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ARTICLE

Purification and Characterization of Nattokinase from *Bacillus subtilis* Natto B-12

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Bacillus subtilis natto B-12 was isolated from natto, a traditional fermented soybean food in Japan. A fibrinolytic enzyme (B-12 nattokinase) was purified from the supernatant of *B. subtilis* natto B-12 culture broth and showed strong fibrinolytic activity. The enzyme was homogenously purified to 56.1-fold, with a recovery of 43.2% of the initial activity. B-12 nattokinase was demonstrated to be homogeneous by SDS-PAGE and was identified as a monomer of 29000 ± 300 Da in its native state by SDS-PAGE and size exclusion methods. The optimal pH value and temperature were 8.0 and 40 °C, respectively. Purified nattokinase showed high thermostability at temperatures from 30 to 50 °C and alkaline stability within the range of pH 6.0−9.0. The enzyme activity was activated by Zn^{2+} and obviously inhibited by Fe^{3+} and Al^{3+}. This study provides some important information for the effect factors of fibrinolytic activity, the purification methods, and characterization of nattokinase from *B. subtilis* natto B-12, which enriches the theoretical information of nattokinase for the research and development of nattokinase as a functional additive of food.

KEYWORDS: Nattokinase; purification; characterization; fibrinolytic activity; stability

INTRODUCTION

Fibrin is formed from fibrinogen by thrombin and lysed by plasmin, a serine protease activated from plasminogen by tissue plasminogen activator (t-PA). Formation and fibrinolysis of fibrin are maintained in balance by the biological system; however, under an unbalanced situation due to some disorders, fibrin cannot be hydrolyzed. The accumulation of fibrin in the blood vessels usually results in thrombosis. Thrombus in blood vessels or in a chamber of the heart usually leads to myocardial infarction and other cardiovascular diseases (CVD). According to a report by the World Health Organization, 17 million people die of CVD every year. It is presumed that the global market sales potential of thrombolytic agents will be nearly $14 billion until 2010 (*1*−*3*).

For thrombolytic therapies, both injection and oral administration of thrombolytic agents used for lysing thrombus in blood vessels have been extensively investigated. On the basis of their different mechanisms, the thrombolytic agents are classified into two types. One is plasminogen activators, which activate plasminogen into active plasmin to degrade fibrin, such as t-PA and urokinase (*4*, *5*). Streptokinase from *Streptococcus hemolyticus* (*6*, *7*) and staphylokinase from *Staphylococcus aureus* (*8*, *9*) have also been reported. The other type is plasmin-like fibrinolytic enzymes, which can directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly. Lumbrokinase from

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earthworm and fibrolase from snake venom are well-known plasmin-like proteins (*10*, *11*). Although some of the enzymes mentioned above are widely used in thrombolytic therapy currently, they have some disadvantages, such as high cost, short half-life, allergic reactions, and excessive bleeding risk within the intestinal tract when orally administered (*12*, *13*). A variety of thrombolytic agents in algae, fungi, and fermented foods were studied to provide safer and cheaper antithrombosis medicine sources. For instance, some fibrinolytic enzymes were found in marine green alga by Matsubara et al. (*14*−*16*). Another fibrinolysin was produced by fungi, such as *Cordyceps militaris*, a Chinese traditional medicinal mushroom (*17*). As traditional foods for hundreds years, fermented foods are attracting more interest in the development of fibrinolytic enzyme sources. Nattokinase (NK) was first found and extracted from natto, a traditional fermented soybean food in Japan (*18*). As an extracellular fibrinolysin produced by *Bacillus subtilis* natto (*B. natto*), NK can both hydrolyze fibrin in blood clots directly and hasten the production of t-PA, which activates plasminogen into active plasmin to hydrolyze fibrin (*19*). Following NK, different fibrinolytic enzymes were purified from Asian traditional foods, such as a Chinese fermented soybean food (douchi) (*20*) and brewing rice wine (*21*), Indonesian fermented soybean (tempeh) (*22*), Korean fermented soybean sauce (chungkook-jang) (*23*), salty fermented fish (*24*), and fermented shrimp paste (*25*).

As NK is commonly purified from the traditional food, it has high safety for thrombus therapy and, therefore, has potential for a new oral thrombolytic medicine. Oral administration of NK extracted from Japanese natto can enhance fibrinolysis in dogs with experimentally induced thrombosis. More importantly, fibrinolytic activity, the amounts of t-PA, and fibrin degradation byproduct in the plasma are doubled when nattokinase is given to human subjects by oral administration (*25*). It was also reported that dietary supplementation of natto could shorten the lysis time of euglobulin clot, which is used to evaluate the total intrinsic fibrinolytic activity in plasma. At the same time, dietary natto extract did not prolong bleeding time, indicating the excellent safety of natto as a functional food (*26*). In this study, the production conditions of nattokinase by *B. subtilis* natto B-12 were optimized. In addition, the fibrinolytic enzyme was also purified and characterized.

