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PURIFICATION AND CHARACTERIZATION OF NATTOKINASE FROM CULTURAL FILTRATE OF RED ALGA *PORPHYRA DENTATA* FERMENTED BY *BACILLUS SUBTILIS* N1

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Shun-Hsien Chang¹, and Guo-Jane Tsai^{1,3}

Key words: *Bacillus subtilis*, fermentation, nattokinase, *Porphyra dentata*.

ABSTRACT

Fibrinolytic enzyme nattokinase was first extracted from a traditional Japanese fermented soybean food, Natto. Edible red alga *Porphyra dentata* shares similar high protein content with soybean. In this study, we successfully purify and characterize nattokinase from the cultural filtrate of *P. dentata* fermented by *Bacillus subtilis* N1. The crude enzyme was purified by ion-exchange and gel filtration to reach electrophoretic homogeneity. The nattokinase, which has a molecular weight of 46.5 kDa and an isoelectric point of 8.35, was stable from pH 5 to 9 and at temperatures up to 55°C, and it showed optimum enzyme activity at pH 8 and at 55°C. This enzyme is characterized as a serine-protease, and its activity can be stimulated by adding CuSO₄ or FeCl₃. Our results identified the fibrinolytic nattokinase in the cultural filtrate of *P. dentata* fermented by *Bacillus subtilis* N1 and provided affecting factors to its fibrinolytic activity.

I. INTRODUCTION

Intravascular thrombosis, the formation of blood clots in blood vessel, is one of the main causes of cardiovascular disease (CVD), a major reason for disability and premature death worldwide. Fibrin, the major protein component of blood clots,

is formed from fibrinogen via the proteolytic action of thrombin. To avoid thrombosis, a process called fibrinolysis, which involves the dissolution of insoluble fibrin clots by the hydrolytic action of plasmin, is activated by tissue plasminogen activators (Cesarman-Maus and Hajjar, 2005). If the fibrin clots fail to be hydrolyzed because of some disorders, thrombosis can occur and lead to myocardial infarction and other CVDs (Meade et al., 1993; Chandler et al., 1997). Furthermore, tissue-type plasminogen activator (t-PA) (Collen and Lijnen, 2004), streptokinase (Reed et al., 1999), and urokinase (Duffy, 2002), which are widely used in the treatment of thrombosis, can activate plasminogen into active plasmin for degrading fibrin. However, these enzymes are expensive or have side effects, prompting scientists to look for safer and cheaper alternatives.

Nattokinase (formerly called subtilisin NAT) is a well-studied protease of microbial origin that possesses fibrinolytic (anti-clotting) activities (Sumi et al., 1987; Chang et al., 2000; Lee et al., 2001; Ko et al., 2004; Paik et al., 2004; Wang et al., 2009; Yin et al., 2010). It was first extracted and purified from a traditional Japanese soybean-fermented food, natto (Sumi et al., 1987), and sources and properties of nattokinase are described well by Peng et al. (2005). It has been shown that nattokinase has a greater fibrinolytic activity than plasmin, and it supports the body in degrading and dissolving the unhealthy coagulation of blood (Chang et al., 2000; Suzuki, 2003). Moreover, nattokinase hydrolyzes active recombinant prokaryotic plasminogen activator inhibitor-1 (PAI-1), indicating that fibrin clot lysis by nattokinase also involves the cleavage and inactivation of PAI-1 (Urano, 2001). These findings, along with the observation that nattokinase can be absorbed across the intestinal tract after oral administration (Sumi et al., 1990; Fujita et al., 1995), make it a promising anti-clotting agent for the prevention and treatment of CVDs. Furthermore, a recent study has revealed that nattokinase can degrade amyloid (Hsu et al., 2009), which is believed to be associated with various neurodegenerative diseases.

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The marine red alga *Porphyra* spp. are widely distributed and are traditionally used as food in East Asian countries (Wei et al., 2003). They are attracting attention as a valuable food source in Western societies because of their high mineral, vitamin, and protein contents (MacArtain et al., 2007; Smith et al., 2010). Antioxidant phenolic compounds, such as rutin, catechol, epigallocatechin gallate (EGCG), hesperidin, morin, caffeic acid, and catechin, have been identified in *Porphyra* spp. (Yoshie et al., 2000; Yoshie-stark et al., 2003; Kazłowska et al., 2010). Furthermore, *P. dentata* powder is a folk medicine used for treating various types of allergies (Kimiya et al., 2008) and inflammatory diseases, such as lymphadenitis and bronchitis. Several ethnopharmacological studies have surveyed various *Porphyra* spp. and have identified their potential for treating aging (Zhao et al., 2008), diarrhea, abdominal cramps, vomiting (Kim et al., 2006), hypersensitivity (Kimiya et al., 2008), acute liver injury (Guo et al., 2007), and cancers (Ichihara et al., 1999). In addition to the high contents of bioactive compounds in *Porphyra* spp., the alga is exempt from the negativity associated with terrestrial biomass resources, which is said to be responsible for the rising food prices. In this study, *Bacillus subtilis* N1 was grown on a medium containing only *P. dentata* powder for the production of nattokinase. The nattokinase in the culture filtrate was purified and characterized to determine its fibrinolytic activity and biological properties.

