Cloning and heterologous expression of bovine pyroglutamyl peptidase type-1 in Escherichia coli : purification , biochemical and kinetic characterisation.

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Abstract

We describe the cloning, expression and purification of the bovine **XM866409** form of pyroglutamyl-aminopeptidase I. The amino acid sequence, deduced from the nucleotide sequence, revealed that it consists of 209 amino acid residues and showed to have 98% homology with the human **AJ278828** form of the enzyme. Three amino acid residues at positions 81, 205 and 208 were found to vary among the two sequences. The bovine enzyme was expressed in XL10-gold *Esherichia coli* cells. Immobilizied Ni-ion affinity chromatography was used to purify the expressed protein resulting in a yield of 3.3mg of PAP1 per litre culture. The purified enzyme had a specific activity of 1700 units/ml. SDS-PAGE produced a single band for bovine PAP1 with a molecular weight of ~23-24 kDa which is in good agreement with previously reported data on PAP1. Kinetic constants Km and Kcat were 59μM and $3.5s^{-1}$, respectively. It possessed an optimum pH between 9-9.5, a temperature of 37°C and showed an absolute requirement for a thiol-reducing agent (10mM DTT). EDTA didn't prove to have an effect on enzyme activity. Competitive inhibition was seen with pyroglutamyl peptides pGlu-His-Pro-NH2 (TRH; Ki= 44.1 uM), pGlu-Ala-OH (Ki=141 uM) and pGlu-Val-OH (Ki=652.17).

Introduction

Pyroglutamyl aminopeptidase (PAP ; EC 3.4.19.3) type 1 is an omega peptidase that has the ability to remove/hyrolyse pyroglutamic acid (pGlu) from the amino-terminus of biologically active peptides and proteins with an apparent specificity for l-pGlu-L-amino acid optical isomers. The enzymatic activity has been reported to be widely present in several bacteria, plants, and animal tissues **[1,2,3,4** and **5].** PAP I is typically a monomeric cysteine peptidase with a low relative molecular mass (average 23 kDa), a soluble or cytosolic location, and a pH optimum between 6.5 and 8.0 (see Cummins and O'Connor for review **[6]**). Barrett and Rawlings suggest that the cysteine peptidases have come from at least seven different evolutionary origins, each of which has produced a sub-group with distinctive structures and properties but almost all depend for activity on catalytic dyads of cysteine and histidine **[7]**. Pyroglutamyl aminopeptidase peptidase I (C15) is the sole member of their Clan CF.

PAP 1 has been shown to have the ability to degrade/inactivate a broad spectrum of pGlu-containing peptides including Thyrotropin Releasing Hormone (TRH), Luteinizing Hormone Releasing Hormone (LH-RH), Bombesin, Neurotensin, the Anorexogenic peptide and Gastrins**.** Despite its broad substrate specificity, however, PAP I is highly specific for N-terminal pGlu residues.

While the mammalian PAP1s have been implicated in the regulation of neuropeptide activity (e.g. the TRH pathway **[10]** and the neurotensin system **[11])** the exact nature of the physiological role of PAP I still remains unclear. Within the human brain the highest PAP1 activity was found in the cortices with the lowest in the cerebellum **[10] .** Falkous and co-workers have observed significantly increased levels of PAP I in the spinal cord of patients suffering from motor neuron disease and that PAP I from human cerebral cortex is extremely susceptible to inhibition by potentially neurotoxic

metal ions **[11].** Faivre-Bauman and coworkers **[12]** showed that addition of specific inhibitors of PAP I and Prolyl Endopeptidase (EC 3.4.21.26) to TRH-synthesizing hypothalamic cells in primary culture results in a significant increase in both their TRH content and in the amount of TRH being released from these cells.

Recent studies carried out with the aid of a chemically synthesised TRH analogue (which is resistant to PAP degradation) have indicated a 'neuromodulating' role for the PAPs as they can control the formation of cyclo(His-Pro) from TRH **[13]**.

Yoshimoto et al. **[14]** have cloned the gene for the *B*. *amyloliquifaciens* enzyme and, following its expression in *Escherichia coli*, have shown that the recombinant enzyme appears to exist as a dimer, suggesting differences between the recombinant and wild type forms of the enzyme. Other studies indicate that the recombinant PAPs from *B*. *subtilis* and *S*. *pyogenes* are probably tetramers, whilst the recombinant PP from *P*. *fluorescens* appears to be dimeric in nature **[6]**.

