

Full Length Research Paper

Isolation, purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus thuringiensis*-SH-II-1A

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In the present study, the isolation, purification and partial characterization of thermostable serine alkaline protease produced from *Bacillus thuringiensis* SH-II-1A was reported. The culture was isolated from soil of slaughter house waste and identified further from ribosomal sequence. The crude enzyme was purified by ammonium sulfate precipitation, dialysis and Sephadex G-200 gel permeation chromatography up to 17.04 fold with recovery of 8.47%. Relative molecular weight (67 kDa) of purified enzyme was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Maximum production of enzyme and cell biomass was observed for 48 h of incubation period at 45°C. Strong activity of enzyme was observed at pH 10 to 11; also stability of up to 2 and 20 h incubation at the same pH range confirms alkaline protease. Optimum temperature recorded for protease activity was 45°C, and 100% thermal stability up to 350 min of incubation was recorded. Among different natural substrates tried, casein was found as ideal substrate. Enzyme activity was strongly enhanced by metal ions like Ca²⁺, Mg²⁺ and Mn²⁺ whereas, 100% enzyme activity was inhibited by phenylmethylsulphonyl fluoride (PMSF), and up to 92% inhibition by diisopropyl fluorophosphates (DFP) confirmed serine protease. Detergent compatibility of the enzyme was studied in the presence of 10 mM CaCl₂ and 1 M glycine at 45°C. This indicates 80 to 100% stability for a period of 0.5 to 2.5 h incubation. Improved washing performance and removal of blood stains from the cotton cloth was observed when detergent Surf excel was used with enzyme. Overall, the observed properties of isolated protease conclude its commercial application in detergent and leather industries.

Key words: Thermostable serine alkaline protease, *Bacillus thuringiensis*, purification, partial characterization.

INTRODUCTION

Among the large number of microbial enzymes, proteases occupy a pivotal position owing to their wide applications. The current estimated value of the worldwide sales of microbial enzymes is \$ 1 billion; proteases alone account for about 60% of the total

worldwide sales (Immaculate and Jamila, 2014) and were the first enzymes to be produced in bulk (Beg et al., 2003; Ellaiah et al., 2003). Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. They play

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a specific catalytic role in the hydrolysis of proteins. This enzyme accounts for 40% of the total worldwide enzyme sales. It is expected to be an upward trend in the use of alkaline proteases in the future (Adinarayana et al., 2003; Moreira et al., 2003). Proteases are used in laundry detergents for over 50 years to facilitate release of proteinaceous materials in stains (blood and milk) and account for approximately 25% of total worldwide sales of enzymes (Ahmed et al., 2011). Thermostable alkaline proteases are having application in detergent industry as additive (Lagzian et al., 2012)

In the present study, the authors produced protease enzyme from *Bacillus thuringiensis* SH-II-1A (Foda et al., 2013). The bacillus strain was obtained from natural source of slaughter house waste drainage soil of different locations (Joachim et al., 2008). Maximum growth of culture and production of enzyme was observed at 48 h of incubation period. Isolation and purification of the enzyme was carried out by sequential steps of ammonium sulphate precipitation, DEAE-cellulose dialysis and Sephadex G-200 gel permeation chromatography. Isolated protease enzyme was identified as serine type after maximum inhibition of enzyme activity by phenyl methyl sulfonyl fluoride (PMSF) and di-isopropyl fluoro phosphate (DFP). Maximum hydrolysis of casein as a substrate and maximum stability was observed at pH 10 to 11, which supports alkaline type protease. Proteolytic efficiency of the enzyme was observed at 45°C, which supports thermal stability of enzyme at higher temperature. Activity of enzyme was enhanced by the use of metal ions like calcium (CaCl₂) supporting cofactor requirement. Enzyme has shown greater compatibility and stability with commercial detergent formulations like surf excel up to 1.5 h of incubation. Improved washing performance and removal of blood stains from the cotton cloth was observed upon incubation of enzyme along with detergent powder. Molecular weight of the purified enzyme was up to 68 kDa estimated with SDS-PAGE and supported by gelatin zymogram activity.

As it was desirable to search for new proteases with novel properties from as many different sources as possible, the present enzyme could become one of the ideal sources to fulfill all the required properties. Overall, the isolated protease enzyme could be considered as an additive for commercial detergent formulations. Commercially, it would have many industrial applications like hair removal property in leather industry, as a laundry additive in detergent industry, and breakdown of X-ray films to remove trapped silver particles. It would have therapeutic applications as a digestive aid, fibrinolytic agent, dissolution of blood clots and treatment of atherosclerosis in the future scope.

MATERIALS AND METHODS

The following chemicals were obtained from Sigma-Aldrich, St.

Louis, MO (USA): Casein, Sephadex G-200, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol (β-ME), DEAE-Cellulose equilibrium dialysis Harvard apparatus. Heavy metal ions, surfactants, organic solvents and chemicals for electrophoresis were purchased from Merck, Germany. Molecular mass markers (20 to 205 kDa) were obtained from Biotech-Genie Pvt Ltd., Bangalore, India. Commercial detergent powder and electrophoresis Vertical Slab Gel System were from Asian Scientific Instruments Ltd. All other chemicals used such as Ariel, Tide, Surf, Surf excel, Nirma and Rin were purchased from local market of high analytical grade.

