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Vol. 7(19), pp. 1983-1992, 7 May, 2013
DOI: 10.5897/AJMR12.041
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Identification of fibrinogen-induced nattokinase WRL101 from *Bacillus subtilis* WRL101 isolated from *Doenjang*

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Accepted 8 January, 2013

To increase the fibrinolytic enzyme (nattokinase WRL101) in *Bacillus subtilis* WRL101, bovine fibrin or fibrinogen (1.0%, w/v) were added in the tryptic soy broth as a substrate. The fibrinolytic activity cultured in the fibrinogen-contained medium was increased. On the other hand, fibrin decreased the activity. Using the medium condition, nattokinase WRL101 (fibrinogen-induced nattokinase WRL101, FIN-WRL101) was isolated by commercial chromatographic techniques and its biochemical characteristics were investigated. The molecular weight of FIN-WRL101 was estimated to be 29 kDa. FIN-WRL101 was optimally active at pH 11.0 and 47°C. It had high degrading activity for the Aα-chain and Bβ-chain of human fibrinogen, but did not affect the γ-chain, indicating that it is an α-fibrinogenase. FIN-WRL101 was completely inhibited by phenylmethylsulfonyl fluoride, indicating that it belongs to the serine protease. FIN-WRL101 exhibited high specificity for Meo-Suc-Arg-Pro-Tyr-*p*NA (S-2586), a synthetic chromogenic substrate for chymotrypsin. Its nucleotide and amino acid sequences were determined.

Key words: *Bacillus subtilis*, *Doenjang*, fibrinolytic enzyme, nattokinase.

INTRODUCTION

Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (Kim et al., 1996). Fibrin is formed from fibrinogen by activated thrombin (EC 3.4.21.5) and lysed by plasmin (EC 3.4.21.7), which is generated from plasminogen by tissue type plasminogen activator (tPA). The major thrombolytic agents are classified into two types. One is plasminogen activators, such as urokinase, tPA (tissue type plasminogen activator), and streptokinase, which activate plasminogen to plasmin. The other type is plasmin-like proteins, such as fibrolase from snake venom and lumbrokinase (Mihara et al., 1991) from earthworm, which can directly degrade the fibrin. The former three 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. Many bacterial fibrinolytic enzymes were discovered from fermented foods, such as

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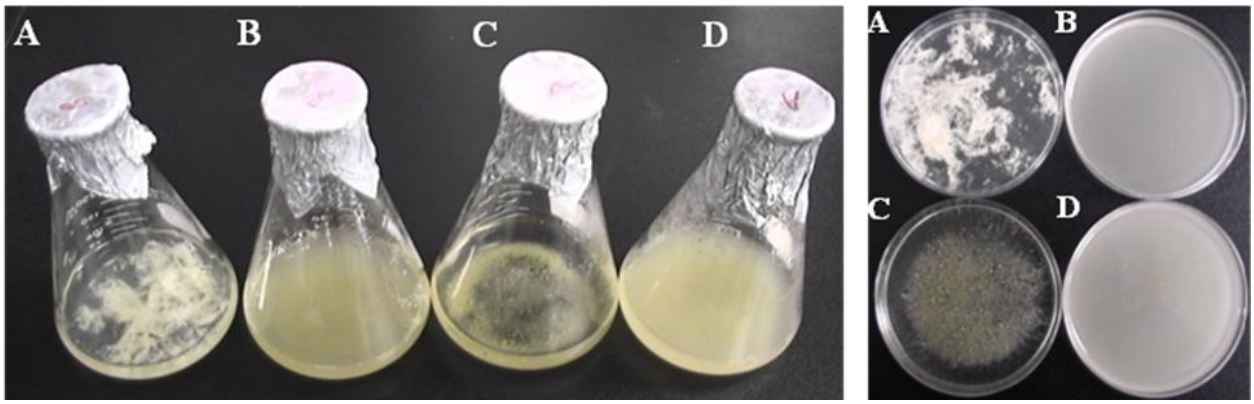

Figure 1. Fibrinogenolysis or fibrinolysis shown by *Bacillus subtilis* WRL101. Fibrinogen (A and B) or fibrin (C and D) (1.0 %) contained TSB media. A and C show control media (before seeding of *B. subtilis* WRL101). B and D show the degradation of fibrinogen (B) or fibrin (D) by *B. subtilis* WRL101.

