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Construction of a genetically engineered strain of nattokinase and assessment of its fibrinolytic activity

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A strain of *Bacillus natto* was isolated from natto and found to have a high yield of nattokinase. The aprN and pro-aprN gene fragments, encoding nattokinase from *Bacillus natto*, were amplified using two pair of primers and were expressed in *Bacillus subtilis* WB800N host cells using the pHT43 plasmid as a vector. In this system, the effect of leader peptides on nattokinase activity, revealing that these leader peptides mediate the folding function of nattokinase was explored. After optimizing the expression conditions (1 mmol/L IPTG inducer at 37°C, pH 7.5, cell culture OD_{600} of 0.6), maximal nattokinase enzymatic activity of 848.52 IU/mL after induction of fermentation for 4 h, was achieved, at which time maximal extracellular protein had been produced. The fermentation medium of the engineering strain was optimized, and purified nattokinase via salt precipitation and ultrafiltration was isolated. Relative to fermentation supernatants, the purification ratio of nattokinase reached 6.63, with a total recovery of 80%, and a specific enzyme activity of 11507.92 IU/mg. These results indicate that the nattokinase overexpression using the pHT43 vector in WB800N cells is an effective means of achieving efficient nattokinase production, and the engineered strains constructed herein have great promise as potential industrial strains for nattokinase production.

Key words: Nattokinase, *aprN* gene, *Bacillus subtilis* WB800N, Fibrinolytic activity, engineered strain, fermentation.

INTRODUCTION

Sumi et al. (1987) first demonstrated the presence of a strong fibrinolytic enzyme in natto which they named nattokinase (NK) (Sumi et al., 1987; Sumi et al., 1988). Subsequently, researchers from various countries have conducted research on nattokinase, determining that the open reading frame encoding of the nattokinase gene consists of 381 amino acids, of which the 29 N-terminal amino acids form a signal peptide, the next 77 constitutes a leader peptide, and the following 275 amino acids form a mature peptide (Nakamura et al., 1992). Nattokinase is an alkaline protease with a molecular weight of about 27.7 kDa and the protein isoelectric point（PI）of 8.6 (Fujita et al., 1993).

In both animal and human studies, NK has exhibited significant and potent fibrinolytic and antithrombotic activity (Fujita et al., 1995; Nagata et al., 2017; Chen et

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al., 2018). NK not only directly dissolves fibrin, but also promotes the release of tissue-type plasminogen activator (tPA) from cells and catalyzes the release of prourokinase from the liver, thereby increasing the formation of plasminogen (Dabbagh et al., 2014). In clinical trials, oral NK increased tPA, significantly reduced ELT, and significantly reduced levels of factor VII and factor VIII in human blood, suggesting that NK can be used as a fibrinolytic/anticoagulant agent with the potential to reduce the risk of thrombosis and CVD in humans (Sumi et al., 1990; Hsia et al., 2009; Kurosawa et al., 2015). In addition, NK has been found to possess antihypertensive (Lee et al., 2015), anti-atherosclerotic, lipid-lowering, anticoagulant (Park et al., 2012) and neuroprotective effects (Metkar et al., 2017). Recent toxicological studies (both *in vivo* and *in vitro*) provide strong evidence for the safety of NK oral consumption (Lampe et al., 2016). Therefore, studies have shown that NK is safe, economical, has a long half-life, is easy to produce in large quantities, and has a variety of beneficial cardiovascular effects, making it a feasible drug for the treatment of cardiovascular diseases (Dabbagh et al., 2014; Ren et al., 2017).

To enhance nattokinase activity and simplify downstream operations, heterologous expression of nattokinase has been studied in several microbial expression systems, including in *Escherichia coli*, Pichia pastoris, *Bacillus subtilis* and *Lactococcus lactis* (Wei et al., 2015; Wu et al., 2011). Nattokinase achieves high levels of expression in *E. coli* model systems, but most recombinant proteins in them are in the form of inactive inclusion bodies (Ni et al., 2016). Although active nattokinase can be expressed in recombinant *P. pastoris* and *L. lactis*, expression is relatively low and product purification is difficult (Dabbagh et al., 2014). As a Gram-positive bacterium, *B. subtilis* is an attractive host for the production of heterologous secreted proteins, as it is non-pathogenic and capable of secreting functional extracellular proteins directly into the culture medium (Nishito et al., 2010).

In this study, we used the pHT43 shuttle plasmid to construct two genetically engineered strains of *B. subtilis* WB800N cells, and we then optimized the expression conditions of these genetically engineered strains. Finally, we established optimal protocols for the downstream separation and purification of NK through salt precipitation and ultrafiltration.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents

B. *subtilis* natto and *E. coli* DH5α are laboratory-preserved strains. *B. subtilis* WB800N and the pHT43 vector were purchased from Wuhan Miaoling Biotech. All enzymes used for DNA manipulations were purchased from Takara (Dalian). Isopropyl-β-D thiogalactopyranoside (IPTG) was obtained from Bio Basic Inc. Urokinase standard (1240 IU) was purchased from Beijing Zhongke Quality Biotech. Fibrinogen and thrombin were purchased from Shanghai Yuanye Biotech.

Amplification of the nattokinase gene

The reference nattokinase gene sequence from *B. subtilis* YF038 (GenBank accession number AY219901) was used to design specific primers (Table 1) using the biological software Clone Manager, and these primers were synthesized by Sangon Biotech (Shanghai). PCR amplification of the gene sequence was carried out using genomic DNA from *B. subtilis* natto as a template. The optimized PCR program for amplification of the gene was as follows: denaturation at 94°C for 5 min, then 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension for 5 min at 72°C.

Construction of engineered bacterial strains

The amplified aprN (containing only the mature NK peptide sequence), pro-aprN (containing both the leader and mature NK peptide sequence) gene products and the pHT43 plasmid were digested using BamH I and Sma I, and were then ligated using T4 DNA ligase. The ligated pHT43-aprN and pHT43-pro-aprN plasmids were then transformed individually into *E. coli* DH5a cells and propagated in the medium supplemented with 100 μg/mL ampicillin (Tu et al., 2016; Zhou et al., 2018). The recombinant plasmid DNA of pHT43-aprN or pHT43-pro-aprN from single colonies was extracted and confirmed by restriction enzyme digestion and sequencing. The DNA sequences of these pHT43-aprN and pHT43-pro-aprN inserts were analysed by Sangon Biotech (Shanghai).

