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ORIGINAL ARTICLE

An alkaline protease from *Bacillus circulans* BM15, newly isolated from a mangrove station: characterization and application in laundry detergent formulations

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Abstract An investigation on the properties of an alkaline protease secreted by *Bacillus circulans* BM15 strain isolated from a mangrove sediment sample was carried out in order to characterize the enzyme and to test its potency as a detergent additive. The protease was purified to apparent homogeneity by ammonium sulphate precipitation and was a 30-kDa protease as shown by SDS-PAGE and its proteolytic activity was detected by casein zymography. It had optimum activity at pH 7, was stable at alkaline pH range (7 to 11), had optimum temperature of activity 40°C and was stable up to a temperature of 55°C after incubation for one hour. Hg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}completely inhibited the enzyme activity, while Ca^{2+}, Mg^{2+}, K^{+} and Fe^{3+} were enhancing the same. The serine protease inhibitor PMSF and metal chelator EDTA inhibited the activity of this protease while the classic metalloprotease inhibitor 1, 10 phenanthroline did not show inhibition. The enzyme was stable in SDS, Triton-X-100 and H_2O_2 as well as in various commercial detergents after incubation for one hour. The extracellular production of the enzyme, the pH and temperature stability and stability in presence of oxidants, surfactants and commercial detergents suggest its possible use as a detergent additive.

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Introduction

Proteases are enzymes catalyzing the total hydrolysis of proteins. They represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes[1]. Proteases have a long history of application in food and detergent industries with the detergent alkaline proteases holding the largest share of the enzyme market. Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH range[2]. Despite the large number of protease producing microorganisms, only a few are considered as appropriate producers for commercial exploitation being 'generally regarded as safe' (GRAS). Bacteria are the most important alkaline protease producers with the genus *Bacillus* being the most prominent source, because of their ability to produce large amounts of alkaline proteases having significant proteolytic activity and stability at high pH and temperature. An alkaline protease from *Bacillus circulans* has been reported previously[3]. Even though a number of proteases have been described and used as detergent additives, they have some limitations with respect to their stability and activity in oxidants and surfactants, which are common ingredients of the detergent formulations. Therefore, we have attempted to isolate an alkaline protease more suitable for this application, from bacteria isolated from a mangrove. Though many different exotic environments have been explored and exploited for alkaline protease producing microbes, the mangrove ecosystem is one among the least studied in this regard. Therefore a mangrove, which is a part Cochin estuary, was

selected for the present study. In this study, the purification and characterization of an alkaline protease from a *Bacillus* sp. newly isolated from this mangrove area and its possible use in detergent formulations has been described.

Materials and methods

Microorganism and culture conditions: Bacteria were isolated from sediment samples of a mangrove that is a part of Cochin estuary and the protease producers were screened using the gelatin agar and casein agar media of Harrigan and McCance[4]. Plates were incubated at 28°C for 24 h and then flooded with 15% $HgCl_2$ in concentrated HCl. A clear zone of protein hydrolysis around the colony indicated protease production. The isolate *Bacillus circulans* BM15 strain showing a very prominent clearing zone (3.8 cm and 3.2 cm diameter on gelatin and casein agar plates respectively) was selected for the study of protease production. Production of protease was carried out in a medium containing (g l^{-1}) casein, 10; peptone, 1; molasses, 4; NaCl, 5; $CaCl_2$, 0.05; $MgSO_4$, 0.05; (pH 7). The medium (50 ml) was inoculated with 0.5 ml of 24 h seed culture in 250 ml Erlenmeyer flasks and incubated at 28(± 2)ºC with shaking at 100 rev min^{-1} for 36 h. The cell free supernatant was collected by centrifugation for 15 min at 4°C and 12,000 g and used for the assay of protease activity.

