

## TITLE, AUTHORS AND AFFILIATIONS

### **Purification, Identification and Characterisation of Seprase from Bovine Serum**

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#### ABBREVIATIONS

AMC, 7-amino-4-methylcoumarin; BCA, bicinchoninic acid; CPC, calcium phosphate cellulose; DFP, diisopropylfluorophosphate; FPLC, fast protein liquid chromatography; HIC, hydrophobic interaction chromatography; PO, prolyl oligopeptidase; TFA, trifluoroacetic acid; Z, *N*-benzyloxycarbonyl; ZIP, Z-Pro-prolinal-insensitive Z-Gly-Pro-AMC-hydrolysing peptidase.

#### ABSTRACT

The study and identification for the first time of a soluble form of a Seprase activity from bovine serum is presented. To date, this activity has only been reported to be an integral membrane protease but has been known to shed from its membrane. The activity was purified 30,197-fold to homogeneity, using a combination of column chromatographies, from bovine serum. Inhibition by DFP, resulting in an  $IC_{50}$  of 100nM confirms classification as a serine protease. The protease after separation and visualisation by native PAGE was subjected to tryptic digestion and the subsequent peptides sequenced. Each peptide sequenced was found to be present in the primary structure of Seprase/Fibroblast Activation Protein (FAP), a serine gelatinase specific for proline-containing peptides and macromolecules. Substrate specificity studies using kinetic, RP-HPLC and LC-MS analysis of synthetic peptides suggest that this peptidase has an extended substrate-binding region in addition to the primary specificity site  $S_1$ . This analysis revealed at least five subsites to be involved in enzyme-substrate binding,

with the smallest peptide cleaved being a tetrapeptide. A proline residue in position P<sub>1</sub> was absolutely necessary therefore showing high primary substrate specificity for the Pro-X bond, while a preference for a hydrophobic residue at the C-terminal end of the scissile bond (P<sub>1</sub>') was evident. The enzyme also showed complete insensitivity to the prolyl oligopeptidase specific inhibitors, JTP-4819, Fmoc-Ala-pyrrCN and Z-Phe-Pro-BT. To date, no physiological substrate has clearly been defined for this protease but its ability to effectively degrade gelatin suggests a candidate protein substrate in vivo and a possible role in extracellular matrix protein degradation.

**Key Words: Z-Pro-prolinal Insensitive Peptidase; prolyl oligopeptidase; seprase/fibroblast activation protein; serine gelatinase; substrate specificity; Z-Gly-Pro-AMC.**

## INTRODUCTION

Proline as an imino rather than an amino acid results in most peptidases being unable to hydrolyse the peptide bond at proline residues. The prolyl peptidases are a unique class of proteases, gaining much attention of late, that possess the ability to cleave proteins and peptides after proline residues. This group includes dipeptidyl peptidase IV, seprase/fibroblast activation protein  $\alpha$ , DPP7, DPP8, DPP9, prolyl carboxypeptidase while more distant members include prolyl oligopeptidase (Rosenblum & Kozarich, 2003). Seprase or fibroblast activation protein (FAP) is an integral membrane serine peptidase, which has been shown to have gelatinase activity. It appears to act as a proteolytically active 170-kDa dimer, consisting of two 97-kDa subunits. It is a member of the group type II integral serine proteases, including dipeptidyl peptidase IV (DPPIV/CD26), that seprase shows considerable sequence homology with, and related type II trans-membrane prolyl serine peptidases, which are inducible, specific for proline-containing peptides and macromolecules and exert their mechanisms of action on the cell surface. DPPIV and Seprase exhibit multiple functions due to their abilities to form complexes with each other and to interact with other membrane-associated molecules (Chen, Kelly & Ghersi, 2003; Chen & Kelly 2003). Localization of these protease complexes at cell surface protrusions, called invadopodia, may have a prominent role in processing soluble factors (such as chemokines, hormones, bioactive peptides) and in the degradation of extracellular matrix components that are essential to the cellular migration and matrix invasion that occur during tumour invasion, metastasis and angiogenesis (Chen & Kelly, 2003; Ghersi et al. 2002). Seprase has also been shown to be one of a number of serine proteases required for microvascular endothelial cell reorganization

