

*New functional food produced from traditional Japanese "natto"*

**NKCP<sup>®</sup>**

*PRACTITIONERS*

**GUIDE**

*Purified Filtrate of Bacillus subtilis natto Culture*

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## What is NKCP?

*NKCP is a functional food that makes it easy for anyone to take natto bacillus-produced protein, regardless of their taste preferences.*

## Introduction

*NKCP, a partial purified product obtained by fermentation of *Bacillus subtilis natto*, is a functional food material that incorporates the antithrombotic activity of Natto. Natto itself is a traditional food produced by fermentation that has been used in Japan for many generations. NKCP is partially purified to remove most of the distinctive odor of Natto and its vitamin K2 content, thus permitting NKCP to have a wide variety of uses as a functional food.*

*The functional protein secreted by *Bacillus subtilis natto* that is contained in NKCP enables it to fulfill its function in a consistent manner. This functional protein, which has been purified and isolated, has been confirmed to be a fragment of bacillopeptidase F, which differs from conventional nattokinase (subtilisin protease). Quantitative assay using the ELISA method supports this result.*

*Researches on the function of NKCP have demonstrated that when taken regularly over a period, it helps to reduce the clotting tendency of blood and maintain normal circulation. The safety of NKCP is confirmed by non-clinical and clinical studies.*

## Characteristics of NKCP

- *NKCP is free of the characteristic odor and viscid texture of *B. subtilis natto*.*
- *Since the vitamin K2 has been largely removed it is less antagonistic to other drugs such as warfarin.*
- *The body of bacterial cells has been removed.*
- *NKCP has been adjusted to contain a fixed amount of functional protein (bacillopeptidase F) capable of peptide degradation. Its activity can be confirmed by quantitative assay of the functional protein.*
- *The functional protein in NKCP is stable at pH 6.0-10.0 and temperature below 60°C.*
- *Three functions have been confirmed through several studies, 1 action of preventing thrombus formation, 2 action of lysing blood clots, and 3 action of decreasing the viscosity of blood is the subject of a patent application concerning NKCP as a blood-viscosity reducing agent [JP2004331559]).*
- *The safety of NKCP has been confirmed in many animal and human studies.*
- *This purified filtrate of *B. subtilis natto* culture and its production process are registered under Patent No.3532503.*

## 1. Background

The „People’s Health Promotion Campaign for the 21st Century (Health Japan 21)“ was launched in 2000 by Ministry of Health and Labor Welfare (MHLW). The campaign through various health promotion activities focused on improving the quality of life (QOL) as well as on prolonging life. Simply put, it focused on „longevity in a healthy state“. The risk caused by blood clots is an important health factor that can shorten „longevity in a healthy state“. Cardiovascular and cerebrovascular diseases, two of the three major causes of death in Japan, account for about 30% of all deaths in Japan, demonstrating the dangers of thrombosis, resulting from current lifestyles. Even if patients escape death, thrombosis can result in sequelae which leave them bedridden, often for the rest of their lives. Symptoms that reduce QOL in daily life, such as neck stiffness, chronic headache, cold hands and feet and dizziness, appear to be associated with thrombosis tendency. Reducing the risk of thrombosis by stabilizing the coagulation-fibrinolysis balance should keep QOL at a high level and should help to promote the nation’s health.

## 2. Development of NKCP

The development of NKCP was based on the finding that the traditional Japanese food Natto contains an ingredient that affects the fibrinogenolysis/coagulation system of blood. The active ingredient responsible has been reported to be a peptidase produced by *B. subtilis natto*, which is used for the production of natto. This finding indicates that continuous ingestion of natto may reduce the risk of blood clotting. However, there are some drawbacks to take Natto for health purposes: it has a strong distinctive odor and flavor; it also has a high vitamin K2 content that may interfere with anticoagulants; and the peptidase content varies greatly according to the manufacturer or production batch.

NKCP (purified filtrate of *Bacillus subtilis natto* culture) was therefore developed to provide a raw material for food products which corrects the drawbacks of natto. NKCP is produced by fermenting the bacillus in a liquid medium containing soybean extract and then partially purifying the peptidase.

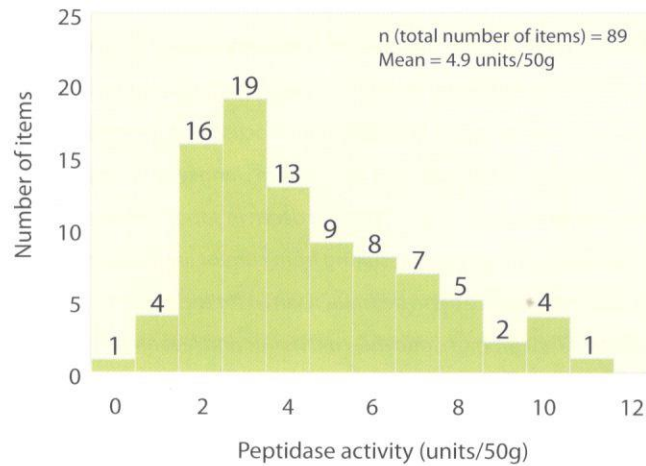
The odor, bacterial body and vitamin K2 content are reduced to a negligible level. NKCP is designed to contain a constant amount of peptidase.

This fermentation process and products are registered under Patent No.3532503.

