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• *BASIC RESEARCH* •

Secretory expression and characterization of a recombinant-deleted variant of human hepatocyte growth factor in *Pichia pastoris*

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Abstract

AIM: To study the secretory expression of human hepatocyte growth factor (hdHGF) gene in *Pichia pastoris*.

METHODS: The full-length gene of human cDNA encoding the deleted variant of hdHGF was cloned by RT-PCR and overlapping-fragment PCR technique using mRNA of human placenta as a template. The cloned hdHGF cDNA was inserted into the *Escherichia coli-yeast* shuttle vector of pPIC9. The constructed plasmid, pPIC9-hdHGF, was transformed into the GS115 cells of the methylotrophic yeast, *P pastoris,* using a chemical method. The Mut$^+$ transformants were screened to obtain high-expression strains by the test and analysis of expressed products of shake-flask culture. A secretory form of rhdHGF was made with the aid of the leader peptide sequence of *Saccharomyces cerevisiae* α-factor.

RESULTS: The expressed products, which showed a band of molecular mass of about 80 ku, were observed on 15% SDS-PAGE and identified by Western blotting and N-terminal amino acid sequencing. In the high cell density culture of 5 L fermentor by fed-batch culture protocol, the cell biomass was reached at approximately 135 g (DCW)/L. The productivity of secreted total supernant protein concentration attained a high-level expression of more than 8.0 g/L and the ratio of rhdHGF band area was about 12.3% of the total band area scanned by SDS-PAGE analysis, which estimated that the product of rhdHGF was 500-900 mg/L.

CONCLUSION: The *P pastoris* system represents an attractive tool of generating large quantities of hdHGF for both research and industrial purposes.

Key words: Hepatocyte growth factor; *Pichia pastoris*; Secretory expressing

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INTRODUCTION

Hepatocyte growth factor (HGF) was identified initially as a mitogen for hepatocytes, called as scatter factor (SF) and fibroblast-derived tumor cytotoxic factor (F-TCF) as well as fibroblast-derived growth factor called plasminogen-like growth factor (PLGF)[1-3]. Nakamura *et al*[16]. reported that HGF could be purified from the serum of partially hepatectomized rats. Subsequently, HGF has been purified from rat platelets and its subunit structure is determined. The purification of human HGF from human plasma is described by Godowski *et al*[5].

The gene locus of human HGF is assigned to chromosome 7q21.1. The genomic gene consists of 18 exons and 17 introns, and spans about 70 kb. The whole length form of human hepatocyte growth factor (preproHGF) consists of 727 amino acids and the mature form of hHGF is composed of 674 amino acids, corresponding to the major form purified from human serum[5]. HGF is a disulfide-linked heterodimer derived by proteolytic cleavage of the human pro-hormone between amino acids R494 and V495. This cleavage process generates a molecule composed of an alpha-subunit of 440 amino acids (MW 69 ku) and a beta-subunit of 234 amino acids (MW 34 ku). The nucleotide sequence of hHGF cDNA reveals that both the alpha-and the beta-chains are contained in a single open reading frame coding for a pre-pro precursor protein. In the predicted primary structure of mature hHGF, an interchain S-S bridge is formed between Cys 487 of the alpha-chain and Cys 604 in the beta-chain. The N-terminus of the alpha-chain is preceded by 54 amino acids, starting with a methionine

group. This segment includes a characteristic hydrophobic leader (signal) sequence of 31 residues and the prosequence. The alpha-chain starts at amino acid (aa) 55 and contains four Kringle domains. The Kringle 1 domain extends from about aa 128 to about aa 206, the Kringle 2 domain is between about aa 211 and about aa 288, the Kringle 3 domain extends from about aa 303 to about aa 383, the Kringle 4 domain extends from about aa 391 to about aa 464 of the alpha-chain. It will be understood that the definition of the various Kringle domains is based on their homology with Kringle-like domains of other proteins (prothrombin, plasminogen). Therefore, the above limits are only approximate. Until now, the function of these Kringles has not been determined. The beta-chain of hHGF shows high homology to the catalytic domain of serine proteases (38% homology to the plasminogen serine protease domain). However, two of the three residues, which form the catalytic triad of serine proteases, are not conserved in hHGF. Therefore, despite its serine protease-like domain, hHGF appears to have no proteolytic activity and the precise role of the beta-chain remains unknown. HGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the alpha-chain and at positions 566 and 653 of the beta-chain. Wild-type human HGF gene *in vivo* exists in the polymorphism. It has been observed that human HGF has a few natural variants. For example, hdHGF-encoded HGF molecule lacking five amino acids in the Kringle 1 domain (FLPSS) is fully functional[6-8].

