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Purification and Characterization of *Bacillus cereus* Protease Suitable for Detergent Industry

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Abstract

An extracellular alkaline protease from an alkalophilic bacterium, *Bacillus cereus*, was produced in a large amount by the method of extractive fermentation. The protease is thermostable, pH tolerant, and compatible with commercial laundry detergents. The protease purified and characterized in this study was found to be superior to endogenous protease already present in commercial laundry detergents. The enzyme was purified to homogeneity by ammonium sulfate precipitation, concentration by ultrafiltration, anion-exchange chromatography, and gel filtration. The purified enzyme had a specific activity of 3256.05 U/mg and was found to be a monomeric protein with a molecular mass of 28 and 31 kDa, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing PAGE, respectively. Its maximum protease activity against casein was found to be at pH 10.5 and 50°C. Proteolytic activity of the enzyme was detected by casein and gelatin zymography, which gave a very clear protease activity zone on gel that corresponded to the band obtained on SDS-PAGE and non-denaturing PAGE with a molecular mass of nearly 31 kDa. The purified enzyme was analyzed through matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) and identified as a subtilisin class of protease. Specific serine protease inhibitors, suggesting the presence of serine residues at the active site, inhibited the enzyme significantly.

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Index Entries: Alkaline protease; *Bacillus cereus*; detergent; purification; characterization.

Introduction

Proteases (EC 3.4) are enzymes that hydrolyze proteins to short peptides and free amino acids and are obtained through a wide variety of sources such as plants, animals, and microorganisms *(1,2)*. Proteases are very important industrial enzymes and account for about 60% of the total world enzyme market *(3–5)*. Alkaline proteases have a large variety of industrial applications, mainly in the detergent, food, pharmaceutical, silk, and leather industries *(6–8)*. In the 1960s, the economic importance of alkaline proteases came to light when bacterial alkaline proteases from *Bacillus* sp. were introduced into the detergent industry, accounting for about 35% of total microbial enzyme sales *(4–6)*. Alkaline proteases from various *Bacillus* sp., such as *Bacillus brevis (9)*, *Bacillus* sp. SSR1 *(10)*, and *Bacillus stearothermophilus (11)*, have been reported to be used in laundry detergent formulations. The most common class of bacterial proteases is that of the serine proteases, which is already known from many *Bacillus* species *(10,12,13)*. We produced an alkaline protease from an alkalophilic bacterial strain, *B. cereus*, that showed better compatibility and better stability in a wide temperature range in comparison with endogenous proteases present in commercial laundry detergents *(14)*.

This article reports the purification and characterization of the alkaline protease produced from *B. cereus*, which is most suitable for the detergent industry. The enzyme that was purified to homogeneity was characterized by zymography and matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS). Further characterization of the enzyme was carried out by testing the effect of various protease class inhibitors on the activity of the enzyme at its optimum pH and temperature.

Materials and Methods

Bacterial Strain and Culture Conditions

B. cereus MTCC 3105 was maintained at 4°C on nutrient agar medium containing 1.0% beef extract, 0.5% NaCl, 1.0% peptone, and 2.0% agar. The organisms were grown in a medium having the following composition (% w/v): 3.0% glucose, 1.0% soybean meal, 0.04% $CaCl_2$, and 0.2% $MgCl_2$ *(15)*. The pH of the medium was adjusted to 7.5 by dissolving the medium's constituents in 0.2 *M* Tris-HCl buffer. The growth medium was inoculated with the organism and incubated for 24 h at 30°C and 200 rpm in an orbital shaker to develop inoculum. Alkaline protease was produced in a large amount by extractive fermentation using a polyethylene glycol 4000/ KH_2PO_4 aqueous two-phase system *(16)*. The liquid medium used for the production of alkaline protease had the following composition (% w/v):

2.0% glucose, 2.0% soybean meal, 0.04% $CaCl_2$, and 0.02% $MgCl_2$ *(15)*. The pH of the medium was adjusted to 8.5 by dissolving the medium's constituents in 0.2 *M* Tris-HCl buffer. The production medium was inoculated with 5% inoculum. The flasks were incubated for 72 h in a temperature-controlled (30°C) shaking incubator (200 rpm). The contents were then centrifuged (10,000*g*, 30°C, 20 min), and the cell-free supernatant was used for determining extracellular protease activity.

