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Technical paper

Natto Bacillus as an Oral Fibrinolytic Agent: Nattokinase Activity and the Ingestion Effect of *Bacillus subtilis natto*

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Extraction of nattokinase, a fibrinolytic enzyme in natto bacillus, was attempted by the following 4 methods: 1) extraction with saline, 2) treatment with the organic solvents acetone, toluene and hexane prior to extraction, 3) alkaline treatment at pH 11.0 and, 4) autolysis in the presence of 0.1% NaN_3 at 4°C. Each fraction showed not only a strong fibrinolytic activity, but also H-D-Val-Leu-Lys-pNA and Suc-Ala-Ala-Pro-Phe-pNA amidolytic activities. Doses of 50–200 mg/kg natto bacillus (1×10^{11} active cells/g) were orally administered for rat experimental pulmonary thrombosis and to healthy human volunteers. A decrease in thrombus count and plasma euglobulin lysis time (ELT), as well as an increase in tissue plasminogen activator (t-PA), indicate that natto bacillus serves to activate plasma fibrinolysis *in vivo*.

Keywords: nattokinase, menaquinone-7, *Bacillus subtilis natto*

In extensive studies on oral fibrinolytic therapy, nattokinase, a strong fibrinolytic agent, has been found in commercially available natto products and cultured media of natto bacillus (Sumi *et al*., 1980; Sasaki *et al*., 1985; Toki *et al*., 1985; Mihara *et al*., 1991). Also reported are the characteristics and effects of nattokinase (Sumi *et al*., 1987; 1990; 1993; Sumi, 1988; 1999a). Since the enzyme triggers a mild but sustainable fibrinolytic effect by oral administration, the natto products have drawn attention from the health food industry and several clinical organizations (Sumi, 1999a; 2000). In the field of ophthalmology, nattokinase has been used as a fibrinolytic agent especially for treatment of retina central vein thrombosis (Nishimura *et al*., 1994). However, until now it has been thought that nattokinase is an extracellular enzyme produced by natto bacillus and found in the sticky ingredient of natto (so-called "threads"), therefore, there has been a lack of investigation into the fibrinolytic enzyme of natto bacillus.

Natto, a Japanese traditional fermented soybean, is known for its various positive effects (Oota, 1975). Especially, attention has been paid to vitamin K_2 (menaquinone-7) produced by natto bacillus, a special vitamin which is rarely found in other food products (Kaneki *et al*., 2001; Orimo, 1996; Sumi, 1999b). After being taken into the blood, vitamin K_2 plays an active role in the synthesis of calcium-binding Gla protein, for example, osteocalcin, and is regarded as an effective preventative of osteoporosis. The authors have shown that natto bacillus increases and is metabolized, to a certain degree, inside the digestive tract because ingestion of natto products and natto bacillus increases plasma menaquinone-7 concentration (Sumi, 1999b; Sumi *et al*., 2003). This study attempts to clarify nattokinase activity within natto bacillus and the effect of natto bacillus on the plasma fibrinolytic system *in vivo*.

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Materials and Methods

Preparations of natto bacillus (*Bacillus subtilis natto*) used were the powder products supplied by Meguro Institute (Osaka) and Nitto Pharmaceutical Co., Ltd. (Kyoto). They were the highest level of purity (viable cell numbers: BN-1 ca. 1×10^{11}/g dry weight, and Nitto ca. 1.8×10^{10}/g wet weight), both of which were prepared by soypeptone-base fermentation. A starter used for natto production was purchased from Miyagino Natto Institute (Tokyo). The procured natto bacilli were cultivated for 2 days while being shaken at 100 rpm in 500 ml Erlenmeyer flasks, which contained 150 ml of 2% polypepton-S (Product of Wako Pure Chemical Industries, Ltd., Osaka) and 2% glycerin at a temperature of 37°C. To control its administration in the experiment, freeze-dried lactobacillus and yeast cells were used . *Lactobacillus acidophilus* TUA002L was cultured with GYP medium (1.0% glucose, 0.5% peptone, 0.5% yeast extract and 1.0% CH_3COONa), and *Saccharomyces cerevisiae* TUA465Y was cultured with YM medium (1.0% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract), respectively. All other materials used were of high quality.

