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Purification and Characterization of a New Fibrinolytic Enzyme of *Bacillus licheniformis* KJ-31, Isolated from Korean Traditional *Jeot-gal*

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Abstract *Jeot-gal* is a traditional Korean fermented seafood and has long been used for seasoning. We isolated 188 strains from shrimp, anchovy, and yellow corvina *Jeot-gal*, and screened sixteen strains that showed strong fibrinolytic activities on a fibrin plate. Among those strains, the strain that had the largest halo zone was chosen and identified as *Bacillus licheniformis* by using 16S rDNA sequencing and an API CHB kit. The fibrinolytic activity of *Bacillus licheniformis* was characterized and designated as bpKJ-31. The active component of bpKJ-31 was identified as a 37 kDa protein, designated bacillopeptidase F, by internal peptide mapping and N-terminal sequencing. The optimum activity of bpKJ-31 was shown at pH 9 and 40°C, with a chromogenic substrate for plasmin. It had high degrading activity for the Bβ-chain and Aα-chain of fibrin(ogen), and also acted on thrombin, but not skim milk and casein. The amidolytic activity of bpKJ-31 was inhibited by 1 mM phenylmethanesulfonyl fluoride, but 1 mM EDTA did not affect the enzyme activity, indicating that bpKJ-31 is an alkaline serine protease, like a plasmin. The bpKJ-31 showed approximately 14.3% higher fibrinolytic activity than the plasmin. These features of bpKJ-31 make it attractive as a health-promoting biomaterial.

Keywords: *Jeot-gal*, *Bacillus lichenifomis* KJ-31, fibrinolytic enzyme, zymography

Fibrin is a fibrillar protein that is polymerized to form a "mesh" that forms a hemostatic clot over a wound site. However, the accumulation of fibrin in the blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases under abnormal conditions [27]. According to the report by the World Health Organization (WHO) in 2001, the proportion of deaths caused by cardiovascular diseases is 29%. In terms of embolism-related diseases like cerebral thrombosis and pulmonary thrombosis, fibrin is related to a high proportion of deaths. Because of its lethality, thrombolytic therapy has been extensively investigated as a medical remedy for these diseases.

Fibrinolysis is the process in which a fibrin clot, the product of coagulation, is broken down. Its main enzyme, plasmin, cuts the fibrin mesh at various places, leading to the production of circulating fragments that are cleaved by other proteases of the kidney and liver. Based upon their mechanisms of activation of the fibrinolytic system, fibrinolytic (thrombolytic) agents are classified into two types. One is a plasminogen activator, such as tissue-type plasminogen activator (t-PA) [7] and urokinase [8]. These activators are serine proteases that activate plasminogen and lead to systemic lysis of fibrin. The other type of fibrinolytic agent is a plasmin-like protease that degrades the fibrin clot directly. Examples of that type of protease are nattokinase [30] and lumbrokinase [21]. The fibrinolytic agents that are currently being used for thrombosis are urokinase, streptokinase, and genetically engineered t-PA. In the cases of t-PA and urokinase, they are of human origin and are generally safe, but they are also very expensive. Streptokinase, which is originated from microbes, is used as an effective and cheap clot-dissolving medication [6]. However, the body will eventually build up immunity to this agent, and patients may suffer from undesirable side effects such as bleeding and allergic responses because of its strong activity. Therefore, the search for safer thrombolytic agents from other sources is ongoing.

Recently, potent fibrinolytic enzymes have been developed from fermented food products such as Japanese *Natto* [9, 25, 30], Korean *Chungkook-Jang* [13, 24], *Doen-Jang*

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soy sauce [3–5, 18], and mushroom [16]. In particular, oral administration of *natto* or *natto*kinase can effectively enhance fibrinolysis and the release of an endogenous plasminogen activator in both animal models and in humans [30]. Because of the promising biological benefits for reducing blood clotting through the consumption of food sources containing fibrinolytic enzymes, many researchers have extensively explored new sources of fibrinolytic enzymes in the form of Asian fermented food products, with the purpose of providing potential health benefits for humans through the use of nutraceutical ingredients.

In this paper, we attempted to screen and identify the microorganism that has strong fibrinolytic activity from a Korean fermented food, *Jeot-gal*. The biochemical properties of the fibrinolytic enzyme, bpKJ-31, were also discussed.