natto (Sumi et al., 1987, 1990; Fujita et al., 1993; Chang et al., 2000; Kwon et al., 2011; Wang et al., 2011), *shiokara* (Sumi et al., 1995) in Japanese food, *Chungkook-Jang* (Kim et al., 1996), *Doenjang* (Kim and Choi, 2000) and *Jeot-Gal* (Kim et al., 1997; Choi et al., 2009b) in Korean food, *douchi* (Peng et al., 2003) in Chinese food and Tempeh (Kim et al., 2006) in Indonesian food. The fibrinolytic enzymes were successively obtained from different microorganisms, the most important among which is the genus *Bacillus*. In particular, nattokinase produced by *Bacillus natto* screened from *natto* was reported that the enzyme not only hydrolyzed thrombi *in vivo*, but also converted plasminogen to plasmin (Sumi et al., 1990). Oral administration of the enzyme showed that it could enhance fibrinolytic activity in plasma and the production of tPA, and its fibrinolytic activity was retained in the blood for more than 3 h. 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 *Chungkook-Jang* (Kim et al., 1996) and subtilisin DJ-4 of *Bacillus* sp. DJ-4 from *Doenjang* (Kim and Choi, 2000) in Korea, a fibrinolytic enzyme of *B. subtilis* IMR-NK1 from Taiwanese soil (Chang et al., 2000), subtilisin DFE of *B. amyloliquefaciens* DC-4 from *douchi* in China (Peng et al., 2003) and a subtilisin-like fibrinolytic protease of *B. subtilis* TP-6 from Tempeh in Indonesia (Kim et al., 2006).

So far, many researchers have focused their efforts on isolating and screening of microorganisms for fibrinolytic enzyme production with higher activity and on purifying and characterizing newly found enzymes. To achieve high product yields, it is a prerequisite to select a proper medium. Little information, however, is available on the optimized media for fibrinolytic enzyme production. Here, we applied fibrinogen (or fibrin) as substrate for production of fibrinolytic enzyme (nattokinase WRL101) from *B. subtilis* WRL101 isolated from *Doenjang*, a popular soybean fermented food in Korea. With a fibrinogen-contained TSB medium, FIN-WRL101 was easily purified and its nucleotide and amino acid sequences were determined.

Also, the amino acid sequence of FIN-WRL101 and its enzymatic properties with those of other subtilisins (BPN', Carlsberg, and DJ-4) were compared.

MATERIALS AND METHODS

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

Strain and culture conditions

B. subtilis WRL101-producing fibrinolytic enzyme (nattokinase WRL101) was isolated from *Doenjang*. The isolated bacterium was identified using 16S ribosomal DNA analysis (SolGent Co., Daejeon, Korea; http://www.solgent.co.kr/). The strain was cultured at 37°C in Tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) containing bovine fibrin or fibrinogen (1.0%, w/v) in 250 ml flasks with shaking (150 rpm) (Figure 1) for the time as indicated in Figure 2. The resulting culture supernatants were then used for fibrinolytic activity assay.

Enzyme assay

Quantitative analysis of fibrinolytic activity was conducted by the standard fibrin plate method (Asrup and Müllertz, 1952). 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 h at room temperature to form a fibrin clot layer. Caseinolytic activity was assayed by using the casein plate