Nattokinase activity was determined by the standard fibrin plate method or amidolytic method with H-D-Val-Leu-Lys-pNA and Suc-Ala-Ala-Pro-Phe-pNA (Sumi *et al*., 1980). The substrate concentrations used were 5×10^{-4} M in 0.1 M phosphate buffer, pH 7.4.

SDS-polyacrylamide gel electrophoresis was performed by the method established by Summaria *et al*. (1975). Coomassie brilliant blue was used for gel protein staining. For molecule weight determination, a standard protein MW-SDS-200kit (Sigma) was used.

Experimental pulmonary thrombosis was triggered by a 2 h intravenous drip of 1.5 g/kg of lactic acid to the femoral artery of male Wistar rats (age 8–9 weeks, weight 250–300 g), which is a

method established by Tomikawa *et al.* (1974). Natto bacillus (50 mg/kg and 200 mg/kg) was ingested 3 h before lactic acid injection. For the thrombus count using an optical microscope, 5 lung tissues were tinctured by HE (hematoxylin orange) and then transversely amputated for observation at a magnification ratio of 200. All the thrombi with a diameter equal to or greater than 25 μm in 3 fields of each tissue specimen were counted and regarded as the overall number of thrombus.

An ingestion experiment of natto bacillus for humans was performed using volunteers with no hematologic abnormality in strict accordance with the Helsinki Pact. Plasma euglobulin lysis time (ELT) was determined as reported previously (Sasaki *et al.*, 1985). Tissue plasminogen activator (t-PA) was determined by the method reported by Stephen *et al.* (1989). Activated plasmin's H-D-Val-Leu-Lys-pNA hydrolyzing activity was converted to the IU using the standard t-PA preparation (Kowa Pharmaceutical Co., Ltd., Nagoya).

For the blood coagulation system, a clot digitam TE-20 (Elma Optics) was used. Plasma recalcification time was measured with the addition of 0.2 ml plasma and 0.1 ml of 50 mM $CaCl_2$. Prothrombin time was measured by adding 0.1 ml of plasma as well as 5 mg/ml of thromboplastin 0.2 ml (Mochida).

Results

Nattokinase activity in the commercial natto bacillus Nattokinase activity, found in the extracted fraction, was determined after treating natto bacillus by the methods described below;

(1) Extraction with saline One gram of dry natto bacillus (BN-1) was suspended in 15 ml of saline, and centrifuged for 10 min at 3000 rpm. A supernatant obtained after the centrifugation was regarded as Extract No. 1. Then, the remaining precipitate was rinsed with 15 ml of saline for another suspension inside saline, which was regarded as Extract No. 2. Nattokinase activity in each extract was determined by the standard fibrin plate method. As shown in Table 1, nattokinase activity can be found in Extract No. 1. Only small amounts of activity were observed with Extract No. 2, which was a bacillus suspension liquid. When H-D-Val-Leu-Lys-pNA was used, which is known as a sensitive substrate of nattokinase, even Extract No. 2 showed a strong hydrolyzing activity. It was also found that a part of the activity was inhibited by aprotinin or soybean trypsin inhibitor (SBTI).

Table 1. Nattokinase activity determined in the natto bacillus extract.

	No. 1	No. 2
Fibrin plate lysis (mm^2)[a]	230.5	6.0
H-D-Val-Leu-Lys-pNA		
Amidolysis (nmol/min/ml)[b]	285.6	101.6
+SBTI	185.3	34.0
+Aprotinin	169.2	24.8

[a] Fibrinolytic activity of natto bacillus extract (10 μl) was determined by the standard fibrin plate after incubation for 18 h at 37°C. No. 1 is the extract of 1×10^{10} natto bacillus with 15 ml saline, and No. 2 is mixture of the residue (ppt) and saline.
[b] Reaction mixture (1.0 ml) was consisted with 5×10^{-4} M substrate/0.1 M phoshate buffer, pH 7.4. After stopping the reaction by addition 0.5 ml of 50% acetic acid, released *p*-nitroaniline was determined by the absorption at 405 nm.
For inhibition, 1 mg/ml of soybean trypsin inhibitor (SBTI) or 10,000 units/ml of aprotinin were added in the reaction mixture.

