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Comparative Fibrinolytic Activities of Nattokinases from *Bacillus subtilis* var. *natto*

Amira Abdel Motaal [1], Inas Fahmy [2], Ali El-Halawany [1], Nahed Ibrahim [2]

[1]*Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr-El-Ainy St. Cairo11562, Egypt.*
[2]*Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt.*

Abstract: Nattokinase is a potent fibrinolytic enzyme which was first found in a traditional Japanese soybean food. Two Nattokinase genes were cloned using chromosomal DNA from *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872. Recombinant *Natto-1.3 kb* (GenBank accession number KF734090) and *Natto-1.1 kb* were expressed using a pQE-30 expression vector, and their fibrinolytic activities were studied. *Natto-1.3 kb* nucleotide sequence contained an open reading frame of 1332 base pairs encoding 106 amino acids for signal peptide and 275 amino acids for mature subtilisin. It showed 100, 99.74 and 98.69 % identities with subtilisin NAT, subtilisin E and subtilisin J from *Bacillus subtilis*, respectively. While the *new Natto-1.1 kb* sequence contained 1088 base pairs encoding only 87 amino acids for signal peptide and 275 amino acids for mature subtilisin. Nattokinase was produced by *Bacillus subtilis* at pH 7.0 in a fermentation medium (g%): 1.0 glucose, 5.5 peptone, 0.5 $CaCl_2$, 0.2 $MgSO_4$. Quantitative analysis of the fibrinolytic activity was conducted by the fibrin plate method using urokinase (20000 U/mg) as the reference standard. The fibrinolytic activities of *Natto-1.3 kb* and *Natto-1.1 kb* were 1000 ± 101 and 1187.5 ± 134 U/mg, respectively, compared to 400 ± 97 U/mg of the supernatant of the fermented culture broth of *B. subtilis*.

***Keywords*:** *Bacillus subtilis*, fermented broth, fibrinolytic activity, Nattokinase

INTRODUCTION

Atherosclerosis, hypertension and several cardiovascular diseases are ever prevailing in Egypt and the Middle East due to bad eating habits and lack of exercise. This requires a continuous search for safe, effective and cheap thrombolytic agents. One known potent fibrinolytic enzyme is Nattokinase (NK) from *Bacillus subtilis* var. *natto* which was first found in the traditional Japanese soybean food natto [1]. NK possesses several advantages over several other conventional clot dissolving drugs. This includes safety, efficacy, preventive and prolonged effects, low cost, convenience in oral administration, as well as stability in the gastrointestinal tract [2, 3]. These properties make from NK a promising thrombolytic therapy especially in developing countries.

The three-dimensional structural model of NK from *Bacillus natto* was previously constructed by homology modeling [4]. Weng et al., 2009 [5] compared between the specific activity of wild type subtilisin NAT and two of its mutants (T220S and M222A). The protein coding regions of subtilisins NAT, E and J were identified to code for a 29-residue signal peptide for protein secretion from the cell membrane, a 77-residue propeptide which functions as an intramolecular chaperone that organizes the *in vivo* folding to achieve the active conformation and a 275-mature subtilisin [6, 7].

The fermented soybeans produced using *B. subtilis* bacteria, however, may have a preventive effect on not only thrombosis but also cancer. Previous reports proved that using the fermentation medium components glucose, peptone, calcium chloride and magnesium sulphate resulted in a maximum NK activity of 3194.25 U/ml [8]. Therefore, with the increase in yield and activity and simultaneous cost reduction, the industrial NK production by *B. subtilis* fermentation can be regarded as economically attractive.

The aim of our study was to clone and express two new nattokinase enzymes (*Natto-1.3 kb* and *Natto-1.1 kb*), compare their amino acid sequences to those of subtilisins NAT, E and J, and compare their fibrinolytic activities to the more economically produced fermentation product using *B. subtilis* culture broth.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Bacillus subtilis (Ehrenberg 1835) Cohn 1872 was purchased from RIKEN BioResource Center, Ibaraki, Japan (Catalogue#20036). The bacterial strain was grown overnight on LB agar plates. The culture was maintained at 4 ^{o}C. LB medium: trypton 10g / L, yeast 5 g / L and sodium chloride 5 g / L.

