Characterization of a Fibrinolytic Enzyme Secreted by *Bacillus velezensis* BS2 Isolated from Sea Squirt Jeotgal^S

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Bacillus sp. BS2 showing strong fibrinolytic activity was isolated from sea squirt (munggae) jeotgal, a traditional Korean fermented seafood. BS2 was identified as *B. velezensis* by molecular biological methods. *B. velezensis* BS2 grows well at 15% NaCl and at 10°C. When *B. velezensis* BS2 was cultivated in TSB broth for 96 h at 37°C, the culture showed the highest fibrinolytic activity (131.15 mU/μl) at 96 h. Three bands of 27, 35 and 60 kDa were observed from culture supernatant by SDS-PAGE, and fibrin zymography showed that the major fibrinolytic protein was the 27 kDa band. The gene (*aprEBS2*) encoding the major fibrinolytic protein was cloned, and overexpressed in heterologous hosts, *B. subtilis* WB600 and *E. coli* BL21 (DE3). *B. subtilis* transformant showed 1.5-fold higher fibrinolytic activity than *B. velezensis* BS2. Overproduced AprEBS2 in *E. coli* was purified by affinity chromatography. The optimum pH and temperature were pH 8.0 and 37°C, respectively. K_m and V_{max} were 0.15 mM and 39.68 μM/1/min, respectively, when N-succinyl-Ala-Ala-Pro-Phe-pNA was used as the substrate. AprEBS2 has strong α-fibrinogenase and moderate β-fibrinogenase activity. Considering its high fibrinolytic activity, significant salt tolerance, and ability to grow at 10°C, *B. velezensis* BS2 can be used as a starter for jeotgal.

Keywords: *Bacillus velezensis*, fibrinolytic activity, sea squirt jeotgal, salt tolerance

Introduction

Jeotgal is a traditional Korean fermented seafood prepared from various fishes, fish eggs, fish intestines, squids, and shellfishes. Most types of jeotgal are made simply by mixing the raw materials with salt and preserving them for several months before consumption [1]. Various microorganisms have been isolated from jeotgal, and identified by cultural methods and/or culture-independent methods [2–6]. Due to the high salinity of most jeotgal types (20–30%, NaCl), halophilic and halotolerant microorganisms can grow during jeotgal fermentation, and they are believed to contribute to the development of the varied and unique flavors of jeotgal. Halophiles such as *Tetragenococcus* sp. or *Halanaerobium* sp. and halotolerant organisms such as *Staphylococcus* sp. are the most often isolated organisms [2,3]. Even though the growth of nonhalophiles is inhibited, some nonhalophiles such as

Bacillus sp. still persist during the whole fermentation process [2, 3]. Various species have been identified as dominant species for jeotgal by both culture dependent and independent methods, but the exact roles of each species for jeotgal fermentation are not understood well.

It is generally believed that microorganisms with strong proteolytic activities can accelerate fermentation processes, thus reducing the time required for the completion of fermentation of fish sauce and jeotgal [7]. Proteases from both raw materials and microorganisms hydrolyze the proteins of the raw materials, generating amino acids, peptides and important metabolites and also affect the overall quality of fermented foods [8, 9]. In this respect, *Bacillus* sp. are promising candidates as starters for jeotgal because they secrete several active proteases into the culture medium. Some bacilli secrete proteases with fibrinolytic activities into the culture medium, and nattokinase produced by some *B. subtilis* strains is the most well known

