JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Enhancement of the Catalytic Activity of a 27 kDa Subtilisin-Like Enzyme from *Bacillus amyloliquefaciens* CH51 by in Vitro Mutagenesis

Jieun Kim,[†] Jong-Hyun Kim,[†] Kyoung-Hwa Choi,[†] Jeong Hwan Kim,[‡] Young-Sun Song,[§] and Jaeho Cha*[,†]

[†]Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735

[‡]Institute of Agriculture & Life Science, Gyeongsang National University, Jinju 660-701

[§]School of Food and Life Science, Inje University, Gimhae 621-749, Korea

ABSTRACT: AprE51 from *Bacillus amyloliquefaciens* CH51 is a 27 kDa subtilisin-like protease with fibrinolytic activity. To enhance the catalytic activity of AprE51, two residues, Gly-169 and Ser-101, which, according to the three-dimensional structural model of subtilisin, are located in the P1 substrate-binding site and S3 subsite, respectively, were mutated by site-directed mutagenesis. Results of the mutational analysis showed that substitution of alanine for Gly-169 increased the fibrinolytic activity 1.4-fold. All four Ser-101 mutations, that is, replacements with arginine, leucine, lysine, and tryptophan, also increased the fibrinolytic activity up to 3.9-fold. The S101W mutant with a bulky side chain was more active than mutants with a positively charged or nonpolar small side chains. The fibrinolytic activity of the S101W mutant was further increased by error-prone polymerase chain reaction. The AprE51−6 mutant (S101W/G169A/V192A) had stronger fibrinolytic activity than the S101W mutant. Purified AprE51−6 had a 2.5-fold higher k_{cat} and a 2.3-fold lower K_m, which resulted in a 6-fold increase in catalytic efficiency (k_{cat}/K_m) relative to that of wild-type AprE51. In addition, AprE51−6 showed a relatively broader pH range and increased thermostability as compared to AprE51.

KEYWORDS: *Bacillus amyloliquefaciens*, cheonggukjang, fibrinolytic enzyme, in vitro mutagenesis, subtilisin-like protease

INTRODUCTION

Cardiovascular diseases, such as acute myocardial infarction, ischemic heart disease, and high blood pressure, are the leading causes of death worldwide.[1] Among the different cardiovascular diseases, thrombosis is currently one of the most widely occurring. Fibrin is a major protein component of the thrombus, and its improper accumulation in the blood vessels induces thrombosis. Drugs using fibrinolytic enzymes are the most effective for the treatment of thrombosis. A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and streptokinase, a bacterial plasminogen activator, have been studied extensively and used as thrombolytic agents.[2,3] However, these agents sometimes cause excessive bleeding and recurrence at the site of residual thrombosis.[4] They also have a short window of therapeutic efficacy and are very costly to produce. Therefore, several lines of investigation are presently being pursued to enhance the efficacy and specificity of fibrinolytic therapy.

Over the last 10 years, safer thrombolytic agents have been developed from Asian-fermented foods such as natto, douchi, doenjang, and jeotgal.[5−9] These agents mainly function as plasmin-like proteases, for example, subtilisin, which can directly degrade fibrin or fibrinogen, thereby rapidly and completely dissolving thrombi. Nattokinase (formerly designated subtilisin NAT, E.C. 3.4.21.62), which was purified from natto, a traditional Japanese fermented food, is used to directly lyse thrombi in vivo.[5,10,11] The mechanism by which nattokinase potentiates fibrinolysis has been studied.[12] In the literature, nattokinase was reported not to possess plasminogen activator activity; however, it appears to directly digest fibrin through limited proteolysis. Cheonggukjang, a traditional Korean fermented soybean-based food, possesses several health-promoting properties, such as the ability to lower blood pressure and prevent both myocardial and cerebral infarction, anticancer activity, and hypocholesterolemic effects in serum.[13−17] The fibrinolytic activity in cheonggukjang is produced by proteases secreted by bacilli.[18] *Bacillus amyloliquefaciens* CH51, previously isolated from cheonggukjang, showed higher fibrinolytic activity than other isolates when analyzed by the fibrin plate method.[19] *B. amyloliquefaciens* CH51 secretes at least five fibrinolytic enzymes (70, 66, 50, 32, and 27 kDa) into the culture supernatant during growth. Among these enzymes, a 27 kDa subtilisin-like protein exhibits higher affinity for fibrin. Previously, this enzyme, designated as AprE51, was partially purified, and its properties were examined. Its structural gene, *aprE51*, was cloned and successfully expressed in the heterologous hosts, *B. subtilis* WB600 and ISW1214, two bacterial strains that lack fibrinolytic activity.[20] AprE51 is indeed a fibrinolytic enzyme because it possesses a relatively higher ratio of fibrinolytic/caseinolytic activity than other subtilisin-type proteases such as cheonggukjang CK, subtilisin BPN′, and subtilisin Carlsberg.[18]

In this study, we attempted to enhance the catalytic activity of AprE51 by in vitro mutagenesis. The production and purification of AprE51 variants and the fibrinolytic activities of these variants as potential thrombolytic agents in comparison with other fibrinolytic enzymes, including nattokinase, are presented.

MATERIALS AND METHODS

Materials. Fibrinogen (from human bovine plasma), thrombin (from human plasma), and plasmin were purchased from Sigma-Aldrich

Received: May 17, 2011
Accepted: July 24, 2011
Revised: July 23, 2011
Published: July 24, 2011

(St. Louis, MO). The chromogenic substrate *N*-Succ-Ala-Ala-Pro-Phe-*p*NA (Succ, succinyl; *p*NA, *p*-nitroaniline) was also purchased from Sigma-Aldrich. Restriction endonucleases were purchased from Beamsbio (Sungnam, Korea). PCR Premix for polymerase chain reaction (PCR) amplification was purchased from Genetbio (Daejeon, Korea). *Taq* polymerase, *Pfu* polymerase, and deoxynucleotide triphosphates (dNTPs) for error-prone PCR and site-directed mutagenesis were purchased from Takara (Shiga, Japan). A purification kit for PCR products and DNA restriction fragments and the QIAquick Gel Extraction kit were obtained from Qiagen (Hilden, Germany). Bradford reagent kit and protein standard markers were purchased from BioRad (Richmond, CA) and Elpis-biotech (Daejeon, Korea). Enzymes were purified using HiTrap Q HP and HiTrap Phenyl HP, purchased from Pharmacia (Amersham Biosciences, Sweden). All other chemicals used were of analytical grade.

Bacterial Strains and Culture Conditions. *Escherichia coli* DH5α was used for transformation and DNA manipulation. *B. subtilis* strain ISW1214 (Takara, Shiga, Japan) was used as a host for the expression of AprE51. Cells containing pHY300PLK (Takara, Shiga, Japan) or pHY51 derivatives were cultivated in LB supplemented with 100 μg/mL ampicillin or 10 μg/mL tetracycline.

