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Bioproperties of Potent Nattokinase from *Bacillus subtilis* YJ1

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Fibrinolytic enzyme activity was observed during cultivation of *Bacillus subtilis* YJ1 in a medium containing 1% skim milk, 1% rice husk, 0.5% NaCl, and 0.25% glucose. It was purified to electrophoretical homogeneity after CM-sepharose FF chromatography. The specific activity and yield were 1791.9 FU/mg and 9.5%, respectively. This purified fibrinolytic enzyme had M of 27.5 kDa, optimal temperature and pH at 50 °C and 8.5, respectively. It was stable at pH 6.0−10.0 and 10−40 °C and inhibited by Fe^{3+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, and PMSF. Compared the N terminal of amino acids and full DNA sequence with those in NCBI, it was considered to be a nattokinase.

KEYWORDS: ***Bacilus subtilis*; nattokinase bioproperties and purification**

INTRODUCTION

Intravascular thrombosis, the accumulation of fibrin in blood vessels or in a chamber of heart, easily causes myocardial infarction and other cardiovascular diseases (CVD), resulted in 17 million of people dying every year as reported by the World Health Organization (*1*). Nowadays, the thrombolytic therapies of both injection and oral administration of thrombolytic agents have been intensively investigated (*2*, *3*).

Natto, a fermented soybean food, has been consumed by oriental people for more than 1000 years. Its fermentation microbe is a Gram-positive endospore forming bacterium, *Bacillus subtilis* natto (formerly called *Bacillus natto*) (*4*), which can secret nattokinases (formerly designated as subtilisin NAT) (*5*, *6*). They first discovered and extracted from Japanese traditional fermented soybean foods were of particular interest due to their effective biological thrombolysis of fibrin and clots of blood in blood vessels (*5*, *6*). They could also be purified from Chinese fermented soybean food (douchi) (*7*) and brewing rice wine (*8*), Indonesian fermented soybean (tempeh) (*9*), Korean fermented soybean sauce (chungkook-jang) (*10*), salty fermented fish (*11*), and fermented shrimp paste (*12*). The catalytic center of nattokinase, with 275 amino acids, contains three conserved residues, Asp-32, His-64, and Ser-221 (*13*), while the molecular mass and isoelectric point were 27.7 kDa and 8.7, respectively (*14*). Its gene sequence is homologous to those of other members of subtilisin family (99.5% homology with subtilisin E, 86% with subtilisin BPN′, and 72% with subtilisin Carlsberg) (*15*). It not only degrades fibrin in thrombi (*16*) but also cleaves plasminogen activator inhibitor type I (*16*, *17*). Nattokinase has greater thrombolytic activity than plasmin (*14*, *16*, *18*), a natural thrombolytic protease in blood, and increases the production of plasmin from plasminogen due to its action on plasminogen activator. These observations, together with the fact that it can be absorbed across the intestinal tract after oral administration (*19*, *20*) and subsequently induce fibrinolysis (*20*), make nattokinase to be a potential clot-dissolving agent for the treatment of cardiovascular disease. Because nattokinase suppresses the intimal thickening of arties and leads to the lysis of mural thrombi observed after endothelial injury (*2*), the dietary supplementation with nattokinase-related foods has been considered to be safe and healthful for circulation system in human body (*2*, *6*). Other clinically thrombolytic agents, such as urokinase and streptokinase, are costly and unstable in the intestinal tract (*21*). The use of oral administration of nattokinase in fibrinolytic therapy for thrombosis and prevention of atherosclerosis is therefore of interest. Accordingly, nattokinase is currently used as a nutrient supplement to improve the blood circulation in humans (*2*, *6*, *20*).

Although many bacteria, actinomyces, algae, and fungi have been found to have fibrin-digestion abilities, the genus *Bacillus*, important GRAS strains from traditional fermented foods, could produce a high yield of fibrinolytic enzymes for further commercial application (*5*). In our laboratory, a nattokinase-producing bacterium *Bacillus subtilis* YJ1 has been isolated from fermented soybean foods, identified and found to have high fibrin-hydrolysis ability. This study aimed to purify and characterize the nattokinase from this strain. The bioproperties of nattokinase will provide useful references or clues for the further studies on the application of this enzyme.

