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Purification, kinetic and functional characterization of membrane bound dipeptidyl peptidase-III from NCDC 252: a probiotic lactic acid bacteria

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Abstract

Pediococcus acidilactici is a probiotic lactic acid bacteria possessing studied in-vitro probiotic properties. Study of membrane proteins is crucial in developing technological and health applications of probiotic bacteria. Genome analysis of *Pediococcus acidilactici* revealed about more than 60 proteases/peptidases which need characterization. Dipeptidyl peptidase-III (DPP-III) is studied for first time in prokaryotes and it is a membrane protein in *P. acidilactici* that has been purified to apparent homogeneity. The enzyme was purified 81.66 fold with 36.75% yield. The specific activity of purified DPP-III was 202.67 U/mg. The protein moved as single band on native PAGE. The purity was also confirmed by in-situ gel assay. However SDS–PAGE analysis revealed it as high molecular weight heterotetramer with molecular weight of 108 kDa. The enzyme was maximally active at pH 8.5 and at 37 C. Purified DPP-III specifically hydrolyzed Arg-Arg-4-βNA with micromolar affinity ($K_m = 9.0 \, \mu M$) and none of studied endopeptidase and monopeptidase substrate was hydrolyzed. Inhibition study revealed purified DPP-III to be a serine protease with involvement of metal ion at active site. The significance of this enzyme as membrane protein is yet to be studied.

Keywords DPP-III · Serine protease · Lactic acid bacteria · Probiotic · *Pediococcus acidilactici*

Introduction

Proteases are ubiquitous to all living organisms. Exopeptidases are less studied as compared to endopeptidases. Amongst enzymes of DPP-series, only DPP-II is studied in *Pediococcus acidilactici* [1] while others are yet to be explored in *P. acidilactici*. Since proteases possess both degradative and synthetic properties, they are of immense physiological and commercial importance.

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Pediococcus acidilactici is a lactic acid bacteria (LAB) with all probiotic properties [2]. LAB are traditionally used in food fermentation and yet need to be explored for their potential health benefits. Bacterial aminopeptidases may be cytosolic, membrane associated or in extracellular media. Understanding of membrane proteins is crucial for development of technology and health applications of probiotic bacteria because surface proteins are most likely to interact with its environment and host's immune system. There are only few reports of membrane proteins of probiotic bacteria.

DPP-III (EC 3.4.14.4) is an exopeptidase which is distributed through all phylogenetic domains including plants and insects and studied in various different organisms [3–12]. DPP-III was found to be a membrane associated in studied Gram positive bacteria including *P. acidilactici* [13]. DPP-III is a dipeptidase that hydrolyzes Arg-Arg-4-βNA and several unsubstituted dipeptides from amino terminus and may generates new biologically important peptides. The enzyme is also involved in enkephalin and angiotensin degradation thus participating in pain and blood pressure regulation respectively [14–16]. Recently crystal structure of recombinant DPP-III from *Bacteroides thetaiotaomicron*

was studied [17]. It is shown to have post proline cleaving activity [18]. Many natural cytokines have proline in second position. So this enzyme is important to be studied in probiotics as action of enzymes will generate bioactive molecules that affect immunomodulation.

There is lack of information about proteolytic and peptidolytic enzymes of *P. acidilactici*. Complete genome of *Pediococcus* revealed that at least 60 proteases or peptidases need to be characterized biochemically. Purification and characterization of enzymes is important to understand the role of enzyme. This paper envisages the purification and characterization of DPP-III from probiotic *P. acidilactici*.

Materials and methods

Chemicals and bacterial strain

DEAE-Sephadex, Sephadex G-100, inhibitors and substrates were purchased from Sigma. Marker proteins were purchased from Himedia. The bacterial strain *P. acidilactici* was purchased from National Dairy Research Institute, Karnal (India) and is now being maintained in our lab. The cells were revived as per protocol provided by NDRI.

Assay of DPP-III

The enzyme was assayed as described by Dhanda et al. [19] using 20 µl Arg-Arg-4-βNA as substrate (4 mg/ml in DMSO) in 0.880 ml of assay buffer (50 mM Tris–HCl, pH 8.5 having 1% Triton X-100 and 300 mM NaCl) and 0.1 ml of enzyme. After incubation at 37 °C for 20 min the reaction was stopped by adding stopping reagent (1 M Na-acetate buffer, pH 4.2). One unit of enzyme activity was defined as amount of enzyme that releases one nanomole of 4-methoxy-βNA per minute from substrate under assay conditions.

Protein estimation

The protein content was estimated by method of Lowry et al. [20] using bovine serum albumin as standard.

Concentration of protein sample

The protein samples eluted from different column were concentrated using ultrafiltration cell using YM10 membrane.

Purification of enzyme

DPP-III was purified using different chromatographies successively. All the steps were carried out at 4 °C and 1% Triton X-100 was added at each step to maintain the enzyme in active form.

Extraction of DPP-III

The enzyme was extracted from the membrane as described by Attri et al. [13]. Sonication in the presence of 300 mM NaCl and 1% TritonX-100 with repeated freezing and thawing resulted in extraction of DPP-III from bacterial membranes.

Gel filteration chromatography

Enzyme homogenate was loaded on Sephadex G-100 column (30 × 1 cm). The fractions were eluted with 50 mM Tris–HCl buffer having 0.1% Triton X-100 of pH 7.4. The fractions positive for DPP-III were pooled, concentrated and dialyzed against 50 mM sodium phosphate buffer, pH 6.8.

Hydrophobic interaction chromatography

The concentrated sample of above step was loaded on phenyl Sepharose CL-4B column (15 × 1 cm), pre-equilibrated with 50 mM phosphate buffer having 1.0 M ammonium sulphate and 1% Triton X-100, pH 6.8. Unbound proteins were eluted with same equilibration buffer and bound proteins including DPP-III were eluted with a decreasing gradient (1.0–0 M) of $(NH_4)_2SO_4$. Fractions with DPP-III activity were pooled, concentrated and dialyzed against 50 mM sodium phosphate buffer, pH 6.4.

Anion exchange chromatography

The dialyzed sample of above step was loaded on DEAE-Sephadex A-25 column (30 × 2 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.4. Unbound proteins were eluted from the column with same buffer. The bound proteins were eluted with 0.0–1.0 M linear NaCl gradient. The fractions positive for DPP-III were pooled, concentrated and dialyzed against 50 mM sodium phosphate buffer containing 1% Triton X-100, pH 6.4. Purified sample was stored at 4 °C and analyzed for purity and further characterized.

Polyacrylamide gel electrophoresis (PAGE) and in-situ gel assays

The apparent homogeneity, purity and activity staining of the enzyme were assessed by 10% Davis gel electrophoresis [21]. For activity staining, polymerized gel was pre run for 2 h before loading the sample. After sample loading the gel was run at 4 °C. After the run was complete, the gel was cut into two equal halves. One-half was subjected to Coomassie Brilliant Blue staining and another half was stained for DPP-III enzyme activity with assay buffer and Arg-Arg-4mβNA substrate at 37 °C and colour was developed using Fast garnet GBC as described by Attri et al. [22].

