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A Newly Derived Protein From *Bacillus subtilis natto* With Both Antithrombotic and Fibrinolytic Effects

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Abstract. *Natto*, steamed soybeans fermented by *Bacillus subtilis natto*, is a traditional Japanese food. We derived a purified protein layer, called NKCP as a trade mark, from *B. subtilis natto* fermentation. In the present study, we examined the fibrinolytic and antithrombotic effects of NKCP and identified its active component to clarify the fibrinolytic effect of NKCP observed in preliminary clinical trials previously. The active component of NKCP was identified as a 34-kilodalton protein designated bacillopeptidase F. NKCP showed direct degradation of artificial blood clot in saline. The protease activity, accounting for the fibrinolytic effect of NKCP, was examined with a chromogenic substrate for plasmin. Dose-dependent prolongations of both prothrombin time and active partial thromboplastin time were observed in rats with intra-duodenum administration of NKCP. Our in vitro and in vivo studies suggest that NKCP has both a fibrinolytic effect and an antithrombotic effect similar to heparin. Because NKCP is derived from food and has safety data demonstrated by previous animal experiments and preliminary clinical trials, NKCP is considered as safe for clinical use.

Keywords: bacillopeptidase F, antithrombosis, fibrinolysis, *Natto*, *Bacillus subtilis natto*

Introduction

Natto, a traditional Japanese food made from fermented soybeans, has attracted attention as a nutritious food containing many peptides and amino acids. *Bacillus subtilis natto*, a bacterium isolated from *natto*, secretes several proteases during the fermentation process. Nattokinase, a fibrinolytic enzyme extracted from *natto*, has been investigated for possible clinical use (1 – 3). In a canine model, oral administration of Nattokinase capsules enhanced fibrinolysis in plasma (1). Although fibrinolytic effects of extracellular proteases derived from *B. subtilis natto* have been reported, antithrombotic effects have not (4 – 6).

We derived a purified protein layer, called NKCP as a trade mark of Daiwa Pharmaceutical Corp., from fermentation of the *B. subtilis* subspecies *Subtilis* 168 strain, supplied as a powder without cell bodies and vitamin K. Previously, we had observed the fibrinolytic effect with shortering euglobulin lysis time of the human blood in preliminary clinical trials of which NKCP has been administered to 28 volunteers for two weeks and 23 volunteers for several months respectively (7).

To clarify detail mechanisms of NKCP for blood coagulation or fibrinolytic system, we examined antithrombotic and fibrinolytic effects of NKCP and identified its active component in the present study.

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Materials and Methods

Identification of the main component of NKCP

NKCP (Daiwa Pharmaceuticals Corp., Tokyo) was fermented by *Bacillus subtilis* subsp. Subtilis 168 strain derived from *Natto* fermentation that was frozenly preserved and colonized on plate agar. After the fermentation in fine granulated soy beans and glucose medium for two days at 42°C, a refined protein layer was obtained as a supernatant from the medium by centrifugation and ultra filtration of 10,000 mol weight mesh. NKCP was provided as a powder by decompressed dehydration of the supernatant.

NKCP was fractionated for protein collection with S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroaniline dihydrochloride; Daiichi Pure Chemicals Co., Ltd., Tokyo) hydrolytic activity, described below. When the proteins were analyzed with 10% – 20% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Daiichi Pure Chemicals Co., Ltd.), one major band of 45 kilodaltons was detected. For isolating the major band on SDS-PAGE, NKCP was fractionated with repeated column chromatography using SuperQ-Toyopeal 650M, Butyl-Toyopearl 650M, and Toyopearl HW-50F (Tosoh Corp., Tokyo).

The molecular weight of the protein was determined by the matrix-assisted laser desorption ionization-time of a flight mass spectrometer (Voyager Elite/STR; PerSeptive Biosystems, Inc., Framingham, MA, USA). The amino-acid sequence of the protein was determined with the Edman degradation method using an amino-acid sequencer (PPSQ21; Shimadzu Corp., Kyoto). For internal amino-acid sequence analysis, the protein was degraded gently with lysyl endopeptidase (Wako Pure Chemical Industries, Osaka).

