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The Purification and Characterisation of novel Dipeptidyl Peptidase IV-like activity from Bovine Serum.

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Running Title

Purification and Characterisation of Bovine DPIV-like activity.

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Abbreviations

Aβ, β-amyloid peptide; ADA, adenosine deaminase; APMSF, 4-amidino-phenylmethylsulfonyl fluoride; CD26, cluster of differentiation molecule 26; CRP, c-reactive protein; DPIV, dipeptidyl peptidase IV (EC 3.4.14.5); DTT, dithiothreitol; IGF, insulin-like growth factor; MCA, 7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; PO, prolyl oligopeptidase (EC 3.4.21.26); PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; TRH, thyrotropin-releasing hormone; ZIP, Z-Pro-Prolinal-insensitive Z-Gly-Pro-MCA-hydrolysing peptidase.

Key words

Proline Specific Peptidases, DPIV, DPIV-like enzymes, Bovine Serum, Substrate Specificity

Abstract

The discovery of a potentially novel proline-specific peptidase from bovine serum is presented which is capable of cleaving the dipeptidyl peptidase IV (DPIV) substrate Gly-Pro-MCA. The enzyme was isolated and purified with the use of Phenyl Sepharose Hydrophobic Interaction, Sephacryl S300 Gel Filtration, and Q-Sephacryl Anion Exchange, producing an overall purification factor of 257. SDS PAGE resulted in a monomeric molecular mass of 158 kDa while Size Exclusion Chromatography generated a native molecular mass of 328 kDa. The enzyme remained active over a broad pH range with a distinct preference for a neutral pH range of 7-8.5. Chromatofocusing and Isoelectric Focusing revealed the enzyme's isoelectric point to be 4.74. DPIV-like activity was not inhibited by serine protease inhibitors but was by the metallo-protease inhibitors, the phenanthrolines. The enzyme was also partially inhibited by Bestatin. Substrate Specificity studies proved that the enzyme is capable of sequential cleavage of bovine β-Casomorphin and Substance P. The peptidase cleaved the standard DPIV substrate, Gly-Pro-MCA with a K_M of 38.4 μM, while Lys-Pro-MCA was hydrolysed with a K_M of 103 μM. The DPIV-like activity was specifically inhibited by both Diprotin A and B, non-competitively, generating a K_i of $1.4x10^{-4}$ M for both inhibitors. Ile-Thiazolidide and Ile-Pyrrolidide both inhibited competitively with an inhibition constant of $3.7x10^{-7}$ M and $7.5x10^{-7}$ M respectively. It is concluded that bovine serum DPIV-like activity share many biochemical properties with DPIV and DPIV-like enzymes but not exclusively, suggesting that the purified peptidase may play an important novel role in bioactive oligopeptide degradation.

INTRODUCTION

Dipeptidyl Peptidase IV (DPIV) was first reported as **glycylproline naphthylamidase** in rat liver during a commercial preparation of acylase I by Hopsu-Havu and Glenner [1] and has been named **dipeptidyl aminopeptidase IV** (DAPIV) and/or **post proline dipeptidyl peptidase IV** in earlier work [2]. DPIV has been reported to be identical to CD26, the surface marker on T- and B-lymphocytes and as an adenosine deaminase (ADA) –binding protein. DPIV is a member of the peptidase family S9, which along with prolyl endopeptidase (PEP) and acylaminoacyl peptidase form the prolyl oligopeptidase family. It is also the fourth member of the Dipeptidyl Peptidase sub-group and has been assigned the enzyme classification number EC 3.4.14.5.

The enzyme is ubiquitously distributed and its functionality is varied and numerous. It has a dual function as a regulatory protease and as a binding protein. Since its discovery over 35 years ago, due to its unique ability to liberate Xaa-Pro or Xaa-Ala dipeptides from the N-terminus of regulatory peptides, many more examples are emerging from both *in vivo* and *in vitro* studies. The specific conformation of the proline residue can impose many structural restrictions on peptides and proteins and confer particular biological properties upon a wide range of physiologically important biomolecules as well as reducing the potential to protease hydrolysis [3]. DPIV's immunological role has become obvious since its presence on the surface of human peripheral lymphocytes was demonstrated [4]. In addition, it is concluded that the T-lymphocyte surface antigen, designated CD26 and a leukocyte activation marker (Tp103) were in fact DPIV [5]. Dipeptidyl peptidase IV has been widely reported as being expressed in healthy, benign and malignant cells. This has led to the enzyme being described as a useful tumour burden marker. For example, squamous-cell lung carcinomas showed significantly high DPIV levels [6].

