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Expression, fermentation, purification and lyophilisation of recombinant Subtilisin QK in *Pichia pastoris*

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ABSTRACT

Subtilisin QK (QK), which is highly homologous to nattokinase (NK), has comparatively high thrombolytic activity on both heated plasma and fibrin plate. The aim of this study was to produce QK in *Pichia pastoris* (*P. pastoris*) and establish improved techniques for its fermentation, purification and lyophilisation. QK was expressed as a secretory protein by *P. pastoris*; the thrombolytic activity of QK reached 112,000 IU (urokinase unit) per mL and the specific activity was 14,679 IU/mg under optimal culture and fermentation conditions. After purification by hydrophobic interaction chromatography (HIC) and gel filtration chromatography (GFC), 91.73% of the thrombolytic activity of recombinant QK was recovered, the specific activity reached 16,272 IU/mg, and the purity of QK was 95%. Various agents were evaluated for their protective effects during freeze-drying at −50 °C. Trehalose and skim milk powder showed the highest lyoprotective effects. This study provides useful data regarding the potential use of QK in the treatment of cardiovascular diseases.

1. Introduction

Microbial thrombolytic enzymes have recently attracted great attention for their potential in thrombolytic therapy. Subtilisin QK (QK), produced by *Bacillus subtilis QK02*, was identified by screening of fermented soybeans using the fibrin plate method. QK has comparatively high thrombolytic activity against both heated plasma and fibrin plates and is highly homologous to nattokinase (NK) [1]. Subtilisin QK is composed of 274 amino acids (MW = 27.8 kDa) and very efficiently degrades directly crosslinked fibrin *in vitro*, according to the SDS-PAGE and fibrin plate methods. Subtilisin QK also shows strong thrombolytic ability in mice, and its effect is better than the clinically used lumbrokinase [2]. In addition, Subtilisin QK can inhibit tyrosine nitration induced by nitrite, hydrogen peroxide, and haemoglobin *in vitro* and *in vivo* and protects human umbilical vein endothelial cells (ECV-304) from damage caused by nitrite and hydrogen peroxide [3]. Therefore, QK is an effective thrombolytic agent with great exploitable potential, similar to nattokinase.

However, the low yield and difficulty of purifying QK from the fermentation of the natural *B. subtilis* QK02 limits its further investigation and application. Many attempts have been made to improve production of the enzyme and simplify its downstream manipulation [4,5]; however, its production remains low. Thus, heterologous expression of QK is required. *Pichia pastoris* is an excellent host for expressing heterologous proteins [6,7]. Compared to *Escherichia coli* expression systems, in which the target protein being expressed is typically inactive in inclusion bodies [8], the proteins expressed in *P. pastoris* are typically biologically active molecules and are secreted into the supernatant. *Pichia pastoris* expression systems are also generally regarded as being fast, simple, and inexpensive.

Freeze-drying (FD) is widely used in the pharmaceutical industry for the manufacture of protein drugs [9]. To preserve the activity and structure of proteins, addition of lyoprotectants during FD process is required. Sugars are widely used as lyoprotectants during FD [10].

In this study, the sequences of QK genes were optimized and QK protein was expressed in *P. pastoris* GS115. Fermentation, mass purification and FD were evaluated.

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2. Experiments

2.1. Strains, plasmids, enzymes

The *P. pastoris* strain GS115 (His4-deficient) and the expression vector pPICZαA were purchased from Invitrogen (Carlsbad, CA, USA). *Escherichia coli* strain DH5α was stored in our lab. Enzymes were purchased from Takara Bio Inc. (Shiga, Japan). *Escherichia coli* cells were cultured at 37 °C in Luria–Bertani medium (yeast extract, 5 g/L; tryptone, 10 g/L; NaCl, 10 g/L; agar, 15 g/L) containing 25 μg/mL Zeocin (Invitrogen).

2.2. Codon optimization and construction of pPICZαA–QK expressing strains

The codon-optimized gene was designed based on the protein sequence of QK (GenBank accession number: AJ579472.2) according to the codon bias of *P. pastoris* (http://www.kazusa.or.jp/codon). Codon optimization was performed using the online software DNAWORKS. Codon-optimized QK was synthesized by Wuhan Qinke Co., Ltd. (Wuhan, China). The synthetic gene was ligated into the pUC57 plasmid to produce pUC57–QK. The QK gene fragment was released from the pUC57–QK vector by digestion with EcoRI/XbaI and then cloned into the *P. pastoris* expression vector pPICZαA, along with the open reading frame of the α-factor signal under control of the AOX1 promoter. The recombinant pPICZαA vector was transformed into competent *E. coli* DH5α cells. Positive clones containing pPICZαA–QK were screened using Zeocin as the selection agent and identified by restriction analysis and sequencing. The recombinant vector pPICZαA–QK was linearized with SacI and electroporated into competent *P. pastoris* GS115 cells. pPICZαA-QK-positive transformants were screened on YPD plates containing 0.1 mg/mL Zeocin, and the presence of the QK insert in these clones was further confirmed by PCR using the 5′AOX1 primer, 5′-CAGGCTGCCGGAAAAAGCA-3 and the 3′AOX1 primer, 5′-TTGTGCAGCTGCTTGTACGT-3′.

