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Cloning of *aprE86-1* Gene Encoding a 27-kDa Mature Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* CH86-1

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A gene encoding the major secreted fibrinolytic protein of *Bacillus amyloliquefaciens* CH86-1 was cloned from genomic DNAs. DNA sequencing showed that the gene, *aprE86-1*, could direct the synthesis of a mature protein 275 amino acids in length after processing. When *aprE86-1* was introduced into *B. subtilis*, a mature 27-kDa protein was produced as expected. The fibrinolytic activity of the *B. subtilis* transformant (TF) was higher than that of *B. amyloliquefaciens* CH86-1, showing the possibility of increasing the fibrinolytic activity of *Bacillus* strains through genetic engineering.

Keywords: *Bacillus amyloliquefaciens*, fibrinolytic enzymes, gene expression

B. amyloliquefaciens CH86-1 was isolated from *Cheonggukjang* prepared by traditional methods [7]. Among the strains isolated, it showed the highest fibrinolytic activity and also produced a large amount of sticky materials on soybeans. Therefore, this strain would seem to be a good starter for the production of *Cheonggukjang*. Detailed characterization of its useful properties is necessary if the advantages of this strain are to be fully utilized. *B. amyloliquefaciens* CH86-1 secretes several fibrinolytic enzymes into the culture supernatant during growth; among them, a 27-kDa protein is the major product. We previously purified this enzyme, designated AprE86-1, and examined its properties [8]. In this report, its structural gene, *aprE86-1*, was cloned and successfully expressed in the heterologous host *B. subtilis* WB600.

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For the cloning of *aprE86-1*, primers 51F (5'-AGGATCC CAAGAGAGCGATTGCGGCTGTGTAC-3', *Bam*HI site underlined) and 51R (5'-AGAATTCTTCAGAGGGAGC CACCCGTCGATCA-3', *Eco*RI site underlined) were used. The primers were designed based on the sequence of a tentative *aprE* homolog of *B. amyloliquefaciens* FZB42 and were used for the cloning of *aprE51* from *B. amyloliquefaciens* CH51 [5]. PCR was performed using an MJ Mini gradient thermal cycler (Bio-RAD, Hercules, CA, U.S.A.) and *ExTaq* DNA polymerase (Takara, Shiga, Japan). The amplification conditions were as follows: 94°C for 5 min; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s; and a final extension at 72°C for 5 min. The resulting 1.5-kb fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). The nucleotide sequence was determined (Fig. 1) and was deposited in GenBank under Accession No. FJ882063. DNA sequencing confirmed that the cloned gene was indeed a homolog of *aprE* from *B. amyloliquefaciens* and *B. subtilis* strains. *aprE86-1* encodes a protein of 382 amino acids. The first codon, GTG, starts at nucleotide (nt) 214, and the ribosome-binding site (RBS; GGAGAGGG) is located 6 nt upstream. Putative -35 (TCTACT) and -10 (TACAAT) promoter sequences, identical to those of *aprE* [9], are underlined in Fig. 1. The tentative transcription start site (TSS) is A, located 7 nt downstream of the -10 sequence. When translated amino acid sequences were compared, AprE86-1 was different from AprE from *B. amyloliquefaciens* FZB42 at four positions; 105 (Q in AprE86-1, K in AprE), 168 (N in AprE86-1, Y in AprE), 251 (S in AprE86-1, A in AprE), and 254 (I in AprE86-1, V in AprE). AprE86-1 also differs from AprE51 from *B. amyloliquefaciens* CH51 at four positions; 26 (S in AprE86-1, T in AprE51), 27 (P in AprE86-1, S in AprE51), 251 (S in AprE86-1, A in