Construction and Expression of *aprE51* Variants. The *aprE51* gene was previously amplified from *B. amyloliquefaciens* CH51 and was cloned into the *Bam*HI and *Eco*RI sites of pHY300PLK to generate pHY51. The protein sequence encoded by the cloned gene is identical to the amino acid sequence of AprE51 (accession no. EU414203).[20] The oligonucleotides (Table 1) for site-directed mutagenesis were synthesized by Bioneer (Daejeon, Korea). Site-directed mutagenesis was performed using the overlap extension method.[21] One pair of primers was used to amplify the DNA containing the mutation site together with upstream sequences. The forward mutant primer contained the mutation to be introduced into the wild-type template DNA, whereas the reverse primer, aph-R, contained the wild-type sequence. A second pair of primers was used to amplify the DNA that contained the mutation site together with downstream sequences. The reverse mutant primer of this pair contained the mutation to be introduced into the template DNA, while the forward primer, aph-F, had the wild-type sequence. The two sets of primers were used in two separate amplification reactions to amplify overlapping DNA fragments. The overlapping fragments were mixed, and the *aprE51* mutant genes were amplified into full-length DNA by using two primers (aph-F and aph-R) that bind to the extremes of the two initial fragments. In each case, the entire encoding gene was sequenced to confirm that only the expected mutation was present. The amplified *aprE51* mutant genes were inserted into the *Hin*dIII and *Bam*HI sites of pHY300PLK to generate pHY51 derivatives.

The wild-type and AprE51 variants were expressed in the protease-deficient host *B. subtilis* ISW1214. Preparation and electroporation of competent *Bacillus* cells were carried out as previously described.[19] *Bacillus* transformants were grown in LB at 37 °C for 40 h. When an A_{600} of approximately 4.0 was reached, the cells were recovered by centrifugation at 5000*g* for 20 min at 4 °C, and the supernatant was used for the enzyme assay.

Purification of AprE51 Variants. The culture supernatant was concentrated by ammonium sulfate precipitation (80%, w/v). The precipitate was resuspended in 10 mL of 20 mM Tris-HCl, pH 8.0 (buffer A), and then dialyzed against the same buffer for 24 h with four buffer changes. The dialyzed enzyme preparation was applied to a HiTrap Q HP column (0.7 cm × 2.5 cm) equilibrated with buffer A. The column was washed with the same buffer, and the bound proteins were eluted by applying a linear gradient of 0−1.0 M NaCl in the same buffer at a flow rate of 0.7 mL/min. Fractions exhibiting fibrinolytic activity were pooled and lyophilized. The concentrated enzymes were dissolved in a minimal volume of buffer A, dialyzed against the same buffer containing 1.0 M $(NH_4)_2SO_4$, and then loaded onto a HiTrap phenyl HP column (1.6 cm × 2.5 cm) equilibrated with buffer A containing 1.0 M $(NH_4)_2SO_4$. Proteins were eluted by sequential application of buffer A containing $(NH_4)_2SO_4$ at a concentration of 1.0 to 0 M. Active fractions were pooled, dialyzed against buffer A, and lyophilized. Protein concentrations were determined by the Bradford method, and BSA was used as the standard.[22] During chromatographic purification, the protein concentration was estimated by observing the absorbance at 280 nm.

Enzyme Assay. The fibrinolytic activity was determined using the fibrin plate method.[23] The fibrin plate was prepared by mixing 7 mL of fibrinogen solution [0.5% (w/v) human fibrinogen in phosphate buffered saline (PBS); pH 7.4], 50 μL of thrombin solution (100 NIH units/mL; Sigma), and 18 mL of 2% (w/v) agarose. The fibrin plate was left at room temperature for 1 h to allow the formation of a fibrin clot layer. The filtered supernatant from *bacilli* cultivated for 40 h or purified enzyme (0.3 μg) was spotted onto the plate and incubated at 37 °C for 16 h. The fibrinolytic activity was determined by measuring the diameters of the clear zone and was expressed in plasmin units (U) by comparing it to the zones formed by known quantities of plasmin. A standard curve, which showed the relationship between the area of the clear zone formed and the number of plasmin units, was prepared over a range of 2−60 mU.

The caseinolytic activity was determined using the casein plate method. The casein plate was prepared by mixing equal volumes of a 2% casein solution in phosphate-citrate buffer (35 mM citric acid, 65 mM sodium phosphate; pH 7.4) and a 2% agarose solution. The purified sample (0.3 μg) was spotted onto the plate, and the plate was incubated at 37 °C for 16 h. The caseinolytic activity was determined by measuring the dimensions of the clear zone, as described above.

Kinetic parameters were measured using the chromogenic substrate *N*-Succ-Ala-Ala-Pro-Phe-*p*NA. Samples (0.3 mL) from the reaction mixture containing the enzyme (AprE51, 1.6 nM; AprE51−6, 0.9 nM) and substrate (0.1−1.0 mM) in 50 mM Tris-HCl buffer (pH 8.0) at 37 °C were taken at various time points over the course of the reaction. The reaction was stopped by addition 100 μL of ice-cold 0.2 M acetic acid. The absorbance of released *p*NA was measured at 405 nm. The kinetic constants were determined using the Michaelis−Menten equation on the basis of the initial reaction rates.

Properties of Purified Enzyme. The optimal pH for the fibrinolytic activity of the enzyme was determined using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA at 37 °C within a pH range of 3.0−12.0 by using the following buffer systems: 50 mM citrate buffer (pH 3.0−5.0), sodium phosphate buffer (pH 6.0−7.0), Tris-HCl buffer (pH 8.0−9.0), and glycine-NaOH buffer (pH 10.0−12.0). The mixture (0.3 mL) contained the enzyme (AprE51, 12 μM; AprE51−6, 2.1 μM) and 0.25 mM substrate in various buffers. Samples were incubated for 5 min, and then, the reaction was stopped by adding 100 μL of 0.2 M acetic acid on ice. The absorbance of released *p*NA was measured at 405 nm. The relative activity was expressed as a percentage of the maximum enzyme activity. All experiments were performed at least three times. The effect of temperature on enzyme activity was tested by heating samples in 50 mM Tris-HCl (pH 8.0) from 25 to 55 °C for 5 min.

The thermostability of AprE51 and AprE51−6 was determined by incubating the enzyme solution (AprE51, 8.0 nM; AprE51−6, 3.7 nM) in 50 mM Tris-HCl buffer (pH 8.0) at different temperatures ranging from 45 to 60 °C. After various time intervals, samples were withdrawn and clarified by centrifugation, and the fibrinolytic activity was measured as described above.

