Influence of Long - Term Ingestion of the Japanese - Traditional Food Natto on the Fibrinolytic Potential in Rat

Hiroyuki SUMI, Yasutaka KOZAKI* and Hiroki HAMADA**

Summary Nattokinase so named after its discovery in natto, a traditional fermented soybean food of Japan, is the most potent and effective fibrinolytic enzyme found amoung the approximately 200 foods the authors have investigated for "oral thrombolytic therapy". The present studies have shown that nattokinase-rich natto ingestion induce the fibrinolytic state in plasma of Wistar rats (♂,b.w. 420-470 g), as indicated by a clear shortening of the ELT and elevation of the PA activity, whereas there is almost no change in plasma inhibitor activities (PAI, α_2-AP and AT-Ⅲ) and coagulation parameters (APTT, Re-Ca^{++}T and TEG patterns). Enhancement of the t-PA activity was also confirmed in kidney and lung tissues.

KEY WORDS : Fibrinolytic enzyme, Nattokinase, Thrombolysis, Tissue plasminogen activator

Introduction

The oral thrombolytic therapy is different from the comventional *i.v.* infusion therapy via the activation of fibrinolytic enzymes from the living body, to dissolve the thrombus or the enhancement of t-PA synthesis in the cells of living body [1-3]. Since 1980, we have screened about 200 foods for safe fibrinolytic enzymes of higher activity for oral administration. Recently, we demonstrated the very strong fibrinolytic activity in Japanese traditional food, natto (fermented soybean, generally so called Itohiki-natto) [4]. The novel fibrinolytic enzyme, named nattokinase (NK) not only digested fibrin but also activated the conversion of pro-urokinase (pro-UK) to urokinase (UK) [5], and induced the fibrinolytic state in human plasma and experimental dog thrombus by oral administration [3,6-9].

In the present study, we report the effect of long-term ingestion of NK-rich natto on the plasma and tissue enzyme systems in rat.

Materials and Methods

As NK-rich natto a liophilyzed powder of natto extract was used. The procedure of saline extraction was as reported previously [4] using a commercial natto (Takano Foods. Co. Ltd., Ibaraki). It had about 20,000 NK units and 113 mg poly-Glu (a peculiar viscous matter)/g dry weight.

Animal experiments: Over a period of 3 months Wistar rat (♂, b.w. 420-470 g) orally received a daily dose of 2.4 g NK-rich natto/kg body weight. As control, the same amount of non-fermented boiled soybean was ingested. Before starting the experiment and after 1.5 and 3 months, the animals were killed and citrated blood (3.8 % Na_3 citrate, 1/10 vol) was taken from vena cava.

Determinations of plasma fibrinolytic and coagulation activities: Citrated blood was centrifuged at 1,800×g for 10 min. Plasma (500 μl) was di-

Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Kuboki, Soja-shi, Okayama 719-11, Japan

* Faculty of Pharmacy, Tokushima University of Arts and Science, Tokushima-shi,Tokushima 770, Japan

** Department of Fundamental Natural Science, Okayama University of Science, Okayama-shi, Okayama 700, Japan

luted in a proportion of 1:10 with cold distilled water (4℃). Under stirring the pH value was adjusted to 5.3 by addition of 1 % acetic acid. The euglobulin was sedimented by centrifugation at 2,000×g for 15 min (4℃). After decantation the sediment was dissolved in 500 μl of borate-buffered saline (0.15M, pH 7.8). The euglobulin lysis time (ELT) was measured by the method of Milstone [11] using a clot lysis time recorder (Ricoh Shoji Co., Japan). The fibrinolytic activity was determined by the standard fibrin plate method of Astrup and Müllertz [12]. The activity was expressed as the lysis area obtained with 30 μl of euglobulin solution at 37℃ for 18 hr. Plasma amidolytic activity was determined by the method of Claeson *et al.* [15] using a relatively specific UK substrate, pyro-Glu-Gly-Arg-pNA (S-2444 ; Kabi Diadnostica) and a plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251 ; Kabi Diagnostica). Activated partial thromboplastin time (APTT) was determined by the method of Lenahan and Phillips [14], and plasma recalcification time (Re-Ca++T) was of Caldwell [13] using a Clot Digitim TE-20 (Eram Optical Works Co., Japan). Thromboelastography (TEG) was performed by TEG apparatus (Hellige) according to the routine procedure.