HGF biological activity refers to any mitogenic, motogenic or morphogenic activities exhibited by wild-type human HGF or hdHGF, which have a broad spectrum of mitogenic cell specificity that can promote the proliferation of hepatocytes, endothelial cells, fibroblasts, melanocytes, and epithelial cells etc.[1,4], inhibit the growth of some tumor cell lines such as $HepG_2$, B6/F1, and KB from tumorigenic target cell lines[9]. Recent studies displayed that hdHGF can exert many important biological effects mediated via their specific tyrosine kinase receptor, C-met. Even to the extent, hdHGF has more significant biological effects on promoting the regeneration of hepatocytes and kidney epithelial cells compared to wild-type human HGF, suggesting that hdHGF has the therapeutic effect *in vivo* on liver injury[2].

Recently, methylotrophic yeast *Pichia pastoris* has become a dominant tool in molecular biology for the production of recombinant proteins. *P pastoris* is known for its high-level expression of heterologous proteins and its tightly regulated alcohol oxidase 1 (AOX1) gene promoter[10]. *P pastoris* can be easily grown to high cell densities using defined minimal media and is able to introduce eukaryotic post-translational modifications[11,12]. The techniques for molecular genetic manipulation are similar to those well established for *Saccharomyces cerevisiae*. At present, *P pastoris* as an efficient protein expression system can be fermented routinely in large scale to meet the industrial demands of interest proteins[13-15]. In the present report, we have described the recombinant production of hdHGF in *P pastoris* and its characterization.

MATERIALS AND METHODS

Strain, vector, reagents, and enzymes

P pastoris host strain GS115 (His^+Mut^+) and secretion expression vector pPIC9 were purchased from Invitrogen (San Diego, CA, USA). *E coli* DH5α was used for routine plasmid amplification and the cloned vector of pUC19 was maintained in our laboratory. Superscript™ II RNase H⁻Reverse transcriptase was purchased from GibcoBRL. Human placenta mRNA was obtained from Clontech Co. Expand™ High Fidelity PCR System was purchased from Boehringer Mannheim Co. Yeast nitrogen base, D-biotin, yeast extract and tryptone were obtained from Sangon (Shanghai, China). *EcoRI*, *NotI, SalI, XbaI, SphI,* T4 DNA ligase, and *Taq* DNA polymerase were obtained from TaKaRa Biotechnology (Dalian, China). Anti-hHGF antibody was purchased from Santa Cruz Biotechnology Co.

Molecular cloning of hdHGF

The whole length gene of human cDNA encoding the deleted variant of hdHGF was amplified by RT-PCR and overlapping fragments were amplified by PCR technique using mRNA of human placenta as the template. Three pairs of PCR primers for amplified hdHGF fragments were designed as follows.

In primer M1, single bottom line stands for SphI and two lines for SalI. Oblique boldface capital letters (included 8 codons) represent the frequently used codons in the highly expressed *P pastoris* genes. In primer M2, single bottom line stands for *XbaI* and two lines for *NotI*. Its complementary chain encodes the sequence for *TACAAGGTTCCACAGTCTTAA* (included 6 codons) and the oblique boldface capital letters represent the bias of codons in the highly expressed *P pastoris* genes. Therefore, the codons encoding N-and C-terminal amino acids of the deleted variant of hdHGF were amplified by RT-PCR and overlapping-fragment PCR technique, using mRNA of human placenta as the template, which has the advantage to acquire high-level expression of foreign genes in *P pastoris*.