Isolation and cultivation of microorganisms

The soil samples were collected in the form of soil from the waste drainage areas of slaughter house at six different locations of Pune district region (MS) India, and were diluted in sterile saline solution (Palsaniya et al., 2012). The clear supernatant liquid of the diluted samples was plated onto skim milk agar plates containing peptone (0.1% wt/vol), NaCl (0.5% wt/vol), agar (2.0% wt/vol) and skim milk (10% vol/vol). Plates were incubated at 37°C for 24 h. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms (Shata, 2005; Shieh, 2009). Depending on the maximum zone of hydrolysis, strain SH-II-1A was selected for further experimental studies. The pure isolated proteolytic strain was further subjected to identification of nucleotide sequence at National Centre for Cell Science (NCCS), Pune (MS), India. It was identified as *B. thuringiensis* and it was designated as *B. thuringiensis* SH-II-1A used in further study. Production of protease from *B. thuringiensis* SH-II-1A was carried out in a medium containing the following components; glucose 1% (wt/vol); yeast extract 0.5% (wt/vol); tryptone, 0.5% (wt/vol); casein, 1.0% (wt/vol); soluble starch, 1.0% (wt/vol); NaNO₂ 1.0% (wt/vol); CaCl₂, 1.0% (wt/vol); NH₄Cl₂ 1.0% (wt/vol); it was maintained at 45°C for 72 h in a shaker incubator (150 rpm). The pH of the medium was preadjusted with 0.1 N NaOH or 0.1 N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged at 5000 xg at 4°C for 15 min, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to purification and further studies (Amrita et al., 2012; Akolkar 2009).

Enzyme purification

Ammonium sulphate precipitation

As described above, fermentation was carried out for 72 h at 45°C and clear supernatant was separated from the cells by centrifuge at 5000 xg for 15 min. Further supernatant was fractionated by precipitation with ammonium sulfate between 40 and 60% of saturation. All subsequent steps were carried out at 5 to 8°C. The protein mass obtained after precipitation was resuspended again in 0.1 M Tris-HCl buffer, pH 10.0, and dialyzed using diethyl amino ethyl (DEAE) cellulose membrane against the buffer having same composition (Asker et al., 2013).

Sephadex G-200 gel permeation chromatography

Sephadex G-200 (5 gm) gel was added to 0.1 M Tris-HCl (pH 10.0) and allowed to swell overnight; and column (1.5 × 65 cm) (Sigma-Aldrich, St Louis, MO) was packed. The column (1.5 × 65 cm) was equilibrated with 0.1 M Tris-HCl buffer and pH 10.0. The protein pellet obtained after saturation with ammonium sulphate between 50 and 70% was dissolved in 0.1 M Tris-HCl buffer and pH 10.0, and a total of 5 mL of protein precipitate was loaded onto a column of Sephadex G-200 (1.5 × 65 cm). The column was equilibrated

with Tris-HCl buffer and pH 10.0. The process of elution was started at a flow rate of 1 mL/min with a 1:1 volume gradient flow from 0.1 to 1 M NaCl in the same buffer. Protein concentration from each fraction was determined by Lowry method using bovine serum albumin as standard. Such fractions were pooled, dialyzed and concentrated to be used for further studies (Asker et al., 2013)

Molecular mass determination

Molecular mass of the purified enzyme fractions showing greater activity was determined on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) and Ali et al. (2014) with 15% acrylamide concentration. Approximately, 10 µg of purified protein was loaded on 15% SDS-PAGE with standard molecular mass markers into two side wells of the gel. The dragging process of loaded protein was started at initial current of 15 mA and then constant current of 30 mA. After electrophoresis, the gel was stained with Co-omassie Brilliant Blue R-250 (CBB R-250) and destained in glacial acetic acid to visualize protein bands.

Determination of proteolytic activity (caseinase determination)

Caseinase activity was assessed by the modified procedure (Tsuchida et al., 1986) using 2% casein substrate dissolved in 0.2 M carbonate buffer pH 10. Casein solution (0.5 ml) with an equal volume of suitably diluted enzyme solution was incubated at 37°C. After 15 min, the reaction was terminated by addition of 1 ml of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged and 5 ml of 0.44 M Na₂CO₃ was added to the supernatant. Addition of two fold diluted (1 mL) Folin CioCalteu reagent to the mixture resulted in blue color. After 45 min at room temperature, the reaction mixture was subjected to centrifugation (Remi, 4000 rpm, 4°C). The intensity of color developed in the supernatant mixture was measured at 660 nm against a reagent blank prepared in the same manner. Tyrosine served as the reference standard and optical density of colored solution was measured by UV spectrophotometer (Shimadzu, Japan).

Protein assay

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280 nm (Singhal et al., 2012).

Effect of pH on enzyme activity and stability

The activity of the enzyme (10 µl) was measured at different pH values in the presence of 10 mM CaCl₂. The pH was adjusted using the following buffers (0.05 M): phosphate buffer (pH 6.0 to 7.0), Tris-HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0 to 12.0). Reaction mixtures containing 0.5% casein (1 mL) were incubated at 45°C for 30 min. The reaction was stopped by addition of 0.5% Trichloro acetic acid (TCA). Absorbance of TCA soluble peptides was measured at 280 nm. The purified enzyme was diluted in different relevant buffers (pH 6.0 to 12.0) and incubated at 45°C for 2 and 20 h for checking of enzyme stability. The relative activity at each exposure was measured as per assay procedure (Siala et al., 2009).

