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Chapter 4

HOW A NOVEL PROTEASE PRODUCED BY *BACILLUS SUBTILIS NATTO* IMPROVES BLOOD FLOW: SUBJECTIVE SYMPTOMS IN LIFESTYLE-ASSOCIATED DISEASE

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ABSTRACT

Recently, traditional Japanese foods have attracted attention because of Japan's lower prevalence of cardiovascular disease and a national life expectancy exceeding that of Western countries. Natto is a traditional solid fermented soybean product popular in Japan. Notably, the main component of the novel supplement NKCP® is a 34-kilodalton fragment of bacillopeptidase F derived from natto. As whole bacteria, bacterial

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components, and vitamin K are removed from the preparation it is considered safe for users. Both the antithrombotic and fibrinolytic effects of NKCP® were demonstrated by *in vitro* experiments. Additionally, when administered to the alimentary tract of rats, these properties were confirmed. Furthermore, when administered orally to humans, NKCP® improved subjective symptoms associated with dysregulated blood flow, including stiffness of the shoulders, lower back pain, and coldness of extremities. Moreover, when used for 4 weeks by patients with lifestyle-associated diseases, both systolic and diastolic blood pressure were significantly decreased. Thus, NKCP®, a novel dietary supplement derived from the Japanese traditional food natto improved patients symptoms and blood pressure by improving blood flow through its antithrombotic and fibrinolytic properties. Therefore, functional foods such as natto which could prevent the development of chronic diseases associated with dysregulated blood flow may become attractive to people worldwide.

Keywords: *Bacillus subtilis natto,* bacillopeptidase F, coagulation, fibrinolysis, blood pressure

TRADITIONAL JAPANESE FOOD, NATTO

Recently, traditional Japanese foods have attracted much attention because of Japan's low prevalence of cardiovascular diseases and longer life expectancy compared with Western countries [1, 2]. In 2013, the traditional cuisine of Japan, washoku, was added to the United Nations Educational, Scientific, and Cultural Organization (UNESCO) List of Intangible Cultural Heritage. The principle of washoku is a rice dish complemented by a variety of side dishes, soup, and pickles. The relatively small portion size of the main and side dishes helps to avoid overeating. Furthermore, as the main cooking methods in washoku are steaming, boiling and stewing, liquid tends to be included in the food, subsequently increasing water intake while avoiding excess intake of calories [3]. There are multiple potential healthy traits in washoku: frequent consumption of fish which enhances the intake of eicosapentaenoic acid, docosahexaenoic acid and omega-3 fatty acids; inclusion of large quantities of vegetables; and common use of soybean-based foods. Foods based on soybeans, which

famously include fermented miso and tofu, are known to reduce both blood pressure and blood glucose levels [4, 5].

Natto is a traditional solid fermented soybean product that is popular in Japan. When making natto, the soybeans are first soaked in water and cooked, then friendly starter bacteria are added, and the soybeans are fermented under warm and damp conditions. Natto is considered a dietary health supplement because of its high level of nutrients: 100 g of natto contains a variety of amino acids (16 g), vitamin B1 (0.07 mg), vitamin B2 (0.56 mg), vitamin B6 (0.24 mg), and fiber (6.7 g). People usually mix natto with soy sauce, chopped green onions, and mustard, and eat it with hot rice. Interestingly, the results of an internet questionnaire in Japan showed that almost half of the Japanese population eats natto more than once every 3 days [6]. However, many people find natto difficult to consume because of its strong, unique flavor and slick texture. Moreover, people in Western countries may take some time to get used to its distinct smell. Therefore, natto, which includes valuable nutrients, is not consumed widely globally. Recent research has clarified the reason that traditional Japanese food, or washoku, is good for health, may be associated with novel peptides derived from *Bacillus subtilis natto*. The author and colleagues have extracted a novel peptide from natto which influenced blood flow. The evidence shown in this chapter indicates that this peptide can serve as a functional food supplement. If a person hates to eat natto, by taking this supplement they can still obtain its health benefits. Furthermore, through this chapter, the authors highlight the medical properties of bacillopeptidase F and hypothesize why Japan has a lower prevalence of cardiovascular diseases than Western countries.

