Constitutive Expression of Human Goose-type Lysozyme 2 in Pichia pastoris Using the GAP Promoter Postprint

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Abstract

Using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, human goose-type lysozyme 2 (hLysG2) was expressed in *Pichia pastoris*, and an effective production process for recombinant hLysG2 (rhLysG2) was established at pilot scale. The hLysG2 gene was designed and artificially synthesized according to the codon bias of *P. pastoris* and cloned into the pGAPZ A plasmid to construct the recombinant expression plasmid pGAPZ A-hLysG2. The recombinant expression vector was linearized and electroporated into *P. pastoris* GS115 competent cells, and high-copy recombinant strains were obtained through Zeocin resistance screening and cultivated in a 5 L bioreactor. After 60 h of fermentation, the enzyme activity in the fermentation supernatant reached its maximum, and SDS-PAGE and Western blot analysis confirmed the expression of rhLysG2 in the supernatant. Compared with inducible expression, the constitutive expression shortened the fermentation time by 48 h and increased the total activity of rhLysG2 in the supernatant by 23.8%; after purification of rhLysG2 by chitin affinity chromatography and size exclusion chromatography, 187.4 mg of recombinant protein could be purified per liter of fermentation supernatant, with the purity of the purified product exceeding 99.0%; turbidity assay analysis showed that under conditions of pH 5.6, 30 °C, and 0.1 mol/L Na , rhLysG2 achieved a maximum enzyme activity of 13500 U/mg. Using the GAP promoter, high-purity and high-activity rhLysG2 was successfully expressed in *P. pastoris*, avoiding the use of methanol, shortening fermentation time, increasing protein yield, and laying the foundation for developing rhLysG2 as a novel antimicrobial agent against drug-resistant bacteria.

Full Text

Constitutive Expression of Human Goose-type Lysozyme 2 in *Pichia pastoris* Using the GAP Promoter

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Abstract

This study aimed to achieve constitutive expression of human goose-type lysozyme 2 (hLysG2) in *Pichia pastoris* using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and to establish an efficient bench-scale production process for recombinant hLysG2 (rhLysG2). The hLysG2 gene was designed and artificially synthesized according to the codon usage bias of *P. pastoris*, then cloned into the pGAPZ A plasmid to construct the recombinant expression vector pGAPZ A-hLysG2. After linearization, the vector was electroporated into competent *P. pastoris* GS115 cells, and high-copy recombinant strains were selected via Zeocin resistance screening and cultivated in a 5 L bioreactor. The enzyme activity in the fermentation supernatant peaked after 60 h of cultivation, and SDS-PAGE and Western blot analysis confirmed rhLysG2 expression. Compared with inducible expression, the constitutive expression system reduced fermentation time by 48 h and increased total rhLysG2 activity in the supernatant by 23.8%. Following purification by chitin affinity chromatography and size-exclusion chromatography, 187.4 mg of recombinant protein was obtained per liter of fermentation supernatant, with purity exceeding 99.0%. Turbidimetric analysis revealed that rhLysG2 achieved maximum specific activity of 13,500 U/mg under conditions of pH 5.6, 30 °C, and 0.1 mol/L Na . This study successfully expressed high-purity, high-activity rhLysG2 in *P. pastoris* using the GAP promoter, avoiding methanol usage, shortening fermentation time, and increasing protein yield, thereby laying the foundation for developing rhLysG2 as a novel anti-drug-resistant bacterial agent.

Keywords: Human goose-type lysozyme 2; *Pichia pastoris*; GAP promoter; Constitutive expression; Bactericidal activity

Introduction

Lysozyme is a hydrolytic enzyme widely distributed in various organisms throughout nature, having been identified in animals, plants, fungi, bacteria, and bacteriophages. Based on amino acid composition, molecular structure, and enzymatic properties, lysozymes can be classified into six types: chicken type (C-type), goose type (G-type), invertebrate type (I-type), as well as phage lysozymes, bacterial lysozymes, and plant lysozymes [1-2]. The innate immune system is widely recognized as the first line of defense against pathogenic microorganisms, and numerous studies have demonstrated that various lysozyme types (such as C-type and G-type) represent crucial innate immune defense factors. These enzymes hydrolyze the glycosidic bond between N-acetylmuramic acid C-1 and N-acetylglucosamine C-4 in bacterial peptidoglycan, disrupting the peptidoglycan backbone and causing bacterial lysis through internal osmotic pressure, thereby exerting protective functions [3].