NKCP



*Distribution of peptidase activity of commercial natto products*



### **3. Evidence of NKCP as a functional food**

#### **(1) Functional ingredient**

The functional ingredient of NKCP responsible for reducing the risk of thrombosis is a peptidase secreted by natto bacillus (*B. subtilis natto*). This peptidase is one of the proteins which are contained in natto that we, Japanese ingest on a daily basis. The activity of the peptidase can be ascertained by its promotion of hydrolysis using a synthetic substrate for plasmin or artificial fibrin. The improved thrombolytic activity of natto and partial purified product obtained from fermentation of *Bacillus subtilis natto* has been demonstrated in studies of human and dog.

NKCP has stable peptidase activity and its major active ingredient is a 34,000-45,000 dalton protein secreted by *B. subtilis natto*, belonging to the serine protease. The activity is stable at pH 6.0-10.0 and optimum pH is 9.0. NKCP is stable below 60°C. The peptidase activity is measured by determining the ability to hydrolyze a highly specific synthetic substrate for plasmin (S-2251), and its quantitative determination is carried out using ELISA, by measuring the amount of antigen reacting with an antibody specific for the fragment of bacillapeptidase F.

**Protease secreted by *Bacillus subtilis* after exponential multiplication**

<b>Protease</b>	<b>Gene</b>	<b>Properties</b>
Bacillopeptidase F	<i>bpr</i>	Molecular weight by SDS-PAGE: 47 kDa <sup>1)</sup> , 48 kDa <sup>2)</sup> Secreted as 92 kDa protein and converted into 80 kDa and 48 kDa proteins <sup>2)</sup> . Has high esterase activity as well as proteinase activity <sup>3)</sup>
Neutral protease	<i>npr</i>	Major exoproteinase as with <i>apr</i> .
Extracellular protease	<i>epr</i>	Molecular weight by SDS-PAGE:40-34 kDa
Metallo protease	<i>mpr</i>	Molecular weight by SDS-PAGE:28 kDa <sup>1)</sup>
Subtilisin (alkaline) protease	<i>apr</i>	Molecular weight by SDS-PAGE:20 kDa <sup>4)</sup> , 28 kDa <sup>5)</sup> Nattokinase. Its casein decomposing activity and direct fibronolytic activity have been confirmed. The ability to decompose and inactivate plasminogen activator inhibitor type 1 (PAI-1) has also been reported. <sup>5)</sup>

1) *Journal of Bacteriology*, Vol. 172, pp. 1019-1023, 1990.

2) *The Journal of Biological Chemistry*, Vol.265, pp.6845-6850, 1990.

3) *The Journal of Bacteriology*, Vol.172, pp.1470-1477, 1990.

4) Sumi, et al., *Experientia*, Vol. 43, pp. 1110-1111, 1987

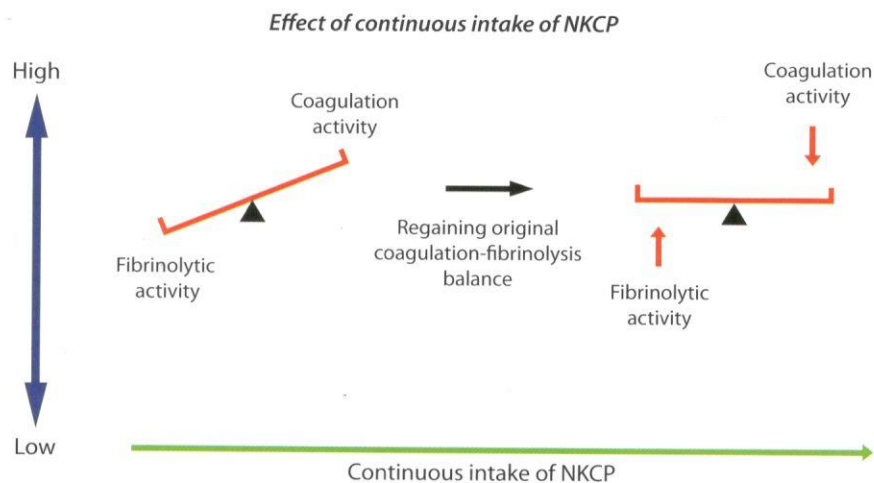
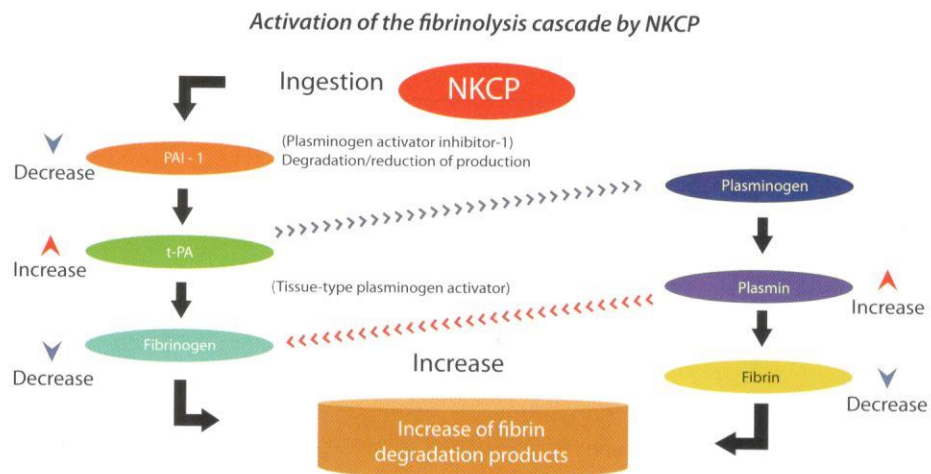
5) *The Journal of Biological Chemistry*, Vol.276, pp.24690-24696, 2001.

