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Membrane Bound Aminopeptidase B of a Potential Probiotic *Pediococcus acidilactici* NCDC 252: Purification, Physicochemical and Kinetic Characterization

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Abstract
An arginine aminopeptidase (EC 3.4.11.6) called aminopeptidase B was purified to apparent homogeneity from membrane extract of a potential probiotic *Pediococcus acidilactici* NCDC 252 using successive chromatographies on sephadex G-100 and phenylsepharose CL-4B. Purified enzyme was a heterotrimer with molecular mass of ~ 101.36 kDa. Predicted molecular weight of the enzyme from its gene (93.9 kDa) was close to the calculated molecular weight. The enzyme was optimally active at pH 7.5 and 40 °C. It was strongly inhibited by metal chelating agent and thiol protease inhibitors suggesting that enzyme is a metalloprotease with involvement of thiol. The K_m and V_{max} of enzyme for Arg-4mβNA were calculated to be 26 µM and 19.9 nmol/ml/min respectively. Its 3-D structure was modeled and validated using in-silico approach. In-silico analysis revealed Ser, His, Phe, Tyr and Thr to be present at active site of aminopeptidase B. Docking studies revealed that Arg-4mβNA binds with high affinity to the enzyme followed by Lys-4mβNA. The enzyme also hydrolyzed dipeptide-4mβNA derivatives containing hydrophobic amino acids and diaminocarboxylic acids (Arg, Lys and Asp) at the N-termini but not tripeptides, endopeptidase substrates and -βNA derivatives or peptides with proline and phenyl at their N-termini or C-termini.

Keywords Aminopeptidase B · Metalloprotease · Lactic acid bacteria · *Pediococcus acidilactici* NCDC 252 · Probiotic

Introduction

Various *Pediococcus* species are commonly used as beneficial microbes (starter culture) in producing cheese and yoghurts (Casquete et al. 2012; Bintsis 2018). The starter cultures are bacterial strains that are rich in enzymes that breakdown proteins into assimiable amino acids and peptides that provide flavor and taste to food products and also help in food ripening more quickly. Enzyme action also improves taste and flavour. This degradation and utilization require the concerted action of proteinases, peptidases, aminopeptidases, amino acid and peptide uptake systems. This whole set of enzymes constitute proteolytic system.

Pediococcus acidilactici NCDC 252 is an acidophilic facultative anaerobe possessing all in vitro probiotic attributes (Attri et al. 2015). Its genome sequence was found to be novel (Bansal et al. 2019). Several enzymes i.e. endopeptidases and exopeptidases were screened and characterized (Attri et al. 2012, 2015, 2018; Gandhi et al. 2016, 2020; Chanalia et al. 2018) while many are yet unidentified. Enzymes of proteolytic system hydrolyze milk proteins and release free amino acids required for bacterial cell growth. Exopeptidases catalyse the selective removal of amino acid residues from N-terminus of oligopeptides, polypeptides and proteins. Aminopeptidases are ubiquitously distributed in animals, plants and bacteria.

Aminopeptidases are important for specific as well as general bacterial physiological processes viz. catabolising the exogenous peptides, final stages of protein turnover and

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protein maturation. Aminopeptidases may be cytoplasmic, membrane associated or secreted in extracellular fluid. Aminopeptidases are also very important from a biotechnological point. These enzymes cleave simple amino acids from specific proteins. Arginine aminopeptidases are expected to be involved in removing bitterness causing peptides as Arg and Pro containing peptides cause bitterness. It also removes Arg/Lys from N- terminus of several peptides including Leu-enkephalin. Occurrence of aminopeptidase B is demonstrated in different bacteria (Goldstein et al. 2002; Ohishi et al. 2005) but it is yet to be characterized in detail. This enzyme is also reported in closely related genus i.e. *Streptococcus mitis* and *Lactobacilluc sakei* (Sanz and Toldra 2002).

In this paper we report the purification and biochemical characterization of the membrane bound aminopeptidase B from a probiotic *P. acidilactici* NCDC 252. Being of probiotic origin, it may have dual advantage of (i) contribution of bioactive peptides and (ii) it is expected to be safe for food and dairy industry.

Materials and Methods

Reagents

NCDC 252 was initially purchased from National Dairy Research Institute (NDRI), Karnal (India) and now being maintained in our lab. Arg-4-methoxy-β-naphthylamide (Arg-4mβNA), Sephadex G-100, Phenysepharose CL-4B, Fast Garnet GBC (o-aminoazotoluenediazonium salt) were from Sigma. Tris, TritonX-100 (TX-100), lysozyme, β-mercapatoethanol (β-ME), Man Rogosa Sharpe (MRS), ammonium sulphate, disodium hydrogen phosphate and sodium phosphate monobasic were from Himedia, India. NaCl, DMSO and HCl were obtained from Rankem, India. Protein samples were concentrated using Amicon stirred cell with YM 10 membrane. Protein markers (14.3–97.4 kDa) for were from Bangalore Genei, India.

Bacterial Growth

Autoclaved MRS medium was inoculated with NCDC 252 cells and grown at 32 °C on shaker at 250 rpm. The culture was centrifuged at 10,000 rpm for 10 min for collection of biomass. Cells were harvested in log phase after 36 h.

In Silico Characterization of Aminopeptidase

Aminopeptidase N gene was searched in NCDC 252 genome (Accession No. PTXW00000000) using DIAMOND BLASTX mode which is a BLAST-compatible local aligner for mapping translated DNA query sequences against a protein reference database (Buchfink et al. 2015). In silico analysis of gene encoding for aminopeptidase N was done by using BLAST2GO platform. The gene sequence was translated by Gene translator (http://www.scfbioiitd.res.in/chemgenome/genetranslator.jsp).

Homology Modeling

Homology modeling of translated sequence was done by MODELLER, used for comparative and homology modeling of protein's three-dimensional structure. The modeled protein was validated by Protein Structure Validation Software Suite (PSVS) (https://mybiosoftware.com/psvs-1-5-protein-structure-validation-software-suite.html).

Active Site Residues

Ligand binding site of modelled protein structure were predicted by 3D Ligand Site (http://www.sbg.bio.ic.ac.uk/3dligandsite/index.cgi). It is ligand binding site server and is based upon successful manual methods used in eighth round of Critical Assessment of techniques for protein Structure Prediction (CASP8) (Wass et al. 2010).

Docking Studies

Binding interactions were studied by Autodock vina using exhaustiveness value of 8. All other parameters of software were kept as default and all bonds contained in ligand were allowed to rotate freely, considering receptor as rigid. The final visualization of the ligated enzyme structure was performed using Pymol2.3 and Discovery Studio 4.0.

Aminopeptidase B Assay

Aminopeptidase B was assayed spectrophotometrically (Bogra et al. 2009) with slight modifications. To 0.880 ml of assay buffer (50 mM Tris–HCl, pH 7.5 having 0.1% Triton X-100 and 50 mM NaCl), 0.1 ml enzyme was added and pre-incubated for 10 min at 37 °C. The reaction was started by adding 20 µl of the substrate (Arg-4mβNA, 4 mg/ml DMSO) and incubated at 37 °C for 20 min. The reaction was stopped by adding 1 ml of stopping reagent (1.0 M sodium acetate buffer, pH 4.2). The 4mβNA liberated was coupled using 0.5 ml of Fast Garnet GBC (1 mg/ml in water). The colour was extracted with 2 ml of n-butanol and estimated at 520 nm. The A_{520} was converted into activity units. One unit of enzyme activity was expressed as amount of enzyme

which released one nanomole of 4-methoxy-βNA per minute from substrate under assay conditions.

Protein Quantification

Protein content at each step of purification was quantified by Lowry's method using bovine serum albumin (BSA) as standard.

Concentration of Protein Sample

The fractions positive for aminopeptidase B from different chromatographic columns were concentrated using ultrafiltration cell using YM10 membrane.