2.3. Expression of recombinant protein

Positive transformants were grown in 10 mL YPD medium overnight and sub-cultured into 50 mL BMGY (1%yeast extract, 2% peptone, 1.34% yeast nitrogen base, 1% glycerol, 4×10^{-5}% biotin, and 100 mM potassium phosphate, pH 6.0) at 30 °C with constant shaking (300 rpm) until the culture reached an OD_{600} = 6.0. The yeast cultures were harvested by centrifugation at $3000 \times g$ for 5 min and resuspended to an OD_{600} of 1.0 in 50 mL BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.5% methanol, 4×10^{-5}% biotin, and 100 mM potassium phosphate, pH 6.0) in 500-mL flasks. The flasks were incubated at 30 °C and 300 rpm. Methanol (100%) was added to the culture to a final concentration of 0.5% (v/v) every 24 h. A negative control containing the expression vector pPICZαA without the QK gene insert was used. After 72 h of induction, the culture supernatants and cell lysates were collected and subjected to SDS-PAEG (5% stacking gel and 12% separating gel).

2.4. Thrombolytic activity assay

The thrombolytic activity of QK was assayed by the standard fibrin plate method using urokinase [1]. A total of 50 μL of samples at 10-fold dilution was added to each well. The plate was incubated for 18 h at 37 °C and thrombolytic activity was determined based on the standard curve of urokinase.

2.5. Optimization of QK recombinant strain cultivation in shaking flasks

To prepare for fermentation, the parameters for QK recombinant strain cultivation in shaking flasks were evaluated. Based on the initial cultivation conditions (pH 5.5, 28 °C induction temperature, culture medium volume 100 mL in 1-L flasks, 10% inoculum size, and 0.5% methanol) in shaking flasks, each parameter was evaluated individually and only one parameter was changed during each test. The parameters in the methanol induction phase including initial pH (pH 4.5–7.0), temperature (24, 26, 28, 30 and 32 °C), initial culture medium volume (50–250 mL in 1-L shaking flask), inoculum size (5–15%), and methanol concentration (0.5–2%) were studied to evaluate their effects on the thrombolytic activity of recombinant QK. After 72 h of induction, thrombolytic activity of recombinant QK in the culture supernatants was analyzed. Each experiment was performed in triplicate three times. The results are shown as the mean ± SD from three independent experiments.

2.6. Fermentation

Fermentation was performed in a 7-L fermenter (Baoxing, Shanghai, China). Fermentation with 3 L of fermentation medium [to the basal salt medium BSM 5 g/L KH_2PO_4, 1 g/L $CaSO_4{\cdot}2H_2O$, 10 g/L K_2SO_4, 10 g/L $MgSO_4$, 40 g/L $NH_4H_2PO_4$, 1.5 g/L KOH, 40 g/L glycerin, 1.25 mL/L antifoaming agent, 4.35 mL/L sterile-filtered trace element solution PTM1 (0.6 g/L $CuSO_4{\cdot}5H_2O$, 0.09 g/L KI, 0.3 g/L $MnSO_4{\cdot}H_2O$, 0.2 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 20 g/L $ZnCl_2$, 65 g/L $FeSO_4{\cdot}7H_2O$, 0.2 g/L biotin, 0.5 mg/L conc·H_2SO_4)] was added after autoclaving. The temperature was maintained at 28 °C and the pH was adjusted to 5.0 by adding 28% NH_4OH. The dissolved oxygen (DO) was maintained at nearly 100%. The stirrer speed was adjusted to 280 rpm.

The fermentation inoculum of the recombinant QK from *P. pastoris* was prepared by cultivating the cells at 28 °C with shaking at 250–300 rpm for 18–24 h in a 1-L shaking flask containing 100 mL of YPD medium. Next, 10% (v/v) of the culture was inoculated into the fermenter. The fermentation process of *P. pastoris* with recombinant QK comprised three phases. During the first phase, the DO level was maintained at greater than 20%, and the cells were incubated at 28 °C and pH 5.0 until the glycerol in the medium was consumed. The expected wet cell weight (WCW) at the end of this phase was 90–150 g/L. Phase 2 (glycerol fed-batch phase) was initiated by providing feeding medium containing 50% (w/v) glycerol and 12 mL/L PTM1 solution. The glycerol was added at a rate of 45 mL/h. The added glycerol lasted over 4 h and the DO level was maintained at greater than 20%. At the end of this phase the WCW was expected to be 180–220 g/L. When the expected WCW was reached, the supply of glycerol was stopped. The DO level was increased to 100% and maintained for 40 min after the glycerol in the medium was consumed. The temperature was then adjusted to 26 °C. Phase 3 was the methanol induction phase. At the beginning of the stage, 10.8 mL/h methanol with 12 mL/L PHT1 was added and the DO level was adjusted to greater than 20%. While the DO level was constant, the supply rate of methanol was increased to 21.9 mL/h. Two hours later, the supply rate was increased to 32.7 mL/h and then maintained until the end of fermentation. The third phase required approximately 96 h and the WCW at the end was expected to be 350–450 g/L. In the third phase, 3 tubes of 2 mL fermentation liquor were collected every 12 h and analyzed for biomass, thrombolytic activity, total protein concentration, and other experiments. Each experiment was performed in triplicate three times. The results are shown as the mean ± standard deviation (SD) from three independent experiments.

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