Effect of temperature on enzyme activity and stability

The activity of the enzyme was determined by incubating the

reaction mixture at different temperatures ranging from 35 to 65°C in the presence of 10 mM CaCl₂ for 30 min. To determine the enzyme stability with changes in temperature, purified enzyme was incubated at different temperatures (35, 40, 45, 50, 55, 60 and 65°C) in the presence of 10 mM CaCl₂ and relative protease activities were assayed at standard assay conditions (Bhunja et al., 2012).

Effect of protease inhibitors on enzyme activity

The effect of different protease inhibitors on purified protease activity was studied using phenyl methyl sulfonyl fluoride (PMSF) (1 mM), ethylene diamine tetra acetic acid (EDTA) (5 mM), *p*-chloro mercuric benzoate (*p*-CMB) (5 mM), Iodoacetate (5 mM), Di-isopropyl fluoro phosphate (DFP) 5 mM and β-mercaptoethanol (β-ME) (5 mM) (Sigma et al., 1975). The reaction mixture was prepared by pre-incubating the purified enzyme with inhibitors without substrate for 10 min at 45°C. The protease assay was performed by addition of casein as a substrate for 30 min. Protease activity obtained without inhibitor was considered as 100% (Usharani et al., 2010).

Effect of various metal ions on enzyme activity

Effect of monovalent (Na⁺ and K⁺), divalent (Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺, Cd²⁺, Zn²⁺ and Hg²⁺) and trivalent (Al³⁺, Fe³⁺) metal ions on enzyme activity at a concentration of 5, 10 and 15 mM was investigated by using casein as substrate (Sigma et al., 1975). The reaction mixture was prepared by pre-incubating the purified enzyme with metal ions at each concentration for 10 min at 45°C and the proteolytic activity was determined for 1 h by the above mentioned method. Enzyme activity in the absence of metal ions was considered as 100% (George et al., 2012).

Hydrolysis of protein substrates

Protease activity with different protein substrates including BSA, casein, egg albumin and gelatin was assayed by mixing 100 ng of the enzyme and 200 µL of assay buffer containing the protein substrates (2 mg/mL). After incubation at 45°C for 30 min, the reaction was stopped by adding 200 µL of 10% (wt/vol) trichloro acetic acid (TCA) and allowed to stand at room temperature for 10 min. The undigested protein was removed by centrifugation and peptides released were assayed. The specific protease activity towards casein as a substrate was considered as a control (Iqbal et al., 2011).

Detergent stability

The compatibility of SH-II-1A protease with local laundry detergents was studied in the presence of 10 mM CaCl₂ and 1 M glycine. Detergents used were Nirma (Nirma Chemical, India); Henko (Henkel Spic, India); Surf, Surf Excel, Super Wheel, Rin (Hindustan Lever Ltd, India); and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% wt/vol) and incubated with protease for 3 h at 45°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100% (Ire et al., 2011).

Washing test with protease preparation

Application of protease (5000 U/mL) as a detergent additive was studied on white cotton cloth pieces (4 × 4 cm) stained with blood.

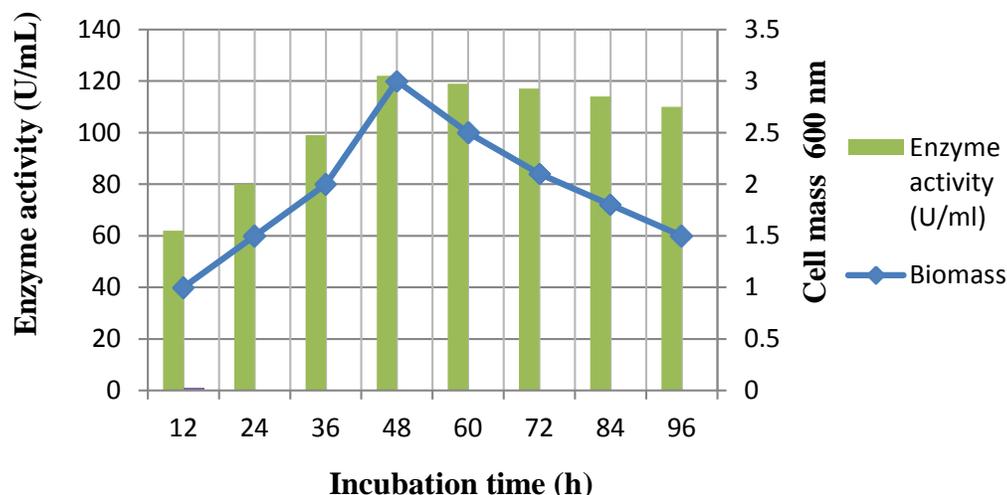


Figure 1. Maximum growth of culture and enzyme production.

The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied: 1. Flask with distilled water (100 mL) + stained cloth (cloth stained with blood); 2. Flask with distilled water (100 mL) + stained cloth (cloth stained with blood) + 1 mL Wheel detergent (7 mg/mL); 3. Flask with distilled water (100 mL) + stained cloth (cloth stained with blood) + 1 mL Wheel detergent (7 mg/mL) + 2 mL enzyme solution.

The above flasks were incubated at 45°C for 15 min. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of cloth pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood were taken as control for comparison (Ram et al., 2012).

RESULTS AND DISCUSSION

Identification of bacterial culture

The most significant characteristics of the isolate SH-II-1A are summarized: these are vegetative cells and characterized as large stout rods that are straight or slightly curved with rounded ends (Gopala, 2011). They usually occur in pairs or short chains. It is Gram positive, non-capsulated and motile with peritrichous flagella. Classification of *B. thuringiensis* strains has been accomplished by H serotyping the immunological reaction to the bacterial flagellar antigen (Ash et al., 1991). The *hag* gene encodes flagellin, which is responsible for eliciting the immunological reaction in H serotyping. This showed that the isolate belongs to the class of *Bacillus* sp.

