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Research Article

A Fibrinolytic Enzyme Produced by *Bacillus subtilis* Using Chickpea (*Cicer arietinum* L.) as Substrate

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Abstract: A Fibrinolytic Enzyme (BSFE) was isolated from fermented chickpeas using *Bacillus subtilis*. BSFE was purified with ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The fibrin (ogen) olytic activity of BSFE was investigated by means of fibrinolysis plate and hydrolysis of fibrinogen. Through these steps, the purity of the enzyme increased with 74.60-fold with 6.88% recovery activity. The molecular weight of the BSFE was estimated to be 30 kDa by SDS-PAGE. The optimum pH, optimum temperature, pH stability and thermal stability of BSFE were measured, respectively as 8.0, 55°C, 6.0-8.0 and less than 45°C. The activity was inhibited by serine protease inhibitor PMSF as well as metalloprotease inhibitor EDTA, indicating that the BSFE is a serine metalloprotease. In fibrin plate assay, BSFE showed more stronger fibrinolytic activity than that of nattokinase and it specifically hydrolyzed Aα and Bβ chains followed by γ chain of fibrinogen. Therefore, this study provided a method and it for the preparation of multifunctional food of chickpeas which has strong fibrinolytic activity.

Keywords: *Bacillus subtilis*, chickpea fermentation, fibrinolytic activity, Fibrinolytic Enzyme (BSFE)

INTRODUCTION

Different enzymes from various microbial, animal and plant sources have been popularly used in wide ranges of physiological, medicinal and industrial applications. Among them, fibrinolytic enzyme is one which possesses significant medical applications for the treatment of Cardiovascular Diseases (CVDs) caused by intravascular thrombosis (Mander *et al*., 2011). Intravascular thrombosis due to accumulation of fibrin in arteries is one of the major causes of CVDs. Based on the different working mechanisms, the thrombolytic agents are classified into two types: Plasminogen Activators (PA) and plasmin-like proteins (Peng *et al*., 2005). PA uniformly increase the risk of bleeding complications especially intracranial hemorrhage and no PA has shown clinical efficacy without bleeding risk (Yang *et al*., 2011; Marder, 2009). Plasmin is a "direct-acting" thrombolytic agent that had been considered to contain greater thrombolytic efficacy and safety than can be afforded by PA, but it is ineffective when administered by the intravenous route because it is neutralized by plasma α2-antiplasmin (Marder, 2009; Choi *et al*., 2013). Therefore, a safer and cheaper fibrinolytic enzyme from various sources has been receiving huge attention.

The 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 cardiovascular diseases and other related diseases (Agrebi *et al*., 2009). In 1897 nattokinase was extracted from natto (Sumi *et al*., 1987), from then on much more attention was paid to the development of new types of fibrinolytic enzyme from food sources such as Korean chungkook-jang soy sauce (Kim *et al*., 1996), Korean doen-jang (Kim and Choi, 2000), Chinese douchi (Peng *et al*., 2003) and Asian fermented shrimp paste (Wong and Mine, 2004).

The predominant starters used in these above mentioned foods are all of *Bacillus* spp. and the fibrinolytic enzymes isolated from *Bacillus* spp.-fermented soybean have been well documented. However, fewer reported about the fibrinolytic enzymes extracted from other fermented beans. Chickpea, an annual herbage plant, is the third most important grain legume in the world on the basis of total grain production (Kou *et al*., 2013). In addition, chickpeas have been applied as a Uighur traditional medicine in the Xinjiang province of China for a long time (Wei *et al*., 2011). However, about 20% of chickpea seeds are damaged during harvesting and processing and is considered as a by-product that is sold at low prices for

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livestock feeding (Torres-Fuentes *et al*., 2011). Furthermore, chemical analysis showed that chickpeas are rich in organic and inorganic compounds, suggesting it as a good candidate for culture media (Fu, 2012; Zhang *et al*., 2011). Considering the production cost and improvement of enzyme activity, utilizing *Bacillus subtilis* as a microbial alternative for chickpeas fermentation for the production of fibrinolytic enzyme seems to provide a promising approach. Liquid fermentation of chickpeas with a newly isolated *Bacillus subtilis* was investigated for the first time and the fibrinloytic enzyme purification, biochemical characterization and thrombolysis ability were evaluated.