Protease assay: The proteolytic activity was monitored by modification of casein digestion method of Kunitz [5]. Suitably diluted protease enzyme (50 µl) was added to 3 ml casein (0.6% w/v) prepared in phosphate buffer (0.05 M, pH 7.0). After incubation (30 min, 40°C) the undigested substrate was precipitated by adding 3ml of 5% (w/v) trichloroacetic acid (TCA) and allowed to stand for 15 min at room temperature, followed by filtration through Whatman No. 1 filter paper. The absorbance of the supernatant was measured at 280 nm. A control was run simultaneously, in which TCA was added prior to the addition of enzyme solution. A standard curve was generated with pure tyrosine as standard. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1 µg of tyrosine per ml per minute under the specific conditions of assay. Protein content of the sample was measured by the method of Lowry *et al* [6] with bovine serum albumin (BSA) as standard.

Partial purification of protease: Enzyme in the cell free supernatant portion of the culture was precipitated by ammonium sulphate (up to 80% saturation) and kept overnight at 4°C. The precipitate was recovered by centrifugation for 15 min at 4°C and 25,000 g and re suspended in minimum quantity of 0.05 M phosphate buffer (pH 7.0) and dialysed against the same buffer. This dialysed fraction was tested for homogeneity and protease activity and this dialysate was used for further characterization studies.

SDS – PAGE and Zymography: SDS PAGE (10%) was performed as described by Laemmli[7], under reducing conditions. The molecular weight was determined by interpolation from a linear semi- logarithmic plot of relative molecular weight versus the relative mobility using broad range standard molecular weight markers (3.5, 15.9, 35.5, 89.1, 141.3 and 205 kD). Casein zymography was performed to test for the proteolytic activity of the purified fraction, as described by Schmidt *et al*.[8] with minor modifications. Polyacrylamide gel (10%) was co-polymerized with casein to get a final concentration of 1% casein. The samples were applied in non-reducing Laemmli buffer without heat denaturation and run at 90Volts. The gel was washed in 2.5% Triton- X- 100 (3×15 min) to remove SDS, and was incubated in 50 mM Tris HCl buffer (pH 7.6) with 10mM CaCl2, and 0.1 M NaCl, for 1 h at 37°C and stained with 0.5% Coomassie brilliant blue for 30 minutes. The gel was de-stained with the de-staining solution, methanol/ acetic acid/ water (50:10:40, by vol.).

Effect of pH and temperature on the enzyme activity and stability: The effect of pH on protease activity was tested by measuring the activity of the purified enzyme at different pH values. Stability of the enzyme at various pH values was studied by pre- incubating 10µl of the enzyme in buffers of different pH (7 to 12) for 1h and assaying the residual enzyme activity at optimum conditions of assay. Buffers used were Sodium phosphate 0.1M (pH 6-8), Glycine-NaOH 0.1M (pH 9-11). Effect of temperature on the enzyme activity was determined by incubating the reaction mixture at different temperatures (15 to 80°C). To determine the temperature stability, purified enzyme was pre incubated at different temperatures (15 to 70°C), for 1 h and then residual activity was assayed under standard assay conditions.

Effect of metallic salts and inhibitors on protease activity. The dialysed enzyme was pre incubated for 1h in various concentrations (1 mM, 2.5 mM, 5 mM) of different metallic salts ($HgCl_2$, $ZnCl_2$, $CoCl_2$, $CuSO_4$, $CaCl_2$, $MnSO_4$, $MgSO_4$, NaCl, KCl and $Fe(PO_4)_3$) and the residual activity (%) was measured under optimum conditions. For the inhibition studies, the enzyme was pre-incubated for 1h with 1mM concentration of different protease inhibitors like ethylene-diamenetetraaceticacid (EDTA), 1,10 phenanthroline, phenylmethylsulphonylfluoride (PMSF), iodoaceticacid (IAA), urea, dithiothreitol and β-mercaptoethanol and assayed the residual activity at optimum conditions of assay.