MATERIALS AND METHODS

Materials

Samples of a Korean fermented food, *Jeot-gal* (shrimp, anchovy, and yellow corvenia), were collected from Jeonju Fermented Food Expo, 2004. Growth media including marine agar and R2A agar were purchased from Difco (Detroit, U.S.A.). Fibrinogen (from bovine plasma), thrombin (from bovine plasma), plasmin, *p*-nitroanilide (*p*NA), phenylmethylsulfonyl fluoride (PMSF), and chromogenic substrates (D-Val-Leu-Lys-*p*NA for plasmin substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA for subtilisin and chymotrypsin substrates) were purchased from Sigma. AccuPower PCR Premix for PCR amplification was purchased from Bioneer (Daejon, Republic of Korea). A purification kit for PCR products, QIAquick Gel Extraction kit, was obtained from Qiagen (Hilden, Germany). API 50CHB kit was purchased from bioMerieux (France). EDTA (disodium salt) and DEAE-Sepharose Fast Flow were obtained from Pharmacia Biotech. Co. (Uppsala, Sweden), and gel filteration HiPrep 16/60 Sephacryl S-200 HR was purchased from Amersham Biosciences.

Screening of Fibrinolytic Bacterial Strains

The *Jeot-gal* sample was homogenized using a Stomacher homogenizer, and a loopful of each slurry was streaked on LB and Marine 221 agar plates and incubated at 30°C for five days. A total of 108, 46, and 34 isolates were collected from shrimp, anchovy, and yellow corvina *Jeot-gal*, respectively, based on their morphological and cultural characteristics. Among them, sixteen isolates with fibrinolytic activity were screened using a fibrin plate. Single colonies were picked and cultivated in enrichment media whose supernatants were spotted on a 0.3% fibrin plate using 0.85-cm paper discs. After incubating the fibrin plates at 37°C for 18 h, colonies with surrounding clear zones were selected and classified as fibrinolytic isolates. The single strain showing the largest halo zone on the fibrin plate was selected and further identified.

Identification of Fibrinolytic Microorganism

The selected isolate was enriched, and its 16S rDNA was analyzed in order to identify the isolate at the species level. The genomic DNA of the isolate was extracted by the modified method of Rodrigues and Tait [28]. Amplification of the 16S rDNA was performed with universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), using a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer). PCR products were isolated from an agarose gel using a QIAquick Gel Extraction Kit (Qiagen, U.S.A.) and sequenced with an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). A sequence similarity search was performed using BLAST in the NCBI database. Based upon the result of the 16S rDNA sequence of the isolate, an API 50CHB kit and a 20E kit for *Bacillus* spp. identification were used. The biochemical profile, obtained for the strain after the final reading, was identified using the identification software APILAB.

Purification of Fibrinolytic Enzyme

The strain producing a fibrinolytic enzyme was aerobically cultured in 500 ml of LB at 30°C for 72 h. The culture was centrifuged at 10,000 ×*g* for 15 min. The fibrinolytic enzyme was purified by chromatographic procedures, including anion-exchange and gel filtration chromatographies. All purification steps were performed at 4°C. The crude enzyme was precipitated at 75% saturation of ammonium sulfate, and the protein was collected by centrifugation (10,000 ×*g*, 20 min) and dissolved in 50 mM Tris-HCl (pH 7.5, buffer A). The crude enzyme solution was applied to a DEAE-Sepharose FF column (1.6×40 cm). The column was washed with buffer A, and was then eluted with a linear gradient of 0–1 M NaCl in buffer A at a flow rate of 5.0 ml/min. Active fractions were collected and concentrated by ultrafiltration with a YM-10 membrane (Amicon, Stirred Cell 8200). The concentrated enzyme was dialyzed against buffer A with three buffer changes, and loaded on a gel filtration HiPrep 16/60 Sephacryl S-200 HR column using Tris-HCl buffer containing 0.15 M NaCl (pH 7.5), at a flow rate of 0.4 ml/min. Finally, active fractions were pooled and concentrated by ultrafiltration.

The prepared protein samples were analyzed by SDS-PAGE as described by Laemmli [15]. Protein concentration was determined according to the method of Bradford [2], using bovine serum albumin as a standard and measuring the absorbance at 595 nm.

