Purification and thrombolytic effects in vivo of recombination Nattokinase on carrageenan-induced tail thrombosis in a rat model

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ABSTRACT

Nattokinase is a serine protease with fibrinolytic activity and has been proven clinical efficacy and safe for human use by the oral route. In this study, conducted purified recombinant nattokinase from *Bacillus subtillis* DB104 strain and thrombolytic effects of nattokinase have been observed in vivo. The result of the enzyme purification by membrane filtration method for enzyme recovery efficiency is 82.45%. Then the enzyme was purified to Q-Sepharose ion-exchange chromatography with a purification factor of 1.82 and at a yield of 66.91%. Enzyme activity was 5,457 FU/mg protein. With the carrageenan-induced tail thrombosis in a rat model, results were significantly reduced by 67.3 to 83.6% at oral doses and injected nattokinase compared with the control group at 48h. At 72 hours the rate increased to 82-89% in the oral dose and up to 91% in the injected dose nattokinase and comparable to 90% for heparin-positive hepatitis.

1. Introduction

Thrombosis is a leading cause of morbidity and mortality throughout the world. Hence, the search for a fibrinolytic agent to treat thrombosis continues. Nattokinase, a potent fibrinolytic enzyme, was primarily isolated from a traditional Japanese fermented food 'Natto' by Sumi, Hamada, Nakanishi, and Hiratani (1990). It has 4 times greater fibrinolytic activity than plasmin (Sumi et al., 1990). The enzyme was reported not only to possess tissue plasminogen activator activity (Kotb, 2012) but also to directly digest fibrin by limited proteolysis (Fujita et al., 1995). Nattokinase has been shown to potentiate fibrinolysis by cleaving the t-PA inhibitor type I into low molecular weight fragments, leading to efficient lysis of blood clots in the body (Urano, Ihara, & Umemura, 2001).

In the process of nattokinase purification, the traditional protein separation and purification techniques, such as organic solvent fractionation, salting out and protein chromatography, have been tested with a number of disadvantages, such as long separation and purification time required, more operation units and less activity recovery (Nakanishi, Nomura,

Tajima, & Hiratani, 1998; Urano et al., 2001). Nakanishi produced nattokinase by a procedure consisting of alcohol or ammonium sulfate precipitation, hydrophobic interaction chromatography, ion-exchange chromatography and gel filtration (Nakanishi et al., 1998).

In the present study, we report on the purification and characterization of recombinant nattokinase produced from *Bacillus subtilis* DB104 (Tran, Le, Dinh, & Tran, 2013). The fibrinolytic enzyme was purified to electrophoretic homogeneity and its biochemical and thrombolytic activity were investigated. Carrageenan was intravenously administered to the tails of rats at 30 minutes after gavage administration of nattokinase, thereby inducing thrombus formation, and the length of the infarcted region was measured. The efficacy of carrageenan in inducing thrombus formation was evaluated by comparing the lengths of infarcted regions in the tails of rats in the nattokinase administered group with those in the tails of control rats. The action of nattokinase against thrombosis was evaluated by performing these experiments.

2. Materials and methods

2.1. Materials

2.1.1. Microorganism and its maintenance

B. subtilis pBG01-aprN/BD104, containing the gene coding for nattokinase under the control of the inducible promoter P_{Sgrac} was preserved in our laboratory (Tran et al., 2013). Bacteria were maintained as spores suspended in 50% (v/v) glycerol, stored at -25°C. Bacteria were cultured at 37°C in Luria–Bertani (LB) medium for culture collection and in fermentation medium (yeast extract 10 g l^{-1}, $CaCl_2$ 5 g l^{-1}, glucose 2 g l^{-1} and NaCl 10 g l^{-1}) for enzyme purification. Chloramphenicol (10 µg ml^{-1}) was added to the growth medium when necessary.

