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Purification and Characterization of a Subtilisin D5, a Fibrinolytic Enzyme of *Bacillus amyloliquefaciens* DJ-5 Isolated from *Doenjang*

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Abstract The fibrinolytic enzyme, subtilisin D5, was purified from the culture supernatant of the isolated *Bacillus amyloliquefaciens* DJ-5. The molecular weight of subtilisin D5 was estimated to be 30 kDa. Subtilisin D5 was optimally active at pH 10.0 and 45°C. Subtilisin D5 had high degrading activity for the Aα-chain of human fibrinogen and hydrolyzed the Bβ-chain slowly, but did not affect the γ-chain, indicating that it is an α-fibrinogenase. Subtilisin D5 was completely inhibited by phenylmethylsulfonyl fluoride, indicating that it belongs to the serine protease. The specific activity (F/C, fibrinolytic/caseinolytic activity) of subtilisin D5 was 2.37 and 3.52 times higher than those of subtilisin BPN' and Carlsberg, respectively. Subtilisin D5 exhibited high specificity for Meo-Suc-Arg-Pro-Tyr-*p*NA (S-2586), a synthetic chromogenic substrate for chymotrypsin. The first 15 amino acid residues of the N-terminal sequence of subtilisin D5 are AQSVPYGISQIKAPA; this sequence is identical to that of subtilisin NAT and subtilisin E.

Keywords: *Bacillus amyloliquefaciens*, *doenjang*, fibrinolytic enzyme, subtilisin D5

Introduction

Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (1,2). Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by activated thrombin (EC 3.4.21.5). The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin (EC 3.4.21.7), which is generated from plasminogen by plasminogen activators, such as urokinase, tissue type plasminogen activator (tPA), and streptokinase. These 3 agents are currently being used as thrombolytic agents; however, they are expensive and have undesirable side effects such as gastrointestinal bleeding and allergic reactions. Therefore, the search for alternative, safer thrombolytic agents from various sources is ongoing.

Recently, potent fibrinolytic enzymes have been isolated and characterized from fermented food products, such as Korean *cheonggukjang* (1), *jeotgal* (3), and *doenjang* (2), Japanese *natto* (4-7), and *shiokara* (8), Chinese *douchi* (9), and Indonesian *tempeh* (10). The fibrinolytic enzymes were successively obtained from different microorganisms, the most important among which is the genus *Bacillus*. Sumi *et al*. (5) isolated a fibrinolytic enzyme, nattokinase (NK), from *Bacillus natto*. This enzyme not only hydrolyzed thrombi *in vivo*, but also converted plasminogen to plasmin. In particular, the oral administration of *natto* or NK capsules can effectively enhance fibrinolytic activity in plasma and the release of an endogenous plasminogen activator in both animal models and human subjects. Moreover, the fibrinolytic activity was retained in the blood for more than 3 hr. These results suggest that NK may be a potent natural agent for oral thrombolytic therapy. Subsequently, many fibrinolytic enzymes were identified in different traditional fermented foods, such as CK of *Bacillus* sp. strain CK 11-4 from *cheonggukjang* (1) and subtilisin DJ-4 of *Bacillus* sp. DJ-4 from *doenjang* (2) in Korea, a fibrinolytic enzyme of *B. subtilis* IMR-NK1 from Taiwanese soil (7), subtilisin DFE of *B. amyloliquefaciens* DC-4 from *douchi* in China (8), and a subtilisin-like fibrinolytic protease of *B. subtilis* TP-6 from *tempeh* in Indonesia (9).

Doenjang is a typical and popular soybean fermented food in Korea that requires a manufacturing process more complicated than that of *natto* in Japan. In the present work, subtilisin D5, the fibrinolytic enzyme from *B. amyloliquefaciens* DJ-5, which was isolated from *doenjang* (11), was purified and characterized. Here we compare the amino acid sequence of subtilisin D5 and its enzymatic properties with those of other subtilisins (BPN', Carlsberg, and DJ-4).

Materials and Methods

Materials Human fibrinogen, thrombin, plasmin, various chromogenic substrates, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl aminoethyl (DEAE)-Sepharose CL-6B and CM-cellulose were purchased from Pharmacia (Uppsala, Sweden). Other chemicals were of analytical grade.

