Determination of the Thrombolytic Activity of Natto Extract

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INTRODUCTION

Bacillus subtilis, the species to which natto bacillus belongs, has been reported to secrete several proteases extracellularly during growth (1-4). Among these proteases, the physiological activity or clinical applications of antithrombotic substances contained in natto, such as the peptidase subtilisin, have been reported (5-8). Based on the results of this research, functional foods have been developed and some thrombolytic activity assay methods have been proposed for use in quality specifications. One of these methods is "the standard of natto bacillus culture extract food (January 15, 2003)" distributed to its members by the Japan Health Food & Nutrition Food Association (Table 1). This standard classifies the products into processed foods, supplemented foods, and raw materials, according to the activity per reference amount for one day as measured by the described method.

Classification	Fibrinolytic Activity
Processed Foods	Containing 2000 FU or more of Nattokinase per Reference Amount for One Day
Supplemented Foods	Containing not less than 1000 FU and less than 2000 FU of Nattokinase per Reference Amount for One Day
Raw Material	Containing 5000 FU/g or more of Nattokinase

***Table 1**: Food standard classification including fibrinolytic activity for natto bacillus culture supplementation.*

In this assay, fibrinogen and thrombin, extracted from bovine plasma, are reacted to produce fibrin polymer. The sample is allowed to act on and degrade this polymer; the obtained acid soluble low-molecular-weight degradation product is measured by its ultraviolet absorbance at 275 nm. However, the method requires several corrections since the substrate is from a natural product, and the method requires the difference in absorbance to measure between 0.04 and 0.08. This range is extremely narrow. Therefore, the method should be improved to be used as a quantification system.

This method was studied to determine the thrombolytic activity of natto extract, and was improved. Moreover, this method was compared with the common enzyme activity assay method of plasmin using a synthetic chromogenic substrate.

METHODS

Plasmin-like activity was measured by the hydrolytic activity on S-2251 (H-D-Val-Leu-Lys-pNA•2HCl / Daiichi Pure Chemicals). The sample was dissolved and diluted with 0.05 M Tris-HCl buffer (pH 9.0) such that the absorbance at 405 nm would not exceed 1.0. A 0.1 mL aliquot of sample solution and 0.2 mL of 0.1 M Tris-HCl buffer (pH 9.0) were incubated with 0.2 mL of 5 mM substrate solution (S-2251 dissolved in purified water), at 37°C for 5 min. The reaction was arrested by adding 0.5 mL of 10%(v/v) acetic acid, and the incubated solution was centrifuged at 10,000 g for 5 min. The absorbance of the obtained supernatant was measured at 405 nm to determine the concentration of p-nitroanilide (pNA). One unit was defined as the enzyme activity required to release 1 nmol of pNA per 1 min of reaction.

Fibrinolytic activity was examined in accordance with "the standard of natto bacillus culture extract food" distributed to its members by the Japan Health Food & Nutrition Food Association. Fibrinogen solution was prepared by dissolving bovine plasma fibrinogen (SIGMA) in 0.05 M boric buffer (pH 8.5) at a concentration of 96 mg/L. Thrombin solution was prepared by dissolving bovine thrombin (SIGMA) in 0.05 M boric buffer (pH 8.5) at 20 IU/mL. The sample was dissolved in acetic buffer containing calcium sulfate, sodium chloride, and triton X-100 at a concentration such that the difference of absorbance at 275 nm would become 0.04 - 0.08 when measured. To form a fibrin mass, 1.4 mL of 0.05 M boric buffer (pH 8.5) and 0.4 mL of fibrinogen solution were incubated with 0.1 mL thrombin solution in a test tube at 37°C for 10 min (after agitation). A 0.1 mL aliquot of sample solution was added to this, the mixture was agitated immediately and again for 5 seconds at 20 and 40 min while incubating at 37°C for 60 min. After the incubation, 2.0 mL of 1.0 M trichloroacetic acid was added, and the mixture was agitated. After further incubation at 37°C for 20 min to arrest the reaction, it was centrifuged at 15,000 g for 5 min. The obtained supernatant was measured for absorbance at 275 nm. As a blank control, another mixture was prepared by adding the sample solution only after adding trichloroacetic acid and incubating, and this was measured as well. The difference between the absorbance of the two mixtures was used as the assay result. One unit (FU) was defined as the enzyme level required to increase the absorbance by 0.01 per 1 min of reaction.

