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Heterologous Expression of Human Granulocyte-Colony Stimulating Factor in *Pichia pastoris*

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Abstract: Human granulocyte-colony stimulating factor (hG-CSF) was expression in a eukaryotic system, *Pichia pastoris*. For this, hG-CSF cDNA was amplified using reverse transcription-polymerase chain reaction (RT-PCR) and inserted into pPIC9 vector. The recombinant plasmid was electroporated into the *P. pastoris*. The recombinant hG-CSF was successfully expressed by in this system. The protein of approximately 18 kDa was secreted into the culture supernatant when induced with methanol. Western blot showed that the recombinant hG-CSF expressed in *P. pastoris* had a high specificity to monoclonal antibody against hG-CSF. The expression of hG-CSF was determined 3.1 mg L^{-1} in shake flask culture by ELISA method.

Key words: Eukaryotic expression system, gene cloning, human granulocyte-colony stimulating factor; *Pichia pastoris*

INTRODUCTION

Human granulocyte-colony stimulating factor (hG-CSF), is a hematopoietic growth factor that stimulates the proliferation and differentiation process of neutrophil precursor cells and it enhances some of the functional properties of mature neutrophils. The hG-CSF has been widely used hematopoietic growth factor due to its proven efficacy against different forms of neutropenia and chemotherapy induced leucopenia (Dale, 2002). Furthermore, hG-CSF stimulates the mobilization of progenitor cells for autologous or allogenic transplantation (Viret *et al*., 2006). The hG-CSF has been cited for use in treatment of other human health problems, such as myocardial infarction (Oh *et al*., 2006) and cerebral ischaemia (Lu and Xiao, 2006).

The methylotrophic yeast *Pichia pastoris* is a powerful tool for the heterologous expression of proteins (Cereghino and Cregg, 2000). The increasing popularity of this expression system can be attributed to several factors, such as the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris*, many eukaryotic posttranslational modifications (Hasslacher *et al*., 1997; Hollenberg and Gellissent, 1997; Sreekrishna *et al*., 1997). Protein expression in *P. pastoris* is based on the use of the alcohol oxidase 1 (AOX1) promoter. This promoter is strongly induced by methanol (Li *et al*., 2007).

The aim of this study was the expression of hG-CSF as a secretory protein in *P. pastoris*. It is, because, this yeast has several advantages in comparison of prokaryotic and eukaryotic expression systems. Culture and genetic manipulation of the system is simple. Unlike prokaryote expression systems, posttranslational modifications such as glycosylation, are performed by *P. pastoris*. G-CSF is a glycosylated protein, therefore, it seems that yeast expression system is better than *E. coli*. Expression of hG-CSF in a secretory manner, may be, has more advantages than intracellular and preiplasmic expression in prokaryotes. It is because of, native G-CSF is secreted into blood. Sectetory proteins need a simple strategy for purification. Yeast expression systems are faster, simpler and less expensive to use than mammalian expression systems.

MATERIALS AND METHODS

Bacteria, yeast and growth media: *E. coli* strain TOP10F was used for the propagation of recombinant plasmid. *E. coli* transformants were selected on Luria-Bertani (LB) plates pH 7 (0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 1.5% bacteriological agar) with 100 µg mL^{-1} ampicilin. *P. pastoris* strain GS115 (Invitrogen) was used for hG-CSF protein expression. The *P. pastoris* GS115 strain was cultured in YPD medium

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(1% yeast extract, 2% peptone, 2% dextrose plus 2% agar in plates). *P. pastoris* transformants were selected on minimal dextrose medium (MD) (1.34% yeast nitrogen base without amino acids, 4×10^{-5}% biotin, 2% dextrose and 2% agar) and minimal methanol medium (MM) (1.34% yeast nitrogen base without amino acids, 4×10^{-5}% biotin, 0.05% methanol and 2% agar). *P. pastoris* growth and induction media were Buffered Minimal Glycerol-Complex Medium (BMGY) (1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin and 1% (v/v) glycerol) and buffered minimal methanol complex medium (BMMY) (same as BMGY except that glycerol was replaced by 2% (v/v) methanol), respectively.