II. MATERIALS AND METHODS

1. Source of *Porphyra dentata*

Dried *P. dentata* was purchased from Don-Xie-Chen Manufacturing Factory (Makung city, Penghu Hsien, Taiwan). The dried *P. dentata* was ground, screened through a mesh (0.42 m/m) and stored in a desiccator for use.

2. Analytical Chemicals

All chemicals used in this research were of analytical grade. Trichloroacetic acid (TCA) and sodium carbonate were obtained from Merck Chemical Industries, Ltd. (Darmstadt, Germany). Acrylamide, Coomassie Brilliant Blue R 250, fibrinogen, inhibitors of iodoacetic acid (IAA), ethylenediamine tetraacetic acid (EDTA), N-ethylmaleimide (NEM), phenylmethane sulfonyl fluoride (PMSF) and tosylphenylalanine chloromethyl ketone (TPCK), resins of CM-Sepharose Fast Flow and Sephadex G-50, salts of calcium chloride, copper sulfate, ferric chloride, magnesium chloride, potassium chloride, sodium chloride and zinc chloride, sodium dodecyl sulfate (SDS), thrombin, tris (aminomethane) hydrochloride, tyrosine, protein markers and protein assay kit were purchased from Sigma Chemical Industries, Ltd. (Saint Louis, MO, U.S.A.). PhastGel IEF 3-9, IEF buffer and IEF calibration kit were purchased from GE Healthcare Life Sciences, Ltd. (Uppsala, Sweden).

3. Analysis of Proximate Composition of *P. dentata*

The proximate composition including moisture, crude protein, crude lipid, crude fiber and ash of *P. dentata* was analyzed according to AOAC official methods of analysis (AOAC, 1998). Moisture content was determined using a hot-air oven (Sanyo Electric Co., Ltd., Kaizuka, Osaka, Japan) at 100°C for 24 h. Nitrogen content was measured using a Kjeltac TM 2100 (Foss, Sweden) and the crude protein content was calculated by multiplying nitrogen content by a factor of 6.25. Crude lipids were extracted using a Soxhlet System HT 1043 Extraction Unit (Tecator, Sweden) and the crude lipid content was determined after oven-drying (100°C) the extract for 2 h. Crude fiber content was determined by filtering with a Fibertec system (Fibertec Inc., Scotland). Ash content was measured by heating the dried powder of *P. dentata* in a Furnace (Risen Instruments Co., Ltd., Taiwan) at 550°C for 12 h.

4. Microorganism and Cultivation for Nattokinase Production

B. subtilis N1 was isolated from a commercial natto product (Typhula natto, Dounanhiratsukashokuhin Co., Ltd., Noboribetsu, Hokkaido, Japan) by our group. Its potential for nattokinase production was identified by the presence of clear zone on the fibrin plate, based on the method described by Ko et al. (2004). This strain was cultivated in tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, MD, USA) at 37°C for 24 h and added with equal volume of sterile glycerol for storage at -80°C. It was sub-cultured twice in TSB at 37°C for 24 h before the use as an inoculum for the production of nattokinase.

A 5-L-fermentor containing 2 L *P. dentata* suspension (3%, w/v) was inoculated with the culture of *B. subtilis* N1 to have an initial cell density of 10⁶ CFU/mL and fermented at 37°C with an agitation rate of 150 rpm and an aeration rate of 2.0 vvm for 48 h. After centrifugation (9,600 ×g, 15 min) the supernatant was collected and defined as crude enzyme solution for following experiments.

5. Enzyme Assay

The nattokinase activity was determined by measuring its fibrinolytic activity, according to the method described by Chang et al. (2000) with some modifications. This assay was carried out by incubating the reaction mixture containing 2.5 mL of 1.2% (w/v) fibrin solution, 2.5 mL of 0.1 M Tris buffer (pH 7.8) and 1 mL of suitable diluted crude or purified enzyme solution at 37°C for 15 min before adding 5 mL of 0.1 M TCA to stop reaction. The reaction mixture was kept at room temperature for 20 min. After centrifugation (12,000 ×g, 10 min), the supernatant of reaction mixture was collected and measured for the absorbance at 275 nm. A standard curve was obtained by measuring the absorbance at 275 nm of 0-100 µg/mL tyrosine solution. One unit of the enzyme activity was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 µg of tyrosine per minute at 37°C.

6. Enzyme Purification

The crude enzyme solution was concentrated by vacuum evaporation at 40°C and filtered by 0.22 µm filter membrane to remove any bacterial cells. The concentrated crude enzyme solution was loaded onto a CM-Sepharose Fast Flow column (1.6 × 10 cm, bed volume 17 mL). The column was washed with 6 bed volumes of 10 mM Tris buffer (pH 7.4) and eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 0.2 mL/min. The fractions with fibrinolytic activity were pooled, concentrated and further loaded onto a Sephadex G-50 gel filtration column (2.6 × 90 cm). The column was eluted with 10 mM Tris buffer containing 0.5 M NaCl (pH 7.4) at a flow rate of 0.4 mL/min. The fractions containing the enzyme activity were combined, dialyzed against 50 mM Tris buffer (pH 7.8) and stored until further use. The protein concentration was determined by Lowry method using bovine serum albumin as the standard (Lowry et al., 1951).