Isolation of plasma plasminogen activator : Plasma plasminogen activator (PA) was purified by Celite affinity chromatography as reported previously [16,17]. A column of Celite activated with 4 N HCl was used and adsorbed enzymes was eluted with 0.1 % liquid ammonium. Molecular weight of PA was determined with zymography according to the method of Tissot et al. [18], combining the electrophoretic potencies in SDS-polyacrilamide and fibrin plate. As standards, human UK mixtures (containing high molecular form: mol. wt. 53,000 and low molecular form: mol. wt. 32,000, Green Cross Co. Ltd., Japan) were used for estimation.

Assay of plasminogen activator in tissues: Tissue plasminogen activator (t-PA) was extracted by a modification of the method of Mihara *et al.* [19]. The freshly taken organs (kidneys and lungs) were washed with physiological saline and cut into small pieces. They were homogenized in 9 volume of ice-cold KCl (0.15 mol/l) using a glass homogenizer. The supernatant of the centrifugates (2,000×g, 15 min) contained the"free form" of activator. The sediment was suspended in 9 volume of cold KCl solution (2 mol/l) and homogenized. The supernatant after centrifugation contained the"bound form" of activator. The supernatants were diluted in a proportion of 1:10 with cold distilled water, adjusted to a pH of 5.3 with 1 % acetic acid. The sediment collected by centrifugation was dissolved in borate-buffered saline (0.15M, pH 7.8). This was assayed for the activator activity by the standard fibrin plate method. The t-PA activity was expressed as International Unit (IU) per gram wet weight of the organs.

Assay of plasminogen activator inhibitor : Assay of plasminogen activator inhibitor (PAI) was performed by the method of Chmielewska and Wiman [20]. After 25 μl plasma were incubated with 25 μl of t-PA (100 IU/ml) for 10 min at room temperature, 50 μl of acetate buffer (1.0 mol/l, pH 3.9) were added and the reaction mixture was incubated for 10 min at room temperature, and immediately freezed at -80℃. To the thawed sample 300 μl of Tris buffer (0.05 mol/l, pH 8.3, 0.01% Tween 80) were added and 100 μl of the mixture were diluted with 900 μl of Tris buffer. Two hundred μl of this diluted plasma (20 to 25℃), 200 μl fresh prepared mixture of S-2251 (3.8 mmole/l), plasminogen (10 CU/ml Trisbuffer) and Tris buffer (v/v/v = 1/1/3) and 100 μl t-PA stimulater [1 volume of stock solution (5 mg human fibrinogen fragments in 1.5 ml sterile water) with 4 volumes of Tris buffer] were mixed and incubated at 37℃ for 1 hr. By means of a standard curve PAI concentration was calculated from the extinction of the plasma sample to be tested.

Plasminogen (Plg) [21], α_2-antiplasmin (α_2-AP) [22], and antithrombin Ⅲ (AT-Ⅲ) [23] were determined as described in the ref [24]. Statistical significance was calculated by Student's *t*-test.

Results & Discussion

As shown in Fig. 1, the activation of plasma fibrinolysis was confirmed by the shortening of ELT by NK-rich natto ingestion. The plasma fibrinolysis continuously increased during 1.5 months in natto treatment groups, finally reaching about two-fold enhanced PA activity at the end of the experiment. Activation was also confirmed by plasma amidolysis during natto ingestion (Fig. 2). The activated plasma enzymes isolated by Celite affinity chromatography showed the molecular weights of about 73,000 (main form), 54,000 and 110,000 (minor forms) by zymography (Fig. 3).

It is well known that different molecular forms of PA (t-PA) are present in tissues. Recent report of Padrō *et al* [25] shoued the highest activity is in lung, followed by kidney and other organs. In the present study, NK-rich natto produced an increase (1.5-fold) of t-PA activity in kidney (Fig. 4 left). An increase of t-PA activity was also observed in lung (Fig. 4 right). These were due to the increase of "free form" enzyme extracted with 0.15 M KCl. In contrast, the activity of "bound form" t-PA was unchanged, 42 ± 13 IU in kidney and 3 ± 2 IU/g (wet weight) in lung, respectively ($n=5$, $p > 0.5$). Only after a daily dose of natto over 3 months an increase in the inhibitor level (PAI) was observed which, however, did not significantly differ from that before treatment ($n=5$, $p > 0.5$) (Fig. 5). No change of plasminogen, α_2-antiplasmin and anitithrombin Ⅲ activity was obserbed.

In other few experiments (not shown), there was also no significant alternations regarding the coagulation parameters of APTT, Re-Ca^{++}T and TEG patterns during the natto treatment.

