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Cloning and characterization of a thermostable and alkaline fibrinolytic enzyme from a soil metagenome

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A sequence-based polymerase chain reaction (PCR) was employed to screen fibrinolytic enzymes from soil metagenomes. A basic alignment search tool (BLAST) sequence homology analysis of 15 positive amplicons indicated a high degree of nucleotide sequence identity (>98%) to a fibrinolytic enzyme, nattokinase in *Bacillus* sp. Among the positive clones, KSL79_FE was selected for further characterization. Sequence analysis showed that its open reading frame (ORF) consisted of 1,146 nucleotides encoding 375 amino acids, of which two differed from the nattokinase (T268S and V298A). To overexpress the fibrinolytic enzyme, we transformed the plasmid pET28a/KSL79_FE into *E. coli* BL21 Codon (+) cells, leading to yield optimal expression by using a 9-h induction with 30 uM isopropyl thio-β-D-galactoside (IPTG) at an OD_{600} of 0.5. The resulting KSL79_FE enzyme exhibited caseinolytic and fibrinolytic activities similar to those of nattokinase. In contrast to the nattokinase which showed the optimal conditions for proteolytic activity at 37°C and pH 8.0, KSL79_FE enzyme displayed maximal proteolytic activity at 50°C and pH 9.0. In addition, the enzyme activity of KSL79_FE was inhibited by Zn^{+2} ions, but not by Cu^{+2} ions, not similar to nattokinase. The two residues varied from amino acid sequence of nattokinase which might change the biochemical properties and optimal enzyme activity of KSL79_FE.

Key words: Nattokinase, proteolytic activity, metagenome, fibrinolytic activity, cloning and expression.

INTRODUCTION

Blood clots form when thrombin proteolytically converts fibrinogen into fibrin, which subsequently forms insoluble clots. A balance between fibrin clot formation and fibrinolysis is critical for maintaining healthy circulation, but improper fibrin accumulation in the blood vessels (thrombosis) can seriously impair blood circulation, leading to acute myocardial infarction, ischemic heart disease, and high blood pressure. Thrombosis is

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Abbreviations: tPA, Plasminogen activator; **PCR,** polymerase chain reaction; ***FE*,** fibrinolytic enzyme gene; **IPTG,** isopropyl thio-β-D-galactoside; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **PVDF,** polyvinylidene fluoride; **IgG,** immunoglobulin G; **TCA,** trichloroacetic acid; **EDTA,** ethylenediaminetetraacetic acid; **EGTA,** ethylene glycol bis (2-aminoethyl ether) tetraacetic acid; **PMSF,** phenylmethylsulfonyl fluoride; **DTT,** DL-dithiothreitol; **BLAST,** Basic Alignment Search Tool; **ORF,** open reading frame; **NA,** *p*-nitroaniline; **DGGE,** denaturing gradient gel electrophoresis.

accumulation in the blood vessels (thrombosis) can seriously impair blood circulation, leading to acute myocardial infarction, ischemic heart disease, and high blood pressure. Thrombosis is prevalent in modern life and is the main contributor to death throughout the world.

As therapeutic agent for thrombosis, fibrinolytic enzymes, such as tissue plasminogen activator (tPA), urokinase, and the bacterial plasminogen activator streptokinase have been extensively studied. Among them, urokinase has been used to prevent and treat cardiac and cerebrovascular diseases for many years. However, this enzyme has a short half-life, low specificity to fibrin, and causes excessive bleeding and recurrence (Wong and Mine, 2004).

In the search for potential alternatives to traditional anti-thrombosis drugs, previous studies have identified and characterized several effective thrombolytic agents from microorganisms (Kim et al., 1996), earthworms (Wang et al., 2005), snake venom (De-Simone et al., 2005; Jia et al., 2003), centipede venom (You et al., 2004), insects (Ahn et al., 2003), and leeches (Chudzinski-Tavassi et al., 1998). These intriguing agents, which are usually plasminogen activators or plasmin-like proteases, have proven useful in understanding the fibrinolytic mechanism and have shown promise of potential therapeutic drugs. In recent, fibrinolytic enzymes were discovered from traditional fermented product, such as Natto in Japan, Chungkookjang in Korea, and Douchi in China (Peng et al., 2005).

According to a previous report (Sumi et al., 2003), oral intake of the fibrinolytic enzymes in fermented foods and tPA levels in plasma is also increasing the solubilization of fibrin in the blood, suggesting that oral intake of anti-thrombotic agents may be effective or more effective than intravenous injection. As a result, recent intensive research has sought to develop efficient methods to screen for fibrinolytic enzymes which are suitable for oral administration.

In this regard, traditionally fermented foods are recognized as a source of fibrin-specific fibrinolytic enzymes. However, harvest of fibrinolytic enzymes directly from fermented foods is hampered by the difficult isolation and cultivation steps, such as expression of the proteins and characterization of their biological functions. Therefore, it is worthwhile to investigate with new approaches and useful environmental resources that may yield interesting fibrinolytic enzyme (Hugenholtz and Pace, 1996).

For many decades, microbiologists have been fascinated by the observation that the vast majority of natural environmental microorganisms (99% of the organisms in some environments) cannot yet be cultivated *in vitro* (Lu et al., 2013; Liles et al., 2003). Only one percentage of microorganisms has been analyzed from biosphere and yet most microbes in nature have not been studied. Conventional culturing methods have the limits to those that grow under laboratory conditions (Sharma et al., 2010). The recent research provides compelling evi-evidence for the existence of many novel types of micro-organisms in the environment in numbers and varieties that dwarf those of the comparatively few microorganisms amenable to laboratory cultivation. Countable numbers of enzymes, such as protease, lipase and amylase, have been isolated and characterized from collective genomes of the total microbiota found in nature, termed as the metagenome. The pool of combined genomes obtained from communities of microbial organisms in their natural environments provides access to the world of uncultured microorganisms and vastly more genetic information than one of the cultivable subset of microbiota (Lee et al., 2006). Here we isolated and characterized a fibrinolytic enzyme from environmental soil metagenomic libraries beyond conventional fermented foods.

MATERIALS AND METHODS

Materials

Enzymes and chemicals were purchased from BioRon (Ludwigshafen, Germany), and vectors and reagents for the cloning process were purchased from Takara Korea Biomedical (Seoul, Korea). Polymerase chain reaction (PCR) products and plasmid purification kits were from Bioprogen (Daejeon, Korea). Other fine chemicals and reagents used in this study were purchased from commercial grade.