A forward primer(P1: 5'TTCTTTCACCCAGGCATCTC3') and a reverse primer (P2: 5'CTATGTTTGTTCGTGTTGG AATCC3') as well as another forward primer (P3: 5'GTGG GACAAAGAACATGGAAGACTTAC3') and its reverse primer (P4: 5'GCTTCAGACACACTTACTT CAGCTA3') were designed to synthesize the two cDNA fragments based on the hdHGF sequence reported by Nakamura *et al*[16], namely one fragment (F1, about 1.6 kb) was amplified using a pair of primers P1 and P2 and the other fragment (F2, approximately 1.0 kb) was amplified using a pair of primers P3 and P4. Overlapping-fragment amplification using F1 and F2 fragments as templates was performed by routine PCR procedure using a pair of primers M1 and M2 (Figure 3). The cDNA product obtained from RT-PCR was modified by introducing *SphI* and *SalI* sites at the 5'end and *XbaI* and *NotI* sites as well as a TAA stop codon at the 3'end. Thirty-five cycles of PCR were performed: denaturation at 94 ℃ for 60 s, annealing at 55 ℃ for 60 s,

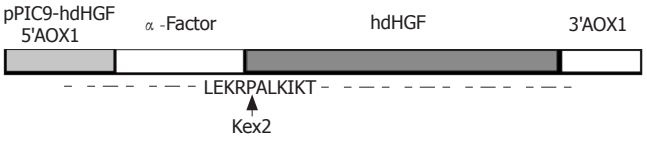

Figure 1 Schematic representation of hdHGF expression cassettes used. Arrowhead indicates the cleavage site of Kex2 protease.

extension at 72 ℃ for 90 s, and then a further extension at 72 ℃ for 10 min. The PCR procedures were carried out according to the standard procedures published earlier[17].

Construction of expression plasmid

The PCR products were digested with *SphI* and *XbaI*, and cloned into the same enzyme digested vector pUC19. The recombinant vectors were transformed into *E coli* DH5 α . The recombinant transformants were acquired via the blue-white colony screening in the agar medium containing X-gal and characterized using restriction endonucleases *SphI* and *XbaI*. The gene sequence analysis of the recombinant pUC-hdHGF was carried out by Sanger's dideoxynucleotide DNA sequencing. The verified hdHGF cDNA fragment with *SalI* and *NotI* was cloned into the site of expression vector pPIC9 digested with *XhoI* and *NotI* enzymes. The recombinant plasmids were transformed into *E coli* strains of JM109. Screening and selection of expression plasmid clones containing hdHGF cDNA fragments through the identification with restriction endonucleases *Bam*HI and *NotI*, resulted in the plasmid pPIC9-hdHGF containing hdHGF gene under the control of AOX1 promoter and in-frame with α -factor signal sequence (Figure 1).

Yeast transformation

Plasmids used for transformation were linearized with *SalI*. The *SalI*-linearized pPIC9-hdHGF or parent pPIC9 was transformed into *his4* competent *P pastoris* GS115 cells by a chemical method. After the growth on minimal dextrose medium (MD) plates at 30 ℃ for 3 d, several colonies containing the linearized pPIC9-hdHGF fragment were selected for PCR confirmation by colony PCR, which was designed to amplify the 200-bp special sequence of pPIC9-hdHGF using a pair of primers, namely P5 (sense, 5'GTGGGACAAGAACATGGAAGA CTTA3') and P6 (antisense, 5'CTATGTTTGTTCGTGTTGGAATCC3').

Expression of hdHGF by recombinant Pichia in shake flask

Ten colonies were used to inoculate 10 mL buffered minimal glycerol-complex medium (BMGY) in a 50 mL shake flask, respectively. After being shook at 250 r/min for 2 d at 30 ℃, the cells were pelleted and resuspended in a 2 mL buffered minimal methanol-complex medium (BMMY). Following the additional 2 d of induction at 30 ℃, the samples of expressed hdHGF in culture supernatants were determined and hdHGF in culture supernatants was also analyzed by SDS-PAGE.

Fed-batch cultivation of P pastoris in a 5-L bioreactor

The clones exhibiting the highest level expression of hdHGF were selected for fed-batch fermentations which were carried out in a 5-L working volume bioreactor using a BIOFLO 3000 (New Brunswick Scientific) interfaced with AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific) for data acquisition and supervisory control.

