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Cloning of Fibrinolytic Enzyme Gene from *Bacillus subtilis* Isolated from *Cheonggukjang* and Its Expression in Protease-deficient *Bacillus subtilis* Strains

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Abstract *Bacillus subtilis* CH3-5 was isolated from *cheonggukjang* prepared according to traditional methods. CH3-5 secreted at least four different fibrinolytic proteases (63, 47, 29, and 20 kDa) into the culture medium. A fibrinolytic enzyme gene, *aprE2*, encoding a 29 kDa enzyme was cloned from the genomic DNA of CH3-5, and the DNA sequence determined. *aprE2* was overexpressed in heterologous *B. subtilis* strains deficient in extracellular proteases using a *E. coli-Bacillus* shuttle vector. A 29 kDa AprE2 band was observed and AprE2 seemed to exhibit higher activities towards fibrin rather than casein.

Keywords: *Cheonggukjang*, fibrinolytic enzyme, *Bacillus subtilis*, gene expression

Cheonggukjang, traditional Korean fermented soybeans, has some important health promoting effects, including the prevention of high blood pressure, a hypocholesterolemic effect, fibrinolytic activities, antimutation and anticarcinogenic effects, and antioxidative effects [10]. The *Bacillus* species responsible for *cheonggukjang* fermentation secrete several different types of protease into the culture media and the proteases then degrade the soy proteins into peptides and amino acids. Certain of these peptides are known to function as bioactive compounds [5, 11, 13]. For example, Paik *et al.* [19] purified a fibrinolytic enzyme from a *B. subtilis* strain isolated from *cheonggukjang*, and the enzyme was identified as a serine protease with a molecular mass of 45 kDa, and the optimum pH and temperature for fibrin degradation were pH 7.0 and 60°C, respectively. Chang *et al.* [3] also screened 220 bacilli isolates from *cheonggukjang* looking for strains with fibrinolytic and immunostimulating activities. Therefore, the amount of nitric oxide (NO), tissue necrosis factor (TNF), and interleukin-1α were examined in the RAW 264.7 cell line with added bacilli cells resulting in the selection of 2 *B. licheniformis* and 1 *B. subtilis* strains, which efficiently induced immunostimulating factors. Lee *et al.* [15] purified a 44 kDa fibrinolytic enzyme from a *Bacillus* strain isolated from *doenjang*, and Yoo *et al.* [25] purified a 29 kDa fibrinolytic enzyme from *B. subtilis* K-54 isolated from *cheonggukjang*, where the activity of the 29 kDa enzyme was severely inhibited by PMSF (phenylmethylsulfonyl fluoride), similar to other serine proteases, and the first eight amino acids from the N-terminus were determined. Understanding the role and contribution of each fibrinolytic enzyme to the overall fibrinolysis by an organism is important when selecting *Bacillus* strains for commercial production of *cheonggukjang*, especially as *cheonggukjang*.and related products have scientifically provable biofunctionalities and improved sensory properties [14]. Accordingly, the isolation of diverse *Bacillus* strains and characterization of their fibrinolytic enzymes are important, as the ability to compare many different fibrinolytic enzymes will generate useful information towards developing strains with enhanced fibrinolytic capabilities. Therefore, this study isolated a *B. subtilis* strain from *cheonggukjang* prepared according to traditional methods and cloned a fibrinolytic gene encoding a 29 kDa mature enzyme. The gene, designated as *aprE2*, was then characterized and successfully overexpressed in other *B. subtilis* hosts.

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Table 1. Bacterial strain and plasmids.

