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RESEARCH PAPER

Gene Cloning, Expression, and Properties of a Fibrinolytic Enzyme Secreted by *Bacillus pumilus* BS15 Isolated from Gul (Oyster) Jeotgal

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Abstract A *Bacillus* strain, BS15, showing strong fibrinolytic activity, antibacterial activity, and salt tolerance was isolated from gul (oyster) jeotgal, a Korean fermented sea food. BS15 was identified as *B. pumilus*. *B. pumilus* BS15 was able to grow in LB broth with 18% (w/v) NaCl. When culture supernatant was analyzed by SDS-PAGE, 22, 27, 35, and 60 kDa proteins were observed. The 27 kDa protein was determined to be major fibrinolytic enzyme by fibrin zymography. The gene (*aprEBS15*) was cloned in pHY300PLK, a *Bacillus-E. coli* shuttle vector. A *B. subtilis* transformant (TF) harboring pHYBS15 showed higher fibrinolytic activity than *B. pumilus* BS15, and produced the same 27 kDa protein. *aprEBS15* was overexpressed in *E. coli* BL21 (DE3), and recombinant enzyme (AprEBS15) was purified. The optimum pH and temperature of AprEBS15 were pH 8.0 and 40°C, respectively. Km and Vmax values were 0.26 mM and 21.88 μmol/L/min, respectively. *B. pumilus* BS15 can be used as a starter for jeotgals and other fermented foods with high salinities.

Keywords: *Bacillus pumilus,* fibrinolytic enzyme, jeotgal, starter

1. Introduction

Bacillus pumilus is often isolated from marine environments including seaweeds and intestines of fishes and shellfishes [1,2]. Some *B. pumilus* strains produce antimicrobial substances, and have been tested as probiotics for aquaculture and domestic animals such as pigs [3,4]. *B. pumilus* is generally considered non-pathogenic and safe for use as probiotics but few cases of infection have been reported [5]. Potential of *B. pumilus* as a plant growth stimulator was tested, and one of the mechanisms was the production of antimicrobial substances such as bacteriocines and lipopeptides [6]. Although few researches have been done on the metabolites of *B. pumilus*, potential of *B. pumilis* as a host for production of industrially important enzymes has been suggested recently [7].

Jeotgals are Korean traditional fermented seafoods prepared from various fishes, fish eggs, fish intestines, and shellfishes [8]. Gul (oyster, *Crassostrea gigas*) jeotgal (GJ) is prepared by mixing gul, salt, and some seasonings such as red pepper powder, garlic, and ginger. Unlike saeu (small shrimp) jeotgal and myeolchi (anchovy) jeotgal, no studies have been done on the microbial communities of GJ during fermentation. One reason is that GJ is consumed in a short period after preparation whereas other jeotgals are typically fermented for months [8]. The presence of various seasonings in GJ is another reason because microorganisms of different groups are associated with each seasoning [9]. Bacilli are often isolated from jeotgals and fish sauces. Although most bacilli are not halophilic or halotolerant, still bacilli have been isolated in significant numbers from jeotgals and fish sauces with high NaCl contents. Fukui *et al.* prepared fish sauce (15% NaCl, w/w) from deep sea smelt (*Glossanodon semifasciatus*) and fermented for 8 months at room temperature [10]. When fish sauce samples were plated onto plate count agar, bacilli, especially *B. subtilis*, were detected at 10^6 CFU/g. But bacilli were not detected or not a major group when a

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culture independent method (16S rRNA gene amplification) was used [10]. Similarly, bacilli were not one of major groups when jeotgals or fish sauces were analyzed by culture-independent methods such as 16S rRNA gene clone library or pyrosequencing of DNA samples [11,12]. But bacilli were detected by metatranscriptome analysis of shrimp sauce [13]. Considering these reports, *Bacillus* species seem to be not a major group like *Tetragenococcus* or *Halanaerobium* in jeotgals with high salinities, but still present in significant numbers, and likely to play some roles during fermentation of jeotgals. Future studies on the roles of *Bacillus* for jeotgal fermentation are necessary.

We isolated *B. pumilus* BS15 from GJ, and the strain showed significant salt tolerance, antimicrobial activity, and strong fibrinolytic activity. A fibrinolytic gene, *aprEBS15*, was cloned and overexpressed in *E. coli* and *B. subtilis*. Recombinant AprEBS15 was purified and the properties were studied. Our results indicate that *B. pumilus* BS15 is promising as a starter for jeotgals.

2. Materials and Methods

2.1. Isolation and identification of BS15

Bacilli with fibrinolytic activities were isolated from GJ samples prepared at our lab. Fresh gul was purchased at a local fishery processing shop, Tongyeong, Gyeongnam, in the December of 2016. Gul was washed with tap water and excess water was drained. Washed gul was mixed with salt (23%, w/w). No other seasonings were added and GJ was fermented at 15°C for 6 months. GJ samples were taken out at intervals during fermentation, and screened for bacilli with protease activities. Colonies on tryptic soy agar (TSA, Becton, Dickinson and Company, Sparks, MD, USA) plates with NaCl (10%, w/v) were spotted onto Luria-Bertani (LB, tryptone 10 g, yeast extract 5 g, NaCl 5 g, per l, pH 7.0) agar plates with skim milk (1%, w/v). Colonies with halos were further tested for fibrinolytic activities by fibrin plate method [14].

