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Expression of nattokinase in *Escherichia coli* and renaturation of its inclusion body

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ABSTRACT

Nattokinase is an important fibrinolytic enzyme with therapeutic applications for cardiovascular diseases. The full-length and mature nattokinase genes were cloned from *Bacillus subtilis* var. natto and expressed in pQE30 vector in *Escherichia coli*. The full-length gene expressed low nattokinase activity in the intracellular soluble and the medium fractions. The mature gene expressed low soluble nattokinase activity and large amount insoluble protein in inclusion bodies without enzyme activity. Large amount of refolding solutions (RSs) at different pH values were screening and RS-10 and RS-11 at pH 9 were selected to refold nattokinase inclusion bodies. The recombinant cells were lysed with 0.1 mg/mL lysozyme and ultrasonic treatment. After centrifugation, the pellete was washed twice with 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100 to purify the inclusion bodies. The inclusion bodies were dissolved in water at pH 12.0 and refolded with RS-10. The refolded proteins showed 42.8 IU/mg and 79.3 IU/mg fibrinolytic activity by the traditional dilution method (20-fold dilution into RS-10) and the directly mixing the protein solution with equal volume RS-10, respectively, compared to the 52.0 IU/mg of total water-soluble proteins from *B. subtilis* var. natto. This work demonstrated that the inclusion body of recombinant nattokinase expressed in *E. coli* could be simply refolded to the natural enzyme activity level by directly mixing the protein solution with equal volume refolding solution.

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1. Introduction

Nattokinase (E.C. 3.4.21.62) is a serine protease produced by *Bacillus subtilis*. The enzyme directly cleaves cross-linked fibrins in vitro, catalyzes the conversion of plasminogen to plasmin or inactivates the fibrinolysis inhibitor (plasminogen activator inhibitor 1, PAI-1) (Urano et al., 2001; Yongjun et al., 2011). As its high fibrinolytic activity, the enzyme can be used as a therapeutic drug for the treatment of cardiovascular diseases, such as hypertension, high cholesterol osteoporosis (Dubey et al., 2013; Mine et al., 2005). Unlike existing thrombolytic drugs which can be used only as injection, nattokinase has a low molecular weight (275 amino acid residues and 28 kDa of its mature protein) and high stability to low pH environment. It can safely pass through the gastrointestinal tract, be absorbed by the intestine into bloodstream and can be used for oral administration (Peng et al., 2003; Wei et al., 2011). The high activity and ideal therapeutic application of nattokinase for various diseases make it attractive worldwide.

Although nattokinase capsules are commercial available on the market, its application is limited due to the low yield and difficulty in purification from *B. subtilis* natto (Peng et al., 2005). Many attempts have been made to improve microbial production of the fibrinolytic enzyme, including screening of high yield microbial strains or species, optimizing culture medium and conditions, and purification technologies (Borah et al., 2012; Jaques et al., 2011; Liu et al., 2005; Xiao-Lan et al., 2005).

To improve enzyme production and simplify the downstream manipulation, heterologous expression of nattokinase has been investigated in several microbial expression systems, such as in *Escherichia coli*, *Pichia pastoris*, *Bacillus subtilis* and *Lactococcus lactis* (Wei et al., 2015; Wu et al., 2011). Although active nattokinase can be expressed in recombinant *P. pastoris* and *L. lactis*, and many efforts were invested to increase the recombinant nattokinase production, such as altering sequences, DNA family shuffling (Dabbagh

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et al., 2014). The expression is low and the product is difficulty in purification (Cereghino and Cregg, 2000; Liang et al., 2007a).

Nattokinase had high expression in recombinant *E. coli* (Han et al., 2009; Liang et al., 2007b), but most of the recombinant protein is in the form of inactive inclusion bodies which are hardly to be renatured. Nattokinase or pronattokinase as an insoluble recombinant protein linked to the C-terminus of oleosin, soluble nattokinase was subsequently released through self-splicing of intein induced by temperature alteration, and fibrinolytic activity was observed in the recombinant nattokinase (Zhang et al., 2005). Secretory expression is supposed to be an effective way to avoid the inclusion bodies and to produce active protein in *E. coli*. With the help of two different signal peptides, the native signal peptide of nattokinase and the signal peptide of PelB, active nattokinase was successfully expressed in *E. coli* with periplasmic secretion, but fibrinolytic activity was much lower than natural nattokinase in *Bacillus subtilis* (Liang et al., 2007a).

In this research, the full-length and mature nattokinase genes were cloned from *B. subtilis* and expressed in *E. coli*. The full-length gene expressed low nattokinase activity, and mature gene expressed low nattokinase activity in the intracellular soluble fraction but large amount of insoluble inclusion bodies in the *E. coli* expression systems. Then, the renaturation of nattokinase inclusion body was systematically investigated. After a series of screening of refolding solutions (RSs), two solutions, RS-10 and RS-11, were selected as potential refolding solutions for nattokinase inclusion bodies. Using RS-10, the inactive recombinant nattokinase was successfully renaturated, which yielded high enzyme activity equivalent to natural nattokinase, using the traditional dilution method and the direct method by mixing equal volumes of protein solution and refolding solution.