6) *Mol Gen Genet* 1990 May; 221 (3):486-490

## (2) Mechanism of action

The thrombolytic effect of NKCP has been demonstrated by *in vitro* and *in vivo* studies. By adding NKCP, the inactivation of plasminogen activator inhibitor (PAI-1) and the reduction of blood viscosity are observed *in vitro* studies.

As shown in the following figure, ingestion of NKCP reduces the plasminogen activator inhibitor and helps the plasminogen activator to work efficiently. Tissue plasminogen activator (t-PA) activates plasmin and reduce fibrinogen and fibrin clot. Thus, NKCP induce the activation of fibrinolysis cascade by reducing PAI-1.



Because of activation of the fibrinolysis, NKCP helps in maintaining the balance between coagulation and fibrinolysis of the blood.

NKCP reduces blood viscosity and coagulation activity *in vitro* and *in vivo*. These effects on the blood are likely to contribute to maintain sound blood conditions since it prevents stagnant blood flow.

### (3) Scientific data on NKCP

#### 1/ Animal study

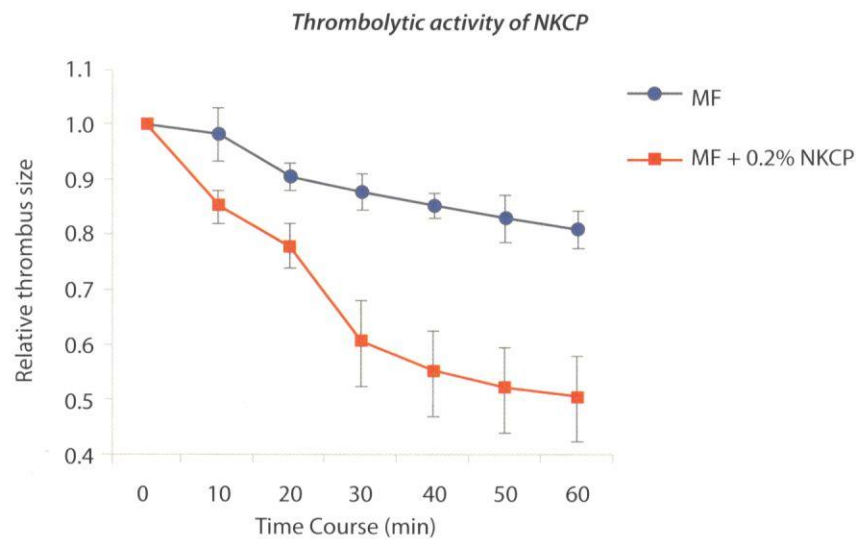
##### a) In vivo thromolytic effect of oral NKCP in experimental rat thrombosis model

Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University

The thromolytic activity of NKCP after 14 weeks administration of standard feed (MF) containing 0.2% NKCP was evaluated in rat experimental thrombolysis models for arterial blood clot mainly consisting of platelets using mesenteric microvessels.

The NKCP group showed increase of thromolytic activity compared to the MF group.

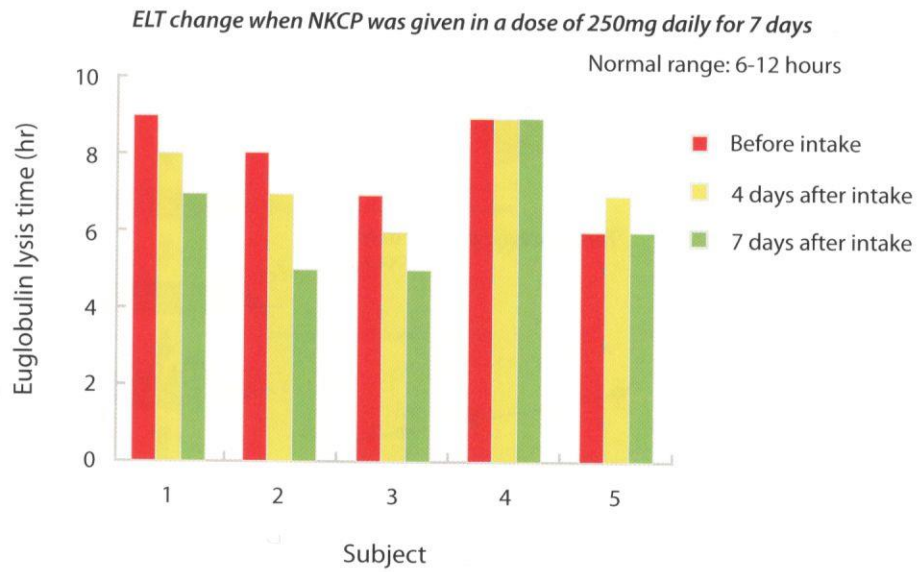
The thromolytic activity of 0.2% NKCP corresponds to 0.2 mg/kg of tissue plasminogen activator (t-PA).



b) Relation between NKCP ingestion and its effect in humans

Daiwa Pharmaceutical Report

The effectiveness and safety of NKCP was evaluated in 40 healthy adult volunteers by daily oral administration of NKCP from a dose of 125mg per day to a dose of 1000mg per day. The subjects administered NKCP for 3 weeks. Various clinical laboratory tests were performed to determine the recommended dosage using the euglobulin lysis time (ELT) as the main indicator. In the dose of 250mg per day or more, the administration of NKCP showed a stable activity after day 4.