Seed culture for the bioreactor was started from the fresh glycerol stock and inoculated directly into 500-mL shake flasks (50-mL working volume) containing a minimum glycerol medium (1.34% YNB, 1% glycerol, and 1.61 μmol/L biotin). After 24 h of growth, seed culture was inoculated with 1% inoculum. After 16-20 h of growth, seed culture was used to inoculate the bioreactor. Ten percentage of the inoculum was used for the inoculation of a 5-L bioreactor containing 2.-L medium of high-cell density fermentation, comprising of 10× basal salts (42 mL/L 85% H_3PO_4, 1.8 g/L $CaSO_4{\cdot}2H_2O$, 28.6 g/L K_2SO_4, 50 g/L glycerol, 23.4 g/L $MgSO_4{\cdot}7H_2O$, 6.5 g/L KOH, and 4.35 mL/L 10·PTM_1 salts, 6.0 g/L $CuSO_4 \cdot 5H_2O$, 0.08 g/L KI, 3.0 g/L $MnSO_4{\cdot}H_2O$, 200 g/L $ZnCl_2$, 0.02 g/L HBO_4, 65 g/L $FeSO_4{\cdot}7H_2O$, 0.2 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.5 g/L $CoCl_2$, 0.2 g/L biotin, and 5 mL/L H_2SO_4, buffered to pH 5.5 using 2 mol/L NH_4OH). Dissolved oxygen was maintained at over 20% air saturation at 30 ℃ and aeration was maintained at 2 vvm. pH was maintained at 5.5 and fermentation was carried out in two phases. Growth phase consisted of a glycerol batch phase and cells were grown batch-wise until glycerol in the medium was utilized. To achieve a high cell density, the glycerol (50% glycerol, 4.3 mL/L PTM_1, feeding rate: 18 mL/h·L) fed-batch phase was initiated and lasted for 6-10 h. Production phase consisted of a methanol fed-batch phase when cells were induced by methanol (100% methanol plus 12 mL/L PTM_1 salts). The methanol feed rate was gradually increased over a period of 6 h-6 mL/h and the fermentation continued for an additional 46-92 h. Expressed hdHGF in culture supernatants was analyzed by SDS-PAGE and the concentration of secreted total supernant proteins was also determined at different intervals of induction phase using the standard curve analysis of human serum albumin (HSA).

Western blotting of rhdHGF

The purified recombinant hdHGF was run in 15% Tris-tricine electrophoresis[20] and then transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with rabbit anti-hdHGF antibody as described previously[19].

RESULTS

Molecular cloning of hdHGF fragments

Based on the hdHGF sequence reported by Nakamura *et al*[16], we designed three pairs of PCR primers for amplified hdHGF fragments. A forward primer P1 and a reverse primer P2 and another forward primer P3 and

its reverse primer P4 were designed to synthesize the two fragments. The two fragments of hdHGF PCR products about 1 570 (F1) and 970 bp (F2), respectively, were also clearly seen in 1% agarose gel electrophoresis stained with 5 mg/mL ethidium bromide amplified with P1-P2 and P3-P4 primer pairs (Figure 2A). Overlapping-fragment PCR amplification using F1 and F2 fragments as templates was performed by routine PCR procedure using a pair of primers M1 and M2 and the PCR product of full-length gene of hdHGF was revealed (Figure 2B).

Construction of the expression plasmid

A cDNA encoding the mature form of hdHGF was used in these experiments. This cDNA consisted of 2.1 kb (Figure 2B) with an open-reading frame encoding a 669 amino acid peptide. The DNA fragment encoding the mature hdHGF was digested with *XhoI* and *NotI* from pUC19 vector and cloned into the same enzyme digested vector pPIC9 downstream of the alcohol oxidase I (AOXI) promoter (Figures 1 and 3). The resultant

