Purification and Characterization of an Extracellular Low Temperature-Active and Alkaline Stable Peptidase from Psychrotrophic *Acinetobacter* sp. MN 12 MTCC (10786)

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Abstract An extracellular low temperature-active alkaline stable peptidase from *Acinetobacter* sp. MN 12 was purified to homogeneity with a purification fold of 9.8. The enzyme exhibited specific activity of 6,540 U/mg protein, with an apparent molecular weight of 35 kDa. The purified enzyme was active over broad range of temperature from 4 to 60 °C with optimum activity at 40 °C. The enzyme retained more than 75 % of activity over a broad range of pH (7.0–11.0) with optimum activity at pH 9.0. The purified peptidase was strongly inhibited by phenylmethylsulfonyl fluoride, giving an indication of serine type. The K_m and V_{max} for casein and gelatin were 0.3529, 2.03 mg/ml and 294.11, 384.61 µg/ml/min respectively. The peptidase was compatible with surfactants, oxidizing agents and commercial detergents, and effectively removed dried blood stains on cotton fabrics at low temperature ranging from 15 to 35 °C.

Keywords *Acinetobacter* · Low temperature-active · Detergent · Extracellular peptidase · Protease

Introduction

Microbial peptidases also known as proteases constitute one of the most important groups of commercially and industrially relevant enzymes, accounting for approximately 60 % of total global enzyme sales [15]. Though peptidases can be produced by plants and animals, microorganisms represent an excellent source of enzymes due to their broad

biochemical diversity, easy and fast cultivation, ease in isolation of enzyme, and genetic manipulation for strain improvement [12, 15]. Peptidases find vast applications in the detergents, dairy, food, leather, and pharmaceutical industries, peptide synthesis and so forth [11, 14]. Majority of enzymes produced by microorganisms do not suit well under the harsh reaction conditions required in industrial/environmental processes. Keeping in view these limitations and the industrial requirement for highly active preparations of peptidases with appropriate activity, specificity and stability at different pH, temperatures, and in presence of metal ions, surfactants, and organic solvents, in last couple of decades research focus have diverted towards extremophiles in search for new enzymes functioning under extreme conditions [11]. Among extreme environments, low temperature is very common and more than 80 % of earth's biosphere has temperature below 5 °C. These low temperature environments are inhabited by cold-adapted microorganisms: psychrotrophs and psychrophiles, but comparatively less attention has been paid to them.

As revealed from the literature, the peptidases are wide spread among the psychrotrophs but less explored. High catalytic activity at low temperature and low thermostability at elevated temperature are the two most important properties of low temperature active proteases making them suitable candidate for biotechnological applications. These low temperature active enzymes provide financial benefits through energy savings by eliminating costly heating step, function in cold environments and during the winter season provide increased yields, thermal lability for easily inactivating the enzyme when required [5]. Extracellular peptidases have been purified and characterized from various psychrotrophs like *Alteromonas*, *Azospirillum*, *Bacillus*, *Colwellia*, *Curtobacterium*, *Exiguobacterium*, *Flavobacterium*, *Pseudomonas*, *Pseudoalteromonas*, *Serratia*, *Sphingomonas*, and *Stenotrophomonas*

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[11]. But there is no report on extracellular peptidase production from the genus *Acinetobacter*. On the basis of ecologic and industrial interest in peptidases from cold adapted microbes, the screening of microorganisms from cold regions of the western Himalaya was performed and peptidase producing psychrotrophs were isolated [17]. The novel psychrotrophic *Acinetobacter* sp. MN 12 showing growth and enzyme production at low temperature and alkaline conditions was used for purification and characterization of the peptidase.