2.1.2. Animals

The healthy male and female Swiss rats weighing 18-22 g approximately 4-6 weeks old were provided by the Ho Chi Minh City Pasteur Institute. The animals were maintained in humidity-controlled rooms with a 12-hour light/dark cycle (light period 7:00 am to 7:00 pm) at 22 ± 3°C. All the animals were maintained on tap water and a normal pellet diet for at least 3 days before the following experiments were performed.

2.1.3. Chemicals and reagents

Fibrinogen, thrombin, k-carrageenan were purchased from Sigma Aldrich (USA). Q-Sepharose and Sephadex - G75 were purchased from GE Healthcare Life Sciences, Ltd. (Uppsala, Sweden). All other reagents were of the highest quality commercially available.

2.2. Analytical methods

2.2.1. SDS-PAGE and fibrin zymography Protein concentration was determined by

Bradford (1976) method using bovine serum albumin (BSA) as the standard protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5%) was performed to determine the molecular mass of the enzyme's purity following the methods of Laemmli (1970).

Nattokinase activities were assessed using fibrin zymography as previously described methods Kim, Choi, and Lee (1998). Briefly, resolving gel solution (12%) was prepared with 0.12% (w/v) fibrinogen and thrombin (1 U/ml). After PAGE, the gel was treated with 2.5% Triton X-100, 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.5) for removing SDS and incubated 3 times in the same buffer for 15 min each, followed by incubation overnight (16 h) at 37 ◦C in 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 7.5). The gel was stained with Coomassie Brilliant Blue G (0.5%, w/v) and destained with methanol/acetic acid/water (45:10:45). The area of fibrin degradation on the gel zymography was depicted as clear bands against a blue background of undegraded fibrin.

2.2.2. Determination of the fibrinolytic nattokinase activity

Nattokinase activity was measured using a fibrin degradation assay developed by Japan Bio Science Laboratory Co., Ltd. (JBSL) (Japan Nattokinase Association, 2000). To 1.4 mL of 50 mM sodium borate buffer (pH8.5), a 0.4 mL of 0.72% (w/v) fibrinogen solution was added and kept at 37^{o}C for 5 min. To the resulting, fibrinogen solution was then added 0.1 mL of thrombin (20U/mL) and kept at 37^{o}C for another 10 min. After the addition of 0.l mL of the enzyme, the proteolytic reaction was performed at 37^{o}C for 60 min. After the reaction was ceased by adding 2 mL of 0.2M trichloroacetic acid (TCA), samples were kept at ambient temperature for 20 min and then centrifuged at 14000 rpm for 5 min. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution.

2.2.3. Purification of nattokinase

All steps in the purification procedure were operated at room temperature except for centrifugation, which was conducted at 4°C. The supernatant of the crude enzyme was obtained by centrifugation at 7000 rpm for 30 min, and then the precipitate was precipitated by the addition of solid ammonium sulfate at 40%-70% saturation. The precipitate was allowed to form at 4°C overnight and was collected by centrifugation at 10,000 rpm for 20 min at 4°C for further purification. The precipitate was dissolved in 20 mM Tris-HCl (pH 9.4) buffer. The solution was dialyzed against the same buffer overnight. Enzyme activity and protein content were determined in the fraction.

The concentrated crude enzyme solution was loaded onto a Q-Sepharose column (1.6 × 15 cm, bed volume 25 mL) (AKTA system) which was preequilibrated with buffer. The column was washed with 6-bed volumes of 10 mM Tris buffer (pH 9.4) to remove all unbound proteins and eluted with a linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 1 mL/min. The fractions with fibrinolytic activity were pooled, concentrated with ammonium sulfate. The precipitate was collected by centrifugation at 10,000rpm for 20 min at 4°C, dissolved in 10 mM Tris buffer (pH 7.8), and dialyzed against the same buffer overnight. The sample was loaded onto a Sephadex G-75 gel filtration column (2.6 × 90cm) (AKTA system) which was preequilibrated with 10 mM Tris buffer (pH 7.8). The column was eluted with the same buffer at a flow rate of 0.4 mL/min.