16S rRNA and *recA* genes were amplified and sequenced as described previously [15].

BLAST was used to find homologous sequences in the database (National Center for Biotechnology Information). Phylogenetic analyses were performed using MEGA 5.05 [16]. Phylogenetic trees were inferred based on the neighbor-joining methods, and bootstrap analysis was performed with 1,000 replications [17].

2.2. Growth, fibrinolytic activity, and salt tolerance of *B. pumilus* BS15

Growth and fibrinolytic activity of *B. pumilus* BS15 was examined by using 4 different media: LB broth, Brain Heart Infusion (BHI, Becton, Dickinson and Company), nutrient broth (NB, Becton, Dickinson and Company), and tryptic soy broth (TSB, Becton, Dickinson and Company). Growth was estimated by OD_{600} values and fibrinolytic activity of filtered supernatant (FS) was measured by fibrin plate method [14]. Aliquot of culture was centrifuged at 4,000 × g for 10 min at 4°C, and the supernatant was filtered using a 0.45 μm filter (Sartorius Stedim, Goettingen, Germany). The size of a lysis zone on a fibrin plate was measured and converted into a plasmin unit (U) by comparing it with those formed by plasmin standards with different units (Sigma, St. Louis, MO, USA). For salt tolerance test, *B. pumilus* BS15 was grown overnight in LB and inoculated (1%, v/v) into TSB with NaCl (15-20%, w/v). Cultures were incubated for 72 h with shaking at 37°C and growth was measured at 12 h intervals. All measurements were repeated 3 times and the means are shown with SD (standard deviations) values.

2.3. SDS-PAGE and fibrin zymography

B. pumilus BS15 was cultivated in LB for 96 h at 37°C with aeration. Aliquots of the culture were centrifuged at 8,000 × g for 10 min at 4°C. The supernatant was analyzed by SDS-PAGE and fibrin zymography. The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard. For SDS-PAGE, 10 μg of protein was concentrated by TCA (trichloroacetic acid) and loaded onto a 10% acrylamide gel. Fibrin zymography was done using 1 μg of protein and loaded onto a polyacryamide gel containing fibrin, which was prepared by mixing fibrinogen (0.2%, w/v) and 100 μL of thrombin (1 NIH unit/μL) with acrylamide solution [14]. After electrophoresis at a constant current of 15 mA in a cold room, the fibrin gel was soaked in 50 mM Tris-HCl buffer (pH 7.4) containing 2.5% Triton X-100 for 30 min at room temperature on a rotary shaker. The gel was washed with distilled water for 30 min to remove the triton X-100 and soaked in zymogram reaction buffer (30 mM Tris-HCl, pH 7.4, 0.02% of NaN_3) for 12 h at 37°C. Finally, the gel was stained with Coomassie Blue R-250 for 1 h, and destained for 4 h.

2.4. Cloning of *aprEBS15* gene

An *aprEBS15* gene was amplified from *B. pumilus* BS15 genome by using a primer pair: CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', *Bam*HI site underlined) and CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3', *Eco*RI site underlined). PCR was performed as described previously [14]. Amplified fragment was ligated with pHY300PLK (4.87 kb, Tc^R) (Takara, Shiga, Japan), and the recombinant plasmid was introduced into *B. subtilis* WB600 by electroporation. A

B. subtilis transformant (TF) was inoculated into LB broth with tetracycline (15 μg/mL). Growth and fibrinolytic activity of culture were measured during 96 h cultivation. SDS-PAGE and fibrin zymography were done for culture supernatant from *B. subtilis* TFs.

2.5. Overexpression of *aprEBS15* in *E. coli* BL21(DE3)

An *aprEBS15* gene without its own signal sequence (pro *apr*EBS15) was amplified using following primer pairs: pET-F (5'-AGAGGATCCGATGGCAGGGAAATC-3' *Bam*HI site underlined) and pET-R (5'-AGACTCGAG CTGAGCTGCCGCCTG-3' *Xho*I site underlined). The PCR fragment was inserted into pET26b(+) (Merck Millipore, Darmstadt, Germany), and the ligation mixture was introduced into *E. coli* BL21 (DE3) competent cells by electroporation. A TF harboring pETBS15 (pET26b(+) with pro *aprEBS15*) was cultivated in LB (250 mL) containing kanamycin (Km, 30 μg/mL) at 37°C with aeration until the OD_{600} reached 0.8. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added (1 mM), and culture was further cultivated for 20 h at 30°C. AprEBS15 was purified as described below.

2.6. Purification and properties of AprEBS15

Culture was centrifuged at 8,000 × g for 10 min at 4°C, and cell pellet was resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4). Cells were disrupted by sonication (5 cycles of 1 min each, with cooling for 2 min between cycles). Cell extract was centrifuged at 12,000 × g for 15 min at 4°C and the pellet (insoluble fraction) was used for purification of AprEBS15. The insoluble fraction was first dissolved in 6 M guanidine-HCl, stood at room temperature for 4 h, and then dialyzed against an excess volume of buffer (2 M Tris-HCl, pH 7.0, 5 mM cysteine, 1 mM cystine) for 12 h at 4°C. Buffer change of the dialyzate was done using binding buffer and Amicon filters (MWCO 10K; Millipore, Billerica, MA, USA). Dialysate was centrifuged at 12,000 × g for 20 min at 4°C to remove precipitate. Purification of renatured AprEBS15 was done by using a HiTrap IMAC FF column (GE healthcare, Uppsala, Sweden) as described previously [14].