B. subtilis WB800N cells prepared from glycerol stocks were next streaked onto neomycin-containing agar plates and incubated overnight at 37°C. Competent WB800N cells were then prepared as previously described by Chityala et al. (2015), and the sequence-verified recombinant pHT43-aprN or pHT43-pro-aprN plasmids were introduced into the cells via electroporation (Vojcic et al., 2012). These electrotransformed cells were quickly added into 500 μL of resuscitation medium and incubated for 40 min. These cells were then added to fresh sterile Luria–Bertani medium (LB) (1 mL) and incubated for another 45 min. Cells were the centrifuged at 3000 rpm for 2 min, and the cell pellets were re-suspended in 100 μL LB medium and spread over LB agar plates containing chloramphenicol, which were then incubated at 37°C overnight. The transformed colonies were further selected using a dual-antibiotic plate. The recombinant plasmid DNA from single colonies was then extracted and confirmed by PCR amplification. The verified engineered strains, named WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN, were then stored in glycerol at -80°C.

Induced expression of engineered nattokinase

Single colonies of WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN were inoculated in 25 mL LB containing chloramphenicol (5 μg/mL) and incubated at 37°C, with 200 rpm shanking overnight. The pH was maintained at a fixed value of 7.5 during subsequent culturing. Fresh LB media (25 mL) was inoculated with this overnight culture as a 2% inoculum, and was then incubated at 37°C with 200 rpm shaking. NK expression in these cells was induced using 0.1 mM IPTG after the cell density reached 0.6–0.8 OD_{600} (optical density at 600 nm), and cells were then incubated at 30°C for 24 h. Samples were then collected and centrifuged at 8000 rpm for 2 min at 4°C, and supernatants were stored at 4°C for subsequent SDS-PAGE analysis and determination of NK enzymatic activity.

Table 1. Primers used for PCR amplification.

Primer	Sequence (5' to 3')	Restriction site
aprN-F	CGGGATCCATGGCGCAATCTGTTCCTTATGG	BamH I
aprN-R	TCCCCCGGGTTATTGTGCAGCTGCTTGTA	Sma I
pro-aprN-F	CGGGATCCATGGCCGGAAAAAGCAGTACAGA	BamH I
pro-aprN-R	TCCCCCGGGTTATTGTGCAGCTGCTTGTA	Sma I

Determination of the molecular weight of engineered nattokinase

SDS-PAGE was performed as previously described (Laemmli et al., 1970). To evaluate the expression levels of NK in samples, the same amount of supernatant from each culture was taken to prepare identically diluted loading samples, and these samples were run on a gel along with a molecular weight ladder. The molecular weight of engineered NK proteins was then determined with a standard protein ladder, and the expression levels of this protein were semi-quantitatively measured.

Fibrinolytic activity assay

Engineered NK enzymatic activity was evaluated using the fibrin plate method as previously described (Man et al., 2018) with some minor adjustments. Urokinase, thrombin, agarose, and fibrinogen solutions were all prepared in 10 mmol/L phosphate buffer (pH 7.5). 5 mL agarose solution (10 g/L), 5 ml fibrinogen solution (2.2 g/L) and 100 µL (10 IU) thrombin were all mixed in a 50 mL Erlenmeyer flask, and then poured into sterile petri dishes. The solution in the plate was left undisturbed for 1 h to form fibrin clots, and then 2 mm diameter wells were made in the plate to allow for sample application. 1 mL of fermentation broth was centrifuged at 8000 r/min for 2 min at 4°C, and the supernatant was stored at 4°C for analysis of enzymatic activity. To observe fibrinolytic activity, 4 µL of this sample solution was carefully dropped into each well, plates were incubated at 37°C for 18 h, and the diameter of the fibril transparent circle was then measured. Eight different concentrations of a urokinase standard solution were diluted to prepare a nattokinase activity standard curve, allowing for the quantification of the NK fibrinolytic activity.

Optimizing fermentation parameters for engineered nattokinase expression

A test of fermentation parameters was performed in order to maximize engineered nattokinase production. Test parameters, which were modulated one by one, included: culture temperatures (18, 25, 30, 37, and 40°C), media pH (4, 5, 6, 7, 7.5, 8, 9, 10, 11, and 12), IPTG concentrations for induction of NK expression (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mmol/L) and cell growth stage at time of IPTG induction (cell culture OD_{600} of 0.2, 0.6, 1.0, 1.4, and 1.8). There was one control sample and three parallel replicates in each group of experiments. Each sample was measured 3 times on average, and the average value was analyzed as statistical data.

Isolation and purification of nattokinase

The genetically engineered seed solution was inoculated with 3% inoculum into 100 mL of optimized fermentation media (containing 26.05 g/L peptone, 29.29 g/L glucose, 1.5 g/L $MgSO_4$, 0.74 g/L $CaCl_2$, 1.5 g / L KH_2PO_4, 10 g / L NaCl, pH 7.2 ~ 7.5), in a 200 r / min, 37°C shaker and grown to a cell density of OD_{600nm} = 0.6, after which 1 mM IPTG was added to induce fermentation for 4 h. After fermentation, samples were spun at 8000 r/min for 10min to collect the fermentation supernatant. Ammonium sulfate was added to the supernatant to 30%, and samples were allowed to stand at 4°C overnight, followed by centrifugation at 12000 r / min for 20 min to remove the supernatant. Ammonium sulfate was then added to a 60% saturation, after which it was allowed to stand at 4°C overnight, followed by spinning at 12000 r / min After 20 min, the precipitate was dissolved in 5 mL of 0.04 mol/L pH 8.0 barbital sodium-HCl buffer, and the crude enzyme solution was obtained after dialysis.