MATERIALS AND METHODS

Microorganism and Culture. The *Bacillus subtilis* YJ1, isolated from fermented soybean foods, was cultured in a medium containing 1% skim milk, 1% rice husk, 0.5% NaCl, and 0.25% glucose. The viable cell counts (CFU/mL), fibrinolytic activity, and pH were measured during incubation. After 96 h incubation at 37 °C with 150 rpm shaking, the cultured broth was used for the further fibrinolytic enzyme purification.

Enzyme Activity Assay. Fibrinolytic enzyme activity was determined by measuring the hydrolysis ability on fibrin according to Chang et al. (*18*)

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with some modifications. To 1.4 mL of 50 mM sodium borate buffer (pH 8.5), a 0.4 mL of 0.72% (w/v) fibrinogen solution was added and kept at 37 °C for 5 min. To the resulting fibrinogen solution was then added 0.1 mL of thrombin (20 U/mL) and kept at 37 °C for another 10 min. After the addition of 0.1 mL of enzyme, the proteolytic reaction was performed at 37 °C for 60 min. After the reaction was ceased by adding 2 mL of 0.2 M trichloroacetic acid (TCA), samples were kept at ambient temperature for 20 min and then centrifuged at 14000*g* for 5 min. One unit of fibrinolytic activity was defined as the amount of enzyme that caused an increase of 0.01 in the absorbance at 275 nm within 60 min reaction at 37 °C.

Determination of Protein Concentration. Protein concentration was determined by dye binding method (*22*) using bovine serum albumin as standard.

Purification of Fibrinolytic Enzyme. After 30 min centrifugation at 8000*g* and filtered through a 0.45 μm membrane, crude fibrinolytic enzyme in broth was precipitated by 40−60% saturation of ammonium sulfate. The precipitate was centrifuged at 8000*g* for 15 min and dialyzed against 20 mM Tri-HCl buffer (pH 7.0; buffer A) overnight. The resulted crude enzymes were eluted by CM-sepharose FF (2.6 cm × 30 cm) with buffer A containing a linear gradient of 0.0 to 1.0 M NaCl at a flow rate of 0.5 mL/min. Fractions with fibrinolytic activity were collected and subjected to fibrinolytic activity assay and electrophoresis (SDS-PAGE).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was performed mainly according to Laemmli (*23*). To the purified enzyme, an equal volume of sample buffer (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 2% SDS in 0.25 M Tris−HCl buffer; pH 6.8) was added and boiled at 100 °C for 5 min. The resulted samples were then subjected to SDS-PAGE (resolving gel: 12.5%, stacking gel: 4%) by using a Mini-Protein II system (Bio-Rad, USA). Electrophoresis was performed under 100 V at room temperature for 1.5 h.

N-Terminal Amino Acid Sequence Analysis. Purified fibrinolytic enzyme was subjected to SDS-PAGE and then electro-transferred onto polyvinylidene difluoride membrane (PVDF). Proteins were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences were analyzed by Edman degradation (*24*) and performed by using an Applied Biosystems Procise Sequencer.

Determination of Activation Energies (E_a). After 30 min incubation at 30−70 °C, the purified enzymes were cooled down in ice water for 5 min and then measured the residual fibrinolytic activity. Arrhenius plot was drawn using $10^3/T$ (where T is the absolute temperature in °K) as X-axis and K_D as Y-axis (K_D: the rate constant of thermal inactivation, calculated from the equation of $K_D = (\ln C_0 - \ln C_t)1/t$, where C_0 and C_t are fibrinolytic activities before and after heating treatment, respectively, and t is heating time in second). The activation energy (E_a) was determined from the slope of Arrhenius plots (slope = $E_a/2.303R$ where R (1.976 cal/mol) is the ideal gas constant).

