

Partial Purification of Alkaline Protease as Thrombolytic Agent from Mutant Strain *Bacillus licheniformis* EMS250-O-1

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Received: July 31, 2016; Accepted: September 18, 2016; Published (web): December 27, 2016

ABSTRACT: Thrombosis leads to myocardial infarction, stroke and other cardiovascular complications. Microbial thrombolytic agents such as urokinase, streptokinase etc. are used to treat complications related to thrombosis. To search for new microbial enzymes as thrombolytics having better efficacy and specificity, *Bacillus licheniformis* EMS-O-1 mutant strain was cultured in modified urea-molasses media followed by purification using ammonium sulphate precipitation and ultrafiltration through centricon tube of 100 MWCO value. The yield of crude enzyme was 11129.14 U/mg and after purification 40180.46 U/mg. Purification process increased the specific activity of purified enzyme to 12.28 fold with a recovery of 17.79%. The purified enzyme was a serine protease with molecular weight of 25.5 kDa as confirmed by irreversible inhibition of activity with phenylmethylsulfonyl fluoride (PMSF) followed by SDS-PAGE gel image and by LC-MS analyses. *In vitro* clot lysis assay of the purified enzyme exhibited 38.30% thrombolytic activity. The crude enzymes from the mutant strain EMS-O-1 were found to be stable up to 50 °C and showed maximum stability between pH range 7.5 to 8.5. These findings signify that proteases produced by *B. licheniformis* mutant have the potential to be developed as a viable thrombolytic agent.

Key words: *Bacillus licheniformis*; Protease; Purification; Thrombolytic activity

INTRODUCTION

Proteolytic enzymes or proteases are very important constituents in living systems. They are related in almost every aspects of life such as protein turnover, sporulation, germination, enzyme modification, blood clotting, tumor progression etc.¹ Their industrial applications include detergents, food industry, leather industry, manufacture of aspartame etc.² Instead of complicated protein biology, proteases are relatively safe and well tolerated which enables their applications in thrombolysis, correction of coagulation disorders, sepsis, neuromascular problems, digestion, etc.¹

Formation of clots (thrombus) in blood vessels can lead to serious disease to human such as myocardial infarction and stroke. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis.^{3,4} A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase (u-PA), and bacterial plasminogen activator streptokinase have been extensively studied and used as thrombolytic agents. Their major functions have been described as plasminogen activators or plasmin-like proteases which can directly degrade fibrin or fibrinogen, thereby dissolving thrombi rapidly and completely.^{5,6}

Over the last 10 years, several effective thrombolytic agents have been identified and characterized from microorganism. Therefore,

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microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases.⁷⁻⁹ Fibrinolytic enzymes were successively discovered from different microorganisms, the most important among which is the genus *Bacillus* from traditional fermented foods. These bacterial strains have been found to effectively produce extracellular and intracellular proteases. Many previous researches have shown to successfully isolate and purify the fibrinolytic enzymes from *Bacillus* sp. for examples, *Bacillus* sp. strain CK 11-4 was discovered from traditional Korean fermented-soybean sauce, named Chung-Kook-Jang¹⁰, *Bacillus subtilis* DC33 was isolated from Chinese traditional Douchi¹¹ and *Bacillus licheniformis* KJ-31¹² isolated from Korean traditional Jeot-gal. The physicochemical properties of these enzymes have been characterized, and their effectiveness in thrombolysis in vitro has been further evaluated.

Thrombolytic agents such as microbial streptokinase and urokinase, and recombinant tissue plasminogen activators such as altepase are used to dissolve the clots.¹³⁻¹⁵ Unfortunately, these agents are often expensive, thermolabile and produce undesirable side effects. Therefore, further research needs to be carried out to find new fibrinolytic enzyme as potential antithrombotic drug. Recently, proteases produced by *Bacillus* species have been identified to possess fibrinolytic and thrombolytic activities.^{16,17} These finding has opened a gateway to isolate newer thrombolytic agents from *Bacillus* species as future antithrombotic therapy. The present study has been designed to develop the process to produce and purify the proteases produced by *Bacillus* species obtained from different sources such as foods, soils, etc.

