Full Paper

Genome sequencing, purification, and biochemical characterization of a strongly fibrinolytic enzyme from *Bacillus amyloliquefaciens* Jxnuwx-1 isolated from Chinese traditional douchi

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A strongly fibrinolytic enzyme was purified from *Bacillus amyloliquefaciens* Jxnuwx-1, found in Chinese traditional fermented black soya bean (douchi). The molecular mass of the enzyme, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was 29 kDa. The optimal pH and temperature for the enzyme were 7.6 and 41°C, respectively. The enzyme was inhibited by phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, ethylenediaminetetraacetic acid, Fe³⁺, and Fe²⁺. The highest affinity exhibited by the enzyme was towards N-Succinyl-Ala-Ala-Pro-Phe-pNA. These results indicated that it is a subtilisin-like serine metalloprotease. The enzyme degraded both fibrinogen and fibrin, displaying its highest degrading activity towards the A α -chains followed by B β chains and C γ chains. The enzyme was also activated by plasminogen, indicating its ability to degrade fibrinogen and fibrin in two ways: (a) by activating plasminogen conversion into plasmin, or (b) by direct hydrolysis. It degraded thrombin, suggesting that it may act as an anticoagulant to prevent thrombosis. Taken together, our results indicate the potential of this enzyme in controlling cardiovascular disease.

Key Words: *Bacillus amyloliquefaciens*; douchi; fibrinolytic enzyme; purification; subtilisin-like serine metalloprotease

Abbreviations: CVDs, Cardiovascular diseases; u-PA, urokinase-type plasminogen activator; t-PA,

tissue plasminogen activator; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; TLCK, N-Tosyl-L-Lysine chloromethyl ketone; TPCK, N- α -Tosyl-L-phenylalanine chloromethyl ketone; pNA, p-nitroaniline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GO, Gene Ontology

Introduction

Cardiovascular diseases (CVDs) such as ischemic heart disease, high blood pressure, and acute myocardial infarction are a leading cause of death, accounting for approximately a third of all deaths worldwide (Mine et al., 2005). Thrombus formation is one of the main causes of CVDs. Fibrin, the main component of thrombus, is formed via catalysis of fibrinogen by thrombin (EC 3.4.21.5). Fibrin is degraded by plasmin (EC 3.4.21.7), derived from plasminogen by the action of activators. Under normal physiological conditions, the formation and degradation of fibrin remain in equilibrium. However, any disturbance of such equilibrium, may lead to the accumulation of fibrin, resulting in thrombus formation (Choi et al., 2011).

Fibrinolytic agents are currently categorized into plasmin-like proteins, which can directly degrade the fibrin, and plasminogen activators, including urokinase-type plasminogen activator (u-PA) (Duffy, 2002), tissue plasminogen activator (t-PA) (Collen and Lijnen, 2004), and bacterial plasminogen activators, which indirectly degrade fibrin by activating plasminogen conversion into plasmin. However, in addition to being very expensive, clinical use

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of these agents result in undesirable side effects, such as a short *in vivo* half-life, low specificity for fibrin, and excessive bleeding (Choi et al., 2011; Liu et al., 2015; Lu et al., 2010; Wang et al., 2006). Therefore, a search for alternatives, with fewer or no side effects, may prove useful.

Over the past decade, fibrinolytic enzymes in fermented foods have been investigated (Fujita et al., 1993; Rajendran et al., 2016; Yong et al., 2003). Examples of this include Japanese natto (Sumi et al., 1987), Asian fermented shrimp paste (Hua et al., 2008), Chinese douchi (Wang, S. H. et al., 2008; Yong et al., 2003), red beans (Chang et al., 2012), and Korean Chungkook-Jang (Kim et al., 1996). These may be considered useful sources for exploring mechanisms underlying fibrinolysis, as well as for identifying potential drugs for clinical use.

Douchi is a fermented food used in food flavoring, which has been produced in China and several other countries for thousands of years (Chen et al., 2011b). Douchi is also used in Chinese traditional medicine to treat dyspepsia, restlessness, and asthma and to stimulate sweating (Chen et al., 2007; Chen et al., 2011a). In 1994, Barnes's team found that douchi may inhibit prostate and breast cancer (Messina et al., 1994). It also displays anti-diabetic activity (Wang, D. et al., 2008) and provides protection against osteoporosis and cardiovascular diseases (Ishida et al., 1998). Several studies have antecedently indicated that fibrinolytic enzymes have been purified from douchi and characterized (Wang et al., 2006; Wang, S. H. et al., 2008; Yong et al., 2003). However, studies conducted on fibrinolytic enzymes in douchi are scarce. The objective of the present study was to isolate, purify, and characterize a strongly fibrinolytic enzyme from douchi using submerged fermentation culture.