MATERIALS AND METHODS

Chemicals and Reagents. Urokinase (standard, 12000 IU/mg), fibrinogen (bovine), thrombin (bovine), and plasminogen (bovine) were purchased from China's National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sephadex G-75 and Phenyl-Sepharose 6 fast flow were purchased from Pharmacia Co. (Amersham Biosciense, Uppsala, Sweden). Size exclusion standards were purchased from Sigma-Aldrich Co. (Beijing, China). All other reagents were of analytical grade.

Optimization of Medium Composition and Culture Conditions for Nattokinase Production. *B. natto* B-12 strain was previously isolated from natto food by our group. The strain was cultured at 37 °C in starter culture medium (pH 7.0) containing 1% peptone, 0.5% beef extract, and 0.5% NaCl. After 24 h of cultivation, 2% (v/v) starter was transferred into basal medium (pH 7.0) containing 2% (w/v, the following is the same) glucose, 2% soybean meal, 0.5% NaCl, 0.1% KH_2PO_4, 0.4% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. In the present study, medium components including various carbon and nitrogen sources were optimized, and the concentrations of carbon and nitrogen sources were 2%. Then the concentrations (1−5%) of selected carbon and nitrogen sources were optimized. Moreover, the cultivation conditions including incubation temperature (25−40 °C), initial pH (5−10), volume of the resultant medium (50−150 mL), rotated speed of flask shaker (90−210 rpm), and incubation time (24−120 h) were investigated. The culture medium in a 250 mL Erlenmeyer flask was aerobically cultured at 37 °C for 48 h on a rotary shaker (120 rpm) (THZ-82, Huafeng Instrument Co. Ltd., Jintan, China). At the end of cultivation, the supernatant of crude enzyme was obtained by centrifugation (8000*g*, 4 °C, 20 min) (Himac CF15RX, Hitachi, Honshu, Japan).

Fibrinolytic Activity Evaluation. Nattokinase activity was defined and expressed in units of urokinase activity (IU/mL). A standard curve of urokinase activity was plotted according to the method introduced in the Chinese Pharmacopoeia (*27*). One milliliter of urokinase enzyme standard was mixed with 0.3 mL of fibrinogen (6.67 mg/mL), 0.2 mL of thrombin (6.0 IU/mL), and 0.2 mL of plasminogen solution (1.2 casein units/mL) in 0.04 mol/L barbitone−0.1 mol/L sodium chloride buffer (BSC) (pH 7.8) immediately, and then the mixture was incubated at 37 °C. The reaction mixture became a fibrinogen clot within 30−40 s (time for clot forming, t_f), and then the clot dissolved automatically. Reaction end point was controlled when the air bubble rose to half of the mixture volume (total reaction time, t_t). The difference between t_t and t_f was contrasted with a double-logarithm curve made by a series of contents of standard urokinase (10−100 IU/mL). Subsequently, NK activity was evaluated according to the standard curve of urokinase activity. This means that NK activity is equivalent to the urokinase activity (IU/mL) in that they dissolve the fibrinogen clot in same period of time. One unit of urokinase activity was defined as the amount of enzyme dissolving 1 μg of fibrinogen clot per minute per milliliter at 37 °C (*27*). The specific activity of NK was defined as activity units per milligram of protein (IU/mg). Protein concentration was determined according to the Bradford method (*28*) using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard protein.

Purification of NK. All of the purification procedures were operated at 4 °C and conducted according to the report of Ueda, Kubo, and Miyatake (*2*). The supernatant of crude enzyme was adjusted to pH 8.6, and then ammonium sulfate was added to the solution to make different saturations ranging from 30 to 80% (in increments of 10%). The precipitate was collected by centrifugation (8000*g*, 20 min) and dissolved in BSC (pH 7.8); the solution was dialyzed against the same buffer overnight. The enzyme dialysate was applied to a Sephadex G-75 column (1.5 cm × 35 cm) previously equilibrated with BSC. The column was eluted with BSC at a flow rate of 0.5 mL/min. Elution peaks were collected, and the enzyme activity and protein concentration in each fraction were determined, respectively. The fraction containing the enzyme with highest activity was collected and concentrated by ultrafiltration and then was loaded onto a Phenyl-Sepharose 6 Fast Flow column (1.6 cm × 25 cm) previously equilibrated with Tris-HCl buffer containing 1.0 mol/L ammonium sulfate. The bound proteins were eluted with a discontinuous gradient of ammonium sulfate ranging from 1.0 to 0 mol/L at a flow rate of 1 mL/min. The elution peaks were collected, and the enzyme activity and protein concentration in each fraction were determined, respectively. The active fraction was collected and concentrated by ultrafiltration; meanwhile, the ammonium sulfate was removed and then dried by freeze-dryer.

