

Thrombolytic Activity of Alkaline Protease Purified from a Mutant Strain *Bacillus licheniformis* MZK05M9

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Abstract

Investigations were performed to find out new microbial enzymes as thrombolytics having better efficacy and specificity. Mutant strain of *Bacillus* species, *B. licheniformis* MZK05M9 was cultured in modified urea-glucose media followed by purification using ammonium sulphate precipitation and ultrafiltration through centricon tube of specific MWCO value. The production method yielded 823.42 units/mg of the crude enzyme from mutant strain MZK05M9 and after purification 37695.64 units/mg. The molecular weight of the purified enzyme was estimated as 27.2 kDa and purification increased its specific activity to 16.5 fold with a recovery of 10%. The purified proteases were identified as serine proteases by irreversible inhibition of activity with phenylmethylsulfonyl fluoride (PMSF) and it exhibited 32.84% thrombolytic activity, by *in vitro* clot lysis assay. Stability studies showed that crude enzyme from mutant strain MZK05M9 remained stable up to a temperature of 45°C and showed maximum stability at pH range 7.5 to 8.5. Our observation indicates that proteases produced by *Bacillus licheniformis* mutant have the potential to be developed as a viable thrombolytic agent.

Key words: *Bacillus licheniformis*, protease, purification, thrombolytic activity.

Introduction

Accumulation of fibrin in the blood vessels usually results in thrombosis. Various types of thrombosis lead to different cardiovascular diseases (CVD) such as myocardial infarction (commonly known as heart attack), stroke, transient ischemic attack (TIA), venous thromboembolism (VTE) (e.g., deep vein thrombosis (DVT), pulmonary embolism (PE), etc. CVDs are the primary cause of death globally and are projected to remain the single leading cause of death (Mathers and Loncar, 2006).

Thrombolytic agents are used to dissolve a blood clot (thrombus) by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. Because of these

actions, thrombolytic drugs are also called "plasminogen activators", "fibrinolytic drugs", "clot-dissolving medication" and "clot buster" etc. The available thrombolytic agents are of two types, based on their different working mechanisms. One is plasminogen activator, such as tissue-type plasminogen activator (t-PA) (Collen and Lijnen, 2004), urokinase (Duffy 2002), bacterial streptokinase (s-PA), which activate plasminogen into active plasmin to degrade fibrin. Recombinant forms of normal human plasminogen activators t-PA and u-PA are used in clinical intervention. The other type is plasmin-like proteins, which directly degrade the fibrin in blood clots, thereby dissolving the thrombus rapidly and completely. Lumbrokinase from earthworm and fibrolase from snake venom are well-known plasmin-like proteins (Mihara *et al.*, 1991; Chen *et al.*, 1991).

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Although t-PA and urokinase are still widely used in thrombolytic therapy today, their expensive prices and undesirable side effects, such as risk for internal hemorrhage within the intestinal tract, their uses are often limited and investigations are being pursued to search for cheaper and safer resources. The microbial sourced streptokinase is the least expensive, but immunogenic as it is a non-human protein (Lee, 1995; Jennings, 1996). This immunogenicity restricts multiple treatments with streptokinase (Collen *et al.*, 1992; Bick, 1982). Microbial fibrinolytic enzymes have attracted much more medical interest in recent decades (Tough, 2005, Goldhaber and Bounameaux, 2001). Recently, protease produced by *Bacillus* species have been identified to possess thrombolytic activity such as nattokinase (NK) from *Bacillus natto* and subtilisin DFE and subtilisin DJ-4 from *Bacillus amyloliquefaciens* (Sumi *et al.*, 1987; Kim and Choi, 2000; Peng *et al.*, 2003). These proteases are mainly serine (e.g., Nattokinase) or metalloproteases (e.g., *Armillaria mellea* metallo protease) or mixture of both serine and metallo protease (e.g., Protease from *Streptomyces*) (Liu *et al.*, 2005; Wang *et al.*, 1999). In 1987, *B. natto* producing NK was first screened from a traditional Japanese soybean-fermented food named natto. Subsequently, some other *Bacilli* such as *B. Amyloliquefaciens* DC-4 (Peng *et al.*, 2003), *Bacillus* sp. CK (Kim *et al.*, 1996), *Bacillus* sp. strains DJ-2 and DJ-4 (Kim and Choi 2000; Choi *et al.*, 2005), and *Bacillus* sp. KA38 (Kim *et al.*, 1997) from different fermented foods were discovered to produce fibrinolytic enzymes. These exciting findings imply the possibility of consuming fermented foods to prevent cardiovascular diseases (Suzuki *et al.* 2003). These findings have opened a gateway to isolate newer thrombolytic agents from *Bacillus* species as future antithrombotic therapy. The present study has been designed to produce and purify the proteases produced by *Bacillus* species obtained from different sources such as foods, soils, etc to be developed as thrombolytics agent to prevent or cure thrombosis and other related diseases.

