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ScienceDirect
Enzyme and Microbial Technology 41 (2007) 307–311
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Solvent and thermal stability, and pH kinetics, of proline-specific dipeptidyl peptidase IV-like enzyme from bovine serum

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Received 10 July 2006; received in revised form 1 February 2007; accepted 22 February 2007

Abstract

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 $0.071 \pm 0.0034\,\text{min}^{-1}$. Over longer times, residual activities fit to a double exponential decay with k_1 and k_2 values of $0.218 \pm 0.025\,\text{min}^{-1}$ ($46 \pm 4\%$ of overall decay) and $0.040 \pm 0.002\,\text{min}^{-1}$ ($54 \pm 4\%$ 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

1. 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

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-ethanesulfonic 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

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doi:10.1016/j.enzmictec.2007.02.007

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. Ref. [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.

2. Experimental

2.1. 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).

2.2. 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 50 mM HEPES pH 8.0, as described by Buckley et al. [7].

2.3. 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 50 mM HEPES, pH 8.0 containing 5 mM EDTA. Absorbances of samples were determined at 560 nm using a Labsystems Multiskan MS microplate reader.

2.4. Enzyme assays

The standard determination for dipeptidyl peptidase IV activity was performed by using 0.1 mM of Gly-Pro-AMC as substrate in 50 mM HEPES, pH 8.0, containing 5 mM 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 370 nm and an emission wavelength of 440 nm. 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 1 μmol of AMC released per minute at 37 °C.

2.4.1. 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).

2.5. 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 2 l ultrapure water, then further dialysed into each buffer (50 mM 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 5 M HCl), MES (pH 5.5–6.5; pH adjusted with 5 M NaOH), MOPS (pH 6.5–7.0; pH adjusted with 5 M NaOH), HEPES (pH 7.0–8.0; pH adjusted with 5 M HCl), Tris–HCl (pH 8.0–9.0; pH adjusted with 5 M HCl), CHES (pH 9.0–10.0; pH adjusted with 5 M 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.

3. Results

3.1. 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% and 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.

3.2. Temperature profile and thermoinactivation

Activity at 37 °C (50 mM HEPES, pH 8.0) was defined as 100%. Apparent activity increased with temperature to a peak of 134% at 50 °C. 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