Effects of pH and Temperature. The effects of pH and temperature on fibrinolytic enzyme were determined using fibrin as substrate. The optimal pH was determined by measuring the activity of purified fibrinolytic enzyme at pH 3.0−11.0 (pH 5.0−6.0 using 20 mM citrate buffer, pH 5.0−7.0 using 20 mM sodium phosphate buffer, pH 7.0−9.0 using 20 mM Tris-HCl buffer, and pH 9.0−12.0 using 20 mM sodium carbonate buffer), while the optimal temperature of fibrinolytic enzyme in buffer A was measured at temperatures from 10 to 70 °C according to Chang et al. (*18*).

The pH stability was determined by incubating fibrinolytic enzyme in various buffers with pH 3.0−11.0 (pH 5.0−6.0 using 20 mM citrate buffer, pH 5.0−7.0 using 20 mM sodium phosphate buffer, pH 7.0−9.0 using 20 mM Tris-HCl buffer, and pH 9.0−12.0 using 20 mM sodium carbonate buffer) at 37 °C for 30 min. The residual activity was determined according to Chang et al. (*18*).

The thermal stability was determined by incubating fibrinolytic enzyme in buffer A at temperatures from 10 to 70 °C for 30 min. The residual activity was then determined according to Chang et al. (*18*).

Effects of Metal Ion or Inhibitor. Purified fibrinolytic enzyme in buffer A with various concentrations (1.0, 5.0, and 10 mM) of metal ions (Ag^+, K^+, Na^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{3+}; counterion: chloride), and various concentrations (0.5, 1.0, and 2.0 mM) of chemicals [ethylene diamine tetraacetic acid (EDTA), iodoacetic acid (IAA), leupeptin, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonyl fluoride (PMSF), *N*-toyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanineable (TPCK)] were incubated at 37 °C for 30 min. The residual activity was measured according to Chang et al. (*18*).

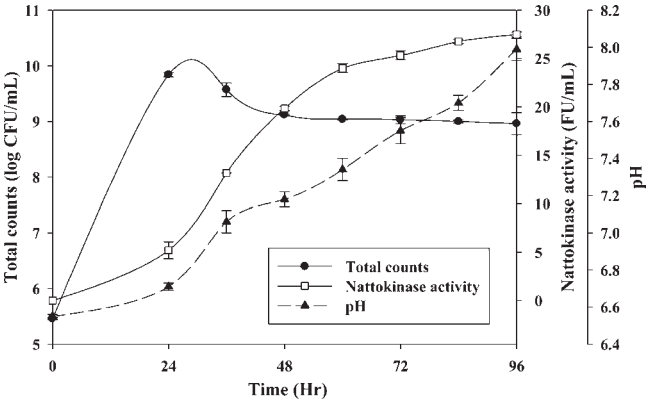

Figure 1. Changes in pH, growth, and fibrinolytic activity during 96 h incubation of *Bacillus subtilis* YJ1 at 37 °C.

Fibrin Plate Assay. Fibrin plates assay was carried out according to Chang et al. (*18*) with slight modification. The fibrin plates were prepared by adding 1.0% of agarose to 1.5% fibrin. After the agarose was dissolved with heating at 70 °C, 10 mL of the mixture was poured into a Petri dish. It was cooled at room temperature and two holes with a diameter of 2 mm on fibrin plate were punched. After the addition of 20 μL of purified fibrinolytic enzyme and commercial nattokinase into holes individually, samples were incubated at 37 °C for 24 h. The transparent zones were recorded.