Purification of extracellular protease of *B. thuringiensis* SH-II-1A

In *B. thuringiensis*, maximum growth and maximum enzyme production was observed at 48 h (Figure 1). Fermentation was carried out at 45°C and clear

supernatant was separated from the cells by centrifuging at 5000 \times g for 15 min. Further supernatant was fractionated by precipitation with ammonium sulfate between 40 and 60% of saturation. All subsequent steps were carried out at 5 to 8°C. The protein mass obtained after precipitation was resuspended in 0.1 M Tris-HCl buffer, pH 10; it was dialyzed using diethyl amino ethyl (DEAE) cellulose membrane against the buffer having same composition in order to achieve desired purity of enzyme.

The protein pellet obtained after dialysis using DEAE membrane was loaded onto a column of Sephadex G-200 (1.5 \times 65 cm) equilibrated with Tris-HCl buffer, pH 10. The elution profile of gel filtration chromatography is shown in Figure 2. Elution profile of column was monitored and it was observed that the protease enzyme was eluted in the form of well resolved single peak, showing casein hydrolysis (caseinase) activity coinciding with a single protein peak at a NaCl concentration of 0.6 M. Eluted fractions sequence numbers (25 to 30) were observed with high protease activity. The summary of purification steps involved for alkaline protease is presented in Table 1 (Zhou et al., 2009; Fakhfakh et al., 2010).

When the ammonium sulphate precipitation and purified protease was analyzed by SDS-PAGE, seven bands were observed in the presence of ammonium sulphate precipitation (Figure 3); while purified protease showed a single band on SDS-PAGE, indicating a homogenous preparation. The molecular weight of the protease was determined by comparison of the migration distances of standard markers protein. The molecular mass standards were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa) and α -lactalbumin (14 kDa) on SDS-PAGE. The molecular mass was determined by extrapolation from a linear semi logarithmic plot of

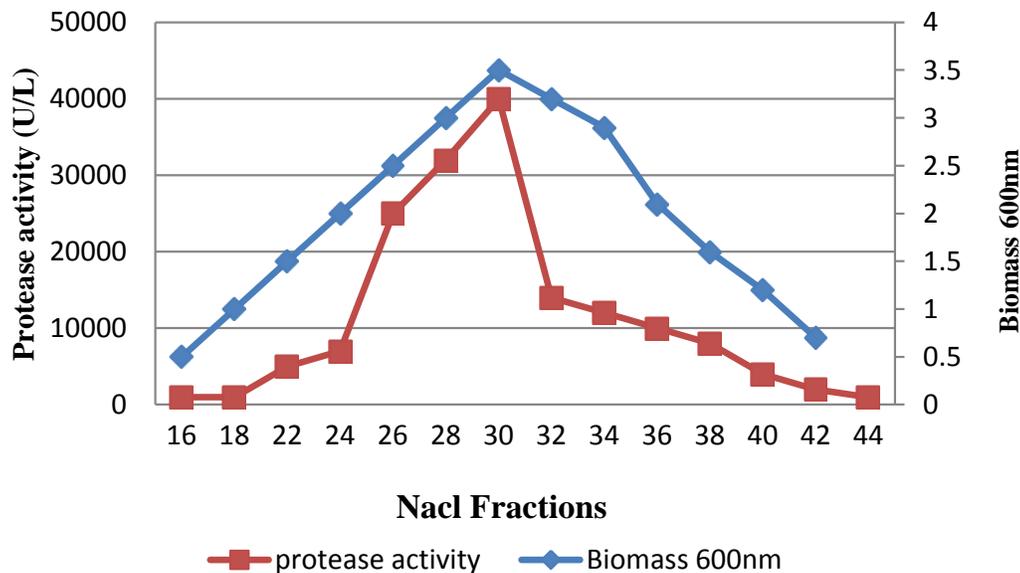


Figure 2. Elution profile of protease SH-II-1A by Sephadex G-200 column chromatography.

Table 1. Summary of purification steps for isolated enzyme.

Purification step	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification folds	Recovery (%)
Crude enzyme	105000	6025	17.4	1	100
Ammonium sulphate ppt. and dialysis	90500	4000	22.62	1.29	86.19
Sephadex G-200	8900	30	296.66	17.04	8.47