Enzyme Purification

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

1. Crude enzyme preparation: Cells were harvested by centrifugation at 10,000 rpm for 10 min and washed thrice with distilled water. The enzyme was extracted from the membrane as described by Attri et al. (2012) using Sodium phosphate buffer (50 mM pH 8.0 with 300 mM NaCl and 1.0% TritonX-100) as extraction buffer. This resulted in 90% extraction of aminopeptidase B from bacterial membranes.
2. Gel filtration chromatography: Sephadex G-100 column (30 × 1.0 cm) was pre equilibrated with 50 mMTris-HCl buffer having 0.1% TritonX-100, pH 7.4. Crude enzyme extract (of above srep) was loaded and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 2 ml each were collected. All fractions were screened for protein(s) at 280 nm and aminopeptidase B (using Arg-4mβNA) and also for DPP-III using Arg-Arg- 4mβNA as described by Dhanda et al. (2007). The active fractions of aminopeptidase B from gel filtration were pooled, concentrated and subjected to heat inactivation of DPP-III at 40 °C for 1 h. It was kept overnight at 4 °C. The sample was centrifuged at 10,000 rpm for 30 min and supernatant was dialyzed against 50 mM sodium phosphate buffer, pH 6.8 and concentrated.
3. Hydrophobic interaction chromatography on phenyl sepharose CL-4B: One molar $(NH_4)_2SO_4$ was added to above enzyme preparation and loaded on phenyl sepharose column (15 × 1.0 cm) pre-equilibrated with 50 mM sodium phosphate buffer [containing 1.0 M $(NH_4)_2SO_4$, pH 6.8]. Fractions of 1.0 ml each were collected. Unbound proteins were eluted with same buffer and bound proteins were eluted with decreasing $(NH_4)_2SO_4$ gradient. For eluting aminopeptidase B, two gradients were run. First gradient with 40 ml of 50 mM sodium-phosphate buffer pH 6.8 containing 0.2 M $(NH_4)_2SO_4$ and 1% TritonX-100, in non-stirred column and 40 ml of 50 mM sodium phosphate buffer pH 6.8, containing 1.0 M $(NH_4)_2SO_4$ and 1% TritonX-100, pH 6.8 buffer in stirred column. Second gradient [after 0.2 M $(NH_4)_2SO_4$] was run with NaCl and slight rise in pH by taking 40 ml of 50 mM sodium phosphate buffer, pH 8.0 containing 1.0 M NaCl and 1% TX-100 in non-stirred vessel and equal volume of 50 mM sodium phosphate buffer containing 0.2 M $(NH_4)_2SO_4$, 1.0 M NaCl and 1% TritonX-100, pH 8.0 in stirred vessel. The fractions were analyzed for protein at A_{280} and aminopeptidase B activity. Fractions positive for aminopeptidase B were pooled, concentrated and dialyzed against 50 mM Tris–HCl buffer pH 7.4. This dialyzed protein was stored at 4 °C for further studies.

Polyacrylamide Gel Electrophoresis (PAGE) and In-Situ Gel Assays

Apparent homogeneity, purity and in-situ gel assay of aminopeptidase B was assessed by 10% Davis gel electrophoresis. 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 completion of gel run, it was cut into two equal halves. One half was subjected to Coomassie Brilliant Blue staining and another half was assyed for aminopeptidase B with assay buffer and Arg-4mβNA at 37 °C. Colour was developed using Fast garnet GBC as described by Attri et al. (2011).

Biochemical Characterization of Purified Aminopeptidase B

Determination of Molecular Weight by SDS-PAGE

Molecular weight and subunit composition of purified enzyme was determined by SDS-PAGE (10%) using medium range molecular weight markers (phosphorylase B (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (44.287 kDa), and carbonic anhydrase (29 kDa), β-lactoglobulin (20 kDa), lysozyme (14 kDa) as standard.

pH Optima and Stability

pH optima of purified aminopeptidase B was determined by assaying it 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 assayed at optimum pH (7.5). Enzyme activity was expressed as percent of maximum activity.

Temperature Optima and Stability

Temperature optima was determined by assaying aminopeptidase B at different temperatures (0–70 °C). 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 40 °C using the standard assay. Enzyme activity was expressed as percent of maximum activity.

Kinetic Characterization

Kinetic parameters viz K_m and V_{max} of the purified enzyme were determined from Michaelis– Menton, Lineweaver–Burk plot and Hanes plot using Arg-4mβNA as substrate in the concentration range of 0 to 150 µM. The assay was carried out at pH 7.5 and 40 °C.

Substrate Specificity

The enzyme was incubated at 40 °C in the standard reaction mixture with different mono, di and tripeptide β-naphthylamide as well as some endopeptidase substrates. Relative enzyme activity was calculated with respect to Arg-4mβNA (control).

Effect of Different Inhibitors

Purified enzyme was pre-incubated for 10 min at 40 °C with effective concentration of different inhibitors. Residual enzyme activity was calculated by standard assay.

Investigation of Active Site by Varying pH

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

Effect of DMSO and Ethanol

Effect of DMSO and ethanol on enzyme activity was studied by incubating the purified enzyme (100 µl) separately with different concentrations of ethanol and DMSO [1–15% (v/v)] at 40 °C for 10 min. Appropriate volume of assay buffer was added and enzyme was assayed. The residual aminopeptidase B activity was expressed as percentage of maximum activity.

Effect of Urea and NaCl

The enzyme was incubated with different concentration of urea (0.1–2.5 M) and NaCl (50–1000 mM) at 40 °C for 10 min and residual activity was calculated as percentage of maximum activity.

Effect of Metal Ions

The effect of different metal ions on enzyme activity was studied by adding chloride salts 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+}) to assay buffer and pre-incubating the enzyme at 40 °C for 10 min. The reaction was initiated by adding 20 µl of substrate and enzyme activity was expressed as percent activity as compared to control.

Reversal of *O*-Phenanthroline Inhibition by Metal Ions

Reversibility of purified aminopeptidase B was studied with respect to o-phenanthroline inhibition. About 5 ml enzyme was treated with 1.0 mM o-phenanthroline for 10 min. This pretreated enzyme was extensively dialyzed at 4 °C for 24 h against 50 mM Tris–HCl buffer, pH 7.0. The dialyzed enzyme (100 µl) was mixed with assay buffer and assayed using standard assay procedure. Residual enzyme activity was calculated and expressed as percent activity in comparison to control. Reversibility of inhibition was measured in the presence of different metal ions at different concentration using standard enzyme assay.

Effect of Thiol Compounds

Effect of thiol compounds (DTE, DTT, cysteine, reduced glutathione, thioglycolic acid and β- ME) was studied on aminopeptidase B by preincubating with each thiol compound in assay buffer at 40 °C for 10 min. The reaction was initiated by adding substrate and enzyme activity was expressed as percent activity in comparison to control.

Reversal of PCMB Inhibition by Thiol Compounds

Reversibility of purified aminopeptidase B was studied with respect to PCMB inhibition. The enzyme was treated with 0.5 mM PCMB for 10 min. PCMB pretreated enzyme was dialyzed at 4 °C for 24 h against 50 mM Tris–HCl buffer, pH 7.0. The dialyzed aminopeptidase B was assayed as per standard assay. Residual enzyme activity was calculated and expressed as percent activity in comparison to control. The reversibility of inhibition was

measured in the presence of different thiol compounds at different concentration.

Results and Discussion

PepN is a broad-specificity metallo-exopeptidase capable of hydrolysing a broad range of peptides containing Lys, Arg and Leu at N-terminus. PepN from *Streptococcus lividans* hydrolysed Arg-ρNA (Butler et al. 1994) and PepN from *Lactococcus lactis* subsp. cremoris Wg2 hydrolysed susbstrates containing Arg as N terminus residue (Niven et al. 1995). Therefore NCDC 252 genome (Bansal et al. 2019) was searched for aminopeptidase B/pepN and translated into its protein sequence. Homology modeling of aminopeptidase B was done by comparing the query sequence with available protein structures and template sequence was selected. Crystal structure of porcine aminopeptidase N ectodomain in functional form (PDB ID: 5Z65) was used as template for homology modeling. The 3-D structure of aminopeptidase B was depicted graphically by the PyMOL visualization tool (Fig. 1). The modeled structure was further validated. Ramachandran plot drawn using PROCHECK revealed 92.9% residues to be present in most favoured regions, 6.5% in additionally allowed regions, 0.2% residues in generally allowed and 0.4% disallowed regions. The overall G-factor score of − 0.10 suggested that the model was accepted as it was equal to recommended value (− 0.5). Verification of 3D structure showed overall model average positive scores (cut-off score > 0.2), indicating the reliability of the proposed model. The 3-D model was also verified by PROVE to measure the average magnitude of volume irregularities in terms of Z-score root mean square deviation (Z-score RMS). All these studies indicated 3-D structure of aminopeptidase B to be valid and comprised of three different polypeptide chains (Fig. 1).