The first documented crystallized structure of PAP 1 from *Bacillus amyloliquefaciens,* was viewed at 1.6 A resolution and was documented as folding in an α/β globular domain with a hydrophobic core consisting of a twisted β sheet surrounded by five α helices. This structure allows the function of most of the conserved residues in the PPI family to be identified, and it has been concluded that the catalytic triad consists of Cys^{144}, His^{168} and Glu^{81} **[15]**. Ito and co-workers,using X-ray crystallography and site-directed mutagenesis, have shown that the molecular recognition of pyroglutamic acid is achieved through two hydrogen bonds and an insertion in a hydrophobic pocket where, Phe^{10} is more important to the hydrophobic interaction than Phe^{142} with Phe^{13} serving as an "induced fit" mechanism **[16]**.

Methods and Materials

Strains, plasmids and enzymes

The *E.coli* strain XL10-Gold (Invitrogen) was for cloning experiments and protein expression. The plasmid vectors pCR2.1 (Invitrogen) was used for cloning and amplification of *rBosT-pap1* cDNA and the plasmids pQE-60 (Qiagen) and pKK223-3 (Amersham Pharmacia) were used for protein expression. Restriction enzymes, RED*Taq* DNA polymerase, and T4 ligase were purchased from Invitrogen and were used according to suppliers recommendations.

Chemicals

Bovine brain tissue samples were obtained from Keypak, Clonee, Co Meath, Ireland. Oligonucleotides were purchased from MWG Biotech AG, Germany. Luria-Bertani (LB) broth media was obtained from the Medical Supply Company, Dublin, Ireland. Peptides (pGlu-His-Pro-NH_2 ,pGlu-Ala-OH and pGlu-Val-OH) and fluorogenic substrate pGlu-AMC were purchased from Bachem, UK. Ni-NTA resin used for purification was purchased from Qiagen, UK. All other chemicals, including buffers and antibiotics were purchased from Sigma-Aldrich Chemical.

Isolation of RNA

RNA was isolated using Trizol Reagent (Invitrogen), a mono-phasic solution of phenol and guanidine isothiocyanate.100 mg of bovine brain tissue was homogenised in 1 ml of Trizol reagent using a glass-Teflon homogeniser (treated with RNase AWAY, Molecular *Bio*-Products, *inc.*) and transferred to a microfuge tube. The sample was incubated at room temperature for 5 min to allow complete dissociation of

nucleoprotein complexes. 200 µl of chloroform was added, mixed by brief vortexing and incubated at room temperature for 3 min. The phases were separated by centrifugation at 13,000 rpm for 15 min. The upper aqueous layer was transferred to a fresh microfuge tube. 500 µl of isopropanol was added and mixed by inversion. The sample was incubated at room temperature for 10 min to precipitate the RNA and then centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the RNA pellet was washed with 1 ml of 70% ethanol. The RNA pellet was air-dried, dissolved in 30 µl RNase-free water and stored at -80°C.

Reverse transcription

The sequence for the putative bovine pyroglutamyl aminopeptidase 1 gene was obtained from the GenBank nucleotide sequence database at NCBI (http://www.ncbi.nlm.nih.gov), accession number **XM866409**.

1 µg of bovine RNA was combined with 1 µl of 25 µM specific reverse primer 5' CAGCAAGGATCCGTGTTCATGGCAACAGTTG 3' and the volume made to 5 µl with dH_2O. The mixture was incubated at 70ºC for 10 min and then placed on ice for 5 min. Reverse transcription was initiated by adding specific enzyme buffer, a dNTP mixture and MMLV reverse transcriptase. The volume was brought to 20 µl with dH_2O. The first strand was synthesised at 37ºC for 60 min followed by inactivation of the transcriptase at 95ºC for 2 min. This was used as template for PCR.

Polymerase Chain Reaction

cDNA was synthesised from total bovine RNA isolated from bovine brain tissue and the region encoding the gene was amplified using primers of 5'

GAACCCGCCATGGAGCAGCCCAGGAAGGCGG 3' (forward) and 5' CAGCAAGGATCCGTGTTCATGGCAACAGTTG 3' (reverse). The polymerase chain reaction was performed using a PTC-200 Peltier Thermal Cycler. The PCR reaction volume was 50 μl containing 1 μl template (10-100 ng), 1-2.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP, 1 unit RED*Taq* polymerase and 5 μl specific 10x enzyme buffer. The parameters for amplification were as follows: denaturation at 95°C for 10 min for one cycle, followed by 30 cycles at 95°C for 1 min, 64.5°C for 30 sec and 72°C for 1 min. A final extension at 72°C for 10 min was also included.

Cloning and sequencing of the *rBosT-pap1* gene

The amplified DNA was cloned into the TA cloning vector pCR2.1 (pZK1). The gene was amplified using gene specific primers 5' GAACCCGCCATGGAGCAGCCCAGGAAGGCGG 3' (forward primer containing the *Nco*1 restriction site CCATGG) and 5' CAGCAAGGATCCGTGTTCATGGCAACAGTTG 3' (reverse primer replacing the native stop codon with a *Bam*H1 restriction site GGATCC). The gene was restricted from this intermediate clone with *Nco*1 and *Bam*H1 and ligated into the vector pQE60 (pZK2) resulting in a construct that possessed a 6xHis tag at the 3' end of the gene that was exploited during protein purification. This gene-6xHis construct was sub-cloned using the pQE-60 encoded *Eco*R1 and *Hin*d III sites, into the expression vector pKK223-3 (PL-Pharmacia) generating the pZK3 expression plasmid (Fig.1). The pZK3 plasmid was verified by DNA sequencing (MWG Biotech AG, Germany).

