Isolation of *Bacillus subtilis* SJ4 from Saeu (Shrimp) Jeotgal, a Korean Fermented Seafood, and Its Fibrinolytic Activity

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A *Bacillus* strain, SJ4, exhibiting strong fibrinolytic activity was isolated from saeu (shrimp, *Acetes chinensis*) jeotgal, a Korean traditional fermented food and was identified as *B. subtilis*. The *B. subtilis* SJ4 strain can grow at a NaCl concentration of up to 15% (w/v). The fibrinolytic activity of *B. subtilis* SJ4 (152.0 U/ml) cultured in Luria-Bertani (LB) broth for 48 h at 37°C with aeration was higher than that of *B. subtilis* SJ4 cultured in TSB (124.5 U/ml) under same culture conditions. The major proteins in the LB culture supernatant of *B. subtilis* SJ4 were analyzed by SDS-PAGE, which revealed three major bands (23, 25, and 28 kDa). The band (23 kDa) with strong fibrinolytic activity, analyzed on fibrin zymogram, was observed at 60–96 h of cultivation. The *aprESJ4* gene encoding the major fibrinolytic enzyme, AprESJ4, was cloned by PCR. The *aprESJ4* gene sequence exhibited high similarities with the fibrinolytic gene sequences of other *Bacillus* species. The amino acid sequence of AprESJ4 exhibited 98.9 and 98.4% similarity with subtilisin NAT and AprE2 of *B. subtilis*, respectively. Hence, *B. subtilis* SJ4 can be a potential starter culture for jeotgal products.

Keywords: *Bacillus subtilis*, saeu jeotgal, fibrinolytic enzyme, starter, *aprESJ4*

Introduction

Bacillus subtilis is one of the most industrially important microorganisms, and used as a host for producing enzymes such as amylases and proteases [1, 2]. *B. subtilis* is also well-known as a producer of various antimicrobial substances including bacteriocins and lipopeptides [3]. *B. subtilis* has served as a model organism representing gram-positive bacteria and spore forming bacteria. *B. subtilis* and closely related *Bacillus* species have been used for fermented soyfoods such as doenjang, meju, douchi, miso, and tempeh in Asian countries including Korea, China, Japan, and Indonesia. They are also

actively utilized as probiotics for human and animals [4, 5]. Isolation of *B. subtilis* strains with useful properties are important because such strains can be used to improve current production processes for metabolites or to develop new processes if isolates produce some novel products with commercial values [6].

We screened jeotgals, Korean traditional fermented seafoods, to isolate *Bacillus* species with useful properties. Since marine environments are different from terrestrial environments, some bacilli from jeotgals were expected to possess some novel and useful properties [6]. Among the bacilli isolates, one strain (SJ4) showed strong fibrinolytic activity together with antibacterial activity. SJ4 was identified as *B. subtilis* and its fibrinolytic activity was studied in details. Some *B. subtilis* strains are known to produce a strong fibrinolytic enzyme known as “Nattokinase”. Nattokinase can dis-

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solve fibrin clots *in vitro* and is believed to play the same function in human body [7]. Products containing Nattokinase are sold as health supplements to prevent or treat vascular diseases caused by fibrin accumulation in blood vessels [8]. Other *Bacillus* species closely related to *B. subtilis* also secrete fibrinolytic enzymes with similar properties [9–11]. Nattokinase and closely related fibrinolytic enzymes are functional materials with potentials as functional food ingredients and medicines replacing current thrombolytic agents such as streptokinase, urokinase, or t-PA (tissue plasminogen activator). More studies are desirable for the production of functional foods and bioactive ingredients from *Bacillus* species isolated from marine environments. In this respect, *B. subtilis* SJ4, as a GRAS organism, seems useful as a starter for fermented soyfoods and jeotgals considering its strong fibrinolytic and antimicrobial activities in addition to its significant salt tolerance.

