Fibrinolysis and anticoagulant potential of a metallo protease produced by *Bacillus subtilis* K42

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In this study, a potent fibrinolytic enzyme-producing bacterium was isolated from soybean flour and identified as *Bacillus subtilis* K42 and assayed *in vitro* for its thrombolytic potential. The molecular weight of the purified enzyme was 20.5 kDa and purification increased its specific activity 390-fold with a recovery of 14%. Maximal activity was attained at a temperature of 40°C (stable up to 65°C) and pH of 9.4 (range: 6.5–10.5). The enzyme retained up to 80% of its original activity after pre-incubation for a month at 4°C with organic solvents such as diethyl ether (DE), toluene (TO), acetonitrile (AN), butanol (BU), ethyl acetate (EA), ethanol (ET), acetone (AC), methanol (ME), isopropanol (IP), diisopropyl fluorophosphate (DFP), tosyl-lysyl-chloromethylketose (TLCK), tosyl-phenylalanyl chloromethylketose (TPCK), phenylmethylsulfonylfluoride (PMSF) and soybean trypsin inhibitor (SBTI). Aprotinin had little effect on this activity. The presence of ethylene diaminetetraacetic acid (EDTA), a metal-chelating agent and two metallo protease inhibitors, 2,2′-bipyridine and *o*-phenanthroline, repressed the enzymatic activity significantly. This, however, could be restored by adding Co^{2+} to the medium. The clotting time of human blood serum in the presence of this enzyme reached a relative PTT of 241.7% with a 3.4-fold increase, suggesting that this enzyme could be an effective antithrombotic agent.

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1. Introduction

Disorders of blood clotting and fibrinolysis are serious medical problems. Thrombosis, which is particularly serious, can lead to cerebral and myocardial infarction due to un-lysed blood clots (Holden 1990). A number of proteases that can interfere with blood clotting have been purified and characterized from various sources including microorganisms. Some of these proteases are fibrinolytic enzymes capable of digesting fibrin (Sumi *et al.* 1995). The fibrinolytic agents available for clinical use are mostly plasminogen activators, such as tissue-type plasminogen activator, urokinase-type plasminogen activator and the bacterial plasminogen activator, streptokinase. In spite of their widespread use, these agents display low specificity to fibrin, are very expensive and cause undesired effects. Consequently, the search continues for plasmin-like fibrinolytic enzymes from various sources for use in thrombolytic therapy (Holden 1990).

As cardiovascular diseases are one of the leading causes of death throughout the world (WHO 2000), we undertook to study a fibrinolytic enzyme-producing bacterium isolated from soybean flour, which was given the name *Bacillus subtilis* K42. Additionally, we purified this enzyme and assayed it *in vitro* for its thrombolytic potential.

2. Materials and methods

2.1 *Isolation, incubation and screening*

B. subtilis K42 was isolated from soybean flour and identified according to Holt *et al.* (1994). Samples collected were plated onto casein agar plates containing (g/l): bacteriological agar 15, casein 5, peptone 5 and yeast extract 1. Plates were then incubated 24 h at 37°C. A clear zone gave an indication of proteolysis. Different colonies from the plates were purified through repeated streaking on

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fresh agar plates. Purified colonies were finally streaked onto fibrin agar plates composed of (g/l): fibrin 5, ammonium sulfate 2, $CaCl_2$ 1, K_2HPO_4 0.1, KH_2PO_4 0.1, $MgSO_47H_2O$ 0.2 and agar 18, pH 8.0. Isolates, which formed a clear zone around their colonies, were selected, and K42 was retained for this study.

2.2 *Fibrinolytic enzyme production*

Production of fibrinolytic enzyme was carried out in medium containing (w/v): 1% galactose, 0.5% NaCl, 0.5% $CaCO_3$, 0.5% tryptone, 0.3% soybean, 0.15% $MgSO_47H_2O$, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.005% $CuSO_4$ and 0.001% $FeSO_4$ with pH adjusted to 6. Inocula were routinely grown in LB broth composed of (g/l): peptone, 10.0; yeast extract, 5.0; NaCl, 5.0, with pH adjusted to 7. Media were autoclaved at 121°C for 20 min. Cultivations were performed on a rotatory shaker (200 rpm) for 54 h at 37°C, in 250 ml Erlenmeyer flasks with 50 ml medium. The cultures were centrifuged and the supernatants were used for estimation of protein content and fibrinolytic activity.

