Cloning and Expression of a Fibrinolytic Enzyme Gene, *aprECJ1*, from *Bacillus velezensis* CJ1 Isolated from *Myeolchi Jeotgal*

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Bacillus velezensis CJ1, showing significant fibrinolytic activity, was isolated from *Myeolchi Jeotgal*, a popular Korean fermented seafood. When *B. velezensis* CJ1 was grown on four different culture media, the culture on the Luria-Bertani (LB) broth showed the highest fibrinolytic activity (102.94 mU/μl) at 48 h. LB was also the best medium for growth. SDS-PAGE of culture supernatant showed four major bands, 38, 35, 27, and 22 kDa in size. Fibrin zymography showed four active bands, 50, 47, 40, and 30 kDa in size. A gene homologous to *aprE* of the *Bacillus* species was cloned by PCR. DNA sequencing showed that *aprECJ1* can encode a protease consisting of 382 amino acids. The translated amino acid sequence of AprECJ1 showed high identity values with those of *B. velezensis* strains and other *Bacillus* species. The *aprECJ1* gene was introduced into *B. subtilis* WB600 using an *E. coli*-*Bacillus* shuttle vector, pHY300PLK, and overexpressed. A 27 kDa band corresponding to the mature form of AprECJ1 was produced and confirmed by SDS-PAGE and fibrin zymography. *B. subtilis* WB600 [pHYaprECJ1] showed 1.8-fold higher fibrinolytic activity than *B. velezensis* CJ1 at 48 h.

Keywords: Fibrinolytic enzyme, *Bacillus velezensis*, *Myeolchi Jeotgal*, gene expression

Introduction

Many *Bacillus* species secrete several proteases into culture medium, and some enzymes possess strong fibrinolytic activities [1]. Recently, these bacilli fibrinolytic enzymes gain interests due to their potential as an alternative to drugs such as t-PA (tissue plasminogen activator), streptokinase or urokinase used for treating thrombosis, one of the top leading causes of human death worldwide [2]. These drugs have some shortcomings, i.e. expensive, short half-lives inside body, and may cause internal bleeding. Scientists have been trying to isolate more safe and effective fibrinolytic enzymes from

plants, animals and microorganisms [3]. Among them, fibrinolytic enzymes from bacilli seem to have the most desirable properties. *Bacillus* species have been used for various fermented foods for a long time, and the organisms and their enzymes are considered generally recognized as safe (GRAS) class. In addition, bacilli can be cultivated easily in large-scale and economically [4]. Nattokinase produced by some *B. subtilis* strains is the most well-known fibrinolytic enzyme and currently sold as a nutritional supplement [5]. Some clinic studies showed the effectiveness of Nattokinase for treating thrombosis in vivo [6].

Isolation of novel *Bacillus* species with strong fibrinolytic activities is essential for extending applications of bacilli fibrinolytic enzymes. Among the diverse natural environments, traditional Asian fermented foods are the best sources for bacilli [7]. Fibrinolytic *Bacillus* strains

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including *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* have been isolated from *Cheonggukjang* and *Doenjang* from Korea [8, 9], *Natto* from Japan [10], and *Douchi* from China [11].

B. velezensis is closely related to *B. subtilis* and *B. amyloliquefaciens*, and synonymous to various strains of *B. amyloliquefaciens* ssp. *plantarum* and *B. methylotrophicus* strains [12].

B. velezensis is an important member of PGPR (plant growth promoting rhizobacteria), and currently sold as a plant growth stimulating agent. We isolated bacilli showing fibrinolytic activities from *Myeolchi* (anchovy) *Jeotgal*, a popular traditional Korean fermented seafood prepared from anchovy (*Engraulis japonica*) and salt [13]. Among the isolates, CJ1 showed the strongest activity. We identified CJ1 as *B. velezensis*, and studied its main fibrinolytic enzyme. We cloned the structural gene, *aprECJ1*, from the genome of *B. velezensis* CJ1, and successfully overexpressed in *B. subtilis* WB600.