NOVEL SUBSTANCES DERIVED FROM NATTO

Bacillus subtilis natto, a bacterium isolated from natto, secretes proteases during fermentation (Table 1). At the end of the exponential growth phase, *B. subtilis natto* produces several neutral proteases (encoded by the structural gene, npr), subtilisin or alkaline protease (encoded by

apr), extracellular protease (encoded by epr), and bacillopeptidase F (encoded by bpf) [7]. A fibrinolytic enzyme called nattokinase isolated from *B. subtilis natto* and its clinical uses have been investigated previously [8, 9]. Nattokinase is highly homologous to subtilisin. However, we derived a purified protein layer, called NKCP® as a trademark of Daiwa Pharmaceutical Corp., from fermentation of the *B. subtilis* subspecies *subtilis* 168 strain. The main component of NKCP® was identified as a 34-kilodalton fragment of bacillopeptidase F. We removed bacterial cells, cellular components, and vitamin K and formulated the peptide as a powder. Therefore, it is easy to be used by people with underlying diseases or on medicines like warfarin. According to the results of the fibrinolytic activity test discussed below, NKCP® was stable when heated up to 60°C and at a pH range from 6.0 to 10.0 when incubated at 37°C for an hour.

Table 1. Enzymes extracellularly secreted by *Bacillus subtilis*

Protease	**Gene**
Bacillopeptidase F	*bpr*
Subtilisin (alkaline) protease	*apr*
Neutral protease	*npr*
Extracellular protease	*epr*
Metallo protease	*mpr*

ANTITHROMBOTIC AND FIBRINOLYTIC EFFECTS OF BACILLOPEPTIDASE F

In Vitro Experiments

When blood is removed from the body, the coagulation cascade is initiated and the blood coagulates. As an index of blood coagulation, levels of fibrin monomer (FM) were measured using a latex immunoassay with a specific antibody (clone F405). The values of FM in the control blood samples containing either normal saline or sodium heparin were

160.1 ± 29.3 μg/ml, suggesting the progression of coagulation, and 6.0 ± 1.1 μg/ml, suggesting the suppression of coagulation, respectively [10]. NKCP® dose-dependently decreased the FM values with anticoagulation activity at concentrations between 0.005 and 0.5 mg/ml (Figure 1) [10].

The serine protease activity of NKCP® was evaluated using S-2251 as a chromogenic substrate for plasmin and streptokinase-activated plasminogen. The serine protease activity was determined based on the difference in absorbance per minute at 405 nm between the formed *p*-nitroaniline and the original substrate. One unit of the serine protease activity was defined as the amount of enzyme which produced a change of l nmol of *p*-nitroaniline per minute at 37°C. The average serine protease activity of NKCP® was l800 units/g [11]. Based on quantification by ELISA, the average serine protease activity was 11.1 units/ 239.9 μg of 34-kilodalton bacillopeptidase F.

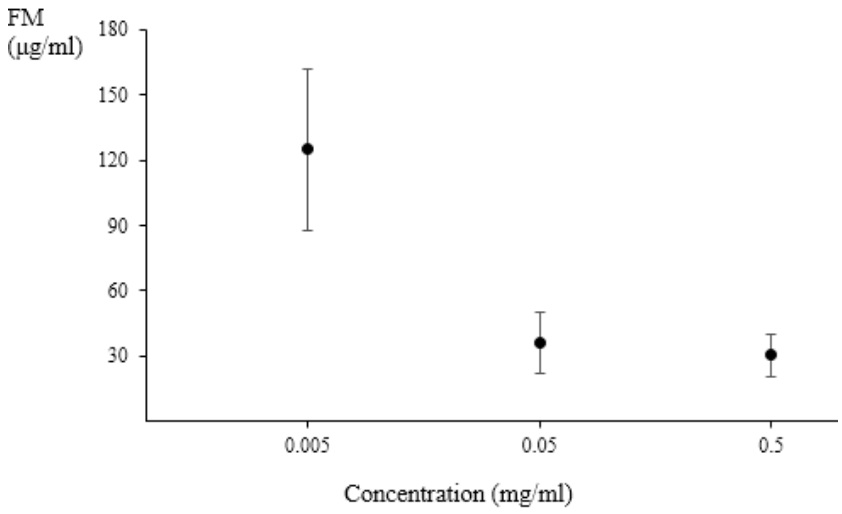

Figure 1. NKCP® decreases fibrin monomer concentration in a dose-dependent manner [10].

As both anticoagulation and fibrinolytic properties were confirmed by *in vitro* analyses, these plasmin-like activities were compared between two

proteases derived from *Bacillus subtilis natto*, NSK-SD® and NKCP®. The main component of NSK-SD® is subtilisin while that of NKCP® is bacillopeptidase F. Initially, the anticoagulant effects on human blood were compared. Although both NSK-SD® and NKCP® reduced FM levels, the anticoagulant effect of NKCP® was more than 100-fold greater than that of NSK-SD® [10]. Next, the plasmin-like activities were compared using S-2251. The results indicated that the specific plasmin-like activity of NKCP® was approximately 2.5-fold higher than that of NSK-SD®, indicating a greater fibrinolytic effect [10]. The results suggest that these proteases produced by *Bacillus subtilis natto* clearly exert different physiological effects on blood coagulation and fibrinolysis systems. These differences might be due to the differences in biological properties between subtilisin and bacillopeptidase F.