[10]. Other *Bacillus* species such as *B. amyloliquefaciens* and *B. licheniformis* also secrete enzymes quite similar with nattokinase [11]. Bacilli producing such fibrinolytic enzymes have an advantage as starters for fermented foods since the enzymes not only contribute to successful progress of fermentation by hydrolyzing proteins but also increase the functionality of fermented foods [12]. Another essential requirement for a starter is the ability to grow under the high salinity of jeotgal. Many bacilli can't grow in high salt environments (10% NaCl or above) but remain as inactive or dormant cells [3]. Some bacilli cells can persist under high salinity conditions and resume rapid growth when conditions are changed favorably. *B. subtilis* JS2 was isolated from saeu (small shrimp) jeotgal and the strain grew in LB broth with 20% (w/v) NaCl [13]. When *B. subtilis* JS2 was inoculated into gul (oyster) jeotgal (23% NaCl, w/v) as a starter (1×10^6 CFU/g jeotgal), it did not grow but some cells remained viable during 24 weeks of fermentation [13]. Considering their strong proteolytic activities, halotolerant bacilli are promising as starters for jeotgal and other fermented foods such as soy sauce. In addition to strong proteolytic activities, many bacilli produce antimicrobial substances, which effectively prevent the growth of pathogenic fungi and bacteria. Also, bacilli form spores, which can last for long periods under adverse conditions.

Bacillus velezensis BS2 was isolated from sea squirt (munggae) jeotgal, and the strain showed significant fibrinolytic activity, salt tolerance, antimicrobial activity, and ability to grow at 10°C. A fibrinolytic gene, *aprEBS2*, was cloned and overexpressed in *B. subtilis* and *E. coli*. *AprEBS2* overproduced in *E. coli* was purified and its properties were studied. Considering these results, *B. velezensis* BS2 seems promising as a starter for jeotgal, accelerating fermentation and improving final quality.

Materials and Methods

Isolation and Identification of *Bacillus* sp. BS2

Bacilli with fibrinolytic activities were isolated from sea squirt (munggae) jeotgal. Sea squirt was purchased at a local fish store at Tongyeong, Gyeongnam, Korea in the spring of 2018. After washing with tap water, sea squirt was mixed with salt, and the final salt concentration was 12% (w/w). Sea squirt jeotgal was fermented for 42 days at 10°C. During fermentation, 10 g of jeotgal was taken out, mixed with 90 ml of 0.1% peptone water, and homogenized by Stomacher 80 (Seward, Worthing, UK). Diluted samples were spread on tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, USA) agar plates with NaCl (10%, w/v). Plates were incubated for 48 h at 37°C, and colonies were spotted

onto Luria-Bertani (LB, tryptone 10 g, yeast extract 5 g, NaCl 5 g/l, pH 7.0) agar plates with skim milk (1%, w/v). Colonies showing big halos were further examined for fibrinolytic activities by fibrin plate method (see below).

16S rRNA genes were amplified by using the following primers: bac-F (5'-CGGCGTGCCTAATACATGCAAG-3') and bac-R (5'-GGCATGCTG ATCCGCATTAC TA-3') [14]. A partial *recA* gene was amplified using primers: recA-F (5'-TGAGTGATCGTCAGG CAGCCTTAG-3') and recA-R (5'-CYTBRGATAAGAR TACCAW GMACCGC-3') [14]. Amplification was done using a thermocycler (MJ Mini Personal Thermal Cycler, BioRad, USA). Reaction mixture (50 µl) contained 1 µl of template DNA, 1 µl of each primer (10 µM), 5 µl of dNTPs (0.25 mM), and 0.5 µl of ExTaq DNA polymerase (Takara, Japan). Amplification conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 40 sec, and a final extension at 72°C for 5 min. Nucleotide sequences of the amplified fragments were determined and searched for homologous genes by BLAST (NCBI, USA).

RAPD-PCR was done using S30 (5'-GTGATCGCAG-3') as a primer and chromosomal DNA as a template. Chromosomal DNA was prepared from 18 h culture on LB broth by phenol-chloroform extraction method, and PCR was done as reported previously [15].

