J. Microbiol. Biotechnol. (2009), **19**(9), 997–1004
doi: 10.4014/jmb.0811.600
First published online 26 March 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of a 27 kDa Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* CH51 Isolated from *Cheonggukjang*

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Received: November 3, 2008 / Revised: February 5, 2009 / Accepted: February 12, 2009

Bacillus amyloliquefancies **CH51 isolated from *cheonggukjang*, a traditional Korean fermented soy food, has strong fibrinolytic activity and produces several fibrinolytic enzymes. Among four different growth media, tryptic soy broth was the best in terms of supporting cell growth and fibrinolytic activity of this strain. A protein with fibrinolytic activity was partially purified from the culture supernatant by CM-Sephadex and Phenyl Sepharose column chromatographies. Tandem mass spectrometric analysis showed that this protein is a homolog of AprE from *B. subtilis* and it was accordingly named AprE51. The optimum pH and temperature for partially purified AprE51 activity were 6.0 and 45°C, respectively. A gene encoding AprE51, *aprE51*, was cloned from *B. amyloliquefaciens* CH51 genomic DNA. The *aprE51* gene was overexpressed in heterologous *B. subtilis* strains deficient in fibrinolytic activity using an *E. coli–Bacillus* shuttle vector, pHY300PLK.**

Keywords: Fibrinolytic enzymes, bacilli, subtilisins, *Bacillus amyloliquefaciens, cheonggukjang*

Strains of *Bacillus amyloliquefaciens* are some of the most common bacilli isolated from fermented soybean foods. Some of these strains possess fibrinolytic activity [4, 18] conferred by certain proteolytic enzymes that are secreted by the bacilli, nattokinase being the most well-known example [20]. Fibrinolytic enzymes are able to resolve fibrin clots, thus being responsible for an important functionality of soybean foods fermented by bacilli.

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Cheonggukjang, a traditional Korean fermented soy food, is an important source of bacilli with fibrinolytic activity [11]. In traditional *cheonggukjang* preparation, cooked soybeans are covered with rice straw, which is a rich source of *Bacillus* spores. During fermentation for 2 or 3 days at around 40°C, soybean proteins are digested, releasing oligopeptides and amino acids. In some cases, sticky materials are produced on the surface of the soybeans. After fermentation, *cheonggukjang* is usually served as a side dish with rice after being boiled with other seasonings, such as garlic and pepper.

Cheonggukjang is known to possess several health-promoting properties, such as anticancer activity, abilities to lower blood pressure and prevent myocardial and cerebral infarction, antioxidant activity, and probiotic activity [15].

B. amyloliquefaciens CH51, previously isolated from *cheonggukjang*, showed higher fibrinolytic activity than other isolates when examined by the fibrin plate method [9]. It produces several fibrinolytic enzymes (see Fig. 1) but these have not yet been characterized. In an effort to understand the fibrinolytic system of *B. amyloliquefaciens* CH51, we attempted to purify fibrinolytic enzymes from its culture supernatants. As a result, the major fibrinolytic enzyme, AprE51 was partially purified, its properties were examined, and its structural gene was cloned.

Materials and Methods

Bacterial Strains and Culture Conditions

B. amyloliquefaciens CH51 was previously isolated from *cheonggukjang* and identified by molecular methods including 16S rRNA gene and *recA* sequencing [12].

B. amyloliquefaciens CH51 was grown in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, U.S.A.) or LB at

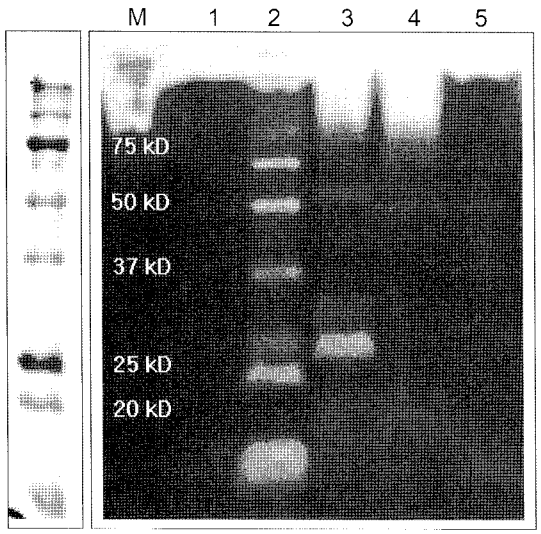

Fig. 1. Fibrin zymogram of extracellular proteins from *Bacillus* strains isolated from *cheonggukjang*.
Bacillus strains were grown in LB for 48 h at 37°C with shaking. The culture supernatant was filtered and concentrated by ammonium sulfate precipitation (80% saturation). One mg was applied to a 15% acrylamide gel. Lanes 1. *B. subtilis* 168 (lab strain); 2, *B. subtilis* CH3-5; 3, *B. amyloliquefaciens* CH51; 4, *B. amyloliquefaciens* CH86-1; 5. *B. licheniformis* CH3-17.