MATERIALS AND METHODS

Microorganism and culture. *Bacillus licheniformis* EMS-O-1, mutant of *Bacillus* species isolated from feather decomposed soil was used to purify the protease¹⁸ and stock culture was

maintained at -70°C in nutrient broth containing 10% glycerol. Initially, *B. licheniformis* EMS-O-1 was streaked onto TSA plates and incubated for 2 days at 37°C and then the organisms were transferred to screw cap test tubes containing 5 ml sterile TSB and incubated overnight at 37°C and used as seed culture. Then seed culture was transferred in each 500 ml Erlenmeyer flask containing 95ml of modified urea-molasses medium (10g molasses, 5g urea, 5g K₂HPO₄ and 1g MgSO₄, initial pH 7.5) and incubated at 37 °C and 150 rpm in a thermostated orbital shaker for 48h.

Purification of enzyme. Alkaline protease rich culture supernatant was separated from biomass by centrifugation of ferment at 5,000 g for 10 min. Then it was subjected to ammonium sulphate precipitation at a saturation level from 50% upto 80% (w/v) followed by centrifugation at 10,000 g at 4°C for 30 minutes. The precipitate obtained was dissolved in 50 mM Tris-HCl buffer (pH 7.0).^{19,20} This partially purified enzyme was then concentrated by ultrafiltration passing through centricon tubes (Amicon®, Millipore)^{21,22} of 100 kDa MWCO and 30 kDa MWCO.

Estimation of extracellular soluble protein and protease activity. Concentration of extracellular soluble protein was estimated by Bradford method.²³ Bovine serum albumin (BSA) was used as standard and absorbances were recorded at 595nm using a spectrophotometer (GENESYS™ 5, Thermo Scientific). The protein concentration was calculated by drawing a standard calibration curve of bovine serum albumin. On the other hand, protease activity was estimated by a modified procedure described by Krieger and Lockwood using azo-casein (Sigma, USA) as a substrate.²⁴ For this study, 400 µl of culture supernatant was mixed with 400 µl of 1% Azo-casein solution in 0.05 mM Tris-HCl buffer (P^H 8.5) and incubated for 1 hour at 37 °C in a shaking water bath. After stopping the reaction with 135 µl of 35% trichloroacetic acid (TCA), the mixture was kept for 10 min at 4 °C and then centrifuged at 10,000 g for 10 min. The supernatant was mixed with NaOH (1 M) at 1:1 ratio and absorbance was recorded at

440 nm immediately. A control reaction was carried out at the same time by adding TCA before mixing the culture supernatant with azo-casein solution. An increase in absorbance of 0.01 at 440 nm was considered as one unit of protease activity which reflected the amount of enzyme and expressed in U/ml. Specific activity (U/mg) was calculated from enzyme activity and extracellular protein concentration (mg/ml).

Protein molecular weight determination.

Permeates from centricon tubes (both 100 kDa MWCO and 30 kDa MWCO) were separated by electrophoresis on sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) using 12% gradient polyacrylamide gel and 4% polyacrylamide stacking gel. The gel sheet was then stained with coomassie brilliant blue (CBB)^{25,26} and molecular weight of the partially purified enzyme on gel image was estimated using Alpha View software. To further confirm, separated protein band was excised from the gel, destained and then extracted using buffer 5% formic acid/acetonitrile (1:2, v/v). The extracted protein was subjected to ESI LC/MS analysis (LC parameters: solvent-water/acetonitrile with 0.1% formic acid, flow rate - 0.5 ml/min, Column- Poroshell 120 EC-C18, Agilent Technologies, System: Shimadzu Prominence UFLC/UFLCxR; MS parameters: System: AB Sciex Triple TOF 5600, Scan for TOF MS: 600 – 3000 Da).²⁷

Stability of enzymes. Stability of crude protease was determined by pre-incubating the protease at different temperatures and pH. To assess the thermal stability crude proteases were incubated at 25°C, 30°C, 40 °C, 50 °C, 60 °C and 70 °C for 1 hr. On the other hand, to assess the pH stability crude protease was incubated by diluting the enzyme in different buffers (pH 5.0 - 11.0) and incubated for 1 hr at room temperature. Buffers used for this study were citric acid buffer (pH 5.0 - 6.0), phosphate buffer (pH 7.0 - 8.0), carbonate buffer (pH 9.0 - 10) and sodium bicarbonate buffer (pH 11). After incubation, the enzyme activity was assayed as described above.