Application of the protease in detergent formulations: The suitability of the *Bacillus circulans* BM15 protease as a detergent additive was determined by testing its stability in oxidants, surfactants and commercial detergents, in addition to its pH stability, temperature stability and substrate affinity. The protease was incubated with different concentrations of surfactants like SDS (0.05, 0.1, 0.2 and 0.4%), Triton X-100 (0.1, 0.4, 0.7 and 1%) and oxidizing agent H_2O_2 (1, 2, 3 and 4%) for 30 minutes, and residual activity of the protease was measured by the standard assay procedure. Stability of the protease in commercial detergents was tested by incubating measured quantity of the enzyme (500 µl) with solutions of different commercial detergents at a detergent concentration of 7mg/ml (to simulate washing conditions) for one hour. The detergents tested were Ariel, Tide (Procter and Gamble Ltd), Rin, Surf, Sunlight (Hindustan Lever Ltd), and Henko (SPIC India Ltd). The enzyme without adding any detergent and incubated under the same conditions was taken as the control. The detergent solutions in the above concentration without added enzyme were assayed for protease activity to rule out the possibility of any protease as an ingredient of the detergent. Suitable aliquots were withdrawn at different time intervals (at 15, 30 and 60 min), for one hour. Residual activity was measured by standard assay procedure and compared with the control and the relative activity was expressed in percentage taking the value given by control as 100%.

All the experiments were carried out in triplicate and the data was statistically analyzed for significance by analysis of variance.

Results and discussion

Partial Purification of the protease: The dialysed enzyme showed a single band of approx. 30 kDa on SDS- PAGE. The crude culture filtrate had a specific activity of 5.2 Umg^{-1} while the ammonium sulphate precipitaed fraction showed a specific activity of 5.57 U mg^{-1}. A detergent protease from Antarctic krill had a molecular weight of 30 kDa and specific activity of 0.5 U/mg [9]. A *Bacillus subtilis* strain has been reported to have 3.88 U mg^{-1} specific activity[10]. The zymogram analysis of BM15 protease revealed a single band of proteolytic activity on casein zymogram gel system (Fig.1). The single band on the zymogram and the SDS-PAGE together suggests the homogeneity of the dialysed enzyme. Proteolytic activity of a *Bacillus cereus* protease with a molecular mass of 31 kDa, has been detected by casein and gelatin zymography in a previous study[11].

Effect of pH and temperature on the activity and stability of the protease: The protease of BM15 was active in the pH range 7 to 10, showing the optimum activity at pH 7. At pH 8, 9 and 10 it had 75% of the maximum activity and it showed maximum stability at pH 8. It was stable between pH 7 and 11 for one hour. Up to pH 11 it retained about half of its activity (Fig. 2). The effect of pH on the stability and activity of BM15 protease was in agreement with the

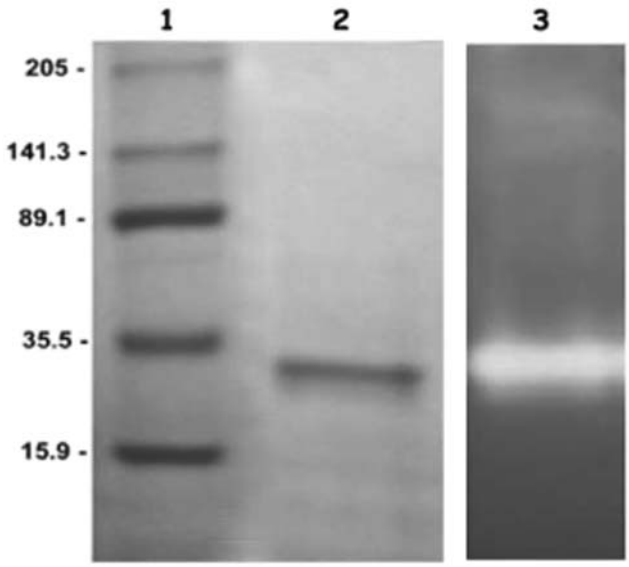

Fig. 1 SDS-PAGE and Zymogram analysis of BM15 protease. Lanes; 1) Molecular weight markers; 2) BM15 protease band on SDS-PAGE; 3) BM15 protease zymogram showing the white band of proteolysis against a blue background.