The activity of purified AprEBS15 was examined at pH 3-11 by using different buffer systems: citrate-NaOH (50 mM), pH 3-5, sodium phosphate (50 mM), pH 6-8, and Tris-HCl (50 mM), pH 9-11. One μg of AprEBS15 was resuspended in each buffer and incubated for 1 h at 40°C and then the activities were measured by the fibrin plate method. For pH stability test, 1 μg of AprEBS15 was resuspended in each buffer and incubated for 6 h at 40°C and then the activities were measured at 1, 3, and 6 h. One μg of AprEBS15 was incubated in sodium phosphate buffer (pH 8) for 30 min at 37-60°C, and then the remaining activities were measured. For thermal stability test, 1 μg of AprEBS15 was incubated in sodium phosphate buffer (pH 8) for 3 h at 37-60°C, and the remaining activities were measured at 0.5, 1, 2, and 3 h. AprEBS15 was exposed to 5 mM metal ions or 1 mM inhibitors for 30 min at 40°C and pH 8. Then the remaining activities were measured.

2.7. Amidolytic activity

The amidolytic activity of AprEBS15 was measured by using N-succinyl-ala-ala-pro-phe-p-nitroanilide (S7388, Sigma) as the substrate. Fifty μL of substrate in sodium phosphate buffer (50 mM, pH 8.0) was mixed with 10 μL of AprEBS15 (1 μg) and 440 μL of sodium phosphate buffer. The mixture was incubated at 40°C for 10 min, and 500 μL of citrate-NaOH buffer (pH 3.0) was added and put on ice immediately. The mixture was centrifuged at 12,000 × g for 5 min and the absorbance of the supernatant was measured at 410 nm. The degree of hydrolysis was calculated from the absorbance value and the molar extinction coefficient value of p-nitroanilide (8,800 M^{-1} cm^{-1}). Kinetic parameters of AprEBS15 were calculated by measuring the amount of released p-nitroanilide from N-succinyl-ala-ala-pro-phe-p-nitroanilide at 40°C. Vmax and Km values were determined from measurements at different substrate concentrations (0.01 to 0.5 mM). Kcat was determined from the relationship, Kcat = Vmax / [enzyme].

2.8. Hydrolysis of fibrinogen

One mg of fibrinogen (bovine, MP Biochemicals, Illkirch, France) was mixed with AprEBS15 (50 ng) and the mixture in 1 mL of 20 mM Tris-HCl (pH 8) was incubated at 40°C up to 12 h. Aliquots were taken out at intervals and mixed with 5× SDS sample buffer. After boiling for 5 min, samples were analyzed by SDS-PAGE using a 10% acrylamide gel.

3. Results and Discussion

3.1. Isolation and identification of BS15

Among 63 bacilli isolated from GJ, BS15 showed the strongest fibrinolytic activity. BS15 was Gram +, rod-type, and the colony showed a typical *Bacillus* morphology on LB agar plates. BS15 showed 99% identity with *Bacillus pumilus* by API CHB kit (bioMérieux, Marcy l'Etoile, France) test (results not shown). BLAST analysis of its 16S rRNA gene sequence (1,226 nucleotides, MF461323) showed that it was 100% identical to those of *B. pumilus* strains (results not shown). A partial *recA* gene sequence (754 nucleotides, MF461328) also showed 100% identity to *recA* genes of *B. pumilus* strains (results not shown). The

16S rRNA gene sequences from BS15 shared very high sequence similarity with other *B. pumilus* strains. The close relatedness was supported by the phylogenetic trees, as indicated by a solid monophyletic clade within the cluster enclosed by the genus *B. pumilus* (Suppl. Fig. 1). Based on these results, BS15 was positively identified as s *B. pumilus* strain. *B. pumilis* BS15 possesses strong antibacterial activity, which is due to production of a bacteriocin (results not shown).

3.2. Fibrinolytic activity and salt tolerance of *B. pumilus* BS15

B. pumilus BS15 grew well in LB, BHI and TSB, and OD_{600} values reached to 1.2-1.4 at 24 h. But the OD_{600} value of culture in NB decreased rapidly after 12 h (Fig. 1A). Culture in LB at 96 h showed the highest fibrinolytic activity (242.5 U/mL). In LB, the activity increased steadily until 48 h, and then increased rapidly, reaching to the highest activity at 96 h (Fig. 1B). LB was the best medium for the growth and fibrinolytic activity of *B. pumilus* BS15 among the tested media and used for further experiments. *B. pumilus* BS15 grew well in the presence of NaCl up to 17% (w/v), and the OD_{600} values were above 1.1 after 48 h (results not shown). The OD_{600} values decreased slowly after 48 h. *B. pumilus* BS15 grew slowly at 18% NaCl, reaching to OD_{600} value of 0.4 at 48 h and then increased slowly until 72 h.