Construction of plasmids for the expression of the recombinant fibrinolytic enzme

The PCR primers used in this study are listed in Table 1. The putative fibrinolytic enzyme gene (*FE*) from the KSL79 clone was generated by PCR using the Pro/Bam_F plus Pro/Eco_R primer set, as previously described (Cho et al., 2004). The gene product was purified with a PCR purification kit (Bioprogen) and cloned into the pMD18-T vector (Takara Korea Biomedical). The resulting plasmid was transformed into *Escherichia coli* DH5 DH5α. The coding fragment was excised from pMD18/KSL79_FE by treatment of *Bam*HI and *Eco*RI, and the pET28a vector (Novagen; Madison, WI, USA) was linearized with the same restriction enzymes. After the purification, the two fragments were ligated together using T4 DNA ligase (Takara). The final recombinant pET28a/KSL79_FE was confirmed by sequencing analysis (Genotech Co.; Daejeon, Korea).

Induction and preparation of the recombinant fibrinolytic enzyme

E. coli cells harboring pET28a/KSL79_FE were cultured in Luria-Bertani (LB) medium containing 50 µg/ml kanamycin at 37°C, and cell growth was determined by measuring the optical density of the culture at 600 nm (OD_{600}) with a spectrophotometer (U-2800 UV-Vis-Spectrophotometer; Hitachi High-Technologies Co.; Tokyo, Japan). To express the recombinant fibrinolytic enzymes, 30 µM of isopropyl thio-β-D-galactoside (IPTG) was added to the cultures, and subsequently incubated for 9 h under aerated conditions until the OD_{600} of the cultures reached 0.5. After centrifugation at 10,000 rpm for 15 min, the cell pellet was resuspended in a phosphate buffer (20 mM sodium phosphate in 500 mM NaCl (pH 7.4)) and subjected to three cycles of sonication for 10 min with a Vibra-Cell VCX 500 (SONICS; Newtown, CT, USA) at 40% maximum amplitude followed by a 10-min chill. The lysate was centrifuged at

Table 1. Primers used in this study.

Primer	Sequence (5' → 3')
Pro0771_F	CGC W[1]GC CGG AAA CGA AGG TTC
Pro0998_R	ACG TGA GGA GTC GCC ATK[2] GAC G
Pro0044_F	CGT TAA TCT TTA CGA TGG CGT TC
Pro0419_R	CCG CTG TCG ATA ACM GCT AC
Pro0001_F	GCG TGA GAA GCA AAA ATT GTG G
Pro1146_R	GCT TAT TGT GCA GCT GCT T
Pro/Bam_F	GCG GAT CC[3]G TGA GAA GCA AAA AAT TGT GGA TCA GC
Pro/Eco_R	GCG AAT TCT TAT TGT GCA GCT GCT TGT ACG TTG A

[1] W, A or T; [2] K, G or T. [3] Underlined capitals mean restriction sites of corresponding enzyme.

10,000 rpm for 15 min, and the supernatant was used as the crude enzyme preparation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

Cell pellets were incubated with 1 x lysis buffer (Novagen) at 37°C for 1 h. SDS-PAGE was carried out on a 10% polyacrylamide gel at 80 V for 5 h, and the proteins were transferred at 80 V for 2 h onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences; Buckinghamshire, England). The membrane was blocked with 5% (w/v) fat-free milk in TBST buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.01% (v/v) Tween-20) for 1 h, followed by incubation for 1 h in a 1:5,000 dilution of an anti-nattokinase antibody, which was provided from Professor Gal in Jinju Industry University, Korea (Lee et al., 2005). After three washes with TBST, the membrane was incubated for 1 h in a 1:2,000 dilution of an anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (HRP; Koma Biotech Inc.; Seoul, Korea). Immunoreactive proteins were visualized using an ECL Western blotting detection reagent (Amersham) and photographed with a fluorescence scanner (LAS 3000; Fuji Film Life Science; Stamford, CT, USA).

Assay for caseinolytic and fibrinolytic activity

Caseinolytic activity was determined colorimetrically as follows (Lee et al., 2006): A reaction mixture containing 50 µl of 3.0% (w/v) casein, 400 µl of 67 mM sodium phosphate buffer (pH 7.4), and 50 µl enzyme solution was incubated for 1 h at 37°C. To colorimetrically observe fibrinolytic activity, a reaction mixture containing 250 µl of 1.2% (w/v) fibrin, 200 µl of 67 mM sodium phosphate buffer (pH 7.4), and 50 µl enzyme was incubated at 37°C for 3 h (Lee et al., 2006). Both reactions were stopped by the addition of 750 µl trichloroacetic acid (TCA) solution (0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) for 30 min at room temperature. The reaction mixture was centrifuged at 15,000 rpm for 15 min, and 80 µl of culture free supernatant was incubated with 200 µl of 0.55 M Na_2CO_3 and 20 µl Folin-Ciocalteu (Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 30 min. The absorbance of the mixtures was read at 660 nm. One unit of caseinolytic or fibrinolytic activity was defined as the amount of enzyme that, in one minute, yielded an increase in absorbance at 660 nm equivalent to 1 g of tyrosine.

Effects of metal ions and chemical reagents on caseinolytic activity

The influence of various metal ions was investigated by pre-incubation the enzymes in presence of $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ at final concentration of 5 and 10 mM in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 30 min. To examine the effect of protease inhibitors, the enzymes were preincubated with the different chemical reagents such as ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis (2-aminoethyl ether) tetraacetic acid (EGTA) (metal-chelating agents), phenylmethylsulfonyl fluoride (PMSF) (a serine protease inhibitor), and DL-dithiothreitol (DTT) (a reducing agent) at final concentration of 5 and 10 mM in 50 mM Tris-HCl buffer (pH 7.0) at 37°C for 30 min. The percentage of relative activity was calculated by considering the activity of the enzymes without metal ions or chemical reagents as 100%. Under the standard conditions with 3% (w/v) casein.