Assay of Protease Activity

Alkaline protease activity was estimated using a modified procedure of Hagihara with casein as the substrate *(17)*. The reaction mixture (11 mL), containing 5.0 mL of 1.2% casein solution in buffer solutions of appropriate pH, and suitably diluted enzyme (1.0 mL), was incubated at 50°C for 10 min. The reaction was terminated by adding 5.0 mL of 0.3 *M* trichloroacetic acid, and after 30 min of incubation (30°C) the reaction mixture was centrifuged (10,000*g*, 30°C, 20 min). The absorbance of clear supernatant was measured spectrophotometrically at 275 nm. Enzyme activity was expressed as protease unit, in which 1 U of protease activity was defined as the quantity of the enzyme that liberated the digestion product not precipitated by protein precipitating reagent and gave an absorbance at 275 nm equivalent to 1 µg/mL of tyrosine/min under assay conditions.

Protein Assay

Protein content in culture filtrates was determined by the method of Bradford *(18)* using Bio-Rad protein dye reagent concentrate and bovine serum albumin (BSA) as the standard.

Purification of Alkaline Protease

The fermented broth (200 mL) was centrifuged at 10,000 rpm for 30 min at 4°C. Solid ammonium sulfate was added to the supernatant for 30% saturation and centrifuged at 4°C for 20 min. Solid ammonium sulfate was again added to the supernatant for 80% saturation and centrifuged at 4°C for 20 min. The precipitate was resuspended in 20 mL of 25 m*M* Tris-HCl buffer (pH 8.0). The precipitated sample was desalted and concentrated on an Amicon Ultra-15 filter unit (10-kDa cutoff). The desalted sample was loaded onto a Mono-Q spin column (Vivapure) for anion-exchange chromatography equilibrated with a loading buffer of 25 m*M* Tris-HCl (pH 8.0). The spin column was washed with the same loading buffer, and the enzyme was eluted with a linear gradient of 0–0.5 *M* NaCl. Fractions containing protease activity were pooled and concentrated on an Amicon Ultra-15 filter unit (10-kDa cutoff).

The concentrated enzyme sample was then subjected to a size-exclusion chromatography on a Superdex-200 (2 × 60 cm) gel filtration column (Pharmacia) equilibrated with 25 m*M* Tris-HCl buffer (pH 8.0) and eluted with the same buffer containing 150 m*M* NaCl. Fractions of 1 mL each were

collected at a flow rate of 30 mL/h. The active fractions were pooled and concentrated on an Amicon Ultra-15 filter unit (10-kDa cutoff) for further analysis.

The molecular weight of the purified enzyme was determined using a Superdex-200 column calibrated previously with a range of reference proteins: catalase (250 kDa), BSA (69 kDa), and cytochrome (12.3 kDa).

Gel Electrophoresis

Purity of the sample and estimation of molecular mass were determined by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) based on the Laemmli *(19)* method. Proteins were visualized by Coomassie Brilliant Blue staining (Novagen stain kit). The marker proteins for reference molecular mass determination were myosin (245 kDa), β-galactosidase (123 kDa), serum albumin (77 kDa), ova albumin (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (25.4 kDa), and lysozyme (17.0 kDa).

Nondenaturing PAGE was also performed by the method of Laemmli on 12% polyacrylamide gel. Proteins were detected using Coomassie Brilliant Blue.

pH and Temperature Studies

To determine the optimum pH of the protease produced by *B. cereus*, the activity of purified enzyme was measured at different pH values. Protease enzyme activity was measured at pH 7.5, 8.5, 9.5, 10.5, and 11.5 using Tris-HCl buffer and Na_2CO_3/NaOH buffer. Reaction mixtures of different pH values were incubated at 50°C, and the activity of the enzyme was measured.

The effect of temperature on protease activity was studied by incubating the reaction mixture at different temperatures ranging from 30 to 60°C. The reactions were carried out at the optimum pH of 10.5, and the activity of the enzyme was measured.

Determination of Thermostability of B. cereus *Protease*

To determine its stability, *B. cereus* protease was incubated at 40 and 50°C for different time intervals up to 7 h.

