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Secretory Expression of Human Growth Hormone in *Saccharomyces cerevisiae* Using Three Different Leader Sequences

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Abstract A recombinant human growth hormone (hGH) was expressed as a secretory product in the yeast *Saccharomyces cerevisiae*. Three different leader sequences derived from the mating factor α1 (MFα1), inulinase and invertase were used to direct the secretion of hGH into the extracellular medium. Among three leader sequences tested, the inulinase leader sequence was found to be the most efficient in the secretory expression of hGH. In contrast, no hGH was detected in the extracellular medium with the invertase leader sequence. After 48 h shake-flask culture, the yields of hGH secreted into the medium by the invertase, MFα1, inulinase and invertase leader sequences were approximately 0, 0.3 and 0.9 mg/L, respectively. The secretion efficiencies were also found to be 0, 3.8 and 13% for the invertase, MFα1 and inulinase leader sequences, respectively.

Keywords: human growth hormone, secretion, leader sequence, *Saccharomyces cerevisiae*

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been widely used as a recombinant host for the expression of a variety of heterologous proteins because of its advantages over other host systems. It is non-pathogenic, produces no endotoxins, and can be cultivated on simple media to a high-cell density [1]. In addition, yeast has been considered as a preferred host system for the production of human secretory proteins due to the similarities between yeast and animal cell secretion pathways.

Human growth hormone (hGH) is a pituitary-derived polypeptide with biological activities such as protein synthesis, cell proliferation and metabolism. Because of these biological activities, the hGH has therapeutic applications in the treatment of dwarfism, bone fractures, skin burns and bleeding ulcers. To date, *Escherichia coli* has been more frequently used as an expression host for the mass production of recombinant hGH [2-6]. However, direct expression of hGH in *E. coli* cytoplasm results in the synthesis of methionyl hGH (m-hGH). It has been reported that the methionyl residue at the N-terminus of m-hGH may play a role in antibody formation in patients treated with m-hGH [7]. To overcome this problem, several attempts have been made, including the secretion of hGH [8-10] and the enzymatic cleavage of N-terminal fusion partner followed by fusion protein expression [11].

In the present work, we describe the secretory expression of hGH from *S. cerevisiae* under the control of a galactose-inducible promoter. To investigate the effect of the leader sequence on the secretion of hGH, the studies with three different leader sequences, the mating factor α1 (MFα1), the inulinase, and the invertase leader, were carried out.

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MATERIALS AND METHODS

Strains and Transformation

The yeast strain used in this study was *Saccharomyces cerevisiae* 2805 (*MATα pep4::HIS3 pro 1-δ can1 GAL2 his3 δ ura3-52*) [12]. Yeast transformation was carried out by the lithium acetate method [13]. *Escherichia coli* DH5α was used for bacterial transformation and plasmid propagation.

Construction of Human Growth Hormone Expression Vectors

Construction of pYGMF-hGH

A 250bp fragment encoding MFα prepro leader sequence was amplified by polymerase chain reaction (PCR) using pYGLP10 [14] as a template and two primers: 5'-CAATGAATTCGATTAAAAGAATGAGATTTC-3' (new *Eco*RI site, 5' to start codon) and 5'-TTTATCTAGAGATACCCCTTCTTC TTTAGC-3' (new *Xba*I site). Primers were synthesized by DNA synthesizer (Model 391, Applied Biosystems Co., USA), and PCR was carried out in a thermocycler (Perkin Elmer 2400, USA). DNA was amplified by 20 cycles under standard conditions with annealing at 55°C and elongation at 72°C. The PCR product was gel purified using QIAEX II gel extraction kit (QIAGEN, Germany) and digested with *Eco*RI and *Xba*I (Boehringer Mannheim, Germany).

The hGH cDNA-coding sequence used was isolated from human pituitary cDNA library. The coding region of hGH was amplified by PCR with primers: 5′-CATTAGTCTAGATTCCCAACCATTCCCTTATCCAG G-3′ which adds *Xba*I site and 5′-CATCGCGTCGA-CCTAGAAGCCACAGCTGCCCTCCACACA-3′ which adds *Sal*I site. The PCR–amplified products of 580 bp was gel purified, digested with *Xba*I and *Sal*I and cloned into pBluescript SK+ with a piece of MFα1 prepro leader sequence, resulting in the plasmid pBMF-hGH. To achieve expression of fusion protein, the pBMF-hGH was digested with *Eco*RI and *Sal*I. The fragment isolated from the plasmid pBMF-hGH was directly ligated into pYGLP10 digested with the same restriction enzymes, resulting in the plasmid pYGMF-hGH.