Growth, Fibrinolytic Activity, and Salt Tolerance of *B. velezensis* BS2

B. velezensis BS2 was grown in 4 different media: LB broth, Brain Heart Infusion (BHI, Becton, Dickinson and Company), nutrient broth (NB, Becton, Dickinson and Company), and TSB. Aliquots were taken at 12 h intervals during 96 h cultivation at 37°C with shaking, and OD_{600 nm} values were measured using a spectrophotometer (UV-1601, Shimadzu, Japan). Aliquots were centrifuged at 4,000 ×g for 10 min at 4°C, and the supernatant was filtered using a 0.45 µm filter (Sartorius Stedim, Germany). Filtered supernatant (FS) was assayed for fibrinolytic activity by fibrin plate method [14]. The size of lysis zones on fibrin plates were converted into plasmin units (U) by comparing them with those of plasmin with different units (Sigma, USA). Salt tolerance of *B. velezensis* BS2 was examined by checking growth in LB broth with NaCl. *B. velezensis* BS2 was grown overnight in LB (1%, v/v), and then inoculated into fresh TSB broth with different NaCl concentrations (10, 12, 15, 17, 20%, w/v). Cultures were incubated for 96 h with shaking at 37°C, and OD₆₀₀ values were measured at 12 h intervals.

SDS-PAGE and Fibrin Zymography

B. velezensis BS2 was grown in TSB for 96 h at 37°C. Aliquots were taken at intervals, and FSs were obtained. FSs were boiled for 5 min in 4 × SDS sample buffer (12 g Tris base, 57.6 g glycine, 4 g/l SDS). SDS-PAGE was done using a 10% acrylamide gel, and fibrin zymography was done using a polyacrylamide gel containing fibrin, which was prepared by mixing fibrinogen (0.12%, w/v) and 100 µl of thrombin (10 NIH units/ml) with

acrylamide solution. After electrophoresis at a constant current of 10 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. Gel was washed with distilled water for 30 min to remove 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 [14].

Cloning of *aprEBS2* Gene

aprEBS2 was amplified from *B. velezensis* BS2 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) [16]. PCR was done as described above except annealing temperature was 65°C. Amplified fragment was ligated with pHY300PLK (4.87 kb, Tc^R) (Takara) after being digested with *Bam*HI and *Eco*RI. Ligation mixture was used to transform *B. subtilis* WB600 competent cells by electroporation. A *B. subtilis* transformant (TF) was grown in LB broth containing tetracycline (10 µg/ml). Growth and fibrinolytic activity were measured at intervals during 96 h cultivation. Preparation of *B. subtilis* WB600 competent cells and electroporation (200 Ω, 18 kV/cm) were done according to published methods [17]. Plasmid DNA preparation, restriction enzyme digestion, and agarose gel electrophoresis were done according to published methods [18].

Overexpression of *aprEBS2* in *E. coli* and Purification of Recombinant AprEBS2

aprEBS2 without its own signal sequence was amplified using the following primer pairs: pETBS2-F (5'-AGAGGATCCGATGGCAGGGAAATC-3' *Bam*HI site underlined) and pETBS2-R (5'-AGACTCGAGCTGAGCTGCCGCTG-3' *Xho*I site underlined). Amplified gene was inserted into pET26b (+) (Merck Millipore, Germany), resulting in pETBS2. An *E. coli* BL21 (DE3) TF harboring pETBS2 was obtained by electroporation, and grown in LB (250 ml) containing kanamycin (30 µg/ml). When the OD_{600} reached 0.8, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added (1 mM), and culture was incubated for 20 h. Cells were obtained by centrifugation, resuspended in 5 ml of PBS, and then disrupted by sonication (UW 2070, Bandelin, Germany). Cell extract was centrifuged at 8,000 ×g for 15 min at 4°C. Soluble fraction (supernatant) and insoluble fraction (pellet) were examined by SDS-PAGE. Insoluble fraction was resuspended with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and loaded onto a HiTrap IMAC FF column (GE Healthcare, Sweden). Sodium phosphate buffer (20 mM, pH 7.4) containing imidazole and 0.5 M NaCl was used as elution buffer, and the imidazole concentration was increased from 50 mM to 500 mM with 50 mM intervals. One microliter of each fraction (1 ml) was spotted onto a fibrin plate and incubated at 37°C. Active fractions were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.4) for 24 h. AprEBS2 was

concentrated by using an Amicon filter (MWCO 12,000; Millipore). Protein concentration was measured by Bradford method using bovine serum albumin (BSA) as a standard [19].

