Solvent and Thermal Stability, and pH Kinetics, of Proline-specific Dipeptidyl Peptidase IV-like Enzyme from Bovine Serum.

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Short title: Bovine DPP IV Solvent and Thermal Stability and pH Kinetics

Summary

Proline-specific dipeptidyl peptidase-like (DPP IV; EC 3.4.14.5) activity in bovine serum has attracted little attention despite its ready availability and the paucity of useful proline-cleaving enzymes. Bovine serum DPP IV-like peptidase is very tolerant of organic solvents, particularly acetonitrile: upon incubation for 1 h at room temperature in 70% acetonitrile, 47% dimethylformamide, 54% DMSO and 33% tetrahydrofuran (v/v concentrations) followed by dilution into the standard assay mixture, the enzyme retained half of its aqueous activity. As for thermal performance in aqueous buffer, its relative activity increased up to 50°C. Upon thermoinactivation at 71°C, pH 8.0, (samples removed periodically, cooled on ice, then assayed under optimal conditions) residual activities over short times fit a first-order decay with a k*-value of 0.071 ± 0.0034 min^{-1}. Over longer times, residual activities fit to a double exponential decay with* k_1 *and* k_2 *values of 0.218 ± 0.025 min^{-1} (46 ± 4% of overall decay) and 0.040 ± 0.002 min^{-1} (54 ± 4% of overall decay) respectively.*

The enzyme's solvent and thermal tolerances suggest that it may have potential for use as a biocatalyst in industry. Kinetic analysis with the fluorogenic substrate Gly-Pro-7-aminomethylcoumarin over a range of pH values indicated two pK values at 6.18 ± 0.07 and at 9.70 ± 0.50. We ascribe the lower value to the active-site histidine; the higher may be due to the active site serine or to a free amino group in the substrate.

Keywords: Dipeptidyl peptidase IV; bovine serum; solvent stability; thermal stability; pH kinetics

Abbreviations: ACN, acetonitrile; ADAbp, Adenosine deaminase binding protein; AMC, 7-amino-4-methylcoumarin; BCA, bicinchoninic acid; CD26, cluster of differentiation molecule 26; CHES, 2-(Cyclohexylamino)ethanesulfonic acid; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DPP IV, dipeptidyl peptidase IV (EC 3.4.14.5); EDTA, diaminoethanetetra-acetic acid; HEPES, N-(2-Hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid); Hyp, hydroxyproline; MES, 2-(N-Morpholino)ethanesulfonic acid; MOPS, 3-(N-Morpholino)propanesulfonic acid; RANTES, regulated on activation normal T-cell expressed and secreted; T_{50}, half-inactivation temperature; THF, tetrahydrofuran; Tris, Tris(hydroxymethyl) aminomethane.

Introduction

Proline frequently occurs near the amino termini of many biologically active peptides. Due to the cyclic, rigid nature of the Pro residue, however, only a limited number of enzymes can cleave Pro residues within peptides [1]. Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5, a member of the S9 prolyl oligopeptidase family [2,3]) is one of these: it selectively cleaves dipeptides from the N-terminus of peptides with a Pro, hydroxyproline (Hyp) or Ala in the penultimate position [2,4,5].

In vivo, DPP IV is ubiquitous, occurring in both membrane-bound and soluble forms [6] and has diverse roles in various cell types [7]. It participates in the post-translational processing of chemokines (such as RANTES) and in the inactivation of neuropeptides (such as substance P) [4,5,8]. High DPP IV levels are associated with inhibition of tumour progression [9]. In contrast, inhibitors of DPP IV activity show promise in therapy of Type 2 diabetes [10]. DPP IV is a type II multifunctional cell surface protein and is identical to CD26 (a costimulatory molecule found on activated T cells) and to adenosine deaminase binding protein (ADAbp), indicating a function distinct from its enzymatic role [6,8,11]. Contrasts between DPP IV and the related proteins fibroblast activation protein and seprase are discussed in ref. [6].

