Isolation of 2 *Bacillus* Strains with Strong Fibrinolytic Activities from *Kimchi*

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Received: March 17, 2020 / Revised: May 5, 2020 / Accepted: May 7, 2020

Two *Bacillus* strains, K3 and K208, both demonstrating strong fibrinolytic activities were isolated from *Kimchi*, a traditional Korean preparation of fermented vegetables. Isolates were subjected to various molecular biology based identification methods including RAPD-PCR and identified as *B. subtilis* and *B. velezensis*, respectively. Tryptic soy broth (TSB) was found to best maintain both the growth and the fibrinolytic activity of these strains. Culture supernatants were analyzed by SDS-PAGE and fibrin zymography, and the results indicate that a 40 and 27 kDa band seem to be responsible for the fibrinolytic activities of these two isolates and the 27 kDa band was subsequently identified as the mature form of AprE, the major fibrinolytic enzyme. Thus the *aprE* genes were cloned and the translated amino acid sequences demonstrated 99.3% identity with each other, and 86.5% identity with BsfA, a fibrinolytic enzyme from *B. subtilis* ZA400 also isolated from *Kimchi*, and AprE2, a fibrinolytic enzyme from *B. subtilis* CH3-5 isolated from *Cheonggukjang*, a traditional Korean fermented soy. Given this *B. subtilis* K3 and *B. velezensis* K208 may be promising starter cultures in the production of fermented foods.

Keywords: *Bacillus subtilis*, *Bacillus velezensis*, fibrinolytic enzymes, *aprE* gene cloning

Introduction

Bacilli are widely present among diverse environments including soil, water, fermented foods, and intestines of animals [1, 2]. Bacilli secrete various hydrolytic enzymes into surrounding environments, and amylases and proteases are the most important enzymes widely utilized for various industrial applications [3]. Many commercial products including fermented foods depend on the strong enzyme activities of bacilli. Bacilli play important roles during fermentation of soy products such as doenjang (fermented soy paste), ganjang (soy sauce), and cheonggukjang (fermented and boiled soy)

due to their high proteolytic activities [4–6]. During fermentation, bacilli contribute to the development of unique flavor and texture of foods by producing peptides and amino acids from proteins, functional materials such as γ -PGA (polyglutamic acid) [7]. Bacilli also produce a variety of different antimicrobial compounds including antibiotics, bacteriocins, and lipopeptides, which inhibit growth of pathogens [8]. Some secreted proteases show strong fibrinolytic activities, and nattokinase and Bacillopeptidase-F (Bpr) are the enzymes with strong fibrinolytic activities and currently used as nutritional supplements or alternatives replacing drugs for treating or preventing thrombolysis caused by fibrin accumulation in blood vessels [9, 10].

Bacillus species with strong fibrinolytic activities, especially classified as GRAS (generally recognized as safe), have potentials to be used as starters for the pro-

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duction of fermented foods. Since such *Bacillus* species are widely present among fermented foods, it is necessary to isolate novel strains with strong fibrinolytic activities, and test their potentials as starters for fermented foods. Unlike fermented soy foods, *Bacillus* species have been rarely isolated from *Kimchi*. Here, we report isolation of 2 *Bacillus* strains from kimchi and their fibrinolytic activities.

Materials and Methods

Isolation and identification of bacilli with strong fibrinolytic activities

Fermented foods including various *Jeotgal* and *Kimchi* products were purchased at Jungang market in Jinju in July, 2019, and used as sources for the isolation of bacilli with fibrinolytic activities. Samples were homogenized by using Stomacher 80 (Seward, UK), and then serially diluted with sterile water. Diluted samples were spreaded onto Luria-Bertani (LB, tryptone 10 g, yeast extract 5 g, NaCl 5 g per liter, pH 7.0) agar plates with skim milk (MB cells, Korea, 2%, w/v). Plates were incubated for 2 days at 37°C. Colonies showing halos were selected, and examined for fibrinolytic activities by fibrin plate method as described previously [11].