Appropriate amounts of crude enzyme solution after salting out were added to a 30 kDa ultrafiltration centrifuge tube and spun at low temperature for 20 min at 8000 r/min. The ultrafiltrate was then transferred to a 10 kDa ultrafiltration centrifuge tube and centrifuged at 8000 r/min for 20 min. The ultrafiltered solution was stored at 4 ℃ and subjected to subsequent analysis.

RESULTS

Generation of recombinant plasmids and engineered strains

Two sets of DNA fragments encoding the nattokinase gene were amplified by PCR from the genomic DNA of *B. subtilis* natto. The single bands in Figure 1 confirmed that the PCR products of aprN (828 bp in size) and pro-aprN (1059 bp in size) are of the expected size. The pHT43-aprN and pHT43-pro-aprN recombinant plasmids were then extracted from *E. coli* DH5a cells and verified by restriction enzyme digestion using BamH I and Sma I (Figure 2), and the DNA sequence of the pHT43-aprN and pHT43-pro-aprN inserts were analyzed. The results revealed that the DNA sequence of cloned genes were identical to that of nattokinase from the precursor *B. subtilis* YF038 strain (GenBank accession number AY219901), confirming successful construct generation.

Engineered nattokinase expression and assessment of fibrinolytic activity

As the cloned gene was under the control of lac operon, which is induced by IPTG, induction of engineered nattokinase expression was evaluated via the supplementation of 0.1 mM IPTG in the media of the transformed *B. subtilis* WB800N cells. The IPTG was added to the fermentation broth when the culture OD_{600}

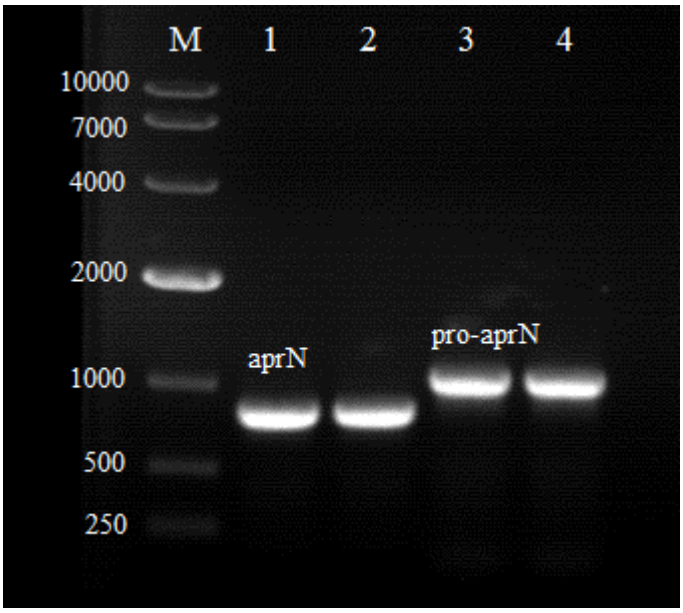

Figure 1. Amplification of the nattokinase gene by PCR from the genomic DNA of *B.subtilis* natto. Lane M, DNA maker 10000; Lane 1-2, aprN fragment of the nattokinase gene; Lane 3-4, pro-aprN fragment of the nattokinase gene.

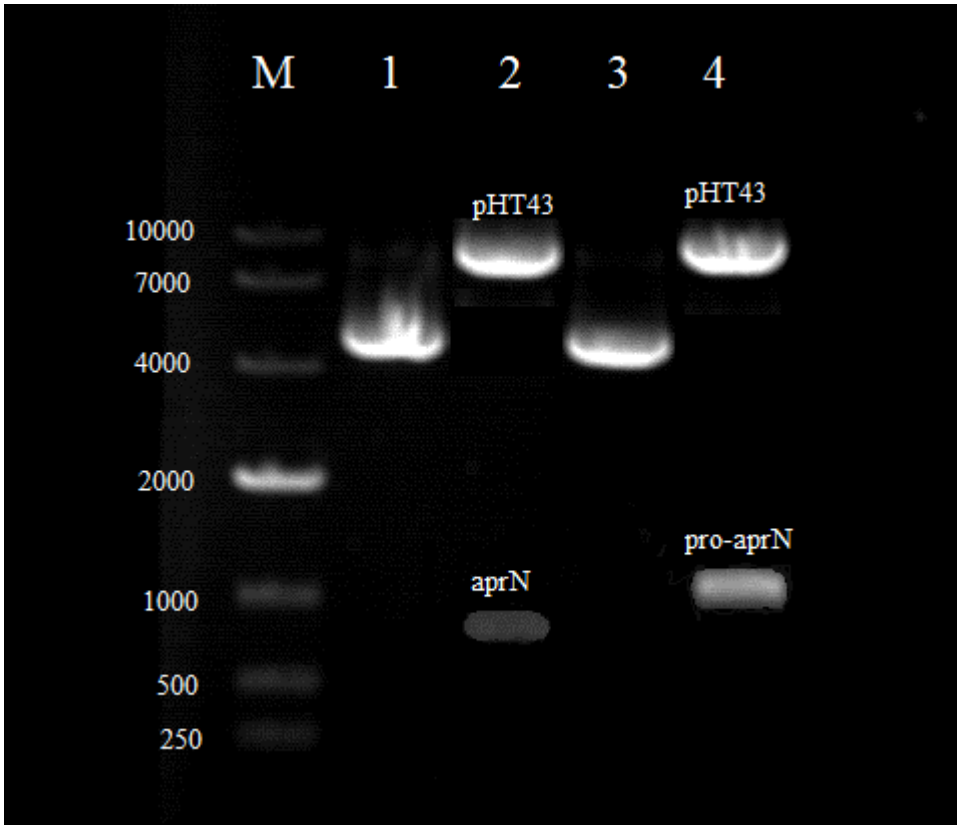

Figure 2. Verification of the recombinant pHT43-aprN and pHT43-pro-aprN plasmids by restriction enzyme digestion with BamH I and Sma I. Lane M, DNA maker 10000; Lane 1, intact pHT43-aprN; Lane 2, digested pHT43-aprN; Lane 3, intact pHT43-pro-aprN; Lane 4, digested pHT43-pro-aprN.