Elution peaks were collected, and the enzyme activity and protein concentration in each fraction were determined, respectively. The fraction containing the enzyme with the highest

activity was collected and concentrated by ultrafiltration. The active fraction was pooled, concentrated, analyzed for purity by SDS-PAGE, then dried by freeze-dryer.

2.2.4. Rat model of carrageenan-induced thrombosis

Rats with tails longer than 9 cm were selected and anesthetized using diethyl ether for the experiments. To assess the in vivo thrombolytic activity, a total of 30 rats were randomly subdivided into 5 groups, each group containing 6 rats. Group 1 rats were injected with 10 mM Tris buffer (pH 7.8), which served as the control (placebo). The animals in groups 2 and 3 were treated with graded concentrations (200 and 400 ul nattokinase, respectively). The rats in groups 4 and 5 were injected into the tail vein from the tip of the rat tail with a 50ul dose of nattokinase and 50 ul of 100 IU heparin sodium, respectively. After 30 minutes of intravenous doses of nattokinase, the tails were ligated and 1mg/kg body weight of k-carrageenan was induced through intravenous injection. The ligatures were removed after 15 minutes of injection. The length of the infarcted region was measured, and the appearance of the wine-colored thrombus formation in the tails was photographed after 24, 48 and 72 hours of treatment by the above-mentioned thrombolytic agents.

3. Results and discussion

3.1. Purification of nattokinase

Nattokinase produced by *B.subtilis* DB104 was purified by a simple method consisting of precipitation (Table 1). Most of the enzyme activity was achieved at 40-70% saturation ammonium sulfate fraction having 298,500 FU, in which specific activity was 3,142 FU/mg protein with a yield of 75.65% (Table 1). It is noted that ammonium sulfate salts are the most widely used in the concentration of enzymes due to high solubility and low cost, compared with the other organic solvents and no effect on pH or the stability of the enzyme (Whitaker, 1972).

In other studies, the activity of precipitates in the crude enzyme supernatants with 30-80% saturations $(NH_4)_2SO_4$ was contrasted. To preserve the fibrinolytic activity of fibrinolytic enzyme as much as possible, according to the activity assay, the precipitate formed in 30-60% saturation of $(NH_4)_2SO_4$ was collected (Wang et al., 2009). The fibrinolytic enzyme was also purified by ammonium sulfate saturation. The protein fraction was precipitated with 85% ammonium sulfate (Dubey, Kumar, Agrawala, Char, & Pusp, 2011).

In the present study, the activity of the precipitates in the crude enzyme supernates with 30-90% saturations $(NH_4)_2SO_4$ was contrasted. To preserve the fibrinolytic activity of nattokinase as much as possible, according to the activity assay, the precipitate formed in 50-70% saturation of $(NH_4)_2SO_4$ was collected. Then the dialyzed precipitate was applied to Q-sepharose.

