Thrombolytic Activities of Nattokinase Extracted from *Bacillus Subtilis* Fermented Soybean Curd Residues

Xiaoyan Zu, Zhenya Zhang, YingnanYang, Haitao Che, Guihua Zhang & Ji Li
Graduate School of Life and Environmental Sciences, University of Tsukuba
Tsukuba 305-8572, Japan
E-mail: xiaoyanzu@gmail.com

Abstract

To recycle food wastes, soybean curd residue (SCR) was used as substrate in *Bacillus subtilis* fermentation to extract nattokinase (NK). In researches, the extract conditions of Natto-NK, as well as the fibrinolytic activities of SCR-NK were investigated. In fibrinolytic activities, Natto-NK and wet corn distillers' grain (WDG)-NK were regarded as comparisons. It was showed that the highest Natto-NK yield could reach 0.108 g per 150 g Natto (wet), when extract conditions were involved with saline (0.9%) 375 ml, $(NH_4)_2SO_4$ 30 g and the ratio of 3/4 of ethanol to saline. After fibrin hydrolysis for 4 h, hydrolyzed areas of SCR-NK were 49 mm^2 in one hole, consistent with that of Natto-NK. Apart from this, as a result calculated according to the dried substrate, the SCR-NK yields (0.415 g/150 g) were significantly ($p<0.05$) higher than Natto-NK yields (0.270 g/150 g), although in activities, SCR-NK (1.3 fibrinolytic unit/ml (FU/ml)) showed slight superiorities to that of Natto-NK. It suggested that SCR could be regarded as a promising fermentation source for NK production.

Keywords: SCR, WDG, Nattokinase, Extraction, Fibrinolytic activity

1. Introduction

NK is primarily found from a traditional fermented soybean food named as "Natto" in Japan (Sumi, et al., 1987). It is a subtilisin-like serine protease with molecular weight of 27,000-42,000 Da (Chang, et al., 2000; Fujita, et al., 1993; Kim et al., 1996; Peng, et al., 2003). As fibrinolytic protease, NK digested not only fibrin but also the plasmin substrate H-D-valyl-L-leucyl-L- lysine-*p*- nitroanilide dihydrochloride (H-D-Val-Leu- Lys-pNA, S-2251) (Sumi et al., 1987), and the oral administration of NK produced a mild and frequent enhancement of the fibrinolytic activity in the plasma (Sumi, et al., 1990). Subsequently, in a rat mode, Fujita et al. (1995a) demonstrated that NK absorbed from the intestinal tract can cleave fibrinogen in plasma after intraduodenal administration of the enzyme, and the thrombolytic activity of NK is stronger than that of plasmin (Fujita, et al., 1995b; Fujita, et al., 1995). Furthermore, NK can cleave active recombinant prokaryotic plasminogen activator inhibitor into low molecular weight fragments (Urano et al., 2001). Those researches imply that NK is useful as a promising thrombolytic agent.

Soybean curd residue (SCR) and wet corn distillers' grain (WDG), are protein wastes from food manufacturing. In order to treat those kinds of wastes, they have been widely studied as inexpensive fermentation sources for several years. For example, WDG can be used as fermentation substrate to culture the *Ganoderma lucidum* (Yang, et al., 2003). On the other hand, SCR can be fermented by cellulolytic *Aspergillus* (Khare, et al., 1995), *Bacillus subtilis* (Ohno, et al., 1995; Zhu, et al., 2008) and *Ganoderma lucidum* (Hsieh & Yang, 2004) to produce citric acid, antioxidant, iturin A or fruit body of *G. lucidum*. Those utilizations, expected to reduce both environment load and production costs of high-value materials, are also important in microbial fermentation for production of industrial enzymes, such as Nattokinase (NK). However, to the best of our knowledge, it is still lack of the reports concerning SCR or WDG fermentation by *Bacillus subtilis (natto)* to obtain the NK. Moreover, optimum conditions during NK extraction and evaluative method of NK activity are incomplete as well. Therefore, we optimized the extract conditions of Natto-NK, and fermented NK using SCR or WDG as substrates. Subsequently the fibrinolytic activities of SCR-NK, WDG-NK and Natto-NK were well compared and evaluated.