(2) Organic solvent treatments As the second step in the experiment, dry natto bacillus (BN-1) was treated with several organic solvents. The water extraction ratio of nattokinase from the bacillus was significantly increased by the treatment using toluene and hexane when compared with those by water and methanol (Fig. 1).

(3) Alkaline treatment Nattokinase was extracted from natto bacillus (Nitto) using a high-density $NaClO_4$, KSCN and urea. It was also treated at various levels of pH. The highest extraction ratio was achieved at pH 11.0, followed by 2 M $NaClO_4$, 6 M urea, and 2 M KSCN treatments. Nattokinase activity was slightly decreased after an extended period of alkaline treatment.

(4) Autolysis Five milliliters of distilled water, containing 0.1% of NaN_3, was added to 1.0 g wet weight of natto bacillus (Miyagino) cultivated by shaking in a Erlenmeyer flask. The mixture was then left for a certain period at 4°C for autolysis of natto bacillus (Table 2). This process resulted in the detection, of a considerably strong nattokinase activity in the supernatant. This activity was also determined using two synthetic amide substrates, H-D-Val-Leu-Lys-pNA and Suc-Ala-Ala-Pro-Phe-pNA (Table 2). These data indicate a significant promotion of nattokinase activity in the supernatant. The concentration of protein in the extract solution (absorbance 280 nm) increased with the passage of time, to 3.64 on the first day, 6.65 on the third day, 9.85 on the fifth day, and to 11.74 on the seventh day, whereas the protein concentration of the control (0 day) was 1.24. The autolysis of the cells could also be confirmed by SDS-polyacrylamide gel electrophoresis.

Oral administration of natto bacillus The effect of oral administration of natto bacillus (BN-1) on plasma fibrinolysis

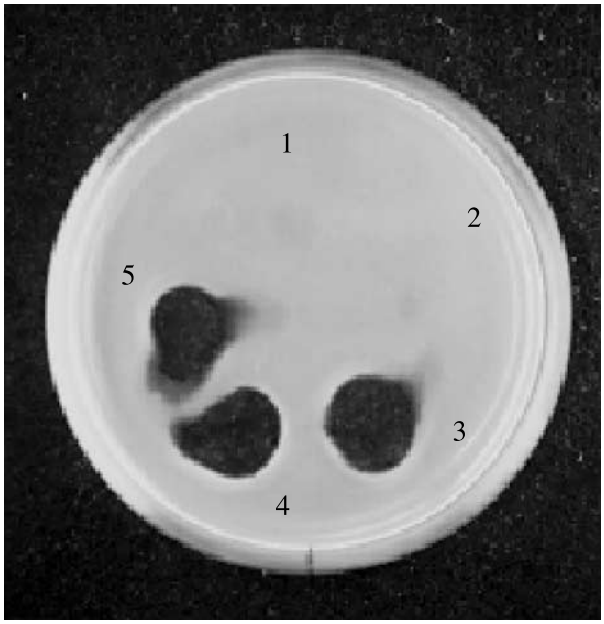

Fig. 1. Effect of several organic solvents on the pre-treatment of natto bacillus for the extraction of nattokinase. 0.1 g of natto bacillus (BN-1) was dissolved with 5.0 ml of No.1–5 organic solvents. After stirring for 2.5 h at 4°C each precipitate was dried by heating at 50°C. These were powdered, extracted with distilled water, and 30 μl of the supernatant was applied on a fibrin plate. This photograph is after incubation for 8 h at 37°C. Organic solvents used were 1, distilled water (control); 2, methanol; 3, hexane; 4, toluene and 5, acetone, respectively.

Table 2. Nattokinase activity in the autolysate of natto bacillus.

Autolysis time (4°C)	Fibrin plate mm^2/24 h	H-D-Val-Leu-Lys-pNA ΔOD_{405}/4 h	Suc-Ala-Ala-Pro-Phe-pNA ΔOD_{405}/4 h
0 d (control)	182	0.478	0.005
1 d	400	0.705	0.008
3 d	456	0.969	0.020
5 d	426	1.103	0.167
7 d	441	1.288	0.218

The volume of 30 μl natto bacillus lysate was used for the fibrin plate sample, which was extracted with 0.1% NaN_3. Amidolytic activity was determined with 5×10^{-4} M substrate in the 1 ml of reaction mixture. Sample volume used was 0.1 ml for H-D-Val-Leu-Lys-pNA amidolysis or 0.01 ml for Suc-Ala-Ala-Pro-Phe-pNA amidolysis.