Sequence analysis of 16S rRNA The chromosomal DNA was isolated using a method described elsewhere (12). Polymerase chain reaction (PCR) amplification of the 16S rRNA was conducted using 2 primers according to lane (13), 5'-AGAGTTTGATCMTGGCTCAG-3' (position 8 to

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27, in *Escherichia coli* 16S rRNA numbering) and 5'-AAGGAGGTGATCCAGCC-3' (position 1,541 to 1,525, in *E. coli* 16S rRNA numbering), as described previously (14). The cloning and sequencing of the amplified 16S rRNA were performed using the V3 region sequencing primer (5'-GCTCGTTGCGGGACTTAACC-3'). The 16S rRNA sequence of *B. amyloliquefaciens* DJ-5, as determined in this study, was aligned using the PHYLIP package (15). The similarity values of 16S rRNA genes were calculated from the alignment and the evolutionary distances were calculated using the model proposed by Jukes and Cantor (16).

Sequence analysis of *gyrA* gene The *gyrA* fragment, corresponding to *B. subtilis gyrA* numbering positions 43-1,065 (17), were PCR-amplified using 2 oligonucleotide primers, 5'-CAGTCAGGAAATGCGTACGTCCTT-3' and 3'-CAAGGTAATGCTCCAGGCATTGCT-5', as described previously (14). The resultant amplicon was purified using Wizard PCR Prep kits (Promega, Madison, WI, USA), and sequenced in both directions using an ABI 310 automated DNA sequencer and BIG-dye cyclic sequencing kits (PE Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The same primers were used for the sequencing reactions. The *gyrA* sequence of *B. subtilis* subsp. *subtilis* strain 168 was obtained from the Genbank database (accession number Z99104) (17). Nucleotide similarities were calculated using the PHYDIT program (18). Phylogenetic trees were inferred using the neighbor-joining method (19). Evolutionary distance matrices for the neighborjoining method were generated according to the model proposed by Jukes and Cantor (16). The resultant unrooted tree topology was evaluated using bootstrap analyses (20) of the neighbor-joining method based on 1,000 resamplings. The trees were rooted using the *gyrA* sequence of *Bacillus* sp. C125 (accession number AB010081) as an out-group. All analyses were performed using the PHYLIP package (15).

Bacterium and culture conditions *B. amyloliquefaciens* DJ-5-producing fibrinolytic enzyme (subtilisin D5) was isolated from *doenjang* (11). The isolated bacterium was identified using 16S ribosomal DNA analysis (SolGent Co., Daejeon, Korea; http://www.solgent.co.kr/). Bacteria were cultured at 37°C in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) in 1-L Erlenmeyer flasks with shaking (150 rpm) for 2 days. The resulting culture supernatants were then used for enzyme purification.

Enzyme assay Quantitative analysis of fibrinolytic activity was conducted by the standard fibrin plate method (21). Fibrinogen [5 mL of 0.6%(w/v)] solution in a 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2%(w/v) agarose solution and 0.1 mL of thrombin (10 NIH units/mL) in a petri dish. The solution was left for 1 hr at room temperature to form a fibrin clot layer. Caseinolytic activity was assayed by using the casein plate method. Casein [5 mL of 0.6%(w/v)] solution in a 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2%(w/v) agarose solution in a petri dish. Twenty μL (0.1 μg) of sample solution were applied to a fibrin plate and incubated at 37°C for 12 hr. The same volume of plasmin solution (1 NIH unit/mL) was also incubated on a fibrin plate as a positive control for fibrinolytic protease activity.

Amidolytic assay The amidolytic activity was colorimetrically estimated with a Beckman DU-70 spectrophotometer by using various chromogenic substrates. Assays were carried out in 50 mM glycine-NaOH buffer (pH 10.0), 0.2 mL of 0.5 mM substrate, and purified enzyme (0.3 μg/0.2 mL). The mixture was incubated at 37°C for 5 min, and the reaction was stopped by adding 0.1 mL of 50% acetic acid. Activity was determined from the change in absorbance at 405 nm due to the formation of *p*-nitroaniline. One unit was defined as the amount of enzyme releasing 1 μmol of substrate/min.