Materials and Methods

Isolation and identification of *Bacillus* strains from jeotgal

Three different types of jeotgals, saeu (shrimp, *Acetes chinensis*), myeolchi (anchovy, *Engraulis japonicus*) and hwangseok-eo (big head croaker, *Colichthys lucidus*) jeotgals, were purchased at a local market of Jinju, Gyeongnam, Korea, in during the Spring of 2019. Ten grams of each jeotgal was mixed with 90 ml of sterile 0.1% peptone water and homogenized using a stomacher (Seward, UK). The homogenate was serially diluted using 0.1% peptone water. Aliquots (0.1 ml) of diluted samples were spread onto Luria-Bertani (LB) agar plates (tryptone 10 g, yeast extract 5 g, NaCl 5 g, per liter, pH 7.0) and the plates were incubated at 37°C until colonies appeared.

Colonies showing typical *Bacillus* morphology were selected and spotted onto LB agar plates with skim milk (1%, w/v), and the plates were incubated at 37°C. Colonies showing large lysis zones were selected and their fibrinolytic activities were examined by spotting on fibrin plates as described previously [9].

For identification of *Bacillus* sp. SJ4, 16S rRNA genes were amplified using primers: bac-F (5'-CGGCGTG-CCTAATACATGCAAG-3') and bac-R (5'-GGCATGCT-GATCCGCATTACTA-3') [12]. A *recA* gene was amplified using the following primers: recA-F (5'-TGAGTGATC-

GTCAGGCAGCCTTAG-3') and recA-R (5'-CYTBRGA-TAAGAR TACCAWGMACCGC-3') [12]. Amplification was done using a thermocycler (MJ Mini personal thermal cycler, BioRad, USA). The reaction mixture (50 µl) contained 2 µl of template DNA, 2 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 s, 58°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 5 min. Chromosomal DNA was prepared from culture grown in LB broth for 18 h by using a phenol-chloroform extraction method [12]. Nucleotide sequences of the amplified fragments were determined at Cosmogenetech (Korea). BLAST program (National Center for Biotechnology Information) was used to find homologous sequences in the data library (<http://www.ncbi.nlm.nih.gov>).

Growth and fibrinolytic activity of *B. subtilis* SJ4 in different culture media

B. subtilis SJ4 was cultivated in 4 different media: LB broth, brain heart infusion broth (BHI, Becton, Dickinson and Company, USA), nutrient broth (NB, Becton, Dickinson and Company), and tryptic soy broth (TSB, Becton, Dickinson and Company). Aliquots were taken at 12 h intervals during 96 h cultivation at 37°C with shaking, and OD₆₀₀ values were measured (UV-1601, Shimadzu, Japan). An aliquot of the culture was centrifuged at 4,000 ×g for 10 min at 4°C, and the supernatant was filtered using 0.45 µm filter (Sartorius Stedim, Germany). The filtered supernatant (FS) was used for fibrinolytic activity measurement by fibrin plate method [9]. The size of a lysis zone on a fibrin plate was measured and converted into a plasmin unit (U) by comparing its size with those formed by plasmin (Sigma) at different units. All measurements were done in triplicates and the mean values were used.

Salt tolerance of *B. subtilis* SJ4

Salt tolerance of *B. subtilis* SJ4 was studied. *B. subtilis* SJ4 was inoculated (1%, v/v) into TSB with 5, 8, 10, 12, 15, and 18 % (w/v) NaCl, and incubated for 96 h at 37°C with aeration. Aliquots of cultures were taken at 12 h intervals, and the growth was checked by measuring the absorbance at 600 nm. All measurements were done in triplicates and the mean values were used.

SDS-PAGE and fibrin zymography

FS was prepared from *B. subtilis* SJ4 culture grown for 96 h in LB broth, and analyzed by SDS-PAGE. A 10% acrylamide gel was used and 20 µg of FS was loaded after being boiled for 5 min in 4 × SDS sample buffer. Fibrin zymography was done using a polyacrylamide gel containing fibrin, which was prepared by mixing fibrinogen (0.1%, w/v) and 100 µl of thrombin (1,000 NIH units/ml) with acrylamide solution. After electrophoresis at a constant current of 10 mA on ice, the fibrin gel was soaked in 50 mM Tris-HCl (pH 7.4) buffer containing 2.5% Triton X-100 for 30 min at room temperature on a rotary shaker. The gel was then 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, and 0.02% of NaN₃) for 12 h at 37°C. Finally, the gel was stained with coomassie blue R-250.