To study the time course of cultivation, 50 ml of the fermentation medium (pH 6) was used, and the relationships between incubation time (6–96 h) against protease activity and growth were investigated.

2.3 *Protein determination*

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

2.4 *Assay of fibrinolytic activity*

This was basically measured by the method of Anson (1939), but with a few modifications. The reaction mixture contained 1 ml of 1.2% of bovine fibrin solution in boric acid buffer (pH 7.8) and 1 ml of cell-free supernatant (CFS). The reaction mixture was incubated for 2 h at 37°C. Then the reaction was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid. This was followed by centrifugation and assaying the solubilized proteins in the supernatant. One unit of fibrinolytic activity (U) was defined as the amount of enzyme required to liberate 1 μg of L-tyrosine/ml/min at 37°C.

2.5 *Enzyme purification*

The enzyme was purified by three steps including fractional precipitation with ethanol (40–80% saturation levels), ion-exchange chromatography with DEAE-Sepharose FF and gel filtration chromatography with Sephadex G-100. All purification steps were performed at 4°C. Culture broth was centrifuged at 7000*g* for 10 min, and the supernatant was first mixed with chilled ethanol (−15°C), drop-wise, till 80% alcohol concentration, with constant stirring. Protein was collected by centrifugation (10000*g*, 15 min) and dissolved in 20 mM Tris– HCl buffer (pH 7.8, Buffer A). After removing of insoluble material, the crude enzyme solution was applied to a DEAE-Sepharose FF column (1.5×20 cm^2). The column was washed with Buffer B (20 mM Tris–HCl buffer pH 9.4) at a flow rate of 0.5 ml/min and active fractions were pooled and dialysed against buffer B. After dialysis, active

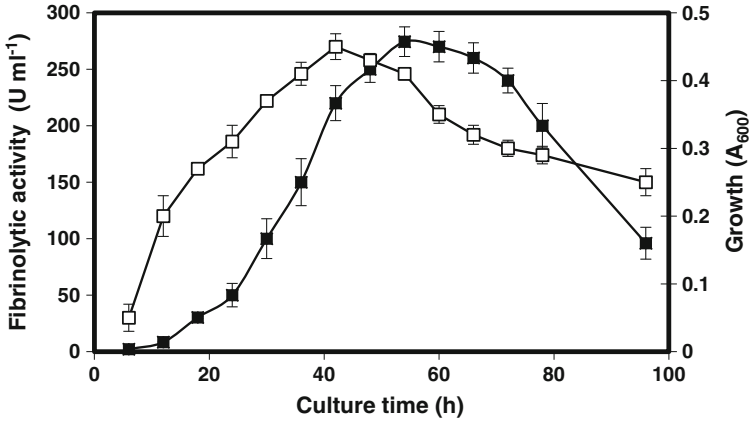

Figure 1. Time courses of growth and enzyme production in a culture of *B. subtilis* K42: (-■-) enzyme activity (U/ml); (-□-) growth. For the production of enzyme, *B. subtilis* K42 was grown in 50 ml of liquid medium in an Erlenmeyer flask (250 ml) containing (w/v): 1% galactose, 0.5% NaCl, 0.5% $CaCO_3$, 0.5% tryptone, 0.3% soybean, 0.15% $MgSO_4.7H_2O$, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.005% $CuSO_4$, and 0.001% $FeSO_4$ with pH adjusted to 6. Each value represents the mean±SD (n=3).

Table 1. Summary of purification steps

Purification step	Total activity (U)	Protein content (mg)	Sp. activity (U/mg protein)	Purification folds	Recovery (%)
Culture broth	371200	7600.0	48.8	1.0	100
80% ethanol	293700	2130.0	137.9	2.8	79
DEAE-Sepharose FF	174684	10.8	16174.4	331.2	47
Sephadex G-100 FF	51370	2.7	19025.9	389.6	14

fractions were passed through a Sephadex G-100 FF column (2.5×100 cm^2) using buffer A. Finally, active fractions were pooled and concentrated by lyophilization.

SDS-PAGE was carried out to determine the purity and molecular weight of the enzyme, as described by Laemmli (1970), using 5 and 15% (w/v) stacking and separating gels, respectively.