Properties of Purified AprEBS2

The effect of pH on AprEBS2 was examined by using different buffer systems (50 mM): citrate-NaOH, pH 3–5; sodium phosphate, pH 6–8; and Tris-HCl, pH 9–11. AprEBS2 (1 µg) resuspended in each buffer was incubated for 1 h at 37°C, and the activity was measured by the fibrin plate method. For measuring pH stability, AprEBS2 (1 µg) in each buffer was incubated up to 6 h at 37°C, and activity was measured at 1, 3, and 6 h. AprEBS2 (1 µg) in sodium phosphate buffer (pH 8.0) was incubated for 30 min at 37–60°C, and then the activity was measured. For measuring thermal stability, AprEBS2 (1 µg) in sodium phosphate buffer (pH 8.0) was incubated up to 3 h at 37–60°C, and the remaining activity was measured at 0.5, 1, 2, and 3 h. AprEBS2 was exposed to 5 mM metal ions or 1 mM inhibitors for 30 min at 37°C and pH 8.0, and the remaining activity was measured.

Amidolytic Activity Measurements and Enzyme Kinetics

The amidolytic activity of AprEBS2 was examined by using *N*-succinyl-L-alanyl-L-alanyl-L-proline-*p*-nitroanilide (S7388, Sigma). Fifty µl of substrate in sodium phosphate buffer (50 mM, pH 8.0) was mixed with AprEBS2 (1 µg), and the total volume was adjusted to 500 µl with sodium phosphate buffer. After 10 min incubation at 37°C, 500 µl of citrate-NaOH buffer (pH 3.0) was added and the tube was put on ice immediately. The mixture was centrifuged at 12,000 ×g for 5 min and $\text{OD}_{410\text{ nm}}$ of the supernatant was measured. The degree of hydrolysis was calculated from the absorbance value and the molar extinction coefficient of *p*-nitroanilide (8,800 $\text{M}^{-1}\text{cm}^{-1}$). Kinetic parameters of AprEBS2 were determined by measuring the release of *p*-nitroaniline from *N*-succinyl-L-alanyl-L-alanyl-L-proline-*p*-nitroanilide in sodium phosphate buffer at 37°C. V_{max} and K_m values were determined from measurements at different substrate concentrations ranging from 0.03 to 0.9 mM. V_{max} value was converted to K_{cat} from the relationship $K_{\text{cat}} = V_{\text{max}}/[\text{enzyme}]$.

Hydrolysis of Fibrinogen by AprEBS2

One microgram of fibrinogen (Bovine, MP Biochemicals, Illkirch, France) was mixed with AprEBS2 (50 ng) in 1 ml of sodium phosphate buffer and incubated at 37°C up to 6 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.

Results and Discussion

Isolation and Identification of *Bacillus* sp. BS2

Among 47 tentative bacilli isolates showing fibrinolytic activities, BS2 produced the largest lytic zone on a fibrin plate (results not shown). BS2 was Gram-positive rod, and

the colony possessed a typical *Bacillus* morphology on LB agar plates. 16S rRNA genes were amplified and sequenced (1,225 nucleotides, MH378167). BLAST analysis indicated that the gene showed 99% identities to genes from *B. amyloliquefaciens* and *B. velezensis* strains. A partial *recA* gene was amplified and the sequence (758 nucleotides, MH378166) also showed 99% identities to *recA* genes from *B. amyloliquefaciens* and *B. velezensis* strains. Sequencing of 16S rRNA and *recA* genes was not sufficient for accurate species identification. Thus, RAPD (randomly amplified polymorphic DNA)-PCR was done using a S30 primer. Two bands of 800 and 2,000 bp in size were amplified from BS2 chromosomal DNA, and they matched with bands from a *B. velezensis* reference strain (Fig. S1). Another isolate (*Bacillus* sp. SS2) also showed the same pattern. In contrast, a 1.5 kb band was amplified from *B. amyloliquefaciens* reference strain, and the 1.5 kb band is one of the unique bands of *B. amyloliquefaciens* strains [15]. From these results, BS2 was positively identified as a *B. velezensis* strain.