Animal Experiments

There was a concern whether the antithrombotic effects demonstrated in *in vitro* experiments could be obtained with oral intake of NKCP®. Therefore, NKCP® was administered to rats with a thrombotic tendency. A closed loop of duodenum was created in anesthetized rats and various concentrations of NKCP® were administered. Blood was obtained six hours later and both prothrombin time (PT) and active partial thromboplastin time (APTT) were examined. The results indicated a dose-dependent prolongation of both PT and APTT (Figure 2) [11]. As both the extrinsic and intrinsic coagulation pathways were inhibited in a dose-dependent manner with intra-duodenal administration of NKCP®, the results confirmed the antithrombotic properties of NKCP® in rats and suggest it is not a secondary action due to an excess fibrinolysis.

Next, thrombolytic activity was examined in a rat thrombosis model. After 14 weeks administration of standard feed containing NKCP®, the relative thrombus size was compared with that of the control group. When NKCP® was added, the relative thrombus size was markedly decreased

suggesting NKCP® has thrombolytic activity [12]. The fibrinolytic efficacy of oral administration of NKCP® was thus also confirmed *in vivo* in rats.

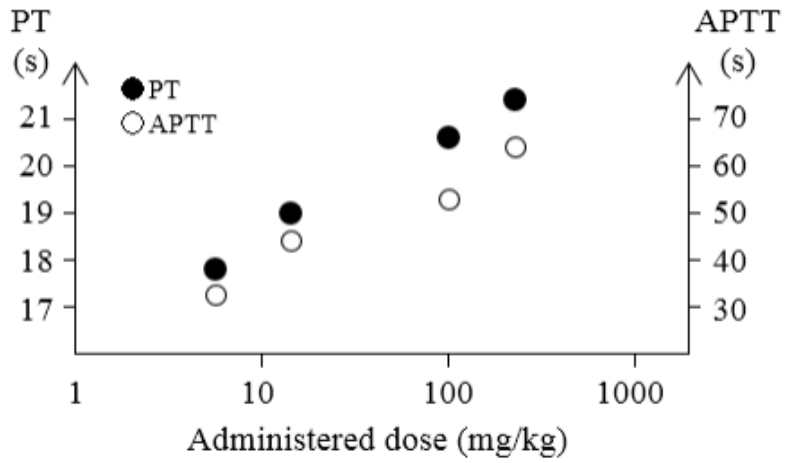

Figure 2. NKCP® prolongs the intrinsic and extrinsic pathways ways of coagulation in a dose-dependent manner [11].

Clinical Experiments

The fibrinolytic effect of NKCP® was confirmed by *in vitro* and animal experiments as shown above. Therefore, its fibrinolytic effect in humans was examined next. As an index of fibrinolytic activity, euglobulin lysis time (ELT) was used. ELT is a measure of the ability of plasminogen activators and plasmin to lyse a clot; specifically, the time from the formation of a blood clot to its dissolution. To evaluate the fibrinolytic effects in humans orally administered NKCP®, changes in ELT were examined. In the initial clinical experiment, a daily oral dose of 250 mg NKCP® was administered to 28 healthy volunteers for 2 weeks. Significant shortening of the ELT, from 9.8 ± 2.0 to 8.4 ± 1.7 sec, was obtained ($p < 0.01$) [13]. Regarding long term administration of NKCP®, similar results were also obtained after 1 month and 2 months of administration (9.0 ± 1.3 to 8.1 ± 1.5 sec, and 9.0 ± 1.3 to 8.0 ± 1.5 sec, respectively, $p < 0.01$) [13]. The fibrinolytic effect of NKCP® with oral intake was thus

confirmed. However, some issues remain to be resolved, including how NKCP® is absorbed from the alimentary tract, and what substances influence the secretion of fibrinolytic peptidases.

CLINICAL USES OF BACILLOPEPTIDASE F

Blood Pressure Depressant

Both anticoagulation and fibrinolytic activity are associated with blood fluidity. Because the ELT was shortened by oral intake of NKCP®, blood fluidity was improved which influenced blood pressure. Currently, a large proportion of adults suffer from lifestyle-associated diseases, including hypertension, hyperlipidemia, or type 2 diabetes mellitus, and are referred to doctors to receive medications as necessary.