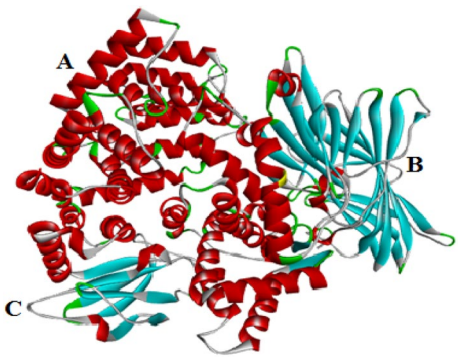

Fig. 1 Modeled 3-D structure of aminopeptidase

Purification of Enzyme

Aminopeptidase B was extracted as explained by Attri et al. (2012) that resulted in more than 90% extraction. Addition of 1% Triton X-100 was essential to maintain the enzyme in soluble form. The enzyme was purified to apparent homogeneity by successive chromatographies on gel filtration and hydrophobic chromatographic column. DPP-III was the major contaminant of aminopeptidase B therefore it was also assayed on gel filtration column fractions (Fig. 2a).

Both enzymes were eluted in overlapping fractions. But DPP-III is thermolabile (Bogra et al. 2009) and it was separated from aminopeptidase B by subjecting concentrated active fractions of aminopeptidase B to 40 °C for 1 h. The co-eluted DPP-III was heat inactivated and supernatant was chromatographed further on phenyl sepharose chromatographic column. Aminopeptidase B was eluted using two gradients. In first gradient, concentration of ammonium sulphate was gradually lowered from 1.0 to 0.2 M. Lowering upto 0 M of ammonium sulphate did not elute aminopeptidase B. As NaCl and raised pH are known to disturb hydrophobic interaction, therefore second gradient was run with increase in NaCl (upto 1 M) and increase in pH (upto 8) (Fig. 2b).

The results of purification are summarized in Table 1.

The enzyme was purified 63.98 fold with a yield of 33.26%. Arginyl aminopeptidases have also been previously purified from bacteria. Fold purification of 63.98 is comparable to that of 80.9 from *Toxoplasma gondii* (Berthonneau et al. 2000) though it is much less than of goat brain i.e. 280 (Bogra et al. 2009), 158,433 fold and 12.0% yield in *Capnocytophaga granulosa* ATCC 51,502 (Ohishi et al. 2005), 3506 fold with a 2.8% yield in *Streptococcus gordonii* FSS2 (Goldstein et al. 2002) and 500-fold with yield of 4.2% from *Lactobacillus sakei* (Sanz and Toldra 2002). The difference in yield and purification fold of aminopeptidase B from different sources may be because of differential expression of enzyme and different methods employed for purification.

Native PAGE and In- Situ Gel Assay

The apparent homogeneity and purity of enzyme was confirmed by single band on native PAGE (Fig. 3a) that corresponded to the activity band of *in- situ* gel assay (Fig. 3b).

Molecular Weight

Three bands of ~ 14.79 kDa, 31.62 kDa and 54.95 kDa (total mol. wt. ~ 101.36 kDa) were obtained on SDS-PAGE (under denaturing and reducing conditions) confirming the heterotrimeric nature of aminopeptidase B (Fig. 4a and b). Molecular weight of aminopeptidase B predicted from pepN gene identified in NCDC 252 genome was found to be 93.9 kDa.

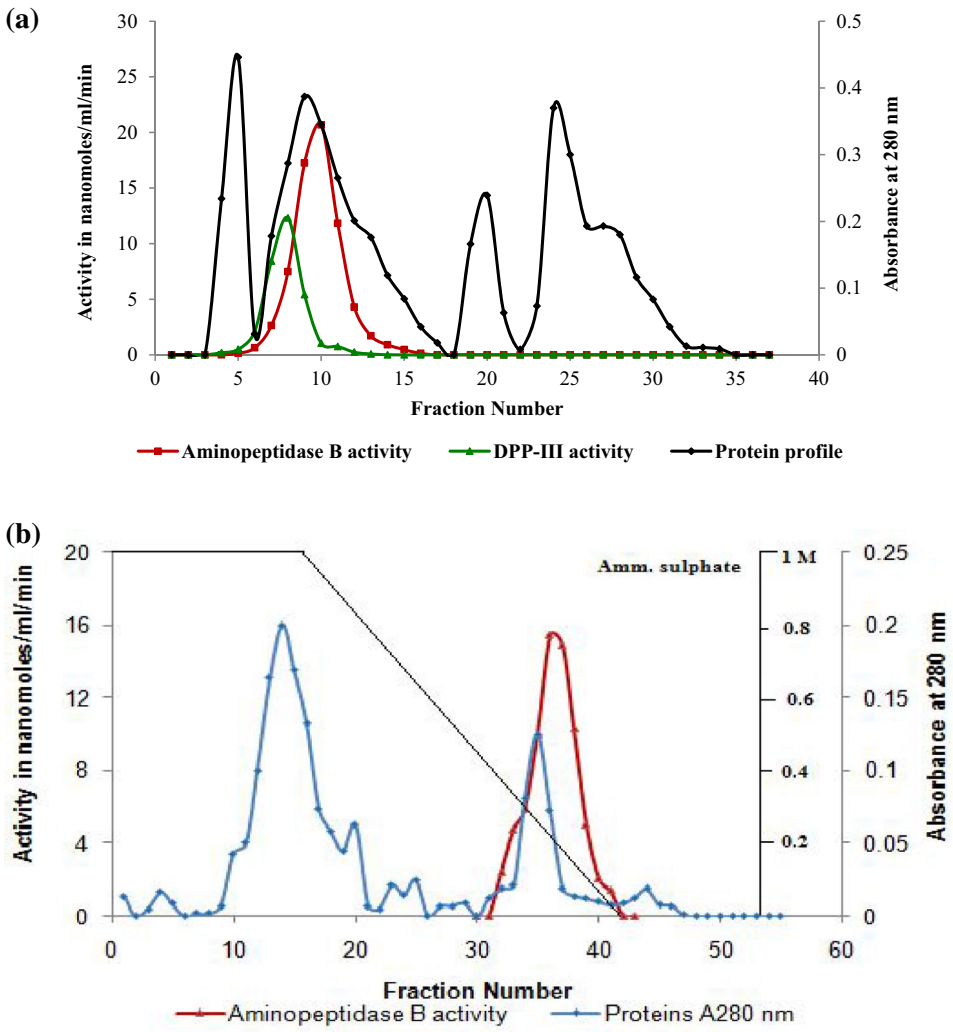

Fig. 2 **a** Protein and activity profile of DPP-III and aminopeptidase B on gel filtration chromatographic column. **b** Protein and activity profile of aminopeptidase B on Phenyl-Sepharose chromatography

Table 1 Purification table of aminopeptidase B from *P. acidilactici*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Membranes	5522 ± 0.28	11,500 ± 0.19	2.083 ± 0.06	1 ± 0.08	100 ± 0.12
Crude extract	5308.35 ± 0.34	10,446.83 ± 0.21	1.968 ± 0.3	1.968 ± 0.10	90.84 ± 0.39
Sephadex G-100	1258 ± 0.05	8763.12 ± 0.11	6.966 ± 0.17	3.344 ± 0.09	76.2 ± 0.37
Heat inactivation	168 ± 0.22	6593.07 ± 0.08	39.244 ± 0.24	18.84 ± 0.3	57.33 ± 0.06
Phenyl sepharose CL-4B	28.7 ± 0.17	3825.56 ± 0.04	**133.27 ± 0.14**	**63.98 ± 0.21**	**33.26 ± 0.02**

Values are mean ± SD of three different experiments

Bold values indicate purification of enzyme

Predicted (93.9 kDa) and calculated (101.3 kDa) molecular weight are close to each other, the difference might be because of amino acid composition of aminopeptidase B and post translational modifications.

