

Research Article

Activity Staining and Inhibition Characterization of Dipeptidylpeptidase-III Enzyme from Goat Brain

Pooja Attri, Jasbir Singh, Suman Dhanda, and Hari Singh

Department of Biochemistry, Kurukshetra University, Haryana, Kurukshetra 136119, India

Correspondence should be addressed to Suman Dhanda, dhanda.suman999@gmail.com

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Dipeptidylpeptidase-III (DPP-III) from goat brain was purified and characterized using Arginyl-Arginyl-4-methoxy- β -naphthylamide (Arg-Arg-4m β NA) substrate. This enzyme retained its activity in native 10% polyacrylamide gel when stained using Arg-Arg-4m β NA. The activity was significantly increased by 100 mM chloride. Studies for its inhibition with some peptides and chemical inhibitors revealed that Leu-Trp-Met-Arg-Phe-Ala was most potent inhibitor followed by Arg-Phe-Ala and Gly-Phe-Leu. All the studied chemical inhibitors caused 40–50% inhibition at 1 mM. Metal ions helped to regain activity of EDTA pretreated enzyme. ZnCl₂ at 50 μ M almost completely restored the enzyme activity. Further ZnCl₂ and CoCl₂ exerted protective effects on EDTA pretreated enzyme for its susceptibility to DTNB inhibition. Therefore, DPP-III is a metalloprotease with the involvement of cysteine residues either located at the catalytic site or involved in regulation.

1. Introduction

DPP-III (EC 3.4.14.4) of the goat brain is a dipeptidylaminopeptidase which removes N-terminal dipeptide from Arg-Arg-4m β NA at pH 8.5. The enzyme was recently purified and characterized [1] and found to be affected by thiol compounds and metal ions [2]. The enzyme is cytosolic and ubiquitous in all studied tissues [3]. It hydrolysed Leu-enkephalin and other bioactive peptides and displayed micromolar affinity for enkephalins thereby suggesting its involvement in regulating enkephalin disposition [2, 4]. It is also reported in the cerebrospinal fluid of patients with acute pain [5]. All the studied analgesic and antihypertensive drugs inhibited the enzyme [6]. In the present study, it has been demonstrated that the enzyme activity is retained in Davis gel electrophoresis at pH 8.3. Understanding of catalytic mechanisms, structural features of protease, and their inhibitors are very important to explore their applications in medicinal field. So present study is carried out to understand its inhibitors.

2. Materials

The goat brains were obtained from a local slaughter house in Kurukshetra. Arg-Arg-4m β NA was of Bachem Feinchemikalien Co., Budendorf, Germany. Ethylene diamine tetraacetic acid (EDTA), Tris-HCl, dialysis bags, Polyacrylamide, N, N'-methylene bisacrylamide, ammonium persulphate, and TEMED were procured from Himedia. Fast Garnet GBC was from Sigma Chemical Co., St. Louis, Mo, USA. The routine pipetting was done with micropipettes from Tarson and very small volumes were pipetted out using Hamilton syringes. The digital spectrophotometer from Systronics was used to record the absorbance at 520 nm.

3. Methods

3.1. Purification and Assay of DPP. DPP was purified and assayed with Arg-Arg-4m β NA as substrate at pH 8.5 (Tris-HCl, 50 mM, containing 100 mM NaCl and 1 mM β -mercaptoethanol (β -ME) [1]. One unit of enzyme activity

was defined as the amount of enzyme that liberated 1 nanomole of 4 m β NA from the substrate per minute under assay conditions.

3.2. Determination of Optimum Concentration of Chloride Ions. The enzyme was incubated with Tris-HCl buffer (50 mM, pH 8.5 containing 1 mM β -ME) with different concentrations of NaCl (0 to 1000 mM) at 37°C for 10 min. The activity is expressed as percentage of maximum.

3.3. Activity Staining. For activity staining, the 10% Davis gel was prepared [7]. Before loading the protein samples, the polymerized gel was pre-electrophoresed for 2 h at a current of 4 mA by using resolving gel buffer. Purified protein (25 μ g) was loaded on gel and run at a constant current of 4 mA at 4°C. The gel was cut in two halves. One half was stained with Coomassie Brilliant dye and other half was stained for enzyme activity by thoroughly washing the gel with assay buffer and incubating the gel with substrate at 37°C and then putting the gel in Fast Garnet GBC (1 mg/mL) for 15 min for colour development.