Materials and Methods

Culture Conditions and Peptidase Production

The peptidase producing psychrotrophic *Acinetobacter* sp. MN 12 (GenBank, accession number FM213383) was isolated from the cold environment alkaline soil sample from Mane latitude 32°1'42'', longitude 78°14'22'' [17]. The peptidase production was checked on skim milk agar plates of different pH at 5 °C. The strain was conserved and stored at –80 °C and submitted to the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh (accession number MTCC 10786). For peptidase production *Acinetobacter* sp. MN 12 was grown in peptone dextrose broth (PDB) medium containing peptone (2 %), yeast extract (1 %) and dextrose (2 %). Culture was incubated on a rotary shaker (200 rpm) at 28 °C for 36 h. The peptidase was extracted from the culture broth by centrifugation for 10 min at 16,000×g and 4 °C. The supernatant was used as a crude enzyme for the estimation of proteolytic activity.

Peptidase Assay

Peptidase activity was determined by a modified method of Folin & Ciocalteu. The reaction was carried out by using 25 µl of the cell free filtrate/purified enzyme mixed with 0.6 % casein in 130 µl of distilled water. The mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 130 µl of 10 % trichloroacetic acid (TCA) and centrifuged at 16,000×g for 5 min. The amino acids present in the supernatant were determined by the Folin and Ciocalteu's phenol reagent. One unit of enzyme activity was defined as the enzyme that liberated 1 µg of tyrosine per min under assay conditions.

Purification of Peptidase

For the purification of peptidase, 20 ml of overnight grown culture was inoculated into 2 l of PDB medium (in 250 ml flasks containing 100 ml medium at 2 % concentration). After 36 h of incubation in incubator shaker, cells were harvested by centrifugation and the supernatant was used

for enzyme purification. The supernatant was precipitated by slowly adding 70 % solid ammonium sulfate. After 6 h, the solution was centrifuged (16,000×g for 30 min), and the pellet was resuspended in 20 mM Tris–HCl buffer (pH 7.0). The resuspended sample was dialyzed against the same buffer and tested for peptidase activity. For ion-exchange chromatography, the dialysed proteins were loaded onto an anion-exchange DEAE-Cellulose column (1.6 × 24 cm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.0). The bound proteins were eluted with same buffer containing a linear gradient of 0–0.5 M NaCl at a flow rate of 60 ml/h by using AKTApurifier plus. Fractions showing peptidase activity were pooled and lyophilized. For size exclusion chromatography the lyophilized (fractions showing peptidase activity) sample was loaded onto a gel column Sephacryl S-200 pre-equilibrated with 20 mM Tris–HCl buffered saline pH-7.0. The column was washed and the bound protein was eluted with the same buffer at a flow rate of 30 ml/h. Fractions of 1 ml were collected and positive fractions were pooled together and concentrated by lyophilization and used for further characterization. Protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as standard.

SDS-PAGE and Zymography

The homogeneity of the purified protein was determined by using 12 % SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue R250. The molecular weight of the peptidase was estimated using standard protein molecular weight marker. The zymography was performed using 10 % polyacrylamide gel copolymerized with 0.1 % casein at 4 °C. Following electrophoresis, gel was washed for 30 min in 50 mM Tris–HCl buffer (pH 7.6) containing 2.5 % Triton X-100. The gel was incubated in 50 mM Tris–HCl (pH 7.6) containing 0.2 M NaCl and 5 mM CaCl₂ for 4 h. The gel was Coomassie stained and activity was visualized as zones of clearance.

Effect of Temperature and pH on Activity and Stability of Purified Peptidase

The effect of temperature on peptidase activity was examined by performing the assay at different temperatures ranging from 4 to 70 °C. To determine the enzyme stability at different temperatures, enzyme was incubated at 4–70 °C for 1 h and residual peptidase activity was measured by peptidase assay. Similarly for the determination of optimum pH, substrate solutions were prepared in 0.1 M buffers of various pH: potassium phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0) and glycine NaOH (pH 10.0–12.0). The activity was measured under standard assay conditions. For determination of pH stability, the enzyme was incubated in buffers of

different pH (6–12) for 1 h. Subsequently, enzyme activity was measured and compared under standard assay conditions.

Effect of Various Metal Ions on Peptidase Activity

The effect of various metal ions like Ca^{2+} , Mn^{2+} , Na^{+} , Zn^{2+} , Hg^{2+} , Cu^{2+} and Mg^{2+} was studied at a concentration of 5 mM against control. After incubation at 25 ± 2 °C for 1 h, the remaining activity was measured by the standard assay method.