## 2/ Action of antithrombosis

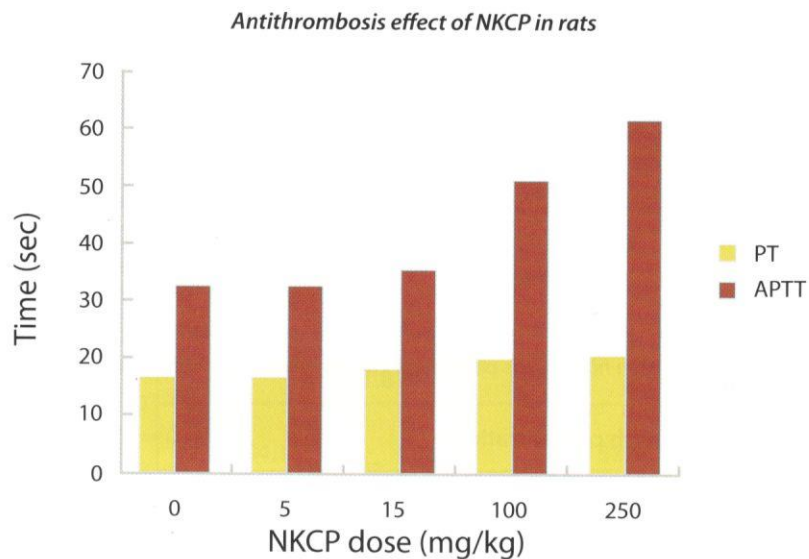
### a) Antithrombosis effect of NKCP in rats

Daiwa Pharmaceutical, research and development department

A comparative study was conducted to evaluate the anti-thrombosis of NKCP by the *in situ* loop method using thrombosis model.

Thrombosis was induced by injured in the endothelial cells of the abdominal descending aorta. Six hours after the induction of thrombosis, the activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured as indicator for blood clotting activity.

The APTT values for the control, NKCP 100mg/kg and 250mg/kg group were 33.5±2.4 sec, 52.0±4.5 sec and 63.3±2.9 sec respectively, indicating a significant prolongation for NKCP. The PT values for the control, NKCP 100mg/kg and 250mg/kg group were 16.7±0.5 sec, 20.6±0.9 sec and 21.3±1.7 sec respectively, indicating a similar result for APTT. Since NKCP showed a significant prolongation of the APTT and PT, as indicated above, its possible role in inhibition of clot formation was suggested.



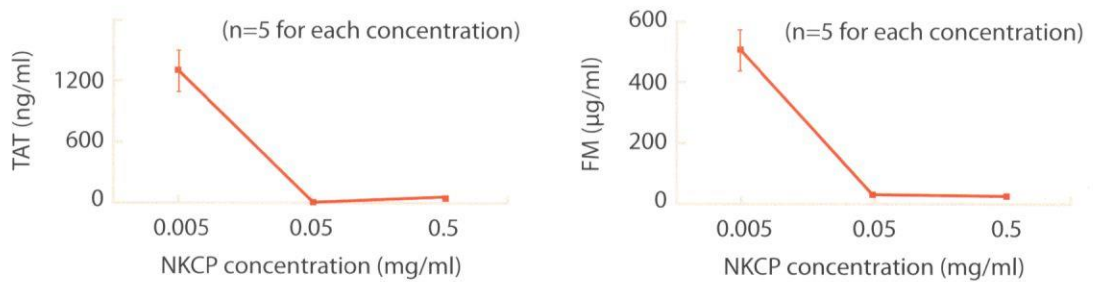
b) Antithrombosis/fibrinolytic effect of NKCP

The 26th meeting of the Japanese Society of Biorheology 2003

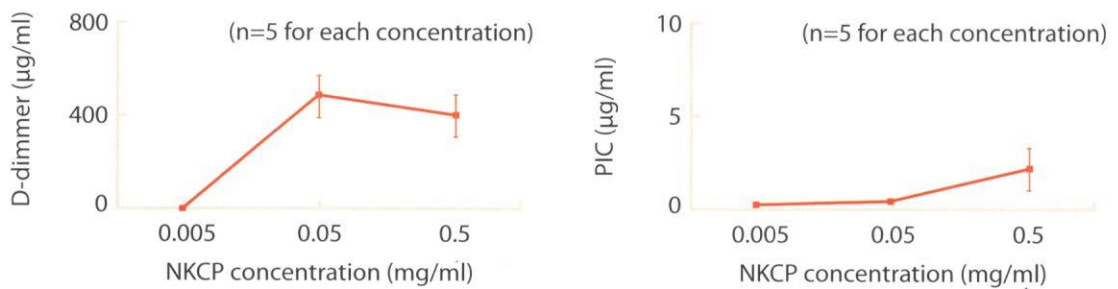
Dokkyo University School of Medicine, Department of Legal Medicine