Expression of bovine pyroglutamyl aminopeptidase type 1.

A 1L volume of Luria-Bertani broth containing ampicillin (100 μg/ml), tetracycline (10 μg/ml) and chloramphenicol (25 μg/ml) was inoculated with 10 mls of a stationary phase culture of *E.coli* XL10-Gold (Tet^R, Δ(μχρA)183 Δ(mcrCB-hsdSMR-mrr)173, *end*A1, *sup*E44, *thi-1, rec*A1, *gyr*A96, *rel*A1, *lac* Hte[F' *pro*AB *lacI*^{q}ZΔM15 Tn10(tet^R) Amy Cam^R]) transformed with the PAP1 expression plasmid pZK3. Cultures were incubated at 37ºC until an OD_{600} of 0.35 was reached. For induction, IPTG was added to a final concentration of 50 μM. After 4 hours the cells were harvested at 4000 rpm for 10 min at 4ºC. The cell pellet was washed with 200 ml buffer A (50 mM potassium phosphate, pH 8.0) and stored at –20ºC. The cells were re-suspended in 50 ml buffer A and disrupted with a 3 mm microtip sonicator using 2.5 sec, 40 kHz pulses for 40 sec. The cell debris was removed by centrifugation at 4000 rpm for 20 min at 4ºC yielding a crude lysate.

Purification of His-tagged PAP1

3 ml of Ni-NTA resin was gently mixed with 50 ml of crude lysate for 60 min at 4°C. The mixture was poured into a 0.7 x 15 cm column, allowing the resin to settle as the liquid passed through. The column was washed four times with 15 ml buffer A containing 20 mM imidazole and eluted with 3 x 5 ml fractions buffer A containing 200 mM imidazole. The elute was dialysed overnight against 1 L buffer A and stored at 4 ºC. The purification scheme was analysed by SDS-PAGE. Protein concentration was determined by the standard BCA assay and PAP1 activity was determined fluorimetrically.

Standard fluorimetric activity assay

PAP1 activity was determined according to the method by Fujiwara and Tsuru (1978) [17], as modified by Browne and O'Cuinn (1983)[18]. The quantitative assay was performed in triplicate using 96-well microtitre plates. 25 µl PAP1 sample was incubated at 37°C for 15 min with 100 µl pGlu-AMC substrate (final concentration 250 µM) in buffer A containing, at final concentration: DTT (10 mM), EDTA (2 mM) and 5% (v/v) DMSO. The reaction was terminated by the addition of 100 µl 1.5 M acetic acid. Negative controls were also included in each assay by adding acetic acid to the enzyme prior to the substrate. Liberated AMC was detected using a Perkin Elmer LS-50B plate reader spectrophotometer using excitation and emission wavelengths of 370 and 440 nm, respectively. The fluorescence readings were converted to nanomoles of AMC released per minute using a standard curve of AMC prepared under identical assay conditions.

Determination of protein concentration

All samples were dialysed extensively against ultrapure water prior to assay in order to remove interfering substances. Protein was determined by the enhanced bicinchoninic acid (BCA) assay based on the method of Smith *et al*. (1985) [19]. Sample (50 µl) was incubated with 200 µl BCA reagent at 37°C for 30 min, after which the absorbance at 570nm was determined. BSA was used as a standard for this assay.

SDS-PAGE and Zymogram

Samples were analysed by SDS-PAGE using 15% acrylamide/bis-acrylamide gels (10 x 12 cm) and stained using 0.25% (w/v) Coomassie blue solution. For zymogram

analysis, samples were electrophoresed at 4ºC on 15% acrylamide/bis-acrylamide gels without SDS. The gels were subsequently incubated for 10 min at 37ºC with buffer A containing 5 μM pGlu-AMC and 10 mM DTT. The gel was visualised under UV light.

Effect of pH on activity of recombinant pyroglutamyl peptidase

A pH range 6.0-10.5 was used to observe the effect of pH on PAP1 activity. The standard assay was carried out using the following buffers: Buffer A (50 mM potassium phosphate, pH 6.0-8.0), buffer B (50 mM Tris/HCl, pH 7.5-9.5) and buffer C (50 mM NaOH/glycine, pH 9.5-10.5). PAP1 samples were pre-incubated at 37°C for 10 min prior to addition of the substrate solution.

