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Effect of a Novel Substance from *natto* on Tissue-type Plasminogen Activator (t-PA) Release in Perfused Rat Hindlegs

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Abstract: *Natto* is a traditional Japanese food made from soybeans fermented by *Bacillus subtilis natto*. *Natto* and natto fungi contain many physiologically active substances, including dipicolinic acid, nattokinase, and vitamin K2. We used a rat hindleg perfusion model to show that the novel substances present in *natto* improve thrombolytic activity in the blood. The perfusion sample was extracted by mixing *natto* with twice the amount of water and heating at 121°C for 30 min. The effect of the extract on fibrin dissolution was measured using the rat hindleg perfusion test and the fibrin plate method. In blood vessels perfused with the *natto* extract made using the Miyagino strain, tissue-type plasminogen activator(t-PA) activity increased by 4.8 ± 2.1 times (n = 8). Five varieties of commercially available *natto* were examined. The average increase in fibrinolytic activity was 5.8 ± 3.0 times (n = 20).

Keywords: *Bacillus subtilis natto*, tissue-type plasminogen activator, fibrinolysis, *natto*, hindleg perfusion, soybean.

1. INTRODUCTION

Natto, a traditional fermented soybean food consumed in Japan, contains many physiologically active substances. In an earlier report, we indicated the presence of nattokinase [1], vitamin K2 (menaquinone-7) [2], and dipicolinic acid (2,6-pyridinedicarboxylic acid; DPA) obtained from *natto* [3, 4]. Blood circulation in the human body is mediated by 2 systems, namely a coagulation cascade and a fibrinolysis system, that are controlled by numerous factors [5]. Blood clotting involves the conversion of the soluble protein fibrinogen to insoluble fibrin by thrombin. Conversely, the system to which fibrin is resolved by plasmin is a fibrinolysis system. Nattokinase has been widely reported as being an enzyme like a plasmin that can dissolve fibrin clots [6].

As a dietary component, *natto* is widely consumed in Japan for preventing thrombus formation. The market for nattokinase as a dietary supplement has recently increased and spread worldwide. Several studies have reported the fibrinolytic activity of nattokinase but not other components derived from *Bacillus subtilis natto*. We reported that DPA exhibits anti-platelet aggregation activity through adenosine 5′-diphosphate (ADP) and collagen, and it inhibits fibrinogen conversion by thrombin [7].

In this study, we confirmed that in addition to nattokinase, other biochemical substances derived from *natto* could influence blood circulation through other pathways. In blood vessels, vascular endothelial cells release tissue-type plasminogen activator (t-PA) that dissolve the fibrins that are responsible for thrombus formation. In the present study, we used perfusion tests using rat hindlegs and confirmed that increase of t-PA secretion within blood vessels. Here we present the results of these experiments.

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2. MATERIALS AND METHODS

2.1. Materials

The different types of *natto* used in our study included Okame (Takano Foods Co. Ltd., Ibaraki, Japan), Ushiwaka (Moriguchi Kako Shokuhinn Co. Ltd., Kyoto, Japan), Genki (Marukin Foods Co. Ltd., Kumamoto, Japan), Tanzei (Kume Quality Products Co. Ltd., Ibaraki, Japan), and Hiruzen (Hiruzen Shokuhin Kako Co. Ltd., Okayama, Japan), all of which are generally available in Japan. The viscous substances were extracted from these *natto* samples by mixing with twice the amount of water. Each extract was autoclaved at 121°C for 30 min and centrifuged at 9000 × *g* and the supernatant was filtered through a 0.2μm filter. Steamed Hokkaido-grown Tsurunoko soybeans (Hokuren Federation of Agricultural Cooperatives, Hokkaido, Japan) were subjected to the same treatment.