Moreover, SDS-PAGE gives approximate molecular weight of protein (Windhorst et al. 2002). Modeled structure of this protein was also formed of three different chains (Fig. 1). Aminopeptidase B reportedly had different molecular weight in the range of 52–360 kDa with different subunit composition (Goldstein et al. 2002). Trimeric aminopeptidase B was also reported in *L. sakei* (Sanz and Toldra 2002). However it was reported to be monomeric in *Streptococcus* sp. (Goldstein et al. 2002) and cloned arginyl aminopeptidase from *Bos taurus*, *Mus musculus*

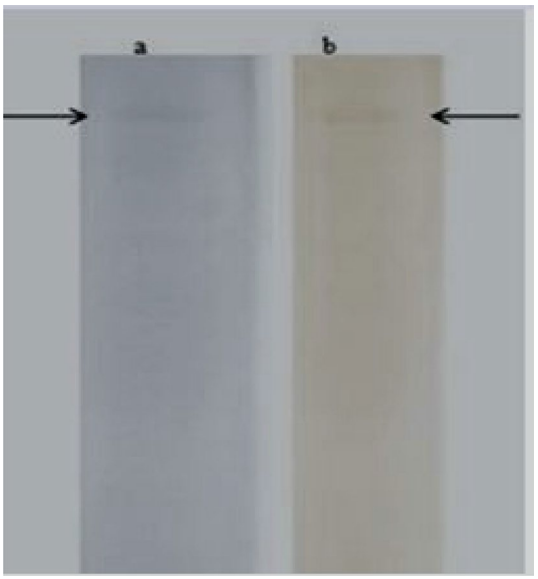

Fig. 3 Analysis of purified aminopeptidase B on 10% PAGE stained with Coomassie Brilliant Blue (**a**) and in-situ gel assay (**b**)

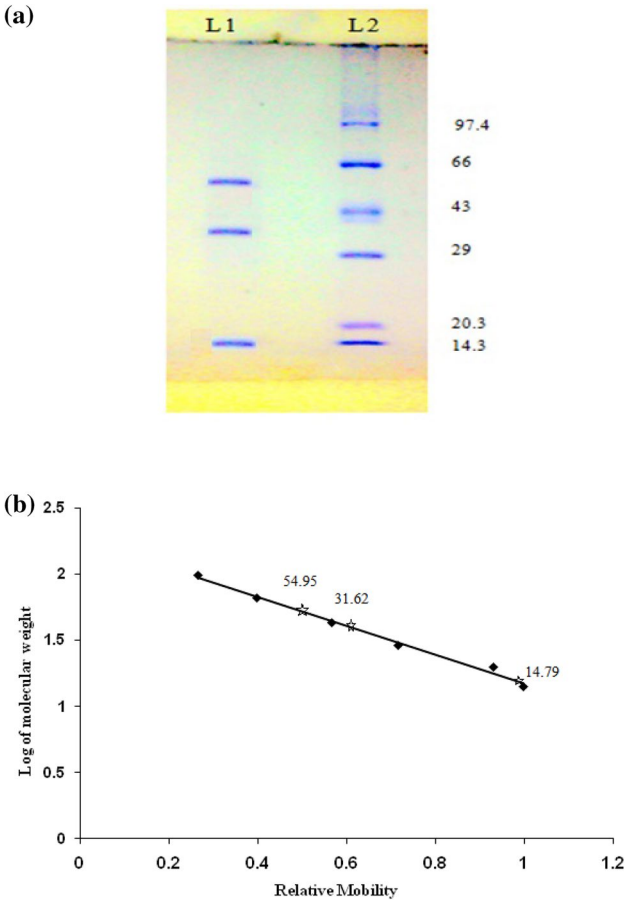

Fig. 4 **a** SDS-PAGE (10%) of purified aminopeptidase B with β-ME (Lane 1) and Lane 2 shows molecular weight markers in the range of 14.3–97.4 KDa. **b** Relative mobility Vs. Molecular weight graph

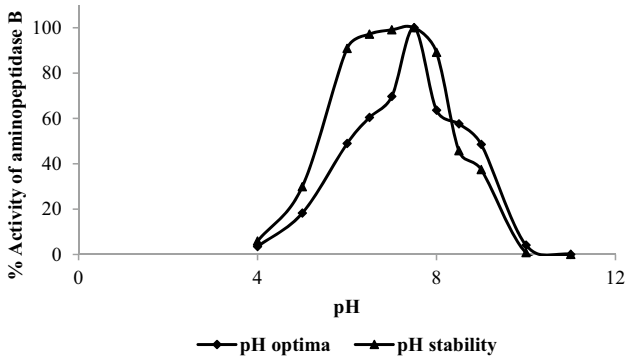

Fig. 5 pH optima and stability of aminopeptidase B. The enzyme was optimally active at 7.5 with stability in narrow pH range of 5.8–7.8

and *Rattus norvegicus* were also monomeric (Cadel et al. 1995).

pH Optima and Stability

Aminopeptidase B worked optimally at pH 7.5 with only 50% activity at pH 6.0 and pH 9.0 (Fig. 5).

pH optima of 7.5 was also reported for aminopeptidase B of porcine liver, human and rat erythrocytes (Hirose et al. 2006). pH optima of 7.4 was observed for goat brain (Bogra et al. 2009) and rat liver recombinant enzyme (Fukasawa et al. 1996). The general aminopeptidases (PepN and PepC) from dairy lactic acid bacteria, which removes Arg from the N-termini of oligopeptides exhibited optimal activity around pH 7.0 (Kunji et al. 1996).

Aminopeptidase B was most stable in pH range 5.8–7.8 and retained 50% activity at pH 5.0 and 8.0. Similar observations were made for enzyme of rat and *Bos Taurus* (Hwang et al. 2007) and by other workers (Berthonneau et al. 2000).

Temperature Optima and Stability

Temperature optima of aminopeptidase B was 40 °C and it was stable up to 40 °C, then activity declined abruptly (Fig. 6a).

Purified enzyme retained ~ 80% activity at 45 °C and ~ 40% at 50 °C. It was completely inactivated at 70 °C. The temperature optima of aminopeptidase B from NCDC 252 is close to 37 °C as reported in *Microsporidia* (Millership et al. 2002), pea seedlings (Cook and Adam 1997), endothelial cells (Fukasawa et al. 2006), rat retina (Piesse et al. 2004) *L. sakei* (Sanz and Toldra 2002), *C. granulosa* ATCC 51,502 (Ohishi et al. 2005). The energy of activation for aminopeptidase B was calculated to be 13.51 Kcal/mol from Arrhenius plot (Fig. 6b) for the first time and thus cannot be compared.