Enzyme purification All purification steps were carried out at 4°C. The buffers used were as follows: Buffer A, 50 mM Tris-HCl buffer (pH 7.4); Buffer B, 50 mM Tris-HCl buffer (pH 8.4); and Buffer C, 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl.

To purify the fibrinolytic enzyme, enzyme in the 2 L culture supernatant was concentrated by ultrafiltration with PM-10 membrane (Amincon, Inc., Beverly, MA, USA). The concentrated sample was dialyzed against 20 volumes of Buffer A for 1 day with 3 buffer changes. The dialyzed suspension was loaded onto a CM-cellulose column (2.0×10 cm) equilibrated with Buffer A. Proteins were eluted with a 300 mL of linear gradient of 0 to 1 M NaCl in Buffer A. Fractions showing fibrinolytic activity were pooled and then dialyzed against Buffer B. The dialyzed enzyme was loaded onto a DEAE-Sepharose CL-6B column (2.0×10 cm) equilibrated with Buffer B. Proteins were eluted with a 100 mL of linear gradient of 0 to 1 M NaCl in Buffer B. Active fractions were pooled and concentrated by lyophilization, and further purified by TSK gel filtration column (2.0×110 cm) (Toyopearl HW-55F; Tosoh, Kyoto, Japan) using Buffer C.

Fibrinogenolytic activity Fibrinogenolytic activity (22) was assayed by incubating 0.1 mL of a 0.2%(w/v) human fibrinogen solution (Sigma-Aldrich) with 0.05 mL of enzyme solution (containing 0.1 μg of enzyme) in 50 mM glycine-NaOH buffer (pH 10.0) at 37°C. At different times (0 to 60 min), 0.15 mL of denaturing sodium dodecylsulfate (SDS) sample buffer [0.125 mM Tris-HCl, pH 6.8, 0.1%(w/v) SDS, and 1%(v/v) β-mercaptoethanol] was added and the mixture heated at 95°C for 4 min. For each sample, 25 μL containing approximately 15 mg of fibrinogen were analyzed by SDS-PAGE.

SDS-PAGE and determination of N-terminal amino acid sequence of purified enzyme SDS-PAGE was performed by the Laemmli method (23). Protein samples were diluted 5 times with SDS sample buffer comprised of 0.5 M Tris-HCl (pH 6.8), 10%(w/v) SDS, 20%(v/v) glycerol, and 0.03%(v/v) bromophenol blue. After SDS-PAGE, the purified enzyme on the gel was transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting (24) and stained with Coomassie blue. The stained material was excised and used for direct N-terminal sequencing by the automated Edman degradation method using a gas-phase

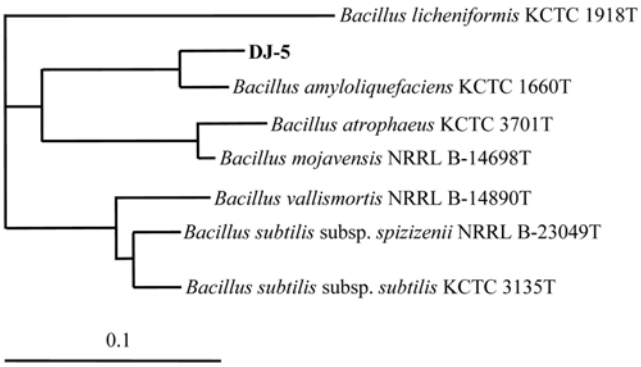

Fig. 1. Rooted neighbor-joining tree based on the partial *gyrA* nucleotide sequences. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets. The scale bar indicates 0.1 nucleotide substitutions/nucleotide position.