Expression vector construction: The hG-CSF cDNA was amplified by RT-PCR using F (5'-AAACTCGAG-AAAAGAAC ACCCCTAGGCCCTGC-3') and R (5'-CGGAATTCTTACTAGGGCTGGGCAAGGTG G-3') primers, which were designed in this study. The F primer contained XhoI site and the R primer contained EcoRI site and two stop codons. Primers were designed to generate the 569 bp of PCR fragment containing an open reading frame for hG-CSF lacking its signal peptide. The RT-PCR reaction was carried out as described earlier (Saeedinia *et al*., 2003). The hG-CSF cDNA was digested by XhoI and EcoRI restriction enzymes (Fermentas) and it was ligated with pPIC9 vector (Invitrogen) under the control of AOX1 promoter with *Saccharomyces cerevisiae* alpha factor secretion signal (α-MF). The resultant recombinant vector was named as pPIC9/hG-CSF (Fig. 1). The α-MF present in the vector upstream to the hG-CSF gene was used to make the target protein to secrete into the medium. The recombinant vector was transformed into *E. coli* for mass production. Plasmid DNA was extracted and analyzed for the presence of hG-CSF fragment. A recombinant plasmid was sequenced using vector specific primers as per the manufacturer's recommendation.

Transformation of *P. pastoris*: *P. pastoris* strain GS115 cells were made electrocompetent following manufacturer's recommendations (Invitrogen). Approximately 10 μg of recombinant expression plasmid pPIC9/hG-CSF was linearized by digesting with *Bgl*II enzyme to get His^+Mut^+ transformant in *P. pastoris* GS115 cells. Electroporation was carried out by a Gene Pulser (Bio-Rad) with pulse settings of 25 μF, 1.5 kV and 200 Ω. Transformants were plated onto MD plates and incubated at 30°C for 4 days. The parent pPIC9 without insert, linearized with BglII was also transformed as negative control. Transformants of methanol utilization plus (Mut^+)

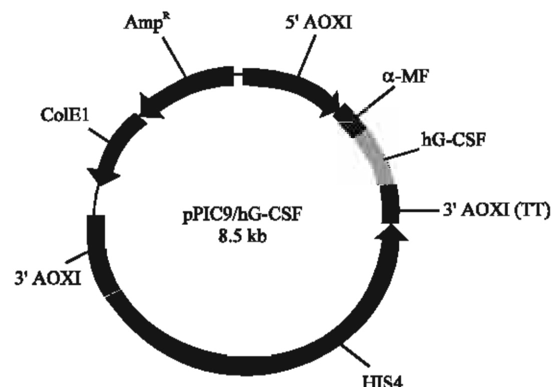

Fig. 1: Structure of pPIC9/hG-CSF expression vector. The hG-CSF cDNA was cloned in pPIC9 plasmid under the control of AOX1 promoter with *S. cerevisiae* α-mating factor secretion signal

phenotype were selected by growing on MM and MD plates. Transformants bearing the chromosomally integrated copies of the pPIC9/hG-CSF were then detected by a genomic PCR assay using the 5' and 3' AOX1 primers.

Expression of hG-CSF protein: The colonies that were found positive with genomic PCR assay were selected for induction. Positive His^+ Mut^+ colonies were inoculated into 50 mL BMGY medium taken in 250 mL conical flask along with negative control (*Pichia* transformed with pPIC9 without insert) and were incubated at 28°C in a shaker incubator at 250 rpm until the culture reached an A600 of 2-6 as per the manufacturer's recommendation. The cells were harvested by centrifugation at 3,000 g for 5 min at room temperature and the cell pellet was resuspended in BMMY medium to an A600 of 1.0 in a conical flask for induction with proper aeration. Incubation was continued at 28°C in a shaker incubator at 250 rpm with the addition of methanol to achieve concentration 2% at every 24 h to sustain the induction. Different induction periods ranging from 24-144 h were also tested along with different methanol concentrations to find the optimal expression conditions. After every induction with varied methanol concentration and incubation periods, proteins in the culture supernatants were precipitated using 30% ammonium sulfate solution and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot: The expressed protein samples were separated by electrophoresis on a 12% SDS-PAGE gel. The expression of hG-CSF was confirmed with the anti-hG-CSF specific monoclonal antibody (Sigma) through

western blotting. Briefly, proteins were transferred from the gel onto nitrocellulose membrane. After transfer, the membrane was probed sequentially with the anti-hG-CSF specific monoclonal antibody (2 μg mL^{-1}) and 1:1000 dilution of horse radish peroxidase (HRP) labeled goat anti-mouse immunoglobulin G (IgG). Protein samples were visualized with DAB/H_2O_2 (Diaminobanzidine/hydrogen peroxide) chromogen/substrate solution.