In an improved assay of fibrinolytic activity, 96-well flat-bottom microplates were used. Fibrin polymer was prepared by distributing 0.2 mL of fibrinogen, dissolved in 0.05 M boric buffer (pH 8.5) - 0.15 M NaCl solution (BSB, pH 7.8) at a concentration of 5 mg/mL, and 0.01 mL of thrombin, dissolved in 0.15 M NaCl solution at 50 IU/mL, into each well, and agitating. 0.03 mL of the sample solution was added to each well and incubated at 37°C. Subsequently, absorbance at 655 nm was measured against time. One unit (IFU = improved FU) was defined as the enzyme level required to decrease absorbency by 0.1 per 60 min of reaction.

RESULTS AND DISCUSSION

To verify plasmin-like activity, the present research was conducted with refined cultured natto bacillus, NKCP (Daiwa Pharmaceutical, Co., Ltd.). The main active substance of NKCP was identified as fragments of bacillopeptidase F4) secreted by natto bacillus. NKCP shows plasmin-like activity, and its antithrombotic activity has been thoroughly studied; therefore, it was suitable for this study.

In NKCP, plasmin-like activity and reaction time showed a linear relation for over 10 min and no obvious saturation was observed, so a 5 min reaction time was determined to be suitable for this method *(Figure 1)*. The enzyme level correlated well with the activity, and was linearly related at low to high doses, verifying that this assay yielded quantitative results *(Figure 2)*.

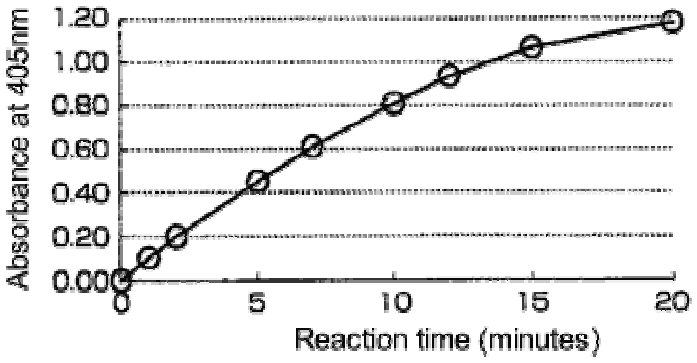

***Figure 1**: The relation between plasmin-like activity and reaction time.*

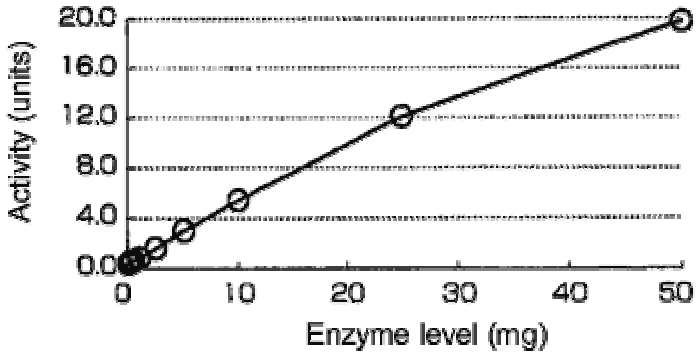

***Figure 2**: The relation between plasmin-like activity and reaction time.*

Fibrinolytic activity and plasmin-like activity were compared using four commercially produced enzymes: urokinase (Funakoshi), lumbrokinase (Waki Pharm.), trypsin (DIFCO), and papain (SIGMA). Urokinase, a serine protease that functions as a tissue plasminogen activator, is obtained from urine. "The standard of natto bacillus culture extract food" states that "1 FU is equivalent to about 50 IU of urokinase"; however, urokinase alone cannot display fibrinolytic activity. Lumbrokinase is obtained from the earthworm (*Lumbricus rubellus*), and is reported to have thrombolytic activity, and trypsin is a protease contained in animal digestive fluid; all of these enzymes have serine protease-like activity. Papain is a protease obtained from papayas and is classified as a cysteine protease.

Urokinase (2,000 units) showed only 1.25 FU of fibrinolytic activity, while the other proteases not obtained from natto were highly active. Enzymes including lumbrokinase and trypsin were not correlated in terms of their thrombolytic activity and plasmin-like activity, and the enzymes which showed higher plasmin-like activity had exponentially low thrombolytic activity. Papain had high fibrinolytic activity *(Figure 3)*.