Zymography

Fibrin zymography was carried out in 10% polyacrylamide gels containing 0.12% fibrinogen and 0.1 μg of thrombin

(10 NIH units). The samples were diluted in 5× 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 washed with distilled water to remove Triton X-100, and was then incubated with 50 mM Tris-HCl (pH 7.4) at 37°C for 12 h. The gel was stained with Coomassie blue for 2 h and then destained. The bands, corresponding to the areas where fibrin was digested, were visualized as nonstained regions of the zymogram gel [12].

Enzyme Assay

Fibrinolytic activity was determined using a fibrin plate according to the previously described method [1]. Five ml of 0.3% fibrinogen solution in 50 mM sodium phosphate buffer (pH 7.4) was mixed with 5 ml of 2% agarose solution along with 0.1 ml of a thrombin solution (10 NIH units). The solution was applied to a Petri dish and left for 1 h at room temperature to form a fibrin clot layer. Twenty μl of the sample solution was dropped onto a fibrin plate and incubated at 37°C for 12 h. The activity of fibrinolytic enzyme was estimated by measuring the dimensions of the clear zone on the fibrin plate and plotting a calibration curve based on plasmin standard solutions. A quantitative assay for fibrinolytic activity was performed as follows. Fibrin clots in liquid suspension were incubated with fibrinolytic enzyme, and the released tyrosine was monitored. A total of 100 μl of reaction solution was prepared with 0.6% bovine fibrin solution and 10 μl fibrinolytic enzyme (0.5 mg/ml). The reaction was carried out at 37°C for 20 min and centrifuged at 10,000 ×*g* for 20 min, and the amount of tyrosine released in the supernatant was determined using a spectrophotometer. Fifty μl of the supernatant was mixed with 500 μl of 20 mM Tris-HCl (pH 8.0) and 100 μl of 1 N Folin reagent, and then the observance was measured at 650 nm [34].

The amidolytic activity of the fibrinolytic enzyme was measured spectrophotometrically using chromogenic substrates for the plasmin (D-Val-Leu-Lys-*p*NA) and subtilisin (*N*-succinyl-Ala-Ala-Pro-Phe-*p*NA). A 200 μl mixture (pH 9.0) containing a 0.5 mM concentration of synthetic substrate and 2 μl of enzyme (0.5 mg/ml) was incubated in a 96-well microplate at 40°C. After 5 min, the absorbance of released *p*NA was measured at 415 nm (Benchmark 170-6850, BIO-RAD). One unit of proteolytic activity was defined as the amount of enzyme releasing 1 μmole of *p*NA equivalent per minute per milliliter.

The effect of pH on the enzyme activity was determined as described above, in the pH range of 4.0–12.0. The buffers used were 20 mM citrate buffer (4.0–6.0), 20 mM sodium phosphate buffer (6.0–7.0), 20 mM Tris-HCl buffer (8.0–9.0), and 20 mM glycine-NaOH buffer (10.0–12.0) at 40°C. The pH stability of the fibrinolytic enzyme was determined under the same conditions described above after sitting at 4°C for 12 h. A total of 200 μl of reaction solution was incubated in a 96-well plate under a given condition, and was subsequently withdrawn and assayed for residual activity using *p*NA.

Substrate Specificity

The substrate specificity of the enzyme was assayed by the procedure described by Peng *et al.* [26] using fibrin, fibrinogen, BSA, and thrombin as a substrate. The degradation of each substrate was tested by incubating 0.5 mg of bovine-purified fibrin (fibrinogen) in 1 ml of Tris-HCl (20 mM, pH 8.0) containing 0.5 μg of purified fibrinolytic enzyme at 37°C. At various time intervals, aliquots were taken from the reaction mixture and boiled after mixing with 5× SDS sample buffer. The aliquots were then centrifuged at 12,000 ×*g* for 10 min. The supernatant was electrophoresed by 10% SDS-PAGE to examine the continuous breakdown of the substrates. Degradation of casein and skim milk was carried out by placing paper discs on 2% casein and skim milk plates, respectively.

Protease Inhibition Assay

To determine the class to which the fibrinolytic enzyme belonged, the effect of different reagents on the fibrinolytic activity was examined. Twenty μl of enzyme solution (10 μg/ml) was preincubated with PMSF and EDTA at concentrations of 0.1 and 1 mM, respectively. The residual activity was determined using a 0.3% fibrin plate.