Cloning of the *aprESJ4* gene

The major fibrinolytic enzyme gene, *aprESJ4*, was cloned from *B. subtilis* SJ4 genome by PCR. A primer pair was used, CH51-F (5'-AGGATCCCAAGAGAGC GATTGCGGCTGTGTAC-3', *Bam*HI site underlined) and CH51-R (5'-AGAATTCCTTCAGAGGGAGCCACCCGTC GATCA-3', *Eco*RI site underlined) [13]. PCR reaction mixture (50 µl) contained 2 µl of template DNA, 2 µl of each primer (10 µM), 5 µl of dNTPs (0.25 mM), and 0.5 µl of *ExTaq* DNA polymerase (Takara). The amplification conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 40 s; a final extension at 72°C for 4 min. The amplified fragment was ligated into pGEM-T Easy Vector (Promega, USA) and *E. coli* DH5α competent cells (Enzynomics, Korea) were transformed with the ligation mixture. Plasmid DNA was prepared by using a kit (iNtRON Biotech., Korea). DNA sequencing and BLAST analyses were done as described previously.

Results and Discussion

Isolation and identification of *Bacillus* sp. SJ4

One bacilli isolate, SJ4, was obtained from saeu jeotgal. It was gram-positive, rod-typed, and the colony showed a typical *Bacillus* morphology on LB agar plates. *Bacillus* sp. SJ4 showed strong protease and fibrinolytic activities together with antimicrobial activ-

ity. Total DNA was prepared and 16S rRNA and *recA* genes were amplified and sequenced. BLAST analyses showed that 16S rRNA gene sequence (1,179 nucleotides, MK648141) of *Bacillus* sp. SJ4 showed 99% identities to those from *B. amyloliquefaciens*, *B. velezensis*, *B. subtilis*, and *B. siamensis*. Partial *recA* gene (772 nucleotides, MK648143) sequence showed 99% identities to those from *B. subtilis* strains. From these results, *Bacillus* sp. SJ4 was positively identified as *B. subtilis*, and named accordingly *B. subtilis* SJ4.

Growth and fibrinolytic activity of *B. subtilis* SJ4 in different culture media

B. subtilis SJ4 grew well in LB, BHI, and TSB media,

Fig. 1. Observation of *B. subtilis* SJ4 (A) growth and (B) fibrinolytic activity in different culture media. -●-, LB; -○-, TSB; -▼-, BHI; -△-, NB. FS at each time point was applied to a fibrin plate and the fibrinolytic activity was expressed as U/ml. Plasmin (4, 8, 16, and 60 mU) was used as the standard.

showing similar growth curves in these 3 culture media. The OD₆₀₀ values reached 1.43–1.57 at 24 h, and gradually increased to 1.61–1.65 at 48 h (Fig. 1A). The final values were 1.35–1.58 at 96 h. In contrast, the OD₆₀₀ value of NB culture reached 1.37 at 24 h and then decreased continuously. The final value at 96 h was 0.67, which was significantly lower than those of other cultures. The highest fibrinolytic activity, 152.0 U/ml, was observed from culture in LB at 48 h followed by culture in TSB (124.5 U/ml) at the same time point. Culture in BHI showed 97.5 U/ml and culture in NB showed the lowest activity (84.3 U/ml) at 48 h. After 48 h, fibrinolytic activities decreased continuously in all cultures.

Changes in fibrinolytic activities during growth are variable depending upon hosts. Like *B. subtilis* SJ4, *B. amyloliquefaciens* RSB34 isolated from doenjang (a Korean fermented soyfood) showed the highest activity at 48 h when grown in LB [9]. In contrast, other *Bacillus* isolates showed the highest enzyme activities at 96 h or later during cultivation [14, 15]. The activity profile is also variable depending upon culture medium as shown in this work.