MATERIALS AND METHODS

Materials: Bovine fibrinogen, bovine thrombin, Folin-Phenol reagent, Phenyl-Methylsulfonyl (PMSF) and Ethylenediamine Tetraacetic Acid (EDTA) were obtained from Sigma-Aldrich (MO, USA). Sepharose CL-4B and DEAE-cellulose were obtained from Pharmacia Co (Amersham Bioscienses, Sweden). Other reagents and chemicals used were of analytical grade and were commercially available.

Strain and cultivation: *Bacillus subtilis* was isolated along with other several strains from the soybean fermented food. The strain was identified as a strain related to *Bacillus subtilis* based on morpholigical and 16S rDNA sequences. It was grown for 48 h at 37°C on Luria-Bertani broth medium (LB) containing (in g/L): peptone, 10.0; yeast extract, 5.0; NaCl, 5.0; agar 20 with pH adjusted to 7.2. A loopful of spores were scraped from the plate and inoculated into seed medium. After 8 h cultivation, the seed culture broth 3% (v/v) was transferred into fermentation medium which consisted of (in g/L): sucrose, 50; chickpea, 34.8; K_2HPO_4, 5; KH_2PO_4, 6; $CaCl_2$, 0.4; $MgSO_4$, 0.3 and further cultivated in a shaking incubator (200 rpm) at 37°C for 48 h.

Protein estimation: Protein concentration was determined by the method of Bradford (1976) and Murata *et al*. (1963) using bovine serum albumin as standard. The concentration of protein was determined by measuring the absorbance at 595 nm.

Measurement of enzyme activity: Proteolytic activity was assayed according to the method of (Bradford, 1976) using casein as reaction substrate. Reaction mixture was prepared by mixing 1 mL enzyme sample with 1 mL of 2% (w/v) casein in 20 mM phosphate buffer (pH 7.2). Following incubation at 55°C for 10 min, 2 mL of ice-cold 0.4 M trichloroacetic acid was added and then immersed in ice water for 20 min. After centrifugation at 10000×g for 10 min, the supernatant was mixed with 1ml Folin-Phenol reagent (0.33 M) and 5 mL Na_2CO_3 (0.4 M) solution. The mixture was incubated at 40°C for 20 min and the optical density of the produced color was measured at 660 nm using spectrophotometer. To assess the enzyme activity, a standard curve was generated using tyrosine solutions with different concentrations, ranging from 0 to 100 μg/mL. Protease activity was quantified in Unit (U), where 1 U is defined to be the amount of enzyme required to release 1 μg tyrosine/min under the assay condition.

Purification of fibrinolytic enzyme: The culture broth of *Bacillus subtilis* was centrifuged at 5000×g for 10 min at 4°C and $(NH_4)_2SO_4$ at 80% saturation was added to the supernatant to precipitate the proteins. Precipitated proteins were recovered by centrifugation at 10000×g for 20 min at 4°C. The precipitate, suspended in 20 mM phosphate buffer (pH 7.2), was dialyzed against the same buffer. The crude enzyme solution was applied to a DEAE-cellulose column (1.6×30 cm) which was pre-equilibrated with 20 mM phosphate buffer (pH 7.2). The column was eluted with a linear gradient of NaCl from 0 to 1 M at a flow rate of 1 mL/min. The unadsorbed fractions were combined and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in the 20 mM phosphate buffer (pH 7.2). The obtained enzyme solution was purified using gel filtration with Sepharose CL-4B column (1.0×60 cm) equilibrated with 20 mM phosphate buffer (pH 7.2) at a flow rate of 0.6 mL/min. After monitoring the enzyme purity by gel electrophoresis, various biochemical properties of the enzyme were determined.

Determination of molecular weight: The molecular weight of the purified enzyme was determined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970) using a 7.5% (w/v) stacking and 15% (w/v) polyacrylamide resolving gels.

Effect of pH on enzyme activity and stability: To determine the optimal pH, enzyme assay was carried out with casein at various pH values (pH 5.0-11.0) under the standard assay protocol described earlier. Standard pH buffers (20 mM) were: citric acid-sodium citrate buffer (pH 5.0-6.0), Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0-8.0), glycine-NaOH buffer (pH 9.0-10.0) and Na_2HPO_4-NaOH buffer (pH 11.0). Likewise stable pH range was determined by measuring the residual activities after incubating the enzyme for 1 h at 25°C in the respective pH buffer (20 mM). The residual enzyme activities were calculated with reference to initial activity considering 100%.

