Increasing the Production of Nattokinase and Vitamin K_2 in Natto with Dipicolinic Acid

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Abstracts: It was found that when dipicolinic acid is added to the culture solution of *Bacillus subtilis natto*, the area of fibrin dissolved by nattokinase (standard fibrin plate) and the amidase activity of nattokinase against Suc-Ala-Ala-Pro-Phe-pNA increased. For example, when manufacturing natto using steamed soybeans, the addition of 10-64 mM of dipicolinic acid increases amidase activity by more than 10 times.

The concentration of vitamin K_2 (menaquinone-7) also increased by about 4 times with the addition of 10 mM of dipicolinic acid. No other food contains such a high concentration of vitamin K_2. The results were the same for the shaking culture as well as for the stationary culture. If the concentration of dipicolinic acid is appropriately controlled, a product with excellent levels for both nattokinase activity and vitamin K_2 concentration could be manufactured.

INTRODUCTION

Natto is a traditional fermented soybean food in Japan that is said to have a history of about 1,000 years. The current annual production of natto is more than 300,000 tons. Biotin is required as an essential nutrient for growth of the bacteria used in production of natto. We have conducted research on nattokinase [1-3], a strong fibrinolytic enzyme contained in natto, and dipicolinic acid (DPA; 2,6-pyridinedicarboxylic acid), known as an antibacterial substance. In a living organism, plasmin acts directly on fibrins to cause fibrinolysis, but it has been found that nattokinase not only directly causes fibrinolysis but also has a strong effect in activating pro-urokinase molecules [4], thus showing that nattokinase also has the indirect activity of promoting fibrinolysis [5, 6].

Although the mechanism is not understood, natto has been taken as a vitamin K_2 rich food [7]. Epidemiological data reportedly indicates that the intake of natto may play a role in the prevention of osteoporosis. A trial study showed increased vitamin K_2 and r-carboxirated osteocalcin concentrations in the plasma of normal, healthy individuals following dietary intake of natto containing vitamin K_2 over a long period [8].

Dipicolinic acid has sometimes been described as a substance eliminating radioactivity, but it is essentially an antibacterial substance with strong inhibitory activity against yeast and the growth of O-157, among other substances [9]. Dipicolinic acid has also drawn attention recently for its inhibitory effects on the anti-platelet aggregation activity of blood, with those effects per weight being stronger than those of aspirin [10]. Furthermore, dipicolinic acid is well-known as a chelate substance existing within spores that is closely related to the heat resistance of bacteria [11]. However, there has been no previous study comparing the two biologically active substances of nattokinase and vitamin K_2 in the presence of a substantial amount of dipicolinic acid (0-100mM).

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MATERIALS AND METHODOLOGY

DPA from Nacalai Tesque, Inc. was used and an aqueous solution was prepared with sodium hydroxide at pH 7.4 for use in the test. Bovine fibrinogen, which was used as the artificial thrombus, and Suc-Ala-Ala-Pro-Phe-pNA, an amide substrate, of Sigma Co., Ltd. were used. Bovine thrombin from Mochida Pharmaceutical Co., Ltd. was used. For the culture medium, Nissui Pharmaceutical Co., Ltd. dry bouillon was used. For *Bacillus subtilis natto*, the Miyagino and Naruse strains, the typical strains used in food, and the Meguro strain, used as a digestive, were used. *Bacillus subtilis* IAM 12118 (type Marburg), a representative strain of *Bacillus subtilis* in the genus Bacillus, was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo.

For the culture of *Bacillus subtilis natto*, the following three methods were employed. Inside a test tube (18 mm×105 mm), 0.5 mL of DPA was added to 0.5 mL of the culture medium, so that the final concentration would be 0-4 mM. 50 μL of 1 platinum loop/10 mL of *Bacillus subtilis natto* (Miyagino strain, about 1.0×10^8 bacteria/mL) was then inoculated onto the mixture and stationary culture was performed at 37°C for 24 hours. Next, steamed soybeans and DPA were added to a 90 mm-square of polystyrene paper (PSP), so that the final concentration would be 0-100 mM (wet weight of 50 g). The sample (0.5 mL) of 0.1 g/10 mL *Bacillus subtilis natto* (Meguro strain, about 1.0×10^9 bacteria/mL) was then inoculated onto the mixture and stationary culture in this PSP was performed at 37°C for 24 hours. 1% glycerin/3% polypepton S and DPA were added to a 500 mL Erlenmeyer flask, so that the final concentration would be 0-20 mM (wet weight of 150 g), and inoculated with 1.0mL of 1 platinum loop/10 mL of *Bacillus subtilis natto* (Miyagino strain, about 0.5×10^7 bacteria/mL). Shaking culture was then performed for 72 hours at 37°C and 100 rpm.