Materials and Methods

Microorganism and culture: The microorganism used was a mutated *Bacillus* species, *Bacillus licheniformis* MZK05M9 isolated from feather decomposed soil (Hoq *et al.*, 2005). Stock culture of the organism was maintained at -70°C in nutrient broth containing 10% glycerol. A loopful of the spore suspension from the lyophilized seed-lot of mutant *B. licheniformis* MZK05M9 was streaked onto TSA plates and incubated for 2 days at 37°C . One loopful of the organisms from these plates were transferred to screw cap test tubes containing 5 ml sterile TSB and incubated overnight at 37°C . These were used as inocula for fermentation in Erlenmeyer flasks.

The seed culture (5 ml of mutant *B. licheniformis* MZK05M9) was transferred to 95 ml of modified urea-glucose medium in each 500 ml Erlenmeyer flask. 1000 ml modified urea-glucose medium contained 20 g glucose, 10 g urea, 5 g K_2HPO_4 , 1 g CaCl_2 and 1 g MgSO_4 (initial pH 7.5). The inoculated flasks were placed in a thermostated orbital shaker for 48 hrs, at 37°C and 150 rpm.

Purification of enzyme: After 48 h of fermentation in the modified urea-molasses medium, the extracellular alkaline protease rich fraction was harvested by centrifugation of ferment at 6,000 rpm for 10 min. The culture supernatant obtained after centrifugation was subjected to ammonium sulphate precipitation at a saturation level from 50% upto 80% (w/v) and after centrifugation at 10,000 g at 4°C for 30 minutes the resulting precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.0) (Dawson *et al.*, 1986; Taylor, 1953). The crude enzyme as well as partially purified enzyme was evaluated for protease activity and thrombolytic activity. Furthermore, partially purified enzyme was concentrated by ultrafiltration through centricon tubes (Maity *et al.*, 2007; Guerrieri *et al.*, 2000). In this purification purpose, 100 kDa MWCO and 30 kDa MWCO centricon tubes (Amicon®, Millipore) were used and proteins were concentrated by centrifugation at 5,000 g for 15 minutes (2.5×30 cm, Amersham Biosciences, Sweden). The permeate enzymes were then used for thrombolytic activity study.

Estimation of extracellular soluble protein and protease activity: The extracellular soluble protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Absorbances were recorded at 595 nm using a spectrophotometer (GENESYS™ 5, Thermo Scientific) and protein concentration was determined based on the standard curve.

Enzyme activity was determined using azo-casein (Sigma, USA) as a substrate by a modified procedure described by Krieger and Lockwood (Krieger and Lockwood, 1981). Briefly, 400 µl of 1% Azo-casein solution in 0.05 mM Tris-HCl buffer (pH 8.5) was added to 400 µl of culture supernatant and kept for 1 hour at 37°C in a shaking water bath. The reaction was stopped by the addition of 135 µl of 35% trichloroacetic acid (TCA) and the mixture was kept at 4°C for at least 10 min. After centrifugation at 13,000 rpm for 10 min, 0.75 ml of supernatant was mixed with 0.75 ml of 1 M NaOH and the absorbance was recorded at 440 nm within one minute in spectrophotometer, (GENESYS™ 5, Thermo Scientific). The control was prepared by adding TCA before mixing the culture supernatant with azo-casein solution. One unit of protease activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 at 440 nm under the above assay condition. The value obtained is expressed in U/ml

Protein molecular weight determination: To estimate the molecular weight, permeate protein from 100 kDa MWCO and 30 kDa MWCO centricon tube was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gradient polyacrylamide gel and 4% polyacrylamide stacking gel. The gel sheet was stained with 0.25% coomassie brilliant blue (CBB) [Laemmli, 1970; Merrill, 1990]. The molecular weight of the partially purified enzyme was determined by gel image using Alpha View software.

