

A Study on Ingredient Production using Cultured Cells from Microorganisms, Animals, and Plants: Isolation of Nattokinase-producing Bacteria and Nattokinase Production Conditions

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Introduction

Natto is a fermented food that has been eaten for a very long time. Although some people cannot eat it because of its distinctive stickiness and smell, many people enjoy it for the same features. It is well known that natto contains many useful ingredients such as vitamin K2. Nattokinase, an ingredient of natto, has recently been reported to have thrombolytic activity. We planned a cooperative study on nattokinase produced by *Bacillus subtilis natto*, and as part of the study, we first tried to define the basic facts about nattokinase production including proliferation of *Bacillus subtilis natto*.

1. Determination of nattokinase activity

Nattokinase is known to have a plasmin-like activity and a fibrinolytic activity. In the present study, the highly reproducible plasmin-like activity was used to evaluate the activity of nattokinase. The fibrinolytic activity is important for providing a high level of activity, which will be reported later. The plasmin-like activity was determined with a commercially available laboratory test agent, which defines for descriptive purposes 1 unit as the activity that increases absorbance at 405 nm by 0.01/min.

2. Determination of nattokinase contained in commercially available natto

The activity of nattokinase in commercially available natto was studied. One pack of commercially available natto contains 40-80 g of natto. The same quantity of a buffer solution was added to the natto for extraction and the activity in the extract was determined. Prior to determination, enzyme solutions at various concentrations were measured to confirm the dose-dependent activity. The activity per gram of natto was calculated for comparison between products. As a result, 1 pack containing 45g of natto, which was the most common size of pack, contained about 20-25 units/g.

Apart from eating quality, including taste, smell, and stickiness, nattokinase activity tended to be low in natto products taken as having high quality and high in cheaper products.

3. Isolation of bacteria with high nattokinase producing activity

Bacillus subtilis natto was isolated from commercially available natto. Common *Bacillus subtilis natto* includes Takahashi bacteria and Miyagino bacteria. In Japan, natto manufacturers purchase bacteria from natto bacteria suppliers and use it in fermentation. The seed bacteria are a mixture of *Bacillus subtilis natto* and many kinds of bacteria such as lactobacillus. To obtain *Bacillus subtilis natto* with low stickiness and high nattokinase producing activity, the target bacteria was isolated from commercially available natto. Briefly, colonies on an agar plate were isolated and they were separately cultured in a liquid medium. After removing bacterial cells, the culture medium was freeze-dried, a fixed weight of the freeze-dried medium was dissolved in water, and the activity in the solution was determined as mentioned above.

This method allowed isolation of *Bacillus subtilis natto* with high nattokinase producing activity. As the bacteria with high activity were continuously cultured, however, the colony appearance changed or nattokinase activity disappeared in many cases, showing a high rate of mutation. This is why other bacteria such as lactobacillus are mixed in with the seed bacteria sold.

4. Nattokinase production by liquid-cultured *Bacillus subtilis natto*

Ten types of bacteria isolated were liquid-cultured and the culture supernatants were freeze-dried to examine the nattokinase activity, stickiness, and smell.

Stickiness and smell are very important for nattokinase as an ingredient of natto. These two factors make some people hesitate to try natto but make others enjoy it. To obtain a culture ingredient without distinctive stickiness and smell, we tried to product a culture medium, eventually freeze-dried culture medium with minimized stickiness and smell. The stickiness leads to moisture absorption and an uncomfortable feeling on the tongue. The cause of the smell is bacteria-produced ammonia. As ammonia cannot be removed during culture, a treatment was given during freeze-drying to obtain a product with less ammonia smell. The bacteria probably produced nattokinase after the number of cells reached a peak and no nattokinase was produced in a culture liquid without a soybean ingredient. After selecting culture conditions such as medium composition and freeze-drying conditions, the obtained freeze-dried extract from *Bacillus subtilis natto* culture contained an amount per gram of nattokinase corresponding to that contained in 1 pack of the commercially available product.

5. Future challenges

As the used *Bacillus subtilis natto* tend to mutate, it is necessary to select more stable bacteria. The smell was not completely eliminated. This should be improved. In addition, there is room for improvement in the induction of nattokinase.