Nattokinase-Promoted Tissue Plasminogen Activator Release from Human Cells

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Key Words

Nattokinase · *Bacillus subtilis natto* · Tissue plasminogen activator · Human vascular endothelial cell

Abstract

When heated to a temperature of 70°C or higher, the strong fibrinolytic activity of nattokinase in a solution was deactivated. Similar results were observed in the case of using Suc-Ala-Ala-Pro-Phe-pNA and H-D-Val-Leu-Lys-pNA, which are synthetic substrates of nattokinase. In the current study, tests were conducted on the indirect fibrinolytic effects of the substances containing nattokinase that had been deactivated through heating at 121°C for 15 min. *Bacillus subtilis natto* culture solutions made from three types of bacteria strain were heat-treated and deactivated, and it was found that these culture solutions had the ability to generate tissue plasminogen activators (tPA) from vascular endothelial cells and HeLa cells at certain concentration levels. For example, it was found that the addition of heat-treated culture solution of the Naruse strain (undiluted solution) raises the tPA activity of HeLa cells to about 20 times that of the control. Under the same conditions, tPA activity was raised to a level about 5 times higher for human vascular endothelial cells (HUVEC), and to a level about 24 times higher for nattokinase sold on the market. No change in cell count was observed for HeLa cells and HUVEC in the culture solution at these concentrations, and the level of activity was found to vary with concentration.

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Introduction

In the autumn of 1982, Sumi et al. [1] discovered that nattō, a traditional fermented food in Japan, possesses a hitherto unidentified and very strong fibrinolytic activity. Because the isolated fibrinolytic enzyme had a very strong fibrinolytic activity similar to that of plasmins, the enzyme was named ‘nattokinase’.

Nattokinase was extracted from nattō sold on the market, purified and packed in enteric-coated capsules, and the 1.3-gram capsules were orally administered to test subjects three times a day. Plasma euglobulin fractions were obtained over a period of time by drawing blood from the test subjects. It was found that with the gradual rise in euglobulin fibrinolytic activity of the plasma euglobulin fractions, there was a significant increase ($p < 0.001$) in fibrin degradation products in the blood, starting from the first day on which nattokinase was orally administered. An increase in tissue plasminogen activator (tPA) was also observed [2]. It should be noted that since bacteria (*Bacillus subtilis natto*) are the source of nattokinase, the mass production of nattokinase is possible at a very low cost. It was also found that nattokinase is relatively stable in relation to heat and acidity, and that its separation and purification is quite easy. One of the most noteworthy characteristics of nattokinase is the fact that nattō itself has been consumed in daily life by common Japanese people for more than 1,000 years, which provides strong reassurance on the safety of the oral ad-

ministration of nattokinase [3–5]. The three strains by Naruse, Miyagino and Takahashi have been used as seed bacteria for a long time in fermentation processes for food preservation in Japan, which means the safety of these strains is also assured [4].

In the current study, the effects of nattokinase on human vascular endothelial cells (HUVEC) and HeLa cells were observed after the nattokinase was heat-treated and deactivated. As a result, it was found that the activity of nattokinase is not limited to that of conventional active types, and that heat-treated and deactivated nattokinase also shows strong activity in releasing tPA from living cells.

Materials and Methods

For *B. subtilis natto*, the following three representative strains of seed bacteria used for food in Japan were used: the Naruse strain (Naruse Fermentation Chemical Laboratory KK, Tokyo, Japan), the Miyagino strain (Miyagino Natto Seizoujo, Sendai, Japan) and the Takahashi strain (Yuzo Takahashi Laboratory, Yamagata, Japan). The IFO 3301 strain of *Escherichia coli* was purchased from the Institute for Fermentation, Osaka, Japan (IFO). The nattokinase that currently has the highest level of purity was supplied by Honda Trading Corporation (Tokyo, Japan; about 20,000 FU/g). Bovine fibrinogens and fetal bovine serum (FBS) were purchased from Sigma-Aldrich, and bovine thrombins from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan).