The hydrolysis of fibrinogen by purified AprE51 and AprE51−6 was examined. A fibrinogen solution (20 μg of purified human fibrinogen in 50 mM Tris-HCl; pH 8.0) was mixed with purified enzyme (10 ng) and incubated at 37 °C for up to 12 h. At each time interval, samples were mixed with 5 × SDS sample buffer, boiled for 5 min, and then analyzed by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1. Synthetic Oligonucleotides Used for Site-Directed Mutagenesis[a]

substitution	oligonucleotide sequences
wild-type	aph-F: 5′-GAGCGATTGAAGCTTTGTACAAATACTC-3′, *Hind*III site
	aph-R: 5′-TCTTCAGAGGGATCCACCCGTCGA-3′, *Bam*HI site
mutant	
G169A	ap169A-F: 5′-GCTACCCTGCTAAATACCCTTCTG-3′
	ap169A-R: 5′-CAGAAGGGTATTTAGCAGGGTAGC-3′
S101R	ap101R-F: 5′-CTGACGGTCGCGGCCAGTA-3′
	ap101R-R: 5′-TACTGGCCGCGACCGTCAG-3′
S101L	ap101L-F: 5′-GCTGACGGTCTCGGCCAGTAC-3′
	ap101L-R: 5′-GTACTGGCCGAGACCGTCAGC-3′
S101W	ap101W-F: 5′-GCTGACGGTTGGGGCCAGTAC-3′
	ap101W-R: 5′-GTACTGGCCCCAACCGTCAGC-3′
S101K	ap101K-F: 5′-CGCTGACGGTAAGGGCCGTAC-3′
	ap101K-R: 5′-GTACTGGCCCTTACCGTCAGCG-3′

[a] Base changes from the wild-type AprE51 gene are underlined.

Polyacrylamide Gel Electrophoresis and Fibrin Zymography. To concentrate proteins for SDS-PAGE analysis, the protein solution was precipitated by addition of trichloroacetic acid (20%), and then, the precipitate was resuspended in 10 μL of 20 mM Tris-HCl (pH 8.0). SDS-PAGE was carried out according to the method of Laemmli.[24] Fibrin zymography was performed as previously described.[9,25] The polyacrylamide gel solution (12%, w/v) contained 0.12% (w/v) fibrinogen and 100 μL of thrombin (100 NIH units/mL). The samples were diluted 5-fold in a zymogram sample buffer, which consisted of 0.5 M Tris-HCl (pH 6.8), 10% SDS, 20% glycerol, and 0.5% bromophenol blue. After electrophoresis, the gel was soaked in 50 mM Tris-HCl (pH 7.4), which contained 2.5% Triton X-100, for 30 min at room temperature. The gel was then washed with distilled water for 30 min to remove the Triton X-100 and was then incubated in zymogram reaction buffer (50 mM Tris-HCl; pH 7.4) at 37 °C for 12 h. Finally, the gel was stained with 0.25% Coomassie brilliant blue R-250 for 1 h, after which it was destained, and the bands with fibrinolytic activity were visualized as nonstained regions on the gel.

Error-Prone PCR Mutagenesis. Random mutagenesis was carried out by error-prone PCR, as previously described.[26] The primers aph-F and aph-R were used to amplify an approximately 1.5 kb AprE51 fragment. The reaction mixture contained 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 5.5 mM $MgCl_2$, 0.08 mM $MnCl_2$, 0.2 mM each of dGTP and dATP, 1 mM each of dTTP and dCTP, 10 pmol of each primer, 5 ng of template DNA, and 1 U of *Taq* DNA polymerase in a 20 μL reaction volume. PCR was performed in a GeneAmp 2400 PCR System (Perkin-Elmer, Waltham, MA). A 2 μL sample of the PCR mixture was electrophoresed on a 0.8% (w/v) agarose gel and stained with ethidium bromide (0.25 mg/mL).

RESULTS

Design of Mutations. AprE51 is synthesized as a preproprotein, as are all subtilisins. It consists of 382 amino acids, with 107 amino acids at the N terminus corresponding to the prepro-sequence.[20] The active form of AprE51 is a single chain structure composed of 275 amino acids with no intramolecular disulfide bonds. Because AprE51 belongs to the subtilisin family of serine proteases, it has the same conserved catalytic triad (Asp-32, His-64, and Ser-221) and oxyanion hole (Asn-155).[20] AprE51 shares the highly homologous characteristics of most subtilisins. It has been reported that the substitution of alanine for Gly-169 in subtilisin BPN′ improved substrate specificity.[27,28] An earlier mutagenesis study of *B. amyloliquefaciens* subtilisin DFE from the traditional Chinese fermented soybean food douchi also indicated that substitution of alanine for Gly-169 resulted in a 36% increase in fibrinolytic activity (unpublished result), thus indicating that Gly-169 is important for catalysis. Sequence alignment of AprE51 with subtilisin DFE and nattokinase showed that a glycine at this position in AprE51 and subtilisin DFE, while an alanine in nattokinase. According to the homology model of nattokinase, Ala-169 is located in the P1 substrate-binding site.[29] Previous attempts to study the substrate-binding region of nattokinase have shown that substitution of Ser-101 in the S3 subsite could enhance fibrinolytic activity.[12] Comparison of the amino acid sequences of AprE51 and nattokinase showed that the 101st amino acid residue in AprE51 was also serine. On the basis of this information, we chose Gly-169 and Ser-101 as initial targets for site-directed mutagenesis of AprE51. Gly-169 was replaced with alanine, and Ser-101 was replaced with tryptophan, lysine, leucine, and arginine. Error-prone PCR mutagenesis was then used to further improve the fibrinolytic activity of these AprE51 variants.

Construction, Expression, and Purification of AprE51 Variants. Five mutant plasmids containing the Gly-169 and Ser-101 mutants were constructed using the overlap extension method. The pHY51 derivative constructs containing *aprE51* mutant sequences were transformed into *B. subtilis* ISW1214 for expression. After the A_{600} reached approximately 4.0, the fibrinolytic activity was detected in the culture supernatant by the fibrin plate assay as a clear zone around each spot on the plate. The level of gene expression for the mutant and wild-type enzymes, as determined by densitometric estimation of the amount of the enzyme secreted in the cell culture, was nearly identical. The enzymes secreted by all five mutants were active. The mutant enzymes were purified to homogeneity after ammonium sulfate precipitation followed by HiTrap Q HP ion-exchange and HiTrap phenyl HP hydrophobic interaction chromatography. A single band was detected upon SDS-PAGE (Figure 1) at approximately 27 kDa. Fibrin zymography confirmed that the 27 kDa protein was the main protein with fibrinolytic activity. This protein had the same size as nattokinase, subtilisin DFE from *B. amyloliquefaciens* and subtilisin DJ-4 from *Bacillus* sp. DJ-4.[5,6,8] Fibrin zymography failed to resolve AprE51−6 as a separate band in a gel according to its molecular weight. Instead, a big smear appeared at the top of the gel, which