We have reported that a new fibrinolytic enzyme nattokinase activates plasma fibrinolysis by the release of endogenous PA [5, 6], and that almost the same activation effect is observed by ingestion of the parent fermented soybean natto, whereas the control boiled soybean has no effect [6]. The most important difference between such an oral fibrinoly-

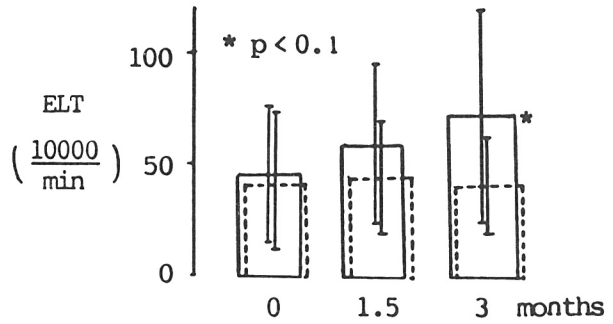

Fig. 1 Activation of plasma fibrinolysis by NK-rich natto ingestion.
The activity is expressed as reciprocal number of lysis time (min) × 10,000 at 37℃. Solid line shows the natto treated group and dotted line shows the control group, respectively. Each value represents the mean ± SD (n=15).

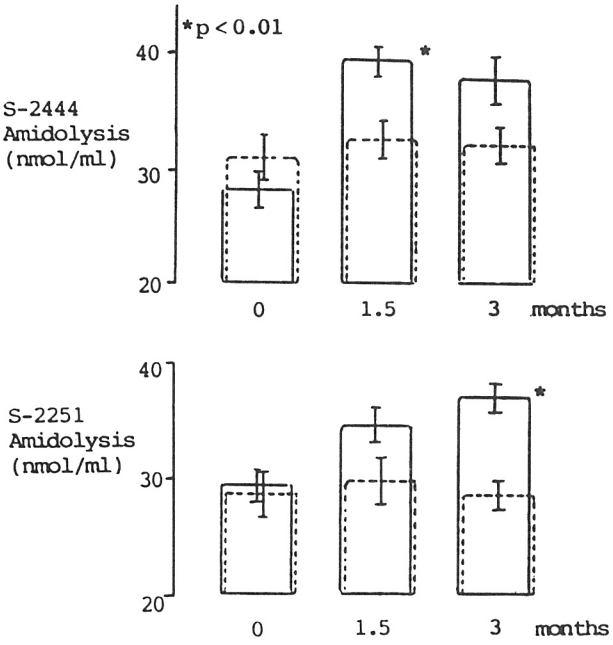

Fig. 2 Activities of plasma amidolysis by NK-rich natto ingestion.
The activity is expressed as nmoles substrate hydrolyzed/min/ml plasma at 37℃. Each value represents the mean ± SD (n=15).

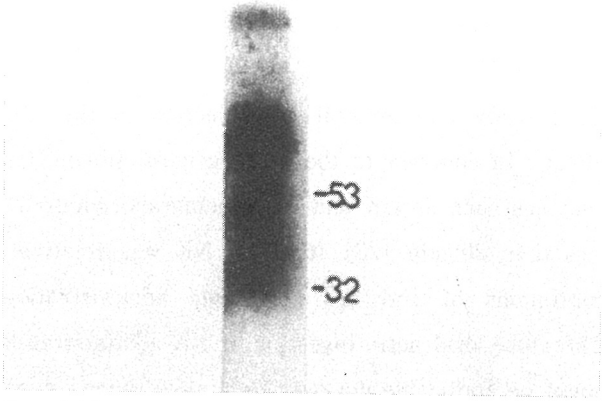

Fig. 3 Typical zymographic pattern of rat plasma PA fraction after 3 months NK-rich natto ingestion.
The volume of 20 μl of the eluate from Celite affinity chromatography was applied. The numbers on the right indicate mol. wt. (5×10^4) using UK as the standards.