Materials and Methods

Isolation and Identification of *B. velezensis* CJ1 from *Myeolchi Jeotgal*

Myeolchi (anchovy, *Engraulis japonica*) *Jeotgal* was purchased at a local traditional market in Jinju, Gyeongnam province, Republic of Korea, in 2020. Ten g of *Myeolchi Jeotgal* was mixed with 90 ml of 0.1% peptone water, homogenized by using a stomacher (Stomacher®80, USA), and serially diluted with peptone water. Diluted samples were spreaded on Luria-Bertani (LB, tryptone 10 g, yeast extract 5 g, NaCl 10 g per liter, pH 7.0) agar plates. The plates were incubated for 48 h at 30°C. Colonies with typical *Bacillus* morphologies were selected and spotted onto LB plates with skim milk (1%, w/v) (Acumedia, USA). Isolates showing strong proteolytic activities were further investigated for the fibrinolytic activities by fibrin plate method [14]. Colonies showing strong fibrinolytic activities were identified by molecular biological methods. Chromosomal DNA was prepared from 18 h culture on LB broth by phenol-chloroform extraction method. 16S rRNA genes were amplified using primers: bac-F (5'-CGGCGTGCCTA-ATACATGCAAG-3'), and bac-R (5'-GGCATGCTGATC-CGCATTACTA-3') [15]. *recA* was amplified using primers: recA-F (5'-TGAGTGATCGTCAGGCAGCCT-

TAG-3'), and recA-R (5'-CYTBRGATAAGAR TACCAW-GMACCGC-3') [15]. PCR was done in 50 µl volume consisting of 2 µl template DNA, 2 µl primers (10 µM each), 5 µl dNTPs (0.25 mM each), and 0.5 µl Ex *Taq* DNA polymerase (Takara, Japan). PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 4 min. Nucleotide sequences of the amplified fragments were determined and analyzed for homologous genes by BLAST (NCBI, USA).

Randomly amplified polymorphic DNA (RAPD)-PCR was done using S30 primer (5'-GTGATCGCAG-3') and Go-*Taq*® DNA polymerase (Promega, USA) [15]. PCR was done in 30 µl volume consisting of 2 µl template DNA, 2 µl S30 primer (10 µM), and 15 µl Go *Taq*® green master mix. PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 40 cycles consisting of 94°C for 15 s, 32°C for 15 s, and 72°C for 2 min, and final extension at 72°C for 4 min.

Growth, fibrinolytic activity and salt tolerance of *B. velezensis* CJ1

B. velezensis CJ1 was cultured on 4 different media: LB broth, Brain Heart Infusion (BHI, Becton, Dickinson and Company, USA), nutrient broth (NB, Becton, Dickinson and Company), and tryptic soy broth (TSB, Becton, Dickinson and Company) at 37°C with shaking for 96 h. Growth of each culture was monitored by measuring the OD₆₀₀ value at 12 h intervals. Aliquot of culture was taken at each time point, and centrifuged at 12,000 ×g for 10 min at 4°C, and the supernatant was filtered using a 0.45 µm filter (Sartorius Stedim, Germany). The filtered supernatant was used as a sample for fibrinolytic activity measurement by fibrin plate method [14]. Fibrinogen (Sigma, USA) (1.2%, w/v) was mixed with 7 ml 1 M phosphate-buffered saline (PBS, pH 7.0), and 0.1 ml thrombin solution (100 NIH units/ml, Sigma) and the solution was mixed with 7 ml 2% (w/v) agarose solution. Next, the mixed solutions were poured into a plate, and stood for 1 h at room temperature (RT) to allow fibrin clot formation. Holes on a fibrin plate were made using a capillary glass tube, then samples were applied into each hole and the plate was incubated for 18 h at 37°C. The fibrinolytic activity was measured from the diameter of transparent zone on the fibrin plate

and compared with those obtained from plasmin at 2–40 mU.