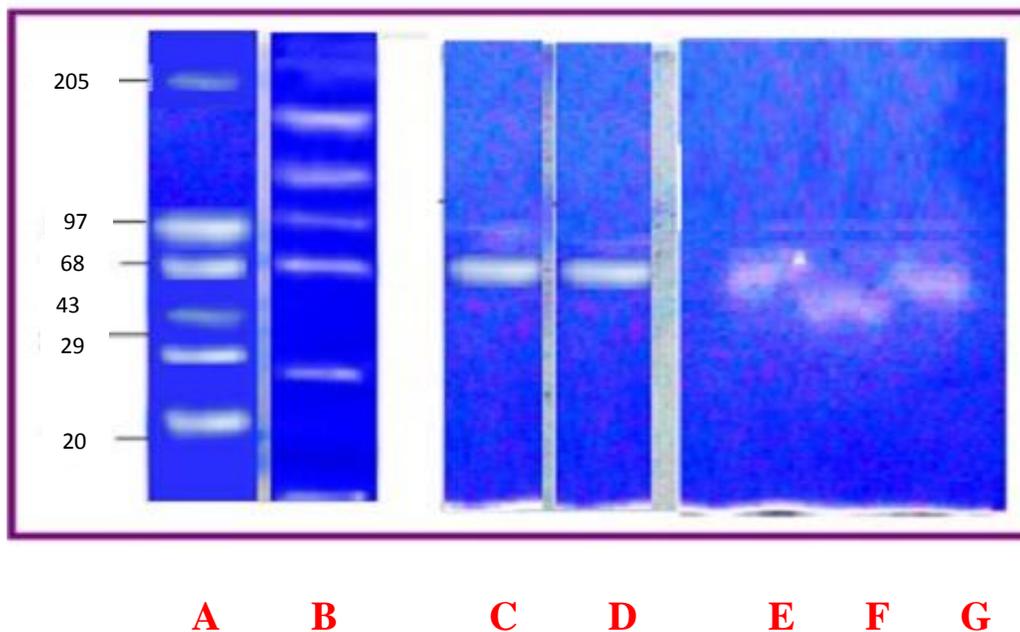


Figure 3. SDS-PAGE and Gelatin zymogram for enzyme. Lane A, Molecular mass marker; Lane B, crude enzyme; Lane C, purified enzyme; Lane D, standard BSA; Lane E and G, gelatin zymogram for standard BSA; Lane F, gelatin zymogram for purified enzyme. The molecular mass markers are- myosin 205 kDa, phosphorylaseB-97 kDa, bovine serum albumin- 68 kDa, ovalbumin-43 kDa, carbonic anhydrase-29 kDa, tyrosine-20 kDa.

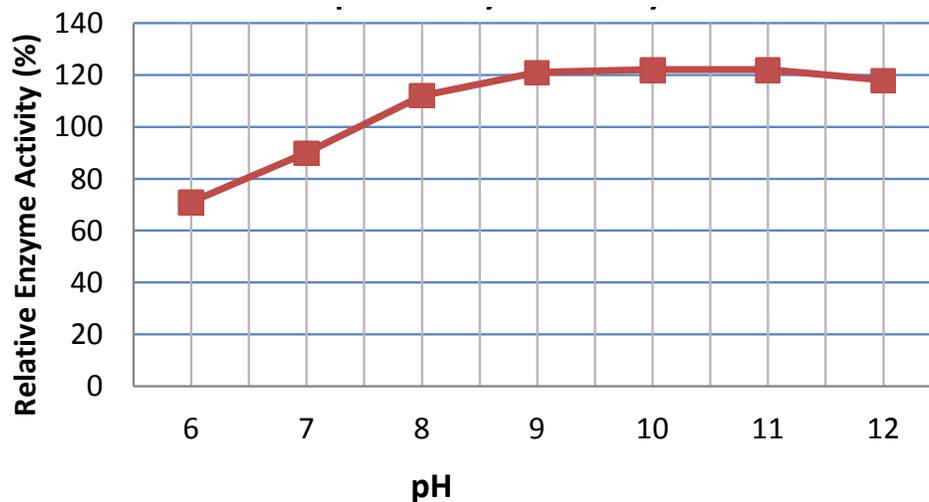


Figure 4. Relative enzyme activity (%) observed at different pH level.

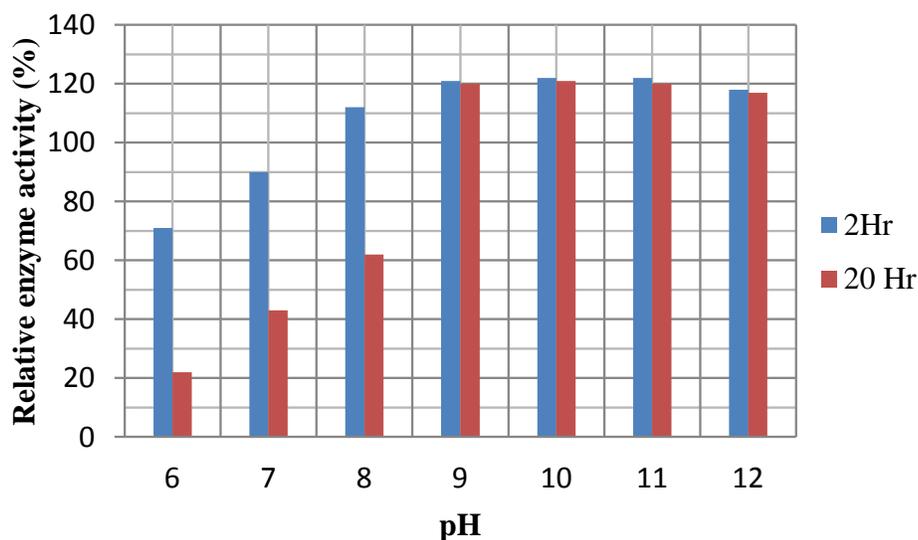


Figure 5. Stability of enzyme at different pH level.

relative molecular mass versus the Rf value (relative mobility). Depending on the relative mobility, the molecular weight of the protein band was calculated to be 68 kDa, which coincided with the band of bovine serum albumin (BSA) marker protein (Prashant et al., 2013).