Bovine fibrinogen was purchased from Sigma Co. Ltd. (MO, USA) and bovine thrombin was obtained from Mochida Pharmaceutical Co. Ltd. (Tokyo, Japan). All other reagents used in the study were of special grade. A UHP-43K filter device (Advantec Toyo Kaisha Ltd., Tokyo, Japan) was used for the ultrafiltration. Ultrafilters with molecular weight cutoffs of 200,000, 50,000, 20,000, and 10,000 were used. The fractions filtered using ultrafilters of molecular weight >10,000 were washed twice with 50 mL of ion-exchange water to adjust the concentration.

The *natto* extract was subjected to anion exchange chromatographic separation on a column (2.5 × 10 cm) of

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MARATHON MSA (Muromachi Technos Co. Ltd., Tokyo, Japan). The anion exchange column was washed with ion-exchange water until the absorbance at 280 nm was 0.01, and it was then followed by successive elution with 1.0 M sodium chloride solutions. The fractions were then dialyzed overnight against water and stored at −20°C.

2.2. Methods

2.2.1. Rat hindleg perfusion

The rat hindleg perfusion test was performed according to the method described by Emeis [8]. A cannula was inserted into the abdominal aorta of a male Wistar rat (7 weeks of age) under urethane anesthesia, and the aorta was perfused with Tyrode's buffer solution at a rate of 10 mL/min. A sample of the *natto* extract was dissolved in the same buffer solution, and perfusion with the buffer and *natto* sample solutions was conducted alternately. The perfusion solution was collected using a fraction collector (130 drops/tube) connected to the inferior vena cava to ensure that the perfused solution had passed through the blood vessels of the hindlegs (Fig. **1A**). The fibrinolytic activity of the fractions was measured using the standard fibrin plate method. All experiments involving the use of animals were performed in accordance with the Animal Experiment Guidelines of Kurashiki University of Science and the Arts.

2.2.2. Standard fibrin plate method

The fibrinolytic activity of each sample collected during the perfusion period was measured using the standard fibrin plate method [9]. In a square petri dish (144 mm × 104 mm), 20 mL of 0.5% bovine fibrinogen and 0.1 mL of 50 U/mL bovine thrombin were mixed to induce fibrin formation. The fibrin plate was incubated with 30 μL of the samples at 37°C and the dimensions of the fibrinolytic areas were measured.

2.2.3. Zymographic analysis

For zymographic analysis of the perfusion solution, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed. The gel was then washed with Triton-X 100 for 30 min. The gel was then placed atop the fibrin plate prepared in the square petri dish. After incubation at 37°C, the formed fibrinolytic bands were identified [10].

2.2.4. Statistical analyses

Statistical analysis was performed of the experiments with $n \geq 3$. Values are presented as mean ± standard deviation. Comparisons were made using Student's *t* test. Differences of $P < 0.05$ were considered statistically significant.