Colonies showing strong fibrinolytic activities on fibrin plates were selected and identified by molecular biological methods. 16S rRNA genes were amplified using primers: bac-F (5'-CGGCGTGCCTAATACATGCAAG-3'), and bac-R (5'-GGCATGCTG ATCCGCATTACTA-3') [12]. Primers for *recA* was amplified using primers: recA-F (5'-TGAGTGATCGTCAGGCAGCCTTAG-3'), and recA-R (5'-CYTBRGATAAGAR TACCAWGMACCGC-3') [12]. PCR was done in 50 µl volume consisting of 2 µl of template DNA, 2 µl of primers (10 µM each), 5 µl of dNTPs (0.25 mM), and 0.5 µl of Ex *Taq* DNA polymerase (Takara, Japan). PCR conditions were as follows: initial denaturation at 94°C, 5 min followed by 30 cycles consisting of denaturation at 94°C, 30 s, annealing at 58°C, 30 s, and extension at 72°C, 1 min, and final extension at 72°C, 4 min.

RAPD-PCR was done using S30 (5'-GTGATCGCAG-3') primer and Go-Taq® DNA polymerase (Promega, USA) [12]. PCR was done in 30 µl volume consisting of 2 µl of template DNA, 2 µl of S30 primer (10 µM), and 15 µl of Go Taq® green master mix. PCR conditions were

as follows: initial denaturation at 94°C, 5 min followed by 40 cycles consisting of denaturation at 94°C, 15 s, annealing at 32°C, 15 s, and extension at 72°C, 2 min, and final extension at 72°C, 4 min.

Growth and fibrinolytic activities of bacilli isolates

Bacilli isolates were grown in different culture media: LB broth, brain heart infusion (BHI, Becton, Dickinson, and Company, USA) broth, nutrient broth (NB, Neogen, USA), and tryptic soy broth (TSB, Becton, Dickinson, and Company) at 37°C with shaking. Aliquots of culture were taken at 12 h intervals, and OD₆₀₀ values were measured. Culture was centrifuged at 12,000 ×g, 4°C for 5 min, and the supernatant was obtained and used as a sample for fibrinolytic activity measurement. Fibrinolytic activity (FA) was measured by fibrin plate method as described previously [11]. The size of the clear zone that formed was converted into plasmin units (U) by comparing it to zones formed by known quantities of plasmin (USA). A standard curve showing the relationship between the clear zone formed and the number of plasmin units was prepared in the range of 2–40 mU.

SDS-PAGE and fibrin zymography

B. subtilis K3 and *B. velezensis* K208 were grown in TSB at 37°C for 96 h. Aliquots were taken at 12 h intervals. Culture supernatant was prepared and analyzed by SDS-PAGE and fibrin zymography. 10% acrylamide gels with 5% stacking gels were used for SDS-PAGE and fibrin zymography. For SDS-PAGE, culture supernatant was first concentrated by trichloroacetic acid (TCA) precipitation method and then 10 µg was loaded into each well. For fibrin zymography, 1 µg of sample was loaded without TCA concentration. SDS-PAGE and fibrin zymography were done according to a method previously reported [11].

Cloning of *aprE* genes

aprE genes were cloned by PCR. Primers used were as follows: CH51-F (5'-AGGATCCCAAGAGAGC-GATTGCGGCTGTGTAC-3', *Bam*HI site underlined) and CH51-R (5'-AGAATTCTTCAGAGGGAGCCAC-CCGTCGATCA-3', *Eco*RI site underlined) [13]. PCR was done in 50 µl volume consisting of 1 µl of template DNA, 2 µl of each primers (10 µM each), 5 µl of dNTPs

(0.25 mM), and 0.5 µl of Ex *Taq* DNA polymerase. PCR conditions were initial denaturation at 94°C, 5 min followed by 30 cycles consisting of denaturation at 94°C, 30 s, annealing at 60°C, 30 s, and extension at 72°C, 1 min, and final extension at 72°C, 4 min. Amplified fragments (1.5 kb) were ligated with pGEM-T-Easy (Promega). Ligation mixture was used to transform *E. coli* DH5α competent cells by electroporation, and *E. coli* cells harboring recombinant plasmid were screened on LB agar plates with ampicillin (50 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 80 µg/ml), and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 mM). Plasmid from a white colony was extracted and sent for sequencing. Nucleotide sequences were analyzed by BLAST at national center for biotechnology (NCBI).