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CAAAAACCTAATGTCCTTCCATCGGTTTTTTCCATTAAAATTTAAATATTTCGGGTTCCT 60
ATTAAACGAAAGAGAGATGATATACCTAAATAGAAATAAAACAAACTGAAAAAAATTGGG 120
TCTACTAAAATATTATTCCATGCTATACAATTAATCCACAGAATAATCTGTCTATTGGTT 180
-35                    -10      +1
                                  M  R  G  K  K  V  W  I  S   9
GTTCTGCAAATGAAAAAAAGGAGAGGGTAAAGAGTGAGAGGCAAAAAGGTATGGATCAGT 240
                    RBS
L  L  F  A  L  A  L  I  F  T  M  A  F  G  S  T  S  P  A  Q   29
TTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGCGTTCGGCAGCACGTCTCCTGCCCAG 300
A  A  G  K  S  N  G  E  K  K  Y  I  V  G  F  K  Q  T  M  S   49
GCGGCAGGGAAATCAAACGGGGAAAAGAAATACATTGTCGGATTTAAACAGACAATGAGC 360
T  M  S  A  A  K  K  K  D  V  I  S  E  K  G  G  K  V  Q  K   69
ACGATGAGCGCCGCTAAGAAAAAAGATGTCATTTCTGAAAAAGGCGGGAAAGTGCAAAAG 420
Q  F  K  Y  V  D  A  A  S  A  T  L  N  E  K  A  V  K  E  L   89
CAATTCAAATATGTAGACGCAGCTTCAGCTACATTAAATGAAAAAGCCGTAAAAGAGCTG 480
K  K  D  P  S  V  A  Y  V  E  E  D  H  V  A  Q  A  Y  A  Q  109
AAAAAAGACCCTAGCGTCGCTTACGTTGAAGAAGATCACGTTGCACAGGCGTACGCGCAG 540
S  V  P  Y  G  V  S  Q  I  K  A  P  A  L  H  S  Q  G  F  T  129
TCCGTGCCTTACGGCGTATCACAGATTAAAGCCCCTGCTCTGCACTCTCAAGGCTTCACC 600
G  S  N  V  K  V  A  V  I  D  S  G  I  D  S  S  H  P  D  L  149
GGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGATTCTTCTCATCCTGATTTA 660
K  V  A  G  G  A  S  M  V  P  S  E  T  N  P  F  Q  D  N  N  169
AAGGTAGCAGGCGGAGCCAGCATGGTTCCTTCTGAAACAAATCCTTTCCAAGACAACAAC 720
S  H  G  T  H  V  A  G  T  V  A  A  L  N  N  S  V  G  V  L  189
TCTCACGGAACTCACGTTGCCGGTACAGTTGCGGCTCTTAATAACTCAGTCGGTGTATTA 780
G  V  A  P  S  A  S  L  Y  A  V  K  V  L  G  A  D  G  S  G  209
GGCGTTGCGCCAAGCGCATCTCTTTACGCTGTAAAAGTTCTCGGCGCTGACGGTTCCGGC 840
Q  Y  S  W  I  I  N  G  I  E  W  A  I  A  N  N  M  D  V  I  229
CAGTACAGCTGGATCATTAACGGAATTGAGTGGGCGATCGCAAACAATATGGACGTTATT 900
N  M  S  L  G  G  P  S  G  S  A  A  L  K  A  A  V  D  K  A  249
AACATGAGCCTCGGCGGACCTTCTGGTTCTGCAGCGTTAAAAGCGGCAGTTGACAAAGCC 960
V  S  S  G  I  V  V  V  A  A  A  G  N  E  G  T  S  G  G  S  269
GTTTCTTCCGGCATCGTAGTCGTTGCGGCAGCCGGTAACGAAGGCACTTCCGGCGGCTCA 1020
S  T  V  G  Y  P  G  K  Y  P  S  V  I  A  V  G  A  V  N  S  289
AGCACAGTGGGCTATCCTGGTAAATACCCTTCTGTCATTGCGGTAGGCGCTGTTAACAGC 1080
S  N  Q  R  A  S  F  S  S  V  G  S  E  L  D  V  M  A  P  G  309
AGCAACCAACGAGCATCTTTCTCAAGCGTAGGTTCTGAGCTTGATGTCATGGCACCAGGC 1140
V  S  I  Q  S  T  L  P  G  N  K  Y  G  A  Y  N  G  T  S  M  329
GTCTCTATCCAAAGCACGCTTCCTGGAAACAAGTACGGCGCGTACAATGGTACGTCAATG 1200
A  S  P  H  V  A  G  A  A  A  L  I  L  S  K  H  P  N  W  T  349
GCATCTCCGCACGTTGCCGGAGCGGCTGCTTTGATTCTTTCTAAGCACCCGAACTGGACA 1260
N  T  Q  V  R  S  S  L  E  N  T  T  T  K  L  G  D  A  F  Y  369
AACACTCAAGTCCGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATGCTTTCTAC 1320
Y  G  K  G  L  I  N  V  Q  A  A  A  Q  *382
TACGGAAAAGGGCTGATCAACGTACAGGCGGCAGCTCAGTAAAACATAAAAAACCGGCGT 1380
CGGGCATGGCCCCGCCGGTTTTTTATATCGGTTTTTTCCATTAAAATTTAAATATTTCGG 1440
GTTCCTATTAAACGAAAGAGAGATGATATACCTAAATAGAAATAAAACAAACTGAAAAAA 1500
ATTGGGTCTACTAAAATATTATTCCATGCTATACAATTAATCCACAGAATAATCTGTCTA 1560
TTGGTTGTTCTGCAAATGAAAAAAAGGAGAGGATAAAGAGTGAGAGGCAAAAAGGTATGG 1620
ATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATG 1659
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Fig. 1. Nucleotide sequence of *aprE86-1*.
Translated amino acid sequences are also shown. The first codon, GTG, starts at nt 214 and the RBS (GGAGAGGG) is located 6 nt upstream. Putative -35 and -10 promoter sequences are underlined and the presumptive transcription start site is marked as +1. The transcription terminator is also underlined.

