[BIO21] Biosynthetic production of human growth hormone gene in methylotrophic yeast, *Pichia pastoris*

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Introduction

Human growth hormone (hGH) is secreted from the anterior pituitary gland and exerts a wide variety of functions such as, IGF-1 production, protein synthesis, glucose metabolism, lipolysis, lipogenesis, and cell proliferation/differentiation (Isaksson *et al.*, 1985; Press, 1988; Thorner and Vance, 1988; Strobl and Thomas, 1994). Moreover, recently, hGH has attract much interest from scientists because it is found to not only retard biological aging, but also to significantly reverse many of the effects of aging (Klatz and Kahn, 1998). Besides 22K-hGH (22,000 Dalton), which is a major component composed of 191 amino acids, 20K-hGH (20,000 Dalton) is also known to be naturally secreted, being encoded by the same gene as 22K-hGH and lacking 15 amino acids (residues 32–46) by alternative messenger RNA (mRNA) splicing (DeNoto *et al.*, 1981; Lewis, *et al.*, 1980).

The methylotrophic yeast, *Pichia pastoris* has become the second most important yeast expression system after *S. cerevisiae*. *P. pastoris* has been developed as an expression system for high-level production of recombinant proteins (Buckholz & Gleeson, 1991; Cregg *et al.*, 1993). *Pichia* offers the features of (i) methanol-induced expression of heterologous genes via integration of the gene into the genome adjacent to the alcohol oxidase I (*AOX1*) gene promoter, (ii) growth to high cell density in inexpensive, chemically defined media, and (iii) the capacity to carry out post-translational modifications resembling those of mammalian cells (Cregg *et al.*, 1993; Romanos, 1995). Due to the absence of stable episomal vector in *Pichia*, recombinant plasmid has to be recombined into *Pichia* chromosome via gene conversion or integration events, thus generating two types of methanol utilization phenotype (Romanos, 1995). Integration of recombinant plasmid at *his4* or *AOX1* locus will revealed methanol utilization plus (Mut+) phenotype in GS115 and SMD1168 strains; on the other hand, replacement of *AOX1* locus via gene conversion will generate methanol utilization slow (Mut^S) phenotype in GS115 and SMD1168 strains.

In this report, we describe the construction of synthetic hGH gene and the expression of recombinant hGH in *Pichia* strains GS115 and SMD1168 via shake-flask culture.

Methods and materials

Human growth hormone (hGH) gene construction

The synthetic hGH gene was constructed using single-step assembly polymerase chain technique derived from DNA shuffling (Stemmer, 1994; Stemmer *et al.*, 1995). Twenty hGH oligonucleotides (Alpha DNA, Canada), each about 54 nucleotides in length, which collectively encode both strands of hGH gene were designed. The *Bam*HI and *Eco*RI sites were introduced at the 5'-end and 3'-end of hGH gene, respectively for the directional cloning into the *P. pastoris* expression vector, pPIC3.5K (Invitrogen, CA, USA). Apart from that, the Kozak consensus sequence *5'-GCCACCATG-3'* has been appended at the 5'-end downstream of the *Bam*HI site as initiation of translation. In this research project, *P. pastoris* GS115 and SMD1168 (Invitrogen, USA) were used as the expression hosts; thus, the oligos were designed with the consideration of *P. pastoris* codon usage.

The PCR product amplified by *Taq* DNA polymerase was purified using Qiagen QIAquick™ Gel Extraction kit and was ligated into the pGEM®-T vector (Promega). Subsequently, the recombinant plasmid was transformed into *E. coli* JM109 according to the standard procedure described in Sambrook *et al.* (1989). The plasmid extracted was then subjected to sequencing using ABI PRISM®

BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Biosystem) for nucleotide sequence confirmation.

Mutagenesis of synthetic hGH gene

Among four clones, where the recombinant plasmids have been verified by sequencing, the fourth clone's plasmid showed the most promising result with six mutations along the 612 bp hGH gene. The mutations comprised of a base insertion, base deletion at two locations, and base misincorporation at three different locations. Stratagene QuikChange® site-directed mutagenesis kit was used to repair the frame-shift and point mutations found in the DNA fragment. Thus, six pairs of PAGE purified mutagenic oligonucleotide primers (Alpha DNA, Canada), each about 45 nucleotides in length, were designed according to the protocol provided in the Stratagene QuikChange® site-directed mutagenesis instruction manual for the repair of six mutations at different locations.