2. Materials and methods

2.1 Materials and reagents

The fresh SCR (pH 6, moisture 75.0±0.3%), was purchased from Tsuchiura Tofu Processing Factory (Japan). In dry matter, its crude protein (CP) reached by 28.2±2.5%. The corn WDG (pH 4), containing 74.3±0.1% moisture

and 34.86±0.03% CP (in dry matter), was provided by Huaren Food Co., Ltd (Tianjing, China). The moisture contents were calculated by weight difference of initial and dried samples which were dried by windy oven (WFO-700, Tokyo Rikakikai Co., Ltd, Japan) at the chamber temperature of 60 ^{o}C for 24 h. The contents of CP were measured using cellulase hydrolysis at 50 ^{o}C for 3 h (pH 6.5), in which the dry matter was regarded as the material that only contained protein and cellulose (Food Engineering Group, Agricultural Division of Kyoto University, 1975). The reagents of Bovine thrombin and 0.4% fibrinogen were purchased from Sigma Chemical Co. (St. Saint Louis, MO, USA), other chemicals used were analytical grade, and purchased from Wako pure chemical industries, Ltd (Japan).

2.2 Solid state fermentation of SCR and WDG

One hundred and fifty grams of fresh SCR (adjusted to pH 8.0), in 250-ml Erlenmeyer flask, were covered with silicone stopple, and autoclaved at 121 ^{o}C for 30 min. After that, the SCR (moisture was adjusted to 80% using sterile distilled water) was inoculated with 20% (v/v) bacterial suspension (in 10^6 cfu/ml level) of *Bacillus subtilis*, and incubated at 37 ^{o}C for 20 hours in a ventilated control-temperature incubator with the relative humidity at around 95%. The ferment processes and conditions of corn WDG were consistent with that of SCR.

2.3 Natto-NK Extraction and optimization

Fresh Natto food 150 g (60% moisture) were firstly smashed into slurry by the juice blender (MJ-781, Matsuden Co., Ltd, Japan) at 4 ^{o}C for 10 min, and mixed 0.9% saline in 300 ml (control), 375 ml, 450 ml or 525 ml levels, respectively. After the obtained solution stirred for 40 min and filtered for 2 times by cotton cloth, 95% ethanol was added into the filtrate with the ratio of 1/1(standard), 3/4, 5/8, 1/2 (v/v), corresponding to the optimum saline volume. It was centrifugated with 8000 rpm at 4 ^{o}C. Forty minute later, $(NH_4)_2SO_4$ (5-70 g, 40 g was regarded as standard) were supplemented into the supernatant for salting out the crude NK. The solution was then centrifugated at 12,000 rpm for 30 min and removed the supernatant. After that, we dissolved the crude NK into 300 ml buffer solution (pH 7.4, contained 0.1 M phosphate buffer and 0.2 M NaCl in the ratio of 1:1 (v/v)). Furthermore, the ultra filter units with membrane UF-1 OPS and UF-5 OPS (molecular weight cut off: 1×10^4 Da and 5×10^4 Da, provided by Tosoh Co., Japan), were used for excluding inorganic salts and impurities and obtaining target NK with molecular weight of $1\text{-}5\times10^4$ Da. Finally, crude NK powder was obtained after dried by freeze dryer (FDU-506, Tokyo Rikakikai Co., Ltd, Japan). In this experiment, the three variables were adjusted one by one along with the extract sequence, and standard values were used before those variables were fixed. All levels in one variable were performed with three replications.

2.4 NK activity assay

2.4.1 Fibrinolytic efficiency of NK in fibrin plate (Jorge, et al., 1993)

Fibrinogen (0.4%) 10 ml dissolved by boric acid-saline buffer (contained 0.05 M H_3BO_3, 0.05 M KCl, pH 7.8) was separated averagely into 4 plates (grid type, 100×20 mm), and added with bovine thrombin (50 U/ml, dissolved in 0.9% normal saline) of 0.5 ml/plate. The mixture was stirred and heated at 85 ^{o}C for 30 min. After cooling down, NK (10 mg/ml, dissolved in 0.9% normal saline) 0.02 ml were dropped on the surface of concretionary fibrin, and those fibrin plate were incubated at 37 ^{o}C for 4 h. Furthermore, the circle areas of the holes were calculated, at the same time, their depths of the holes were observed visually. In this assay, Natto-NK (as control), SCR-NK, and WDG-NK were measured, and fibrinolytic efficiency was expressed as the average area of those holes in one fibrin plate.