SDS-PAGE and fibrin zymography

B. velezensis CJ1 was cultivated in LB broth for 36 h at 37°C. Aliquots taken at 12 h intervals were centrifuged and filtered as described above. Filtered supernatants were used for SDS-PAGE and fibrin zymography. For SDS-PAGE, supernatant was concentrated by trichloroacetic acid (TCA) precipitation method, and 10 µg was loaded into each well. For fibrin zymography, supernatant (1 µg) was loaded without TCA concentration. Electrophoresis was done using acrylamide gels consisting of 5% stacking gel and 12% separating gel. Fibrin gel was prepared by mixing 0.12% (w/v) fibrinogen and 10 µl thrombin (10 NIH units/ml) with the 12% polyacrylamide gel solution. After electrophoresis at 10 mA and 4°C, the gel was incubated for 30 min at RT in 50 mM Tris-HCl buffer (pH 7.4) containing 2.5% (v/v) Triton X-100. Gel was washed with distilled water for 30 min and incubated overnight at 37°C in 30 mM Tris-HCl buffer (pH 7.4) containing 0.02% (w/v) of NaN₃. The gel was stained with coomassie blue R-250.

Cloning of *aprECJ1*

B. velezensis CJ1 was cultivated in LB broth for 12 h at 37°C. Cells were collected by centrifugation at 12,000 ×g for 10 min at 4°C. Cell pellet was resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% SDS) with 40 µl of proteinase K (Sigma) and stood for 30 min at 55°C. Suspension was extracted with phenol and chloroform mixture, and ethanol precipitation was done for DNA recovery. DNA was resuspended in small volume of sterile water and used as a template for PCR. To amplify *aprECJ1* from *B. velezensis* CJ1 genome, PCR was done by using a primer pair: CH51-F (5'-AGGATCCAAGAGAGCGATTGCG-GCTGTGTAC-3', *Bam*HI site underlined) and CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA3', *Eco*RI site underlined) [16]. The PCR reaction mixture (50 µl) contained 1 µl template DNA, 1 µl each primer (10 µM), 5 µl dNTPs (0.25 mM), and 0.5 µl Ex *Taq* DNA polymerase (Takara, Tokyo, Japan). Amplification conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 40 sec, and a final extension at 72°C for 5 min. The amplified fragment was

ligated with pHY300PLK, an *E. coli*-*Bacillus* shuttle vector (4.87 kb, Tc^R) after digested with *Bam*HI and *Eco*RI. Ligation mixture was used to transform *E. coli* DH5α competent cells by electroporation (200 Ω, 21 kV/cm). Plasmid DNA preparation from *E. coli* DH5α, restriction enzyme digestion, preparation of competent cells, and agarose gel electrophoresis were performed according to the published methods [17].

Heterologous expression of *aprECJ1* in *B. subtilis* WB600

A 1.3 kb *aprECJ1* gene amplified as above was ligated into pHY300PLK and the resulting recombinant plasmid was introduced into *B. subtilis* WB600 competent cells by electroporation (200 Ω, 21 kV/cm). A *B. subtilis* WB600 transformant (TF) was obtained and inoculated into LB broth containing tetracycline (15 µg/ml) for 96 h at 37°C. Growth and fibrinolytic activity of culture was measured at 12 h intervals. *B. velezensis* CJ1 and *B. subtilis* WB600 [pHY300PLK] were used as controls. SDS-PAGE and fibrin zymography were done for culture supernatant as described above.

Results and Discussion

Isolation and Identification of *B. velezensis* CJ1 from *Myeolchi Jeotgal*

Among about 1,000 bacilli isolates, 15 isolates with strong proteolytic activities were selected first, and then tested for fibrinolytic activities. Finally, one isolate, CJ1, showing the strongest fibrinolytic activity was selected for further studies (data not presented). CJ1 was Gram positive, rod shaped and appeared as the typical *Bacillus* colony on LB agar plates. The 16S rRNA gene sequence (1,219 nucleotides, MW792221) was determined and BLAST analysis indicated that the gene showed 99% identities to those of *B. velezensis* and *B. amyloliquefaciens* strains. *recA* gene sequence (749 nucleotides, MW792222) showed 100% identities to those of *B. velezensis* and *B. amyloliquefaciens* strains. 16S rRNA and *recA* genes sequencing could not identify the species of CJ1. It is well-known that closely related *Bacillus* species are difficult to be identified either by traditional classification methods or molecular biological methods such as 16S rRNA gene sequencing [15]. To clarify its species status, RAPD-PCR was done using S30 primer [15]. Two bands of 700 and 1,800 bp in size were amplified from CJ1