Aminopeptidase B was stable up to 40 °C. With further increase in temperature, activity declined. About 80%

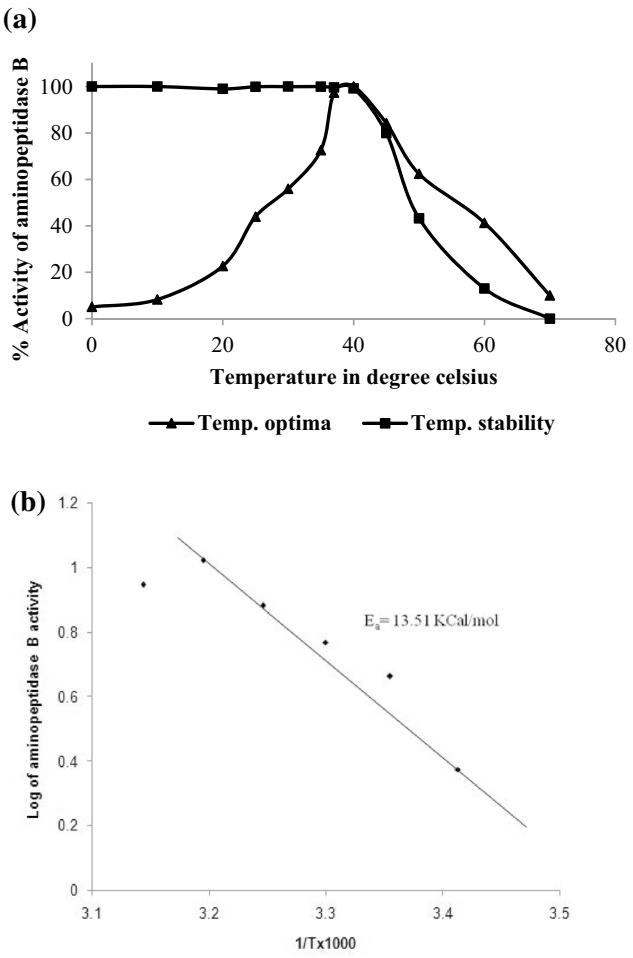

Fig. 6 **a** Temperature optima and stability of aminopeptidase B. The enzyme exhibited temp optima of 40 °C. **b** Arrhenius plot for aminopeptidase B to calculate Energy of activation (E_a). E_a for aminopeptidase B was 13.51 kcal/mol

activity was retained at 45 °C, 50% at 50 °C but only 10% was left at 60 °C (Fig. 6a). Similar temperature limit was also observed for *L. sakei* (Sanz and Toldra 2002). The enzyme loses activity at high temperature due to denaturation and loss of tertiary/quaternary structure.

Kinetic Characterization

K_m and V_{max} for aminopeptidase B were 26 µM and 19.9 nmol/ml/min respectively as reported by Michaelis–Menten, Lineweaver Burk and Hanes plot (Fig. 7a–c).

Though the enzyme exhibited micromolar affinity for its substrate but K_m for purified aminopeptidase B from NCDC 252 is higher than the K_m of 15.9 µM from *L. sakei* (Sanz and Toldra 2002). K_m of 26 µM in this study is much lower than K_m of 51 µM for extracellular arginine aminopeptidase from *Streptococcus* sp. (Goldstein et al. 2002). The catalytic constant K_{cat} and catalytic efficiency, K_{cat}/K_m of aminopeptidase B for Arg-4mβNA were found to be 0.0298 s^{-1} and 1.2 × 10–3 s^{-1} μM^{-1} respectively. The values are very low as compared to that of 2.3 s^{-1}, 34 s^{-1} and 20 s^{-1} in human leukocyte, *Bacillus subtilis* and rat testes respectively (Tanioka et al. 2003; Fundoiano-Hershcovitz et al. 2005; Pham et al. 2007). Mostly studied aminopeptidases reportedly follow Michaelis–Menten kinetics and so is aminopeptidase B of NCDC 252.

Substrate Specificity

Aminopeptidases either exhibit broad or narrow substrate specificity. The preference of bacterial enzymes for Arg over other amino acids at N-terminus of different substrates was remarkably higher than mammalian enzymes. Aminopeptidase B of NCDC 252 exhibited narrow substrate specificity and Arg-4mβNA was most preferred substrate. Similar results were reported in previous studies (Sanz and Toldra 2002; Bogra et al. 2009). So enzyme activity with Arg-4mβNA was considered as 100%.

Aminopeptidase B of NCDC 252 preferred Arg at N-terminus of different substrates (Table 2). It also hydrolyzed dipeptide-4mβNA derivatives containing basic and acidic residues at N-terminus (Arg-Arg-4βmNA 11%, Lys-Ala-4βmNA 4%, Asp-Ala-βNA 6%) but not -βNA derivatives or peptides with Pro and Phe at their N-termini or C-termini, such as Pro-βNA, Pro–Leu-βNA, Phe-Arg-βNA, Phe-βNA, Gly-Phe-βNA. Among tested substrates, Leu- βNA and Ile-βNA were hydrolyzed up to 3.0% only.

Similar specificities were also reported for aminopeptidases from *L. helveticus* sp. (Degraeve and Martial-Gros 2003). In *Lactobacillus plantarum* CRL 775 and *Pediococcus pentosaceus* CR2142L 792, highest activity of tri- and dipeptidases were reported (Gerez et al. 2008). Trp-Met-4mβNA, His-Ser- βNA, Ala-Ala- βNA, Ser-Tyr- βNA, Leu-Ala-βNA, Ser-Met-βNA, Ser-βNA, Asp-Arg-NA, Val-βNA, Asp-βNA, Leu-Trp-βNA, Tyr-βNA, Trp-βNA, Gly-βNA and Gly-Arg-βNA were also not acted upon by purified enzyme. None of the tested tripeptides and endopeptidase substrates viz Gly- Pro-Leu-βNA, N-Benzoyl DL-Arg-βNA, Z-Phe-Arg-4mbNA were hydrolyzed by the enzyme under study.

Active Site Residues and Docking Studies of Aminopeptidase B

Active site residues of aminopeptidase B in modeled structure were identified as Ser, His, Phe, Tyr and Thr. These might act as binding or catalytic residues. Docking studies indicated strong interaction of aminopeptidase B with Arg-4mβNA and Lys-4mβNA. After successful binding of these substrates with aminopeptidase B, various modes of protein–ligand interactions generated with particular binding energy were obtained. The