Recently, many DPIV-like enzymes have been reported, such as Attractin, Fibroblast activation protein α (FAPα) and DP8, displaying DPIV-like activity [7-9], as well as inactive proteins [10]. This potentially undermines the many previous reports of the influence of DPIV in biological processes and disease states. It is assumed that the effect of DPIV-like enzymes play a more important role than was previously considered. Therefore any report of DPIV-like activity from various tissues of different species should be treated with added interest.

Attractin was originally called DPPT-L and thought to be a larger form of DPIV in human serum with a molecular monomeric weight of 175 kDa [11]. The protein is expressed and secreted by activated T cells and appears to play a significant role in T-cell proliferation. It has also been called the Mahogany protein due to its potential in murine colour and obesity mutations as a result of its ability to bind to the agouti protein. Although Attractin displayed some CD26 antigenic epitopes, the inhibition profiles of both Attractin and DPIV are quite different [12]. In addition, no significant sequence homology is shared between the two proteins.

FAPα is generally classified as a 95 kDa monomeric serine protease with 49% amino acid sequence identity to CD26 [13], however a 175 kDa gelatinase has also been reported as FAP [14]. It is expressed as a cell surface antigen with a high expression rate in human malignant tumours, suggesting that the enzyme could be a potential marker for tumour invasiveness [8].

Abbott *et al.*, [9] cloned a 882-amino acid protein that has 27% identity and 51% similarity to DPIV which they term DPP8. The cloned enzyme has similar biochemical characteristics to DPIV such as a 100 kDa monomeric weight but seems to exist as a monomer in the cytoplasm. An interesting difference between the two enzymes is that the cloned, recombinant DPP8 enzyme is not glycosylated but was still capable of hydrolysis of DPIV substrates, Ala-Pro, Arg-Pro and Gly-Pro.

The aim of this study was to isolate and purify, for the first time, a DPIV-like activity from bovine serum and characterise its biochemical properties in an effort to distinguish it as Dipeptidyl Peptidase IV or one of the DPIV-like enzymes.

MATERIALS AND METHODS

All peptides and fluorimetric substrates were supplied by Bachem Feinchemikalein AG (Bubendorf, Switzerland). All chromatography resins and polybuffers were purchased from Pharmacia Fine Chemical Company (Uppsala, Sweden). BCA reagent and Gelcode Blue Protein stain were supplied by Pierce Chemical Company (Illinois, USA). A range of tri- and tetra-peptides were kindly donated by Dr. G. O'Cuinn (National University of Ireland, Galway, Ireland). Ile-Thiazolidide and Ile-Pyrrolidide were both kindly donated by Dr. Hans Ullich Demuth (Probiodrug, Halle, Germany). All other materials utilised were obtained from Sigma Chemical Company (Poole, Dorset, England).

Serum Preparation

Bovine whole blood was collected from a freshly slaughtered animal and kept in a 4^{o}C cold room for 24 hours to allow clot formation. The remaining unclotted blood was then decanted and centrifuged at 6,370 x g for 60 mins at 4^{o}C using a Beckman J2-MC refrigerated centrifuge fitted with a JL-10.5 rotor. The supernatant and loose cellular debris was decanted and re-centrifuged at 48,400 x g for 15 mins at 4^{o}C using a JA-20 rotor. The serum was collected, pooled and stored in 20 ml aliquots at –17^{o}C.

Protein Determination

Protein concentration was determined by Biuret and BCA (standard and enhanced) assays as previously described [15, 16]. BSA was used as the protein standard. Prior to the assay, samples were dialysed against 50 mM HEPES, pH 8.0. Absorbances of samples were determined at 570 nm using a Tecan Spectra Classic photometric plate reader.

Determination of DPIV-like activity

Dipeptidyl Peptidase IV-like activity was determined by the degradation of 0.1 mM Gly-Pro-MCA in 1% v/v Ethanol (EtOH) with 50 mM HEPES, pH 8.0 as the diluant. 100 μl of enzyme sample was added to 400 μl of thermally equilibrated substrate in triplicate and incubated at 37^{o}C for 60 minutes. The reaction was terminated by the addition of 1 ml of 1.5 M acetic acid. Suitable negative controls and blanks were included. Liberated MCA was fluorimetrically determined using a Perkin-Elmer LS-50 Luminescence Spectrometer at excitation and emission wavelengths of 370 and 440 nm, respectively. DPIV-like activity was quantified by reference to an MCA standard curve. One unit of enzyme activity is defined as nanomoles of MCA released per minute at 37^{o}C. A non-quantitative assay was developed to assist in the rapid detection of Gly-Pro-MCA degrading activity in post column chromatography fractions. 200 μl of 0.1 mM Gly-Pro-MCA in 50 mM HEPES, pH 8.0 was added to 100 μl of sample in each well and the micro-plate was incubated at 37^{o}C for 60 minutes. Fluorescence intensities were determined as previously described using the Perkin-Elmer LS-50 Luminescence Spectrometer plate reader attachment.