Detection of Protease Activity by Zymography

Zymography is a simple, sensitive, and functional assay method for analyzing protease activity on SDS-PAGE impregnated with a protein substrate. The alkaline protease produced from *B. cereus* was analyzed on 12% SDS polyacrylamide gel containing 0.1% casein as copolymerized substrate as described by Oliver et al. *(20)* with some modifications. Zymography was performed on 12% SDS-PAGE by the same method using 0.1% gelatin. After electrophoresis, the gel was washed three times with deionized water for 1 h with gentle shaking to remove the SDS. The gel was

Table 1
Purification of Alkaline Protease From *B. cereus*

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture broth	200	13,045.5	67.5	193.26	100	1.0
$NH_4(SO_4)_2$ precipitate	20	11,836	40.0	295.9	90.7	1.53
Mono-Q	2.0	6994.16	6.8	1028.55	53.61	5.32
Superdex-200	0.5	1732.22	0.532	3256.05	13.27	16.8

incubated in activation buffer made of 50 m*M* Tris-HCl and 10 m*M* $CaCl_2$ (pH 8.0) at 37°C for 15 h with gentle shaking. Staining and destaining of the gel was performed by Coomassie Brilliant Blue (Novagen stain kit) in a single step based on the method given by Leber and Balkwill *(21)*. The protease activity band was visualized as a clear colorless band against blue background.

MALDI-TOF-MS Analysis

The identification of protease by MALDI-TOF-MS was performed by cutting the protease band from SDS-PAGE. The gel sample was digested with trypsin and the resulting peptides were spotted on a MALDI target plate *(22)*. The peptides were then analyzed by MALDI-TOF (BRUKER Reflex III), and the peptide fingerprint was compared with the National Center for Biotechnology Information (NCBI) protein database (Mascot search).

Effect of Protease Inhibitors

Inhibitors known to be specific to the different classes of proteases were used to determine the nature of protease produced from *B. cereus.* The enzyme was incubated with each inhibitor at different concentrations for 2 h at 37°C, followed by the measurement of residual activity under the standard protease assay conditions.

Results

An alkaline protease produced from *B. cereus* was purified to its homogeneity, and the results of purification are summarized in Table 1. The purified fractions obtained from different purification steps were loaded onto SDS-PAGE, as shown in Fig. 1. One hundred fractions were collected from a Superdex-200 column, and protease activity was obtained from fraction numbers 83–88, as plotted in Fig. 2. Analysis of the active peak fractions from the Superdex-200 column on SDS-PAGE revealed a homogeneous protease with a molecular mass 28 kDa (Fig. 3). Purified protease preparation was concentrated by an Amicon Ultra-15 centrifugal unit

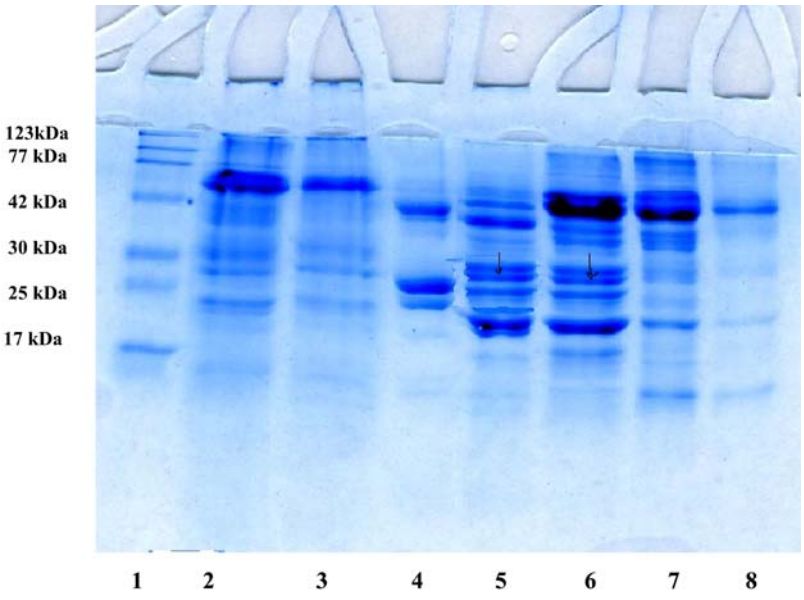

Fig. 1. Fractions from different purification steps loaded onto 15% SDS-PAGE: lane 1, molecular mass marker; lane 2, ammonium sulfate–precipitated enzyme; lane 3, ultrafiltration fraction; lane 4, first flow-through of mono-Q column; lane 5, 100 m*M* NaCl fraction; lane 6, 250 m*M* NaCl fraction; lane 7, 500 m*M* NaCl fraction; lane 8, 2 *M* NaCl fraction from mono-Q column.

