontinuous Assay
Purified PO activity which had been stored in the presence and absence of 0.5% w/v BSA, was assayed as outlined in section 2.4.1. Reactions were stopped with 1mL of 1.5M acetic acid after 10, 20, 30, 40, 50 and 60 min. Plots of fluorescent intensity versus time were prepared.

2.8.2.2. Continuous Assay
Purified PO activity (with 0.5% v/v BSA) was diluted 1/5 with 100mM potassium phosphate buffer with 0.5% v/v BSA, pH 7.4 and was preincubated for 15 min at 37°C. 100µM and 200µM Z-Gly-Pro-MCA in 4 and 8% v/v DMSO respectively was also preincubated until it reached thermal equilibrium. 300µL of enzyme was added to 1.2mL of substrate in a cuvette holder which was kept at 37°C. Enzyme activity/MCA liberated was continuously monitored over 40 minutes, with readings taken every 2 seconds. Plots of fluorescent intensity versus time were prepared.

2.8.3. Linearity of Purified Prolyl Oligopeptidase Assay with Respect to Enzyme Concentration
A range of dilutions of purified PO were prepared in 50mM potassium phosphate buffer with 5mM DTT, 0.5mM EDTA, pH 7.4. Enzyme dilutions were assayed in triplicate as outlined in section 2.4.1.
2.8.4. Effect of DTT on Prolyl Oligopeptidase Activity Assay Sensitivity

2mL aliquots of 200μM Z-Gly-Pro-MCA, 8% v/v DMSO were prepared incorporating 0-100mM DTT. Purified PO activity was assayed in triplicate as outlined in section 2.4.1.

2.8.5. Effect of EDTA on Prolyl Oligopeptidase Activity Assay Sensitivity

2mL aliquots of 200μM Z-Gly-Pro-MCA, 8% v/v DMSO, were prepared incorporating 0-150mM EDTA and purified PO activity was assayed in triplicate as outlined in section 2.4.1.

2.8.6. Effect of NaCl on Prolyl Oligopeptidase Activity Assay Sensitivity

2mL aliquots of 200μM Z-Gly-Pro-MCA, 8% v/v DMSO were prepared incorporating 0-1 5M NaCl and PO activity was assayed in triplicate as outlined in section 2.4.1.

2.9. Characterisation of Purified Prolyl Oligopeptidase Activity

2.9.1. Relative Molecular Mass Determination

The relative molecular mass of prolyl oligopeptidase was determined using gel filtration chromatography and polyacrylamide gel electrophoresis.

2.9.1.1. Sephacryl S-200 HR Gel Filtration Chromatography

A 230mL (2.5cm x 47cm) Sephacryl S-200 HR gel filtration column was used for this procedure. Column was run at 4°C at a flow rate of 1mL/min for the entire experiment.

2.9.1.1. Void Volume Determination

Sephacryl S200 column was equilibrated with 300mL of 50mM potassium phosphate buffer with 150mM NaCl, pH 7.4. 2mL of a 2mg/mL blue dextran solution containing 10% v/v glycerol was applied directly onto the column resin, under buffer, using a syringe. Column was washed with equilibration buffer until blue dextran had visibly eluted. 2.5mL fractions were collected throughout the procedure. The absorbance of each fraction was measured at 620nm and the volume at which this absorbance reached a maximum was taken to be the void volume (Vo).

2.9.1.2. Column Calibration using Molecular Mass Standards

Sephacryl S-200 column was prepared and equilibrated as outlined in section 2.9.1.1. 2mL of each standard containing 10% v/v glycerol was applied onto column which was then washed with...
equilibration buffer 2.5mL fractions were collected and assayed for protein using the standard BCA
assay as outlined in section 2.3.2. The elution volume (Ve) for each standard was taken as the volume
in which the absorbance at 595nm reached a maximum. A calibration curve of Log molecular mass
versus Ve/Vo was prepared. Protein standards used in this procedure were as follows: Cytochrome C
(12,400Da), Carbonic Anhydrase (29,000Da), Bovine Serum Albumin (66,000Da), Alcohol
Dehydrogenase (150,000 Da) and β-Amylase (200,000Da).

2.9.1.3. Determination of Relative Molecular Mass of Purified Enzyme

Sephacryl S-200 column was prepared and equilibrated as outlined in section 2.9.1.1. 2mL of
purified prolyl oligopeptidase sample with 10% v/v glycerol was applied onto column which was then
washed with equilibration buffer. 2.5mL fractions were collected and assayed for PO activity using
the fluorimetric assay outlined in section 2.4.1. The elution volume (Ve) for the enzyme was taken as
the volume at which maximum PO activity was observed. The molecular mass of the enzyme was
determined using the calibration curve constructed in section 2.9.1.2.

2.9.1.2. SDS Polyacrylamide Gel Electrophoresis

The SDS polyacrylamide gel electrophoresis of samples and standards has already been outlined in
section 2.7.1. In order to determine the molecular mass of the purified enzyme a calibration curve of
the Log molecular mass versus the Relative Mobility (Rf) of each standard was constructed. The Rf
value was determined by dividing the distance migrated by each standard, by the distance migrated by
the bromophenol blue dye front. It was then possible, from this calibration curve to estimate the
molecular mass of the purified enzyme, using its calculated Rf value.

2.9.2. pH Effects

2.9.2.1. pH Activity Profile

10mL of post phenyl sepharose PO pool was dialysed overnight against distilled water. 50µL of
dialysate was preincubated at 37°C for 15 minutes with 50µL of appropriate 50mM buffer at various
pHs. 200µM Z-Gly-Pro-MCA was prepared in a range of 50mM buffer systems at various pH values
outlined in table 2.9.1. Enzyme activities were determined in triplicate as outlined in section 2.4.1,
using substrate prepared in a pH range of 2.5-10.5.

2.9.2.2. pH Inactivation Profile.

10mL of post phenyl sepharose PO pool was dialysed overnight against distilled water. 50µL of
dialysate was preincubated at 37°C for 15 minutes with 50µL of 50mM buffer at various pHs (2.5-
10.5). 200µM Z-Gly-Pro-MCA was prepared as outlined in section 2.4.1, using 50mM potassium

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phosphate buffer pH 7.4. Enzyme activities were determined in triplicate as outlined in section 2.4.1. The various buffer systems used are outlined in table 2.9.1.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid / Dibasic Potassium Phosphate</td>
<td>2.5-6.0</td>
</tr>
<tr>
<td>Potassium Phosphate</td>
<td>6.0-8.0</td>
</tr>
<tr>
<td>Tris / HCl</td>
<td>8.0-9.0</td>
</tr>
<tr>
<td>Glycine / NaOH</td>
<td>9.0-10.0</td>
</tr>
</tbody>
</table>

Table 2.9.1.  
Buffer Species and Ranges used in pH Activity and Inactivation Profiles

2.9.3. Thermostability Studies on Purified Prolyl Oligopeptidase Activity

2.9.3.1. Effect of Assay Temperature on Purified Prolyl Oligopeptidase Activity
Purified PO activity was assayed at 4, 20, 37, 45, 50 and 60°C, by a modification of the assay described in section 2.4.1. Both the enzyme sample and the substrate used were preincubated at the appropriate temperature for 15 minutes prior to the assay.

2.9.3.2. Effect of Preincubation of Purified Prolyl Oligopeptidase Activity at Various Temperatures for Different Times
Purified PO activity was preincubated for 0, 15, 30, and 45 min. at 20, 37, 40, 50 and 60°C and assayed as outlined in section 2.4.1.

2.9.4. Effect of Divalent Metal Salts on Purified Prolyl Oligopeptidase Activity
Purified PO activity was dialysed for 18 hours against 1.5L of either 20mM potassium phosphate buffer, pH 7.4 or 20mM Tris/HCl pH 7.4 (see table 2.9.2.), with a buffer change after 12 hours. 2mM stocks of divalent metal salts were prepared as outlined in table 2.9.2. pH of stock solutions were adjusted to 7.4 using 20mM monobasic or dibasic potassium phosphate where appropriate or 15M HCl when Tris buffer was used. 50μL of dialysate was preincubated for 15 minutes with 50μL of...
metal salt and assayed as outlined in section 2.4.1 with the exception that DTT and EDTA were
omitted from substrate. Suitable negative and positive controls were prepared and standard curves
incorporating each divalent metal salt were prepared to allow for any quenching effects.

<table>
<thead>
<tr>
<th>Metal Salt</th>
<th>Concentration (mM)</th>
<th>Preparation and Dialysis Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>CoSO₄*</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>2</td>
<td>20mM Potassium Phosphate buffer</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>2</td>
<td>20mM Potassium Phosphate buffer</td>
</tr>
<tr>
<td>HgSO₄**</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>2</td>
<td>20mM Potassium Phosphate buffer</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>NiSO₄</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>2</td>
<td>20mM Tris/HCl</td>
</tr>
</tbody>
</table>

Table 2.9.2. *Preparation of Divalent Metal Salts*

*Metal salt dissolution assisted by boiling water bath

**Metal salt initially dissolved in 1.5M HCl and then diluted to appropriate concentration with Tris
buffer

2.9.5. **Effect of Functional Reagents on Purified Prolyl Oligopeptidase Activity**

40mL of purified PO activity was dialysed for 18 hours against 2.5L of 100mM potassium phosphate
buffer, pH 7.4 Dialysis buffer was changed after 6 and 12 hours. 50μL of dialysed enzyme sample
was preincubated at 37°C for 15 minutes with 50μL of appropriate functional reagent. Functional
reagents were prepared as outlined in Table 2.9.3. After the incubation period, enzyme assays were
carried out in triplicate as outlined in section 2.4.1. Suitable negative and positive controls were also
prepared. Positive controls incorporated 50μL of relevant diluent without functional reagent, for
example 5% v/v acetone, to replace the functional reagent. Quenched standard curves incorporating
each relevant functional reagents were also prepared. Results were converted into enzyme units/mL
and expressed as a percentage of positive control activity (100%).
<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Compound Name</th>
<th>Concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine Protease</td>
<td>Iodoacetamide</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>Iodoacetate</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>N-Ethylmaleimide (NEM)</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Dithio-bis-nitro-benzoic acid (DTNB)</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Cysteine Protease</td>
<td>Dithiothreitol (DTT)</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Activators</td>
<td>2-Mercaptoethanol</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Serine Protease</td>
<td>Aminoethyl-Benzene sulfonylfluoride</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>N-Ethylmaleimide (NEM)</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Dithio-bis-nitro-benzoic acid (DTNB)</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Metallo Protease</td>
<td>EDTA</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>EGTA</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>CDTA</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>8-Hydroxyquinoline</td>
<td>20mM</td>
<td>5% v/v Acetone</td>
</tr>
<tr>
<td></td>
<td>Imidazole</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>1, 10-Phenanthroline</td>
<td>20mM</td>
<td>5% v/v Acetone</td>
</tr>
<tr>
<td></td>
<td>1, 7-Phenanthroline</td>
<td>20mM</td>
<td>5% v/v Acetone</td>
</tr>
<tr>
<td></td>
<td>4, 7-Phenanthroline</td>
<td>20mM</td>
<td>5% v/v Acetone</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Aprotinin</td>
<td>1mg/mL</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Bacitracin</td>
<td>2mg/mL</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Benzamidine</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Carnitine</td>
<td>20mM</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>N-Decanoyl Coenzyme A</td>
<td>2mg/mL</td>
<td>Buffer</td>
</tr>
<tr>
<td></td>
<td>Pepstatin</td>
<td>1mg/mL</td>
<td>5% v/v Acetone</td>
</tr>
<tr>
<td></td>
<td>Soyabeen Trypsin Inhibitor</td>
<td>2mg/mL</td>
<td>Buffer</td>
</tr>
</tbody>
</table>

**Table 2.9.3. Preparation of Functional Reagents**

1 All functional reagents were prepared in 50mM potassium phosphate buffer with or without 5% v/v acetone. Reagents prepared in acetone were initially dissolved in 100% acetone and adjusted to stock concentration upon addition of 50mM potassium phosphate buffer. Adjustment of pH to 7.4 was achieved through use of 50mM monobasic and dibasic potassium phosphate buffer which were added, where appropriate during preparation of stock solutions.

2 Complete dissolution was achieved by heating in a boiling water bath.
2.9.6. **Substrate Specificity Studies on Purified Prolyl Oligopeptidase Activity**

The substrate specificity of purified bovine brain PO activity for a range of both natural and synthetic proline containing peptides was determined using reverse phase HPLC, MALDI-TOF mass spectrometry and fluorometry. Some comparative studies were carried out using the purified bovine brain, *F. meningosepticum*, porcine kidney and partially purified bovine serum prolyl oligopeptidase activities.

2.9.6.1. **Substrate Specificity Studies Using Reverse Phase HPLC**

The HPLC system used consisted of a Beckman System Gold Programmable Dual Pump Module 126, Photodiode Array Detector Module 128 and Autosampler 507. The column used was a Beckman Ultrasphere C-8 reverse phase column (4.6mmx200mm) and a Beckman Ultrasphere C18 reverse phase guard column (4.6mmx45mm). All solvents and buffers used in sample preparation and chromatography were filtered and degassed. Ultrapure water was used in buffer preparation.

2.9.6.1.1. **Preparation of Stock Substrates and Standards**

3mL of 1.3mM substrate/standard was prepared as outlined in Table 2.9.4. Briefly, samples were initially dissolved in 300μL of appropriate solvent (when required), and diluted to 3mL with 100mM potassium phosphate buffer pH 7.4.

2.9.6.1.2. **Reaction of Substrates With Prolyl Oligopeptidase Enzyme Activities**

50μL of enzyme sample was added to 200μL of substrate. The reaction was allowed to proceed at 37°C for 24 hours. Reactions were then terminated by the addition of 25μL of 5% (v/v) TFA. Suitable negative controls and blanks were prepared.

2.9.6.1.3. **Reverse Phase HPLC of Samples**

Mobile phases for the HPLC consisted of solvent A, 98.8% MeOH, 0.2% (v/v) TFA and solvent B, 98.8% Ultrapure water, 0.2% (v/v) TFA. The reverse phase C8 column was equilibrated with 8mL of 15% solvent A/85% solvent B. 20μL of sample was injected onto the column followed by a 15mL 15%-70% linear gradient of solvent A. This was followed by a 10mL wash of 70% solvent A/30% solvent B. A flow rate of 1mL/min was used throughout the procedure. The absorbance of the eluent was continuously monitored at 214nm.
<table>
<thead>
<tr>
<th>Substrate/Fragment</th>
<th>Concentration (mM)</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>TRH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>TRH-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Glu²TRH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Phe³TRH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>TRH-Gly</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Substance P</td>
<td>1 3</td>
<td>10% v/v 1 5M Acetic Acid²</td>
</tr>
<tr>
<td>+ADNF-14</td>
<td>1 3</td>
<td>Buffer³</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>1 3</td>
<td>Buffer³</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>1 3</td>
<td>Buffer³</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>1 3</td>
<td>Buffer³</td>
</tr>
<tr>
<td>Arg⁵-Vasopressin</td>
<td>1 3</td>
<td>Buffer³</td>
</tr>
<tr>
<td>H-Gly-Pro-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>H-Gly-Gly-Pro-Ala-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>H-Gly-Ala-Phe-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Z-Pro-Pro-OH</td>
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<td>10% MeOH¹</td>
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<tr>
<td>Z-Pro-Gly-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Z-Pro-Ala-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Z-Pro-Leu-Gly-OH</td>
<td>1 3</td>
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<tr>
<td>H-Leu-Gly-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
<tr>
<td>Z-Gly-Pro-Ala-OH</td>
<td>1 3</td>
<td>10% MeOH¹</td>
</tr>
</tbody>
</table>

Table 2.9.4. Preparation Of HPLC Substrate Solutions:

¹ Substrate/fragment was initially dissolved in 300μL of methanol and was diluted to 1 3mM by the addition of 100mM potassium phosphate buffer pH 7.4.

² Substrate/fragment was initially dissolved in 1 5M acetic acid and diluted to 1 3M by the addition of 100mM potassium phosphate buffer pH 7.4.

³ Substrate/fragment was dissolved in 100% 100mM potassium phosphate buffer pH 7.4. Sonication was required to get complete dissolution.

*ADNF-14, Activity Dependent Neurotrophic Factor
2.9.6.2. Substrate Specificity Using MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectrometry was carried out using a Hewlett Packard G2025 mass spectrometer with a linear time of flight analyser. The instrument was equipped with a 337 nm nitrogen laser, a high-potential acceleration source (5 kV), and a 10 m flight tube. Detector operation was in the positive ion mode and signals were recorded and filtered using a LeCroy 9350M digital storage oscilloscope interfaced to a computer. The spectrometer was internally calibrated using Hewlett-Packard low molecular weight standards.

