

The Expression of Recombinant Human Hepatocyte Growth Factor in *Pichia pastoris*

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Abstract—The aim of this study was to construct a stable and efficient eukaryotic expression system for the secretion of biologically active recombinant human hepatocyte growth factor (rhHGF). The eukaryotic expression vector pGAPZ α A was chosen to express rhHGF. To ensure the presence of the secondary structure, we inserted the enterokinase sequence between Arg494 and Val495. After digesting the rhHGF and pGAPZ α A plasmid with Xho I and Xba I, we connected and transformed them into *E. coli* Trans10 competent cells. This resulted in the successful construction of the shuttle plasmid, pGAPZ α A-rhHGF. After sequencing, we transformed the linearized pGAPZ α A-rhHGF plasmid into *Pichia pastoris* GS115 using electroporation for subsequent protein expression. The expressed rhHGF samples were collected at 0, 24, 48, 72 and 96 h, purified by affinity chromatography, and tested using Western blotting. As a result, the pGAPZ α A-rhHGF shuttle plasmid was constructed successfully. A positive band of approximately 80 kDa was observed in the Western blotting indicating successful expression of rhHGF. The highest expression abundance of rhHGF protein was observed at 48 h. Furthermore, we isolated and cultured primary rat hepatocytes, the harvested rhHGF protein exhibited high biological activity. This research provides experimental evidence for the eukaryotic expression of rhHGF protein and theoretical support for large-scale manufacturing.

Keywords: hepatocyte growth factor, *Pichia pastoris*, eukaryotic expression system, shuttle plasmid, pleiotropic cytokine

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Hepatocyte growth factor (HGF) is a pleiotropic cytokine composed of α - and β -chains containing 4 kringle domains and the serine protease-like structure, respectively [1]. It is produced by mesenchymal cells and plays vital roles in stimulating the growth, motility, and multicellular tissue-like structure induction of different kinds of epithelial cells [2]. The human HGF was initially purified from the plasma of a patient with fulminant hepatic failure in 1988 [3]. Subsequently, researchers cloned the cDNA of human HGF, elucidated its primary structure, and identified it as a novel growth factor with unique structural characteristics [4, 5]. HGF may have important implications in organogenesis, morphogenesis, carcinogenesis, and organ regeneration [6]. It holds clinical significance in liver fibrosis, hepatitis, hepatotoxin poisoning, ischaemia, and liver regeneration after transplantation [7]. Additionally, HGF acts as a renoprotective factor in renal regeneration and a plumeotropic factor in lung regeneration following various renal and lung injuries [8, 9]. Furthermore, HGF has been reported to function as an angiogenetic factor. The clinical trials have demonstrated that the recombinant

human HGF (rhHGF) plasmid can effectively salvage limbs during the treatment of patients with chronic limb-threatening ischemia. However, it is important to note that the long-term safety of this treatment still needs to be assessed [10]. Moreover, it has been observed that HGF and its receptor, Met, have the ability to modulate immune cell functions and inhibit the progression of chronic inflammation and fibrosis [1].

In recent years, scientists have discovered that HGF plays a significant role in diseases of the central nervous system. HGF, along with its Met receptor, is expressed in both the central and peripheral nervous system from prenatal development to adulthood. It has positive effects on neuronal growth and survival, making it one of the neurotrophic growth factors [11]. HGF has the ability to promote neural regeneration due to its anti-inflammatory and anti-fibrotic activities, which has led to its consideration as a candidate medicine for spinal cord injury (SCI) [12]. Research conducted by Jeong et al. [13] demonstrated that HGF could inhibit the secretion of glial scar formation inducers and enhance axonal growth beyond glial scars resulting in improved nerve function recovery in

animal models of SCI. Additionally, the exogenous HGF treatment has shown anti-fibrotic effects in the animal model of transient middle cerebral artery occlusion reducing glial scar formation and scar thickness [14]. These findings collectively support the notion that HGF exhibits anti-glia scar effects. HGF is a crucial factor in preventing neuronal death and promoting survival through pro-angiogenic, anti-inflammatory, and immune-modulatory mechanisms [11]. Tsuzuki et al. [15] reported that HGF may prevent apoptotic neuronal cell death and play angiogenic effects in rat transient focal cerebral ischemia. Furthermore, HGF has the potential to act on neural stem cells and produce biological effects of enhancing neuroregeneration in neurodegenerative diseases [16]. Human clinical trials investigating rhHGF or HGF-based therapy have been conducted in several neurological diseases including SCI [17], diabetic neuropathy [18], Alzheimer's disease [19, 20], amyotrophic lateral sclerosis [21, 22], all of which have shown relatively satisfactory results.