RESULTS AND DISCUSSION

Preparation of clones encoding fibrinolytic enzyme

In a previous study, we have constructed metagenomic libraries from soil specimens. Using these metagenomic libraries, we employed a sequence-based polymerase chain reaction (PCR) method to screen for genes encoding fibrinolytic enzymes from soil metagenomic libraries. The specific PCR primers for fibrinolytic enzyme gene were Pro0001_F, Pro0044_F, Pro0771_F, Pro0419_R, Pro0998_R, and Pro1146_R originated from genes encoding fibrinolytic enzymes that belong to the subtilisin group of serine/threonine proteases (Table 1)(Nakamura et al., 1992). The fibrinolytic enzyme gene from *B. subtilis* var. *natto* (nattokinase) was used as a positive control.

Analysis of clone pools of soil metagenomic libraries with each primer set was carried out and their positive PCR products revealed the bands ranged from 228 to 1,146 bps in size, depending on used primers. Pro0001_F and Pro1146_R primer set yielded the full sequences of 15 putative fibrinolytic enzymes.

Analysis of the putative fibrinolytic genes

We subsequently analyzed the DNA sequences of the 15 PCR products generated with Pro0001_F and Pro1146_R by direct complete sequencing. Using a Basic Alignment Search Tool (BLAST) program analysis, these PCR

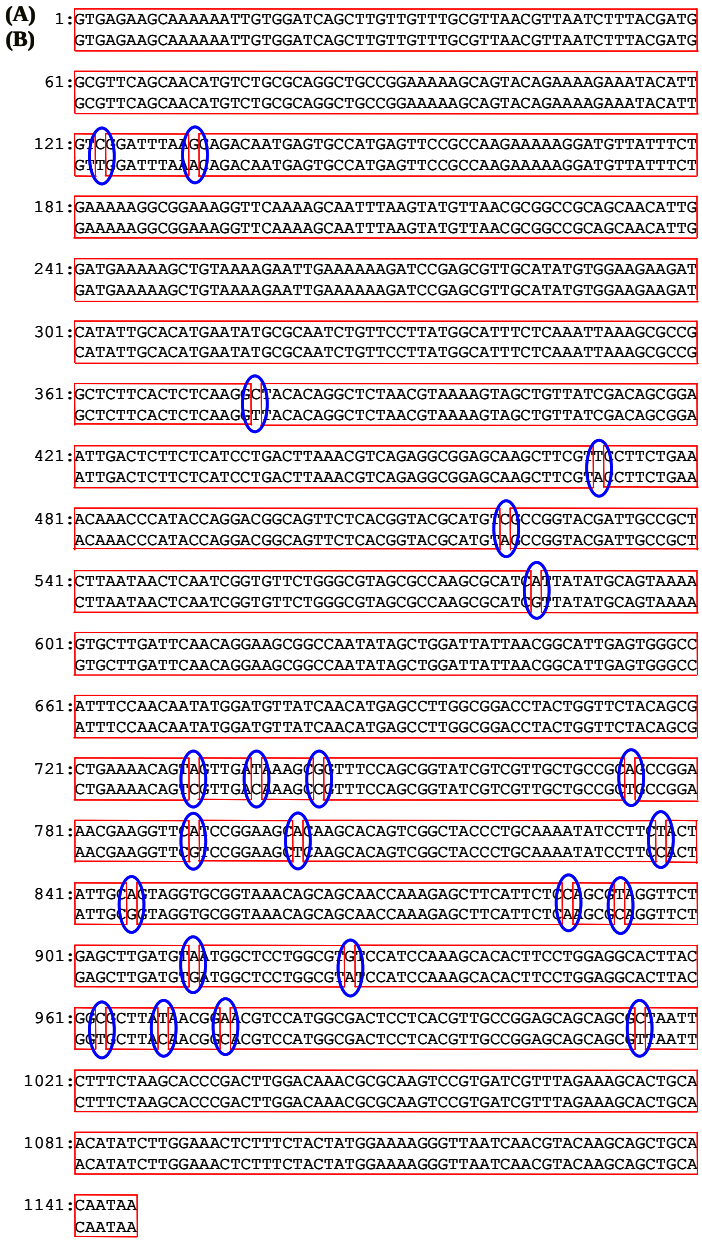

Figure 1. Comparison of nucleotide sequences of KSL79_FE and nattokinase from *B. subtilis* var. *natto*. Nucleotide sequences of KSL79_FE (Genbank accession number FJ950748) (A) and nattokinase (Genbank accession number AF368283) (B) were aligned and numbered from the GTG start codon at the 5' end of the fibrinolytic enzyme gene. The circle indicated difference sequences.

products showed 98% (964-965 bases matched/979 bases total) and 99% (950 bases matched/955 bases total) sequence similarities to the *nattokinase* gene from *Bacillus subtilis* var. *natto* (GenBank accession number AF368283). One clone, KSL3_FE, was 100% identical to the *B. subtilis* gene *aprE*, which encodes a subtilisin precursor (GenBank accession number AJ539133); the 14 other clones were 99.5% identical to the *B. subtilis* gene *Nk1*, encoding a thermostable mutant of the nattokinase (GenBank accession number AY940162).

The KSL79_FE PCR product, one of *Nk1*-like clones, consisted of 1,146 bps open reading frame (ORF), extending from a GTG initiation codon to a TAA termination sequence, which encodes a polypeptide comprised of 382 amino acid residues. We found 22 and 6 nucleotide differences between the ORF regions of KSL79_FE and the *nattokinase* and *Nk1* genes, respectively (Figure 1), but the deduced amino acid