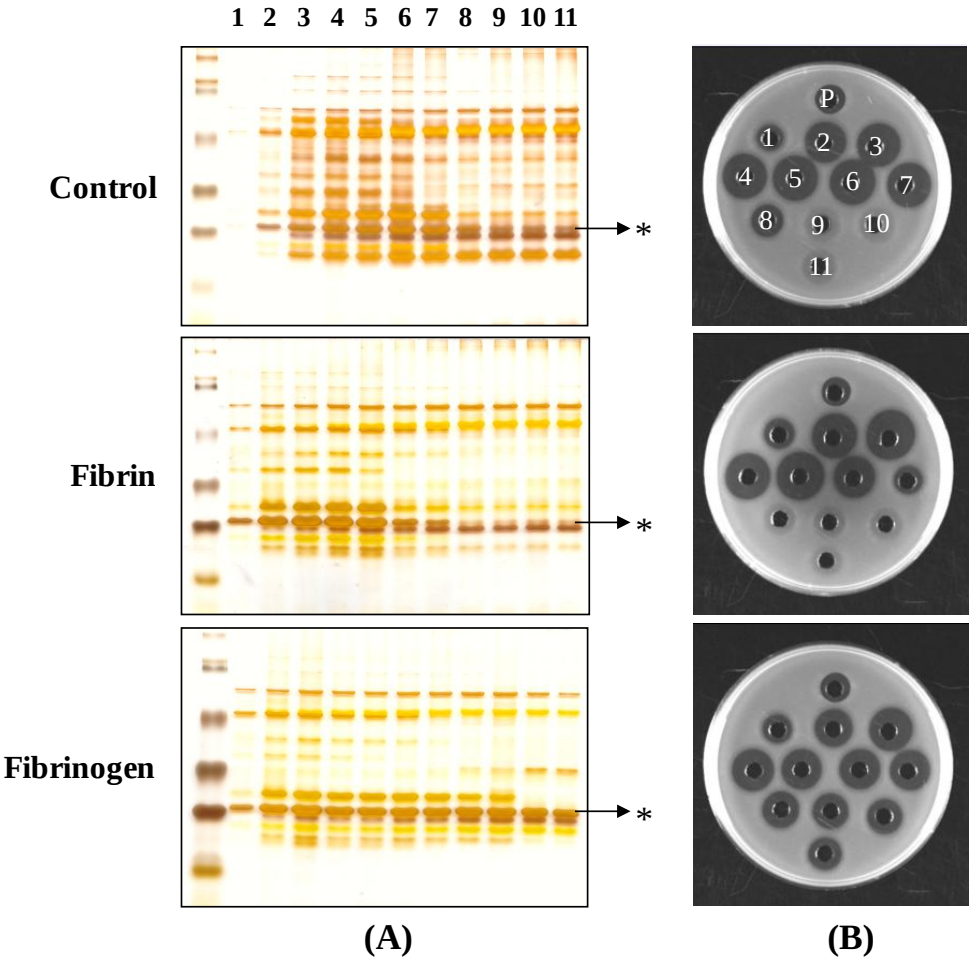

Figure 2. Comparison of extracellular protein pattern and fibrinolytic activity of *B. subtilis* WRL101 cultured in fibrin or fibrinogen contained TSB media. After TCA precipitation, the samples (40 μg) were loaded on the SDS gel (12%), and the gels were silver-stained (a). The activity of each fraction was assayed with fibrin plate (b). 1-11 are 6, 12, 18, 24, 36, 48, 72, 96, 120, 144, and 168 h cultivation, respectively. Asterisked bands (*) are subtilisin determined by N-terminal amino acid sequencing analyzer.

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. 20 μl (0.1 μg) of sample solution were applied to a fibrin plate and incubated at 37°C for 12 h. 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.

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) and buffer B, 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl.

To purify the fibrinolytic enzyme, *B. subtilis* WRL101 was cultured at 37°C in TSB containing bovine fibrinogen (1.0%, w/v) in 2-L Erlenmeyer flasks with shaking (150 rpm) for 2 days. The fibrinolytic enzyme in the 500 ml 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 one day with three 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 100 ml of linear gradient of 0 to 0.5 M NaCl in Buffer A. Fractions showing fibrinolytic activity were pooled and then dialyzed against Buffer B. The dialyzed enzyme was concentrated by lyophilization, and further purified by TSK gel filtration column (2.0 × 110 cm) (Toyopearl HW-55F, TOSOH, Kyoto, Japan) using Buffer B.

Amidolytic assay

The amidolytic activity was colorimetrically estimated with a Beckman DU-70 spectrophotometer by using various chromogenic substrates (Choi et al., 2009b). 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 per minute.

Table 1. Purification steps of FIN-WRL101 from *B. subtilis* WRL101.

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture broth	50.0	650	13.0	1.0	100
Ultrafiltration (PM-10)	31.5	625	19.8	1.5	96.2
CM-cellulose	5.1	308	60.4	4.6	47.4
Toyopearl HW-55F	2.3	258	112.2	8.6	39.7

Fibrinogenolytic activity

Fibrinogenolytic activity (Hung et al., 1994) was assayed by incubating 0.1 ml of a 0.2% human fibrinogen solution (w/v, Sigma) 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 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 µg 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 (1970) method. 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 (Matsudaira, 1987) 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 protein sequencer model Procise 491 (ABI, Foster City, CA, USA).

PCR cloning of FIN-WRL101 gene

Using the amino acid sequence of FIN-WRL101, we searched the nucleotide sequence database of National Center for Biotechnology Information for a subtilisin homologue and found the nattokinase clone (Accession No.: AF368283.1). Chromosomal DNA from *B. subtilis* WRL101 was prepared by the method of Rochelle et al. (1992), and used as the template for PCR. FIN-WRL101 gene was amplified by PCR using *Nde*I-linked sense primer (5'-GGAATTCCATATGAGAAGCAAAAAATTGTGGATCA-3') and *Bam*HI-linked antisense primer (5'-CGCGGATTCTTATTGTGCAGCTGCTTGT-3'). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1.5 min. The PCR-amplified 1143 bp DNA fragment was extracted from agarose gel and then ligated into pGEM-T Easy vector (Promega) to generate pT-FIN-WRL101 plasmid. The FIN-WRL101 gene and its deduced amino acid sequences were determined (SolGent Co.).

RESULTS AND DISCUSSION

Identification of a fibrinolytic enzyme-producing bacterium

In the present study, the bacterial strain (WRL101) producing a fibrinolytic enzyme, fibrinogen induced nattokinase WRL101 (FIN-WRL101), was isolated from *Doenjang*, a traditional Korean soybean fermented food.