and capillary morphogenesis in vitro (Aimes et al. 2003). It is overexpressed by invasive tumour cells (Pineiro-Sanchez et al. 1997; Monsky et al. 1994) in particular it has been shown to be expressed by activated fibroblasts in the stroma of various epithelial cancers, mesenchymal tumours and breast-cancer cells (Ariga et al. 2001). Clinicopathologic analysis has revealed increased Seprase expression in cases of invasive ductal carcinoma is associated with longer overall and disease-free survival (Ariga et al. 2001; Kelly et al. 1998). Seprase activity is most often assessed by zymography, which is not a quantitative assay, but can be assessed qualitatively by measuring the release of gelatin fragments from radiolabelled gelatin (Kelly, 1999). It has been suggested that the cell surface localization of Seprase would allow it to be used to target therapeutic agents to malignant breast cells. However, to date, the pathophysiologic significance of its expression remains poorly understood.

In this report we describe the purification and identification of a prolyl peptidase from bovine serum resulting in a surprising primary structure related to the cell surface-associated serine integral membrane peptidase Seprase, along with the first primary and secondary substrate specificity studies performed on this enzyme.

## **EXPERIMENTAL PROCEDURES**

### **Materials**

Bachem U.K. supplied all fluorometric peptide substrates. All other peptides were synthesised by the Royal College of Surgeons in Ireland. Phenyl Sepharose CL-4B and Cibacron blue 3GA chromatography resins were obtained from Sigma Chemical Company (Poole, Dorset, England). A HiPrep Sephacryl S-300 column was obtained from Pharmacia. HPLC grade ammonium acetate and acetic acid were obtained from Fluka. HPLC grade acetonitrile and methanol were obtained from Riedel-de-Haen.

### **Methods**

#### ***Serum Preparation***

Bovine whole blood was collected from a freshly slaughtered animal and stored at 4°C over 24 hours to allow clot formation. The remaining un-clotted whole blood was decanted and centrifuged at 6000rpm for 1 hour at 4°C using a Beckman J2-MC centrifuge fitted with JL-10.5 rotor. The supernatant and loose cellular debris was decanted and re-centrifuged at 20,000rpm for 15mins using a JL-20 rotor. The final serum was collected and stored at -17°C in 20ml aliquots.

### ***Enzyme Activity Assay***

ZIP activity was determined with 0.1mM AMC fluorometric substrate Z-Gly-Pro-AMC in 4% v/v MeOH containing 500mM sodium chloride according to the method of Birney and O'Connor, 2001 with slight modification. JTP-4819 (20 $\mu$ l 2.5 x 10<sup>-4</sup>M in 10% v/v MeOH) was added to 100 $\mu$ l enzyme sample followed by the addition of 400 $\mu$ l substrate. The assay was performed at 37°C for 60 min. End point measurements were permitted, as the enzyme assay was linear with respect to time and enzyme concentration up to 60 min. The reaction, prepared in triplicate, was terminated by the addition of 1ml 1.5M acetic acid. Negative controls and blanks were included. The release of AMC from the substrate Z-Gly-Pro-AMC was spectrofluorometrically measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer (excitation, 370nm; emission, 440nm). Fluorometric intensities observed were converted to nanomoles AMC released per minute per ml using appropriate AMC standard curves. One unit of enzyme activity is defined as nanomoles of AMC released per minute at 37°C.

A non-quantitative fluorometric microtitre plate assay was developed to assist in the rapid identification of Z-Gly-Pro-AMC degrading activities in post-column chromatography fractions. 200 $\mu$ l of 100 $\mu$ M Z-Gly-Pro-AMC in 4% MeOH containing 500mM NaCl at 37°C was added to 100 $\mu$ l of sample and 20 $\mu$ l 2.5 x 10<sup>-4</sup>M JTP-4819 in each well. The microtitre plate was incubated at 37°C for 30 minutes. AMC released was determined fluorometrically as outlined previously using the Perkin Elmer LS-50B fluorescent plate reader attachment.

### ***Protein Determination***

Protein concentration was determined by Biuret, BCA (Ohnishi & Barr, 1978; Smith et al. 1985) and Coomassie Plus assays, using bovine serum albumin as standard.

### ***Enzyme Purification***

All purification steps were performed at 4°C except for FPLC separations, which were performed at room temperature. The following purification protocol of ZIP from bovine serum shows significant modification of the original procedure reported by Birney and O'Connor, 2001. Details of the additional Cibacron Blue column are detailed below. These modifications were deemed necessary in order to obtain an increased level of purity.