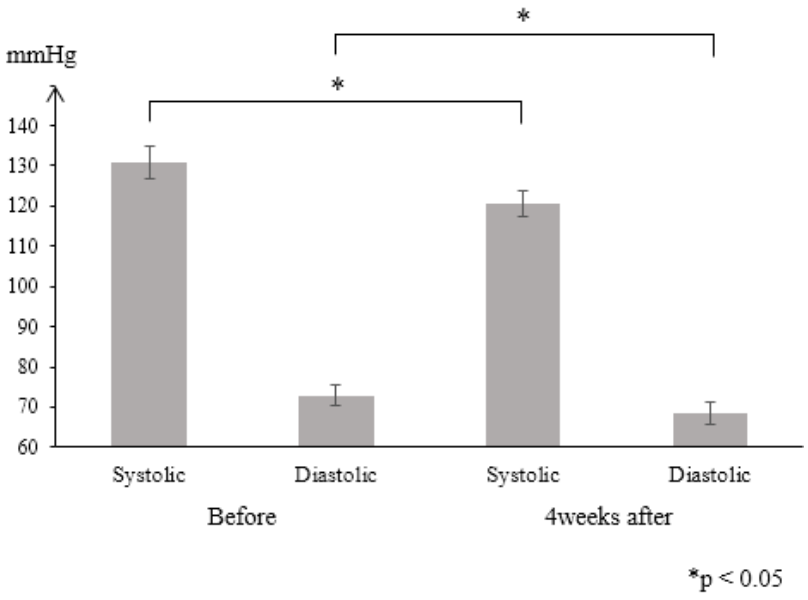

Figure 3. NKCP® decreases mean diastolic and systolic blood pressure after 4 weeks of treatment [14].

Therefore, the effect of NKCP® on blood pressure in patients with lifestyle-associated diseases was examined. A cross-over, double-blind study was performed with 21 patients who regularly visit hospitals or clinics for the treatment of lifestyle diseases [14]. As an active control, NSK-SD® containing subtilisin as the active main component was used. A 4-week course of NKCP® significantly decreased the average value of systolic blood pressure from 130.9 to 120.5 mmHg (p = 0.001) and that of diastolic blood pressure from 72.9 to 68.6 mmHg (p = 0.024) (Figure 3) [14], confirming the ability of NKCP® to decrease blood pressure. For the active control group, no significant difference in either systolic or diastolic blood pressure was found after 4 weeks. We hypothesize that the difference in effect between these two substances is due to the differences seen in the *in vitro* experimental results above. An increase in blood fluidity by NKCP® is thus considered the most probable reason for the decrease in blood pressure.

Relief of Subjective Symptoms

In developed countries, neck or shoulder stiffness, lower back pain, and coldness or fatigue of the extremities are typical subjective symptoms of lifestyle-associated diseases. These symptoms are due to use of visual display terminals (VDTs), prolonged sitting, and long-term exposure to air conditioning when performing routine tasks. Because the etiology of these symptoms is related to local blood flow, the effects of NKCP® on these subjective symptoms were investigated.

First, 250 mg of NKCP® was orally administered daily for 3 months to 23 people with a mean age of 53.7 years and no acute diseases [13]. The changes in the degree of headache, stiffness of the shoulder and the dizziness were then examined. Notably, after 2 months of treatment, only stiffness of the shoulder was significantly improved [13].

Although the positive results were considered to be due to improving of local blood flow by NKCP® intake, further clinical testing was performed to confirm the result. A double-blind crossover study to examine the effects of NKCP® on 29 healthy participants (mean age of 39.0 years) with neck and shoulder stiffness and pain was performed [15]. Participants were randomly divided into 2 groups and ingested 250 mg of NKCP® or placebo daily for 4 weeks. The NKCP® group had a significantly lower visual analogue scale (VAS) score for neck and shoulder stiffness (4.0 ± 2.3, $p = 0.008$) and pain (2.4 ± 1.8, $p = 0.004$), and stiffness in the neck muscles (6.2 ± 0.8, $p = 0.009$) compared with the VAS score prior to ingestion (4.7 ± 2.1, 3.2 ± 2.4, 6.5 ± 0.8, respectively) [15]. The headache score for the NKCP® group was also significantly decreased compared with the placebo group ($p = 0.041$, Table 2). Interestingly, significant increases in skin surface temperatures of the neck and shoulder were shown in the NKCP® group but not in the placebo group. These results suggest that NKCP® alleviated the symptom of neck and shoulder stiffness due to improved peripheral circulation.