Results and Discussion

Isolation and identification of bacilli with strong fibrinolytic activities

Bacilli were isolated from various *Jeotgal* and *Kimchi* products, and many of them showed proteolytic activities on LB agar plates with skim milk (data not shown). Strains with strong proteolytic activities were further tested for the fibrinolytic activities by fibrin plate method. Two isolates, K3 and K208, showed strong

fibrinolytic activities, and both strains were isolated from *Kimchi*. Blast analyses of 16S rRNA and *recA* genes of K3 indicated that K3 was either *B. subtilis*, *B. amyloliquefaciens*, *B. siamensis*, or *B. velezensis* (Table 1). Similarly, K208 was either *B. amyloliquefaciens*, *B. methylotrophicus*, *B. pumilus*, *B. siamensis*, *B. subtilis*, or *B. velezensis*. Considering that the 16S rRNA sequences among *Bacillus* species are highly identical (> 99%), *recA* gene sequences were used for accurate identification of isolates. Due to the fundamental role of RecA, *recA* gene is ubiquitous, and has been used as a phylogenetic marker for distantly related species [14]. The results showed that both 16S rRNA and *recA* gene sequences are highly conserved among these closely related *Bacillus* species, and the sequencing data alone was not enough for the accurate identification of bacilli isolates at species level [15]. RAPD-PCR was done to determine the species of K3 and K208. The RAPD-PCR profiles clearly showed that K3 was *B. subtilis*, and K208 *B. velezensis*, and the merit of RAPD-PCR for distinguishing closely related *Bacillus* species (Fig. 1). Accordingly, K3 was named as *B. subtilis* K3, and K208 as *B. velezensis* K208. Other isolates, K52 and M7, showed the same profiles with that of K208, thus they were probably *B. velezensis*, too. The Genbank accession numbers for 16S rRNA gene and *recA* gene were

Table 1. Identification of K3 and K208 by 16S rRNA and *recA* genes sequencing.

Strain	Gene	Length (bp)	Description	Identities (%)
K3	16S rRNA	1,234	<i>Bacillus subtilis</i>	100.00
			<i>Bacillus amyloliquefaciens</i>	
			<i>Bacillus siamensis</i>	
			<i>Bacillus velezensis</i>	
	<i>recA</i>	763	<i>Bacillus velezensis</i>	99.87
			<i>Bacillus amyloliquefaciens</i>	99.74
			<i>Bacillus subtilis</i>	99.48
			<i>Bacillus vallismortis</i>	99.21
K208	16S rRNA	1,198	<i>Bacillus amyloliquefaciens</i>	100.00
			<i>Bacillus velezensis</i>	
			<i>Bacillus subtilis</i>	
			<i>Bacillus siamensis</i>	
			<i>Bacillus methylotrophicus</i>	
			<i>Bacillus pumilus</i>	
	<i>recA</i>	705	<i>Bacillus velezensis</i>	100.00
			<i>Bacillus amyloliquefaciens</i>	99.72
			<i>Bacillus subtilis</i>	99.29
			<i>Bacillus vallismortis</i>	99.15

Fig. 1. RAPD-PCR profiles of some isolates and reference strains. M, size marker (iVDye 1kb DNA ladder, GenDEPOT, USA); 1, K52; 2, K208; 3, M7; 4, *B. velezensis* ATCC17177; 5, *B. subtilis* ATCC33234; 6, *B. circulans* ATCC4513; 7, K3; 8, *B. amyloliquefaciens* ATCC23845; 9, *B. thuringiensis* ATCC33679; 10, *B. subtilis* ATCC6051A; 11, *B. mycoides* ATCC6465; 12, *B. tequilensis* ATCC15944; 13, K117.

MT093345 and MN974487 for *B. subtilis* K3, and MT093346 and MN974488 for *B. velezensis* K208.