Phylogenetic analysis of WRL101, based on the levels of similarity of the 16S rRNA sequences (deposited in GenBank under Accession No. JN400257), indicate that the strain belonged to the genus *Bacillus* and is closely related to the type strain of *Bacillus subtilis* strain JSU-2 (100%), and the strain was then named *B. subtilis* WRL101.

Effect of fibrin or fibrinogen on fibrinolytic enzyme production

Based on the observation, fibrin decreased FIN-WRL101 production. After 72 h cultivation in the fibrin-contained medium, the fibrinolytic activity was completely inhibited (Figure 2). Plus, no FIN-WRL101 band was detected at this time on the SDS gel. On the other hand, both the fibrinolytic activity and FIN-WRL101 band cultured in the fibrinogen-contained medium were increased. Using this medium, we can easily isolate FIN-WRL101 as follows:

Purification of FIN-WRL101 from *Bacillus subtilis* WRL101

To purify the fibrinolytic enzyme (FIN-WRL101), *B. subtilis* WRL101 was cultured in bovine fibrinogen (1.0%, w/v) containing TSB medium. FIN-WRL101 from *B. subtilis* WRL101 was purified to electrophoretic homogeneity using commercial chromatographic techniques (Table 1). FIN-WRL101 migrated as a single band with an apparent molecular mass of 29 kDa on SDS-PAGE under both reducing and nonreducing conditions (Figure 3). The purified FIN-WRL101 had a specific activity of 112.2 U/mg. This is similar to that reported by Takahashi et al. (2004) for SMCE from *B. pumilus* TYO-67, by Choi et al. (2009a) for subtilisin D5 from *B. amyloliquefaciens* DJ-5, and by Wang et al. (2006) for subtilisin FS33 from *B. subtilis* DC33 (30.0 kDa), but lower than the values published for subtilisin E (55.8 kDa) (Wong et al., 1984), subtilisin IMR-NK1 (31.5 kDa) (Chang et al., 2000), KK (38 kDa) (Seo and Lee, 2004) and KA (41 kDa) (Kim et al., 1997) and higher than those for subtilisin NAT (27.7 kDa, formerly designated

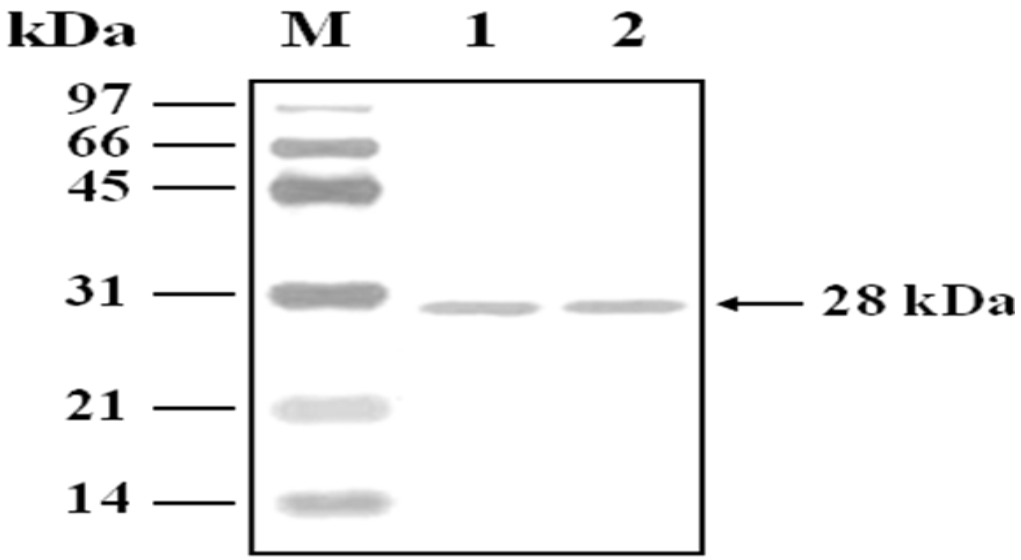

Figure 3. SDS-PAGE of purified FIN-WRL101. Reducing (1) or non-reducing (2) FIN-WRL101 were applied 12% gel.