Materials and Methods

Materials. DEAE-Sepharose fast flow and Superdex 75, PD-10 columns were purchased from GE Healthcare Co. (USA). Bovine fibrinogen, bovine thrombin, human fibrinogen, human thrombin, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), pepstatin A, aprotinin, ethylenediaminetetraacetic acid (EDTA), N-Tosyl-L-Lysine chloromethyl ketone (TLCK), N- α -Tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-Succinyl-Ala-Ala-Pro-Phe-pNA, N-Benzoyl-Phe-Val-Arg-pNA, N-(p-Tosyl)-Gly-Pro-Lys-pNA, N-Succinyl-Ala-Ala-Ala-pNA, and p-nitroaniline (pNA) were purchased from Sigma-Aldrich Co. (USA). Protein standard markers (ranging from 14.3 to 97.2) were purchased from Takara Biotechnology (Dal Lan) Co. Ltd. All other chemicals used were of analytical grade.

Bacterial and culture condition. We prepared a suspension by soaking and shaking the douchi with sterile saline. Douchi suspension was cultured in a nutrient broth medium (peptone 10 g/L, beef dipping powder 5.0 g/L, NaCl 5.0 g/L) for 48 h at 37°C. Subsequently, the obtained strains were inoculated to a fibrinolytic enzyme-producing strain screening medium (Na₂HPO₄ 0.2%, NaCl 0.5%, skim milk powder 1%, agar 2%), and cultured at 37°C for 48 h to observe whether the colony produced a transpar-

ent hydrolyzed circle. Twenty-four bacterial colonies with different activities were isolated from the douchi fermentation process. The strain, Jxnuwx-1, showed the highest fibrinolytic enzyme activity and was identified as *B. amyloliquefaciens* following alignment with NCBI database sequences. *B. amyloliquefaciens* Jxnuwx-1 was deposited with the China Center for Type Culture Collection (CCTCC No.: M2014638). Fermentation was carried out with the fermentation medium, comprising 30 g/L corn starch, 20 g/L beef powder, 1 g/L NaCl, 1 g/L K₂HPO₄·3H₂O, 0.5 g/L MgSO₄·7H₂O, 1 g/L CaCl₂, for 72 h at 37°C and 170 rpm. The supernatant from centrifuged (10,000 rpm, 15 min, 4°C) fermentation broth was considered as the crude enzyme extract.

Genome Sequence of *B. amyloliquefaciens* Jxnuwx-1.

Genome sequencing was performed using Illumina Solexa HiSeq4000 at Novogene Bioinformatics Technology Co., Ltd, Beijing, China. A library containing 350-bp inserts was constructed. Sequencing was performed with the pair-end strategy of (150, 150)-bp reads to produce 2.0 Gb of filtered sequences, representing a 500-fold coverage of the genome.

Genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline 2.0. Open reading frames were identified by Glimmer 3.02 (Delcher et al., 2007) and GeneMark (Besemer et al., 2001). The resulting translations were used in a BLASTP (Altschul et al., 1990) search against the GenBank NR database, as well as against KEGG (Kanehisa et al., 2008) and COG (Tatusov et al., 2000) databases. tRNA and rRNA genes were identified via tRNAscan-SE (Lowe and Eddy, 1997) and RNAmmer (Lagesen et al., 2007), respectively.

Enzyme assay and protein determination. The fibrinolytic enzyme content of each solution was determined using the method of Astrup and Müllertz (1952), with slight modifications. The fibrin plates consisting of a fibrinogen solution (2 mg of fibrinogen in 8 mL of 40 mM sodium barbital buffer (pH 7.8), 20 IU of thrombin solution, and 8 mL of 12 g/L agarose) in petri dishes (10 cm in diameter), were left to stand for 1 h at room temperature to facilitate clotting, following which 10 μ L of the enzyme solution was carefully added onto each fibrin plate and subsequently incubated at 37°C for 18 h. Activity of the fibrinolytic enzyme was estimated by measuring the diameter of the lytic cycle according to the calibration curve, using urokinase as a standard. Protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

Purification of fibrinolytic enzyme. The fibrinolytic enzyme was purified using chromatographic procedures including anion exchange and gel filtration chromatography. Unless otherwise stated, all purification steps were performed at 4°C. The crude enzyme extract was precipitated at 65% saturation of (NH₄)₂SO₄, and protein precipitation was facilitated by centrifugation (12,000 rpm for 15 min at 4°C) followed by dissolving in 20 mM Tris-HCl buffer (pH 7.8). The supernatant thus obtained by centrifugation was desalted on a PD-10 column using 20 mM Tris-HCl buffer (pH 7.8). After desalting, the enzyme solution was loaded onto a DEAE-Sepharose fast flow col-

umn, previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8). Fractions displaying fibrinolytic activity were collected, concentrated by ultrafiltration and further purified by gel filtration. Purification by gel filtration was performed on a fast protein liquid chromatography (FPLC)-Superdex 75 gel filtration column (1.6 cm × 60 cm) using 20 mM Tris-HCl buffer (pH 7.8) containing 0.3 mol/L NaCl at a flow rate of 1 ml/min. Fractions containing fibrinolytic activity were pooled, concentrated, and used for further characterization.