Internal Mapping and N-Terminal Amino Acid Sequencing

The fibrinolytic enzyme was digested by trypsin, and the trypsin-digested peptides were analyzed using a MALDI-TOF mass spectrometer (Amersham Biosciences). After SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidene difluoride membrane by electroblotting and stained with Coomassie blue. The stained material was excised and used for N-terminal sequencing by the automated Edman degradation method at the Korea Basic Science Institute (KBSI, http://www.kbsi.re.kr/).

Degradation of the Artificial Blood Clots

The *in vitro* fibrinolytic effect of the enzyme was examined by artificial blood clot degradation. An artificial blood clot was formed by spontaneous coagulation in a glass test tube using fresh rabbit blood. After 1 h, the artificial blood clot was rinsed out thoroughly. The artificial blood clot was dipped in 2 ml of PBS (phosphate-buffered saline, pH 7.4) containing 0.5 mg/ml purified enzyme at room temperature. As a control, normal saline was used without the fibrinolytic enzyme.

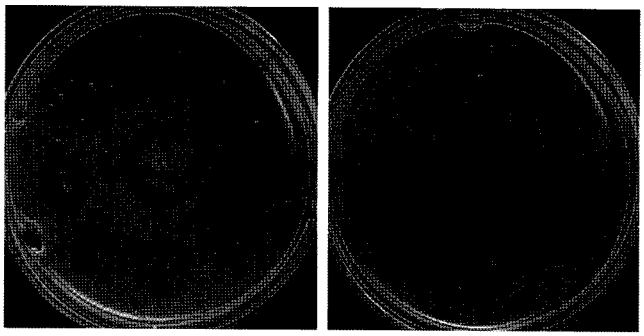

Fig. 1. Fibrin plate showing clear zones resulting from fibrinolysis of isolates.
Cell-free culture supernatant of the isolates was tested.

RESULTS AND DISCUSSION

Screening of a Fibrinolytic Strain from *Jeot-gal*

Microbial fibrinolytic enzymes have attracted increasing medical interest in recent decades because of their expensive prices and undesirable side effects, such as the risk of internal hemorrhage within the intestinal tract when t-PA and urokinase were orally administrated. Potent fibrinolytic enzymes have recently been developed from Asian fermented food products such as Japanese *Natto* [9, 30] and Korean *Chungkook-Jang* [13, 24] and *Doen-Jang* soy sauce [3–5, 18]. In particular, dietary supplementation with *natto* may shorten the euglobulin clot lysis time, which is used to evaluate the total intrinsic fibrinolytic activity in plasma. Therefore, the application of *natto*kinase in commercial food products has prompted us to screen fermented *Jeot-gal* for a new fibrinolytic strain and its enzyme. In this study, three *Jeot-gal* samples (shrimp, anchovy, and yellow corvina) were screened for microorganisms showing strong fibrinolytic activity. Among one-hundred eighty-eight isolates (108 shrimp, 46 anchovy, and 34 yellow corvina, respectively), sixteen isolates showed fibrinolytic activity. A typical fibrin plate having fibrinolytic activity of isolates is shown in Fig. 1. Isolates from anchovy samples showed strong fibrinolytic activity compared with the isolates from shrimp and yellow corvina samples. Based on the extent of the activity, one strain from an anchovy sample was chosen for further characterization (Table 1).

Table 1. Fibrinolytic activities of 16 preliminary selected isolates.

Source	Name	Fibrinolytic activity[a] (cm)
Shrimp	S37	None
	S77	1.75±0.1
Anchovy	A13	1.55±0.2
	A14	1.00±0.1
	A15	1.15±0.2
	A17	1.20±0.3
	A27	1.35±0.2
	A28	1.60±0.1
	A30	1.25±0.2
	A31	2.15±0.3
	A33	1.35±0.1
Yellow corvina	Y55	1.05±0.1
	Y57	2.00±0.2
	Y58	0.95±0.1
	Y69	0.90±0.2
	Y72	0.80±0.1
	Y80	1.75±0.3

[a]The fibrinolytic activity of isolates was determined by measuring the clear zone around the spot.