7. Molecular Weight (MW) Determination

The MW of the enzyme was determined by using SDS-PAGE and gel filtration chromatography. The enzyme was analyzed by SDS-PAGE with standard protein markers of myosin (170 kDa), β-galactosidase (130 kDa), phosphorylase B (95 kDa), albumin (72 kDa), ovalbumin (43 kDa), carbonic anhydrase (34 kDa), myoglobin (26 kDa) and lysozyme (17 kDa) by using 15% acrylamide gel and the gel was stained with Coomassie Brilliant Blue R 250. The relative mobility (R_f) for each protein standard was plotted on a logarithmic scale against the corresponding M.W. and a line of best fit was drawn for the calculation of the MW of the enzyme. In addition, the MW of enzyme was checked by the Sephadex G-50 gel filtration column (2.6 × 90 cm) described above using bovine serum albumin (BSA, 67 kDa) and RNase (13.7 kDa) as standard proteins. A calibration curve was obtained for the calculation of the MW by plotting the fraction number of each protein standard against the logarithmic MW of each protein.

8. Isoelectric Point (pI) Determination

The pI of the purified nattokinase was investigated by isoelectric focusing (IEF) on PhastGel IEF 3-9, and compared with the pI of each standard protein (phycocyanin, pI 4.45; β-lactoglobulin B, pI 5.10; bovine carbonic anhydrase, pI 6.00; human carbonic anhydrase, pI 6.50; equine myoglobin, pI 7.00; human hemoglobin A, pI 7.10; human hemoglobin C, pI 7.50; lentil lectin, pI 8.20; cytochrome c, pI 9.60) according to the manufacturer's instructions (PhastSystem user's manual, GE Healthcare). The relative mobility (R_f) values for the protein standards were plotted on a logarithmic scale against the corresponding pI and a line of best fit was drawn for the calculation of the pI of the enzyme.

9. Effects of pH and Temperature on Enzyme Activity

The effect of pH on the activity of the purified enzyme was determined at 37°C and various pH, from 4 to 10. Various

buffers at a concentration of 50 mM in the reactions were used accordingly: citric acid buffer (pH 5-6), phosphate buffer (pH 5-7), Tris buffer (pH 7-9), and sodium carbonate (pH 9-12). The effect of temperature on the purified enzyme activity was determined at the temperature of 20, 30, 37, 45, 55, 65, 75 or 85°C in 50 mM Tris buffer (pH 7.8).

10. Effects of pH and Temperature on Enzyme Stability

The effect of pH on the stability of the purified enzyme solution was carried out by incubating the enzyme solution at 37°C for 1 hour in the 50 mM various buffers with pH from 4 to 10, as described above. The residual fibrinolytic activity was analyzed after re-adjustment of the enzyme solution to pH 7.8 by HCl or NaOH. The thermal stability of the purified enzyme solution was evaluated by measuring the residual enzyme activity after incubation of enzyme solution (in 50 mM Tris buffer, pH 7.8) at various temperatures (20, 30, 37, 45, 55, 65, 75 and 85°C) for 10 to 120 min.

11. Effects of Metal Ions and Inhibitors on Enzyme Activity

The effects of mono-, di- and trivalent metal ions, and inhibitors on the enzyme activity were carried out by pre-incubating the enzyme solution with the chemicals of KCl, NaCl, MgCl₂, CuSO₄, CaCl₂, ZnCl₂ and FeCl₃, and the inhibitors of IAA, EDTA, NEM, PMSF and TPCK at a concentration of 1 or 5 mM in 50 mM Tris buffer (pH 7.8) at 37°C for 1 hour. The fibrinolytic activity of the enzyme solution at each incubation condition was assayed. The relative activity was expressed as a percentage of the original enzyme activity without any effectors.

12. Statistical Analysis

Data were analyzed statistically using SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to determine statistical differences between sample means, with the level of significance set at $p < 0.05$ or 0.01. Multiple comparisons of means were done by Duncan's or Student's tests. All data are expressed as mean ± SD.

III. RESULTS AND DISCUSSION

1. Proximate Composition of Dried *P. dentata* and Nattokinase Production from *P. dentata* Suspension

The proximate composition of dried *P. dentata* was shown in Table 1. The most abundant 2 components of carbohydrate and crude protein in *P. dentata* were 50.80% ± 0.45% and 32.30% ± 0.22%, respectively. Dependent on seaweed species, their protein contents varied widely, with brown seaweeds being lower (3%-15% dry weight) than that of green or red seaweeds (10%-47% dry weight) (Nguyen et al., 2011). The protein content (dry weight) of commercial *P. dentata* used in this study was higher than that of red algae of *H. japonica* and *H. charoides* (18%-19%) (Wong and Cheung, 2000) and other *Porphyra* species (24.11%) (Sanchez-Machado et al., 2004).