37°C with aeration. Nutrient broth (NB) and brain heart infusion (BHI) were used for comparision. *E. coli* and *B. subtilis* strains were grown in LB at 37°C with shaking. Cells containing pHY300PLK (Takara, Shiga, Japan) or pHY51 were cultivated in LB supplemented with 10 μg/ml tetracycline (LBTc).

Assay of Fibrinolytic Activity

Bacillus cells were cultivated in TSB at 37°C with shaking. At the appropriate growth stage, the culture was centrifuged at 5,000 ×*g* for 20 min at 4°C. The supernatant was filtered and assayed for fibrinolytic activity. If necessary, the supernatant was further concentrated by ammonium sulfate precipitation (80%, w/v). The ammonium sulfate pellet was redissolved in a small volume of buffer A (20 mM Tris-HCl, pH 7.0). Protein concentration was determined according to the Bradford method [2] using bovine serum albumin (BSA) as a standard.

Fibrinolytic activity was determined using the fibrin plate method [1]. In a Petri dish, 7 ml of 1.2% (w/v) fibrinogen (Sigma, St. Louis, MO, U.S.A.) solution in 1 M phosphate-buffered saline (PBS) was mixed with an equal volume of 2% (w/v) agarose solution along with 0.1 ml of thrombin solution (100 NIH units/ml; Sigma). The Petri dish was left at room temperature for 1 h to allow a fibrin clot layer to form. Twenty μl of sample was dropped into a hole that had been made in a fibrin plate using a capillary glass tube, and the plate was incubated at 37°C for 6 h. 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. A standard curve showing the relationship between the area of the clear zone formed and the number of plasmin units was prepared in the range of 1.5–30 mU.

SDS–PAGE and Fibrin Zymography

Culture supernatant was concentrated by ammonium sulfate precipitation (80%, w/v), and then the pellet was resuspended in a small volume of buffer A and dialyzed against the same buffer for 24 h at 4°C with four buffer changes. SDS–PAGE was performed according to Laemmli's method [13], and fibrin zymography was carried out as previously described [6, 10]. The separating gel solution (12%, w/v) contained fibrinogen (0.12%, w/v) and 100 μl of thrombin (10 NIH units/ml).

Partial Purification of AprE51

Culture supernatant (1 l) was concentrated by ammonium sulfate precipitation (80%, w/v). The precipitate was resuspended in 10 ml of buffer A and then dialyzed against 20 volumes of the same buffer for 24 h with four buffer changes. The protein sample was loaded onto a CM-Sephadex (Amersham Pharmacia Biotech, Uppsala, Sweden) column (2.5×8 cm), and proteins were eluted by sequential application of six 200-ml volumes of buffer A containing NaCl at a concentration of 0 to 1 M, increased stepwise in 0.2 M increments. Twenty μl of each 15-ml fraction was spotted onto a fibrin plate. Fractions showing fibrinolytic activity were pooled, dialyzed against buffer A, and then lyophilized. Hydrophobic interaction chromatography using Phenyl Sepharose 6 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for further purification. The column (2.5×12 cm) was washed and pre-equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. Proteins were eluted by sequential application of 200-ml volumes of buffer A containing $(NH_4)_2SO_4$ at a concentration of 1 to 0 M, decreased stepwise in 0.2 M increments. Active 15 ml fractions were pooled, dialyzed against buffer A, and lyophilized prior to analysis by SDS–PAGE and zymography. Protein bands were excised from the stained SDS gel. Trypsin digestion and mass spectrometric analysis of peptides were performed at Genomine (Pohang, Gyungbuk, Korea).

Properties of Partially Purified AprE51

Effects of pH, temperature, metal ions, and inhibitors on the fibrinolytic activity of AprE51 were examined by the fibrin plate method. Eight mg of AprE51 was incubated in either 0.1 M citrate–NaOH buffer (pH 3.0 to 5.0), 0.1 M sodium phosphate buffer (pH 6.0 to 7.0), 0.1 M Tris-HCl (pH 8.0 to 9.0), or 0.1 M glycine–NaOH buffer (pH 10.0) at 37°C for 2 h, and then the amount of fibrinolytic activity remaining was measured. Heat stability was examined by incubating 8 μg of AprE51 in 0.1 M sodium phosphate buffer (pH 6.0) at 37–55°C for 30 min and measuring the activity that remained. For the effects of metal ions and inhibitors, AprE51 was incubated in 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM metal ions (KCl, $CaCl_2$, $CoCl_2$, $MgCl_2$, $CuSO_4$, $MnCl_2$, or $ZnCl_2$) or 1 mM inhibitor (phenylmethylsulfonylfluoride [PMSF], SDS, or EDTA) at 45°C for 1 h and the activity that remained was measured.