Salt tolerance of *B. subtilis* SJ4

B. subtilis SJ4 can grow well in TSB with NaCl up to 12% (w/v). Culture with 5–12% NaCl reached the highest OD₆₀₀ values of 1.50–1.68 at 48 h and then decreased gradually. It took 12 h for *B. subtilis* SJ4 to start to grow rapidly at 12% NaCl (Fig. 2). At 15% NaCl,

Fig. 2. Growth of *B. subtilis* SJ4 in TSB with NaCl (5–18%, w/v). —●—, 5%; —○—, 8%; —▼—, 10%; —△—, 12%; —■—, 15%; —□—, 18% NaCl.

it took 48 h before *B. subtilis* SJ4 started to grow but the OD₆₀₀ value was 0.70 at 96 h, quite lower than the cultures with 5–12% NaCl (1.14–1.17 at 96 h). *B. subtilis* SJ4 could not grow at 18% NaCl concentration. Considering its significant salt tolerance, *B. subtilis* SJ4 can be used as a starter for fermented foods such as jeotgals where the salt concentration does not exceed 15%. *B. subtilis* SJ4 produced antimicrobial substances against some food pathogens such as *B. cereus* (results not shown). These days, consumers prefer foods with less salt because high salt diet is known to cause several serious health problems [16]. For this, fermented foods such as soy paste, soy sauce, and jeotgals are produced with reduced salt contents compared to traditional fermented foods where lots of salts are added to prevent putrefaction by spoilage bacteria during fermentation. Growth of pathogenic bacteria is a serious problem for low salt foods. Use of *Bacillus* strains with antimicrobial activity as starters can reduce the risk of bacterial contamination.

SDS-PAGE and fibrin zymography

FS samples from culture grown for 96 h in LB were analyzed by SDS-PAGE (Fig. 3A). Three proteins, 23, 25, and 28 kDa in size, were the major bands on a coomassie stained gel. Minor bands of ca 42 and 58 kDa in size were also observed (Fig. 3A). The intensity of the 23, 25, and 28 kDa bands reached the highest around 60 h. On a zymogram gel loaded with the same samples, 23 kDa band was clearly observed among samples collected from 60–96 h time points (Fig. 3B). Faint band of ca 42 kDa was also observed on the zymogram, and the band intensity increased around 60 h although it appeared at 36 h of incubation.

Considering the size of reported fibrinolytic enzymes, the 28 kDa band seems as the major fibrinolytic protein in *B. subtilis* SJ4. The fibrin zymogram results were different from the fibrinolytic activity measurement results (Fig. 1) where the highest activity was observed at 48 h. Also, the band intensity of 28 kDa protein was higher at 60 h on a zymogram. The 23 kDa band showing strong fibrinolytic activity on a zymogram appeared around 60 h and similar intensity was maintained until 96 h. The intensity of 23 kDa band seemed at the highest at 72 h. A growth stage, at which the highest fibrinolytic activity was observed, was usually the time point where

Fig. 3. Analysis of FS from *B. subtilis* SJ4 using (A) coomassie blue stained gel and (B) fibrin zymogram. M, Dokdo-marker broad-range (EBM-1034, Elpis-Biotech., Daejeon, Korea). *B. subtilis* SJ4 was grown in LB broth for 96 h at 37 °C. 1, 12 h; 2, 24 h; 3, 36 h; 4, 48 h; 5, 60 h; 6, 72 h; 7, 84 h; 8, 96 h.

the intensity of the 27 kDa protein was the highest on a fibrin zymogram [13, 14]. It is not known why the highest fibrinolytic activity of *B. subtilis* SJ4 culture was observed at 48 h but at that time point, intensity of the

27 kDa band was not at the highest. Purification and characterization of fibrinolytic enzymes are necessary to better understand the fibrinolytic enzymes in *B. subtilis* SJ4.