Despite the continuous emergence of various new therapy methods, such as HGF activators, HGF plasmids, HGF gene transfer, the direct administration of biologically active rhHGF, this compound has several advantages in clinical practice. It is easier to use, safer, cheaper, and more readily applicable in comparison with other therapy methods. Considering the potential extensive medical application of HGF, exploring stable and efficient protein production methods holds significant clinical significance. The aim of the study was to construct a stable and efficient eukaryotic expression system for secreting biologically active recombinant rhHGF. The present technique is valuable as it enables the production of recombinant, two-chain HGF with promising biological activities, thereby expanding the choice of expression systems for recombinant production.

MATERIALS AND METHODS

Materials. The *Escherichia coli* Trans 10 competent cells used in the laboratory were purchased from Novagen (USA). *Pichia pastoris* GS115 and the secretion expression vector pGAPZ α A were obtained from Invitrogen (USA). The following reagents were purchased from TransGen Biotechnology (China): 2 \times Eco Taq PCR Supermix, ProteinFind $\text{\textcircled{R}}$ Goat Anti-Mouse IgG, and ProteinFind $\text{\textcircled{R}}$ Anti-His Mouse Monoclonal Antibody. The DNA Marker, DNA Ligation Kit, TaKaRa MiniBEST Agarose Gel DNA Extraction Kit, TaKaRa MiniBEST Plasmid Purification Kit, and Dr.GenTLE TM Precipitation Carrier were obtained from TaKaRa (Japan). Restriction endonucleases were purchased from Thermo Fisher Scientific (USA), and Ni Sepharose TM excel was obtained from GE HealthCare (USA). Recombinant enterokinase was purchased from Shenzhen Haiwang Intlong Biotechnology Co. (China). The primers in

the study were synthesized by GenScript Biotech Corporation (China).

Gene optimization of HGF. The full-length sequence of the human HGF gene was obtained from the GenBank and aligned with the Vector NTI software. Three template sequences, BC130286, HUMHGFHL, and HUMSCFA1, were used for the alignment. The open reading frames of these three HGF sequences were found to be completely consistent. The finalized full-length sequence of the human HGF gene includes the Xho I sequence at the 5' terminal, and His-tag, and the Xba I sequence at the 3' terminal. Additionally, an enterokinase sequence was inserted between Arg494 and Val495. The optimized human HGF gene was artificially synthesized in GenScript Biotech Corporation (China) using preferred codons in *P. pastoris*. The bacterial fluid pUC57-HGF was obtained as a result.

Construction of recombinant pGAPZ α A-rhHGF plasmid. The pUC57-HGF plasmid was extracted using the TaKaRa MiniBEST Plasmid Purification Kit. It was then digested with Xho I and Xba I, and the HGF fragment was recovered using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit. Similarly, the pGAPZ α A plasmid was digested with Xho I and Xba I, and the fragment was recovered using the gel extraction kit. The recovered HGF fragment and pGAPZ α A fragment were mixed in a specific molar ratio and connected at 16 $^{\circ}$ C for 4 h. The mixture was then transformed into *E. coli* Trans10 competent cells. The bacteria were inoculated on solid low salt Luria-Bertani (LB) medium containing (g/L): tryptone—10.0, yeast extract—5.0, sodium chloride—5.0 and agar—15.0 (pH 7.5) supplemented with 25 μ g/mL Zeocin TM and cultured upside down at 37 $^{\circ}$ C until colonies formed (approximately 12–16 h). Ten randomly selected colonies were subjected to bacterial fluid PCR identification. The colonies were placed in 200 μ L of low salt LB liquid medium containing 50 μ g/mL ampicillin and shaken at 37 $^{\circ}$ C and 250 rpm for 2 h. A mixture was prepared by combining 2 μ L of bacterial fluid, 10 μ L of 2 \times Eco Taq PCR Supermix, 0.25 μ L 10 μ M forward primer, 0.25 μ L 10 μ M reverse primer, and 7.5 μ L of ddH $_2$ O (the primers were listed in Table 1). After centrifugation at 500 g for 15 s, the mixture was subjected to PCR reaction in a PCR instrument. The reaction parameters included pre-denaturation at 94 $^{\circ}$ C for 3 min, denaturation at 94 $^{\circ}$ C for 30 s, annealing at 60 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 2 min (\times 30 cycles), final extension at 72 $^{\circ}$ C for 5 min and termination at 16 $^{\circ}$ C. The resulting product was analyzed using 1% agarose gel electrophoresis. After the analysis with digital gel image processing system, bacterial fluids with positive results were selected for identification of pGAPZ α A-rhHGF. The recombinant pGAPZ α A-rhHGF plasmid was extracted using plasmid purification kits, and then subjected to double enzyme digestion with Xho I and Xba I.

