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High-level expression of Proteinase K from *Tritirachium album Limber* in *Pichia pastoris* using multi-copy expression strains

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ABSTRACT

Proteinase K is widely used in scientific research and industries. This report was aimed to achieve high-level expression of proteinase K using *Pichia pastoris* GS115 as the host strain. The coding sequence of a variant of proteinase K that has higher activity than the wild type protein was chosen and optimized based on the codon usage preference of *P. pastoris*. The novel open reading frame was synthesized and a series of multi-copy expression vectors were constructed based on the pHBM905BDM plasmid, allowing for the tandem integration of multiple copies of the target gene into the genome of *P. pastoris* with a single recombination. These strains were used to study the correlation between the gene copy number and the expression level of proteinase K. The results of quantitative polymerase chain reaction (qPCR) indicated that the tandem expression cassettes were integrated into the host genome stably. Meanwhile, the results of qPCR and enzyme activity assays indicated that the mRNA and protein expression levels of the target gene increased as the gene copy number increased. Moreover, the effect of gene dosage on the expression level of the recombinant protein was more obvious using high-density fermentation. The maximum expression level and enzyme activity of proteinase K, which were obtained from the recombinant yeast strain bearing 5 copies of the target gene after an 84-h induction, were approximately 8.069 mg/mL and 108,295 U/mL, respectively. The recombinant proteinase was purified and characterized. The optimum pH and temperature for the activity of this protease were approximately pH 11 and 55 °C, respectively.

1. Introduction

Proteinase K (E.C.3.4.21.64), named because it can digest native keratin, is an extracellular serine endoproteinase produced by *Tritirachium album Limber* [1]. This fungus is thought to secrete the protease to hydrolyze environmental proteins which supply carbon and nitrogen for cell growth. Proteinase K belongs to the peptidase family S8 [2]. Its predominant cleavage site is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked α-amino groups [2]. Proteinase K is a broad-spectrum protease that functions at wide pH and temperature ranges. In addition, Ca^{2+} can increase its stability, while sodium dodecyl sulfate (SDS) and carbamide can increase its activity [3]. The combination of these unique features makes proteinase K an important tool in scientific research. Proteinase K can be used in the preparation of biological macromolecules by degrading the constitutive proteins of cells and removing nucleases such as DNases and RNases. For industrial purposes, proteinase K can be used as a detergent additive in the washing industry, and it can also be used to treat keratin-containing waste from the poultry and leather industries [4].

Abbreviations used: qPCR, quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; MD, minimal dextrose medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *GAP*, glyceraldehydes-3-phosphate dehydrogenase; C_t, cycle threshold; BSM, skerman's basal mineral salt medium; DO, dissolved oxygen; TCA, trichloroacetic acid; UPR, unfolded protein response.

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As the market for this enzyme is large, developing low-cost methods for the large-scale purification of proteinase K is crucial. Heterologous expression is a common method used to increase the yield of the target proteins; however, proteinase K is toxic to cells and causes cell lysis when large amounts of the active enzyme accumulate in cells. Using an *Escherichia coli* expression system, an inactive form of proteinase K was shown to accumulate in inclusion bodies, making recombinant expression possible [5]. However, this cytoplasmic localization necessitates complicated downstream renaturation and purification procedures.

In this report, we employed an expression system using *Pichia pastoris* as a host to simplify the production of proteinase K. The strong and tightly regulated *AOX*1 promoter was used to control the expression of the target protein, and a secretory expression system was used to alleviate the toxicity of proteinase K to cells and simplify the downstream purifying process. Additionally, a multi-copy expression strategy was used to elevate the yield of the target protein. Our results demonstrated that proteinase K was expressed at approximately 8.069 mg/mL (108,295 U/mL) using this system with high-density fermentation.

2. Materials and methods

2.1. Strains, plasmids, media and reagents

E. coli XL10-Gold and *P. pastoris* GS115 were from Invitrogen (USA). The pMD18-T and pMD20-T vectors were from TaKaRa (China). And the *P. pastoris* expression vector pHBM905BDM was derived from pHBM905A [6]. To construct pHBM905BDM, the *AOX*1 promoter and the α-MF leading sequence of pHBM905A were replaced with the d1+2 × 201 *AOX*1 promoter [7] and the MF4I leader sequence [8], respectively. Buffered glycerol-complex media (BMGY), buffered methanol-complex media (BMMY), and minimal dextrose media (MD) were prepared as described in the Invitrogen *Pichia* expression kit (USA). Skerman's basal mineral salt (BSM) media for the high-density fermentation were prepared according to the Invitrogen *Pichia* fermentation process guidelines (USA). Oligonucleotides and enzymes were obtained from Shengong (China), NEB (USA) and Takara (China), respectively.