G-type lysozyme was first discovered in 1967 in the egg white of the Emden goose and was identified as an enzyme with bactericidal function [4]. Subsequently, G-type lysozyme genes have been cloned from various vertebrates (including mammals, birds, and fish) and invertebrates (such as mollusks) [5-11]. For example, two G-type lysozymes exist in turbot (*Scophthalmus maximus*), and upon bacterial infection, the expression level of G-type lysozyme 2 in mucosal tissues is upregulated to exert bactericidal effects [7]. Two G-type lysozymes are distributed in humans: human G-type lysozyme 1 and human G-type lysozyme 2 (hLysG2). Current findings indicate that only hLysG2 retains the substrate-binding sites and key catalytic residues in the active center associated with bactericidal activity [1, 12-13]. Our previous research demonstrated that hLysG2 is expressed in ocular tissues and the male reproductive system and can be secreted into tears and seminal plasma to exert bactericidal effects [12, 14-15].

In recent years, the emergence and rapid spread of various super drug-resistant bacteria have highlighted the challenges in treating infectious diseases, making the search for novel antimicrobial agents with mechanisms distinct from traditional antibiotics an urgent priority [16-17]. Since the bactericidal mechanism of lysozyme differs from that of antibiotics, pathogenic bacteria cannot readily develop resistance. Additionally, hLysG2 and human C-type lysozyme are encoded by different genes and exhibit distinct enzymatic activities, endowing hLysG2 with unique application prospects as a candidate molecule for novel anti-drug-resistant bacterial agents. To establish an efficient production process for hLysG2, our research group employed the *Pichia pastoris* expression system, which offers numerous advantages including stable inheritance of foreign genes, proper post-translational processing, appropriate glycosylation of products, secretory expression, and high-density fermentation [18-22]. Beyond these inherent system advantages, promoter selection represents a critical factor affecting protein expression and production processes. In 1997, Waterham et al. first isolated the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter [23].

Compared with inducible expression using the alcohol oxidase 1 (AOX1) promoter, constitutive expression using the GAP promoter eliminates the need to switch carbon sources from glycerol to methanol, reduces fermentation duration, and increases productivity, making it more suitable for large-scale production of foreign proteins [24-26].

This study aimed to design and artificially synthesize the hLysG2 gene according to *P. pastoris* codon preference, achieve high-level expression of rhLysG2 using the GAP promoter, and establish an effective bench-scale production process, thereby laying the groundwork for applied research on rhLysG2.

Materials and Methods

1.1.1 Strains and Plasmids

Staphylococcus aureus, *Enterococcus faecalis*, *Salmonella* spp., *Pasteurella multocida*, and *Streptococcus agalactiae* were purchased from the China Center for Type Culture Collection (Wuhan University). *Pichia pastoris* GS115 strain, *Escherichia coli* TOP10F strain, and *Micrococcus lysodeikticus* were maintained in our laboratory. The yeast constitutive expression vector pGAPZ A was purchased from Invitrogen.

1.1.2 Reagents

Plasmid mini-prep kits, DNA fragment purification kits, and gel extraction kits were purchased from Qiagen. DNA and protein molecular weight markers, Pyrobest DNA polymerase, T4 DNA ligase, and restriction enzymes EcoR I, Not I, and Avr II were purchased from NEB. The hLysG2 gene and all primers were synthesized by Sangon Biotech. Yeast Nitrogen Base (YNB) and Zeocin were purchased from Invitrogen. Rabbit anti-hLysG2 polyclonal antibody and chitin affinity chromatography media were prepared and maintained in our laboratory. The ECL detection kit was purchased from Santa Cruz Biotechnology. Amicon® Ultra centrifugal filters were purchased from Millipore.

1.1.3 Culture Media

LB medium: 1% yeast extract, 2% tryptone, 1% NaCl. YPD medium: 1% yeast extract, 2% tryptone, 2% glucose. YPDS solid medium: 1% yeast extract, 2% tryptone, 2% glucose, 1 mol/L sorbitol, 2% agar. Fermentation medium: 1% yeast extract, 2% tryptone, 2% glucose, 0.5% KH PO , 0.05% MgSO ·7H O, pH adjusted to 6.0.