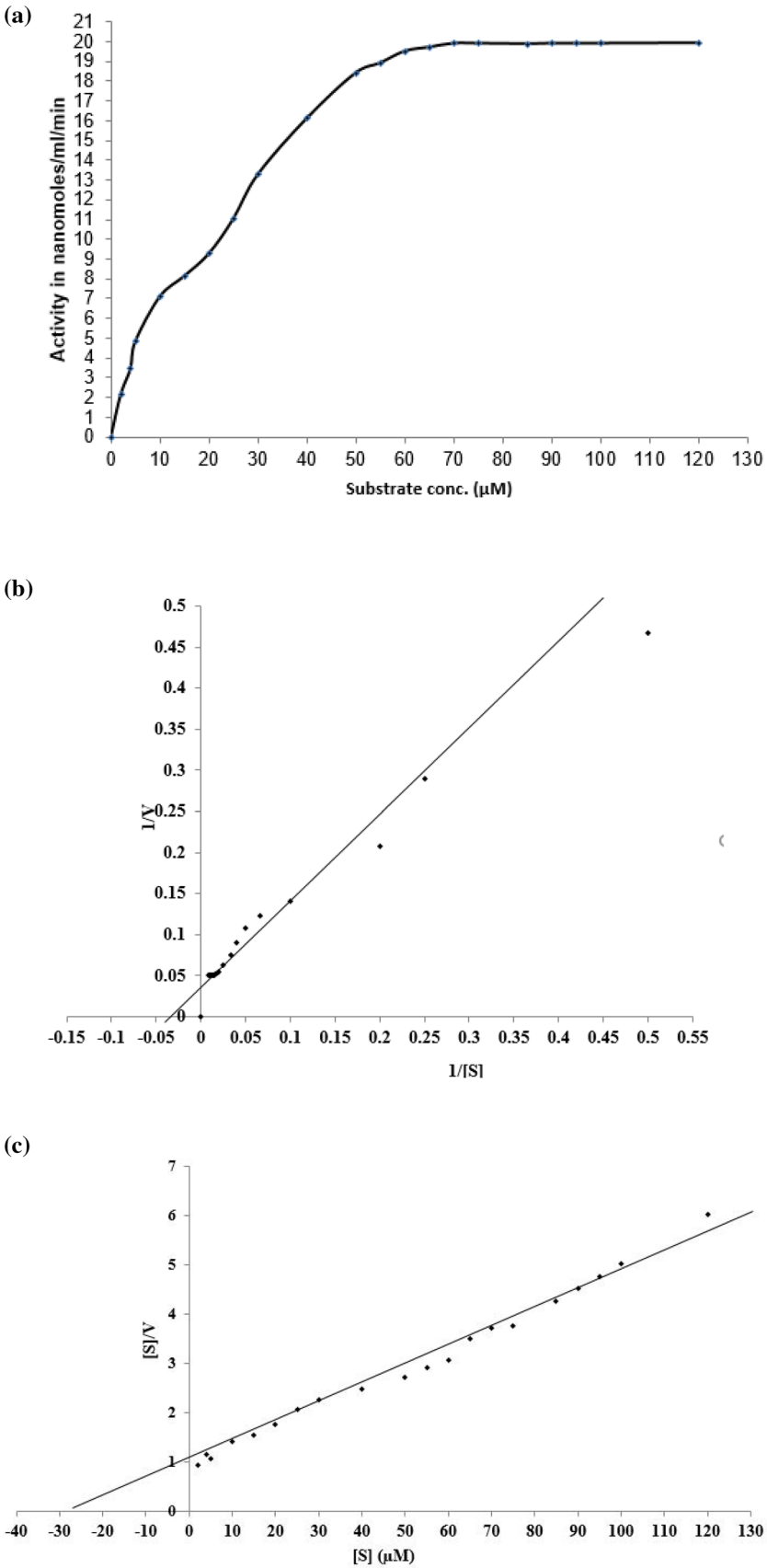

Fig. 7 Determination of kinetic parameters (K_m and V_{max}) for hydrolysis of Arg-4mβNA by aminopeptidase B. Michaelis–Menton plot (**a**), Lineweaver–Burk plot (**b**), Hanes plot (**c**)

Table 2 Substrate specificity of purified aminopeptidase B

Substrates	% Activity
Arg-4mβNA	100 ± 0.35
Arg-Arg-4βmNA	11 ± 0.23
Lys-Ala-4βmNA	4 ± 0.09
Asp-Ala-βNA	6 ± 0.39
Leu- βNA	3 ± 0.16
Ile-βNA	3 ± 0.24

Values are mean ± SD of three different experiments

binding mode with least binding energy indicated best mode of binding. Binding energy for Arg-4mβNA and Lys-4mβNA was − 8.6 kcal/mol and 7.5 kcal/mol respectively that indicated higher affinity of aminopeptidase B for Arg-4mβNA than Lys-4mβNA. Arg-4mβNA interacted through two H-bonds with Pro369, Ser635 residue and two π–π interactions through Trp884 and Tyr638 residues. It also formed π-alkyl interaction (Fig. 8a). Lys-4mβNA formed two H-bond with Thr604 and two π–π interaction through Tyr638 residue. It also formed π-alkyl interaction (Fig. 8b).

Effect of Organic Solvents

Ethanol and DMSO are used as solvent for dissolution of substrates and inhibitors therefore these were tested for their effect on enzyme's activity. Aminopeptidase B activity increased slightly at 1% (v/v) ethanol but declined with further increase in ethanol concentration and retained about 65% activity at 5% (v/v) of ethanol (Fig. 9). This might be due to altered active site structure of enzyme. The results of NCDC 252 enzyme are in agreement to earlier reports (Bogra et al. 2009). DMSO initially activated the aminopeptidase B upto 4% (v/v) (Fig. 9) and thus substrate was prepared so as that its addition resulted in 4% (v/v) DMSO in assay mixture.

Decline in enzyme activity was gradual beyond 4% (v/v) DMSO. Initial increase in enzyme activity at low DMSO concentration might be due to increased substrate solubility. Goat brain DPP-III was also activated by DMSO (Dhanda et al. 2008a). DMSO probably facilitates the movement

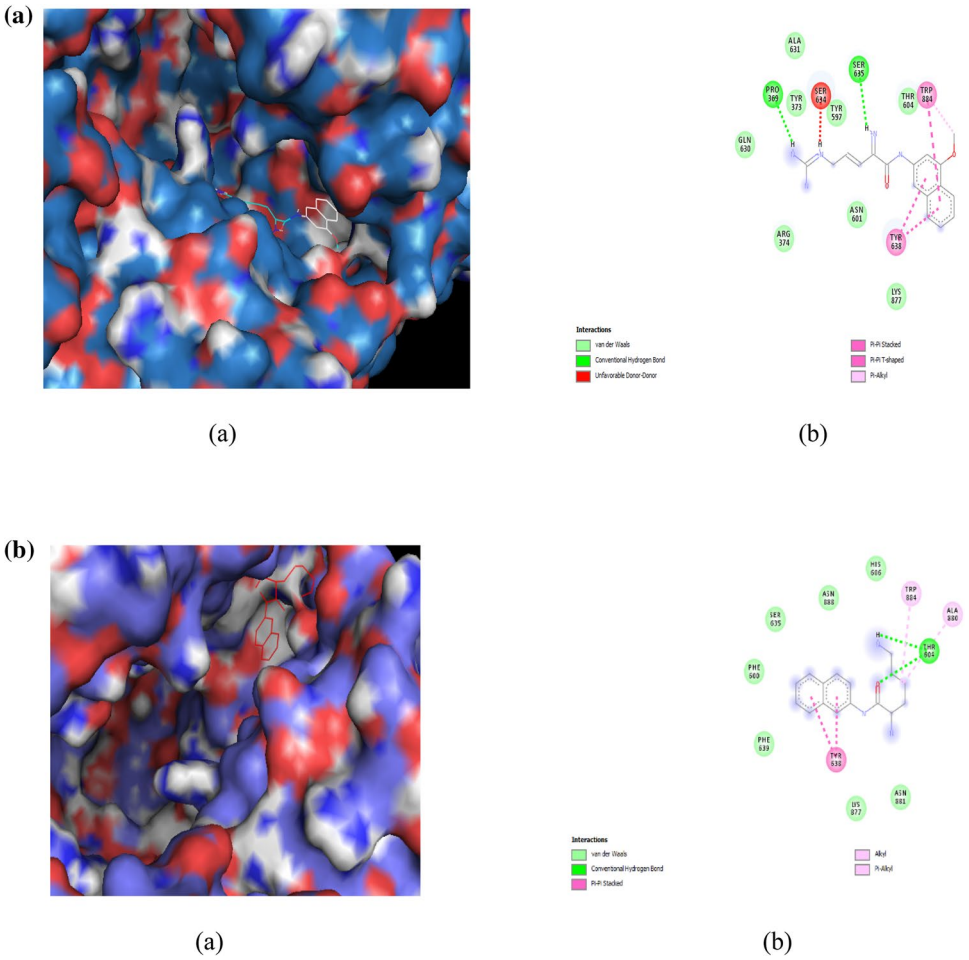

Fig. 8 **a** Arg-4mβNA bind with aminopeptidase. Best binding mode in the pocket of aminopeptidase (**a**) and Binding interactions of Arg-4mβNA with amino acid (**b**). **b** Lys-4mβNA bind with aminopeptidase. Best binding mode in the pocket of aminopeptidase (**a**) and Binding interactions of Lys-4mβNA with amino acid (**b**)

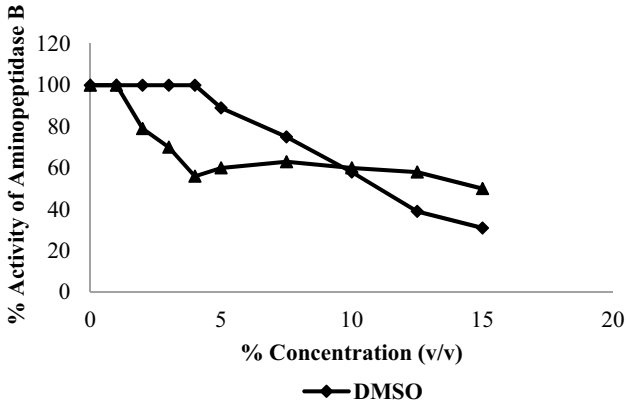

Fig. 9 Effect of organic solvents on aminopeptidase B. Aminopeptidase B retained about 65% activity at 5% (v/v) of ethanol

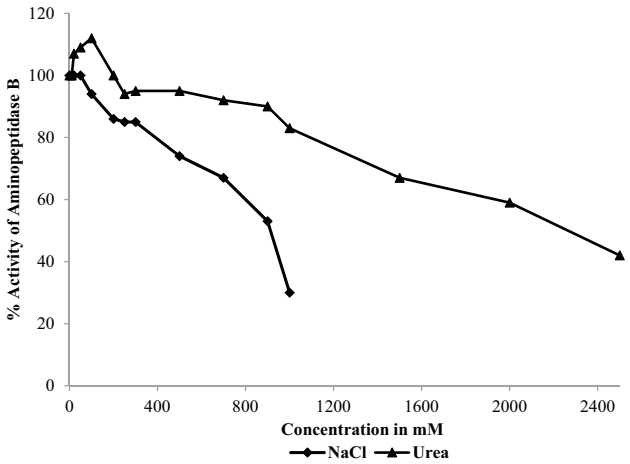

Fig. 10 Effect of urea and NaCl on aminopeptidase B

of enzyme subunits in solution. DMSO may also alter the hydrophobic sites on enzyme and thus induce more favorable conformation for its active site (Barberis et al. 2006). Decreased enzyme activity at high DMSO concentration might be due to disturbance of structure of water around hydrophobic sites of enzyme thereby resulting in conformational change and subsequent decreased enzyme activity (Dhanda et al. 2008a and Bogra et al. 2009).