2.6 *Biochemical properties*

2.6.1 *Effect of pH on fibrinolytic enzyme activity and stability:* The purified enzyme reaction mixtures were adjusted to different pH values using three buffer system; citrate-phosphate buffer, pH 2–6, sodium phosphate buffer, pH 7–8, and glycine-NaOH buffer, pH 9–13, at 37°C using fibrin as a substrate. The pH stability in the range of 2.0–13.0 was examined by incubating the enzyme solution for 2 h at 37°C with different buffers, and then residual activity at pH 7 was determined.

2.6.2 *Effect of temperature on fibrinolytic enzyme activity and stability:* The effect of temperature on fibrinolytic activity was studied from 15 to 65°C in 0.2 M sodium phosphate buffer, pH 7.8, using fibrin as a substrate. The thermal stability of K42 enzyme was determined by allowing the enzyme solution in 50 mM phosphate buffer (pH 7) to stand for 2 h at various temperatures. At the end of incubation period, at those temperature degrees, the replicate tubes were cooled and assayed for residual activity.

2.6.3 *Effects of metal ions and enzyme inhibitors on enzyme activity:* The effect of various metal ions on the activity of the purified K42 enzyme was investigated by adding metal ions (Co^{2+}, Fe^{3+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+} , Mg^{2+} or Hg^{2+}).

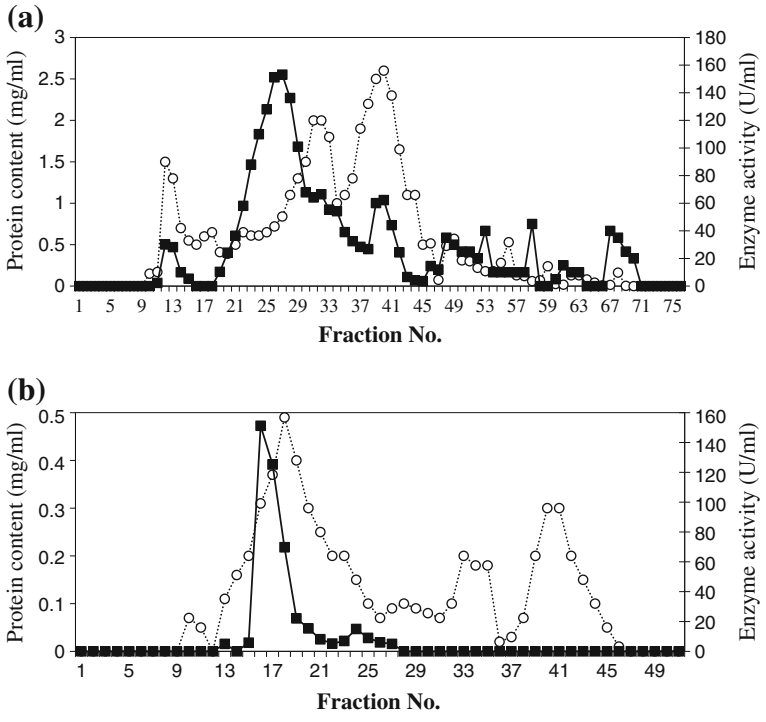

Figure 2. **(a)** Elution profile of K42 protease on DEAE-Sepharose FF: (..○..) protein content (mg/ml) and (-■-) enzyme activity (U/ml). **(b)** elution profile of K42 protease on Sephadex G-100 FF: (..○..) protein content (mg/ml) and (-■-) enzyme activity (U/ml).

The effects of protease inhibitors were also assessed, using EDTA, PMSF, SBTI, DFP, TPCK, TLCK, aprotinin, 2,2′-bipyridine and *o*-phenanthroline. The activity of the enzyme in the absence of metal ions and inhibitors was taken as 100%.

2.6.4 *Influence of organic solvents on fibrinolytic enzyme activity and stability:* Preparations were incubated with solvents (DE, TO, AN, BU, EA, ET, AC, ME and IP) at 20%, v/v at 37°C for 30 min, and the remaining enzymatic activities were measured under normal assay conditions. In this study, phosphate buffer (pH 7) was used as control and the effects of various organic solvents on the stability of fibrinolytic enzyme were investigated at 4°C and room temperature (25°C) for 30 days.