Thrombolytic activity assay. Thrombolytic activity of the partially purified enzymes were

assessed in vitro by clot lysis method. Streptokinase was used as standard and PBS was used as control.²⁸ Briefly, fresh venous blood was transferred in different pre-weighed microcentrifuge tube (500 µl/tube) and allowed to form clot incubating at 37°C for 45 minutes. Then serum was completely removed from each tube and weight of the clot in each tube was calculated. Then, 100 µl of partially purified enzymes were added in each tube. Commercially available streptokinase and PBS were used as control. All tubes were incubated at 37°C for 90 minutes for the lysis of clot. Then lysed clot was removed from each tube and weighed again. The percentage of weight loss in each tube was calculated. The assay was repeated thrice and the values were expressed as mean ± SEM. The significance between % clot lysis by streptokinase and purified protease was analyzed by *t*-test.

RESULTS AND DISCUSSION

The production method yielded crude enzyme with specific activity of 1129.14 U/mg from the *Bacillus* sp. mutant strain EMS250-O-1 and total activity was found to be 405900.00 U (Table 1). The culture supernatant of EMS250-O-1 mutant was subjected to ammonium sulphate precipitation at 60% saturation which concentrated the proteins with specific activity of 40180.46 U/mg, while total activity enzyme was estimated as 135916.50 U. The initial total protein content was estimated 359.52 mg which reduced to 41.54 mg after salting out. Further ultra filtration with 100 kDa MWCO centricon tube reduces the protein concentration to 1.78 mg/ml. This purification process increased the specific activity by 12.28 folds with a recovery of 17.80% (Table 1). The successive purification through salt precipitation and ultrafiltration process increased the specific activity to 3272.75 U/mg and 40180.46 U/mg, respectively (Table 1).

The total protein content of the culture supernatant decreased when the crude enzymes were subjected to ammonium sulphate precipitation. The purified enzyme obtained after ammonium sulphate precipitation and ultrafiltration through centricon

tube suggested that the ultrafiltration could retain the specific molecular weight proteins suggesting that our approach for purification was effective. The

purified protease was identified as serine protease by irreversible inhibition of activity with phenylmethylsulfonyl fluoride (PMSF).

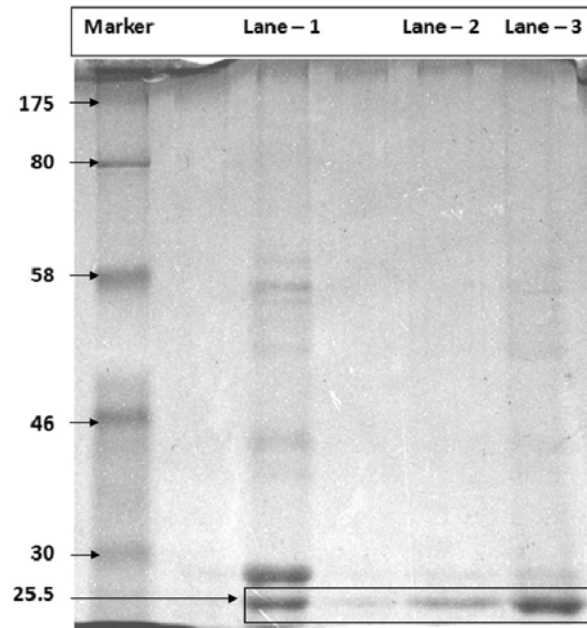


Figure 1. Molecular weight determination of the purified protease enzyme from *Bacillus sp.* EMS250-O-1 by polyacrylamide gel electrophoresis. Lane 1: Ammonium sulfate precipitated enzyme, Lane 2: 100 kDa Centricon tube retained enzyme, Lane 3: 30 kDa MWCO Centricon tube permeate enzyme.