Determination of molecular weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% SDS–PAGE) was performed by the method of Laemmli et al. [23] to determine molecular weight and subunit composition of purified enzyme.

pH optima and stability of DPP-III

pH optima of enzyme was determined by carrying out standard assay in 50 mM sodium acetate (pH 4.0–5.0), sodium-phosphate (pH 6.0–7.5), Tris–HCl (pH 8.0–9.0), Glycine-NaOH (pH 9.5–10.0) buffers. To assess the pH stability, the enzyme was pre-incubated in buffers of different pH (4.0–10.0) at 37 °C for 10 min and then enzyme was assayed at optimum pH 8.5. Enzyme activity was expressed as percent of maximum activity.

Temperature optima and stability of DPP-III

Temperature optima was estimated by assaying DPP-III at different temperatures (0–70 °C) with Arg-Arg-4mβNA as a substrate. Thermal stability of the purified enzyme was assessed by pre-incubating the enzyme for 10 min at different temperatures (0–70 °C) and then by assaying the residual enzyme activity at 37 °C using the standard assay. Enzyme activity was expressed as percent of maximum activity.

Determination of kinetic parameters

Kinetic parameters viz K_m and V_{max} of purified enzyme were determined from Lineweaver–Burk plot [24] and Hanes plot [25] using Arg-Arg-4mβNA in the substrate concentration range of 0–150 μM. The enzyme was assayed using standard assay procedure.

Substrate specificity

The enzyme was incubated at 37 °C in the standard reaction mixture with different mono, di and tripeptide β-naphthylamide as well as endopeptidase substrates (complete list of substrates is provided in Table 2) and relative enzyme activity was calculated with respect to Arg-Arg-4mβNA as a substrate using standard assay procedure described by Dhanda et al. [19].

Effect of different inhibitors

Purified enzyme was pre-incubated for 10 min at 37 °C with effective concentration of different inhibitors (complete list of inhibitors is provided in Table 3). Residual activity was calculated by assaying the enzyme with Arg-Arg-4mβNA substrate.

Investigation of active site by varying pH

To investigate catalytic residues at active site, log V_{max} was plotted against different pH values. The curve was extrapolated to find the pK_a values of amino acids involved in enzyme catalysis.

Effect of organic solvents

As DMSO and ethanol were used as solvents for different substrates and inhibitors, therefore effect of DMSO and ethanol on enzyme activity was studied by incubating the enzyme with different concentration of ethanol and DMSO (1–15%) at 37 °C for 10 min. The activity was expressed as percentage of the maximum activity.

Effect of urea and NaCl

The enzyme was incubated with different urea (0.1–2.5 M) and NaCl (50–1000 mM) concentration at 37 °C for 10 min and the residual enzyme activity was estimated using standard assay procedure and expressed as percentage of control.

Effect of different metal ions

Effect of different metal ions on enzyme activity was studied by addition of different metal ions (K^+, Fe^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+}, Ba^{2+} and Mg^{2+}). The enzyme was preincubated with chloride salt of different ions in assay buffer at 37 °C for 10 min. The reaction was initiated by adding 20 μl of substrate and the enzyme activity was expressed as the percent activity in comparison to control.

Reversal of o-phenanthroline inhibition by different metal ions

Reversibility of purified DPP-III was studied with respect to o-phenanthroline inhibition. About 5 ml enzyme was treated with 1.0 mM o-phenanthroline for 10 min (resulted in 70% inhibition, Table 3). This pretreated enzyme was extensively dialyzed at 4 °C for 24 h against 50 mM Tris–HCl buffer of pH 7.0. This dialyzed enzyme was used in DPP-III assay. Enzyme assay was run as per standard assay procedure. The residual enzyme activity was calculated and expressed as percent activity as compared to control. The reversibility of inhibition was measured in the presence of different metal ions at different concentration.

Effect of different thiol compounds

The effect of thiol compound (DTE, DTT, cysteine, reduced glutathione, thioglycolic acid and β-ME) on enzyme activity was studied. The enzyme was pre-incubated with each

thiol compound in assay buffer at 37 °C for 10 min. Then the enzyme was assayed using standard assay procedure and enzyme activity was expressed as percent activity in comparison to control.

Results and discussion

For last decades, LAB and probiotics are being studied for their effective biomolecules and health benefits. DPP-III is a therapeutically important membrane protein purified from probiotic LAB i.e. *P. acidilactici*. This enzyme has never been described in LAB. Screening of different LAB revealed significant DPP-III activity in *P. acidilactici*. Being surface protein of probiotic bacteria, its study might be important for understanding the adhesion with host cell and signaling.

DPP-III was assayed using Arg-Arg-4mβNA as substrate. The enzyme was extracted from the membranes by method of Attri et al. [13]. Crude enzyme preparation resulted in two pH optima at 7.5 and 8.5. This might be due to aminopeptidase B which sequentially removes Arg from Arg-Arg-4mβNA. Therefore it was simultaneously assayed.

Purification of enzyme

Addition of 1.0% Triton X-100 was essentially required for maintaining the enzyme in active form. The enzyme was purified to apparent homogeneity by successive chromatographies on gel filtration (Fig. 1a), hydrophobic interaction (Fig. 1b) and anion exchange chromatography (Fig. 1c). The elution profile on gel chromatography (Fig. 1a) shows both DPP-III and aminopeptidase B activities in same fractions. Differential elution pattern from hydrophobic interaction chromatography (Fig. 1b) confirm that both are non-identical. The conditions for eluting DPP-III from hydrophobic chromatography column did not result in elution of aminopeptidase B, which remained bound to the column so, the two were separated. The results of purification are summarized in Table 1.

The enzyme was purified 81.66 fold with percent yield and specific activity of 36.75% and 202.67 U/mg respectively. The yield of DPP-III from *P. acidilactici* is comparable to 34% in human placenta [26], 35% in human erythrocytes [27] and 49% in porcine spleen [28]. Fold purification of purified DPP-III of *P. acidilactici* is significant as compared to that obtained in *Saccharomyces cerevisiae* [12] while it is much less as compared to that of 12,000 in porcine spleen [28], 1775 in human placenta [26], 762 and 754 in goat brain and guinea pig brain [19, 29].

The purity of enzyme was checked and confirmed by the presence of single band on Davis gel electrophoresis (Fig. 2a) which was further confirmed by in-situ gel assay (Fig. 2b). The Coomassie band was corresponding to the

band obtained during in-situ gel assay. This confirmed the purity of enzyme.