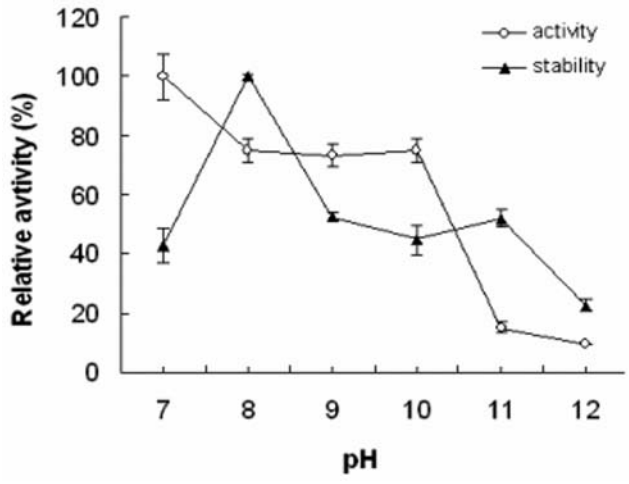

Fig. 2 The effect of pH on the activity and stability of the BM15 protease. (—○—)The effect on the activity of the protease, which is determined by carrying out the reaction at different pH values. (—▲—) The effect on the stability which is determined by incubating the enzyme in different pH buffers and measuring the residual activity. Both are expressed as relative activity, setting the maximum enzyme activities observed as 100%.

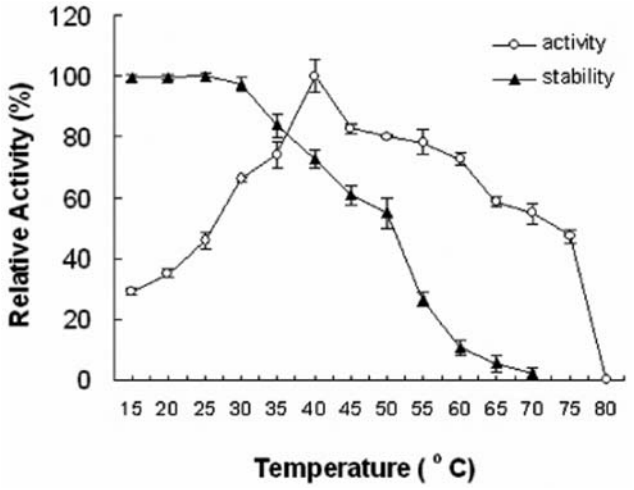

Fig. 3 The effect of temperature on the activity and stability of the BM15 protease. (—○—)The effect on the activity of the protease, which is determined by carrying out the reaction at different temperatures. (—▲—) The effect on the stability which is determined by incubating the enzyme at different temperatures and measuring the residual activity. Both are expressed as relative activity, setting the maximum enzyme activities observed as 100%.

earlier studies on proteases from other bacteria that the pH range of most alkaline proteases was generally between pH 6–9[12], or between 9 and 11[13]. Optimum temperature of activity of BM15 protease was 40°C and it showed activity over a wide range of temperatures from 15°C to 75°C having 29% and 49% of the maximum activity respectively. It had more than 50% activity over a temperature range of 30°C to 70°C. BM15 protease was stable up to a temperature of 50°C retaining about 55% of the maximum activity (Fig. 3). The BM15 protease had more than 50% activity at a range of 30°C to 70°C. This protease was stable up to 50°C after 1 h of incubation, as observed from the stability studies. It was almost stable up to 30°C (66% retained activity) and it gradually decreased to 55% at 50°C and 26% at 55°C. In general all currently used detergent-compatible proteases are thermo stable in nature with varying thermo stabilities at laundry temperatures (50–70 °C). This protease is a promising detergent additive at alkaline conditions and activity over a broad temperature range would facilitate washing even at room temperatures which is a present day requisite to maintain fabric quality at low energy demands.