Effect of temperature

Purified enzyme was assayed in triplicate for 15 minutes at seven different temperatures, 4, 22, 30, 37, 40, 50 and 60°C.

Kinetic Studies

The Michaelis constant (K_m) was determined using a range of concentrations of pGlu-AMC (10-500μM), prepared in buffer A containing at final concentration 10mM DTT , 2mM EDTA and 5% (v/v) DMSO. Purified PAP1 activity was assayed in triplicate with each substrate concentration. K_m , V_{max} (maximal velocity) and K_{cat} (turnover number) values of PAP1 for the substrate pGlu-AMC were obtained by fitting the data to Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf kinetic models. These parameters were also determined using Enzfitter software from Biosoft, Cambridge, UK. Dissociation constant (K_i) was determined for three peptides pGlu-His-Pro-NH_2

(TRH), pGlu-Ala-OH and pGlu-Val-OH using a range of concentrations of pGlu-AMC (10-300μM), prepared in buffer A containing at final concentration 10mM DTT , 2mM EDTA and 5% (v/v) DMSO. K_i values were determined using the Lineweaver and Burk method.

Results and Discussion

Sequencing of bovine pyroglutamyl peptidase

The *rBos-Pap1* clone which we obtained was subjected to sequencing and compared with the human pap1 mammalian sequence available in GenBank **AJ278828**.The 680bp DNA fragment (Fig.1) had 71 nucleotides base changes differing from the human form. The bovine pap1 sequence encodes proteins of 209 amino acids and shows 98% homology with the human pap1 sequence [20]. Three amino acid residues at positions 81, 205 and 208 are found to vary among the two sequences (Fig.2). Catalytic residues Glu85, Cys149 and His168 were fully conserved between the two sequences which is a common feature with members of the C15 family of cysteine peptidases.

Expression and purification of recombinant bovine pyroglutamyl aminopeptidase 1

Competent XL10-Gold E.coli cells were used for expression of bovine pap1. Induction for *rBos-Pap1* was performed at 37ºC with 0.05mM IPTG for 4 h. Cells from the induced culture were harvested, suspended in buffer A, sonicated and purified. Purification was carried out as previously described and samples were analysed for activity and protein concentration. The purification scheme was visualised by SDS-PAGE (Fig.3). SDS-PAGE of recombinant bovine pap1 showed a

single band with an estimated size of 23kDa. This agrees well to previously reported data. [6]. This expression run yielded 3.3mg of bovine PAP1 protein and a specific activity of 1700.22 units/mg (Table 1). Zymogram analysis which was carried out on the purified preparation showed that purified enzyme was indeed active (Fig.4).

Biochemical characterisation of recombinant bovine pyroglutamyl-peptidase 1

Effect of pH

Bovine pap1 displayed an optimum pH of 9.0-9.5 (Fig.5). This compares well with data obtained from other researchers which examine the effect of pH on pyroglutamyl aminopeptidase activity. Cummins and O'Connor [6] found optimal pH of bovine brain to be 8.5 and Dando et al.[20] obtained maximal activity in the range of pH 7-9.0 for human pyroglutamyl. Awadé et al. [21] found bacterial pyroglutamyl peptidase to display an optimum pH between 7-9.0.

Effect of temperature

Optimum temperature for bovine pyroglutamyl peptidase activity was obtained at 37°C (Fig.6). 50% of activity was lost at 50°C and 60°C while 50°C was found to be the optimal temperature for human pyroglutamyl peptidase activity [20].

Effect of DTT and EDTA

As with all cysteine peptidases [22], there was an absolute requirement for a thiol-reducing agent for recombinant bovine pyroglutamyl peptidase enzyme activity. Optimum activtity was obtained using 10mM DTT (Fig.7). EDTA is thought to have a stabilising effect on mammalian pap1 [22, 23], however it did not have any effect on bovine pyroglutamyl peptidase activity.

Kinetics

The kinetic parameters of purified recombinant bovine pyroglutamyl aminopeptidase were determined various methods (Table 2). A K_m value of 59 μM was determined for the recombinant pyroglutamyl peptidase using various pGlu-AMC substrate concentrations ranging between 10 and 500μM. Dando et al. obtained a K_m value of 50 μM for human pyroglutamyl peptidase.

V_{max} and K_{cat} values obtained for bovine pyroglutamyl peptidase were 3.15 units/ml and 3.5 s^{-1} respectively.

A number of pyroglutamyl peptides were tested with the recombinant bovine enzyme (Table 3). All of the peptides behaved as competitive inhibitors. TRH produced the lowest K_i of 44.117μM, while K_i values for pGlu-Ala-OH and pGlu-Val-OH were 141μM and 652.17μM respectively.

Conclusion

In this study bovine pyroglutamyl aminopeptidase was cloned, expressed and purified……...