Construction of pYGINU-hGH

The *INU1A* signal sequence was amplified by PCR using pYGILP10 [14] as a template with two following oligonucleotides, 5′-CACGAATTCGGATCCATGAAG-TTAGCATACTCCCTCTTG-3′ and 5′-TGAGATT CTC-TTGTAATTAATAACTGAAGCACTGAC-3′. The amplified 70 bp fragment was digested with EcoRI and AsnI. The hGH cDNA coding sequence was amplified using pYGMF-hGH. The primers used were 5′-TCAGTTA-TTAATTACAAGAGATTCCCAACCATTCCCTTATCC-3′ which adds AsnI site and 5′-CATCGCGTCGACCTA-GAAGCCACAGCTGCCCT CCACACA-3′ which adds SalI site to it. The PCR product was gel purified and digested with AsnI and SalI. The fragments, INU1A signal sequence and hGH-coding sequence, were inserted into pBluescript SK+ and then ligated into pYGLP10 cut with EcoRI and SalI. The construct was named pYGINU-hGH.

Construction of pYGINV-hGH

A synthetic gene of invertase signal sequence was constructed from the following oligonucleotides: (I) 5′-ATGCTTTTGCAAGCTTTCC TTTTCCTTTTG-3′, (II) 5′-GCTGGTTTTGCAGCCAAAATATCTGCA–3′, (III) 5′-ACCAGCCAAAAGGAAA AGGAAAGCTTGCA-AAAGCAT-3′, and (IV) 5′-TGCAG ATAT TTTGGCTG-CAAA-3′. These oligonucleotides were 5′-phosphorylated with T4 polynucleotide kinase, except 3′ ends, annealed and ligated using T4 DNA ligase. The introduction of restriction enzyme site was achieved by PCR using synthetic invertase signal sequence and two primers: 5′-TACGAATTCATGCTTTTGCAAGCTTTC-3′ (new 5′ *Eco*RI site) and 5′-TGCAGATATTTTGGCT-GCAAA-3′ (3′ blunt end). The PCR product using pfu polymerase was gel purified and digested with *Eco*RI.

The coding region of hGH was also isolated by PCR with primers: 5′-TTCCCAACCATTCCCTTATCCA-GGCTTTTT-3′ (5′ blunt end) and 5′-CATCGC GT-CGACCTAGAAGCCACAGCTGCCCTCCACACA-3′ (3′ *Sal*I site). The PCR – amplified products of 580 bp was gel purified, digested with *Sal*I and cloned into pBluescript SK+ with a piece of invertase signal sequence, resulting in the plasmid pBINV-hGH. To achieve expression of fusion protein, the pBINV-hGH was digested with *Eco*RI and *Sal*I. The fragment isolated from plasmid was directly ligated into pYGLP10 digested with the same restriction enzymes. This construct was named pYGINV-hGH.

Media and Culture Conditions

The YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) was used for the cultivation of host and yeast transformants. The YNBCAD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.5% casamino acids) was used for the selection of yeast transformants and also for the seed culture. For the induction of hGH gene, the yeast transformants were grown in shake-flasks containing the YPDG medium (1% yeast extract, 2% bactopeptone, 1% glucose, and 1% galactose). Yeast extract, bactopeptone, yeast nitrogen base without amino acids, and casamino acids were purchased from Difco (USA).