Molecular weight determination SDS–PAGE

Molecular weight was determined using SDS–PAGE (Fig. 3a) and it was calculated from the graph between relative mobility of standard protein vs. corresponding log of molecular weight (Fig. 3b). DPP-III is an oligomer of four subunits having different molecular mass i.e. 33.02, 30.31, 25.01 and 19.9 kDa. The calculated molecular mass of purified DPP-III was 108.24 kDa. DPP-III was earlier reported to be a monomeric enzyme with molecular weight in the range of 80–260 kDa [17, 19, 27, 30]. DPP-III was homodimer of 66 kDa in porcine spleen [28] and heterodimer of 60 kDa (32.8 and 27 kDa) in *Vigna radiata* [7]. Higher molecular weight i.e. 158 kDa was reported in *Dictyostelium discoideum* [4]. Whether multimeric DPP-III has any advantage is obscure however being membrane protein, multimeric proteins form quaternary structure that allows the hydrophobic regions to be buried within protein structure. This reduces contact surface and limits the quantity of water required to stabilize this protein.

Mainly bacterial aminopeptidases described are monomers [31] and mostly enzymes that are secreted in external environment are also monomeric. Other bacterial aminopeptidases have multimeric structure comprised of 2, 4 or 6 subunits. Formation of multimeric or quaternary structure might allow hydrophobic region to be buried within that interact with the hydrophobic region of membrane.

Effect of pH on enzyme activity

The optimum pH for the purified enzyme was determined by assaying DPP-III at different pHs 4–12 using Arg-Arg-4mβNA as substrate. Percent activity was calculated with respect to highest activity. The optimum pH of DPP-III was 8.5 and enzyme exhibited only 50% activity at pH 6.0 and 10.0 (Fig. 4). pH optima of 8.0 was reported in *B. thetaiotaomicron* [17]. Similar pH optima was also reported for this enzyme from different tissues [19, 26, 28, 29, 32]. DPP-III of *D. discoideum* exhibited pH optima of 10.2 [4], 9.0 for lens [33] and rat brain DPP-III [34] and 8.0 for DPP-III of *Vigna radiata* [7]. The studied DPP-III exhibited about 50% activity at pH 6.0 and 10.5 (Fig. 4).

DPP-III was stable in very narrow and slightly alkaline pH range of 7.5–9.5. Other workers have also reported similar pH stability for DPP-III from different sources viz. *Vigna radiata*, 7.5–9.5 [7] and goat brain, 7.5–9.0 [19]. DPP-III from *Saccharomyces cerevisiae* was stable in pH range of 7.6–8.0 [12] whereas rat liver enzyme was stable over 5.0–7.5 [35]. Relatively higher stability was observed at pH 4.5 for goat brain DPP-III [19].

Table 1 Hydrolytic activities of purified DPP-III on various synthetic substrates

Substrates	% Activity
Arg-Arg-4βmNA	100 ± 0.083
Lys-Ala-4βmNA	8 ± 0.48
Gly-Arg-βNA	6.1 ± 0.03
Ala-Ala-βNA	5.7 ± 0.065
Gly-Phe-βNA	4.3 ± 0.73
Leu-Ala-βNA	2.8 ± 0.021
Asp-Ala-βNA	2 ± 0.51
Leu-Trp-βNA	Nil
Asp-Arg-NA	Nil
Trp-Met-4mβNA	Nil
His-Ser-βNA	Nil
Ser-Tyr-βNA	Nil
Ser-Met-βNA	Nil
Phe-Arg-βNA	Nil
Arg-4mβNA	Nil
Ser-βNA	Nil
Leu- βNA	Nil
Pro-βNA	Nil
Val-βNA	Nil
Asp-βNA	Nil
Ile-βNA	Nil
Phe-βNA	Nil
Tyr-βNA	Nil
Trp-βNA	Nil
Gly-βNA	Nil
Gly-Pro-Leu-βNA	Nil
Z-Phe-Arg-4mβNA	Nil
N-Benzoyl DL-Arg-βNA	Nil

Values are mean $\pm$ SD of three different experiments. Arg-Arg-4βmNA is the preferred substrate for DPP-III

Fig. 2 Analysis of purified DPP-III on 10% PAGE stained with Coomassie Brilliant Blue (**a**) and in-situ gel assay (**b**). The purity of enzyme was checked and confirmed by the presence of single band on Davis gel electrophoresis (Fig. 2a) which was further confirmed by in-situ gel assay (Fig. 2b). The Coomassie stained band corresponds to activity band in in-situ gel assay thereby confirming enzyme purity

Effect of temperature on enzyme activity

Purified DPP-III worked optimally at 37 °C. The enzyme activity increased gradually up to 37 °C and then abruptly declined. The enzyme retained 50% activity at 20 and 40 °C. The enzyme was completely inactivated at 50 °C and thus said to be thermosensitive (Fig. 5a). The temperature optima of 37 °C was also reported earlier for DPP-III of bovine lens [33] and human RBCs [27]. Energy of activation for DPP-III was calculated to be 7.109 kCal/mol from Arrhenius plot (Fig. 5b) which is lesser than the values of 15.18 kJ/mol and 12.46 kCal/mol for DPP-III of *Vigna radiata* and goat brain respectively [7, 19]. Temperature stability of enzyme was determined with reference to highest activity DPP-III. DPP-III was very sensitive to temperature beyond the optimum temperature. Enzyme was stable up to 37 °C and then activity fell
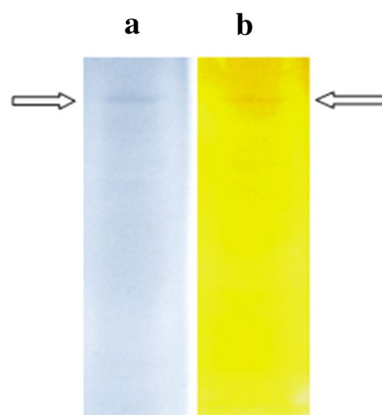
abruptly (Fig. 5a). 90 and 95% activity was lost at 40 and 45 °C respectively. Loss of enzyme activity at temperature beyond 40 °C was reported by Dhanda et al. [19] and Abramic et al. [27].