Bovine pyroglutamyl aminopeptidase displayed typical biochemical properties to that of other forms of PAP1 including human and bacterial.

Acknowledgments

We thank…….

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Abbreviations

PAP1	pyroglutamyl aminopeptidase type 1
pGlu-AMC	pyroglutamyl-7-amino-4-methyl coumarin
AMC	7-amino-4-methyl coumarin
DTT	dithiothreitol
EDTA	ethylenediaminetetra Acetic Acid
DMSO	dimethyl sulfoxide
BSA	bovine serum albumin
IPTG	Isopropyl-β-D-thiogalactosidase
Ni-NTA	Nickel Nitrilotriacetic acid
TRH	

Figures

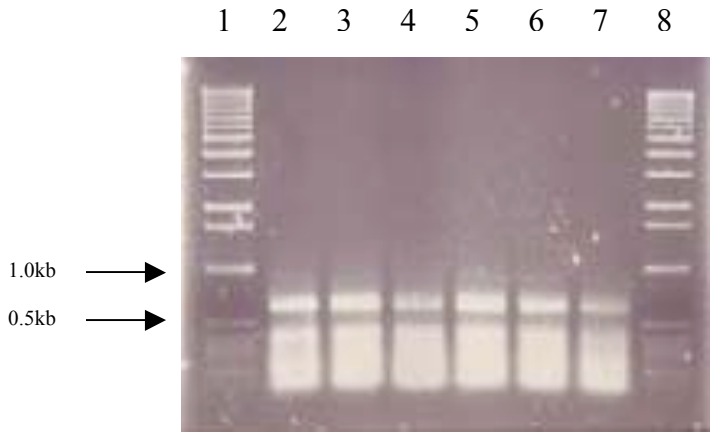


Fig.1. PCR analysis of bovine pap1 gene by 1% agarose gel. Lane 1 and 8 marker, lane 2-7, pap1 DNA.

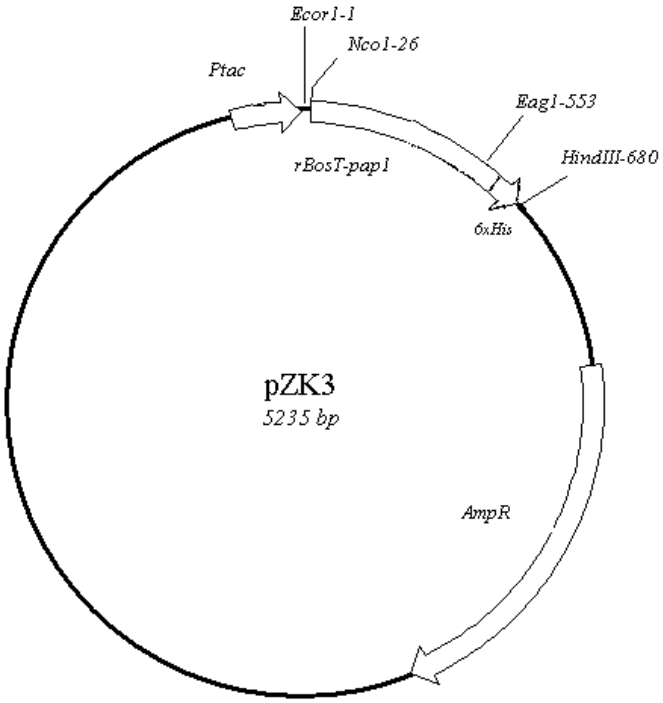


Fig.2. Map of plasmid pZK3 used to express *rBosT-pap1* in XL10-Gold cells. The construction of this plasmid is described under Materials and methods.