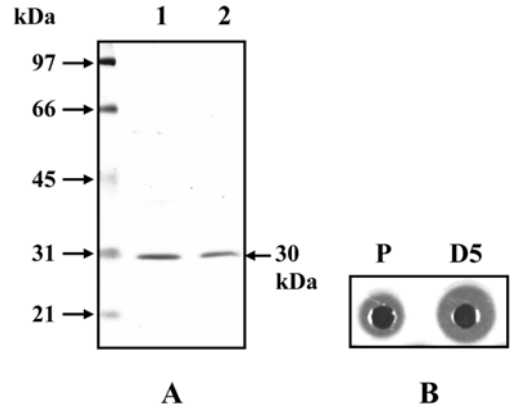

Fig. 2. SDS-PAGE (A) and fibrinolytic activity (B) of purified enzyme from *B. amyloliquefaciens* DJ-5. SDS electrophoresis [12%(w/v) acrylamide] was performed. Lane 1 and 2 represent reduced and nonreduced purified subtilisin D5, respectively. P and D5 mean 1.0 NIH plasmin and subtilisin D5, respectively.

protein sequencer model Procise 491 (ABI, Foster City, CA, USA).

Results and Discussion

Identification of a fibrinolytic enzyme-producing bacterium Many fibrinolytic enzymes have been isolated and characterized from traditional Asian fermented foods, such as *cheonggukjang* (1), *jeotgal* (3), and *doenjang* (2) (Korea), *natto* (4-7), and *shiokara* (8) (Japan), *douchi* (9) (China), and *tempeh* (10) (Indonesia). In addition, some fibrinolytic enzymes were discovered from the root tissue of *Stemona japonica* (Blume) Miq, a Chinese traditional medicine (25), and marine alga (e.g., *Codium latum*, *C. divaricatum*, and *C. intricatum*) (26-28). In the present study, the bacterial strain producing a fibrinolytic enzyme was isolated from *doenjang*, a traditional Korean soybean fermented food.

Phylogenetic analysis of DJ-5, based on the levels of similarity of the 16S rRNA sequences (deposited in GenBank under Accession No. AY769247), indicated that the strain belonged to the genus *Bacillus* and is closely related to the type strain of *B. amyloliquefaciens* ATCC 23350^T (99.65%), *Bacillus atrophaeus* NCIB 12899^T (99.45%), *B. subtilis* ATCC 6051^T (99.14%), *B. subtilis* subsp. *spizizenii* NRRL B-23049^T (98.79%), and *Bacillus licheniformis* ATCC 14580^T (97.24%) with sequence similarity values of 97.24 to 99.65%. Using this result, it is difficult to determine the type species of DJ-5. Thus, a comparative analysis of the partial *gyrA* gene was performed as described by Chun and Bae (17). The maximum pairwise similarity between the *Bacillus* sp. DJ-5 *gyrA* gene (deposited in GenBank under Accession No. AY822026) and *B. amyloliquefaciens* KCTC 1660^T was 94.90%. The phylogenetic tree based on the neighbor-joining analysis of the *gyrA* sequences is given in Fig. 1.

Purification of subtilisin D5 from *B. amyloliquefaciens* DJ-5 Subtilisin D5 from *B. amyloliquefaciens* DJ-5 was purified to electrophoretic homogeneity using commercial chromatographic techniques (Table 1). Subtilisin D5 migrated as a single band with an apparent molecular mass of 30 kDa on SDS-PAGE under both reducing and nonreducing conditions (Fig. 2A). The fibrinolytic activity of subtilisin D5 was measured to be 4.21 plasmin NIH units using the fibrin plate method (Fig. 2B). This is similar to that reported by Takahashi *et al.* (29) for SMCE from *B. pumilus* TYO-67 and by Wang *et al.* (30) for subtilisin FS33 from *B. subtilis* DC33 (30.0 kDa), but lower than the values published for subtilisin E (55.8 kDa) (31), subtilisin IMR-NK1 (31.5 kDa) (7), KK (38 kDa) (32), and KA (41 kDa) (3) and higher than those for subtilisin NAT (27.7 kDa, formerly designated nattokinase, NK) (6), CK (28.2 kDa) (1), and subtilisin DFE (28.0 kDa) (9).