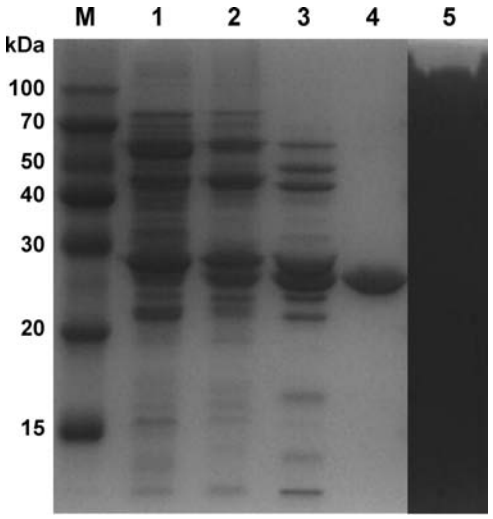

Figure 1. SDS-PAGE and zymography of the purified AprE51−6 expressed in *Bacillus subtilis* ISW1214. Lane M, molecular weight standard; lane 1, culture supernatant from a *B. subtilis* ISW1214 transformant; lane 2, after ammonium sulfate precipitation; lane 3, ion exchange chromatography; lane 4, hydrophobic interaction chromatography; and lane 5, zymography of purified enzyme. For SDS-PAGE, 5 μg (in 1 × SDS buffer) was loaded after boiling for 5 min. For zymography, 0.3 μg was loaded without boiling.

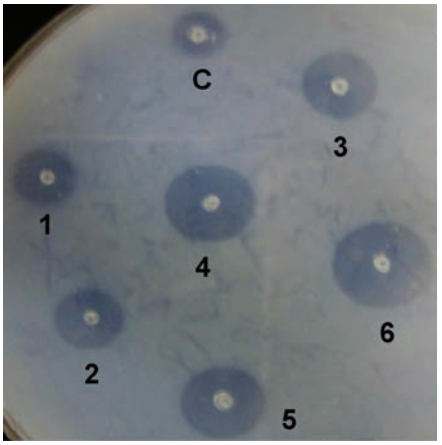

Figure 2. Fibrinolytic activity of the purified AprE51 variants in a fibrin plate. C, wild-type AprE51 as a control; 1, AprE51−1; 2, AprE51−2; 3, AprE51−3; 4, AprE51−4; 5, AprE51−5; and 6, AprE51−6. The resulting data are shown in Table 2.

indicated that AprE51−6 was stuck in the upper region of the gel (Figure 1). This phenomenon, so-called "a binding mode", was caused by the tight binding of enzyme to fibrin in the gel. The same phenomenon has also been observed for wild-type AprE51[20] and other fibrinolytic enzymes.[25]

Fibrinolytic Activities of AprE51 Variants. The fibrinolytic activities of wild-type AprE51 and the AprE51 variants were determined by the fibrin plate assay. AprE51−1 (G169A) showed a larger clear zone than the wild-type enzyme (Figure 2). A semiquantitative analysis of fibrinolytic activity was conducted by the fibrin plate method by using plasmin as a standard. Substitution of Gly-169 with Ala increased the fibrinolytic activity approximately 1.4-fold (Table 2). Site-directed mutagenesis at

Table 2. Fibrinolytic Activity of Purified AprE51 Variants[a]

variant	amino acid substitutions	fibrinolytic activity (units/mg)	fold
AprE51 (wild-type)		310.3	1
AprE51−1	G169A	419.2	1.4
AprE51−2	S101R/G169A	692.5	2.2
AprE51−3	S101L/G169A	882.9	2.9
AprE51−4	S101W/G169A	1182.2	3.9
AprE51−5	S101K/G169A	1018.9	3.3
AprE51−6	S101W/G169A/V192A	1618.5	5.0

[a] The fibrinolytic activity was determined by the fibrin plate method by using plasmin as a standard, as described in the Materials and Methods. Assays were done at 37 °C for 16 h at pH 7.4. The fold activity was calculated as the ratio of the fibrinolytic activity of each mutant to that of the wild-type enzyme.

Table 3. Kinetic Constants of Wild-Type AprE51, the AprE51-6 Mutant, and Other Fibrinolytic Enzymes[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
AprE51	231.3 ± 18.4	0.35 ± 0.01	6.61×10^5
AprE51−6	586.2 ± 96.7	0.15 ± 0.03	3.91×10^6
nattokinase[b]	45.2 ± 3.2	0.32 ± 0.01	1.41×10^5
FS33[c]	37.04	0.21	1.76×10^5
FE from *B. subtilis* SK006[d]	28.75 ± 0.23	0.45 ± 0.03	6.4×10^4
FE from *B. subtilis* IMR-NK1[e]	21.08	0.34	6.2×10^4

[a] Kinetic constants were determined using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA as a substrate in 50 mM Tris-HCl buffer (pH 8.0) at 37 °C. Data are expressed as mean values of three replicate experiments with the corresponding standard deviations. [b] Ref 30. [c] Ref 7. [d] Ref 35. [e] Ref 36.

position Ser-101 was conducted using AprE51−1 as a template, and the resulting four mutants (AprE51−2, S101R; AprE51−3, S101L; AprE51−4, S101W; and AprE51−5, S101K) had increased fibrinolytic activity, as estimated by the plate assay (Figure 2). Mutants with a bulky side group were more active than those with a positively charged or nonpolar side chain, and the order of activity was S101W > S101K > S101L > S101R (Table 2). Error-prone PCR mutagenesis was then carried out by using AprE51−4 as a template to acquire a mutant with further improved fibrinolytic activity. Approximately 400 clones were screened for activity, and of the positives clones, 1, named AprE51−6, showed a specific fibrinolytic activity of 1619 U/mg, which was approximately 5-fold higher than that of the wild-type AprE51 enzyme. Sequence analysis of the entire *aprE51−6* gene showed that in addition to G169A and S101W, Val-192 was replaced with alanine.