Kinetic parameters

Stock solution (10 mM) of Arg-Arg-4mβNA was prepared in DMSO and diluted accordingly to get the lower concentrations. To assay buffer of DPP-III substrate stock solution was added so as to get the final concentration in the range of 1–150 µM. The reaction was started by adding 100 µl of purified enzyme. Kinetic parameters i.e. K_m and V_{max} calculated for DPP-III by Michaelis Menton (Fig. 6a) Lineweaver–Burk plots (Fig. 6b) and Hanes plot (Fig. 6c) were 9.0 µM, 12.5 nanomoles/ml/min respectively. DPP-III exhibited micromolar affinity for Arg-Arg-4mβNA. The catalytic constant K_{cat} and catalytic efficiency i.e. K_{cat}/K_m were 0.0292 s^{-1} and 3.2×10^{-3} s^{-1} $µM^{-1}$ respectively. Kinetic parameters of DPP-III are close to that of measured for DPP-III of *B. thetaiotaomicron* [17]. K_m values varied considerably for DPP-III from different sources. Reported K_m for *P. acidilactici* is close to that of rat DPP-III (7.8 µM), human (8.4 µM) [36] and *Saccharomyces cerevisiae* DPP-III (8.8 µM) [37]. Higher values were reported for DPP-III of rat liver, bovine lens, goat brain, guinea pig and rat brain [29, 33–35]. K_{cat} and K_{cat}/K_m for *P. acidilactici* are comparable to 0.039 s^{-1} and 9.75×10^{-4} s^{-1} $µM^{-1}$ in goat brain [19], 0.09 s^{-1} and 10.21 s^{-1} mM^{-1} in *Saccharomyces cerevisiae* [37] and very low as compared to the values of 18.0 s^{-1} and 6.21×10^4 s^{-1} M^{-1} rat liver [35].

Fig. 3 **a** SDS–PAGE (15%) of purified DPP-III with β-ME (Lane 2) and Lane 1 shows molecular weight markers in the range of 14.3–97.4 kDa. Four bands of ~ 19.9, 25.01, 30.31 and 33.02 kDa revealed to be a heterotetramer. The calculated molecular weight was 108.24 kDa. **b** Relative mobility vs. molecular weight graph. DPP-III is heterotetrameric with overall molecular weight of 108.24 kDa. DPP-III is an oligomer of four different subunits i.e. 33.02, 30.31, 25.01 and 19.9 kDa (indicated as star in the graph). The overall molecular mass of purified DPP-III was 108.24 kDa
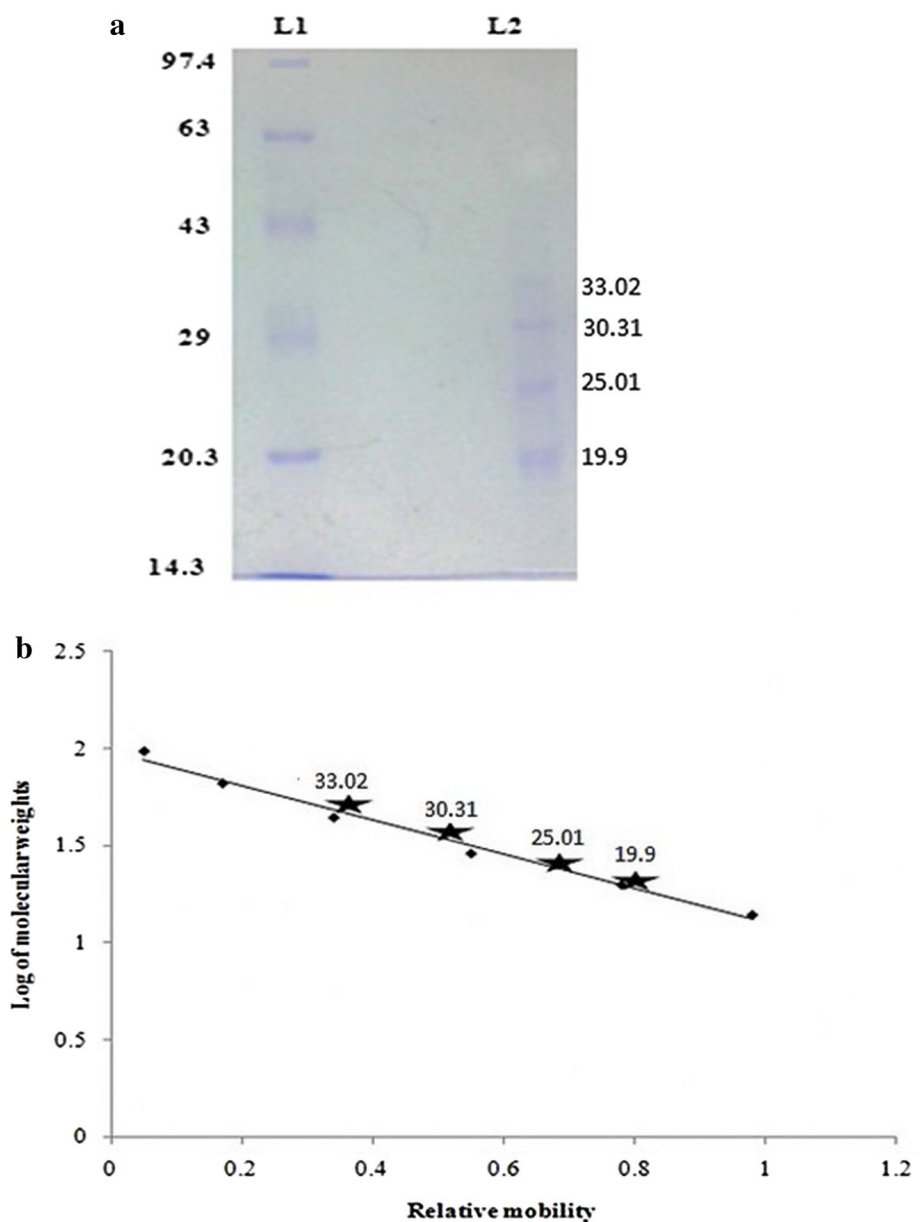
Substrate specificity

Studies of DPP-III with various dipeptide-βNA revealed highest affinity towards Arg-Arg-4mβNA (Table 2). On the other hand enzyme did not hydrolyze any aminopeptidase substrate viz. Arg-4mβNA, Ser-βNA, Leu- βNA, Pro-βNA, Val-βNA, Asp-βNA, Ile-βNA, Phe-βNA, Tyr-βNA, Trp-βNA, Gly-βNA. Different tripeptidase and endopeptidase substrates (Arg-Phe-Ala-βNA, Gly-Pro-Leu-βNA, Z-Phe-Arg-4mβNA, N-Benzoyl DL-Arg-βNA) were also not hydrolyzed by bacterial DPP-III. The enzyme exhibited highest substrate specificity towards Arg-Arg-4mβNA and other studied substrate were hydrolyzed. Similar results were reported from other sources [7, 19, 27, 32]. Present study

also suggests that purified DPP-III from *P. acidilactici* also cleave only dipeptide moieties from the N-termini of substrates thus suggesting this enzyme as dipeptidyl exopeptidase in nature.