Table 1. Proximate composition of commercial dried *P. dentata* (g/100g)

Composition	Amount (%) (% based on dry weight)*
Moisture	6.86 ± 0.03
Crude protein	30.07 ± 0.20 (32.30 ± 0.22)
Crude lipid	3.96 ± 1.09 (4.25 ± 0.09)
Crude fiber	3.37 ± 0.32 (3.61 ± 0.35)
Ash	8.42 ± 0.06 (9.04 ± 0.07)
Carbohydrate**	47.32 ± 1.34 (50.80 ± 0.45)

* Data are expressed as mean ± SD (n = 3).

** Calculated by difference (= 100-crude protein-crude lipid-crude fiber-ash).

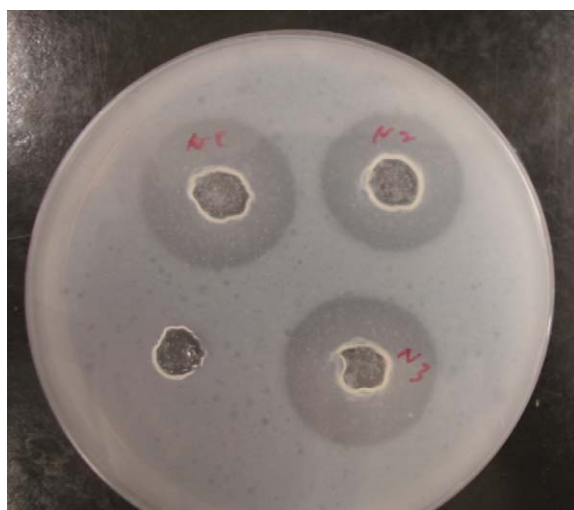


Fig. 1. Fibrinolytic activity of the culture filtrate of *Bacillus subtilis* N1. Ten microliter of culture filtrate of *Bacillus subtilis* N1 in TSB at 37°C for 24 hours were applied to a fibrin plate for observation of clear zone. The commercial TSB medium without inoculum was served as the control.

The fact of high protein content in *P. dentata*, similar as that in soybean (36%-42% dry weight) (Guillon and Champ, 2002; Jeff-Agboola and Oguntuase, 2006), indicates that *P. dentata* may be a promising candidate for nattokinase production since *B. subtilis* natto metabolizes proteins in soybean for the production of nattokinase in natto. The crude lipid, crude fiber and ash contents were 4.25% ± 0.09% 3.61% ± 0.35% and 9.04% ± 0.07%, respectively. In general, seaweeds from red algae of *Porphyra* sp. (Sanchez-Machado et al., 2004), *Hypnea japonica* and *H. charoides* (Wong and Cheung, 2000), brown algae of *Himantalia elongate* and *Laminaria ochroleuca* (Sanchez-Machado et al., 2004) and green algae of *Ulva lactuca* and *Caulerpa lentillifera* (Ratana-arporn and Chirapart, 2006; Nguyen et al., 2011) were rich in minerals (19.1%-46.2% dry weight) and carbohydrate/dietary fiber (25.1%-55.4% dry weight) but low in lipid (0.29%-1.64% dry weight). The reason that the ash content in commercial *P. dentata* used in this study was much lower than that in referred seaweeds mentioned above was probably due to the thoroughly

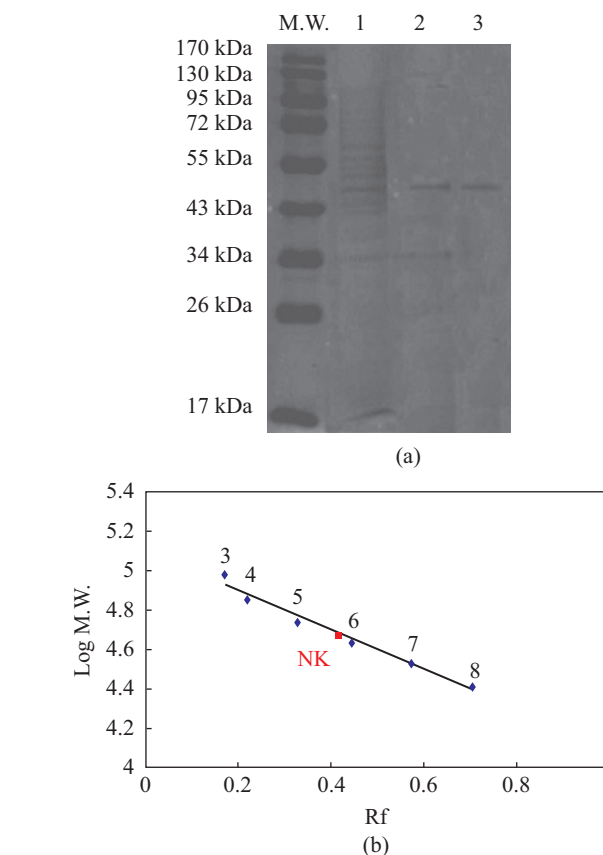


Fig. 2. SDS-PAGE (a) and the molecular weight determination (b) of the nattokinase from *Bacillus subtilis* N1. Standard proteins: (1) myosin (M.W. 170 kDa); (2) β -galactosidase (M.W. 130 kDa); (3) phosphorylase B (M.W. 95 kDa); (4) albumin (M.W. 72 kDa); (5) glutamate dehydrogenase (M.W. 55 kDa); (6) ovalbumin (M.W. 43 kDa); (7) carbonic anhydrase (M.W. 34 kDa); (8) myoglobin (M.W. 26 kDa) and (9) lysozyme (M.W. 17 kDa). Lane 1: Crude enzyme solution; Lane 2: After CM-Sepharose FF ion exchange chromatography; Lane 3: After Sephadex G-50 gel filtration chromatography.