Table 1. The primer sequences (5' to 3') were used in PCR identification

		Primer sequence
Specific primer	Forward primer	ATGCTCGAGATGTGGGTTACTAAATTGCT
	Reverse primer	CCGTCTAGAAGATTGTGGAACCTTGTAAG
Universal primer	pGAP-Forward	GTCCTATTTC AATCAATTGAA
	3'AOX1	GCAAATGGCATTCTGACATCC

Finally, the positive clones were sequenced by Gen-Script Biotech Corporation (China).

Transformation of recombinant pGAPZ α A-rhHGF plasmid into *P. pastoris* GS115 by electroporation. The recombinant pGAPZ α A-rhHGF plasmid was sequenced and extracted using plasmid purification kits. The pGAPZ α A-rhHGF plasmid was linearized with Avr II and then transformed into *P. pastoris* GS115 competent cells through electroporation. After successful linearization, the plasmid was concentrated using nucleic acid precipitant Dr. GenTLE Precision Carrier (TaKaRa, Japan) to obtain a concentration of 5–10 μ g linearized pGAPZ α A-rhHGF plasmid in 10 μ L ddH₂O. The *P. pastoris* GS115 competent cells were prepared. The linearized pGAPZ α A-rhHGF plasmid (10 μ L) was mixed gently with 100 μ L of these cells, and the mixture was transferred into 2-mm-gap electroporation cuvette on ice for 5 min. Finally, the cuvette was placed in a shock chamber and subjected to electric shock using the following parameters: 400 Ω , 250 μ f, 1.5 KV, 7.3 ms (discharge time). To begin, 1 mL of antibiotic-free YPD fluid culture medium containing (g/L): tryptone—20.0, yeast extract—10.0 and glucose—20.0 was added to the electroporation cuvette in the super clean bench. The mixture was then transferred to 2 mL-microcentrifuge tube. After incubation at 30°C and 250 rpm for 2 h, it was centrifuged at 1000 g for 3 min at room temperature. A portion of the supernatant was discarded, but 200 μ L was retained to resuspend the bacterial cells. The bacteria were then inoculated on YPD plates containing 100 μ g/mL ZeocinTM and cultured upside down at 30°C for 2–3 days until colonies formed. Afterwards, 24 detached bacterial colonies were randomly selected for PCR detection. The bacterial colonies were smeared on the bottom of 2 mL centrifuge tubes. The tubes were then heated in a microwave at medium heat for 1 min and frozen at –20°C for 2 min. After that, the bacteria were resuspended with 20 μ L of sterile water. The suspensions were centrifuged at 1800 g at room temperature for 1 min. Next, 2 μ L of the supernatant was mixed with 10 μ L 2 \times Eco Taq PCR Supermix, primers (both 0.5 μ L with a concentration of 10 μ M), and 7 μ L ddH₂O. Two sets of primers were used in the reaction system: specific primers (forward primer and reverse primer) for 12 colonies, and universal primers (pGAP Forward and 3'AOX1) for another 12 colonies (Table 1 and Fig. 1c). After centrifugation at 500 g for 15 s, the mixtures were

placed in a PCR instrument for the reaction. The reaction parameters were as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 150 s (\times 30 cycles), final extension at 72°C for 10 min and termination at 16°C. Finally, the products were detected using 1% agarose gel electrophoresis and a digital gel image processing system.

Expression and purification of rhHGF protein. The bacterial suspensions of positive colonies having the brightest band in agarose gel electrophoresis were inoculated into 50 mL YPD liquid culture medium and cultured at 30°C and 250 rpm. Samples were taken at 0, 24, 48, 72 and 96 h for SDS-PAGE detection. The bacterial suspension of positive clones was then inoculated into 50 mL YPD liquid culture medium and incubated at 30°C and 250 rpm for 48 h. The bacteria were collected by centrifugation at 18000 g and 4°C for 10 min. The supernatant was collected and filtered using 0.45 μ m microporous filter membrane (XinYa, China). The protein was purified using Ni²⁺–NTA–agarose affinity chromatographic column (particle size: ~90 μ m, column size: 5 mL; GE HealthCare, USA). Gradient elution was performed using imidazole elution solutions containing 20, 40, 80, 120, 200 and 500 mM. SDS-PAGE was conducted for detection.

Concentration measurement and Western blotting. The concentration of purified rhHGF protein was measured using the BCA method and BSA as a standard. The purified HGF protein was then subjected to SDS-PAGE and transferred to a PVDF blotting membrane using a Semi-Dry Transfer Unit (Biometra, Germany). The membrane was sealed with 5% skim milk powder and incubated with anti-His mouse monoclonal antibodies (3000 : 1) at 37°C for 1 h. Subsequently, the membrane was incubated with secondary antibodies after cleaning. Finally, bands were developed using an enhanced HRP-DAB substrate chromogenic reagent kit.