Effect of Urea and NaCl

The enzyme under study was sensitive to even very low concentration of NaCl. It retained maximum activity up to 50 mM NaCl, but further increase was accompanied with decreased enzyme activity (Fig. 10). Similar results were also reported by other workers (Bogra et al. 2009).

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 (Zangi et al. 2009). Low urea concentration enhanced aminopeptidase B activity (Fig. 10).

Enzyme retained about 40% activity at 2.5 M urea. Increased activity at low urea concentration is probably because active site of the membrane enzyme (hydrophobic nature) is more easily altered by denaturants and activated enzyme appears more open and flexible at active site (Hongjie et al. 1998). Addition of urea to aqueous protein solution breaks the water structure and makes it a better solvent for hydrophobic groups (Idrissi 2005; Zangi et al. 2009). This can trigger a folded protein to unfold by exposing hydrophobic side chains to more accommodating solvent.

Effect of Different Inhibitors

Interaction of aminopeptidase B with different inhibitors revealed its strong inhibition by bestatin (65.89% inhibition at 100 µM), NEM (74.75% at 1 mM), DTNB (94% inhibition at 1 mM) and 4-nitrophenyl iodoacetate (79.76% at 0.5 mM) and p-chloro mercuricbenzoic acid (83.58% inhibition at 0.5 mM). Iodoacetamide and o-phenanthroline both showed 85% inhibition at 1.0 mM concentration (Table 3).

Inhibition with thiol protease inhibitors was also reported previously (Bogra et al. 2009). EDTA caused 60% inhibition and o-phenanthroline caused 87% inhibition at 5 mM and 1 mM respectively. These studies suggested this enzyme to be a metalloprotease with involvement of thiol in catalysis and/or regulation. Our results are in agreement to previous findings (Bogra et al. 2009). A number of peptidases have been reported which are metalloenzymes and have sulphydryl group at the active site of enzyme. Aminopeptidase B like placental, rat liver and goat brain DPP-III seem to require both metal ion and cysteine for catalytic process (Dhanda et al. 2007). Most bacterial aminopeptidases are cysteine and metalloproteases (Gonzales and Robert-Baudouy 1996). Though sulphydryl group did not directly participate in catalytic process yet it was essential for full manifestation of enzyme's activity.

Investigation of Active Site Residues

Catalytic residues at active site were also identified by plotting log V_{max} vs pH. pK_a values of amino acids involved in catalysis were found to be 6.1 and 8.3 which correspond to pK_a of His and Cys respectively (Fig. 11). His is reported to co-ordinate metal ion in aminopeptidase B. His was also identified at active site by in-silico analysis. These results are in agreement with inhibition studies

Table 3 Effect of inhibitors on purified aminopeptidase B

Inhibitor	Concentration (in mM)	%Inhibition
Control	–	Nil
4-nitrophenyl iodoacetate	0.1	51.55 ± 0.37
	0.5	79.76 ± 0.21
Leupeptin	0.1	29.69 ± 0.55
	0.5	82.68 ± 0.41
Aminoethylbenzoylsulfonyl fluoride (AEBSF)	0.5	17.4 ± 0.11
	0.1	25.99 ± 0.03
	1.0	32.3 ± 0.41
Iodoacetamide	0.1	78.76 ± 0.05
	0.5	80.62 ± 0.3
	1.0	85.56 ± 0.22
1,10-Phenanthroline	0.1	60.42 ± 0.53
	0.5	72.31 ± 0.32
	1.0	87.22 ± 0.26
EDTA	0.5	25.36 ± 0.05
	1.0	34.64 ± 0.51
	2.5	44.4 ± 0.43
	5.0	60.02 ± 0.46
Pepstatin-A	0.5	Nil
p-chloromercuric benzoic acid	0.1	10.31 ± 0.01
	0.5	83.58 ± 0.46
Bestatin	1 (μM)	16.71 ± 0.26
	5	45.77 ± 0.17
	10	54.23 ± 0.01
	100	65.89 ± 0.08
DTNB	0.1	35.67 ± 0.4
	0.5	47.91 ± 0.25
	1.0	94.23 ± 0.19
PMSF	0.5	28.25 ± 0.07
NEM	0.1	15.05 ± 0.1
	0.5	51.96 ± 0.07
	1.0	74.75 ± 0.03

Values are mean ± SD of three different experiments

but microenvironment in which catalytic residues exist in enzyme may also affect the pK_a of side chain.

Effect of Different Metal Ions

Amongst all studied metals, Cu^{2+} strongly inhibited aminopeptidase B. Enzyme retained 54% and 94% activity at 1 mM Fe^{3+} and Co^{2+}respectively. All other metals had inhibitory effect to different extent (95% inhibition by Cu^{2+}, 88% by Fe^{2+}, 75% inhibition by Zn^{2+}, Mn^{2+} and 50% inhibition was observed by Fe^{3+}). The enzyme inhibition increased with increase in metal ion concentration (Fig. 12).

Inhibition of aminopeptidase B by Cu^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+} and Zn^{2+} ions was also reported earlier (Sanz and

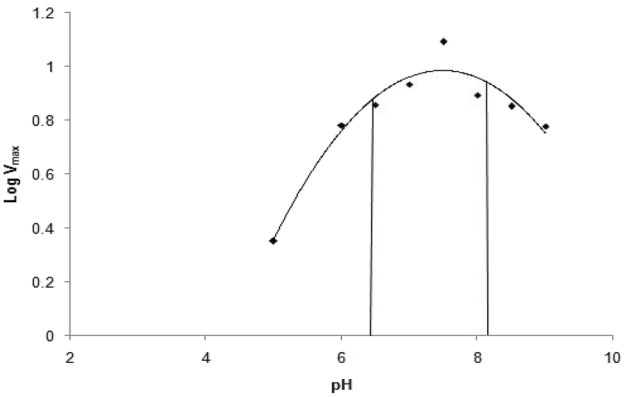

Fig. 11 log V_{max} vs. pH for aminopeptidase B. pKa of 6.1 and 8.3 suggests involvement of His and Cys respectively in enzyme catalysis