Aside from any intracellular role, aminopeptidases have applications in debittering casein hydrolysates [12,13]. In the food industry, Pro-containing peptides are associated with bitter flavours, yet few Pro-cleaving enzymes have been exploited to date in debittering [12-15]. The proline specificity of DPP IV suggests that it may have potential as a biocatalyst for peptide processing in-vitro. Persistence of DPP IV activity, i.e. its stability, will be an important factor in any such application. Recently Mittal et al. described the effects of immobilization on the stability of goat brain DPP IV in

calcium alginate beads [16]. Bovine serum, readily available in quantity as a by-product of the beef industry, is a good source of soluble DPP IV-like peptidase [6] but this bovine serum enzyme has received scant attention to date (e.g. refs. [7]). Here we show that bovine serum DPP IV-like peptidase is very stable to water-miscible organic solvents and possesses good thermal stability characteristics. In addition, we investigate its pH kinetics and show that a single ionizing group influences its catalysis.

Experimental

Materials Kepak Meats (Clonee, County Meath, Ireland) supplied whole bovine blood. Gly-Pro-AMC was obtained from Bachem Feinchemikalein AG (Bebendorf, Switzerland). Fisher Scientific UK Ltd., (Loughborough, England) supplied HPLC grade acetonitrile (ACN), dimethylformamide (DMF), dimethylsulfoxide (DMSO) and tetrahydrofuran (THF). Bicinchoninic acid (BCA) protein assay kit and Gelcode Blue Protein stain were supplied by Pierce Chemical Company, (Illinois, USA). All chromatography resins and other materials were obtained from Sigma Chemical Company (Poole, Dorset, England).

Enzyme Preparation Dipeptidyl Peptidase IV-like activity was purified from whole bovine serum to near homogeneity (specific activity 1.1 U/mg) using hydrophobic interaction (Phenyl Sepharose 4B), gel filtration (Sephacryl S-300) and anion-exchange (Q-Sepharose) chromatographies in buffers based on 50mM HEPES pH 8.0, as described by Buckley *et al*. [7].

Protein Determination Biuret [17] or standard BCA assays were used to determine the protein concentration of samples as previously described [18]. Bovine serum albumin was used as standard. Prior to assay, samples were dialysed against 50mM HEPES, pH

8.0 containing 5mM EDTA. Absorbances of samples were determined at 560nm using a Labsystems Multiskan MS microplate reader.

Enzyme Assays The standard determination for dipeptidyl peptidase IV activity was performed by using 0.1mM of Gly-Pro-AMC as substrate in 50mM HEPES, pH 8.0, containing 5mM EDTA. Enzyme sample (25 µl) was added to 100 µl of thermally equilibrated substrate in triplicate wells of a white microtitre plate. The reaction mixture was incubated at 37°C for 60 min after which time the reaction was terminated by the addition of 175 µl of 1.7 M acetic acid. (The reaction had been shown to proceed linearly up to 120 min.) Suitable negative controls and blanks were included. The fluorescence of AMC liberated by hydrolysis was determined using a Perkin-Elmer LS-50 Luminescence Spectrometer at an excitation wavelength of 370nm and an emission wavelength of 440nm. Standard plots of fluorescence intensity versus 7-aminomethylcoumarin (AMC) concentration were run in different buffers, in the presence of crude bovine serum, or of solvents, to take account of quenching or inner filter effects. One unit of enzyme activity was defined as one micromol of AMC released per minute at 37°C.

Solvent and thermal stabilities To assess stability to organic solvents, DPP IV-like peptidase was incubated in 0-90% (v/v) mixtures of the solvents acetonitrile, DMF, DMSO and THF with 50 mM HEPES pH 8.0 (pH adjusted with 5.0 M HCl) as the aqueous component for 1 h at room temperature; residual activity was then measured by dilution of a 25 µl aliquot into the standard assay mixture above. All assays were performed in triplicate. To determine thermal stability, aliquots of purified DPP IV-like peptidase were incubated at increasing temperatures (37-92°C) for 10 min. Samples

were then cooled and stored on ice, and later warmed to 37°C and assayed under optimal conditions (above) and expressed as percentage of activity at optimum temperature (37°C). The half-inactivation temperature, T_{50}, was determined by inspection of the plot of percent activity against temperature. To determine heat stability over time, the purified enzyme was incubated at 71°C from 0 to 60 min. Aliquots were removed at appropriate time intervals, cooled and stored on ice, then warmed to 37°C and assayed for residual activity under optimal conditions as described above. Data were fitted to exponential decay functions using Enzfitter software (Biosoft, Cambridge, UK).