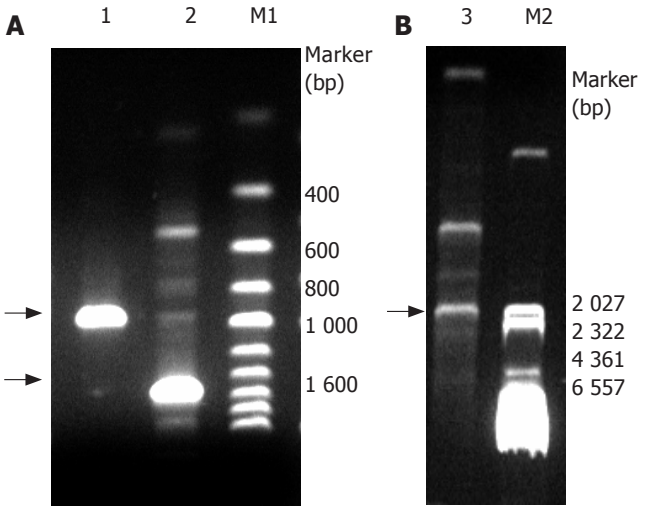

Figure 2 Analysis of PCR products by 1% agarose electrophoresis (**A** and **B**). Lane 1: fragment (F2) amplified with P3-P4 primer pair; lane 2: fragment (F1) amplified with P1-P2 primer pair; lane 3: fragment (whole length) amplified with M1-M2 primer pair; M1: 200-bp ladder marker; M2: λ+*Hind*III marker. The arrowheads show the bands of the interesting fragments

construct harbored a single open reading frame encoding a 85 amino acid translation product consisting of the α-factor secretion leader peptide (Figures 1 and 3). Prior to the secretion of the peptide into the culture medium, the signal peptide should be cleaved off by the *KEX2* gene products at the site (Glu-Lys-Arg-X) (Figure 1). The integrity of the recombinant plasmid was confirmed by direct DNA sequencing. Enzyme identification of the recombinant plasmids of pPIC9-hdHGF digested by *BamHI* and *NotI* is shown in Figure 4. This constructed vector was linearized with *SalI* and transformed into the competent cells of *P pastoris* GS115. The transformants were selected on MD plates and confirmed by colony PCR. Forty-one colonies presenting strong amplification

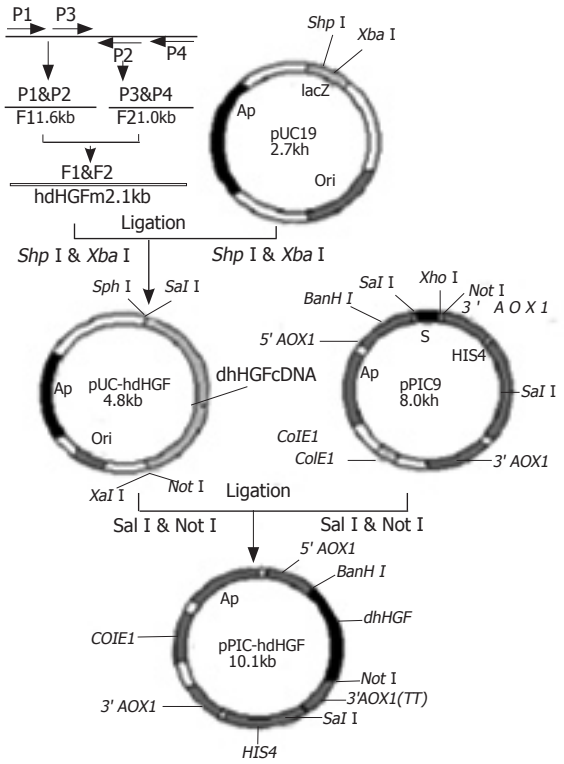

Figure 3 Cloning of hdHGF cDNA and construction of expression vectors pUC-hdHGF and pPIC9-hdHGF.

products were used for small-scale expression trials and the amount of the recombinant peptide was determined by SDS-PAGE.

Expression of recombinant hdHGF in shake flask

We investigated the expression of recombinant hdHGF by both Mut^s and Mut^+ (GS115) strains in shake flasks. Since SDS-PAGE analysis revealed that the hdHGF level expressed by Mut^s strain was much higher than that of Mut^+ (data not shown), Mut^s was chosen for the expression of th e growth factor. In addition, Mut^s phenotype was selected over the Mut^+ phenotype because