Effect of different inhibitors

In order to investigate amino acid residues involved in catalysis, different inhibitors were studied for their effect on DPP-III activity and results are summarized in Table 3. DPP-III of *P. acidilactici* was strongly inhibited by serine protease inhibitors. PMSF and AEBSF resulted in 88% and 84% inhibition at 0.2 mM concentration. Leupeptin is inhibitory for Cys, Ser and Thr proteases and it caused 71% at
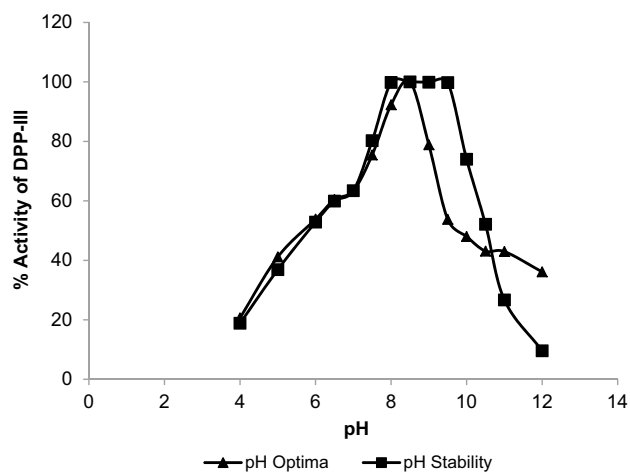
Fig. 4 pH optima and stability of DPP-III. DPP-III was optimally active at 8.5 with stability in narrow pH range of 7.5–9.5
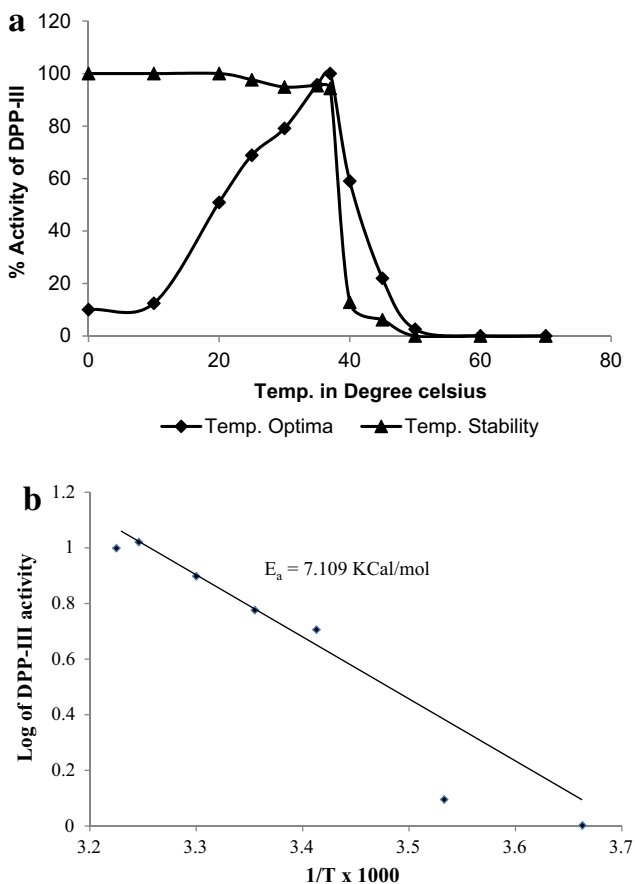

Fig. 5 **a** Temperature optima and stability of DPP-III. DPP-III exhibited temp optima of 37 °C and activity declined abruptly with complete loss of enzyme activity at 50 °C. Enzyme exhibited thermo stability only up to 37 °C. **b** Arrhenius plot for DPP-III. Arrhenius plot to calculate Energy of activation (Ea) for DPP-III. Ea for DPP-III is 7.109 kCal/mol

0.1 mM. EDTA at 10 mM and *o*-phenanthroline at 1 mM resulted in 60.32 and 69.84% inhibition respectively thereby indicating presence of metal ion at active site. Inhibition of DPP-III by metal chelating agents was also reported earlier by many workers [7, 19, 38]. DPP-III of rat skin was insensitive to EDTA, which may be because of very low EDTA concentration used by them [39, 40]. DPP-III from bovine pituitary required preincubation with β-ME before sensitivity to EDTA could be demonstrated [32]. Sensitivity towards inhibitors suggests that microbial DPP-III is a serine protease as reported for DPP-III from human erythrocyte, placenta, seminal plasma, rat erythrocyte and in guinea pig brain [26, 29, 38, 41]. DPP-III from *P. acidilactici* was least affected by thiol protease inhibitors i.e. DTNB. Contrarily DPP-III from number of sources was reported to be thiol protease [19, 30, 32–35, 42]. Some workers have reported the role of cysteine in DPP-III activity regulation. Bovine pituitary DPP-III was completely inhibited by 0.01 mM NEM [32] whereas enzyme from *P. acidilactici* and DPP-III of goat brain [19] showed slight inactivation even at 2.0 mM. From inhibition studies it was concluded that DPP-III of *P. acidilactici* is a serine protease with involvement of metal ion(s) at the active site.

Investigation of active site residues

Investigation of catalytic residues was performed by plotting log V_{max} vs. pH. pK_a values of amino acids involved in catalysis were found to be approximately 6.5 and 10.5 which corresponded to pK_a of His and Tyr respectively (Fig. 7). Inhibition studies revealed DPP-III to be a serine protease, pK_a of Ser is 13.0. This study shows the pK_a of Tyr instead of Ser. The results may be ± 3.0 pH unit, depending upon microenvironment of active site. Microenvironment of enzyme's active site where catalytic residues exist may also affect the pK_a of side chain. Moreover Ser and Tyr both have –OH group and thus Tyr may also have some role in binding/regulation/catalysis at active site. Histidine is known to co-ordinate metal ions and thus may play some catalytic role in DPP-III activity. These results are in agreement with inhibition studies.

Effect of organic solvents

As substrate solution was prepared in DMSO, therefore effect of DMSO was studied on DPP-III. The enzyme was assayed and percent activity was calculated. Initially at low concentration (2%) DMSO activated DPP-III activity. Then activity declined upto 5% DMSO concentration. From 5 to 15% of DMSO, decline was negligible (Fig. 8). Initial increase in enzyme activity at low concentration may be due to increased substrate solubility. DMSO being diprotic solvent facilitates nucleophilic reactions. DMSO may also alter