Characterization of the AprE51−6 Mutant. The kinetic constants K_m and k_{cat} were determined for the highly active AprE51−6 enzyme from the initial rate measurements for hydrolysis of the synthetic subtilisin substrate *N*-Succ-Ala-Ala-Pro-Phe-*p*NA. As shown in Table 3, AprE51−6 had an increased catalytic rate constant compared to the wild-type. The k_{cat}/K_m of AprE51−6, which reflects the specific activity, exceeded that of the wild-type AprE51 enzyme by a factor of 6. An increase in k_{cat}/K_m is affected by both the k_{cat} and the K_m values. As compared to the catalytic efficiency (k_{cat}/K_m) of nattokinase, the efficiencies of AprE51 and AprE51−6 were 4.7- and 27.7-fold higher, respectively.[30]

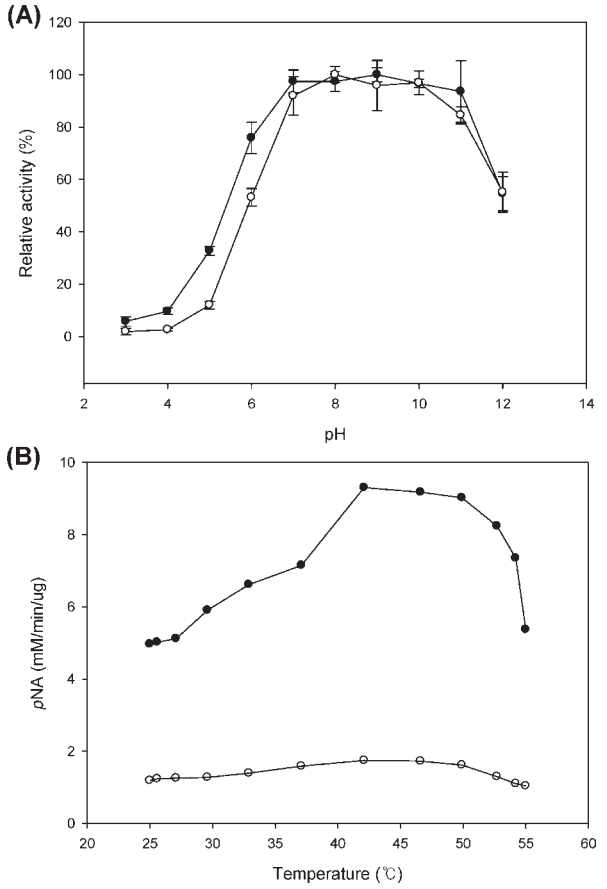

Figure 3. Effect of pH (A) and temperature (B) on the activity of wild-type AprE51 and the AprE51−6 mutant. The fibrinolytic activities of AprE51 (○) and AprE51−6 (●) were determined with *N*-Succ-Ala-Ala-Pro-Phe-*p*NA at 37 °C using various 50 mM buffers (citrate, pH 3.0−5.0; sodium phosphate, pH 6.0−7.0; Tris-HCl, pH 8.0−9.0; and glycine-NaOH, pH 10.0−12.0).

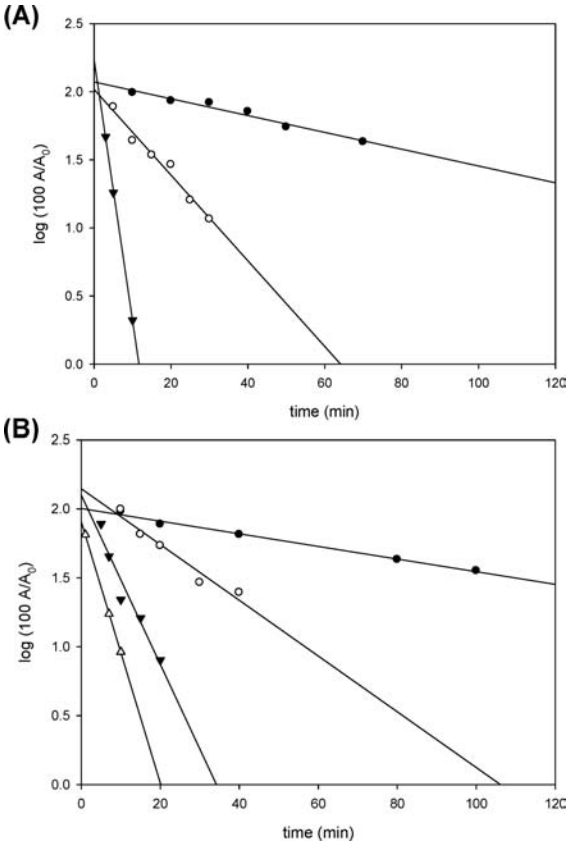

Figure 4. Thermostability of wild-type AprE51 enzyme (A) and the AprE51−6 mutant (B). The thermostability of the enzymes was determined after incubation in 50 mM Tris-HCl buffer (pH 8.0) at 45 (●), 50 (○), 55 (▼), and 60 °C (△) for different incubation times. The residual activity was measured at 37 °C in 50 mM Tris-HCl (pH 8.0) with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA.

The results for the determination of optimum pH are shown in Figure 3A. Both AprE51 and AprE51−6 were active at neutral and alkaline pH values, and the optimal reaction occurred at pH 7−11. The enzyme activity decreased more rapidly in acidic conditions than in alkaline conditions. These results are consistent with reports using nattokinase.[5] The nattokinase from *B. subtilis* NK 040823 was stable at pH values ranging from 6.0 to 9.0.[31] AprE51 and AprE51−6 had much broader pH optimum than nattokinase. Both enzymes lost activity at pH 4.0, while nearly 60% of initial activity was retained at pH 12.

The optimum temperature and thermostability of AprE51 and AprE51−6 are shown in Figures 3B and 4. The optimum temperature of both enzymes was 45−50 °C. At all temperatures tested, the AprE51−6 had a specific activity for *N*-Succ-Ala-Ala-Pro-Phe-*p*NA that was 3−6-fold greater than that of AprE51. However, a remarkable loss of activity was observed above 50 °C. This phenomenon was confirmed by the thermostability measurements at 45, 50, 55, and 60 °C (Figure 4 and Table 4). After a 60 min incubation period, AprE51 and AprE51−6 were stable at 45 °C, but after 10 min at 60 °C, the fibrinolytic activity of both enzymes was completely lost. Through the temperatures we tested, AprE51−6 was found to be more stable than AprE51. The half-life of denaturation at 50 °C is 10.6 min for AprE51 and 22.0 min for AprE51−6 (Table 4).

Table 4. Thermostabilities of Wild-Type AprE51 and the AprE51-6 Mutant[a]

	half-life (min)			
	45 °C	50 °C	55 °C	60 °C
AprE51	60.4	10.6	3.3	
AprE51−6	67.8	22.0	6.9	2.3

[a] The fibrinolytic activities of the enzymes were determined at various temperatures by using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA. After various time intervals, samples were withdrawn, and the residual activity was measured at 37 °C in 50 mM Tris-HCl buffer (pH 8.0).

The Aα and Bβ chains of fibrinogen were cleaved rapidly by AprE51 and AprE51−6 (Figure 5). The Aα chain was the most sensitive and hydrolyzed immediately, while the Bβ chain degraded within 30 min. The γ-chain degraded slowly but was completely degraded within 2 h. The degradation pattern for fibrinogen against Aα-, Bβ-, and γ-chains showed that AprE51−6 had higher fibrinolytic activity than the wild-type AprE51.