*Cibacron Blue 3GA Chromatography.* 100ml of 20mM potassium phosphate, pH 7.4 was used to equilibrate a 20ml Cibacron blue 3GA resin. The post calcium phosphate cellulose ZIP fraction was concentrated and then extensively dialysed overnight against 2 liters of 20mM potassium phosphate, pH 7.4. After sample application the column was washed with 100ml of 20mM potassium phosphate, pH 7.4 to remove any unbound protein. Elution was performed using a 100ml linear 0-2M NaCl gradient in 20mM potassium phosphate, pH 7.4. Loading, washing and elution were all performed at 1ml/min. 5ml fractions were collected and assayed for ZIP activity and protein content as described previously. Fractions containing ZIP activity were pooled and enzyme activity and protein content were quantified using the fluorometric assay and coomassie plus assay respectively.

### ***Assessment of Purity***

*Electrophoretic Technique* The SDS-PAGE system of Laemmli, 1970 was used to monitor enzyme purification. Gels were stained as recommended with coomassie blue and silver (Novex).

*Fluorometric Analysis* Purified Seprase activity was assayed in the presence of a number of targeted fluorometric substrates to confirm the absence of potentially interfering/contaminating peptidases. Each substrate was prepared as a 10mM stock in 100% v/v MeOH, then diluted in 100mM potassium phosphate yielding a final substrate concentration of 0.1mM in 4% v/v MeOH. Assays were performed as described previously.

### ***Catalytic Classification***

A range of concentrations of DFP was prepared from a stock concentration of 20mM using 100mM potassium phosphate containing 500mM NaCl as diluant. A stock substrate of 200 $\mu$ M Z-Gly-Pro-AMC in 8% v/v MeOH and containing 500mM NaCl was prepared using 100mM potassium phosphate, pH 7.4. This stock was preincubated at 37°C until completely dissolved and thermal equilibrium was reached. 1ml of substrate stock was added to 1ml of appropriate DFP concentration to give a final substrate concentration of 100 $\mu$ M Z-Gly-Pro-AMC in 4%v/v MeOH containing 500mM NaCl. ZIP activity using these substrate: inhibitor mixtures was assayed in triplicate as outlined previously. Suitable negative and positive controls were prepared.

### ***Protease Identification***

*Electrophoretic Technique* A 10% native PAGE gel was prepared, with the exception that no SDS was present in the gel. Purified ZIP was obtained from four separate 20ml purifications, dialysed extensively (18hr) into ultra-pure water and finally concentrated to 500µl using a speedvac. 10x (non-denaturing) solubilisation buffer was prepared as follows: 3.2ml 10% SDS, 2ml 0.5M Tris/HCl pH 6.8, 1.6ml glycerol, 0.05%(w/v) bromophenol blue and 1.2ml of ultra-pure water, in a final volume of 8ml. Running buffer was prepared without SDS present. 90µl of concentrated ZIP sample was added to 10µl 10x (non-denaturing) solubilisation buffer and applied directly to the gel. The gel was run at a constant voltage of 125V at 4°C for up to 2.5hrs.

*U.V. Zymogram development* After native page electrophoresis, the gel was rapidly removed and placed in 50ml of 100µM Z-Gly-Pro-AMC in 100mM potassium phosphate pH 7.4 containing 4%v/v MeOH and 500mM NaCl. It was incubated with shaking for 10-15mins at 37°C, after this excess substrate was removed and the gel placed under ultraviolet light in an image analyser for visualisation. It was possible to detect a significant fluorescent band where the synthetic substrate Z-Gly-Pro-AMC was being hydrolysed. The fluorescent band was excised and sent to the Harvard Microchemistry Facility where sequence analysis was performed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer.

### ***Gelatin Zymography***

Gelatin zymography was performed to observe enzyme activity against the protein substrate gelatin. The gel was prepared by incorporating the protein substrate of interest (gelatin) within the polymerised acrylamide matrix. The enzyme sample was resolved by 10% native PAGE gel in the presence of 1mg/ml gelatin (denatured type 1 collagen; Sigma) (Aoyama & Chen, 1990). The method of Laemmli was followed, excluding any reducing agents or boiling procedures. Samples were mixed 3:1 with 4x (non denaturing) solubilisation buffer, which consisted of 16% v/v SDS, 40% v/v Glycerol and 0.08% Bromophenol Blue. The gels were run at 20mA per gel in running buffer (25mM Tris, 192mM Glycine) for up to 4 hours at 4°C. After electrophoresis, the gel was washed for 30 mins in 2.5% Triton X-100 at room temperature, with one wash change. The gel was then incubated overnight at 37°C in reaction buffer (100mM potassium phosphate, pH 7.4; 500mM NaCl). After staining with Coomassie

Brilliant Blue R-250 (Biorad) for 2 hours with shaking, the gel was destained in water until clear bands were visible (Yao et al. 1996). Gelatin degrading activity was identified as a clear zone of lysis against a blue background.