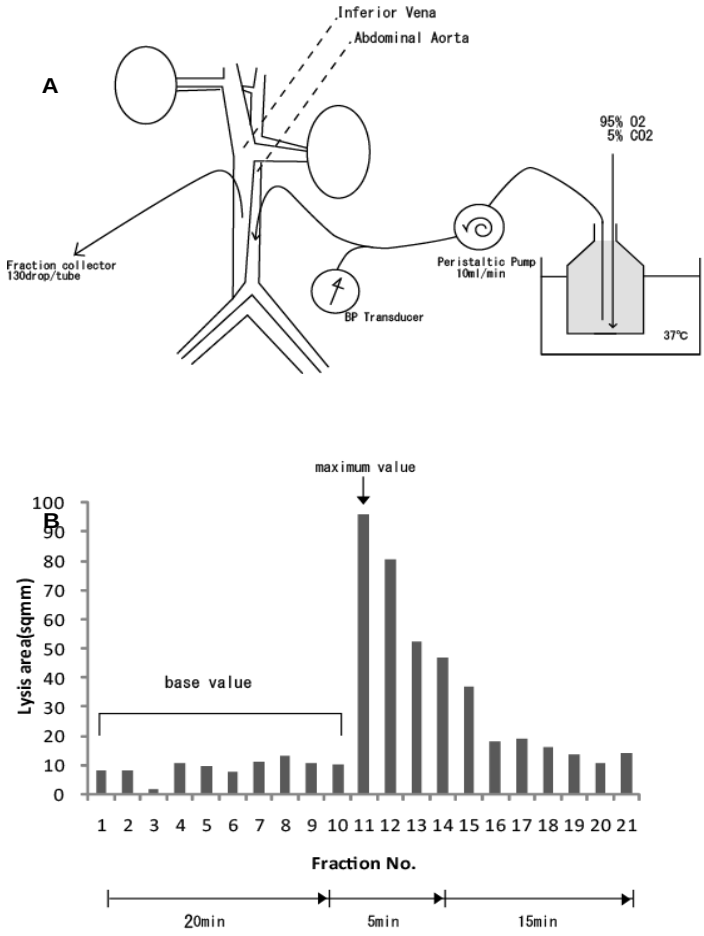

Fig. (1). Effect of fibrinolytic activity of the *natto* extract in the rat hindleg perfusion model.
A, rat hindleg perfusion model; **B**, perfusion eluted patterns. The fibrinolytic activity was measured using the standard fibrin plate method. We evaluated it by dividing the maximum value of the dissolution area caused by sample perfusion by the mean value (baseline) before sample perfusion.

3. RESULTS

Fig. (**1B**) shows the typical result of a blood vessel perfusion experiment performed using the *natto* extract. The fibrinolytic activity of the fractions at 20 min before perfusion with the sample solution was almost constant, and this mean value was assumed as the baseline value. The increase in fibrinolytic activity reached its peak after perfusion with the sample diluted 1000 times with Tyrode's buffer, and the maximum fibrin dissolution and revitalization caused by this stimulation was assumed as the maximum value.

The influence of various *natto* samples on the fibrinolytic activity in the blood vessel perfusion experiment was compared with respect to the maximum value/base value ratio. Fig. (**2**) shows a comparison between the fibrinolytic activity of a sample solution of nonfermented steamed soybean and fermented soybean (*natto*) prepared from the Miyagino strain. The increase in the fibrinolytic activity of the fermented soybean was 4.8 ± 2.1 (n = 8) times, a level greater than the increase in the fibrinolytic activity of the nonfermented soybean (2.9 ± 2.5 times; n = 6).

Increased fibrinolytic activity was confirmed for all 5 varieties of commercially available *natto* that were examined. The maximum increase in fibrinolytic activity, 7.4 ± 3.2 times, was observed with the Okame *natto*. The average increase in fibrinolytic activity caused by the 5 analyzed *natto* varieties was 5.8 ± 3.0 times (Fig. **3**). The Okame *natto* extract was ultrafiltered using UHP-43K. The sample solution was fractionated to yield fractions with the following molecular weights: >200,000, F-1; 200,000–50,000, F-2; 50,000–20,000, F-3; 20,000–10,000, F-4; and <10,000, F-5. The concentration of each sample was adjusted to that of the control, which was not ultrafiltered. The highest fibrinolytic activity, 5.8 times, was shown by F-1, whereas the lowest, 3.9 times, was shown by F-5 (Fig. **4**). Two or more related substances appeared to be responsible for the increase in fibrinolytic activity since it increased after the administration of all samples. The *natto* extract was subjected to ion-exchange chromatography using MARATHON MSA-1 as the anion-exchange resin. The increase in the fibrinolytic activity of the unabsorbed fraction was 3.2 ± 1.0 times. However, this increase was 50% less than that shown by the