2.9.6.2.1. Preparation of Stock Substrates

1 mL of each substrate was prepared as outlined in Table 2.9.5. Buffer used in all preparations was 100 mM Tris-HCl, 10 mM DTT, 1 mM EDTA, pH 7.6

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mg/mL)</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLIP (ACTH 18-39)</td>
<td>2 mg/mL</td>
<td>Buffer</td>
</tr>
<tr>
<td>Amyloid A4 Precursor Protein</td>
<td>1 mg/mL</td>
<td>Buffer</td>
</tr>
<tr>
<td>(Gly-Pro-Ala) polymer</td>
<td>2 mg/mL</td>
<td>Buffer</td>
</tr>
</tbody>
</table>

Table 2.9.5. Preparation of Stock Substrates for Mass Spectrometry

2.9.6.2.2. Reactions of Substrates with Purified Prolyl Oligopeptidase Activity

50 µL of substrate was added to 50 µL of purified brain PO activity which had been concentrated 10-fold (volume) using Amicon 15 mL Centricon centrifugal concentrators (50). Negative controls were prepared by adding 50 µL of water to 50 µL of peptide. Positive and negative controls were incubated in a water bath at 37°C. At various time intervals 10 µL of incubated sample was taken and added to an equal volume of 2',6'-dihydroxyacetophenone (matrix solution).

2.9.6.2.3. MALDI-TOF Mass Spectrometry of Samples

A small volume (>1 µL) of sample matrix solution mix was transferred to a probe tip and evaporated in vacuum chamber (Hewlett-Packard G2024A sample preparation accessory) ensuring a homogenous sample crystallisation. Spectra were collected by accumulating data generated by a number of single laser shots with a laser power of between 5.7-8.3 µJ.

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2.9.63. Kinetic Studies

Substrate specificity studies, on bovine brain, serum and *F. meningosepticum* PO activities, based on kinetic analysis were performed.

2.9.63.1. Km Determination for Z-Gly-Pro-MCA

The Michaelis Menten Constant (Km) was determined for the reaction of purified bovine brain, bovine serum and *F. meningosepticum* PO activity, with the substrate Z-Gly-Pro-MCA. A range of concentrations (0-200μM) of Z-Gly-Pro-MCA were prepared from a 200μM stock solution, 8% v/v DMSO, 100mM potassium phosphate buffer, 5mM DTT, 0.5mM EDTA, pH 7.4. Stock was prepared as described in section 2.4.1. Enzyme activities were determined in triplicate and suitable negative controls were prepared. (section 2.4.1) The Km and Vmax values for this substrate were determined using Michaelis-Menten, Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf and Direct Linear Plot analysis.

2.9.63.2. Km Determination for pGlu-His-Pro-MCA

The Michaelis-Menten Constant (Km) was determined for the reaction of bovine brain, bovine serum and *F. meningosepticum* PO activity, with the fluorimetric substrate pGlu-His-Pro-MCA. A range of concentrations of pGlu-His-Pro-MCA (0-200μM) were prepared using 100mM potassium phosphate buffer, 5mM DTT, 0.5mM EDTA, pH 7.4 as diluent. Enzyme activities were determined in triplicate in a similar method as outlined in section 2.4.1. Suitable negative controls were included. The Km and Vmax values for this substrate were determined using Michaelis-Menten, Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf and Direct Linear Plot analysis.

2.9.63.3. Ki Determination for Proline-Containing Peptides

The effects of a variety of proline-containing peptides on Km values obtained for purified brain, serum and recombinant PO activities for the substrate Z-Gly-pro-MCA was determined. The peptides and their preparation is outlined in table 2.9.6. A 400μM stock solution of Z-Gly-Pro-MCA, 16% v/v DMSO, was prepared as outlined in section 2.4.1. From this stock solution a range of dilutions were prepared (0-400μM) using 100mM potassium phosphate buffer, 5mM DTT, 0.5mM EDTA, pH 7.4 as diluent. 2mL of appropriate peptide was added to an equal volume of substrate dilution. PO activity was assayed in triplicate using these substrate mixtures as outlined in section 2.4.1. The Km and apparent Km values were determined using the Lineweaver-Burk analysis model.
Table 2.9.6. Preparation of Peptides For Kinetic Studies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Stock Concentration</th>
<th>Assay Concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRH</td>
<td>1mM</td>
<td>400μM</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>TRH</td>
<td>500μM</td>
<td>200μM</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>Glu2-TRH</td>
<td>400μM</td>
<td>160μM</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>Phe2-TRH</td>
<td>500μM</td>
<td>200μM</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>400μM</td>
<td>160μM</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>Substance P</td>
<td>400μM</td>
<td>160μM</td>
<td>5% v/v 1 5M acetic acid</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>250μM</td>
<td>100μM</td>
<td>Buffer^2</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>250μM</td>
<td>100μM</td>
<td>Buffer^2</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>250μM</td>
<td>100μM</td>
<td>Buffer^2</td>
</tr>
<tr>
<td>Arg4-Vasopressin</td>
<td>250μM</td>
<td>100μM</td>
<td>Buffer^2</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>250μM</td>
<td>100μM</td>
<td>5% MeOH</td>
</tr>
</tbody>
</table>

1Substrate/fragment was initially dissolved in methanol and was diluted to appropriate concentration by the addition of 100mM potassium phosphate buffer, 5mM DTT, 0.5mM EDTA pH 7.4 (final methanol concentration of 5% v/v)
2Substrate/fragment was dissolved in 100% 100mM potassium phosphate buffer, 5mM DTT, 0.5mM EDTA, pH 7.4
3Substrate/fragment was initially dissolved in 1.5M acetic acid and diluted to 1.3M by the addition of 100mM potassium phosphate buffer pH 7.4
*Sonication was required to achieve complete dissolution.

2.9.7. Effect of Specific Prolyl Oligopeptidase Inhibitors on Purified Prolyl Oligopeptidase Activity

2.9.7.1. Determination of IC50 Values for Specific Prolyl Oligopeptidase Inhibitors

A range of dilutions of specific PO inhibitors was prepared as outlined in Table 2.9.7 A stock substrate of 400μM Z-Gly-Pro-MCA, 16% v/v DMSO in 100mM potassium phosphate buffer pH 7.4 with 10mM DTT and 1mM EDTA was prepared and preincubated at 37°C until completely dissolved and thermal equilibrium was reached. 2mL of substrate stock was added to 2mL of appropriate inhibitor giving a final substrate concentration of 200μM with 8% DMSO v/v PO activity (Bacterial
brain and serum), using substrate-inhibitor mixtures was assayed in triplicate as outlined in section 2.4.1. Suitable negative and positive controls were prepared.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stock Concentration (M)</th>
<th>Preparation</th>
<th>Assay Concentration (M)</th>
</tr>
</thead>
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<tr>
<td>Z-Pro-Prolinal</td>
<td>5x10⁻²</td>
<td>100% v/v MeOH</td>
<td>1x10⁻⁵-1x10⁻¹³</td>
</tr>
<tr>
<td>Z-Pro-Prolinal-Dimethylacetate</td>
<td>1x10⁻²</td>
<td>100% v/v MeOH</td>
<td>1x10⁻³-1x10⁻¹²</td>
</tr>
<tr>
<td>Cyclohexyl-Prolinal</td>
<td>2.008x10⁻²</td>
<td>50% v/v Dioxane</td>
<td>1x10⁻⁵-1x10⁻¹⁴</td>
</tr>
<tr>
<td>Z-Indolinyl-Prolinal</td>
<td>8.6x10⁻³</td>
<td>50% v/v Dioxane</td>
<td>1x10⁻⁵-1x10⁻¹⁴</td>
</tr>
<tr>
<td>Fmoc-Ala-Pyr-Nitrile</td>
<td>5x10⁻³</td>
<td>20% v/v DMSO</td>
<td>1x10⁻⁴-1x10⁻¹³</td>
</tr>
<tr>
<td>Fmoc-Pro-Pyr-Nitrile</td>
<td>2.6x10⁻⁵</td>
<td>100% v/v Dioxane</td>
<td>1x10⁻³-1x10⁻¹³</td>
</tr>
<tr>
<td>Z-Phe-Pro-Methylketone</td>
<td>1.6x10⁻²</td>
<td>Buffer</td>
<td>1x10⁻⁵-1x10⁻¹⁴</td>
</tr>
<tr>
<td>Postatin</td>
<td>8.1x10⁻³</td>
<td>20% v/v Dioxane</td>
<td>1x10⁻⁴-1x10⁻¹³</td>
</tr>
<tr>
<td>α-Ketobenzothiazole</td>
<td>6.9x10⁻⁵</td>
<td>50% v/v Dioxane</td>
<td>1x10⁻⁵-1x10⁻¹⁵</td>
</tr>
</tbody>
</table>

Table 2.9.7. Preparation of Prolyl Oligopeptidase Specific Inhibitors.

Stock concentrations of inhibitors were prepared in appropriate solvents before dilution with 100mM potassium phosphate buffer, pH 7.4 to give appropriate dilutions. Dilutions were prepared two-fold concentrated and then diluted with 400μM Z-Gly-Pro-MCA, 16% v/v DMSO, prepared as outlined in section 2.4.1. Purified PO activity was assayed in triplicate using substrate/inhibitor mixture as outlined in section 2.4.1., incorporating suitable negative and positive controls.

2.9.7.2. Effect of Z-Phe-Ala-Chloromethylketone on Purified Bovine Brain and Recombinant Prolyl Oligopeptidase Activity With Time

A 1x10⁻⁴M solution of Z-Phe-Ala-CMK was prepared in 50%v/v acetonitrile. Using this as the inhibitor mixture the following reaction mixtures were prepared: 122μL of bovine brain/recombinant PO activity, 24μL of inhibitor mixture and 94μL of 50mM hepes buffer with 1mM DTT, 1mM EDTA, 150mM NaCl, pH 7.6. Positive controls with 24μL of 50%v/v acetonitrile instead of inhibitor mixture were also prepared. These reactions mixtures were left at room temperature (20°C) and at
appropriate time intervals up to 470 minutes 12μL of this reaction mixture was taken and assayed fluorometrically as follows. 6μL of 50μM Z-Gly-Pro-MCA was added to reaction mixture with 102μL of 50mM hepes buffer with 1mM DTT, 1mM EDTA and 150mM NaCl, pH 7.6. After 1 hour at 37°C 100μL of this solution was added to 500μL of 1M sodium acetate/HCl, 5% v/v DMSO, pH 4.4 and fluorescent intensities were measured at excitation and emission wavelengths of 370nm and 440nm respectively. Results were expressed as a percentage of activity of samples taken at a time of 0 minutes.
3. RESULTS
3. Results

3.1. MCA Standard Curves-Quenched and Unquenched

MCA standard curves were prepared as outlined in section 2.2.1 and 2.2.2. Plots of fluorimetric intensity versus MCA concentration are presented in figures 3.1.1 to 3.1.3. Figures 3.1.1. and 3.1.2 illustrate the quenching effect of undiluted and diluted soluble bovine brain supernatant on fluorescence. Figure 3.1.3 illustrates the quenching effect of undiluted serum. Table 3.1 is a summary of the slopes obtained from each curve.

3.2. Protein Standard Curves

Protein standard curves incorporating BSA were prepared as outlined in sections 2.3.2, 2.3.3 and 2.3.4. Figures 3.2.1 and 3.2.2, plots of absorbance at 560nm versus BSA concentration, represent BCA standard and enhanced microplate procedure. Figure 3.2.3, absorbance at 595nm versus BSA concentration, represents the Biorad microassay procedure.

3.3. Measurement of Prolyl Oligopeptidase and Z-Pro-Prolinal Insensitive Z-Gly-Pro-MCA Hydrolysing Activity

Figures 3.3.1 and 3.3.2 represent PO and ZIP activity in bovine serum and bovine brain supernatant which were measured as outlined in section 2.4.1 and 2.4.2. Bovine serum was found to contain 277 units of PO activity and 51 units of ZIP activity per mL of serum (5542 and 1020 units of PO and ZIP activity respectively in 20mL of serum). Brain supernatant was found to contain 45882 units of PO activity per mL (1835292 units in 40mL of supernatant) but no ZIP activity was detectable. Units of activity are picomoles of MCA released per minute at 37°C.
Figure 3.1.1. MCA Standard Curves. Plots of fluorometric intensity versus MCA concentration. 100μL of buffer (●) or 100μL of brain supernatant (○) was combined with 400μL of standard concentration of MCA and 1mL of acid was added before measurement of fluorescent intensity as outlined in section 2.2.1. The emission slit width was set at 2.5nm. Error bars represent the SEM of readings in triplicate.

Figure 3.1.2 MCA Standard Curves. Plots of fluorometric intensity versus MCA concentration. 100μL of buffer (●) or 100μL of a one in twenty dilution of brain supernatant (○) was combined with 400μL of standard concentration of MCA and 1mL of acid was added before measurement of fluorescent intensity as outlined in section 2.2.1. The emission slit width was set at 2.5nm. Error bars represent the SEM of readings in triplicate.
Figure 3.1.3. MCA Standard Curves. Plots of fluorimetric intensity versus MCA concentration 100µL of buffer (●) or 100µL of a serum (○) was combined with 400µL of standard concentration of MCA and 1mL of acid was added before measurement of fluorescent intensity as outlined in section. The emission slit width was set at 10 nm. Error bars represent the SEM of readings in triplicate.

Table 3.1. Slope Determinations for Quenched and Unquenched Standard Curves

<table>
<thead>
<tr>
<th>Figure</th>
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<th>Quenched</th>
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<td>7570</td>
<td>7570</td>
</tr>
<tr>
<td>313</td>
<td>68207</td>
<td>46924</td>
</tr>
</tbody>
</table>

Table 3.1. Slope Determinations for Quenched and Unquenched Standard Curves
Figure 3.2.1. BSA Standard Curve Plot of absorbance at 560nm versus BSA concentration obtained using the BCA assay procedure as outlined in section 2.3.2. Error bars represent the SEM of triplicate readings.

Figure 3.2.2. BSA Standard Curve Plot of absorbance at 560nm versus BSA concentration obtained using the BCA enhanced assay procedure as outlined in section 2.3.3. Error bars represent the SEM of triplicate readings.
Figure 3.2.3. BSA Standard Curve. Plot of absorbance at 595nm versus BSA concentration obtained using the Biorad microassay procedure as outlined in section 2.3.4. Error bars represent the SEM of triplicate readings.
Figure 3.3.1. and 3.3.2. Measurement of PO and ZIP Activity in Serum and Brain Supernatant. Enzyme activities of samples were measured as outlined in section 2.4.1 and 2.4.2 respectively. Total Activity* refers to activity expressed in terms of total enzyme units i.e. Units or pmoles MCA released per minute at 37°C. ZPP represents 5x10^-4 M Z-Pro-Prolinal. This inhibitor was incorporated into the assay as outlined in section 2.4.2.
3.4. Partial Purification of Prolyl Oligopeptidase From Bovine Serum

3.4.1. Serum Preparation
2L of serum was collected from 10L of whole blood

3.4.2. Phenyl Sepharose Hydrophobic Interactions Column Chromatography I
Figure 3.4.1. illustrates the elution of PO activity, ZIP activity and protein from a phenyl sepharose column which was prepared and run as outlined in section 2.5.2. While two peaks of Z-Gly-Pro-MCA activity, measured as in section 2.4.3., were evident, the first run through peak was found to be completely sensitive to inhibition by Z-Pro-prolinal using the assay described in section 2.4.4. The second activity peak which was eluted in distilled water was totally insensitive to Z-pro-prolinal. Fractions 4-10 were combined to give a 35mL post phenyl sepharose chromatography I pool. 1mL of this pool was retained for quantitative PO activity (section 2.4.1) and protein (section 2.3.2) measurements.

3.4.3. Phenyl Sepharose Hydrophobic Interactions Column Chromatography II
Post phenyl sepharose chromatography I PO pool was salted and applied to second phenyl sepharose column as outlined in section 2.5.3. Figure 3.4.2. illustrates the elution of PO activity and protein, measured as outlined in sections 2.4.3 and 2.3.1 respectively. Fractions 38-45 were combined to give post phenyl sepharose chromatography II PO pool (40mL). 1mL of this pool was retained for quantitative PO activity (section 2.4.1) and protein measurements (section 2.3.2). The remainder was aliquoted and stored at -80°C for future use. The effectiveness of bovine serum PO partial purification is outlined in table 3.4.1.
Figure 3.4.1 Elution Profile of Z-Gly-Pro-MCA Degrading Activity from Phenyl Sepharose Hydrophobic Interaction Chromatography. 20mL of bovine serum, containing 200mM ammonium sulphate, was applied to column as outlined in section 2.5.2. The column was eluted isocratically with distilled water containing no ammonium sulphate. Fractions were assayed for protein (●) using the absorbance at 280nm as outlined in section 2.3.1, PO activity (●) and ZIP activity was assayed as outlined in sections 2.4.3 and 2.4.4 respectively.
Figure 3.4.2. Elution Profile of Prolyl Oligopeptidase Activity From Phenyl Sepharose Hydroporphic Interaction Column II. 20mL of post phenyl sepharose I PO pool, containing 1M ammonium sulphate was applied to column as outlined in section 2.5.3 The column was eluted with a linear 1-0M ammonium sulphate gradient (-----) and 0-15% glycerol gradient. Fractions were assayed for protein (... using the absorbance at 280nm as outlined in section 2.3.1. PO activity was assayed as outlined in section 2.4.3
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific Activity (Umts/mg)</th>
<th>Purification factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1,210.4</td>
<td>5,542</td>
<td>4.57</td>
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<td>100</td>
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<td>Phenyl Sepharose I</td>
<td>344.9</td>
<td>3,002</td>
<td>8.70</td>
<td>1.90</td>
<td>54</td>
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<tr>
<td>Ammonium Sulphate</td>
<td>305.6</td>
<td>2,873</td>
<td>9.40</td>
<td>2.06</td>
<td>52</td>
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<tr>
<td>Phenyl Sepharose II</td>
<td>45.9</td>
<td>2,059</td>
<td>44.9</td>
<td>9.82</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 3.4.1. Partial Purification of PO from Bovine Serum. Units are expressed as pmoles MCA released per minute at 37°C. Pools were assayed for PO activity as outlined in section 2.4.1. Protein was estimated using the BCA standard microplate assay as outlined in section 2.3.2.
3.5. Purification of Prolyl Oligopeptidase Activity from the Cytosolic Fraction of Bovine Brain

3.5.1. Bovine Brain Preparation
50g brain slice yielded 240mL of post ultracentrifugation cytosolic brain supernatant

3.4.2. Ammonium Sulphate Precipitation
45% and 75% ammonium sulphate precipitation steps were performed as outlined in section 2 6 2. PO activity remained in the supernatant (38mL) following the 45% step and centrifugation. PO activity was precipitated after the 75% step.