Stability of enzymes: Thermal stability of crude protease was determined by pre-incubating the protease at temperatures of 25, 30, 40, 50, 60 and 70°C for 1 hrs. After incubation, the enzyme was

assayed by incubating with 1% (w/v) azo-casein as substrate at 37 °C and the protease activity was measured at 440 nm taking untreated enzyme as control. The control was prepared by adding TCA before mixing the culture supernatant with azo-casein solution. On the other hand, the pH stability of crude protease was determined by diluting the enzyme in different buffers (pH 5.0 – 11.0) and incubated for 1 h at room temperature. Following buffers were used for this study: 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 11.0). After incubation, the enzyme activity was assayed as described above.

Thrombolytic activity assay: To assess the thrombolytic activity of enzyme *in vitro* clot lysis model was adopted (Al-Mamun et al., 2012). Streptokinase was used as standard and PBS was used as control. In this method, fresh venous blood collected from healthy volunteers at the Dhaka University Medical Center following institutional ethical guidelines and transferred in different pre-weighed sterile microcentrifuge tube (500 µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed and each tube having clot was again weighed to determine the clot weight. Each microcentrifuge tube containing clot was properly labeled and 100 µl of partially purified enzymes from 100 MWCO centricon tube permeate, commercially available streptokinase (of different dilutions) and PBS was added to the respective tubes. After incubation of all tubes at 37°C for 90 minutes, fluid released due to lysis of clot was removed from each tube and weighed again. Difference in weight taken before and after clot disruption was considered to calculate the clot lysis in percentage. The percentage of weight loss in each tube was considered as an evidence of thrombolytic activity of partially purified enzyme by comparing with streptokinase and control. The assay was repeated thrice and the values were calculated as mean ± SEM. The significance between % clot lysis by streptokinase and purified protease was tested by the paired *t*-test analysis.

Results and Discussion

The supernatant from the culture media of strain MZK05M9 was subjected to fractional ammonium sulphate precipitation, where the crude enzymes precipitated at 60% of ammonium sulphate saturation. The initial total protein content was 264.286 mg which reduced to 28.696 mg after salting out. Ultrafiltration with 100 kDa MWCO centricon tube reduces the protein concentration to 0.589 mg/ml. The initial specific activity of crude enzyme was 849.811 mg/ml and after successive purification through salt precipitation and ultrafiltration, the final specific activity increased to 2286.863 units/mg and

37707.028 units/mg, respectively (Table 1). The total protein content in crude enzymes was decreased due to ammonium sulphate precipitation. However, purification process yielded 16.5 folds increase in specific activity with a recovery of 10%. The partially purified enzyme obtained by ultrafiltration through centricon tube could retain the specific molecular weight proteins. This suggests that our approaches for purification were effective. Furthermore, irreversible inhibition of enzyme activity with phenylmethylsulfonyl fluoride (PMSF) confirmed that purified enzyme was serine protease.

Table 1. Purification of thrombolytic protease from mutant Strain *B. licheniformis* MZK05M9.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude enzyme	222030	264.286	849.811	1	100
Ammonium sulfate precipitated enzyme	65629.8	28.696	2286.863	2.777	29.559
100 kd Centricon tube permeate	22213.5	0.589	37707.028	16.482	10.004

Table 2. Thrombolytic enzymes from *Bacillus* species.

Author	Microorganism	Specific activity (U/mg)	Purification Fold	Recovery (%)	M.W. (kDa)
Kim et al. 1996	<i>Bacillus</i> sp. CK 11-4	143.3	7.5	95	28.2
Lee et al. 2010	<i>B. amyloliquefaciens</i> CH86-1	100.2	25.96	1.22	27
Cheng et al. 2006	<i>B. subtilis</i> DC33	15,494.90	34.6	13	30
Yin et al. 2010	<i>B. subtilis</i> YJ1	1791.9	9.2	9.5	27.7
Chang et al. 2000	<i>B. subtilis</i> IMR-NK1	4400	9.2	6.5	31.5
Park et al. 2013	<i>B. subtilis</i> WRL101	112.2	8.6	39.7	29
Zhang et al. 2005	Subtilisin DFE in <i>E. coli</i>	4597	5.4	5.2	
Jeong et al. 2001	<i>B. subtilis</i> BK-17	63165	929	29	31
Balaraman et al. 2006	<i>B. sphaericus</i>	4258	91	25	18.6
Kotb et al. 2011	<i>B. subtilis</i> K42	19025.9	389.6	14	20.5
Sanusi et al. 2012	<i>Bacillus</i> sp. strain B1	1.17	59	0.51	45
Jo et al. 2011	<i>B. amyloliquefaciens</i> MJ5-41	11.58	3.99	33.81	27
Hwang et al. 2007	<i>B. licheniformis</i> KJ-31	242.8	19.0	0.2	37
Asad uz Zaman et al. 2016	<i>B. licheniformis</i> EMS250-O-1	40180.46	12.28	17.79	25.5