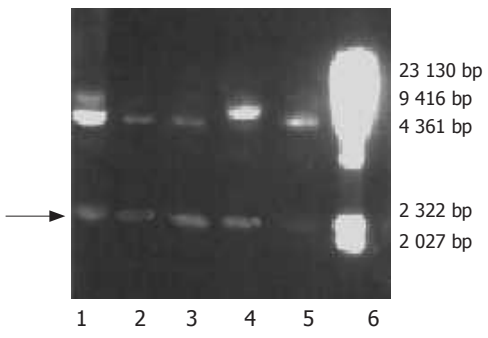

Figure 4 Agarose electrophoresis analysis of restriction enzyme mapping of recombinant plasmids of pPIC9-hdHGF. Lanes 1-5: recombinant plasmids of pPIC9-hdHGF digested by *Bam*HI and *Not*I; lane 6: λ-DNA/*Hind*III marker. The arrowhead reveals bands of the interesting fragment.

of the latter's higher oxygen requirement that could result in oxygen-deficient conditions within the bioreactor. Fifty transformants (Mut^s) were used for the expression studies in shake flask experiments and secretion of the recombinant hdHGF into the culture medium was determined by SDS-PAGE analysis. The expression experiments were performed to screen out four high-level expression strains of hdHGF, which were named as HG209, HG211, HG305, and HG309 (Figure 5).

Expression of recombinant hdHGF in fermenter cultures

The selected clones with the highest expression level were chosen for fed-batch cultivation. A time-course study of

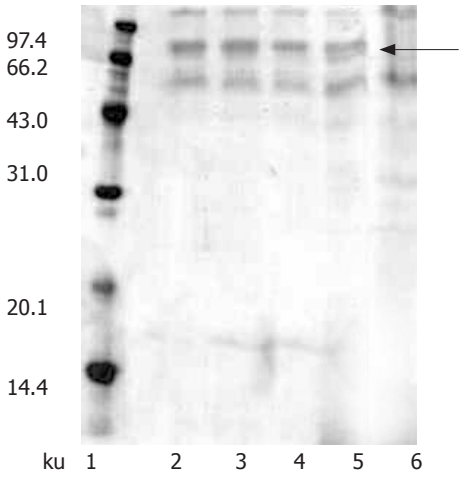

Figure 5 Fifteen percentage SDS-PAGE analysis of high expression strains in shake-flask culture.Lane 1: LMW marker; lane 2: GH209; lane 3: HG211; lane 4: HG305; lane 5:HG309; lane 6: GS115. The arrowhead shows bands of the interesting protein.

secretion of hdHGF revealed a gradual accumulation of recombinant cytokines. The effect of induction pH on the production of recombinant cytokines was investigated. *P pastoris* is known to grow over a wide pH range from 3 to 7, with a minimal effect of pH on the growth rate. However, pH could significantly affect the productivity of secreted recombinant proteins in the fermentation broth. To find out the optimal pH for the expression of recombinant hdHGF, we conducted experiments with pH between 3.5 and 6.5 during the fed-batch production phase. The highest yield of recombinant hdHGF was observed at pH 5.5 (Figure 6).

Protein expression was initiated by changing carbon source from glycerol to methanol. At first, we attempted to express hdHGF in baffled shake flasks and obtained about 50 mg/L of hdHGF secreted into the medium after 72-h induction. For more systematic production of hdHGF, we expressed the protein using fermenter cultures. Figure 6 shows the secretion level of hdHGF using 5 L fermenter. Upon depletion of glycerol, the dry cell weight reached 45.6 g/L. A glycerol fed-batch phase was performed for an additional 6-12 h and the cell biomass reached at

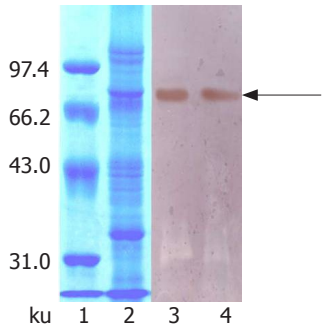

Figure 6 Fifteen percentage SDS-PAGE and Western blotting analysis of the 5-L fed-batch high cell density fermentation of GH209. Lane 1: LMW marker; lane 2: 10% SDS-PAGE result; lanes 3 and 4: Western blotting result. The arrowhead shows bands of the interesting protein.