3.5.3. DEAE Sepharose Fast Flow Anion Exchange Chromatography
Following dialysis, 7mL of post ammonium sulphate precipitate PO pool was applied to DEAE Sepharose column as outlined in section 2 6 3. Figure 3 5 1 shows the elution profile of PO activity in a linear 0-350mM NaCl gradient. Fractions 32-36 were combined to give the post DEAE Sepharose chromatography pool (30mL). 1mL of this pool was retained for activity (section 2 4 1) and protein measurements (2 3.2).

3.5.4. Sephacryl S200 Sepharose Gel Filtration Chromatography
Following concentration by reverse osmosis, 2mL of the post DEAE Sepharose chromatography pool was applied to a sephacryl S200 gel filtration column as outlined in section 2 6 4. Figure 3 5 2 shows the elution of PO activity and protein from the column, qualitatively measured as in sections 2 4 3 and 2 3 3, respectively. Fractions 31-37 were combined to give the post sephacryl S200 gel filtration chromatography pool. 1mL of this pool was retained for quantitative PO activity and protein measurements as outlined in sections 2 4 1 and 2 3 3, respectively.

3.5.5. PBE 94 Chromatofocusing
Post sephacryl S200 gel filtration chromatography pool was dialysed and applied to chromatofocusing column as outlined in section 2 6 5. PO activity was eluted in a decreasing, linear, 6-4 pH gradient. PO activity eluted approximately at pH 4.8 (see figure 3 5 3). Fractions 37-43 were combined to give the post chromatofocusing pool (35mL). 1mL of this pool was retained for quantitative PO activity and protein measurements (see sections 2 4 1 and 2 3 4, respectively).

3.5.6. Phenyl Sepharose Hydrophobic Interactions Column Chromatography
Post chromatofocusing pool was salted and applied to phenyl sepharose column as outlined in section 2 6 6. Figure 3 5 4 shows the elution of PO activity during a 10mM potassium phosphate buffer with 15% v/v glycerol wash. Fractions 25-38 were combined to give the post phenyl sepharose...
chromatography pool (25mL). 1mL of this pool was retained for quantitative protein and activity measurements as outlined in sections 2.4.1 and 2.3.4 respectively. The effectiveness of the purification is shown in Table 3.5.1.
Figure 3.5.1. Elution profile of Prolyl Oligopeptidase from DEAE Sepharose Anion Exchange Chromatography. 75mL of post ammonium sulphate precipitation dialysate was applied to column at pH 8.0 as outlined in section 2.6.3. The column was eluted with a linear 0-350mM sodium chloride (- - - -) gradient. Fractions were assayed for PO activity (•) as outlined in section 2.4.3. and protein using the BCA standard microplate assay procedure as described in section 2.3.2.
Figure 3.5.2. Elution Profile of Prolyl Oligopeptidase Activity From Sephacryl S200 Sepharose Gel Filtration Chromatography. 2mL of reverse dialysed post DEAE sepharose pool with 10% (v/v) glycerol was applied to column as outlined in section 2.6.4. The column was eluted with 25mM imidazole-HCl with 150mM NaCl, pH 6.3. Fractions were assayed for PO activity (•) as outlined in section 2.4.3. Protein was determined using the BCA enhanced microassay procedure as outlined in section 2.3.3. Vo represents the void volume (105mL).
Figure 3.5.3. Elution Profile of Prolyl Oligopeptidase Activity from PBE 94 Chromatofocusing Column. 35mL of post sephacryl S200 aliquot was applied to column at pH 6.0. Column was then eluted with a linear 6.0-4.0 pH gradient. Fractions were assayed for PO activity (•) as outlined in section 2.4.3. Protein ( . . . ) was measured as outlined in section 2.3.4. The pH of each fraction was also monitored.
Figure 3.5.4. Elution Profile of Prolyl Oligopeptidase Activity From Phenyl Sepharose Hydrophobic Interactions Column. Post chromatofocusing pool was applied to column in 1M ammonium sulphate Prolyl oligopeptidase activity was eluted with a linear 1-0M ammonium sulphate (----) gradient, followed by a 100mL wash with 10mM potassium phosphate buffer with 5mM DTT, 0.5mM EDTA and 15% (v/v) glycerol Fractions were assayed for PO activity (●) and protein (----) according to sections 2.4.3 and 2.3.4 respectively
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (Units/mg)</th>
<th>Purification Factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
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<tr>
<td>Brain Cytosol</td>
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<td>Ammonium sulphate</td>
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<tr>
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<td>Sephacryl S200</td>
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<td>23</td>
</tr>
</tbody>
</table>

Table 3.5.1. Purification of Prolyl Oligopeptidase from Bovine Brain  Units are expressed as pmoles MCA released per minute at 37°C

Pools were assayed for PO activity as described in section 2.4.1. Protein was estimated using ¹ BCA Standard microplate assay (section 2.3.2), ² BCA Enhanced microplate assay (section 2.3.3) and ³ Biorad protein microassay (section 2.3.4)
3.5.7. Determination of Purity of Bovine Brain Prolyl Oligopeptidase

3.5.7.1. Polyacrylamide Gel Electrophoresis

SDS polyacrylamide electrophoresis was performed as outlined in section 2.7.1. Figure 3.5.5 represents the silver stained gel image showing the various steps in bovine brain PO purification and molecular weight markers.

<table>
<thead>
<tr>
<th>Lane Number</th>
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</tr>
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<tbody>
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<td>1</td>
<td>DEAE Sepharose</td>
</tr>
<tr>
<td>2</td>
<td>S200</td>
</tr>
<tr>
<td>3</td>
<td>PBE 94</td>
</tr>
<tr>
<td>4</td>
<td>Phenyl Sepharose</td>
</tr>
<tr>
<td>5</td>
<td>Markers</td>
</tr>
</tbody>
</table>

A  B-Galactosidase (116kDa)
B  Fumarase (48.5kDa)
C  Carbonic Anhydrase (29kDa)

Figure 3.5.5 SDS-Polyacrylamide Gel Electrophoresis For Bovine Brain PO Purification

Silver stained polyacrylamide gel prepared as outlined in section 2.7.1, illustrates the purification process with a major and minor band in the post phenyl sepharose pool. Location of the major band relative to the molecular weight bands indicates a molecular weight of 66,000 Daltons.
3.5.7.2. Fluorimetric Assays for Interfering Peptidase Activity
The presence of contaminating peptidase activity was determined by monitoring for hydrolysis of a range of fluorimetric substrates which were prepared and tested as outlined in sections 2.7.2 and 2.4.1, respectively. Table 3.5.2 illustrates that none of the substrates were cleaved by the purified PO enzyme with the exception of Z-Gly-Pro-MCA and pGlu-His-Pro-MCA in the absence of Z-pro-prolylinal (see section 2.7.2).

<table>
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<tr>
<td>Ala-MCA</td>
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</tr>
<tr>
<td>Arg-MCA</td>
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</tr>
<tr>
<td>Gly-Arg-MCA</td>
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</tr>
<tr>
<td>Gly-Pro-MCA</td>
<td>No</td>
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<tr>
<td>pGlu-His-Pro-MCA</td>
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<tr>
<td>Z-Arg-MCA</td>
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</tr>
<tr>
<td>Z-Gly-Pro-MCA</td>
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Table 3.5.2. Cleavage of Quenched Fluorimetric Substrates by Contaminating Peptidases.
* Cleavage due to PO activity was obtained in the absence of Z-Pro-prolylinal

3.6. Purified Prolyl Oligopeptidase Activity Assay Development

3.6.1. Solvent Effects on Purified Prolyl Oligopeptidase Activity and Assay Sensitivity
The effect of solvents such as DMSO, DMF, dioxane, methanol and ethanol on PO activity and their effectiveness in solubilising PO fluorimetric assay substrate was investigated as outlined in section 2.8.1. Figure 3.6.1 illustrates the effects of these solvents on PO activity. DMF, while the most effective substrate solubilisation solvent, was most inhibitory of enzyme activity with only 20% residual PO activity at 10% v/v. Methanol was least inhibitory of PO activity but in contrast to DMF was least effective in solubilising substrate. DMSO, dioxan and ethanol displayed similar trends in inhibition of enzyme but DMSO was found to be the optimal solvent in terms of both substrate solubility and enzyme activity.
3.6.2. Linearity of Purified Prolyl Oligopeptidase Activity Assay With Respect to Time

The linearity of the purified PO activity assay as a function of time was examined as outlined in section 2.8.2.1 and 2.8.2.2. Firstly, the linearity of the PO activity assay in the presence and absence of 0.5% w/v BSA (included in sample before storage), as a function of time was examined discontinuously over 60 minutes using 200µM substrate. Figure 3.6.2a illustrates that in the presence of BSA, the assay was found to be linear over 60 minutes. In the absence of BSA, the assay stopped being linear in less than 10 minutes. Figure 3.7.2b shows the linearity of PO activity assay as a function of time using 100µM and 200µM Z-Gly-Pro-MCA with 4% v/v and 8% v/v DMSO respectively. Over 40 minutes, the assay is quite clearly linear using 200µM substrate.

3.6.3. Linearity of Purified Prolyl Oligopeptidase Activity Assay With Respect to Enzyme Concentration

A range of dilutions of purified PO activity were prepared and assayed as outlined in section 2.8.3 in order to examine the linearity of purified PO activity assay as a function of enzyme concentration. Figure 3.6.3 illustrates that over a 40 minute time period, the purified PO activity assay is linear with respect to enzyme concentration.

3.6.4. Effect of DTT on Purified Prolyl Oligopeptidase Activity Assay Sensitivity

The effect of a range of DTT concentrations on purified PO activity assay sensitivity was investigated as outlined in section 2.8.4. From Figure 3.6.4, it was observed that 5mM DTT included in the assay substrate resulted in just over 2.5 times more activity than that of a control with no DTT. Triplicate readings were also found to be more reproducible in the presence of DTT.

3.6.5. Effect of EDTA on Purified Prolyl Oligopeptidase Activity Assay Sensitivity

The effect of various EDTA concentrations in PO assay substrate, on purified PO activity assay sensitivity was investigated as outlined in section 2.8.5. As illustrated in Figure 3.6.5, a slight increase in activity was observed in PO activity in the presence of 5mM EDTA, followed by a steady decline in activity.

3.6.6. Effect of NaCl on Prolyl Oligopeptidase Activity Assay Sensitivity

The effect of various NaCl concentrations on purified PO activity assay was studied as outlined in section 2.8.6. Figure 3.8.6 illustrates that PO activity was optimal in the presence of 400mM NaCl, with activity of 118% of the control sample (contained no NaCl).
Figure 3.6.1. Effect of Solvents on PO Activity Assay Sensitivity

This investigation was performed as outlined in section 2.81. Plot shows the effect of 3-10% v/v DMF (○), 5-10% v/v DMSO (●), dioxane (■), ethanol (▲) and methanol (▼) on assay sensitivity/enzyme activity. DMF appears to be most inhibitory of PO activity with 6% resulting in 60% inhibition of PO activity. Methanol gave the highest sensitivity in comparison to ethanol, dioxane and DMSO. Enzyme activity* expressed as a % of the activity obtained using 6% v/v DMSO.
Figures 3.6.2a. and 3.6.2b. Linearity of Purified PO Assay With Respect To Time.

Figure 3.6.2a. shows linearity of purified PO enzyme assay with (●) and without (○) 0.5% w/v BSA over 60 minutes. Figure 3.6.2b. shows linearity of purified PO assay with 100μM and 200μM substrate.
Figure 3.6.3. Linearity of Prolyl Oligopeptidase Assay With Respect to Enzyme Concentration.

Plot of enzyme activity versus enzyme concentration. Enzyme activity refers to % of activity in undiluted sample.
Figure 3.6.4. Effect of DTT on Prolyl Oligopeptidase Assay Sensitivity.
Plot shows effect of 0-100mM DTT on PO activity. Investigation was carried out as outlined in section 2.6.4. Enzyme activity* expressed as % of activity in the absence of DTT.
Figure 3.6.5. Effect of EDTA on PO Assay Sensitivity

Plot shows the effect of EDTA on PO assay sensitivity/activity. Experiment was performed as outlined in section 2.6.5. Enzyme activity* expressed as % of activity in the absence of EDTA.
Figure 3.6.6. Effect of NaCl on PO Assay Sensitivity

Plot show the effect of NaCl on the sensitivity of PO assay. Experiment was performed as outlined in section 2.8.6. PO activity/assay sensitivity increases at NaCl concentrations up to 400mM and declines at higher NaCl concentrations. Enzyme activity* is an expression of the % of activity in the absence of NaCl.
3.7. Characterisation of Purified Prolyl Oligopeptidase Activity

3.7.1. Relative Molecular Mass Determination

3.7.1.1. Sephacryl S200 HR Gel Filtration Chromatography
The relative molecular mass of the purified PO activity was determined using a calibrated Sephacryl S200 gel filtration column which was prepared and run as described in section 2.9.1.1. Using a plot of Log(molecular mass) versus elution volume (molecular mass standards/void volume as illustrated in Figure 3.7.1.1, the molecular mass of PO was found to be 69,440 kDa.

3.7.1.2. Polyacrylamide gel Electrophoresis
Using electrophoresis the relative molecular mass of the purified PO activity was determined as described in section 2.9.1.2. From a plot of Log (molecular mass) vs Rf value (distance travelled by mass standard or unknown/distance travelled by bromophenol blue dye front), illustrated in figure 3.7.1.2, the relative molecular mass of purified PO activity was found to be 69,501 kDa. The gel used in the molecular mass determination is shown in figure 3.5.5.

3.7.2. pH Effects
The effects of assaying PO activity at a range of pHs and preincubating the enzyme at a range of pHs prior to assaying for activity at pH 7.4 was investigated as described in sections 2.9.2.1 and 2.9.2.2 respectively. It can be concluded from figure 3.7.2a. that the optimum pH for assay of PO activity is 7.4 using potassium phosphate buffer. When PO activity was preincubated at a range of different pHs, using different buffer systems, and then assayed at pH 7.4 (potassium phosphate) it was found that the enzyme was almost completely inactivated at pHs of 4.5 or less and 10.0 or greater. Preincubation of the enzyme at pHs between 5 and 9 prior to assay at pH 7.4 resulted in no loss of activity. This is illustrated in figure 3.7.2b.
Figure 3.7.1.1. Sephacryl S-200 Molecular Mass Calibration Curve. Plot of Log molecular mass versus Ve/Vo where Ve is the elution volume of the molecular mass standard (●) or unknown (PO activity), (○) and Vo is the void volume (105mL). Ve and Vo were determined as outlined in sections 2.9.1.1 and 2.9.1.2. Linear regression analysis of the data obtained from the molecular mass standards produced the following calibration equation:

$$\log(\text{molecular mass}) = -2.6(\text{Ve/Vo}) + 9.86$$

From this equation the molecular weight of PO was found to be 69.44kDa
Figure 3.7.1.2. SDS PAGE Molecular Mass Calibration Curve. Plot of Log molecular mass versus Rf value where the Rf value is the distance travelled by mass standard(*) or unknown (PO), (o), divided by the distance travelled by bromphenol blue dye front. Linear regression analysis of the data obtained from the molecular mass standards produced the following calibration equation

\[
\text{Log (molecular mass)} = -1.017 \times \text{Rf} + 5.2
\]

From this equation the molecular weight of PO was found to be 69,501 Daltons
Figures 3.7.2a. and 3.7.2b. Effect of pH on Prolyl Oligopeptidase Activity. Plots show the effect of pH on PO activity. Investigations were carried out as in sections 2.9.2.1 and 2.9.2.2. Figure 3.7.2a represents the activity of PO when assayed at different pHs. It is evident that PO activity has a broad pH/activity range with a pH optima of 7.4 in potassium phosphate. Figure 3.7.2b represents the inactivation profile of PO (pH at which PO activity is completely inactivated even when assay performed at pH 7.4). PO activity is completely inactivated below pH 4.5 and 10.0. Buffer systems used were citrate/phosphate (●), phosphate (○), tris/HCl (▲) and glycine/NaOH (♦). Enzyme activity* expressed as % of activity obtained in potassium phosphate, pH 7.4.
3.7.3. **Temperature Effects**

The effect of assaying PO activity at different temperatures and preincubating the enzyme at a range of temperatures, for different times, prior to assaying at 37°C was investigated as outlined in sections 2.9.3.1 and 2.9.3.2 respectively. The optimal temperature for the assay of PO activity was determined to be 37°C. This is illustrated in figure 3.7.3a. Figure 3.7.3b. illustrates that PO activity was completely inactivated by preincubation at 50°C and 60°C, for 15 minutes or more, prior to assay at 37°C. Preincubation of PO samples at 37°C and 40°C at various times up to 45 minutes, had little effect on activity but samples left at room temperature (20°C) for more than 30 minutes lost greater than 30% of activity upon assay at 37°C.
Figures 3.7.3a. and 3.7.3b. Effect of Temperature on PO Activity.