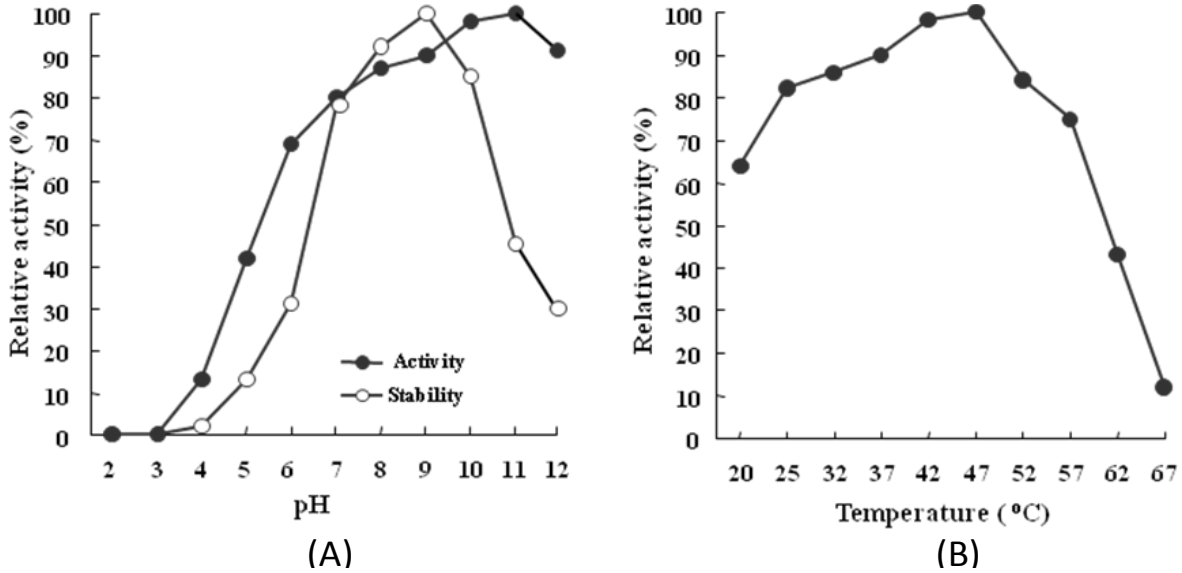

Figure 4. Effects of pH (A) and temperature (B) on the activity of FIN-WRL101. The enzyme solutions were incubated with buffer of various pHs, at 4°C for 24 h. For the effect of temperature, the enzyme solutions were incubated at the indicated temperature for 30 min.

nattokinase, NK) (Fujita et al., 1993), CK (28.2 kDa) (Kim et al., 1996) and subtilisin DFE (28.0 kDa) (Peng et al., 2003).

Effect of pH and temperature on activity and stability

The effect of pH on the activity of FIN-WRL101 was determined in buffers of various pH values, and results show that FIN-WRL101 was active over a wide range of pH values from 5.0 to 12.0 and was most active at pH 11.0 (Figure 4A). The enzyme was very stable in the range of pH 7.0 to 10.0 at 37°C for 60 min, but became unstable out of this range. The effect of temperature on the fibrinolytic activity of the enzyme was examined at pH 11.0 (Figure 4B). The temperature showing the maximal enzyme activity was 47°C, which was comparable to those of nattokinase (Fujita et al., 1993), subtilisin DJ-4 (Kim and Choi, 2000) and subtilisin D5 (Choi et al., 2009a).

Table 2. Effects of metal ions and inhibitors on the activity of FIN- WRL101.

Metal ions (5 mM) / Inhibitor (1 mM)	Activity (%)
None	100
$CaCl_2$	94.8
$CdCl_2$	78.8
$CoCl_2$	105.3
$CuSO_4$	61.7
$FeCl_2$	91.3
$MgCl_2$	109.8
$MnCl_2$	96.5
$NiCl_2$	88.8
$ZnCl_2$	66.4
PMSF	35.8
EDTA	91.2
EGTA	97.5
Leupeptin	91.5
Aprotinin	93.4

Table 3. Comparison of FIN-WRL101 with other proteases for activity.

Protease	FA (U)[a]	CA (U)[b]	SA[c]
FIN-WRL101	98.9±4.2	107.6±3.1	0.92
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

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

Effect of inhibitors and metal ions on fibrinolytic activity

The effects of various inhibitors and metal ions on the fibrinolytic activity of FIN-WRL101 are summarized in Table 2. FIN-WRL101 was inhibited by 1 mM PMSF, but EDTA, EGTA, Leupeptin and Aprotinin did not inhibit the fibrinolytic activity, indicating that FIN-WRL101 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+}, Mn^{2+} or Ni^{2+}, which it is similar to that of subtilisin D5 (Choi et al., 2009a) from *B. amyloliquefaciens* DJ-5. However, FIN-WRL101 was some activated by Mg^{2+}

Comparison of the specific activity of FIN-WRL101 with other proteases

The specific activity (F/C, the ratio of fibrinolytic activity to caseinolytic activity) of FIN-WRL101 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 FIN-WRL101 was 1.88 and 2.79 times higher than those of subtilisin BPN' and Carlsberg, respectively. But, it was 1.42 and 1.26 times lower than that of subtilisin D4 (Kim and Choi, 2000) and D5 (Choi et al., 2009a), respectively.