After the main component protein of NKCP was identified, to standardize its quantity, we used a quantitative method using enzyme-linked immunosorbent assay (ELISA) with rabbit polyclonal antibodies (Sima Laboratories Corp., Tokyo). Samples dissolved in phosphate buffer were added to 96-well microwell plates coated with the immobilized antibody and incubated for 90 min at room temperature. After washing with phosphate buffer to remove any unbound proteins, the peroxidase-conjugated antibody was added and incubated for 60 min at room temperature. After another washing process, the freshly prepared substrate solution containging *o*-phenylenediamine and hydrogen peroxide in citrate-phosphate buffer was added and incubated for 30 min at room temperature. Then 2 N sulfuric acid was added to terminate the reaction. The assay was quantified by measuring absorbance at 490 nm.

Evaluation of the fibrinolytic effect

The fibrinolytic effect of NKCP was observed by artificial blood clot degradation. An artificial blood clot was made by spontaneous coagulation in a glass test tube using fresh human blood obtained from healthy young male volunteers with written informed consents. One hour later, the artificial blood clot was rinsed out repeatedly. The artificial blood clot was dipped in 0.1 mg/mL of NKCP solution at room temperature. As a control, normal saline was used instead of NKCP.

Evaluation of the serine protease activity

The serine protease activity of NKCP was evaluated by using S-2251 as a chromogenic substrate for plasmin and streptokinase-activated plasminogen. The serine protease activity was determined on the basis of the difference in absorbance per minute at 405 nm between the formed *p*-nitroaniline and the original substrate. The mixture of 0.1 mL of sample solution, 0.2 mL of 0.1 M Tris buffer at pH 9.0, and a 5-mM chromogenic substrate solution was incubated at 37°C for 5 min and was citrated to stop the reaction. As a control, 2% citrate solution was used instead of the sample. One unit of the serine protease activity was defined as the amount of enzyme which produced a change of 1 nmol of *p*-nitroaniline per minute at 37°C.

Evaluation of the antithrombotic effect

The antithrombotic effects of NKCP were evaluated in 8-week-old male Sprague-Dawley rats. After the rats were anesthetized, a looped 10-cm-long polyethylene cannula was inserted into the abdominal aorta to induce thrombosis with endothelial damage and blood flow disturbances. A closed loop of duodenum was created by obstructing the pylorus and the distal end of the duodenum (8, 9). For the positive control as hypoprothrombinemia rats, 8-week-old male Sprague-Dawley rats had been given the solution of 0.65 mg/L of warfain potassium (Eisai Corp., Tokyo) as a water supply for the previous eleven days from cannulation.

Solutions with final NKCP concentrations of 0 (control), 5, 25, 100, and 250 mg/kg were prepared with saline and administered directly to the closed loop of duodenum 2 h after cannulation to groups of 6 rats. Six hours after NKCP was administered, blood was drawn from the aorta and immediately citrated. Both the prothrombin time (PT) and the active partial thromboplastin time (APTT), indexes of thrombotic tendency, were determined. Statistical analysis was performed with the Williams test to compare values of blood samples of which control, or any NKCP concentration had been administered. The result was presented as the mean ± S.E.M.

Results

Identification of the main component

The main component of NKCP was identified to have a molecular weight of 34,134 daltons. The amino acid sequence was analyzed for 85 residues from the N-terminal. Three peptides obtained by lysyl endopeptidase degradation were analyzed with the same method. By comparing the obtained sequences to the gene database of *B. subtilis*, the main component of NKCP was identified as a 34-kilodalton fragment of bacillopeptidase F (10, 11), an extracellular serine protease secreted by *B. subtilis* after the cell growth phase (Fig. 1). The assigned structural amino acid sequence also contained the three catalytic residues of bacillopeptidase F (Asp, His, and Ser) which were observed in various serine proteases, such as subtilisin.