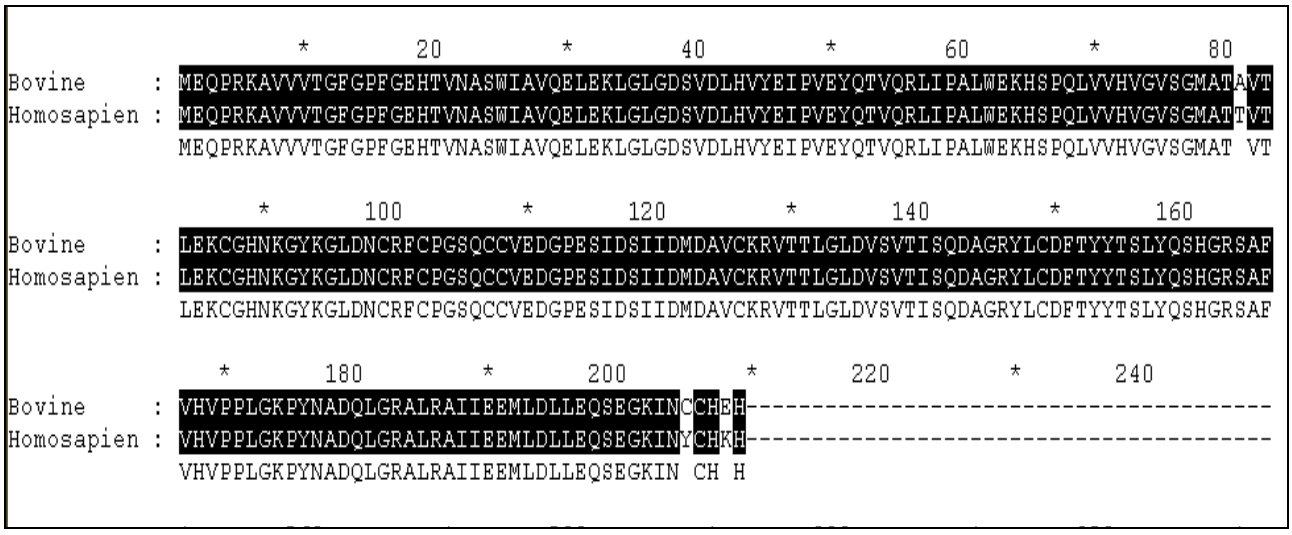


Fig.3. Alignment of the recombinant bovine pyroglutamyl aminopeptidase I amino acid sequence with human pyroglutamyl aminopeptidase I **AJ278828** highlighting the three amino acid variations at positions 81, 205 and 208. The sequences were aligned using the Genedoc program. [24].

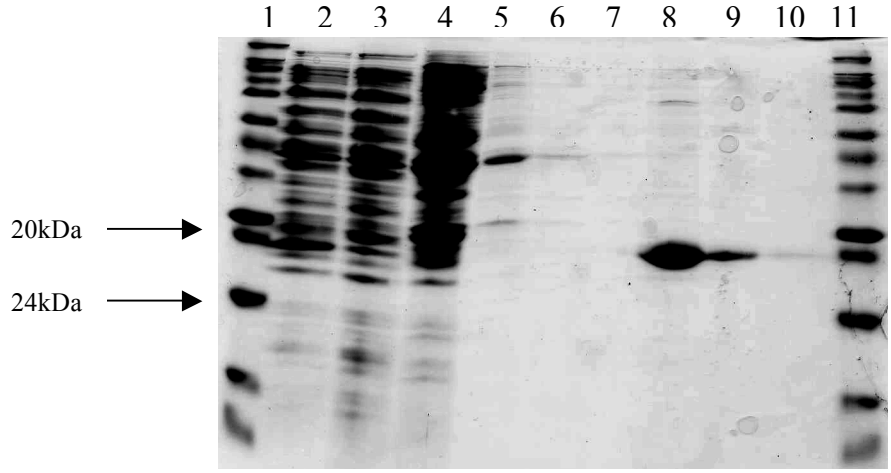


Fig.4. SDS-PAGE of bovine pyroglutamyl aminopeptidase at each stage of purification. Lane 1, protein molecular marker; lane 2, crude supernatant; lane 3, flow-through; lane 4-7, sequential column washings; lane 8-10, elution of recombinant bovine pyroglutamyl aminopeptidase protein from the column

Fig.5. Zymogram analysis of purified bovine pyroglutamyl aminopeptidase highlighting activity.

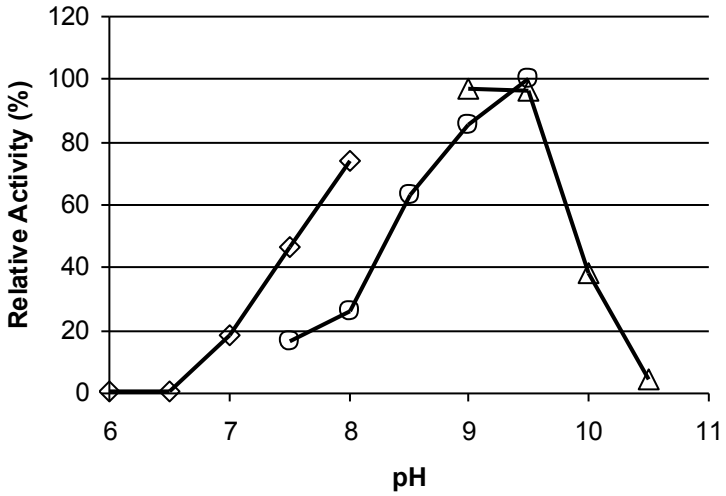


Fig.6. Effect of pH on recombinant bovine pyroglutamyl aminopeptidase I activity. Enzyme activity was measured under standard assay conditions over the range pH 6.0-10.5 with buffers as described in materials and methods. The buffers used were ◊, potassium phosphate; ○, Tris/HCL and Δ, glycine/NaOH.