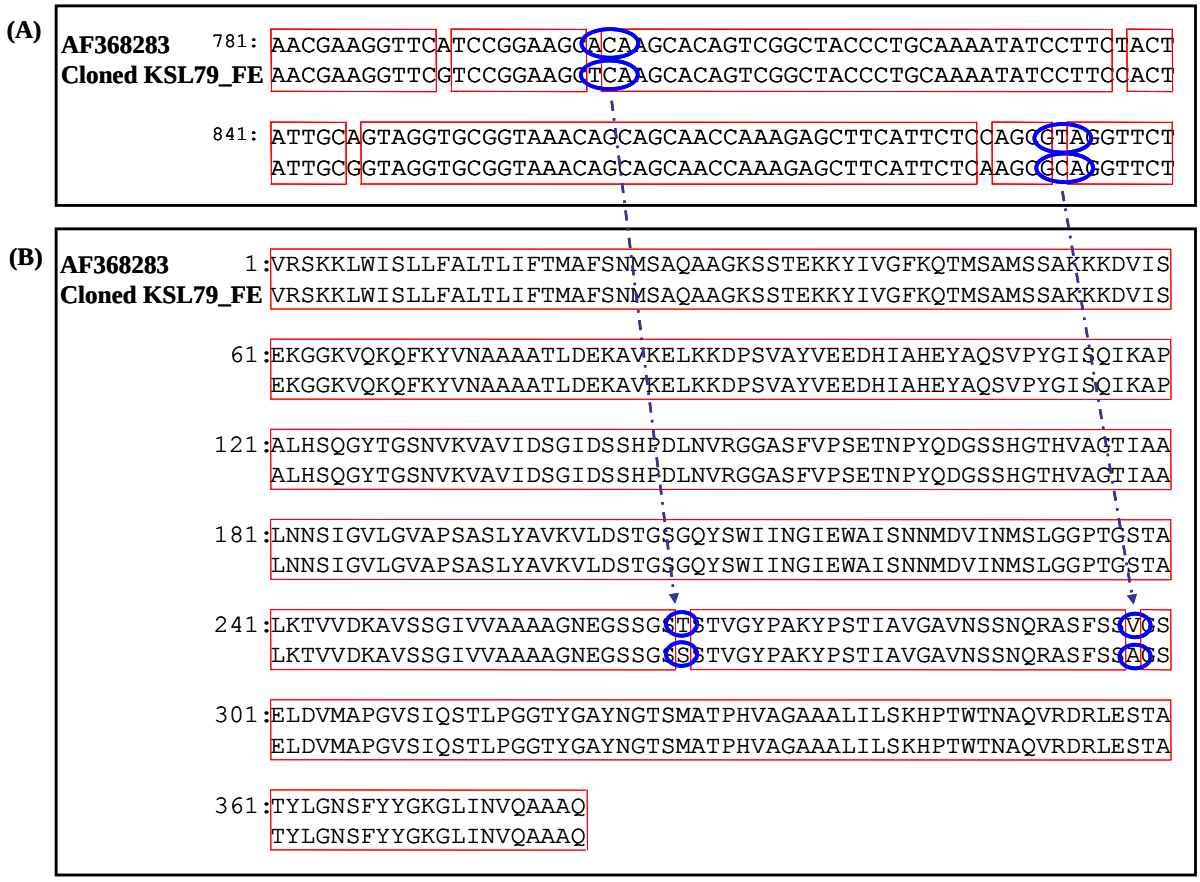

Figure 2. Comparison of the deduced amino acid sequences of KSL79_FE and nattokinase from *B. subtilis* var. *natto*. Differences of nucleotide sequences (A) and deduced amino acid sequences (B) between KSL79_FE (Genbank accession number FJ950748) and nattokinase from *B. subtilis* var. *natto* (Genbank accession number AF368283) were aligned.

sequence of KSL79_FE was 100% identical to the *Nk1* sequence. In contrast, although 20 of the nucleotides different from the *nattokinase* sequence were silent mutations, the remaining two nucleotides created new codons translatable to other amino acids: serine replaced threonine at position 268, and alanine replaced valine at position 298 (Figure 2).

A previous report identified the *arpN* as a novel fibrinolytic enzyme in *B. subtilis*, even though its sequence was almost 98% homologous to one of subtilisins in *B. subtilis* (Wong and Doi, 1986). Also another report isolated, purified, and characterized the thermostable fibrinolytic Nk1 protein (Chang et al., 2000). Deduced amino acid analysis of KSL79_FE showed that it had a typical 29-amino-acid-long signal peptide (pre-sequence) with the signal peptidase cleavage sequence of AlaGlnAlaAla at the N terminus as that of subtilisin. It was also followed by a 77-amino-acid-long hydrophilic sequence (pro-sequence) required for the production of enzymatically active form and the appropriate folding of the enzyme molecule. These results suggest that KSL79_FE protease belongs to subtilisin family protease.

Expression of the fibrinolytic enzyme gene KSL79_FE

To amplify the encoding region of putative fibrinolytic enzyme from the selected KSL79 clone, we performed PCR using the Pro/Bam_F primer, which contained a *Bam*HI restriction site just before the start codon, and the Pro/Eco_R primer, which contained an *Eco*RI site after the stop codon (Table 1). We then digested these restriction sites-anchored amplicon, ligated it into the pMD18-T vector, and transformed the resulting plasmid (pMD18-T/KSL79_FE) into *E. coli* DH5α (Figure 3). After DNA sequence verification, we subcloned the *KSL79_FE* ORF into the pET28a expression vector (Figure 3).

SDS-PAGE and Western blotting analyses were performed to determine the pET28a/KSL79_FE expression level in *E. coli* BL21 Codon Plus, as regulated by the *lac* promoter and induced by IPTG. We observed