Plots show the effect of assay temperature on PO activity (3.7.3a) and the effect of preincubation at different temperatures for different times on PO activity under normal assay conditions (3.7.3b). Experiments were performed as outlined in section 2.9.3.1 and 2.9.3.2 respectively. Plots illustrate that PO has a narrow optimal assay temperature range being most active at 37°C. In figure 3.9.3a enzyme activity is expressed as a % of activity at 37°C. In figure 3.9.3b enzyme activity* is expressed as a % of activity obtained when preincubated for 15 min at 37°C.
3.7.4. **Effect of Divalent Metal Salts on Purified Prolyl Oligopeptidase Activity**

The effect of divalent salts on purified prolyl oligopeptidase activity was investigated by incorporating the appropriate metal salt in fluorometric PO activity assay as outlined in section 2.9.4. Results presented in Table 3.7.1 show that PO activity was inhibited by almost 100% by 1mM HgSO₄ and CuSO₄. PO activity was also very strongly inhibited (greater than 50% inhibition) by CdSO₄, CoSO₄ and NiSO₄, and to a lesser extent, by ZnSO₄.

<table>
<thead>
<tr>
<th>Metal Salt</th>
<th>% Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSO₄</td>
<td>102.4 ± 1.5</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td>CoSO₄</td>
<td>24.7 ± 0.05</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>96.34 ± 0.29</td>
</tr>
<tr>
<td>HgSO₄</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>94.9 ± 0.37</td>
</tr>
<tr>
<td>NiSO₄</td>
<td>37.7 ± 0.04</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>59.7 ± 1.2</td>
</tr>
</tbody>
</table>

**Table 3.7.1. Effect of Divalent Metal Salts on Purified PO Activity**

Actual concentration of metal salts during preincubation was 1mM and during assay was 200μM. Residual activity is an expression of the percentage of activity remaining (pmoles/min) following incubation with metal salt. Positive controls were taken as enzyme activity following incubation with appropriate diluent buffer.
3.7.5. **Effect of Functional Reagents on Purified Prolyl Oligopeptidase Activity**

The effect of various functional reagents on purified PO activity was investigated as outlined in section 2.9.5. Table 3.7.2 illustrates the percentage residual activity after preincubation with appropriate functional reagent. The percentage residual activity is an expression of enzyme activity (units/mL) as a percentage of positive control activity (100%). In Table 3.7.2, concentrations of functional reagents are the actual concentrations during the incubations. Of the reagents tested, the cysteine protease inhibitors, DTNB (10, 5mM and 0.05mM) NEM (10mM) and serine protease inhibitor, AEBSF (10mM) were found to be the most potent inhibitors of PO activity, resulting in complete inhibition. PO activity was also significantly inhibited by the cysteine protease inhibitors iodoacetamide (10mM) and to a lesser extent iodoacetate (10mM) with only 14.84% and 40.40% activity remaining respectively. Activity was also inhibited by over 50% by 10mM 1,10 phenanthroline, bacitracin and N-Decanoyl CoA and to a lesser extent by 1,7 and 4,7 phenanthroline (10mM). A two-fold increase in PO activity following incubation with 10mM DTT was observed.
<table>
<thead>
<tr>
<th>Functional Reagent</th>
<th>% Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10mM</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>14.84</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>40.40</td>
</tr>
<tr>
<td>N-Ethylmaleimide (NEM)</td>
<td>0.36</td>
</tr>
<tr>
<td>Dithionitrobenzocacid (DTNB)</td>
<td>0</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>201.3</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>85.09</td>
</tr>
<tr>
<td>AEBSF</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>112.65</td>
</tr>
<tr>
<td>EGTA</td>
<td>95.81</td>
</tr>
<tr>
<td>CDTA</td>
<td>94.38</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>89.71</td>
</tr>
<tr>
<td>Imidazole</td>
<td>89.05</td>
</tr>
<tr>
<td>1, 10-Phenanthroline</td>
<td>43.08</td>
</tr>
<tr>
<td>1, 7-Phenanthroline</td>
<td>74.35</td>
</tr>
<tr>
<td>4, 7-Phenanthroline</td>
<td>70.90</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>93.12</td>
</tr>
<tr>
<td>Carnitine</td>
<td>101.88</td>
</tr>
<tr>
<td></td>
<td>1mg/mL</td>
</tr>
<tr>
<td>Bactracin</td>
<td>21.59</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>89.15</td>
</tr>
<tr>
<td>Soybean Trypsin Inhibitor</td>
<td>77.75</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>86.00</td>
</tr>
<tr>
<td>Puromycin</td>
<td>92.71</td>
</tr>
<tr>
<td>N-Decanoyl CoA</td>
<td>46.05</td>
</tr>
</tbody>
</table>

Table 3.7.2. *Effect of Functional Reagents on Prolyl Oligopeptidase Activity*

Functional reagents were prepared according to section 2.9.5. % Residual activity is an expression of the remaining activity relative to positive controls. Concentrations given were the actual concentrations during the 15 minute pre-incubation.
3.7.6. Substrate Specificity Studies on Purified Prolyl Oligopeptidase Activity

Substrate specificity studies on purified bovine brain prolyl oligopeptidase activity were performed as described in section 2.9.6. Using reverse phase HPLC and MALDI-TOF mass spectrometry, the cleavage of some proline containing peptides was monitored qualitatively. Fluorescence spectrometry was used to determine kinetic parameters for fluorescent substrates and proline containing peptides. Some comparative investigations were carried out using partially purified bovine serum PO activity and purified recombinant Flavobacterium meningosepticum PO activity.

3.7.6.1. Substrate Specificity Studies using Reverse Phase HPLC

Reverse phase HPLC was used to determine whether brain, serum and F. meningosepticum PO activities cleaved a variety of proline containing peptides, listed in table 2.9.6.1. Reactions of peptides with enzyme samples and reverse phase HPLC were carried out as outlined in sections 2.9.6.1.2 and 2.9.6.1.3 respectively. The optimal wavelength for detection of peptides and cleavage products was found to be 214 nm. Table 3.7.3 illustrates the peptides tested and whether or not they were cleaved by the various PO activities. Figures 3.7.6.11 to 3.8.6.110 represent chromatograms of absorbance at 214 nm versus retention time (minutes) for ADNF-14, angiotensin I, angiotensin II, angiotensin III, Arg8-vasopressin, bradykinin, Glu2-TRH, Gly-Gly-Pro-Ala, TRH and TRH-Gly respectively for brain and F. meningosepticum activities.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Brain PO</th>
<th>Serum PO</th>
<th>F. meningosepticum PO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADNF-14</td>
<td>Yes</td>
<td>N D</td>
<td>N D</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Arg⁸-Vasopressin</td>
<td>No</td>
<td>N D</td>
<td>Yes</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>H-Gly-Ala-Phe-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>H-Gly-Gly-Pro-Ala-OH</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>H-Gly-Pro-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Glu⁵TRH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Phe⁵TRH</td>
<td>Yes</td>
<td>N D</td>
<td>Yes</td>
</tr>
<tr>
<td>TRH-Gly</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>TRH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LHRH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Substance P</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Z-Gly-Pro-Ala-OH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Z-Pro-Ala-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Gly-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Leu-Gly-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Z-Pro-Pro-OH</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3.7.3. Substrate Specificity studies-Reverse Phase HPLC of Proline Containing Peptides
Reverse Phase HPLC was carried out on peptides which had been incubated with brain, serum and bacterial PO activities as outlined in section 2.9.6.1 Table states whether or not cleavage was obtained. N D -not determined
Figure 3.7.6.1.1. Chromatogram of absorbance at 214nm versus retention time in minutes for ADNF-14. Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of ADNF-14 before (---) and after (—) incubation with purified bovine brain PO, is illustrated. Cleavage product *, although not well resolved from ADNF-14 peak, is also shown.
Figures 3.7.6.1.2a and 3.7.6.1.2b. Chromatograms of absorbance at 214nm versus retention time in minutes for Angiotensin I. Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of Angiotensin I before (---) and after (—) incubation with purified bovine brain PO (3.7.6.1.2a) and bacterial PO (3.7.6.1.2b) is illustrated. Cleavage products *, are also shown.
Figures 3.7.6.1.3a. and 3.7.6.1.3b. Chromatograms of absorbance at 214nm versus retention time in minutes for Angiotensin II Substrate specificity studies performed as outlined in section 2.9.6.1 The presence of Angiotensin II before (----) and after (---) incubation with purified bovine brain PO (3.7.6.1.3a) and bacterial PO (3.7.6.1.3b) is illustrated Cleavage products *, are also shown.
Figures 3.7.6.1.4a. and 3.7.6.1.4b. Chromatograms of absorbance at 214 nm versus retention time in minutes for Angiotensin III. Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of Angiotensin III before (••••••) and after (---) incubation with purified bovine brain PO (3.7.6.1.4a) and bacterial PO (3.7.6.1.4b) is illustrated. Cleavage products *, are also shown.
Figures 3.7.6.1.5a. and 3.7.6.1.5b. Chromatograms of absorbance at 214nm versus retention time in minutes for Arg⁸-vasopressin. Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of Arg⁸-vasopressin before (---) and after (—) incubation with purified bovine brain PO (3.7.6.1.5a.) and bacterial PO (3.7.6.1.5b.) is illustrated. Cleavage products * are also shown.
Figures 3.7.6.1.6a and 3.7.6.1.6b. Chromatograms of absorbance at 214nm versus retention time in minutes for Bradykinin. Substrate specificity studies performed as outlined in section 2961. The presence of Bradykinin before (---) and after (→) incubation with purified bovine brain PO (37616a) and bacterial PO (37616b) is illustrated. Cleavage products *, are also shown.
Figures 3.7.6.1.7a and 3.7.6.1.7b. Chromatograms of absorbance at 214nm versus retention time in minutes for Glu²-TRH Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of Glu²-TRH before (-----) and after (----) incubation with purified bovine brain PO (3.7.6.1.7a) and bacterial PO (3.7.6.1.7b) is illustrated.
Figures 3.7.6.1.8a and 3.7.6.1.8b. Chromatograms of absorbance at 214nm versus retention time in minutes for Gly-Gly-Pro-Ala. Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of Gly-Gly-Pro-Ala before (----) and after (—) incubation with purified bovine brain PO (3.7.6.1.8a) bacterial PO (3.7.6.1.8b) is illustrated. The major cleavage product *, Gly-Gly-Pro, is also shown.
Figures 3.7.6.1.9a. and 3.7.6.1.9b. Chromatograms of absorbance at 214nm versus retention time in minutes for TRH Substrate specificity studies performed as outlined in section 2.9.6.1. The presence of TRH before (-----) and after (---) incubation with purified bovine brain PO (3.7.6.1.9a) and bacterial PO (3.7.6.1.9b) is illustrated. The major cleavage product *, TRH-OH, is also shown.
Figures 3.7.6.1.10a. and 3.7.6.1.10b. Chromatograms of absorbance at 214nm versus retention time in minutes for TRH-Gly Substrate specificity studies performed as outlined in section 2 9 6 1. The presence of TRH-Gly before (---) and after (—) incubation with purified bovine brain PO (3 7 6 1 10a) and bacterial PO (3 7 6 1 10b) is illustrated. The major cleavage product * , TRH-OH, is also shown.
3.7.6.2. Substrate Specificity With MALDI-TOF Mass Spectrometry

MALDI-TOF Mass Spectrometry was used to determine whether purified brain PO cleaved ACTH$_{18-39}$, (Gly-Pro-Ala) polymer and amyloid precursor protein fragment 708-715. It was also intended to confirm if bovine brain PO behaved in a similar manner to the bacterial and the porcine kidney forms of the enzyme. Mass spectrometry was performed as outlined in section 2.9.6.3. Brain PO was found to cleave ACTH$_{18-39}$ at 2 positions to give ACTH$_{18-24}$, ACTH$_{25-39}$ and ACTH$_{25-36}$. Cleavage sites were marked by the presence of proline. PO was also found to cleave the (Gly-Pro-Ala) polymer over time. Amyloid fragment was cleaved to give APP$_{708-713}$. Table 3.7.4 summarises the cleavage sites deduced for these peptides and figures 3.7.6.21-3.7.6.23 represent the mass spectra (relative intensity versus mass/charge ratio) obtained. Comparisons with the bacterial and porcine enzymes are discussed in section 4.7.6.2.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP$_{708-715}$</td>
<td>Gly-Gly-Val-Val-Ile-Ala-Thr-Val</td>
</tr>
<tr>
<td>CLIP (ACTH$_{18-39}$)</td>
<td>Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe</td>
</tr>
<tr>
<td>(Gly-Pro-Ala)$_{15}$</td>
<td>(Gly-Pro-Ala-Gly-Pro-Ala-Gly-Pro-Ala)$_{5}$</td>
</tr>
</tbody>
</table>

Table 3.7.4. Prolyl Oligopeptidase Cleavage Sites as Determined by MALDI-TOF Mass Spectrometry

APP  Amyloid A4 Precursor Protein
CLIP  Corticotropin Lobe Intermediate Peptide
Figures 3.7.6.2.1a. and 3.7.6.2.1b. MALDI-TOF MS Analysis of CLIP Cleavage by Prolyl Oligopeptidase with Time

Figures 3.7.6.2.1a. and 3.7.6.2.1b represent spectra obtained after incubation of enzyme with CLIP for times of 0 and 60 minutes respectively as outlined in section 2.9.6.2. M2505.40, M1665.8 and M858.2 represent ACTH\textsubscript{18-39}, 25-39 and 18-24 respectively.
Figure 3.7.6.2.1c. MALDI-TOF MS Analysis of CLIP Cleavage by Prolyl Oligopeptidase with Time. Figure 3.7.6.2.1c. represents the spectrum obtained after incubation of enzyme with CLIP for 120 minutes as outlined in section 2.9.6.2. M2505.40, M1665.8, M1277.1 and M858.2 represent ACTH_{18-39}, 25-39, 25-36 and 18-24 respectively.
Figure 3.7.6.2.2a and 3.7.6.2.2b. MALDI-TOF MS Analysis of APP<sub>708-713</sub> Cleavage by Prolyl Oligopeptidase with Time. Figures 3.7.6.2.2a and 3.7.6.2.2b. represents the spectra obtained after incubation of enzyme with APP for 0 and 120 minutes respectively as outlined in section 2.9.6.2. M715.8 and M515.7 represent APP<sub>708-713</sub> and APP<sub>708-713</sub> respectively.
Figure 3.7.6.2.3a and 3.7.6.2.3b. MALDI-TOF MS Analysis of (Gly-Pro-Ala)polymer Cleavage by Prolyl Oligopeptidase with Time. Figures 3.7.6.2.3a and 3.7.6.2.3b represent the spectra obtained after incubation of enzyme with (Gly-Pro-Ala)polymer for 0 and 200 minutes respectively as outlined in section 2.9.6.2. Peaks 1-12 represent (Gly-Pro-Ala)_{i+17} (fig 3.7.6.2.3a) and peaks 6-11 represent (Gly-Pro-Ala)_{i+19} (figure 3.7.6.2.3b).
Figure 3.7.6.2.3c. MALDI-TOF MS Analysis of (Gly-Pro-Ala)polymer Cleavage by Prolyl Oligopeptidase with Time.

Figure 3.7.6.2.3c and represents the spectrum obtained after incubation of enzyme with (Gly-Pro-Ala)polymer for 300 minutes as outlined in section 2.9.6.2. Peaks 4-8 represent (Gly-Pro-Ala)14-19.
3.7.6.3. Kinetic Studies

3.7.6.3.1 Km Determination for Z-Gly-Pro-MCA

Michaelis Menten Constants were determined for the reactions of purified bovine brain, bovine serum and recombinant bacterial PO activities using the substrate Z-Gly-Pro-MCA as outlined in section 2.9.6.3.1. Km values were determined using Michaelis Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Wolf analysis. Figures 3.7.6.3.1 to 3.7.6.3.3 represent data obtained experimentally as Lineweaver-Burk plots for brain, serum and bacterial PO respectively. Table 3.7.5 illustrates the Km results.