Enzyme-linked immunosorbent assay: The expression level of recombinant hG-CSF was measured by Enzyme-Linked Immunosorbent Assay (ELISA). Standard curve was designed by serial dilutions of the commercial hG-CSF from 0.1 to 50 μg mL^{-1}. Briefly, the expression level of hG-CSF was determined by coating of culture supernatants in microtiter plate for 2 h at 37°C. Following three washes with Phosphate Buffered Saline (PBS), blocking buffer was added and incubated at room temperature for an hour. Anti-hG-CSF monoclonal antibody was added to each well at a dilution of 1/1000 in PBS and incubated for an hour at room temperature. A polyclonal goat anti-mouse immunoglobulins/HRP (Dakocytomation) was diluted 1:2000 in PBS and added to each well and incubated as before. Then, freshly prepared substrate-chromogen mixture (hydrogen peroxide and tetramethyl-benzidine) was added to each well. Incubation was carried out at room temperature for 15 min. The reaction was stopped by adding an equal volume of 1 N H_2SO_4 to each well.

RESULTS

Gene cloning: The 569 bp fragment of hG-CSF gene was isolated through PCR (Fig. 2A). The gene was cloned in *Pichia* expression vector pPIC9 at XhoI and EcoRI sites and the recombinant construct; pPIC9/hG-CSF was transformed into *E. coli* TOP10F' for its mass production before transforming in *Pichia*.

Yeast transformation: The hG-CSF expression construct, as well as the intact parent vector (pPIC9) were linearized by BglII and used to transform the *P. pastoris* GS115 strain. The Mut^+ phenotype of yeast transformants were isolated by their normal growth on both MM and MD plates. The PCR amplification with AOX1 primers verified that the hG-CSF gene had been integrated into the AOX1 locus on the chromosome of the transformed *P. pastoris*. For Mut^+ clones two expected bands were detected, one 1056 bp pertaining to the G-CSF expression cassette flanked by AOX1 sequences and the other 2200 bp corresponding to the native AOX1 gene of yeast genome (Fig. 2B).

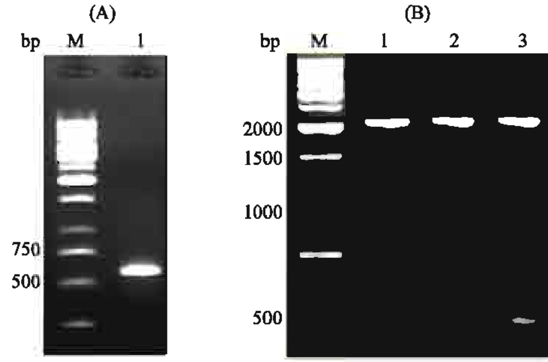

Fig. 2: (A) PCR amplification of hG-CSF cDNA. Lane 1: 569 bp hG-CSF gene amplicon; lane M: Generuler 1 kb DNA ladder (Fermentas). (B) PCR detection of expression cassette in the transformed yeast by 5' and 3' AOX1 primers. Lanes 1, 2: revealed a PCR band of 1056 bp corresponding to hG-CSF; lane 3: revealed a PCR band around 492 bp corresponding to without insert vector (pPIC9); lanes 1, 2, 3: have the additional 2200 bp AOX1 gene band indicating Mut^+ phenotype; lane M: Generuler 1 kb DNA ladder (Fermentas)

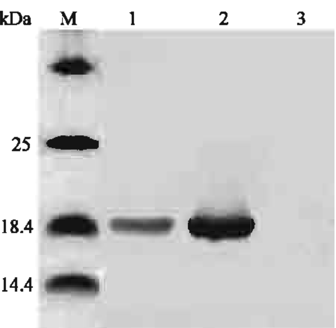

Fig. 3: Western blot analysis of recombinant hG-CSF protein. Lane 1: Expression of target protein in culture supernatant after 144 h induction; Lane 2: Positive control-Commercial hG-CSF; Lane 3: Expression of target protein in culture supernatant at 0 h induction; Lane M: protein molecular weight marker