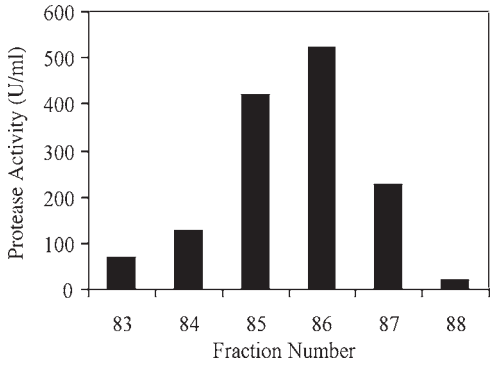

Fig. 2. Protease activity in active fractions of Superdex-200 gel filtration column.

(10-kDa cutoff) and appeared as a single band on nondenaturing PAGE with a molecular mass of 31 kDa (Fig. 4). The apparent molecular mass of the alkaline protease was also estimated at about 31 kDa by a Superdex-200 gel filtration column. Approximately 16-fold purification from the initial culture broth was achieved, with a recovery of 13%. The specific activity of the finally purified enzyme was 3256.05 U/mg (Table 1).

Enzyme is active over a broad pH range and a maximum activity was found at pH 10.5 (Fig. 5). A crude preparation of the enzyme had the same

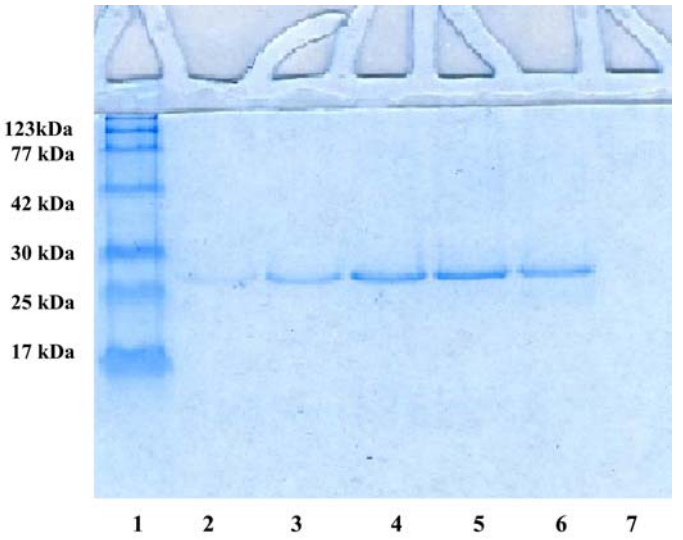

Fig. 3. Fractions from Superdex-200 column on 12.5% SDS-PAGE: lane 1, protein molecular mass marker; lane 2, fraction no. 83; lane 3, fraction no. 84; lane 4, fraction no. 85; lane 5, fraction no. 86; lane 6, fraction no. 87; lane 7, fraction no. 88.

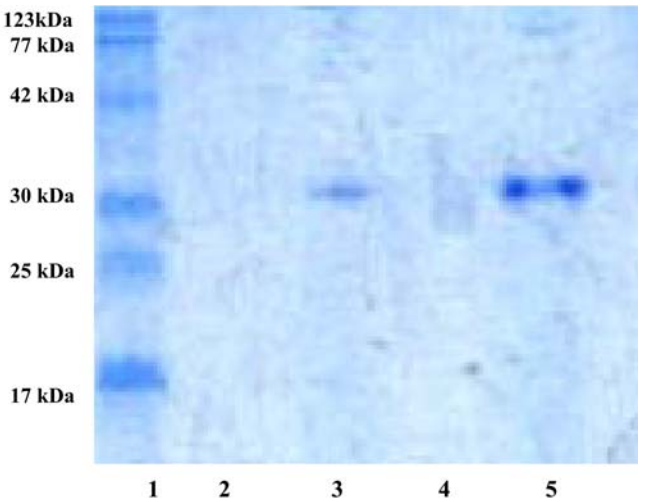

Fig. 4. Purified protease giving single band on 12% native PAGE: lane 1, molecular mass marker; lane 3, 10 µg of purified enzyme; lane 5, 20 µg of purified enzyme; lanes 2 and 4, blank.