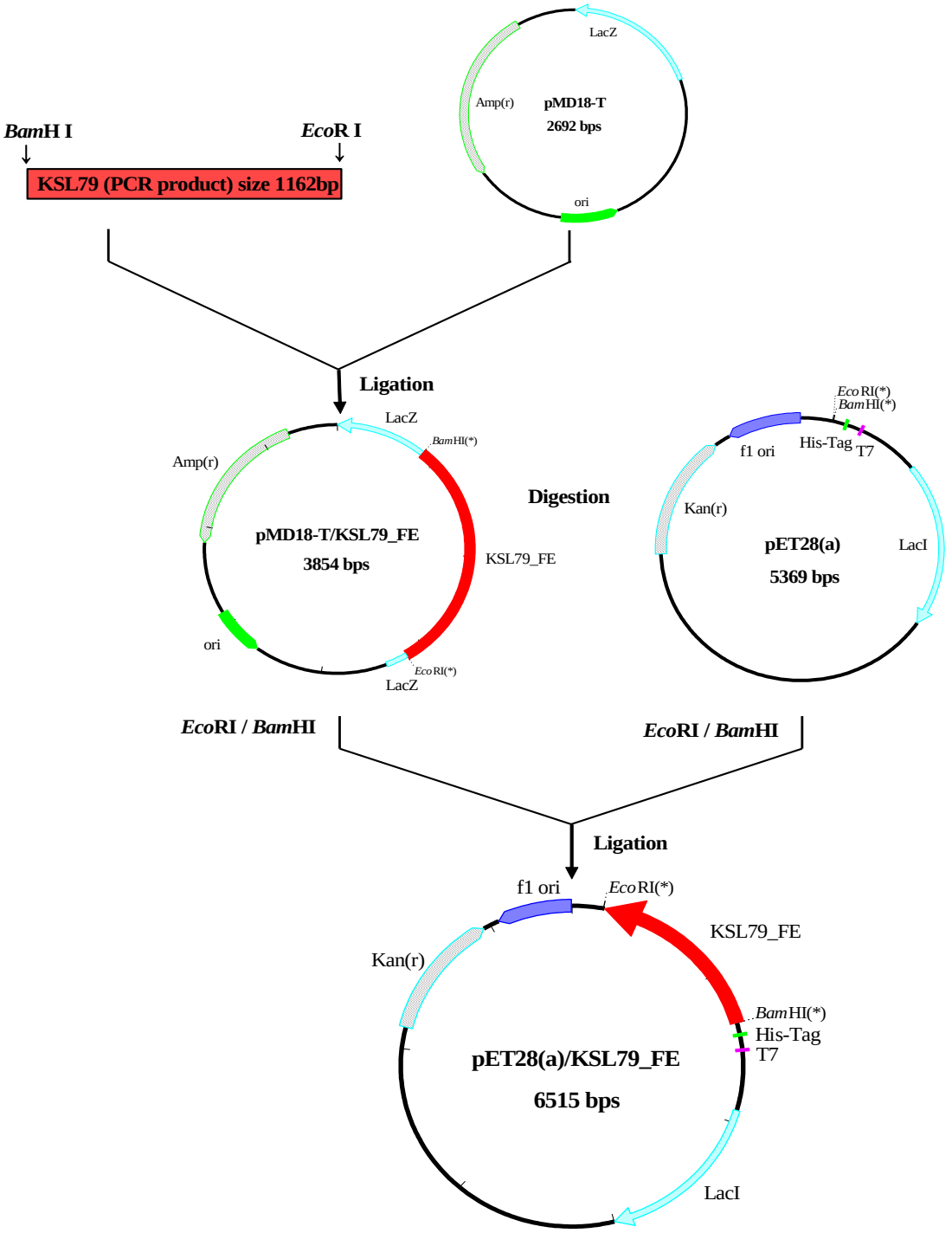

Figure 3. Construction of pET28a/KSL79_FE recombinant plasmid. The KSL79_FE PCR fragment excised from the pMD18/KSL79_FE plasmid was ligated into the multi-cloning site of pET28a expression cloning vector for prokaryote genes.

an optimal recombinant enzyme expression in *E. coli* BL21 Codon Plus cell harboring pET28a/KSL79_FE after optimal induction with 30 μM IPTG for 9 h (Figure 4A and B), exhibiting a molecular weight of approximately 41.8 kDa, which was immunoreactive with a nattokinase antibody (Lee et al., 2005). In studies using *Bacillus* expression systems, it has been reported that the active fibrinolytic enzyme such as subtilisin-type protease is produced in form of 275 amino acid residues, upon deletion of the pre-sequence and autolysis of pro- sequence,

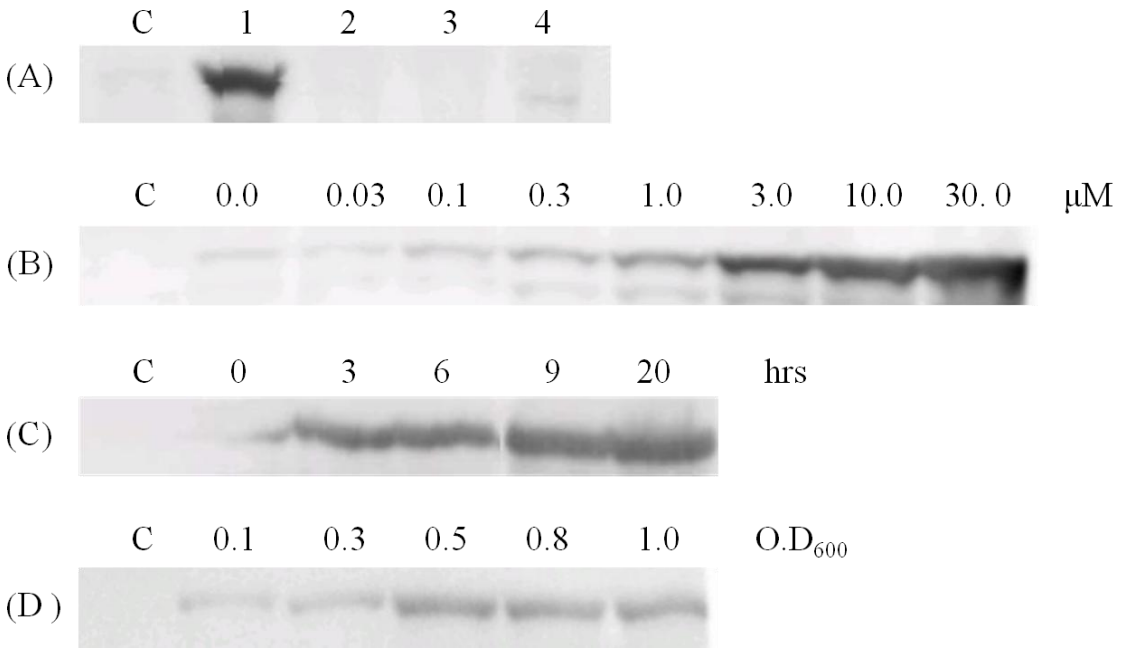

Figure 4. Effect of host cell strain, IPTG concentration, induction time, and cell density on pET28a/KSL79_FE expression. (A) BL21 Codon Plus (1), XL1-blue (2), JM109 (3), or DH5α was transformed with recombinant pET28a/KSL79_FE. Transformants were induced at 0.5 of $O.D_{600}$ with 50 μM IPTG for 5 h; (B) BL21 Codon Plus transformed with recombinant pET28a/KSL79_FE was induced for 5 h with 0.0, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 μM IPTG, respectively. (C) Transformants were induced with 30 μM IPTG at an OD_{600} of 0.5 for 0, 3, 6, 9, and 20 h, respectively. (D) Transformanst were induced with 30 μM IPTG for 5 h after the OD_{600} reached 0.1, 0.3, 0.5, 0.8, and 1.0, respectively. Control(C) means untransformed BL21 Codon Plus.

exhibiting two major characteristic protein bands with molecular masses of approximately 40 and 43 kDa on SDS-PAGE analysis, even though subtilisin would be synthesized as a pre-pro-enzyme (Staley and Konopka, 1985). However, in this experiment with *E. coli* as host cells, only one protein band with a molecular mass of approximately 43 kDa was observed as an active enzyme (Figure 4B).