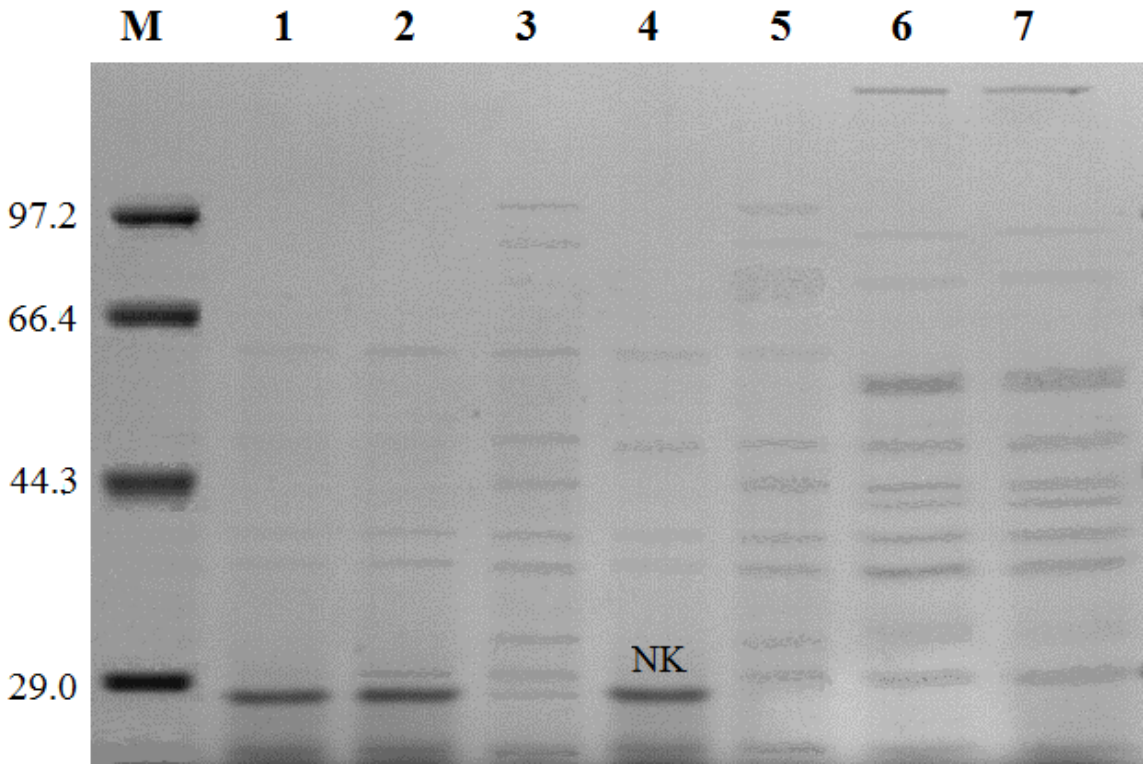

Figure 3. SDS-PAGE revealing that a 28 kDa protein for in the *B.subtilis* WB800N cells engineered to express nattokinase. Lane M, protein molecular weight standards; Lane 1-2, IPTG-induced WB800N/pHT43-pro-aprN; Lane 3, Non-induced WB800N/pHT43-pro-aprN; Lane 4, IPTG-induced WB800N/pHT43-aprN; Lane 5, Non-induced WB800N/pHT43-aprN; Lane 6, IPTG-induced *B.subtilis* WB800N/pHT43; Lane 7, Non-induced *B.subtilis* WB800N/pHT43.

reached 0.6 to 0.8. Extracellular protein produced at 30°C within 24 h post-induction was then collected for SDS-PAGE and for a fibrinolytic activity assay, with *B. subtilis* WB800N cells transformed with an empty pHT43 vector as a control.

The presence of engineered nattokinase protein in culture supernatants was first verified by SDS-PAGE analysis. A major protein band of approximately 28 kDa, similar to the molecular mass of nattokinase from *B. subtilis* natto, was observed in the pHT43-aprN and pHT43-pro-aprN samples, while no corresponding band was observed in the pHT43 samples (Figure 3), indicating that both the WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN strains successfully expressed nattokinase.

Fibrinolytic activity of the engineered nattokinase protein was next detected via a fibrin plate-based method. Samples from the aprN-expressing cells did exhibit any enzymatic activity, while those from the pro-aprN-expressing cells exhibited robust activity, indicating that the WB800N/pHT43-pro-aprN strain successfully expressed active nattokinase (Figure 4). The apparent difference in enzymatic activity of the nattokinase expressed by the WB800N/pHT43-aprN and WB800N/pHT43-pro-aprN strains confirmed that the leader peptide sequence of nattokinase may direct the proper folding of the protein in order to facilitate normal enzymatic activity.

The effect of IPTG concentrations on nattokinase activity

IPTG was added to the fermentation flask when the WB800N/pHT43-pro-aprN cell density reached 0.6-0.8 OD_{600}. No effects of different IPTG concentrations on cells growth within the first 26 h of fermentation at 30°C were observed (Figure 5a). An evaluation of enzymatic activity 24 h post-induction with IPTG revealed that increasing of the IPTG concentration up to 1.0 mM improved the engineered nattokinase production, which peaked at 701.59 IU/mL at an IPTG concentration of 1.0 mM (Figure 5b). These data are similar to a previous report in which 1.0 mM of IPTG was found to be optimal for inducing the expression of recombinant enzymes, such as procerain B and the antimicrobial peptide cathelicidin-BF (Nandana et al., 2014). Further increases in IPTG concentrations to 1.6 mM resulted in a decrease of NK expression to a level of 504.82 IU/mL (Figure 5b).