Identification of Fibrinolytic Strain KJ-31

Out of sixteen isolates, the A31 isolate obtained from an anchovy sample showed the largest halo zone. For the identification of the A31 fibrinolytic isolate, 16S rDNA sequencing and API CHB were carried out. The PCR product amplified by universal 16S rDNA primers was sequenced and compared using the BLAST program. The A31 isolate showed high identity to that of *Bacillus licheniformis* (99.9%). The API 50CHB/20E kit, which is used to differentiate *Bacillus* species, was selected for the biochemical identification of the isolate. The data from the API 50CHB/20E kit were read in the database (APILAB), and the A31 isolate showed 93.7% homology with *B. licheniformis*. Combined with the results of 16S rDNA

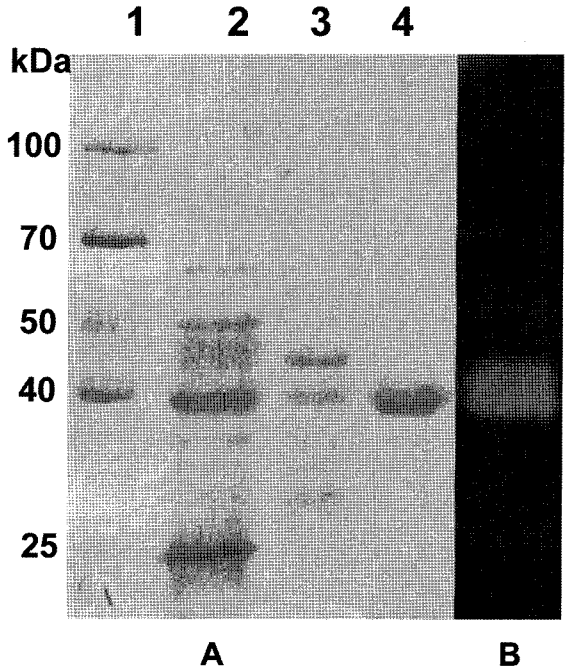

Fig. 2. SDS-PAGE and fibrin zymography of the purified fibrinolytic enzyme from *B. licheniformis* KJ-31.
SDS-PAGE (**A**) and fibrin zymography (**B**) were performed. Lane 1, molecular mass markers; lane 2, after ammonium sulfate precipitation; lane 3, after DEAE-Sepharose FF; lane 4, after gel filtration chromatography.

sequencing and the API 50CHB/20E kit, the A31 isolate was found to belong to *B. licheniformis*. Therefore, the novel fibrinolytic microorganism was referred to as *B. licheniformis* KJ-31. Members of the genus *Bacillus* from traditional fermented foods are important among the microorganisms that have been found to produce fibrinolytic enzymes [4, 10–14, 18, 21–24, 26, 29]. However, to our knowledge, the fibrinolytic enzyme of *B. licheniformis* has not been reported previously.

Purification of Fibrinolytic Enzyme from *B. licheniformis* KJ-31

The fibrinolytic enzyme from *B. licheniformis* KJ-31 was purified to electrophoretic homogeneity by anion-exchange chromatography and gel filtration chromatography. After concentration of the protein eluted from gel filtration, the purified protein was subjected to SDS-PAGE and zymography. A single band was observed in the purified sample, with a molecular mass of 37 kDa on SDS-PAGE (Fig. 2A). The fibrinolytic enzyme degraded fibrin at the same position in fibrin zymography (Fig. 2B). Based on the standard curve of released *p*NA, the final specific activity of the fibrinolytic enzyme increased up to 19-fold, with a 0.2% recovery compared with the initial culture supernatant (Table 2).

Effect of pH and Temperature on Fibrinolytic Activity and Stability

The enzyme activities were measured at various pHs and temperatures for the chromogenic substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. The enzyme showed optimum activity at around pH 9.0–10.0. The fibrinolytic enzyme was very stable in the range of pH 8.0–11.0 for 1 h, but the stability rapidly decreased at above pH 11.0 and below pH 7.0 (Fig. 3A). It was found that the fibrinolytic enzyme was highly unstable under acidic conditions. The enzyme activity was increased with increasing temperature up to 40°C, and then sharply decreased by 60°C (Fig. 3B). The fibrinolytic enzyme was stable at 40°C for 30 min, but unstable over 50°C. Based on the results of pH and temperature dependence, the fibrinolytic enzyme purified from *B. licheniformis* KJ-31 was considered to be a relatively alkalophilic and thermolabile enzyme.