Effect of temperature and pH on the proteolytic activity of KSL79_FE

To compare the proteolytic activity of KSL79_FE to that of nattokinase, we first evaluated the temperature dependency of proteolytic activity under standard conditions at various temperatures (20, 30, 37, 50, 60, and 70°C) and pH 7.0, using casein or fibrin as substrates. As shown in Figure 5A, the optimum temperatures for caseinolytic activity of the KSL79_FE enzyme and nattokinase were 50 and 37°C, respectively. Likewise, we observed similar optimum temperatures for fibrinolytic activity of KSL79_FE enzyme and nattokinase (Figure 5B). We next determined the optimal pH for proteolytic activity of KSL79_FE and nattokinase enzymes by varying the pH of the reaction mixture from pH 3.0 to 11.0, using 0.5 mM citrate buffer (pH 3.0 to 5.0), 0.1 mM Tris-HCl buffer (pH 5.0 to 9.0), or 0.1 mM carbonic acid buffer (pH 9.0 to 11.0). In contrast to nattokinase that exhibited maximum activity at pH 7.0 - 8.0, the optimum enzyme activity of KSL79_FE for fibrin or casein hydrolysis was observed at pH 9.0 (Figure 6A and B). Taken together, these results indicate that the KSL79_FE enzyme requires higher temperature and pH than nattokinase to maximally hydrolyze casein or fibrin.

Substrate specificity of KSL79_FE

To compare substrate specificity of the two enzymes, we investigated the ratio of casein hydrolysis to fibrin hydrolysis. As shown in Table 2, the KSL 79_FE enzyme hydrolyzed both casein and fibrin with substrate specificities that resembled those of nattokinase, which is known to be more specifically hydrolyzing to fibrin. Enzymatic kinetics was determined by measuring the release of *p*-nitroaniline (NA) from the chromogenic substrate N-succinyl-AAPF-*p*NA. The Michaelis-Menten constrants (K_m and V_{max}) of the KSL 79_FE were obtained from the plot of the fibrinolytic activity with fibrin concentration. All of these kinetic parameters were similar with previous data of Nk1 protein, which was identical to amino acid sequences of the KSL 79_FE (data not shown) (Chang et al., 2000).

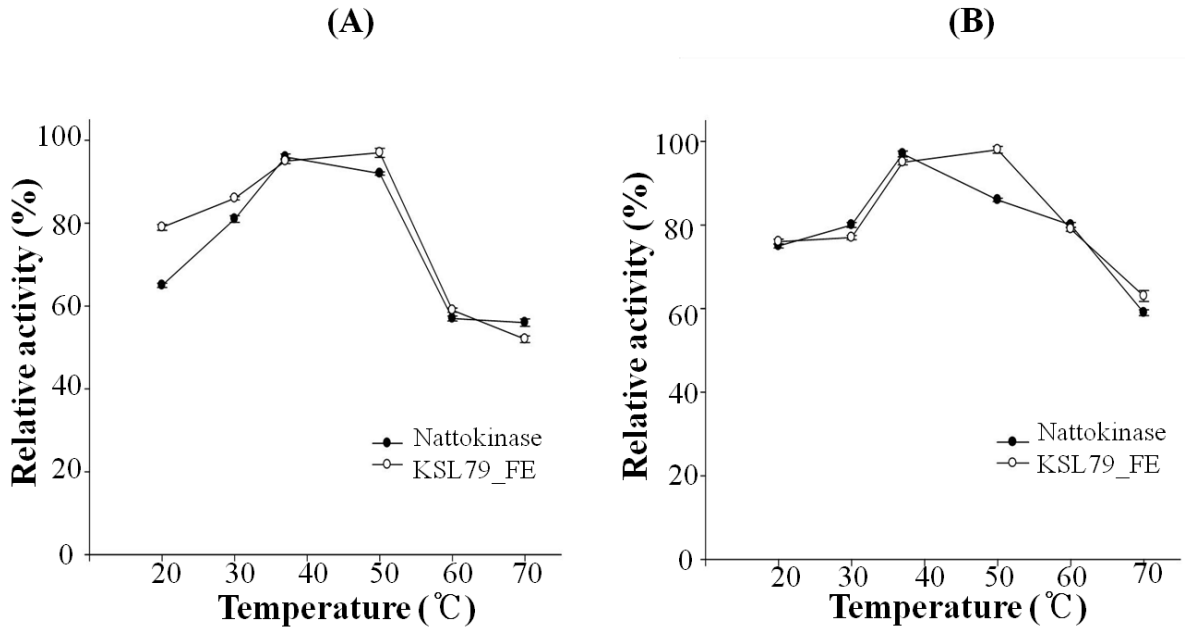

Figure 5. Optimal temperatures of enzymes for maximal proteolytic activity. The optimal temperature for proteolytic activity of the KSL79_FE and nattokinase enzymes was determined under standard conditions at different temperatures (20, 30, 37, 50, 60, and 70°C) and pH 7.0, using casein (A) or fibrin (B) as substrates.