Effect of Inhibitors and Chelators on Peptidase Activity

The effects of standard peptidase inhibitors and chelators: PMSF, dithiothreitol (DTT), 2-mercaptoethanol (2-ME), iodoacetamide, pepstatin A, ethyleneglycol tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid (EDTA) at two concentrations of 5 and 10 mM on peptidase activity was studied. The activity was determined after pre-incubation of enzyme with various compounds for 1 h at room temperature using standard assay method.

Peptidase Activity on Various Protein Substrates

Peptidase activity with various protein substrates; BSA, casein, gelatin, skim milk and azocasein (2 mg/ml) was assayed following standard peptidase assay. The specific peptidase activity towards casein was taken as control.

Compatibility with Surfactants, Oxidizing Agents and Commercial Detergents

The suitability of peptidase as a detergent additive was determined by testing its stability in oxidants, surfactants and commercial detergents. The peptidase was incubated with 1 % of surfactants like SDS, Triton X-100, and oxidizing agent like H_2O_2 , and with commercial detergents: Ariel, Tide, Rin and Surf excel at 7 mg/ml for 1 h and the residual activity was measured.

Determination of K_m and V_{max}

The effect of increasing substrate concentrations on the reaction velocity of peptidase was investigated by using two substrates; casein and gelatin, and the concentrations ranged from 2 to 32 mg/ml. The K_m and V_{max} were determined by plotting Lineweaver–Burk plot.

Wash Performance Analysis of Peptidase

The application of peptidase as a detergent additive was studied on white cotton cloth pieces (4×4 cm) stained

with blood and dried overnight. The pieces were stained with 100 μl of blood and fixed properly. The stained cloth pieces were taken in flasks containing 100 ml of water under three different conditions; (i) stained cloth in water (ii) stained cloth in water supplemented with detergent (7 mg/ml) and (iii) stained cloth in water supplemented with detergent (7 mg/ml) and 2 ml of enzyme. The flasks were incubated at 15, 25 and 35 °C for 2 h. After incubation, cloth pieces were taken out and dried. Visual examination of the pieces was done by comparing against the cloth pieces kept in water alone.

Statistical Analysis

All experiments were done in triplicates and standard error is indicated by vertical bars in figures. The effects of various parameters on the enzyme activities were evaluated using ANOVA to identify means that differed significantly by STATISTICA version 7.

Results and Discussion

Purification of Peptidase from *Acinetobacter* sp. MN12

Acinetobacter sp. MN12 produced peptidase at low temperature and broad range of pH. The enzyme was purified to 9.8-fold with specific activity of 6,540 U/mg (Table 1). The SDS-PAGE revealed the presence of a single protein band with an apparent molecular weight 35 kDa (Fig. 1a). The proteolytic activity of the purified enzyme was confirmed by zymogram (Fig. 1b). The molecular weight of this peptidase was found similar to *Exiguobacterium* sp. 36 kDa [10], but was different from protease of other psychrotrophic microorganisms [11].

Effect of Temperature and pH on Enzyme Activity and Stability

The peptidase from *Acinetobacter* sp. was active over broad range of temperature from 4 to 60 °C with optimum activity at 40 °C. Peptidase showed more than 65 % of the relative activity at 4 °C (Fig. 2a). The optimum temperature for cold active enzymes from psychrotrophs generally ranges from 10 to 50 °C [7]. Protease from *Colwellia psychrerythraea* had optimum activity at 19 °C [8] and protease from *Stenotrophomonas* showed significant activity at 10–40 °C, with maximum activity at 15 °C [16]. Thermostability of protease is another important character, which is a useful criterion for application as a detergent additive due to the requirement of retention of activity during storage. The peptidase from *Acinetobacter* sp. is stable over broad range of temperature retaining more than 60 % activity at

Table 1 Purification of low temperature active alkaline stable peptidase from *Acinetobacter* sp. MN 12