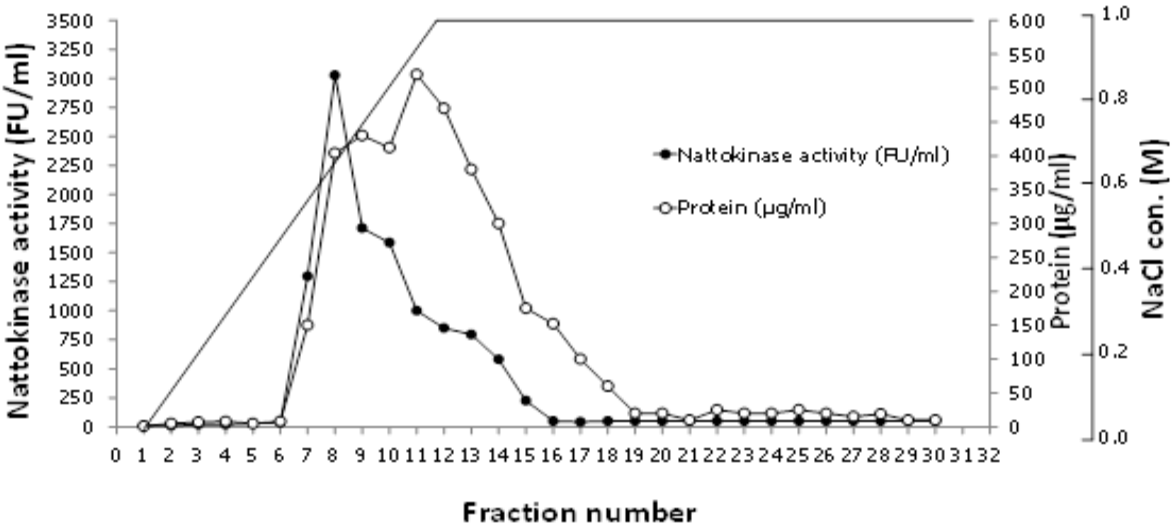

Figure 1. Q-sepharose ion exchange chromatography for purified nattokinase from B. subtilis DB104 by using Q-sepharose column. Nattokinase activity (FU/ml) (●), Protein (µg/ml) (○)

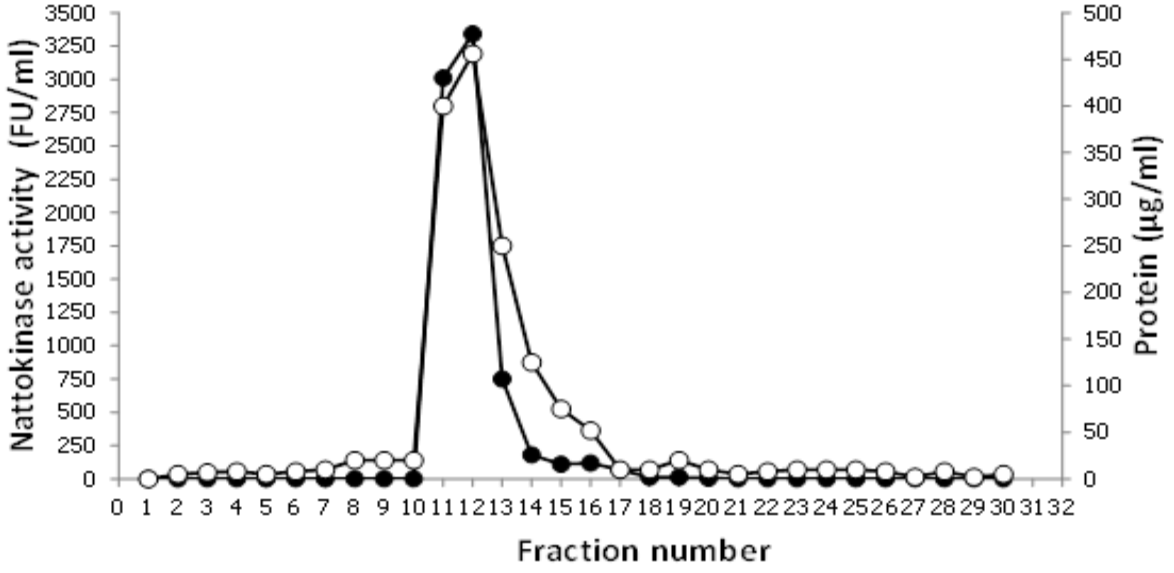

Figure 2. Gel filtration chromatography for purified nattokinase from B. subtilis DB104 by using Sephadex G75 column. Nattokinase activity (FU/ml) (●), Protein (µg/ml) (○)