B. pumilus BS15 possesses significantly higher salt tolerance than other bacilli strains. For example, *B. subtilis* CH3-5 and *B. amyloliquefaciens* CH86-1, both isolated from cheonggukjang [18,19], grow slowly at 13% NaCl and do not grow at 16% NaCl (Suppl. Fig. 2). High salt tolerance of *B. pumilus* BS15 is an important advantage when the strain is used as a starter for jeotgals and other fermented foods with high salinities.

3.3. SDS-PAGE and fibrin zymography

Four bands of 22, 27, 38, and 60 kDa in size were observed

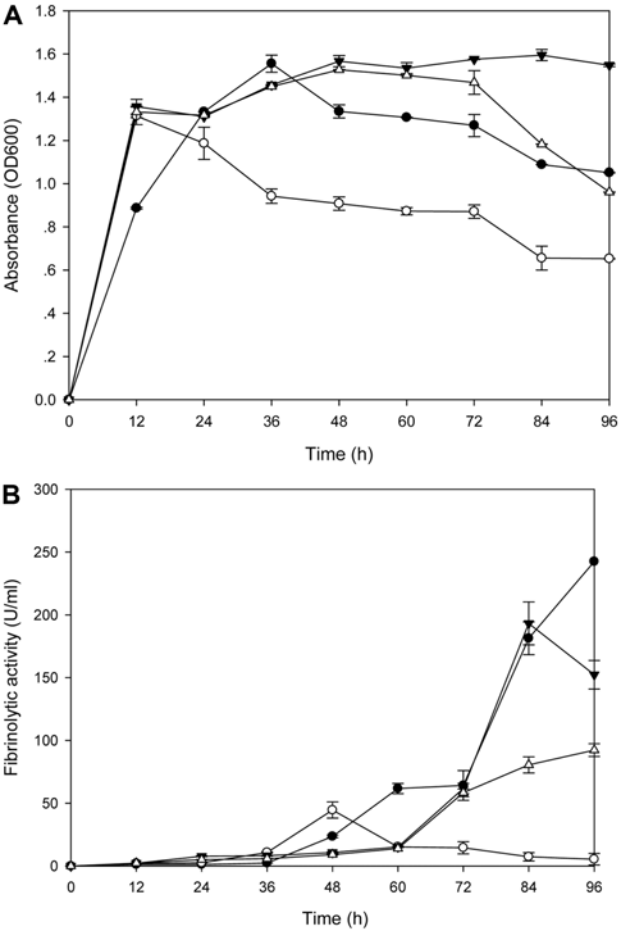

Fig. 1. Growth (A) and fibrinolytic activity (B) of *B. pumilus* BS-15 on different media. -●-, LB; -○-, NB; -▼-, BHI ; -△-, TSB. Filtered culture supernatant (20 μL) at each time point was applied to a fibrin plate and the fibrinolytic activity was expressed as U/mL.

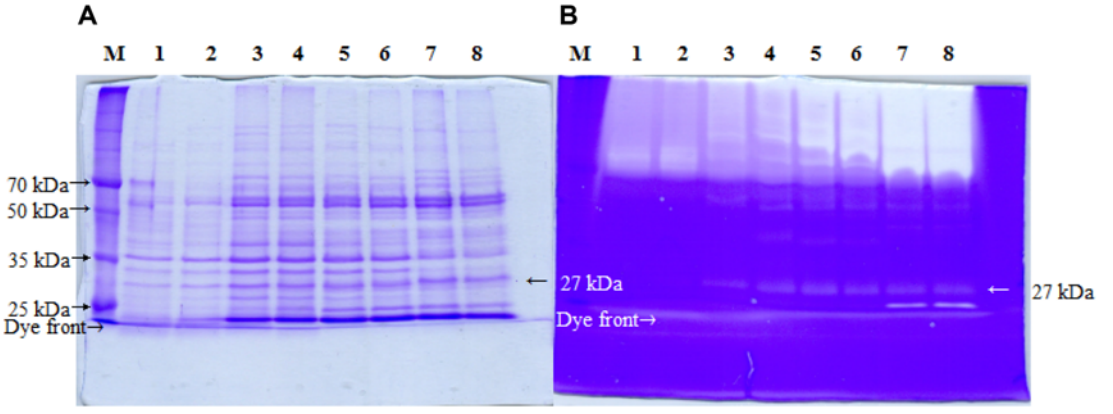

Fig. 2. Coomassie blue stained gel (A) and fibrin zymogram (B) of culture supernatant of *B. pumilus* BS15. M, Dokdo-marker broad-range (EBM-1034, Elpis-Biotech., Daejeon, Korea). *B. pumilus* BS15 was grown in LB broth at 37°C up to 96 h. 1, 12 h; 2, 24 h; 3, 36 h; 4, 48 h; 5. 60 h; 6, 72 h; 7, 84 h; 8, 96 h.

in addition to minor bands on a coomassie blue stained gel (Fig. 2A). The 27 kb band observed by fibrin zymography (Fig. 2B) is the major fibrinolytic protein. The fibrinolytically active band was observed at 36 h and the band intensity increased thereafter. A smaller band, 23 kDa, appeared at 84 h and present at 96 h (Fig. 2B, lanes 7-8). The 23 kDa band seemed a degradation product from 27 kDa band.