approximately 135 g (DCW)/L. Induction of hdHGF was initiated by the addition of 100% methanol containing 12 mL of PTM1 trace salts/L. Sample analysis at different intervals was also performed to show the increasing amounts of recombinant hdHGF presented in the culture medium with increasing induction time until 96 h. Dissolved oxygen (DO) was monitored by DO sensor and the oxygen transfer rate 1 min after turning off the carbon source feed. Dissolved oxygen was maintained between 20% and 30% air saturation in the two-phase fermentation. The secreted total supernant protein concentration in the induction phase was traced and observed to attain the high-level expression of more than 8.0 g/L after 84-96 h of induction cultivation, which was determined using the standard curve analysis of human serum albumin (HSA). The scanning result showed that the secreted rhdHGF protein band (in lane 3 of Figure 6) achieved about 12.3% of the total supernatant proteins. By comparison with the standard protein markers, the estimated product of rhdHGF was 500-900 mg/L. The maximum secretion yield was approximately 980 mg/L (Figure 6). Upon induction by methanol, four clones secreted a specific 80-ku protein with the same size as the standard HGF. The productivity varied among the four high-level expression strains of hdHGF.

These clones indicated that the highest product of rhdHGF was named HG209 (GS115/pPIC9-hdHGF) and selected for further analysis. Western blotting analysis showed that the 80-ku protein band from HG209 (GS115/pPIC9-hdHGF) reacted specifically with the rabbit anti-hHGF antiserum (Figure 6). The N-terminal sequence of the recombinant hdHGF was determined to be PALKI, which was identical to the N-terminal sequence of native hdHGF (Figure 1).

DISCUSSION

hdHGF is a large complex protein comprising of 669 amino acids. It is the most potent multifunctional cytokine on the regeneration of hepatocytes and kidney epithelial cells compared to wild-type human HGF[20]. It can promote cell division, migration, and differentiation.

Its receptor is the product of oncogene c-met. Besides being a nutritional factor of liver and kidney, hdHGF also promotes angiogenesis for peripheral artery disease and myocardial ischemia[20] and can affect synthesis of extracellular matrix and matrix metalloproteinases and tissue inhibitor of metalloproteinases in autosomal dominant polycystic kidney disease cyst-lining epithelial cells[21].

Since the concentration of native HGF in plasma is very low, purification of HGF from plasma is very difficult. It was reported that HGF is expressed in foreign gene expression systems such as mammalian cells, CHO cells[22,23], insect cells[24], and gene therapy[25]. Dang *et al*[26] and Li *et al*[27]. reported that the HGF gene is expressed in *E coli* and *P pastoris*. However, the expression of recombinant-deleted variants of human hepatocyte growth factor (hdHGF) has not been reported in yeast expression system. Therefore, in this investigation, we used the methylotrophic yeast *P pastoris* as the host for the high-level expression and secretion of recombinant hdHGF. Recombinant hdHGF was successfully secreted by *P pastoris* and the productivity of secreted total supernatant protein concentration attained high-level expression of more than 8.0 g/L and the ratio of rhdHGF band area was about 12.3% of the total band area scanned by SDS-PAGE analysis, which estimated the product of rhdHGF to be 500-900 mg/L. It had an approximately fivefold increase in productivity compared to that of HGF expressed in *P pastoris*[27]. Western blot analysis showed that the 80-ku protein band of GS115 (pPIC9-hdHGF) reacted specifically with the rabbit anti-hHGF antibody. N-terminal sequencing revealed that recombinant rhdHGF had the correct N-terminal amino acid sequence. These results suggest that the optimization of bias codons of *P pastoris* encoding N-and C-terminal amino acids of hdHGF via PCR-mediated codon replacement can acquire high-level expression of foreign genes in *P pastoris*. Nakamura *et al*[16]. reported that native hdHGF is composed of an alpha-subunit of 440 amino acids (MW 69 ku) and a beta-subunit of 234 amino acids (MW 34 ku). However, we found that the recombinant hdHGF produced by *P pastoris* was translated as a single-chain polypeptide comprising of 669aa, which was not cut by the proteolytic cleavage of the human pro-hormone between amino acids R494 and V495 in host cells of *P pastoris*.

In conclusion, though further characterization, bioassay, and optimization of the expression and cultivation of recombinant hdHGF by *P pastoris* are required, this expression system of hdHGF is expected to be a powerful tool in the industrial production of this foreign protein.

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