For SDS-PAGE analysis of purified protein, protein sample was taken from 100 kDa MWCO centricon tube retained enzyme, 100 kDa MWCO

centricon tube permeate enzyme and 30 kDa MWCO centricon tube permeate enzyme. Gel imaging analysis by Alpha View software predicted the

presence of approximately 27.2 kDa protein band present in both protein sample taken from strain MZK05M9 (Figure 1). The purified enzymes were tested for its thrombolytic activity followed by the method (Al-Mamun *et al.*, 2012) described earlier.

The temperature stability of the protease from strain MZK05M9 showed that the activity of *Bacillus* sp. MZK0509 remained more or less stable up to a temperature treatment of 45°C, after which the activity began to decrease slowly and after 55°C, it

dropped rapidly reaching the lowest value of 14.7 U/ml at 70°C (Figure 2). The highest enzyme activity was obtained at the lowest temperature treatment of 25°C and the value recorded was 519.8 U/ml. The organism, MZK05M9 showed highest enzyme activity and stability at the pH range between 7.5 to 8.5 and there is decrease in activity or stability with decreasing or increasing pH from that point. The highest activity obtained at the point of highest stability was 532 U/ml (Figure 2).

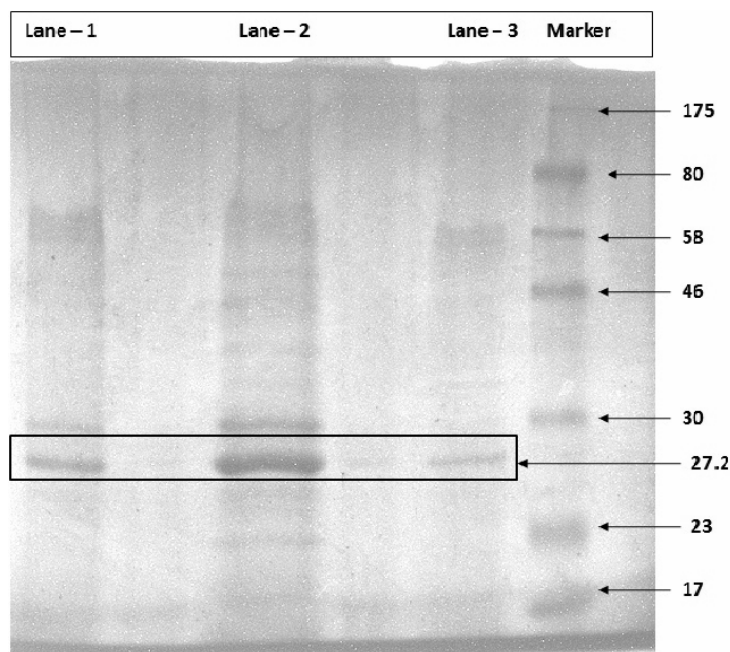


Figure 1. Molecular weight of the purified protease from *Bacillus* sp. MZK05M9. Lane 1: 100 kDa MWCO Centricon tube retained enzyme, Lane 2: 100 kDa Centricon tube permeate enzyme, Lane 3: 30 kDa MWCO Centricon tube permeate enzyme.