Effect of temperature on enzyme activity and stability: To determine the optimal temperature, enzyme assay was carried out at pH 8.0 and various temperatures ranging from 20 to 65°C. Likewise, the thermal stability was evaluated by measuring the

residual activities after incubating the enzyme sample at pH 8.0 and various temperatures for 30-120 min. The initial enzyme activity was considered to be 100%.

Effects of enzyme inhibitors and metal ions: In order to characterize the nature of the purified enzyme, effect of PMSF and EDTA protease inhibitors were examined. The purified enzyme was pre-incubated with inhibitors for 30 min at 25°C before measuring the residual activity. The effect of various metal ions was investigated by adding 5 mM metal ions such as Ca^{2+}, Na^{+}, Al^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} to the reaction mixture. Enzyme activity measured in the absence of inhibitors or metal ions was considered to be 100%.

Fibrinolytic assays in fibrin plate: Quantitative analyses of fibrinolytic activity was conducted using fibrin plate, following the method described by Astrup and Mullertz (1952) with slight modifications. Fibrin plate was prepared by pouring the solution composed of 0.75% fibrinogen in 10 mM Tris-HCl buffer (pH 7.2), 1.0% agarose and 42 U/mL thrombin into the petri dish. The plate was left for an hour at room temperature to form fibrin clot. Ten microliters of the purified enzyme was carefully loaded onto each circular well (2 mm in diameter) on the plate and then the plate was incubated at 37°C. Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the well.

Table 1: Purification summary of BSFE from *Bacillus subtilis*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	314.37	951579	3027	100	1.00
Ammonium sulfate	197.20	933053	4732	98.05	1.56
DEAE-cellulose	0.75	86429	115239	9.08	38.07
Sepharose CL-4B	0.29	65477	225783	6.88	74.60

Protease activity was assessed by folin-phenol method using casein as reaction substrate (Murata *et al*., 1963)