washing and soaking treatment of fresh *P. dentata* with water before drying operation. *B. subtilis* N1 was shown the potential to produce nattokinase by creating clear zone on the fibrin plate (Fig. 1). Accordingly, this strain was used in this study for the production of nattokinase in *P. dentata* suspension. After culturing *B. subtilis* N1 in a fermentor containing *P. dentata* suspension (pH 7.0) as the sole substrate at 37°C for 48 h, the nattokinase activity in the culture filtrate was 987 U/mL.

2. Nattokinase Purification

The crude enzyme solution was purified to electrophoretic homogeneity (Fig. 2(a)) by two steps of liquid chromatography. As summarized in Table 2, crude enzyme was first purified by using an ion-exchange column CM Sepharose Fast Flow to obtain a purification fold of 2.7 and a yield of 9.73%. The crude enzyme suspension from the 3% (w/v) *P. dentata* fermented solution was highly viscous and might have caused

Table 2. Summary of purification of nattokinase from *Bacillus subtilis* N1-fermented *Porphyra dentata*.

Purification steps	Total activity* (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	1,120,000	23,900	46.9	1	100
CM-Sepharose FF	109,000	870	125	2.67	9.73
Sephadex G-50	29,100	108	269	5.74	2.6

*The assay for enzyme activity was carried out with 0.5% fibrin in 50 mM Tris buffer (pH 7.8) at 37°C for 15 min. The reaction was stopped by 0.1 M trichloroacetic acid. The supernatant of the reaction mixture was collected after centrifugation (12,000 ×g, 10 min) and measured for the absorbance at 275 nm. A standard curve was obtained by measuring the absorbance at 275 nm of 0-100 µg/mL tyrosine solution. One unit of the enzyme activity was defined as the amount of enzyme that gave an increase in absorbance at 275 nm equivalent to 1 µg of tyrosine per minute at 37°C.

Table 3. The summary of *Bacillus*-originated nattokinases/fibrinolytic enzymes.

Enzyme	Mol. Wt. and pI	Optimum pH and temp	Substrate used	References
Fibrinolytic enzyme	20.5 kDa	pH 9.4, 40°C	Fibrin	(Hassanein et al., 2011)
Fibrinolytic enzyme	26 kDa, pI 9.0-9.2	pH 8.0,	Fibrin	(Huang et al., 2013)
Nattokinase	27.5 kDa	pH 8.5, 50°C	Fibrin	(Yin et al., 2010)
Nattokinase	27.7 kDa	-	Synthetic substrate for plasmin	(Sumi et al., 1987)
Fibrinolytic enzyme	28 kDa	pH 9.0, 50°C	Synthetic substrate for nattokinase	(Mahajan et al., 2011)
Fibrinolytic enzyme	28 kDa, pI 8.0	pH 9.0, 48°C	Fibrin	(Peng et al., 2003)
Fibrinolytic enzyme	28.2 kDa	pH 10.5, 70°C	Synthetic substrate for plasmin	(Kim et al., 1996)
Fibrinolytic enzyme	29 kDa	pH 10, 40°C	Fibrin	(Kim & Choi, 2000)
Nattokinase	29 kDa	pH 8.0, 40°C	Fibrinogen clot	(Wang et al., 2009)
Fibrinolytic enzyme	31.5 kDa, pI 8.3	pH 7.8, 55°C	Fibrinogen clot	(Chang et al., 2000)
Fibrinolytic enzyme	32.3 kDa	pH 7.4, 37°C	Fibrinogen clot	(Mukherjee et al., 2012)
Fibrinolytic enzyme	44 kDa	pH 8.0, 50°C	Fibrin	(Lee et al., 2001)
Fibrinolytic enzyme	45 kDa	pH 7.0, 60°C	Fibrin	(Paik et al., 2004)
Fibrinolytic enzyme	43-46 kDa	pH 7.2, 30°C	Fibrin	(Hua et al., 2008)
Fibrinolytic enzyme	46.5 kDa, pI 8.35	pH 8.0, 55°C	Fibrin	In this study

insufficient binding of nattokinase to the column, leading a relatively low protein yield. The nattokinase was further purified by using a gel filtration Sephadex G-50 column to obtain a pure nattokinase sample with a purification fold of 5.74 and a yield of 2.6% for the following studies of mass and isoelectric point of nattokinase.