A big bright zone was observed in the upper part of the fibrin gel, which is often observed during fibrin zymography. The phenomenon, known as binding mode, is caused by binding of fibrinolytic enzymes to fibrin in the gel [20,21]. The intensity of upper zone increased as incubation of culture continued. The intensity apparently increased at 60 h (Fig. 2B, lane 5), and further increased at 84 h (Fig. 2B, lane 7), and the result matched well with the fibrinolytic activity measurement results of culture grown in LB. The fibrinolytic activity of *B. pumilis* BS15 culture increased at 60 h, and the activity rapidly increased at 84 h (Fig. 1B). From these observations, it was concluded that fibrinolytically active proteins including the 23 kDa band were present at highest concentration during late stage of growth. The activity was highest at 96 h.

3.4. Cloning of *aprEBS15*

Bacilli actively secrete several proteases into culture medium, and alkaline protease (AprE) is the major enzyme [22]. Among bacilli, the nucleotide sequences of *aprE* genes are well conserved. A primer pair designed for an *aprE* gene from a *Bacillus* species can be used for the cloning of *aprE* genes from other *Bacillus* species. In this work, a primer pair was used which was initially designed for the amplification of *aprE51* from *B. amyloliquefaciens* CH51 [23], and *aprE34* from *B. amyloliquefaciens* RSB34 [14]. A 1.4 kb fragment containing *aprEBS15* was amplified and inserted into pHY300PLK. *B. subtilis* WB600 TFs harboring recombinant plasmid, pHYBS15 (6.2 kb, Ap^r, Tc^r), were obtained. BLAST analysis of the sequence (1,485 nucleotides, MF943247) confirmed that the gene was a homolog of *aprE* genes from *Bacillus* sp. An ORF of 1,149 bp in size, capable of encoding a protein of 382 amino acids, was located. The first 30 amino acids corresponded to a signal peptide as judged by SignalP 4.1 Server (Technical University of Denmark) and the next 77 amino acids corresponded to a prosequence as judged by comparison of amino acid sequence with those of other fibrinolytic enzymes (results not shown). The calculated pI and molecular weight of proenzyme were 8.93 and 35,900.24 Da, respectively. The first amino acid of mature enzyme was the alanine at 108^{th}. The pI and molecular mass of mature enzyme were 6.65 and 27,490.62 Da, respectively. The calculated size of mature enzyme matched well with the 27 kDa band observed by SDS-PAGE (Fig. 2).

aprEBS15 showed 99% nucleotide sequence identities to other genes from *B. amyloliquefaciens* CH86-1 (FJ882063,