Cloning of the *aprE51* Gene

The primer pair CH51-F (5'-AGGATCCCAAGAGAGCGATTGCG GCTGTGTAC-3', BamHI site underlined) and CH51-R (5'-AGAAT TCTTCAGAGGGAGCCACCCGTCGATCA-3', EcoRI site underlined), was designed based on the sequence of *aprE* (Accession No. CP000560.1) from *B. amyloliquefaciens* FZB42. PCR was performed using a GeneAmp 2400 PCR system (PerkinElmer, Waltham, MA, U.S.A.). The reaction mixture (50 μl) contained 1 ml 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, Shiga, Japan). The amplification conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 64°C for 30 sec, 72°C for 40 sec; a final extension at 72°C for 5 min. The amplified fragment was ligated into pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.)

and *E. coli* DH5α cells were transformed with the ligation mixture. Preparation and electroporation of *E. coli* competent cells were performed as previously described [9]. Plasmid DNA preparation, restriction enzyme digestion, and agarose gel electrophoresis were performed according to published methods [19].

Overexpression of *aprE51* in Protease-Deficient *B. subtilis* Strains

aprE51 was amplified using primer pair CH51-F and CH51-R, and then digested with BamHI and EcoRI. The 1.5 kb fragment was ligated into pHY300PLK, resulting in pHY51. Preparation and electroporation of *Bacillus* competent cells were carried out as previously described [9]. *B. subtilis* WB600 [21] and ISW 1214 (Takara) were used as hosts for *aprE51* gene expression. *B. subtilis* transformants (TFs) were grown in LBTc at 37°C for 2 days. Cells were recovered by centrifugation at 5,000 ×*g* for 20 min at 4°C and the supernatant was used for the enzyme assay.

For SDS-PAGE and zymography, protein samples were prepared from *B. subtilis* TFs using different methods. Culture supernatant was mixed with an equal volume of cold 20% (w/v) trichloroacetic acid (TCA) solution and kept on ice for 15 min prior to centrifugation [7]. The protein pellet was washed four times with 100% ethyl alcohol, dried, and then resuspended in 50 mM Tris-HCl (pH 7.4). Ten μg of protein was subjected to SDS-PAGE (12% acrylamide) as described above. For zymography, the supernatant was concentrated by ammonium sulfate precipitation (80%, w/v) and 1 mg was applied to the gel.

RESULTS AND DISCUSSION

Fibrinolytic Activity of *B. amyloliquefaciens* CH51

We previously isolated bacilli exhibiting fibrinolytic activity from *cheonggukjang* prepared by traditional methods in Sunchang County, North Jeolla Province, Korea [12]. Among these strains, CH51 and CH86-1, which were identified as *B. amyloliquefaciens*, showed the highest fibrinolytic activities. Analysis of culture supernatant by zymography on a fibrin gel revealed that *B. amyloliquefaciens* CH51 produces 6 fibrinolytic enzymes (see Fig. 1, lane 3). Distinct bands with sizes of 70, 66, 50, 32, and 27 kDa were observed. In addition, a large smear was observed at the top of the separating gel. The smear was caused by either binding of fibrinolytic enzymes to fibrin in the gel [14] or by their high pI values [4], which could have prevented them from migrating to the points in the gel that correspond to their molecular masses. It will be necessary to characterize each component of the fibrinolytic system and evaluate the contribution of each enzyme to the observed fibrinolytic activity of *B. amyloliquefaciens* CH51 if this strain is to be fully utilized for the production of functional foods or medicines.

The growth and fibrinolytic activity of *B. amyloliquefaciens* CH51 in four different media were compared. Each medium was inoculated with 2% (w/v) overnight culture and incubated at 37°C with shaking. Growth in TSB showed the best result, reaching an absorbance value of 1.9 by the end of cultivation at 48 h (Fig. 2). Growth in other media was less