Determination of molecular weight. Molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method using 50 g/L stacking and 120 g/L resolving polyacrylamide gel (Laemmli, 1970). Molecular weight was determined using the protein standard marker comprising rabbit phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), bovine carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and chicken egg white lysozyme (14.3 kDa).

Effect of pH and temperature on fibrinolytic enzyme. The optimal pH of the purified enzyme was determined by estimating fibrinolytic enzyme activity at different pH levels (pH 7.2–8.0) in sodium barbital buffer (pH 7.8) at 37°C. The pH stability of the enzyme was determined by measuring residual enzyme activity, using the fibrin plate method, prior to incubating the enzyme in different buffers ranging from pH 3–12 for 1 h. The buffers used were: sodium acetate buffer (pH 3.0–6.0), sodium barbital buffer (pH 7.0–9.0), carbonate buffer (pH 10.0–11.0), Na₂HPO₄-NaOH buffer (pH 11.0–12.0).

The optimum temperature for enzyme activity was determined by measuring activity at temperatures ranging from 23–55°C in sodium barbital buffer (pH 7.8). In order to assess thermal stability of the enzyme, residual enzyme activity was determined by incubating the enzyme over a temperature range of 23–63°C for 0.5 h in sodium barbital buffer (pH 7.8) using the fibrin plate method.

Effect of inhibitors and metal ions on enzyme activity. The effect of inhibitors on the fibrinolytic enzyme was investigated using PMSF, TLCK, TPCK, SBTI, pepstatin A, EDTA, and aprotinin at different concentrations in 20 mM sodium barbital buffer (pH 7.8) for 1 h at 37°C (Table 4). Residual activity of the enzyme was measured using the fibrin plate method. The effect of various metal ions such as FeCl₂, FeCl₃, CaCl₂, CoCl₂, CuCl₂, MgCl₂, ZnCl₂, and MnCl₂ at concentrations of 5 mM was estimated by incubating these ions with the enzyme at 37°C for 30 min, followed by the measurement of residual fibrinolytic enzyme activity using the fibrin plate method.

Activation of plasminogen (plasminogen activation activity). Plasminogen activator activity was determined using the plasminogen-rich and plasminogen-free fibrin plate method with slight modifications (Astrup and Müllertz, 1952). The plasminogen-rich fibrin plate was prepared as previously described, whereas the plasminogen-free fibrin plate was prepared by heating at 85°C for 30 min to inactivate other fibrinolytic factors (Wang et

al., 2006). Plasminogen activity was estimated using the Student's *t*-test to compare fibrinolytic enzyme activity between the plasminogen-rich and plasminogen-free plates.

Degradation of fibrinogen and fibrin. Fibrinogenolytic activity was tested via a fibrinogenolytic assay (Koh et al., 2001) with slight modifications. Fibrinogen (46 µL of 2% human fibrinogen in 20 mM Tris-HCl buffer (pH 7.8)) was mixed with the purified enzyme (46 µL of 2.3×10^{-3} mg/mL), and incubated at 37°C. At time intervals of 1 min, 2 min, 3 min, 4 min, 15 min, 30 min, 1 h, and 4 h, aliquots were placed on ice, mixed with loading buffer, boiled, and analyzed by SDS-PAGE to assess cleavage patterns in fibrinogen chains.

Next, fibrinolytic activity was measured via a fibrinolytic assay with slight modifications (Liu et al., 2015). Human fibrin (46 µL of 2% fibrin in 20 mM Tris-HCl buffer (pH 7.8)) was mixed with 10 µL of human thrombin (20 IU/mL), and left to stand for 1 h at room temperature to facilitate coagulation, and purified enzyme (46 µL of 2.3×10^{-3} mg/mL) was added, following which the preparation was incubated for 1 min, 2 min, 3 min, 4 min, 15 min, 30 min, 1 h, and 4 h. At each indicated time interval, aliquots were placed on ice, and analyzed to examine cleavage patterns in fibrin.

Proteolytic effect of purified enzyme on blood proteins. To investigate the effect of the purified enzyme on blood, partial blood protein components were selected for analysis. The purified enzyme (46 µL of 2.3×10^{-3} mg/mL) in 20 mM Tris-HCl buffer (pH 7.8) was incubated with an equal volume of immunoglobulin G (Ig G) (10 mg/mL), human serum albumin (HSA) (10 mg/mL) and thrombin (200 IU/mL) at 37°C for 4 h. Samples were analyzed using SDS-PAGE.

Amidolytic activity of the enzyme. Amidolytic activity of the purified enzyme was investigated via spectrophotometry, using N-Succinyl-Ala-Ala-Pro-Phe-pNA, N-Benzoyl-Phe-Val-Arg-pNA, N-(p-Tosyl)-Gly-Pro-Lys-pNA and N-Succinyl-Ala-Ala-Ala-pNA as substrates. The purified enzyme (100 µL of 2.3×10^{-3} mg/mL in 20 mM Tris-HCl buffer (pH 7.8)) was mixed with 150 µL (5 mM) of each of substrate and incubated at 37°C for 5 min. The amount of p-nitroaniline released was determined by measuring the change in absorbance at 405 nm ($\epsilon = 9.65 \times 10^3$ /M/cm, Tris-HCl buffer (pH 7.8)) using a spectrophotometer.