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ATTGCCGAGACTCAGGAGCATTTAACCTAAAAAGCATGACATTTGAGCATATGAACATTTACTCATGTCTATTTCGTTCTTTCTG 90
TATGAAAAATAGTTATTTTCGAGTCTCTACGGAAATAGCGAGAGATGATATACCTAAATAGAGATAAAATCATCTCAAAAAAATGGGTCTAC 180
                                     -35
TAAATATTATTCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAGTCTACTCTGAATTTTCAAAAGGGAGAGGGTAAAGA 270
                                     -10                                     RBS
M R S K K L W I S L L F A L T L I F T M A F S N M S A Q A▼A 30
GTGAGAAGCAAAAATGTGGATCAGCTTGTGTGTTGCGTTAACGTAACTCTTACGATGGCGTTCAGCAACATGTCTGCCGAGGCTGCC 360
G K S S T E K K Y I V G F K Q T M S A M S S A K K K D V I S 60
GGAAAAAGCAGTACAGAAAAGAAATACATTGTGCGATTAAACAGACAATGAGTCCCATGAGTTCCGCCAAGAAAAAGGATGTTATTTCT 450
E K G G K I Q K Q F K Y V N A A A A T L D E K A V K E L K K 90
GAAAAAGCGGAAAGATTCAAAAGCAATTTAAGTATGTAAACGTGCTCGACCAACATGGATGAAAAAGCTGTAAAGAATGAAAAA 540
D P S V A Y V E E D H I A H E Y▽A Q S V P Y G I S Q I K A P 120
GATCCGAGCGTTGCATATGTGAAGAAGATCATATTGCACATGAATATGCGCAATCTGTTCTTATGGCATTCTCAAATTAAGCGCG 630
A L H S Q G Y T G S N V K V A V I D S G I D S S H P D L N V 150
GCTCTCACTCTCAAGGCTACACAGGCTCTAACGTAAAGTAGCTGTTATCGACAGCGGAATGACTCTTCTCATCCTGACTTAAAGCTC 720
R G S A S F V P S E T N P Y Q D G S S H G T H V A G T I A A 180
AGAGCGGAGCAAGCTTCGTACCTTCTGAAACAACCATACACAGGACGGCAGTTCTCACGGTACGCATGTAGCCGGTACGATTCCGCGT 810
L N N S I G V L G V A P S A S L Y A V K V L D S T G S G Q Y 210
CTTAATAACTCCATCGGTGTTCTGGGCGTAGCGCAAGCGCATCATATATGCGAGTAAAGTGCTTGATTCAACAGGAAGCGGCAATAT 900
S W I I N G I E W A I S N N M D V I N M S L G G P T G S T A 240
AGCTGGATTATTAACGCGATTGAGTGGGCCATTTCCAACAATATGGATGTTATCAACATGAGCCTTGGCGGACCTACTGGTTCTACAGCG 990
L K T V V D K A V S S G I V V A A A A G N E G S S G S T S T 270
CTGAAAACAGTAGTTGATAAAGCGGTTCCAGCGGTATCGTCGTGCTGCCGAGCGGAAACGAAGGTTGTCGCGAAGCACAAGCACA 1080
V G Y P A K Y P S T I A V G A V N S S N Q R A S F S S A G S 300
GTGCGCTACCTGCAAAATATCCTTCTACTATGTCAGTAGGTGCGGTAACAGCAGCAACCAAGAGCTTCATTCTCCAGCGCAGGTTCT 1170
E L D V M A P G V S I Q S T L P G G T Y G A Y N G T S M A T 330
GAGCTTGATGTAATGGCTCCTGGCGTGTCCATCCAAAGCACACTTCCTGGAGGCACCTACGGCGCTATAACGGAACGTCCTAGCGACT 1260
P H V A G A A A L I L S K H P T W T N A Q V R D R L E S T A 360
CCTCACGTTGCCGAGCAGCAGCGTAATTCTTCTAAGCACCGGACTTGGACAAATGCGCAAGTCGTCGATGCTTTAGAAAGCACTGCA 1350
T Y L G S S F Y Y G K G L I N V E A A A Q * 382
ACATACCTTGGAAAGCTCTTCTACTATGGAAGGGTTAATCAATGTAGAAGCAGCTGCCCAATAATATCAAAAAGAGCAGGTTCTCTCC 1440
ATACCTGCTCTTTTATTGCGCCGATCCTGTTCTGTGGCAGGATGTCGGTGCATCCCTTCTTCTCCGCATGTTGAATCGGTTTC 1529

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Fig. 4. Nucleotide sequence of *aprESJ4*. The deduced amino acid sequences are shown below the nucleotide sequences. Putative -35 and -10 promoter sequences are underlined. Putative ribosome binding site (RBS) and transcription terminators are also underlined. The ends of pre (▼) and pro sequences (▽) are marked. The stop codon is marked as the asterisk above the nucleotide sequence.