2.4.2 Fibrinolytic activity of NK

SCR-NK (the same batch) and Natto-NK (as control) were used in this assay. NK samples (10 mg/ml) were diluted 100 times before test. *Procedure 1*: the solution containing 1.4 ml boric acid-saline buffer (pH 7.8) and 0.4 ml fibrinogen (0.4%), was firstly pre-incubated at 37±0.3 ^{o}C for 5 min, and incubated at same temperature for 10 min after mixed the bovine thrombin 0.1 ml. *Procedure 2*: the mixture added NK sample 0.1ml was stirred for 5 second at 20 min and 40 min respectively. After 60 min, we added 2 ml trichloroacetic acid (0.2 M) into the mixture and placed it at 37±0.3 ^{o}C for 20 min to stop the reaction. *Procedure 3*: the absorbency of the 1-ml supernatant obtained from centrifugating (12,000 rpm) was determined at 275 nm and named as A_1. At the same time, the solution obtained from *procedure 1* was directly stopped by 0.2 M trichloroacetic acid and added with NK sample 0.1ml. After stirred for 5 second and placed for 20 min at 37 ^{o}C, its absorbency at 275 nm was also measured and named A_2. One fibrinolytic unit (FU) was defined as the amount of NK that released an increase of 0.01/min in absorbency at 275 nm. NK activity was calculated using the following formula.

$FU/ml=((A_1-A_2)/0.01)\times(1/60)\times(1/0.1)\times d$

(d- diluent times of NK samples)

2.5 Statistical analysis

The obtained data were analyzed using a two-tailed Student's *t*-test, and results were expressed as mean±SD. Statistic difference was considered to be significant at $p<0.05$(*).

3. Results and discussion

3.1 Effect of saline volume during Natto-NK extraction

During Natto-NK extraction, saline (0.9%) were used as the first solutions to dissolve Natto sample in four levels. In this step, fresh Natto (150 g each group) was solved in saline. The group added 300 ml saline was acted as control. As the result (Fig. 1) shown, there was no significant difference in NK yield when Natto samples solved in 375 ml, 450 ml or 525 ml saline, respectively. However, those three saline levels have more advantages on NK yield than the group control ($p<0.05$). It implied that a critical value of the saline volume was existed in NK extraction. Below the value, the NK would be lost due to saline deficiency and Natto sample incomplete dissolved, and there was also disadvantageous when saline volume higher than the critical value. From this experiment, the critical volume of saline was about 375 ml (Fig. 1).

3.2 Effect of ethanol and $(NH_4)_2SO_4$ during Natto-NK extraction

Natto slimes could disturb the NK extractions, which contained poly glutamic acid (PGA) and mucin (polysaccharide) (Kunioka and Goto, 1994; Nagai *et al.,* 1997). Different concentrations of Ethanol solutions thereby were used to eliminate Natto slimes, and the crude NK were subsequently salted out by diverse dose of $(NH_4)_2SO_4$. As results shown in Fig. 2, salting-out curve was moved to the left with ethanol increasing, and $(NH_4)_2SO_4$ was mostly saved at the ratio of 3/4 of ethanol to saline. In this curve, crude NK was salted out 0.108±0.012 g in the highest by using 30-35 g $(NH_4)_2SO_4$, then decreased greatly after crossed the peak value.