SDS-PAGE. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in Laemmli's system to determine the enzyme's purity (*29*) and the molecular mass of purified enzyme, using 4% acrylamide in the stacking gel and 12.5% acrylamide in the separating gel. Protein bands were visualized by staining with Coomassie R 250 brilliant blue. Standard proteins (Amersham Biosciences) were used for molecular mass determination as follows: myoglobulin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), albumin egg (44.3 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa).

Determination of Molecular Mass. The molecular mass of purified enzyme was determined by two methods. One is SDS-PAGE described as above, and the other one is size exclusion chromatography as previously reported (*30*). The modified parts are shown as follows: Sephadex G-75 column (1.5 cm × 35 cm), size exclusion standards contain blue dextran and five other kinds of proteins (12,400−200,000 Da) in equilibration buffer (0.05 mol/L Tris-HCl, pH 7.5, 0.1 mol/L KCl). The flow rate was 0.1 mL/min, and the operation was performed at 4 °C.

Properties of Purified Enzyme. The effect of pH on NK activity was determined by adjusting the prepared nattokinase solution (urokinase activity = 50 IU/mL) to pH values ranging from 4.0 to 12.0 at 37 °C for 30 min in the following buffer solutions: HAc−NaAc buffer (pH 4.0−8.0),

Table 1. Effects of Carbon and Nitrogen Sources, Temperature, and pH Value on NK Production[a]

nutritional components		urokinase activity (IU/mL)	cultivation conditions		urokinase activity (IU/mL)
carbon source	glucose	317.41 ± 27.44 a	incubation temperature	25 °C	205.67 ± 16.90 c
	sucrose	63.46 ± 8.78 f			
	fructose	205.92 ± 16.33 b		30 °C	424.94 ± 36.80 a
	maltose	344.93 ± 26.44 a			
	xylose	94.18 ± 9.92 e		37 °C	317.41 ± 27.44 b
	corn starch	175.24 ± 10.84 c			
	soluble starch	127.62 ± 11.70 d		40 °C	43.56 ± 5.769 d
	beef extract	206.62 ± 13.82 b			
nitrogen source	soybean meal	317.41 ± 27.44 b	initial pH	5	13.95 ± 2.28 f
	soy protein isolate	216.92 ± 19.46 c		6	62.13 ± 6.72 e
	carbamide	135.49 ± 18.45 d		7	317.41 ± 27.44 a
	$(NH_4)_2SO_4$	83.72 ± 10.11 e		8	268.82 ± 24.19 b
	peptone	246.12 ± 26.75 c		9	150.89 ± 17.66 c
	wheat bran	429.54 ± 29.16 a		10	84.32 ± 9.45 d

[a] All values are mean ± SD ($n = 3$). In each testing group (carbon source, nitrogen source, incubation temperature, and initial pH), values followed with different letters are significantly different ($p < 0.05$).

Na_2CO_3 buffer (pH 9.0−10.0), and NaOH solution (pH 11.0−12.0). Analysis of its pH stability was carried out by incubating the enzyme for 1 h in the buffer systems. The surviving fiborinolytic activity was determined right after readjustment of the pH of the enzyme solution by HCl or NaOH (pH 7.8). The effect of temperature on the activity of NK was tested by heating samples (prepared nattokinase solution in BSC buffer, pH 7.8, 50 IU/mL) from 25 to 65 °C for 30 min. The thermal stability was investigated by assaying the residual NK activity at 30−60 °C for 20, 40, 60, 80, 100, and 120 min, respectively. The effects of nine kinds of metal ions, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, and Al^{3+} at a final concentration of 5.0 mmol/L, on the nattokinase's activity were tested by adding them to the prepared nattokinase solution (BSC buffer, pH 7.8, 50 IU/mL), respectively. The purified enzyme solution without these ions added was used as control.

Statistical Analysis. Data were statistically analyzed using the SPSS (Statistical Product and Service Solutions) 11.0 package software (SPSS Inc., Chicago, IL) for the analysis of variance and *F* test. Significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Effects of Medium Composition and Culture Condition on Enzyme Production. The effects of carbon and nitrogen sources on NK production are shown in **Table 1**. The highest NK production was obtained when maltose was used. In contrast, very low enzyme production was observed when sucrose was used as carbon source. It was reported that a high concentration of sucrose usually prevented bacteria from producing protease, whereas maltose could decrease the catabolite repression and induce enzyme production (*31*). On the basis of this result, maltose was selected as the carbon source for subsequent experiments. Carbamide and ammonium sulfate gave a lower NK production. On the contrary, wheat bran was most helpful to the enzyme yield. Bran is a biological complex, which is made of starch (12−18%), protein (15−18%), dietary fiber (35−50%), fat (3−5%), and ash (4−6%) (*32*). Therefore, the positive effect of wheat bran may be attributed to the fact that it enriches aminophenol, vitamins, mineral elements, and enzymes. As shown in **Table 1**, the optimal temperature and pH value for NK production were 30 °C and pH 7, respectively. The optimal culture conditions are different from the fibrinolytic enzyme produced by *B. amyloliquefaciens* DC-4 screened from *douchi* (*33*), the optimal pH value and temperature of which are 9.0 and 48 °C, respectively. The result indicates that the optimal culture conditions of fibrinolytic enzyme significantly depend on the different sources.