Biological activity tests of the rhHGF protein. Primary SD rat hepatocytes were isolated, purified, and cultured [23, 24]. Cell viability was measured using the 3-(4,5)-dimethylthiazolohiazole(-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) assay. A suspension of primary hepatocytes was inoculated into a 96 well plate at a concentration of 1×10^5 /mL, with 100 μ L in each well. Five treatment levels of rhHGF protein were set, with

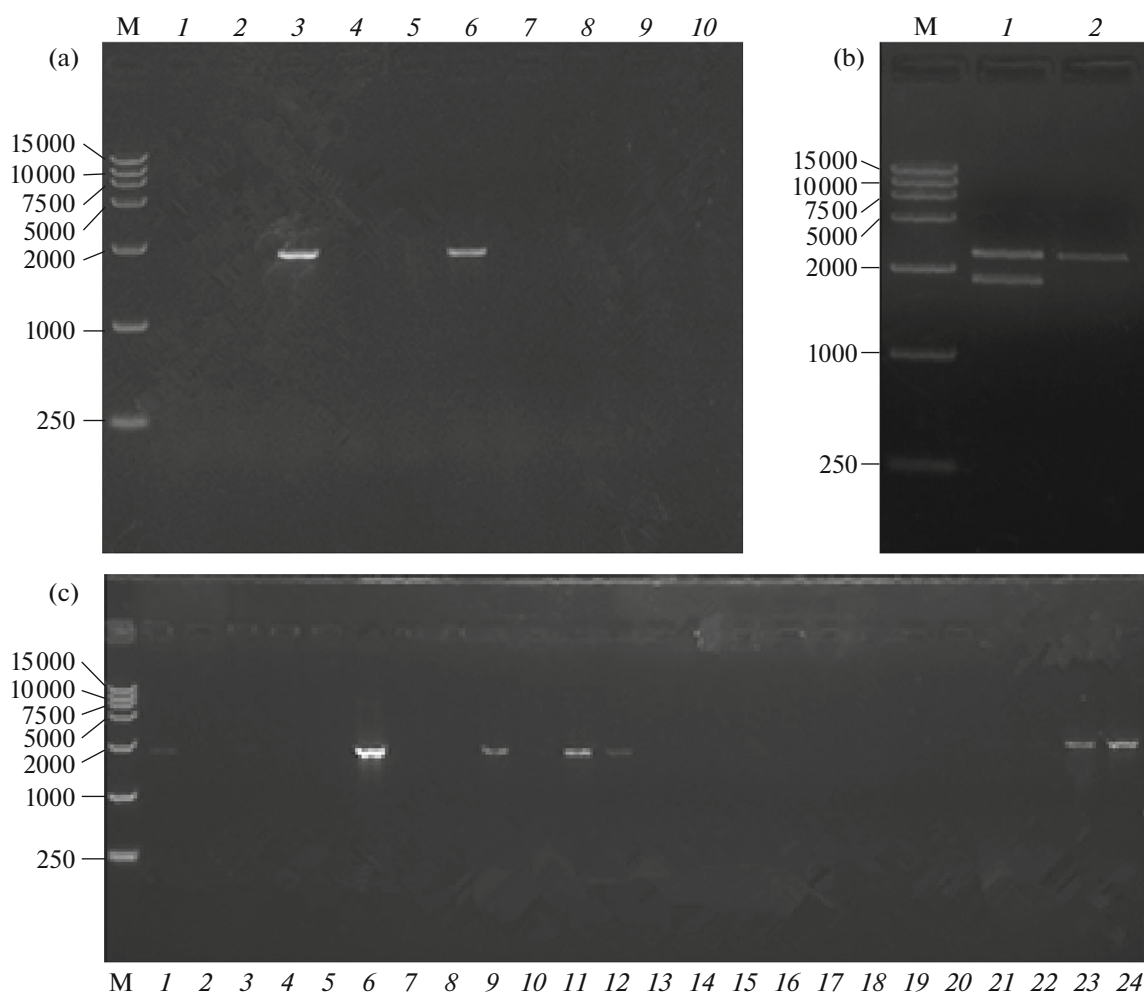


Fig. 1. (a) The positive bands in agarose gel electrophoresis represent that the bacteria contain recombinant pGAPZ α A-rhHGF plasmid. 1–10: the gel imaging of PCR products from the 10 randomly selected colonies. (b) The PCR results of possibly positive plasmid digested by Xho I and Xba I. The one with two bands of 2250 bp (the target gene) and 3100 bp (the vector) is true positive, and another with one band is false positive. (c) The PCR results of cultured bacterial colonies after transforming recombinant pGAPZ α A-rhHGF plasmid into *P. pastoris* GS115 by electroporation. The positive bands were revealed in channels 6, 9, 11, 12, 23 and 24. Specific primers were applied to bacterial colonies in channels 1–12, while universal primers were used for the other 12 bacterial colonies in channels 13–24. The bands in channels 6, 9, 11, and 12 had a size of approximately 2250 bp which matched the size of the target gene. On the other hand, the bands in channels 23 and 24 had a size of around 2790 bp, including 540-bp fragment resulting from pGAPZ α . M: marker.