activity profile with respect to pH as purified enzyme. Temperature optimization studies at pH 10.5 showed 50°C to be the optimum temperature for alkaline protease from *B. cereus* (Fig. 6). *B. cereus* protease was stable up to 50°C and the activity rapidly decreased with higher temperature. The thermostability of *B. cereus* protease was determined at 40 and 50°C. Protease showed nearly 100% activity up to 3 h and 67% activity up to 7 h

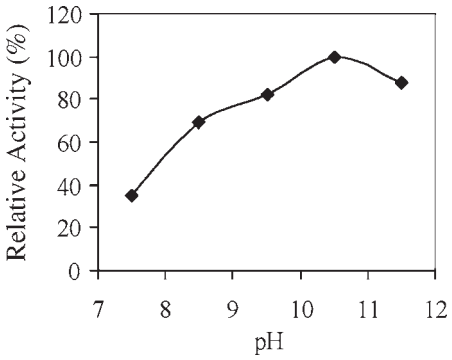

Fig. 5. Effect of pH on alkaline protease activity by *B. cereus* at constant temperature of 50°C.

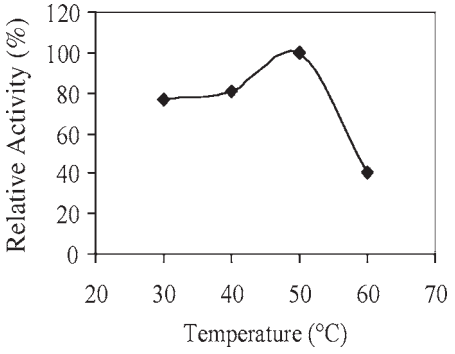

Fig. 6. Effect of temperature on alkaline protease activity by *B. cereus* at constant initial pH of 10.5.

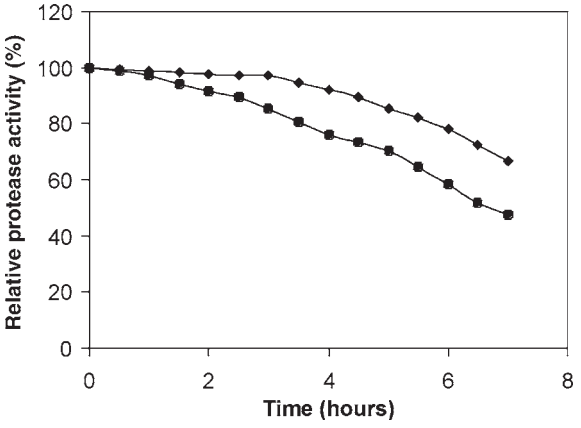

Fig. 7. Effect of temperature on stability of *B. cereus* protease: (◆) 40°C; (●) 50°C.

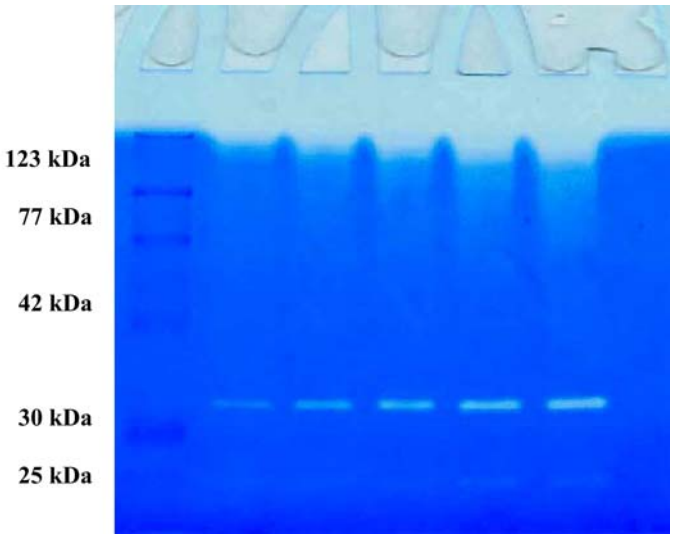

Fig. 8. Casein zymogram showing bands of protein cleavage on 12% SDS-PAGE according to increase in enzyme concentration. Lanes (left to right). Lane 1: molecular mass marker; lanes 2–6: 1, 2, 4, 6, and 8 µg of purified protease, respectively.