3. Determination of Molecular Mass and Isoelectric Point of Nattokinase

The molecular weight of the purified nattokinase was 46.5 kDa, as estimated by SDS-PAGE (Fig. 2(b)). This value is quite close to the value (46.0 kDa) estimated by gel filtration on Sephadex G-50 (data not shown). This demonstrated that this enzyme is a monomeric protein. The molecular weights of some *Bacillus*-originated nattokinase/fibrinolytic enzymes are ranged from 20.5 to 46.5 kDa, as shown in Table 3. Based on molecular weight, it seems that most nattokinase/fibrinolytic enzymes produced by *B. subtilis* or *Bacillus* species from various sources are divided into two categories, one with smaller molecular weight of 26-32 kDa (Sumi et al., 1987; Kim et al., 1996; Chang et al., 2000; Kim and Choi, 2000;

Peng et al., 2003; Wang et al., 2009; Yin et al., 2010; Mahajan et al., 2011; Huang et al., 2013), and the other with larger molecular weight of 43-46 kDa (Lee et al., 2001; Paik et al., 2004; Hua et al., 2008). The molecular weight of the nattokinase obtained in this study is similar in molecular weight with the fibrinolytic enzyme from a *Bacillus* sp. that isolated from Korean traditional food Chungkookjang (45 kDa), soybean paste (44 kDa) and fermented shrimp paste (43-46 kDa) (Lee et al., 2001; Paik et al., 2004; Hua et al., 2008). The isoelectric point (pI) of the nattokinase was estimated to be 8.35, by using IEF electrophoresis (Fig. 3). As shown in Table 3, the pI of some *Bacillus*-originated nattokinase/fibrinolytic enzymes are ranged from 8 to 9.2 (Sumi et al., 1987; Chang et al., 2000; Peng et al., 2003; Huang et al., 2013) although the fibrinolytic enzyme published by Mukherjee et al. (2012) had a relatively low pI of 5.8.

4. Effects of pH and Temperature on the Activity and Stability of the Nattokinase

The effect of pH on the enzyme activity and stability was investigated over a range of from 4 to 10, as represented in

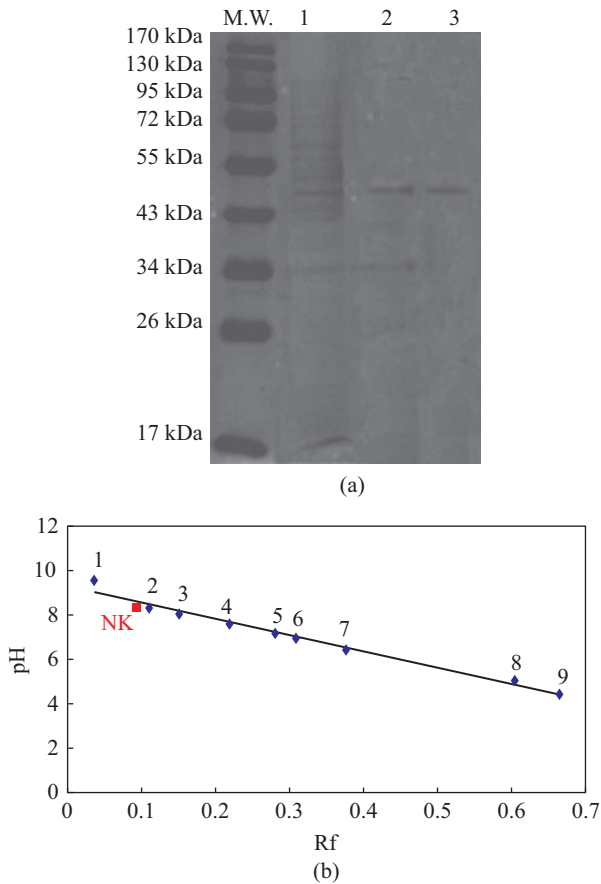


Fig. 3. Isoelectrofocusing electrophoresis (a) and the determination of pI (b) of the nattokinase from *Bacillus subtilis* N1. Standard proteins: (1) cytochrome c, pI 9.60; (2) lentil lectin, pI 8.20; (3) human hemoglobin C, pI 7.5; (4) human hemoglobin A, pI 7.10; (5) equine myoglobin, pI 7.00; (6) human carbonic anhydrase, pI 6.50; (7) bovine carbonic anhydrase, pI 6.00; (8) β -lactoglobulin B, pI 5.10; (9) phycocyanin, pI 4.45. Lane 1: the purified nattokinase. IEF was carried out on PhastGel IEF 3-9 according to the manufacturer manual.

Fig. 4. The optimum pH for enzyme activity at pH 8, and the enzyme remained over 90% activity at other tested pH value, indicating that this enzyme is active over a wide pH range. Similar results have been reported in other studies of serine proteases (Seong et al., 2004; Pande et al., 2006). The pH stability of the enzyme was also examined in a pH range of 4 to 10 by measuring the residual enzyme activity after 1 hour incubation at different pH values. As shown in Fig. 4, the pH stability of the nattokinase was stable (over 80% residual activity) in a pH range of 5 to 9. A previous report indicated that Subtilisin DJ-4 stayed active at pH 4-11 for 48 hours (Kim and Choi, 2000). However, the activity of nattokinase obtained in this study was completely lost when this enzyme was outside the pH range of 5 to 9.