The optimal media components and culture conditions are demonstrated in **Figure 1**. It was noted that the highest NK activity in the crude enzyme supernate was obtained from the culture with 2% maltose and 3% wheat bran as shown in **Figure 1A**. A higher sugar concentration caused the osmosis of the environment around the microorganism and was detrimental to metabolite biosynthesis (*34*). As shown in **Figure 1B**, NK activity obviously decreased with increasing medium volume when the medium volume was less than one-third of the total flask cubage (250 mL). Whereas the medium volume increased sequentially, the enzyme activity did not change significantly. *B. natto* is an aerophilic bacteria (*35*, *36*); less medium provides a greater amount of oxygen for microorganism multiplication and metabolism. As shown in **Figure 1C**, the acceleration of the flask shaker led to a remarkable enhancement of enzyme production, and the highest enzyme activity was gained at 180 rpm. Higher rotating speed gave richer air for aerophilic bacteria, which is advantageous to enzyme production. The results are consistent with the results in an investigation of medium volume. As shown in **Figure 1D**, the enzyme production peak was at 60 h.

Previously, extensive studies on the optimization of medium composition and culture conditions for nattokinase production by *B. subtilis* have been conducted (*33*, *37*, *38*). Nattokinase yield varied in different optimized fermentation media. However, comparison of these fibrinolytic activities is difficult due to different activity assay methods and the absence of specific activity. In our present study, after the optimization of contents in culture medium and incubation conditions, 2% starter of *B. natto* B-12 was added into 50 mL of resultant medium containing 2% maltose, 3% bran, 0.5% NaCl, 0.1% KH_2PO_4, 0.4% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0, in 250 mL flasks, and the flasks were incubated on a flask shaker at 30 °C and 180 rpm for 60 h. At the end of culture, the enzyme activity in the supernate from centrifugation was 903 IU/mL (urokinase activity units), which is nearly 3 times higher than the original medium.

Purification of NK. In the present study, the activity of the precipitates in the crude enzyme supernates with 30−80% saturations $(NH_4)_2SO_4$ was contrasted. To preserve the fibrinolytic activity of NK as much as possible, according to the activity assay, the precipitate formed in 30−60% saturation of $(NH_4)_2SO_4$ was collected. Then the dialyzed precipitate was applied to Sephadex G-75; the urokinase activity of proteins in peak B was 50 times as high as the sum of proteins in peaks A and C (**Figure 2**). After the Sephadex G-75 step, the enzyme was purified by 22.7-fold with an activity recovery of 47.6%. Proteins in peak B were pooled and concentrated and then subjected to