NKCP was prepared to hydrolyze S-2251, a synthetic substrate specific to plasmin at 10U/mg. A NKCP solution in physiological saline was added to human blood immediately after drawing to measure clinical laboratory values related to coagulation/fibrinolysis. As a result, NKCP decreased both concentration of thrombin-antithrombin III complex (TAT) and fibrin monomer (FM) and showing that it has an anticoagulant action like heparin. Moreover, the concentration of D-dimer (D-d) was high and that of fibrinogen (Fbn) was low, showing that it has a fibrinolytic effect like alteplase. Different from alteplase, however, the fibrinolytic effect was accompanied by no increase in the concentration of  $\alpha 2$  plasmin inhibitor - plasmin complex (PIC), suggesting the fibrinolytic effect is independent of plasmin. As the concentration of NKCP was higher, the effect became larger. These results demonstrated that the new extract from natto fermentation has an anticoagulant effect and a plasminindependent fibrinolytic effect on human blood.

Changes in coagulation parameters against NKCP concentration



Changes in fibrinolysis parameters against NKCP concentration



### 3/ Action of decreasing the viscosity of blood

#### a) Effect of NKCP on blood viscosity

Journal of the Japanese Society of Hemorheology Vol. 5 (1), 2002

NKCP was administered to 13 healthy adult volunteers for a week to measure the euglobin lysis time (ELT) as the indicator for fibrinolytic activity. One healthy adult volunteer, who took 1,000mg of NKCP daily continuously after meals, showed a remarkably shortened whole blood passage time as determined by a micro channel array flow analyzer (MC-FAN) on Day 7 or later.

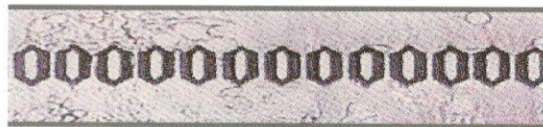
#### Effect of NKCP on the improvement in whole blood fluidity

Intake: 1g after each meal(hard capsule with enteric coating)

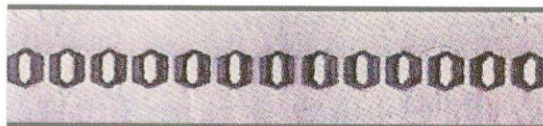
Intake period: 2 weeks

Measurement method: Measuring whole blood passage time in capillary models with MC FAN

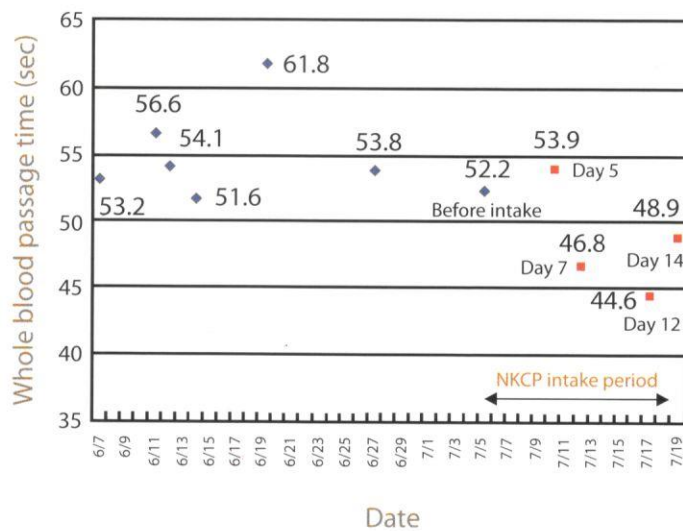
<Before intake>



<After intake>



Changes in whole blood passage time with NKCP



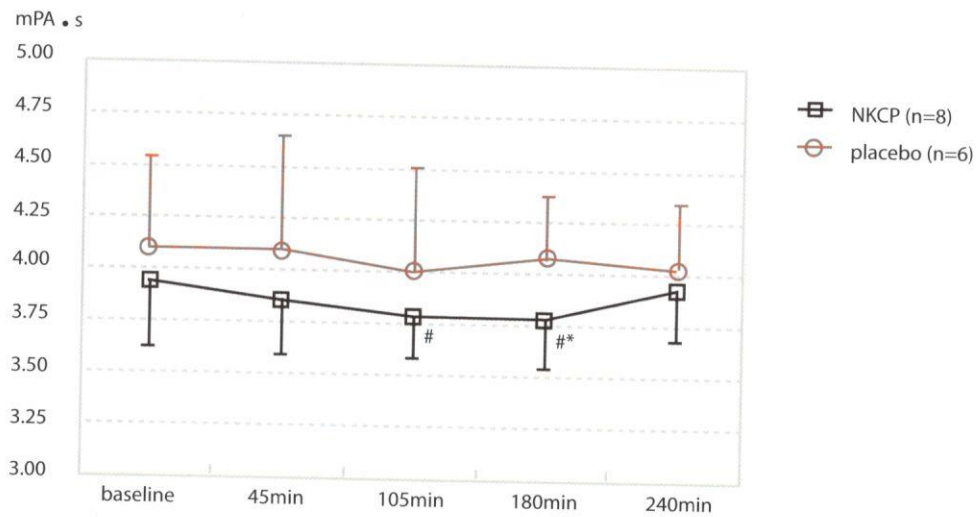
b) Study on blood viscosity in volunteers

Dokkyo University School of Medicine, Department of Legal Medicine

Fourteen healthy volunteers, a control group of 6 male and NKCP group of 8 male were given a placebo and 1,250mg of NKCP, respectively. Blood viscosity was measured successively up to 240 minutes after the administration.