Determination of kinetic constants. The K_m and V_{max} of the purified enzyme were determined by the Lineweaver and Burk method (Lineweaver and Burk, 1934), with N-Succinyl-Ala-Ala-Pro-Phe-pNA as substrate at concentrations of 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mM. The reactions were performed in 20 mM Tris-HCl buffer (pH 7.8) at 37°C.

Identification of fibrinolytic enzyme protein sequence by LC-MS/MS. Trypsin solution (20 ng/mL) was used to swell SDS-PAGE strips and hydrolyze the purified fibrinolytic enzyme at 37°C overnight. Next, extraction, vacuum concentration, and identification of peptides by LC-MS/MS (chromatographic column: C18, 3 µm, 150 mm × 75 µm, flow rate 300 nL/min, gradient 5–7% B (0–

Fig. 1. Histogram presentation of clusters of orthologous groups (COG) classification.

Table 1. General features of *B. amyloliquefaciens* Jxnuwx-1 and DSM7 (T) genome.

Feature	Jxnuwx-1	DSM7(T)
Number of contigs	48	1
Length of the genome assembly (Mb)	4.09	3.98
GC content (%)	46.0%	46.1%
tRNA number	58	94
Number of protein-coding genes	3,870	3,870
Number of genes	4015	4120
Average CDS size (bp)	906.1	883.7
Coding percentage (%)	85.7	85.9
Number of pseudo genes	85	121
GenBank No.	LMAT00000000	NC_014551

1 min), 7–40% B (1–45 min), B solution of 95% CAN, 0.1% FA, mass spectrometry scanning model DDA) was performed.

Results and Discussion

Genome sequencing and general features

The complete genome information of *B. amyloliquefaciens* type strain DSM7 (T) was obtained from NCBI/GenBank database. Genomic comparative analysis between strain Jxnuwx-1 and *B. amyloliquefaciens* type strain DSM7 (T) revealed a certain difference in the genomes (Table 1). Sequences of strain Jxnuwx-1 were assembled into 48 contigs using the Velvet software with an N50 length of 321,522 bp. The Jxnuwx-1 chromosome is approximately 4.09 Mbp in length, with an average G+C content of 46.0%. A total of 3,870 protein coding genes and 58 tRNA coding genes were identified. The genome sequence represents a valuable shortcut, which aids scientists to locate and identify genes. Putative coding genes were found in the Jxnuwx-1 genome. The Total Genome

Shotgun project was deposited at DDBJ/EMBL/GenBank under the accession LMAT00000000. The version described in this paper is LMAT01000000 (Table 1).

COG and GO clustering analysis

Based on gene prediction and annotation results, *B. amyloliquefaciens* Jxnuwx-1 was predicted to contain 3,870 protein-coding genes. Protein sequences were searched against the COG database using BLAST, with E-value $\leq 1e-5$ and identity $\geq 40\%$ as filters. A total of 2,739 genes (70.8%) were assigned to 25 COG categories. Among the 25 COG categories, the cluster representing General function prediction (428; 13.33%) was the largest group, followed by Amino acid transport and metabolism (335; 10.43%), Function unknown (277; 8.62%), Transcription (271; 8.44%), Carbohydrate transport and metabolism (239; 7.44%), Inorganic ion transport and metabolism (204; 6.35%), Energy production and conversion (173; 5.39%), and Cell wall/membrane/envelope biogenesis (169; 5.26%), whereas only a few genes were assigned to cell cycle control, cell division, and chromosome partitioning (Fig. 1).

Gene Ontology (GO) is a major bioinformatics initiative that unifies gene and gene product attributes across all species. GO has three ontologies: Molecular function, Cellular component, and Biological process (Consortium, 2008). In this study, the Interproscan (Jones et al., 2014) program was used to provide GO annotation for the proteins of *B. amyloliquefaciens* Jxnuwx-1 and *B. amyloliquefaciens* DSM7 (T). WEGO software (Ye et al., 2006) was used to establish GO functional classification for genes encoding these proteins.

In total, 2,405 (62.1%) protein encoding genes, matched to known proteins by BLAST, were assigned to GO classes with 8,845 functional terms. Of these, assignments to the

Table 2. Summary of purification steps of fibrinolytic enzyme from submerged culture.

Purification step	Volume (mL)	Protein (mg)	Activity (IU)	Recovery (%)	Specific activity (IU/mg)	Fold
Culture supernatant	100	11456.83	40194.84	100	3.51	1
65% (NH ₄) ₂ SO ₄ precipitation	5.40	725.52	31327.86	77.94	43.18	12.31
DEAE-Sepharose FF	54.00	11.52	5235.40	13.03	454.46	129.48
Superdex 75	4.60	1.03	1280.22	3.19	1240.30	353.36

Fig. 2. Gene Ontology classifications of *B. amyloliquefaciens* Jxnuwx-1 and DSM7 (T) protein-coding genes.