Characterization of purified enzyme

For the determination of the pH optimum, phosphate (pH 6 to 7), Tris-HCl (pH 8), and glycine-NaOH (pH 9 to 12) buffers were used in the absence and presence of 10 mM CaCl₂. The highest protease activity was found to be at pH 10 to 11 using glycine-NaOH buffer (Figure 4). These

findings are in line with several earlier reports showing pH optima of 10 to 10.5 for protease from *Bacillus* sp., *Thermus aquaticus*, *Xanthomonas maltophilia* and *Vibrio metschnikovii*. The important detergent enzymes, subtilisin Carlsberg and subtilisin Novo or BPN (Dhandapani et al., 1994) also showed maximum activity at pH 10.5. The stability of enzyme was checked at various pH 6 to 12. 100% stability was achieved at pH 9 to 11 for a period of 2 and 20 h incubation (Figure 5).

The activity of the crude and purified enzyme was determined at different temperatures ranging from 35 to 65°C in the absence and presence of 10 mM CaCl₂. The optimum temperature recorded was at 45°C for protease activity. The enzyme activity gradually declined at

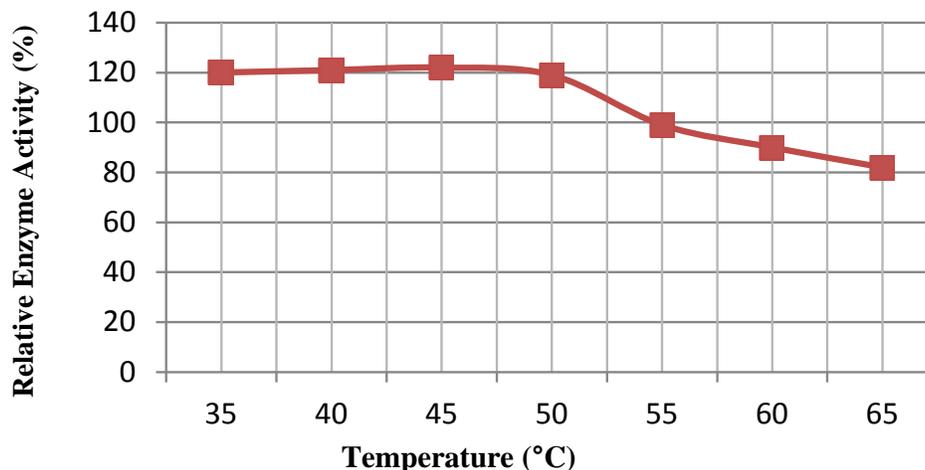


Figure 6. Effect of temperature on protease activity.

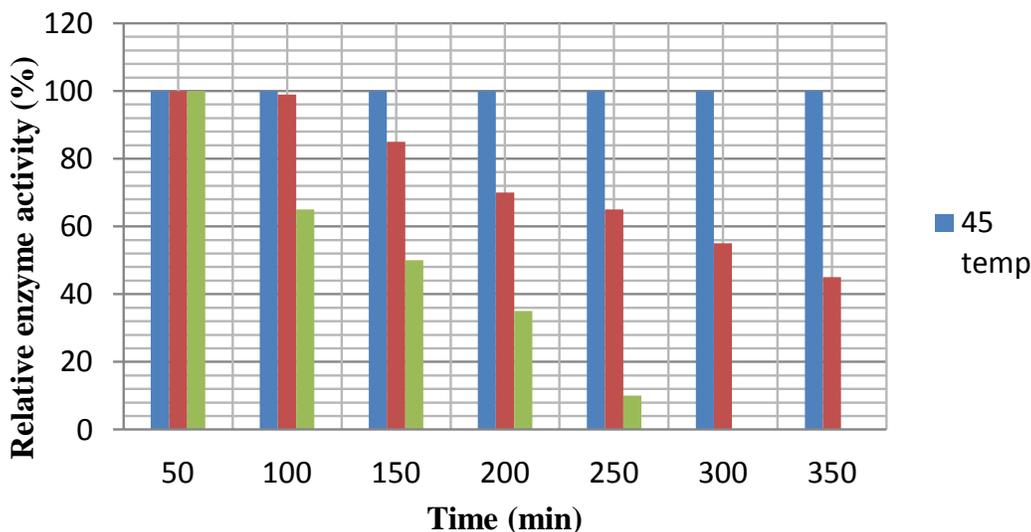


Figure 7. Thermal stability of enzyme at different temperature.

temperatures beyond 50°C (Figure 6). A similar type of result was observed by other investigators where a maximum temperature of 55°C was recorded for an alkaline protease from *Bacillus stearothermophilus* AP-4 and 60°C for a protease (Jellouli et al., 2009) derived from *Bacillus* sp B21-2. The thermal stability of the purified protease was tested at different temperatures of 45, 50 and 55°C for different periods (50 to 350 min) in the presence of 10 mM CaCl₂. The enzyme was almost 100% stable at 45°C even after 350 min of incubation (Figure 7). Previous reports on thermostability have shown half-lives of >200 min at 50°C and 2 to 22 min at 60°C for heat stable serine proteases, and half-lives of 3.4 and 2.4 min at 50°C have been recorded for subtilisin Carlsberg and subtilisin BPN, respectively.

Effect of protease inhibitors on enzyme activity

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center. The effect of different inhibitors on the enzyme activity of the purified protease was studied. Among the inhibitors tested (at 5 mM concentration), PMSF was able to inhibit the protease completely, while DFP exhibited 94% inhibition. In this regard, PMSF sulphonates, the essential serine residue in the active site of the protease and has been reported to result in the complete loss of enzyme activity. Relative enzyme activity by different inhibitors is described in Figure 8. Our findings are similar to those of Tsuchida et al. (1986) where the protease was completely inhibited by PMSF.