Bacteria or plasmid	Description	Source or reference
E. coli		
DH5α	Φ80*dlacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hdR17* (r_k^-, m_k^+), *supE44*, *relA1*, *deoR*, Δ(*lacZYA-argF*)U169	Gibco BRL
Bacillus subtilis CH3-5	Isolated from *cheonggukjang*	This study
Bacillus subtilis WB600	Em^r, Lm^r, Δ*npr*, Δ*aprA*, Δ*epr*, Δ*bpf*, Δ*mpr*, Δ*mprB*	Wu *et al.* [23]
Bacillus subtilis ISW1214	*hsrM*1, *leuA*8, *metB*5, Tet^S	Takara
Plasmids		
pGEM-T easy	TA cloning vector, 3 kb, Ap^r	Promega
pHY300PLK	*Bacillus-E. coli* shuttle cloning vector, pACYC177 and pAMα1 derivative origins, 4.87 kb, Ap^r, Tc^r	Takara
pHY3-5	pHY300PLK::fibrinolytic gene from *B. subtilis* CH3-5, 6.7 kb, Ap^r, Tc^r	This study

MATERIALS AND METHODS

Isolation of Bacilli with Fibrinolytic Activities

Cheonggukjang, prepared according to traditional methods [1], was screened for *Bacillus* strains with fibrinolytic activities. Ten g of *cheonggukjang* was homogenized with 90 ml of sterile water, and serially diluted samples were spread on LB plates containing 1% (w/v) skim milk. Colonies with halos were then selected and spotted onto fibrin plates. LB plates containing 025% (w/v) fibrinogen from bovine plasma (Sigma, U.S.A.) were prepared according to the method described by Noh *al.* [18]. The bacterial strains and plasmids used in this study are described in Table 1. The *E. coli* and *Bacillus* strains were cultivated in LB at 37°C with vigorous shaking. When necessary, antibiotics were included in the culture media at the following concentrations: Em (erythromycin) 5 μg/ml, Lm (lincomycin) 5 μg/ml, Tc (tetracycline) 10 μg/ml, and Ap (ampicillin) 100 μg/ml.

Identification of Isolate CH3-5

The isolate was first identified using an API kit (API 50 CHB, API 20E kit, BioMerieux, France). Then, for more accurate identification, the 16S rRNA gene and 16-23S intergenic region were amplified and the sequences were determined. To amplify the 16S rRNA gene, a primer pair based on a conserved region in 16S rDNA sequences from *Bacillus* sp. (GenBank numbers: AB286650, AB286649, DQ866837, EF029070, EF010982, DQ371241, DQ371229, DQ304770, AY956951, AB201796, AB201799) was used: bac-F (5'-CCGCGTGCCTAATACATGCAAG-3') and bac-R (5'-GGCATGCTGATCCGCGATTACTA-3'). For the amplification of the 16-23S intergenic region, the following primer pair was used: L516SF (5'-TCGCTAGTAATCGCGGATCGGC-3') and L523SR (5'-GCATATCGGTGTTAGTCCCGTCC-3') [25]. The chromosomal DNA was prepared from a 1.5 ml overnight culture grown on LB using the phenol-chloroform extraction method.

Cloning of *aprE2* Gene

The primer pair, aprEF (5'-GAGAACAGAGAAGCCGCTGTTA-3') and aprER (5'-GCCGCATCTGATGTCTTTGCTT-3'), were designed based on the published *aprE* sequences [20]. The PCR was performed using a GeneAmp 2400 PCR system (Perkin Elmer). The reaction mixture (50 μl) contained 1 μl of template DNA, 1 μl of each primer (10 μM), 1 μl of deoxynucleoside triphosphates (0.25 mM), and 0.5 μl of Ex *Taq* DNA polymerase (Takara, Japan). The amplification conditions were as follows: 94°C for 5 min; 30 cycles at 94°C for 0.25 min, 60°C for 0.5 min, 72°C for 1.5 min; a final extension at 72°C for 5 min. The amplified fragment was ligated with a pGEM-T Easy vector (Promega, U.S.A.) and the ligation mixture introduced into *E. coli* DH5α competent cells. The *E. coli* competent cell preparation and electroporation were performed as described previously [9]. The plasmid DNA preparation, restriction enzyme digestion, and agarose gel electrophoresis were all performed according to the published methods [22].