Molecular Cloning with pGEM-T Vector. Total Genomic DNA was extracted from *B. subtilis* YJ1 according to Ausubel et al. (*25*), while cloning was performed according to Sambrook et al. (*26*). Polymerase chain reaction (PCR) was used to amplify nattokinase DNA with 33 cycles (30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s extension at 72 °C) and then 10 min of DNA thermal cycler at 72 °C (2720 Thermal Cycler; Applied Biosystems, Foster, CA). Primer was designed according to the alignment between N-terminal amino acid of purified nattokinase and functional sequences from NCBI Database (http://www.ncbi.nlm.nih.gov/) and used for PCR. The forward primer was 5′-(RTG AGA RGC AAA AAR KTR TRG ATC AG)-3′ (R: G or A; K: G or T), while the reverse primer was 5′-(*TTA* YTG TGC WGC TGC TTG TAC GTT G)-3′ (Y: T or C; W: A or T). The underlined sequences were start codons, while the *italic* sequences were stop codons. After amplification, the PCR product was ligated with pGEM-T easy vector and transformed into cloning host, *E. coli* Top 10F′, according to Hanahan and Meselson (*27*). After blue/white selection, plasmid from transformed *E. coli* was extracted and prepared for DNA sequencing using T7 and SP6 as sequencing primers.

RESULTS AND DISCUSSION

Enzyme Production and Purification. As indicated in **Figure 1**, the highest pH and maximum fibrinolytic activity were observed after 24 and 96 h incubation, respectively. The steady phase appeared between 24 and 96 h incubation at 37 °C. This phenomenon suggested that this strain produced maximum proteases between logarithmic and stationary phases. Therefore, broth of *B. subtilis* YJ1 after 96 h incubation at 37 °C was collected for further fibrinolytic enzyme purification and characterization.

As shown in **Table 1**, about 42% and 9.5% of fibrinolytic enzymes were recovered after precipitation with 40−60% saturation of ammonium sulfate and CM sepharose FF chromatography, respectively. Most of contaminant was excluded during CM sepharose FF ion exchange chromatography (data not shown), and the fibrinolytic enzyme was purified to electrophoretical homogeneity (**Figure 2**) with specific activity of 1791.9 FU/mg and purification fold of 9.2, respectively (**Table 1**).

Table 1. Summary of Purification of Fibrinolytic Enzyme (Nattokinase) from *Bacillus subtilis* YJ1

procedure	total activity (FU)	total protein (mg)	specific activity (FU/mg)	purification (fold)	yield (%)
crude enzyme	10575.0	54.0	195.8	1.0	100.0
fraction of ammonium sulfate (40−60%)	4450.5	15.0	296.7	1.5	42.1
CM sepharose fast flow	1003.5	0.6	1791.9	9.2	9.5

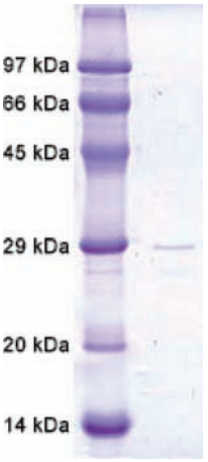

Figure 2. SDS-PAGE of purified fibrinolytic enzyme from *Bacillus subtilis* YJ1 (M: marker; I: purified enzyme after CM Sepharose FF ion exchange chromatography).

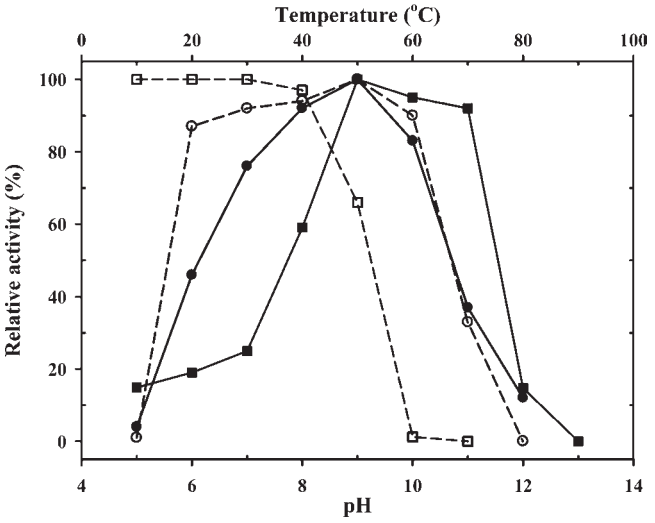

Figure 3. Effect of pH and temperature on the purified fibrinolytic enzyme (○ and ●, pH; □ and ■, temperature; − − −, optimum; — — —, stability).