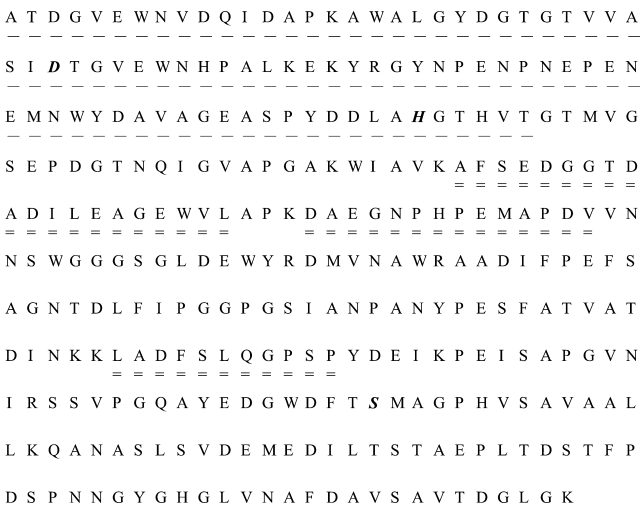

Fig. 1. Structural amino-acid sequences of the 34-kilodalton bacillopeptidase F. Characters underlined with a single line were determined from the NH_2-terminal, and those underlined with a double line were determined with lysyl endopeptidase degradation. Bold and italic characters are the three residues of the catalytic center: Ser, His, and Asp, the active sites of serine protease.

Evaluation of the fibrinolytic effect

The blood clot degradation was observed in the test tube of NKCP with spreads of red blood cells trapped by multiple fibrins net. In the test tube of normal saline, the blood clot degradation was not observed until 60 min (Fig. 2).

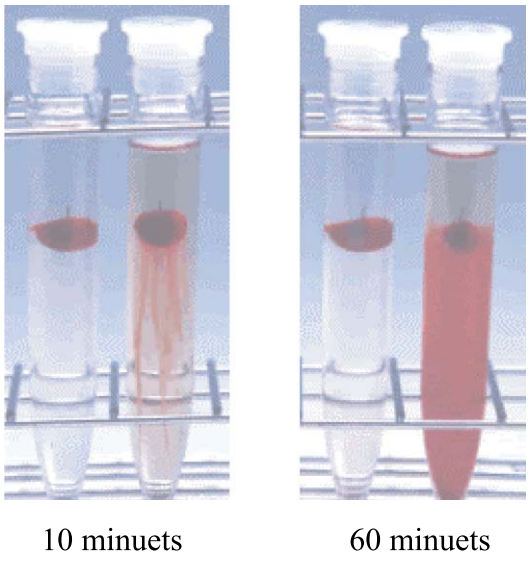

Fig. 2. The direct fibrinolytic effect was observed in the NKCP solution. The tubes containing the artificial blood clots were filled with either normal saline (left side) or NKCP solution (right side) in each figure. In the right side tubes, the blood clot degradations were observed by spreading of the red blood cells in the solution after 10 min and after 60 min.

Evaluation of the serine protease activity

The average of serine protease activity was 1800 units per gram of NKCP. Based on ELISA identification, the average of serine protease activity was 11.1 units per 239.9 μg of 34-kilodalton bacillopeptidase F (n = 6).

Evaluation of antithrombotic effects

The PT and APTT of untreated rats were 16.2 ± 0.3 and 33.4 ± 1.7 s, respectively (n = 6). The PT and APTT of hypoprothrombinemia rats pretreated by warfarin potassium were 35.4 ± 5.4 and 52.9 ± 6.8 s, respectively (n = 6). The PT was prolonged by NKCP in a dose-dependent manner from 17.4 ± 0.4 s with a dose 5 mg/kg to a maximum of 21.3 ± 1.7 s with a dose of 250 mg/kg. The APTT was also prolonged in a dose-dependent manner from 36.6 ± 4.3 s with a dose of 5 mg/kg to a maximum of 63.3 ± 2.9 s at a dose of 250 mg/kg (Fig. 3). The PT was significantly greater than control with doses of 5 mg/kg or more, and the APTT were significantly greater than the control with doses of 25 mg/kg or more (P<0.05).