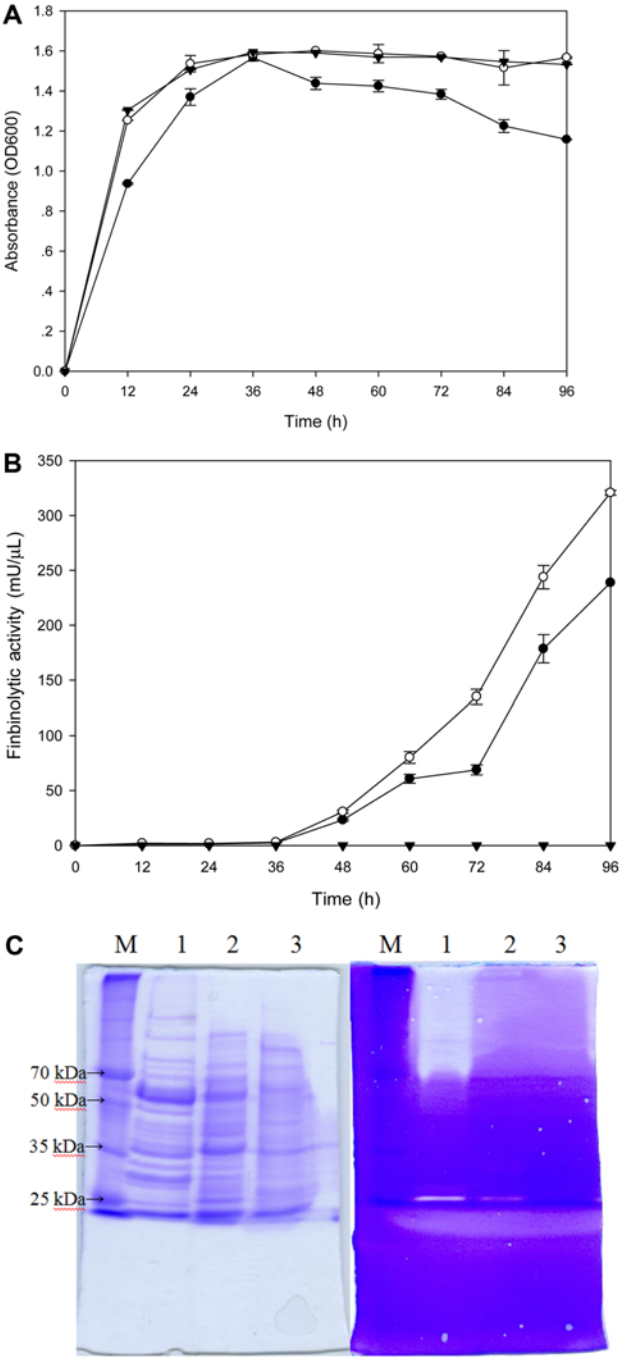

Fig. 3. Growth (A) and fibrinolytic activity (B) of *B. subtilis* TFs. *B. subtilis* WB600 TFs were cultivated in LB broth for 96 h at 37°C. *B. pumilus* BS15 (control) was grown under the same conditions. -●-, *B. pumilus* BS15; -○-, *B. subtilis* WB600 [pHYBS15]; -▼-, *B. subtilis* WB600 [pHY300PLK]. (C) SDS-PAGE (left) and fibrin zymogram (right) results of culture supernatant prepared from *B. subtilis* TFs at 96 h. Lane 1, *B. pumilis* BS15; lane 2, *B. subtilis* WB600 [pHYBS15]; lane 3, *B. subtilis* WB600 [pHY300PLK]. M, Dokdo-marker broad-range.

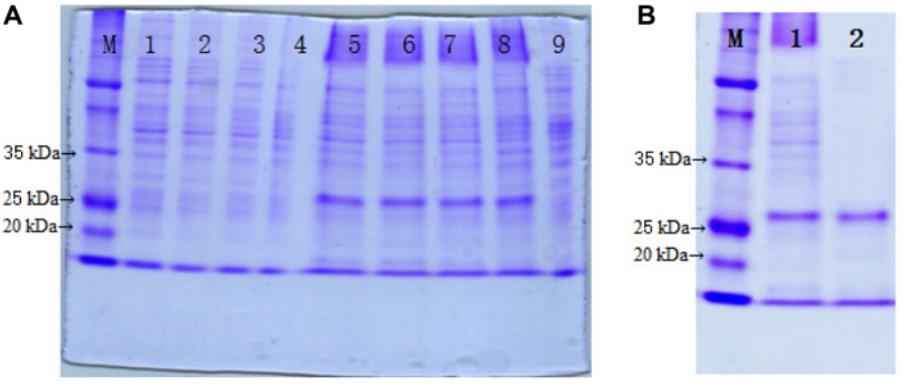

Fig. 4. Overexpression of *aprEBS15* in *E. coli* BL21(DE3). M, DokDo-Marker broad-range; lanes 1-4, soluble fraction from cells grown after induction for 2 (1), 4 (2), 10 (3), and 20 h (4); lanes 5-8, insoluble fraction from cells grown after induction for 2 (5), 4 (6), 10 (7), and 20 h (8); 9, a negative control, insoluble fraction from *E. coli* BL21 [pETBS15] grown for 20 h without induction.

1,144/1,149) [19], *B. subtilis* HK176 (KJ572414, 1,129/1,149) [24], and 98% identities to genes from *B. amyloliquefaciens* CH51 (EU414203, 1,137/1,149) [23]. AprEBS15 showed high similarities with other fibrinolytic enzymes: 99% with AprE86-1 from *B. amyloliquefaciens* CH86-1 (ACS45325), AprE5-41 from *B. amyloliquefaciens* MJ5-41 (AEE81297), AprE51 from *B. amyloliquefaciens* CH51 (ACA34903), and AprE176 from *B. subtilis* HK176 (AHN52401). Amino acids consisting of catalytic triad (Asp^{32}, His^{64}, and Ser^{221}) are conserved in AprEBS15 like other similar enzymes. *B. subtilis* WB600 harboring pHYBS15 showed the same growth profile with *B. subtilis* WB600 [pHY300PLK] (control) (Fig. 3A). TF showed the highest fibrinolytic activity (408.2 U/mL) at 96 h whereas control did not show any significant activity (Fig. 3B). *B. subtilis* WB600 [pHYBS15] showed 1.34 fold higher activity than *B. pumilus* BS15. SDS-PAGE and fibrin zymography were done for the culture supernatant from TFs. *B. subtilis* carrying pHYBS15 produced the 27 kDa band (Fig. 3C, lane 2 of coomassie stained gel), and the fibrinolytically active degradation product (Fig. 3C, lane 2 of fibrin zymogram) which were not observed in control, *B. subtilis* [pHY300PLK]. The results confirmed again that the cloned *aprEBS15* encodes the 27 kDa major fibrinolytic enzyme and the same enzyme was produced in a heterologous host, *B. subtilis* WB600.

3.5. Purification of recombinant AprEBS15

Bound AprEBS15 was eluted from a HiTrap IMAC FF column at the immidazol concentration of 200 mM. The size of eluted AprEBS15 was 27 kDa on a acrylamide gel (Fig. 4B), and the size matched well with the calculated size of mature AprEBS15.