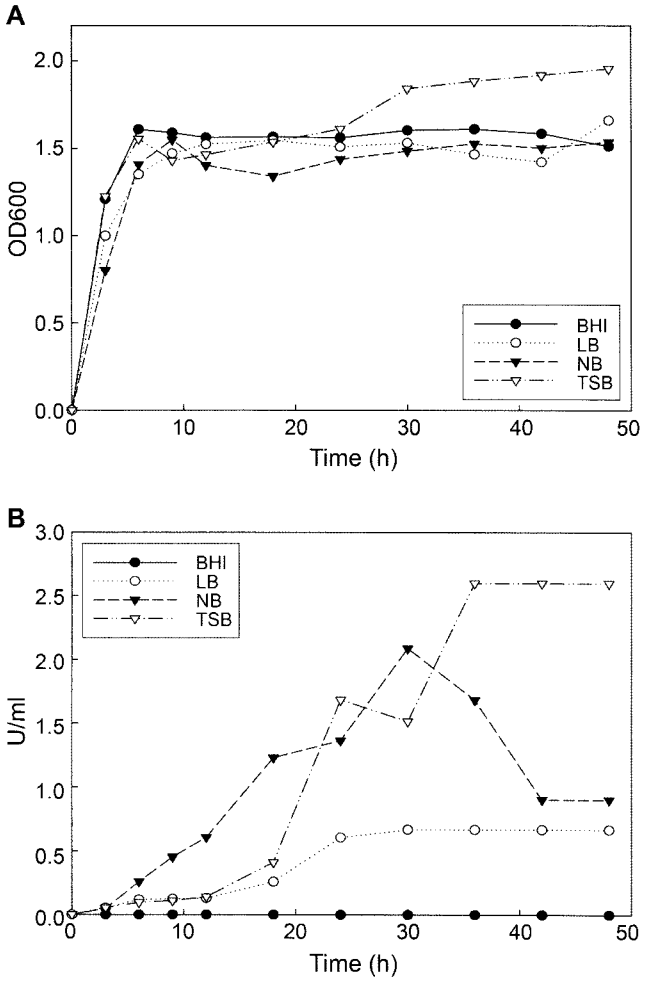

Fig. 2. Changes in absorbance (**A**) and fibrinolytic activity (**B**) of *B. amyloliquefaciens* CH51 during growth on four different media. ●, BHI; ○, LB; ▼, NB; ▽, TSB. Filtered culture supernatant (20 μl) at each time point was applied to a fibrin plate and the fibrinolytic activity was expressed as U/ml.

efficient than in TSB, reaching about 1.5. Cells grown in TSB showed the highest fibrinolytic activity, followed by those grown in NB and LB. Interestingly, cells did not show any significant fibrinolytic activity when grown in BHI although the medium supported cell growth as well as NB and LB did (Fig. 2). This result, the cause of which is unknown, indicates that the choice of growth medium and possibly cultivation conditions are important for the production of fibrinolytic enzymes by *B. amyloliquefaciens* CH51.

Partial Purification of AprE51

Fibrinolytic enzymes in the culture supernatant were purified by CM-Sephadex and Phenyl-Sepharose column chromatographies. A fibrin plate assay was used to detect active fractions. Purification results are summarized in Table 1 and the overall purification was 20.3-fold. When active fractions from the Phenyl-Sepharose column were pooled

Table 1. Partial purification of AprE51.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification (χ fold)	Yield (%)
Culture supernatant	425.2	7156.2	16.8	1.0	100
80% Ammonium sulfate precipitation	71.2	3851.2	54.1	3.2	54
CM-Sephadex	5.2	1,173.5	227.5	13.5	16
Phenyl-Sepharose	2.7	940.1	342.4	20.4	13

and examined by SDS-PAGE, four major bands (45, 27, 23, and 19 kDa in size), in addition to a few minor bands, were detected when the gel was stained with Coomassie blue (Fig. 3). The four major bands were excised from the gel and subjected to trypsin digestion and mass spectrometric analysis. The 27 kDa protein (AprE51 in Fig. 3) was identified as a homolog of subtilisin DJ-4 from *Bacillus* sp. DJ-4 [5], subtilisin DFE from *B. amyloliquefaciens* [18], hypothetical protein AprE from *B. amyloliquefaciens* FZB42 (protein id=ABS73414), and AprE from *B. subtilis* (protein id=ABY83469).

These results confirmed that the 27 kDa protein is a subtilisin-type protease with fibrinolytic activity; it has been accordingly named AprE51. *B. subtilis* secretes several proteases into the culture medium, subtilisin being the most predominant [3]. Therefore, AprE51 is the major protease with fibrinolytic activity secreted by *B. amyloliquefaciens* CH51. The 23 kDa protein (BglS in Fig. 3) was identified as a β-1,3-1,4-endoglucanase precursor, and the 19 kDa protein (XynA in Fig. 3) as endo-1,4-beta-xylanhydrolase. The 45 kDa protein (YnfF in Fig. 3) might be a homolog of YnfF, a precursor of dimethyl sulfoxide reductase, from *Bacillus* species. Zymography results (Fig. 3B) also support the conclusion that AprE51 is the major fibrinolytic enzyme of *B. amyloliquefaciens* CH51. In lane 4, two minor fibrinolytic enzymes were observed that were later removed by CM-Sephadex column chromatography. These minor fibrinolytic enzymes will need to be characterized in the future. Cultivation conditions for the production of fibrinolytic enzymes must be chosen carefully since they may affect the activity of each enzyme differently.