Fig. 1. RAPD-PCR profiles of *Bacillus* spp. CJ1 and related *Bacillus* spp. M, size marker (iVDye I kb DNA ladder, GenDEPOT, Katy, TX, USA); 1, *Bacillus* spp. CJ1; 2, *B. velezensis* ATCC17177; 3, *B. subtilis* ATCC33608; 4, *B. subtilis* ATCC33234; 5, *B. amyloliquefaciens* ATCC 23350. Agarose gel (0.8%) was used to show the RAPD-PCR profiles.

chromosomal DNA, which was the same profile with that from *B. velezensis* ATCC17177 (Fig. 1). From these results, CJ1 was identified as *B. velezensis* and accordingly named *B. velezensis* CJ1.

Growth, Fibrinolytic Activity and Salt Tolerance of *B. velezensis* CJ1

B. velezensis CJ1 grew well on LB, BHI, and TSB, and the OD₆₀₀ values reached 1.4–1.6 at 12 h cultivation. Culture on NB remained at lower values, and the OD₆₀₀ value at 96 h was 0.98 whereas those of other cultures were 1.7–1.8 at 96 h (Fig. 2A). LB was the best medium for the fibrinolytic activity. Culture on LB showed the highest fibrinolytic activity (102.94 mU/μl) at 48 h (Fig. 2B). After 48 h, the activity decreased gradually until 60 h and very low activities were detected between 72 and 96 h. The fibrinolytic profiles of *B. velezensis* CJ1 were variable depending upon culture medium, indicating components of culture affected the fibrinolytic activities.

B. velezensis CJ1 grew well at 10–12% NaCl, and the OD₆₀₀ values were 1.4–1.6 at 144 h (Fig. 3). *B. velezensis* CJ1 grew slowly at 15% NaCl and the highest OD₆₀₀ value was 1.07 at 96 h. *B. velezensis* CJ1 did not grow until 48 h at 17% NaCl, and then grew slowly, and the OD₆₀₀ value was 0.16 at 144 h. *B. velezensis* CJ1 didn't grow at 20% salt for 144 h. Considering its salt tolerance, *B. velezensis* CJ1 could be used as a starter for

Fig. 2. Growth (A) and fibrinolytic activity (B) of *B. velezensis* CJ1 for 96 h cultivation on different culture media. ●, LB; ○, TSB; ▼, BHI; △, NB.

Fig. 3. Growth of *B. velezensis* CJ1 on LB broth with different NaCl contents. ●, 10% NaCl; ○, 12% NaCl; ▼, 15% NaCl; △, 17% NaCl; ■, 20% NaCl.

Fig. 4. SDS-PAGE (A) and fibrin zymography (B) of culture supernatant of *B. velezensis* CJ1. *B. velezensis* CJ1 was grown in LB broth at 37 °C up to 96 h. Aliquot was taken at 12 h intervals. M, Dokdo-marker broad-range (EBM-1034, ELPIS, Deajeon, 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.

fermented foods where the salt concentration does not exceed 15%.

SDS-PAGE and fibrin zymography

On a coomassie blue stained gel, the intensities of 4 bands (38, 35, 27, and 22 kDa) increased as the culture entered into the stationary phase of growth between 24 h and 36 h (Fig. 4 A). The intensities of the bands decreased after 36 h. The intensities of 22 and 27 kDa bands slightly increased at 84 h and then decreased again. Fibrinolytic activity of *B. velezensis* CJ1 on LB broth was at the highest between 24 h and 36 h (Fig. 2B), which period overlapped the time points where band intensities of 4 proteins were stronger.