Purification of Gly-Pro-MCA cleaving activity

Phenyl Sepharose hydrophobic interaction chromatography.

A 25 ml Phenyl Sepharose CL-4B hydrophobic interaction column (2.5cm x 7cm) was equilibrated at 1 ml/min with 100 ml 50 mM HEPES, pH 8.0 containing 5 mM EDTA and 500 mM ammonium sulphate. Crude serum was thawed at 37^{o}C and solid ammonium sulphate was added to a final ammonium sulphate concentration of 500 mM. 5 ml of salted serum was applied to the equilibrated column followed by a 100 ml wash of the equilibration buffer to wash through any unbound protein. Bound protein was eluted by a 50 ml linear gradient of 50 mM HEPES, pH 8.0 containing 5 mM EDTA and 500 mM ammonium sulphate to 50 mM HEPES, pH 8.0 containing 5 mM EDTA. A further 25 ml wash of 50 mM HEPES, pH 8.0 containing 5 mM EDTA ensured all bound protein was eluted. 5 ml fractions were collected and assayed for DPIV-like activity and protein content as previously described. Fractions containing Gly-Pro-MCA degrading activity were combined and the volume recorded to yield post-Phenyl Sepharose DPIV-like activity and stored at –17^{o}C.

HiPrep 16/10 Sephacryl S-300 HR Gel Filtration Chromatography.
A 120 ml Hiprep 16/10 Sephacryl S300 High Resolution Gel Filtration column was equilibrated at room temperature with 150 ml 50 mM HEPES, pH 8.0 containing 5 mM EDTA at 1 ml/min using the Bio-Rad Biologic system and until a steady conductivity and UV baseline was observed. The post-Phenyl Sepharose DPIV-like pool was concentrated via reverse dialysis using Polyethylene Glycol at 4^{o}C. Glycerol was added to the concentrated sample to generate a final glycerol concentration of 10% (v/v). 1.5 ml of concentrated post-Phenyl Sepharose DPIV-like activity was loaded onto the column and washed with 150 mM of equilibration buffer at room temperature. 3 ml fractions were collected and assayed for DPIV activity and protein content as previously described, parallel with online UV monitoring at 280nm. Fractions that contained DPIV-like activity were pooled and stored at -17^{o}C.

Q-Sepharose Fast Flow Anion Exchange Chromatography.
A 20 ml pre-packed Q-Sepharose column attached to a BioRad BioLogic Chromatography system was equilibrated with 50 ml 50mM HEPES, pH 8.0 containing 5 mM EDTA. 5 ml of post-Sephacryl S300 HR DPIV-like activity was applied onto the column followed by a 50 ml wash with equilibration buffer. Any bound protein was eluted with a 50 ml linear gradient of 50 mM HEPES, pH 8.0 containing 5 mM EDTA to 50 mM HEPES, pH 8.0 containing 5 mM EDTA and 500 mM NaCl. A further 25 ml wash of 50 mM HEPES, pH 8.0 containing 5 mM EDTA and 500 mM NaCl ensured that all bound protein was eluted. 3 ml fractions were collected and assayed for DPIV activity and protein as previously described, parallel with online UV monitoring at 280nm. Fractions that contained DPIV-like activity were pooled and stored.

G100 Gel Filtration Chromatography.
A 50 ml G100 Sephadex Gel Filtration column was equilibrated with 100 ml 50 mM HEPES, pH 8.0 containing 5 mM EDTA. 10 ml of the post QAE pool was applied onto the column and the resin was washed with 150 ml 50 mM HEPES, pH 8.0 at 0.5 ml/min containing 5 mM EDTA. 3 ml fractions were collected and were analysed for Gly-Pro-MCA degrading activity and protein content as previously described. The conductivity was measured with a Jenway 4071 Conductivity Meter which gave an accurate 'real time' conductivity reading in units of mS/cm which was directly proportional to the salt content. Pooled post G100 fractions typically had protein levels of 3 μg/ml (1.5 units/ml) which was concentrated 10-fold before storage at –17^{o}C. As a consequence, characterisation studies were performed on 30 μg/ml of purified enzyme unless stated otherwise.

Assessment of Purity

SDS-PAGE.