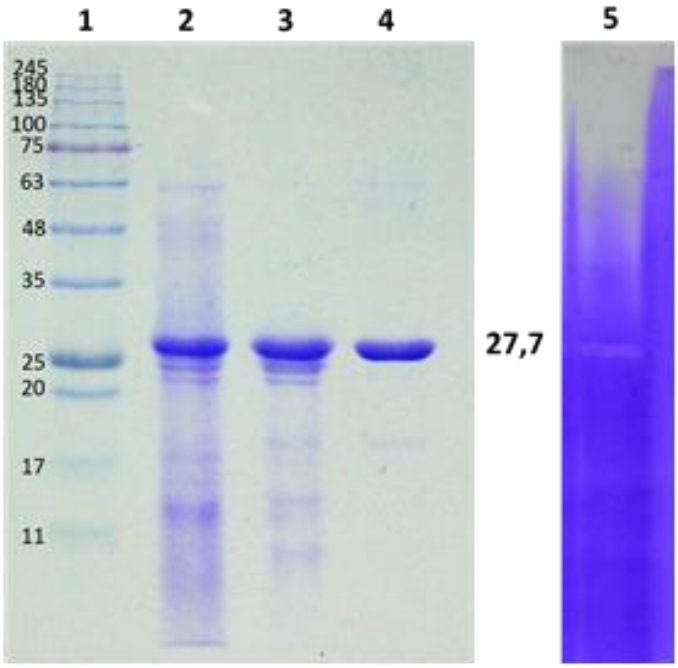

Figure 3. SDS-PAGE analysis of the purified nattokinase expressed in B. subtilis DB104. Lane 1, protein molecular weight marker; lane 2, concentrated enzyme from ammonium sulphate precipitation steps; lane 3, purified nattokinase from Q-sepharose anion exchange column; lane 4, purified nattokinase from Sephadex G-75 column; lane 5, activity staining (zymography) clear zone showed the hydrolysis of fibrin by the purified enzyme presented in the lane 4. The arrow indicates the band of nattokinase.

Table 1

Purification stages of nattokinase from *B. subtilis* DB104

Steps	Total protein (mg)	Specific activity (FU/mg)	Total activity (FU)	Purification (fold)	Yield (%)
Culture supernatant	190	2,026	385.300	1.0	100
Ammonium sulfate fraction (40%-70% saturation)	95	3,142	298,500	1.55	75.65
Q-sepharose	34.9	5,457	190,450	2.69	49.43
Sephadex-G75	21.4	7,421	158,800	3.67	41.22

Source: The researcher's data analysis

Table 1 shows the salinization enzyme having an effective recovery rate compared to the original crude enzyme 75.65%, the recovery efficiency was 50%. The purity is 1.5 times that of the original enzyme. After chromatography of the Q-sepharose enzyme, the recovery

activity was 49.43% and the purity was 2.7 times higher than that of the original enzyme. Compared to the Balaraman and Prabakaran study (2007), the activity-recovery efficiency was 30% when purifying tyrosinase from the broth after fermentation with *Bacillus sphaericus* (Balaraman & Prabakaran, 2007).

Following gel filtration, the enzyme had a recovery activity of 41.22% and purity was 3.7 times that of the original enzyme. The result is equivalent to Wang's (2009) study of recovery efficiency of 47.6%. As summarized in Table 1, the fibrinolytic enzyme was easily and quickly purified by the combination of various steps. The finally eluted proteins were subjected to SDS-PAGE, and only one band was observed in the purified sample on SDS-PAGE (Figure 3, lanes 2, 4). The molecular weight of the enzyme was approximately 27,7 kDa. With nattokinase as a standard, the final specific activity of the fibrinolytic enzyme increased more than 3.7-fold with a 41% recovery based on the initial culture supernatant.