The viscosity of blood was measured with a viscosity meter of the vibration type that was able to measure the viscosity change to the blood clot in real time without adding an anti-coagulant. As the result, decrease in blood viscosity was observed mainly at 180 minutes after the administration of NKCP. Blood viscosity at 180 minutes showed significantly lower compared with the control group.

Study on blood viscosity in volunteers



# : difference from baseline by Duncan's multiple test,  $p < 0.05$

\* : difference between NKCP and placebo by paired t test,  $p < 0.05$

#### 4/ Generalization (human clinical test)

##### Fibrinolytic and antithrombotic effect of NKCP by the oral administration

Journal of the Japanese Society of Biorheology Vol. 18 (1), 2004

The effect on the coagulation/fibrinolysis system of NKCP at the dose of 250mg daily for 2 weeks was evaluated in 28 adults including patients of metabolic disorder associated with the risk of thrombosis.

The ELT decreased by 10.1% at the end of this study.

The effect of chronic use of NKCP at the dose of 250 mg daily for 2 months was evaluated in 23 adults including patients. The ELT was decreased significantly at 1 and 2 month's examination, and t-PA showed a significant increase at 2 month's examination. As to the objective symptoms, significantly improved neck stiffness was observed at 1 and 2 month's interview.

Changes in subjective symptoms with NKCP intake

Symptom	Conditions	Before intake	At 1month	At 2 month
Headache	Severe	1	1	1
	Moderate	7	3	4
	No symptom (including mild headache)	15	16	16
	Remarkable improvement	-	3	2
	Shirley Williams multiple test	-	N.S.	N.S.
Neck stiffness	Severe	5	1	1
	Moderate	10	9	10
	No symptom (including mild neck stiffness)	8	9	11
	Remarkable improvement	-	4	1
	Shirley Williams multiple test	-	P<0.05	P<0.05
Dizziness	Severe	0	0	0
	Moderate	6	4	4
	No symptom (including mild dizziness)	17	18	18
	Remarkable improvement	-	1	1
	Shirley Williams multiple test	-	N.S.	N.S.

Figures represent the number of patients

Significant difference by Multiple Range Test: N.S. Not Significant, p<0.05 Significant difference (5% of the danger rate)

Changes in fibrinolysis/coagulation parameters with NKCP intake (n=23)

Parameters	Normal values	Before intake	At 1 month	At 2 month
ELT <sup>1)</sup>	6-12hr	9.0 ± 1.3	8.1 ± 1.5 **	8.0 ± 1.5 **
t-PA <sup>2)</sup>	≤ 10 ng/mL	5.4 ± 2.6	5.8 ± 2.8	6.4 ± 2.2 *
FDP <sup>3)</sup>	≤ 4 µg/mL	3.0 ± 0.7	2.0 ± 0.6 *	3.0 ± 0.7

Figure represents mean ± S.D.

Significant difference by Duncan's Multiple Comparison: \*:p<0.05, \*\*:p<0.01

(1) Upper limit of measurement (ULM) is 12 hrs.

(2) Lower limit of measurement (LLM) is 1.5 ng/ml

(3) LLM is 2 µg/mL.

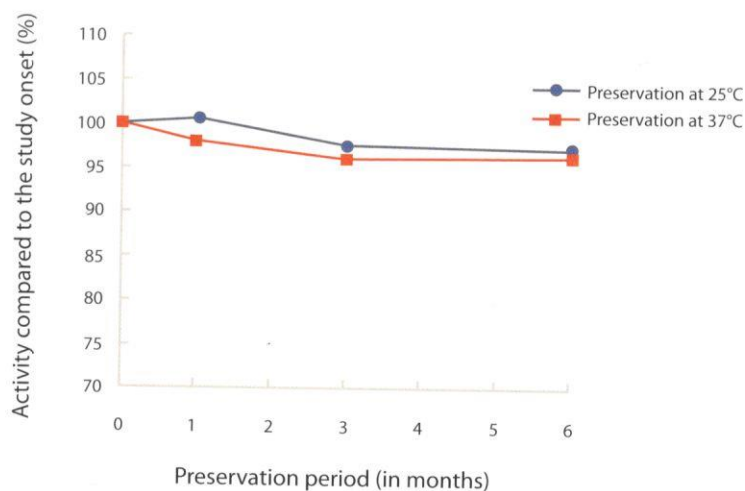
## 4. Stability

### (1) Storage stability

NKCP was examined for changes in the content of natto bacillus-produced proteins and bacillopeptidase activity during storage. Changes in bacillopeptidase activity of NKCP powder were ± 5% or less when stored at 25°C or 37°C for 6 months.