Comparison of the AprE51−6 Mutant with Other Fibrinolytic Enzymes. The fibrinolytic activity of the AprE51−6 mutant was compared with those of other fibrinolytic enzymes. The kinetic value of AprE51−6 for the synthetic subtilisin substrate was 27.7-fold higher than that of nattokinase (Table 3). The caseinolytic and

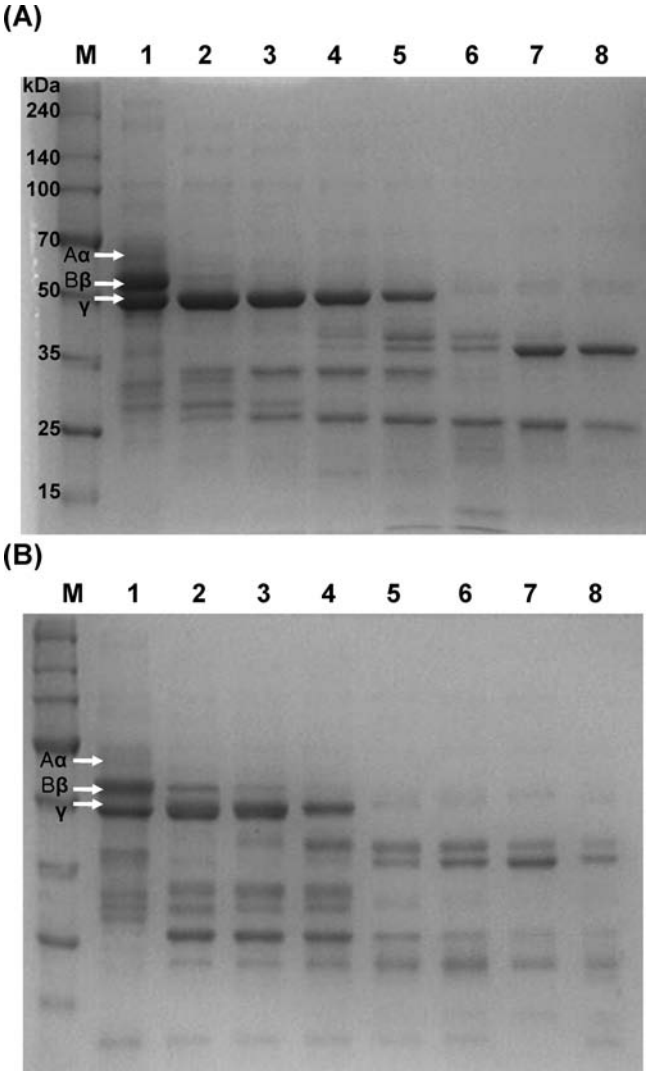

Figure 5. Hydrolysis of fibrinogen by wild-type AprE51 (A) and the mutant AprE51−6 (B). The fibrinogen solution (1 mg of purified human fibrinogen in 50 mM Tris-HCl, pH 8.0) was mixed with purified enzyme (10 ng) and incubated at 37 °C. After designated time intervals, the samples were boiled in 5 × SDS sample buffer and separated by 12% SDS-PAGE. Lane M, molecular weight standard; lane 1, control (no enzyme treatment); lane 2, 15 min; lane 3, 30 min; lane 4, 1 h; lane 5, 2 h; lane 6, 3 h; lane 7, 6 h; and lane 8, 12 h.

fibrinolytic activities of AprE51 and AprE51−6 were measured, and the specificity of the enzymes for fibrin was compared. As shown in Table 5, the specific F/C ratio for AprE51−6 was 1.32, which is 1.8, 4.1, and 14.7 times higher than the ratios of CK 11−4 from cheonggukjang, subtilisin BPN′, and subtilisin Carlsberg, respectively, but 1.4 times lower than that of subtilisin DFE. This indicates that AprE51−6 is indeed a fibrinolytic enzyme, which has a relatively high specificity for fibrin as substrate.

DISCUSSION

We previously isolated a *B. amyloliquefaciens* CH51 strain with fibrinolytic activity from the Korean fermented food cheonggukjang, which was prepared by traditional methods in Korea.[20] The subtilisin-like enzyme AprE51, which was purified from this strain, is a serine protease, and it showed relatively high fibrinolytic activity as compared to nattokinase, which is the most well-known fibrinolytic enzyme isolated from the traditional Japanese soybean food natto. In this study, we attempted to genetically engineer AprE51 to improve its fibrinolytic activity because of its considerable commercial potential comparable to nattokinase. The gene encoding AprE51 was previously cloned, and the deduced amino acid sequence was determined.[20] The primary sequence of AprE51 is highly homologous to those of other members of the subtilisin family. It is 99% identical to subtilisin DJ-4, 98% identical to subtilisin DFE, 96% identical to subtilisin BPN′, and 85% identical to nattokinase.[5] The three-dimensional structures of subtilisin BPN′ and the subtilisin from *B. amyloliquefaciens* (BAS) have been determined, and the detailed microenvironment of the catalytic site and substrate-binding sites has been described.[32,33] On the basis of substrate specificity studies of nattokinase[12] and subtilisin BPN′[28] and structural information of subtilisin BPN′ and BAS, Gly-169 and Ser-101 were targeted for mutagenesis to increase the fibrinolytic activity of AprE51.

Table 5. Comparison of AprE51 and AprE51−6 with Other Fibrinolytic Enzymes

enzyme	fibrinolytic activity (U)	caseinolytic activity (U)	F/C[a] (%)	sources
AprE51	310	224	1.38 (71)	*B. amyloliquefaciens*
AprE51−6	1618	1230	1.32 (69)	*B. amyloliquefaciens*
subtilisin DFE[b]	138	72	1.92 (100)	*B. amyloliquefaciens*
DJ-4[c]	146	148	0.98 (51)	*Bacillus* sp.
CK 11−4[d]	257	352	0.73 (38)	*Bacillus* sp.
subtilisin BPN′[d]	142	438	0.32 (17)	*B. amyloliquefaciens*
subtilisin Carlsberg[d]	30	325	0.09 (4.7)	*B. licheniformis*

[a] F/C, fibrinolytic activity/caseinolytic activity. [b] Ref 6. [c] Ref 8. [d] Ref 18.