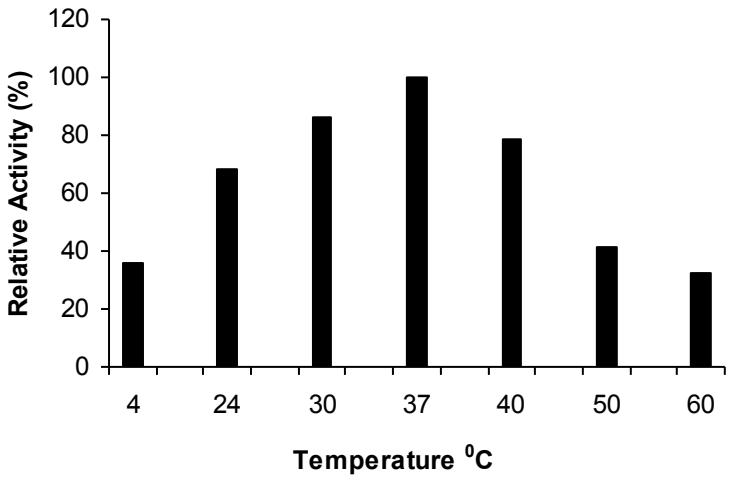


Fig.7. Effect of temperature on bovine pyroglutamyl aminopeptidase I activity. Assays were carried out with substrate pGlu-AMC at 4, 24, 30, 37, 50 and 60°C.

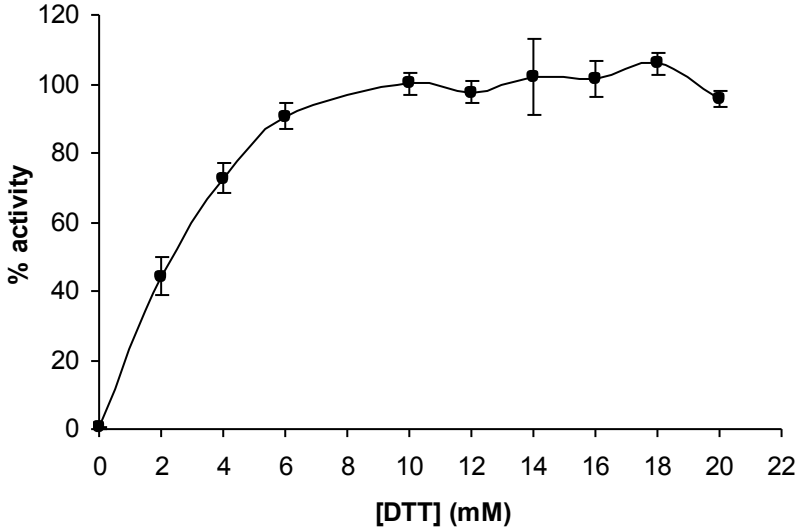


Fig.8. Effect of DTT on recombinant bovine pyroglutamyl aminopeptidase I activity. Enzyme activity was measured under standard assay conditions using a range of DTT concentrations from 0-20mM.

Tables

Table1

Summary of recombinant bovine pyroglutamyl aminopeptidase purification.

	Volume ml	Total Activity[a] Units	Total Protein mg	Specific Activity Units/mg
Purified PAP1	5	5593.783	3.3	1700.22

Summary of recombinant bovine pyroglutamyl aminopeptidase purification. [a] Units = nmoles.min^{-1} i.e. Units are expressed as nanomoles of AMC released per minute at 37°C.

Table 2

Kinetic parameters of purified bovine pyroglutamyl aminopeptidase type 1 using methods listed below.

Method	K_m μM	V_{max} Units/ml	K_{cat} s^{-1}
Lineweaver-Burk	66	3.3	3.6
Eadie-Hofstee	55	2.93	3.25
Hanes-Woolf	50	3.04	3.37
Enzfitter Software	63	3.22	3.57
Average	59	3.12	3.5

Enzyme assays were carried out as described in Materials and methods.