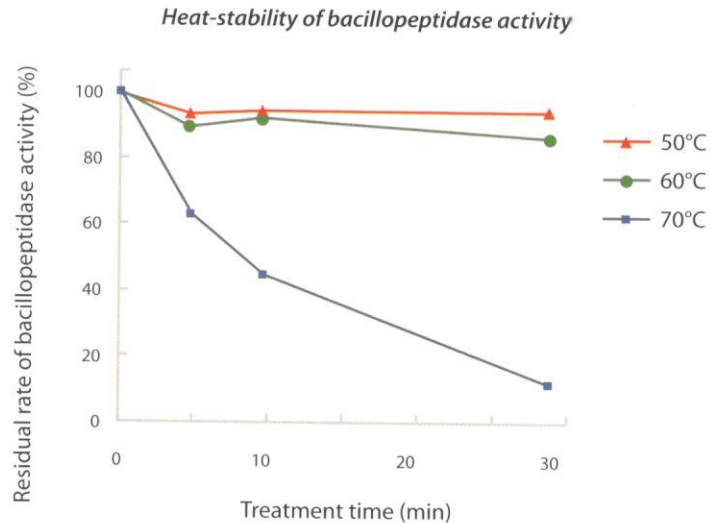
No changes in the content and activity were observed after 6 months of storage at 37°C or 24 months of storage at room temperature.

Stability of NKCP bacillopeptidase activity



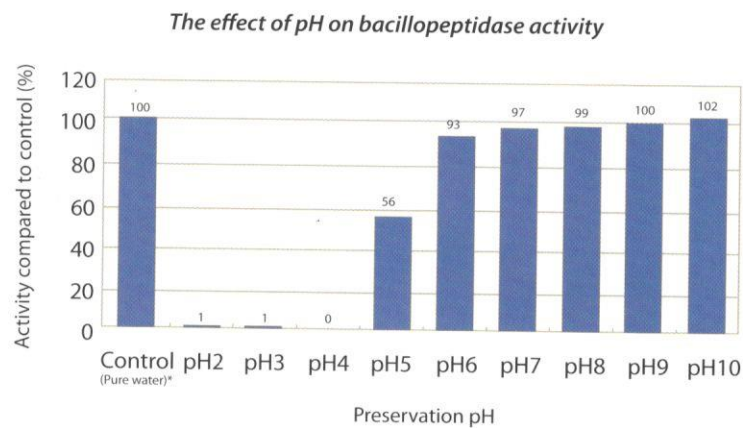
## 2) Heat-stability

A solution of 1g of NKCP in 10ml of 50m mol/L tris-hydrochloric acid buffer (containing 100m mol/L of NaCl, pH 9.0) was used in a heat-stability test for bacillopeptidase activity. The NKCP solution, treated under different temperatures and times, was examined for the residual rate of bacillopeptidase activity of *B. subtilis* natto produced protein using a synthetic chromatogenic substrate, S-2251. As shown in the figure below, the bacillopeptidase in NKCP was stable when heated up to 60°C.



## (3) The effect of pH

The bacillopeptidase activity of NKCP powder was determined by buffer solution decomposition of respective pH for an hour of 37°incubation. The activity is stable in the pH range of 6.0 – 10.0.



\* Bacillopeptidase activity immediately after dissolution with pure water

## 5. Safety Studies

Study	Results
Single dose toxicity (rat)	LD <sub>50</sub> > 5.000 mg/kg
Repeated dose toxicity (rat, 90 days p.o)	Male : NOAEL > 1.325 mg/kg Female: NOAEL > 1.541 mg/kg
Mutagenicity (AMES test)	Negative (± S-9 Mix)
Antigenicity (guinea pig)	None (subcutaneous, intravenous)
Excess administration (rat)	Rat models of prolonged coagulation given five times the normal dose through the duodenum showed no bleeding, and NKCP induced no remarkable symptoms in the coagulation system
Effect on bleeding time (rat)	An oral dose of a maximum 300 mg/kg body weight had no effect on the prolongation of the bleeding time
Repeated administration in humans	Twenty-three healthy adults were given 250 mg of NKCP daily for 12 weeks and no clinical adverse effects were observed
Excess administration in humans	Five healthy adults were given 750mg of NKCP daily for 6 weeks and no clinical adverse effects were observed. Eight healthy adults were given 1.250mg of NKCP daily for 7 days and no clinical adverse effects were observed

## 6. Standard dose

Based on evaluation of effectiveness and overdose tests on humans, 125-500mg of NKCP is recommended as the standard dose per day.

## 7. Specification of NKCP

Appearance	Light yeallow powder
Odor	No odor or sight fermented odor
Moisture	8% or less
Arsenic	1ppm or less
Heavy metal	10ppm or less
Aerobic plate count	3000FU/g or less
Coliform bacteria	Negative
Protein produced from <i>B.subtilis natto</i>	90mg/kg or more

Storage condition: Avoid high temperature and humidity and ensure containers are tightly sealed due to hygroscopic property.

## 8. Determination of activity and functional ingredient

### (1) Assay for bacillopeptidase activity

The sample solution is incubated at 37°C for 5 minutes with the synthetic chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-p-nitroaniline dihydrochloride) as substrate and the p-nitroaniline (pNA), which is released due to hydrolysis, is determined by measuring absorption at 405nm.

The bacillopeptidase activity in NKCP (unit/g) is defined as 1 unit when 1nmol of pNA per minute is freed from the substrate of 2m mol/L S-2251 at 37°C by 1g of NKCP.

### (2) Assay for the determining functional ingredient (ELISA)

The *B. subtilis* natto-produced protein (BNPP) responsible for NKCP's bacillopeptidase activity is purified to prepare antibodies specific for rabbits. The amount of antigen reacting with the specific BNPP antibodies is determined using the sandwich ELISA method. Measurement values are represented as the weight of the functional ingredient.