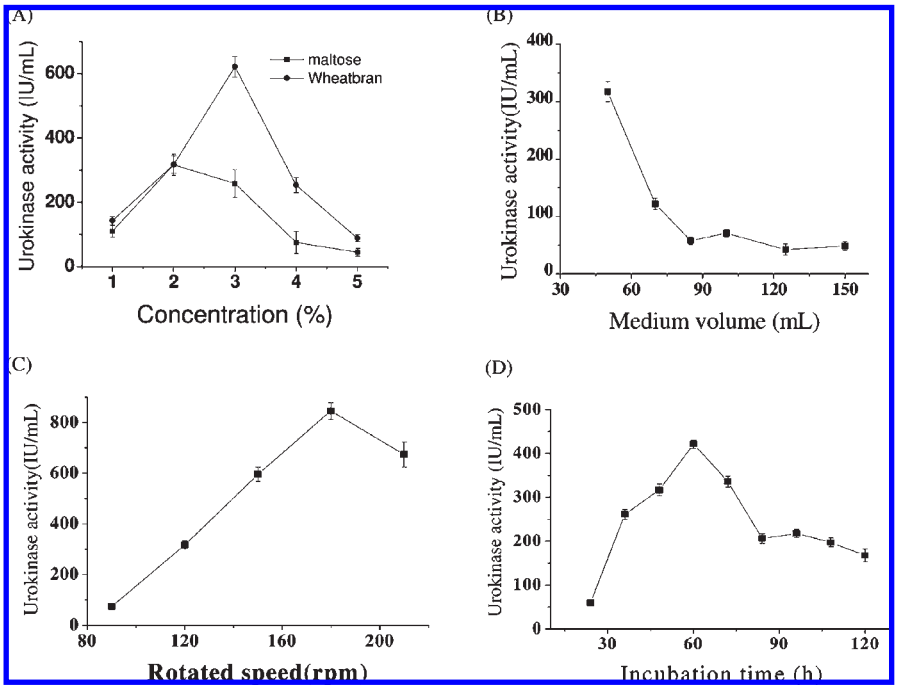

Figure 1. Effects of culture conditions on nattokinase production by *B. natto* B-12: (**A**) concentrations of maltose and wheat bran; (**B**) medium volume; (**C**) rotation speed; (**D**) incubation time. Values are means ± SD of three independent experiments.

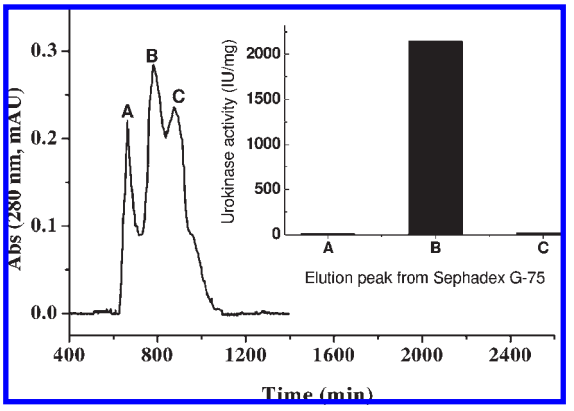

Figure 2. Elution diagram of Sephadex G-75 column of the enzyme. After precipitation by $(NH_4)_2SO_4$ solution and dialysis, the enzyme was eluted with barbitone−sodium chloride buffer (pH 7.8) at a flow rate of 0.5 mL/min at 4 °C.

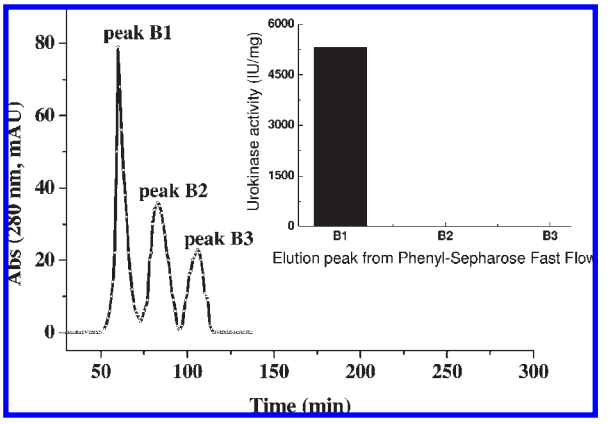

Figure 3. Elution diagram of Phenyl-Sepharose Fast Flow column of peak B from a Sephadex G-75 column. The elution was run with discontinuous gradient of $(NH_4)_2SO_4$ ranging from 1.0 to 0 mol/L at a flow rate of 1 mL/min.

hydrophobic chromatography. The elution of a Phenyl-Sepharose Fast Flow column resulted in three separate peaks, B1, B2, and peak B3 (**Figure 3**). According to the activity assay, proteins in peak B1 rather than peaks B2 and B3 exhibit fibrinolytic activity. The purity of enzyme in peak B1 was determined by SDS-PAGE (**Figure 4**). After treatment of protein sample from peak B1 with β-mercaptoethanol, SDS-PAGE shows a single band with an apparent molecular mass (M_r) of 29,000 Da, indicating that the protein in peak B1 is purified to homogeneity and contains a single subunit or more than one subunit with the same M_r of 29,000 Da. The yield and purity of the protease in each purification procedure are summarized in **Table 2**. The ammonium sulfate precipitation of the culture supernatant resulted in a 2.3-fold increase in the specific activity. After the purification procedure on Sephadex G-75, 22.7-fold was obtained. After the final purification on Phenyl-Sepharose Fast Flow, 56.1-fold purification and 43.2% yield were obtained.