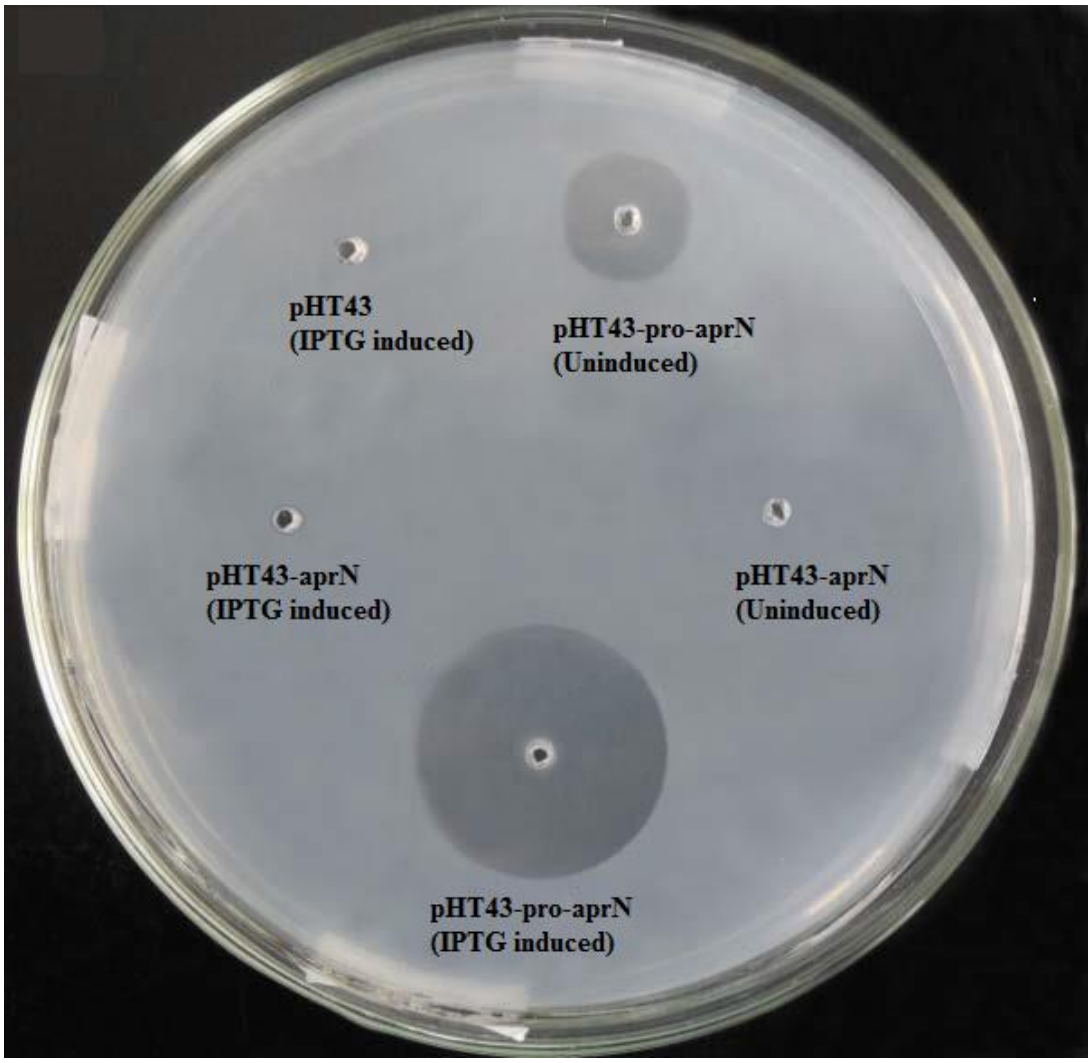

Figure 4. Engineered nattokinase enzymatic activity was evaluated using the fibrin plate method in strains induced with 0.1 mM IPTG at 30°C for 24 h, using *B. subtilis* WB800N cells transformed with an emply pHT43 vector as a control. The right one of each pair of samples was induced using IPTG, while the other was not.

This reduction of enzyme yield at higher concentrations of IPTG may be attributable to an IPTG-induced metabolic burden, as previously suggested (Glick et al., 1995). Examination of the supernatants from these samples by semi-quantitative SDS-PAGE further confirmed that the optimal IPTG concentration was 1.0 mM (Figure 5c).

Effect of temperature on nattokinase activity

The yield of NK from the WB800N/pHT43-pro-aprN cells was investigated at a range of different incubation temperatures from 18 to 40°C. The engineered nattokinase production at these temperatures ranged from 46.78-848.52 IU/mL. It was found that enzyme yield rose with increases in incubation temperature, up to a maximal enzyme production (848.52 IU/mL) that was achieved at 37°C. Further increases in temperature up to 40°C decreased the enzyme production to a level of 691.23 IU/mL (Figure 6a). These results were consistent with a previous finding that 37°C is an optimal temperature for the production of nattokinase from *B. subtilis* WB800 (Nguyen et al., 2013). In addition, different temperatures have a significant effect on cell growth, and consistent with this, it was found that cells grew more slowly at temperatures below 25°C (Figure 6b).