Cloning and expression of Nattokinase

The nucleotide sequence database of National Center for Biotechnology Information was searched for a Nattokinase homologue and the Nattokinase NAT from *B. subtilis* (P35835) was found. Chromosomal DNA from *B. subtilis* (Catalogue#20036) was prepared by the method of Rochelle et al., 1992 [9], and was used as the template for PCR. *Natto-1.3 kb* and *Natto-1.1 kb* genes were amplified by PCR using BamHI-linked sense primers (5'- CGCGGATCCGTATGAAAATAGTTATTTCG - 3' for *Natto-1.3 kb* and CGCGGATCCATGGCGTTCAGCAACATGTCTGCG for *Natto 1.1 kb*) and PstI-linked antisense primer (5'- AAAACTGCAGTTATT- GTGCAGCTGCTTGTACGTTG - 3'). PCR amplification was performed under the following conditions: 35 cycles of 95 ^{o}C for 2 min, 95 ^{o}C for 30 s, 50 ^{o}C for 30 s, and 72 ^{o}C for 3 min and 72 ^{o}C for 10 min. The PCR-amplified 1330 bp (*Natto-1.3 kb*) and 1088 bp (*Natto-1.1 kb*) DNA fragments were extracted from agarose gel and then ligated into pBluescript II SK (-) plasmid (Stratagene). After digestion with BamHI and PstI, the *Natto-1.3 kb* and *Natto-1.1 kb* fragments were inserted into the bacterial expression vector pQE-30 (Qiagen).

Escherichia coli M15[pREP4] was the host bacterial strain for the expression vector. Transformed cells were then grown at 37 ^{o}C in LB-medium, supplemented with kanamycin (25µg/ml) and ampicillin (100 µg/ml) until an OD_{600} of 0.6 was reached. Protein expression was induced by 1.0 mM IPTG (Sigma) and incubation was continued for 4-5 h at 37^{o}C. The cells were harvested by centrifugation (20 min, 4000 x g, 4^{o}C). Protein purification was carried out through the N- terminal hexa histidine residue (coded in the pQE-30 vector, 6xHis-tag). The purification was done according to QIA expressionist protocol (Qiagen, Fifth Edition, 2001) using Ni^{2+}-NTA agarose. The purified protein was stored at -20^{o}C then lyophilized to be used for the activity assay.

Determination of the concentration of the purified protein

After purification of the protein to homogenity, 2 µl of the protein in 800 µl water were mixed with 200 µl Biorad reagent and mixed. Samples were measured at 595 nm and the protein concentration was determined by means of a standard calibration curve established using 4, 6, 8 and 12 µg of bovine serum albumin (BSA) [10].

Production of Nattokinase by fermentation

The bacterial strain *B. subtilis* subsp. *natto* grown overnight on an LB plate, was washed in 5ml LB medium and transferred into a 1L flask containing 500 ml LB medium. The culture was incubated with shaking at 300 rpm at 30° C for 6 hours. A 70 L fermentor (BIO F10 5000, New Brunswick Scientific, New Brunswick, NG, USA), containing 50 L of the fermentation medium (g%): 1.0 glucose, 5.5 peptone, 0.5 $CaCl_2$, 0.2 $MgSO_4$ and pH was adjusted to 7.0 [8], was inoculated with 1 L of the seed culture. Cells were grown at 30° C with an air flow rate of 1.5 slpm and an agitation speed between 200-300 rpm under an overhead pressure of 1 psi. The cell culture was harvested by centrifugation at 4000g, 4^{o}C for 10 min, where the clear supernatant was lyophilized and assayed for the NK activity.

Fibrinolytic activity assay

Quantitative analysis of the fibrinolytic activity was conducted by the fibrin plate method [11-13], using urokinase as the reference standard. In brief, 15 ml of 0.8 mg/ml fibrinogen solution (in 0.1M sodium phosphate buffer, pH 7.4) were mixed with 20 ml of 2% agarose solution and warmed up in a 45 °C water bath for 15 min, followed by the addition of 1 ml of thrombin solution (7.5 U/mL) in a 90 mm Petri dish. The mixture was left for 1 h at room temperature to form a fibrin clot layer. The enzyme protein was lyophilized into a powder form and dissolved in 50 µl of phosphate buffer, pH 7.4. Each 10 µl of the enzyme sample solutions were placed on a filter disc (5 mm in diameter) and incubated at 37^{o}C for 18 h. After measuring the diameter of the clear zone, the units (U) of the enzyme activities were determined according to the urokinase established standard calibration curve (Fig. 1). Specific activity was denoted by urokinase units of fibrinolytic activity in each milligram of enzyme. Urokinase from human urine, fibrinogen from human plasma and thrombin from bovine plasma were purchased from Sigma Aldrich.