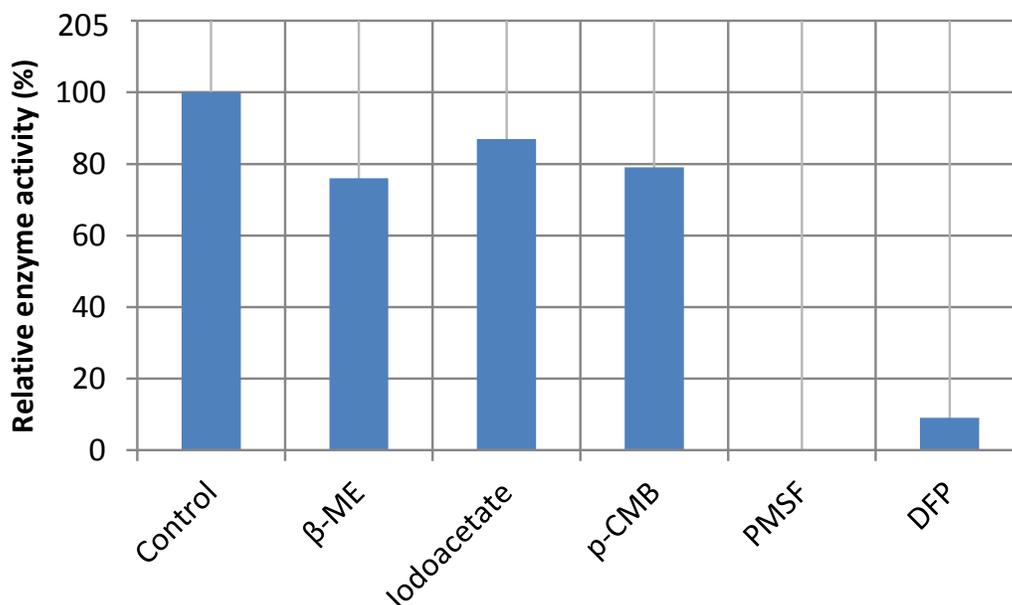


Figure 8. Effect of various enzyme inhibitors on enzyme activity. *β-ME indicates β-mercaptoethanol; *p*-CMB, *p*-Chloro mercuric benzoate; PMSF, Phenyl methyl sulfonyl fluoride; DFP, di-isopropyl fluoro phosphate.

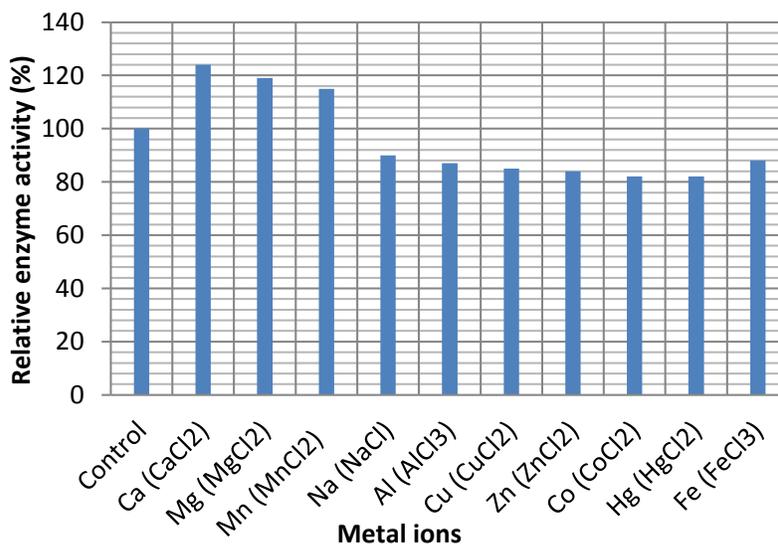


Figure 9. Effect of various metal ions on enzyme activity.

This indicated that it is a serine alkaline protease. Slight inhibition was observed with other inhibitors like iodoacetate, *p*-CMB and β-ME.

Effect of various metal ions on enzyme activity

Most of the metal ions tested had a stimulatory effect (Ca²⁺, Mg²⁺ and Mn²⁺) or a slight inhibitory effect (other ions) on enzyme activity. Some of the metal ions such as Ca, Mg²⁺ and Mn²⁺ increased and stabilized the protease

activity of the enzyme; this is possible because of the activation by the metal ions. These cations also have been reported to increase the thermal stability of other *Bacillus* alkaline proteases. These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures. Other metal ions such as Zn²⁺, Cu²⁺, Hg²⁺, Co²⁺, N²⁺, Cd²⁺, Al³⁺ and Fe³⁺ did not show any appreciable effect on enzyme activity. Relative enzyme activity is described in Figure 9.