Overexpression of *aprE2* in Protease-deficient *Bacillus subtilis* Strains

The *aprE2* with EcoRI sites was amplified using a primer pair (5'-GCGAATTCGCCGCATCTGTGTCTTTG-3', 5'-GCGAATTCGAGAACAGAGAAGCC GCT-3', EcoRI site underlined). The 1.8 kb fragment was then ligated with pHY300PLK, an *E. coli-Bacillus* shuttle vector (Takara), after EcoRI digestion. The *Bacillus* competent cells were prepared according to a protocol provided by Takara. *B. subtilis* ISW1214 and WB600 were cultivated in LB (ISW1214) or LB with Em and Lm (WB600, each antibiotic, 5 μg/ml) at 37°C with shaking. Two 32-ml quantities of LB containing 0.5 M sorbitol were inoculated with 2 ml of each culture and grown until the OD_{660} reached 0.85–0.95. The cells were then left to stand on ice for 10 min and then recovered by centrifugation (5,000 ×*g*, 5 min at 4°C). Next, the cells were washed four times with an equal volume of cold solution A (0.5 M sorbitol, 0.5 M mannitol, 10% glycerol),

resuspended in 0.8 ml of solution A, and 60 μl aliquots were dispensed into freeze vials and stored at –70°C. The electroporation was performed by adding 100 ng of pHY3-5 to the cells thawed on ice, and then the cells were transferred into a 0.1-cm electroporation cuvette (BioRad). After a single pulse (25 μF capacitance, 200 Ω resistance, and 1.8 kV/cm), 0.9 ml of solution B (L-broth, 0.5 M sorbitol, 0.38 M mannitol) was added immediately and the cells left to recover for 3 h at 37°C with shaking. LB plates with Tc (10 μg/ml) and LB plates with Tc (10 μg/ml), Em (5 μg/ml), and Lm (5 μg/ml) were used to select the *B. subtilis* ISW1214 and WB600 transformants, respectively.

SDS-PAGE and Zymography

SDS-PAGE was performed for the culture supernatant (secreted proteins) and whole cells (total cellular proteins) from *B. subtilis* CH3-5 and transformants harboring pHY3-5. The cells were cultivated in 5 ml of an LB broth for 24 h and the supernatant obtained by centrifugation followed by filtration using a 0.45-μm syringe filter. The filtered supernatant was mixed with an equal volume of a cold 20% (w/v) TCA solution and left to stand on ice for 15 min before centrifugation [7]. The protein pellet was dried after being washed four times with 100% ethyl alcohol, and then resuspended in 50 mM Tris-HCl (pH 7.4). For the total cellular proteins, the cell pellet obtained by decanting the supernatant was washed and resuspended in 50 mM PBS (phosphate-buffered saline, pH 7.0). The cells were then disrupted by sonication (30 s, three times), and the soluble fraction obtained by centrifugation was used as the sample for the total cellular proteins. The protein concentration was determined using the Bradford method [2] with BSA (bovine serum albumin) as the standard. Each protein sample (4.6 μg) was analyzed by SDS-PAGE (15% acrylamide). A fibrin zymogram was also obtained for the secreted proteins. Ammonium sulfate (Amresco, U.S.A.) was added to the culture supernatant to a final concentration of 80% saturation and the proteins recovered as an ammonium sulfate pellet, which was then resuspended in a small volume of 30 mM Tris-HCl (pH 7.4) and dialyzed against the same buffer for 24 h at 4°C. Each sample was analyzed using a fibrin-polyacrylamide gel (10% acrylamide) that was prepared by mixing fibrinogen (0.12%, w/v, Sigma, U.S.A.), a polyacrylamide solution, and thrombin (100 NIH, Sigma, U.S.A.). Then, 12 μg of each sample, except for CH3-5 (1.5 μg), was loaded onto the fibrin-containing arcylamide gel together with plasmin (0.6 mU, Sigma, U.S.A.) and the electrophoresis performed at a constant current of 12 mA. Thereafter, the gel was briefly washed with 50 mM Tris-HCl (pH 7.4) containing 2.5% Triton X-100 to remove any SDS and incubated for 12 h at 37°C with a 30 mM Tris-HCl (pH 7.4) buffer containing 0.02% sodium azide. After the renaturation steps, the gel was stained with Coomassie brilliant blue [18].