B. velezensis is closely related to *B. amyloliquefaciens*, *B. siamensis*, and *B. methylotrophicus*. Because of very high similarities among these species, the exact distinction between these species is controversial [20, 21]. Some *B. velezensis* strains are used to promote plant growth by inhibiting pathogenic fungi [22, 23]. In addition to plant and soil environments, *B. velezensis* strains have been isolated from marine environments such as sea water and sea mud [24, 25]. But so far, there has been no report on the isolation of *B. velezensis* from jeotgal. Difficulty in correct species identification among very closely related *Bacillus* species might be the reason. Considering this, polyphasic

classification schemes are necessary for the accurate species identification of bacilli isolates from environmental samples including jeotgal.

Growth, Fibrinolytic Activity, and Salt Tolerance of *B. velezensis* BS2

B. velezensis BS2 formed a film at the top of broth when statically cultivated in TSB. The film formation was most obvious at 37°C (result not shown), but not observed at 15°C and 10°C. *B. velezensis* BS2 grew quickly for the first 12 h at 45°C and OD₆₀₀ value was at the highest point, 1.01, at 12 h. Then the value decreased gradually, reaching 0.86 at 96 h. *B. velezensis* BS2 grew slowly but steadily at 10°C, reaching 0.52 at 96 h (Fig. 1A). Culture at 37°C showed the highest fibrinolytic activity, and culture at 25°C showed the second highest activity (Fig. 1B). *B. velezensis* BS2 produces a bacteriocin that inhibits growth of important food pathogens such as *Listeria monocytogenes* and *B. cereus*, an advantage when used as a starter (results not shown).

B. velezensis BS2 grows well in the presence of NaCl up to 15% (w/v), and slowly at 17% (Fig. 2). At 15% NaCl, BS2 did not grow until 48 h, and OD₆₀₀ was just 0.047. Then growth started rapidly, and OD₆₀₀ value was 1.47 at 96 h, the same as those of cultures at 10% (1.49) or 12% (1.52) NaCl (Fig. 2). At 17% NaCl, *B. velezensis* BS2 did not grow until 84 h. Then it grew slowly, and OD₆₀₀ value was 0.26 at 96 h.

Among the 4 culture media tested, TSB was the best medium in terms of cell growth and fibrinolytic activity, and used for further experiments. Culture in TSB showed the highest fibrinolytic activity (131.15 mU/μl) at 96 h, whereas NB was the poorest medium (results not shown).

Fig. 1. Effect of temperature on the growth (A) and fibrinolytic activity (B) of *B. velezensis* BS2. -●-, 10°C; -○-, 15°C; -▼-, 25°C; -△-, 37°C; -■-, 45°C.

FS (filtered culture supernatant) at each time point was applied onto a fibrin plate and the fibrinolytic activity was expressed as plasmin unit/ml.

Fig. 2. Salt tolerance of *B. velezensis* BS2.

B. velezensis BS2 was grown in TSB with NaCl for 96 h with shaking. -●-, 10%; -○-, 12%; -▼-, 15%; -△-, 17%; -■-, 20% NaCl (w/v).

SDS-PAGE and Fibrin Zymography

When FS was analyzed by SDS-PAGE, 27, 35, and 60 kDa bands were observed in addition to other bands (Fig. 3A). The 27 kDa band was also observed on a fibrin zymogram and it was the main fibrinolytic protein (Fig. 3B). One notable observation was that the band intensity increased after 72 h, and the strongest intensity was observed at 96 h (Fig. 3B, lanes 6–8). The result agreed with the fibrinolytic activity measurement results (Fig. 1B). Cultures at 72–96 h showed higher fibrinolytic activities and the cultures showed the strong 27 kDa band by SDS-PAGE. The fibrinolytic activity of culture increased as cells entered into stationary growth phase.