The kinetics of nattokinase production

In order to investigate the kinetics of NK production in

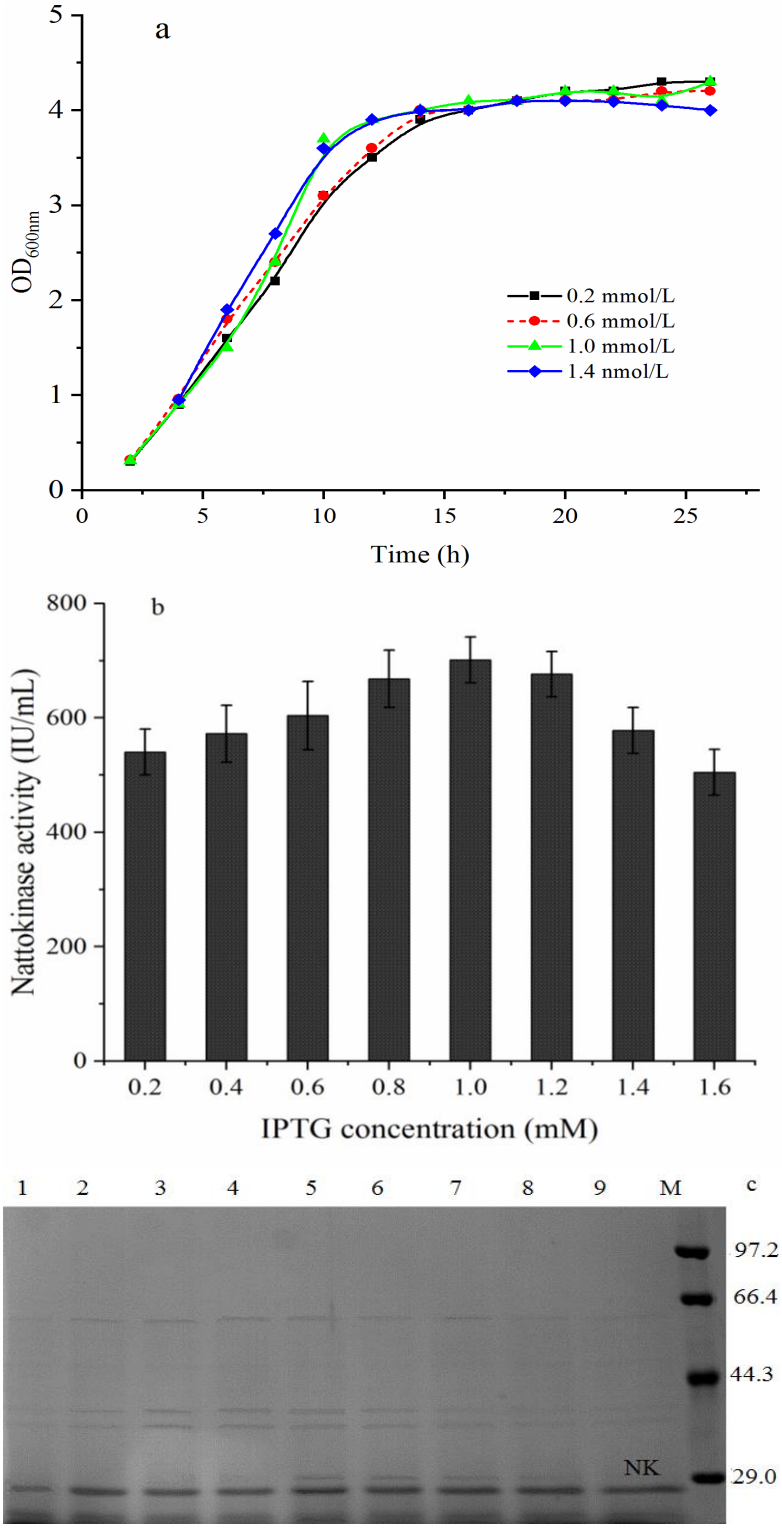

Figure 5. The effect of different IPTG concentrations on WB800N/pHT43-pro-aprN cell growth (a), on nattokinase activity in WB800N/pHT43-pro-aprN cells induced with IPTG for 24 h (b), and on nattokinase production (analyzed by semi-quantitative SDS-PAGE) in WB800N/pHT43-pro-aprN cells induced with IPTG for 24 h (c). All samples were cultured at 30°C.. Lane M, protein molecular weight standards; Lane 1-9 , cell samples induced with IPTG concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mM in that order.

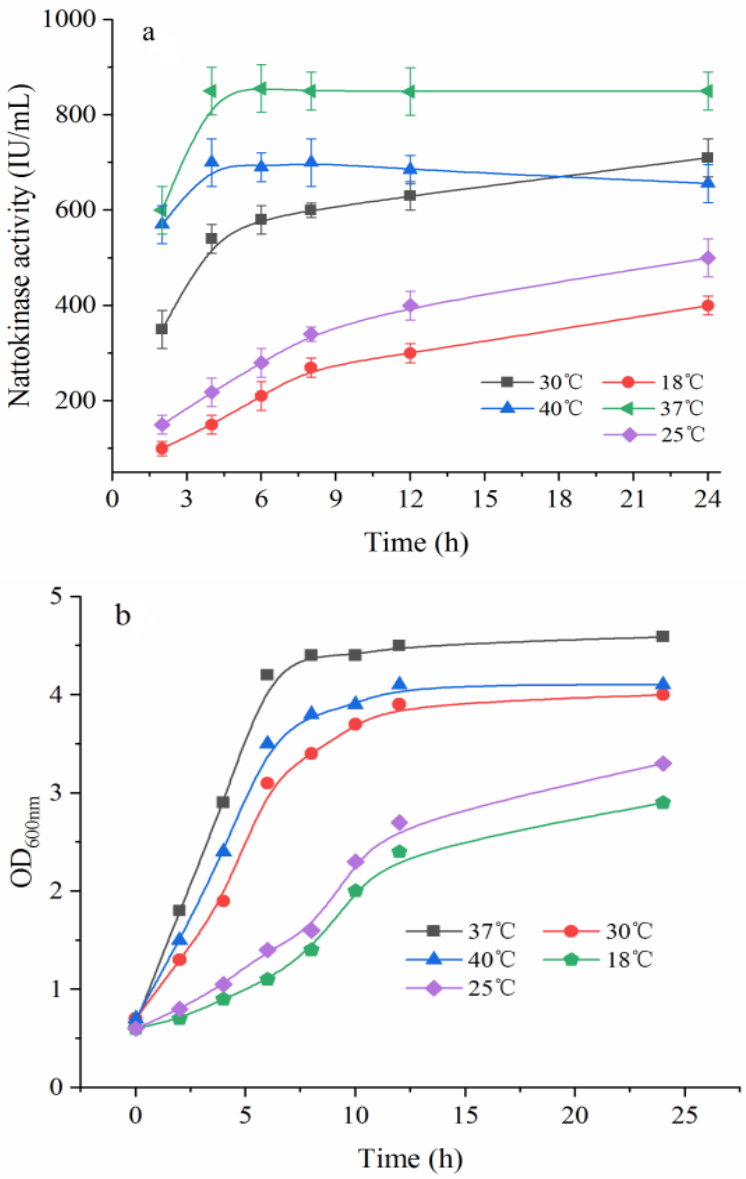

Figure 6. Effect of temperature on nattokinase activity changes (a) and on cell growth dynamics (b) of WB800N/pHT43-pro-aprN cells within 8 h after induction with IPTG.