Molecular Mass (*M*) and N-Terminal Amino Acid Sequence. The molecular mass (M) of purified fibrinolytic enzyme was 27.5 kDa, determined by SDS-PAGE (**Figure 2**) and Sephacryl S-100 HR chromatography (data not shown). This M was similar to that from the vegetable cheese natto (*14*) but lower than those from *Bacillus* species, 30.0 kDa from *Bacillus subtilis* DC33 (*28*), 31.5 kDa from *Bacillus subtilis* IMR-NK1 (*18*), and 44.0 kDa from *Bacillus* sp. KDO-13 (*29*). The N-terminal amino acid sequence of the purified fibrinolytic enzyme is AQSVPYGI. Comparing with those from other species in GenBank by using Basic Local Alignment Search Tool (BLAST), the purified fibrinolytic enzyme had higher homology with those from different strains of *Bacillus* species (data not shown). This information was, therefore, employed to design the primer for amplification of fibrinolytic enzyme (nattokinase) DNA from *B. subtilis* YJ1.

Table 2. Effects of Metal Ions and Chemicals on the Purified Fibrinolytic Enzyme

	relative activity (%)		
	concentration (mM)		
metal ions[a]	1.0	5.0	10.0
none	100	100	100
Ag^{+}	104	102	118
K^{+}	103	114	117
Na^{+}	96	105	114
Ba^{2+}	102	129	113
Ca^{2+}	90	103	108
Cd^{2+}	100	110	105
Co^{2+}	112	160	150
Cu^{2+}	95	18	10
Hg^{2+}	84	19	0
Mg^{2+}	100	100	117
Mn^{2+}	89	88	88
Ni^{2+}	92	106	113
Zn^{2+}	11	0	0
Fe^{3+}	40	0	0
chemicals[b]	0.5	1.0	2.0
EDTA	77	77	75
IAA	100	100	100
leupeptin	99	98	44
NEM	100	100	100
*p*CMB	100	100	100
PMSF	6	0	0
TLCK	96	96	92
TPCK	100	100	100

[a] The counterion for the tested metals was chloride. [b] EDTA: ethylene diamine tetraacetic acid; IAA: iodoacetic acid; NEM: N-ethylmaleimide; *p*CMB: *p*-chloromercuribenzoate; PMSF: phenylmethyl sulfonyl fluoride; TLCK: *N*-toyl-L-lysine chloromethyl ketone; TPCK: *N*-tosyl-L-phenylalanineable.

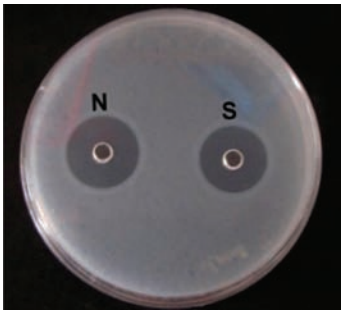

Figure 4. Fibrinolytic activity of the purified enzyme (N: purified enzyme, 0.0287FU/μL, 20 μL; S: commercial nattokinase, 0.0267FU/μL, 20 μL).

The Activation Energies (E_a). The thermal inactivation rate constants (K_D) of purified fibrinolytic enzyme at 30°, 40°, 50°, and 60 °C were 5.56×10^{-6}, 2.5×10^{-5}, 3.67×10^{-4}, and 8.5×10^{-3} $FU \cdot g^{-1} \cdot s^{-1}$, respectively. Using these thermal inactivation rate constants K_D to draw the Arrhenius plots, the slope and activation energy (E_a) were −24.786 and 49.30 (kcal/mol), respectively.

