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

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Received: June 10, 2019 / Revised: July 6, 2019 / Accepted: July 10, 2019

**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 \textit{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 \textit{Bacillus} species closely related to \textit{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 \textit{Bacillus} species isolated from marine environments. In this respect, \textit{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**

\textbf{Isolation and identification of \textit{Bacillus} strains from jeotgal}

Three different types of jeotgals, saeu (shrimp, \textit{Acetes chinensis}), myeolchi (anchovy, \textit{Engraulis japonicus}) and hwangseok-eo (big head croaker, \textit{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 \textit{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 \textit{Bacillus} sp. SJ4, 16S rRNA genes were amplified using primers: bac-F (5'-CGGGCTTG-CCTAATACATGCAAG-3') and bac-R (5'-GGCATGCTGATCCGCATTACTA-3') [12]. A recA gene was amplified using the following primers: recA-F (5'-TGAGTGATC-GTCAGGCAGCTTGA-3') and recA-R (5'-CYTBRGAT-TACCAWGMACC-5') [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 \textit{B. subtilis} SJ4 in different culture media**

\textit{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 OD\textsubscript{600} 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 \textit{B. subtilis} SJ4**

Salt tolerance of \textit{B. subtilis} SJ4 was studied. \textit{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'-AGGATCCCAAGAGCAGTTGCGGCTGTGAC-3', BamHI site underlined) and CH51-R (5'-AGAATTCATCGAGGGAGCCACCGTCGATCA-3', EcoRI 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 activity. 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,
showing similar growth curves in these 3 culture media. The OD_{600} 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 OD_{600} 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 OD_{600} 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, it took 48 h before B. subtilis SJ4 started to grow but the OD_{600} 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
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.

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.

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. pl 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 (108\(^{th}\)), and pl 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

![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.](http://dx.doi.org/10.4014/mbl.1906.06003)
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.

**Acknowledgments**

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A1B03030037). Zhuang Yao, Meng Yu, and Jeong A Kim were supported by BK21 Plus program, MOE, Republic of Korea.

**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

**References**