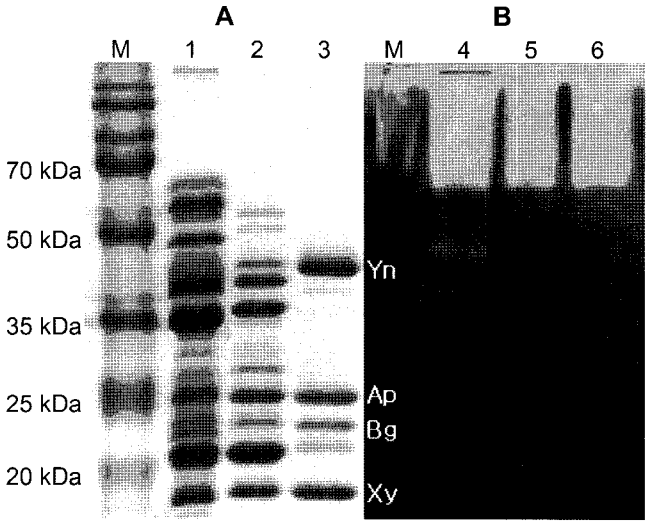

Fig. 3. SDS-PAGE (**A**) and zymography (**B**) of partially purified AprE51.
A 10-μg boiled sample was subjected to SDS-PAGE and a 2-μg unboiled sample to zymography. Lanes 1 and 4, 80% ammonium sulfate precipitation of supernatant; lanes 2 and 5, active fractions from CM-Sephadex column; lanes 3 and 6, active fractions from Phenyl-Sepharose 6 Fast Flow column; M, Precision Plus protein standard (BioRad, U.S.A.). Four bands in lane 3 were identified and the name marked on the right side of each band: Yn (=YnfF), Ap (=AprE51), Bg (=BglS), and Xn (=XynA).

Properties of Partially Purified AprE51

The effects of pH, temperature, and metal ions on the fibrinolytic activity of AprE51 were examined using the fibrin

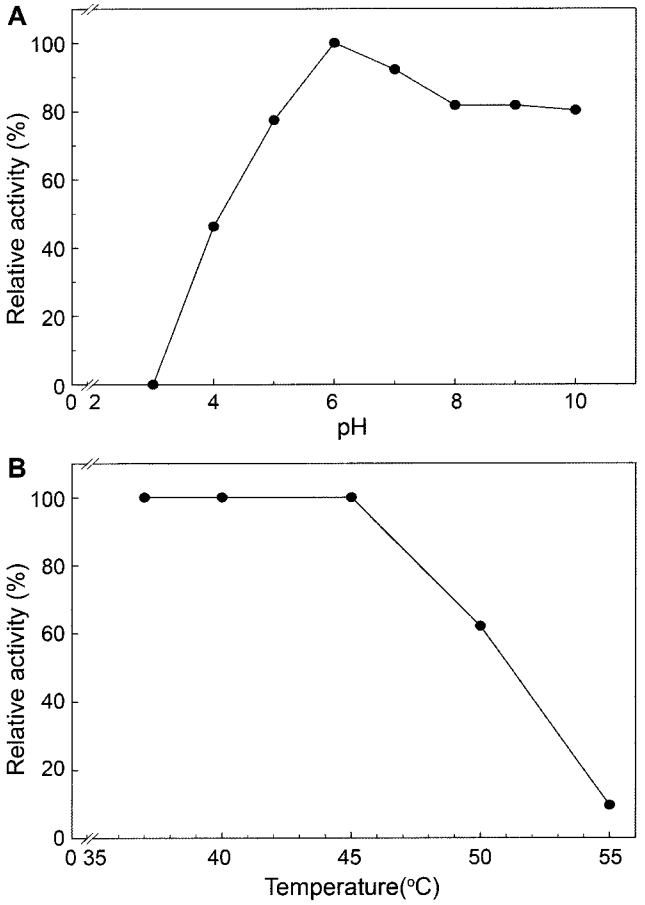

Fig. 4. Effects of pH (**A**) and temperature (**B**) on the fibrinolytic activity of AprE51.

Table 2. Effect of metal ions and inhibitors on the fibrinolytic activity of AprE51.