Samples generated during the purification procedure (serum, post-phenyl Sepharose, post-S300, post-QAE and post-G100 DPIV-like) were resolved by electrophoresis under denaturing conditions according to the method of Laemmli [17]. Samples were dialysed for 18 hours at 4^{o}C against 2 litres of 62.5 mM Tris/HCl, pH 6.8 with buffer changes after 3 and 6 hours. Dialysed samples were subsequently diluted in sample solubilization buffer, denatured after a 60 second treatment in a boiling water-bath and 10 μg/well was subjected to electrophoresis on a 10% acrylamide SDS gel with appropriate High Range molecular weight markers (Sigma). Gels were stained as recommended by the Gelcode Reagent protocol (Pierce).

Fluorimetric Analysis.

Purified DPIV-like activitiy was assayed for the presence of various peptidase activities by incubating the enzyme with different specific fluorimetric substrates (Table 2). Each substrate was prepared as a 10 mM stock in 100% v/v EtOH and diluted in 50 mM HEPES, pH 8.0, yielding a final concentration of 0.1 mM in 1% v/v Ethanol. 100 μl purified enzyme was incubated with 400 μl of each substrate and activity was detected as described previously.

Relative Molecular Mass Determination

SDS-PAGE:

The relative molecular weight of the purified DPIV-like enzyme was determined using SDS-PAGE as previously described. A standard curve of the log of molecular mass versus the relative mobility (R_f) of each molecular weight marker was constructed. The log of the molecular mass of the purified enzyme was calculated from the calibration curve using its R_f value and its relative molecular mass could be estimated.

Size-exclusion FPLC chromatography.

The enzyme's relative molecular weight could also be determined with the use of a Hiprep 16/10, Sephacryl S-300 (16 x 60mm) size exclusion column on a Bio-Rad Biologic Chromatography FPLC system. The column was equilibrated with 200 ml 50 mM HEPES, pH 8.0 with 100 mM NaCl. Molecular weight standards or purified enzyme were injected onto the column followed by a 200 ml wash with equilibration buffer. The elution volume (V_e) was evaluated via the continuous online monitoring at 280 nm. Enzyme elution was determined using the specific substrate, Gly-Pro-MCA as previously described. A graph of V_e/V_o versus log molecular weight was generated and the native molecular weight of the DPIV-like enzyme determined (where V_o is the void volume of the column, estimated using blue dextran).

Optimum pH Determination

5 ml of purified enzyme was dialysed overnight against 2 litres of distilled water at 4^{o}C. 50 μl of dialysate was pre-incubated at 37^{o}C for 15 minutes with 50 μl of a range of 50 mM buffers at varying pH values. 100 μM Gly-Pro-MCA was prepared in each of the 50 mM buffers at each pH. The effect of the pH and buffer was determined by assaying enzyme activities in triplicate as previously outlined.

Isoelectric Point Determination

Chromatofocusing chromatography.

A 25 ml polybuffer exchanger (PBE 94) column was equilibrated with 300 ml 50 mM Imidazole/HCl, pH 7.8. Polybuffer 74 was diluted 8-fold in distilled water and the pH adjusted to 4.5 using HCl. 15 ml of the diluted polybuffer was applied to the column after equilibration. 5 ml purified DPIV-like enzyme was dialysed overnight against 3 litres of Imidazole/HCL, pH 7.8 at 4^{o}C and applied onto the chromatofocusing resin. The resin was washed with 300 ml polybuffer, pH 4.5. 3 ml fractions were collected throughout and assayed for DPIV-like activity and their pH's measured.

Isoelectric focusing (IEF).

A vertical pre-cast Isoelectric Focusing (IEF) gel system was employed to determine the isoelectric point of the purified enzyme as previously reported [18]. The solution was dialysed overnight against 2 litres of 50 mM HEPES, pH 8.0 to remove any IEF contaminants. The dialysed sample was diluted with an equal volume of sample buffer. The gels were prepared in an ATTO vertical electrophoresis system (160mm x 160mm x 1mm). After the bottom of the chamber was filled with 1x anode running buffer, the gels were placed in position and the upper chamber filled with cathode buffer. 20 µl of each prepared sample and reconstituted markers were loaded into the wells under the cathode buffer. Electrophoresis was carried out at 100V for 60 min, 200V for the second 60 min and 500V for the final 30 min with the current running from 5-6 mA/gel. Staining was performed on the gel using brilliant blue G colloidal stain. The stained gel image was captured using a UVP white/UV transluminator camera unit driven by Image-Store 7500 software. A standard curve was constructed of pI versus the R_f for each of the IEF markers. The R_f represents the distance migrated by the standard or sample divided by the distance to the anoidic lip of the gel. The pI of the enzyme was estimated using the calibration curve by calculating its R_f value from the stained gel.