Immunoblot Analysis of Human Growth Hormone

After 1 mL of the culture broth was taken from the shake-flasks, the cell precipitates and extracellular medium were separated by centrifugation. To investigate the secretion of hGH, the cell precipitates was suspended in 400 μL of lyticase buffer (1.2 M sorbitol, 0.1% β-mercaptoethanol, 10 mM NaN_3, 50 mM potassium phosphate; pH 7.5), treated with 200 μg of the lyticase (Sigma Chem. Co., USA) for 60 min at 37°C and distrupted by vigorous mixing with glass bead (0.4 to 0.5 mm). After centrifugation at 15,000 rpm for 10 min, the supernatant (intracellular fraction) was harvested, added into 100 μL of 5× Laemmli lysis buffer and boiled. The extracellular medium was precipitated with TCA and DOC. After centrifugation, the precipitates were suspended in 30 μL of 1× Laemmli lysis buffer and boiled. The intracellular and extracellular fractions were subjected to 15% SDS-PAGE and immunoblotting. The proteins separated by SDS-PAGE were immunoblotted with a mouse anti-hGH monoclonal antibody purchased from Sigma Chem. Co. The amount of hGH was quantified by measuring the staining intensity of recombinant hGH on a nitrocellulose filter with a densitometer (Bio-Rad GS-700, CA, USA) by using the purified hGH as a standard.

RESULTS AND DISCUSSION

Design and Construction of hGH Secretion Vectors

Proteins secreted from yeast possess a hydrophobic amino-terminal extension, called a leader peptide, which mediates cotranslational translocation of the proteins into the endoplasmic reticulum which is the first stage in the secretion pathway. The leader peptide is known to influence the quantity and quality of the

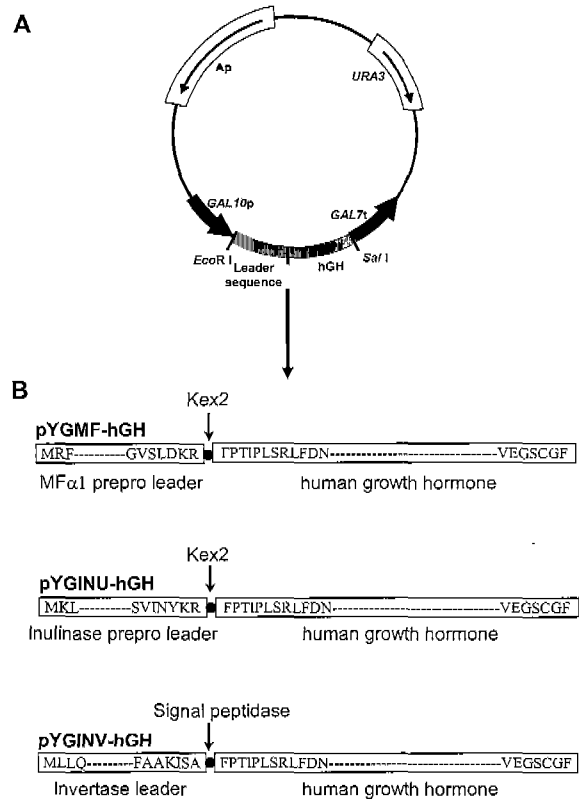

Fig. 1. (A) Basic structure of the plasmid used in this study and (B) amino acid sequences representing the junction regions between three different leader peptides and human growth hormone.

secreted protein. Hence, the choice of an appropriate leader sequence is very important in designing secretion systems for heterologous proteins to maximize the yield and fidelity of the product.

In this study, three different expression vectors were constructed to investigate the effect of leader sequence on the secretion of hGH in *S. cerevisiae*. They were designed to direct the secretion of hGH by the aid of one of the following leader sequences, the MFα1 prepro leader, the inulinase (INU1A) prepro leader and the invertase (INV) leader sequence, and named pYGMF-hGH, pYGINU-hGH and pYGINV-hGH, respectively (Fig. 1B). The transcriptions of the fused genes in all the plasmids were designed to be regulated by *GAL10* promoter and *GAL7* transcription terminator (Fig. 1A). In cases of MFα1 prepro leader- and inulinase leader-directed secretion, the carboxy-terminal dibasic residue, Lys-Arg, of the leader peptides is processed by the action of KEX2 endoproteinase. On the other hand, the invertase leader peptide is processed by the signal peptidase.

Effects of Leader Sequences on Expression and Secretion of hGH

To date, two different types of leader sequences have been used to secrete heterologous proteins from yeast: the use of a heterologous (mammalian or non-yeast) leader sequence; and the use of a homologous (yeast) leader peptide. Although some successful results have been reported in protein secretion with heterologous leader sequences [15], very low secretion efficiency has been often observed in the secretory expression of various heterologous proteins such as α-interferon [16] and α_1-antitrypsin [17]. This results from the difference in the secretory pathway in yeast and the higher eukaryotes. In this study, therefore, three different homologous leader sequences were employed to direct the secretion of hGH in *S. cerevisiae*, and their efficiency in hGH secretion was compared.