Metal ions (5 mM)/Inhibitors (1 mM)	Activity (%)
None	100
KCl	102.2
$CaCl_2$	104.5
$CoCl_2$	93.4
$MgCl_2$	103.4
$CuSO_4$	60.5
$MnCl_2$	95.6
$ZnCl_2$	87.1
PMSF	33.4
SDS	69.4
EDTA	83.0

plate method. The optimal pH was 6.0 (Fig. 4). The enzyme activity was maintained at 45°C but was decreased at higher temperatures. At 50°C, 62% activity remained, but at 55°C, only 12% activity remained. Enzyme activity was not inhibited by K^+, Ca^{2+}, or Mg^{2+} but was inhibited by Co^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} ions (Table 2). Among the metal ions tested, Cu^{2+} showed the greatest inhibition (39.5%). PMSF, a well-known inhibitor of serine-type proteases, inhibited fibrinolytic activity by 66.6%. EDTA also inhibited the activity by 17%, indicating that inhibition of another minor protease, probably a metalloprotease, might be responsible for this loss of activity, even though its presence was not confirmed by SDS–PAGE.

Cloning and Sequencing of *aprE51*

The *aprE51* gene encoding AprE51 was cloned from *B. amyloliquefaciens* CH51 genomic DNA by PCR. The amplified 1.5 kb DNA fragment was ligated into pGEM-T Easy Vector and introduced into *E. coli* DH5α cells. TFs harboring the recombinant plasmid pTaprE51 were obtained,

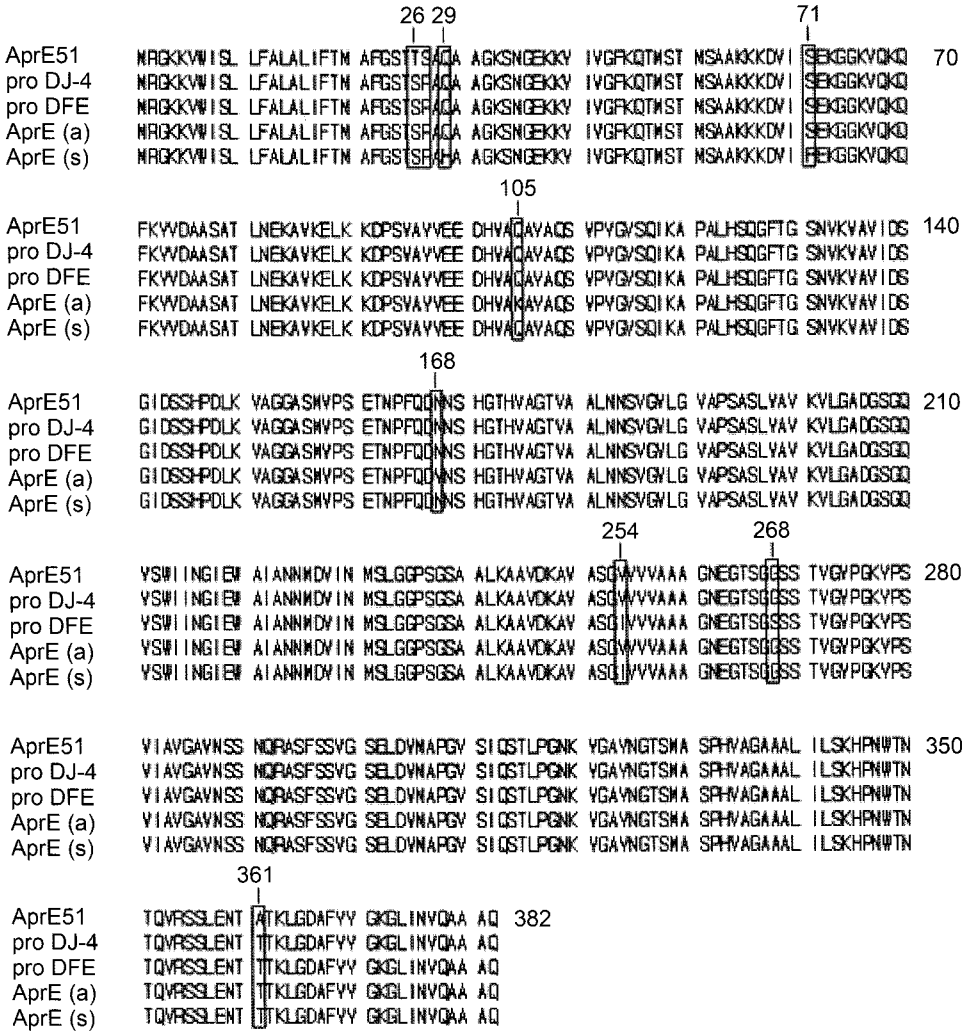

Fig. 5. Comparison of amino acid sequence of AprE51 with sequences of other homologous proteins.
Pro-subtilisin DJ-4 (AAT45900), subtilisin DFE precursor (AAZ66858), AprE from *B. amyloliquefaciens* FZB42 [AprE (a), ABS73414], and AprE from *B. subtilis* [AprE (s), ABY83469] were aligned with AprE51. Amino acids showing variation are marked.