pH properties The pH-activity profile utilized a single substrate concentration (0.1 mM). Purified DPP IV-like peptidase was dialysed for 12 h against 2L ultrapure water, then further dialysed into each buffer (50mM in each case) over the pH range 4.0-10. The buffers used were acetic acid-sodium acetate (pH 4.0–5.5; pH adjusted with 5M HCl), MES (pH 5.5–6.5; pH adjusted with 5M NaOH), MOPS (pH 6.5–7.0; pH adjusted with 5M NaOH), HEPES (pH 7.0–8.0; pH adjusted with 5M HCl), Tris-HCl (pH 8.0–9.0 pH adjusted with 5M HCl), CHES (pH 9.0–10.0 pH adjusted with 5M NaOH); each replaced 50 mM HEPES in the assay protocol above. Michaelis-Menten kinetics were determined in each of these buffers using substrate concentrations ranging 0.05-0.5 mM. Enzfitter software was used to estimate pK_a values from plots of V_m, $1/K_m$ and V_m/K_m versus pH.

Results

Effect of organic solvents on DPP IV-like activity Fig. 1 shows the effects of acetonitrile (ACN), DMF, DMSO and THF on the enzyme's stability. In ACN, the enzyme

retained >50% of its original activity up to and above 70% (v/v) solvent. Activity was stable in the presence of 0-40% (v/v) DMF but sharply declined thereafter. DMSO concentrations > 50% (v/v) led to inactivation. Activation effects were observed in THF between 10-20% but THF was the most potent denaturing solvent overall. Concentrations of half-inactivation (C_{50}) in ACN, DMF, DMSO and THF were 77 ± 0.5, 47 ± 0.5, 54 ± 1.0 and 33 ± 0.5 % (v/v) respectively.

Temperature profile and thermoinactivation Activity at 37ºC (50mM HEPES, pH 8.0) was defined as 100%. Apparent activity increased with temperature to a peak of 134% at 50ºC. Above 58°C, activity decreased gradually but at 64°C still equalled that at 37°C (Fig. 2). The half-inactivation temperature T_{50} was estimated as 71°C and this temperature was used for thermoinactivation over 60 min. Up to 28 min, data fitted a single exponential decay to give a *k*-value of 0.071 ± 0.003 min^{-1} (apparent half-life 10 min) but deviated above 30 min. The full time course fitted a double exponential decay, yielding values of 0.218 ± 0.025 min^{-1} (46.5 ± 4.0 % of overall decay) and 0.040 ± 0.002 min^{-1} (54.5 ± 4.1% of overall decay) for k_1 and k_2 respectively.

Effect of pH on DPP IV-like activity and kinetics Fig. 3 illustrates the effect of both buffer and pH on enzyme activity. The buffers used (50 mM in each case) were acetic acid-sodium acetate (pH 4.0–5.5), MES (pH 5.5–6.5), HEPES (pH 7.0–8.0), Tris-HCl (pH 8.0–9.0), CHES (pH 9.0–10.0). Each was adjusted as follows: sodium acetate adjusted with 5M HCl; MES adjusted with 5M NaOH; MOPS adjusted with 5M NaOH; HEPES adjusted with 5M HCl; Tris adjusted with 5M HCl; CHES adjusted with NaOH. Overlapping pH values were assayed when changing from one buffer to another to

distinguish between pH and buffer effects. The enzyme exhibited a broad pH-activity profile in the range of 7.0-10 at 37^{o}C; however, this depended on the buffer.