Catalytic Classification

Functional reagents were prepared in 50 mM HEPES/HCl, pH 8.0 and 50 µl purified DPIV-like activity (with the EDTA dialysed out) was pre-incubated in triplicate for 15 min at 37^{o}C with an equal volume of each functional reagent concentration (Table 3). After pre-incubation, the enzyme and functional reagent were added to 0.1 mM Gly-Pro-MCA and incubated for 60 min at 37^{o}C. DPIV-like activity was calculated as previously described.

Substrate Specificity

Reverse-Phase HPLC Analysis

A range of synthetic and bioactive proline-containing peptides were incubated with 100 µl purified enzyme at 37^{o}C for 24 h and the reaction was terminated by the addition of 25 µl 0.5% v/v TFA. Blanks, standards and post-incubation samples (20 µl) were applied at 0.5 ml/min to the C_{18} column, equilibrated with 100% buffer A (5% Acetonitrile, 0.2% TFA). After application, the column was washed with 4 ml of 100% buffer A, followed by a 10 ml linear gradient of 100% buffer A to 100% buffer B (80% Acetonitrile, 0.2% TFA). A 5 ml wash of 100% buffer B eluted any remaining hydrophobic peptides. The absorbance of the eluant was continuously monitored online at 214nm and 280nm using a diode array detector.

Kinetic Analysis

K_M Determinations

The purified enzyme was assayed with increasing concentrations (0-0.5 mM) of Gly-Pro-MCA and Lys-Pro-MCA, in triplicate as previously described. The data obtained was applied to Michaelis-Menton, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf kinetic models. The Michaelis-Menton constant, (K_M) and the maximum velocity value, (V_{Max}) were subsequently determined for both substrates.

K_I Determinations

The inhibition of the purified DPIV-like activity by a variety of peptides was determined. A range of Gly-Pro-MCA dilutions (0-1 mM) was prepared and aliquoted (200 µl). An equal volume of each peptide concentration was added to the substrate, in triplicate, resulting in a final substrate concentration range of 0-0.5 mM. 100 µl of purified enzyme was added to each triplicate and blank and incubated at 37^{o}C for 60 min. Enzymatic activity was determined as previously described and the resultant data was applied to a number of kinetic models, allowing for the estimation of the mode of inhibition and the determination of the inhibition constant *(K_i)* [19].

RESULTS

Purification of the Dipeptidyl Peptidase IV-like activity

DPIV-like activity was divided into two Gly-Pro-MCA cleaving split peaks after Phenyl Sepharose Hydrophobic Interaction chromatography as shown in Figure 1A. The first peak is unbound DPIV-like activity while the second peak is bound activity which is eluted at the end of the negative Ammonium Sulphate gradient. Interest was focused on the main 'run-through' peak due to the separation from Z-Gly-Pro-MCA degrading activity such as Prolyl Oligopeptidase which is present in the eluted pooled fractions (data not shown). Table 1 shows a 48-fold increase in purity of the pooled unbound DPIV-like activity. Further contaminating protein could be removed from this enzymatic activity with use of Sephacryl S-300 Gel Filtration Chromatography (Fig. 1B.) increasing the purification factor to 74. When using the Q anion exchanger, DPIV-like activity bound and eluted sharply on inclusion of 0.5 M sodium chloride as shown in Figure 1C. This allowed for a significant removal of contaminating protein (11.5-fold reduction) which increased the enzyme's purity to 250.5-fold. The removal of sodium chloride from the post-QAE DPIV-like pool was performed using a G100 Gel Filtration column (Fig. 1D) which also served to increase the enzyme's overall purification factor to 257.3 (Table 1).

Purity Assessment

SDS PAGE of post column pools visualised numerous protein bands especially for crude serum and post-Phenyl Sepharose samples. These bands were considerably reduced after QAE Sepharose and one band was observed in the post-G100 pool, at 150 kDa. Contaminating peptidase activity in the purified sample was absent, proven by the absence of hydrolysis of a range of relevant fluorimetric substrates (Table 2).