A novel fibrinolytic enzyme from *B. amyloliquefaciens* DC-4 isolated from *douchi*, a traditional Chinese soybean fermented food, has been purified by ammonium sulfate treatment followed by chromatography columns on CM-Sepharose Fast Flow, DEAE-Sepharose Fast Flow, Phenyl-Sepharose 6 Fast Flow, and Sephadex G-50 (*33*). The enzyme is a serine protease and similar to nattokinase from *B. natto*. The enzyme was purified 11.5-fold with a 2.8% yield. The specific activity of the final enzyme preparation was estimated to be 4664 IU/mg (urokinase activity units). Two fibrinolytic enzymes were purified from the supernatant of *B. subtilis* QK02 culture broth by ammonium sulfate precipitation, gel filtration chromatography on Sepacryl S-100, ion-exchange chromatography on CM Sepharose Fast Flow, and gel filtration on Superdex 75. The final specific fibrinolytic activities of the enzymes were 1930 and 4100 IU/mg with the specific activity increased 2.91- and 61.9-fold (*39*). Compared with these studies, the specific activity of purified

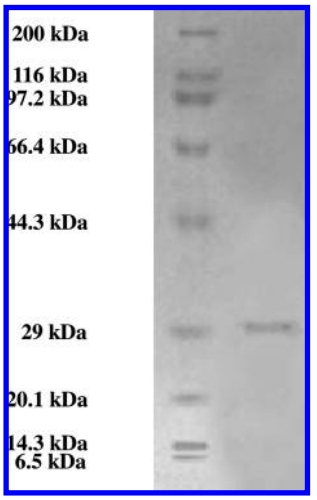

Figure 4. SDS-PAGE analysis of peak B1 from hydrophobic chromatography: left lane, molecular mass standards [myoglobulin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), albumin egg (44.3 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa)]; right lane, peak B1 from hydrophobic chromatography.

Table 2. Summary of the Purification Procedures

purification procedure	total protein (mg)	total activity (IU)	specific activity (IU/mg)	yield (%)	purification fold
culture supernate	285.7	27076	94.78	100	1
$(NH_4)_2SO_4$ precipitation	64.0	13863	216.61	51.2	2.3
Sephadex G-75	6.3	12879	2146.53	47.6	22.7
Phenyl-Sepharose Fast Flow	2.2	11696	5316.36	43.2	56.1

NK and the purification factor in the present study are relatively high.

Molecular Mass of NK. Figure 5 shows a diagram of a size exclusion chromatogram of purified NK with that of a set of protein standards. A plot of the log M_r versus the size exclusion elution coefficient (K_{av}) of protein standards is drawn as a standard curve (plot is not shown). The term K_{av} represents the fraction of the stationary gel volume that is available for diffusion of a given solute species and is calculated from the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$. In this case $V_t = V_z$ (interstitial volume) + V_p (pore volume) + V_m (matrix volume); V_0 is the void volume; V_e is the elution volume of molecules. On the basis of the standard curve, a molecular mass of 29,000 ± 300 Da (n = 3) is estimated for the purified NK in its native form. The molecular mass is approximately the same as the value from SDS-PAGE in the presence of 2-mercaptoethanol (**Figure 4**), which suggests that the purified protein in its native form is a monomer of 29,000 Da.

The molecular mass of B-12 nattokinase was obviously different from most of the other microbial nattokinases (fibrinolytic enzymes), such as the nattokinase of *Pseudomonas* sp. TKU015 (21 KDa) (*40*); the metalloprotease of *R. chinensis* 12 (18 kDa) (*21*), *Bacillus* sp. KA38 (41 kDa) (*24*), *Bacillus* sp. KDO-13 (45 kDa) (*41*); the serine protease of *B. subtilis* BK-17 (31 kDa) (*3*), *B. subtilis* IMR-NK1 (31.5 kDa) (*42*), *S. megasporus* SD5 (35 kDa) (*43*), the serine protease (QK-1) of *B. subtilis* QK02 (42 kDa) (*39*), and *B. subtilis* KCK-7 (44 kDa) (*44*). The microbial nattokinases that had a molecular mass similar to that of the B-12 nattokinase included the serine protease (QK-2) of *B. subtilis*

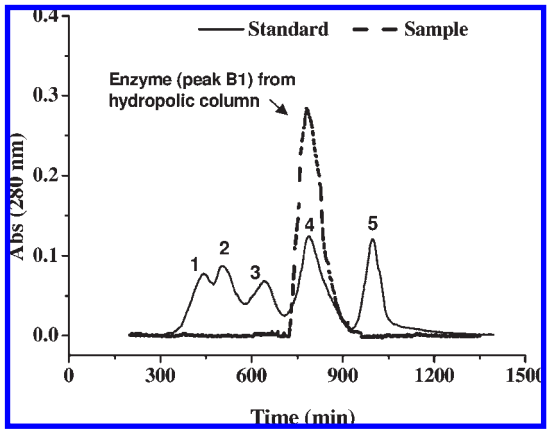

Figure 5. Overlay of a size exclusion chromatogram of the purified enzyme and protein standards. After precipitation by $(NH_4)_2SO_4$, sequential purification was carried out on Sephadex G-75 and Phenyl-Sepharose Fast Flow columns at 4 °C. Peaks: 1, β-amylase (200,000 Da); 2, yeast alcohol dehydrogenase (150,000 Da); 3, bovine serum albumin (66,000 Da); 4, carbonic anhydrase (29,000 Da); 5, horse heart cytochrome *c* (12,400 Da). The enzyme (open circle, peak B) was estimated to have a molecular mass of about 29,000 ± 300 Da (n = 3).