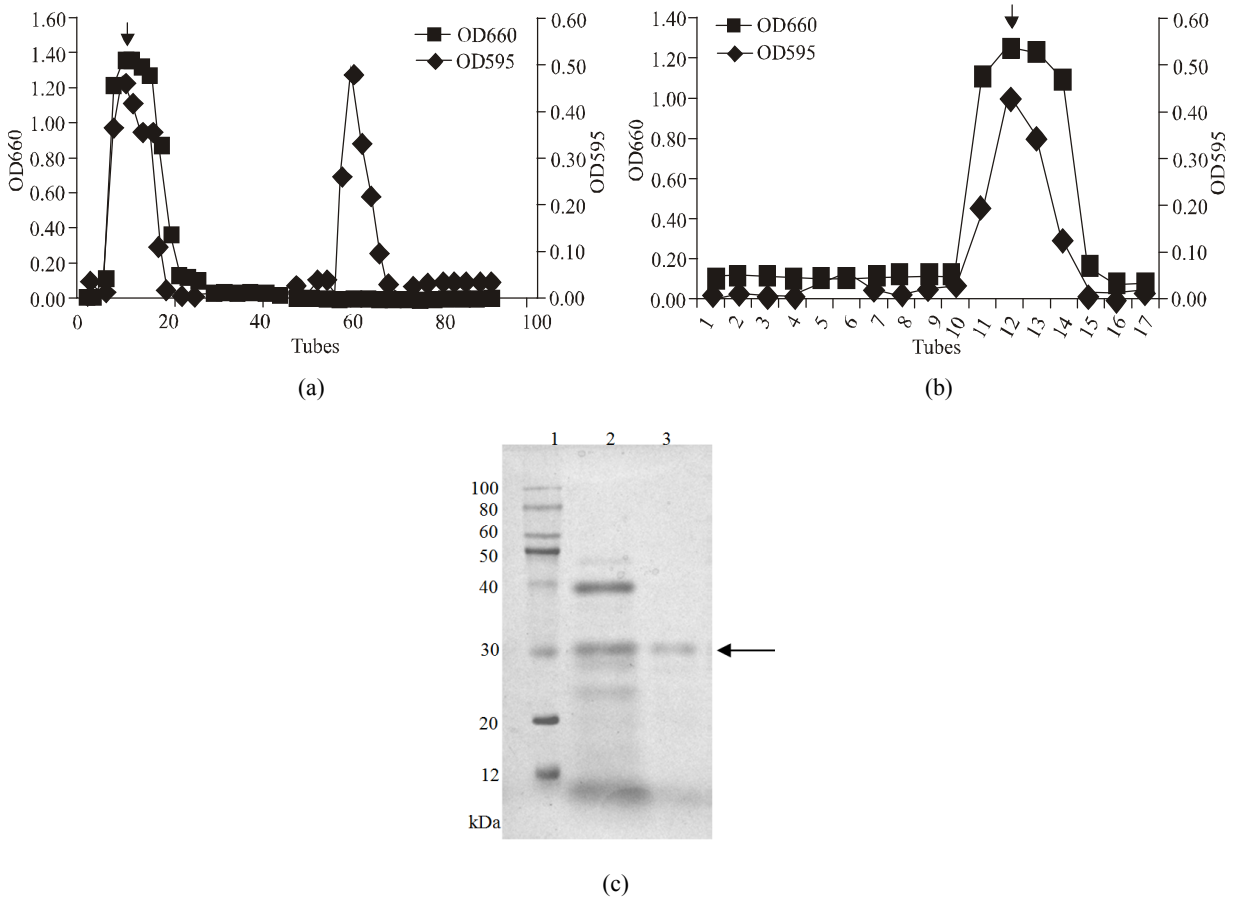


Fig. 1: Purification of BSFE from *Bacillus subtilis*-fermented chickpea, (a) ion-exchange chromatography of BSFE using DEAE-cellulose column (1.6×30 cm) with 20 mM phosphate buffer (pH 7.2) and was eluted with a linear gradient of NaCl from 0-1 M, (b) gel filtration chromatography with sepharose CL-4B column (1.0×60 cm), (c) SDS-PAGE of the protein bands from the various purification steps
Lane 1: Standard protein marker (100, 80, 60, 50, 40, 30, 20 and 12 kDa, respectively); Lane 2: Purified by ion chromatography with DEAE-cellulose; Lane 3: Purified BSFE electrophoretic analysis was carried out in 15% (w/v) gel