Discussion

NKCP was derived from the fermented medium of *B. subtilis natto*. *B. subtilis natto* produces several extracellular proteases after exponential growth ends. It is considered that the large quantities of secreted extracellular proteases from *B. subtilis* are attracted to produce heterogonous proteins using for a host organism. However, the numerous extracellular proteases cause the rapid degradation of foreign proteins. Bacillopeptidase F is one of these extracellular proteases and has

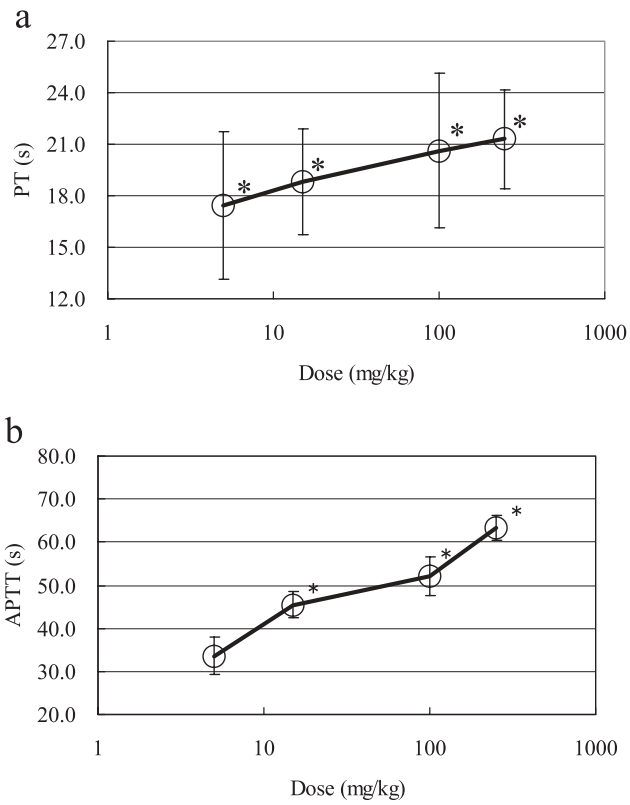

Fig. 3. Changes in PT and APTT values with NKCP. a: Changes in PT values (n = 6, mean ± S.E.M.) with NKCP. As a negative control, untreated rats had PT values of 16.2 ± 0.3 s. As a positive control, hypoprothrombinemia rats pretreated by warfarin potassium had PT values of 35.4 ± 5.4 s. The values with NKCP were prolonged dose-dependently. The values with asterisks were significantly greater than that of untreated rats (P<0.05). b: Changes in APTT values (n = 6, mean ± S.E.M.) with NKCP. As a negative control, untreated rats had APTT values of 33.4 ± 1.7 s. As a positive control, hypoprothrombinemia rats pretreated by warfarin potassium had APTT values of 52.9 ± 6.8 s. The values with NKCP were prolonged dose-dependently. The values with asterisks were significantly greater than that of untreated rats (P<0.05).

been isolated in various forms from *B. subtilis* (10 – 12).

We concluded that the main component of NKCP was a 34-kilodalton serine protease, bacillopeptidase F, due to the identification of amino-acid sequence and the presence of catalytic center, that is, Ser, His, and Asp, which was the active site of the serine protease (10, 11). Furthermore, NKCP is considered to be a fibrinolytic enzyme because of the direct fibrin clot degradation and the chromogenic substrate degradation. Therefore, these results are in good accordance with previously reported clinical trials (7).

As fibrinolysis is also activated by an activation of the blood coagulation under the well-balanced regulation between blood coagulation and fibrinolysis, we further examined the effect of NKCP on the blood coagulation system.

Our result suggests that NKCP has an antithrombotic effect by the prolongation of both PT and APTT values with direct administration to the duodenum of rats. Because both the extrinsic and intrinisic coagulation pathways are inhibited with NKCP in a dose-dependent manner respectively, the antithrombotic effect is not a secondary action following an excess fibrinolytic effect. Although our result shows the antithrombotic effect of NKCP, detailed mechanisms should be investigated in further studies.

To our knowledge, our studies are the first to report that the newly derived protein has both antithrombotic and fibrinolytic effects. Our present results are performed to examine the beneficial effect with oral administration of NKCP derived from *Natto*.

Because NKCP is derived from food and has safety data demonstrated by animals, that is, acute and subacute toxicity test using rats, antigenicity test using guinea pig, and by preliminary clinical trials for human volunteers, NKCP is considered to be safe for clinical use (7). Our in vitro and in vivo studies suggest that NKCP has both fibrinolytic effects and antithrombotic effects similar to heparin (13). In vivo studies should be performed to examine the ability of NKCP to prevent thrombotic diseases.

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