Bacillus subtilis natto Propagation Product

For the heat-treated product of the *B. subtilis natto* culture solution, 3% dry bouillons were used as medium. 250 ml of the medium was placed in a 500-ml Erlenmeyer/shaker flask, and after performing heat sterilization at 121°C for 15 min in an autoclave, 1 platinum loop of the slant-cultured bacteria (5×10^5 /ml) was inoculated. Then the culture was shaken at 100 rpm for 96 h at 37°C. After the preparation of the culture was complete, the supernatant was heat-treated in an autoclave and filtered through a 0.2- μ m membrane filter (Advantec KGS-47-TF), with the resulting product used as the test sample.

Cell Culture

FBS, Eagle's amino acids and vitamins medium, L-glutamine and sodium bicarbonate were added to Eagle's MEM. In this culture solution, at 37°C under partial pressure of 5% CO₂, HeLa S3 cells (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) were multiplied and subcultured in 25- or 75-cm² culture flasks (Falcon). The multiplied HeLa S3 cells were separated by using 0.25% trypsin and suspended in fresh culture solution. A measurement of the cell count was conducted using a hemacytometer. These cells were put in a 24-well plate (Falcon) at a rate of 1×10^5 cells/cm². After the culture was performed and reached the confluent state, the used culture solution was disposed of. After washing the cells twice with 500 μ l of FBS, 450 μ l of fresh culture solution and 50 μ l of reagents were added. The culture solution collected after a culture of 24 h was established as the '1st medium'. Centrifugal

separation was performed on the collected culture solution at 4°C and 4,000 rpm for 10 min. The supernatant was collected and stored at –20°C until it was used for each test [6].

The multiplication medium for human endothelial cells was used for HUVEC (Toyobo Co., Ltd., Osaka, Japan) [7]. In a process similar to that for the HeLa cells, the cells were placed in a 24-well plate and cultured at 37°C under partial pressure of 5% CO₂ until reaching the confluent state. After disposing of the used culture solution and washing the cells with FBS, 450 μ l of fresh culture solution and 50 μ l of reagents were added and the culture solution collected, after a culture of 24 h was established as the '1st medium'. For the collected culture solution, centrifugal separation was performed at 4°C and 4,000 rpm for 10 min. The supernatant was recovered and stored at –20°C until used for each test.

Cell Count

After the culture solution was collected, the cells remaining on the plate were separated using 0.25% trypsin-EDTA and suspended in culture solution and PBS for a measurement of the cell count based on an absorbance at 600 nm. The cell count was calculated based on a calibration curve ($A_{600} 1.0 = 13.4 \times 10^5$ cells/ml).

Fibrinolytic Activity

Nattokinase activity was shown by the lysis area (in mm²) in artificial thrombi through the standard fibrin plate method [8]. An amount of 30 μ l of the culture solution collected from the well plate was placed on each 0.5% fibrin plate, which was created by using fibrinogens, borate-buffered saline (pH 7.8) and thrombins. After incubation at 37°C, the lysis area was measured after 4 h for nattokinase and after 48 h for other substances.

Amidase Activity

Nattokinase activity was also determined by the colorimetric method by using the synthetic substrate Suc-Ala-Ala-Pro-Phe-pNA or H-D-Val-Leu-Lys-pNA. The reaction mixture (1 ml) contained 100 μ l of the substrate (final concentration of 5×10^{-4} M) and 0.1 M borate-buffered saline at pH 7.8. The amount of pNA released was determined based on the absorbance at 405 nm.

Zymography

To conduct zymography on the culture solutions, SDS-PAGE was performed on 10% gel slabs, after which the gel was washed with Triton X-100 for 30 min. Then the gel was layered on top of the fibrin plate containing agar that was created inside a square petri dish, and after incubation at 37°C, the bands that were formed were identified [9]. As the molecular weight standard, tPA (68 kDa; hapase, 3,600,000 U/vial; Kowa Pharmaceutical Co., Ltd.) was used.