G169A, S101R, S101L, S101K, and S101W mutants were constructed, and each transformant was expressed in an active form in *B. subtilis* ISW1214. The G169A mutation enhanced the fibrinolytic activity of AprE51 1.4-fold, as determined by the fibrin plate assay. It seems that substitution of Gly-169 with alanine reduces substrate-binding capacity. It is possible that the effect of the Ala-169 mutation on the P1 substrate-binding site is mediated by a change in the hydrophobicity of the site, which has been shown to be important in substrate binding.[27] Mutation of the S3 subsite (Gly-100, Ser-101, and Leu-126) of nattokinase has been reported to affect the substrate specificity.[12] The results of mutation analysis in nattokinase showed that mutation of Gly-100 and Leu-126 impaired the structure of the active site and drastically decreased activity, while bulky or positively charged residues at position 101 enhanced fibrinolytic activity. As expected, in AprE51, all four mutations of Ser-101 increased the fibrinolytic activity. In particular, AprE51−4, in which a tryptophan was substituted for the serine, had an activity that was 3.9-fold higher than that of the wild-type. Structure prediction and comparison to related structures suggested that the increased fibrinolytic activity might be because of the introduction of a bulky side chain at position 101; this may help stabilize both the transition state and the formation of the Michaelis complex during the reaction. Error-prone PCR further increased the fibrinolytic activity, and the evolved enzyme, that is, AprE51−6, had 5-fold higher fibrinolytic activity than the wild-type enzyme, as determined by the fibrin plate assay. Substitution of alanine for Val-192 in AprE51−6 was confirmed by

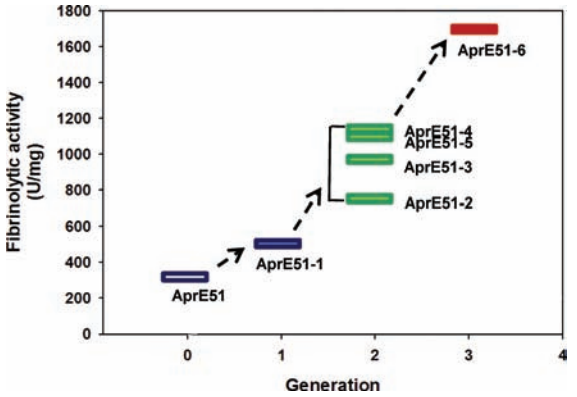

Figure 6. Progression of fibrinolytic activity improvement of AprE51 by mutagenesis. Site-directed and error-prone PCR mutagenesis yielded six AprE51 variants.

sequencing. Val-192 appears to be located in a hydrophobic pocket of the S2′ subsite; therefore, Ala-192 may slightly affect the enzyme−substrate interaction.[34] The introduction of small hydrophobic residue alanine instead of valine might broaden the S2′ subsite pocket; thus, the fibrin or synthetic substrate can be more easily accessible to the substrate binding site. This hypothesis is supported by the 2.3-fold lower K_m value of the AprE51−6 mutant as compared to that of the wild-type enzyme (Table 3). Most of the thermostabilizing mutations are located in loops connecting elements of secondary structure. The substitution of Val-192 with alanine probably reduces the entropy of the flexible loop. The progression toward improved fibrinolytic activity of AprE51 is summarized in Figure 6.

As shown in Table 3, AprE51−6 had the highest affinity for *N*-Succ-Ala-Ala-Pro-Phe-*p*NA, which is a well-known substrate for subtilisin and chymotrypsin. The kinetic constant of AprE51−6 for *N*-Succ-Ala-Ala-Pro-Phe-*p*NA indicated that AprE51−6 was the most efficient as compared to nattokinase from natto, FS33 from traditional Chinese douchi, the fibrinolytic enzyme from *B. subtilis* SK006 isolated from traditional Asian fermented shrimp paste, and the fibrinolytic enzyme from *B. subtilis* IMR-NK1.[7,35,36] For the development of effective targeted thrombolytic agents, the goal is to increase the specificity for fibrin as well as the fibrinolytic activity. It was unexpected that AprE51−6 would have a high specificity for fibrin, since subtilisin BPN′, which is very similar to AprE51, has no such specificity.[18] Clearly, the change of critical amino acid residues in the substrate-binding site may account for this difference, and further comparison between these two enzymes might provide some hints about the structure−function relationship in this enzyme family. Additional site-directed mutagenesis and functional analysis of AprE51 will be important to identify the amino acids critical for fibrin specificity.

Although active AprE51−6 enzyme could be expressed and secreted in *Bacillus* sp., the level of expression was not high enough. Optimization of enzyme production is necessary for the clinical application of this enzyme. *Bacillus* sp. isolated from traditional Asian food has been recognized to be safe for humans. We previously reported that the cheonggukjang kinase (CGK) (AprE2) purified from *B. subtilis* CH3−5, which was coisolated with *B. amyloliquefaciens* CH51 from the same cheonggukjang source, showed thrombolytic effect in a rat model of cerebral embolic stroke.[37] Therefore, it can be given orally as a thrombolytic agent. At present, whether purified AprE51−6 induces the lysis of thrombi in vivo or causes side effects is still under investigation.

AUTHOR INFORMATION

Corresponding Author

*Tel: +82-51-510-2196. Fax: +82-51-514-1778. E-mail: jhcha@pusan.ac.kr.

Funding Sources

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (no. 2007-0055330).

REFERENCES

(1) Mine, Y.; Wong, A. H. K.; Jiang, B. Fibrinolytic enzymes in Asian traditional fermented foods. *Food Rev. Int.* **2005**, *38*, 243–250.

(2) Moukhametova, L. I.; Aisina, R. B.; Lomakina, G. Y.; Varfolomeev, S. D. Properties of the urokinase-type plasminogen activator modified with phenylglyoxal. *Russ. J. Bioorg. Chem.* **2002**, *28*, 278–283.

(3) Baruah, D. B.; Dash, R. N.; Chaudhari, M. R.; Kadam, S. S. Plasminogen activators: A comparison. *Vascul. Pharmacol.* **2006**, *44*, 1–9.

(4) Bode, C.; Runge, M.; Smalling, R. W. The future of thrombolysis in the treatment of acute myocardial infarction. *Eur. Heart J.* **1996**, *17*, 55–60.

(5) Sumi, H.; Hamada, H.; Tsushima, H.; Mihara, H.; Muraki, H. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese *Natto*; a typical and popular soybean food in the Japanese diet. *Experientia* **1987**, *43*, 1110–1111.

(6) Peng, Y.; Huang, Q.; Zhang, R. H.; Zhang, Y. Z. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from *douchi*, a traditional Chinese soybean food. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2003**, *134*, 45–52.

(7) Wang, C. T.; Ji, B. P.; Li, B.; Nout, R.; Li, P. L.; Ji, H.; Chen, L. F. Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional *Douchi*. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 750–758.

(8) Kim, S. H.; Choi, N. S. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from *Doen-Jang*. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1722–1725.

(9) Hwang, K. J.; Choi, K. H.; Kim, M. J.; Park, C. S.; Cha, J. Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31, isolated from Korean traditional *Jeot-gal*. *J. Microbiol. Biotechnol.* **2007**, *17*, 1469–1476.

(10) Sumi, H.; Hamada, H.; Nakanishi, K.; Hiratani, H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* **1990**, *84*, 139–143.