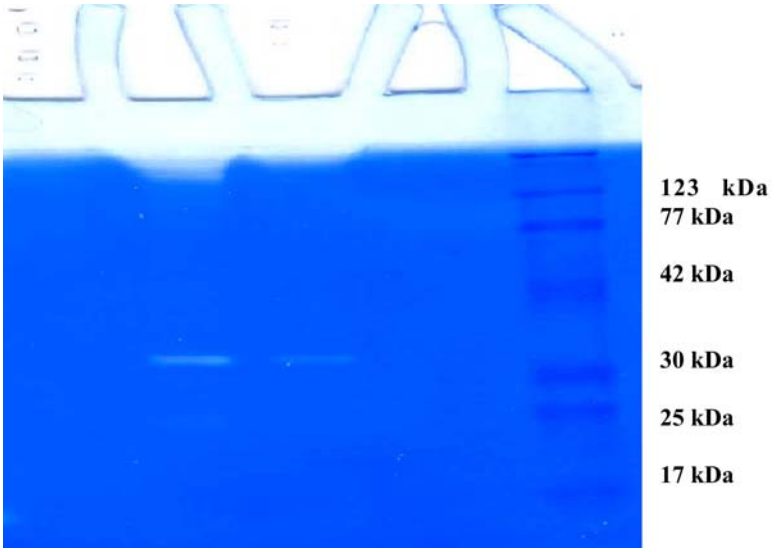

Fig. 9. Gelatin zymogram showing zone of proteolysis on 12% gel. Lanes (left to right). Lanes 2 and 3: 20 and 10 µg of purified protease, respectively; lane 5: molecular mass marker; lanes 1 and 4: blank.

at 40°C. At 50°C *B. cereus* protease retained 47% activity after 7 h. The protease had a half-life of 6.7 h (402 min) at 50°C (Fig. 7).

The purified protease was detected and characterized by zymography using casein and gelatin as substrates (Figs. 8 and 9). Casein zymography

Table 2
Matched Amino Acid Sequences of Tryptic Fragments of *B. cereus* Protease Obtained From MALDI-TOF-MS With Tryptic Fragments of Subtilisin (*Bacillus* sp.) Obtained From NCBI Protein Database

Start	End	Peptide
238	247	HPTWTNAQVR
1	12	AQSVPYGISQIK
13	27	APALHSQGYTGSNVK
250	265	LESTATYLGSSFYYGK
28	45	VAVIDSGIDSSHPDLN
248	265	DRLESTATYLGSSFYY
142	170	AVSSGIVVAAAAGNEG
46	94	GGASFVPSETNPYQDG

Table 3
Effect of Inhibitors on Protease Activity

Compound	Concentration (m*M*)	Relative activity (%)
Control	—	100
PMSF	1	28.73
	5	4.89
Pefabloc	1	0.6
	5	0.0
Dithiothreitol	1	94.78
	5	83.80
β-MER	1	96.70
	5	74.72
Iodoacetic acid	1	93.40
	5	93.0
Aprotinin	1	71.42
	5	70.0
EDTA	1	83.20
	5	75.0

gave a more prominent protease activity band in comparison to gelatin in 15 h of incubation. The molecular mass of *B. cereus* protease on zymography gel was found to be 31 kDa.

Purified protease was identified by MALDI-TOF-MS and the peptide fingerprint compared with the NCBI protein database. It was found from the result that five proteins were matched with *B. cereus* protease by significant scores (more than 74). Those matched proteins were subtilisin from *Bacillus* sp., subtilisin (alkaline mesentericopeptidase), chain A mesentericopeptidase, nattokinase (*Bacillus subtilis*), and subtilisin NAT precursor.

The highest matching (score: 148) of the tryptic fragments of *B. cereus* protease was found with tryptic fragments of subtilisin from *Bacillus* sp. That subtilisin has accession no. gi/80421 and a molecular mass of 27.369 kDa. Table 2 provides the matched amino acid sequence of subtilisin with *B. cereus* protease.

To determine the nature of the *B. cereus* protease, activity was measured in the presence of different protease inhibitors (Table 3). Protease was strongly inhibited by the serine protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and Pefabloc at 5 m*M*. By contrast, there was no inhibition by the cysteine-type inhibitors *p*-chloromercuribenzoic acid, β-mercaptoethanol (β-ME), and iodoacetic acid. No inhibition was detected when the metalloprotease inhibitor EDTA and the aspartate protease inhibitor aprotinin were added to *B. cereus* protease.