3.7.6.3.2 Km Determination for pGlu-His-Pro-MCA

Michaelis Menten Constants were determined for the reactions of purified bovine brain, bovine serum and recombinant bacterial PO activities using the substrate pGlu-His-Pro-MCA as outlined in section 2.9.6.3.1. Km values were determined using Michaelis Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Wolf analysis. Figures 3.7.6.3.2 to 3.7.6.3.3 represent data obtained experimentally as Lineweaver-Burk plots for brain, serum and bacterial PO respectively. Table 3.7.6.3.1 illustrates the Km results.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Brain PO</th>
<th>Serum PO</th>
<th>Recombinant PO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM</td>
<td>LB</td>
<td>HW</td>
</tr>
<tr>
<td>Z-Gly-Pro-MCA</td>
<td>39.5</td>
<td>62.5</td>
<td>48.5</td>
</tr>
<tr>
<td>pGlu-His-Pro-MCA</td>
<td>82.3</td>
<td>99.8</td>
<td>91.3</td>
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</tbody>
</table>

Table 3.7.5. Km values (µM) obtained for Fluorimetric Substrates Z-Gly-Pro-MCA and pGlu-His-Pro-MCA

Km values were obtained for brain, serum and recombinant bacterial PO activities. MM-Michaelis-Menten, LB-Lineweaver-Burk, EH-Eadie-Hofstee, HW-Hanes-Wolf.
**Ki Determination for Proline-Containing Peptides**

Ki values were determined using brain serum and recombinant PO for a range of proline-containing peptides as outlined in section 2.9.6.3.3, using the fluorimetric substrate Z-Gly-Pro-MCA. Inhibitor constants (Ki values) were determined using Lineweaver-Burk analysis and are outlined in Table 3.7.6. Figures 3.7.6.3.1 to 3.7.6.3.5 represent Lineweaver-Burk reciprocal plots for angiotensin II, AVP, TRH, LHRH and neurotensin using bpvme brain activity.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>*Concentration (µM)</th>
<th>Brain (1)</th>
<th>Serum (2)</th>
<th>Recombinant (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRH</td>
<td>200</td>
<td>90 91</td>
<td>83.3</td>
<td>642</td>
</tr>
<tr>
<td>LHRH</td>
<td>400</td>
<td>30 02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>160</td>
<td>33 3</td>
<td>12 0</td>
<td>133.5</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>100</td>
<td>21 2</td>
<td>3.8</td>
<td>155.3</td>
</tr>
<tr>
<td>Arg⁸-Vasopressin</td>
<td>100</td>
<td>77 8</td>
<td>3.3</td>
<td>333.3</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>100</td>
<td>14 3</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>100</td>
<td>12 7</td>
<td>1.8</td>
<td>19.56</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>100</td>
<td>23 1</td>
<td>2.7</td>
<td>17.8</td>
</tr>
<tr>
<td>Glu²TRH</td>
<td>160</td>
<td>2797 2</td>
<td>221.4</td>
<td>1285.4</td>
</tr>
<tr>
<td>Phe²TRH</td>
<td>200</td>
<td>493 2</td>
<td>128 80</td>
<td>631.91</td>
</tr>
</tbody>
</table>

Table 3.7.6. **Ki Values Determined for a Range of Proline-Containing Peptides**

Ki values were determined using Z-Gly-Pro-MCA as substrate. All peptides were found to be competitive inhibitors of PO and Ki values were estimated from Km and apparent Km values (Km in the presence of (inhibitor/substrate).

* Actual concentration of peptide during assay
Figures 3.7.6.3.1.1 to 3.7.6.3.1.3. *Kinetic Analysis of brain, serum and bacterial PO respectively using Z-Gly-Pro-MCA*

Lineweaver-Burk reciprocal plots of reaction velocity versus Z-Gly-Pro-MCA concentration $K_m$ results illustrated in table 3.7.6.3.1.
Figures 3.7.6.3.2.1 to 3.7.6.3.2.3. Kinetic Analysis of brain, serum and bacterial PO respectively using pGlu-His-Pro-MCA

Lineweaver-Burk reciprocal plots of reaction velocity versus Z-Gly-Pro-MCA concentration. Km results illustrated in table 3 7 6 3 1
Figure 3.7.6.3.3.1. Kinetic analysis of the effect of Angiotensin II (○) on bovine brain PO activity towards Z-Gly-Pro-MCA (●). Figure illustrates Lineweaver-Burk reciprocal plots of reaction rate versus substrate concentration. Procedure was performed as outlined in section 2.9.6.3.3. Rates* were an expression of fluorescent intensities.

Figure 3.7.6.3.3.2. Kinetic analysis of the effect of Arg⁴-Vasopressin (○) on bovine brain PO activity towards Z-Gly-Pro-MCA (●). Figure illustrates Lineweaver-Burk reciprocal plots of reaction rate versus substrate concentration. Procedure was performed as outlined in section 2.9.6.3.3. Rates* were an expression of fluorescent intensities.
Figure 3.7.6.3.3. Kinetic analysis of the effect of TRH (○) on bovine brain PO activity towards Z-Gly-Pro-MCA (●). Figure illustrates Lineweaver-Burk reciprocal plots of reaction rate versus substrate concentration. Procedure was performed as outlined in section 2.9.6.3. Rates* were an expression of fluorescent intensities.

Figure 3.7.6.3.4. Kinetic analysis of the effect of LHRH (○) on bovine brain PO activity towards Z-Gly-Pro-MCA (●). Figure illustrates Lineweaver-Burk reciprocal plots of reaction rate versus substrate concentration. Procedure was performed as outlined in section 2.9.6.3. Rates* were an expression of fluorescent intensities.
Figure 3.7.6.3.3.5. Kinetic analysis of the effect of Neurotensin (□) on bovine brain PO activity towards Z-Gly-Pro-MCA (●). Figure illustrates Lineweaver-Burk reciprocal plots of reaction rate versus substrate concentration. Procedure was performed as outlined in section 2.9.6.3.3. Rates* were an expression of fluorescent intensities.
3.7.7. **Effect of Specific Inhibitors on Purified Prolyl Oligopeptidase Activity**

3.7.7.1. Determination of IC50 Values For a Range of Specific Prolyl Oligopeptidase Inhibitors

The effect of various dilutions of specific inhibitors on purified bovine brain, bovine serum and recombinant flavobacterial PO activities was investigated as outlined in section 2.9.7.1. Figures 3.7.7.1.1 to 3.7.7.1.9 illustrate the effect of these inhibitors on purified bovine brain PO activity. Table 3.7.7 summarizes the IC50s determined for brain, serum and recombinant activities. α-Ketobenzoazolone displayed the most potent inhibitory activity, with an IC50 of \(6.3 \times 10^{-11}\) M for the brain activity. All three PO activities were strongly inhibited by Z-Pro-proinal, Z-indolmyl proinal, Fmoc-Ala-Pro-nitrile and Fmoc-Pro-Pro-nitrile. However, in the case of Fmoc-Pro-Pro-nitrile the IC50 obtained for the recombinant activity was almost 10-fold higher than the IC50s obtained for the mammalian activities.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Z-Pro-proinal</td>
<td>10nM</td>
</tr>
<tr>
<td>Z-Pro-proinal-Dimethylyacetate</td>
<td>44μM</td>
</tr>
<tr>
<td>Cyclohexyl-proinal</td>
<td>649nM</td>
</tr>
<tr>
<td>Z-Indolmyl-proinal</td>
<td>10nM</td>
</tr>
<tr>
<td>Fmoc-Ala-Pyr-Nitrile</td>
<td>10nM</td>
</tr>
<tr>
<td>Fmoc-pro-Pyr-Nitrile</td>
<td>89nM</td>
</tr>
<tr>
<td>Z-Phe-Pro-Methylketone</td>
<td>13μM</td>
</tr>
<tr>
<td>Postatim</td>
<td>1 7μM</td>
</tr>
<tr>
<td>α-Ketobenzoazolone</td>
<td>63μM</td>
</tr>
</tbody>
</table>

Table 3.7.7. **IC50 Values Determined for Proline Specific Peptidase Inhibitors**

The effect of specific inhibitors on purified bovine brain, serum and recombinant flavobacterial PO activities was investigated as outlined in section 2.9.7.1. Results are expressed as IC50s (M) which were taken as the concentration of inhibitor required to inhibit enzyme activity by 50%. Enzyme activities* were expressed as percentages of activities without inhibitor.
Figures 3.7.7.1.1 and 3.7.7.1.2. Effect of Z-Pro-prolinal and Z-Pro-prolinal dimethylacetate on bovine brain prolyl oligopeptidase. Figures 3.7.7.1.1 and 3.7.7.1.2 illustrate the effect of Z-Pro-prolinal and Z-Pro-prolinal dimethyl acetate respectively on purified bovine brain PO IC50 values determined for each inhibitor are illustrated in table 3.7.7. Experiments were performed as outlined in section 3.7.7.1. Enzyme activity* is an expression of the % of activity obtained in the absence of inhibitor.
Figures 3.7.1.3. and 3.7.1.4. Effect of Fmoc-Pro-Pyr-CN and Fmoc-Ala-Pyr-CN on bovine brain prolyl oligopeptidase. Figures 3.7.1.3 and 3.7.1.4 illustrate the effect of Fmoc-Pro-Pro-Nitrile and Fmoc-Ala-Pro-Nitrile respectively on purified bovine brain PO. IC50 values determined for each inhibitor are illustrated in table 3.7.7. Experiments were performed as outlined in section 3.7.7.1. Enzyme activity is an expression of the % of activity obtained in the absence of inhibitor.
Figures 3.7.7.15 and 3.7.7.16. Effect of Cyclohexyl-Proline and α-Ketobenzothiazole on bovine brain prolyl oligopeptidase. Figures 3.7.7.15 and 3.7.7.16 illustrate the effect of Cyclohexyl-Proline and α-Ketobenzothiazole respectively on purified bovine brain PO IC50 values determined for each inhibitor are illustrated in table 3.7.7. Experiments were performed as outlined in section 3.7.1. Enzyme activity* is an expression of the % of activity obtained in the absence of inhibitor.
Figures 3.7.7.1.7. and 3.7.7.1.8. Effect of Postatin and Z-Phe-Pro-Chloromethylketone on bovine brain prolyl oligopeptidase. Figures 3.7.7.1.7. and 3.7.7.1.8. illustrate the effect of Postatin and Z-Phe-Pro-Chloromethylketone respectively on purified bovine brain PO IC50 values determined for each inhibitor are illustrated in table 3.7.7. Experiments were performed as outlined in section 3.7.7.1. Enzyme activity* is an expression of the % of activity obtained in the absence of inhibitor.
Figure 3.7.7.19. Effect of Z-Indolinyl-Prolinal on bovine brain prolyl oligopeptidase. Figure 3.7.7.19 illustrates the effect of Z-Indolinyl-Prolinal on purified bovine brain PO IC50 values determined for each inhibitor are illustrated in table 3.7.7. Experiments were performed as outlined in section 3.7.7.1. Enzyme activity is an expression of the % of activity obtained in the absence of inhibitor.
3.7.7.2. Effect of Z-Phe-Ala-Chloromethylketone on Purified Bovine Brain and Recombinant Prolyl Oligopeptidase Activity with Time

The effect of Z-Phe-Ala-Chloromethylketone on purified bovine brain and recombinant Flavobacterium meningosepticum prolyl oligopeptidase activity was investigated as outlined in section 2.8.7.2. Enzyme activity, expressed as a percentage of activity in the absence of inhibitor, over time is illustrated in figure 3.7.7.2.1. The inhibitor which was found to completely inhibit the recombinant activity after 45 minutes, had no effect on the bovine brain form of the enzyme.
Figure 3.7.7.2.1. Effect of Z-Phe-Ala-CMK on purified Bovine Brain and recombinant Flavobacterium meningosepticum Prolyl Oligopeptidase Activity.

The effect of Z-Phe-Ala-CMK on bovine brain (●) and recombinant PO (■) activities over time was investigated as outlined in section 2.8.7.2. Enzyme activities* were expressed as a percentage of activities measured at 0 minutes. Positive controls with no inhibitor for brain (○) and recombinant (□) samples were included in the experiment.
4. DISCUSSION
4.1. Fluorescence Spectrophotometry and 7-Amino-4-Methyl Coumarin

In order to detect low levels of peptidases, the use of high affinity substrates is essential. Early studies on PO made use of radiometric substrates such as radiolabelled oxytocin (Walter et al., 1971). Fluorimetric assays offer increased safety and ease of use over radiometric assays and increased sensitivity and selectivity over colorimetric assays (Rendell, 1987). The enzyme assays outlined in section 2.4 are all based on the use of an internally quenched MCA molecule attached to an N-blocked dipeptide, Z-Gly-Pro. This substrate was first synthesised by Yoshimoto et al., 1979, and was reported to have a higher affinity for PO and the greatest sensitivity over substrates such as Z-Gly-Pro-ONp. Km and kcat values of 20µM and 557 s⁻¹ were reported for this substrate, by this group.

Fluorescence is a form of luminescence involving the re-emission of previously absorbed radiation. In the assay system for PO, this peptidase mediates peptide bond cleavage at the carboxyl end of proline leading to the liberation of MCA. Electromagnetic radiation at a wavelength of 370nm is absorbed by MCA elevating it to an excited state. Then on returning to the ground state, radiation of 440nm is emitted.

4.2. Quenching and the Inner Filter Effect

With regard to fluorescence, a variety of forms of quenching can affect sensitivity leading to misleadingly low fluorescence. For instance, self quenching is the result of fluorescing molecules colliding and losing their excitation energy by radiationless transfer. Collisional impurity quenching involves the formation of a complex between excited MCA and a ground state impurity again resulting in radiationless energy transfer. This type of quenching can be usually attributed to the presence of dissolved oxygen or compounds containing heavy atoms such as halogens (Willard et al., 1988). It is a form of quenching that is the result of the presence of impurities that absorb either the exciting or emitted radiation, leading to a reduction of the fluorescent power that is particularly problematic in the case of crude biological samples such as serum and brain extracts. This type of quenching is also known as the “inner filter” effect. In order to compensate for this effect, it was necessary in the preparation of MCA standard curves to replicate assay conditions. Therefore, serum and brain supernatant were incorporated into MCA dilutions during the preliminary assays of crude samples for PO and ZIP activities (for preparation see section 2.2). Figures 311 and 313 illustrate the quenching effect of neat brain supernatant and serum on MCA fluorescence. This influence is also reflected in the slopes obtained for the quenched and unquenched curves. In the case of neat brain supernatant a 23% reduction in the slope value was observed at an emission slit width of 2.5nm, but in the case of serum a 31% decrease in the slope value was observed at an emission slit width of 5nm (see table 31). Another possibility in the minimisation of the quenching effect is dilution of the sample. This proved successful in the case of brain supernatant as the PO activity.
present was too high to measure at the lowest emission slit width of 2.5nm. Therefore a one in twenty
dilution was necessary to make the measurement of liberated MCA. This dilution also eliminated the
quenching effect as identical slopes were obtained for standard curves prepared with buffer and a
working dilution of brain supernatant (see table 3.1.). Although dilution of serum would have been
feasible to minimise the quenching effect, it would have meant expanding the emission slit width.
Expanding the emission slit width would consequently broaden the bandwidth over which the
fluorimeter integrates light emitted from a particular sample. For instance for an emission wavelength
and slit width of 440nm and 2.5nm respectively, the fluorimeter integrates light radiated from 438.75
to 441.25nm. If the slit width is increased to 10nm, light radiated from 435-445nm is integrated.
Although an increase in slit widths would undoubtedly lead to an increase in sensitivity this would be
accompanied by a parallel increase in inner filter effect interference.
Although the quenching effect of bovine brain supernatant on fluorescence was eliminated by dilution,
quenched standard curves were still prepared throughout purification and characterisation
procedures. Many compounds other than biological samples can contribute to quenching. These
include buffers such as Tris-HCl and imidazole-HCl. Decreasing the pH of these buffers can cause a
corresponding decrease in slope. A variety of functional reagents and divalent metals can also
contribute to quenching. For instance slopes obtained for standard curves prepared in the presence of
10mM 8-hydroxyquinoline, a metal chelator, are 57% lower than those calculated for standard curves
prepared with buffer. As a precautionary measure quenched standard curves were prepared for all
functional reagents and metal salts.