Reverse Transcriptase Polymerase Chain Reaction

HeLa cells were cultured in 25-cm² culture flasks until reaching the confluent state. After treatment with the heated product of *B. subtilis natto* culture solution for 24 h, the conditioned medium was replaced with a fresh one. The conditioned medium was collected after incubation for 24 h. The cells were washed twice with PBS, and total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method [10]. The cDNA templates were synthesized using SuperScript II. To prepare the PCR

Fig. 1. Deactivation by heat treatment as observed for fibrinolytic activity. The lysis area (mm²) on the fibrin plate was measured for 30 μ l of the sample after 4 h at 37°C. Results for 20 FU/ml (1 mg) nattokinase before and after heat treatment, for the control (20°C) and at 70, 80, 90 and 100°C (10 min). 1 = Control; 2 = 70°C; 3 = 80°C; 4 = 90°C; 5 = 100°C.

to amplify the tPA gene, the following primers were used as forward and reverse primers, respectively: 5'-ATC TTG GGC AGA ACA TAC CG-3' and 5'-TGC ACT CTT CCC TCT CCT GT-3'. Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as the internal standard, we performed RT-PCR with 5'-GAG TCA ACG GAT TTG GTC G-3' and 5'-TCG CTG TTG AAG TCA GAG GA-3' as the forward and reverse primers, respectively [11].

Statistical Analysis

The differences between mean values were analyzed by the Student t test [12].

Results

Nattokinase was diluted to 20 FU/ml (1 mg; 30°C) with saline and was heat-treated for 10 min at temperatures between 70 and 100°C, which resulted in deactivation at 70°C for each of the samples (fig. 1). The results were similar with heating solutions of *B. subtilis natto* culture made from the three strains of bacteria. Similarly, the strong amidase activity of nattokinase for Suc-

Fig. 2. Activity of 20 FU/ml nattokinase for Suc-Ala-Ala-Pro-Phe-pNA. ○ = 100 μ l/ml; ● = 10 μ l/ml; △ = 1 μ l/ml.

Fig. 3. Activity of 20 FU/ml nattokinase for H-D-Val-Leu-Lys-pNA. ○ = 1 mg/ml; ● = 100 μ l/ml; △ = 10 μ l/ml.

Ala-Ala-Pro-Phe-pNA, H-D-Val-Leu-Lys-pNA and other substrates [9] was deactivated when heat-treated for 10 min at 100°C (fig. 2, 3; cases with no activity observed have been omitted).

Zymography was applied to the heat-treated product of *B. subtilis natto* culture solution made from the Naruse strain, a representative *B. subtilis natto* strain, and a check was conducted on the molecular species of enzymes affecting HeLa cells (fig. 4). Although no activity was observed for any of the enzyme solutions, it was found that the addition of 1/8–1 (undiluted solution) of the heat-

Fig. 4. Zymography (active enzymes after SDS-PAGE) of HeLa medium. 1–1/64: Dilution of heated original culture (Naruse strain). tPA: shown for standard tPA.

Table 1. tPA-generating effects of heat-treated products of *B. subtilis natto* culture solutions containing the major strains of seed bacteria

	Sample mm ²	Control mm ²	S/C ratio	n	p
<i>B. subtilis natto</i> culture solutions					
Naruse strain	326.2 ± 121.5	16.4 ± 20.2	19.9	38	*
Miyagino strain	486.8 ± 6.6	24.4 ± 7.2	20.2	3	*
Takahashi strain	237.3 ± 121.6	23.5 ± 6.3	10.1	3	
<i>E. coli</i> culture solution					
<i>E. coli</i> IFO 3301	31.3 ± 5.7	17.3 ± 2.3	1.8	3	

S/C ratio = Sample/control ratio. Values denote means ± SD unless otherwise stated. The lysis area (mm²) on the fibrin plate was measured for 30 µl of sample after 48 h at 37°C. * p < 0.001.

Fig. 5. Effects of heat-treated product of *B. subtilis natto* culture solution (Naruse strain) on HeLa culture solution. The media collected from cultured HeLa S3 cells were applied on a fibrin plate. **Inset** Results of a Northern blot analysis of RNA from HeLa S3 cells. The Northern blot analysis used a tPA mRNA hybridization probe and, as a loading control, a GAPDH mRNA hybridization probe.

treated product of *B. subtilis natto* culture solution to HeLa cells considerably increased tPA, as shown in the concentrations on a fibrin agar plate. Also, bands were identified in the region, showing the same molecular weight. At the same time, Northern blot analysis results showed increases in the amount of tPA RNA contained in the sample although no change was observed in the

amount produced for the internal standard of GAPDH mRNA (fig. 5).