Enzyme Characterisation

SDS-PAGE was also employed to determine the relative molecular mass of the purified enzyme. The high molecular weight band observed from the post-G100 sample represented a molecular mass of 158 kDa. Size-exclusion FPLC was subsequently employed to evaluate a native molecular mass of 328 kDa. As a consequence of these findings, it was deduced that the purified peptidase exists as a dimer of similar sized subunits. Chromatofocusing resulted in the elution of the Gly-Pro-MCA cleaving activity after a decreasing linear pH gradient, corresponding to an elution pool of pH 4.8. Isoelectrofocusing (IEF) of the purified enzyme showed a single band with a corresponding pI of 4.74 (Fig. 2). The enzyme showed a distinct optimum at pH 7 - 8.5,

with a slight preference for Potassium Phosphate as the buffer (Fig. 3). Despite this, it was discovered that the Gly-Pro-MCA substrate autocyclised significantly when formulated in Phosphate buffer. Therefore HEPES, pH 8.0 was selected as the buffer of choice.
Table 3 highlights the effect functional reagents have on the purified enzyme when incubated together. The enzyme did not appear to be inhibited by any of the classic serine protease inhibitors while the metalloprotease inhibitors had a more potent effect. The metal chelators, EDTA and EGTA reduced activity by 19.6 and 18.8% respectively. The phenanthrolines were even more inhibitory with 4,7 Phenanthroline reducing the enzyme activity by 46% at 20 mM. Certain cysteine protease inhibitors also had an inhibitory effect on the enzyme with 10 mM N-ethyl maleimide (NEM) reducing activity by approximately 47%. However the classical thiol group reducing agent and cysteine protease activator, DTT, had little effect on activity while Mercaptoethanol increased activity by 25%. Interestingly, the aminopeptidase inhibitor, Bestatin was capable of reducing the enzymatic activity by almost 25%.

Substrate Specificity
Table 4 outlines the ability of the purified enzyme to hydrolyse synthetic and bioactive peptides, detected using reverse-phase HPLC. The enzyme did not seem capable of cleaving any of the β-amyloid proteins. In general, peptides without a proline at the P1 position were not hydrolysed. Interestingly, sequential dipeptide cleavage was achieved for bovine β-Casomorphin (Fig. 4) with cleavage products of Tyr-Pro (16.967 min), Phe-Pro (17.833 min) and Gly-Pro (9.9 min). Uncleaved β-Casomorphin can be seen as the 18.967 min peak. Similar sequential dipeptide cleavage was observed for Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2). Most peptides containing proline at the penultimate position were cleaved highlighting the enzyme's requirement for the presence of this amino acid.

Kinetic Analysis
The purified enzyme was investigated further with kinetic studies on standard substrates and inhibitors. The K_M was estimated using Michealis-Menton, Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf graphical models. The commercial DPIV substrate, Gly-Pro-MCA was hydrolysed with an average K_M of 38.4 μM. In comparison, Lys-Pro-MCA proved a poor substrate with a K_M of 102.7 μM (data not shown). When proline containing peptides were included with the Gly-Pro-MCA substrate, the Michealis-Menten constant was evaluated, the type of possible inhibition determined and the resulting K_i estimated. The sequential dipeptide hydrolysis of both β-Casomorphin and Substance P could be achieved however their inhibition profiles were different. β-Casomorphin appears to compete with the substrate for enzyme binding (Table 4). As a consequence, a corresponding inhibition constant was estimated as 90 μM. In contrast, Substance P appears to inhibit un-competitively with a K_i of 450 μM. Diprotin A and B, two common DPIV inhibitors, both had a similar effect on the catalytic properties of the enzyme. Both inhibited non-competitively with inhibition constants of approximately 140 μM, while the corresponding IC_{50} values for both inhibitors were also similar such as 36 μM for Diprotin A (Fig. 5) and 50 μM for Diprotin B. Ile-Thiazolidide and Ile-Pyrrolidide proved to be very potent inhibitors of the enzymatic activity. Both inhibited competitively with Ile-Thiazolidide having a particularly potent effect on the enzyme's activity with a K_i of 0.37 μM (Table 4). In addition, Ile-Thiazolidide also returned a potent IC_{50} value of 0.75 μM, while a value of 1 μM was discovered for Ile-Pyrrolidide (Fig. 6).

DISCUSSION

The serine protease, DPIV has been implicated to influence many biological pathways and disorders [13]. However, recent reports of DPIV activity may not be solely attributed to Dipeptidyl Peptidase IV [7-9]. As a consequence, Gly-Pro-MCA cleaving activity discovered in bovine serum was treated with interest. Our group has being studying proline specific peptidases in bovine brain and therefore were interested to compare these tissue activities to those of serum.

Hydrophobic Interaction Chromatography separated two DPIV-like activities. The unbound peak was re-applied onto the Phenyl Sepharose column under the same conditions to investigate the potential of protein overloading of the resin. However, as before, the enzymatic activity did not bind to the column indicating that at least two DPIV-like activities are present in bovine serum. The Hydrophobic Interaction Chromatography step resulted in separation of the main Gly-Pro-MCA cleaving activity from Z-Gly-Pro-MCA cleaving activity attributed to Prolyl Oligopeptidase and ZIP which is also present [20]. This was essential as both activities have similar substrate specificities. The enzyme was further purified with the use of both gel filtration and anion exchange chromatography. The latter column proved particularly successful in increasing the purity of the enzyme due to its acidic isoelectric point (pI) resulting in a final purification factor of **257**. In addition, enzymatic activity was readily detectable allowing for accurate characterisation studies to be employed.