each treatment having 5 replicates. After culturing the plate in a sterile incubator at 5% CO₂ and 37°C for 4 h, different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 μ g/dL) of rhHGF protein were added. The idle bacterial suspension of pGAPZ α A was used as a control group. The plate was continuously cultivated under the condition of 5% CO₂ and 37°C for 24 h. Then, 0.5% MTT 10 μ L was added to each well and the cells were cultured for an additional 4 h. The culture medium was discarded and 150 μ L of dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken at low speed for 10 min, and the OD₄₉₀ was measured using HBS-ScanX microplate reader (DeTie, China) to assess the relative cell viability. The relative cell viability was calculated according to for-

mula: $(OD_T - OD_0)/(OD_C - OD_0) \times 100\%$. OD_T represented the OD value of treatment wells containing rhHGF, cells, culture medium, MTT and DMSO. OD₀ represented the OD value of blank wells containing culture medium, MTT and DMSO. OD_C represented the OD value of control wells containing idle bacterial suspension of pGAPZ α A, cells, culture medium, MTT and DMSO. SPSS 26.0 was used for statistical analysis and GraphPad Prism 9.1.1 for graph. Group comparison used one-way ANOVA. Spearman correlation was performed to judge the relationship between rhHGF and the relative cell viability. A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Construction of recombinant pGAPZ α A-rhHGF plasmid. The digested rhHGF and pGAPZ α A fragments were connected and transformed into *E. coli* Trans10 competent cells. The transformed cells were then cultured on low salt LB plates. Ten randomly selected colonies were chosen for PCR identification. The results of the gel imaging showed the presence of two positive bands (Fig. 1a). Plasmid DNA was extracted from the bacteria of the colonies with positive bands and further analyzed by digestion with Xho I and Xba I. After digestion, the true positive sample yielded two bands, one with a size of 2250 bp (size of the target gene) and another with a size of 3100 bp (size of the vector). However, there was also a false positive sample that only produced one band (Fig. 1b). The positive clonal plasmid was sent for sequencing, and no variations were observed. Thus, the recombinant pGAPZ α A-rhHGF plasmid was successfully constructed.

Expression of rhHGF. Recombinant pGAPZ α A-rhHGF plasmid was transformed into *P. pastoris* GS115 by electroporation, and the bacteria were cultured. Twenty-four detached bacterial colonies were randomly selected for PCR identification. Finally, there were 6 positive bands in gel imaging system (Fig. 1c). The 2250-bp fragment, similar in size to the target gene, was obtained using specific primers (channels 6, 9, 11, 12 in Fig. 1c). Additionally, the around 2790-bp fragment was obtained using universal primers (channels 23, 24 in Fig. 1c), with an additional 540-bp fragment compared to the 2250-bp fragment, which was attributed to pGAPZ α . The bacterial suspension with the strongest band was chosen to inoculate the YPD liquid culture medium and culture at 30°C and 250 rpm. Samples were taken at 0, 24, 48, 72 and 96 h for SDS-PAGE detection, and the highest expression abundance of rhHGF protein was observed at 48 h (Fig. 2a). The bacteria were cultured again at 30°C and 250 rpm, and the products were collected at 48 h. After centrifugation, filtration, and chromatographic purification, gradient elution was conducted using imidazole elution solutions containing 20, 40, 80, 120, 200 and 500 mM. SDS-PAGE detection confirmed successful elution of the protein starting from 80 mM, with the most effective elution observed at 120 mM imidazole (Fig. 2b).

Concentration measurement and Western blotting of rhHGF. The absorbance of the rhHGF protein at 595 nm was measured to be 0.094, corresponding to a concentration of 16.6 mg/L. Additionally, Western blotting was performed using anti-His antibodies, and the obtained result showed a positive band around 80 kDa (Fig. 2c).

The expressed rhHGF protein was certified to have biological activity. In this study, primary rat hepatocytes were cultured and exposed to different concentrations of rhHGF protein in the cell culture medium.

The biological activity of the rhHGF protein was assessed by measuring the relative cell viability using MTT assay. The results showed that the rhHGF protein could increase the cell viability of hepatocytes significantly at concentrations of 0.5–2.0 $\mu\text{g/dL}$ ($P < 0.0001$), while the concentration of 0.1 $\mu\text{g/dL}$ had no significant effect ($P > 0.05$) (Fig. 3a). Furthermore, when the concentrations of rhHGF were in the range from 0.1 to 2.0 $\mu\text{g/dL}$, there was a positive correlation between the level of rhHGF and the relative cell viability ($r = 0.951$, $P < 0.0001$) (Fig. 3b). These findings provide evidence that the expressed rhHGF protein has biological activity. In the detecting range of 0.1–2.0 $\mu\text{g/dL}$, the biological activity demonstrated a concentration dependence.

