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Research Article

Expression of the Full-length Human Recombinant Keratinocyte Growth Factor in *Pichia pastoris*

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Abstract

Keratinocyte Growth Factor (KGF) is a paracrine-acting and epithelium-specific growth factor produced by cells of mesenchymal origin. Based on preclinical data, recombinant KGF plays a critical role in protecting and repairing of damaged epithelial tissues. Despite great efforts to express recombinant human KGF(rhKGF) in different organisms, attempts for finding appropriate protein expression system with the ability of producing a properly folded and processed KGF needs further investigation. *Pichia pastoris* has been used successfully and extensively for production of industrial enzymes and pharmaceutical proteins. Herein, we investigated the affect of pro-region-α-factor early deletion on production and secretion of rhKGF in *Pichia pastoris*. Initially, expression of human *KGF* induced in MCF-7 cell line treated with 1, 25-Dihydroxy vitamin D3. The coding sequence of full-length $rhKGF_{194}$ was then cloned into the yeast integrative expression vector, downstream of α-factor and was integrated into *P. pastoris* genome. KGF protein was expressed in *P. pastoris x33* cells, usingα-factor signal peptide for translocation of KGF to ER. An internal human signal peptide was also arranged after α-factorfor early removal of the pro-region in ER. RT-PCR results demonstrated that KGF mRNA was expressed successfully after induction by methanol. Recombinant KGF protein expression was detected by Western blotting in cell lysats, but not in conditioned media. A molecular weight of 17 kD for $rhKGF_{194}$ indicates that the α-factor and internal human signal peptideshad been removed in *x33* cells. The results indicate that in the absence of pro-region-α-factor, the recombinant KGF protein was not efficiently processed and transported within the biosynthesis-secretory pathway. As KGF protein is an unstable growth factor and tend to aggregate because of some native properties, It seems that presence of a chaperon molecule fusion with KGF is necessary for efficient secretion of the recombinant protein.

***Keywords*:** Keratinocyte growth factor, Pichiapastoris, Signal peptide

Introduction

Keratinocyte Growth Factor (KGF, also known as FGF-7) is a member of the fibroblast growth factor familythat binds solely to fibroblast growth factor receptor2b (FGFR2b) (Finch and Rubin, 2004). KGF is produced by mesenchymal cells, and promotes proliferation, DNA repair and migration of different types of epithelial cells. KGF has an important role in repair of injured epithelium and wound healing in various tissues and organs (Yen et al., 2014).

A recombinant truncated KGF, named as Palifermin (Kepivance™), has been approved by U.S. Food and Drug Administration to reduce incidence, duration and severity of mucositis in patients suffering from hematologic malignancies undergoing high doses of radiation and chemotherapy, before haematopoietic stem-cell transplantation (Finch and Rubin, 2006).

These therapeutic applications require large quantities of purified biologically active recombinant FGF7. Due to the low production level of rhKGF in various expression systems, and also the poor stability and the protein's tendency to aggregate rapidly, pharmaceutical applications of the growth factor have been limited (Huang et al., 2012; Chen et al., 1994; Feng et al., 2014; Xue et al., 2014). Attempts for finding an expression system with high production level and appropriate folding of the recombinant protein with simple and high quality purification method needs further investigation.

Due to the growth inhibitory effect of rhKGF in *E.*

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coli host cells, the yield of KGF production in this organism has been low or barely detectable, and the produced proteins sometimes aggregate as inclusion bodies. Palifermin (a commercial N-terminally truncated recombinant KGF protein) produced in *Escherichia coli* host, with a molecular weight of 16.3 kDa. Expression of KGF as a fusion protein with glutathione s-transferase (GST) in bacterial systems improved the yields of protein production. However; relative to the expression of FGF1, FGF2 and FGF10, the production level in bacteria was still low and needs further steps for purification of the overexpressed protein (Luo et al., 2004). Using molecular dynamics simulation of our GST-KGF fusion protein, it was indicated that GST act as a chaperone and interact with positively charged residues in heparin binding sites of the rhKGF and avoid conformational changes caused by repulsion of those positive amino acids, and subsequent aggregation of KGF (unpublished observations).

As *Pichia pastoris* is a notable eukaryotic expression system and is being used frequently for expression and production of different recombinant proteins, we decided to design and optimize a secretory expression system using this organism. The ability to secrete properly folded proteins into the culture media is one of the main advantages of *P. pastoris* as a protein producing host. However, level of secreted foreign proteins varies widely in yeast because of some roadblocks in specific steps in the secretory pathway (Ahmad et al., 2014; Daly and Hearn, 2005). Therefore, it is important to study different factors for improving secretion efficiency of KGF in large scale. One critical question is whether folding of recombinant KGF can be properly achieved in ER, or it needs a molecular chaperon for avoiding aggregation and ER-associated degradation (ERAD).

In this study, the coding sequence of human full-length KGF was cloned and stably expressed in *P. pastoris*. For answer the aforementioned question, we used thenative signal peptide of KGF as an internal second signal peptide, for early removal of pro-region-α-factor sequence.We then investigated the effects of this in the expression and secretion of KGF in *P. pastoris x33*.