The enzyme's molecular mass was determined via SDS-PAGE and size exclusion chromatography. Under reducing conditions, SDS-PAGE resulted in a monomeric mass of 158 kDa. The native protein's molecular mass of 328 kDa indicates that it may exist as a glycosylated dimer which correlates well with the postulated structure of DPIV [21].

The optimum pH for the assay of the purified enzyme was estimated to be in the pH range 7-8.5 with a typical bell-shaped curve. This range fits perfectly with what has been reported for human serum [7, 23]. The inactivation of enzymatic activity at pH's lower than 6.5, eliminates the presence of DPIV-like activity attributed to DPII or Quiescent Cell Proline Dipeptidase (QPP) [24-26]. An isoelectric point of 4.74 was determined by Chromatofocusing and Isoelectric Focusing which is similar to human lymphocyte DPIV [27] and FAP or Seprase from the cell membranes of melanoma cells [14].

Catalytic classification of the purified enzyme surprisingly resulted in no inhibition from the serine protease inhibitors. This deviates from literature which report that DPIV and DPIV-like enzymes are 'unclassical' serine proteases [13]. However the minimal effect of the classical serine protease inhibitor, PMSF, has also been previously reported for human blood DPIV activity [27, 28]. Interestingly, the metallo protease inhibitors tested had a more potent effect on the enzyme's catalytic activity. The most effective compound, 4,7 phenanthroline, reduced activity by over 46%. However, the classical metal chelators, EDTA or EGTA did not have such an inhibitory effect which correlates with the literature [12]. The cysteine protease inhibitor, NEM had a potent effect on reducing the enzyme's activity. The proposed structure of DPIV outlines a cysteine rich region located next to the catalytic domain [21]. Therefore cysteine protease inhibitors and activators could ascertain their influence in DPIV or DPIV-like substrate catalysis both positively and negatively. However, only 2-mercaptoethanol, the cysteine protease activator, had a slight catalytic enhancing effect on the enzyme. This indicates that cysteine is not one of the catalytic residues necessary for substrate hydrolysis but may maintain the enzymes active site tertiary structure and influence its unique accessibility. A similar effect was observed for DPPT-L or Attractin in human serum [12] which further undermines classifying the purified enzyme as a serine protease.

Substrate specificity via reverse phase HPLC indicated that the enzyme prefers bulky, hydrophobic residues but dislikes acidic groups in the P2 position, prefers proline in the P1

position and cannot cleave peptides with proline or hydroxyproline in the P1' position. The most interesting result observed was the sequential dipeptide cleavage of the opioid, β-Casomorphin. Opioids are polypeptides that influence nerve transmission in certain parts of the brain. They are called as such because they bind to specific receptors that bind opiate drugs such as morphine. Therefore they may be regarded as the brains own 'pain killers' [29]. Bovine β-Casomorphin has a proline residue at every second position of the seven amino acid peptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH). Therefore it is a perfect candidate for DPIV-like sequential hydrolysis which proved to be the case when the three dipeptides were cleaved, namely, Tyr-Pro, Phe-Pro, and Gly-Pro. Thus the DPIV-like enzyme could play a role in reducing pain prevention when in the brain. The serum enzyme could hydrolyse the peptide, which would subsequently pass the blood-brain barrier and have a physiological effect which correlates well with the literature [30]. In addition, the peptide's cleavage was confirmed as solely due to the enzyme when Ile-Pyrrolidide, a specific DPIV inhibitor, was added, inhibiting peptide cleavage (data not shown). However, the hydrolysis of the third and final dipeptide has been previously reported as difficult or unattainable [31]. Therefore, we believe that this is the first report of complete peptide hydrolysis of β-Casomorphin by DPIV or a DPIV-like enzyme. Similarly, Substance P is also a perfect candidate for cleavage by a DPIV-like enzyme. The eleven amino acid peptide, (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), contains a proline residue at the second and fourth position at the N-terminus. The peptide has many implications, some of which include its release during headache and migrane [32]. The purified enzyme was capable of cleaving the two dipeptides which is expected for DPIV [30]. The resultant seven amino acid peptide (Gln-Gln-Phe-Phe-Gly-Leu-Met) would have an unpredicted but specific effect on receptor binding and response. Nonetheless, the DPIV-like enzyme may play an influential role on Substance P effects *in vivo*. The enzyme's broad substrate specificity and physiological potential is evident from its influence on Enterostatin (Val-Pro-Asp-Pro-Arg-OH), IGF (Gly-Pro-Glu-) and Neuropeptide $Y_{(1\text{-}24)}$ (Tyr-Pro-Ser-Lys-Pro-Asp-Asn-) hydrolysis. However the enzymes specificity is directly effected by larger peptides when cleavage of the entire Neuropeptide Y molecule was not possible (data not shown).