AprE51), and 254 (I in AprE86-1, V in AprE51) (Fig. 2). AprE86-1, however, differs significantly (52 amino acids) from AprE2 from *B. subtilis* [4]. The calculated pI and molecular mass of AprE86-1 are 9.23 and 39,125.16 Da, respectively. When compared with homologous proteins, AprE86-1 is synthesized as a preproprotein. It has a leader sequence 30 amino acids long and a prosequence 77 amino acids long. The mature enzyme consists of 275 amino acids with a calculated pI and molecular mass of 6.65 and 27,476.60 Da, respectively.

For overexpression, the 1.5-kb fragment containing *aprE86-1* was digested with *Eco*RI and *Bam*HI and ligated into pHY300PLK, an *E. coli–Bacillus* shuttle vector (Takara). The resulting recombinant plasmid, pHY86-1, was introduced into *B. subtilis* WB600 [10]. *Bacillus* competent cell preparation and electroporation were performed as described previously [4]. Luria–Bertani (LB) plates containing tetracycline (Tc, 10 μg/ml) were used to select *B. subtilis* WB600 transformants (TF). *Bacillus* TF was cultivated in LB broth for 24 h. The supernatant was obtained by centrifugation and concentrated by precipitation with ammonium sulfate [80% saturation (w/v); Amresco, Solon, OH, U.S.A.]. The resulting pellet was resuspended in a small volume of 30 mM Tris (pH 7.4) and dialyzed against the same buffer for 24 h at 4°C. A 10 μg protein sample was analyzed on a 12% acrylamide gel. The protein concentration was determined using the Bradford method [1] with bovine serum albumin (BSA) as the standard. A 27-kDa band was produced by *B. subtilis* WB600 carrying pHY86-1, but not by the control (WB600 harboring pHY300PLK) (Fig. 3). Culture supernatants from *B. amyloliquefaciens* CH51 and *B. sutilis* WB600 [pHY51] were also analyzed for comparision. As shown in Fig. 3A, the same 27-kDa band was observed for *B. subtilis* WB600 carrying pHY51. The profile of proteins secreted by CH86-1 was different from that of CH51.

In terms of overall fibrinolytic activity, CH86-1 performed better than CH51, although the difference between the two strains was not great (see Fig. 3B and 3C). However, *B. subtilis* WB600 [pHY51] showed slightly higher fibrinolytic activity than WB600 [pHY86-1], as judged by the fibrin plate assay (see Fig. 3C). This result indicates that other fibrinolytic enzymes contribute to the overall fibrinolytic capacity of CH 86-1 (as well as CH51) and that AprE51 is a more active enzyme than AprE86-1 in *B. subtilis* WB600. For fibrin zymography, 1 μg of filtered culture supernatant was analyzed on a fibrin-polyacrylamide gel (12% acrylamide) prepared by mixing fibrinogen [0.12% (w/v); Sigma, St. Louis, MO, U.S.A.], a polyacrylamide solution, and thrombin (100 NIH; Sigma). Electrophoresis was performed at a constant current of 12 mA. After electrophoresis, the gel was washed with 50 mM Tris-HCl (pH 7.4) containing 2.5% Triton X-100 to remove any SDS, and then incubated for 12 h at 37°C with 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. Zymogram results also showed that only *B. subtilis* WB600 with pHY86-1 (and pHY51) had fibrinolytic activity whereas *B. subtilis* WB600 control cells did not; an active band, however, was not observed at the 27-kDa position. Instead, a large smear appeared at the top of the gel, which has often been observed for