On fibrin zymogram, 4 active bands were observed, and they were 50, 47, 40, and 30 kDa in size (Fig. 4B). The intensity of the 30 kDa band was at the highest at 48 h followed by at 36 h and 60 h. At 72 h and thereafter, the band intensity reduced. A 50 kDa band was observed for the first 36 h only, and a 47 kDa band observed at 60 h and thereafter. A 40 kDa band was observed at 36 h and 48 h. The changes in fibrin zymogram reflect changes in proteolytic enzymes and their activities inside *B. velezensis* CJ1 during growth on LB broth. Several fibrinolytic enzymes are synthesized, and each enzyme may have specific time points where the enzyme becomes fully active. Also a large fibrinolytic enzyme is subjected to posttranslational processings at N- or C-terminus or both, generating small sized derivatives which are still active. More studies are necessary to elucidate how many different fibrinolytic proteases are secreted by *B. velezensis* CJ1 and how their activities

change during growth. It is known that *Bacillus* species secrete several proteases such as Apr, Npr, Epr, Mpr, and Vpr [18]. Some enzymes are subjected to processing at either N-terminus or C-terminus, generating small fragments. To identify the processed derivatives, it is necessary to determine the amino acid sequences of each band on SDS-PAGE gel.

The strength of fibrinolytic activity of a sample can be estimated roughly from the size of the halo at the top part of the fibrin gel. The halo is suspected to be caused by binding of fibrinolytic enzymes to fibrin in the gel [19]. The 30 kDa protein on the fibrin zymogram might be one of the major enzymes responsible for the fibrinolytic activity of *B. velezensis* CJ1 at 36–60 h. It is noteworthy that the 30 kDa band was missing at 12 h and 24 h time points. It might be a degradation or processed product from a fibrinolytic enzyme such as Vpr from *B. subtilis* SJ4 where the mature Vpr (68.5 kDa) was subjected to autoprocessing and smaller proteins in the range of 33–20 kDa were generated [20]. The 30 kDa band was not located on the SDS-PAGE gel, and this might be due to the less sensitivity of coomassie blue stained gel. More studies are necessary on the identification of these bands on fibrin zymogram.

Cloning of *aprECJ1*

A 1.3 kb PCR fragment was cloned and the nucleotide sequence was determined (MW792223). Sequence analysis located an ORF which could encode a protein consisting of 382 amino acids (Fig. 5). BLAST analysis confirmed that the translated gene product was an AprE type protease. AprECJ1 showed 100–99% amino acid