Fibrin and Casein Plate Assays

The fibrin plate was prepared using the method of Noh *et al.* [18]. Equal volumes of a 2% agarose solution and fibrinogen (0.5% in 1 M PBS, Sigma, U.S.A.) were mixed together with 100 μl of thrombin (100 NIH, Sigma, U.S.A.), and the mixture poured into a Petri dish (8.5×1.3 cm). After the agarose had solidified, the protein samples prepared as described above were spotted on the plate (0.5 μg each). As a control, plasmin (0.006 U, Sigma, U.S.A.) was also spotted. The degree of fibrinolysis was then checked after 12 h at 37°C. The casein plate was prepared by mixing equal volumes of a 2% casein solution (in a 50 mM potassium phosphate buffer, pH 7.5; Calbiochem, Germany) and 2% agarose solution. The filtered supernatant from the bacilli cultivated in LB for 24 h was spotted on the plate (0.5 μg) together with proteinase K (0.12 U, Takara, Japan) and the plate incubated for 18 h at 37°C.

RESULTS AND DISCUSSION

Isolation and Identification of Isolate CH3-5

Several bacilli colonies showing the highest fibrinolytic activities on the fibrin plate were isolated from *cheonggukjang*, and one isolate, CH3-5, was selected for further examination. The API test results indicated that CH3-5 may be a *Bacillus circulans* (81.1% identity and T value 0.59). A 1.3 kb fragment, part of the 16S rRNA gene, was PCR-amplified using the primer pair described in Materials and Methods. The sequence was determined and 1,211 nt (nucleotides) used for the sequence analysis. The homology search results using BLAST showed that CH3-5 was either a *B. subtilis* (99% identity) or a *Bacillus licheniformis* (99% identity). When the 900-bp (basepair) 16-23S intergenic region was amplified and the DNA sequences analyzed, a 99% identity was revealed with the 16-23 S intergenic region from *B. subtilis*. Accordingly, CH3-5 was named *B. subtilis* CH3-5.

Cloning of *aprE2* and Its Nucleotide Sequence

A fibrinolytic gene homologous to *aprE* [13] was cloned from the genomic DNA of CH3-5 using a PCR. An *aprE* homolog was chosen, as it is the best known fibrinolytic gene among *B. subtilis* and other closely related species. The amplified 1.8 kb DNA fragment was ligated with a pGEM-T Easy vector (Promega) and the ligation mixture introduced into *E. coli* DH5α by electroporation. The recombinant plasmid pTaprE2 was then selected on LB Ap (100 μg/ml) plates containing X-gal (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, 40 μg/ml) and IPTG (isopropyl-beta-D-thiogalactoside, 0.5 mM). The DNA sequencing results confirmed that the cloned gene was indeed a homolog of *aprE* and thus named *aprE2*. Fig. 1 shows the nucleotide sequence for *aprE2* (GenBank