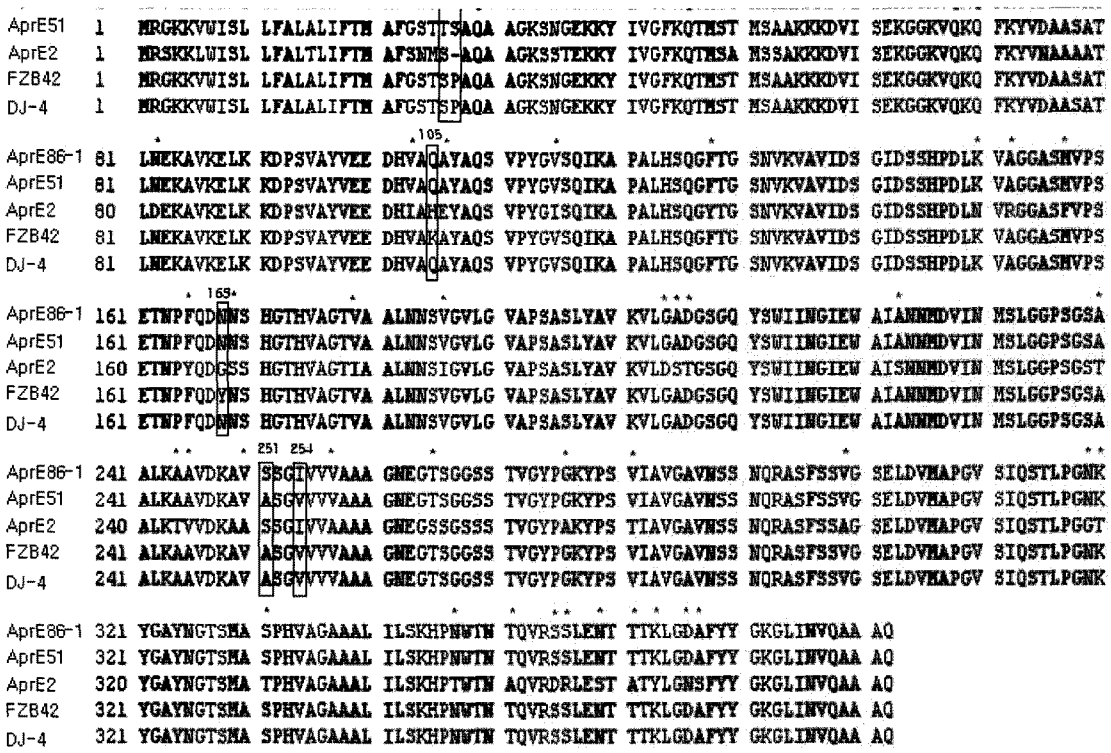

Fig. 2. Comparison of amino acid sequence of AprE86-1 with those of homologous proteins: AprE51 from *B. amyloliquefaciens* CH51 (EU414203), AprE2 from *B. subtilis* CH3-5 (DQ997812), FZB42 from *B. amyloliquefaciens* FZB42 (ABS73414), and pro-subtilisin DJ-4 (AAT45900).
Amino acids showing variation in *B. amyloliquefaciens* strains are marked with boxes, and amino acids showing differences in AprE2 and AprE86-1 are marked with * at the top.

fibrinolytic proteins [3, 6]. The sample used for fibrin zymography was not boiled in SDS sample buffer before loading on the gel because fibrinolytic activity was destroyed by heat treatment even for as short a time as 1 min (data not shown). Without boiling, fibrinolytic enzymes are hardly resolved on an SDS gel according to their molecular weight. Binding of enzymes to the fibrin in the gel and the high pI value of an enzyme might explain the poor resolution [2, 3].

The growth and fibrinolytic activities of *B. subtilis* TFs in LB during 84 h were examined and the results are shown in Fig. 4. No differences were observed in cell