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Number of Bacterial Cells

After centrifugal separation (5000g×10 minutes) was performed for the culture solution, the number of bacterial cells per 1 mL of the supernatant was counted by using a microscope counting chamber.

Measurement of Nattokinase Activity

Standard Fibrin Plate Method [1]

Inside a rectangular petri dish of 144 mm×104 mm, 20 mL of fibrinogen solution with a final concentration of 0.5% and 100 μL of 50 U/mL thrombin were added to create a fibrin plate.

Each sample (30 μL) was placed on the plate and after incubation at 37°C for 4 or 20 hours, the lysis area (mm^2) was measured.

Synthetic Amide Substrate Dissolution Method [12]

To the system of reaction with 0.1 mL of each sample and 0.1 M phosphate buffer-saline (pH 7.8), 0.1 mL of Suc-Ala-Ala-Pro-Phe-pNA prepared so that the final concentra-

Fig. (1). Dipicolinic acid added and nattokinase activity.

Data are presented as a mean±SD of 3 separated measurements. Dipicolinic acid concentration was 0 mM (□), 1 mM (■), 2 mM (■), 4 mM (■).

a; The standard fibrin plate method.

It was compared with *Bacillus subtilis natto* or *Bacillus subtilis* IAM 12118. Nattokinase activity was determined on a fibrin plate at 37°C for 4 hours.

b; Synthetic amide substrate.

Final concentration of 5×10^{-4} M Suc-Ala-Ala-Pro-Phe-*p*NA was used for enzymatic substrate.

The results are expressed as nmol of substrate hydrolyzed per min per mL of each sample.

c; Number of bacterial cells.

The cells were counted by using a counting chamber.

tion would be 5×10^{-4} M was added. After incubation at 37°C for 5 minutes, absorbance of the released pNA was measured at 405 nm. Prior to addition of the substrate, pre-incubation was performed for each sample and 0.1 M phosphate buffer-saline was added at 37°C for 2 minutes. Based on the standard curve, the amount of released pNA (nmol) per minute for 1 mL of the system of reaction was calculated.

Measurement of Vitamin K_2 Concentration

Vitamin K_2 was measured through the already stated HPLC method by drawing on the fact that reduction of menaquinone-7 (MK-7) to hydroquinone is achieved by platinum-alumina catalyst and that it becomes fluorescent [13, 14]. Using ODS-II (Shimadzu Corp.: ϕ4.6×250 mm) bonded with the octadecyl group (C18) for the stationary phase as the reversed phase distribution column; platinum-alumina (Wako Pure Chemical Industries, Ltd.: ϕ4.0×10 mm) as the catalyst column; and 97% ethanol as the developing solvent, the procedures were performed at 40°C and 0.7 mL/minute. 0.1 mL of the sample, 0.9 mL of distilled water and 1.5 mL of isopropanol were mixed. After 5.0 mL of hexane was added and the mixture was stirred, centrifugal separation was performed at 20°C and 1710 ×g for 10 minutes. 4 mL of the supernatant hexane fraction was condensed with an evaporator and dissolved in 100 μL of ethanol, following which the solution was analyzed through HPLC.

Standard Analysis

Differences between mean values were analyzed by Student's t-test.

RESULTS AND DISCUSSION

Fig. (**1**) shows the results of cultivating *Bacillus subtilis natto* with a certain added amount (2mM) of dipicolinic acid. a) shows fibrinolytic activity for the fibrin plate, while b) shows the amidase activity of nattokinase. c) shows the number of bacterial cells (average value of 3 counts). It is clear in particular that for the Miyagino strain and Naruse strain, which are the representative strains of *Bacillus subtilis natto*, there were substantial changes in the amount of nattokinase produced depending on the concentration of dipicolinic acid. On the other hand, there was scarcely any difference observed in the activity of *Bacillus subtilis*, which was used as a control. The number of bacterial cells was the highest for the control at $2089\text{-}2943\times10^5$/mL. Given the weak fibrinolytic activity and amidase activity of the control, however, the results are believed to demonstrate that no correlation exists between the number of bacterial cells and these activities.