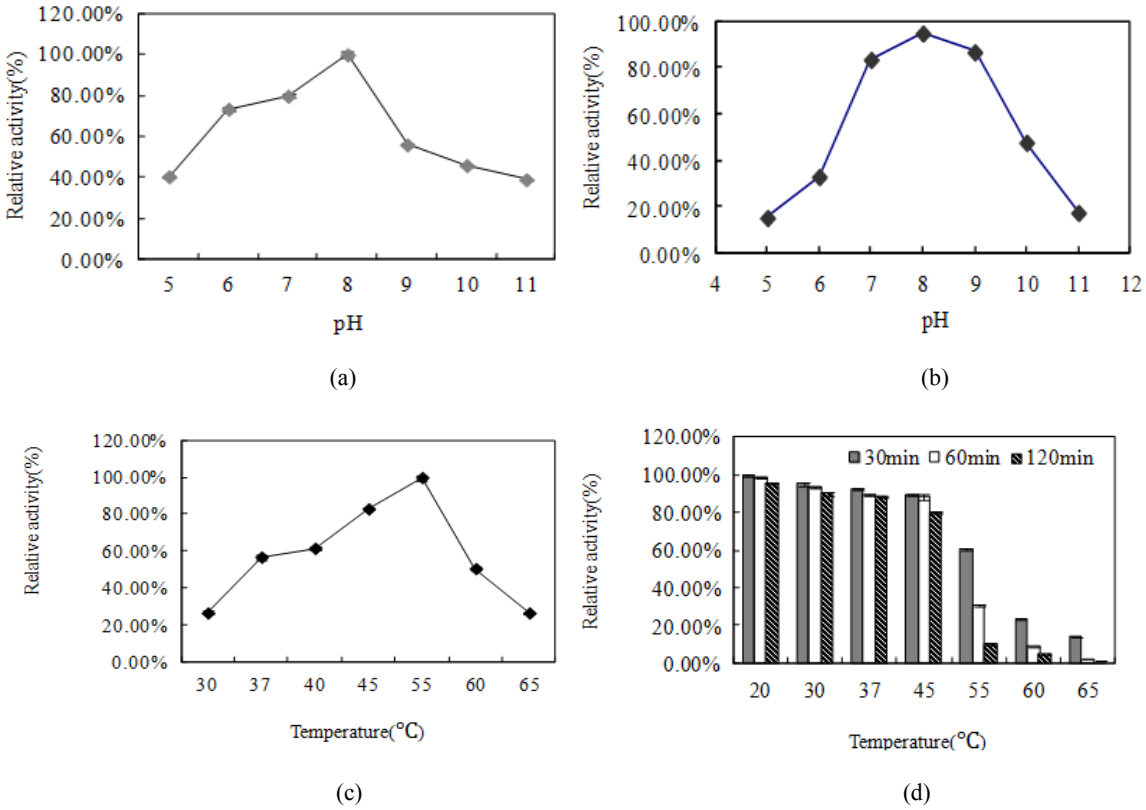


Fig. 2: Effect of pH and temperature on enzyme activity, (a) optimal pH was determined by assessing the enzyme activity in the pH range 5.0-11.0, (b) stable pH range was determined by measuring the residual enzyme activities after incubating the enzyme at various pHs for 1 h, (c) optimal temperature was determined by performing the enzyme assay in different temperature ranging from 20 to 65°C, (d) thermo-stability of the enzyme was evaluated by measuring the residual enzyme activities after incubating the enzyme in various temperatures for 30-120 min at pH 8.0

Fibrinogenolytic activity assay: Quantitative analyses of fibrinogenolytic activity were conducted following the reported method (Mander *et al*., 2011) with slight modifications. Two hundred micro liter fibrinogen (1 µg/µL) in 10 mM Tris-HCl buffer (pH 7.2) was incubated at 37°C with 60 µL purified enzyme (1 µg/µL) for various time durations. The reaction was stopped by addition of denaturing buffer. The resulting degradation products were analyzed by 12% SDS-PAGE according to the specified method (Laemmli, 1970).

RESULTS AND DISCUSSION

Enzyme purification and molecular weight: Purification of BSFE was carried in the three sequential steps which are summarized in Table 1. The crude enzyme was applied to ion exchange chromatography on DEAE-cellulose column and fraction exhibited fibrinolytic activity as indicated by an arrow were collected (Fig. 1a). The active fractions were pooled, concentrated and purified by gel filtration chromatography on the Sepharose CL-4B (Fig. 1b). Overall, 74.60-fold purification and 6.88% activity recovery (yield) was obtained after completion of the purification steps. SDS-PAGE of the purified enzyme was performed in order to verify the enzyme purity and to determine the molecular weight. The protein migrated as a single band and the molecular weight was estimated to be 30 kDa (Fig. 1c). Molecular weight of the reported *Bacillus* spp. fibrinolytic enzymes was the similar molecular masses of 30 kDa. However, the molecular weights of many *Bacillus* spp. fibrinolytic enzymes are different, such as a metallo protease produced by *Bacillus subtilis* K42 (20.5 kDa) (Chang *et al*., 2012). Interestingly, the molecular weight of the enzyme purified from *Bacillus subtilis* QK02 (42 kDa) (Wang *et al*., 2008) and *Bacillus* sp., KA38 (41 kDa) (Hassanein *et al*., 2011) were larger than 30 kDa.

Biochemical properties of BSFE:

Effect of pH and temperature: The optimal pH for BSFE was found to be 8.0 (Fig. 2a). The optimum pH of the BSFE was similar to some *Bacillus* spp. fibrinolytic enzymes, such as *Bacillus subtilis* TKU007 serine proteases (Wang *et al*., 2011) and *Bacillus* sp., KDO-13 metalloprotease (Lee *et al*., 2001). The enzyme was completely stable at pH 8.0 and <90% activity remained between pH 7.0 and 9.0 (Fig. 2b).