The results are summarized in three main categories: Biological process, Cellular component, and Molecular function.

Biological process made up the majority (4237, 47.90%), followed by Molecular function (2981, 33.70%) and Cellular component (1627, 18.39%) (Fig. 2).

Purification and characterization of fibrinolytic enzyme

A submerge culture method was developed to produce the fibrinolytic enzyme. The enzyme was purified by a series of purification steps (Table 2). The fibrinolytic enzyme extract was subjected to 65% ammonium sulfate precipitation, dissolved and desalted, and the supernatant was loaded on to a DEAE-Sepharose FF column. This was followed by gel filtration chromatography on a Superdex 75 column by AKTA Explorer 100, for purification of the fibrinolytic enzyme. In terms of yield, 1.03 mg of the enzyme was purified 353.36-fold, with an overall yield of 3.19% in purity. The specific activity of the purified enzyme was 1240.3 IU/mL (Table 2).

SDS-PAGE was used to determine the molecular weight and verify the purity of the enzyme (Fig. 3A). The purified enzyme appeared as a single homogeneous band on SDS-PAGE (Fig. 3B). Its molecular mass, determined by SDS-PAGE, was 29 kDa, which matched that of enzymes from *Bacillus halodurans* IND18 (29 kDa) (Vijayaraghavan et al., 2016) and *Bacillus subtilis* YU-1432 (29 kDa) (Lee and Lee, 2011), and was closely similar that to that of a fibrinolytic enzyme produced by *B. amyloliquefaciens* DC-4 (28 kDa) (Yong et al., 2003) and *Bacillus subtilis* DC33 (30 kDa) (Wang et al., 2006) from Chinese traditional Douchi, as well as to that of a serine protease enzyme from *B. amyloliquefaciens* An6 (30 kDa) (Agrebi et al., 2010). Its molecular mass was smaller than that of the fibrinolytic enzymes from *Bacillus* sp. (34.4

Fig. 3. Purification and determination of the molecular mass of the fibrinolytic enzyme.

A. SDS-PAGE was performed on a 12% gel. Lane M, protein standard marker; lane 1, the supernatant extract with fibrinolytic enzyme activity; lane 2, ammonium sulfate precipitation from supernatant culture; lane 3, the active fraction from a DEAE-Sepharose FF column; lane 4, the active fraction from Superdex 75 column. B. Determination of the molecular mass of the fibrinolytic enzyme. Lane M, protein standard marker; lane 1, the purified fibrinolytic enzyme.

kDa) (Cheng et al., 2015), *Bacillus cereus* WQ9-2 (37 kDa) (Xu et al., 2010) and *Bacillus* sp. SM2014 (71 kDa) (Jain et al., 2012), but higher than that of subtilisin-like enzymes from *B. amyloliquefaciens* CH51 (27 kDa) (Kim et al., 2011) and *B. amyloliquefaciens* MJ5-41 (27kDa) (Jo et al., 2011), and that of a chymotrypsin-like serine fibrinolytic enzyme from *B. amyloliquefaciens* FCF-11 (18.2 kDa) (Kotb, 2014).

Effects of pH and temperature on enzyme activity

The optimal pH for the purified enzyme was 7.6 (Fig. 4A), while it remained stable within a broad pH range of 7.0–11.0 (Fig. 4B). The maximum activity of the enzyme was detected at 41°C (Fig. 4C). It exhibited thermostability in the temperature range of 23–43°C, retaining >80% of relative activity after a 30 min incubation (Fig. 4D). Relative activity and thermostability of the purified enzyme sharply decreased above 43°C. Human physiological pH of 7.4, is closely similar to the optimum pH value of the purified enzyme (7.6), which was also similar to that of a fibrinolytic enzyme from *Bacillus* sp. AS-S20-I (pH 7.4) (Mukherjee et al., 2012), whereas it was lower than that of a nattokinase from *Bacillus subtilis* TKU007 (pH 8.0) (Wang et al., 2011) and a fibrinolytic enzyme from *B. amyloliquefaciens* FCF-11 (pH 8.0) (Kotb, 2014), *Bacillus subtilis* DC33 (pH 8.0) (Wang et al., 2006), *B. amyloliquefaciens* DC-4 (pH 9.0) (Yong et al., 2003), *Bacillus* sp. SM2014 (pH 10.0) (Jain et al., 2012) and *Bacillus halodurans* IND 18 (pH 9.0) (Vijayaraghavan et al., 2016). The optimal temperature for the purified enzyme was 41°C, which is similar to that for enzymes from *Ba-*

Fig. 4. Effect of pH and temperature on fibrinolytic activity of the enzyme.