Sequencing and cloning

Prior to sequencing for confirmation, the mutagenized recombinant plasmids were extracted using Promega Wizard® *Plus* SV Minipreps DNA Purification System. After sequencing, the recombinant plasmids were digested with *Eco*RI and *Bam*HI restriction enzymes to obtain the synthetic human growth hormone DNA fragment. The digested hGH DNA fragment was purified from ethidium bromide stained agarose gel using QIAquick® Gel Extraction Kit (QIAGEN) and ligated into *P. pastoris* expression vector, pPIC3.5K using T4 DNA ligase (Promega). pPIC3.5K (9004 bp) is an intracellular expression vector containing a strong alcohol oxidase 1 (AOX 1) promoter, which could be induced by methanol for high-level protein expression.

Transformation and selection of P. pastoris clones

P. pastoris strains GS115 (*his4*) and SMD1168 (*his4 pep4*) were used for expression of the hGH protein. Cell culture, electro-competent cells, transformation and selection of recombinant clones were performed as described in the *Pichia* User Manual (Invitrogen, USA). Prior to transformation, recombinant pPIC3.5K vector containing synthetic hGH gene was linearized

using *Bsp*EI restriction enzyme (New England Biolabs). 20 μg of purified *Bsp*EI-linearized recombinant plasmid was used in the electroporation transformation. Electroporated cells were plated on a dextrose-base regeneration medium lacking histidine (RDB) and incubated for 3 days at 30°C for histidine prototrophy screening. Randomly picked clones were subculture and lysed prior to direct PCR screening using 5'-*AOX1* and 3'-*AOX1* primers for methanol utilization (Mut) phenotype.

Induction of expression in shake-flask culture

Each of recombinant GS115 and SMD1168 His⁺ Mut⁺ clones were picked and subjected to expression in shake-flask culture according to method described in *Pichia* User Manual (Invitrogen, USA). Time course study of 0-120 h with interval duration of 24 h was carried out to determine the optimal protein expression time point, mean while, 100% methanol was added daily to a final concentration of 3% until induction was terminated.

SDS-PAGE and Western blots

SDS-PAGE according to a modified version of Laemmli's method (Laemmli, 1970) and Western blot analysis according to Towbin *et al*. (1979) were performed for recombinant hGH analysis. Bands were visualized using horseradish peroxidase-conjugated donkey anti-goat IgG antibody (Promega) and the ECL Western blotting kit (Amersham Pharmacia Biotech). The primary antibody used was goat polyclonal anti-hGH antibody (Santa Cruz Biotechnology, Inc.). 50 μg of total protein was analysed in 12.5% (v/v) SDS-PAGE following by western blot.

Results and discussion

We have sequenced four clones for the hGH DNA fragment amplified by Taq DNA Polymerase. The results showed that 20 oligonucleotides have been assembled accordingly. However, there were a few insertion, deletion and misincorporation of bases, which might be due to improper purification of the oligonucleotides. Hence, solution for reducing mutations in the synthetic gene is to use highly purified oligonucleotides containing no (n-1) failure

sequence. The mutations were successfully repaired using Stratagene QuikChange® site-directed mutagenesis kit. We have also successfully cloned the synthetic human growth hormone gene into *Pichia* expression vector.

By linearizing the recombinant vector at a restriction enzyme site located in *HIS4* gene, using *Bsp*EI enzyme, Mut[+] recombinants were conveniently generated either in GS115 or SMD1168 strains via single crossover integration event. GS115/pPIC3.5K-hGH [2/5] and SMD1168/pPIC3.5K [30] were His[+] Mut[+] recombinant clones randomly picked for protein expression. Direct PCR using both recombinant clones' cell lysates showed two bands on 1.2% (w/v) agarose gel. The ~2.2 kb band and the ~800 bp band correspond to the *AOX1* gene in *Pichia* genome and the synthetic hGH gene, respectively. The presence of *AOX1* gene indicates that the recombinant clones were Mut[+]. The PCR results showed that the synthetic hGH gene was integrated into the genome of recombinant *P. pastoris*, which proved that recombinant *P. pastoris* had excellent genetic stability in terms of the properties of growth and expression of recombinant hGH protein.