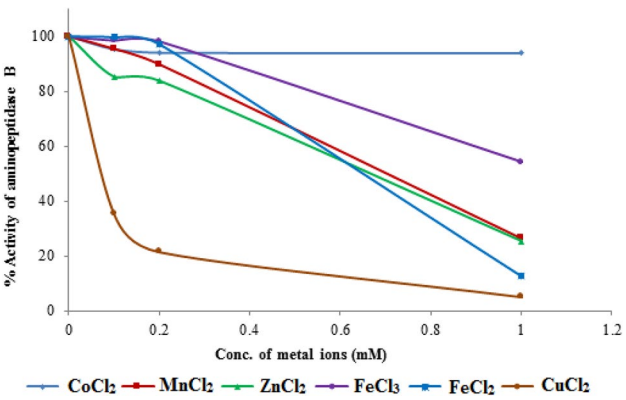

Fig. 12 Effect of different metal ions on aminopeptidase B

Toldra 2002). However Zn^{2+} was an activator of aminopeptidase B of goat brain (Bogra et al. 2009) and secretory vesicle from rat (Hwang et al. 2007). Though reason for activation of metalloenzyme by Co^{2+} is not clear but like aminopeptidase B of NCDC 252, Co^{2+} also activated goat brain DPP-III, leucine aminopeptidase of bovine lens, human and porcine liver aminopeptidase, metalloendopeptidase, enkephalinase B and some microbial peptidases and leukocytes and erytherocytes aminopeptidases (Dhanda et al. 2008a, b). Activation by metal ions is common in aminopeptidases and studies of inhibitors and metal ions suggest aminopeptidase B to be metalloenzyme. Effect of metal ions was also studied in combination with other metals and EDTA. Results are shown in Table 4. Hg^{2+} caused 96% inhibition of the enzyme activity. Hg^{2+} form mercaptide bonds with thiol at active site and cause enzyme inactivation. Other metals viz. Na^{+}, K^{+}, Ca^{2+}, NH^{4+}, Mg^{2+} and Ba^{2+} had no effect at 1 mM concentration. Modified enzyme activity (in the presence of Co^{2+}) by subsequent addition of metal ions indicate the

Table 4 Effect of metal ions in combination with other metal ions and EDTA on the activity of aminopeptidase B

Inhibitor	% Activity
Control	100 ± 0.34
NaCl 1 mM	100 ± 0.81
KCl	96.92 ± 0.46
$CaCl_2$	95.23 ± 0.28
NH_4Cl	94.54 ± 0.19
$MgCl_2$	96.25 ± 0.87
$BaCl_2$	93.86 ± 0.51
$HgCl_2$	4.09 ± 0.39
0.1 mM KCl	98.59 ± 0.75
0.01 mM $ZnCl_2$	93.31 ± 0.69
1 mM EDTA	64.3 ± 0.58
1 mM EDTA + 0.1 mM $ZnCl_2$	24.66 ± 0.27
0.1 mM $CoCl_2$ + 0.01 mM $ZnCl_2$	90.84 ± 0.48
0.1 mM $CoCl_2$ + 0.1 mM $ZnCl_2$	88.74 ± 0.53

Values are mean ± SD of three different experiments

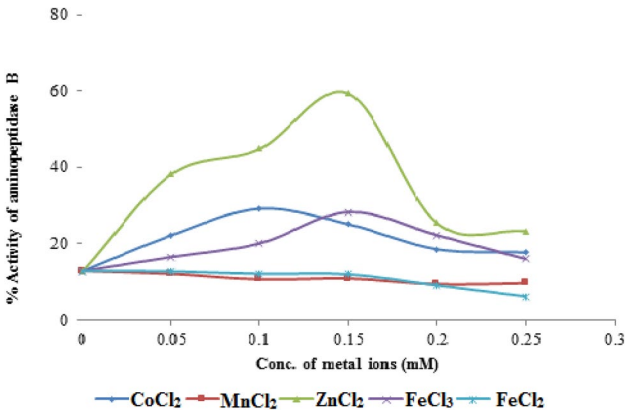

Fig. 13 Effect of different metal ions on o-phenanthroline pretreated aminopeptidase B

metal competition for same binding site i.e. catalytic and regulatory as reported for other aminopeptidases (Dhanda et al. 2008a).

Reversal of O-Phenanthroline Inhibition by Different Metal Ions

Reversal of o-phenanthroline pretreated enzyme (caused 87% inhibition at 1.0 mM concentration) was studied and results revealed that Zn^{2+}, Co^{2+} and Fe^{3+} reversed the inhibition (Fig. 13). Zn^{2+} restored the enzyme activity up to 60% at 0.15 mM, Co^{2+} and Fe^{3+} up to 30% at 0.1 and

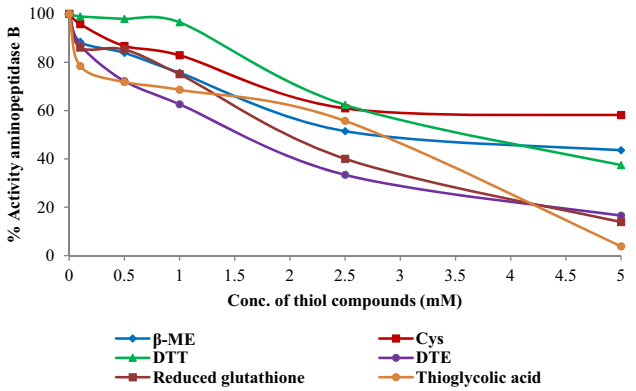

Fig. 14 Effect of thiol compounds on aminopeptidase B

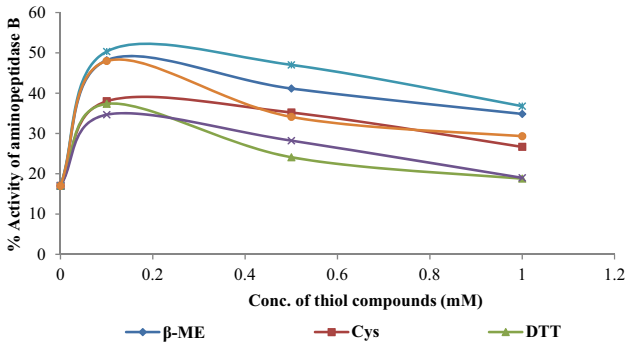

Fig. 15 Effect of thiol compounds on PCMB pretreated aminopeptidase B

0.15 mM concentrations respectively. Similar findings were also reported by other workers (Cadel et al. 1995). These studies confirmed the metalloenzyme nature of aminopeptidase B from *P. acidilactici*.

Effect of Different Thiol Compounds

In addition to inhibition of NCDC 252 aminopeptidase B by sulfhydryl reagents, further evidence of involvement of thiol group(s) in catalysis was supported by the use of thiol compounds. All tested thiol compounds were inhibitory for aminopeptidase B and degree of inhibition increased with increase in concentration of thiol compounds (Fig. 14). Inhibition by DTT was also reported by Bogra et al. (2009). Complete aminopeptidase B inhibition by 10 mM DTT was also reported by Huston et al. (2004). Sulfhydryl compounds partially inhibited arginine aminopeptidases from *Streptococcus* sp. (Goldstein et al. 2002). Based on these studies it can be concluded that aminopeptidase B from NCDC 252 is a metalloprotease with direct or indirect involvement of thiol group(s). Further studies are required to explain the role of cysteine residue(s) and metal ion(s) in the catalytic action of enzyme under study.

Reversal of PCMB Inhibition by Different Thiol Compounds

PCMB at 0.5 mM concentration resulted in 83% inhibition of aminopeptidase B and the ability of different thiol compounds to restore the activity of PCMB pretreated enzyme was investigated and results are shown in Fig. 15. Studies revealed that PCMB inhibition was partially reversed by addition of thiol compounds such as β-ME, reduced glutathione and thioglycolic acid at 0.1 mM whereas reducing agents alone inhibited the enzyme. It was observed that only 50% enzyme activity could be restored and none of them could restore full activity. The results confirmed the involvement of –SH group(s) in the enzyme catalysis. Such studies were not conducted specifically for aminopeptiadse B so results cannot be compared.

Conclusion

Aminopeptidase B was purified to apparent homogeneity from *P. acidilactici*. It is a membrane bound, high molecular weight heterotrimeric enzyme. The enzyme works optimally at physiological pH and 40 °C with Arg-4mβNA substrate. Substrate specificity suggests its capability to hydrolyze bioactive peptides. Docking of modeled protein also suggested high binding affinity for Arg-4mβNA. It is a metalloprotease having thiol residues at active site. Its membrane location suggests its role in catabolism of exogenous peptides and peptides generated from endogenous proteins. But exact role needs to be determined. Supply of enzymes is one of the mechanism of probiotic action because enzymes alter metabolic activities of intestinal microflora, physicochemical conditions in the colon and production of different bioactive compounds etc.

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Declarations

Conflict of interest Authors declare no conflict of interest among themselves.

Informed Consent All the data are available in the manuscript and all the authors agree to publish it.

Ethical Approval The present study does not involve human and animal samples.

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