Fig. 6 Determination of kinetic parameters (K_m and V_{max}) for hydrolysis of Arg-Arg-4mβNA by DPP-III. **a** Michaelis–Menton plot. **b** Lineweaver–Burk plot. **c** Hanes plot. K_m and V_{max} for DPP-III was calculated to be 9.0 μM and 12.5 nanomoles/ml/min respectively
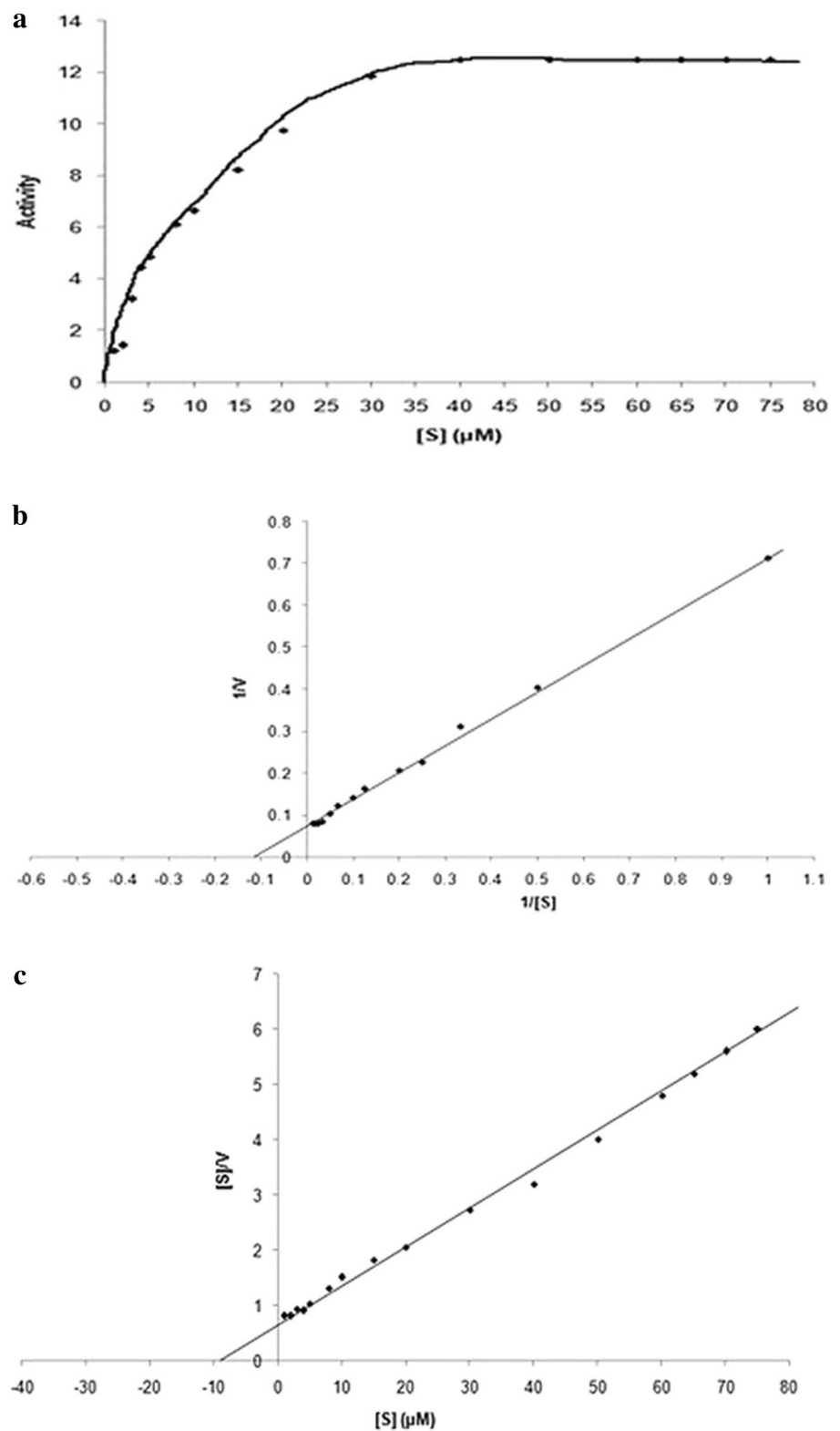
hydrophobic sites on enzyme and thus induce more favorable conformation for the enzyme's active site [43]. This becomes more significant for membrane bound enzyme which possess long stretch of hydrophobic residues. Decrease in DPP-III activity beyond 2% DMSO might be because of disturbance of water layer around hydrophobic sites of enzyme and thus unfavorable change in enzyme's conformation. DMSO probably also facilitates the movement of enzyme subunits in

Table 2 Effect of different inhibitors on activity of purified DPP-III

Inhibitor	Concentration	%Inhibition
Control	–	Nil
4-Nitrophenyl iodoacetate	0.05 mM	1.37 ± 0.19
	0.1	9.8 ± 0.27
	0.2	23.29 ± 0.01
Leupeptin	0.05	31.9 ± 0.53
	0.1	70.7 ± 0.41
Aminoethyl benzoylsulfonyl fluoride (AEBSF)	0.05	42.47 ± 0.12
	0.1	57 ± 0.15
	0.2	83.56 ± 0.23
PMSF	0.05	56.6 ± 0.43
	0.1	60.98 ± 0.61
	0.2	87.8 ± 0.36
1,10-Phenanthroline	0.05	4.11 ± 0.04
	0.1	27 ± 0.38
	0.2	47.4 ± 0.11
	1.0	69.84 ± 0.25
EDTA	1	19.18 ± 0.13
	2.5	33.29 ± 0.29
	5.0	38.36 ± 0.48
	10.0	60.32 ± 0.15
Pepstatin-A	0.05	12.33 ± 0.13
	0.1	22.27 ± 0.57
	0.2	29.58 ± 0.10
p-Chloromercuric benzoic acid	0.05	9.59 ± 0.27
	0.1	19.51 ± 0.19
Bestatin	0.05	6.88 ± 0.79
	0.1	14.63 ± 0.32
DTNB	0.1	15.07 ± 0.02
	0.2	19.5 ± 0.12
NEM	0.1	2.44 ± 0.08
	0.2	12.19 ± 0.12

Values are mean ± SD of three different experiments. DPP-III is a serine protease with requirement of metal ion catalysis

solution. Ribulose bisphosphate enzyme was also activated by 2% DMSO [44].

Activity of DPP-III declined (after 4%) in the presence of high concentration of ethanol. This might be because of

alteration of active site structure of DPP-III. It retained about 90% activity at 5% (v/v) of ethanol (Fig. 8). Similar results were observed for goat brain DPP-III [45].

Effect of urea and NaCl

NaCl stock solution (5.0 M in water) was added to different tubes to affect a final concentration of 0, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900 and 1000 mM and volume was made by adding appropriate volume of assay buffer. DPP-III was activated by Cl^- ions and maximum activation was observed at 300 mM NaCl (Fig. 9). Chloride ions also activated DPP-III of goat brain, *Vigna radiata*, bovine pituitary and human erythrocyte [7, 32, 45].

DPP-III is a membrane bound multimeric protein and urea is denaturant, so effect of urea was studied on purified DPP-III. Urea stock solution (10.0 M in assay buffer) was added to obtain the final urea concentration in the range of 0–2.0 M in 1 ml reaction mixture. It was observed that DPP-III activity slightly increased at 0.1 M but thereafter DPP-III lost half of its activity at 1.5 M urea concentration (Fig. 9). Urea is a denaturant that interacts differently with hydrophobic/hydrophilic groups and protein backbone. These interactions are dominated by formation of hydrogen bonds and other polar interactions [46, 47]. Addition of urea to aqueous protein solution might distort water structure and thus make it a better solvent for hydrophobic groups [48, 49]. This might trigger a folded protein to unfold by exposing the hydrophobic side chains to more accommodating solvent. Urea at low concentration was found to be an activator because, the active site of DPP-III might be altered by denaturants and the activated enzyme appears more open and flexible at the active site.

