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Effect of New Natto Extract on Blood Coagulation and Fibrinolysis

Kazunobu Omura¹, Masahito Hitosugi¹, Munehiro Niwa⁴, Masasi Ikeda², Xia Zhu³, Toshiaki Nagai¹, Shogo Tokudome¹

¹Department of Legal Medicine, Dokkyo University School of Medicine

²Institute of Medical Science, Dokkyo University School of Medicine

³Daiwa Pharmaceutical Co., Ltd.

⁴Faculty of Policy Informatics, Chiba University of Commerce

880 Kita-Kobayashi, Mibu, Tochigi 321-0293, Japan

Phone: +81-282-86-1111 Fax: +81-282-86-7678

E-mail: omura@dokkyomed.ac.jp

Abstract

Fermented soybeans “natto” are traditional functional food that contains bioactive products like Vitamin K, proteases of casein and fibrin lysis. NKCP is refined protein fraction from cultured *Bacillus subtilis* (natto) isolated from common fermented soybeans. We examined the main protein of NKCP and the effects of the coagulation and fibrinolysis.

Our results suggested NKCP is the fragment of Bacillopeptidase F and it would be expected to use for the prevention of thrombosis.

1. Introduction

Natto is a traditional functional food containing enzymes that disintegrate casein and fibrin clots and rich vitamin K involved in bone metabolism and blood coagulation. Focusing on these functions of natto, we separated and purified a protein produced by *Bacillus subtilis natto* that ferments soybeans (hereinafter, called NKCP).

NKCP was analyzed scientifically to identify the main protein and study the effects on blood coagulation and fibrinolysis.

2. Method

2.1. Purification of NKCP

NKCP was produced and purified by Daiwa Pharmaceutical Co., Ltd. Analysis of DNA base sequence showed that the NKCP producing bacteria isolated from natto belong to *Bacillus subtilis*. The bacteria from natto were cultured in a medium with a soybean ingredient and the culture was purified to remove the bacterial cells, smell, stickiness, and vitamin K. The purified powdery protein fraction is NKCP.

2.2 Physicochemical properties of NKCP

The molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (abbreviated as SDS-PAGE) and time of flight-mass spectrometry (abbreviated as TOF-MS). The primary amino acid sequence was analyzed using automated sequential Edman degradation.

The enzyme activity was evaluated by determining the ability to hydrolyze the plasmin colorimetric synthetic substrate S2251 (H-D-Val-Leu-Lys-pNA: Daiichi Pure Chemicals Co., Ltd.) at 2×10^{-3} M in a 0.04 M tris hydrochloric acid buffer (pH 9.0). The heat stability of the enzyme activity was determined after heat treatment of an NKCP solution in tris hydrochloric acid buffer (pH 9.0) at 50, 60, and 70°C for 30 minutes. The acid stability was evaluated after 1-hour treatment at 37°C with an NKCP solution in tris hydrochloric acid buffer set at pH 2.0 - 10.0.

2.3 Effect on blood coagulation and fibrinolysis

The effect of NKCP was studied in a thrombus formation model using an *in situ* loop method. A thrombus formation rat model was produced as follows: after an abdominal median incision was made under anesthesia with urethane at 1.2 g/kg, vascular endothelial cells in the descending aorta were damaged to induce platelet adhesion and aggregation. A NKCP physiological saline solution was injected into the intestine at the same time when thrombus formation was induced, and activated partial thromboplastin time (abbreviated as APTT) and prothrombin time (abbreviated as PT) were determined 6 hours after the induction of thrombus formation.

3. Results

The molecular weight of NKCP was 45 kDa by SDS-PAGE and 34.134 Da by TOF-MS.

Analysis of primary amino acid sequence showed that NKCP was a fragment of bacillopeptidase F that is a protease produced by *Bacillus subtilis*.

The ability to hydrolyze the plasmin synthetic substrate was 1.80×10^{-4} mol/min/L in an NKCP sample solution at 100 mg/mL. The enzymatic activity of NKCP powder stored for 2 years at room temperature varied within 5%. For heat stability, the activity in an NKCP solution was stable up to 50°C, slightly decreased at 60°C, and decreased to 1/3 at 5 minutes and disappeared at 30 minutes at 70°C. For acidity, the activity was stable at pH 7.0 – pH 10.0, gradually decreased from pH 6.0, and disappeared at pH 4.0.

In the study in the thrombus formation model by *in situ* loop method, APTT was significantly delayed in a dose-dependent manner in the NKCP group compared to the control group. PT showed a similar tendency, although the degree of change was lower (Table 1).

4. Discussion

Bacillus subtilis is known to release 5 types of proteases (gene; *apr*, *npr*, *epr*, *mpr*, and *bpr*) outside the cells at the end of exponential proliferation. It was suggested that NKCP is a 34 kDa fragment of Bacillopeptidase F (*bpr*), which is a serine protease, the same as the released main enzyme Subtilisin protease (*apr*).

The NKCP used in this study had a high hydrolyzing activity on the plasmin synthetic substrate, was stable above pH 6.0 and under 60°C, and had high storage stability. Also, NKCP injection into the intestinal tract delayed coagulation in the thrombus formation rat model.

These findings on NKCP suggest that it can be clinically applied as a preventative agent for thromboses. Further study will be needed for clinical application.