Effect of metal ions and inhibitors on the protease activity: Hg^{2+}, Co^{2+}, Cu^{2+} Mn^{2+} and Zn^{2+} were inhibitory to the BM15 protease activity whereas Ca^{2+}, Mg^{2+}, K^{+} and Fe^{3+} were stimulatory (Table.1). For this protease the enhancing effect of Ca^{2+} was profound, giving 195% of retained activity at 5mM concentration, followed by Mg^{2+}(132%), K^{+}(118%) and Fe^{3+}(114%). A number of metal ions were found to have an enhancing effect on this protease, that of calcium being profound. as in a number of previous reports[14]. The BM15 protease was almost completely inhibited by the serine protease inhibitor PMSF (up to 88%) and the metal chelator EDTA (up to 95%) but the classic metalloprotease inhibitor O-Phenanthroline did not show inhibition. This suggests that it could belong to a group of calcium dependent serine protease, which can also be inactivated by EDTA. This fact is also supported by the enhancement of activity with calcium. *Pseudoperkinsus tapetis* protease also showed this kind of inhibition profile, being inactivated by both PMSF and EDTA but not by phenanthroline and it was suggested to be a Ca^{2+} or Mg^{2+} dependent serine protease[15]. It was also true with the two proteases of *Vibrio metschnikovii* [16]. BM15 protease retained about 65% activity with mercaptoethanol. DTT was not inhibitory. IAA also was less inhibitory (Table. 2).

Table 1 The effect of metal ions on the activity of the protease. The maximum activity observed is set as 100% activity.

	Residual activity (%)		
	Concentration (mM)		
Metallic salts	1	2.5	5
$HgCl_2$	3.98±1.58	1.17±1.46	4.56±0.92
$ZnCl_2$	8.29±1.41	4.56±3.85	4.45±1.07
$CoCl_2$	13.65±1.40	9.81±4.12	6.43±1.41
$CuSO_4$	8.17±1.41	0.83±0.20	6.19±0.20
$CaCl_2$	171.08±13.12	193.22±2.33	195.90±1.79
$MnSO_4$	77.51±1.99	38.42±1.58	16.56±4.50
$MgSO_4$	104.64±4.88	115.96±1.01	132.98±3.32
NaCl	76.23±0.70	93.94±3.59	100.23±3.36
KCl	125.87±7.22	127.85±2.22	118.18±2.80
$Fe(PO_4)_3$	112.02±3.85	110.32±2.00	114.72±10.49

Table 2 The effect of inhibitors on the activity of BM15 protease.

Inhibitors	Residual Activity (%)
Phenanthroline	92.60 ± 4.51
EDTA	5.67 ± 1.55
PMSF	11.83 ± 3.38
IAA	64.14 ± 4.15
Urea	37.73 ± 0.64
Dithiothreitol	114.90 ± 9.38
Mercaptoethanol	65.50 ± 2.17

Suitability of BM15 protease as a detergent additive: The proteases and other enzymes used in detergent formulations should be compatible with various detergent

components along with oxidizing and sequestering agents. Broad substrate specificity is a desirable character for detergent protease. In the present study, the effects of an oxidizing agent (H_2O_2), a non-ionic (Triton-X-100) and an anionic (SDS) detergent, as well as commercial detergent formulations, on the BM15 protease, were investigated to evaluate its suitability and effectiveness as a detergent additive. Its stability was tested in presence of H_2O_2 and it was found that the activity increased up to 116% after incubation with 4% H_2O_2 for 30 minutes. This is more effective when compared to a protease reported by Manachini and Fortina [17], which retained 70% of its activity in 3% H_2O_2 after 1 hour at 25°C and 45% after 30 minutes at 40°C. For the detergent protease of a marine shipworm bacterium, at 3% H_2O_2 a significant reduction in the stability of the proteases was observed[18]. There are only a few reports on H_2O_2 stable enzymes[19]. BM15 protease retained 70% activity in 0.05% SDS and 56% in 0.4% SDS. Proteases from *Oerskovia xanthineolytica* TK-1[20] and *Streptomyces* sp. YSA-130[21] were reported to be inhibited in presence of 0.1% SDS. The activity of BM15 protease was found to increase with increasing concentrations of T- X-100 up to retaining about 103% activity at 0.7% after 30 minutes (Table 3).