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1    GCCGCATCTGATGTCTTTGCTTGGCGAATGTTCATCTTATTTCTTCCTCCCTCCCAATAATTTTTTCATTCTATCCCTTTTCTGTAAAGTTTATTTTTCA
101  GAATACTTTTATCATCATGCTTTGAAAAAATATCACGATAATATCCATTGTTCTCACGGAAGCACACGCAGGTCATTTGAACGAATTTTTTCGACAGAAA
201  TTTGCCGGGACTCAGGAGCATTTAACCTAAAAAAGCATGACATTTCAGCATAATGAACATTTACTCATGTCTATTTTCGTTCTTTTCTGTATGAAAATAG
301  TTATTTCGAGTCTCTACGGAAATAGCGAGAGATGATATACCTAAATAGAGATAAAATCATCTCAAAAAAAATGGGTCTACTAAAATATTATTCCATCTAT
                                                                                    -35
401  TACAATAAATTCACAGAATAGTCTTTTAAGTAAGTCTACTCTGAATTTTTCTTAAAAGGAGAGGGTAAAGAGTGAGAAGCAAAAAATTGTGGATCAGCTTG
      -10          +1                                        RBS               M  R  S  K  K  L  W  I  S  L
501  TTCTTTGCGTTAACGTTAATCTTTACGATGGCGTTCAGCAACATGTCTGCGCAGGCTGCCGGAAAAAGCAGTACAGAAAAGAAATACATTGTCGGATTTA
      L  F  A  L  T  L  I  F  T  M  A  F  S  N  M  S  A  Q  A↓A  G  K  S  S  T  E  K  K  Y  I  V  G  F
601  AACAGACAATGAGTGCCATGAGTTCCGCCAAGAAAAAGGATGTTATTTCTGAAAAAGGCGGAAAGGTTCAAAAGCAATTTAAGTATGTTAACGCGGCGC
      K  Q  T  M  S  A  M  S  S  A  K  K  K  D  V  I  S  E  K  G  G  K  V  Q  K  Q  F  K  Y  V  N  A  A
701  AGCAACATTGGATGAAAAAGCTGTAAAAGAATTGAAAAAAGATCCGAGCGTTGCATATGTGGAAGAAGATCATATTGCACATGAATATGCGCAATCTGTT
      A  A  T  L  D  E  K  A  V  K  E  L  K  K  D  P  S  V  A  Y  V  E  E  D  H  I  A  H  E  Y  A  Q  S  V
801  CCTTATGGCATTTCTCAAATTAAAGCGCCGGCTCTTCACTCTCAAGGCTACACAGGCTCTAACGTAAAAGTAGCTGTTATCGACAGCGGAATTGACTCTT
      P  Y  G  I  S  Q  I  K  A  P  A  L  H  S  Q  G  Y  T  G  S  N  V  K  V  A  V  I  D  S  G  I  D  S
901  CTCATCCTGACTTAAACGTCAGAGGCGGAGCAAGCTTCGTACCTTCTGAAACAAACCCATACCAGGACGGCAGTTCTCACGGTACGCATGTAGCCGGTAC
      S  H  P  D  L  N  V  R  G  G  A  S  F  V  P  S  E  T  N  P  Y  Q  D  G  S  S  H  G  T  H  V  A  G
1001 GATTGCCGCTCTTAATAACTCAATCGGTGTTCTGGGCGTAGCGCCAAGCGCATCATTATATGCAGTAAAAGTGCTTGATTCAACAGGAAGCGGCCAATAT
      T  I  A  A  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
1101 AGCTGGATTATTAACGGCATTGAATGGGCCATTTCCAACAATATGGATGTTATTAACATGAGCCTTGGCGGACCTTCTGGTTCTACAGCGCTGAAAACAG
      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  S  G  S  T  A  L  K  T
1201 TCGTTGATAAAGCCGCTTCCAGCGGTATCGTCGTTGCTGCCGCTGCCGGAAACGAAGGTTCGTCCGGAAGCTCAAGCACAGTCGGCTACCCTGCAAAATA
      V  V  D  K  A  A  S  S  G     V  V  A  A  A  A  G  N  E  G  S  S  G  S  S  S  T  V  G  Y  P  A  K
1301 TCCTTCTACTATTGCGGTAGGTGCGGTAAACAGCAGCAACCAAAGAGCTTCATTCTCAAGCGCAGGTTCTGAGCTTGATGTGATGGCTCCTGGCGTATCC
      Y  P  S  T  I  A  V  G  A  V  N  S  S  N  Q  R  A  S  F  S  S  A  G  S  E  L  D  V  M  A  P  G  V  S
1401 ATCCAAAGCACACTTCCTGGAGGCACTTACGGTGCTTACAACGGCACGTCCATGGCGACTCCTCACGTTGCCGGAGCAGCAGCGCTAATCCTTTCTAAGC
         Q  S  T  L  P  G  G  T  Y  G  A  Y  N  G  T  S  M  A  T  P  H  V  A  G  A  A  A  L  I  L  S  K
1501 ACCCGACTTGGACAAACGCGCAAGTCCGTGATCGTTTAGAAAGCACTGCAACATATCTTGGAAACTCTTTCTACTATGGAAAAGGGTTAATCAACGTACA
      H  P  T  W  T  N  A  Q  V  R  D  R  L  E  S  T  A  T  Y  L  G  N  S  F  Y  Y  G  K  G  L  I  N  V
1601 AGCAGCTGCACAATAATAGTAAAAAGAACCAGGTTCCTCCATACCTGCTTCTTTTTATTTGTCAGCATCCTGATGTTCCGGCGCATTCTCTTCTTTCTCC
      Q  A  A  A  Q  *
1701 GCATGTTGAATCCGTTCCATGATCGAAGAATGGCTGCCTCTGAAAATCTTCACAAGCACCGGAGGATCAACCTGGCTCAGCCCCGTCACGGCCAAATCCT
1801 GAAACGTTTTAACAGCGGCTTCTCTGTTCT
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Fig. 1. Nucleotide sequence of *aprE2*.