Effect of pH and Temperature. The optimal pH and temperature for the purified fibrinolytic enzyme was 8.5 and 50 °C, respectively, and stable at pH 6.0−10.0 and 10−40 °C (**Figure 3**). The optimal temperature of purified fibrinolytic enzyme was similar with these from *Bacillus* sp. KDO-13 (*29*), *Bacillus amyloliquefaciens* DC-4 (*30*), and *Bacillus subtilis* IMR-NK1 (*18*). Nevertheless, it was

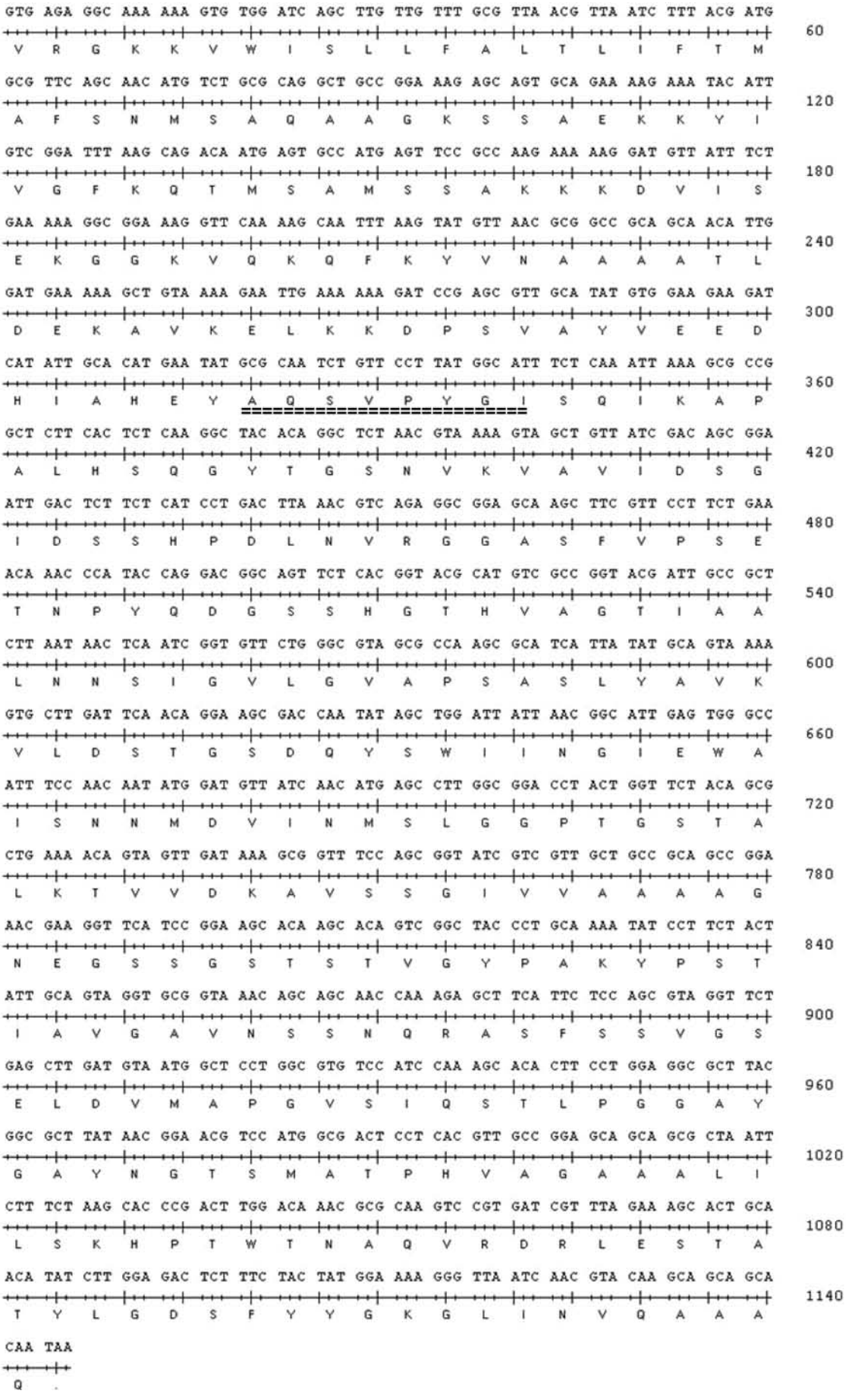

Figure 5. The full DNA sequence of nattokinase amplified from *Bacillus subtilis* YJ1. (===: result of N-terminal sequencing from mature nattokinase).