ELISA kit for BNPP determination



## 9. References

### Academic conferences

- „Anticoagulantfibrinolytic effect of new natto extract“  
26th Meeting of the Japanese Society of Biorheology, Osaka  
Omura K., Hitosugi M., Tokutome S. (Dokkyo University School of Medicine,  
Department of Legal Medicine), Niwa M. (Chiba University of Commerce, Department of Policy  
Informatics), Koike Y. (The Jikei University), Yufu T. and Iida N. (The Jikei University)
- 2003.6 „Effect of New Natto Extract on Blood Coagulation and Fibrinolysis“  
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Omura K., Hitosugi M., Tokutome S. (Dokkyo University School of Medicine,  
Department of Legal Medicine), Maeda H. (Daiwa Pharmaceutical Co., Ltd.) and  
Kaketani K. (Hanzomon Gastrointestinal Clinic)
- 2003.9 „Effect of Natto Extract on Blood Coagulation and Fibrinolysis“  
51st Discussion of Rheology, Nara  
Omura K., Hitosugi M., Tokutome S., Nagai T. (Dokkyo University School of Medicine, Department of  
Legal Medicine), Ikeda M. (Dokkyo University School of Medicine), Zhu X. (Daiwa Pharmaceutical Co.,  
Ltd.) and Niwa M. (Chiba University of Commerce, Department of Policy Informatics)
- 2003.9 „Effect of Purified Natto Culture Filtrate (NKCP) on the Coagulation/Fibrinolysis System“  
2nd General Technical Meeting of the Japanese Academy for Clinical Complementary & Alternative  
Medicine, Tokyo  
Maeda H. (Daiwa Pharmaceutical Co., Ltd.)
- 2003.11 „Effect of Natto Extract (NKCP) on Human Blood“  
5th Meeting of 21st Century Food and Health Forum, Tokyo  
Omura K., Hitosugi M. (Dokkyo University School of Medicine, Department of Legal  
Medicine), Kaketani K. (Hanzomon Gastrointestinal Clinic), Maeda H. and Zhu X.  
(Daiwa Pharmaceutical Co., Ltd.)
- 2003.12 „Fibrinolytic and Anti-thrombotic Effect of NKCP, the Protein Layer from Bacillus Subtilis (Natto)“  
The 3rd International Conference on Food Factors: Physiologic Functions and Disease Risk Reduction  
K. Omura (Dokkyo University School of Medicine, Department of Legal Medicine)
- 2004.5 „Development of Anti-Coagulant food, NKCP of Bacillus subtilis natto“  
1st Meeting of Anti-Coagulant Food, Kobe  
H. Maeda (Daiwa Pharmaceutical Co., Ltd.)

- 2004.6 „Effect of Natto Extract (NKCP) on Human Blood“  
27th Meeting of Japanese Society of Biorheology  
Hitosugi M, Omura K. (Dokkyo University School of Medicine, Department of Legal Medicine), Niwa M. (Chiba University of Commerce, Department of Policy Informatics) and Tokulome S. (Dokkyo University School of Medicine, Department of Legal Medicine)
- 2004.6 „Development of ELISA, enzyme-linked immunosorbent assay and BNPP“  
2004 Symposium of AOAC International Japan Section, Tokyo  
Zhu X. (Daiwa Pharmaceutical Co., Ltd.), Omura K. (Dokkyo University School of Medicine, Department of Legal Medicine), Hashimoto M. (Shima Research Co., Ltd.) and Maeda H. (Daiwa Pharmaceutical Co., Ltd.)
- 2004.8 „The protein layer of *Bacillus subtilis natto* cultured medium changes the activity of both blood coagulation and fibrinolysis“  
21st Symposium 01 Medical and Pharmaceutical Society for WAKAN-YAKU, Toyama  
Omura K., Hitosugi M. (Dokkyo University School of Medicine, Department of Legal Medicine), Kudo M., Zhu X., Maeda H. (Daiwa Pharmaceutical Co., Ltd.), Ikeda M. (Dokkyo University School of Medicine) and Tokutome S. (Dokkyo University School of Medicine, Department of Legal Medicine)
- 2004.8 „A possibility of the influence upon human blood rheology by the competently refined protein layer from cultured *Bacillus subtilis natto*“  
International Congress on Rheology 2004, Korea  
Omura K., Hitosugi M. (Dokkyo University School of Medicine, Department of Legal Medicine), Zhu X. and Maeda H. (Daiwa Pharmaceutical Co., Ltd.)
- 2004.9 „Effect of the abstract from natto (NKCP) on blood rheology and coagulation/fibrinolytic system“  
52nd The Society of Rheology, Hiroaki  
K. Omura, M. Hitosugi, M. Ikeda, X. Zhu, M. Niwa, T. Nagai, S. Tokutome
- 2004.9 „Fibrinolytic and Anti-thrombotic Effect of Purified Filtrate of *Bacillus Culture* (NKCP)“  
American College of Nutrition, 45th Annual Meeting, Long Beach Zhu and H. Maeda (Daiwa Pharmaceutical Co., Ltd.), K. Omura (Dokkyo University School of Medicine, Department of Legal Medicine)

## Papers

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Developed/developed by Daiwa Pharmaceutical Co., Ltd. Japan. (2008)  
Imported and distributed by DHD (Europe) Ltd., Cambridge CB1 1BH, UK

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