The effect of temperature on the enzyme activity and stability was also studied in a range of 20 to 85°C at pH 7.8, as shown in Fig. 5. The optimum temperature for enzyme activity was approximately at 55°C (Fig. 5(a)). As shown in

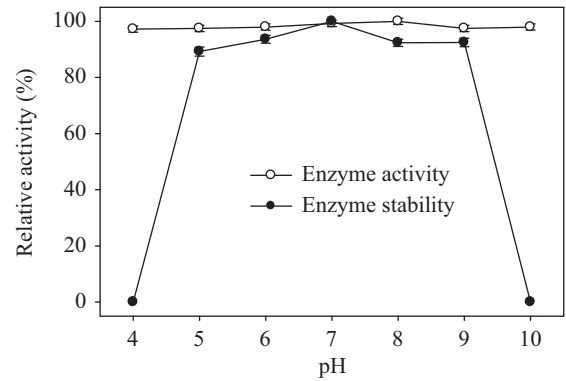


Fig. 4. Effect of pH on the activity and stability of the nattokinase from *Bacillus subtilis* N1. The fibrinolytic activity was measured in a range of pH 4-10, respectively, in 50 mM citric acid buffer (pH 5-6), phosphate buffer (pH 5-7), Tris buffer (pH 7-9) and sodium carbonate buffer (pH 9-12) at 37°C. The enzyme stability was measured by incubating it at 37°C and various pH values for 1 h prior to the determination of remaining fibrinolytic activity in Tris buffer at pH 7.8.

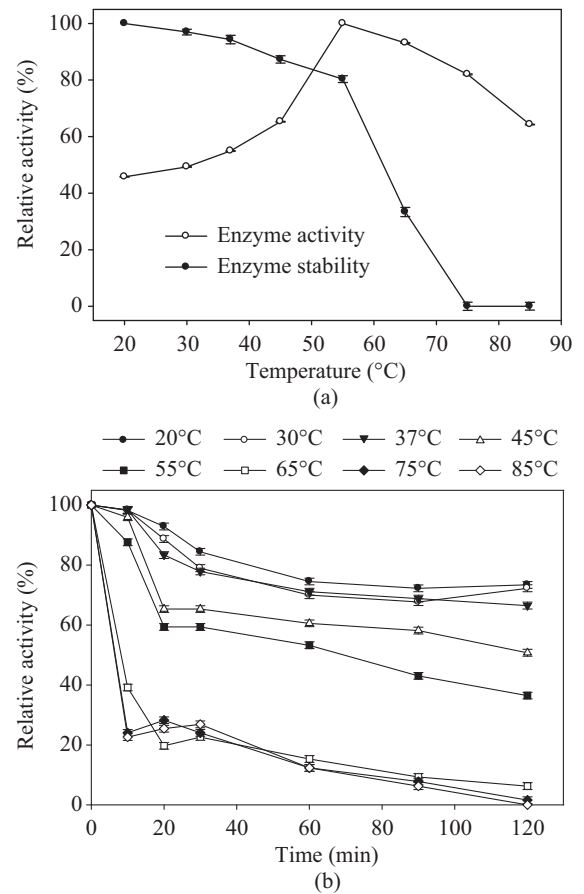


Fig. 5. Effect of temperature on the activity (a) and stability (b) of the nattokinase from *Bacillus subtilis* N1. The enzyme activity was determined at various temperatures (20, 30, 37, 45, 55, 65, 75 and 85°C) and pH 7.8 in 50 mM Tris buffer. The enzyme stability was measured by incubating it for 10 min to 120 min at various temperatures and pH 7.8 prior to measuring remaining fibrinolytic activity in Tris buffer at pH 7.8.

Table 4. Effect of various inhibitors on the fibrinolytic activity of nattokinase from *Bacillus subtilis* N1-fermented *Porphyra dentata*.

Inhibitors**	Relative activity* (%)	
	1 mM	5 mM
None	A100.00 ± 0.00 ^{b,***}	A100.00 ± 0.00 ^c
IAA	B109.42 ± 2.74 ^a	A151.07 ± 2.79 ^a
EDTA	A84.70 ± 2.13 ^d	B28.30 ± 2.11 ^f
Leupeptin	A82.27 ± 2.38 ^d	B47.78 ± 2.03 ^e
NEM	B99.00 ± 2.57 ^b	A137.51 ± 2.53 ^b
PMSF	A71.40 ± 2.42 ^e	B23.56 ± 2.41 ^f
TPCK	A91.11 ± 1.89 ^c	B57.18 ± 2.17 ^d

* The enzyme was pre-incubated with various inhibitors prior to the fibrinolytic enzyme assay. The results were expressed as percentage (%) relative to that of none.

** IAA, iodoacetic acid; EDTA, ethylenediamine tetraacetic acid; NEM, N-ethylmaleimide; PMSF, phenylmethane sulfonyl fluoride; TPCK, tosylphenylalanine chloromethyl ketone.

*** Each value represents means ± SD (n = 3). Means with different superscript in the same column (a-f) are significantly different by Duncan's multiple range test ($p < 0.05$). Means with different letters in the same row (A, B) are significantly different by Student's *t* test ($p < 0.05$).