Discussion

Alkaline proteases play a key role in laundry detergent industries; they allow better removal of proteinaceous stains from cloths when added to laundry detergents. Many researchers have reported the use of alkaline proteases from *Bacillus* origin in laundry detergents *(9–11)*. Detergent industries generally use alkaline proteases in the form of granules in detergent formulations. We found in a previous study that *B. cereus* produces an extracellular alkaline protease that is active at high alkaline pH and temperature and shows better compatibility with most of the commercial laundry detergents with respect to temperature stability and enzyme activity *(14)*. The stability of *B. cereus* protease in commercial laundry detergents was found to be longer than that of the endogenous proteases present in commercial laundry detergents *(14)*.

In the present study, thermostable alkaline protease was purified from the culture supernatant of *B. cereus* by the combination of ammonium sulfate fractionation, Amicon-15 ultrafiltration, Mono-Q anion-exchange column, and Superdex-200 gel filtration column with high fold purification, as shown in Table 1. The purification fractions from each purification step up to the Mono-Q column were loaded onto SDS-PAGE, as shown in Fig. 1. The protease activity from the Mono-Q column was found in 100- and 250-m*M* NaCl fractions. The protein band corresponding to 28 kDa in 100- and 250-m*M* fractions on gel is for *B. cereus* protease, which is not present in the first flow-through and 500-m*M* fraction of the mono-Q column on the gel. Ammonium sulfate precipitation and ultrafiltration fractions on gel also showed a protein band corresponding to 28 kDa, which is for *B. cereus* protease.

The 100- and 250-m*M* fractions from the Mono-Q column were loaded onto a Superdex-200 gel filtration column and 100 fractions were collected. The protease activity was found from fraction numbers 83–88, and the protease activity of these fractions are plotted in Fig. 2. The active fractions

from the Superdex-200 column were loaded onto gel, which showed a single band on nearly 28 kDa, as shown in Fig. 3.

The molecular mass of the purified enzyme was estimated in the native form by nondenaturing PAGE, gel filtration column, and zymography as 31 kDa and on denaturing SDS-PAGE as 28 kDa. A similar molecular mass of proteases from some other bacterial strains was already reported. Singh et al. *(10)* reported a molecular mass of *Bacillus* sp. SSR1 protease of 29 kDa on SDS-PAGE and 35 kDa on native PAGE. Similarly, Kobayashi et al. *(13)* estimated the molecular mass of *Bacillus* strain as 28 kDa on SDS-PAGE.

The enzyme was very active at a high pH and temperature range and also showed very good thermostability at 40 and 50°C up to 7 h. It showed a half-life of 6.7 h (402 min) at 50°C. These characteristics of the enzyme are essential for its use in the detergent industry *(9,10,14)*.

Zymography was performed for the detection of *B. cereus* protease activity, and estimation of the molecular mass of protease on polyacrylamide substrate-loaded gels was also reported by Jobin and Grenier *(23)* and Thangam and Rajkumar *(24)*. Most researchers have reported gelatin zymography on SDS-PAGE and casein zymography on nondenaturing PAGE for proteases *(20,21,24)*. We found that casein zymography on SDS-PAGE is more suitable for *B. cereus* protease in comparison to gelatin, which gave sharper bands of proteolysis on PAGE than the gelatin zymogram in the same incubation time (Figs. 6 and 7). Casein zymography showed a very clear increase in band thickness with an increase in concentration of enzyme loaded onto gel (Fig. 7). Casein zymogram gave a clear band of proteolysis on SDS-PAGE after 15 h of incubation, whereas for casein zymography 36 h of incubation was reported *(20)*.

Purified *B. cereus* protease was further characterized by MALDI-TOF-MS. The tryptic digests read by the MALDI-TOF analyzer were matched with a known protein database (Mascot search). The tryptic digests of *B. cereus* protease were matched by a significant score of 148 with tryptic digests of subtilisin from *Bacillus* spp. obtained from the protein database. Table 2 provides the matched amino acid sequences. Hence, this result confirms that the *B. cereus* protease belongs to the subtilisin family.

B. cereus protease was inhibited by serine protease inhibitors (Table 3), which suggests the presence of serine groups on the active site of the enzyme *(1,12,25)*.

Conclusion

We purified and characterized the alkaline protease from *B. cereus*, which was found to be pH tolerant, thermostable, and laundry detergent compatible. These properties make it suitable for use in commercial laundry detergents.

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