A. Optimal pH of the purified enzyme was determined by measuring the fibrinolytic enzyme activity at different pH (7.2–8.0) in sodium barbital buffer (pH 7.8) at 37°C. The buffers used were sodium acetate buffer (pH 3.0–6.0), sodium barbital buffer (pH 7.0–9.0), carbonate buffer (pH 10.0–11.0), and Na_2HPO_4 -NaOH buffer (pH 11.0–12.0). B. The pH stability of the enzyme was determined by measurement of the residual enzyme activity after incubating the enzyme in different buffers ranging from pH 3–12 at 37°C for 1 h. C. Optimum temperature for enzyme activity was determined by measuring activity in the range of 23–55°C, in sodium barbital buffer (pH 7.8). D. Thermal stability of residual enzyme activity was determined after incubation of the enzyme over a temperature range of 23–63°C for 0.5 h in sodium barbital buffer (pH 7.8).

Table 3. The biochemical characterization of fibrinolytic enzymes isolated and purified from *B. amyloliquefaciens* Jxnuwx-1 were compared with those of other *B. amyloliquefaciens* strains.

Strain	Molecular mass (kDa)	Optimal pH	Optimal temperature (°C)	Reference
<i>B. amyloliquefaciens</i> Jxnuwx-1	29	7.6	41	—
<i>B. amyloliquefaciens</i> DC-4	28	9.0	48	Yong et al. (2003)
<i>B. amyloliquefaciens</i> An6	30	9.0	60	Agrebi et al. (2010)
<i>B. amyloliquefaciens</i> FCF-11	18.2	8.0	40	Kot (2014)
<i>B. amyloliquefaciens</i> MJ5-41	27	7.0	45	Jo et al. (2011)
<i>Bacillus halodurans</i> IND18	29	9.0	60	Vijayaraghavan et al. (2016)
<i>Bacillus subtilis</i> DC33	30	8.0	55	Wang et al. (2006)
<i>Bacillus</i> sp.	34.4	6.5	54	Cheng et al. (2015)
<i>Bacillus cereus</i> WQ9-2	37	8.0	50	Xu et al. (2010)
<i>Bacillus</i> sp. SM2014	71	10.0	60	Jain et al. (2012)
<i>Bacillus</i> sp. AS-S20-I	32.3	7.4	37	Mukherjee et al. (2012)

cillus subtilis TKU007 (40°C) (Wang et al., 2011) and from *B. amyloliquefaciens* FCF-11 (40°C) (Kotb, 2014). It is higher than the value for the fibrinolytic enzyme from *Bacillus* sp. AS-S20-I (37°C) (Mukherjee et al., 2012), but lower than that for the enzyme from *B. amyloliquefaciens* MJ5-41 (45°C) (Jo et al., 2011), *B. amyloliquefaciens* DC-4 (48°C) (Yong et al., 2003), *Bacillus cereus* WQ9-2 (50°C) (Xu et al., 2010), and *Bacillus* sp. SM2014 (60°C) (Jain et al., 2012). Furthermore, the optimum temperature and pH value of the enzyme were similar to those of the fibrinolytic enzyme from *B. amyloliquefaciens* FCF-11 (40°C, pH 8.0).

Effects of inhibitors and metal ions on enzyme activity

The effect of various metal ions and protease inhibitors on the purified enzyme was investigated by assessing residual fibrinolytic activity of the enzyme. Enzyme activity was significantly inhibited by Fe^{2+} (18.96%) and Fe^{3+} (35.63%), but strongly enhanced by Ca^{2+} (131.61%), Co^{2+} (146.16%), Cu^{2+} (131.61%) and Mg^{2+} (131.61%) (Table 3). The enzyme was significantly inhibited by PMSF (19.27%) and SBTI (73.19%), which are serine protease inhibitors. Furthermore, it was also strongly inhibited by EDTA, a metalloprotease inhibitor. However, the enzyme was not inhibited by either the aspartic protease inhibitor, pepstatin A, the cysteine protease inhibitor, TLCK, or the

Table 4. Effect of various metal ions on fibrinolytic enzyme of purified enzyme.

Metal ion (5 mM)	Residual activity (%)
Control	100.00
Fe ²⁺	18.96
Fe ³⁺	35.63
Ca ²⁺	159.67
Co ²⁺	146.16
Cu ²⁺	131.61
Mg ²⁺	131.61
Zn ²⁺	110.44
Mn ²⁺	83.84

Table 5. Effect of various inhibitors on fibrinolytic enzyme of purified enzyme.

Inhibitors	Concentration (mM)	Relative activity (%)
Control		100.00
PMSF	5	19.77
SBTI	0.05	73.19
Pepstatin A	0.05	83.91
EDTA	10	0.68
Aprotinin	0.5	88.09
TLCK	5	99.79
TPCK	5	92.60

chymotrypsin inhibitor, TPCK (Table 4). Therefore, the purified enzyme isolated in this study is most likely a serine metalloprotease. These results are comparable with the observed effects of inhibitors, which are known serine metalloproteases, on fibrinolytic enzymes from *B. amyloliquefaciens* MJ5-41 (Jo et al., 2011), and *Brevibacillus* sp. KH3 (Maeda et al., 2011). However, the enzyme is dissimilar to the fibrinolytic enzymes from *Bacillus* sp. (Cheng et al., 2015) and *Bacillus cereus* WQ9-24 (Xu et al., 2010), which are known to be a serine protease and a metalloprotease, respectively (Tables 4 and 5).