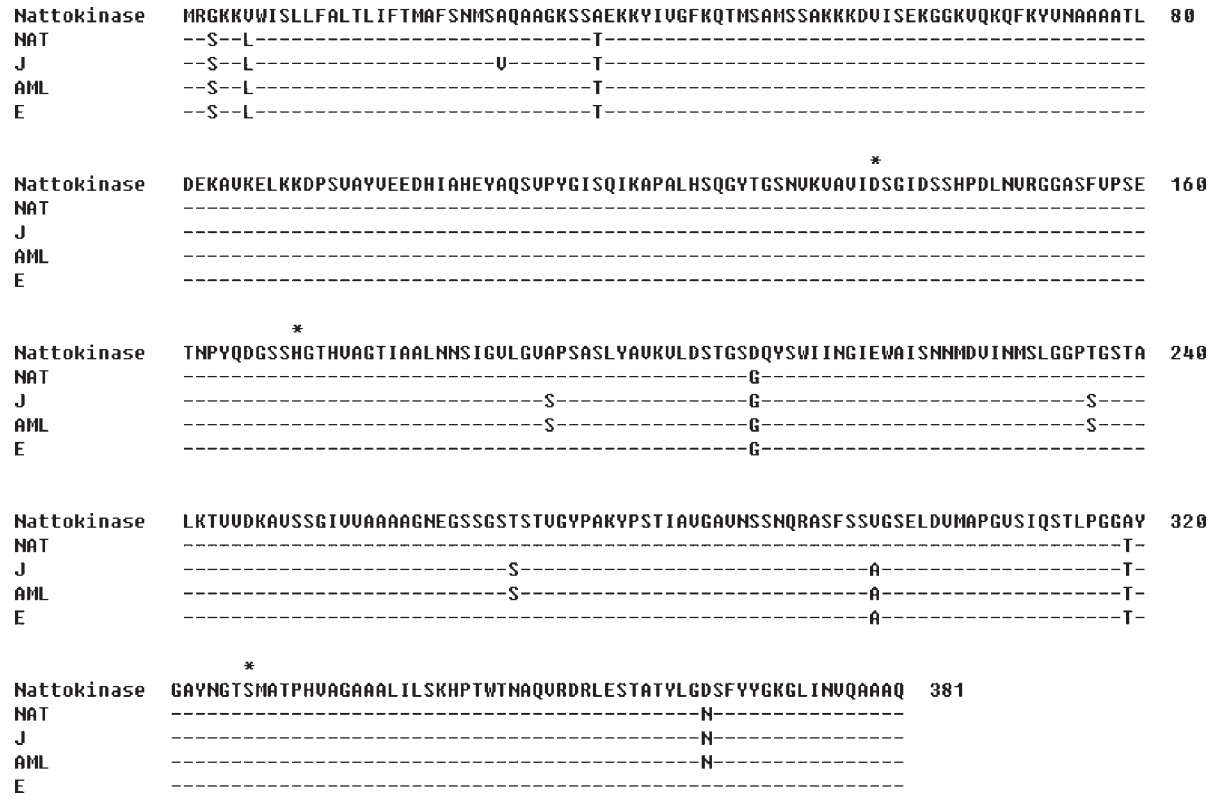

Figure 6. Deduced amino acid sequence alignment of nattokinase from *Bacillus subtilis* YJ1 and other subtilisin proteases. NAT: subtilisin NAT (nattokinase) from *B. subtilis* (*34*); J: subtilisin J from *B. subtilis* (*35*); AML: subtilisin amylosaccharicus from *B. amylosaccharicus* (*36*); E: subtilisin E from *B. subtilis* (*37*); *: active site.

lower than some of other *Bacillus* strains [60 °C (*B. subtilis* KCK-7) (*31*), and 70 °C (*Bacillus* sp. strain CK 11−4) (*10*)]. The thermal stability of purified fibrinolytic enzyme was similar to those from *B. amyloliquefaciens* DC-4 (*30*) but lower than that from *Bacillus* sp. KDO-13 (*29*) and *Bacillus* sp. Strain DJ-4 (*10*).