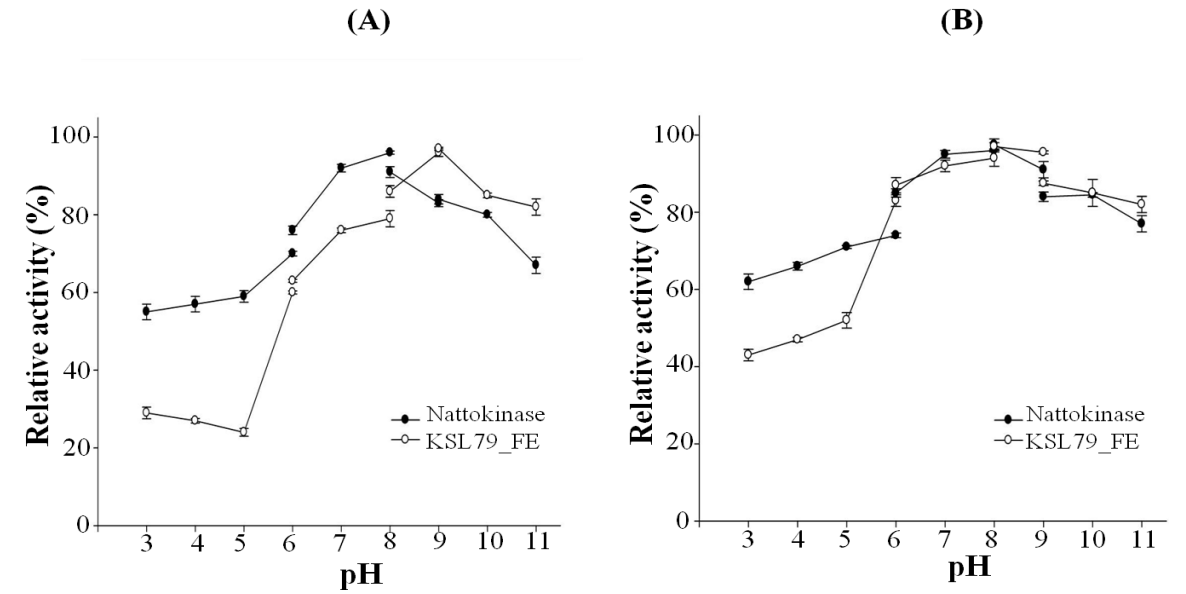

Figure 6. Optimal pH of enzymes for proteolytic activity. The optimal pH for proteolytic activity of KSL79_FE and nattokinase enzymes was determined by varying the pH of the reaction mixture from pH 3.0 to 11.0, using 0.5 M citrate buffer (pH 3.0 to 5.0), 0.1 M Tris-HCl buffer (pH 5.0 to 9.0), or 0.1 M carbonic acid buffer (pH 9.0 to 11.0), and incubating the enzymes at their respective optimal temperatures using casein (A) or fibrin (B) as substrates.

Effects of metal ions and additives on the proteolytic activity of KSL79_FE

The proteolytic activity of KSL79_FE was estimated in the presence of different metal ions. As shown in Figure 7B, all metal ions at 5 mM concentration did not or slightly affect the activity of KSL79_FE protease, whereas it was partially inhibited in the presence of 10 mM Fe^{2+} ions.

Table 2. Substrate specificity of nattokinase and KSL 79_FE enzymes.

Enzyme	Caseinolytic acitivity (U/ml)[2)]	Fibrinolytic acitivity (U/ml)[2)]	Substrate specificity[3)]
Nattokinase[1)]	0.6290	0.4847	1.29
KSL79_FE	0.7555	0.5789	1.30

[1)]A fibrinolytic enzyme produced from *B. subtilis* var. *natto* was used as a control; [2)]proteolytic activity by colorimetric method when samples were treated with equal enzyme concentrations at $O.D_{600}$; [3)]caseinolytic activity/fibrinolytic activity.

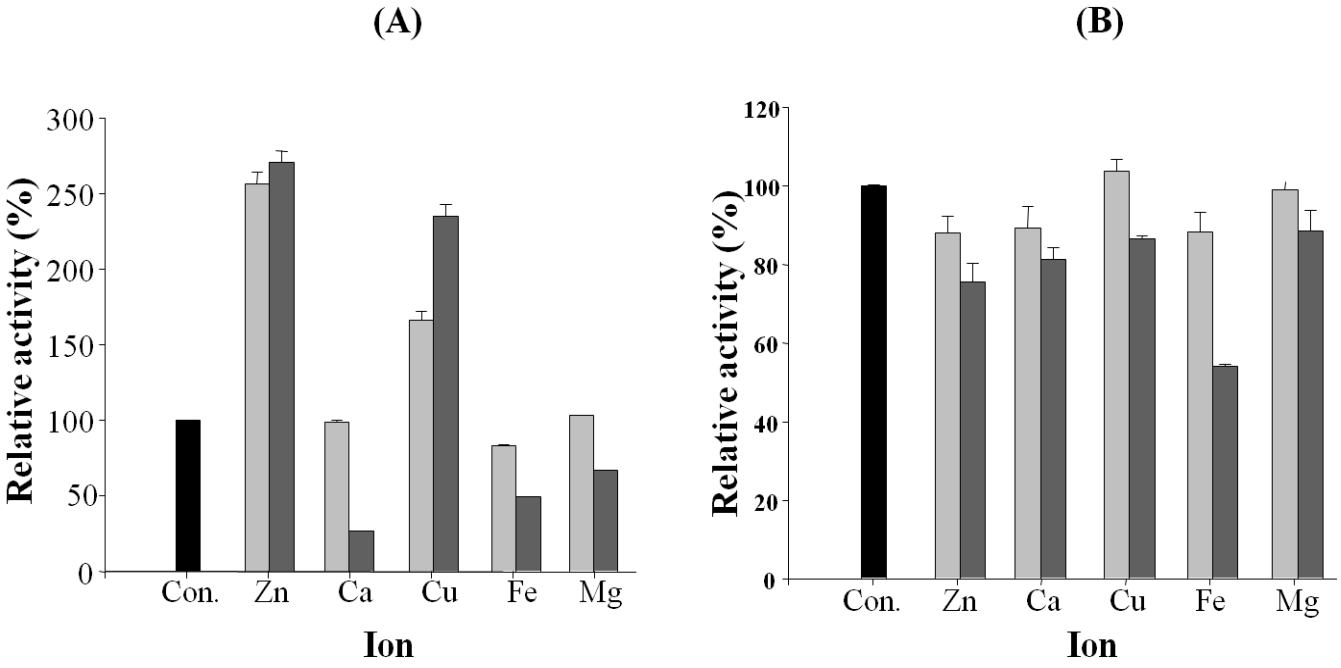

Figure 7. Effects of metal ions on the activity of nattokinase and KSL79_FE. The nattokinase (A) and KSL79_FE (B) were pre-incubated in both the absence and the presence of 5 mM or 10 mM metal ions in 50 mM Tris-HCl (pH 7.0) at 37°C. After 30 min of pre-incubation, residual protease activity was measured with 3.0% (w/v) casein.