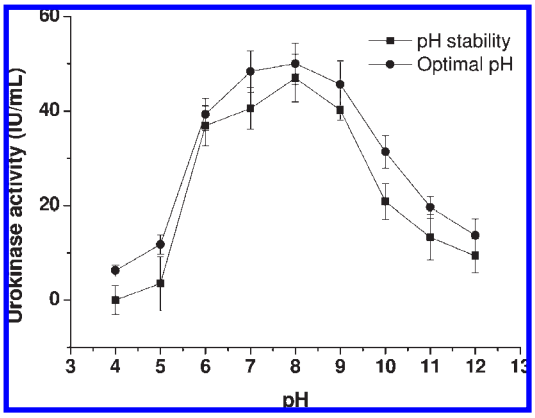

Figure 6. Optimal pH and stability of NK from *B. natto* B-12. NK was added into reaction mixtures in the following buffer solutions: HAc−NaAc buffer (pH 4.0−8.0), Na_2CO_3 buffer (pH 9.0−10.0), and NaOH solution (pH 11.0−12.0). The survival enzyme activity was measured at 37 °C right after readjustment of the pH of the enzyme solution by HCl or NaOH (pH 7.8). Values are means ± SD of three independent experiments.

QK02 (28 kDa) (*39*), the serine protease of *B. amyloliquefaciens* DC-4 (28 kDa) (*33*), the thermophilic alkaline serine protease of *Bacillus* sp. CK 11-4 (28.2 kDa) (*23*), the serine protease of *Bacillus* sp. DJ-4 (29 kDa) (*45*), and the serine protease of *B. subtilis* LD-8547 (30 kDa) (*46*).

Optimum pH, Temperature, and Stability of NK. The results of optimum pH and pH stability are shown in **Figure 6**. NK was active at neutral and alkaline pH values, and optimal reaction for NK was obtained at pH 8.0. The optimized pH value of the nattokinase was higher than many microbial fibrinolytic enzymes, such as pH 7 of *Bacillus* sp. KA38 metalloprotease (*24*), pH 7 of *Bacillus*sp. KDO-13 metalloprotease (*41*), pH 7 of *B. subtilis* A1 metalloprotease (*47*), and pH 7.5 of *B. subtilis* Jin7 nattokinase (*48*). Previous study has shown that the amino acid composition of NK plays an important role in its optimal reaction pH value (*49*). Therefore, it is speculated that, in the present study, the enzyme may contain more basic amino acids than acidic amino acids. The purified NK exhibited reduced

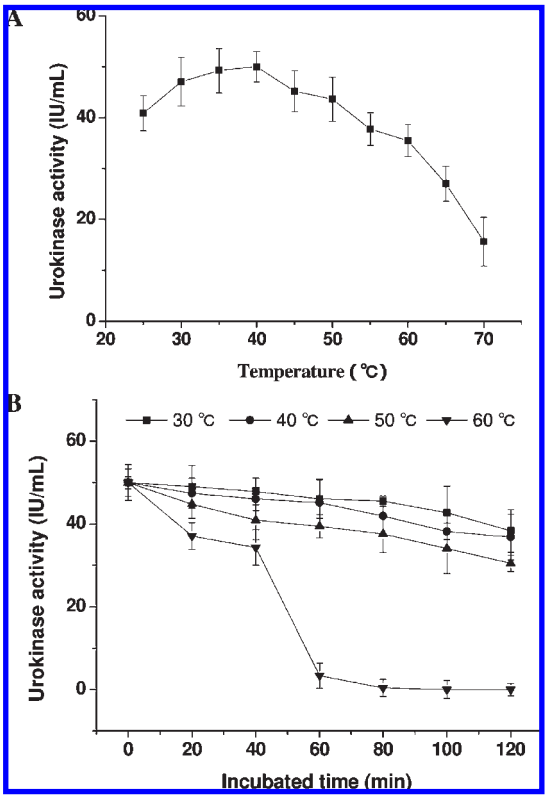

Figure 7. Optimum temperature (**A**) and thermostability (**B**) of NK from *B. natto* B-12. The enzyme reaction was carried out in barbitone−sodium chloride buffer (pH 7.8) at 25−65 °C for 30 min. The thermostability was investigated by assaying the residual NK activity at 30−60 °C for 20−120 min, respectively. Values are means ± SD of three independent experiments.

bioactivity at extremely high and low pH values. In addition, an acid condition made the enzyme activity decrease more rapidly than an alkaline condition did. The results are consistent with some previous reports (*18*, *48*, *50*, *51*). Above 80% of its activity was retained at pH values ranging from 6.0 to 9.0. NK lost all of the activity at pH 4.0, and nearly 20% of initial enzyme activity was retained at pH 12. The nattokinase from *B. subtilis* NK 040823 was stable at pH values ranging from 6.0 to 8.0 (*51*); its alkaline tolerance ability was lower than that of B-12 nattokinse in the present study. A high optimum pH in conjunction with the ability to retain activity at pH values 9−12 together indicate the alkaline tolerance characteristic of B-12 nattokinase.