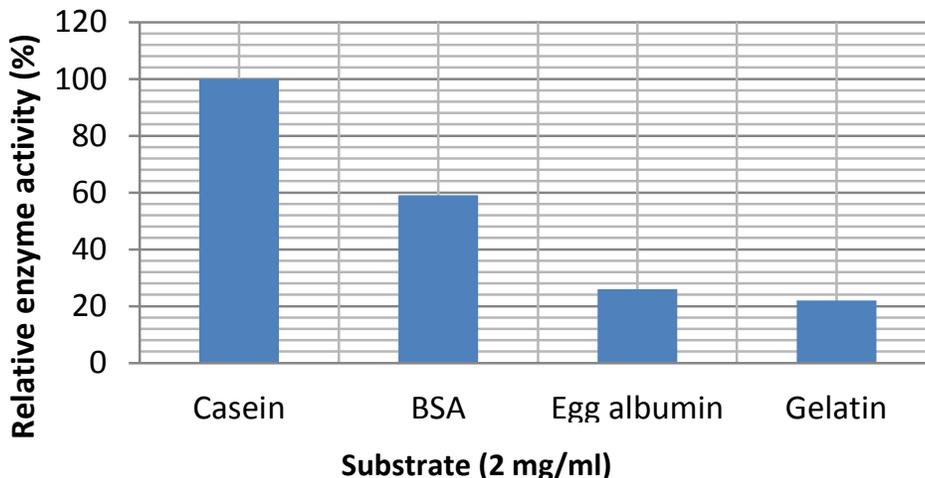


Figure 10. Hydrolysis of different natural substrates.

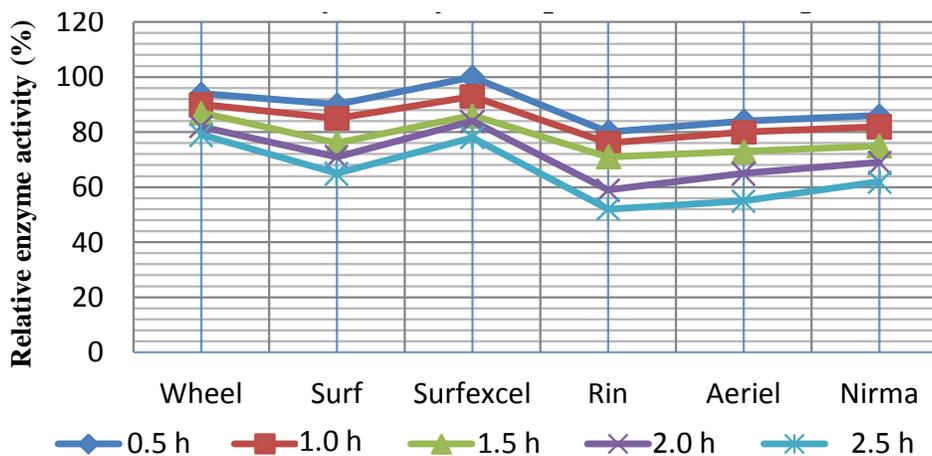


Figure 11. Compatibility and stability of enzyme with various detergent formulations.

Hydrolysis of protein substrates

When assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein and poor to moderate hydrolysis of BSA and egg albumin; although the hydrolysis was hardly observed with gelatin (Figure 10) (Loops et al., 2003).

Compatibility with detergents

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. Protease from *B. thuringiensis* SH-II-1A showed stability and compatibility with a wide range of commercial detergents at 45°C in the presence of CaCl₂ and glycine as stabilizers using distilled water. Isolated protease has shown good stability and compatibility in the presence of

detergent Surf excel. The enzyme retained more than 60% activity with most of the detergents tested even after 2.5 h incubation at 45°C after the supplementation of CaCl₂ and glycine (Annamalai et al., 2013). High activity alkaline protease was reported from *Conidiobolus coronatus* showing compatibility at 50°C, in the presence of 25 mM CaCl₂, with a variety of commercial detergents. Also, 16% activity was reported in Revel, 11.4% activity in Aerial and 6.6% activity in Wheel. Comparing these results, the *Bacillus subtilis* PE-11 enzyme was significantly more stable in commercial detergents. The compatibility of alkaline protease was studied with Surf excel in the presence of 10 mM CaCl₂ and 1 M glycine for different periods (0.5 to 2.5 h) at 45°C. The enzyme retained about 82% activity after 1.5 h in the presence of surf excel at 45°C and was almost inactivated after 2.5 h in the absence of any stabilizer (Figure 11). However, the addition of CaCl₂ (10 mM) and glycine (1 M), individually



Figure 12. Washing performance of protease in presence of detergent (Surf excel). **A.** Cotton cloth stained with blood; **B.** cloth stained with blood and washed with distilled water only; **C.** cloth stained with blood and washed with detergent only; **D.** cloth stained with blood and washed with the detergent and enzyme solution.

and in combination, was very effective in improving the stability, where it retained 60% activity even after 2.5 h. As the protease produced by our isolate *B. thuringiensis* SH-II-1A was stable over a wide range of pH values and temperatures and also showed compatibility with various commercial detergents tested in the presence of CaCl_2 and glycine, it was used as an additive in detergent to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent (that is, Surf excel) could significantly improve the cleansing of the blood stains (Figure 12).

Conclusion

The alkaline protease isolated from *B. thuringiensis* SH-II-1A is a thermostable serine protease. It is stable at alkaline pH, at high temperatures, and in the presence of commercial detergents and is compatible with commercial and local detergents. These properties indicate the possibilities for use of the protease in the detergent industry. This enzyme can be exploited commercially.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

NCCS, National Centre for Cell Sciences; **DEAE**, di ethyl amino ethyl; **CBB**, co-ommassie brilliant blue; **SDS**-

PAGE, sodium dodecyl sulphate poly-acrylamide gel electrophoresis; **TCA**, tri-chloro acetic acid; **UV**, ultra violet; **BSA**, bovine serum albumin; **p-CMB**, para chloro mercuric benzoate; **PMSF**, phenyl methyl sulfonyl fluoride; **β -ME**, β -mercapto ethanol; **DFP**, di isopropyl fluoro phosphate.

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