Amidolytic activity using synthetic substrates

The amidolytic activity of the FIS-WRL101 was investigated with several synthetic substrates. FIN-WRL101 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 *Bacillus natto* (Fujita et al., 1993) and subtilisin D5 from *B. amyloliquefaciens* DJ-5 (Choi et al., 2009a) also showed high activity for this substrate of chymotrypsin.

N-Terminal amino acid sequence of FIS-WRL101

The N-terminal amino acid sequence of FIN-WRL101 was analyzed by the automated Edman degradation

Table 4. Comparative amidolytic activity of the FIN-WRL101 for the hydrolysis of several synthetic substrates.

Synthetic substrate (0.8 mM)	Substrate hydrolysis (nmol/ml/min)
H-D-Pro-Phe-Arg-*p*NA[a] (Plasma kallikrein)	0
N-α-Benzyloxycarbonyl-D-Arg-Gly-Arg-*p*NA (Factor Xa, trypsin)	1.6
H-D-Phe-Pip-Arg-*p*NA (Thrombin)	0
H-D-Ile-Pro-Arg-*p*NA (broad spectrum of serine protease)	0.5
Meo-Suc-Arg-Pro-Tyr-*p*NA (Chymotrypsin)	23.9
Glu-Gly-Arg-*p*NA (Urokinase)	0
H-D-Val-Leu-Lys-*p*NA (Plasmin, Plasminogen)	0.4

[a] *p*NA, *p*-Nitroaniline.

Table 5. N-terminal amino acid sequence and molecular mass of FIN-WRL101, compared with other fibrinolytic enzymes in literature data.

Fibrinolytic enzyme	Sequencing	kDa	Strain	Author
FIN-WRL101	AQSVPYGISQIKA	29	*B. subtilis* WRL101	In this study
Subtilisin D5	AQSVPYGISQIKAPA	30	*B. amyloliquefaciens* DJ-5	Choi et al. (2009a)
Nattokinase (NK)	AQSVPYGISQIKAPALHS	27.7	*B. subtilis* natto	Fujita et al. (1993)
Subtilisin E	AQSVPYGISQIKAPALHS	55.8	*B. subtilis* sp.	Wong et al. (1984)
Subtilisin FS33	AQSVPYGIPQIKAPA	30	*B. subtilis* DC33	Wang et al. (2006)
Subtilisin DFE	AQSVPYGVSQIKAPALHS	28	*B. amyloliquefaciens* DC-4	Peng et al. (2003)
SMCE	AQTVPYGIPQIKAD	30	*B. pumilus* TYO-67	Takahashi et al. (2004)
CK	AQTVPYGIPLIKAD	28.2	*Bacillus* sp. CK 11-4	Kim et al. (1996)
Subtilisin Carlsberg	AQTVPYGIPLIKAD	-	*B. licheniformis*	Smith et al. (1968)
Subtilisin IMR-NK1	AQPVPNGRTAIKA	31.5	*B. subtilis* IMR-NK1	Chang et al. (2000)
KA38	VYPFPGPIPN	41	*B. subtilis* KA38	Kim et al. (1997)
KK	IVGGYEQZAHSQPHQ	38	*B. firmus* NA-1	Seo et al. (2004)

method after SDS-PAGE and electroblotting. The sequence of the first 13 residues was found to be AQSVPYGISQIKA, which is identical to that of subtilisin NAT (formerly designated Nattokinase from *B. subtilis* natto) (Fujita et al., 1993), subtilisin E (from *B. subtilis* sp.) (Wong et al., 1984) and subtilisin D5 (from *B. amyloliquefaciens* DJ-5) (Choi et al., 2009a) (Table 5). Amino acids A-Q (positions 1 and 2) and I-K-A (positions 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 FIN-WRL101 is a subtilisin-like serine-type fibrinolytic enzyme that occurs as a monomer.

Fibrinogenolytic activity of FIN-WRL101

FIN-WRL101 showed high fibrinogenolytic activity, degrading predominantly the Aα-chain of human fibrinogen within 10 min. By comparison, FIN-WRL101 degraded the Bβ-chain slowly and did not cleave the γ-chains (Figure 5), indicating that it is an α-fibrinogenase and different from subtilisin BPN' and FS33 (Wang et al., 2006).

In general, fibrin(ogen)olytic enzymes belong to as two classes, the α(β)-fibrinogenases (known as zinc-metalloproteinases) and the β-fibrinogenases (known as thermostable serine proteinases) (Siigur et al., 1996; Lee et al., 1999). On the basis of these results, FIN-WRL101 is a serine-type alkaline chymotrypsin-like α(β)-fibrinogenase. Furthermore, our results demonstrate that FIN-WRL101 is highly specific for the Aα-chain of human fibrinogen. Hence, this study highlights the potential for FIN-WRL101 as an effective thrombolytic agent. Investigations to further characterize FIN-WRL101 are underway.