Materials and Methods

Cell culturing and RNA extraction

The cell line originated from a breast adenocarcinoma (MCF-7) was obtained from Pasteur Institute of Iran. The cells were grown in RPMI 1640 medium (Invitrogen, UK) supplemented with 10% FBS (Invitrogen, UK), 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 ng/mL amphotericin B, at 37°C and 5% CO2. For minimizing effects of exogenous growth factors, medium was replaced with medium containing1.5% FBS, 24h before treatment. Cells were treated with 1, 25-dihydroxyl vitamin D3(Sigma, St. Louis, MO), at different concentration (150-300nM). 36 hours after treatment, cells were detached from the six-well plates using trypsin/EDTA solution, and total RNA was extracted according to the standard manual of TRIzol solution.

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification and construction of expression vector

The specific primers for RT-PCR to amplify KGF (Gen Bank accession number NM_002009.3) were designed using Gene runner (version 3.02; Hastings Software), Oligo v 6.54, and PerlPrimer v1.1.16 softwares. RT-PCR was carried out using Revert AID cDNA synthesis kit (Fermentas, Canada) and KGF reverse specific primer (table 1), according to manufacturer's protocol. The fragment coding for the full-length KGF (194aa) was amplified using PCR technique. The sequences of the forward and reverse primers were shown in table 1. Forward and Reverse primers contained *XhoI* and *XbaI* restriction sites, respectively, that marked as underline letters. PCR conditions were perform as following: initial denaturation at 94ºC for 2min, followed by 30 cycles of 94°C for 30 seconds, 60ºC for 30 seconds, and 72ºC for 30 seconds; with a final extension at 72ºC for 5 minutes. The PCR products were electrophoresed on 1% agarose gel and visualized with ethidium bromide staining and under UV light. The productwas ligated into the TA vector and transformed in DH5α *E. coli*. The authentisity of the PCR products was confirmed by DNA sequencing (Microgen, South Korea). The KGF coding sequence was then cloned into pPICZαA expression vector,with the cloning sites of *XhoI* at upstream andXbaIat downstream.

P. pastoris transformation

Recombinant (pPICZαA/KGF) and control (pPICZαA) plasmids were linearized with *SacI* restriction enzyme and then purified by purification kit (Gene all, South Korea).

Electro-competent *P.pastoris* strain X-33 was prepared according to the manufacturer's recommendations (Invitrogen). 10µg of linearized plasmids were transformed in 80 µl of electro-competent X-33, using a Bio-Rad gene-Pulser apparatus (1.5 KV voltage, 25 uF capacitance, and 400Ω resistance).

The cells were plated on YPDS plates containing 100 µg/ml zeocin and incubated at 30°C for 2-3 days. To verify the chromosomal integration of the plasmids, the genomic DNA of some of X-33 transformant colonies was analyzed by PCR. Single colonies were suspended in 20µl H_2O, and after being frozen and thawed, were used for PCR as templates. AOX1 universal primers and KGF specific primers (table 1) were used in PCR reactions. The PCR conditions were as following conditions: initiation at 95°C for 5 minutes, amplification for 30 cycles with: denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72ºC for 5 minutes.

Table 1. Name and sequences of the primers used for clonining and PCR.

Primer name	sequence
KGF reverse specific primer	5'CACTTAAAGAAATCTCCCTGCTG -3'
KGF forward specific primer	5'-CATGAACACCCGGAGCACTAC-3'
KGF cloning Forward	5'CCGCTCGAGAAAAGAATGCACA AATGGATACTG-3'
KGF cloning Reverse	5'-TGCT CTAG ATT AA GT TA TT GCCATAGGAAGAAAGTG-3'
AOX1 forward primer	5'-GACTGGTTCCAATTGACAAGC-3'
AOX1 reverse primer	5'-GCAAATGGCATTCTGACATCC-3'

Expression of recombinant KGF in *P. pastoris*

Selected clones were inoculated in BMGY medium (2% peptone, 1% yeast extract, 1.34% yeast nitrogen base (YNB), 100mM potassium phosphate PH6.0, 4×10^{-5}% biotin, and 1% glycerol) and grown at 30°C and 300rpm unit reaching OD_{600} of 3. The cells were centrifuged at 3000×g for 10 minutes at 4°C and resuspended to an OD_{600} of 1.5 in BMMY medium and were grown for 96h at 30°C. the methanol was added to a final concentration of 0.5% (v/v) every 24 hours to maintain induction. For analyzing secretion of recombinant protein, the media was concentrated about 10-fold using a 10 kDa molecular weight cut-off membrane in a stir-cell filtration unit (Amicon).For analyzing intracellular protein expression, cell lysis were prepared with acid-wash 0.5 mm glass beads (Sigma, St. Louis, MO), based on manufacture's instructions.