Activity was higher at pH 8.0 in HEPES than in Tris-HCl and was greatest at pH 7.5 in MOPS. At pH 5.5, DPP IV activity decreased 25% on changing from acetic acid-sodium acetate to MES buffer. Activity decreases of 65% and 67% were observed at pH 7.0 and 7.5 respectively, on changing from MOPS to HEPES. At pH 9.0, the enzyme was more active in CHES than in Tris-HCl. Optimum pH was between 7.5 and 9.0. Complete inactivation occurred at pH 4.0. The decrease in DPP IV-like activity at pH values < 6 rules out contamination by lysosomal dipeptidyl peptidase II (EC 3.4.14.2, pH optimum 5.5 [19]) in the purified sample.

Michaelis-Menten kinetics were determined at each pH point (same buffers as above) to ascertain the pH dependence of DPP IV catalysis. Two p*K* values of 6.18 ± 0.07 and 9.70 ± 0.50 were observed (Enzfitter software: Biosoft, Cambridge, UK). Both occurred in plots of log V_m/K_m (Fig. 4) and log $1/K_m$ versus pH; only the upper value occurred in a plot of log V_m against pH (data not shown). (Upon calculation of V_{max} and V_{max}/K_m, the buffer effects seen in Fig. 3 were much less pronounced and were ignored.)

Discussion

As far as we can ascertain, this is the first detailed study of the stability of DPP IV-like activity from bovine serum. DPP IV-like peptidase was exposed to solvents with different denaturing capacities (DC, ref. [20]; values in brackets): ACN (64.3), DMF (63.3), DMSO (60.3) and THF (100). Overall, DPP IV-like peptidase shows good

solvent tolerance. As expected, THF was the most deleterious solvent (C_{50} 33% v/v) but activation effects were observed up to 20% (v/v) THF. These could be due to conformational changes in the enzyme at low solvent concentrations. ACN is the least harmful of the solvents tested (C_{50} 77% v/v), followed by DMSO (C_{50} 54% v/v) and DMF (C_{50} 47% v/v); while DC values of these three solvents are close (60.3 – 64.3), the exact order is not followed. In contrast, DPP IV from goat brain gave low C_{50} values (v/v) of < 10% in DMSO and approx. 12% in ethanol [16], although different protocols were used. The organotolerance of enzymes is of great interest: there are advantages to using enzymes in non-aqueous or mixed media, including the catalysis of reactions unfavourable in water, such as peptide synthesis [21,22]. Amino acids are typically most soluble in solvents such as DMF and DMSO [22]. The bovine serum DPP IV-like protein tolerates up to 40% (v/v) of both these solvents; however, it was most stable in acetonitrile (up to 70% v/v). The enzyme's tolerance of water-miscible solvents suggests that it may be a potentially useful biocatalyst in peptide processing (in aqueous or mixed media) or in enzymatic peptide synthesis.

Bovine serum DPP IV-like activity increased up to 50°C and remained high up to 64°C. Porcine seminal plasma DPP IV was similarly stable up to 50°C [23]. Goat brain DPP IV showed optimal activity at 50°C but retained only approx. 35% activity at 60°C [16]. Yoshimoto *et al.* [24] reported a higher optimum temperature of 60°C for lamb kidney DP IV, which retained 50% activity up to 72°C. At 71°C (the observed T_{50}) our DPP IV-like peptidase undergoes a straightforward thermal inactivation. At shorter times (up to 28 min), data fitted satisfactorily to a first-order process, allowing estimation of the apparent half-life (10 min). Durinx et al. [25] reported a *k*-value of 0.0370 ± 0.0019 min^{-1} for human serum DPP IV at 65°C in 50 mM Tris buffer pH 8.3. This gives a half-

life of approx. 19 min, longer than the present bovine serum enzyme but obtained at a lower temperature (6^{o}C less).

A double exponential decay becomes evident at longer times. The active form of human DPP IV is a dimer; the monomer is inactive [24,26,27]. Assuming that the present bovine serum DPP IV-like peptidase also exists as a dimer, the biphasic loss of activity at 71°C may proceed via (i) formation of a partially-unfolded, but still catalytically active, dimeric intermediate and (ii) subsequent dissociation of the dimeric intermediate to inactive monomers. Dependence of the inactivation rate on the total protein concentration can give insights into the contribution of dissociation phenomena to an observed kinetically irreversible inactivation [28,29] but we have yet to undertake experiments of this sort.