Purification steps	Total protein (mg)	Total protease activity (U)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Culture supernatant	930	618,458	665	1	100
(NH ₄) SO ₄	11	21,877	1,989	3	3.5
DEAE-cellulose	1.4	8,828	6,305	9.5	1.4
Sephacryl S-200	0.15	981	6,540	9.8	0.16

Fig. 1 **a** SDS-PAGE profile of purified peptidase Lane M Unstained protein molecular weight marker, Lane 1 ammonium sulphate precipitation, Lane 2 purified peptidase. **b** Zymogram analysis of purified peptidase; Lane M prestained protein marker, Lane 1 ammonium sulphate precipitation, Lane 2 purified peptidase

50 °C (Fig. 2a). Protease from *Colwellia* [20] and *Exiguobacterium* [10] had been reported to be stable at 40 and 50 °C respectively.

The pH measurements showed optimal activity of the peptidase at pH 9.0 (Fig. 2b), similar to protease from *Bacillus cereus* [9]. The proteases from *Pseudomonas* strains [21] and *Stenotrophomonas maltophilia* had optimal activity at pH 10.0 and 11.0 respectively [18]. The purified enzyme was most stable at pH 10.0, and at pH 11.0 there was only 12–14 % reduction in the activity (Fig. 2b). These characteristics of low temperature activity, thermo and alkalistability of peptidase under investigation suggested its usefulness in detergent industry.

Fig. 2 **a** Effect of temperature on activity and stability of purified peptidase from *Acinetobacter* sp. MN 12. The temperature profile was determined by assaying peptidase activity at temperatures between 4 and 70 °C. The activity of enzyme at 37 °C was taken as control. The stability of peptidase was determined by pre-incubating at different temperatures for 1 h and the residual activity was determined under standard conditions. **b** Effect of pH on activity and stability of purified peptidase from *Acinetobacter* sp. MN 12. Peptidase activities were evaluated in the pH range of 6–12 using different buffers at 37 °C. Stability at different pH was determined by incubating the enzymes in different buffers for 1 h at room temperature and residual activity was measured at 37 °C

Effect of Various Metal Ions on Peptidase Activity

The effect of metal ions showed that peptidase activity was stimulated by Na⁺, Mn²⁺, Ca²⁺ and Zn²⁺, inhibited by Hg²⁺ and Cu²⁺, while Mg²⁺ did not show much effect on enzyme activity (Table 2). Similarly, the activity of protease from *Exiguobacterium* sp. SKPB5 was stimulated by Mn²⁺, Ca²⁺, Zn²⁺ [10] and from *Curtobacterium luteum* was stimulated by Mn²⁺ and inhibited by Hg²⁺ and Cu²⁺ [13]. The heavy metal ions mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides), as well as with histidine and tryptophan residues thus showing the inhibitory effect.

Effect of Inhibitors and Chelators on Peptidase Activity

The enzyme was completely inhibited by reducing agent 2-ME, and strongly inhibited by PMSF (Table 3), suggesting

Table 2 Effect of various metal ions on peptidase activity

Metal ions	Relative activity (%)
Ca ²⁺	192
Cu ²⁺	87
Hg ²⁺	49
Mg ²⁺	97
Mn ²⁺	248
Na ⁺	111
Zn ²⁺	151
Control	100
SEm	0.78
CD ($p = 0.01$)	2.87

that the peptidase under investigation is serine peptidase. PMSF showed inhibitory effect on protease from *Bacillus cereus* [9] and *Colwellia* sp. [20]. The inhibitory and stimulatory effects of various compounds on protease from psychrotrophs have been reviewed [11].