The inclusion of proline containing compounds with the Gly-Pro-MCA substrate had a significant effect on the apparent K_M obtained. β-Casomorphin inhibited competitively while in contrast, Substance P displayed distinct un-competitive inhibition of the enzyme's activity. The difference in the mode of inhibition is interesting as both peptides have a similar sequence with the second and fourth amino acid occupied by a proline residue. It is probable that sequential cleavage of the peptides is not required to inhibit the enzyme, however the size and the amino acid positions play a major role in the mode of inhibition obtained.

Inhibitor studies using specific DPIV inhibitors proved that the enzyme can be completely inhibited. Diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu) have been synthesised as specific DPIV inhibitors but could also be regarded as good substrates. The hydrophobicity of isoleucine and valine at position P2 is preferred by DPIV, competing with the substrate for hydrolysis, creating a potent inhibitor. The effect of these compounds both show similar potency resulting in complete inhibition but in a non-competitive fashion. Although Diprotin A is most potent in its inhibitory capacity, it is expected that both compounds behave in a similar fashion. This correlates well with what has been reported for DPIV in the literature [31, 33]. Similarly, Ile-Thiazolidide and Ile-Pyrrolidide have both been reported as specific DPIV inhibitors, which provides an important tool in elucidating the structure and function of the proteolytic enzyme [34]. Both compounds proved to be very specific inhibitors of the purified activity. Kinetic analysis of the inhibition showed a significantly different mode of inhibition to that of the Diprotins. Both compounds inhibited in a competitive manner with Ile-Thiazolidide appearing to be the most potent with a K_i of 0.37 μM.

As the structures of both Thiazolidide and Pyrrolidide are almost identical, the only exception being the presence of a sulphur atom in the five membered ring of Thiazolidide, it is expected that the mode of inhibition is also similar or identical. The inhibition constants reported clearly indicate the effect of the compounds on DPIV-like activity and correlates with previous reports [34].

In summary, we document the first purification and characterisation of DPIV-like activity in bovine serum. This peptidase exists as a dimer with a native molecular weight of 328 kDa, larger than that reported for DPIV [13] but similar to a large molecular weight protein reported as Attractin [22]. In an attempt to further classify this peptidase, the Dipeptidyl aminopeptidase activity of the purified enzyme was evaluated with the aminopeptidase inhibitor bestatin where 25% inhibition was observed which has also been reported for DPIV-like activity thought to be attributable to Attractin [22]. Similarily trypsin had no effect on the observed enzymatic activity (data not shown) which also correlates with the Attractin report. Another common distinction between DPIV and Attractin is the latters inability to bind to ADA [7]. This binding inability was also observed for the purified peptidase which suggests that the observed activity is due to Attractin. Even though DPIV has been classified as an unclassical serine protease, the purified enzyme was resistant to serine protease inhibitors. Enzymatic activity was completely inhibited by DPIV specific inhibitors, Diprotin A and B as well as Ile-Thiazolidide and Ile-Pyrrolidide indicating that the mode of substrate hydrolysis and inhibition of DPIV and this peptidase are similar. Similarly, the enzymatic activity observed was identical to that reported for DPIV with a proline residue necessary in the P1 position. The enzyme was capable of sequentially hydrolysing β-Casomorphin and Substance P, suggesting a role in pain modulation within the brain. With ever increasing reports of DPIV-like activity, it is difficult to comprehensively classify and characterise this peptidase. It is interesting to note that while Iwaki-Egawa and co-workers reported no enzymatic difference between membrane-bound and serum forms of human DPIV [35] Duke-Cohan and co-workers found no sequence homology between them [7]. However, one should not rule out the varying effects of enzymes from different species and tissues. These effects could involve alternative splicing as well as many unique post-translational modifications. Future immunodetection studies, using antibodies raised against bovine DASH molecules, would greatly aid in clarifying the exact nature of the relationship between these activities. Nonetheless, it is clear that DPIV activity in bovine serum could play a significant role in the degradation and turnover of bioactive peptides in the body's circulatory system and elsewhere.

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