and DNA sequencing confirmed that the cloned gene is indeed a homolog of *aprE*. The sequence was deposited in GenBank (Accession No. EU414203). Fig. 5 shows the translated amino acid sequence of AprE51 and its alignment with the sequences of other subtilisins. These proteins were found to be 99% identical to AprE51 when conservative amino acid substitutions are taken into consideration. When compared with AprE from *B. amyloliquefaciens* FZB42, AprE51 has four different amino acids, located at positions 26 (T in AprE51, S in AprE), 27 (S in AprE51, P in AprE), 105 (Q in AprE51, K in AprE), and 168 (N in AprE51, Y in AprE). Two changes are conservative (positions 26 and 105), leaving only two significant differences in amino acid sequence (99% positive matches) between the two proteins.

AprE51 is synthesized as a preproprotein, as are all subtilisins. It consists of 382 amino acids, with the 107 amino acids at the N-terminus corresponding to the prepro sequence; tandem mass spectrometric results showed that the active 27 kDa protein sequence begins with AQS--, corresponding to position 108 of AprE51 as inferred from the nucleotide sequence (data not shown). The calculated molecular weight of the active enzyme is 27,446.57, which corresponds well to the SDS-PAGE result. The first 30 amino acids seem to constitute the prepeptide (signal peptide) when comparing the sequence to that of other homologous proteins. This signal peptide is longer than that of AprE2 from *Bacillus subtilis* CH3-5 by one amino acid [9]. Subtilisins BPN' and DJ-4 have signal peptides consisting of 30 amino acids, whereas subtilisins E [16], NAT [17], and J [8] have signal peptides consisting of 29 amino acids.

Although an active ca. 27 kDa band was observed on a zymogram of *B. amyloliquefaciens* CH51 culture supernatant (Fig. 1, lane 3), it was not AprE51. Under nondenaturing conditions, AprE51 failed to migrate through the separating gel to the point corresponding to its size (27 kDa). When slices corresponding to the apparent molecular masses of 70 and 80 kDa were excised from the gel and analyzed by mass spectrometry, one band (80 kDa) turned out to be AprE51 (data not shown). When the eluted protein sample was analyzed by SDS-PAGE after boiling, a 27 kDa band appeared instead of an 80 kDa band (data not shown). These results indicate that, under nondenaturing conditions, AprE51 molecules are not resolved according to size but remain near the top of the acrylamide gel, forming a large smear; this behavior has also been reported for other subtilisins secreted by *Bacillus* species [3, 4]. The same result was obtained when *aprE51* was overexpressed in heterologous *B. subtilis* hosts (see Fig. 6 B, lanes 3 and 6). Zymography cannot clearly distinguish between each enzyme component, especially if their activity has been destroyed by denaturing samples in order to resolve protein components. In many cases, a large smear is observed at the top of a zymogram instead of individually separated active bands. More efficient methods of resolving fibrinolytic enzymes will certainly need to be developed in order to facilitate the understanding of the fibrinolytic capacity of bacilli.

Overexpression of *aprE51* in Protease-Deficient *B. subtilis* Strains

The PCR-amplified 1.5 kb *aprE51* gene was ligated into pHY300PLK, generating pHY51, which was introduced into *B. subtilis* WB600 and ISW1214. Expression of *aprE51* in these hosts was confirmed by SDS-PAGE and zymography (Fig. 6). In Fig. 6A, an arrow on the right side of the gel indicates the position of AprE51, and the results show that only cells harboring pHY51 produced this protein. In *B. subtilis* WB600, a strain deficient in six extracellular proteases [21], the lack of these proteases was probably responsible for the efficient production of AprE51. It is suspected that *B. subtilis* ISW1214 has a similar genetic background to WB600 since similar levels of *aprE51* expression were observed in both strains (Fig. 6B).

Results obtained for cell growth and fibrinolytic activity of *B. subtilis* TFs are summarized in Fig. 7. The fibrinolytic