The Results implied that the saturate of $(NH_4)_2SO_4$ was obviously different after the ratio of ethanol was changed in solution. And optimum conditions of extraction were 0.9% saline 375ml, 3/4 of ethanol to saline, and $(NH_4)_2SO_4$ 30 g. Besides, according to the NK molecular weight reported (Kim *et al.,* 1996), the ultra-filter membranes (with molecular weight cut off: 1×10^4 Da and 5×10^4 Da) were used to remove inorganic salts (e.g. Na^+ and NH_4^+) and needless biological macromolecules. After dried, the target NK obtained was white powder.

3.3 Fibrinolytic efficiency of SCR-NK, WDG-NK and Natto-NK

Nattokinase is a potent fibrinolytic enzyme (Sumi et al., 1990). The properties of Nattokinase closely resemble plasmin. It enhances the body's natural ability to fight blood clots in several different ways (Fujita et al., 1995a). And its efficiency can easily be observed from fibrinolytic area on the fibrin plate (Fujita et al., 1995b; Urano et al., 2001).

The fibrinolytic effects of Natto-NK (control), SCR-NK and WDG-NK were showed as the holes in Fig. 3. As results shown, there was no difference between hydrolyzation effects of SCR-NK and Natto-NK (about 49 mm^2 for average area in one hole). Obviously, the effect of SCR-NK was at least no lower than Natto-NK based on their hydrolytic efficiency. As for WDG-NK, the hydrolysis depth was scarcely observed although its hydrolyzed area was visually consistent with other two groups. On the other hand, the crude WDG-NK obtained was not so efficient in fibrin plate, viewed the matter from our investigations, which possibly was due to the low NK (pure) contents. At fermentation, WDG seemed hardly to be used by *Bacillus subtilis* for NK production because of its high cellulose contents. Its mechanism is still not well understood and the corresponding researches are needed.

3.4 Fibrinolytic activity of SCR-NK and Natto-NK

Fibrinolytic activity is an important index to evaluate the effect of NK. Considering the slight effects of WDG on fibrin plate mentioned above, we eliminated it from the fibrinolytic activity measurements. Miyamura *et al*. (1998) have been reported the fibrinolytic activity of NK from okara and natto. For investigating the effects of SCR-NK and Natto-NK in detail, the fibrinolytic activities were measured as well. We used fresh SCR (containing 80% water) and Natto (containing 60% water) to do this extraction, while for comparing the activities of those extracts, final results were both calculated by 150 g dried samples. Figure. 4 showed that the yields of SCR-NK were reached about 0.415 g per 150 g dried SCR, which significantly ($p<0.05$) higher than Natto-NK yield (0.270 g) after accordant extraction processes.

According to their fibrinolytic efficiency and activity results (Fig. 3), it was found that SCR-NK was efficient, same as Natto-NK in fibrin hydrolysis. Although the fibrinolytic activity of SCR-NK (1.3±0.17 FU/ml, similar as the results of Miyamura *et al*. (1998)) was no statistic superior difference with Natto-NK activity (Fig. 4), the higher extraction yields of SCR-NK also contributed to the cost reduction of NK production. At present, NK in market is generally extracted from Natto (fermented from soybean), which is more expensive than using SCR as

ferment substrate. Therefore, as a practical and cheaper fermented source, SCR has more advantages and convenience to produce NK.

4. Conclusion

In our investigations, NK can be extracted from *Bacillus subtilis* fermented SCR, and its extraction yields were superior to that of Natto-NK. Moreover, the fibrinolytic activities of SCR-NK were slightly higher than that of Natto-NK. Therefore, it was concluded that SCR possessed the potentialities to as a promising fermentation source for industrial NK production.

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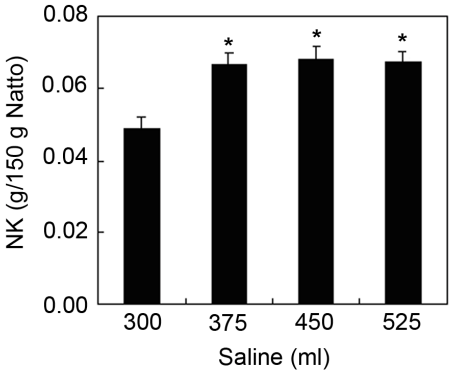