2.7 *In vitro coagulation assays*

Anticoagulant activity *in vitro* was determined as an increase in the relative partial thromboplastin time (RPTT) using Batomunkueva and Egorov (2001) modification of the method described by Strukova *et al.* (1989).The reaction mixture containing 0.1 ml of the human blood serum, 0.1 ml of a thromboplastin suspension and 0.1 ml of kaolin was incubated in a water bath at 37°C for 2 min and then rapidly mixed with 0.1 ml of 0.3% CaCl2. The time of the blood serum clotting was determined using a stopwatch after addition of 0.1 ml of pure enzyme. The control mixture contained an equivalent amount of physiological saline solution. The relative PTT was calculated using the formula $[(A - -B)/B] \times 100\%$, where A and B are the times (in seconds) of the blood serum clotting in the presence and absence of the K42 enzyme, respectively.

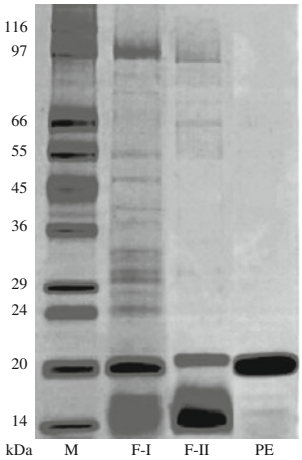

Figure 3. SDS-PAGE profile of fibrinolytic enzyme from *B. subtilis* K42. Fraction I represents the alcohol precipitate recovered with 40% ethanol (lane F-I), fraction II represents the precipitate recovered after 80% alcohol saturation (lane F-II), and the purified enzyme (lane PE) in comparison with protein markers (lane M).

2.8 *Statistical analysis*

The mean values and the standard deviation were calculated from the data obtained with triplicate trials. The data were analysed by Student's paired *t*-test, using the statistical analysis system (SAS Institute Inc., Cary, NC, USA). Significance was determined at $P<0.05$, $P<0.01$ and $P<0.001$ for all analysis.

3. Results

3.1 *Influence of incubation time on enzyme activity*

Figure 1 shows the relationship between incubation time and protease activity of isolate no. 42. Maximum enzyme productivity was found at the 54 h. Enzyme productivity increased along with cell growth (as judged by OD600) and reached a maximum (274.4 U/ml) after cell growth reached a peak at 42 h of incubation.

3.2 *Properties of purified enzyme*

As summarized in table 1, the enzyme was easily and quickly purified by the combination of various steps (figure 2a and b). The final specific activity of the enzyme increased to more than 389-fold with a 14% recovery based on the initial culture supernatant. The finally eluted proteins were subjected to SDS-PAGE that showed one major band with apparent subunit molecular weight of approximately 20.5 kDa (figure 3, lane PE).

The pH activity profile of the purified fibrinolytic enzyme had maximum values at pH 9.4. The optimum temperature for the enzyme was 40°C. The enzyme maintained its initial activity at less than 30°C and retained 76% of it at 60°C, but lost its initial activity after 2 h at 80°C.

3.3 *Effect of organic solvents on enzyme activity and stability*

The effect of different organic solvents (20%, v/v) on the stability of the purified enzyme was also studied. The enzyme activity without any solvent (control) was taken as 100%. The remaining activities were found to be 96.8, 89.0, 94.1, 105.3, 95.8, 101.3, 109.6, 100.5 and 103.5%, in the presence of DE, TO, AN, BU, EA, ET, AC, ME and IP respectively. In general, tested solvents showed stabilizing effect on enzyme activity.

The enzyme was more stable in presence of DE and ET but less stable in presence of AN and BU. As shown in figure 4, after being exposed in 50 mM phosphate buffer (control) at 4 and 25°C for 30 days, the remaining activities in the culture supernatant were 126.3 and 0 U/ml, respectively. In the presence of 20% (v/v) tested organic solvents at 4°C, the enzyme retained more than 80% of its initial activity (stabilizing effect), while at room temperature, enzyme lost most of its activity in presence of AN, but other solvents showed stabilizing effect.

3.4 *Effect of activators and inhibitors on enzyme activity and stability*

Table 2 shows that the pure form of enzyme was inhibited in presence of Cu^{2+} and Mn^{2+}. However, Co^{2+} exerted highly stimulatory effect followed by Mg^{2+} ions, while Zn^{2+}, Fe^{3+}, Ca^{2+}, Ba^{2+} and Hg^{2+} showed a stabilizing effect on the enzyme activity. The effects of protease inhibitors and several chemical reagents are summarized in table 3. Serine protease inhibitors (DFP, TLCK, TPCK and PMSF) and trypsin inhibitors (SBTI and aprotinin) had little effect on the activity. However the metal chelating agent, EDTA, and two metallo protease inhibitors, 2,2′-bipyridine and *o*-phenanthroline, highly repressed the enzymatic activity. The activity of the enzyme was restored when Co^{2+} ions were added in presence of EDTA.