Unlike KSL79_FE, the enzyme activities of nattokinase were enhanced by Zn^{2+} and Cu^{2+} ions and inhibited by Ca^{2+}, Fe^{2+}, and Mg^{2+} ions (Figure 7A).

Next, we determined the effects of chemical reagents on the enzyme activities of nattokinase and KSL79_FE. The results displayed that the addition of EDTA (10 mM), EGTA, PMSF, or DTT strongly inhibited the proteolytic activity of KSL79_FE (Figure 8B), whereas the enzyme activity of nattokinase was reduced by only PMSF treatment (Figure 8A), collectively, these results indicate that KSL79_FE protease from metagenomic library requires different metal ions and stability for proteolytic activity as compared to those of nattokinase.

Nucleotide sequence accession number

Nucleotide sequence of the KSL79_FE fibrinolytic enzyme gene from soil metagenome was deposited in GenBank database under the accession number FJ950748.

Fibrinolytic enzymes have been isolated and studied from various resources, especially from traditional fermented Asian foods. These well-known fibrinolytic enzymes chiefly have been administrated orally to convey their effects. Moreover, recent reports suggested that the original producer isolated from these sources, *B. subtilis*, expresses recombinant fibrinolytic enzymes at a low level. These issues necessitate studies to investigate, isolate, and express novel fibrinolytic enzymes, to characterize new approaches, and to explore various other sources besides fermented food. Our approached one largely untapped resource, the environmental metagenome; as such, the study here in is the first to our knowledge to screen for fibrinolytic enzyme from soil metagenome.

Metagenome analysis is a powerful technique for exploring unknown but useful living cells in the environ-

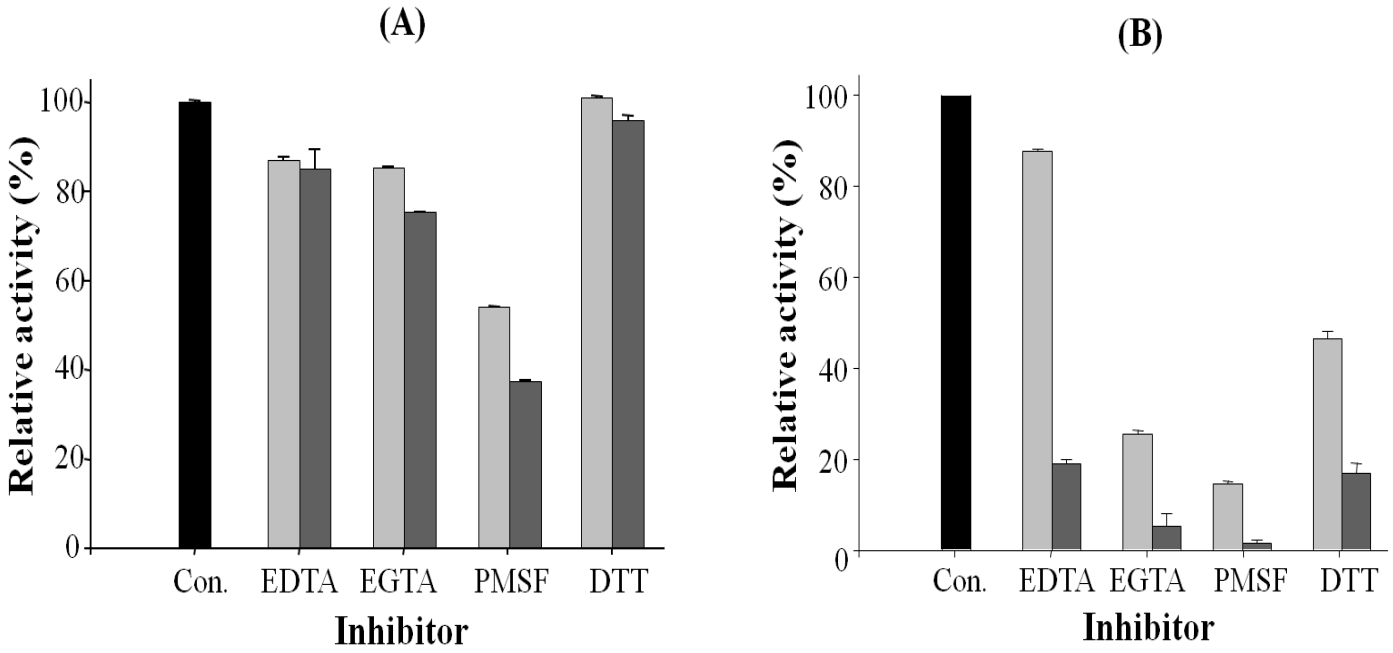

Figure 8. Effects of chemical reagents on the activity of nattokinase and KSL79_FE. The nattokinase (A) and KSL79_FE (B) were pre-incubated in both the absence and the presence of 5 mM or 10 mM chemicals in 50 mM Tris-HCl (pH 7.0) at 37°C. After 30 min of pre-incubation, residual protease activity was measured with 3.0% (w/v) casein.

ment.Existing methods used to conduct molecular metagenomics, such as PCR amplification, PCR denaturing gradient gel electrophoresis (PCR-DGGE) analysis, and microarray using specific probes, represent functional approaches specific to the environmental microorganisms, but they generally yield low active clone recovery rates. Even though current *in vitro* cultivation techniques have improved our ability to grow previously uncultivable microorganisms, the overwhelming majority of microbes in nature have not been characterized (Lee et al., 2006). Moreover, activity-based screening after cultivation is problematic, because there is so little information regarding optimal cultivation and expression conditions.

In this study, we isolated 15 positive clones from this soil DNA library by PCR. One of the clones, KSL79_FE, revealed sequence differences from *B. subtilis* var. *natto* nattokinase but was identical to the thermostable nattokinase mutant Nk1, in which two amino acids at positions 268 and 298 are altered. Nevertheless, the KSL79_FE enzyme exhibited the optimal hydrolytic conditions of 50°C and pH 9.0, compared to 37°C and pH 7.0 to 8.0 for nattokinase, suggesting that the two amino acids that differed from the nattokinase sequence may convey changes in the optimal pH and temperature required for maximal proteolytic activity. Despite these differences, both enzymes displayed similar substrate specificity. Future studies will characterize how the proteolytic activity of the expressed recombinant enzyme depends on temperature and pH, and they will elucidate the relationship between amino acid sequence and proteolytic activity in this enzyme.

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