RESULTS AND DISCUSSION

Cloning of Nattokinase

The subtilisin *Natto-1.3 kb* gene of *Bacillus subtilis* (*natto*) was cloned and its nucleotide sequence was determined (deposited in GenBank under accession number KF734090). The sequence revealed only one open reading frame composed of 1332 base pairs and 381 amino acid residues. The amino acid sequence of nattokinase *Natto-1.3kb* was compared with the published sequences of other subtilisins (Fig. 2) and showed 100, 99.74 and 98.69 % identities with subtilisin NAT, subtilisin E and subtilisin J from *Bacillus subtilis*, respectively. On the other hand, subtilisin *Natto-1.1 kb* gene was cloned and its sequence revealed an open reading frame composed of 1088 base pairs and 362 amino acid residues. It had 19 amino acid residues missing at the N-terminal of the signal peptide compared to other subtilisins. The catalytic domain of 275 amino acids as well as the catalytic triad (Asp-32, His-64, and Ser-221) were well conserved in both genes as many Bacillus subtilisins [4].

Expression and purification of Nattokinase

Cultures of transformed *E. coli* M15[pREP4] was induced with 1.0 mM IPTG at 37^{o}C, which led to the production of major proteins of 40 and 48.8 kDa (Fig. 3). Protein purification was carried out through the N- terminal hexa histidine residue using Ni^{2+}-NTA agarose. The concentrations of the purified proteins were calculated to be 500 and 400 µg/ml for *Natto-1.1 kb* and *Natto-1.3 kb*, respectively, using the established standard calibration curve [10].

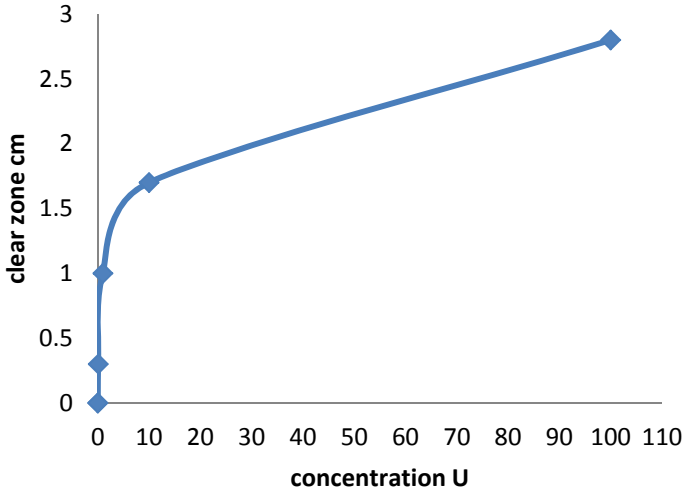

Figure 1. Standard calibration curve for urokinase (standard fibrinolytic enzyme).

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Natto-1.3kb MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
        NAT MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
        E   MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
        J   MRSKKLWISLLFALTLIFTMAFSNMSVQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
            ***************************.********************************

Natto-1.3kb EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
        NAT EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
        E   EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
        J  EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
            ************************************************************

Natto-1.3kb ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
        NAT ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
        E   ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
        J   ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
            ************************************************************

Natto-1.3kb LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
        NAT LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
        E   LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
        J   LNNSIGVLGVSPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPSGSTA
            **********:*************************************************:****

Natto-1.3kb LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGS
        NAT LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGS
        E   LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGS
        J   LKTVVDKAVSSGIVVAAAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGS
            ***************************:****************************.**

Natto-1.3kb  ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
        NAT ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
        E   ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
        J   ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
            ************************************************************

Natto-1.3kb TYLGNSFYYGKGLINVQAAAQ
        NAT TYLGNSFYYGKGLINVQAAAQ
        E   TYLGNSFYYGKGLINVQAAAQ
        J   TYLGNSFYYGKGLINVQAAAQ
            *********************
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Figure 2. Comparison between the amino acid sequence of *Natto-1.3 kb* (KF7340090) and other subtilisin proteases; Subtilisin NAT (P35835), Subtilisin E (P04189), Subtilisin J (P29142).

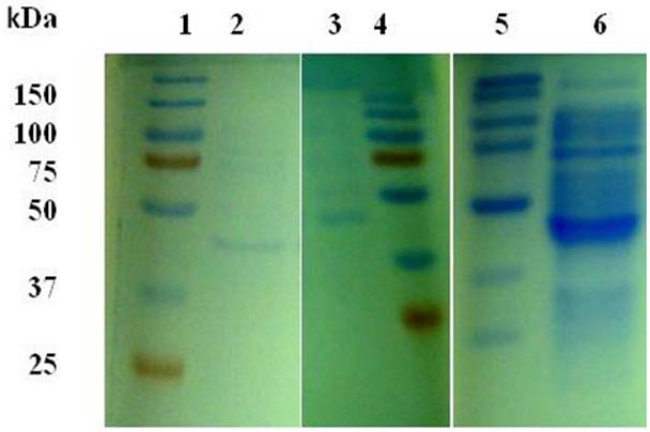

Figure 3. SDS-PAGE (12%) of purified NK and fermentation product. Lanes 1,4 and 5, prestained protein marker fermentas; lane 2, *Natto-1.1 kb*; lane 3, *Natto-1.3 kb*; lane 6, total protein from supernatant of fermentation product.