DISCUSSION

Hepatocyte growth factor (HGF) plays significant roles under both normal physiological and pathological conditions within the body. It is particularly noteworthy for its effectiveness as a mitogen for hepatocytes, making it crucial in partial hepatectomy and live transplantation procedures. Furthermore, HGF's anti-inflammatory and anti-fibrotic properties have shown promise in advancing drug treatments for liver cirrhosis. Apart from its role in promoting regeneration and repair in the liver [7], lung, kidney, and gastrointestinal tract [25], ongoing research is uncovering additional important functions of HGF. It is widely recognized that HGF also plays a significant role in the regeneration and repair of the nervous system. However, the availability of HGF is limited compared to the demand. Fortunately, with the advancements in genetic engineering, HGF proteins can now be synthesized, and various novel substitutes have been designed. While the prokaryotic system allows for large expression quantities, the inclusion bodies produced require denaturation and renaturation, resulting in a tedious and complicated operation procedure with significant protein loss. As a result, the protein yield is low, and the activity is not guaranteed. To overcome these challenges, researchers have explored the use of eukaryotic expression systems, which offer simpler operation and the ability to express exogenous proteins with biological activity. Among these systems, *P. pastoris* has emerged as one of the most useful tools for producing recombinant proteins in molecular biology [26–28]. Despite its high protein productivity, there is still an urgent need to optimize the cultivation of this microorganism due to specific challenges related to strain and product, such as promoter strength, methanol utilization type, and oxygen demand [28]. In this study, we were the first to construct the recombinant plasmid pGAPZ α A-rhHGF and successfully transform it into *P. pastoris* GS115. Additionally, we optimized the rhHGF gene, conditions for protein expression and purification, and confirmed the biological activity of the expressed protein.