Effect of pH and temperature on activity and stability The effect of pH on the activity of subtilisin D5 was determined in buffers of various pH values, and results showed that subtilisin D5 was active over a wide range of pH values from 5.0 to 11.0 and was most active at pH 10.0 (Fig. 3). The enzyme was very stable in the range of pH 6.0 to 9.0 at 37°C for 60 min, but became unstable out of this

Table 1. Purification steps of subtilisin D5 from *B. amyloliquefaciens* DJ-5

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture broth	115.0	980	8.5	1	100
Ultrafiltration (PM-10)	63.4	921	14.5	1.7	94
CM-cellulose	17.5	383	21.9	2.6	39.1
DEAE-Sepharose CL-6B	5.8	265	45.7	5.4	27
Toyopearl HW-55F	1.9	211	111.1	13.1	21.5

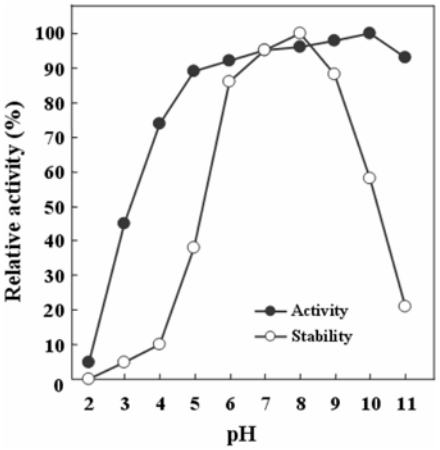

Fig. 3. Effect of pH on fibrinolytic activity and stability of subtilisin D5 from *B. amyloliquefaciens* DJ-5 at 30°C. After incubation of the enzyme over the pH range of 2.0 to 11.0 at 30°C for 2 hr, the residual activity was measured.

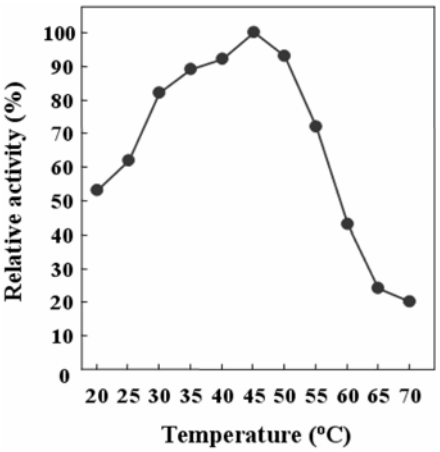

Fig. 4. Effect of temperature on fibrinolytic activity of subtilisin D5 from *B. amyloliquefaciens* DJ-5. The activity was assayed at various temperatures in 50 mM glycine-NaOH buffer (pH 10.0).

range. The effect of temperature on the fibrinolytic activity of the enzyme was examined at pH 10.0 (Fig. 4). The temperature showing the maximal enzyme activity was 45°C, which was comparable to those of nattokinase (6) and subtilisin DJ-4 (2). The enzyme was very stable at 30 and 40°C, and showed 30, 12, and 9% of the initial activity after 1 hr at 50, 55, and 60°C, respectively (Fig. 5).

Effect of inhibitors and metal ions on fibrinolytic activity The effects of various inhibitors and metal ions on the fibrinolytic activity of subtilisin D5 are summarized in Table 2. Subtilisin D5 was inhibited by 1 mM PMSF, but ethylenediamide tetracetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), leupeptin, and aprotinin did not inhibit the fibrinolytic activity, indicating that subtilisin D5 is a serine protease. In addition, the enzyme activity was inhibited by 5 mM of Cd^{2+}, Cu^{2+}, and Zn^{2+}, but not by Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+}.

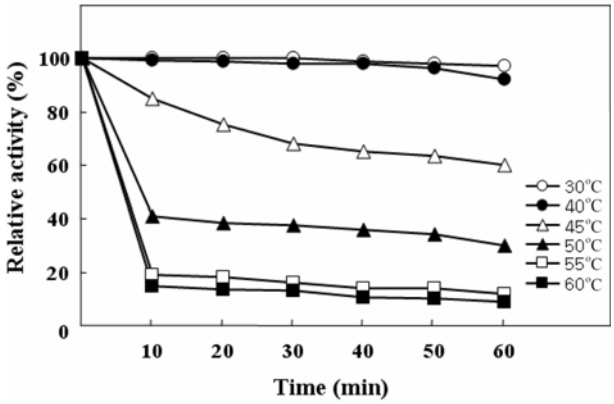

Fig. 5. Effect of temperature on stability of subtilisin D5 from *B. amyloliquefaciens* DJ-5. To establish the thermostability of subtilisin D5, the residual enzyme activity was measured after incubation at the indicated temperatures for 1 hr.