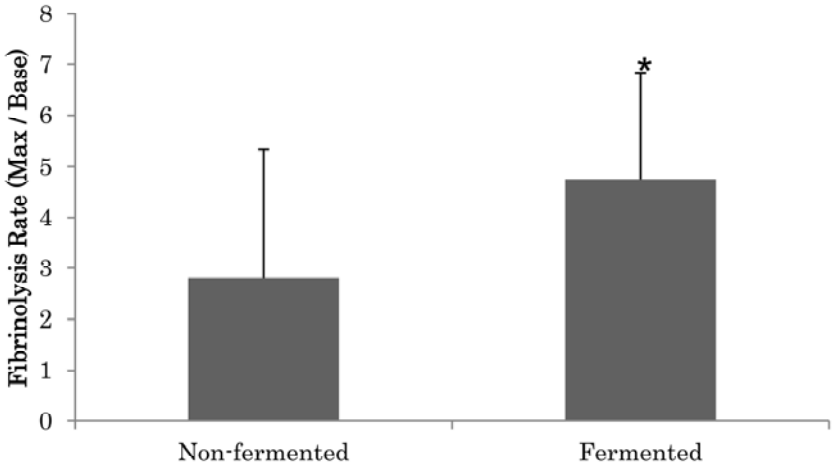

Fig. (2). Comparison of fibrinolytic activity according to fermentation condition.
The fibrinolytic activity was measured using the standard fibrin plate method. The indicated fibrinolysis rate is the maximum value divided by the baseline value. Values are presented as mean ± SD; n (nonfermented) = 6; n (fermented) = 8. *P < 0.05 vs. baseline value.

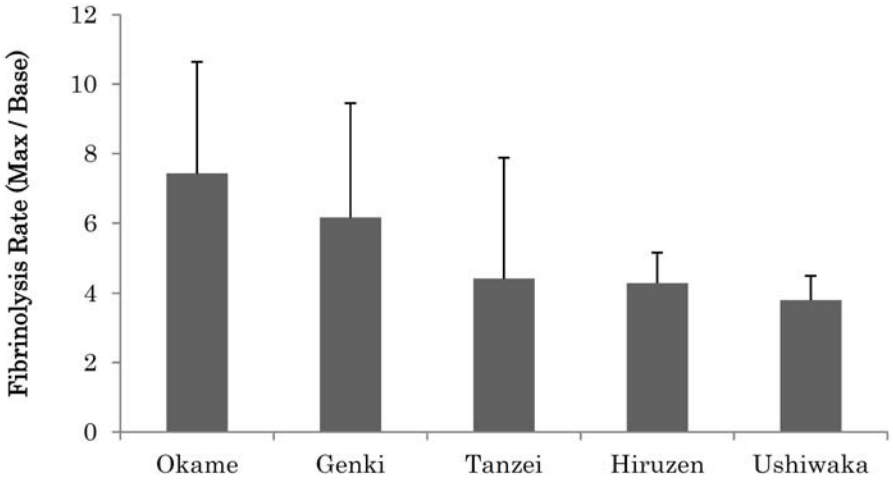

Fig. (3). Comparison of fibrinolytic activity between commercial *natto* types.
The 5 kinds of *natto* generally sold in Japan. Values are presented as mean ± SD; n (Okame) = 8; n (others) = 3.

unprocessed sample. Zymographic examination was performed using special high-activity perfusion solutions as described above. As shown by the zymography pattern in Fig. (**5**), an increase in the t-PA band width (68 kDa) was confirmed for all samples.

4. DISCUSSION

We confirmed that a thrombolysis-inducing substance is present in *natto*. There are many studies on the fibrous dissolution of nattokinase. The substance confirmed to be present in *natto* through this study increased t-PA secretion from endothelial cells. However, the mechanism of action of this substance appeared to be different from the direct action of t-PA and plasmin on the fibrinolysis system. This substance was produced by *B. subtilis natto* fermentation and is present in several commercially available *natto* varieties. In our study, a concentration of 500 μg/mL (wet weight) of the substance in *natto* increased the fibrinolytic activity; this concentration is present in approximately 2.5 g of *natto*, which is rather a very low amount given that the average total body weight of an adult male is 60 kg.