Activation of plasminogen (plasminogen activator activity)

Plasminogen activator activity was determined by comparing the lytic circle diameters of plasminogen free and plasminogen rich plates, using the Student's *t*-test. The diameter of the lytic circle of the plasminogen rich plate was significantly larger than that of the plasminogen free plate (Fig. 5), indicating that the purified fibrinolytic enzyme may act as a plasminogen activator. A similar result was reported for the fibrinolytic enzyme from *Bacillus subtilis* DC33 (Wang et al., 2006), which may act as plasminogen activator by activating the formation of active plasmin from plasminogen.

Mode of hydrolysis of fibrinogen and fibrin by the enzyme

Fibrinogenolytic and fibrinolytic activity and degradation patterns of the enzyme were analyzed using SDS-PAGE. During the degradation of fibrinogen by the purified enzyme, A α -chains of fibrinogen were cleaved within 1 min of incubation, B β -chains within 4 min of incubation, and the C γ -chains, slowly but completely, within 4 h

Fig. 5. Determination of plasminogen activator.

Plasminogen activator activity was analyzed using plasminogen rich (A) and plasminogen free plates (B). Purified fibrinolytic enzyme (10 μ L) was added to each plate, and incubated at 37°C for 18 h. The plasminogen activator activity of the fibrinolytic enzyme was assessed by comparing diameters of the lytic circles of plasminogen rich and plasminogen free plates.

Fig. 6. Effect of the enzyme purified from *B. amyloliquefaciens* Jxnuwx-1 on the activity of fibrinogen and fibrin.

A. The purified enzyme (2.3×10^{-3} mg/mL) was incubated with 2% human fibrinogen and human fibrin, in 20 mM Tris-HCl buffer (pH 7.8) at 37°C for different time periods, respectively, and the aliquots analyzed via SDS-PAGE. Lane C, control, lanes 1–8, the degradation aliquots after 1 min, 2 min, 3 min, 4 min, 15 min, 30 min, 1 h and 4 h of incubation, respectively. B. Degradation of albumin (HSA), thrombin and immunoglobulin G (Ig G) by the purified enzyme. Lanes 1, 3, and 5 represent the controls of albumin (HSA), thrombin, and immunoglobulin G (IgG), respectively. Lanes 2, 4, and 6 represent the digestion fragments of albumin (HSA), thrombin, and immunoglobulin G (Ig G), respectively.

of incubation (Fig. 6A(1)). This indicated that the cleavage site of A α and B β chains may be different from that of thrombin. The fibrinolysis pattern of the enzyme was similar to that of fibrinogenolysis, where the fibrin A α -chains were hydrolyzed within 1 min, B β -chains within 1 h, and C γ -chains within 4 h (Fig. 6A(2)). Based on the findings stated above, the purified enzyme may not only degrade fibrinogen and fibrin directly, and therefore be regarded as a direct fibrinogenolytic and fibrinolytic agent, but may also act as a plasminogen activator, such as urokinase (u-PA) and tissue plasminogen activator (t-PA), by activating plasminogen conversion into plasmin. The fibrinolytic enzymes from *Bacillus* sp. (Cheng et al., 2015) and *Cordyceps militaris* (Liu et al., 2015) showed similar degradation patterns. However, the purified enzyme

hydrolyzed three chains of fibrinogen/fibrin completely within 4 h, which was dissimilar to the fibrinolytic enzyme from *Bacillus* sp. nov. SK006, which degraded B β -chains and C γ -chains, but not the A α -chains (Hua et al., 2008).

Effect of the enzyme on blood plasma proteins

In order to determine the effect of the purified enzyme on different blood plasma proteins, HSA, thrombin and IgG were incubated with the purified enzyme at 37°C for 4 h (Fig. 6B). HSA was marginally hydrolyzed while IgG was partially hydrolyzed by the enzyme. Thrombin was significantly degraded, indicating that the purified enzyme may degrade thrombin and, therefore, function as an anti-coagulant to prevent thrombosis. The results were similar to that of the fibrinolytic enzyme from *Cordyceps militaris* (Liu et al., 2015). These findings indicated that the purified enzyme may cause certain side effects, compared with fibrinolytic enzymes from *Armillariella mellea* (Jun-Ho and Kim, 1999) and *Tricholoma saponaceum* (Kim and Kim, 2001) which showed dissimilar results and did not hydrolyze blood plasma proteins.