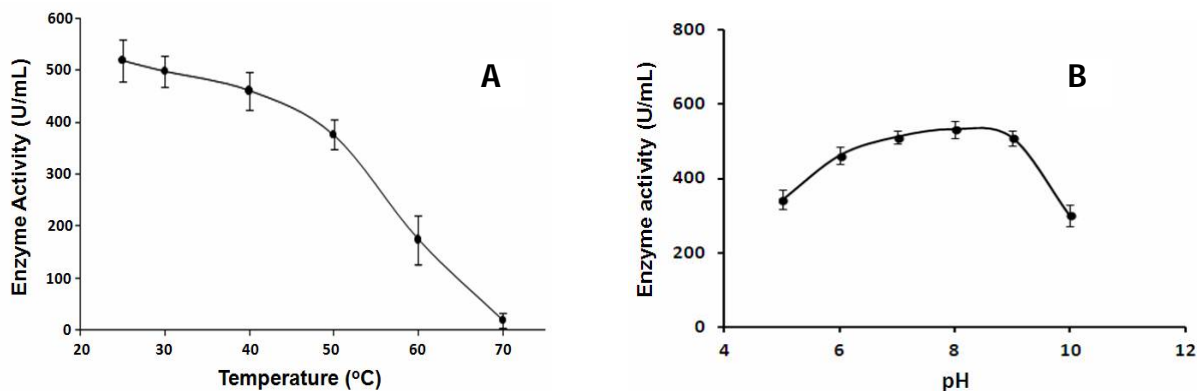


Figure 2. Effect of temperature (A) and pH (B) on the stability of crude alkaline protease

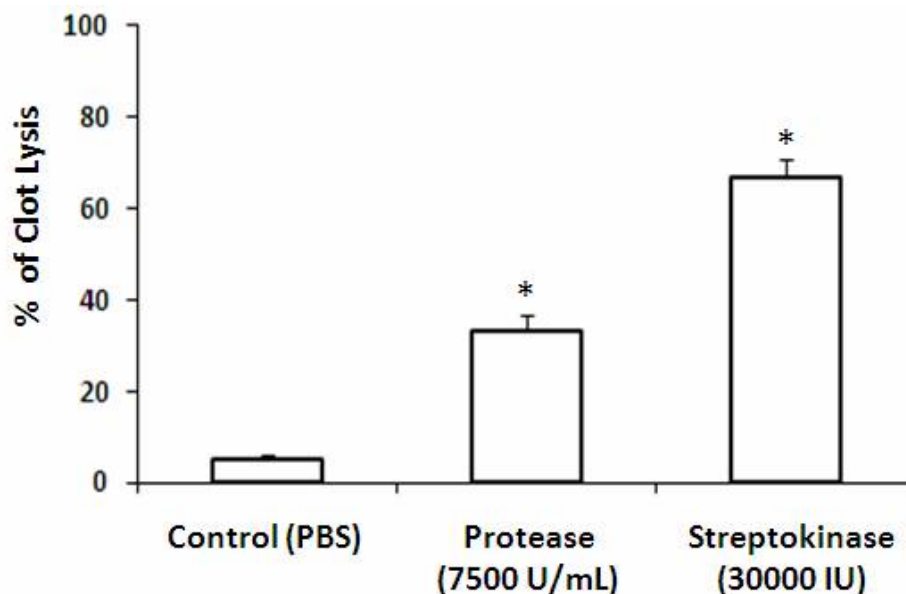


Figure 3. Thrombolytic effects of purified protease from *Bacillus sp.* MZK05M9, with streptokinase and PBS. Data represented as mean \pm SEM of three independent experiments. * $P < 0.001$ vs control (phosphate buffered saline).

To evaluate the thrombolytic activity of the purified enzyme, blood was withdrawn from three healthy volunteers following ethical guidelines set up by Ethical Review Committee of Faculty of Pharmacy, University of Dhaka. Collected blood was allowed to form clots and used for the assay as described under materials and methods. Thrombolytic activity assay data showed that the average % of clot lysis by the enzyme obtained from *Bacillus sp.* MZK05M9 were $28.36 \pm 2.16\%$, $38.21 \pm 1.96\%$ and $31.81 \pm 2.09\%$, respectively for three independent assays carried out in triplicate (Figure 3) with an average activity of 32.79%. The difference in thrombolytic action between the volunteers in response to average activity might be due to body physiological difference (age, weight, % of clotting factor, food habit, etc.). 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 (Table 2). This is an indication of thrombolytic activity of the purified protein.

Conclusion

Thrombolytic enzymes, including plasminogen activators have been reported from various microbes. However, except for streptokinase (a bacterial protein from beta haemolytic *E. coli*), none of them are practically in use as a thrombolytic agent. Although, the beneficial effects of thrombolytic therapy are now well established and the biochemical mechanisms of thrombolytic therapy have been elucidated, but the search for alternative and complimentary therapy is still continuing due to some reasons including availability and diversity of natural resources, easy access and affordability. The nature of thrombolytic activity of the protease on blood clot was similar to that of streptokinase. From the above study, we can conclude that, the thrombolytic proteases obtained from *B. licheniformis* strain MZK05M9 has the potential to be developed as novel and economic therapeutic agents for the treatment of thrombolytic and related diseases with fewer side effects in future.

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