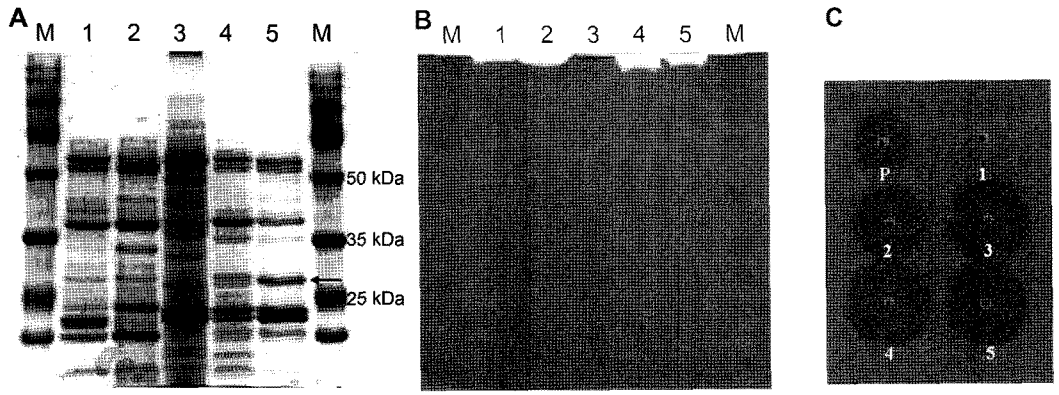

Fig. 3. SDS-PAGE (**A**), fibrin zymography (**B**), and fibrin plate assay (**C**) of culture supernatants from *B. subtilis* TFs.
For SDS-PAGE (**A**), a 10-μg protein sample obtained by 80% ammonium sulfate precipitation of supernatant was applied to the gel after boiling in 1× SDS-PAGE buffer. For fibrin zymography (**B**) and fibrin plate assay (**C**), a 1-μg sample obtained by filtering the supernatant from a 24-h culture in LB was applied without heat treatment. Gels contained 12% acrylamide. Panels **A** and **B**; lanes 1, CH51; 2, CH86-1; 3, WB600 [pHY300PLK]; 4, WB600 [pHY51]; 5, WB600 [pHY86-1]; M, DokDo-MARK protein size marker (Elpisbio, Taejeon, Korea). An arrow indicates the 27-kDa mature AprE86-1 (and AprE51). Panel **C**; P, plasmin (3 mU; Sigma); 1, WB600 [pHY300PLK]; 2, CH51; 3, CH86-1; 4, WB600 [pHY51]; 5, WB600 [pHY86-1].

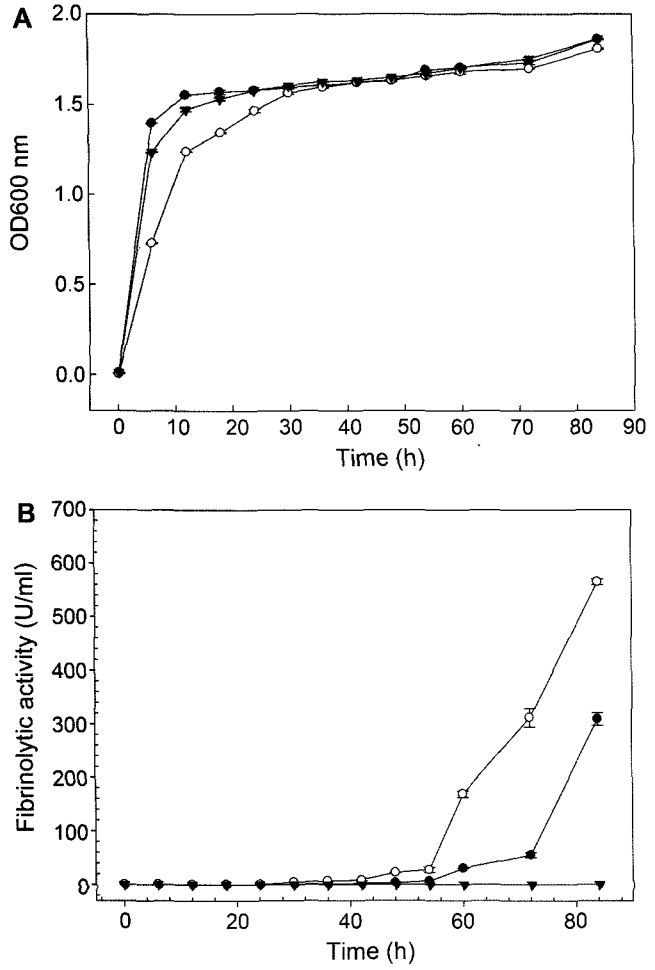

Fig. 4. Growth (**A**) and fibrinolytic activities (**B**) of *B. subtilis* TFs.