Effect of Surfactants, Oxidizing Agents and Commercial Detergents on Peptidase Activity

Low temperature-active and alkaline stable peptidases have been of interest to detergent industries for their ability to remove proteinaceous material under wash conditions. For using an enzyme as detergent additive, it should be active in presence of oxidants, surfactants and bleaching agents. The purified peptidase was found stable in the presence of various agents retaining more than 50 % of activity with Tween 20, Tween 80, urea and H₂O₂ (Table 4). The purified peptidase was found stable in presence of detergents like Ariel, Rin, Surf excel and Tide, retaining more than 60 % activity even after 2 h (Table 4). Proteases from various microorganisms differ in their stability towards different commercial detergents retaining 42–93 % residual activity after 1 h [4, 19]. As far as peptidase from psychrotrophs is concerned, there is meager information on low temperature-active, alkaline, oxidant and detergent stable peptidase.

Peptidase Activity on Various Protein Substrates

The purified peptidase showed high substrate specificity towards casein (100 %) followed by BSA (95 %), azocasein (88 %), skim milk (86 %) and gelatin (50 %) respectively (Table 5). The cold-active protease from psychrotrophic *Serratia marcescens* and alkaline protease from *Bacillus laterosporus*—AK1 showed highest activity toward casein [2] while, protease from psychrotrophic *Arthrobacter* sp. MNPB6 showed highest activity towards BSA and casein [17].

Table 3 Effect of various chelators/inhibitors on peptidase activity

Chelators/inhibitors	Concentration (mM)	Relative activity (%)
EDTA	5	46
	10	42
EGTA	5	79
	10	48
PMSF	5	39
	10	26
2-ME	5	0
	10	0
Iodoacetamide	5	84
	10	31
Pepstatin A	5	60
	10	57
DTT	5	51
Control	–	100
SEm		0.368
CD ($p = 0.01$)		1.211

Table 4 Effect of surfactants, oxidizing agents and commercial detergents on peptidase activity

Surfactants/detergents	Relative activity (%)
Tween 20	55
Tween 80	59
SDS	39
Urea	56
H ₂ O ₂	66
Ariel	65
Rin	71
Tide	61
Surf excel	65
Control	100
SEm	0.596
CD ($p = 0.01$)	2.13

Determination of K_m and V_{max}

The K_m and V_{max} values for casein were 0.3529 mg/ml and 294.11 µg/ml/min while for gelatin were 2.03 mg/ml and 384.61 µg/ml/min. The low value of K_m for casein suggests that it has better affinity for enzyme as compared to gelatin. The K_m and V_{max} for an alkaline protease from *Bacillus mojavensis* with casein as substrate varied from 0.0371–0.0250 mg/ml and 60.61–120.48 µg/ml/min at 45–60 °C [3]. K_m for aminopeptidase produced by *Colwellia psycherythraea* with MCA-L as substrate ranged from 43 µM at –1 °C and 72 µM at 19 °C [8].

Table 5 Peptidase activity on different protein substrates

Substrates	Relative activity (%)
Casein	100
BSA	95
Gelatin	50
Skim milk	86
Azocasein	88
SEm	0.253
CD ($p = 0.01$)	0.989

Fig. 3 Washing performance of peptidase from *Acinetobacter* sp. MN 12 at different temperatures **a** 15 °C, **b** 25 °C, **c** 35 °C; **(i)** Blood stained cloth washed with water **(ii)** Blood stained cloth washed with detergent **(iii)** Blood stained cloth washed with detergent and enzyme

Washing Performance of Peptidase

The peptidase from *Acinetobacter* sp. MN12 acted synergistically with the detergents and effectively removed blood stains at low temperature (Fig. 3). The proteases of *Bacillus* spp. [2] and *Botrytis cinera* [1] removed the blood stains at 60 °C. Also, proteases from *Pseudomonas aeruginosa* [6] were able to remove blood stain at 28–30 °C. The peptidase from *Acinetobacter* sp. MN12 was able to remove blood stain effectively and hence showed its potential for detergent industry.

Conclusion

This is the first report on purification and biochemical characterization of extracellular peptidase from genus

Acinetobacter. This novel extracellular peptidase is a low temperature-active displaying optimum activity at 40 °C. The peptidase is alkaline stable, compatible with surfactants, oxidants, commercial detergents and effectively removes blood stain at low temperature, thus indicating its potential for detergent formulations.

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