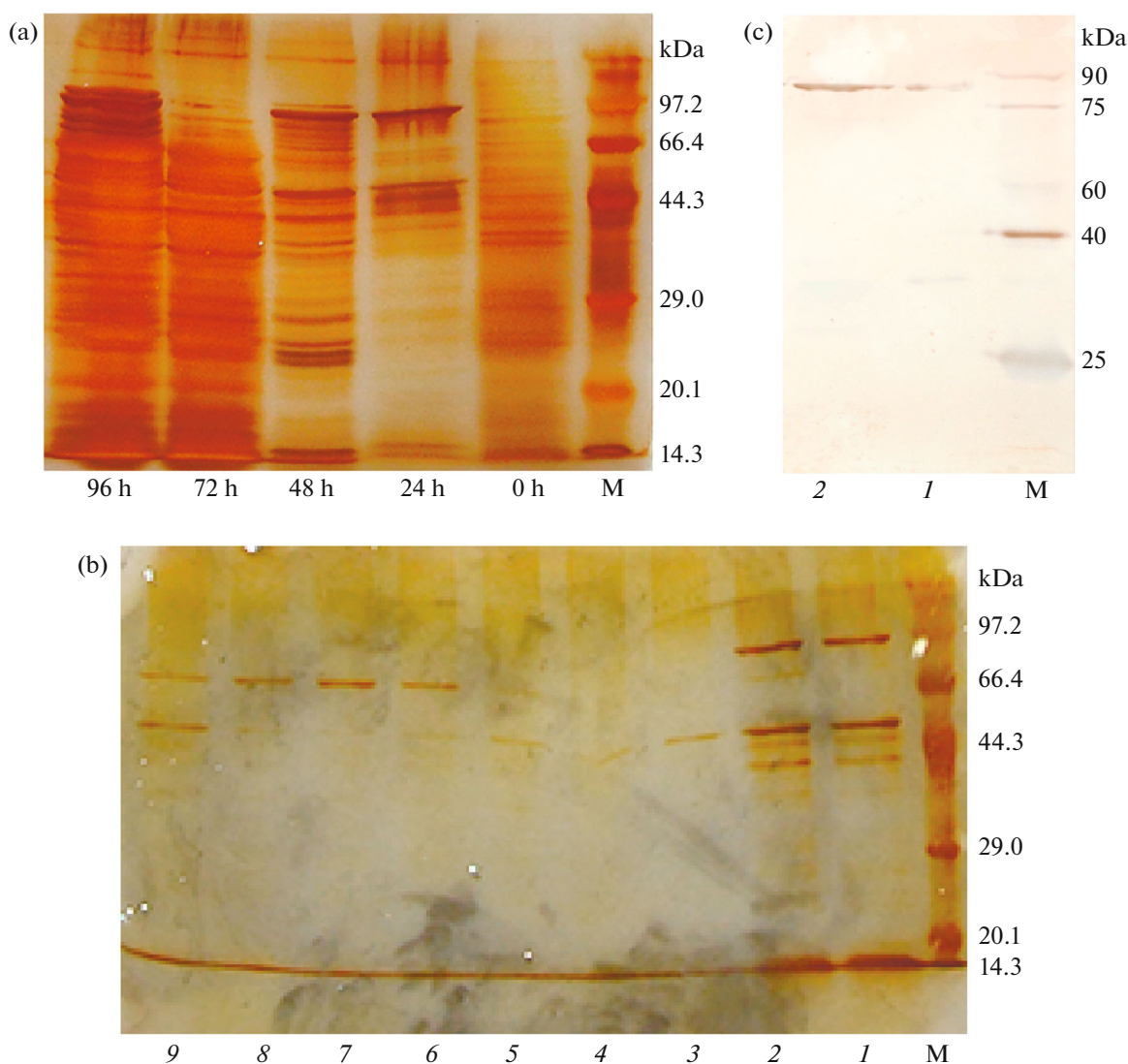


Fig. 2. (a) The expression of rhHGF protein at different culture time. (b) The rhHGF protein was eluted by imidazole of different concentrations. 1: after passing through the affinity chromatography column; 2: before passing through the affinity chromatography column; 3: elution with equilibration buffer; 4: elution with 20 mM imidazole; 5: elution with 40 mM imidazole; 6: elution with 80 mM imidazole; 7: elution with 120 mM imidazole; 8: elution with 200 mM imidazole; 9: elution with 500 mM imidazole. (c) Western blotting of rhHGF. The anti-His antibodies are the first antibodies employed to bind to the His-tag on the rhHGF protein. 1, 2: the positive bands of rhHGF protein. M: proteins markers.

Active HGF is the disulfide-linked heterodimer composed of the 69-kDa α -chain and the 34-kDa β -chain. The *P. pastoris* expression system offers a significant advantage over other expression systems as it allows for post-translational modifications. These modifications include correct protein folding, formation of disulfide bonds, glycosylation, and processing of signal sequence. By utilizing the *P. pastoris* expression system, the rhHGF protein can undergo disulfide bond formation after translation, thereby preserving its biological activity. The signal peptide of *P. pastoris* is PHO1. While it can secrete and express some exogenous proteins, most proteins require the signal peptide of the expression vector. The *P. pastoris* expression

system commonly uses secretory expression vectors and non-secretory expression vectors. Therefore, the selection of yeast expression vectors should be based on the intended purpose of gene expression. Secretory expression vectors include pPIC9, pPIC9k, pHIL-S1, pPICZ α , and pYAM75P. Intracellular expression vectors include pHILD2, pA0815, pPIC3K, pPICZ, pHWO10, pGAPZ, and pGAPZ α . Some studies have utilized the expression vector pPIC9 to produce a large yield of secretory recombinant deleted variant HGF in shake flask and fermenter cultures [29]. However, it should be noted that the pPIC9 expression vectors contain the alcohol oxidase1 gene (AOX1) promoter and rely on methanol as the sole carbon source. Inter-