2. Materials and methods

2.1. Microbial strains, plasmids and chemical reagents

B. subtilis natto was isolated from natto food of local supermarket. *E. coli* BL21 (DE3) was stored in our laboratory. Plasmid pQE30, DNA marker, Taq polymerase, and restriction enzymes (Sac I and BamHI) were purchased from Invitrogen Inc. (Carlsbad, CA, USA). Ampicillin and kanamycin were purchased from Sangon Co. (Shanghai, China). Other chemical reagents are analytical grade and were purchased from local suppliers.

2.2. Cloning and expression of nattokinase gene in E. coli

The genomic DNA of *B. subtilis* natto was prepared according to the Short Protocols in Molecular Biology (Ausubel et al., 2005) and was used as a template to amplify nattokinase gene by PCR. Two pairs of primers were designed based on the sequence of nattokinase gene (gi: 14422312) in the GenBank. Primers P1 (CCCGAGCTCTTATTGTGCAGCTGCTTG) and P2 (GCGGATCCGAATGAGAAGCAAAAAATT) were used to amplify the full-length nattokinase gene, and primers P3 (CCCGAGCTCTTATTGTGCAGCTGCTTG) and P4 (CGCGGATCCATGGCGCAATCTGTTCCT) were used to amplify the mature peptide gene (without signal sequences). All primers had a restriction enzyme site (the underlined sequences). PCR products were sequenced by Sangon Co. (Shanghai, China). The sequences were analyzed using the BLAST programs at NCBI database.

The PCR products were digested with Sac I and BamHI, and inserted into pQE30 vectors. The recombinant plasmids containing full-length nattokinase gene (pQE30-f-NK), and mature peptide gene of nattokinase (pQE30-m-NK) were transformed into *E. coli* BL21 (DE). Positive colonies were selected on Luria-Bertani (LB) solid medium containing 50 μg/mL ampicillin and 50 μg/mL kanamycin and confirmed by sequencing. The recombinant *E. coli* cells were cultured in LB medium containing 50 μg/mL ampicillin and 50 μg/mL kanamycin at 37 °C with 250 rpm shaking. When the cell concentration (OD_{600}) reached 0.8, nattokinase expression was induced for 6 h unless otherwise stated by adding 0.6 mM IPTG (isopropyl β-D-1-thiogalactopyranoside, Sigma-Aldrich Co. Ltd., Poole, United Kingdom) into the cultures. Soluble and insoluble proteins were analyzed by 5% (stacking gel) – 10% (separating gel) SDS-PAGE (sodium dodecyl sulfate polyacrylamidegel electrophoresis).

2.3. Separation and dissolution of nattokinase inclusion body

2.3.1. Cell harvesting and lysis

Cell suspension of the recombinant *E. coli* was centrifuged at 5000*g* for 1 min, and the precipitate was resuspended and centrifuged with phosphate buffered saline (PBS pH 8.0) twice. The precipitate was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 10 mM EDTA, 1% Triton X-100 and 0.1 mg/mL lysozyme and incubated at room temperature for 15 min to lysate the cells, as we described before (Wu et al., 2010). The cell lysis was further ultrasonicated (300 W power) for 30 cycles (each for 5 s with 5 s gap) on ice. After centrifuged at 10,000*g* for 10 min at 4 °C, soluble protein in supernatant and inclusion body in precipitate were separated.

2.3.2. Isolation of the inclusion bodies

Unless otherwise stated, the inclusion bodies from 2.0 g wet *E. coli* cells were washed by resuspending them twice in 2 mL 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100 for 10 min, and once in 2 mL 50 mM Tris-HCl pH 8.0, followed each by centrifugation at 10,000*g* for 10 min. The precipitate was dissolved in pH 12.0 water and ultrafiltrated at 4000 g centrifugation for 30 min using the Amicon Ultra 50 kDa and 30 kDa molecular weight cut-off centrifugal tubes (Millipore, Bedford, MA, USA). The fraction between 50 kDa and 30 kDa was collected as purified protein of nattokinase inclusion body proteins for refolding.

2.3.3. Dissolution of inclusion bodies

The nattokinase inclusion body (10 mg) was suspended in 2 mL 20 mM Tris-HCl at pH 8.0 and centrifuged at 10,000*g* for 5 min at 4 °C. Then H_2O at different pH values were used to dissolve the nattokinase inclusion body for 1 h. As the un-dissolved inclusion body has absorbance at 300–500 nm and dissolved protein has not, the solubility of the protein in the solutions was determined by adding 10 (l solution in the well of the 96-well microplate and detecting in the Bio-Rad Microplate Reader 680 (Bio-Rad Laboratories, Hercules, CA, USA) at 405 nm, based on the method developed by Vincentelli et al. (2004). The protein stability and degradation of the nattokinase inclusion body were analyzed by SDS-PAGE within 24 h.

2.4. Dilution refolding of nattokinase inclusion body

Pre-screening of potential refolding solution (RS) was performed using the dilution refolding method (Basu et al., 2011). A series of RSs were designed (Table 1). 10 μL inclusion body protein dissolved in pH 12 water (10 mg/mL) were added into 190 μL RSs and mixed by pipetting the solution up and down. After mixing for 1 h, the absorbance of the solutions was analyzed in the microplate reader at 405 nm (Vincentelli et al., 2004). As active nattokinase is water soluble, only the RSs that can keep the protein fully dissolved after dilution were selected as potential effective RSs and were used for further refolding experiments.

Two protein folding methods were used to refolding the recombinant protein, the dilution refolding method and the direct mixing

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