chemical and physical properties with tyrosine, it lacks a role in dopamine synthesis. Since dopaminergic terminals comprise only a small percentage of the cellular mass of the striatum, it can be concluded from these data that the loss of tyrosine from these neurons after electrical stimulation would amount to only a small fraction of the tyrosine present in the slices. Thus, when dopamine neurons are forced (by prolonged electrical stimulation in vitro to release their transmitter, and are not provided with an adequate supply of exogenous tyrosine, additional tyrosine can be mobilized from non-dopaminergic cells as well as from stores within dopamine neurons. It is possible that local glial cells provide one such source of tyrosine, thereby providing nutritional as well as structural support for the dopaminergic terminals, as has been suggested for glia in other systems[5].

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A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet

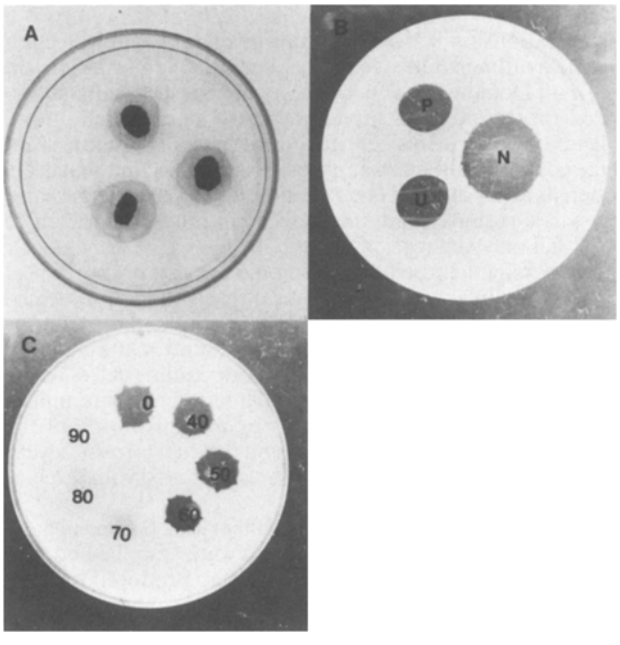
H. Sumi, H. Hamada*, H. Tsushima, H. Mihara and H. Muraki**

*Department of Physiology, Miyazaki Medical College, Miyazaki 889-16 (Japan), *Department of Fundamental Natural Science, Okayama University of Science, Okayama 700 (Japan), and **Department of Fermentation Technology, Faculty of Engineering, Yamanashi University, Kofu 400 (Japan), 23 June 1986*

Summary. A strong fibrinolytic activity was demonstrated in the vegetable cheese Natto, which is a typical soybean food eaten in Japan. The average activity was calculated at about 40 CU (plasmin units)/g wet weight. This novel fibrinolytic enzyme, named nattokinase, was easily extracted with saline. The mol. wt and pI were about 20,000 and 8.6, respectively. Nattokinase not only digested fibrin but also the plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251), which was more sensitive to the enzyme than other substrates tried. Diisopropyl fluorophosphate and 2,2,2-trichloro-1-hydroxyethyl-o,o-dimethylphosphate strongly inhibited this fibrinolytic enzyme.
Key words. Natto; soybean food; fibrinolytic enzyme; serine protease.

The vegetable cheese Natto[1] is a typical and popular soybean fermented food in Japan. It has a history extending back more than 2000 years because the special taste and flavor produced by Bacillus natto are well liked[1,2]. Traditionally, it was also used as a folk medicine for heart and vascular diseases, to relieve fatigue and as an anti-beriberi agent[2]. Moreover, a hypotensive effect on SHR rats[3] and prolongation of the life of Ehrlich sarcoma mice[4] have recently been described. However, there is still no detailed report on its component proteases, except for Miyake's preliminary studies with casein and gelatine as enzyme substrates[5,6]. In this report, we demonstrate the presence of a strong fibrinolytic enzyme in Natto for the first time, and describe investigations of some of the enzymatic properties of this novel enzyme.