Bovine serum DPP IV-like activity persists well above normal body temperature. While not unique in this respect (dimeric bovine erythrocyte Zn-Cu superoxide dismutase, for example, shows no thermal transition below 80^{o}C [30]), the DPP IV-like enzyme is nevertheless more stable than some other oligomeric mammalian enzymes. Dimeric bovine heart creatine kinase, for instance, has a T_{50} <50^{o}C (10 min incubations; [31]), while that of tetrameric rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is <60^{o}C (20 min incubations; [32]). Recombinant tetrameric sheep liver cytosolic serine hydroxymethyl transferase loses some activity after 5 min at 55^{o}C [33] and human IgG begins to denature at 52^{o}C [34]. Dimeric neuronal nitric oxide synthase is unstable at 37^{o}C [35].

DPP IV-like activity is shown over a wide range (pH 6.5-10), with its optimum at pH 7.5, similar to goat brain [16], human serum [25,36] and porcine skeletal muscle DPP IV [3]. DPP IV-like activity isolated from serum would be expected to function

optimally at the pH of the circulatory system i.e. pH 7.4. Processing of many bioactive peptides (substance P) and circulating peptide hormones (growth hormone-releasing hormone GRH) takes place in the blood circulation by DPP IV. Therefore, DPP IV needs to be active and stable at this pH in order to process these bioactive peptides [2,5,7].

Computer fits of pH kinetic data (Enzfitter) revealed two p*K* values at 6.18 and 9.70 in the plot of log V_m/K_m versus pH (Fig. 4). Both values occurred as downward bends in this and in a plot of pK_m versus pH (not shown), indicating that they belong to either the free enzyme (E) or the free substrate (S) [37,38]. The p*K* value at 6.15 is likely due to the catalytic His in the free enzyme. (DPP IV is known to be a serine proteinase [4].) The upper value likely reflects deprotonation of the Gly moiety of the substrate (p*K* of the α-amino group of free Gly-Pro dipeptide is 9.98 [39]) but ionization of the active site serine is another possibility [40]. Further work is required to elucidate this point.

DPP IV has broad substrate specificity for residues at the amino-terminal position (the P_2 position of Gly-Pro-AMC), although aliphatic residues are favoured and a protonated amino group at the P_2 position is a requirement [5,24,26,27]. The active site His residue must also be in the deprotonated form. Hence, changes in pH will affect the protonation states of these residues. At acidic pH (4-5) the active site histidine becomes protonated and activity decreases or is absent, as only one form of the enzyme can bind substrate. Likewise, at pH above the p*K*a of glycine (amino terminal residue of the substrate Gly-Pro-AMC), this residue becomes deprotonated and specificity of DPP IV for the substrate diminishes [5,37] (leaving aside for the moment the possible ionization of the active-site Ser [40]).

Conclusion

A proline-specific DPP IV-like peptidase from bovine serum is a moderately stable protein that shows promising solvent tolerances and inactivates by a complex mechanism at elevated temperatures. Its favourable *in-vitro* stability and broad pH-activity profile, together with its ready availability as a by-product of the beef industry and the relative paucity of useful proline-cleaving enzymes, should facilitate its possible use in biocatalytic applications such as debittering/ processing of proteins and peptides [12]. Its catalysis involves a group ionizing at pH 6.18, most likely a histidine residue.

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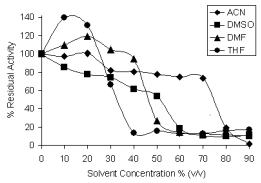
Legends to figures.