Shake flask expression of protein: All the positive His^+ Mut^+ colonies that were found positive with genomic PCR were selected for inducing the expression of the target gene. In order to study the expression of hG-CSF gene fraction, the optimal method and growth conditions necessary for expression were standardized. The recombinant yeast transformed by pPIC9/hG-CSF vector showed expression of the expected 18 kDa protein after 144 h post-induction in western blot, whereas there was

no specific protein band detected in recombinant yeast transformed by pPIC9/hG-CSF vector before induction (Fig. 3). The expression level of recombinant protein versus time was measured by ELISA. The results showed that 144 h of post-induction incubation period with 2% methanol concentration is ideal for hG-CSF protein expression. Ammonium sulfate precipitated protein from the positive transformant was analyzed through western blotting to confirm the specificity of the expressed protein. The expression level of recombinant protein was measured about 3.1 mg L^{-1} of the culture by ELISA.

DISCUSSION

Recombinant G-CSF has wide pharmaceutical applications; therefore we studied its expression in methylotrophic yeast *P. pastoris*, which is a versatile host organism capable of efficient secretion. Recombinant G-CSF has been expressed in various foreign hosts such as *E. coli* (Heidari *et al*., 2001), yeasts (Bae *et al*., 1998; Lasnik *et al*., 2001) and mammalian cells (Rotondaro *et al*., 1997) and is now employed to treat cancer patients undergoing chemotherapy to alleviate the depression of white blood cell levels produced by cytotoxic therapeutic agents. *P. pastoris* is able to utilize methanol as its sole carbon source and has been widely used as a host for the expression of heterologous proteins. In this study, the hG-CSF gene was inserted towards the downstream of AOX1 promoter of the secretory expression vector pPIC9 and the chimeric construct was integrated into the host chromosome through homologous recombination. Preparing pPIC9/hG-CSF plasmid construct, a deletion of Glu-Ala repeats and SnaBI restriction site was made after Lys-Arg signal cleavage originally present in pPIC9 plasmid. It is known that foreign gene fused to α-MF prepro leader at the Lys-Arg processing site without Glu-Ala spacer can lead to properly or non-properly processed protein. On the other hand, Glu-Ala spacer between Lys-Arg site and the N-terminus of the foreign gene can lead to a secreted protein, which is efficiently cleaved after Lys-Arg, but it can retain the Glu-Ala spacer at the amino terminus (Cereghino and Cregg, 2000).

As the target gene is integrated within the genome, it is difficult to lose the target gene when the recombinant yeast is cultured and passaged. Yeast expression systems can also perform many eukaryotic post-translational modifications in the target protein, such as glycosylation. The hG-CSF is a glycosylated protein (Hoglund, 1998), thus, *P. pastoris* expression system may be better than *E. coli*. The native hG-CSF is secreted into blood, hence, it was expressed as a secretory protein. This strategy will be useful for purification of protein in future. Because *P. pastoris* secrets low level of native proteins in medium.

Although all several positive colonies were selected for induction, only a few colonies showed expression of the target protein in Western blotting. The colonies did not show detectable levels of the target protein in the SDS-PAGE. The expression level of recombinant hG-CSF was determined about 3.1 mg L^{-1} in shake flask fermentation. The low expression of the protein may be attributed to several factors. The most important limitation for low expression in shack flask fermentation is found to be inadequate aeration during methanol induction. In this study, hence, the culture volume within the flask was kept as low as 10% of the total volume of flask. In addition, the low expression may be partly attributed to the low copy number of the genes integrated within the yeast genome and partly to the codon usage of the protein. The results can be better in the fermenter, where growth to high cell density was reported. In the fermenter 3-5 times higher transcription levels can be obtained as a consequence of the controlled methanol concentration (Cereghino and Cregg, 2000). Additionally, codon optimization of hG-CSF gene can be further improved the expression level.

In this study, the recombinant hG-CSF was successfully expressed in flask by *P. pastoris* system. The main drawback of the hG-CSF expression in flask is the low level of yield. Hence, the expression of hG-CSF synthetic gene with the use of fermenter is under study in our laboratory.

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