Materials and methods. The following substances were used: Natto from Samejima Shoji Co. Ltd., Japan; swine pancreas trypsin (type 1) and diisopropyl fluorophosphate (DFP) from Sigma Chemical Co., USA; 2,2,2-trichloro-1-hydroxyethyl-o,o-dimethylphosphate (Neguvon) from Wako Pure Chemical Industries, Ltd., Japan; ε-aminocaproic acid (ε-ACA) and trans-4-aminomethyl-cyclohexanecarboxylic acid (t-AMCHA) from Daiichi Seiyaku Co. Ltd., Japan; human plasmin and urokinase from Green Cross Co., Japan; Bz-DL-Arg-pNA from the Protein Research Foundation, Osaka University, Japan; and pyro-Glu-Gly-Arg-pNA (S-2444), pyro-Glu-Pro-Val-pNA (S-2428), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Phe-Pip-Arg-pNA (S-2238), H-D-Val-Leu-Arg-pNA (S-2266) and H-D-Pro-Phe-Arg-pNA (S-2302) from Kabi Group, Inc., USA. All other chemicals were obtained from commercial sources and were of the best grade available. Amidase activity was estimated colorimetrically using several synthetic amido substrates by an end point method[7]: the reaction mixture (1 ml) consisted of the enzyme sample, 5×10^{-4} M substrate and 0.1 M phosphate buffer containing 0.1 M NaCl, pH 7.4. Fibrinolytic activity was determined by the method of Milstone[8] employing standard fibrin plates[9]. Protein concentration was estimated by the method of Lowry al.[10] using bovine serum albumin (Armour Pharmaceutical Co.) as the reference protein. Gel filtration



Fibrinolytic activity of nattokinase. *A* Natto was applied directly to a fibrin plate. *B* Nattokinase was extracted from 300 g of Natto with 220 ml of saline for 15 min with stirring at 4 °C. The material was filtered through gauze and then centrifuged at 3000 rpm for 10 min. N, nattokinase extract, 21.0 mg protein/ml; P, plasmin standard, 4.0 CU/ml; U, urokinase standard, 100 IU/ml, applied to a fibrin plate, respectively. *C* Nattokinase extract was heat-treated at the temperatures indicated (°C) for 10 min, and then applied to a fibrin plate. Each sample volume was 10 µl, and the incubation time was 18 h at 37°C.

Comparative amidolytic activity of nattokinase with several synthetic substrates

Substrate	Substrate hydrolysis (nmol/min/ml)
H-D-Val-Leu-Lys-pNA (S-2251)	68.5
Bz-DL-Arg-pNA	18.0
H-D-Phe-Pip-Arg-pNA (S-2238)	14.0
H-D-Val-Leu-Arg-pNA (S-2266)	13.5
H-D-Pro-Phe-Arg-pNA (S-2302)	11.5
pyro-Glu-Gly-Arg-pNA (S-2444)	0
pyro-Glu-Pro-Val-pNA (S-2484)	0

The reaction mixture (1 ml) contained 20 µl of nattokinase extract (420 µg protein), 5×10^{-4} M substrate and 0.1 M phosphate buffer, pH 7.4. After incubation for 10 min at 37 °C, the p-nitroaniline liberated was determined from the absorption at 405 nm. The results are expressed as nmoles of substrate hydrolyzed per min per ml of nattokinase extract. Each value is the mean of 3 determinations.

was performed with a column (1.0 × 25 cm) of Sephadex G-100 (Pharmacia Chemicals) equilibrated and run with 0.1 M phosphate buffer containing 0.2 M NaCl, pH 7.4. Isoelectric focussing was carried out according to the method of Vesterberg and Svensson[11] using ampholytes of pH 3.5–10.5.