2.2. Yeast transformation

The recombinant plasmids were linearized using *Sal*I and transformed into *P. pastoris* strain GS115 by electroporation (7000 V/cm, 25 μF, 400 × ; Life technologies cell porator, USA). Transformants were selected on MD plates (without histidine), and the expression of the target protein was subsequently identified on BMMY plates containing 1% (w/v) casein (Sigma, USA).

2.3. Expression of the recombinant proteinase K

Transformants were incubated for 2 days at 28 °C in 50 mL of BMGY medium before pelleting the cells centrifugation (4,000 × g, 5 min) and replacing with 50 mL of BMMY. To induce the expression of the foreign protein, methanol was subsequently added to a final concentration of 1% (v/v) every 24 h the supernatant was harvested after 5 days of methanol induction after pelleting the cells by centrifugation at 4 °C and 10,000 × g for 5 min.

2.4. SDS-PAGE and measurement of the protein concentrations

The samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) polyacrylamide gels, followed by staining with Coomassie Brilliant Blue G-250. The protein concentrations were determined using the Bradford kit (Beyotime, China); bovine serum albumin was used as the standard.

2.5. qPCR

The genomic DNA was extracted from cells induced with 1% (v/v) methanol for 2 days using yeast genomic DNA kit (Omega, USA). All primers used for qPCR are listed in Table 1. A modified quantitative real-time PCR method is used to determine copy number of the target gene in cells [9]. Glyceraldehydes-3-phosphate dehydrogenase (*GAP*) gene of *P. pastoris* was used as the reference gene.

The PCR mixtures contained 1 μL of DNA, 1 μL of the forward primer (10 mM), 1 μL of the reverse primer (10 mM), 7 μL of dH_2O and 10 μL of the SYBR Green real-time PCR Master Mix (Toyobo, China). All real-time PCR reactions were carried out in triplicate on the FX96TM real-time PCR Detection System (Bio-rad, USA). The following program was used: a preheat cycle of 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s; annealing at 58 °C for 30 s, and elongation at 72 °C for 20 s. Fluorescent signal measurements were carried out during the elongation step. Recombinant plasmids pMD20T bearing *GAP* and the target gene were used to generate the standard curves. Briefly, the standard curves of *GAP* and the target gene were established using 10-fold serial dilutions of the above-mentioned plasmids ranging from 10^4 to 10^8 copies/mL. The cycle threshold (C_t) values of real-time quantitative PCR in every dilution were performed in triplicate and the average was plotted against the logarithm of the corresponding template gene copy numbers. Each standard curve was generated by linear regression of the plotted points. The total gene copy numbers of *GAP* and target gene in the genomic DNA sample were determined by relating the C_t values to the standard curves. Finally, the target gene copy numbers integrated in the genome of recombinant *P. pastoris* would be calculated by the ratio of the copy numbers of the target gene against *GAP* [10].

2.6. Quantitative PCR to determine the transcription efficiency of the target gene

Total RNA was extracted from cells induced with 1% (v/v) methanol for 2 days using the Yeast RNAiso kit (TaKaRa, China). The absorbance of each sample at 260 nm and 280 nm was then measured to evaluate the concentration and purity of the RNA.

Reverse transcription was performed using the PrimeScript II 1st strand cDNA synthesis kit (TaKaRa, China). Quantitative real-time PCR was carried out using the StepTwo Real-time PCR system with the SYBR Green real-time PCR Master Mix (Toyobo, China). All primers used in qPCR were listed in Table 1. The PCR mixtures included 0.3 μL of the sense primer (10 mM), 0.3 μL of the antisense primer (10 mM), 7.5 μL of the SYBR Green real-time PCR Master Mix, 5.4 μL of dH_2O, and 1.5 μL of cDNA. All real-time PCR reactions were performed in triplicate on the CFX96TM real-time PCR Detection System (Bio-rad, USA). The following program was used: a preheat cycle of 95 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 30 s and annealing at 58 °C for 30 s. The specificity of the

Table 1
Primers used in this study for qPCR.

Gene	Primer	Sequence (5′-3′)
mpk-P	RT mpk-P forward	CCGCTCTGGACGCTGCTATGGAAA
	RT mpk-P reverse	GGAGCGTTAGTTTGTGCAGCGTTTATAGTC
GAP	RT *GAP* forward	ATGGCTATCACTGTCGGTATTAACGGTT
	RT *GAP* reverse	CGCTGGCAGAAACCTCACCCTT
β-actin	RT actin forward	CCAATGAACCCAAAGTCCAA
	RT actin reverse	CCGTCACCAGAGTCCAAAAC