In a comparative test using the heat-treated culture solution of *B. subtilis natto*, the addition of each sample resulted in an activity that increased the production of tPA from human cells to a level of 10 or more times that of the control (237.3–486.8 mm²). In particular, the cul-

Fig. 6. Effects of heat-treated product of *B. subtilis natto* culture solution (Naruse strain) on HUVEC culture solution. □ = Fibrinolysis; ● = cell number.

Fig. 7. Effects of heat-treated product of nattokinase (NK) on HeLa culture solution. □ = Fibrinolysis; ● = cell number.

ture solution for the Naruse strain displayed a strong activity that was about 20 times as much as the level for the control of water (326.2/16.4; $p < 0.001$; $n = 38$). The sample/control ratio was <2.0 when the same test was conducted for comparison purposes with the *E. coli* IFO 3301 strain (table 1).

A check was conducted on the effects of the culture on HUVEC, using the heat-treated product of the culture solution that was obtained by the culture of *B. subtilis natto*. As shown in figure 6, a strong fibrinolytic activity

compared with the control (water) was observed when the sample was added (89/19 or 4.5 times as much as the control, observed for an undiluted solution, with $n = 5$).

Using the heat-treated product of nattokinase, a strong fibrinolytic activity was observed at concentrations of 5 FU/ml and higher (0.25 mg) in terms of the original enzyme amount (fig. 7). A check of the effects of the addition of nattokinase on HeLa cells, with saline as the control, showed a strong fibrinolytic activity of 24 times that of the control (215/9; $n = 4$) when the undiluted solution was added. There were no substantial changes in the cell count at the time of the culture for either the HUVEC or the HeLa cells (fig. 6, 7).

Discussion

Deactivation of the fibrinolytic activity of nattokinase occurred when the nattokinase was heated to 70°C. It was also found that the synthetic substrates of Suc-Ala-Ala-Pro-Phe-pNA and H-D-Val-Leu-Lys-pNA were deactivated in addition to fibrinolysis (fig. 1–3). The activity of vascular endothelial and HeLa cells to generate tPA is well known, but it was found in the current study that both of these reaction systems are greatly stimulated by *B. subtilis natto* culture solution to an enhanced tPA activity (fig. 5; table 1). A Northern blot analysis also confirmed the synthesis of tPA from HeLa cells. The zymography conducted using the culture solution with increased fibrinolytic activity showed that bands could be identified in the same region as that of the tPA standard (68 kDa).

When undiluted solutions were added, an activity of 10 or more times the control was observed for all the nattō samples. This level of activity is noteworthy. Regarding the effects of the Naruse strain on HUVEC and those of nattokinase on HeLa cells, no correlation was found between the amount of the heat-treated product added and the change in cell count (fig. 6, 7). In addition to tPA, a molecular species believed to be the plasminogen activator inhibitor-1/tPA complex was found, but the relationship between urokinase-type plasminogen activator and other proteases is still unclear.

Nattō sold on the market contains 0.6–4.0 mg dipicolinic acid, a substance that is known to be active in antiplatelet aggregation and fibrinolytic systems. This means that someone who eats nattō ingests this amount of dipicolinic acid on a daily basis [13]. Dipicolinic acid is a kind of chelate substance with some notable characteristics such as promoting the growth of *B. subtilis natto* and

increasing the amounts of nattokinase and vitamin K₂ [14, 15]. Hagiwara et al. [16] reported that sitosterol and fucosterol work on bovine vascular endothelial cells to help release tPA. It was also found that the prostacyclin [17] and prostaglandin P₁ [18] derivatives show activity in promoting the release of tPA. It has recently been reported that a large amount of tPA is produced in the central nervous system, and tPA is now drawing more attention to its role in neuron movements, neurite outgrowth, neural development, morphogenesis (including spine formation), fluidity, learning, memory, Alzheimer's disease and nerve cell death from anemia [19–21].

Nattokinase products, which capitalize on the strong fibrinolytic activity of the nattokinase enzyme, are currently sold by about 20 companies around the world. However, the possibility has now been raised that even heat-treated nattokinase with no direct fibrinolytic activity may work effectively on cells of living organisms in an indirect manner. Our current plan is to study the active fractions (probably with a molecular weight of 10,000 or less) responsible for these functions to learn what constitutes these fractions and the mechanism at work.

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