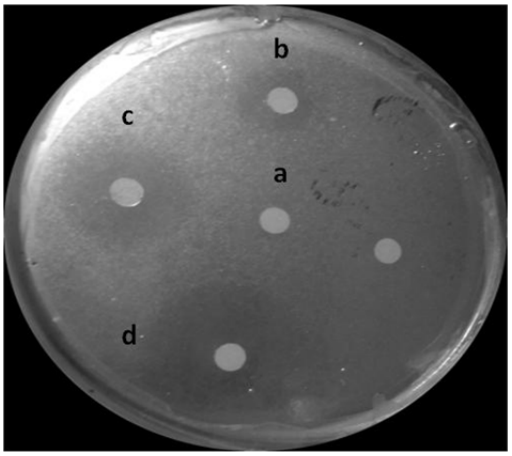

Figure 4. Serial dilutions of urokinase on a fibrin plate; a, 0.0025 µg/disc; b, 0.025 µg/disc; c, 0.25 µg/disc; d, 2.5 µg/disc.

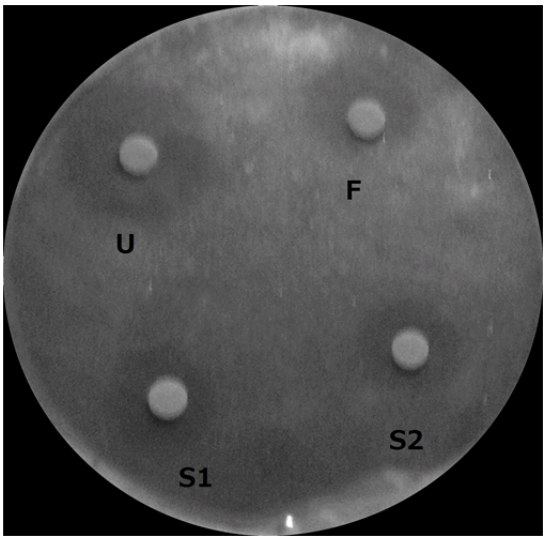

Figure 5. A fibrin plate showing: Ur, urokinase standard; S1 and S2, *Natto-1.1 kb* and *Natto-1.3 kb*, respectively; F, fermentation product.

Table 1. Comparison between the fibrinolytic activities

Enzyme	Specific activity (U/mg)[a]
Natto-1.1 kb	1187.5 ± 134
Natto-1.3 kb	1000 ± 101
Fermentation product (F)	400±97

[a]The data shown are expressed as a mean ± SD, based on three independent experiments.

Comparative fibrinolytic activity

The fibrinolytic activities of the cloned *Natto-1.1 kb* and *Natto-1.3 kb* were compared to that of the fermentation product using urokinase as a reference standard. Serial dilutions of urokinase (100 to 0.001 U) were added to the fibrin plate and the clear zones resulting from hydrolysis of the fibrin clot were recorded (Fig. 4). Ten µl of each of the expressed NK samples (*Natto-1.1 kb* and *Natto-1.3 kb*) and the fermentation product (F) containing 8, 10 and 75 mg/mL protein, respectively, were added to the fibrin clot. All samples revealed fibrinolytic effect as indicated by the clear zone of 1.6 and 1.7 and 1.9 cm corresponding to 95, 100 and 300 U, respectively (Fig. 5). As shown in Table 1, the specific activity of *Natto-19* (1187.5 ± 134 U/mg) was slightly higher than *Natto* but was 3-fold that of the fermentation product. Previously, Weng et al., 2009 [5] expressed a wild type subtilisin NAT and two of its mutants T220S and M222A possessing specific fibrinolytic activities of 1760 ± 154, 1230 ± 90 and 933 ± 97 U/mg, respectively, at pH 7.75.

CONCLUSION

The fibrinolytic activities of the cloned Nattokinases, *Natto-1.1kb* and *Natto-1.3kb*, were more or less the same although 19 amino acid residues were missing at the N-terminal of *Natto-1.1kb*. The lyophilized fermentation product of *Bacillus subtilis* (natto) showed quite a good activity compared with the lyophilized pure expressed Nattokinases. Production of such a fermentation product on a larger scale and its incorporation into pharmaceutical preparations is our next goal.

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