Table 2. Overview of the clinical tests performed with NKCP® [13, 15, 16, 18]

Test	N	Average age	Participants	Significant effect	Ref.
1	23	53.7	Healthy, with subjective symptoms	Stiffness of the shoulder	13
2	29	39.0	Healthy, with subjective symptoms	Stiffness and pain of the neck and shoulder	15
3	17	67.8	Females with life style diseases	Stiffness of the shoulder Low back pain Coldness of the extremities	18
Questionnaire	25	35.5	Healthy, with subjective symptoms	Headache Stiffness of the shoulder Coldness of the extremities	16

People complaining of symptoms without objective or laboratory abnormalities often use NKCP® at a high dose. For people who use

NKCP® at a dose of 500 mg or more daily, changes in subjective symptoms were examined [16]. The study participants were 25 people with a mean age of 35.5 years. The average daily dose of NKCP® was 1300 mg and the average duration of treatment was 21.1 months. With the ingestion of prolonged high dose NKCP®, significant improvements of the symptoms of headache, stiffness of shoulders and coldness of the extremities were observed (Table 2) [16]. Improvement of stiffness of the shoulder was apparent 1.4 months after initiating the intake, headache in 2.5 months, and coldness of the extremities in 4.5 months. Increasing the dose of NKCP® thus also contributes to the improvement of symptoms.

Because endothelial and platelet dysfunction, as well as coagulation and fibrinolysis abnormalities, occur more often in patients with lifestyle-associated diseases (hypertension, diabetes mellitus, or hyperlipidemia) compared with those without, we speculated that changes in blood fluidity improve symptoms caused by dysregulation of blood flow in such patients [17]. Therefore, we investigated whether daily intake of NKCP® helps to improve subjective symptoms in patients with lifestyle diseases or not. Seventeen female patients (mean age of 67.8 years), were included in a cross-over, double-blind study [18]. NSK-SD® with subtilisin as the main active component was used as an active placebo. A 4-week course of NKCP® significantly decreased the VAS score of shoulder stiffness from 42.3 to 32.4 (p = 0.009), that of lower back pain from 25.5 to 18.8 (p = 0.02), and that of coldness of the extremities from 33.1 to 25.7 (p = 0.002, Table 2) [18]. However, no significant difference was found in the VAS score for headache. For the active placebo group, no significant changes in the VAS score for each symptom was found after the 4-week treatment. For patients with lifestyle diseases, taking NKCP® improved blood flow and subsequently relieved lower back pain, shoulder stiffness, and coldness of the extremities, all of which are caused by dysregulated blood flow. The use of dietary supplements derived from the Japanese traditional food natto provides additional benefits of relieving subjective symptoms in patients with lifestyle diseases who are receiving medical care.

IMPORTANCE OF IMPROVING BLOOD FLOW

Antithrombotic agents for human blood are important for improving thrombotic tendencies, and fibrinolytic agents also help maintain the blood flow by depleting formed fibrin. Therefore, dietary supplements with anticoagulant and fibrinolytic properties as determined by *in vitro* and *in vivo* studies play an important role in preventing thrombotic diseases. Therefore, NKCP® might also contribute to preventing cardiac events in patients with atherosclerotic diseases.

With an increasingly aging population in developed countries, chronic diseases, such as diabetes, hypertension, cardiovascular disease, and osteoporosis are also on the rise. Most patients with these diseases suffer from symptoms in accordance with increasing severity of the disease [19]. Although routine blood examination results revealed no marked abnormalities, many patients complained of lower back pain, headache, shoulder stiffness, and coldness of the extremities, all of which may be partly explained by poor blood flow. Various medications are used to reduce the above described symptoms. However, because of their limitations and other concerns such as availability, cost, and adverse effects of these medications, a portion of the population, especially in Asia, has turned to complementary and alternative medicine [20, 21]. Therefore, functional food that prevents the development of such chronic diseases has become attractive to many people. I suggest that dietary supplements derived from the Japanese traditional food natto may improve subjective symptoms associated with dysregulated blood flow. As it does not contain other chemical substances, NKCP® can be considered safe for users. As the major active component, approximately 37.9 μg of bacillopeptidase F is contained per 250 mg of NKCP® [15]. For a person to obtain a similar amount of bacillopeptidase F from food, they must eat nearly two packets of natto daily. Based on the difficulties of this food habit, the use of NKCP® is recommended for people who have any symptoms due to dysregulated blood flow or risk factors for vascular diseases.

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