Effect of Inhibitors on Fibrinolytic Activity

When the enzyme was incubated at 37°C for 18 h on a 0.3% fibrin plate, the enzyme activity was decreased to approximately 33% and 25.4% by 0.1 mM and 1 mM PMSF, respectively, whereas the fibrinolytic activity was not decreased at all by 0.1 mM EDTA, and partially decreased to approximately 77% by 1 mM EDTA (Fig. 4). These results indicate that this enzyme is apparently an alkaline serine protease like a plasmin or *natto*kinase [27].

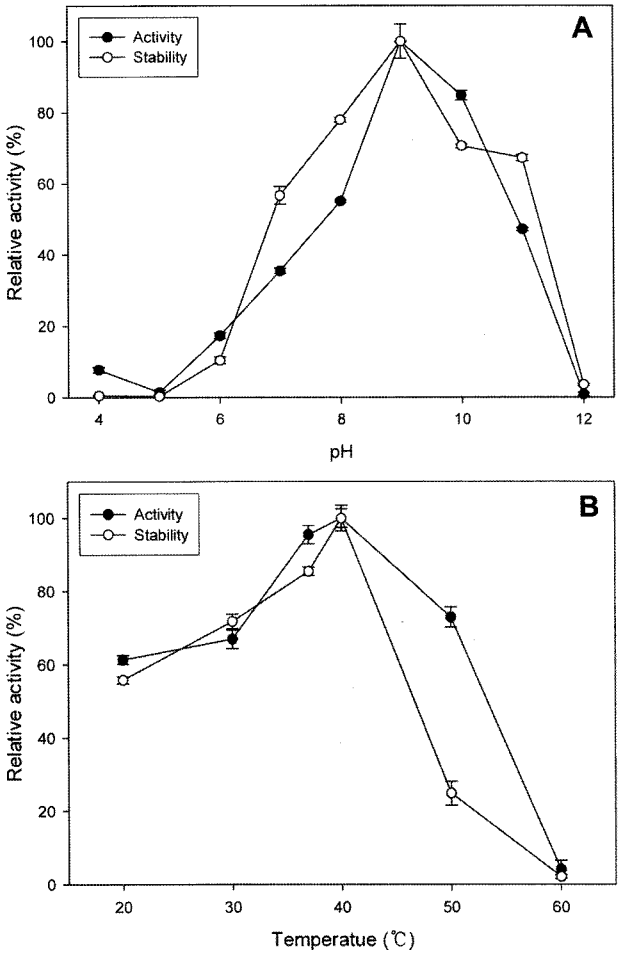

Fig. 3. Effect of pH and temperature on the activity and stability of the purified fibrinolytic enzyme.
A. The measurement of pH activity and stability was carried out as described in Materials and Methods. **B.** The optimum temperature was determined under standard assay conditions, but at various temperatures. The temperature stability was determined by heating the purified enzymes at various temperatures for 30 min. For determination of pH and temperature, the maximum activities obtained under the testing conditions were taken as 100%.

Table 2. Purification of fibrinolytic enzyme from *B. licheniformis* KJ-31.

Steps	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Cell-free supernatant	32,082	12.8	410,067	1.0	100
Ammonium sulfate precipitation	5,249	46.8	241,903	3.6	59
DEAE-Sepharose FF	32	115.0	3,695	9.0	0.9
Sephacryl S-200 gel filtration	3.2	242.8	784	19.0	0.2

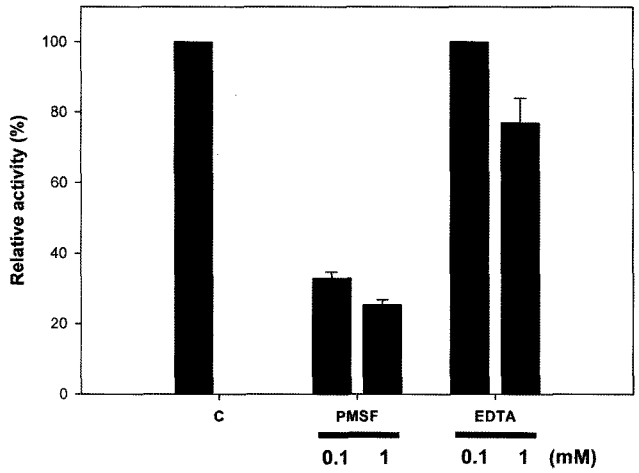

Fig. 4. Effect of inhibitors on the fibrinolytic enzyme activity.