WB800N/pHT43-pro-aprN cells, changes in the NK activity over the course of fermentation time were assessed. As shown in Figure 7a, NK activity increased rapidly with fermentation time within the first 4 h after IPTG induction before plateauing for the next 4 h. At 4 h post induction with an IPTG concentration of 1.0 mM, the NK expression level (as quantified based on enzyme activity per unit of biomass) reached a maximum of 835.83 IU/mL. Extracellular protein from different fermentation time points by semi-quantitative SDS-PAGE analysis, maximal NK production within 4 h of induction was further assessed (Figure 7b). Further examination of kinetics data revealed that NK production was associated with cell growth only during this first 4 h after induction, and as such these cultures could be harvested before entering into the stationary phase of growth while still ensuring maximal NK production.

Effect of growth phase at time of induction on nattokinase activity

To test whether the cell growth phase at time of IPTG induction has any effect on engineered nattokinase activity

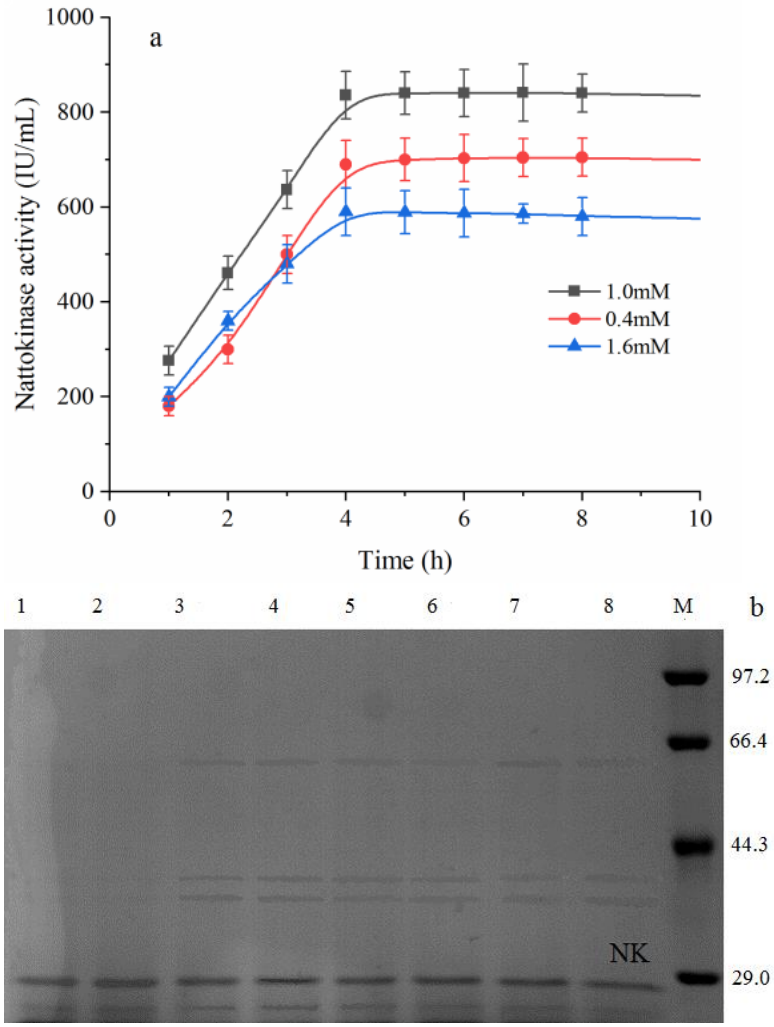

Figure 7. (a) Changes in nattokinase activity/production at 37°C in WB800N/pHT43-pro-aprN cells over a fermentation period of 8 h following 1.0 mmol/L IPTG induction by IPTG, with a corresponding growth curve. **(b)** Semi-quantitative SDS-PAGE analysis of nattokinase production at 37 ℃ in WB800N/pHT43-pro-aprN cells at different fermentation time points after induction with 1.0 mmol/L IPTG. Lane M, protein molecular weight standards; Lane 1-8, cell samples from 1 to 8 h post-induction at 1 h intervals.

in WB800N/pHT43-pro-aprN cells, IPTG induction was carried out at different cell densities reflecting different cell growth phases. Initially, 2% of the seed solution culture was inoculated into fresh media, and then IPTG (1.0 mM) was added at selected cell densities (0.2–1.8 OD_{600}) followed by measurement of enzyme expression 4 h post-induction. When the IPTG induction was carried out from a lower cell density phase to a higher one, the expression of the engineered protein increased, achieving a maximal induction of 835.39 IU/mL at a 0.6 OD_{600}, after which the yield decreased (Figure 8). This finding indicated that early in the logarithmic phase of cell growth, the addition of IPTG can promote the expression of NK, while later in the logarithmic phase of growth IPTG-induced expression gradually decreased. These data indicated that the optimal cell growth phase for IPTG induction of nattokinase expression in these engineered strains is early in the logarithmic phase of cell growth, consistent with a report regarding the production of the antimicrobial peptide cathelicidin-BF expressed in engineered *B. subtilis* WB800N cells (Luan et al., 2014).

Effect of media pH on enzyme activity

The optimal pH for nattokinase expression in WB800N/pHT43-pro-aprN cells was determined to be 7.5, and at this pH the NK enzyme activity reached a

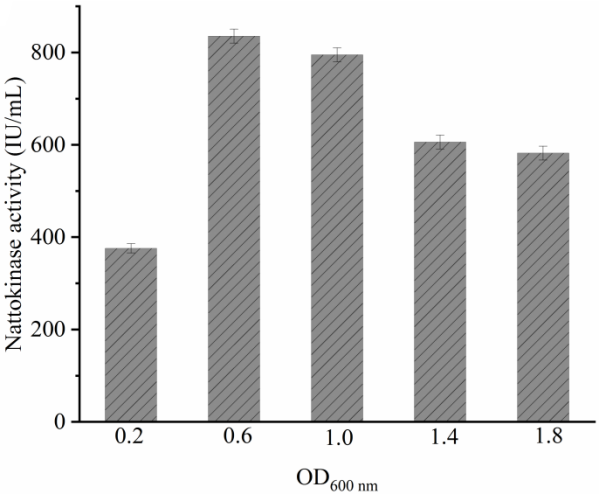

Figure 8. Effect of cell growth stage at time of induction (as determined by OD_{600}) on nattokinase activity in WB800N/pHT43-pro-aprN cells induced with IPTG for 4 h at 37°C.