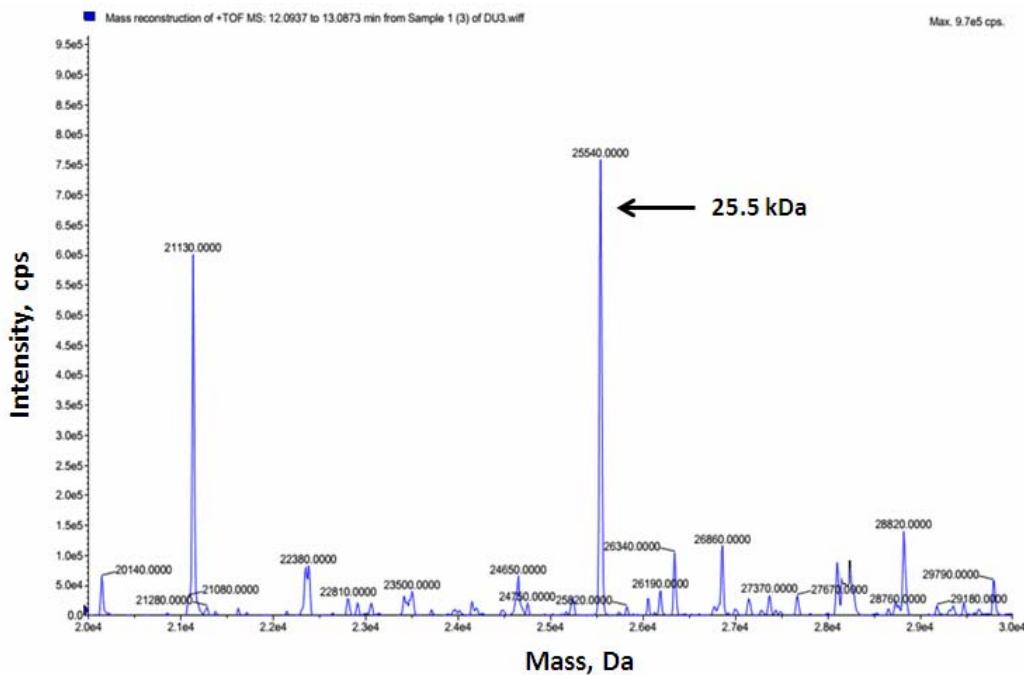


Figure 2. Determination of molecular weight of partially purified proteases resolved on SDS-PAGE by LC-MS analysis. Intense signal at 25.5 kDa and less intense signal at 21.1 kDa indicating existence of two proteins which fairly converge to the molecular range of protease secreted from bacteria.

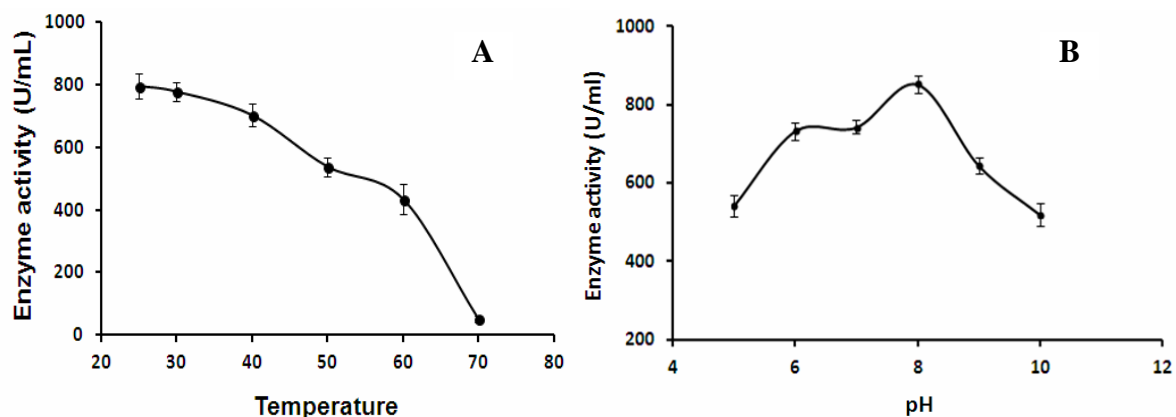


Figure 3. Effect of temperature (A) and pH (B) on the stability of crude alkaline protease