4.3. Measurement of Z-Gly-Pro-MCA Hydrolysing Activity in Crude Bovine
Brain Supernatant and Serum.
Z-Gly-Pro-MCA hydrolysing activity was measured in crude bovine brain supernatant and bovine
serum as outlined in sections 2.4.1 and 2.4.2. in a modification of procedures described previously
(Yoshimoto et al., 1979; Cunningham and O'Connor, 1997b). Assays on both samples were
performed in the presence and absence of 5x10^{-3} M Z-Pro-prolinal, the classical, specific PO inhibitor
(Wilk and Orlowski, 1983). Previously it was concluded that Z-Gly-Pro-MCA was a substrate
specific for PO detection. Many studies, particularly clinical evaluations of PO levels in serum, have
used this fluorimetric substrate but have failed to incorporate a specific PO inhibitor to validate that
all Z-Gly-Pro-MCA hydrolysing activity was entirely attributable to PO (Maes et al., 1994, 1995b).
However the existence of a second Z-Gly-Pro-MCA hydrolysing activity, which was completely
insensitive to Z-Pro-prolinal was recently reported by this laboratory (Cunningham and O'Connor,
1997b). PO and this ZPP insensitive peptidase were clearly distinguishable on the basis of their
behaviour during cation exchange chromatography and sensitivity to DTT, the ZPP insensitive
peptidase activity being unaffected by this reducing agent's presence. Therefore in the case of serum
it was very important to clearly distinguish these two Z-Gly-Pro-MCA hydrolysing activities in order to separate them. It was also necessary to determine whether this ZIP (Z-Pro-prolinal-insensitive Z-Gly-Pro-MCA hydrolysing activity) activity was present in bovine brain. Measurements made in serum confirmed the presence of both PO and ZIP activity, with 17% of total Z-Gly-Pro-MCA hydrolysing activity being Z-Pro-prolinal insensitive (ZIP), (figure 3.3.1). The assay conditions were however conducive to PO and not ZIP activity by the inclusion of DTT and omission of NaCl, which was found in this laboratory to activate ZIP. The total Z-Gly-Pro-MCA hydrolysing activity in brain homogenate was found to be 280% that of activity in serum (see figure 3.3.2.). Interestingly none of this activity was found to be insensitive to Z-Pro-prolinal. This would cast some doubt on the possible role of the ZIP enzyme in the hydrolysis of neuropeptides. However in attempting to assign a particular role for peripheral serum PE in the pathophysiology of depression, Maes et al., 1995b, speculated that PO could exert a central effect by either the degradation of peptides in serum generating bioactive products which could traverse the blood-brain barrier or by degrading smaller peptides, that were able to cross the blood-brain barrier, thus altering their central effects. It is clear that further studies are required of this ZIP enzyme before any possible function in neuropeptide metabolism can be confirmed.

4.4. Partial Purification of Prolyl Oligopeptidase from Bovine Serum.

PO from bovine serum was partially purified from bovine serum as outlined in section 2.5. to allow for comparative work to be completed in substrate specificity and inhibitor experiments. In this purification process it was crucial to ensure removal of all ZIP activity.

4.4.1. Phenyl Sepharose CL-4B Hydrophobic Interactions Chromatography I and II

In order to partially purify PO from bovine serum two consecutive hydrophobic interactions columns were used. The first column functioned in separating PO and ZIP activity. The protein and activity profile for this column illustrate the presence of 2 distinct peaks of Z-Gly-Pro-MCA hydrolysing activity. The first and larger peak appeared to run through the column with a substantial protein peak. The second peak was eluted isocratically with distilled water (see figure 3.4.1.). Fractions, when assayed in the presence and absence of Z-Pro-prolinal were found to contain both PO and ZIP. The first “run-through” peak was completely sensitive to Z-Pro-prolinal (PO), the second peak was completely insensitive to Z-Pro-prolinal (ZIP). While this column was quite efficient in the removal of protein there was a considerable, unexpected loss of activity (46%), (see table 3.4.1.). The higher ammonium sulphate concentration (1M) during the running of the second phenyl sepharose column allowed for binding of PO activity. This activity was eluted during a linear decreasing ammonium sulphate gradient (see figure 3.4.2.). An overall unexceptional purification factor of 9.82 and yield of
37% were obtained overall for this partial purification (table 3.4.1). It was however evident that PO was quite unstable as precautions, such as the inclusion of DTT in all buffers, were taken during the purification procedure.

4.5. Purification of PO from the Cytosolic Fraction of Bovine Brain.

4.5.1. Preparation of the Cytosolic Fraction of Bovine Brain

Although there have been some reports of membrane forms of PO (O'Leary et al., 1996, Dalmaz et al., 1986), this peptidase is thought to be primarily cytosolic in its location (Torres et al., 1986, Dresdner et al., 1982). Preparation of the cytosolic fraction of brain involved a series of homogenisation and centrifugation steps. An osmotic shock step was included at this stage to facilitate the removal of any PO that had occluded in the cell membrane (Dresdner et al., 1982). A final ultracentrifugation step was performed on the combined supernatants to separate any possible remaining membrane material such as microsomes (see section 2.6.1). A report on a membrane form of PO from bovine brain (O'Leary et al., 1996) found that this form of the peptidase had features quite distinct from the cytosolic form such as a sensitivity to thiol reagents and metal chelators which would classify it as a thimet peptidase. Therefore it was necessary to ensure its complete removal. Microsomes are derived primarily from endoplasmic reticulum. Upon cell breakage by homogenisation, the endoplasmic reticulum disrupts and its fragments tend to undergo vesiculation. The resulting membranous vesicles are termed microsomes. It is well known that ultracentrifugation for 60 minutes at 100,000g results in the sedimentation of these microsomes, ensuring their removal (Lambert, 1989).

4.5.2. Ammonium Sulphate Precipitation.

The ammonium sulphate precipitation step performed on the post ultracentrifugation brain supernatant served both as a crude clean up step and a means of concentrating the brain extract prior to column chromatography (see section 2.6.2). The optimal cut with respect to yield and the degree of purification was found to be 45-75% ammonium sulphate with an overall purification factor of 4.2 and yield of 86% (table 3.5.1). An ammonium sulphate step often features in PO purification protocols with varying degrees of success. One protocol which utilised quite a narrow cut of 40-65% reported a 99% yield and a purification of 3.25 (Tate, 1981). In contrast to this Yoshimoto et al., 1983c, performed a 50-80% cut, achieving only a 47% yield and a five-fold purification factor. These results reflect the losses made in activity in order to achieve a higher purification factor.
4.5.3. **DEAE Sepharose Fast Flow Anion Exchange Chromatography**

With the knowledge that mammalian PO is primarily an acidic protein with a pI of around 4.75-4.9 (Yoshimoto et al., 1981, Oliveira et al., 1976, Kalwant and Porter, 1991) anion exchange chromatography was chosen as a suitable step (see section 2.6.3.). Cation exchange chromatography would have required a pH below 4.5, which would have been quite detrimental to activity. As can be seen in the activity-protein profile in figure 3.5.1, a significant portion of protein ran through the column with another substantial portion removed during the linear increasing NaCl gradient used to remove bound PO activity. However, an unremarkable purification factor of 1.4 was achieved for the individual step with an overall yield of 58.7 (table 3.5.1). This is a fairly dramatic loss considering that the peptidase was not exposed to any substantial pH extremes or particularly high NaCl concentrations.

4.5.4. **Sephacryl S-200 Gel Filtration Chromatography**

Following ion-exchange chromatography, the PO pool was concentrated and applied to a sephacryl S200 column (see section 2.6.4). Although this column was not a very effective step with regard to purification, assessed from its activity-protein profile (figure 3.5.2), the decreased purification factor and yield of 57% (see table 3.5.1), it eliminated the need for an overnight dialysis step into imidazole-HCl, pH 6.3, which was found to be extremely harsh on PO activity.

4.5.5. **PBE94 Chromatofocusing**

Because of the difficulties faced in finding a suitable and specific chromatographic procedure (see section 4.5.7), chromatofocusing was chosen due to its wide applicability, potentially very high resolution and its suitability for the late stages of purification (Roe, 1989). In fact, chromatofocusing has been used successfully in previous purification protocols involving PO (Remnex et al., 1991, Browne and O'Cunn, 1983). Binding of PO was achieved at pH 6.3 and was eluted during a pH gradient between pH 4.7-4.9 (see section 2.6.5 for procedure). This roughly confirms the pI value of PO to be between this pH range. Fairly good resolution of protein was obtained with this step (figure 3.5.3), and surprisingly, activity was not adversely affected by the pH extremes with a recovery and purification factor of 83% and 28.5 for the step itself and an overall recovery and purification factor of 24% and 155 (see table 3.5.1).
4.5.6. **Phenyl Sepharose CL-4B Hydrophobic Interactions Chromatography**

Phenyl sepharose was chosen as a final column firstly for its ability to separate PO from the potentially harmful effects of the poly- or amphoteric buffer used in the elution of the chromatofocusing column and secondly as a strategy to stabilise and prepare PO for storage (see section 2.6.6 for procedure). These factors and its value as a purification step made it ideal for a final step. As can be seen from the activity-protein profile (see figure 3.5.4) a significant protein peak ran through the column. Attempts to resolve the bound PO peak from other bound protein through the use of a combined ammonium sulphate, potassium phosphate (decreasing) and glycerol (increasing) gradient were unsuccessful as can be seen in the activity-protein profile. PO and all other protein eluted in 10mM potassium phosphate, pH 7.4 with 15% v/v glycerol, an ideal media for freezing. A purification factor and recovery of 1.64 and 95% was obtained for this particular step (see table 3.5.1).

4.5.7. **Assessment of Purification Process of Prolyl Oligopeptidase from Bovine Brain.**

Overall the purification of PO from bovine brain proved to be a very difficult procedure to develop due to the difficulty in resolving PO from other contaminating protein and secondly the difficulty in maintaining PO activity during steps such as gel filtration. The overall purification factor for the purification was 253.9 with a recovery of 23% which was quite acceptable considering the losses sustained during the initial chromatographic steps. All the usual precautions were taken during this purification scheme with all columns run at 4°C and the inclusion of DTT in all buffers. All columns were run consecutively with minimum time lapses between steps. The SDS-PAGE silverstained gel illustrated in figure 3.5.5 illustrates the presence of a major band corresponding to the molecular mass protein standard BSA which has a molecular weight of 66,000Da. This mass was confirmed using a calibrated S200 column (see section 3.7.1). A faint band was also observed, with a molecular weight of around 50kDa. A range of fluorimetric assays designed for the detection of other peptidases, for example ZIP, PAP I and DAP IV, did not detect any of these peptidases (see table 3.5.2).

A variety of purification regimes were attempted in addition to the procedures outlined in sections 2.6.2-2.6.6 including hydroxylapatite (both commercial and synthesised in our laboratory), a variety of gel filtration resins and dye-ligand resins, metal chelate chromatography and activated thiol sepharose. This latter resin was used in an attempt to exploit PO's dependence on DTT. Although not an absolute requirement for activity, DTT activated PO activity indicating the presence of thiol groups. There have also been a variety of reports relating to mammalian PO's sensitivity to cysteine peptidase inhibitors (Kalwant and Porter, 1991, Tate, 1981, Yoshimoto et al., 1977). Usually the
difficulty with thiol sepharose is the removal of the reducing agent, DTT, in a short time period. The enzyme thiol groups must be reduced, with all the disulphide groups converted to thiols. The reducing agent must be removed quickly in order to minimise reversion of thiol groups to disulphide groups prior to chromatography. Using a method previously developed in our laboratory (Cummins and O'Connor, 1996) for DTT removal it was found that the resin had a very poor affinity for PO and its resolution from contaminating protein was impossible. Another group (Yoshimoto et al., 1983) reported on the successful use of another thiol group affinity resin in the purification of bovine brain PO, PCMB-T sepharose.

Metal chelating chromatography was used to again exploit the presence of cysteine residues in PO. Protein adsorption occurs by the binding of the thiol groups of surface amino acids to transition metals such as zinc (Roe, 1989). Unfortunately no binding of PO or no resolution of PO from other protein took place using zinc and calcium. It is possible that these cysteine residues may be somewhat buried within the enzyme 3-dimensional structure but this would not explain PO's sensitivity to cysteine peptidase inhibitors and some transition metals.

Another method which has been used previously by other groups with some success is hydroxylapatite chromatography (Kalwant and Porter, 1991). Chromatography with this resin was found not to be reproducible due to its sensitivity to phosphate concentrations and the very low flow rates necessary to achieve binding, which were too slow to maintain enzyme activity.

4.6. Prolyl Oligopeptidase Assay Development

Once PO was purified it was necessary to re-evaluate the assay procedure and determine the optimal conditions for PO activity and assay linearity. It was necessary therefore to investigate the effects of solvents and possible activators on enzyme activity and also, the effect of substrate concentration and enzyme concentration on linearity with respect to time. For an enzyme assay to be reproducible and quantitative, substrate hydrolysis must be linear with respect to time and substrate concentration. It is important to point out that all enzyme assay development studies were performed on purified PO. When the assay was found to be optimal the purification was performed with this new assay. All figures presented in Table 35.1 were obtained using the optimised assay.

4.6.1. The Effect of Solvents on Purified Prolyl Oligopeptidase Activity

Because of Z-Gly-Pro-MCA's insolubility in aqueous media and the known inhibitory effects of solvents on this peptidase (Knisatschek et al., 1980), it was necessary to find a solvent optimal for
substrate solubility and PO activity. The relationship between enzymic hydration and catalytic activity is well known. Water is involved in all noncovalent interactions, such as hydrogen bonding, ionic interactions, van der Waals and hydrophobic forces, that help to maintain the catalytically active enzyme conformation (Zaks and Klibanov, 1988). However, some enzymes do function in a naturally hydrophobic environment such as a cell membrane and in some cases can tolerate or even be activated by low solvent concentrations, for instance trypsin activity was found to be enhanced in the presence of 10% v/v acetonitrile and dioxane (Batra and Gupta, 1994). In the case of solvent-enzyme inhibition, there is a correlation between the loss of enzyme activity and the polarity of the solvent in question. Highly polar solvents such as dimethylformamide can interact with an enzyme and associated water molecules to drastically reduce or destroy catalytic activity (Arnold, 1990). Using the procedure outlined in section 2.8.1, it was set out to determine not only which solvent was least detrimental to enzyme activity, but also optimal for substrate solubility. While DMF was found to be the most effective solvent with respect to substrate solubility, it was most inhibitory of enzyme activity. On the other hand, while methanol was the least inhibitory on activity, it was not very efficient for substrate dissolution. Ethanol, dioxane and DMSO were similar in their inhibitory effects on activity, but DMSO was found to be most effective for substrate solubility (see figure 3.6.1). The optimal concentration of DMSO for the working assay concentration of 200μM was 8% v/v. Although the substrate tended to precipitate on removal from a 37°C water bath at this DMSO concentration, 10% DMSO would have been too detrimental to enzyme activity and stability. It is worth noting that none of the solvents tested even slightly activated PO at low concentrations. This may be due to POs naturally hydrophilic environment within the cell cytoplasm. DMSO is thought to disrupt the secondary structure of a protein causing it to unfold. In a study of the effects of DMSO on myoglobin and concanavalin A, low DMSO concentrations had no effect on these proteins' gross secondary structure, but their thermal stability was reduced (Jackson and Mantsch, 1991). It was therefore necessary to minimise any possible adverse effects DMSO could have on PO during characterisation experiments.

2.6.2. Linearity of Purified Prolyl Oligopeptidase Activity Assay with Respect to Time and Enzyme Concentration

In order for an enzyme activity to be quantifiable it is necessary to ensure linearity with respect to time and enzyme concentration. The progress curves of most enzyme reactions with respect to time are generally of the form illustrated in figure 4.1.
Figure 4.1. Linearity of Product Formation with Respect to Time

The time course is linear to begin with but the rate of product formation declines at longer times. There are many reasons for such departure from linearity but in the case of PO and Z-Gly-Pro-MCA non-linearity was thought to be primarily due to substrate depletion and instability of the purified enzyme during assay. This is highlighted by figures 3.6.2a. and 3.6.2b. Firstly activity was monitored over time using purified PO samples which had been stored in the presence and absence of BSA. In the absence of BSA the PO reaction rate departed from linearity in less than ten minutes. This departure from linearity is indicative of PO’s instability in dilute protein solutions. The addition of BSA to the purified sample restores linearity, more than likely by stabilisation of the enzyme. It is also evident from continuous assays performed with 100μM and 200μM Z-Gly-Pro-MCA and monitored over forty minutes that the higher concentration of substrate was necessary to ensure linearity. At this point it was decided to decrease the assay time of one hour, used previously in this laboratory (O’Leary et al., 1996; Cunningham and O’Connor, 1997b) to thirty minutes. Using the conditions determined from these time-course experiments i.e. 200μM substrate, 0.5% w/v BSA, the assay was determined to be linear over thirty minutes with respect to enzyme concentration (see figure 3.6.3).