Cloning of *aprEBS2* Gene

The major fibrinolytic protein (AprE) and its gene (*aprE*) are well conserved among bacilli. The high similarities among *aprE* genes make it possible to use primer pairs designed for cloning of an *aprE* gene from a *Bacillus* species for the cloning of homologous genes from other *Bacillus* species. In this work, CH51-F and CH51-R primers were used to clone *aprEBS2*. The primer pairs were initially designed to clone a fibrinolytic gene (*aprE51*) from *B. amyloliquefaciens* CH51 [16], and later successfully used to clone *aprE34* from *B. amyloliquefaciens* RSB34 and *aprEBS15* from *B. pumilus* BS15 [14, 26].

A 1.4 kb amplified fragment was ligated into pHY300PLK, and pHYBS2 (6.2 kb, Ap^r, Tc^r) was obtained. Sequencing of the insert (1,359 nucleotides, MH378165) (Fig. S2) confirmed that the gene was a homolog of *aprE* genes. An ORF of 1,149 bp in size (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 from comparisons with other fibrinolytic enzymes (results not shown). pI and molecular mass of proAprEBS2 (352 aa) were 8.75 and 35,857.18 Da, respectively. Calculated pI and molecular mass of mature enzyme were 6.65 and 27,429.74 Da, respectively. Nucleotide sequence of *aprEBS2* showed 99% identities to those of *aprE* genes from *B. amyloliquefaciens* CH51 (EU414203, 1,145/1,149) [16], *B. pumilus* BS15 (MF943247, 1,144/1,149) [26], and 98% identities to genes from *B. amyloliquefaciens* CB1 (KM086575, 1,133/1,149) [27], *B. amyloliquefaciens* RSB34 (KX098331, 1,132/1,149) [14], and *B. amyloliquefaciens* MJ5-41 (JF739176, 1,135/1,149) [28]. AprEBS2 showed high similarities with other fibrinolytic

Fig. 3. SDS-PAGE (A) and fibrin zymography (B) of FS from *B. velezensis* BS2.

B. velezensis BS2 was grown in TSB broth at 37°C up to 96 h. FS at each time point was analyzed. M, Dokdo-Marker, broad-range (EBM-1034, Elpis-Biotech., Daejeon, Korea); 1, 12 h; 2, 24 h; 3, 36 h; 4, 48 h; 5, 60 h; 6, 72 h; 7, 84 h; 8, 96 h.

Fig. 4. Growth (A) and fibrinolytic activities (B) of *B. subtilis* TFs.

B. subtilis WB600 TF was grown for 96 h at 37°C in LB broth. OD₆₀₀ and fibrinolytic activities of culture were measured at each time interval. ●, *B. velezensis* BS2; ▼, *B. subtilis* WB600 [pHYBS2]; ○, *B. subtilis* WB600 [pHY300PLK].

enzymes: 99% with AprE5-41 from *B. amyloliquefaciens* MJ5-41 (AEE81297), AprE51 from *B. amyloliquefaciens* CH51 (ACA34903), AprECB1 from *B. amyloliquefaciens* CB1 (AIR72259), AprE34 from *B. amyloliquefaciens* RSB34 (APO13874), and AprEBS15 from *B. pumilus* BS15 (ATD12229). Amino acids consisting of catalytic triad (Asp³², His⁶⁴, and Ser²²¹) are conserved in AprEBS2 like other AprE enzymes [29].

pHYBS2 was introduced into *B. subtilis* WB600 cells by electroporation [30]. *B. subtilis* WB600 TF harboring pHYBS2 showed the same growth pattern with *B. subtilis* WB600 [pHY300PLK] (control) (Fig. 4A), and showed the highest fibrinolytic activity (186.8 U/ml) at 96 h (Fig. 4B).