Table 3. Administration effect of natto bacillus on the experimental acute pulmonary thrombosis in rats.

Dose		*n*	Thrombus count	% Inhibition
Control (saline)	—	8	83.1±5.5	—
Natto bacillus	50 mg/kg, p.o.	5	64.1±5.0[a)]	22.9
	200 mg/kg, p.o.	8	60.3±4.2[b)]	27.4

Values are the mean±S.D. (♂, n=5–8). [a)] $p<0.05$, [b)] $p<0.01$: significantly different from the control.

Table 4. Plasma fibrinolysis after orally administering natto bacillus to human.

	Before (control)	After administration (h)			
		1	4	8	24
Euglobulin lysis time (ELT, min)	31.3±6.0	16.7±7.0[a)]	15.8±6.6	21.3±9.8	30.5±9.8
t-PA (IU/ml)	0.4±0.3	1.1±0.4[a)]	2.7±0.8	1.9±0.8	0.6±0.3

Healthy male volunteers were given 100 mg (dry weight)/kg of natto bacillus. Values are the mean±S.D. (n=5). [a)] $p<0.05$: significantly different from the control.

was studied with rat experimental pulmonary thrombosis and healthy humans.

(1) Administration to the experimental thrombosis A 2 h intravenous drip of 1.5 g/kg of lactic acid to rats triggered an increase in the thrombus count, which cannot be observed in healthy rats (83.1±5.5/5 tissues). Also observed was that prior ingestion of natto bacillus had a positive effect, favorably reducing the number of thrombi (Table 3). Other blood investigations of the experimental animals showed a reduced tendency of plasma ELT (430±18 min in rats without natto bacillus ingestion, 380±23 min for animals with 50 mg/kg ingestion and 268±21 min for those with 200 mg/kg ingestion). In other experiments, we tested the same doses of dry yeast (n=5) and lactobacillus (n=4) in the experimental thrombus model, although no effect was observed (not shown).

(2) Experimental administration to healthy humans Plasma ELT was reduced and t-PA activity was increased 1–4 h after administering 100 mg/kg of natto bacillus to healthy adult volunteers (Table 4).

Blood coagulation activity of animals and humans was investigated according to plasma recalcification time. Almost no change was observed before and after the administration.

Discussion

Nattokinase has been known as an extracellular enzyme (protease) produced by natto bacillus. In the present study, it was found that the enzyme's activity is considerably high in the bacillus. The study also disclosed for the first time that 4 methods—1) extraction with saline, 2) cell treatment with the organic solvents acetone, toluene and hexane, 3) alkaline treatment at pH 11.0 and 4) autolysis of natto bacillus—made it easier to extract nattokinase. This was confirmed by the strong fibrinolytic activity (Fig. 1), synthetic amide substrate hydrolyzing activity (Table 2), and by the experiment using various enzyme inhibitors (Table 1). In one particular report, Suzaki (1960) attempted to clarify autolysis of natto bacillus in terms of antimicrobial property. It is believed that this is the first investigation in terms of a fibrinolytic enzyme.

Several descriptions of the favorable effects of the traditional fermented soybean, natto, can be found in "Honcho Shokkan" written and compiled in the Edo Era (1975). All recent studies investigating the effect on the plasma fibrinolysis were prompted by the discovery of nattokinase. In recent years, it has been confirmed that natto products contain various factors relating to fibrinolysis, i.e., pro-urokinase activator (Sumi *et al*., 1996) and fibrinolysis accelerating substances (FAS) (Sumi *et al*., 2000). Nevertheless, no reports have suggested the activity of natto bacillus itself. Considering the various experiments previously performed, namely the digestive organ model experiment by Ozawa *et al*. (1979), the administration experiment of natto bacillus to pig (Kimura *et al*., 1982), the ingestion experiment to test of plasma vitamin K (menaquinone-7) concentration (Sumi, 1999; Sumi *et al*., 2003), nattokinase produced by natto bacillus inside the digestive tract is also capable of having a positive effect on the plasma fibrinolytic system. Further experiments are now in progress to clarify its reaction mechanism.

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