1.2.1 Target Gene Synthesis

Based on the hLysG2 cDNA sequence (GenBank: NM_175735) and *P. pastoris* codon preference, the hLysG2 coding sequence was designed and synthesized.

EcoR I and Sal I restriction sites were introduced at the 5 and 3 ends, respectively. After synthesis, the gene was cloned into the pMD18-T vector to construct the recombinant plasmid pMD18-T-hLysG2.

1.2.2 Recombinant Plasmid Construction

The pMD18-T-hLysG2 plasmid was transformed into *E. coli* TOP10F , and the recombinant plasmid was extracted after amplification. The pMD18-T-hLysG2 plasmid was digested with EcoR I and Not I, and the target fragment was ligated into similarly digested pGAPZ A vector to construct pGAPZ A-hLysG2 [Figure 1: see original paper]. The pGAPZ A-hLysG2 plasmid was transformed into *E. coli* TOP10F competent cells and plated on LB agar containing 25 g/mL Zeocin. Single positive colonies were selected and cultured in LB liquid medium. The recombinant plasmid was extracted and amplified using pGAP Forward and 3 -AOX1 primers (Table 1), followed by double digestion verification.

1.2.3 Strain Transformation and Screening

The pGAPZ A-hLysG2 plasmid was linearized with Avr II and electroporated into *P. pastoris* GS115 competent cells (1500 V, 25 F, 200 Ω). An equal volume of ice-cold sterile 1 mol/L sorbitol solution was added to the electroporation cuvette, mixed, and plated on YPDS solid medium containing 100 g/mL Zeocin, followed by incubation at 30 °C for 2–3 days. After colonies appeared, positive clones were selected and inoculated onto YPDS plates containing 500, 1000, and 2000 g/mL Zeocin for high-copy strain screening. Genomic DNA was extracted from recombinants using the snailase method, and the target gene was confirmed by PCR amplification using hLysG2 Forward and hLysG2 Reverse primers.

1.2.4 Induction Expression

The selected recombinant *P. pastoris* strain GS115-pGAPZ A-hLysG2 was inoculated into 25 mL YPD medium and cultured at 30 °C for 36 h as seed culture. The seed culture was transferred to a 5 L fermenter containing 2.5 L YPD medium at 1% inoculation and fermented at 30 °C for 5 days, with glycerol supplementation to a final concentration of 1% every 24 h. The recombinant *P. pastoris* strain GS115-pPIC9K-hLysG2 constructed previously in our laboratory using the inducible expression vector pPIC9K was used as a control [12, 27]. All experiments were performed in triplicate.

1.2.5 Recombinant Protein Purification

Purification was performed using chitin affinity chromatography as previously reported [27]. Fermentation broth was centrifuged (3000× g, 30 min) and the supernatant was collected. Twenty milliliters of chitin affinity chromatography medium was mixed with 500 mL supernatant and stirred at room temperature for 10 min. After 5 min of settling, the supernatant was decanted, and the medium was packed into a column. The column was washed with 0.02 M PBS

(pH 6.4) until the A of the effluent reached baseline, then eluted with 0.01 mol/L acetic acid. The eluate was collected, and pH was adjusted to 7.0 with 0.5 mol/L carbonate buffer before dialysis against PBS at 4 °C overnight using a dialysis bag (MWCO 4000–6000). The dialyzed sample was concentrated by ultrafiltration. Sephadex G-75 was fully swollen and packed into a column, equilibrated with PBS. The concentrated enzyme solution was loaded onto the column, and independent elution peaks were collected, concentrated, and analyzed by SDS-PAGE.

1.2.6 HPLC Analysis

Samples were prepared as 5 g/mL rhLysG2 solution with 35 L injection volume. Analysis was performed using an Agilent 1100 series HPLC system with a C18 reverse-phase column. Solution A: 1 mL/L trifluoroacetic acid in water; Solution B: 0.85 mL/L trifluoroacetic acid in acetonitrile. Elution gradient: 0–80% acetonitrile over 45 min at 25 °C.