### ***Substrate Specificity and Kinetic Studies***

#### *Kinetic Analysis*

*K<sub>m</sub> determinations for Z-Gly-Pro-AMC* A 300 $\mu$ M stock solution of Z-Gly-Pro-AMC in 5%v/v MeOH containing 500mM NaCl was prepared. A range of concentrations (0-300 $\mu$ M) was prepared from this stock using 100mM potassium phosphate, pH 7.4, containing 5%v/v MeOH and 500mM NaCl as diluant. Purified ZIP was assayed with each concentration in triplicate as outlined previously. The K<sub>m</sub> of ZIP for the substrate Z-Gly-Pro-AMC was estimated when the data obtained was applied to Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf kinetic models.

*K<sub>i (app)</sub> determinations for selected synthetic peptides* The effect of a variety of selected synthetic peptides on the kinetic interaction between ZIP and the substrate Z-Gly-Pro-AMC was determined. Substrate concentrations in the range 100-300 $\mu$ M Z-Gly-Pro-AMC in 5%v/v MeOH containing 500mM NaCl was prepared in the presence of 200 $\mu$ M peptide, each in a final volume of 2ml. The assay MeOH concentration was maintained at 5%. ZIP activity was assayed in triplicate using these substrate mixtures as outlined previously. The data obtained was applied to the Lineweaver-Burk analysis model, where the inhibition constant (K<sub>i (app)</sub>) and the type of inhibition observed were determined (Henderson, 1992).

*Reverse-Phase HPLC Analysis* The determination of substrate specificity was also based on the separation of the products of peptide hydrolysis by reverse-phase chromatography. Purified ZIP was concentrated 10-fold and dialysed extensively overnight against 50mM ammonium acetate, pH 7.2. 50 $\mu$ l of ZIP in 50mM ammonium acetate, pH 7.2, was added to 200 $\mu$ l of each peptide substrate (1mM) in 50mM ammonium acetate, pH 7.2 containing 2%v/v methanol. Hydrolysis of the peptides was performed at 37°C over a 24hr period. Reactions were terminated with 25 $\mu$ l of 50mM ammonium acetate containing 50%v/v acetonitrile and 5%v/v acetic acid. All samples were then subjected to reverse-phase high performance liquid chromatography using a Waters Spherisorb ODS-2 C-18 column. Mobile phases for the HPLC system consisted of solvent A; 80% (v/v) Acetonitrile, 0.2% (v/v) TFA and solvent B; 5% (v/v) Acetonitrile, 0.2% (v/v) TFA. The reverse phase C-18 column was equilibrated for ten minutes with 100% solvent B. 20 $\mu$ l of sample and controls were injected

onto the column followed by a four-minute wash with 100% solvent B. A linear gradient from 100% solvent B to 100% solvent A over a twenty-one minute period was then applied. Finally, a five-minute wash with 100% solvent A was applied. A flow rate of 0.7ml/min was used throughout. The absorbance of the eluent was monitored at 214nm.

*LC-MS Analysis* Due to the formation of complex standards after hydrolysis of the peptides by ZIP, LC-MS analysis was used to give exact identification of each product formed. Direct infusion MS analysis, which was initially used preferentially ionised some components over others that led to the failure to identify any product formation post incubation. LC-MS analysis using the Bruker/Hewlett-Packard Esquire LC, equipped with an electrospray source (ESI) was then chosen to identify each product of hydrolysis (McMahon et al. 2003). This enabled the compounds of interest to only become singly charged and so their molecular weight being readily obtained.