(11) Sumi, H.; Yanagisawa, Y.; Yatagai, C. Natto bacillus as oral fibrinolytic agent: Nattokinase activity and the ingestion effect of *Bacillus natto*. *Food Sci. Technol.* **2004**, *10*, 17–20.

(12) Wu, S.; Feng, C.; Zhong, J.; Huan, L. Roles of S3 site residues of nattokinase on its activity and substrate specificity. *J. Biochem.* **2007**, *142*, 357–364.

(13) Kim, S. H.; Yang, J. Y.; Song, Y. S. Physiological functions of *Cheonggukjang*. *Food Ind. Nutr.* **1999**, *4*, 40–46.

(14) Lee, J. O.; Ha, S. D.; Kim, A. J.; Yuh, C. S.; Bang, I. S.; Park, S. H. Industrial application and physiological functions of *Cheonggukjang*. *Food Sci. Ind.* **2005**, *38*, 69–78.

(15) Kim, S. H.; Jung, S. H.; Kim, I. H. The effects of *Chungkookjang* biopolymer on blood glucose and serum lipid lowering in streptozotocin-induced diabetic rats. *J. Korean Soc. Food Sci. Nutr.* **2008**, *37*, 35–41.

(16) Kwak, C. S.; Kim, M. Y.; Kim, S. A. Cytotoxicity on human cancer cells and antitumorigenesis of *Chungkookjang*, a fermented soybean product in DMBA-treated rats. *Korean J. Nutr.* **2006**, *39*, 347–356.

(17) Soh, J. R.; Shin, D. H.; Kwon, D. Y. Effect of *Cheonggukjang* supplementation upon hepatic acyl-CoA synthase, carnitine palmitoyltransferase I, acyl-CoA oxidase and uncoupling protein 2 mRNA levels in C57BL/6J mice fed with high fat diet. *Genes Nutr.* **2008**, *2*, 365–369.

(18) Kim, W.; Choi, K.; Kim, Y.; Park, H.; Choi, J.; Lee, Y.; Oh, H.; Kwon, I.; Lee, S. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11−4 screened from *Chungkook-Jang*. *Appl. Environ. Microbiol.* **1996**, *62*, 2482–2488.

(19) Jeong, S. J.; Kwon, G. H.; Chun, J.; Kim, J. S.; Park, C. S.; Kwon, D. Y.; Kim, J. H. Cloning of fibrinolytic enzyme gene from *Bacillus subtilis* isolated from *Cheonggukjang* and its expression in protease-deficient *Bacillus subtilis* strains. *J. Microbiol. Biotechnol.* **2007**, *17*, 1018–1023.

(20) Kim, G. M.; Lee, A. R.; Lee, K. W.; Park, A. Y.; Chun, J.; Cha, J.; Song, Y. S.; Kim, J. H. Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from *Cheonggukjang*. *J. Microbiol. Biotechnol.* **2009**, *19*, 997–1004.

(21) Ho, S. N.; Hunt, H. D.; Morton, R. M.; Pullen, J. K.; Pease, L. R. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **1989**, *77*, 51–59.

(22) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(23) Astrup, T.; Mullertz, S. The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* **1952**, *40*, 346–351.

(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(25) Choi, N. S.; Kim, S. H. The effect of sodium chloride on the serine-type fibrinolytic enzymes and the thermostability of extracellular protease from *Bacillus amyloliquefaciens* DJ-4. *J. Biochem. Mol. Biol.* **2001**, *34*, 134–138.

(26) Pritchard, L.; Corne, D.; Kell, D.; Rowland, J.; Winson, M. A general model of error-prone PCR. *J. Theor. Biol.* **2005**, *234*, 497–509.

(27) Wells, J. A.; Cunningham, B. C.; Graycar, T. P.; Estell, D. A. Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering. *Proc. Natl. Acad. Sci.* **1987**, *84*, 5167–5171.

(28) Wang, C. X.; Shi, Y. Y.; Zhou, F.; Wang, L. Thermodynamic integration calculations of binding free energy difference for Gly-169 mutation in subtilisin BPN′. *Proteins* **1993**, *15*, 5–9.

(29) Zheng, Z. L.; Zuo, Z. Y.; Liu, Z. G.; Tsai, K. C.; Liu, A. F.; Zou, G. L. Construction of a 3D model of nattokinase, a novel fibrinolytic enzyme from *Bacillus natto*. A novel nucleophilic catalytic mechanism for nattokinase. *J. Mol. Graphics Modell.* **2005**, *23*, 373–380.

(30) Weng, M.; Zheng, Z.; Bao, W.; Cai, Y.; Yin, Y.; Zou, G. Enhancement of oxidative stability of the subtilisin nattokinase by site-directed mutagenesis expressed in *Escherichia coli*. *Biochim. Biophys. Acta* **2009**, *1794*, 1566–1572.

(31) Zhu, J.; Du, L.; Lu, F.; Liu, X.; Wang, P. Purification and characterization of a strong fibrinolytic enzyme nattokinase. *Microbiology* **2006**, *33*, 68–71.

(32) Bott, R.; Ultsch, M.; Kossiakoff, A.; Graycar, T.; Katz, B.; Power, S. The three-dimensional structure of *Bacillus amyloliquefaciens* subtilisin at 1.8 Å and an analysis of the structural consequences of peroxide inactivation. *J. Biol. Chem.* **1988**, *263*, 7895–7906.

(33) Almog, O.; Gallagher, T.; Tordova, M.; Hoskins, J.; Bryan, P.; Gilliland, G. L. Crystal structure of calcium-independent subtilisin BPN' with restored thermal stability folded without the prodomain. *Proteins* **1998**, *31*, 21–32.

(34) Rheinnecker, M.; Baker, G.; Eder, J.; Fersht, A. R. Engineering a novel specificity in subtilisin BPN. *Biochemistry* **1992**, *32*, 1199–1203.

(35) Hua, Y.; Jiang, B.; Mine., Y.; Mu, W. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. *J. Agric. Food. Chem.* **2008**, *56*, 1451–1457.

(36) Chang, C. T.; Fan, M. H.; Kuo, F. C.; Sung, H. Y. Potent fibrinolytic enzyme from a mutant *Bacillus subtilis* IMR-NK1. *J. Agric. Food. Chem.* **2000**, *48*, 3210–3216.

(37) Jalin, A. M. A.; Kee, C. K.; Lee, C. Y.; Kang, A. R.; Park, C. M.; Cha, J.; Kim, J. H.; Lee, S. W.; Song, Y. S.; Lee, J. T.; Kang, S. G. Thrombolytic activity of Cheonggukjang kinase in recovery from brain damage in a rat cerebral embolic stroke model. *Neural Regener. Res.* **2010**, *5*, 1875–1882.