Effect of different metal ions

As DPP-III was found sensitive to metal chelators, therefore effect of metal ions was examined. Stock solution (1 and 10 mM) of chloride salts of different metals (K^+, Fe^{3+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+}, Ba^{2+} and Mg^{2+}) was prepared in Tris–HCl buffer of pH 7.0. Only Fe^{2+} was used as $FeSO_4$. Results revealed that DPP-III was activated by

Table 3 Purification table of DPP-III from *P. acidilactici*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Membranes	3981.53 ± 0.05	9882.76 ± 0.05	2.482 ± 0.58	1 ± 0.06	100 ± 0.43
Crude extract	3140.38 ± 0.02	9122.8 ± 0.19	2.905 ± 0.41	1.17 ± 0.1	92.3 ± 0.03
Sephadex G-100	1078.23 ± 0.2	7983.92 ± 0.43	7.405 ± 0.31	2.983 ± 0.28	80.79 ± 0.01
Phenyl Sepharose CL-4B	187 ± 0.13	5896.91 ± 0.41	31.53 ± 0.54	12.70 ± 0.35	59.67 ± 0.08
DEAE-Sephadex A-25	17.92 ± 0.03	**3631.9 ± 0.37**	**202.67 ± 0.18**	**81.66 ± 0.1**	**36.75 ± 0.04**

Values are mean ± SD of three different experiments. The enzyme was purified 81.66 folds with a yield of 36.75
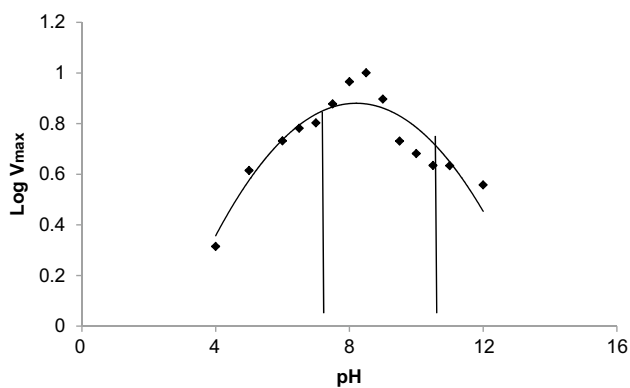
Fig. 7 log V_{max} vs. pH for DPP-III. pKa of 6.5 and 10.5 suggests involvement of His and Tyr respectively in enzyme catalysis
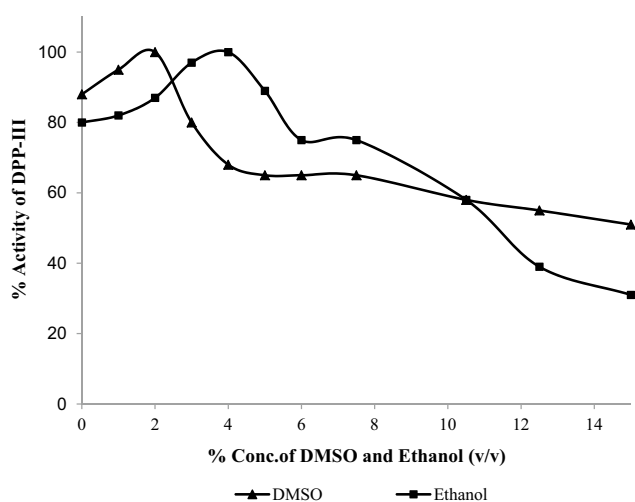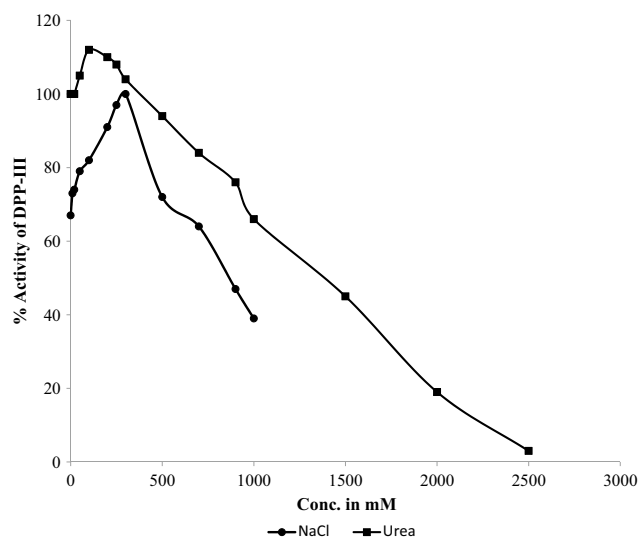

Fig. 9 Effect of urea and NaCl on DPP-III. DPP-III activity was reduced to half at 1.5 M urea. NaCl activated DPP-III up to 300 mM. beyond 300 mM NaCl resulted in decrease in DPP-III activity

Fig. 8 Effect of organic solvents on DPP-III. DMSO (upto 2%) increased DPP-III activity whereas ethanol decreased DPP-III activity

enzyme. Differential response to different metal ions might be because, in metalloenzyme certain metal ions form coordination bonds with certain enzyme residues and bind to substrate. Some stabilize three dimensional structures. Some cations act as regulatory cations and affect in-vivo activity and specificity of metalloenzymes.

Reversal of *o*-phenanthroline inhibition by different metal ions

Extensive dialysis did not revert *o*-phenanthroline inhibition. Reactivation of this suppressed activity by certain metal ions was observed. Up to 55% activity was recovered by Fe^{2+} followed by Co^{2+} and Zn^{2+} (Fig. 11). Whereas Cu^{2+} sand Mg^{2+} had no significant effect. Our studies are in agreement to that of human placental DPP-III [30] and goat brain DPP-III [53] for reversal by Co^{2+} and Zn^{2+}. Divalent metal ions that stimulated human DPP III activity (Co^{2+}, Mg^{2+} and Ca^{2+}) also restored EDTA inhibited DPP-III activity [41]. None of studied metal ion could restore enzyme activity completely. On the basis of metal ion effect, inhibitors study and reversal studies DPP-III from *P. acidilactici* can be assigned as metalloenzyme. Fukasawa et al. [26] also suggested DPP-III as a metalloenzyme having one Zn^{2+} ion in its active site. Regain of enzyme activity by certain metal ion suggest that either metal ion take up the catalytic role at active site or quench metalloprotease inhibitor.