Table 2. Effects of metal ions and inhibitors on the activity of subtilisin D5

Matal ions (5 mM)/Inhibitors (1 mM)	Activity (%)
None	100
$CdCl_2$	73.8
$CoCl_2$	101.3
$CuSO_4$	51.0
$FeCl_2$	99.3
$MgCl_2$	93.8
$MnCl_2$	86.5
$NiCl_2$	98.8
$ZnCl_2$	46.4
PMSF	25.8
EDTA	99.2
EGTA	97.5
Leupeptin	98.5
Aprotinin	96.4

Table 3. Comparison of subtilisin D5 with other proteases for activity

Protease	FA (U)[1)]	CA (U)[2)]	SA[3)]
Subtilisin D5	189.6±2.2	163.4±4.2	1.16
Subtilisin DJ-4	148.9±3.2	113.5±4.2	1.31
Subtilisin BPN'	102.4±2.5	208.6±3.1	0.49
Subtilisin Carlsberg	82.7±3.7	250.4±2.5	0.33

[1,2)]Activity on the fibrin (FA) and casein (CA) plates, respectively; Each value is mean±SEM (n=5).
[3)]Specific activity (F/C, fibrinolytic activity/caseinolytic activity).

Comparison of the specific activity of subtilisin D5 with other proteases The specific activity (F/C, the ratio of fibrinolytic activity to caseinolytic activity) of subtilisin D5 with other proteases was determined by measuring fibrinolytic and caseinolytic activities and calculating the F/C ratios. As shown in Table 3, the specific activity of subtilisin D5 was 2.37 and 3.52 times higher than those of subtilisin BPN' and Carlsberg, respectively.

Table 4. Comparative amidolytic activity of the subtilisin D5 for the hydrolysis of several synthetic substrates

Synthetic substrate (0.8 mM)	Substrate hydrolysis (nmol/mL/min)
H-D-Pro-Phe-Arg-*p*NA[1)] (Plasma kallikrein)	0
N-α-Benzyloxycarbonyl-D-Arg-Gly-Arg-*p*NA (Factor Xa, trypsin)	0.4
H-D-Phe-Pip-Arg-*p*NA (Thrombin)	0
H-D-Ile-Pro-Arg-*p*NA (broad spectrum of serine protease)	0
3-Carbomethoxypropionyl-Arg-Pro-Tyr-*p*NA (Chymotrypsin)	6.8
Glu-Gly-Arg-*p*NA (Urokinase)	0
H-D-Val-Leu-Lys-*p*NA (Plasmin, Plasminogen)	0.3

[1)]*p*NA, *p*-Nitroaniline.

Table 5. N-terminal amino acid sequence of fibrinolytic subtilisin D5, compared with other fibrinolytic enzymes in literature data

Fibrinolytic enzyme	Sequencing	kDa	Strains	Authors
Subtilisin D5	AQSVPYGISQIKAPA	30	*B. amyloliquefaciens* DJ-5	This work
Nattokinase (NK)	AQSVPYGISQIKAPALHS	27.7	*B. subtilis* natto	Fujita *et al.* (6)
Subtilisin E	AQSVPYGISQIKAPALHS	55.8	*B. subtilis* sp.	Wong *et al.* (31)
Subtilisin FS33	AQSVPYGIPQIKAPA	30	*B. subtilis* DC33	Wang *et al.* (30)
Subtilisin DFE	AQSVPYGVSQIKAPALHS	28	*B. amyloliquefaciens* DC-4	Peng *et al.* (9)
SMCE	AQTVPYGIPQIKAD	30	*B. pumilus* TYO-67	Takahashi *et al.* (29)
CK	AQTVPYGIPLIKAD	28.2	*Bacillus* sp. CK 11-4	Kim *et al.* (1)
Subtilisin Carlsberg	AQTVPYGIPLIKAD	-	*B. licheniformis*	Smith *et al.* (35)
Subtilisin IMR-NK1	AQPVPNGRTAIKA	31.5	*B. subtilis* IMR-NK1	Chang *et al.* (7)
KA38	VYPFPGPIPN	41	*B. subtilis* KA38	Kim *et al.* (3)
KK	IVGGYEQZAHSQPHQ	38	*B. firmus* NA-1	Seo *et al.* (32)