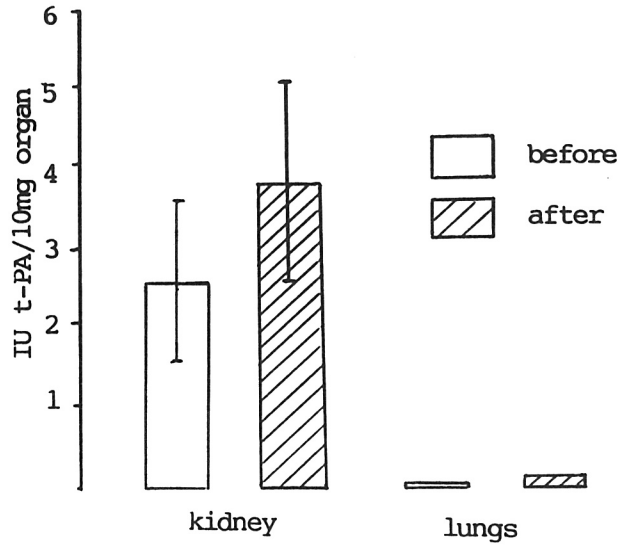

Fig. 4 Plasminogen activator activity in tissues of rats after 3 months NK-rich natto ingestion.
Each value represents the mean ± SD (n=15).

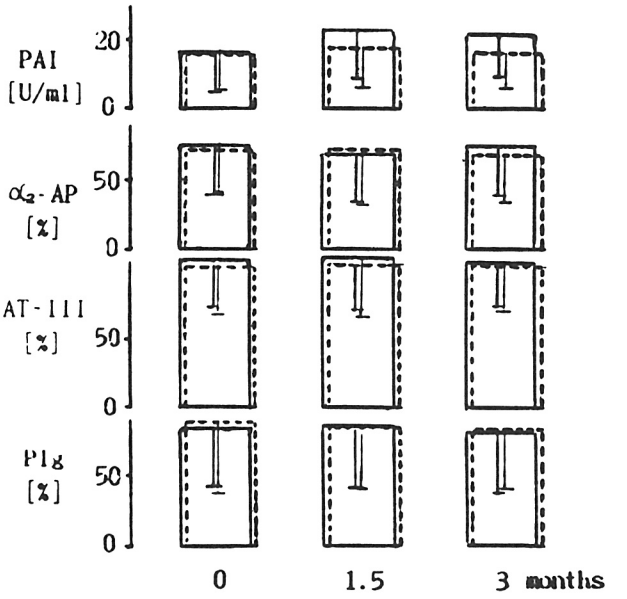

Fig. 5 Activities of plasminogen activator inhibitor (PAI), α_2-antiplasmin (α_2-AP), antithrombin Ⅲ (AT-Ⅲ) and plasminogen (Plg) in plasma during NK-rich natto ingestion.
Each value represents the mean ± SD (n=15).

tic therapy and general i.v. injection is the time effect : in contrast to the other general fibrinolytic enzymes such as UK and t-PA being short half-life less than 20 min [23], the oral NK was relatively continuous of 1-8 hrs by single administration. Therefore, oral natto ingestion or NK administration might be applicable not only to clinical thrombolysis but also to the daily prevention of thrombus.

In the present study, the long-term effect of NK-rich natto suggests the production of some endogenous PAs with the molecular form corresponding to t-PA type (main form, mol. wt. 73,000) in plasma. Increase in PA activity (Figs.1 and 2) was continuous and natto temporal. Furthermore, increases in PA content were also proved in kidney and lung tissues (Fig. 4). Natto is the typical Japanese traditional food and has been used as a folk medicine for more than 2,000 years in Japan. Further application and accumulation of natto data with human many volunteers are now in progress for futural new thrombolytic therapy.

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日本の伝統食品納豆のラットへの長期投与による血中線溶亢進効果

須見　洋行，小崎　泰宣*，浜田　博喜**

岡山県立大学保健福祉学部栄養学科
*徳島文理大学薬学部生理化学
**岡山理科大学基礎理学科

1980年以降、我々は“経口血栓溶解療法”という、これまでの静注投与にはない、線溶酵素を経口化して生体自らが持つ線溶酵素の活性（あるいはt-PAなどの合成）を高め、血栓溶解を起こす投与方法の研究を行い、その目的のもとに約200種類の食品検索で、経口化で最も強力，且つ安全な線溶活性を有する酵素として日本の伝統的発酵食品である納豆にナットウキナーゼを発見した。本報では、一群15匹のウイスター系ラット（♂、420～470ｇ）に、そのナットウキナーゼを高単位含む納豆を３ヶ月間摂取させた場合、投与前に比べてPAⅠ、α_2-APとかAT-Ⅲなどの血漿中のインヒビター活性、あるいはAPTT、Re-Ca^{++}TあるいはTEGで測定した血液凝固系の活性に変化は見られなかったが、血漿ELTの短縮及びPA活性の増加が認められ、血中線溶系酵素の活性化の起こることが初めて分かった。この線溶亢進効果は腎及び肺組織内のt-PA活性の上昇からも確認された。