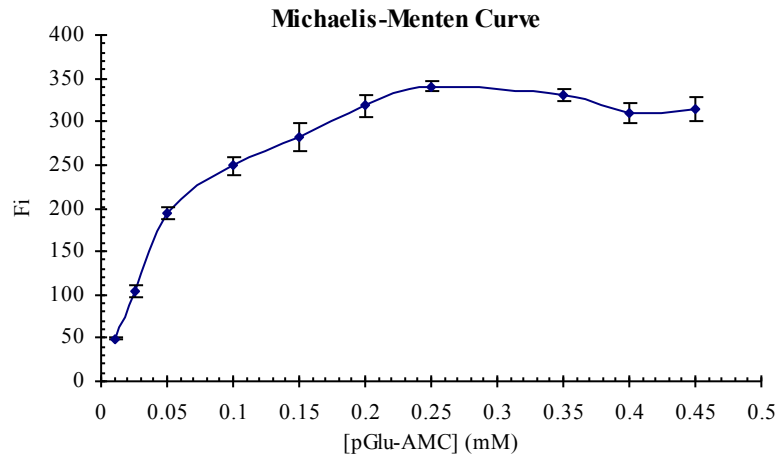
Table 3

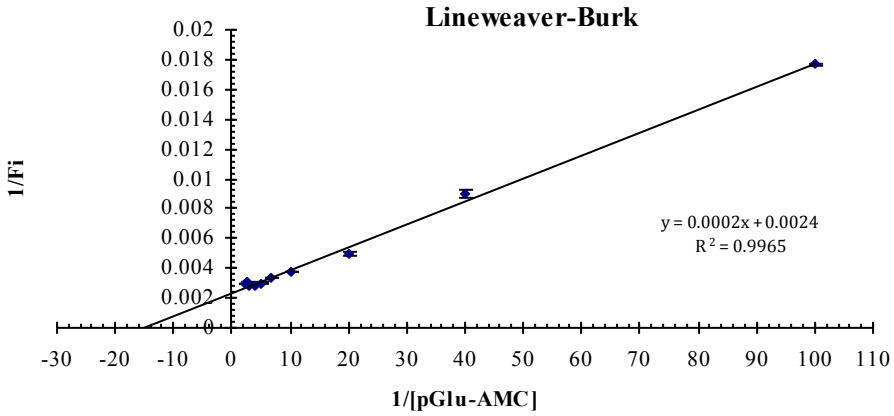
Activity of bovine pyroglutamyl aminopeptidase towards pyroglutamyl peptides.

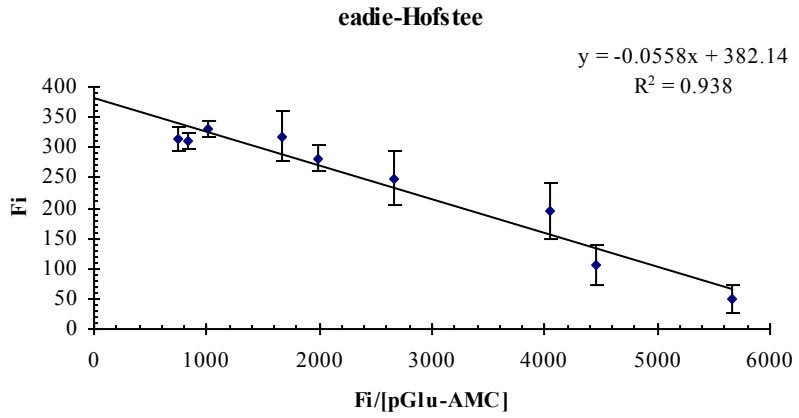
Peptide	K_{mapp} μM	K_i s^{-1}	Inhibition Type
pGlu-His-Pro-NH_2 (TRH)	400	44.1	C
pGlu-Ala-OH	166	141	C
pGlu-Val-OH	83	652.17	C

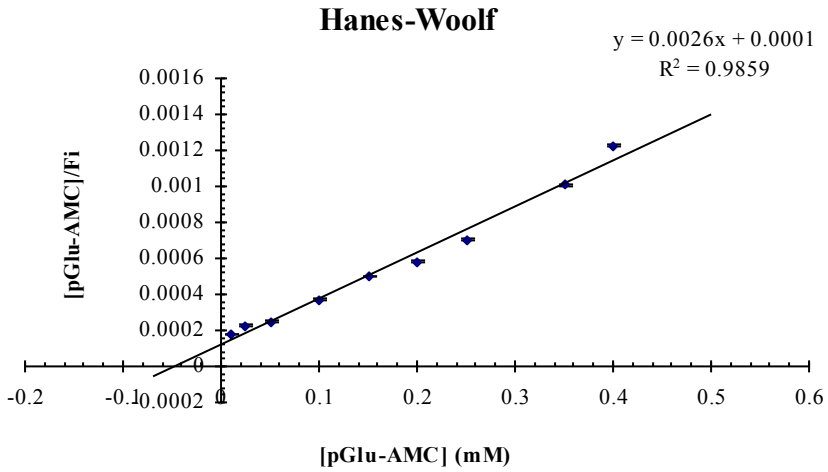
Enzyme assays were carried out as described in Materials and methods. C, competitive.

Other Graphs






eadie-Hofstee
y = -0.0558x + 382.14
R2 = 0.938
Fi
Fi/[pGlu-AMC]
0
50
100
150
200
250
300
350
400
0
1000
2000
3000
4000
5000
6000


Hanes-Woolf
y = 0.0026x + 0.0001
R2 = 0.9859
[pGlu-AMC]/Fi
[pGlu-AMC] (mM)
0.0016
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
-0.0002
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6

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