3.2. In vivo thrombolysis activities

Table 2

The mean ± SD length of the infarcted region in the tails of the rats

Elapsed time (h)	Groups	Infarcted region (cm)
24	1	5.3 ± 0.9
	2	2.4 ± 0.5
	3	0
	4	0
	5	0.5 ± 0.1
48	1	5.5 ± 0.9
	2	1.8 ± 0.4
	3	0.9 ± 0.1
	4	0.9 ± 0.1
	5	0.6 ± 0.1
72	1	5.5 ± 0.9
	2	1.0 ± 0.2
	3	0.6 ± 0.1
	4	0.5 ± 0.1
	5	0.6 ± 0.1

Source: The researcher’s data analysis

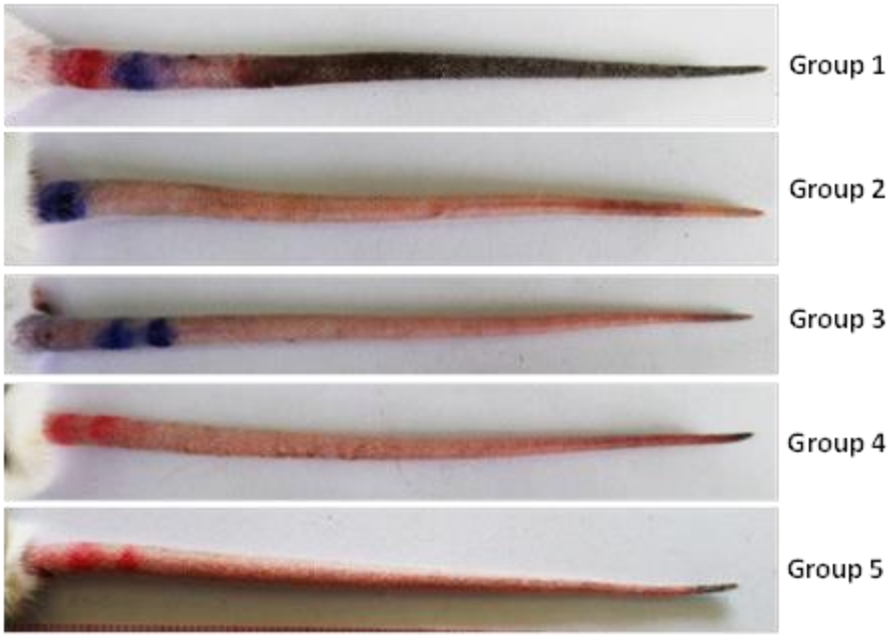

Figure 4. Photographs of the infarcted tails of rats 72 h after carrageenan was injected for the induction of thrombosis

To determine whether or not nattokinase exhibits thrombolytic effect in vivo, different concentrations of the enzyme were treated with graded in the k-carrageenan-induced rat tail thrombus model. After 24, 48, and 72 h, the mean length of the infarcted regions in the tails was found to be significantly shorter for rats in the nattokinase-administered group and group 5 than for control rats (Table 2). As the administered concentration of nattokinase increased, the length of the infarcted area tended to decrease with time. The thrombus length was reduced to 82-89% tail length respectively by nattokinase administration at 72h, similar to the effect produced by heparin. However, Kamiya Hagimori, M., Ogasawara, M., and Arakawa (2010) reported that the frequency of thrombosis in these rats is low (approximately 5%) (Kamiya, et al., 2010).

4. Conclusion

In conclusion, we observed that nattokinase which was isolated from *B.subtilis* DB104 by several steps included ammonium sulfate salt precipitation, then Q-sepharose and gel filtration in Sephadex G-75. The enzyme was purified 3.7-fold with a specific activity of 7,421FU/mg of protein and 41% recovery. The purified enzyme was homogenous on SDS-PAGE and its molecular weight was estimated to be 27.7 kDa. The remarkable thrombolytic activities of recombinant nattokinase were studied, and the enzyme was evaluated for its possible use as a thrombolytic agent. We also recommend additional bioavailability studies using drug delivery systems that target thrombus for developing more effective function-food products.

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