Figure 1. Effect of saline volume on NK yield

In this tests, the 150-g Natto contained 60% water. Data represent mean ± SD from three independent experiments. Two-tailed Student's *t* test was used for statistical analysis and differences were considered significant at $p < 0.05$ (*)

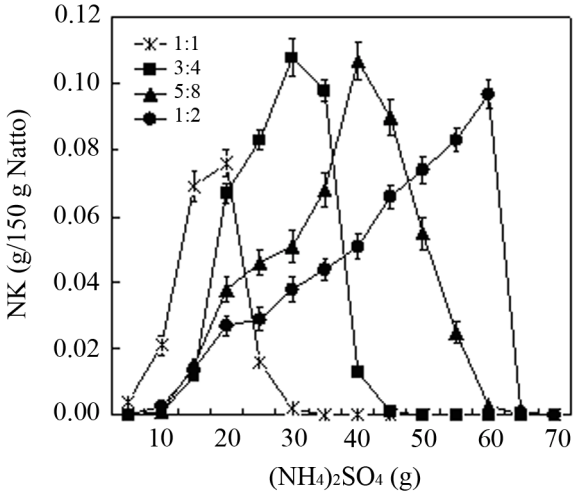

Figure 2. Effect of ethanol and $(NH_4)_2SO_4$ on NK yield

In experiments, the 150-g Natto contained 60% water. Tags of (*), (■), (●) and (▲) represented the ethanol adding levels was submitted to the ratios of 1:1, 3:4, 5:8 and 1:2 (v/v) of saline. Data represent mean ± SD from three independent experiments.

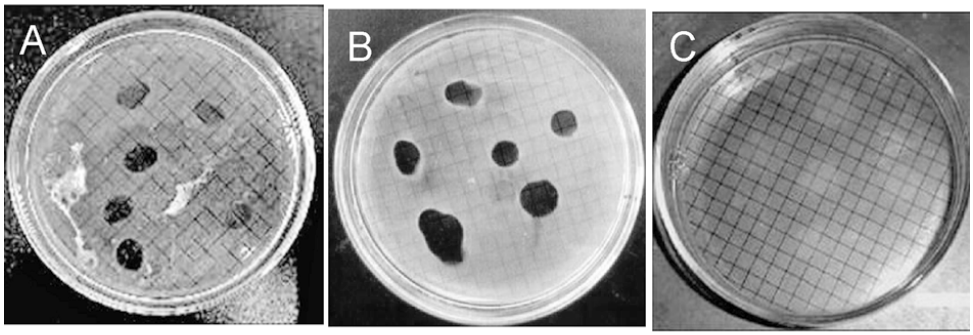

Figure 3. Fibrinolytic effect of Natto-NK (A), SCR-NK (B) and WDG-NK (C) on fibrin plate
Fibrinolytic efficiency was expressed as the average area (πr^2) of those holes in each fibrin plate.

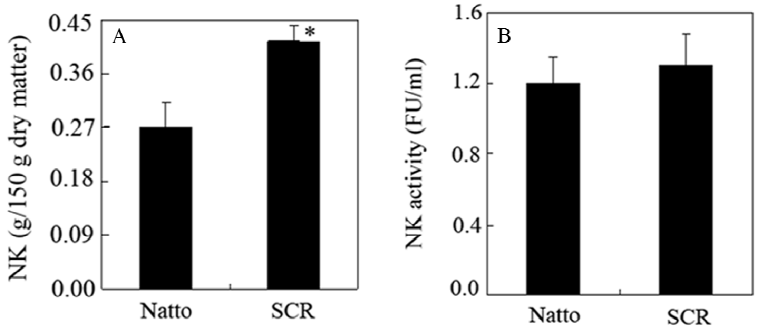

Figure 4. Comparison of extracts yield and fibrinolytic activity of Natto-NK SCR-NK
Data represent mean ± SD from three independent experiments. (A), Yields of Natto-NK and SCR-NK from 150 g samples. Results were calculated according to 150 g dried samples. (B), Fibrinolytic activities of Natto-NK and SCR-NK. Two-tailed Student's *t* test was used for statistical analysis and differences were considered significant at $p < 0.05$ (*).