1.2.7 Enzymatic Property Determination

The optimal pH of rhLysG2 was determined using *M. lysodeikticus* substrate solutions (A 0.8) prepared in acetate buffer (pH 4.0–5.6) and PBS (pH 6.0–8.4). The effect of Na concentration was assessed using substrate solutions containing 0.025–0.2 mol/L NaCl. Temperature effects were examined by incubating substrate solutions at 4–80 °C. Thermal stability was evaluated by incubating enzyme solutions at 30–80 °C for 30 min. The influence of various ions (Na , Co , Ca^2 , Zn^2 , Cu^2 , Hg^2 , Mn^2 , Fe^3) was determined using substrate solutions containing each ion. Ten microliters of 50 g/mL rhLysG2 working solution was mixed with 250 L substrate solution and reacted at room temperature for 1 min. The decrease in A was measured, and relative activity curves for pH, salt concentration, temperature, and thermal stability were plotted using the maximum activity as reference. All experiments were performed in triplicate. Standard enzyme activity was measured under optimal conditions. One unit of enzyme activity (U/mg) was defined as a decrease of 0.001 in A per minute.

1.2.8 Bactericidal Activity Assay

Bactericidal activity was analyzed by colony-forming unit (CFU) assay using two Gram-positive bacteria (*S. aureus* and *E. faecalis*) and three Gram-negative bacteria (*Salmonella* spp., *P. multocida*, and *S. agalactiae*). Bacterial colonies were selected from stock plates, inoculated into 2 mL LB medium, and cultured overnight at 37 °C with shaking. Five hundred microliters of culture was transferred to 25 mL fresh LB medium and cultured at 37 °C with shaking to mid-log phase. Cells were harvested by centrifugation (3000× g, 10 min), washed twice with PBS, and resuspended in 10 g/L peptone medium containing 0.1 mmol/L NaCl (pH 6.0) to a final density of 2 × 10 cells/mL. One hundred microliters of 50 g/mL rhLysG2 solution was mixed with an equal volume of bacterial suspension and incubated at 37 °C for 2 h. One hundred microliters of the mixture

was then plated on LB agar and cultured at 37 °C until colonies appeared. PBS was used as negative control. Experiments were performed in triplicate.

1.2.9 Statistical Analysis

Data from bactericidal activity assays were analyzed using SPSS 17.0 software. Inter-group comparisons were performed using paired-sample t-tests, with $P < 0.05$ considered statistically significant.

Results

2.1 Recombinant Plasmid Construction

Amplification of the recombinant plasmid pGAPZ A-hLysG2 using pGAP Forward and 3 -AOX1 primers yielded a specific band at approximately 1081 bp, consistent with the expected fragment length containing the *Saccharomyces cerevisiae* -factor signal peptide sequence and hLysG2 coding gene. Double digestion of pGAPZ A-hLysG2 with EcoR I and Not I produced a specific band at approximately 589 bp, matching the expected length of the hLysG2 coding gene [Figure 2: see original paper].

2.3 Recombinant *Pichia pastoris* Strain Transformation and Screening

Twenty-eight positive clones were obtained after YPDS resistance plate screening. Genomic DNA was extracted from selected colonies and identified by PCR using hLysG2 Forward and hLysG2 Reverse primers. Approximately 80% of clones amplified a fragment of about 582 bp, consistent with the expected size, confirming integration of the hLysG2 gene into the *P. pastoris* genome [Figure 3: see original paper].

2.4 Recombinant Protein Expression

Activity assays of fermentation supernatant showed that enzyme activity increased progressively with fermentation time, reaching maximum at approximately 60 h. Compared with inducible expression, constitutive expression reduced fermentation time by 48 h and increased total rhLysG2 activity in the supernatant by 23.8% [Figure 4: see original paper]. SDS-PAGE analysis of fermentation supernatant revealed expression of the target protein at 21.5 kDa, consistent with the expected size of rhLysG2. This band was also detected using rabbit anti-hLysG2 polyclonal antibody, confirming effective expression of rhLysG2 in the constitutive *P. pastoris* expression system [Figure 5: see original paper].

2.5 Recombinant Protein Purification

Chitin affinity chromatography of fermentation supernatant yielded a single elution peak [Figure 6: see original paper]. The target protein at 21.5 kDa was

detected by SDS-PAGE and confirmed by Western blot [Figure 7: see original paper]. HPLC analysis showed that purified rhLysG2 had purity greater than 99.0% [Figure 8: see original paper]. The enzyme activity recovery from supernatant reached 42.74% .