Table 2: Effect of protease inhibitors on the fibrinolytic activity of BSFE

Protease inhibitors	Concentration (mM)	Relative activity (%)
Control	0	100
EDTA	5	5.62
PMSF	1	1.70

Purified enzyme was pre-incubated with protease inhibitors at 25°C for 30 min; Enzyme activity measured in the absence of inhibitors was taken as 100%

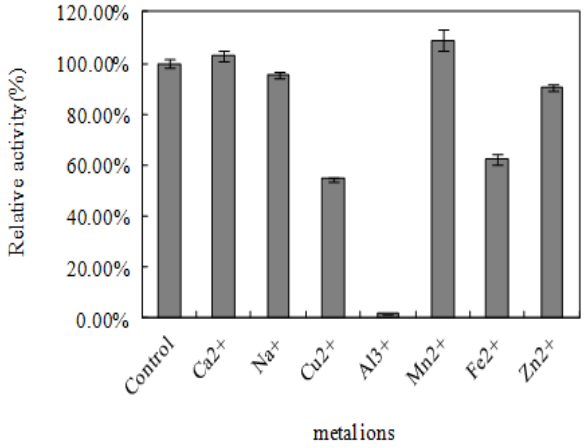


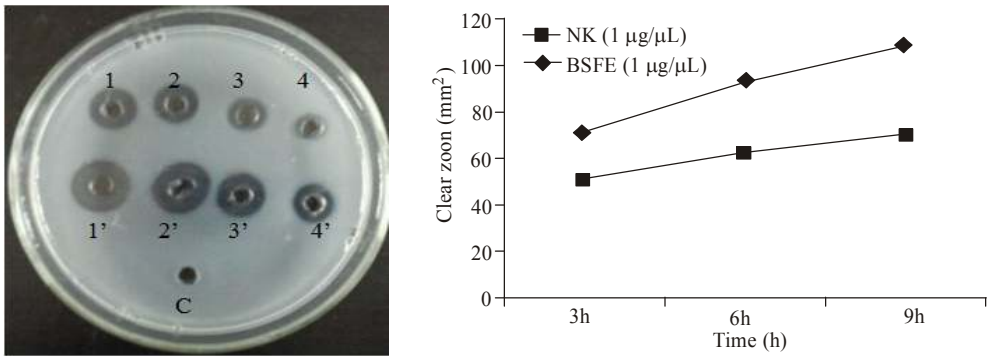
Fig. 3: Effect of metal ions on the enzyme activity; purified enzyme was pre-incubated with various metal ions in 20 mM Tris-HCl buffer (pH 8.0) for 30 min at 25°C and then the residual activity was determined; enzyme activity measured in the absence of any metal ions was taken as 100%; each value represents the mean±S.D. for three determinations

The activity vigorously tends to decrease in acidic range. BSFE showed maximum activity at 55°C and remained stable at or below 45°C (Fig. 2c and d).

Effect of inhibitors and metal ions: The activity was noticeably inhibited by serine protease inhibitor PMSF. It suggests that BSFE may belong to serine protease family which is known to have involvement of serine in the catalytic activity. The activity was also significantly inhibited by metalloprotease inhibitors EDTA suggesting that BSFE aslo belongs to metalloprotease family (Table 2). This type of fibrinolytic enzymes has also been reported from *Streptomyces* sp., CS624 (Mander *et al*., 2011) and *Streptomyces* sp., CS684 (Simkhada *et al*., 2010). But BSFE was different from the *Bacillus* spp. fibrinolytic enzymes that were classified into serine protease or metalloprotease. Furthermore metal ions showed varied effect on the BSFE activity. The activity was completely inhibited by Al^{3+}, significantly inhibited by Cu^{2+}, Fe^{2+} and slightly enhanced by Ca^{2+} and Mn^{2+} (Fig. 3).

Fibrinolytic and fibrinogenolytic activities: The effect of BSFE on fibrin clot lysis was examined by fibrin plate method. Nattokinase (NK) has been explored more extensively than other microbial fibrinolytic enzymes and applied as a functional food. So NK was used as a control in this study. As shown in Fig. 4a, addition of BSFE led to the formation of a clear zone and the size of clear was large than equal amount of NK at any concentration. After 6 h incubation the area of clear zone formed by BSFE was observed to be approximately 1.5-folds bigger than that equal amount of NK (Fig. 4b). Since area of the clear zone is directly proportional to the activity, it can be said that the BSFE offers stronger fibrinolytic activity than that of NK.