Cloning of FIN-WRL101 gene

FIN-WRL101 gene of *B. subtilis* WRL101 was cloned and its nucleotide and deduced amino acid sequences were

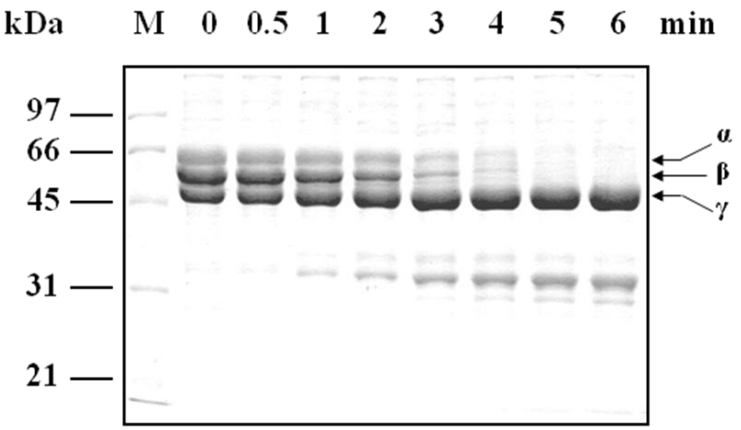

Figure 5. SDS-PAGE of the digested human fibrinogen by FIN-WRL101 under reducing conditions. Fibrinogen consisted of 3-polypeptide chains: Aα (66,000), Bβ (54,000), and γ (48,000). Fifteen mg of fibrinogen incubated with enzymes for each indicated times were subjected to electrophoresis on 10% gel.

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FIN-WRL101    -MRSKKLWISL LFALTLIFTM AFSNMS-AQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ FKYVNAAAAT  79
NAT           -MRGKKLWISL LFALTLIFTM AFSNMS-AQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ FKYVNAAAAT  79
Amylosacch    -MRGKKLWISL LFALTLIFTM AFSNMS-AQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ FKYVNAAAAT  79
J             -MRGKKLWISL LFALTLIFTM AFSNMS-VQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ FKYVNAAAAT  79
DJ-4          -MRGKKVWISL LFALALIFTM AFGSTSPAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI SEKGGKVQKQ FKYVDAASAT  80
BPN'          -MRGKKVWISL LFALALIFTM AFGSTSSAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI SEKGGKVQKQ FKYVDAASAT  80
Carlsberg     MMRKKSFWLGM LTAFMLVFTM AFSDSASAAQ PAKN-VEKDY IVGFKS--GV KTASVKKDII KESGGKVDKQ FRIINAAKAK  78

FIN-WRL101    LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLN VRGGASFVPS 159
NAT           LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLN VRGGASFVPS 159
Amylosacch    LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLN VRGGASFVPS 159
J             LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLN VRGGASFVPS 159
DJ-4          LNEKAVKELK KDPSVAYVEE DHVAQAYAQS VPYGVSQIKA PALHSQGFTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS 160
BPN'          LNEKAVKELK KDPSVAYVEE DHVAHAYAQS VPYGVSQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS 160
Carlsberg     LDKEALKEVK NDPDVAYVEE DHVAHALAQT VPYGIPLIKA DKVQAQGFKG ANVKVAVLDT GIQASHPDLN VVGGASFVAG 158

FIN-WRL101    ETNPYQDGSS HGTHVAGTIA ALNNSIGVLG VAPSASLYAV KVLDSTGSGQ YSWIINGIEW AIANNMDVIN MSLGGPTGST 239
NAT           ETNPYQDGSS HGTHVAGTIA ALNNSIGVLG VAPSASLYAV KVLDSTGSGQ YSWIINGIEW AIANNMDVIN MSLGGPTGST 239
Amylosacch    ETNPYQDGSS HGTHVAGTIA ALNNSIGVLG VSPSASLYAV KVLDSTGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGST 239
J             ETNPYQDGSS HGTHVAGTIA ALNNSIGVLG VSPSASLYAV KVLDSTGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGST 239
DJ-4          ETNPFQDNNS HGTHVAGTVA ALNNSVGVLG VAPSASLYAV KVLGADGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGSA 240
BPN'          ETNPFQDNNS HGTHVAGTVA ALNNSIGVLG VAPSASLYAV KVLGADGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGSA 240
Carlsberg     EAYN-TDGNG HGTHVAGTVA ALDNTTGVLG VAPSVSLYAV KVLNSSGSGT YSGIVSGIEW ATTNGMDVIN MSLGGPSGST 237