A total protein of 50 μg of each cell lysate samples, including the corresponding expression condition control strain GS115 β-gal (Mut[+]) were analysed on 12.5% (v/v) SDS-PAGE. Recombinant hGH was not detected on 12.5% (v/v) SDS gel when compared to the background control, which was the expression product of GS115 strain transformed with parent vector. Nevertheless, the expression condition control, β-galactosidase of GS115 β-gal (Mut[+]) control strain was successfully expressed as detected on the gel at 117 kDa, which indicates that the expression condition was efficient.

Western blot analysis of the cell lysates showed that the recombinant hGH was successfully expressed in both recombinant clones, GS115/pPIC3.5K-hGH [2/5] and SMD1168/pPIC3.5K [30]. Two bands with the size of ~22 kDa and ~20 kDa were present on the X-ray film. The expected size of recombinant hGH is 22 kDa and the ~20 kDa product obtained could be the non-22 kDa growth hormone isoform (Wood, 2001). No reaction was detected over the lanes containing total protein at non-induction time

point (0 h) for both of the recombinant strains; strongly indicate that the binding of the recombinant protein by goat anti-hGH was significant and specific. Time course study was carried out to determine the optimal time post-induction to harvest. Nevertheless, no significant increase of protein expression level was observed for both recombinant clones after 24 h (Figure 1.1 and Figure 1.2).
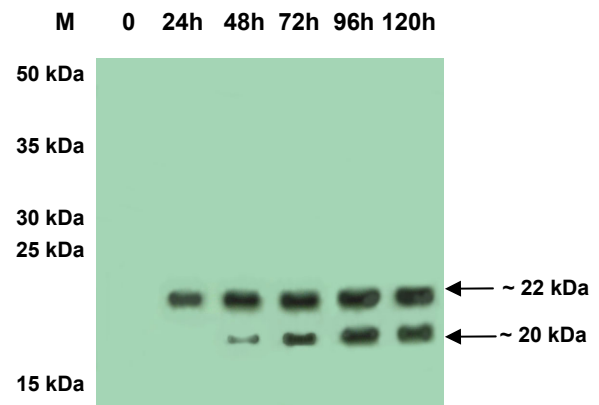

FIGURE 1.1 Immunodetection (Western blot) of recombinant hGH protein expressed in recombinant clone GS115/pPIC3.5K-hGH [2/5] using polyclonal goat anti-hGH. Lane (1), Rainbow protein molecular weight markers (Amersham Pharmacia Biotech); lane (2)-(7), time course study with an interval time of 24 h (from 0-120 h) of recombinant GS115/ pPIC3.5K-hGH [2/5].
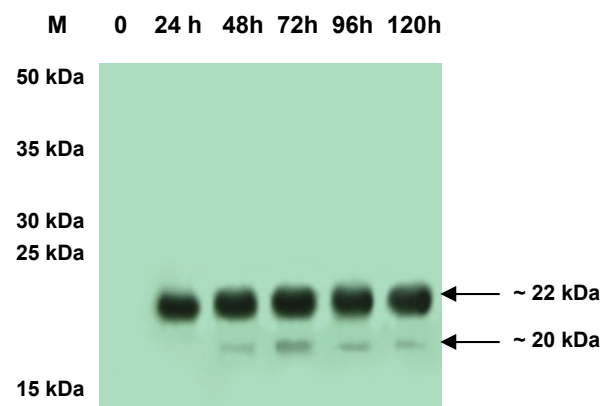

FIGURE 1.2 Immunodetection (Western blot) of recombinant hGH protein expressed in recombinant clone SMD1168/pPIC3.5K-hGH [30] using polyclonal goat anti-hGH. Lane (1), Rainbow protein molecular weight markers (Amersham Pharmacia Biotech); lane (2)-(7), time course study with an interval time of 24 h (from 0-

120 h) of recombinant SMD1168/pPIC3.5K-hGH [30].

In summary, the results obtained in Western blotting revealed that the recombinant hGH protein is expressed stably in *P. pastoris* in shake-flask culture. Further study will be carried out to increase the protein expression level and to purify the recombinant protein for biological activity analysis, as well as to verify the presence of non-22 kDa hGH isoforms in the results obtained.

Acknowledgements:

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