Gene cloning of *aprESJ4*

The major fibrinolytic gene of *B. subtilis* SJ4 was cloned by PCR using a primer pair which was initially used for the cloning of a homologous gene from *B. amyloliquefaciens* [13]. A 1.5 kb fragment was cloned into pGEM-T easy vector and sequenced. A total of 1,529 nucleotides were sequenced (Fig. 4) and analyzed by BLAST. The result confirmed that the cloned gene was indeed a homolog of *aprE* genes. The sequence was deposited in GenBank (Accession No. MK796246). An ORF of 1,146 bp in size, capable of encoding a protein of 381 amino acids in length was found. The first 29 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 pro-sequence as judged from comparison with other similar fibrinolytic enzymes. pI and size of proAprESJ4 (352 aa) were 7.98 and 36,164.37 Da, respectively. The first amino acid of the mature enzyme was suspected to be alanine (108th), and pI and size of the mature enzyme were 6.04 and 27,672.65 Da, respectively. Calculated size of mature enzyme matched well with the observed 28 kDa band on coomassie stained gel (Fig. 3).

Nucleotide sequence of *aprESJ4* showed high similarities with other fibrinolytic genes from *B. subtilis* CH3-5 [17], *B. subtilis* subsp. *natto* BEST195 [18], and

AprESJ4	MRSKKLWISL	LFALTLIFTM	AFSNMSAQAA	GKSSTEKKYI	VGFKQTMSAM	SSAKKKDVIS	60
AprE2	MRSKKLWISL	LFALTLIFTM	AFSNMSAQAA	GKSSTEKKYI	VGFKQTMSAM	SSAKKKDVIS	
subtilisin NAT	MRSKKLWISL	LFALTLIFTM	AFSNMSAQAA	GKSSTEKKYI	VGFKQTMSAM	SSAKKKDVIS	
subtilisin J	MRSKKLWISL	LFALTLIFTM	AFSNMSVQAA	GKSSTEKKYI	VGFKQTMSAM	SSAKKKDVIS	
	*				▽		
AprESJ4	EKGKKIQKQF	KYVNAAAATL	DEKAVKELKK	DPSVAYVEED	HIAHEYAQSV	PYGISQIKAP	120
AprE2	EKGKKVQKQF	KYVNAAAATL	DEKAVKELKK	DPSVAYVEED	HIAHEYAQSV	PYGISQIKAP	
subtilisin NAT	EKGKKVQKQF	KYVNAAAATL	DEKAVKELKK	DPSVAYVEED	HIAHEYAQSV	PYGISQIKAP	
subtilisin J	EKGKKVQKQF	KYVNAAAATL	DEKAVKELKK	DPSVAYVEED	HIAHEYAQSV	PYGISQIKAP	
		●			●		
AprESJ4	ALHSQGYTGS	NVKVAVIDSG	IDSSHPDLNV	RGGASFVPSE	TNPYQDGSSH	GTHVAGTIAA	180
AprE2	ALHSQGYTGS	NVKVAVIDSG	IDSSHPDLNV	RGGASFVPSE	TNPYQDGSSH	GTHVAGTIAA	
subtilisin NAT	ALHSQGYTGS	NVKVAVIDSG	IDSSHPDLNV	RGGASFVPSE	TNPYQDGSSH	GTHVAGTIAA	
subtilisin J	ALHSQGYTGS	NVKVAVIDSG	IDSSHPDLNV	RGGASFVPSE	TNPYQDGSSH	GTHVAGTIAA	
		*			*		
AprESJ4	LNNSIGVLGV	APSASLYAVK	VLDSTGSGQY	SWIINGIEWA	ISNNMDVINM	SLGGPTGSTA	240
AprE2	LNNSIGVLGV	APSASLYAVK	VLDSTGSGQY	SWIINGIEWA	ISNNMDVINM	SLGGPSGSTA	
subtilisin NAT	LNNSIGVLGV	APSASLYAVK	VLDSTGSGQY	SWIINGIEWA	ISNNMDVINM	SLGGPTGSTA	
subtilisin J	LNNSIGVLGV	SPSASLYAVK	VLDSTGSGQY	SWIINGIEWA	ISNNMDVINM	SLGGPSGSTA	
	*		*				
AprESJ4	LKTVVDKAVS	SGIVVAAAAG	NEGSSGSTST	VGYPKYPST	IAVGAVNSSN	QRASFSSAGS	300
AprE2	LKTVVDKAAS	SGIVVAAAAG	NEGSSGSSST	VGYPKYPST	IAVGAVNSSN	QRASFSSAGS	
subtilisin NAT	LKTVVDKAVS	SGIVVAAAAG	NEGSSGSTST	VGYPKYPST	IAVGAVNSSN	QRASFSSVGS	
subtilisin J	LKTVVDKAVS	SGIVVAAAAG	NEGSSGSSST	VGYPKYPST	IAVGAVNSSN	QRASFSSAGS	
		●					
AprESJ4	ELDVMAPGVS	IQSTLPGGTY	GAYNGTSMAT	PHVAGAAALI	LSKHPTWTNA	QVRDRLESTA	360
AprE2	ELDVMAPGVS	IQSTLPGGTY	GAYNGTSMAT	PHVAGAAALI	LSKHPTWTNA	QVRDRLESTA	
subtilisin NAT	ELDVMAPGVS	IQSTLPGGTY	GAYNGTSMAT	PHVAGAAALI	LSKHPTWTNA	QVRDRLESTA	
subtilisin J	ELDVMAPGVS	IQSTLPGGTY	GAYNGTSMAT	PHVAGAAALI	LSKHPTWTNA	QVRDRLESTA	
	*	*					
AprESJ4	TYLGSSFYYG	KGLINVEAAA	Q				381
AprE2	TYLGNSFYYG	KGLINVQAAA	Q				
subtilisin NAT	TYLGNSFYYG	KGLINVQAAA	Q				
subtilisin J	TYLGNSFYYG	KGLINVQAAA	Q				