3.4. Reversal of EDTA Inhibition by Metal Ions. Purified DPP-III was treated with 2.5 mM EDTA for 10 min and then extensively dialysed against 50 mM Tris-HCl buffer, pH 7.0. This enzyme was assayed in the presence of different metal ions at different concentration. The EDTA pretreated enzyme was preincubated with appropriate salts in assay buffer at 37°C for 10 min. The reaction was started by adding 150 μ M of substrate and activity is expressed as percentage of control. Likewise effect of DTNB was studied on EDTA pretreated enzyme in presence of ZnCl₂ and CoCl₂.

4. Results and Discussion

The purified enzyme retained its activity during electrophoresis at pH 8.3, in Davis gel as shown in Figure 1. The activity band obtained with Arg-Arg-4m β NA as specific substrate corresponded well with the protein band stained with Coomassie Brilliant blue. This suggests that polyacrylamide can be used for its immobilization. The enzyme was activated by Cl⁻ ions and maximum activation was achieved at 100 mM final concentration, which accounted for more than 1.5-fold increase in enzyme activity (Figure 2). Therefore 100 mM NaCl was the component of assay buffer. This property is similar to that of DPP-III enzyme of anterior pituitary [8]. Beyond 100 mM, enzyme activity decreased with increase in NaCl concentration.

4.1. Reversal of EDTA Inhibition by Metal Ions. Incubation of pure DPP-III with 2.5 mM EDTA resulted in 60% inhibition of enzyme activity. Dialysis after removal of chelating agent did not restore the activity. The suppressed activity could be partially restored by adding metal ions like ZnCl₂, CoCl₂, NiCl₂, FeSO₄, and MgCl₂ but none of the metal ions could completely restore the enzyme activity. Zn²⁺ at 50 μ M almost completely restored enzyme activity (~97%) (Figure 3). These results support our earlier studies

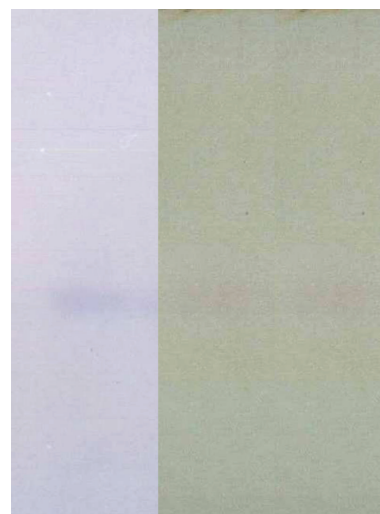


FIGURE 1: Davis gel electrophoresis of DPP-III after staining with coomassie brilliant blue dye (gel 1). Gel 2 shows activity staining of DPP-III.

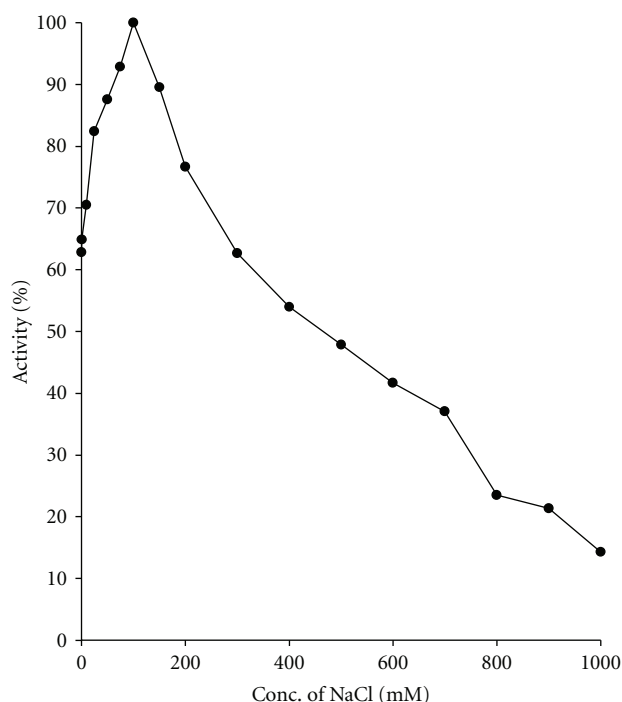


FIGURE 2: Effect of NaCl on activity of DPP-III.