Control did not show any activity. Like the original host, the fibrinolytic activity of *B. subtilis* WB600 [pHYBS2] increased as culture entered into stationary growth phase. The activity was 1.5 fold higher than that of *B. velezensis* BS2. When FS from *B. subtilis* TF was analyzed by SDS-PAGE and fibrin zymography, proAprEBS2 and mature AprEBS2 were observed (Fig. 5). A 35 kDa proAprEBS2 matched with a 35 kDa band on a coomassie blue-stained gel (Fig. 5A), and the mature AprEBS2 band was observed on a polyacrylamide gel (Fig. 5A) and a fibrin zymogram (Fig. 5B). No 27 kDa band was observed from control, *B. subtilis* WB600 carrying intact pHY300PLK (Fig. 5, lanes 7–9).

Fig. 5. SDS-PAGE (A) and fibrin zymography (B) of FS from *B. subtilis* TFs.

M, Dokdo-Marker, broad-range (EBM-1034, Elpis-Biotech., Daejeon, Korea). Bacilli were grown in LB broth at 37°C and FSs were analyzed. Lanes 1–3, *B. velezensis* BS2 grown for 24 h (lane 1), 60 h (2), and 96 h (3); lanes 4–6, *B. subtilis* WB600 [pHYBS2] grown for 24 h (4), 60 h (5), and 96 h (6); lanes 7–9, *B. subtilis* WB600 [pHY300PLK] grown for 24 h (7), 60 h (8), and 96 h (9).

Fig. 6. Overexpression of *aprEBS2* in *E. coli* (A) and purification of AprEBS2 (B).

M, Dokdo-Marker, broad-range (EBM-1034, ELPIS, Daejeon, Korea); Lanes 1–4, soluble fraction from cells grown for 2 (1), 4 (2), 10 (3), and 20 h (4) after induction; Lanes 5–8, insoluble fraction from cells grown for 2 (5), 4 (6), 10 (7), and 20 h (8) after induction; 9, negative control, soluble fraction from *E. coli* BL21 [pETBS2] grown for 20 h without induction. B. M, Dokdo-Marker, broad-range; 1, insoluble fraction; 2, purified AprEBS2.

Overexpression of *aprEBS2* in *E. coli* and Purification of Recombinant AprEBS2

In pETBS2, *aprEBS2* has an additional six His codons at the 3' end immediately after the last sense codon (Gln), which was used for the one-step purification of recombinant AprEBS2. After IPTG induction, soluble and insoluble fractions from *E. coli* BL21 (DE3) with pETBS2 were obtained at different time points and analyzed by SDS-PAGE (Fig. 6A). A thick 27 kDa band was observed from the insoluble fraction (Fig. 6A, lanes 5–8). Recombinant AprEBS2 was purified from the insoluble fraction obtained at 20 h after induction by using a HiTrap IMAC FF column. AprEBS2 was eluted from the column at the imidazole concentration of 350–500 mM, and a single 27 kDa protein was obtained (Fig. 6B, lane 2).

Properties of Purified AprEBS2

The optimum pH for AprEBS2 was pH 8.0 (Fig. 7A). AprEBS2 maintained higher activity at pH 7–10. The relative activity at pH 7, 9, and 10 was 83.8%, 97.9%, and 84.5% of that at pH 8.0, respectively. The enzyme lost activity at pH 5 and below, and the activity declined rapidly at pH above 10. AprEBS2 was most stable at pH 7–8, and relatively stable at pH 9–10. At pH 6 and 11, AprEBS2 still maintained some activity after 6 h (Fig. 7B). Activity was completely lost in 1 h at pH 5 and below. The optimum temperature was 37°C (Fig. 7C). The activity was lost rapidly at 50°C and above, and no activity remained after 30 min incubation at 60°C. AprEBS2 was stable at 37–40°C and 86.3% and 67.4% of activity remained after 3 h at 37°C and 40°C, respectively (Fig. 7D). Considering the results, AprEBS2 has moderate heat stability. The optimum

temperature for AprEBS2 is similar to that for AprEs from other *Bacillus* strains such as *B. subtilis* HK176 [31] and *B. amyloliquefaciens* RSB34 [14], but lower than that for *Bacillus* sp. strain CK11-4 (70°C) [32].