The deduced amino acid sequence of AprE2 is also shown. The amino acids corresponding to a signal peptide and pro sequence are shadowed. The presumptive cleavage site of the signal peptide is marked with an arrow. Three amino acids (Ser221, His64, and Asp32), constituting the conserved catalytic triad of fibrinolytic enzymes belonging to the subtilisin family, are shadowed. Putative -35 and -10 promoter sequences are underlined and the TSS (transcription start site) is marked in bold characters.

Accession Number DQ997812) and its translated amino acid sequence. The first codon, GTG, starts at 471 nt and an RBS (GGAGAGGG) is located 6 nt upstream. Putative -35 and -10 promoter sequences, identical with those of *aprE* [20], are underlined. Thus, judging from its similarity with other subtilisin-type proteases, AprE2 was likely synthesized as a preproenzyme with a 29 aa (amino acid) signal peptide and 77 aa pro sequence. The mature enzyme, 275 aa in size, was suspected to start at the 107th alanine. Subtilisins E [16], NAT [17], J [8], and amylosacchariticus [12] have the same configuration with AprE2, whereas subtilisins BPN' and DJ-4 [4] have signal peptides consisting of 30 aa. The predicted molecular mass of the mature AprE2 was calculated to be 27.64 kDa with a pI of 6.3, whereas the molecular mass of the unprocessed enzyme was 39.39 kDa with a pI of 9.04. When the translated amino acid sequence for AprE2 was compared with that for nattokinase (NK) genes (AY219901, AF368283, AY 895162) in the database, differences were observed at four positions: 130th (S in AprE2, T in NK), 143rd (A in AprE2, V in NK), 162nd (S in AprE2, T in NK), and 192nd (A in AprE2, V in NK). AprE2 appeared essentially identical to the nattokinase produced by *Bacillus* species, and included the conserved amino acids important for catalytic activity, a catalytic triad made up of Ser221, His64, and Asp32 (marked as outlined in Fig. 1), which is commonly present in almost all fibrinolytic enzymes belonging to the subtilisin family [21].

Overexpression of *aprE2* in Proteases-deficient *Bacillus subtilis* Strains

aprE2 was overexpressed in *B. subtilis* WB600 and ISW1214, as confirmed by the SDS-PAGE (Fig. 2) and fibrin plate assay results (Fig. 3). As such, the ensuing increased fibrinolytic activity may have been caused by the increased copy number of *aprE2* in both *B. subtilis* strains. In the case of *B. subtilis* WB600, a strain deficient in six extracellular proteases [23], it is likely that the lack of extracellular proteases contributed to the increased fibrinolytic activities. For *B. subtilis* ISW1214, provided by Takara with pHY300PLK, it was suspected to have similar phenotypes to WB600, as similar levels of *aprE2* gene