Results and discussion. A strong fibrinolytic activity was found when Natto was applied directly to the fibrin plates (fig. A). This fibrinolytic enzyme (nattokinase) could be extracted easily with saline (fig. B). The calculated fibrinolytic activity of the extract obtained from 1 g wet Natto corresponded to about 40 CU plasmin or 1600 IU urokinase, when calculated from each standard fibrinolytic enzyme. Although in a crude state, the nattokinase was relatively stable at neutral pH but was gradually inactivated above 60 °C (fig. C). After 5 rounds of freezing and thawing, more than 95% of the activity remained. At neutral and alkaline pH values (7–12) the nattokinase was stable for 10 min at room temperature, but it was labile below pH 5.0. With some additional substances such as boiled rice extract, boiled meat extract, serum albumin and gastric mucin (1–50 mg dry wt/ml), the stability of nattokinase was much increased and the enzyme activity was not completely inhibited (in any case more than 7.5% of the original activity remained) even in acidic conditions.

The activity was not altered with or without 5 mM Cys, whereas 1 mM DFP and 5 mM Neguvon completely inhibited the fibrinolysis, suggesting that nattokinase is a serine protease. In other experiments, the typical anti-plasminic agents ε-ACA and t-AMCHA[12,13] revealed no effect on the fibrinolysis under the same conditions (final concentration, 50 mM, in the sample applied to fibrin plates).

The amidolytic acivity of nattokinase was investigated with several synthetic substrates. As shown in the table, the most sensitive substrate was found to be the plasmin substrate S-2251, with much lesser effects for Bz-DL-Arg-pNA, S-2238, S-2266 and S-2302. There was virtually no activity on the urokinase substrate S-2444 or the elastase substrate S-2484.

By gel filtration on Sephadex G-100, nattokinase was found to show a mol. wt of about 20000. The active fraction further purified by isoelectric focussing also revealed a single symmetrical fibrinolysis peak with a pI of about 8.6.

In the present study, as outlined above, we first demonstrated the presence of a novel fibrinolytic enzyme, nattokinase, in Natto. The previous utilization of Natto for various purposes in folk medicine may be related to the effects of this strong enzyme. Recently, several fibrinolytic enzymes such as urokinase [14,15] and earthworm protease[16] have been shown to be effective for plasma fibrinolysis by oral administration. They are usually mixed with several stabilizing factors, such as serum albumin and gastric mucin, and enteric-coated. They are not only absorbed into the plasma through the gastrointestinal tract but also induce urokinase-like endogenous plasminogen activator in the plasma, probably from the vascular endothelium or liver[15]. Nattokinase may be an equally good protease for oral fibrinolytic therapy because of its confirmed safety for long-term intake, stability and the strong fibrinolytic activity demonstrated here. Further purification of and in vivo experiments on nattokinase are currently in progress.

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A survey of 3-dehydroretinal as a visual pigment chromophore in various species of crayfish and other freshwater crustaceans

T. Suzuki[1] and E. Eguchi

[1]*Department of Pharmacology, Hyogo College of Medicine, Nishinomiya, Hyogo 663, and Department of Biology, Yokohama City University, Kanazawa-ku, Yokohama 236 (Japan), 16 March 1987*

Summary. 3-Dehydroretinal (vitamin A_2 aldehyde) was found in the eyes of three species among 10 species of freshwater crayfish examined. Since dark-adapted eyes contained the 11-*cis* form of 3-dehydroretinal, this compound must be the chromophore of the visual pigment. 3-Dehydroretinal always coexisted with retinal (vitamin A_1 aldehyde), indicating the presence of a rhodopsin-porphyropsin visual pigment system.
Key words. 3-dehydroretinal; retinal; visual pigment; crayfish.

The visual pigment commonly found in invertebrates is rhodopsin, which has retinal as a chromophore. Recently, two new chromophores have been found in the visual pigment of invertebrates. One is 3-dehydroretinal, which was found in a