3.5 *Clotting time*

Results of *in vitro* study (table 4) showed that clotting time of human blood serum prolonged with *B. subtilis* K42 fibrinolytic enzyme to 82 s; thus relative PTT reached 241.7% with 3.42-fold increase.

4. Discussion

This study describes the purification, partial characterization and *in vitro* application of a fibrinolytic enzyme K42 from *B. subtilis* K42, isolated from soybean flour. The enzyme was purified to electrophoretic homogeneity by anion-exchange and gel filtration chromatography with DEAE-Sepharose and Sephadex G-100, respectively. The molecular mass of the purified enzyme was 20.5 kDa as estimated by SDS-PAGE, with resemblance to NK of *B. subtilis* strain natto (Sumi *et al.* 1987) and AMMP from *Armillaria mellea* (Lee *et al.* 2005). Since the molecular mass of K42 was smaller than that of other known fibrinolytic enzymes such as nattokinase (28 kDa) (Fujita *et al.* 1993), CK (28 kDa) (Kim *et al.* 1996), DJ-4 (29 kDa) (Kim and Choi 2000) and the fibrinolytic enzyme (31.5 kDa) from a mutant of *B. subtilis* IMR-NK1 (Chang *et al.* 2000), it is likely that the fibrinolytic enzyme from *B. subtilis* K42 could result in less antigenicity.

The pH stability profile of K42 enzyme was between 6.5 and 10.5. The optimum temperature for its action was 40°C, but it could maintain its initial activity at less than 30°C and retain 76% of it at 60°C for 2 h just as it is exhibited by FI protease of *Chryseo bacterium taeanense* TKU001 (Wang *et al.* 2008). The optimum pH of K42 was 9.4 and is similar to that of CK of *Bacillus* sp. CK (Kim *et al.* 1996) and subtilis in DJ-4 of *Bacillus* sp. DJ-4 (Kim and Choi 2000).

There are conflicting reports about the effect of organic solvents on enzyme activity and stability. However, solvent-stable proteases have been reported in the case of purified *P. aeruginosa* PseA protease (Gupta *et al.* 2005), *Bacillus* sp. TKU007 protease (Wang and Yeh 2006) and *Bacillus* sp. TKU004 protease (Wang *et al.* 2006). However, in the present study, the solvents tested (DE, TO, AN, BU, EA, ET, AC, ME and IP) showed a stabilizing effect on enzyme activity. It is likely that the stabilization of the enzyme was

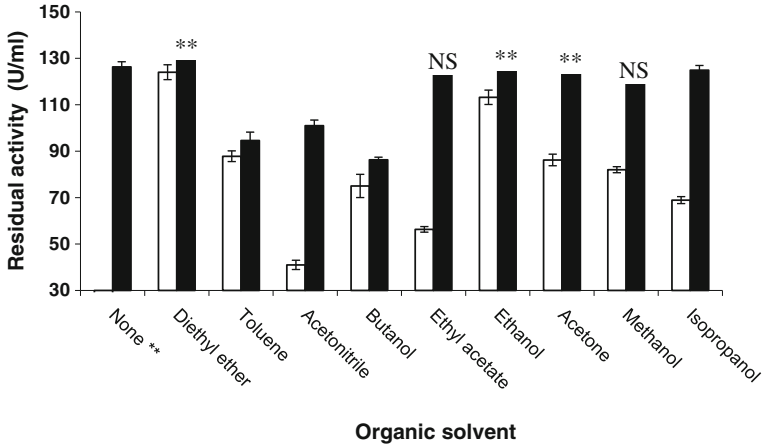

Figure 4. Effect of various organic solvents on the stability of enzyme at 25°C (□) and 4°C (■). Each value represents the mean±SD (n=3). ^{NS}P not significant from the control at $P>0.05$; $^{**}P$ highly significant at $P<0.01$; Student's paired t-test.