Fig. 5. Alignment of amino acid sequence of AprESJ4 with homologous enzymes from bacilli. AprE2 (*B. subtilis* CH3-5, ABJ98765), Subtilisin NAT (*B. subtilis* subsp. *natto* BEST195, AYK86398), and Subtilisin J (*B. stearothermophilus* NCIMB10278, AAA22247). Amino acids different from other enzymes are marked as asterisks (*) above the amino acids. The ends of pre (▼) and pro sequences (▽) are marked. Three amino acids (D32, H64 and S221), constituting the conserved catalytic triad of fibrinolytic enzymes belonging to subtilisin family, are marked as solid circle (●) above the amino acids.

B. stearothermophilus NCIMB10278 [19]. Translated amino acid sequence of AprESJ4 was aligned with those of other homologous enzymes (Fig. 5). AprESJ4 showed 98.43% identity with AprE2 (375/381) from *B. subtilis* CH3-5 (ABJ98765), 98.95% with subtilisin NAT (377/381) from *B. subtilis* ssp. *natto* BEST195 (AYK86398), and 98.16% with subtilisin J (374/381) from *B. stearothermophilus* NCIMB10278 (AAA22247). Conserved amino acids (S221, H64, and D32) were also present in AprESJ4, which are important for catalytic activity, and present in almost all fibrinolytic enzymes [20]. Comparison of amino acid sequence of AprESJ4 with other fibrinolytic enzymes from bacilli indicated that these enzymes are highly conserved among bacilli regardless of their sources. *B. subtilis* SJ4, isolated from marine environments, secretes almost the same enzyme produced by bacilli from soil and plants. This signifies the importance of *aprE* genes for the survival of bacilli under various environments including marine environments.

B. subtilis SJ4 was isolated from saeu jeotgal, a fermented food prepared from saeu (small shrimp) and salt, and saeu jeotgal can be considered as a part of marine environments. Bacilli from marine environments are supposedly different from bacilli originated from terrestrial environments such as soil and plants [21, 22]. For this reason, efforts to isolate novel bacilli from marine environments have been made because they might produce novel metabolites useful for medicine and other bioindustries [6, 23]. *B. subtilis* SJ4 can grow at the maximum salt concentration of 15% (w/v), secrete fibrinolytic enzymes, and the strain possesses antibacterial activities (results not shown). Considering these facts, *B. subtilis* SJ4 and other bacilli from marine environments can be used as starters for salted fermented foods including jeotgals, and hosts for production of functional materials such as fibrinolytic enzymes. Further future studies are desirable for more detailed characterization of fibrinolytic enzymes from *B. subtilis* SJ4.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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