### ***Inhibitor Studies***

The potency of some specific Prolyl Oligopeptidase inhibitors towards purified ZIP and partially purified PO from bovine serum was assessed. IC<sub>50</sub> values were evaluated using steady-state enzyme-substrate reaction. Reactions were initiated by the addition of 100µl enzyme to 1.9ml 20µM Z-Gly-Pro-AMC containing 1%v/v MeOH, giving a final volume of 2ml. The reaction was constantly monitored for 15 minutes until a constant rate was observed. 2µl of each inhibitor concentration was then added and the reaction allowed to proceed for 45 minutes at 37°C. Reaction buffer for purified ZIP was 100mM potassium phosphate containing 500mM NaCl, pH 7.4 and for PO was 100mM potassium phosphate containing 10mM DTT, pH 7.4. Suitable positive and negative controls were also prepared. Enzyme activity was continuously monitored on a Kontron Spectrofluorimeter SFM 25 (excitation wavelength 370nm, emission wavelength 440nm) equipped with a thermostated four-cell changer and controlled by an IBM-compatible computer.

## **RESULTS**

### ***Enzyme Purification and Assessment***

As previously known bovine serum contains two distinct Z-Gly-Pro-AMC hydrolysing activities, Prolyl Oligopeptidase (PO) and Z-Pro-prolinal Insensitive Peptidase (ZIP). The purification of Z-Pro-prolinal Insensitive Peptidase is described in Birney and O'Connor,



2001. However, following concentration and dialysis, post-Calcium Phosphate Cellulose ZIP sample was applied to an additional Cibacron blue 3GA affinity chromatography resin.

Figure 1 presents this elution profile, which clearly shows that ZIP activity actually bound to the resin and eluted during the increasing salt gradient applied. The initial reason for this resin was in the hope that ZIP would run through and thus be separated from any contaminating bovine serum albumin present in the sample. Even though this failed to be the case, the chromatography step proved incredibly effective with a 80-fold decrease in total protein content. The recovery of applied activity was only 37% but it still enabled a 18,735-fold purification to be obtained along with 20% overall recovery of biological activity. This 18,735-fold purification factor compares to the 4023-fold obtained previously for a three step purification of ZIP from bovine serum (Birney and O'Connor, 2001).

The homogeneity of the preparation was confirmed by SDS and native PAGE . The lack of hydrolysis of a range of peptidase substrates confirms the complete absence of potential contaminating peptidase activities in the purified sample (results not shown).

### ***Catalytic Classification***

Previous studies on the catalytic classification of ZIP from bovine serum only possibly confirmed it as a serine protease (Birney & O'Connor, 2001). This was due to 58% inhibition by AEBSF at 22.5mM concentration. To finally catalytically classify ZIP as a serine protease, the irreversible and classic serine protease inactivator DFP was used (Barrett, 1994). The effect of DFP on purified ZIP activity was investigated with an  $IC_{50}$  value of 100nM and a second rate order constant ( $k_2$ ) of  $3.31 \times 10^3 \text{ M}^{-1} \text{ S}^{-1}$  (standard deviation of 0.000943 n=4) being obtained. This shows the high specificity of DFP for the catalytic serine of ZIP, thus confirms catalytically classifying this enzyme as a serine protease.

### ***Protein Sequence Analysis and Identification***

Purified ZIP was resolved on native PAGE, then an in gel (U.V. zymogram) assay was developed. The resulting fluorescent band (Fig. 2) was excised and sequence analysis performed. After proteolytic in-gel digestion of the protein, internal peptide sequences were obtained as described previously. The internal peptides sequenced were used for protein database searching and all the peptides obtained were found to be present in the primary

structure of Seprase/Fibroblast activation protein (Fig. 3) (GenBank accession no. NP004451).

### ***Gelatin Zymography***

As it is known that seprase exhibits gelatinase activity it was important that gelatin zymographic analysis was performed on purified ZIP/Seprase for potential activity. As highlighted by gelatin zymography in Fig. 4 the purified sample showed enzymatic activity towards gelatin, therefore suggesting a candidate protein substrate of this enzyme *in vivo*.

### ***Substrate Specificity***

Previous work on the specificity of ZIP studied proline containing bioactive peptides as potential substrates (Birney & O'Connor, 2001). It is known that the specificity of a protease is determined not only by the two amino acid residues at either side of the scissile bond of a peptide substrate, but also by amino acid residues more distant from the point of hydrolysis. Therefore, this work was performed in order to investigate the secondary specificity of ZIP using peptide substrates of various structural conformation and length (Table 1). These studies involved, **(i)** chain elongation at the amino side of the primary specificity proline residue, **(ii)** amino acid preference at the carboxyl side of the scissile bond, **(iii)** chain elongation at the carboxyl side of the primary specificity proline residue and, **(iv)** a final study was undertaken to investigate the importance of the primary specificity proline residue in ZIP catalysed hydrolysis. These studies were performed using various AMC-containing peptide substrates, kinetic analysis, RP-HPLC and LC-MS.