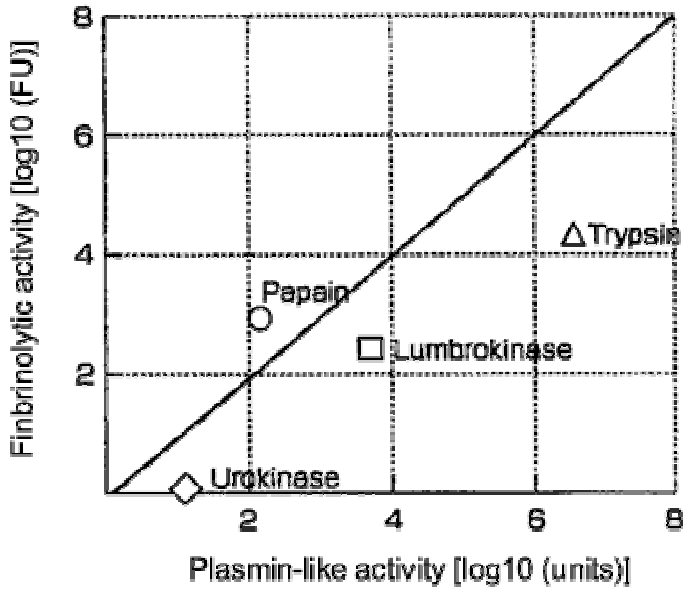

***Figure 3**: Comparison of two assay methods using various enzymes (double logarithmic plot)*

In the assay of fibrinolytic activity, conditions were extremely strictly determined as "the difference of absorbance should be 0.04 - 0.08 when measured"; however, the dilution and the measurement conditions used in this study might not conform to this standard. In addition, fibrinolytic activity could not be converted to plasmin-like activity. The exponentially lower fibrinolytic activity could be caused by some physicochemical quality of the substrate. Fibrin polymerization produces heterogeneous insoluble material with an amorphous surface that would be unlikely to react quantitatively. During the assay, the polymerized material does not disperse sufficiently, and the agitation was inadequate to produce smooth and quantitative reaction with the enzyme. Substances with higher activity may have been more affected by this tendency.

The difference between fibrinolytic activity and plasmin-like activity may have been caused by the assay method used and substrates as distinct from fibrin polymers. The reaction system includes spodogenous fibrinogen and thrombin, and the fibrinopeptides A and B produced during the reaction of fibrinogen and thrombin; any of these can be the substrates used to produce acid soluble low-molecular-weight degradation product. Despite the fact that fibrin degradation products are commonly assayed with the antigen-antibody reaction in medicine, this study uses absorption at 275 nm, which can measure various carbonyl compounds, to measure the amount of reaction. Enzymes such as papain showed high activity, possibly owing to their specificity.

Thus, this represents an improved method to measure fibrinolytic activity. Fibrin polymerization was used to produce a fibrin film to improve the heterogeneous reaction, and the degradation of the fibrin film was measured with transmitted light to improve the assay specificity.

When transmitted light was measured against time at 655 nm using NKCP as the sample, the activity increased linearly with reaction time at each enzyme level *(Figure 4)*. Nevertheless, the activity was high at low enzyme levels, and saturated at high enzyme levels *(Figures 5 and 6)*. After applying the improved method, the relation between the activity and enzyme level was slightly improved, but still not quantitative. This result was brought about by the underlying principle of the measurement, and could not be solved technically.