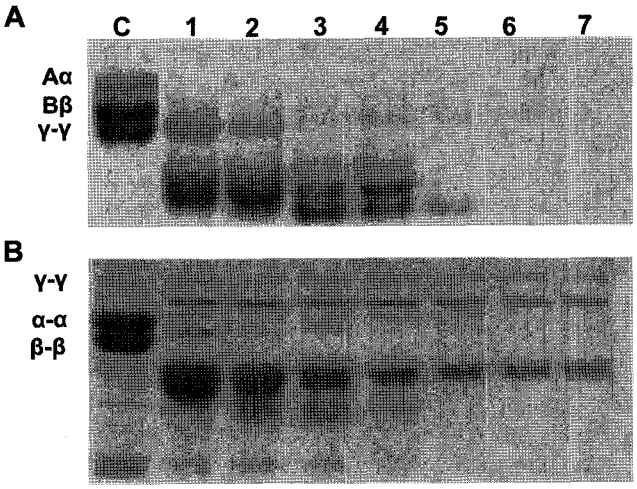

Fig. 5. Hydrolysis of fibrin and fibrinogen by fibrinolytic enzyme.

The hydrolytic products from fibrinogen (**A**) and fibrin (**B**) were separated by 10% SDS-PAGE. Lane C represents the undigested fibrinogen and fibrin. Lanes 1–7 represent hydrolyzed products after 0.2, 0.4, 1, 2, 4, 12, and 24 h, respectively.

Substrate Specificity of the Fibrinolytic Enzyme

Proteolytic activity of the enzyme was measured using 0.5% fibrin, fibrinogen, thrombin, bovine serum albumin, casein, and skim milk as substrates. The fibrin and fibrinogen degraded very well, whereas bovine serum albumin, casein, and skim milk were not degraded (data not shown), indicating that the fibrinolytic enzyme purified from *B. licheniformis* KJ-31 was specific to the proteins involved in blood clotting. To elucidate the mode of action of fibrinolytic enzyme, the degradation products were separated by SDS-PAGE. During the degradation of fibrinogen, Aα-chains and Bβ-chains of fibrinogen were cleaved rapidly to make fibrin-digested products (FDP) within 15 min, followed by a slower release of γ-γ chains (Figs. 5A, 5B). This indicates that the proteolytic action of the enzyme differs from the typical blood clotting enzyme, thrombin, because fibrinogen was hydrolyzed. Similarly, when fibrin was incubated with the enzyme, the α- and β-chains hydrolyzed faster than the γ-chain. Thus, the fibrinolytic enzyme had relatively high substrate specificity to fibrin(ogen).

Internal and N-Terminal Amino Acid Sequences of Fibrinolytic Enzyme

The trypsin-digested peptides were analyzed using a MALDI-TOF mass spectrometer. By submitting the amino acid sequence, the protein could be identified by a homology search with ProteinInfo (http://www.proteometrics.com) by using the ExPASy Molecular Biology Server (http://www.expasy.ch). The amino acid sequences of two major trypsin-digested peaks of the fibrinolytic enzyme were (K)AFSDDGGTD-EDLIAAGEWILAPK and (R)LADFSLQGPSPYDETKP-EISAPGVNIR. Furthermore, the N-terminal amino acid

Table 3. Comparison of the N-terminal amino acid sequence of bpKJ-31 with other fibrinolytic enzymes.

Enzyme	N-terminal amino acid sequence	Reference
Nattokinase	AQSVPYGISQIKAPALHSQGYTGS	[9]
Subtilisin QK-2	AQSVPYGISQIKAPALHSQG	[14]
Subtilisin DFE	AQSVPYGVSQIKAPALHSQGFTGS	[26]
Subtilisin DJ-4	AQSVPYGVSQIKAP	[12]
CK	AQTVPYGIPLIKAD	[13]
Bacillokinase II	ARAGEALDIYD	[10]
KA38	VYPFPGPOPN	[11]
CLP	VVGGKEPP	[19]
CIP	XTPLPQLSGNAVLVEAVLVEAVKA	[20]
SW-1	R/N/FP/DGMTMTAIANQNTQIN	[30]
AMMP	MFSLSSRFFLYTLCLSAVAVSAAP	[17]
NKCP	ATDGVEWNVDQIDAPKAWALGY	[21]
bpDJ-2	TDGVEWNVDQIDAPKAW	[4]
bpKJ-31	KDEKEIEWNINRVDAPKAWK	This work