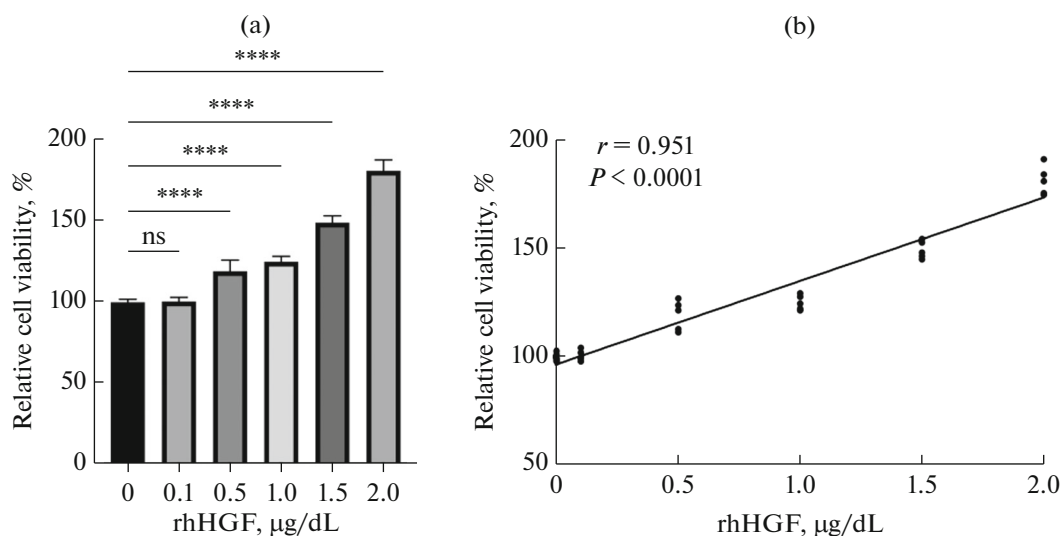


Fig. 3. (a) The biological activity of the rhHGF protein assessed by measuring the relative cell viability using MTT assay. Significantly increased cell viability of hepatocytes was observed after exposition with the rhHGF protein at concentrations of 0.5–2.0 $\mu\text{g}/\text{dL}$ ($P < 0.0001$). When the concentration was 0.1 $\mu\text{g}/\text{dL}$, no significant effect was found ($P > 0.05$). ns: no significance ($P > 0.05$). ****: $P < 0.0001$. (b) Positive correlation revealed between rhHGF level and the relative cell viability. In the detecting range of 0.1–2.0 $\mu\text{g}/\text{dL}$, the biological activity demonstrated the concentration dependence.

estingly, when methanol yeast is grown on a culture medium with glucose or glycerol as the carbon source, the expression of the AOX1 gene is inhibited. Conversely, when methanol is the only carbon source, gene expression is induced. However, the use of methanol poses challenges as it is toxic, volatile, and difficult to separate after protein expression, thereby affecting large-scale fermentation. Consequently, researchers have shifted their focus towards regulating the AOX1 promoter and developing methanol-free systems [28]. One such system is the secretion expression vector pGAPZ α A, which is a shuttle expression vector that replaces the methanol-regulated AOX1 promoter with the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter in the pPICZ carrier. The yield of constitutive expression of foreign proteins in the *P. pastoris* system depends on the toxicity of the protein to the yeast. However, constitutive expression controlled by pGAPZ or pGAPZ α vectors sometimes yields higher than induced expression [30]. At the current stage, the most commonly used α -MF signal peptide is carried by the secretion expression vector pGAPZ α A [31]. During the process of secretion, the α -MF signal peptide is removed by protease Kex-2 to prevent the addition of additional amino acid residues at the N-terminus of exogenous proteins. Additionally, the Kex2 splitting signals promote the secretion, maturation, and post-translation modification of exogenous proteins [31, 32]. The GAP promoter is expressed in a constitutive form and is not inhibited by carbon sources. Therefore, foreign genes can be continuously expressed without changing the carbon source. The process is simple and more suitable for large-scale fermentation. Furthermore, previ-

ous researches found the GAP promoter was more efficient than the AOX1 promoter [33]. Taking into consideration various factors, we chose to use the pGAPZ α A expression vector for the first time to construct the recombinant plasmid of pGAPZ α A-rhHGF and express rhHGF protein with biological activity. The pGAPZ α A expression vector utilizes glycerol or glucose as a carbon source, eliminating the need to change the carbon source during the operation process. Additionally, the fermentation cycle is short, and the extraction process is simple, making it favorable for large-scale fermentation and extraction of exogenous proteins.

Many factors influence protein expression, including protein quality, plasmid stability, gene copy number, codon preference, promoter selection, secretion pathway, glycosylation modification, and fermentation conditions [28, 34]. Among these factors, codon preference and gene copy number have the most significant impact [35]. Optimizing codon usage, gene dosage, promoters, protein secretion pathways, and methanol metabolic pathways have been shown to enhance protein expression levels [28]. In our experiment, we optimized the gene of rhHGF by considering the codon preference of *P. pastoris*. We also added the HIS tag at the 3' terminal and inserted an enterokinase sequence between Arg494 and Val495. Our first successful step was the construction of the pGAPZ α A-rhHGF shuttle plasmid, which did not require methanol as a carbon source. Through Western blotting analysis, we detected a positive band at around 80 kDa, indicating the successful expression of rhHGF. Furthermore, we observed the highest expression abundance of the rhHGF protein at 48 h and confirmed its high

biological activity. However, it is important to note that our experiment has certain limitations. We have only demonstrated the effectiveness of the recombinant pGAPZ α A-rhHGF shuttle plasmid and have not yet validated protein production in shake flask and fermenter cultures. Based on our previous experience, the production capacity of fermenter cultures is expected to be significantly higher, potentially 10 to 100-fold greater than that in shake flask cultures. This would provide both experimental and theoretical support for large-scale manufacturing.

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In this study, we have established a stable and efficient eukaryotic expression system for secreting biologically active rhHGF protein. The pGAPZ α A-rhHGF shuttle plasmid, in combination with *P. pastoris*, may serve as a useful tool in methanol-free systems for the secretion and expression of active exogenous proteins. This study provides experimental evidence for the eukaryotic expression of rhHGF protein and lays the theoretical foundation for large-scale production.

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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