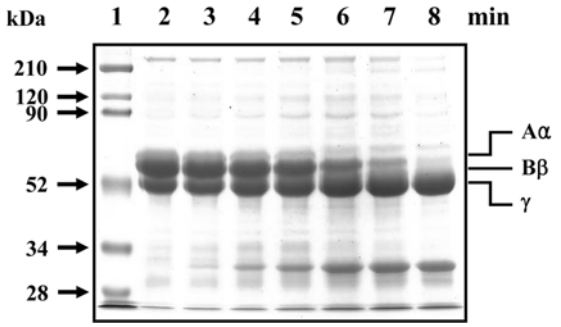

Fig. 6. SDS-PAGE of the digested human fibrinogen by subtilisin D5 under reducing conditions. Fibrinogen consisted of 3-polypeptide chains: Aα (66,000), Bβ (54,000), and γ (48,000). Fifteen mg of fibrinogen incubated with enzymes were subjected to electrophoresis on 10% gel: (1) molecular mass marker; (2) fibrinogen incubated without enzyme for 5 min; (3-8) fibrinogen samples after incubation with enzymes for 0, 1, 5, 10, 15, and 30 min.

Chromogenic assay of amidolytic activity using synthetic substrates The amidolytic activity of the subtilisin D5 was investigated with several synthetic substrates. Subtilisin D5 only hydrolyzed Meo-Suc-Arg-Pro-Tyr-*p*NA (S-2586), a synthetic chromogenic substrate for chymotrypsin, and did not show activity on other tested synthetic substrates (Table 4). NK from *B. natto* (6) also showed high activity for this substrate of chymotrypsin.

N-Terminal amino acid sequence of subtilisin D5 The N-terminal amino acid sequence of subtilisin D5 was analyzed by the automated Edman degradation method after SDS-PAGE and electroblotting. The sequence of the first 15 residues was found to be AQSVPYGISQIKAPA, which is identical to that of subtilisin NAT (formerly designated Nattokinase from *B. subtilis* natto) (6) and subtilisin E (from *B. subtilis* sp.) (31) (Table 5). Amino acids A-Q (position 1 and 2) and I-K-A (position 11, 12, and 13) are the almost conserved amino acid residues of the N-terminal sequence of these subtilisins from *Bacillus* spp. Together, the results for synthetic substrate specificity, effect of inhibitors, and the N-terminal amino acid sequences, indicate that subtilisin D5, the fibrinolytic enzyme of *B. amyloliquefaciens* DJ-5, is a subtilisin-like serine-type fibrinolytic enzyme that occurs as a monomer.

Fibrinogenolytic activity of subtilisin D5 Subtilisin D5 showed high fibrinogenolytic activity, degrading predominantly the Aα-chain of human fibrinogen within 10 min. By comparison, subtilisin D5 degraded the Bβ-chain slowly and did not cleave the γ-chains (Fig. 6), indicating that it is an α-fibrinogenase and different from subtilisin BPN', DJ-4, and FS33.

In general, fibrin(ogen)olytic enzymes belong to as 2 classes, the α(β)-fibrinogenases (known as zinc-metalloproteinases) and the β-fibrinogenases (known as thermostable serine proteinases) (33,34). On the basis of these results, subtilisin D5 is a serine-type alkaline chymotrypsin-like α(β)-fibrinogenase. Furthermore, our results demonstrate that subtilisin D5 is highly specific for the Aα-chain of human fibrinogen. Hence, this study highlights the potential for subtilisin D5 as an effective thrombolytic agent. Investigations to further characterize subtilisin D5 are underway.

Acknowledgments

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