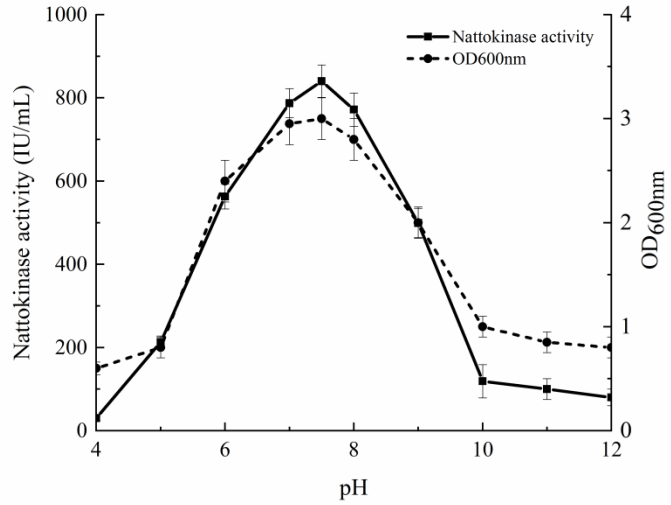

Figure 9. Effect of media pH on nattokinase activity change (solid line) and on cell growth curve (dotted line) of WB800N/pHT43-pro-aprN cells induced with IPTG for 4 h at 37°C.

maximum of 839.17 IU/mL. When the pH was less than 6 or greater than 9, the cell density and the NK enzyme activity decreased dramatically (Figure 9).

Isolation and purification of nattokinase

Compared with the fermentation supernatant, the purification ratio of nattokinase reached 6.63 and the total recovery was 80% (Table 2). The heteroproteins in the fermentation broth were initially removed via salt precipitation, and then nattokinase was isolated and purified via ultrafiltration. An SDS-PAGE analysis revealed that the molecular weight of a single band was about 28 kDa. The purified nattokinase was detected via fibrinolytic plate assay and the results showed good fibrinolytic activity. As such, nattokinase with a high degree of recovery can be obtained via salt precipitation and ultrafiltration.

DISCUSSION

In this study, aprN and pro-aprN encoding nattokinase in

Table 2. Separation and purification results of nattokinase.

Purification step	Protein/mg	Total activity/IU	Specific activity (IU/mg)	Degree of purification	Recovery rate
Fermentation liquid	122.95	213472	1736.25	-	100
Precipitation with ammonium sulfate	56.17	201859.12	3593.72	2.07	94.56
Ultrafiltration	14.84	170777.6	11507.92	6.63	80

Bacillus natto using novel primers was amplified. Two engineered cell lines using the pHT43 plasmid vector, which was expressed in *Bacillus subtilis* was constructed WB800N allowing us to assess enzymatic activity and optimize culture conditions. Although the leader peptide is not part of the functional domain of the NK protein and does not contribute to protein function, it does contribute to the formation of an active 3D structure (Jia et al., 2010). Yabuta et al. (2001) studied the refolding pathway of *B. subtilis* protease E *in vitro*, demonstrating that the leader peptide confers correct folding information for the domain during the folding process. Studies have also shown that specific interactions between the leader peptide and the *B. subtilis* protease domain are important for precursor folding (Weng et al, 2009; Sone et al, 2005). As nattokinase is a serine protease produced by *B. subtilis*, these studies further provide insights into NK folding. In this study, engineered strains expressing NK leader peptide sequences yielded NK protein with high enzymatic activity, while engineered strains expressing only the mature peptide without the leader sequence produced inactive NK protein, consistent with previous studies on the importance of leader peptides.

Man et al. (2018) constructed a *B. subtilis* MX-6 strain expressing nattokinase to maximize nattokinase production 72 h after induction of fermentation, achieving a clear zone diameter on the plasminogen-free fibrin plate of 21.60 mm. Guan et al. (2016) constructed a *B. subtilis* strain expressing nattokinase by optimizing the gene promoter, achieving maximal nattokinase production 36 h after induction of fermentation. Several studies have found that nattokinase production can reach a maximum after 24 h of fermentation. Unlike in these previous studies, in the present study the engineered bacterial strains which we produced achieved maximal nattokinase production just 4 h after induction and initiation of fermentation, with a maximum crude enzymatic activity as high as 848.52 IU/mL, representing a clear improvement in industrialization efficiency. SDS-PAGE based analyses indicated that our engineered strains also produce relatively low levels of heteroprotein species, which will reduce the need for downstream purification processing and will therefore reduce the cost of industrial NK production. We were able to determine that optimal culture conditions for NK production with this bacterial expression system were as follows: media pH at 7.5, 1 mmol/L IPTG induction for 4 h, and fermentation at 37°C, achieving a maximum crude enzyme activity yield of 848.52 IU/mL. These results indicate that overexpression of the pHT43 vector may be an effective and useful strategy for NK production, while the host strain WB800N demonstrates high efficiency and high purity of NK secretion.

In follow-up experiments, the fermentation medium of the engineering strain and isolated and purified nattokinase via salt precipitation and ultrafiltration was optimized. Compared with fermentation supernatants, the purification ratio of nattokinase reached 6.63, with a total recovery of 80%, and a specific enzyme activity of 11507.92 IU/mg. In summary, the engineered strains constructed in this study have great promise as potential strains useful for industrial-scale NK production. Further efforts to optimize and improve NK production are ongoing, with the goal of further improving protein yields and lowering the cost of the industrial production of nattokinase.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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