Cells were cultivated for 84 h at 37°C in LB, and optical densities (600 nm) and fibrinolytic activities were measured at the indicated intervals. ●, *B. amyloliquefaciens* CH86-1; ○, *B. subtilis* WB600 [pHY86-1]; ▼, *B. subtilis* WB600 [pHY300PLK].

growth between *B. subtilis* TFs and *B. amyloliquefaciens* CH86-1. The fibrinolytic activity of *B. subtilis* carrying pHY86-1 was higher than that of *B. amyloliquefaciens* CH86-1. This result might reflect the difference in *aprE86-1* copy number in the two strains. The lack of extracellular proteases in *B. subtilis* WB600 [10] seemed to be partially responsible for the increased activity. The fibrinolytic activities of *B. subtilis* WB600 [pHY86-1] and *B. amyloliquefaciens* CH86-1 remained at basal levels during the exponential growth phase and then increased sharply when cultures entered the stationary phase (after 52 h). We previously showed that extended incubation of a *B. licheniformis* culture overexpressing *aprE2*, a gene encoding the 29-kDa mature fibrinolytic enzyme from *B. subtilis* CH3-5, caused a sharp increase in the fibrinolytic activity and also the degree of protein degradation in the culture supernatant [6]. The same phenomenon was observed in *B. subtilis* WB600 harboring pHY86-1. It is suspected that extended incubation causes increased proteolytic activity in the cell by activation of some proteases that are less active during the growth phase. The increase in proteolytic activity might reflect the necessity of cells to utilize available nitrogen sources in unfavorable environments.

The amount of AprE86-1 could be increased further if careful modifications of an expression vector were to be pursued. pHY300PLK is a general cloning vector, and *aprE86-1* expression was not optimized in this work. Overproduction of AprE86-1 is critical if the protein is intended for use as an ingredient in biofunctional foods or materials. In this respect, development of efficient expression vectors together with appropriate hosts will be necessary.

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References

1. Bradford, M. M. 1976. Rapid and sensitive methods for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
2. Choi, N. S., K. H. Yoo, K. S. Yoon, K. T. Chang, P. J. Maeng, and S. H. Kim. 2005. Identification of recombinant subtilisins. *J. Microbiol. Biotechnol.* **15:** 35–39.
3. Choi, N. S., D. M. Chung, C. H. Ryu, K. S. Yoon, P. J. Maeng, and S. H. Kim. 2006. Identification of three extracellular proteases from *Bacillus subtilis* KCTC 3014. *J. Microbiol. Biotechnol.* **16:** 457–464.
4. Jeong, S. J., G. H. Kwon, J. Chun, J. S. Kim, C. S. Park, D. Y. Kwon, and J. H. Kim. 2007. Cloning of fibrinolytic enzyme gene from *Bacillus subtilis* isolated from *Cheonggukjang* and its expression in protease-deficient *Bacillus subtilis* strains. *J. Microbiol. Biotechnol.* **17:** 1018–1023.
5. Kim, G. M., A. R. Lee, K. W. Lee, J. Y. Park, J. Chun, J. Cha, Y. S. Song, and J. H. Kim. 2009. Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from *Cheonggukjang*. *J. Microbiol. Biotechnol.* **19:** 997–1004.
6. Kwon, G. H., W. J. Jeong, A. R. Lee, J. Y. Park, J. Cha, Y. S. Song, and J. H. Kim. 2008. Heterologous gene expression of *aprE2* encoding a 29 kDa fibrinolytic enzyme from *Bacillus subtilis* in *Bacillus licheniformis* ATCC 10716. *Food Sci. Biotechnol.* **17:** 1372–1375.
7. Kwon, G. H., H. A. Lee, J. Y. Park, J. S. Kim, J. Lim, C. S. Park, D. Y. Kwon, Y. S. Kim, and J. H. Kim. 2009. Development

of a RAPD-PCR method for identification of *Bacillus* species from *Cheonggukjang*. *Int. J. Food Microbiol.* **129:** 282-287.

8. Lee, A. R., G. M. Kim, J. Y. Park, H. D. Jo, J. Cha, Y. S. Song, J. Chun, and J. H. Kim. 2010. Characterization of a 27 kDa Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* CH86-1 Isolated from Cheonggukjang *J. Korean Soc. Appl. Biol. Chem.* 53(1): in-Press.
9. Park, S. S., S. L. Wong, L. F. Wang, and R. H. Doi. 1989. *Bacillus subtilis* subtilisin gene (*aprE*) is expressed from a σ^A (σ^{43}) promoter *in vitro* and *in vivo*. *J. Bacteriol.* **171:** 2657-2665.
10. Wu, X. C., W. Lee, L. Tran, and S. L. Wong. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* **173:** 4952-4958.