Growth and fibrinolytic activities of *B. subtilis* K3 and *B. velezensis* K208

B. subtilis K3 grew well on LB, BHI, and TSB, reach-

ing the OD₆₀₀ values of 1.73 to 1.74 in 60 h, but grew poor on NB, reaching the OD₆₀₀ value of 1.28 at 24 h (Fig. 2A). TSB was the best medium for the fibrinolytic activity and the highest fibrinolytic activity (70.01 mU/μl) was observed at 24 h. The activity decreased slowly until 48 h (62.77 mU/μl) (Fig. 2B), and then the activity decreased rapidly after 48 h, showing only 0.56 mU/μl at 60 h. LB was the second best medium for fibrinolytic activity, and the highest activity (40.40 mU/μl) was shown at 24 h, and then the activity decreased rapidly. Cultures on all 4 media showed basal levels of activities at 60 h and thereafter. The pattern is somewhat different from those of other fibrinolytic bacilli where fibrinolytic activities are increased at the beginning of stationary phase or late log phase, and the activity is maintained during stationary phase, often showing the highest activity around 84–96 h of incubation [16, 17]. The results indicated that the fibrinolytic activity profile is different among bacilli, and thus each isolate should be examined individually.

Fig. 2. Growth and fibrinolytic activities of *Bacillus* isolates in different culture media. *B. subtilis* K3 and *B. velezensis* K208 were grown at 37 °C for 96 h in 4 different media. OD₆₀₀ values and fibrinolytic activities were measured at 12 h intervals. Growth of *B. subtilis* K3 (A) and *B. velezensis* K208 (C). Fibrinolytic activity of *B. subtilis* K3 (B) and *B. velezensis* K208 (D). ●- LB; ○- NB; △- TSB; ▼- BHI.

B. velezensis K208 showed growth curves on 4 media which were quite similar with those of *B. subtilis* K103. But the fibrinolytic profiles of *B. velezensis* K208 were different from those of *B. subtilis* K3. Although TSB was the best medium for the fibrinolytic activity, the highest activity (181.49 mU/ μ l) was observed at 48 h (Fig. 2D). The activity decreased rapidly thereafter, and remained at basal level at 72 h (2.85 mU/ μ l) and thereafter. Unlike *B. subtilis* K3 culture, higher activities were maintained for short period, just around 48 h. LB was the second best medium for the fibrinolytic activity, and the highest activity on LB was observed at 36 h (89.18 mU/ μ l). NB was the poorest medium for the growth and fibrinolytic activity of *B. velezensis* K208 (Fig. 2C, 2D).

SDS-PAGE and fibrin zymography

SDS-PAGE of culture supernatant of *B. subtilis* K3 showed that the band intensity of a 27 kDa protein, corresponding to the mature AprEK3, was higher at 12, 24, 36, and 48 h than that of other time points. The results matched well with the fibrinolytic activity measurements of culture grown on TSB (Fig. 2). The intensity of ca 40 kDa band was also higher at 12–36 h time points

on the SDS-gel, and especially the highest at 24 and 36 h. Since, *B. subtilis* K3 showed higher fibrinolytic activity in TSB at this time points, the 40 kDa protein might contribute to the higher fibrinolytic activity of *B. subtilis* K3. Characterization of the 40 kDa protein will confirm its identity and any role on the fibrinolytic activity of *B. subtilis* K3 in TSB. However, a 27 kDa band was observed on a fibrin zymogram at 60 h. The reason for the discrepancy between fibrinolytic activity measurements (Fig. 2B) and fibrin zymogram (Fig. 3B) is not clear.

SDS-PAGE of culture supernatant from *B. velezensis* K208 showed similar pattern with that from *B. subtilis* K3. Intensity of a 40 kDa protein was higher at 24–48 h, and the same is true for 27 kDa band. Culture showed higher fibrinolytic activities at this time period, too (Fig. 2D). On a fibrin zymogram, the 27 kDa band appeared at 48 h and thereafter, and the band intensity was the highest at 72 h. A 35 kDa band was also observed at stationary phase on a fibrin zymogram. The mature form of AprE, 27 kDa in size, appears at the stationary growth phase for some *Bacillus* species such as *B. amyloliquefaciens* CH86-1 isolated from *Cheongguk-*

Fig. 3. SDS-PAGE and fibrin zymogram of culture supernatant. *B. subtilis* K3 and *B. velezensis* K208 were grown in TSB at 37 °C with aeration. Culture supernatant was taken out at 12 h intervals. SDS-PAGE (A, *B. subtilis* K3; C, *B. velezensis* K208) and fibrin zymography (B, *B. subtilis* K3; D, *B. velezensis* K208) were done. M: Dokdo-marker EBM-1034 (Elpis Biotech, 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. Nucleotide sequence and translated amino acid sequence of *aprEK3*. Possible -35 and -10 promoter sequences are underlined together with ribosome binding site (RBS). Signal peptide cleavage site (▼) and proenzyme cleavage site (▽) are marked. Amino acids consisting of the catalytic triad (Asp³², His⁶⁴, and Ser²²¹) are marked with boxes.

jang [18] as shown by fibrin zymography for the culture supernatant. But the same band appeared at earlier time points for other *Bacillus* species such as *B. subtilis* JS2 isolated from saeu *Jeotgal* [19], indicating the possible operation of different regulatory systems for *aprE* gene expression among *Bacillus* species.