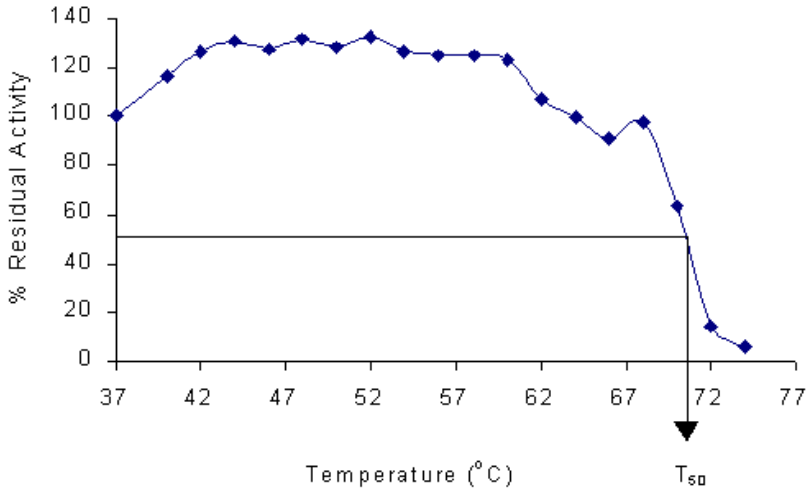
Fig. 1. Effect of organic solvents on bovine serum DPP IV-like peptidase. Enzyme aliquots were incubated for 1 h in the solvent mixtures, then the remaining activity against Gly-Pro-AMC was determined under optimal assay conditions and expressed as a percentage of activity in aqueous buffer, pH 8.0. Each point is the mean of triplicate assays where standard deviations were ± 5%. ACN, acetonitrile; DMF, dimethylformamide; DMSO, dimethylsulphoxide; THF, tetrahydrofuran.

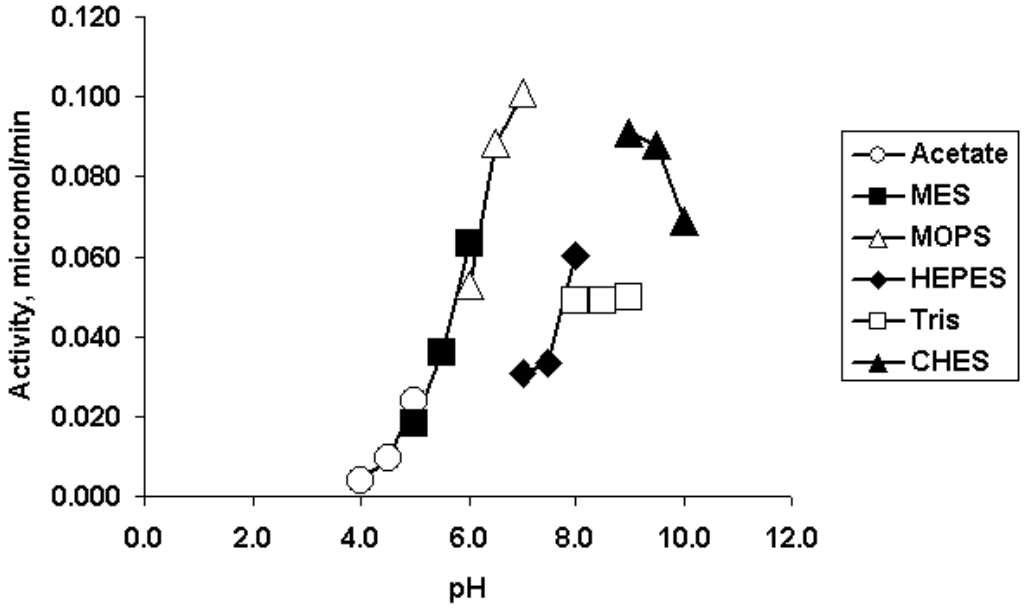
Fig. 2. Temperature profile of bovine serum DPP IV-like peptidase. Enzyme aliquots were incubated for 10 min at various temperatures, cooled and the remaining activity against Gly-Pro-AMC determined at 37^{o}C, pH 8.0, and expressed as a percentage of activity at 37^{o}C. Each point is the mean of triplicate assays where standard deviations were ± 5%. The "blip" at approx. 69oC was reproducible on repeated determinations.

