Technical paper

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

Hiroyuki SUMI,1 Yasuhide YANAGISAWA,1 Chieko YATAGAI1 and Josuke SAITO2

1Department of Physiological Chemistry, Kurashiki University of Science and the Arts, 2640 Tsurajima-cho, Kurashiki, Okayama 712-8505, Japan
2Honda Trading Corporation, 1-8-2, Marunouchi, Chiyoda-ku, Tokyo 100-0005, Japan

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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% NaN3 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 (1×1011 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, 1998; 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 K2 (menaquinone-7) produced by natto bacillus and the effect of natto bacillus on the plasma fibrinolytic system in vivo.

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. 1×1011/g dry weight, and Nitto ca. 1.8×1010/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% CH3COONa), 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 Summara 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 thrombi.

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 CaCl2. 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 by 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.

<table>
<thead>
<tr>
<th></th>
<th>No. 1</th>
<th>No. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrin plate lysis (mm2)</td>
<td>230.5</td>
<td>6.0</td>
</tr>
<tr>
<td>H-D-Val-Leu-Lys-pNA</td>
<td>285.6</td>
<td>101.6</td>
</tr>
<tr>
<td>Amidolysis (nmol/min/ml)</td>
<td>185.3</td>
<td>34.0</td>
</tr>
<tr>
<td>+ Aprotinin</td>
<td>169.2</td>
<td>24.8</td>
</tr>
</tbody>
</table>

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 NaClO4, 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 NaClO4, 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 NaN3, 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...
Table 2. Nattokinase activity in the autolysate of natto bacillus.

<table>
<thead>
<tr>
<th>Autolysis time (4 °C)</th>
<th>Fibrin plate mm²/24 h</th>
<th>H-D-Val-Leu-Lys-pNA ΔOD₄₀₅/4 h</th>
<th>Suc-Ala-Ala-Pro-Phe-pNA ΔOD₄₀₅/4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 d (control)</td>
<td>182</td>
<td>0.478</td>
<td>0.005</td>
</tr>
<tr>
<td>1 d</td>
<td>400</td>
<td>0.705</td>
<td>0.008</td>
</tr>
<tr>
<td>3 d</td>
<td>456</td>
<td>0.969</td>
<td>0.020</td>
</tr>
<tr>
<td>5 d</td>
<td>426</td>
<td>1.103</td>
<td>0.167</td>
</tr>
<tr>
<td>7 d</td>
<td>441</td>
<td>1.288</td>
<td>0.218</td>
</tr>
</tbody>
</table>

The volume of 30 μl natto bacillus lysate was used for the fibrin plate sample, which was extracted with 0.1% NaCl. 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.

<table>
<thead>
<tr>
<th>Dose</th>
<th>n</th>
<th>Thrombus count</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>8</td>
<td>83.1±5.5</td>
<td>-</td>
</tr>
<tr>
<td>Natto bacillus 50 mg/kg, p.o.</td>
<td>5</td>
<td>64.1±5.0⁷</td>
<td>22.9</td>
</tr>
<tr>
<td>200 mg/kg, p.o.</td>
<td>8</td>
<td>60.3±4.2⁷</td>
<td>27.4</td>
</tr>
</tbody>
</table>

Values are the mean±S.D. (n=5–8), ⁷ p<0.05, ⁸ p<0.01: significantly different from the control.

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

<table>
<thead>
<tr>
<th>Before (control)</th>
<th>After administration (h)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Euglobulin lysis time (ELT, min)</td>
<td>31.3±6.0</td>
<td>16.7±7.0⁸</td>
<td>15.8±6.6</td>
<td>21.3±9.8</td>
</tr>
<tr>
<td>t-PA (IU/ml)</td>
<td>0.4±0.3</td>
<td>1.1±0.4⁸</td>
<td>2.7±0.8</td>
<td>1.9±0.8</td>
</tr>
</tbody>
</table>

Healthy male volunteers were given 100 mg (dry weight)/kg of natto bacillus. Values are the mean±S.D. (n=5). ⁸ p<0.05: significantly different from the control.

Discussion

Nattokinase has been known as an extracellular enzyme (pro- tease) produced by natto bacillus. In the present study, it was found that the enzyme’s activity is considerably high in the bacil- lus. 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 nattoki- nase. 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, Suzuki (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 con- firmed 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.

References