with o-phenanthroline thereby confirming this enzyme to be a metalloprotease having Zn²⁺ at the active site. Our results are in agreement with human placental DPP-III [9], whereas Co²⁺ was most effective in restoring the activity of EDTA pretreated DPP-III from human RBCs [10]. Ca²⁺ and Co²⁺ were most effective in restoring the activity of EDTA pretreated guinea pig brain DPP-III [11]. On the other hand, untreated DPP-III was activated by Co²⁺ and inhibited by Zn²⁺, Ni²⁺, and Cu²⁺. Ca²⁺, Mg²⁺, Mn²⁺, and Fe²⁺ were slightly inhibitory [2].

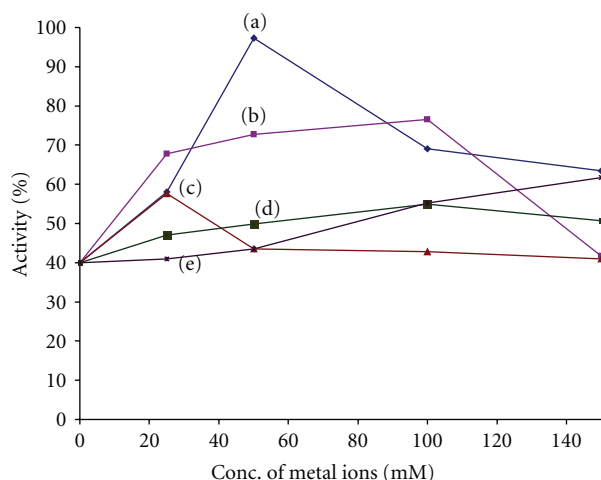


FIGURE 3: Effect of metal ions on 2.5 mM EDTA pretreated enzyme in presence of (a) ZnCl₂, (b) CoCl₂, (c) NiCl₂, (d) FeSO₄, (e) and MgCl₂.

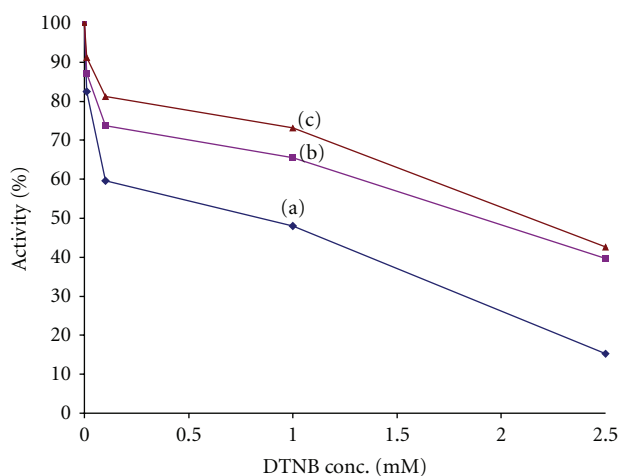


FIGURE 4: EDTA (2.5 mM) pretreated enzyme in the presence of (a), DTNB (b) DTNB and CoCl₂, (c) DTNB and ZnCl₂.

Relationship between binding of metal ions and cysteine residues on DPP-III was investigated by inactivation of EDTA-treated enzyme with DTNB in the presence and absence of Co²⁺ and Zn²⁺. Results are shown in Figure 4. Both metal ions exerted significant protective effect. However Zn²⁺ was more protective than Co²⁺. It appears that binding of metal ions influence reactivity and accessibility of thiol group(s) essential for enzyme activity.

Therefore it can be concluded that enzyme is a metalloprotease with the involvement of cysteine residues either located on the catalytic site or involved in regulation. Several other metalloenzymes having -SH group at the active site have also been reported earlier [12, 13].

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