Cloning of *aprE* genes

aprEK3 and *aprEK208* were cloned after PCR amplification and sequenced. BLAST analyses of both genes confirmed that the 2 genes show very high identities with homologous *aprE* genes from *Bacillus* species (results not shown). Genbank number is MT093822 for *aprEK3*, and MT093821 for *aprEK208*. Both *aprEK3* and *aprEK208* genes encode preproenzyme consisting of 382 amino acids (Fig. 4, only *aprEK3* shown). The first 30 amino acids correspond to signal peptide as judged from Signal P4.1 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). The mature enzyme consists of 275 amino acids. The calculated size and pI of mature AprEK3 were 27,460.60 and 6.65, and 27,490.62 and 6.65 for AprEK208.

When translated amino acid sequences were compared with each other, 2 amino acids were different (99.3% identity). The 91th and 268th amino acid of AprEK3 were N and G whereas they were K and S for AprEK208. Amino acids constituting of the catalytic triad are conserved in AprEK3 and AprEK208, and they are Asp (32th), His (64th), and Ser (221th) (Fig. 4). When the mature AprEK3 was compared with BsfA, a fibrinolytic enzyme from *B. subtilis* ZA400 isolated from *Kimchi*, 86.5% identity (238/275) was observed [20]. Total 37 amino acids were different, and 18 amino acids were located in prepro part. AprEK3 also showed 86.5%

identity (238/275) with AprE2, a fibrinolytic enzyme from *B. subtilis* CH3-5 isolated from *Cheonggukjang* [21]. PreproAprEK3 consists of 382 amino acids, but prepro BsfA and AprE2 consist of 381 amino acids, one amino acid shorter than AprEK3. The signal peptide of prepro AprEK3 consists of 30 amino acids, but that of prepro BsfA is 26 amino acids, and prepro AprE2 is 29 amino acids. Depending upon each strain, *B. subtilis* strains produce similar fibrinolytic enzymes, but still significant differences are observed among them. Therefore further studies are necessary to find out different enzymes in the primary structure have different roles during growth. The highly identical amino acid sequences indicates the highly conserved nature of AprEs among *Bacillus* species, implying the important roles of AprE for *Bacillus* species during stationary growth phase. Proteases such as AprE not only provide peptides and amino acids but also play other important roles. Some proteases are responsible for the conversion of pheromones such as CSF (competence and sporulation factor), responsible for quorum sensing [22]. AprE is also reported to be involved in the processing of subtilin, a peptide antibiotic [23].

Bacilli are often isolated from *Kimchi* as shown in this work and a previous report [20]. But their roles for *Kimchi* fermentation and storage have rarely been examined. This is probably due to the general belief that bacilli are inhibited by acids produced by lactic acid bacteria during *Kimchi* fermentation. But bacilli can remain viable as spores for long period of time and can resume growth later when environmental conditions become favorable for bacilli. Further studies on bacilli during *Kimchi* fermentation and storage might help to better understand any roles of bacilli for *Kimchi* quality. Bacilli with strong fibrinolytic activities are desirable since they have potentials as starters for some types of fermented foods. In this work, we isolated 2 useful *Bacillus* strains with strong fibrinolytic activities, and these isolates are promising starters for *Jeotgals* and other types of fermented foods.

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), and also sup-

ported by a grant 20130290 to Solar Salt Research Center of Mokpo National University from Ministry of Oceans and Fisheries of Korea. Yao Z, Meng Y, Lee SJ, and Yoo JY were supported by BK21 Plus program, MOE, Republic of Korea. Le HG and Jeon HS were supported by full-time graduate scholarship from Gyeongsang National University.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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