The optimum temperature and thermostability of the enzyme are shown in **Figure 7**. The optimum temperature of NK was 40 °C. The protease was relatively stable below 50 °C. However, remarkable loss of activity was observed at 60 °C. The enzyme was relatively stable between 30 and 50 °C, and the enzyme activity decreased slowly with time. As the temperature was raised to 60 °C, the activity of NK showed a rapid decline. Nearly 70% of the enzyme activity was retained at 60 °C for 40 min, 80% activity was retained at 50 °C for 1 h, and the overall initial activity after 60 min of treatment at 60 °C was lost. Some previous studies on the thermostability of NK showed that the fibrinolytic enzyme was relatively stable at temperatures lower than 40 °C and lost all initial activity after 10 min at 60 °C (*42*, *50*) or 1 h at 50 °C (*51*). These results suggest that the thermostability of B-12 nattokinase is relatively higher than that of the nattokinases reported in many studies.

Effects of Metal Ions on NK Activity. To further characterize the purified B-12 nattokinase, we examined the effects of some regular divalent and trivalent metal ions on NK activity. The results are summarized in **Table 3**. Zn^{2+} activated the enzyme activity, Ca^{2+} and Mg^{2+} did not obviously activate or inhibit the enzyme activity. Co^{2+}, Cu^{2+}, Mn^{2+}, and Fe^{2+} inhibited the fibrinolytic enzyme, and the inhibitory effects of Fe^{3+} and Al^{3+} were most obvious among these metal ions. The effects of Cu^{2+} and Al^{3+} on NK activity were consistent with nattokinase from *B. subtilis* NK 040823 (*51*). Ion Zn^{2+} is known to play a major role in the activity of serine protease (*52*), which may account for the positive effect of Zn^{2+} in the present study. These results are different from another nattokinase from *Pseudomonas* sp. TKU015 (*40*) except for the effects of Ca^{2+} and Mg^{2+}. TKU015 nattokinase activity increased significantly in the presence of 5 mmol/L Fe^{2+} and was not affected by many metal ions, such as Zn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+}, and Mn^{2+}. Jin7 nattokinase activity increased significantly in the presence of 10 mmol/L Ca^{2+} and Mg^{2+}. It was partly inhibited by Fe^{2+}, Ba^{2+}, and Mn^{2+} and significantly inhibited by Cu^{2+} and Co^{2+} (*48*). The different effects of metal ions on B-12 nattokinase, TKU015 nattokinase, and Jin7 nattokinase are mainly attributed to the different sources.

Table 3. Effects of Metal Ions on NK Activity[a]

ion	urokinase activity (IU/mL)	ion	urokinase activity (IU/mL)
control	50.03 ± 5.71 b	Cu^{2+}	42.85 ± 6.32 c
Zn^{2+}	56.35 ± 7.62 a	Mn^{2+}	39.65 ± 6.08 c
Ca^{2+}	49.90 ± 7.83 b	Fe^{2+}	36.24 ± 5.43 c
Mg^{2+}	49.20 ± 7.00 b	Fe^{3+}	32.83 ± 4.50 d
Co^{2+}	43.09 ± 6.19 c	Al^{3+}	21.92 ± 3.42 e

[a] The final concentration of metal ions was 5.0 mmol/L. All values are mean ± SD (n = 3). Values followed with different letters are significantly different ($p < 0.05$).

Conclusion. The urokinase activity of crude enzyme from *B. natto* B-12 was significantly improved to 903 IU/mL by optimizing the nutritional compositions and culture conditions; a nattokinase was purified to homogeneity by ammonium sulfate precipitation, Sephadex G-75, and Phenyl-Sepharose Fast Flow column chromatography. The purified enzyme is a monomer with M_r = 29,000 ± 300 Da, which is different from most of the other microbial nattokinases. Its specific activity is 5316.36 IU/mg. Zn^{2+} activates the enzyme at the concentration of 5 mmol/L. It exhibits relatively high thermostability and alkali tolerance ability compared with some other nattokinases in previous studies. Our results indicate that nutritional composition and culture condition, as well as metal ions, have important effects on fibrinolytic activity of nattokinase from *B. natto* B-12. Further study will be conducted to investigate the biochemistry methods of improving the fibrinolytic activity of nattokinase, the molecular structure such as amino acid sequence, and secondary and tertiary structures.

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