According to some previous studies, the optimal pH varied with strains, such as 8.0 for that from *Bacillus* sp. KDO-13 (*29*), 8.5 for that from s *Bacillus subtilis* QK02 (*32*), and 9.0 for that from *B. amyloliquefaciens* DC-4 (*30*). The pH stability of purified enzyme was similar with those from *B. amyloliquefaciens* DC-4 (pH 6.0−10.0) (*30*). It was narrower than those from *B. subtilis* IMR-NK1 (pH 5.0−10.0) (*18*) and the vegetable cheese natto (pH 6.0−12.0) (*14*) but wider that that from *B. subtilis* KCK-7 (7.0−10.0) (*31*).

Effects of Metals and Chemicals. Purified fibrinolytic enzyme was inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, and PMSF and slightly inhibited by ETDA and leupeptin, but activated by Co^{2+} and Ba^{2+}, slightly by K^{+}, Na^{+}, Cd^{2+}, Mg^{2+}, and Ni^{2+} (**Table 2**). This phenomenon suggested that the active site of the purified fibrinolytic enzyme contained SH group. These results are almost similar to that from *Bacillus* sp. strain DJ-4 (inhibited by Fe^{3+}) (*10*), *Bacillus* sp. (inhibited by Hg^{2+}) (*29*), *Bacillus subtilis* KCK-7 (inhibited by Hg^{2+}) (*31*), and *B. subtilis* DC33 (inhibited by Fe^{3+}) (*28*). According to the studies by Lee et al. (*29*) and Choi et al. (*33*), Co^{2+} and Ni^{2+} could activate the nattokinase from *Bacillus* sp. KDO-13, while Cu^{2+} and Zn^{2+} inhibited that from *Bacillus* sp. DJ-2. The purified fibrinolytic enzyme from *B. subtilis* YJ1 was also highly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Fe^{3+}, and PMSF and considered to be a serine protease.

Fibrinolytic Activity and Molecular Characterization of Nattokinase. Fibrinolytic activity of purified enzyme was almost similar to that of commercial nattokinase (**Figure 4**). The N-terminal amino acid sequence of nattokinase was AQSVPYGI, which was completely the same as that of subtilisins or nattokinase from the genus *Bacillus* (data not shown). Accordingly, it was considered to be the subtilisin family of serine protease. The DNA fragment encoded nattokinase was amplified (data not shown) from genomic DNA by PCR and cloned into pGEM-T Easy cloning vector. The full DNA of nattokinase (1146 bp; **Figure 5**) was confirmed by screening and sequencing and compared with those from different microorganisms in GenBank using BLAST. It was different from those produced by *Micrococcus luteus* and *Eisenia fetida* but up to 98% homology to those from different strains of *Bacillus* sp.. Comparison of nattokinase from *B. subtilis* YJ1 with others (**Figure 6**), it belongs to the subtilisin proteases because it has serine catalytic center and is stable at neutral to alkaline pH range. Combined with the result of alignment of N-terminal sequence and deduced amino acids sequence of purified nattokinase, it is further confirmed with the same active sites (Asp, His, and Ser). It was, therefore, considered to be a novel nattokinase from *B. subtilis* YJ1.

From the data obtained in this study, this strain, *B. subtilis* YJ1 could utilize natural wastes such as rice husk as substrate for growth and production of high activity of nattokinase. Furthermore, the nattokinase from *B. subtilis* YJ1 almost had similar fibrinolytic ability to the commercial nattokinase on fibrinolytic analysis, suggesting high potentials for the further applications in health foods and preventive medicine productions (*5*). The further optimization on the commercial scale production of nattokinase is ongoing now.

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