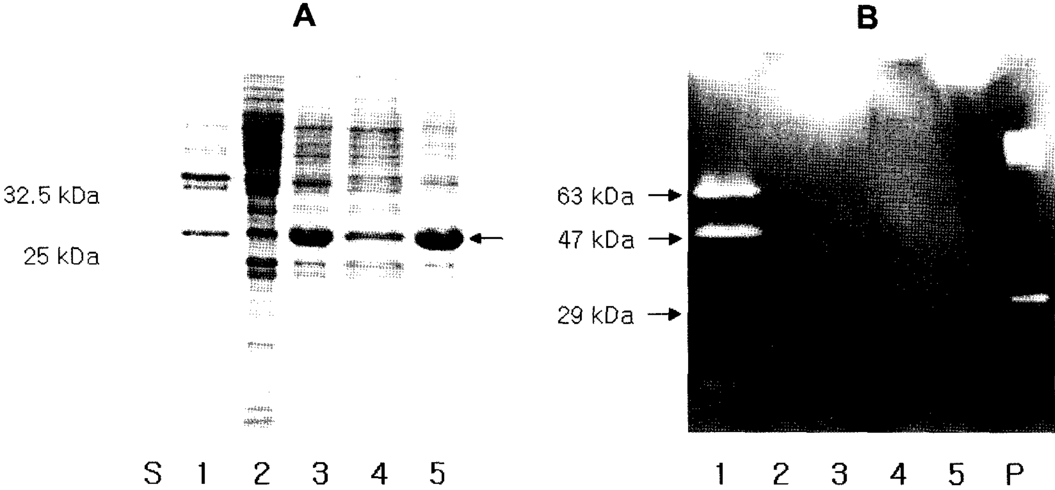

Fig. 2. SDS-PAGE and fibrin zymogram results.

SDS-PAGE (**A**) and fibrin zymogram (**B**) obtained from culture supernatant of recombinant *B. subtilis* strains. 1, *B. subtilis* CH3-5; 2, *B. subtilis* WB600 [pHY300PLK]; 3, *B. subtilis* WB600 [pHY3-5]; 4, *B. subtilis* ISW1214 [pHY300PLK]; 5, *B. subtilis* ISW1214 [pHY3-5]; S, protein size maker (NEB, England, B7709S); P, plasmin (Sigma, P1867). Fifteen % and 10% acrylamide gels were used for the SDS-PAGE and zymography, respectively.

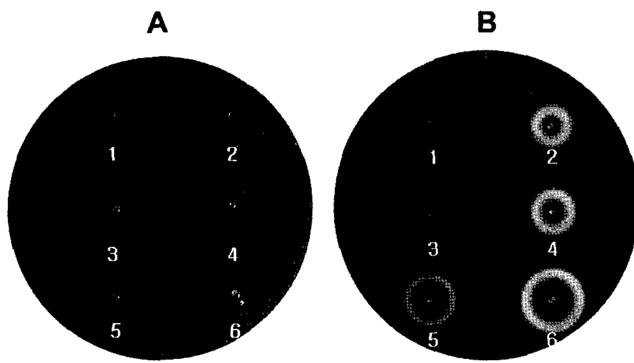

Fig. 3. Fibrin plate assay (**A**) and casein plate assay (**B**).
The filtered supernatant from a 24-h culture in LB was applied onto each spot (0.5 mg). 1, *B. subtilis* WB600 [pHY300PLK]; 2, *B. subtilis* WB600 [pHY3-5]; 3, *B. subtilis* ISW1214 [pHY300PLK]; 4, *B. subtilis* ISW1214 [pHY3-5]; 5, *B. subtilis* CH3-5; 6, plasmin (A, Sigma, 0.006 U) and proteinase K (B, Takara, 0.12 U).

expression were observed in both strains. The results also indicated that careful combination with an appropriate host strain, such as WB600, and expression vector is an efficient way to overproduce AprE2, which could be utilized as an ingredient for biofunctional foods or materials.