sequence of the purified enzyme was also examined after SDS-PAGE and electroblotting. The sequence of the first 20 residues was found to be KDEKEIEWNINRVDAPKAWK, which is identical to that of putative Bacillopeptidase F of *B. licheniformis*, but different from those of bpr (Bacillopeptidase F in *B. subtilis*) and extracellular serine protease [4, 29]. Based on the *bpr* gene coding for Bacillopeptidase F in *B. subtilis*, bpr can be divided into three parts by autolysis. The three domains are composed of subtilisin N, peptidase_S8, and COG4412 (unknown protein). Both the internal and N-terminal sequence revealed that the purified fibrinolytic enzyme belonged to peptidase_S8 of Bacillopeptide F, and it was then referred to as bpKJ-31. The 37 kDa molecular mass of peptidase_S8 was the same as that of the purified fibrinolytic enzyme, bpKJ-31.

Previously, Choi *et al.* [4] purified bpDJ-2 with a molecular mass of 42 kDa, and Roitsch and Hageman [29] also reported two forms of Bacillopeptidase F (bpf) that have molecular masses of 33 and 50 kDa, respectively. All of the reported bpfs were known to be produced by the proteolytic processing of proenzyme forms. As shown in Table 3, the first 10–20 amino acid residues of the N-terminal sequence of the bpKJ-31 showed no homology to the other fibrinolytic enzymes. The common N-terminal sequence observed in subtilisin-type fibrinolytic enzymes was not shown in bpKJ-31. The overall results in relation to the substrate specificity, effect of inhibitors, and the N-terminal amino acid sequence indicate that the bpKJ-31 is a new form of bpf.

Blood Clot Degradation with bpKJ-31

The blood clot degradation was observed in the test tube by spreading the red blood cells in the solution after 60 min (Fig. 6). However, the degradation was not observed in the solution without the addition of fibrinolytic enzyme.

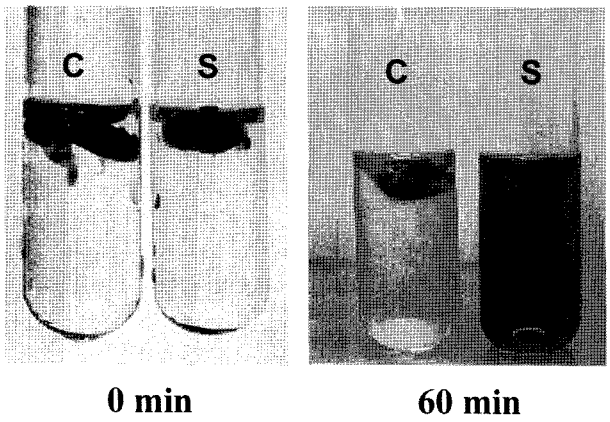

Fig. 6. Lysis of the blood clots by bpKJ-31.
C, Blood clot with saline solution; S, blood clot with 0.5 mg/ml of the fibrinolytic enzyme.

Table 4. Comparison of the specific activity of bpKJ-31 with other fibrinolytic enzymes.

Protease	Source	Specific activity (U/mg)
Streptokinase	*Streptococcus haemolyticus*	5,149.24
Plasmin	Human	212.45
bpKJ-31	*Bacillus licheniformis*	242.80

This result indicates that the bpKJ-31 is, indeed, the fibrinolytic enzyme.

With the *p*NA standard curve, the final specific activity of the fibrinolytic enzyme was increased more than 19-fold, with a 0.2% recovery based on the initial culture supernatant. The enzyme had approximately 14.3% higher fibrinolytic activity than the plasmin, but had much lower fibrinolytic activity than streptokinase, which has strong fibrinolytic activity (Table 4). In the case of streptokinase, it has a high potential to induce side effects such as internal bleeding, owing to its excessively strong fibrinolytic activity. Therefore, our result suggests that bpKJ-31 is a promising candidate as a health-promoting biomaterial that does not induce bleeding. Because bpKJ-31 is derived from food, bpKJ-31 is considered to be safe for clinical use. *In vivo* studies of thrombotic diseases and antithrombotic studies using animal models should be performed to confirm the effect of this enzyme.

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