GATTGATATACCTAAATAGAAATAAAACAACTGAAAAAATTGGGTCTACTAAAATATT 60
 -35
 ATTCCATACTATTACAATTAATCCACAGAATAATCTGTCTATTGGTTATTCTGCAAAATGAA 120
 -10
 M R G K K V W I S L L F A L
 AAAAGGAGTGGAATAAGAGTGAGAGGCCAAAAGGTATGGATCAGTTTGCTGTTTGCTTTA 180
 RBS
 A L I F T M A F G S T T S A Q A▼A G K S
 GCGTTAATCTTTACGATGGCGTTCGGCAGCACGACTTCTGCCAGGCTGCAGGGAAATCA 240
 N G E K K Y I V G F K Q T M S T M S A A
 AACGGGGAAAAGAAATATATTGTCGGATTAAAGCAGACAATGAGCACGATGAGCGCCGCC 300
 K K K D V I S E K G G K V E K Q F K Y V
 AAGAAAAAGATGTCATTCTGAAAAAGCGGGAAAGTGGAAAAGCAATTCAAATATGTA 360
 D A A S A T L N E K A V K E L K K D P S
 GACGCAGCTTCAGCTACATTAATGAAAAAGCTGAAAAGAGCTGAAAAAGAGCCCTAGC 420
 V A Y V E E D H V A Q A Y▽A Q S V P Y G
 GTCGCTTACGTTGAAGAAGATCACGTTGCACAGGCGTACGCGCAGTCCGTGCTTACGGC 480
 V S Q I K A P A L H S Q G F T G S N V K
 GTATCACAGATTAAAGCCCCTGCTCTGCACTCTCAAGGCTTCAACGGATCAAATGTTAA 540
 V A V I D S G I D S S H P D L K V A G G
 GTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTAAAGGTAGCAGCGGA 600
 A S M V P S E T N P F Q D N N S H G T H
 GCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGATAACAACCTCTCACGGAACCTAC 660
 V A G T V A A L N N S V G V L G V A P S
 GTTGCCGGTACAGTTGCGGCTCTTAATAACTCAGTCGGTGTATTAGGCGTTGCGCCAAGC 720
 A S L Y A V K V L G A D G S G Q Y S W I
 GCATCTCTTACGCTGAAAAGTTCTCGGCGCTGACGGTTCCGGCCAGTACAGCTGGATC 780
 I N G I E W A I A N N M D V I N M S L G
 ATTAACGGAATTGAGTGGGCGATCGCAAACAATATGGACGTTATTAACATGAGCCTCGGC 840
 G P S G S A A L K A A V D K A V A S G V
 GGACCTTCTGTTCTGCAGCGTTAAAGCGGCAGTTGACAAAGCCGTTGCTTCCGGCGTC 900
 V V V A A A G N E G T S G G S S T V G Y
 GTAGTGGTTGCGGCAGCCGTAACGAAGGCACTTCCGGCGGCTCAAGCACAGTGGGCTAC 960
 P G K Y P S V I A V G A V N S S N Q R A
 CCTGGTAAATACCCTTCTGTCTTGCAGGCTAGGCGCTGTTAACAGCAGCAACCAAAGAGCA 1020
 S F S V G S E L D V M A P G V S I Q S
 TCTTTCTCAAGCGTAGGTTCTGAGCTTGATGTACGGCACCAGGCGTCTCTATCCAAAGC 1080
 T L P G N K Y G A Y N G T S M A S P H V
 ACGCTTCTGGAACAAATACGGCGCGTACAATGGTACGTCAATGGCATCTCCGCACGTT 1140
 A G A A A L I L S K H P N W T N T Q V R
 GCCGGAGCGGCTGCTTTGATTCTTTCTAAGCACCAGCACTGGACAAACACTCAAGTCCGC 1200
 S S L E N T T T K L G D A F Y Y G K G L
 AGCAGTTTAGAAAACACCACTACAAAACCTTGGTGATGCTTTCTACTACGGAAAAGGGCTG 1260
 I N V Q A A A Q
 ATCAACGTACAGGCGGCAGCTCAGTAAACATAAAAAACCGGCGTCGGCCATGGCCCCGC 1320

Fig. 5. Nucleotide sequence of *aprECJ1*. The nucleotide sequence and deduced amino acid sequence of *aprECJ1* are shown. Tentative promoter sequences (-35 and -10) and RBS (ribosome binding site) are underlined. A possible transcription terminator is also underlined. The end of pre (▼) and pro sequence (▽) are marked. The first amino acid of proenzyme and mature enzyme are marked in bold characters.

identities with multispecies S8 family peptidase of *Bacillus* (100% with WP_003155195; 99% with WP_024085087 and WP_077722018). *AprECJ1* also showed 99–98% amino acid identities with S8 family peptidase of *B. velezensis* strains where the genome sequences were determined (99% with WP_070081588, WP_179155972, WP_044802764, and WP_082997797; 98% with WP_065180663 and WP_173613606). These

AprEs from *B. velezensis* strains are tentative proteins from annotated genes. *AprECJ1* also showed high amino acid sequence identities with fibrinolytic enzymes characterized previously by us, i.e. 99% identity (379/382) with an enzyme from *B. subtilis* (AHN52401.1), an enzyme from *B. pumilus* (ATD12229.1), *AprE3-17* from *B. licheniformis* (378/382, ACU32756.1), an alkaline serine protease from *B. subtilis* (377/392, QIN90910.1), and

a fibrinolytic enzyme from *B. subtilis* (377/382, ATA67131.1).

AprECJ1 is synthesized as a preproform, and the active, mature form accumulates in the culture medium. The first 30 amino acids are signal sequence as judged by SignalP-5.0 (<http://www.cbs.dtu.dk/services/SignalP/>), and the next 77 amino acids are pro-sequence as compared with other similar enzymes. The calculated size and pI of the preproenzyme are 39,100.11 Da and 9.14, respectively. The mature enzyme consists of 275 amino acids, and its molecular weight and pI are 27,446.57 Da and 6.65, respectively.