The effects of metals and inhibitors on the activity of AprEBS2 were studied (Table 1). Mg^{2+} , Ca^{2+} , and Mn^{2+} enhanced the activity by 19.14%, 8.21%, and 4.85%, respectively. But the activity was inhibited by Fe^{3+} (28.77% inhibition), Zn^{2+} (24.62% inhibition), K^{+} (12.76% inhibition), and Co^{2+} (7.35% inhibition). The fibrinolytic activity was completely inhibited by PMSF (phenylmethylsulfonyl fluoride). But EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) and SDS (sodium dodecyl sulfate) did not inhibit the activity significantly. AprEBS2 is a serine protease but not a metalloprotease.

Table 1. Effects of metal ions and inhibitors on the activity of AprEBS2.

Metal ions (5 mM)	Relative activity (%)	Inhibitors (1 mM)	Relative activity (%)
None	100.00	PMSF	0.00
Mn^{2+}	104.85 ± 2.33	EDTA	89.89 ± 1.91
Mg^{2+}	119.14 ± 2.74	EGTA	87.24 ± 1.84
Co^{2+}	92.65 ± 1.99	SDS	92.65 ± 1.99
K^{+}	87.24 ± 1.84		
Zn^{2+}	75.38 ± 1.52		
Na^{+}	101.62 ± 2.24		
Ca^{2+}	108.21 ± 2.42		
Fe^{3+}	71.23 ± 1.41

Fig. 7. The effect of pH and temperature on the fibrinolytic activity of AprEBS2.

(A) Optimum pH, (B) stability against pH variation. -●-, pH 3; -○-, pH 4; -▼-, pH 5; -△-, pH 6; -■-, pH 7; -□-, pH 8; -◆-, pH 9; -◇-, pH 10; -▲-, pH 11. (C) Optimum temperature, (D) stability against temperature. -●-, 37°C; -○-, 40°C; -▼-, 45°C; -△-, 50°C; -■-, 55°C; -□-, 60°C.

Amidolytic Activity Measurements and Enzyme Kinetics

K_m and V_{max} of purified AprEBS2 were determined from the initial rates for the hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-pNA. The K_m and V_{max} values were 0.15 mM and 39.68 $\mu\text{M}/\text{l}/\text{min}$, respectively, and the K_{cat} was 18.14 S^{-1} . K_{cat}/K_m was $1.25 \times 10^5 \text{ S}^{-1} \text{ M}^{-1}$. The values seemed similar to those of some other fibrinolytic enzymes reported elsewhere. The K_{cat}/K_m value was similar to that of subtilisin FS33 ($1.56 \times 10^5 \text{ S}^{-1} \text{ M}^{-1}$) [33].

Hydrolysis of Fibrinogen by AprEBS2

AprEBS2 quickly degraded A_α and B_β chains of fibrinogen (Fig. 8). The A_α chain was the most sensitive and quickly hydrolyzed in 10 min. Most B_β chain was degraded in 20

min and completely degraded in 30 min. AprEBS2 has strong α -fibrinogenase, moderate β -fibrinogenase, and also some γ -fibrinogenase activity. Compared with the control (no enzyme treatment, lane 1), the γ chain was significantly degraded after 6 h incubation (lane 9). The hydrolysis pattern was similar to that observed in AprECB1, a fibrinolytic enzyme produced from *Bacillus amyloliquefaciens* CB1 [27]. But AprEBS2 shows some γ -fibrinogenase activity.

Considering its high fibrinolytic activity, salt tolerance, ability to grow at 10°C, and antimicrobial activity, *B. velezensis* BS2 seems promising as a starter for jeotgal and other fermented foods where NaCl content does not exceed 17% but its effects on the quality of jeotgal needs to be studied in detail in the future.

Fig. 8. Fibrinogen hydrolysis by AprEBS2.

M, DokDo-Marker, broad-range (EBM-1034, ELPIS, Deajeon, Korea); 1, control (no enzyme treatment); 2, 5 min; 3, 10 min; 4, 20 min; 5, 30 min; 6, 1 h; 7, 2 h; 8, 3 h; 9, 6 h. A 10% acrylamide gel was used.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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