3.6. Properties of recombinant AprEBS15

AprEBS15 showed activity at pH 7-10 and the optimum pH was 8 (Fig. 5A). The relative activity was 79.8, 100, 98.1, and 64.6%, at pH 7, 8, 9, and 10, respectively. AprEBS15 had no activity at pH 5 and below, and the activity declined rapidly at pH 6 and 11. The activity remained stable within the first h and then decreased for extended exposure up to 6 h at pH 8 and 9 (Fig. 5B). At pH 6, 10 and 11, the activity decreased rapidly within the first h and then became stable for extended exposure up to 6 h. At pH 7, the activity decreased rapidly within the first 3 h and then became stable for extended exposure up to 6 h. Rapid inactivation occurred at pH 5 and below. The optimum temperature was 40°C at pH 8 (Fig. 5C). When temperature was increased to 60°C and above, no activity remained after 30 min. When AprEBS15 was incubated for 3 h at 50°C or 55°C, the activity decreased rapidly within the first h and then became stable (Fig. 5D). The activities decreased gradually during 3 h exposure at 37°C, 40°C, and 45°C. It was concluded that AprEBS15 had moderate degree of heat stability. The optimum temperature of AprEBS15 is similar to that of *B. subtilis* HK176 [24] but lower than those of other *Bacillus* strains, such as *Bacillus*

Table 1. Effects of metal ions and inhibitors on the activity of AprEBS15

Metal ions (5 mM)*	Relative activity (%)	Inhibitors (1 mM)	Relative activity (%)
None	100.00 ± 0.00	PMSF	0.00 ± 0.00
Mn^{2+}	68.78 ± 1.67	EDTA	92.65 ± 2.36
Mg^{2+}	111.10 ± 5.80	EGTA	83.76 ± 2.36
Co^{2+}	31.99 ± 0.67	SDS	24.64 ± 0.96
K^{+}	113.91 ± 2.99		
Zn^{2+}	105.46 ± 5.46		
Na^{+}	97.50 ± 2.50		
Ca^{2+}	102.65 ± 2.65		
Fe^{3+}	83.76 ± 2.10		

*The counterion for the tested metals was chloride.