#### ***(i) Chain elongation at the amino side of the primary specificity proline residue.***

Effects resulting from an increase of the number of residues of the substrate in the NH<sub>2</sub>-terminal direction from the scissile bond are shown in Table 1. The enzyme was completely inert towards Pro-AMC and Gly-Pro-AMC. Further elongation at the amino terminal by placing an N-benzyloxycarbonyl (Z) group in the P<sub>3</sub> position resulted in hydrolysis. These results are the same for prolyl oligopeptidase where cleavage will not occur if a free  $\alpha$ -amine exists in the N-terminal sequence Yaa-Pro-Xaa or Pro-Xaa (Cunningham & O'Connor 1997a). Furthermore, ZIP failed to cleave Z-Pro-Phe, a similar trait of prolyl oligopeptidase (Wilk, 1983). Therefore it seems a strong interaction in the substrate-ZIP complex at the level P<sub>3</sub>-S<sub>3</sub> in addition to P<sub>1</sub>-S<sub>1</sub> and P<sub>2</sub>-S<sub>2</sub> occurs. Interestingly, replacement of the Z group in the peptide Z-Gly-Pro-Ala with a glycine residue seems to prevent hydrolysis therefore a bulky

hydrophobic residue (Z-group) may be a preference for ZIP in the P<sub>3</sub> position. This interaction at the P<sub>3</sub>-S<sub>3</sub> level may also be the reason for the failure of ZIP to cleave pGlu-His-Pro-AMC, a known substrate of prolyl oligopeptidase. Overall, analysis of substrates with increasing chain length in the NH<sub>2</sub>-terminal direction from residue P<sub>1</sub> reveals that binding of the peptides to ZIP involves interactions between P<sub>1</sub>-S<sub>1</sub>, P<sub>2</sub>-S<sub>2</sub> and P<sub>3</sub>-S<sub>3</sub> and that the minimum hydrolysable peptide size is an N-blocked tripeptide. Initial investigations seem to suggest that the P<sub>3</sub>-S<sub>3</sub> interaction may be a lot more specific in ZIP than prolyl oligopeptidase.

The affinity constant K<sub>m</sub>, for the ZIP catalysed hydrolysis of Z-Gly-Pro-AMC was determined. The hydrolysis followed the normal Michaelis-Menten kinetics allowing Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf kinetic models to be applied to the data. Bovine ZIP was determined to have an average K<sub>m</sub> of 270 μM for the substrate Z-Gly-Pro-AMC.

*(ii) Amino acid preference at the carboxyl side of the scissile bond.* It is well established that the specificity of a protease is determined by the nature of the amino acid residues at both sides of the peptide bond subject to hydrolysis. The effect on the ZIP catalysed hydrolysis of Z-Gly-Pro-AMC of proline-containing substrates with variations of amino acid residues located at the carboxyl-terminal site of the scissile bond (position P<sub>1</sub>' ), but with a constant sequence of Z-Gly-Pro was investigated and their K<sub>i (app)</sub> values presented in Table 1. All peptides inhibited ZIP either in a mixed or non-competitive mode. The lowest K<sub>i (app)</sub> values were obtained for peptides Z-Gly-Pro-Phe and Z-Gly-Pro-Met. The highest K<sub>i (app)</sub> values originated when the charged residues histidine and glutamic acid along with the small hydrophobic alanine residue were in the P<sub>1</sub>' position. Kinetic analysis would therefore seem to show that ZIP has greatest affinity for large hydrophobic residues in the P<sub>1</sub>' position with less affinity for acidic, basic and small amino acids in this position. These results are rather similar to the preference of Prolyl Oligopeptidase for a hydrophobic residue in the P<sub>1</sub>' position with lower specificities for basic and acidic (Koida & Walter 1976). RP-HPLC was performed for peptide digest separations in order to evaluate the ability of ZIP to hydrolyse each peptide, determination of the cleavage point and the effect, as discussed for the kinetic analysis, of the amino acid variation in the P<sub>1</sub>' position on hydrolysis. As shown in Table 1, ZIP hydrolysed each peptide but as shown in Figure 5 cleavage occurred a lot less favourably

with an acidic residue in the P<sub>1</sub>' position. LC/MS analysis highlighted confirmed each product peak obtained.