Amidolytic activity and kinetic constant of the enzyme

Amidolytic activity of the purified enzyme was determined using various chromogenic substrates (Table 6). The purified enzyme showed the highest affinity towards N-Succinyl-Ala-Ala-Pro-Phe-pNA, known as a specific chromogenic substrate for serine proteases. These results were

consistent with the results of the effects of protease inhibitors (Table 5). The above findings indicate that the purified enzyme from *B. amyloliquefaciens* from Chinese traditional douchi may be a subtilisin-like serine metalloprotease. The K_m and V_{max} values for N-Succinyl-Ala-Ala-Pro-Phe-pNA were 0.36 mM and 6 μ mol/min/L, respectively. The K_m value (0.36 mM) was similar to the value of AprE51 from *B. amyloliquefaciens* CH51 (0.35 ± 0.01) (Kim et al., 2011), but lower than the values observed for fibrinolytic enzymes from *Bacillus* sp. nov. SK006 (0.45 mM) (Hua et al., 2008) and *Bacillus subtilis* (0.59 mM) (Chang et al., 2012), respectively (Table 6).

Identification of fibrinolytic enzyme protein sequence by LC-MS/MS

The target protein was successfully identified as peptidase S8 by LC-MS/MS analysis (Fibrinolytic enzyme precursor, GenBank accession number KTF60721). Currently, fibrinolytic enzyme precursors from more than 10 *Bacillus* sp. have been retrieved from the NCBI protein database. Total amino acid residues of peptidase S8 were compared with those of other fibrinolytic enzymes. Total amino acid residues of the enzyme showed high homology with those of pro-subtilisin DJ-4 and AprE3-17, whereas large differences existed between the fibrinolytic enzymes of *Bacillus pseudomycooides* and *Bacillus thuringiensis*, and other fibrinolytic enzymes in terms of protein length and identity (Table 7).

In conclusion, the current study describes the potent fibrinolytic activity of a subtilisin-like serine metalloprotease purified from *B. amyloliquefaciens* Jxnuwx-1, found in Chinese traditional douchi. The enzyme showed a high degree of degradation of fibrinogen and fibrin and exhibited a higher affinity towards N-Succinyl-Ala-Ala-Pro-Phe-pNA, a specific chromogenic substrate for serine proteases. The enzyme can act as a plasminogen activator by activating plasminogen conversion into plasmin and can hydrolyze thrombin. Therefore, it can potentially be developed for use in injected fibrinolytic therapy aimed at controlling cardiovascular disease.

Table 6. Amidolytic activity of the purified enzyme for synthetic substrates.

Synthetic substrates (5 Mm)	Specific activity (μ mol/min/mg)
N-Succinyl-Ala-Ala-Pro-Phe-pNA	33.13
N-(p-Tosyl)-Gly-Pro-Lys-pNA	4.74
N-Benzoyl-Phe-Val-Arg-pNA	1.5
N-Succinyl-Ala-Ala-Ala-pNA	ND

ND: Not detecte.

Table 7. General protein features of *B. amyloliquefaciens* Jxnuwx-1 fibrinolytic enzyme precursor, compared with other fibrinolytic enzymes in NCBI protein database.

ID	Fibrinolytic enzyme	Protein length (aa)	Molecular weight (kDa)	Identity (%) [*]	Enzymes from strains	Accession No.
1	Peptidase S8	382	39.09	100	<i>B. amyloliquefaciens</i>	KTF60721.1
2	Pro-subtilisin DJ-4, partial	382	39.09	99.2	<i>Bacillus</i> sp. DJ-4	AAT45900.1
3	AprE3-17	382	39.15	99	<i>Bacillus licheniformis</i>	ACU32756.1
4	Subtilisin DFE precursor	382	39.14	98.7	<i>B. amyloliquefaciens</i>	AAZ66858.1
5	Fibrinolytic enzyme	381	39.38	85.9	<i>Bacillus subtilis</i>	ANY30161.1
6	Thermostable fibrinolytic enzyme Nk1	381	39.46	85.6	<i>Bacillus subtilis</i>	AAX35771.1
7	Thermostable fibrinolytic enzyme Nk1	381	39.46	85.6	<i>Bacillus</i> sp. LM 4-2	AKE22871.1
8	Fibrinolytic enzyme precursor	381	39.49	84.8	<i>Bacillus</i> sp. ZLW-2	ACE63521.1
9	Fibrinolytic enzyme	362	37.11	80.4	<i>Bacillus subtilis</i>	ABO77900.1
10	Subtilisin, partial	352	36.15	79.6	<i>Bacillus</i> sp. CN	ABU93240.1
11	Fibrinolytic enzyme, partial	275	27.49	71.7	<i>Bacillus</i> sp. Ace02	ABI35684.1
12	34 kDa fibrinolytic enzyme precursor	566	60.92	19.1	<i>Bacillus pseudomycooides</i>	ACK38255.1
13	34 kDa fibrinolytic enzyme	566	60.86	18.9	<i>Bacillus thuringiensis</i>	SCB89176.1
14	Subtilisin, partial	352	35.86	18.9	<i>Bacillus</i> sp. SJ	ABU93241.1

^{*}The total amino acid residues of peptidase S8 compared with that of other fibrinolytic enzymes (the percent of identical amino acid residues are computed using global alignment tool Needle).

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Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article.

Ethics Approval and Consent to Participate

The manuscript does not contain experiments using animals. The manuscript does not contain human studies.

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