4.6.3. Effect of DTT on Purified Prolyl Oligopeptidase Activity

Numerous reports have shown PO to be activated by the thiol-reducing agent DTT (Kalwant and Porter, 1991; Orłowski et al., 1979; Walter, 1976). This is in agreement with the findings that mammalian and plant forms of PO were sensitive to certain thiol peptidase inhibitors such as PCMB, NEM, and 2-iodoacetamide (Kalwant and Porter, 1991; Yoshimoto et al., 1987; Tate, 1981). As these inhibitors were not as potent in eliciting inhibition in comparison to DFP it was suggested PO had a cysteine residue close enough to the active site to influence activity under certain conditions (Polgar,
1991) PO activity was found to increase by over 250% in the presence of 5mM DTT. However, this peak in activity was followed by a steady decline between 10mM and 100mM DTT (see figure 3.6.3). DTT is a thiol reducing agent and can function to reduce disulphide bridges to thiol groups (see figure 4.2). At a particular concentration, DTT could serve to slightly unfold a protein, by disruption of these disulphide bridges, making the active site more accessible to a substrate. If used in excess, DTT could totally disrupt an enzyme structure and this would explain the decline in PO activity at high DTT concentrations (figure 3.6.3.). Alternatively, DTT could activate a cysteine residue that is in close enough proximity to the active site, to assist nucleophilic attack upon a substrate by the catalytically competent cysteine residue.

Another interesting characteristic afforded by the inclusion of DTT in the assay was an improvement in the reproducibility of triplicates and therefore was considered an essential component of the assay system.

![Disulfide-linked chains](image)

**Figure 4.2. Reduction of Disulphide bridges to Thiols by DTT**
4.6.4. Effect of EDTA on Purified Prolyl Oligopeptidase Activity

The reasons for inclusion of EDTA into the assay system were twofold. Firstly metal cations can contribute to the oxidative breakdown of DTT by the formation of free radicals and the inactivation of sulphhydryl groups. There have also been numerous reports of inhibition of PO by divalent metals such as Hg²⁺ and Cu²⁺ (Kalwant and Porter, 1991; Yoshimoto et al., 1987; Tate, 1981). Therefore the inclusion of EDTA minimised the effects of any metal contamination on enzyme activity. As can be seen in figure 3.6.5., 0.5mM EDTA slightly increased PO activity by around 4%. This increase was followed by a steady decline in activity at EDTA concentrations between 10mM and 150mM.

4.6.5. Effect of NaCl on Purified Prolyl Oligopeptidase Activity

NaCl has been reported to frequently stabilise protein structure by preferential hydration, water being involved in all noncovalent interactions that help maintain the catalytically active enzyme conformation (Timasheff and Arakawa, 1989). Prolyl oligopeptidase unlike the members of other serine protease families is very sensitive to ionic strength, its activity enhanced by high concentrations of NaCl (Polgar, 1991). This was confirmed by results obtained for bovine brain PO (figure 3.6.6.) with activation of the enzyme followed by a steady decline in activity at concentrations greater than 400mM. Although this inhibition was also observed by Polgar, 1991, it may have been in part due to insolubility of the substrate observed at high salt concentrations. Because of the difficulties in dissolving Z-Gly-Pro-MCA in the presence of NaCl, it was not included in the assay system.

4.7. Characterisation of Purified Prolyl Oligopeptidase Activity

4.7.1. Relative Molecular Mass Determination

The Relative molecular mass of PO was determined by Sephacryl S-200 gel filtration chromatography and SDS-PAGE as outlined in section 2.9.1. From molecular mass calibration curves prepared from these experiments the molecular weight for PO was confirmed to be 69-70kDa and monomeric (figures 3.7.1.1. and 3.7.1.2). This concurs very well with the majority of investigations which have reported a molecular weight of between 62-80kDa (table 1.3.1.). It is worth noting that some of the higher molecular weights quoted were deduced from an amino acid sequence and did not correct for the presence of an N-terminal signal sequence which is removed to give the mature peptidase (Shirasawa et al., 1994; Chevallier et al., 1992). Two early studies reported molecular weights of 115kDa and 140kDa for PO from lamb kidney and human placenta respectively and suggested that the enzyme was dimeric (Koida and Walter, 1976; Mizutani et al., 1984). It is now widely accepted however that PO is monomeric and a later study on the lamb kidney enzyme confirmed the molecular
weight to be in fact 74-77kDa (Yoshimoto et al., 1981). Further investigations of the human placental peptidase confirmed the molecular weight to be 79kDa (Kalwant and Porter, 1991)

4.7.2. PH Effects on Purified Prolyl Oligopeptidase Activity

In order to investigate the effect of pH and different buffers on PO activity two different pH experiments were performed. The first, described in section 2.9.2.1 served to simply determine the optimum pH and buffer for PO activity. From figure 3.7.2a, it is evident that PO activity was detectable between 4.5 and 9.0 with optimal activity at pH 7.4 in potassium phosphate. This is not surprising considering PO is found naturally at a physiological pH of 7.4 and is similar to the findings of other studies (table 13.1). However, activity in Tris-HCl is 23% lower at pH 8.0 than that measured in phosphate, indicating a general preference for phosphate buffer. The second pH experiment, described in section 2.9.2.2 was used to determine the pHs at which PO was irreversibly inactivated prior to assay at pH 7.4. From figure 3.7.2b, it is evident that PO was inactivated outside the pH range 5-9.5. This was unexpected as during the purification, PO was eluted in an amphoteric buffer of pH 4.5 with only a 5% loss in activity (table 3.51). This discrepancy may have been due to the protocol followed in this experiment which involved a preincubation at 37°C for 15 minutes with the appropriate buffer. However, during the purification the enzyme was kept at 4°C prior to assay. There may also have been variations in PO's sensitivity to the citrate/phosphate used in this procedure and the polybuffer used to elute the chromatofocusing column.

4.7.3. Thermostability Studies on Purified Prolyl Oligopeptidase Activity

Similar to pH studies the effect of temperature on PO was investigated using two different strategies as outlined in section 2.9.3. Firstly, the optimal temperature at which to perform the PO activity assay was determined as described in section 2.9.3.1, which clearly indicated that 37°C was the optimal temperature for activity (figure 3.7.3a). Again, however, this was not surprising considering physiological temperature is 37°C. This result was in agreement with other studies done on mammalian, plant and bacterial forms of the peptidase (see table 13.1). Another temperature experiment was performed to determine the effect of preincubation of PO at various temperatures for different times on activity prior assay at 37°C. It was found that a preincubation at temperatures of 50°C or more for times of 15 minutes or greater caused complete inactivation of the peptidase activity (see figure 3.7.3b)
4.7.4. The Effect of Divalent Metal Salts on Purified Prolyl Oligopeptidase Activity

The effect of divalent metal salts on PO activity was investigated as outlined in section 2.9.4. Previous studies on PO have shown an inhibition of mammalian, plant and bacterial PO by a variety of metal ions such as \( \text{Hg}^{2+} \), \( \text{Zn}^{2+} \) and \( \text{Cu}^{2+} \) (Kalwant and Porter, 1991, Yoshimoto et al., 1987, Walter, 1976). PO activity was severely inhibited by \( \text{Hg}^{2+} \), \( \text{Cu}^{2+} \) and \( \text{Cd}^{2+} \). Activity was also inhibited substantially by \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \) and \( \text{Zn}^{2+} \), but little or no inhibition was observed with \( \text{Fe}^{2+} \), \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), (see table 3.7.1). In contrast to another study which investigated the effects of metal salts on PO from bovine brain, carrot and \( \text{F meningosepticum} \), \( \text{Ni}^{2+} \) was quite potent in its inhibition of bovine brain PO with over 60% inhibition. However this study reported only a 10% inhibition of bovine brain activity (Yoshimoto et al., 1987). \( \text{Zn}^{2+} \) was also found to be not quite as inhibitory as other reports with only 40% inhibition. This is in contrast to some studies that reported complete inactivation of PO activity (Yoshimoto et al., 1987, Orlowski et al., 1979). It is worth noting at this stage that the preparation of these divalent salts solutions required strict monitoring of pH, otherwise any inhibition observed could well have been contributed to by pH extremes. Also quenched standard curves incorporating all metal ions were prepared to allow for quenching effects.

4.7.5. The Effect of Functional Reagents on Purified Prolyl Oligopeptidase Activity.

An investigation into the effects of a variety of functional reagents was investigated as outlined in section 2.9.5. Table 3.7.2 lists the effects of a variety of classes of functional reagents, at different concentrations, on PO activity. The most significant results are discussed as follows.

4.7.5.1. Serine Protease Inhibitors

PO has been classified in the past as a serine peptidase on the basis of its sensitivity to the classical serine protease inhibitor, DFP and analysis of its amino acid sequence (Rennex et al., 1991, Kalwant and Porter, 1991; Yoshimoto et al., 1987a, 1983c, Tate, 1981). In this experiment AEBSF, an irreversible inhibitor of serine proteases such as trypsin and chymotrypsin, was used as a safer, more stable and soluble alternative to the use of DFP (Markwardt et al., 1974). 1mM AEBSF completely inhibited PO and a ten-fold lower concentration inhibited PO by over 80% confirming the presence of an active site serine residue. Another study completed in this laboratory on bovine serum PO reported a lower inhibitory potency for AEBSF, with only 80% inhibition at 10mM (Cunningham and O’Connor, 1998). In contrast to this PMSF only inhibited PO by 10.73% at 1mM (table 3.7.2). This lack of sensitivity to PMSF is a peculiarity that has been found, by other investigators, with all forms.
of PO, mammalian, plant and microbial (Cunningham and O'Connor, 1998; Yoshimoto et al., 1987; Orlowski et al., 1979).

Other serine protease inhibitors aprotinin and bezamidine had minor effects on activity with aprotinin causing maximum inhibition of 14% at 5mM and benzamidine inhibiting PO by 7% at 10mM (table 3.7.2.). Other studies have also found benzamidine to have little or no effect on PO activity (Cunningham and O'Connor, 1998; Browne and O'Cuinn, 1983).

4.7.5.2. Cysteine Protease Inhibitors

Inhibition of mammalian and plant forms of PO by cysteine protease inhibitors has been well documented (Kanatani et al 1993; Yoshimoto et al., 1987; Yoshimoto et al., 1981; Orlowski et al., 1979). However this inhibition seems to be significant only at high concentrations of a particular functional reagent with the size of a reagent determining its inhibitory potency. It has been speculated that PO may have a non-catalytically competent cysteine residue in close vicinity to the active site. Larger reagents were found to exert more of an inhibitory effect on PO activity possibly due to exclusion of a substrate from the active site due to steric hindrance (Polgar, 1991) Reagents such as PCMB, DTNB and NEM should therefore be more inhibitory of PO activity than the smaller iodoacetamide. This effect was confirmed by experiments on the bovine brain PO, with iodoacetate and iodoacetamide exerting moderate inhibition, 60 and 80% respectively at 10mM, considering the high concentrations used. NEM and DTNB were far more effective inhibitors with complete inhibition of PO at concentrations of 10mM (see table 3.7.2).

4.7.5.3. Thiol-reducing Agents

The effect of DTT on PO activity has been discussed previously (see section 4.6.3.) In this procedure DTT (10mM) when preincubated with PO, caused just over a two-fold enhancement of activity (table 3.7.2). This level of activation is significantly lower than that reported for bovine serum (Cunningham and O’Connor, 1998). This discrepancy may however be attributed to the absence of DTT in the substrate buffer. Upon addition of the substrate the total DTT concentration was diluted to 2mM, and it is quite possible that a higher DTT concentration is required for the duration of the assay in order to be effective. The extent of the activation observed is however in accordance with results obtained for section 2.8.4.

4.7.5.4. Metal Chelators and Phenanthrolines

EDTA and 8-hydroxyquinoline (1mM) were both found to slightly activate PO activity, possibly because of their ability to chelate metal cations, such as zinc and nickel, which were previously found to inhibit PO (see table 3.7.1.). These metals may simply be present in an assay component as trace contaminants. Of the three phenanthrolines assessed only 1, 10-phenanthroline is a chelator. All three of these compound were, however found to inhibit PO (see table 3.7.2). Because these three
compounds share a common aromatic ring structure, the inhibition observed may be attributable to non-specific hydrophobic interactions between these compounds and some hydrophobic region of PO. A similar effect was noted with the metallo-protease, pyroglutamyl aminopeptidase II (PAP II), which was inhibited by all three phenanthrolines. This theory was supported by PAP II's ability to interact strongly with phenyl sepharose resin (Czekay and Bauer, 1993), a feature shared by bovine brain PO.

4.7.5.5. Other Agents

Bacitracin was found to possess quite potent inhibitory activity against PO with almost 80% inhibition at 1mg/mL (table 3.72.). There have been some conflicting reports on this antibacterial peptide's ability to inhibit PO with Browne and O'Connor, 1983, reporting no effect on guinea pig brain PO, and Cunningham and O'Connor, 1998, reporting almost a three-fold lower inhibitory potency with bovine serum PO. In another study on soluble bovine brain PO, bacitracin was found to cause strong inhibition with a Kᵢ of 5μM (Tate, 1981). Interestingly, bacitracin has been found to inhibit the degradation of thyroliberin by brain homogenate (McKelvy et al., 1976). N-Decanoyl CoA also inhibited PO by 54% at 1mg/mL (table 3.72.) A previous study on rat liver PO reported that CoA, related compounds, and acyl carnitine were possible endogenous inhibitors of PO with inhibitory potency being dependent on chain length of the acyl group (Yamakawa et al., 1990). N-Decanoyl CoA was found to only inhibit bovine brain PO by 54% at 1mg/mL (see table 3.72).

4.7.6. Substrate Specificity

4.7.6.1. Reverse Phase HPLC

Substrate specificity studies were performed using a range of synthetic and natural peptides by HPLC as outlined in section 2.9.6.1, using brain, serum and *F. meningosepticum* activities. Table 3.73 presents the list of peptides and whether or not hydrolysis occurred in the presence of PO. Figures 3.7.6.1-3.7.6.10 illustrate chromatograms obtained for peptides incubated with and without brain and bacterial activities and table 1.42 lists the amino acid sequences of some of the peptides analysed and the scissile bonds elucidated from previous investigations.

A range of synthetic N-blocked di and tri-peptides were examined. No hydrolysis of peptides with proline at the amino terminus was observed. The mammalian and bacterial enzymes also failed to hydrolyse Z-Pro-Pro-OH, but the peptide Z-Gly-Pro-Ala-OH was hydrolysed by all three enzymes. Of three unblocked peptides tested only H-Gly-Gly-Pro-Ala-OH was hydrolysed by the bacterial enzyme, H-Gly-Ala-Phe-OH and H-Gly-Pro-OH were not hydrolysed by any of the activities. These properties of PO have all been confirmed by earlier studies on the substrate specificity of this peptidase (Walter and Yoshimoto, 1978; Koida and Walter, 1976). It is surprising however that the mammalian...
activities did not hydrolyse this unblocked tetra-peptide. The lack of hydrolysis of this peptide is not in keeping with current understanding of PO specificity, particularly when this peptidase is known to act on tuftsin (Thr-Lys-Pro-Arg) and TRH (pGlu-His-Pro-NH₂) in vitro (Tate, 1981). It is highly likely that the mammalian activities were not concentrated enough to mediate detectable hydrolysis of this peptide.

A range of bioactive peptides were also included in this experiment. Mammalian (brain and serum) and bacterial PO was found to hydrolyse angiotensin I, II and III, bradykinin, substance P, TRH, TRH-Gly, LHRH and Glu²-TRH. Phe²-TRH was hydrolysed by brain and bacterial activities, AVP by bacterial and ADNF-14 by brain activities. Although these investigations were incomplete as the cleavage products generated were not identified, with the exception of TRH and TRH-Gly their hydrolysis by PO has been previously well documented.

Angiotensin I, II, and III have been previously shown to be cleaved at the Pro-Phe bond common to all three peptides (Moriyama et al., 1988). bradykinin at the Pro³-Gly⁸ and Pro⁷-Phe⁸ positions, substance P at the Pro⁴-Gln⁵ position, AVP at the Pro⁷-Arg⁸ position and LHRH at the Pro⁹-Gly¹⁰ position (see table 14 2). (Mendez et al., 1990, Tate, 1981) TRH and its natural precursor TRH-Gly were hydrolysed to liberate TRH-OH and two TRH analogs Glu²-TRH and Phe²-TRH were also hydrolysed, more than likely at the Pro-NH₂ bond. Both of these analogs have been found in the prostate gland and have been linked to human reproductive physiology (Gkonos et al., 1994).

AVP which has been previously shown to be hydrolysed at the Pro⁷-Arg⁸ bond (Walter, 1976), was hydrolysed by the bacterial but not the brain form of the enzyme. This was probably due again to the method sensitivity rather than any inability of the mammalian peptidase to cleave this peptide. Any differences observed in the mammalian and bacterial enzyme activity was probably due to limitations in method sensitivity and not enzyme specificity.