FIN-WRL101    ALKTVVDKAV SSGIVVAAAA GNEGSSGSTS TVGYPAKYPS TIAVGAVNSS NQRASFSSVG SELDVMAPGV SIQSTLPGGT 319
NAT           ALKTVVDKAV SSGIVVAAAA GNEGSSGSTS TVGYPAKYPS TIAVGAVNSS NQRASFSSVG SELDVMAPGV SIQSTLPGGT 319
Amylosacch    ALKTVVDKAV SSGIVVAAAA GNEGSSGSSS TVGYPAKYPS TIAVGAVNSS NQRASFSSAG SELDVMAPGV SIQSTLPGGT 319
J             ALKTVVDKAV SSGIVVAAAA GNEGSSGSSS TVGYPAKYPS TIAVGAVNSS NQRASFSSAG SELDVMAPGV SIQSTLPGGT 319
DJ-4          ALKAAVDKAV ASGVVVVAAA GNEGTSGGSS TVGYPGKYPS VIAVGAVNSS NQRASFSSVG SELDVMAPGV SIQSTLPGNK 320
BPN'          ALKAAVDKAV ASGVVVVAAA GNEGTSGSSS TVGYPGKYPS VIAVGAVDSS NQRASFSSVG PELDVMAPGV SIQSTLPGNK 320
Carlsberg     AMKQAVDNAY ARGVVVVAAA GNSGSSGNTN TIGYPAKYDS VIAVGAVDSN SNRASFSSVG AELEVMAPGA GVYSTYPTST 317

FIN-WRL101    FGAYNGTSMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGSSFYY GKGLINVQAA AQ 381
NAT           YGAYNGTSMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGNSFYY GKGLINVQAA AQ 381
Amylosacch    YGAYNGTSMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGNSFYY GKGLINVQAA AQ 381
J             YGAYNGTSMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGNSFYY GKGLINVQAA AQ 381
DJ-4          YGAYNGTSMA SPHVAGAAAL ILSKHPNWTN TQVRSSLENT TTKLGDAFYY GKGLINVQAA AQ 382
BPN'          YGAYNGTSMA SPHVAGAAAL ILSKHPNWTN TQVRSSLENT TTKLGDSFYY GKGLINVQAA AQ 382
Carlsberg     YATLNGTSMA SPHVAGAAAL ILSKHPNLSA SQVRNRLSST ATYLGSSFYY GKGLINVEAA AQ 379
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Figure 6. Comparison of the amino acid sequence of FIN-WRL101 with those of other subtilisin proteases. FIN-WRL101, fibrin induced nattokinase WRL101 from *Bacillus subtilis* WRL101 (this study); NAT, subtilisin NAT from *B. subtilis* (Nakamura et al., 1992); Amylosacch, subtilisin Amylosaccharicus from *B. amylosaccharicus* (Kurihara et al., 1972); J, subtilisin J from *B. subtilis* (Jang et al., 1992); DJ-4, subtilisin DJ-4 from *Bacillus* sp. DJ-4 (Kim and Choi, 2000); BPN', subtilisin BPN' from *B. amyloliquefaciens* (Smith et al., 1968); Carlsberg, subtilisin Carlsberg from *B. licheniformis* (Smith et al., 1968). Identical amino acids between FIN-WRL101 and others are asterisked.

determined. The nucleotide sequence revealed only one open reading frame (ORF), composed of 1143 base pairs and 381 amino acid residues, which proved to be identical to that of subtilisin NAT (Nakamura et al., 1992), J (Jang et al., 1992) and amylosacchariticus (Kurihara et al., 1972), but differed from that of subtilisin BPN' (Kaneko et al., 1989) (1146 base pairs and 382 amino acids). The amino acid sequence of FIN-WRL101 was compared with the published sequences of the other subtilisins (Figure 6), and was found to show 98.7% identity with subtilisin NAT, 98.2% with subtilisin J, 98.4% with amylosacchariticus, 86.1% with subtilisin BPN' and 65.6% with subtilisin Carlsberg. FIN-WRL101 was found to be identical to subtilisin NAT, except for five amino acid substitutions (boxed); one in the signal peptide region and four in_the mature subtilisin region (Ala-107, underlined). The catalytic triad (Asp-32, His-64 and Ser-221, using the numbering of mature FIN-WRL101) and the amino acid sequences near these amino acid residues were well conserved (Figure 6). Further works should be done concerning the performance of FIN-WRL101 enzyme *in vivo*.

ACKNOWLEDGEMENTS

This research was supported by a HTS-based Integrated Technology Development grant (2008-04171) from the Ministry of Education, Science and Technology through the National Research Foundation of Korea.

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