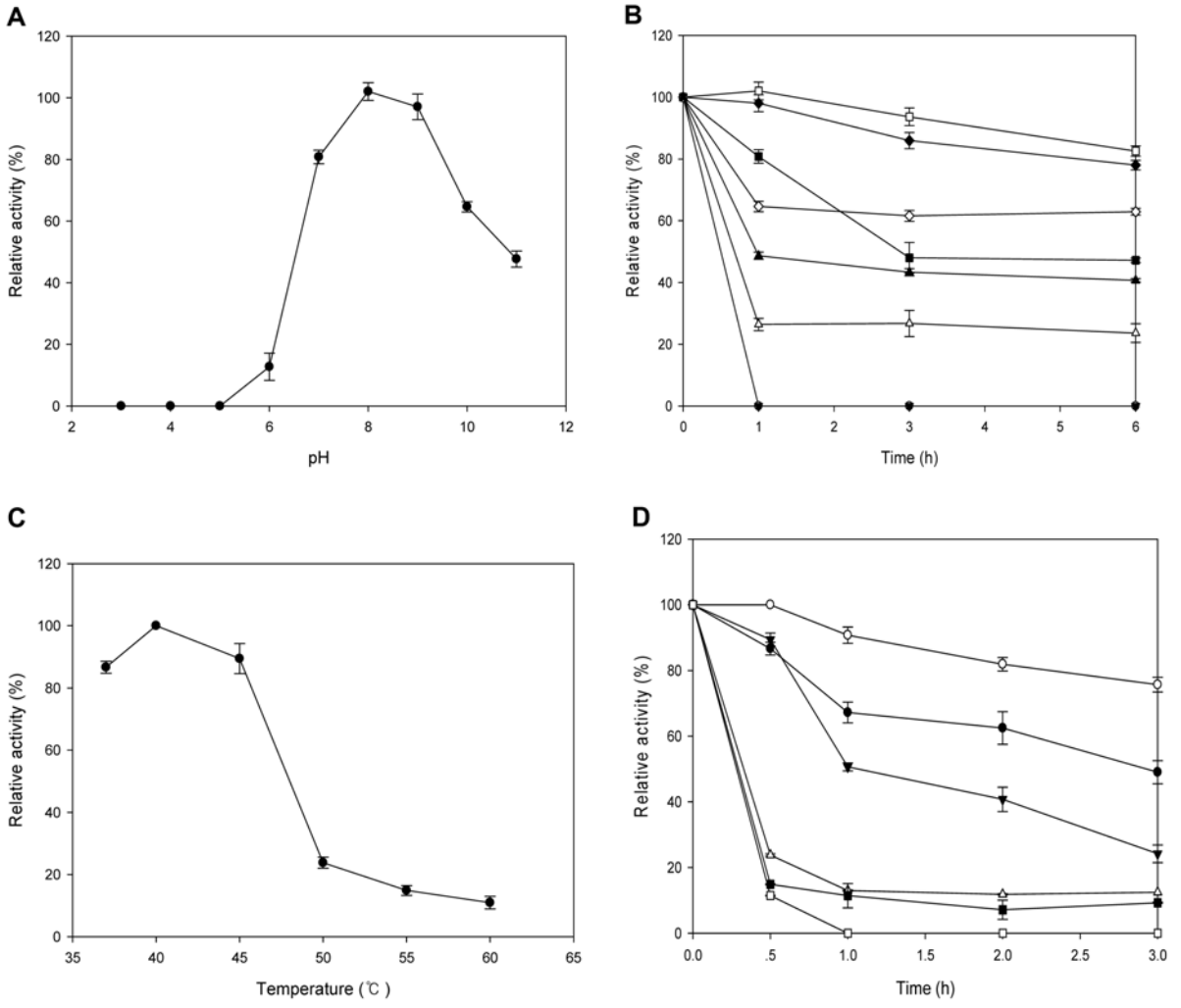

Fig. 5. The effect of pH and temperature on the fibrinolytic activity of recombinant AprEBS15. The optimum pH (A) and stability against pH variation (B): -●-, pH 3; -○-, pH 4; -▼-, pH 5; -△-, pH 6; -■-, pH 7; -□-, pH 8; -◆-, pH 9; -◇-, pH 10; -▲-, pH 11. The optimum temperature (C) and the stability against temperature (D): -●-, 37°C; -○-, 40°C; -▼-, 45°C; -△-, 50°C; -■-, 55°C; -□-, 60°C.

sp. strain CK11-4 (70°C) [25].

The effects of metals and inhibitors on the activity were examined (Table 1). K^+ enhanced the fibrinolytic activity by 13.9%, Mg^{2+} enhanced the fibrinolytic activity by 11.1% and Zn^{2+} slightly enhanced (5.46%). The activity was inhibited by Na^+ (2.9% inhibition) and Fe^{3+} (16.2% inhibition), Mn^{2+} (31.2% inhibition) and Co^{2+} (69.0% inhibition). The activity was completely destroyed by PMSF (phenylmethylsulfonyl fluoride). But SDS, EDTA (ethylenediaminetetraacetic acid), and EGTA (ethylene glycol tetraacetic acid) decreased the activity by 75.3, 7.3, and 16.2%, respectively. The results showed that AprEBS15 is a serine protease.

3.7. Amidolytic activity measurements

Km and Vmax of purified AprEBS15 were determined from the initial rates for the hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-pNA. The Km and Vmax values were 0.26 mM and 21.88 μM min^{-1}, respectively, and the Kcat was 10.02 S^{-1}. Kcat/Km was 3.83×10^4 S^{-1} M^{-1}. The values seemed similar to those of other fibrinolytic enzymes reported. The reported Kcat/Km values were 6.7×10^4, 6.7×10^4, 6.4×10^4, 3.1×10^4, 1.76×10^5, and 5.66×10^4 S^{-1} M^{-1} for fibrinolytic enzyme from *B. subtilis* IMRNK1 [26], a *B. subtilis* strain [27], *Bacillus* sp. nov. SK006 [28], *B. subtilis* CH3-5 [29], *B. subtilis* DC33 [30], and *B. amyloliquefaciens* RSB34 [14]. The amidolytic activity of AprEBS15 was similar to other fibrinolytic enzymes.

3.8. Hydrolysis of fibrinogen by AprEBS15

AprEBS15 quickly degraded Aα and Bβ chains of fibrinogen. The Aα chain was the most sensitive and hydrolyzed in 10 min (Fig. 6). Most Bβ chain was degraded in 1 h, and completely degraded in 3 h. But γ-chain was not

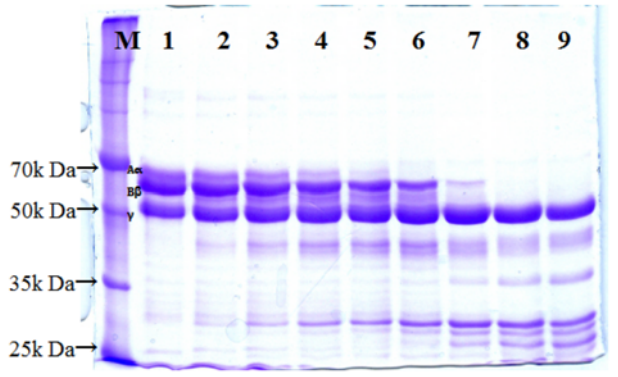

Fig. 6. Fibrinogen hydrolysis by AprEBS15. M, DokDo-Marker broad-range; 1, control (no enzyme treatment); 2, 5 min; 3, 10 min; 4, 20 min; 5, 30 min; 6, 1 h; 7, 3 h; 8, 6 h; 9, 12 h. A 10% acrylamide gel was used.

degraded even after 12 h (Fig. 6). This indicated that AprEBS15 has strong α-fibrinogenase and moderate β-fibrinogenase activities. The degradation pattern was similar to those observed in some other fibrinolytic enzymes such as AprE176 from *B. subtilis* HK176 [24].

4. Conclusion

Considering its significantly high salt tolerance and fibrinolytic activity, *B. pumilus* BS15 can be used as a starter for fermented foods such as jeotgals and soy sauces where salt concentrations are high. Good growth of a starter is essential if high quality fermented foods are intended to be produced by using a starter. Ideal starters can not only improve organoleptic properties of fermented foods but also improve functionalities of foods by producing functional metabolites such as fibrinolytic enzymes during fermentation. Although *B. pumilis* strains have not been utilized often as a host for metabolites production or a starter for fermented foods, potential of *B. pumilus* strains deserves future studies on the metabolites and the effects on the quality of fermented foods. Availability of diverse hosts or starters can accelerate production of fermented foods or valuable metabolites with commercial importance.

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