2.6 Enzyme Activity Analysis

The optimal reaction pH for rhLysG2 was approximately 5.6 [Figure 9a: see original paper]. Ion concentration significantly affected activity, with maximum activity observed at 0.1 mol/L Na [Figure 9b: see original paper]. The optimal temperature was around 30 °C [Figure 9c: see original paper]. rhLysG2 demonstrated high thermal stability, retaining nearly 80% activity after 30 min treatment at 80 °C [Figure 9d: see original paper]. Among eight tested ions (Na , Co , Ca^{2} , Zn^{2} , Cu^{2} , Hg^{2} , Mn^{2} , Fe^{3}), all affected rhLysG2 activity to varying degrees, with Cu^{2} causing complete loss of activity .

2.7 Bactericidal Activity Analysis

Among the two Gram-positive bacteria tested, rhLysG2 exhibited significant bactericidal activity against *S. aureus* but not against *E. faecalis*. Against the three Gram-negative bacteria (*Salmonella* spp., *P. multocida*, and *S. agalactiae*), rhLysG2 showed obvious bactericidal effects with varying potency, demonstrating strongest activity against *P. multocida* and *S. agalactiae* and significant activity against *Salmonella* [Figure 10: see original paper].

Discussion

Our previous studies on hLysG2 tissue expression profiles revealed that, unlike C-type lysozyme which is widely distributed throughout human tissues, organs, and body fluids, hLysG2 distribution is highly restricted, being present only in the eye and male reproductive tract—two critically important organ systems [12, 14-15]. In the eye, lacrimal and accessory lacrimal glands secrete high concentrations of C-type lysozyme into tears, while testicular tissue does not express C-type lysozyme [28, 29]. This differential distribution suggests that hLysG2 functions are not completely identical to those of C-type lysozyme. Enzymatically, hLysG2 can exert bactericidal activity in hypertonic environments, compensating for limitations of human C-type lysozyme [12]. Furthermore, its bactericidal mechanism differs from that of antibiotics. These characteristics endow hLysG2 with potential as a novel anti-drug-resistant bacterial agent.

For antimicrobial protein drug development, achieving high-level recombinant expression is the first critical prerequisite for promoting applied research. Most existing reports on G-type lysozyme have used *E. coli* for recombinant protein preparation [5, 8]; however, target proteins are expressed as inclusion bodies in *E. coli*, requiring cell lysis and protein refolding processes that increase preparation costs and limit scale-up production. Our previous study expressed enzymatically active rhLysG2 in *P. pastoris* using the AOX1 promoter [12, 29]. However,

the AOX1 promoter system requires hazardous methanol as carbon source, necessitates carbon source switching that prolongs fermentation time, and poses potential methanol residue issues in pharmaceutical protein production, all of which increase production costs. To optimize the rhLysG2 production process, this study employed the GAP promoter for constitutive expression in *P. pastoris*. Results demonstrated that compared with the inducible system, GAP promoter-based constitutive expression reduced fermentation time by 48 h and increased total rhLysG2 activity in supernatant by 23.8%, confirming at small-scale fermentation level that this system is suitable for rhLysG2 expression.

For recombinant protein research and application, low-cost, high-yield purification is a key step. Previous studies often added affinity purification tags such as polyhistidine (His-tag), maltose-binding protein (MBP), or glutathione S-transferase (GST) to G-type lysozyme sequences to facilitate protein purification. For instance, researchers introduced MBP tags when expressing seahorse and orange-spotted grouper G-type lysozymes in *E. coli* [5, 8]. In this study, we utilized chitin affinity chromatography to purify rhLysG2 from fermentation supernatant. This approach avoids introducing any affinity tags, preserving the native structure of rhLysG2. Efficient purification was achieved through two steps—chitin affinity chromatography and size-exclusion chromatography—with protein purity >99.0% and enzyme activity recovery of 42.74% from supernatant [29].

In summary, this study successfully expressed rhLysG2 constitutively in *P. pastoris* using the GAP promoter and established an effective bench-scale production process through chitin affinity chromatography. The purified rhLysG2 exhibited high enzymatic activity and bactericidal activity against multiple pathogenic bacteria. This study represents the first optimization of expression conditions for G-type lysozyme and provides a valuable exploration for applied research on rhLysG2, laying the foundation for developing novel anti-drug-resistant bacterial agents.

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Note: Figure translations are in progress. See original paper for figures.