SDS-PAGE and Zymography

aprE2 was overexpressed in the *B. subtilis* WB600 and ISW1214 strains when introduced to these hosts *via* pHY3-5. A mature AprE2 band with an apparent molecular mass of 29 kDa was observed from the supernatant (Fig. 2), yet no clear band corresponding to AprE2 was observed from the total cellular proteins (results not shown), indicating that the AprE2 synthesized in WB600 and ISW1214 was efficiently processed and secreted into the culture media. The size (29 kDa) of the overproduced protein matched well with the value (27.64 kDa) calculated from the sequence data. A secreted protein with the same molecular mass was observed in the WB600 and ISW1214 cells (lanes 2 and 4 in Fig. 2A). However, the protein did not exhibit any fibrinolytic activities, as judged from the fibrin plate assay results (Fig. 3). A zymogram (Fig. 2B) showed that *B. subtilis* CH3-5 secreted at least four fibrinolytic enzymes, and a faint band had an apparent molecular mass of 29 kDa. This protein seemed to be AprE2. It is noteworthy that CH3-5 also secreted other fibrinolytic enzymes, 63 kDa and 47 kDa in size, which were seemingly the main fibrinolytic enzymes. Furthermore, a faint band of ca. 20 kDa was detected in addition to AprE2. However, no 29 kDa band was detected from the supernatant for the WB600 and ISW1214 transformants carrying pHY3-5. Instead, very large smeared bands were observed in the upper part of the gel. As such, the overproduced AprE2 may not have been resolved properly or failed to regain its activity under the conditions used. Although the exact cause of this phenomenon was unknown, a poor resolution on a zymogram was also previously reported for the subtilisin secreted from *Bacillus subtilis* KCTC 3014 [5]. In a previous study, Yoo *et al.* [25] purified a 29 kDa enzyme from a *B. subtilis* isolated from *cheonggukjang* and the first eight amino acids were determined as Ala-Gly-Ser-Val-Pro-Tyr-Gly-Ser. However, the mature AprE2 had two different amino acids (translated from the nucleotide sequence): Gly in the 2nd was replaced with Gln, and Ser in the 8th position was replaced with Ile. The 63 and 47 kDa proteins, apparently the major fibrinolytic enzymes (Fig. 2B), seemed to be produced by many *B. subtilis* strains isolated from *cheonggukjang* in addition to the reference strain 168 (results not shown). Nonetheless, although many fibrinolytic enzymes have already been characterized from diverse strains, there is still a question about how many functionally different fibrinolytic enzymes are produced by each *Bacillus* species. Thus, more thorough and systematic approaches are necessary because if the role of each enzyme is understood, it may be possible to produce *cheonggukjang* and related products with improved biofunctionalities.

Fibrin and Casein Plate Assay Results

Fig. 3 shows the results of the fibrin (Fig. 3A) and casein (Fig. 3B) plate assays. As expected, the diameter of the circle caused by fibrinolysis increased for the protein samples from the *B. subtilis* recombinants harboring pHY3-5. When calculated based on the circle areas from different plasmin concentrations, the protein samples from the WB 600 and ISW1214 cells harboring pHY3-5 showed an activity of 33.0 and 39.4 U/mg, respectively. In contrast, *B. subtilis* 3–5 only showed an activity of 11.9 U/mg. Thus, a three-fold increase was achieved by the overexpression of *aprE2* in the other *B. subtilis* strains. Since *B. subtilis* WB600 (probably the same as ISW1214) lacks six extracellular proteases, the increase in the degree of fibrinolysis seemed to be the direct result of AprE2 overproduction in this host. However, the degree of casein degradation in the two recombinant strains was much less than that in CH3-5. When adjusted to a known protease K concentration, the supernatant samples from the two recombinant cells showed an activity of 45.7 U/mg, whereas the supernatant samples from *B. subtilis* 3–5 showed 65.8 U/mg, indicating that AprE2 may not be efficient for casein hydrolysis. The lack of extracellular proteases in *B. subtilis* WB600 (and probably ISW1214 too) was not complemented by the overproduction of AprE2. Therefore, based on the results, AprE2 seemed to be more active towards fibrin than casein, although the exact substrate preference will not be known until assays using purified AprE2 are performed. It is well known that some fibrinolytic enzymes have different substrate specificities compared with serine proteases [6, 11].

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