**(iii)** *Chain elongation at the carboxyl side of the primary specificity proline residue.*

Taking peptides with Phenylalanine and Methionine in the P<sub>1</sub>' position as in Table 1, the effect of further chain elongation on the carboxyl-terminal site to include P<sub>2</sub>', P<sub>3</sub>' and P<sub>4</sub>' on the ZIP catalysed hydrolysis of Z-Gly-Pro-AMC was evaluated. Table 1 presents the K<sub>i (app)</sub> values obtained and the mode of inhibition observed. A 2-fold increase in affinity was observed in going from Z-Gly-Pro-Phe to Z-Gly-Pro-Phe-His i.e. to include P<sub>2</sub>' position. An affinity constant of 206 μM obtained for Z-Gly-Pro-Phe-His is even greater than the K<sub>m</sub> of 270 μM obtained for ZIP using Z-Gly-Pro-AMC. However, addition of residues in locations P<sub>3</sub>' and P<sub>4</sub>' caused an increase in K<sub>i (app)</sub> values (Table 1), therefore showing a decrease in affinity of ZIP for elongated peptides beyond P<sub>2</sub>' position. An interesting feature showed that ZIP seemed to have a better affinity and hydrolysed peptides to a greater extent when multiple substrate-binding sites are occupied. So overall the important interactions in the substrate-ZIP complex at the carboxyl site of the substrate cleavage point seems to involve P<sub>1</sub>'-S<sub>1</sub>' and P<sub>2</sub>'-S<sub>2</sub>'.

**(iv)** *A final study was undertaken to investigate the importance of the primary specificity proline residue.* Extensive studies on the importance of the P<sub>1</sub> proline residue in catalysis by prolyl oligopeptidase are well reported. These studies conclude that the S<sub>1</sub> subsite is designed specifically to fit proline but tolerates other residues carrying substituent groups provided they do not exceed the size of prolines pyrrolidine ring (Nomura, 1986; Makinen, Makinen & Syed, 1994; Kreig & Wolf, 1995). Therefore it was paramount to study the importance of the P<sub>1</sub>-S<sub>1</sub> interaction in ZIP catalysis. It was decided due to the high affinity of ZIP for Z-Gly-Pro-Phe-His, to replace the P<sub>1</sub> proline residue with leucine in this peptide. Replacement of the proline residue with leucine in the primary specificity position (P<sub>1</sub>) caused a 7-fold decrease in affinity of ZIP for this peptide as shown in Table 1. Analysis of hydrolysis of Z-Gly-Pro-Phe-His by RP HPLC is presented in Figure 6. There seems to be the slightest formation of a Z-Gly-Leu product peak after incubation but LC-MS analysis failed to detect any peak with mass corresponding to the formation of a Z-Gly-Leu product. Therefore, it is evident that the S<sub>1</sub> subsite of ZIP, like prolyl oligopeptidase, is more than likely also designed to specifically fit a proline residue.

### ***Inhibitor Studies***

A number of prolyl oligopeptidase specific inhibitors were incubated with purified ZIP to determine any possible inhibitory effect; partial purified prolyl oligopeptidase was also assayed for comparison. As highlighted in Table 2 all inhibitors tested had little or no inhibitory effect on ZIP activity. On the other hand, partial purified prolyl oligopeptidase was extremely sensitive to all inhibitors; IC<sub>50</sub> values are listed in Table 2. JTP-4819 and Fmoc-Ala-PyrrCN were the most potent inhibitors tested towards Prolyl Oligopeptidase with IC<sub>50</sub> values of 0.8nM and 1.5nM respectively.

### **DISCUSSION**

Due to their cyclic structure, proline residues bestow unique conformational constraints on polypeptide chain structure, affecting the susceptibility of proximal peptide bonds to proteolytic cleavage (Yaron & Naider, 1993). Therefore, for the cleavage of Pro-Xaa peptide bonds (where Xaa is any amino acid) a specialised group of proteases, the prolyl peptidases, have evolved and their activity *in vivo* may have important physiological significance (Rosenblum & Kozarich, 2003). In this report we have described the purification, sequencing and properties of a novel prolyl peptidase from bovine serum.