Fig. (**2**) (top) shows the photograph of a fibrin plate after cultivation at 37°C for 20hours. Steamed soybeans were used as the substrate to create conditions similar to those for the manufacturing of natto. 0-100 mM of dipicolinic acid

Fig. (2). Dipicolinic acid added and nattokinase in natto.

Results of measurement with the standard fibrin plate method. 0-100 mM of dipicolinic acid was added to steamed soybeans. *Bacillus subtilis natto* (Meguro strain) was added and stationary culture was performed in a PSP at 37°C for 24 hours.

Result of measuring amidase activity. Data are presented as a mean±SD of 3 separated measurements. High values shown for 10-64 mM of dipicolinic acid.

Table 1. Concentration of Dipicolinic Acid and Amount of Vitamin K_2 Production

Natto Culture (mM)	Vitamin K_2 (μg/ml)	Shaking Culture (mM)	Vitamin K_2 (μg/ml)
DPA 0	1.25±0.07	DPA 0	1.16±0.08
10	4.53±0.14*	4	2.75±0.24
16	2.84±0.11	10	3.26±0.09**
32	1.31±0.17	20	0.04±0.04
100	0.18±0.14	100	0±0

The amount of vitamin K_2 in the fermented product was though the HPLC.
Data are presented a mean±SD of 5 separated concentrations. *p<0.2, **p<0.1

was added to steamed soybeans, onto which *Bacillus subtilis natto* was then inoculated. The figure shows the results after the cultivation for 24 hours at 37°C. After the completion of the fermentation process, the soybeans were crushed by a mixer together with double its amount of saline, and the supernatant obtained as a result of centrifugal separation was used as the sample. It was clear that activity was particularly high with a high concentration of dipicolinic acid (10-64 mM) compared with the control (the strongest activity being observed with 479 mm^2/mL at 16 mM).

Fig. (**2**) (below) shows changes in the dissolving activity for Suc-Ala-Ala-Pro-Phe-pNA. Rather high values were observed with the addition of 10-64 mM of dipicolinic acid, and it was found that the highest value reached was over 10 times as much as the value for the control.

Table **1** (left) shows the results of a test to check the amount of vitamin K_2 produced under the same conditions. Using the supernatant described above, the amount of vitamin K_2 contained in the fermented product was measured through the HPLC method. It was found that the amount was as much as 4 times the amount for the control (1.25±0.07 μg/mL) in the case of 10mM of dipicolinic acid (4.53±0.14 μg/mL).

Next, using 3% polypepton S containing 1% glycerin as the substrate, *Bacillus subtilis natto* (Miyagino strain) was added and shaking of the culture was performed at 37°C for 3 days (see Table **1** right). The amount of vitamin K_2 was about 3 times (3.26±0.09 μg/mL) as much as the amount for the control (1.16±0.08 μg/mL), in the case of adding 10 mM of dipicolinic acid. The amount of nattokinase increased to the highest level of 753±21 mm^2 in the case of adding 4 mM of dipicolinic acid, compared with the value of 511±9 mm^2 for the control.

Because the Vitamin K_2 content in natto has antagonistic effects on warfarin, clinical guidance has been provided for patients being administered warfarin to refrain from eating natto [15]. On the other hand, Vitamin K_2 in natto has been designated as a food with specific health use in preventing osteoporosis [16]. However, it is said that the amount of vitamin K_2 produced for such use is only 1.5–1.0 times as much as the level contained in ordinary natto. It would be quite interesting to search for the conditions which produce vitamin K_2 at higher concentration levels.

There is no clear understanding about the activity of dipicolinic acid according to the reference documents currently available. However, it was reported in an earlier study that more dipicolinic acid is produced by *Bacillus subtilis natto* when a stock with 0.5% soybean extract is used as the culture medium instead of a stock with 2% soybean extract, meaning that more dipicolinic acid is produced when the nutritional value is actually lower [17]. However, according to that reference, the maximum value for dipicolinic acid concentration in this case was only around 2 μg/mL (1/100 mM) or less. In our study, it was proved by varying the concentration levels of dipicolinic acid that the amount of vitamin K_2 and nattokinase is higher when dipicolinic acid is at certain concentration levels. It was determined that the increases cannot likely be attributed to any multiplication of bacterial cells, because the concentration levels were much higher at 10-64mM in this case. This is because dipicolinic acid shows strong inhibitory activity at such concentration levels [11, 18]. Dipicolinic acid exists mainly in the pores of *Bacillus subtilis natto*, but it has been shown that a small amount of free dipicolinic acid molecules also exist after a long cultivation process [11]. More detailed research is needed regarding these mechanisms.

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