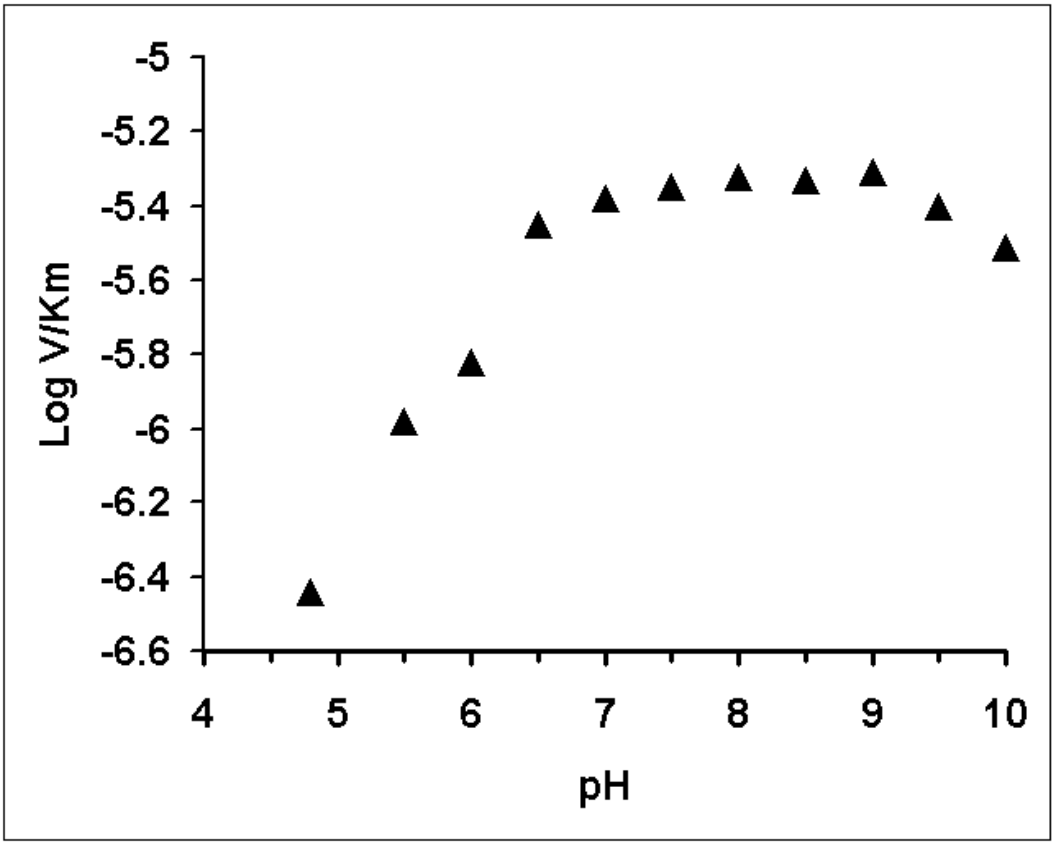
Fig. 3. Effect of pH on the activity of bovine serum DPP IV-like peptidase against Gly-Pro-AMC. Each point is the mean of triplicate assays where standard deviations were ± 5%. The different buffers used are indicated in the insert.

Fig. 4. Effect of pH on V_{max}/K_m for bovine serum DPP IV-like peptidase acting on Gly-Pro-AMC. Each point is the mean of triplicate assays where standard deviations were ± 5%. Computer fits of the data (Enzfitter software) indicated two pK values at 6.18 ± 0.07 and at 9.70 ± 0.50.


ACN
DMSO
DMF
THF
% Residual Activity
Solvent Concentration % (v/v)
0
10
20
30
40
50
60
70
80
90
100
120
140


140
120
100
80
60
40
20
0
% Residual Activity
37
42
47
52
57
62
67
72
77
Temperature (°C)
T50


0.120
0.100
0.080
0.060
0.040
0.020
0.000
Activity, micromol/min
0.0
2.0
4.0
6.0
8.0
10.0
12.0
pH
Acetate
MES
MOPS
HEPES
Tris
CHES


-5
-5.2
-5.4
-5.6
-5.8
-6
-6.2
-6.4
-6.6
Log V/Km
4
5
6
7
8
9
10
pH