Cunningham and O'Connor (1997b) reported the first observation of a second Z-Gly-Pro-AMC hydrolysing activity in bovine serum. The uniqueness of this peptidase was its ability to cleave the specific prolyl oligopeptidase fluorogenic substrate Z-Gly-Pro-AMC, but being completely distinct to this serine protease (Birney & O'Connor, 2001). Hydrophobic Interaction Chromatography was employed initially for separation of PO and ZIP activities, distinguished by JTP-4819, which totally inhibited PO activity with no effect on ZIP activity. The enzyme was further purified to apparent homogeneity using Calcium Phosphate Cellulose, Cibacron Blue 3GA and S-300 Gel Filtration chromatography. All purification steps were performed consecutively leading to the specific activity of purified ZIP being 30,197-fold higher compared to that in crude serum. A 12% conservation of biological activity a reduction of protein from 1766mg to 0.007mg was deemed successful. The homogeneity of the purified sample was analysed by SDS and Native PAGE, with the enzyme migrating as a single band. ZIP was classified as a serine protease based on its inhibition by the typical serine protease inhibitor DFP, showing the high specificity of DFP for the catalytic serine of ZIP.

Identification of this protease was performed by sequencing of internal peptides derived from tryptic digestion of the purified enzyme. Interestingly each of the peptides sequenced were present in the protein primary structure of fibroblast activation protein  $\alpha$ /seprase. Seprase (surface expressed protease), a member of the serine integral membrane peptidases, is a 170kDa membrane serine gelatinase reported to have prolyl endopeptidase activity (Chen, Kelly & Ghersi, 2003). Even though this enzyme is cell surfaced associated, soluble forms of these membrane proteases are known to exist in biological fluids.

Even though conflicting reports exist on possible prolyl dipeptidyl peptidase activity by seprase (Chen, Kelly & Ghersi 2003; Ghersi et al. 2002; Pineiro-Sanchez et al. 1997) no such activity was detected by purified ZIP/Seprase using Gly-Pro-AMC. Further substrate specificity studies suggest ZIP/Seprase has an extended substrate-binding region in addition to the primary specificity site,  $S_1$ . Its possible it is comprised of three sites located at the amino-terminal site ( $S_1, S_2, S_3$ ) and two sites at the carboxyl site from the scissile bond ( $S_1'$  and  $S_2'$ ). Regarding these subsites the  $S_1'$  position has a preference for a bulky hydrophobic residue along with the  $S_3$  subsite. An imino acid residue (proline) in the  $P_1$  position is absolutely necessary and a tetrapeptide  $P_3P_2P_1P_1'$  is the minimum hydrolysable peptide size. Further increase in substrate chain length beyond the  $P_2'$  position causes a continuous decrease in ZIP affinity and hydrolysis ability for substrates. The kinetic, RP HPLC and LC-MS data on the substrate specificity of ZIP suggests it's extremely similar to that of prolyl oligopeptidase. It is noteworthy that the size of the substrate, which is a limiting factor in the activity of oligopeptidases, such as PO (Fulop, Bocskei & Polgar 1998) seems not restricting in the case of ZIP/seprase as the enzyme is capable of degrading gelatin. Furthermore inhibitor studies using specific prolyl oligopeptidase inhibitors were completely inert towards ZIP/Seprase activity.

Birney and O'Connor (2001) reported a relative molecular mass of 174 kDa with a strong indication of dimeric structure for the serum ZIP/Seprase activity. This compares well with the reported human seprase activity (an active 170 kDa dimer). They also reported that the serum activity was a serine protease with oligopeptidase specificity. This again compares well with the human seprase activity. Both the bovine serum and human activities display optimal activity in the pH 7.4 to 7.8 range.

In conclusion, the present study provides new important information on the substrate specificity of Seprase/ZIP that has not been reported previously. This study also includes the first identification of a serum/soluble form of this protease, as it displays a strong homology

with the primary structure and catalytic activity to the cell surface associated serine integral membrane peptidase Seprase. As the expression of this serine matrix protease is closely associated with tumour invasion the identification of a serum form could provide a potential early warning to the onset of tumour progression. In addition, the detailed information on the substrate specificity/active site studies presented should provide valuable information for the design of site directed inhibitors which in turn should help elucidate the biological role of this important endopeptidase which to date is poorly understood.

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