Activity-dependent neurotrophic factor 14, a short fragment derived from ADNF. ADNF is a protein discovered recently to have remarkable protective properties against neurotoxins in the brain. ADNF-14, a fourteen amino acid residue fragment of ADNF has been shown to prevent neuronal cell death from beta amyloid peptide, and gp120, a toxic protein shed by human immunodeficiency virus (Gozes et al., 1997, Gozes and Brenneman, 1996). Cleavage of this peptide by bovine brain PO most likely occurs at the C-terminal Pro-Ala bond. However, this discovery of a possible cleavage of this peptide by PO warrants a more detailed future study to elucidate the fragments generated by this action.
4.7.6.2. MALDI-TOF-Mass Spectrometry

MALDI-TOF MS was performed to firstly determine whether or not the brain form of PO hydrolysed the CLIP fragment, ACTH_{18-39}, a Gly-Pro-Ala polymer and an amyloid A4 precursor protein fragment 708-715. The fragments generated from this reaction were also determinable by their mass/charge ratio (m/z). Secondly the action of the bovine brain activity was compared to the *F meningosepticum* porcine kidney form of the peptidase. The experiments were performed as outlined in section 2.9.6.2. Table 3 lists the PO hydrolysis sites of these three peptides and figures 3, 6, 2, 1 to 3 represent the mass spectra obtained at various times during the incubations. Bovine brain PO was found to hydrolyse the CLIP fragment at the Pro^{24-Asn^{25}} and Pro^{36-Leu^{37}} bonds, generating the fragments ACTH_{18-24}, 25-39 and 25-36 (see figure 3, 6, 2, 1) These fragments are thought to be physiologically relevant in terms of paradoxical sleep. In another study the porcine kidney enzyme had been found to hydrolyse the same bonds (Demuth, H. U., Unpublished data), (see figure 4, 7, 2).

ACTH_{18-39} or CLIP has been shown to exert a very selective paradoxical or REM sleep inducing effect. The full CLIP sequence is not required for this PS inducing effect, as fragments ACTH_{18-24} and ACTH_{25-34} can increase PS to a similar extent. While CLIP and ACTH_{18-24} can only increase the number of PS incidents, the fragment ACTH_{20-24} can increase both the duration and number of these episodes. It has been proposed that the concerted action of PO and DAP IV results in the release of these three fragments (Wetzel *et al.*, 1997). Figure 4, 7, 2 illustrates the proposed PO and DAP IV sites of hydrolysis on ACTH_{18-39} that leads to formation of these fragments. The bacterial activity was found to hydrolyse the Ala^{27-Glu^{28}} and the Pro^{34-Asn^{35}} but not the Pro^{36-Leu^{37}} bonds (see figure 4, 7, 2). This difference in specificity between bacterial and mammalian forms of PO has not been observed previously and is suggestive of some structural difference between the enzyme's subsites. However further investigation of these differences using a range of synthetic substrates would have to be carried out in order to determine exactly what subsites are involved.

The Gly-Pro-Ala polymer, (Gly-Pro-Ala)$_{15}$ was also found to be hydrolysed over time by bovine brain PO. Unfortunately due to time limitations complete hydrolysis was not observed. However figure 4, 7, 3a and 4, 7, 3b represent the hydrolysis of this polymer over time by the porcine kidney and *F meningosepticum* forms of PO. Hydrolysis of this polymer by PO is in conflict with the current opinion that PO specificity is limited to smaller peptides of 25 amino acid residues or less (Moriyama *et al.*, 1988, Camargo *et al.*, 1979) and its classification as an oligopeptidase (Barrett and Rawlings, 1992) as the Gly-Pro-Ala polymer has 45 amino acid residues. This unexpected result means that a more detailed study on PO's ability to hydrolyse larger synthetic and natural peptides is required.

POs ability to cleave this (Gly-Pro-Ala) polymer is of tremendous importance when considering its ability to cleave the amyloid A4 precursor protein fragment 708-715. It was found that the bovine brain
PO hydrolyses this fragment at the Ala\(^{713}\)-Thr\(^{714}\) bond. This ability to cleave this fragment could be of physiological relevance. Amyloid A4 protein, which is found in deposits in senile plaques of patients with Alzheimer’s disease and Down’s syndrome, is derived from the larger amyloid A4 precursor protein. This 42 amino acid peptide is believed to be intrinsically linked to the pathogenesis of this disease because of its ability to aggregate and induce neuronal cell death (Yamaguchi et al., 1988). It is widely believed that proteolytic processing of the precursor protein could lead to the formation of amyloid A4 protein. Recent studies using model synthetic substrates have suggested that PO and a multicatalytic protease (MCP), ingensin, are responsible for hydrolysis of the precursor protein to generate the carboxyl and amino terminal ends of amyloid A4 peptide respectively. This hydrolysis is represented in figure 4.7.4. It has been argued however that PO’s classification as an oligopeptidase would rule out any possible role in amyloid A4 generation, even if a previous proteolytic action had occurred giving rise to the N-terminal portion of this peptide. However, this possibility must be considered with the knowledge that PO is capable of hydrolysing the 45 amino acid polymer, (Gly-Pro-Ala),\(^5\)

![Proposed Prolyl Oligopeptidase Hydrolysis Sites on ACTH 18-39](image)

**Figure 4.7.2. Proposed Prolyl Oligopeptidase Hydrolysis Sites on ACTH 18-39**

Bacterial PO from *F. meningosepticum* was found to hydrolyse the Pro\(^{24}\)-Asn\(^{25}\) and Ala\(^{27}\)-Glu\(^{28}\) bonds and porcine kidney DAP IV the Pro\(^{19}\)-Val\(^{20}\) bond of CLIP18-24 (Unpublished data courtesy of Hans Ulrich Demuth, Hans-Knoll Institute for Natural product Research, Halle, Germany). Bovine brain sites were determined as outlined in section 2.9.6.3.
Figure 4.7.3a. Hydrolysis of Gly-Pro-Ala Polymer by Porcine Kidney (figure 4.7.3a.) and *F. meningosepticum* (figure 4.7.3b) Prolyl Oligopeptidase with Time. * Represents (Gly-Pro-Ala)_{15}, M/Z=mass/charge ratio.
Figure 4.7.4. Proposed Mechanism of Amyloid Precursor Hydrolysis Resulting in Amyloid A4 Peptide Formation

The amyloid A4 peptide 1-42 is generated from the larger amyloid precursor protein APP_1-742. MCP (multicatalytic proteinase) and PO (prolyl oligopeptidase) have been identified as possible enzyme candidates, responsible for APP proteolytic processing (Yamaguchi et al., 1988)

4.7.6.3. Kinetic Studies

47631 Km Determination for Fluorimetric Substrates

Michaelis Menton Constants were determined for brain, serum and bacterial PO activities as outlined in section 29631 using Z-Gly-Pro-MCA and the TRH derivative pGlu-His-Pro-MCA. What is apparent from the Km values determined (see table 375) is firstly the difference in affinities between the three PO activities for a particular substrate. The bovine brain enzyme had a lower affinity for both fluorimetric substrates with Km values of 62.5 μM for Z-Gly-Pro-MCA and 99.8 μM for pGlu-His-Pro-MCA. The Km found for Z-Gly-Pro-MCA is considerably higher than the value of 20 μM reported when this substrate was first synthesised (Yoshimoto et al., 1979), but is comparable to other reports since (Cunningham and O'Connor, 1998, O'Leary and O'Connor, 1995) PO from all sources had a higher affinity for this N-blocked dipeptide than the TRH derivative pGlu-His-Pro-MCA

47632 K_i Determinations for Proline-Containing Peptides

The substrate specificity of the mammalian and bacterial forms of PO was investigated by examining the effect of a range of proline-containing peptides on the Km value determined for Z-Gly-Pro-MCA
Angiotensins I, II and III were found to be potent competitive inhibitors of all three enzyme activities (see Table 3.7.6). This is in agreement with a previous study on porcine muscle PO (Moriyama et al., 1988).

All three activities displayed a very low affinity for the TRH analogs Phe²TRH and Glu²TRH. This low specificity suggests that PO may not play an important physiological role in their degradation (see Table 3.7.6). In contrast to this, both mammalian forms exhibited a strong specificity towards TRH with Ki values (91μM for brain and 83μM for serum) approximate to their Km values for the fluorometric substrate Z-Gly-Pro-MCA (see Table 3.7.6). These values are much lower than the value (680μM) reported previously for the serum enzyme (Cunningham and O'Connor, 1998) and slightly higher than the Ki value reported for the rat brain enzyme (18 9μM) (Hersh, 1981). The competitive action of TRH on the bacterial enzyme activity towards Z-Gly-Pro-MCA produced a Ki of 642μM, illustrating a 20 fold decrease in specificity. This could be reflective of the fact that the bacterial activity could have no possible natural function in the degradation of TRH. This could also explain the bacterial enzyme’s much lower specificity for bradykinin, neuropeptide and AVP, when compared to the mammalian activities.

Both the mammalian activities had high specificities for bradykinin, neuropeptide and AVP with Ki values lower or, in the case of AVP and brain PO, approximate to their specificity towards Z-Gly-Pro-MCA (see Table 3.7.6). The Ki values determined for the serum enzyme for all these peptides were at least one order of magnitude lower than values reported previously for the serum enzyme (Cunningham and O'Connor, 1998). It is not fully understood why the serum PO activity had a slightly higher specificity than the brain PO for all the substrates tested. However, it is unlikely that this difference is due to differences in specificity as serum levels in vivo are much lower than the appreciable levels found in brain (Kato et al., 1980). These high levels in the brain are thought to reflect the significance of the brain enzyme's ability to regulate levels of proline-containing neuropeptides in vivo.

In view of these results and results obtained from other substrate specificity studies (see sections 4.7.6.1 and 4.7.6.2) it is clear that PO is an enzyme with a very broad specificity for proline-containing peptides. However, the physiological significance of this peptidase and its ability to hydrolyse peptides in vivo still remains to be elucidated.
4.7.7. *The Effect of Specific Inhibitors on Prolyl Oligopeptidase Activity*

A range of known specific PO inhibitors were tested on bovine brain, serum and *F. meningosepticum* prolyl oligopeptidase activity as outlined in section 2.9.7. The results are presented in the form of IC50 values in table 3.7.7.

The most potent inhibitor tested was α-ketobenzothiazole with an IC50 value of 63pM for the bovine brain enzyme. This value is in agreement with the value of 42pM, obtained for the bovine serum enzyme (Cunningham and O’Connor 1998). However both of these inhibitor values are considerably lower than that reported by Tsutsumi *et al.*, 1994, for the porcine kidney enzyme (table 1.5.1).

The poorest inhibitor tested was Z-Pro-prolinal dimethylacetate, with IC50s ranging from 32-44μM for mammalian and bacterial forms of PO. This inhibitory potency was over 30 times lower than that reported previously (Goossens *et al.*, 1997) and highlights the importance of the aldehyde group of Z-Pro-prolinal in PO inhibition. Z-Pro-prolinal while not the most potent inhibitor tested was found to have IC50 values of 10, 9 and 7nM for brain, serum and bacterial PO activities respectively. These values are higher than the value of 0.74nM quoted by Tsuru *et al.*, 1988, using bovine brain PO. Cunningham and O’Connor, 1998, reported an IC50 of 16nM using the bovine serum enzyme. Another Z-Pro-prolinal derivative, Z-indolinyl-prolinal was found to be similar in potency to Z-pro-prolinal with IC50s of 10nM for both of the mammalian enzymes, with the bacterial enzyme value being over four-fold higher than that of Z-pro-prolinal. However these values are similar to those obtained by Bakker *et al.*, 1991 (see table 1.5.1). It should be noted that a value of 45pM has been reported previously for the bovine serum enzyme (Cunningham and O’Connor, 1998).

Cyclohexyl-prolinal was found to be a rather poor inhibitor of the bovine brain enzyme relative to Z-pro-prolinal and Z-Indolinyl-prolinal with an IC50 value (649nm) considerably higher than previously reported (see table 1.5.1).

N-blocked dipeptide derivatives using an Fmoc blocking group, Fmoc-Ala-Pyr-nitrile and Fmoc-Pro-Pyr-nitrile had identical IC50s for the mammalian enzymes. For both of these inhibitors however the bacterial enzyme was found to be just over six times less sensitive to their inhibitory activity (see table 3.7.7).

Postatin, an inhibitor of bacterial origin was quite poor in its inhibition of the mammalian and the bacterial enzymes with IC50s in the micromolar range (table 3.7.7), considerably higher than previously reported values of 35nM for the bovine serum enzyme (Cunningham and O’Connor, 1998).

Z-Phe-Pro-methylketone was probably the most interesting inhibitor tested, not because of its inhibitory potency, but the large discrepancy in the sensitivity of mammalian and bacterial forms of PO observed. While both the brain and serum enzymes had IC50s of 13 and 11μM respectively the bacterial enzyme was nearly 3000 times more sensitive to inhibition by this compound.

A timecourse investigation into the effects of Z-Phe-Ala-chloromethylketone found that while the bacterial enzyme was completely inhibited by 1x10⁻⁴M of this inhibitor after 60 minutes, the bovine...
brain enzyme exhibited no sensitivity to this inhibitor. Chloromethylketones, previously thought to be specific for cysteine residues, have been shown to act on PO by binding to the active site serine residue (Stone et al., 1992). Inhibition of bacterial PO confirms this action on the serine residue, as bacterial forms of PO have been previously shown to be insensitive to cysteine protease inhibitors. It is not understood why a compound of this class could distinguish between different forms of PO. In a study of the comparison of inhibition of *F. meningosepticum* and bovine brain by prolinal containing peptide inhibitors, Boc-Pro-prolinal, and Z-Pyr-prolinal were found to be 1000 times more inhibitory of the bacterial enzyme possibly due to some structural difference around S2 and S3 subsites (Yoshimoto et al., 1985). The bacterial activity may have a higher affinity for the peptide portion of this inhibitor, Z-Phe-Ala, in particular the alanine residue at the S1' subsite. In studies on the hydrolysis of CLIP the *F. meningosepticum* activity, unlike the brain activity, hydrolysed the Ala-Glu bond (see section 4 7 6 2). Although previous studies have found that the bacterial and mammalian forms of PO have been shown to have quite similar substrate specificities for synthetic substrates and natural peptides, and have similar subsite widths (Yoshimoto et al., 1980, 1977), it can be now suggested that mammalian and bacterial forms of PO could have slightly different subsite stereospecificities. Further studies are required in order to clarify these possible differences.

### 4.8. Summary

PO was purified from the cytosolic fraction of bovine whole brain. Following preparation of this fraction, PO activity was purified using ammonium sulphate precipitation, DEAE sepharose, sephacryl S200 gel filtration, chromatofocusing and phenyl sepharose chromatography, resulting in a 253-fold purification and a 23% recovery of enzyme activity.

Bovine serum PO was partially purified from bovine serum for comparative substrate specificity and inhibitor studies. The purification consisted of chromatography on two separate phenyl sepharose columns, run under different conditions). The first column served to remove ZIP activity. A 10-fold purification factor and 37% recovery was achieved.

Prior to characterisation work the PO fluorimetric activity assay was optimised. This assay was found to be linear over an assay period of 30 minutes, using 200μM Z-Gly-Pro-MCA prepared in 8% v/v DMSO, 5mM DTT and 0.5mM EDTA were also included to increase assay sensitivity. The inclusion of 0.5% w/v BSA served to stabilise the enzyme for storage and during the assay. While NaCl was found to activate activity and increase the sensitivity of the assay, its adverse effect on substrate solubility warranted its exclusion from the assay procedure.

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The relative molecular mass of the brain activity, determined by gel filtration chromatography and SDS PAGE, was found to be 69.5 kDa. This activity was found to be optimal at 37°C. The optimal pH for activity was found to be 7.4 in phosphate buffer.

The brain enzyme was identified as a serine protease based on its sensitivity to AEBSF. PO activity was strongly inhibited by the cysteine protease inhibitors DTNB and NEM. While the enzyme was activated by DTT, this thiol reducing agent was not essential for catalytic activity. This would suggest the presence of a non-catalytically competent cysteine residue in close proximity to the active site. Enzyme activity was also strongly inhibited by some metal ions, including Hg²⁺, Cu²⁺, Co²⁺ and Ni²⁺.

The brain, serum and Flavobacterium meningosepticum PO activities hydrolysed the substrates Z-Gly-Pro-MCA (Km values of 63 μM, 15 μM and 39 μM respectively) and pGlu-His-Pro-MCA (Km values of 100 μM, 52 μM and 74 μM respectively). All three activities hydrolysed a range of proline-containing synthetic and natural peptides, including angiotensin I, II and III, TRH, bradykinin, neurotensin, APP708-715, a Gly-Pro-Ala polymer and CLIP. A number of these peptides also competitively inhibited PO activity towards Z-Gly-Pro-MCA. The most potent, with respect to brain, serum and bacterial activities, were angiotensin I, II and III, which had Ki values lower than the Km value for Z-Gly-Pro-MCA.

Brain, serum and recombinant activities were inhibited by a range of PO specific inhibitors with α-ketobenzothiaazole, Z-indolyl-proline, and Fmoc-Ala-Pyr-nitrile exerting the greatest inhibitory activity. The bacterial enzyme was found to be 4000 times more sensitive to Z-Phe-Pro-methylketone than the brain enzyme. 1x10⁻⁵ M Z-Phe-Ala-chloromethylketone completely inhibited the bacterial activity, but the brain enzyme was completely insensitive to this inhibitor.

In conclusion, the characteristics of PO purified from bovine brain are similar to those reported previously for other mammalian forms of this enzyme. However, further investigations are required to determine the exact specificity of this enzyme particularly with concern to peptide size. With the development of highly potent and specific inhibitors, it should be possible to elucidate the physiological role of PO in peptide degradation and in the pathogenesis of certain neurological disease states.
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