We have reported the physiological function of DPA that exists in *natto*. DPA is present in the spores of *B. subtilis* and can be separated by ion-exchange chromatography because it tends to get adsorbed onto the ion-exchange resin. A similar hindleg perfusion experiment using 10 μM of the Na salt of DPA (1.67 μg/mL) showed an increase in fibrin dissolution and revitalization and confirmed that DPA accelerated t-PA release from the blood vessels [11].

Considering that the average DPA content in *natto* is 18.7 mg per 100 g (wet weight) [3], the *natto* extract in the solution used in the above-mentioned perfusion experiment contained approximately 90 ng/mL of DPA at a concentration of 0.52 μM. Further, on the basis of the results of ion-exchange chromatography and ultrafiltration, the increase in t-PA–mediated revitalization could be confirmed even in fractions having molecular weights >10,000 that do not contain DPA. Therefore, DPA may not be the only contributing

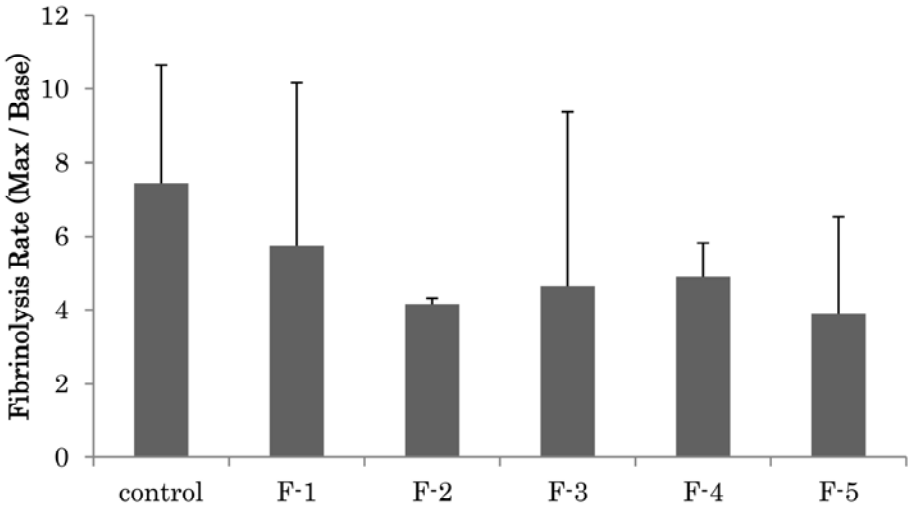

Fig. (4). Comparison of fibrinolytic activity by molecular weight fractionation.
Control, not ultrafiltered; F-1, MW >200,000; F-2, MW 200,000–50,000; F-3, MW 50,000–20,000; F-4, MW 20,000–10,000; F-5, MW <10,000. Values are presented as mean ± SD; n (control) = 8; n (others) = 3.

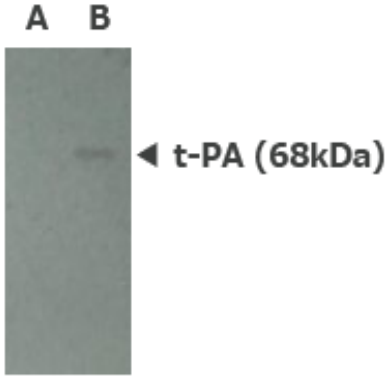

Fig. (5). Zymography patterns of the perfusion solution. t-PA, tissue-type plasminogen activator.
The conditioned medium was observed using fibrin zymography.
A, mixture of baseline fraction; B, maximum fraction.

fibrinolytic factor, and other substances may be primarily responsible for the fibrinolytic activity through an alternative mechanism. However, these materials were not identified in the present study. Further experiments are required to separate and identify fibrinolytic substances other than DPA that are present in *natto*. It may be possible to use *natto* widely as a thrombus-preventing food material because the substance found in *natto* is heat stable and can withstand cooking.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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