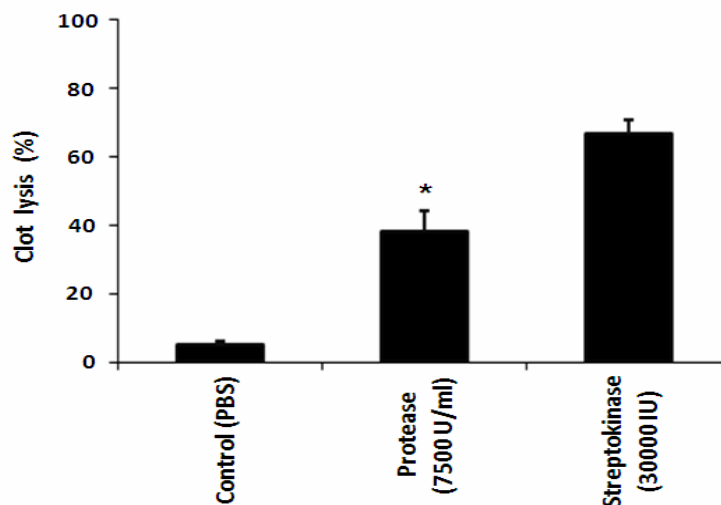


Figure 4. Thrombolytic effects of purified protease from *Bacillus sp.* mutant strain EMS250-O-1 with streptokinase and PBS. Data represented as mean ± SEM of three independent experiments. *P<0.001 vs control (phosphate buffered saline).

Table 1. The purification steps of thrombolytic protease enzyme from mutant strain *B. licheniformis* EMS250-O-1.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	405900.00	359.52	1129.14	1	100
Ammonium Sulfate precipitated enzyme	135916.50	41.54	3272.75	2.90	33.49
100 kd Centricon tube permeate	72233.85	1.78	40180.46	12.28	17.80

Permeates from 100 kDa MWCO tube retained enzymes was revealed by SDS-PAGE analysis of salt precipitated enzyme followed by gel image analysis (Figure 1). The presence of approximately 25.5 kDa protein was confirmed when permeates from 30 kDa MWCO centricon tube was analyzed by SDS-PAGE. Furthermore, gel digestion followed by LC-MS analysis as described above confirmed the molecular

weight of the partially purified enzyme from EMS250-O-1 mutant (Figure 2). The separated protein band of 25.5 kDa on gel, though contained multiple proteins which fairly converge to the molecular range of serine protease, secreted from the strain. Based on SDS-PAGE and LC-MS analysis, it was observed that the estimated molecular weight of partially purified targeted thrombolytic protein is

25.5 kDa approximately. The purified enzyme was tested for its thrombolytic activity as described under materials and methods.

The stability study of the protease from *Bacillus sp.* mutant strain EMS-O-1 showed that the activity remained more or less stable up to 50 °C, after which the activity began to decrease slowly. Activity was found to be decreased rapidly at 55 °C (Figure 3A). The enzyme was found to be highly stable at 25 °C as it showed highest activity (793 U/ml) at this temperature. On the other hand, the enzyme showed highest stability between the pH range 7.5 to 8.5. The highest activity obtained at this point of highest stability was 853.0 U/ml (Figure 3B). The stability of the enzyme was found to be decreased beyond this pH range.

Thrombolytic activity assay of the partially purified protease enzyme showed that the average % of clot lysis by the enzyme obtained from *Bacillus sp.* mutant strain EMS250-O-1 were $32.58 \pm 2.88\%$, $43.11 \pm 3.25\%$ and $38.34 \pm 2.04\%$, respectively for three independent assays carried out in triplicate (Figure 4) with an average activity of 38.30%. This data is an indication of thrombolytic activity of the purified protein. Previous work on purification and characterization of thrombolytic enzymes from bacterial sources reported that the purified thrombolytic enzyme has a molecular weight within the range of 20-45 kDa.^{1,12}

CONCLUSION

A number of microbial enzymes have been reported to have thrombolytic activities. Among them streptokinase (a bacterial protein from beta haemolytic *E. coli*) is used in practice as a thrombolytic agent. The beneficial effects and biochemical mechanisms of thrombolytic therapy have been established and search for new and alternative therapy is still continuing due to diversity of natural resources. *Bacillus* species have been recognized as source of protease enzymes having thrombolytic activities.^{1,11,12} The nature of thrombolytic activity of microbial proteases in clot is similar to that of streptokinase. From our present

study, it is evident that the thrombolytic proteases obtained from *B. licheniformis* strain EMS250-O-1 has significant clot lysis activity. Further research can be conducted to develop a novel and economically viable therapeutic thrombolytic agents using these thrombolytic proteases.

ACKNOWLEDGEMENTS

The authors gratefully acknowledges the Ministry of Science and Technology, Government of the People's Republic of Bangladesh for funding support under special allocation for Science and Technology Programme 2015-2016 (Sl.217, Gr. Sl. 91,MEDI;S). Authors are also thankful to Incepta Pharmaceuticals Ltd., Ashulia, Dhaka, Bangladesh for providing Laboratory support for LC-MS analysis.

CONFLICT OF INTEREST

We declare no conflict of interest.

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