Table 3, the optimum temperature for some *Bacillus*-originated nattokinase/fibrinolytic enzymes are ranged from 30°C to as high as 70°C. The optimal temperature (55°C) of our enzyme is the same as that reported by Chang et al. (2000), and quite close to 50°C reported by Lee et al. (2001), Kimiya et al. (2008), and Yin et al. (2010), or close to 60°C reported by Paik et al. (2004). Fig. 5(b) showed that the enzyme was relatively stable up to 55°C but the stability dropped sharply at higher temperatures. The enzyme incubated at 55°C for 2 hours remained a relative activity of higher than 40% while its relative activity dropped down to less than 30% at 65°C within 20 min. Lee et al. (2001) suggested that the fibrinolytic enzyme remained thermostable up to 60°C.

5. Effects of Inhibitors and Metal Ions on Enzyme Activity

The effects of various inhibitors on the fibrinolytic activity were summarized in Table 4. The data indicated that enzyme activity was inhibited by serine protease inhibitors of PMSF, leupeptin and TPCK, where the former at a concentration of 5 mM showed the strongest inhibitory effect by 76.44%. Our data indicated that the nattokinase obtained in this study is a serine protease, which is equivalent to the results of some previous reports (Sumi et al., 1987; Kim et al., 1996; Kim and Choi, 2000; Ko et al., 2004; Paik et al., 2004; Hua et al., 2008). Metal chelating agent EDTA at a concentration of 5 mM showed a potent inhibitory effect, indicating the metals that involved in enzyme activity or stability are not strongly bound. Intriguingly, irreversible cysteine proteinase inhibitors of IAA and NEM, which can modified the thiol group of cysteine

Table 5. Effect of metal ions on the fibrinolytic activity of nattokinase from *Bacillus subtilis* N1-fermented *Porphyra dentata*.

Metal ions	Relative activity* (%)	
	5 mM	10 mM
None	A100.00 ± 0.00 ^{b,**}	A100.00 ± 0.00 ^c
K ⁺	A88.75 ± 2.63 ^{cd}	A84.59 ± 2.04 ^d
Na ⁺	A87.66 ± 2.47 ^{cd}	A86.40 ± 2.19 ^d
Mg ²⁺	A85.97 ± 2.78 ^{cd}	A86.74 ± 2.69 ^d
Cu ²⁺	B106.57 ± 2.34 ^a	A136.99 ± 2.16 ^a
Ca ²⁺	A83.76 ± 2.61 ^d	A85.68 ± 2.75 ^d
Zn ²⁺	A90.50 ± 2.48 ^c	B85.28 ± 2.15 ^d
Fe ³⁺	B103.15 ± 2.52 ^a	A123.34 ± 2.45 ^b

* The enzyme was pre-incubated with various metal ions prior to the fibrinolytic enzyme assay. The results were expressed as percentage (%) relative to that of none.

** Each value represents means ± SD (n = 3). Means with different superscript in the same column (a-d) are significantly different by Duncan's multiple range test ($p < 0.05$). Means with different letters in the same row (A, B) are significantly different by Student's *t* test ($p < 0.05$).

residues, could increase enzyme activity in a concentration dependent manner; however, the mechanisms are unknown. The effects of mono-, di- and trivalent metal ions on the fibrinolytic activity are represented in Table 5. Under the conditions employed to the purified enzymes, K⁺, Na⁺, Mg²⁺, Ca²⁺, Zn²⁺ ions show some minor inhibitory effect, which might be due to the fact that the substitutions or over loadings of metal ions K⁺, Na⁺, Mg²⁺, Ca²⁺, Zn²⁺ could change enzyme stability or activity (Holland et al., 1995). Cu²⁺ (or SO₄²⁻) and Fe³⁺ ions at 10 mM enhanced the activity in a concentration dependent manner, with being by 36.99% and 23.34%, respectively, at 10 mM. This finding is equivalent to our previous data on the inhibitory effect by the metal chelator of EDTA and similar result has been reported (Paik et al., 2004). There have been some previous reports indicating that transition metals, such as Cu²⁺, Co²⁺ and Zn²⁺, and alkaline metals Ca²⁺ and Mg²⁺ enhanced enzyme activity (Lee et al., 2001; Paik et al., 2004; Hua et al., 2008).

IV. CONCLUSIONS

Based on its proximate composition, we first and successfully introduced marine crop *P. dentata* suspension as the sole medium constituent for the production of nattokinase in fermentor. Crude nattokinase with a fibrinolytic activity of 987 U/mL was obtained by fermenting 3% (w/v) *P. dentata* suspension with *B. subtilis* N1 at 37°C for 48 hours with controlled aeration and agitation. Our biological data indicated that the nattokinase is a serine protease and its fibrinolytic activity can be enhanced by adding CuSO₄ and FeCl₃ and that the enzyme stayed active at as high as 55°C and at a broad range of pH. This study not only gives a brand new idea to use

Bacillus sp. to ferment environmentally friendly sea alga *P. dentata* for the production of nattokinase but provides a practical approach in the development of high nutritional, algae-based fermented food with fibrinolytic activity.

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