Table 2. Effect of metal ions on enzyme activity

Chemicals	Concentration (mM)	Remaining activity (U/ml)
None	–	100±2.2
Ca^{2+}	5	101±3.5*
Cu^{2+}	5	50±1.6
Mg^{2+}	5	118±2.1**
Mn^{2+}	5	66±1.5
Fe^{2+}	5	87±3.1
Zn^{2+}	5	88±2.6
Ba^{2+}	5	85±2.1
Hg^{2+}	5	84±1.9
Co^{2+}	5	137±3.6***

*P significant at $P<0.05$; **P highly significant at $P<0.01$; ***P very highly significant from the control at $P<0.001$; Student's paired t-test.

due to its precipitation in organic solvent that made it possible for its stereo configuration to be retained and thus also its activity. This further suggests that replacement of some water molecules in an enzyme with organic molecules may stabilize the structure of the enzyme (Gupta *et al.* 2005; Wang *et al.* 2006; Wang and Yeh 2006). It is often assumed that enzymes with improved solvent stability also become more resistant to other denaturing agents. Solvent stability may also allow this enzyme to be used in organic solvents to shift the equilibrium of the reversible reaction between hydrolysis and synthesis of peptides to complete the hydrolysis (Gupta *et al.* 2005; Wang *et al.* 2006; Wang and Yeh 2006). Furthermore, recovering back the solvents being used for precipitation ofthis enyzme, which would be on a large scale in industry,would make thisoperation rapid and cost-effective.

Table 3. Effects of protease inhibitors on the enzyme activities

Inhibitors[a]	Concentration (mM)	Relative activity (%)
None	–	100±1.3
Aprotinin	0.1	92±1.5
SBTI	0.1	101±1.2*
TPCK	0.1	112±2.2**
DFP	0.1	115±3.2**
TLCK	0.1	89±1.4
PMSF	10	104±2.4*
EDTA	1	84±1.1
EDTA	5	44±0.8
Co^{2+}+EDTA	5+1	131±3.2***
2,2′-Bipyridine	0.1	4±0.9
o-Phenanthroline	0.1	7±0.4

[a] DFP, Diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; TPCK, tosyl-phenylalanyl chloromethylketose; TLCK, tosyl-lysyl-chloromethylketose; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid. *P significant at $P<0.05$; **P highly significant at $P<0.01$; ***P very highly significant at $P<0.001$; Student's paired t-test.

Table 4. *In vitro* application of *B. subtilis* K42 fibrinolytic enzyme as anticoagulant

Dose (U/0.1 ml)	Clotting time (s)	RPTT (%)
0(B)	24±0.7	0.0
5	25±0.5*	4.2
10	37±3.3**	54.2
20	42±2.1**	75.0
50	57±3.7***	137.5
100	82±0.7***	241.7

*P significant at $P<0.05$; **P highly significant at $P<0.01$; ***P very highly significant at $P<0.001$; Student's paired t-test.

Enzyme activity was inhibited by Cu^{2+} and Mn^{2+}, but was enhanced by the addition of Co^{2+} and Mg^{2+} ions, suggesting that K42 enzyme is a metallo protease. The results from the action of EDTA and 2,2″-bipyridine and *o*-phenanthroline, i.e. inhibition of enzyme activity and its restoration by addition cobalt ions collectively suggest that the fibrinolytic enzyme obtained from the liquid batch cultures of *B. subtilis* K42 is a metallo protease containing cobalt in its active site. This is similar to the properties of fibrinolytic enzyme obtained from *Fusarium pallidoroseum* (El-Aassar 1995).

The *in vitro* application of K42 fibrinolytic enzyme on human blood serum prolonged the clotting time from 24 to 82 s. Thus, relative PTT reached 241.7%. Similar values have been reported for the culture fluid of *Aspergillus ochraceus* 513, which, after dialysis, exhibited a PTT of 205% (Batomunkueva and Egorov 2001). We presume that the antithrombotic effect may due to proteolysis of any one of the blood clotting factors, leading to abolition of both thrombin generation and platelet aggregation.

In conclusion, it is suggested that due to its effectiveness in fibrin degradation seen *in vitro*, pH stability and a small size that could both increase absorption by small intestine villi and reduce antigenicity inside the body, K42 fibrinolytic enzyme could be considered for a more effective fibrinolytic therapy. Further work in animals would focus on the mechanism that potentiates fibrinolysis *in vivo*. It will also be necessary to carry out studies on its effect on plasma proteins, RBC rigidity, surface charge and glycocalyx structure.

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