Heterologous expression of *aprECJ1* in *B. subtilis* WB600

aprECJ1 was overexpressed in *B. subtilis* WB600, a

Fig. 6. Growth (A) and fibrinolytic activities (B) of *B. subtilis* transformants (TFs). TFs were grown on LB broth for 96 h at 37 °C. OD₆₀₀ value and fibrinolytic activity were measured at 12 h intervals. ●, *B. velezensis* CJ1; ○, *B. subtilis* WB600 [pHYaprECJ1]; ▼, *B. subtilis* WB600 [pHY300PLK].

mutated strain where 6 extracellular protease genes were inactivated [21]. *B. subtilis* WB600 TF harboring pHYaprECJ1 (pHY300PLK with *aprECJ1*) showed the same growth pattern with control, *B. subtilis* WB600 [pHY300PLK] on LB broth (Fig. 6A). *B. velezensis* CJ1 grew slower than *B. subtilis* strains, and the absorbance values remained lower than those of *B. subtilis* strains (Fig. 6A). *B. subtilis* WB600 [pHYaprECJ1] showed higher fibrinolytic activity than *B. velezensis* CJ1 (Fig. 6B). *B. subtilis* WB600 [pHYaprECJ1] showed 1.8 fold higher activity than *B. velezensis* CJ1 at 48 h. This was caused by the high copy number of *aprECJ1* in a plasmid vector. If optimization efforts are made to increase gene expression level of *aprECJ1* by promoter replacement with stronger promoters or use of other plasmid vectors, the fibrinolytic activity of *B. subtilis* TF will be increased further. *B. subtilis* WB600 [pHYaprECJ1] showed the highest activity (211.45 mU/μl) at 48 h, and the second highest activity (192.93 mU/μl) at 60 h. After 48 h, the activity decreased rapidly, and showed 75.33 mU/μl at 96 h. For *B. velezensis* CJ1, the highest activity (115.93 mU/μl) was observed at 36 h, and the second highest activity (112.93 mU/μl) at 48 h. *B. subtilis* WB600 [pHY300PLK] did not show any activity during the 96 h incubation period. The 27 kDa mature form of AprECJ1 was observed in both SDS-PAGE gel (Fig. 7A, lane 1 and 2) and fibrin zymogram (Fig. 7B), but was not observed in control (Fig. 7, lane 5, 6). The result showed that AprECJ1 was properly processed in a heterologous host.

B. velezensis CJ1 was isolated from *Myeolchi Jeotgal* and showed strong fibrinolytic activity. The strain grew well in the presence of salt up to 15%. The strain and its fibrinolytic gene, *aprECJ1*, seem promising as a starter for fermented food such as Jeotgal and for production of fibrinolytic enzyme for the possible treatment of thrombosis. Fibrinolytic enzymes secreted by *Bacillus* species such as AprECJ1 show very high amino acid sequence homologies with other proteases. But a few amino acid changes might cause significant differences in the fibrinolytic activities. For example, nattokinase (AprN) and subtilisin E are different in just 2 amino acids but only nattokinase shows strong fibrinolytic activity [22, 23]. For this reason, more studies are necessary on the isolation of novel bacilli from diverse natural environments including fermented foods and characterization of their

Fig. 7. SDS-PAGE (A) and fibrin zymography (B) of culture supernatant from *B. subtilis* TFs. M, Dokdo-marker broad-range (EBM-1034, ELPIS, Daejeon, Korea). *B. subtilis* WB600 [pHYaprECJ1] at 48 h (lane 1), and 60 h (2); *B. velezensis* CJ1 at 48 h (3), and 60 h (4); *B. subtilis* WB600 [pHY300PLK] at 48 h (5), and 60 h (6).

fibrinolytic enzymes. The expected results will help to improve our understanding on the diversity and characteristics of fibrinolytic enzymes, and will pave a road for full utilization of these enzymes.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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