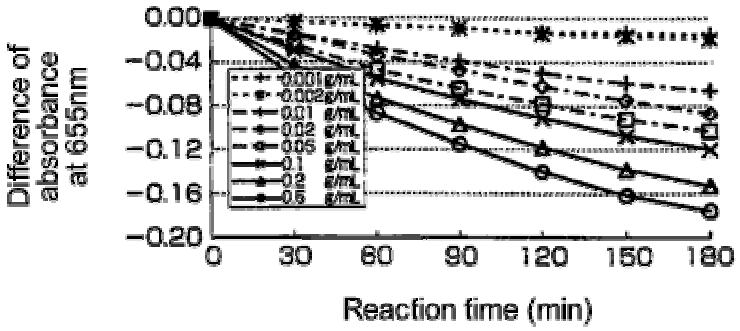

***Figure 4**: Improved fibrinolytic activity (absorption) and reaction time.*

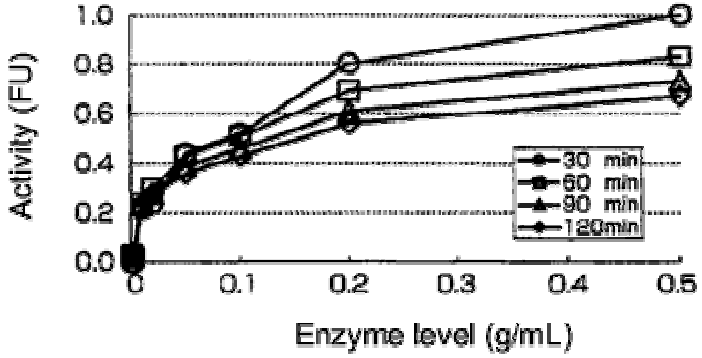

***Figure 5**: Improved fibrinolytic activity.*

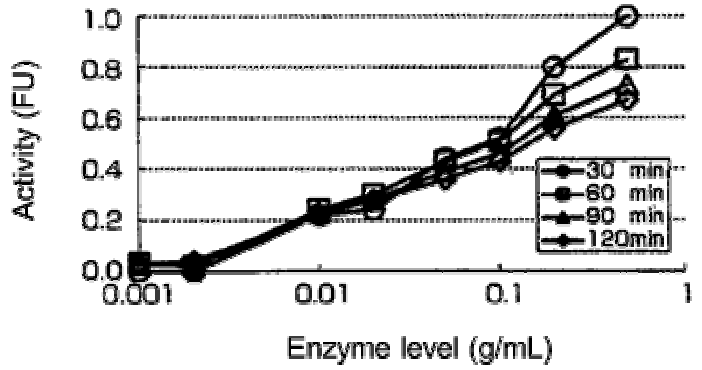

***Figure 6**: Improved fibrinolytic activity (single logarithmic plot)*

Since the assay method from "The standard of natto bacillus culture extract food" uses natural substrates, quality varies considerably from lot to lot. The standard requires that after defining the standard lot of enzyme and substrates, adjustments be made in activity and measurable range according to the combination of reagents used. The method requires that the reference standard should be obtained from the Japan Bio Science Laboratory, but does not provide enough detail to evaluate the suitability of the reference standard for these corrections. Possibly, the plasmin (Plasmin, Human, code 97/536) provided by WHO, which is used in the standard to evaluate some antithrombotic drugs for medical purposes, could be used.

According to Bergey's classification of bacteria, natto bacillus is classified as *Bacillus subtilis* , and several proteases, which are biosynthesized and secreted extracellularly by natto bacillus, have been reported (1-4). While any of the proteases can produce acid soluble low-molecular-weight degradation products, not all the proteases have been reported to have antithrombogenicity. This study revealed high fibrinolytic activity in trypsin and papain. In my view, "The standard of natto bacillus culture extract food" should indicate the quantitative value measured by other quantification methods such as the antibody method, in addition to activity values for substances that have already been reported or researched for their

antithrombogenicity and safety. Otherwise, this standard may end in indicating only fibrinolytic activity, regardless of previously reported research results.

Natto contains Vitamin K in addition to the physiologically active substances having antithrombogenicity. Vitamin K is one of the essential nutrients, known as a blood coagulation activator and with osteoplastic activity. It is present in large amounts in dark-green vegetables, algae, chlorella and so on. According to Japanese nutritional allowance (sixth edition), the nutritional allowance of Vitamin K in individuals 18-69 years old is 65 mg per day for males and 55mg per day for females, and the maximum nutritional intake that poses no risk of adverse effects is 30,000mg per day. When 100g of itohiki-natto (common natto) is taken, the amount of Vitamin K easily exceeds nutritional requirements; however, even if 1 kg of itohiki-natto were to be taken, the amount of Vitamin K would still not exceed the maximum nutritional intake. The liver biosynthesizes Vitamin K dependent blood coagulation factor, and Vitamin K deficiency causes hemorrhagic disease.

The "standard of natto bacillus culture extract food" classifies the products by fibrinolytic activity per reference amount for one day. Nonetheless, it is likely that Vitamin K is mixed into the product by processing. Usually, the reference amount for one day of functional food has been determined by animal experiment, along with clinical research and experience with human use of each product. Surprisingly, the standard does not consider physiologically active substances which can affect activity and safety when the product is actually taken.

CONCLUSION

In this study, a thrombolytic activity assay system for natto extract was examined. As a result, some problems were revealed in the "standard of natto bacillus culture extract food" issued by the Japan Health Food & Nutrition Food Association: homogeneity and quantification of the reaction, specificity for fibrinolytic activity, reaction of proteases which are not from natto, and so on. If functional food based on this standard is scientifically evaluated in terms of activity or safety, the food quality can be ensured. However, undesirable outcomes such as health hazards could occur in the absence of thorough scientific evaluation.

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