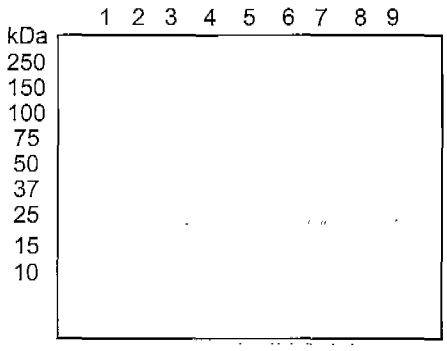

Fig. 2. Immunoblot analysis of the recombinant human growth hormone secreted by transformed *S. cerevisiae* 2805. The secretion of human growth hormone was directed by three different leader sequences. The cells were grown in YPDG medium for 48 h. Lanes: *1*, prestained protein molecular weight marker; *2* authentic hGH (3 μg); *3* authentic hGH (0.3 μg); *4, 5* invertase leader; *6, 7* MFα1 prepro leader; *7, 8* inulinase leader; *4, 6, 8* extracellular; *5, 6, 9* intracellular.

S. cerevisiae 2805 was transformed with each of the plasmids pYGMF-hGH, pYGINU-hGH and pYGINV-hGH, and the resulting transformants were cultured in YPDG medum. After 48 h of culture, the culture supernatants and whole cell extracts were analyzed by the immunoblot method (Fig. 2). Discrete 22-kDa protein bands were detected on the lanes for all the intracellular and extracellular fractions except the lane (Fig. 2, lane 4) for the extracellular fraction of *S. cerevisiae* 2805/pYGINV-hGH culture. This demonstrates that the invertase signal cannot direct the secretion of hGH into the extracellular medium. The expression level and secretion efficiency were compared in Table 1. The secretion efficiencies were found to be 0%, 3.8% and 13.0% for the invertase, MFα1 and inulinase leader sequences, respectively. The highest secreted amount of hGH (0.9 mg/L) was obtained with inulinase leader sequence.

The hGH bands in Fig. 2 comigrated with the authentic hGH purchased from Sigma Chem. Co., indicating correct processing between leader peptides and mature hPTH by processing enzymes. The MFα1 and inulinase leader sequences has a Lys-Arg sequence, which is

Table 1. Expression level and secretion efficiency of human growth hormone

Leader sequence	Human growth hormone conc. (μg/mL)			Secretion efficiency(%)[b]
	Intracelluar	Extracelluar	Total[a]	
Invertase	2.2	–	2.2	–
MFα1	7.5	0.3	7.8	3.8
Inulinase	6	0.9	6.9	13

[a] Total = extracelluar hGH (μg/mL) + intracellular hGH (μg/mL)
[b] Secretion efficiency (%) = extracellular hGH (μg/mL) / total hGH (μg/mL) × 100

a target site of the *KEX2* gene product, at the C-terminus of pro region. On the contrary, the cleavage between invertase leader and hGH occurs by a signal peptidase. The lack of proper processing of leader peptide could result in a defect in transport of the precursor from endoplasmic reticulum and Golgi [18]. Therefore, it appears that no hGH secretion observed in the secretion system with invertase leader sequences has no relation with the proteolytic processing between leader peptide and hGH.

The inulinase leader peptide, originated from *Kluyveromyces marxianus* inulinase, have shown much higher secretion efficiency than the MFα1 leader peptide in secretion of human lipocortin-1 and human interleukin-2 in *S. cerevisiae* [14]. In this study, it was also found that the inulinase leader sequence was the most efficient of the three different leader sequences used in the secretory expression of hGH from *S. cerevisiae*. In general, the secretion efficiency of heterologous proteins in *S. cerevisiae* is affected by a variety of genetic and environmental parameters such as leader peptide, host, promoter, culture conditions, etc. However, based on our studies protein secretion with inulinase leader sequence, it clearly appears to be a powerful leader sequence in directing the secretion of heterologous proteins in *S. cerevisiae*.

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