The target protein of the coagulation cascade is fibrinogen, a 340 kDa soluble plasma protein consisting of disulfide-bonded Aα-, Bβ- and γ-chains (Lu *et al*., 2010). To elucidate the effect on fibrinogen, BSFE was incubated with fibrinogen and the mode of reaction was analyzed by SDS-PAGE. As illustrated in Fig. 5, all the Aα-, Bβ- and γ-chains of fibrinogen were susceptible to cleavage by BSFE. The fibrinogenolytic pattern of BSFE was Aα>Bβ>γ which was similar to other



(a) (b)

Fig. 4: Fibrinolytic activities of BSFE, (a) fibrinolytic activity on the plasminogen-rich fibrin plate at 3 h; numerically marked lytic circles were: 1-4, NK (5, 3, 1 and 0.5 µg/µL, respectively); 1'-4', BSFE (5, 3, 1 and 0.5 respectively); and C, negative control (Tris-HCl buffer), (b) comparison of fibrinolytic activities between the purified enzyme (1 µg/µL) and NK (1 µg/µL) after 3, 6 and 9 h, respectively

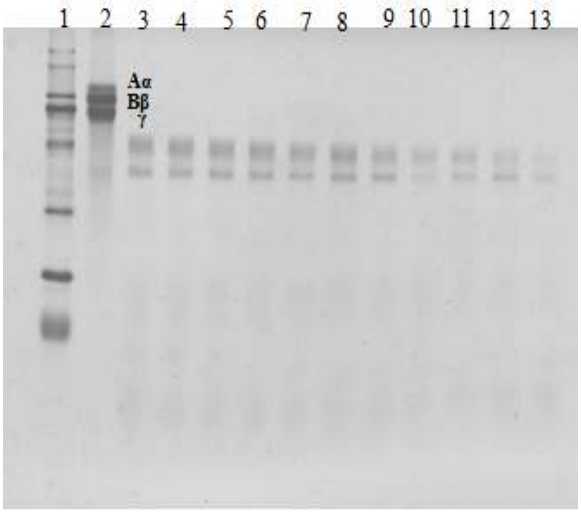


Fig. 5: Fibrinogenolytic activities of BSFE
Lane 1: Protein marker; Lane 2: Fibrinogen; Lane 3-13: Hydrolyzed aliquots after 5, 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 min, respectively

fibrinolytic proteases (Mander *et al*., 2011; Yang *et al*., 2011; Kim *et al*., 2013). Even though the pattern is similar, we used BSFE at the concentration of 3/10 of fibrinogen hydrolyzed all the chains within 5 min (Fig. 5). Mander *et al*. (2011) indicated that *Streptomyces* spp. fibrinolytic Enzymes (FES624) at the concentration of 3/10 of fibrinogen hydrolyzed the Aα-chain and Bβ-chain after 10 min of incubation and then the γ-chain also hydrolyzed after 150 min. Another study showed that treatment of *Paenibacillus polymyxa* EJS-3 (PPFE-1) at the concentration of 5/16 of fibrinogen caused hydrolysis of the Aα-chain, Bβ-chain after 30 h of incubation (Kim *et al*., 2013). These results suggest that BSFE has considerably higher fibrinogenolytic activity than these proteases.

CONCLUSION

The fibrinolytic enzymes from *Bacillus* spp. have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process. In contrast to other reported producing fibrinolytic enzymes, this study is a novel contribution towards the use of chickpeas as a new complex organic substrate for the production of fibrinolytic enzymes by liquid fermentation. In the present study, a profound Fibrinolytic Enzyme (BSFE) was purified from *Bacillus subtilis* fermented-chickpea. One of the most important features of the BSFE that it was a serine metalloprotease which was different from the *Bacillus* spp. fibrinolytic enzymes. Another important feature of the BSFE was its high activity. It can hydrolyze the Aα-chain, Bβ-chain and γ-chain of fibrinogen within 5 min and its plasmin-like activities higher than nattokinase. These effects suggest that BSFE holds a potential to be an antithrombotic agent or functional food.

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