As seen from fig.4, the BM15 protease was considerably stable with all the commercial detergents tested. After one hour, 72% activity was retained with Ariel and Rin, 74% with Henko, 78% with Sunlight, 67% with Surf and 70% with Tide. This protease was almost equally stable with all the detergents tested. An insect protease was found to retain 80% of its activity with Surf and 60% in Nirma, and least stable in Ariel retaining only 30% activity at pH 11 after 60 minutes[22]. Ingale *et al*.[23] reported a *Basidiobolus* protease, which had retained activity only below 40% after 40 minutes with Surf (3 mg/ml) whereas BM15. A *Nocardiopsis* protease was found to have a maximum retained activity of 64% after 1 hr. incubation with commercial detergents[24]. This shows that the *Bacillus circulans* BM15 protease is considerably more stable in commercial detergents than many of the reported proteases. The *Bacillus circulans* protease cleaved different protein substrates like casein, gelatin and BSA (Data not shown) the preferred substrate being casein. Besides the stability in various detergents and detergent components and the substrate affinity, the temperature and pH stability are major factors to be considered. The BM15 protease was stable between pH 7 and 11 for one hour. Up to pH 11 it retained about half of its activity. It had more than 50% activity at a range of 30°C to 70°C. This protease was stable up to 50°C after 1 h. In a report by Banik and Prakash[25] the endogenous protease activity in various commercially available laundry detergents of international companies was studied and the maximum protease activity was found at 50°C in pH range 10.5–11.0 in all the tested laundry detergents. Therefore the present study indicates that the alkaline protease from this newly isolated *Bacillus circulans* BM15 is valuable for application in detergent formulations owing to its pH and temperature stability, detergent compatibility, stability to oxidizing agents and substrate affinity.

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Table 3 The stability of the BM15 protease in SDS, H_2O_2 and T-X-100. The protease was incubated with these compounds for 30 min and the residual activity was measured and expressed in % value setting the value of control as 100%.

Compound	Concentration of compound tested (%)	Residual enzyme activity (%)
H_20_2	1	103.92±6.61
	2	113.00±3.04
	3	128.3±2.49
	4	116.29±4.82
DS	0.05	70.4±6.81
	0.1	66.21±3.07
	0.2	68.57±3.20
	0.4	56.35±2.28
T-X-100	0.1	69.35±1.76
	0.4	94.18±10.48
	0.7	102.81±10.47
	1	72.36±8.14

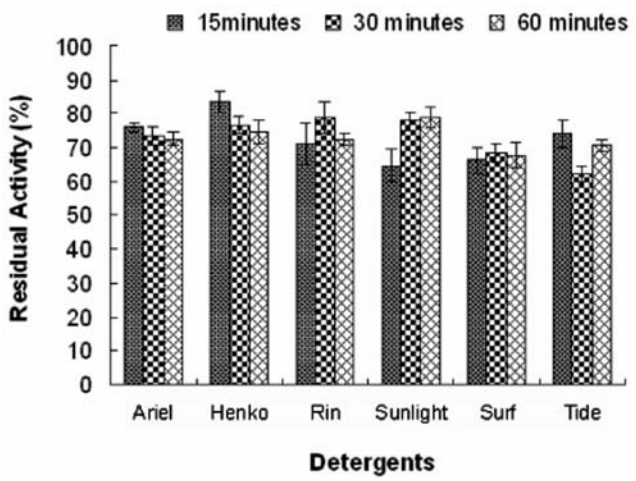

Fig. 4 Stability of the BM15 protease in commercial detergents. The protease was incubated in the detergent solution (7mg/ml w/v) for one hour and the residual activity was measured at 15 min(▩)30 min(▨) and 60 min(▨) .

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