Effect of different thiol compounds

Stock solution (10 and 100 mM) of different thiol compounds (DTE, DTT, cysteine, reduced glutathione,

Co^{2+}, Fe^{2+} and Cu^{2+} up to 0.2 mM concentration. The enzyme is slightly activated by Fe^{3+} and K^+ at low concentration (0.05 mM) and then inhibited with further increase in their concentration (Fig. 10a). Other metals were inhibitory to different extent (40% inhibition by K^+, 30% by Ca^{2+}, 60% by Mg^{2+} and 80% inhibition by Zn^{2+} and Mn^{2+} at 0.2 mM concentration). Mn^{2+} was the most inhibitory amongst all the studied metal ions. These studies support that *P. acidilactici* DPP-III is a metalloenzyme. Inhibition by Zn^{2+}, Mn^{2+} and Hg^{2+} was also reported for DPP-III by other workers [30, 35, 45]. In contrast, a slight activation by 10 μM Zn^{2+} was reported for human RBCs DPP-III [27] Co^{2+} has also been reported as an activator for a metal-endopeptidase [50], enkephalinase B [51] and some microbial peptidases [11, 52]. Activation by Co^{2+} was also reported for bacterial DPP-III [17]. Stimulation of activity by Co^{2+} may partially be attributed due to their interaction with amino acid residues involved in conferring active conformation to this

Fig. 11 Effect of thiol compounds on DPP-III. None of the studied thiol compound (DTE, DTT, cysteine, reduced glutathione, thioglycolic acid and β-ME) affected DPP-III thereby confirming it to be a serine protease
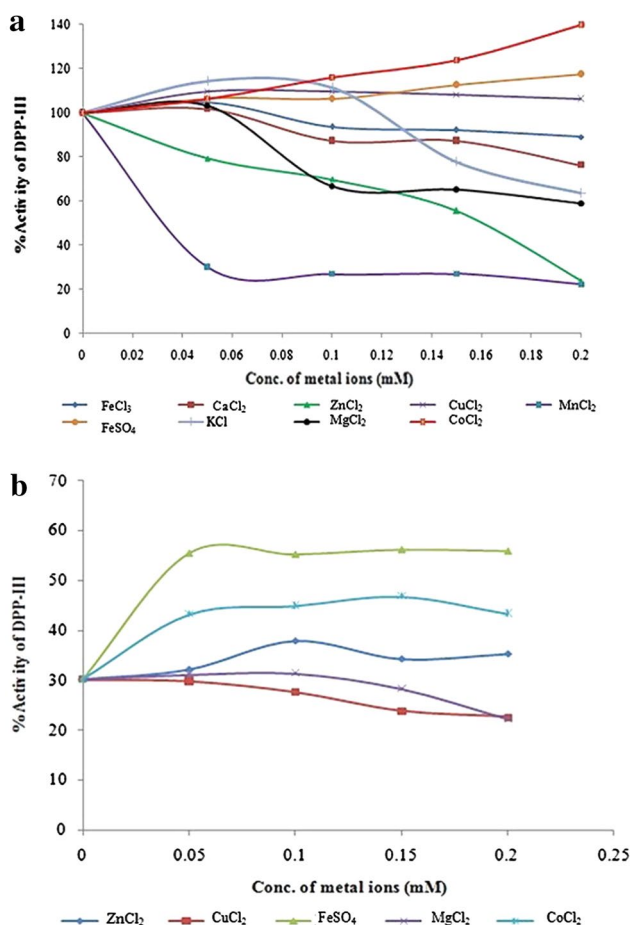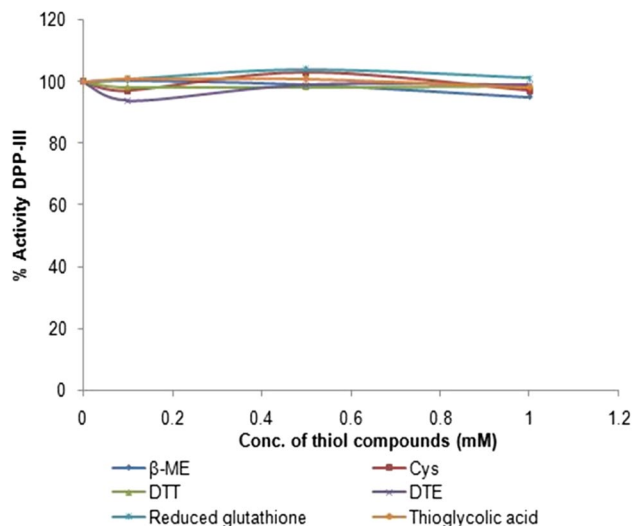
Fig. 10 a. Effect of different divalent metal ions on DPP-III. Enzyme was activated by Co^{2+}, Fe^{2+} and Cu^{2+} up to 0.2 mM concentration. The enzyme is slightly activated by Fe^{3+} and K^+ at low concentration (0.05 mM) and then inhibited with further increase in their concentration. Other metals were inhibitory to different extent. Mn^{2+} was the most inhibitory amongst all the studied metal ions. b Effect of different divalent metal ions on o-phenanthroline pretreated DPP-III. o-phenanthroline (1.0 mM) treatment for 10 min resulted in 70% inhibition of DPP-III. The pretreated DPP-III was dialyzed and incubated with metal ions. Fe^{2+}, Co^{2+} and Zn^{2+} helped in recovery of enzyme's activity

Conclusion

DPP-III was purified to apparent homogeneity from probiotic *P. acidilactici*. This is the first report of this enzyme from prokaryotes. The enzyme is a membrane bound high molecular weight (~ 108 kDa) heterotetramer and works optimally at pH 8.5 at 37 °C with Arg-Arg-4mβNA as substrate. This is a serine protease with involvement of metal ions in enzyme catalysis. Being membrane exopeptidase, it might be involved in generating free amino acids for bacterial nutrition and physiological processes. Trimming of oligopeptides might generate health promoting bioactive peptides (a probiotic attribute). DPP-III possess some characteristics similar to already studied DPP-III but some viz heterotetrameric nature, negligible effects of thiol and membrane association are unique and novel. Crystal structure of *B. thetaiotaomicron* has revealed differences in bacterial DPP-III (in upper and lower domain) from yeast and mammalian DPP-III and that might cause functional differences. Study of membrane protein is a challenge and its exact role in *P. acidilactici* is yet to be determined.

Compliance with ethical standards

Conflict of interest There is no conflict of interest among the authors. The present study does not include any sample subject to any type of ethical approval.

thioglycolic acid and β-ME) was prepared in Tris–HCl buffer, pH 7.0. Results revealed that thiol compounds had no effect on DPP-III (Fig. 11) that further supported that DPP-III from *P. acidilactici* is a serine protease. Though, activity of DPP-III was reduced upto 28% and 90% respectively in guinea pig brain and human RBCs at 1 mM dithiothreitol. While thiol compounds were inhibitory for human placental and goat brain DPP-III [30, 45]. In some studies, thiol compounds (dithiothreitol, dithioerythritol, and β-mercaptoethanol) exhibited protective effects on mammalian DPP-IIIs during their purification and storage [7, 19, 38].

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