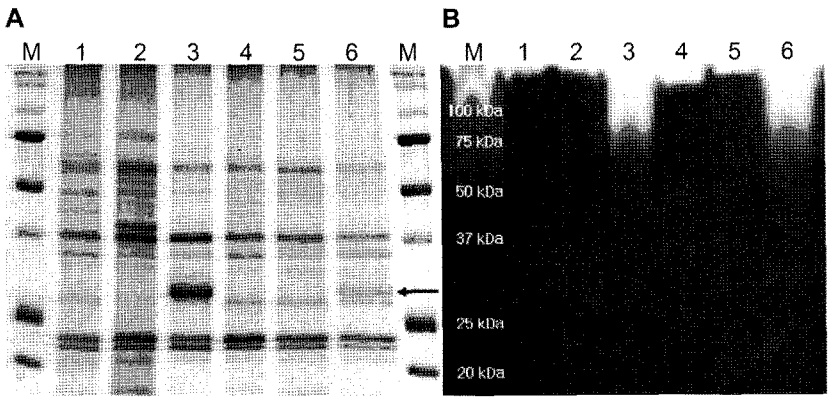

Fig. 6. SDS-PAGE (**A**) and fibrin zymography (**B**) of culture supernatant of *B. subtilis* TFs.
Lanes 1, *B. subtilis* WB600; 2, *B. subtilis* WB600 [pHY300PLK]; 3, *B. subtilis* WB600 [pHY51]; 4, *B. subtilis* ISW1214; 5, *B. subtilis* ISW1214 [pHY300PLK]; 6, *B. subtilis* ISW1214 [pHY51]; M, Precision Plus protein standard. A 15% acrylamide gel was used. A 10-μg protein sample (boiled) was subjected to SDS-PAGE and a 1-μg (unboiled) sample to zymography.

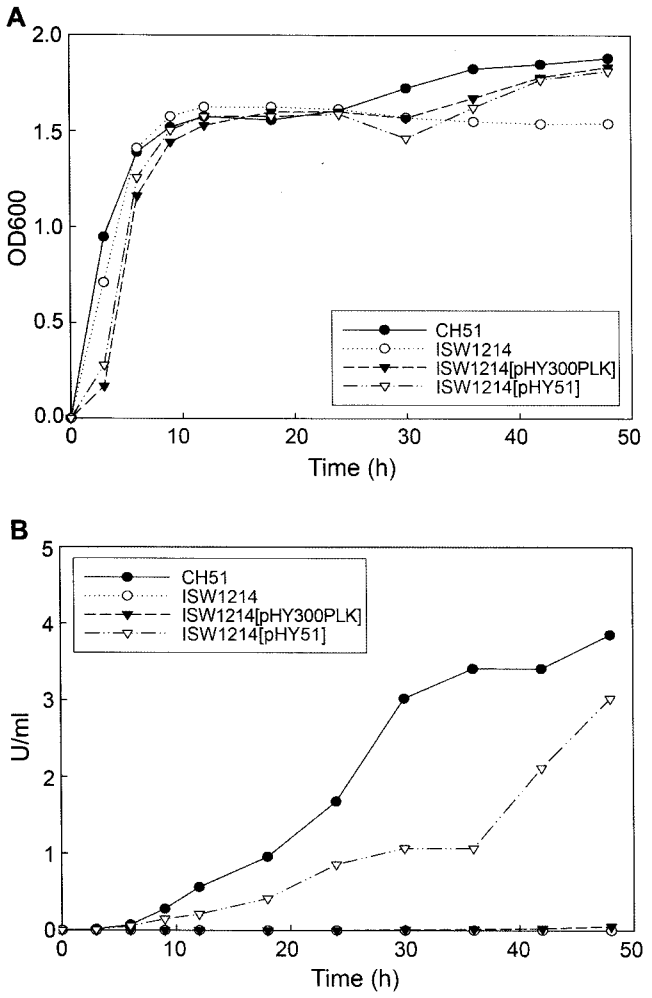

Fig. 7. Growth (**A**) and fibrinolytic activity (**B**) of *B. subtilis* TFs. ●, *B. amyloliquefaciens* CH51; ○, *B. subtilis* ISW1214; ▼, *B. subtilis* ISW1214 [pHY300PLK]; ▽, *B. subtilis* ISW1214 [pHY51].

activities of both *B. subtilis* TFs were lower than that of *B. amyloliquefaciens* CH51. It could be interpreted that AprE51 alone might not be sufficient for *B. subtilis* hosts to be as highly fibrinolytic as *B. amyloliquefaciens* CH51 because the latter synthesizes additional fibrinolytic enzymes. If *aprE51* were to be overexpressed in *B. amyloliquefaciens* CH51 or *B. amyloliquefaciens* CH86-1, another highly fibrinolytic isolate, it might enhance the fibrinolytic activity of the host cells. It is desirable that each component of the fibrinolytic system of *B. amyloliquefaciens* CH51 be characterized and used for the construction of *Bacillus* strains with improved fibrinolytic activity.

Acknowledgments

This work was supported by a research grant (Project No. R01-2007-000-20024-0) from the Korea Science and Engineering Foundation (KOSEF). G. M. Kim, A. R. Lee, K. W. Lee, and J. Chun were supported by the 2nd Stage Brain Korea 21 Program of the Korean Ministry of Education and Human Resources Development. The authors are grateful for the financial support.

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