Western blot analysis

Protein concentration in the medium supernatants and cell lysiswas quantified by Bradford protein determinations. Equal amounts of protein (about 10µg for medium supernatants and 25 µg for cell lysis) were loaded on 12% SDS-PAGE gel and transferred onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, USA). After blocking, the membrane was blotted with a polyclonal goat KGF antibody (C-19) (1:300 dilution, Santa Cruz biotechnology, Santa Cruz, CA).after 1 hour of washing in PBS-T, the membrane was incubated with the rabbit anti-goat IgG horseradish peroxidase-conjugatedantibody (1:3000 dilution, Santa Cruz biotechnology, Santa Cruz, CA).After washing in PBS-T, the peroxidase activity was visualized with a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) ona X-ray film.

Results

Preparing human KGF coding sequence and constructing a pPICZαA/KGF expression plasmid

Based on previous reports, established epithelial tumor cell lines derived from a variety of tissue sources were not expressing KGF protein. Nevertheless, vitamin D3 induced KGF transcript andprotein expression in the human breast cancer cell line MCF-7(Finch & Rubin 2006). For this reason, MCF7 cell linewas treated with different concentrations (150, 200, and 300 nM) of 1,25D3 for 36 hours. Total RNA was extracted and due to low expression of KGF mRNA, KGF reverse primer was used in cDNA synthesis, instead of oligodT primer. Using KGF forward and reverse specific primers, KGF cDNA containing 654 nucleotides was amplified by PCR technique. Results demonstrated that optimum concentration of 1,25D3 was 300 nM (Figure 1B). For producing the fragment containing the coding sequences for KGF protein (194 amino acids) as well as the restriction sites for *XhoI* and *XbaI* enzymes at 5' and 3' ends, respectively, the fragment was amplified using PCR and KGF cloning primers (Figure 1C). This segmentwas cloned into *XhoI* and *XbaI* restriction sites of pPICZαA vector.A schematic representation of rhKGF protein with 194 amino acid is shown in Figure 1A. Positive clones were selected and the authenticity of the recombinant plasmid was confirmed by DNA sequencing.

Integration of hKGF/pPiczα construct into *P. Pastoris* genome

The recombinant plasmid, pPICZαA/KGF_{194}, was linearized with *SacI* restriction enzyme and waselectroporated into the competent *P. pastoris* X-33.

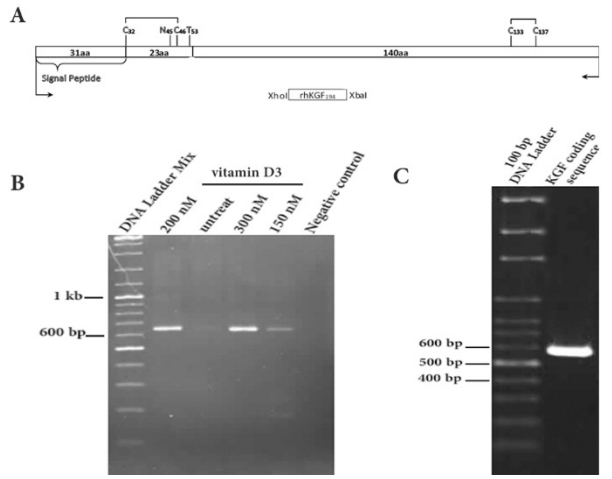

Figure 1. KGF coding sequence. Panel A. Schematic representation of rhKGF protein with 194 amino acid. $RhKGF_{194}$ contains a signal peptide (31 amino acid) and two disulfide bond linking Cys_{32} to Cys_{46} and Cys_{133} to Cys_{137}, besides two N-linked and O-linked glycosylation sites on Asn_{45} and Thr_{53}, respectively. Panel B. induction of KGF mRNA by treating MCF7 cell line with different concentrations of vitamin D3. Panel C. Amplification of the fragment coding KGF protein. Lane 2 amplicon Coding KGF_{196} (608bp).

Integration of the DNA construct into the host genomewas confirmed by PCR, using universal AOX1 and KGF specific primers. X-33 containing pPICZαA vector in its genome, was used as a negative control. The pattern of PCR product confirmed that both constructs were successfully integrated into the host X33 genome (Figure 2A). PCR with AOX1 primers resulted in amplification of two fragments of 1196bp (608bp+ 588bp) and 2.2Kb (AOX1 gene) for positive pPICZαA/full-length KGF transformant. On the other hand, the control transformants containing pPICZαA plasmid showed two fragments with the sizes of 588bp and 2.2Kb (lane 4 in Figure 2A). PCR with KGF specific primers amplified a 608bp fragment for pPICZαA/full-length KGF transformant. As it was expected, there was no amplification for control transformant (Lane 5in Figure 2A).

Confirming human KGF mRNA expression intransformant *P. pastoris* cells

Using RT-PCR, KGF mRNA expression was investigated after 48 hours of induction by 1% methanol.Total RNA was extracted from induced and non-induced transformant expressing KGF. To avoid genomic's DNA amplification, DNase treatments were employed before cDNA synthesis, and no-RT reactions were used as negative controls. The result confirmed the expression of KGF mRNA in *P. pastoris* cells (Figure 2B).

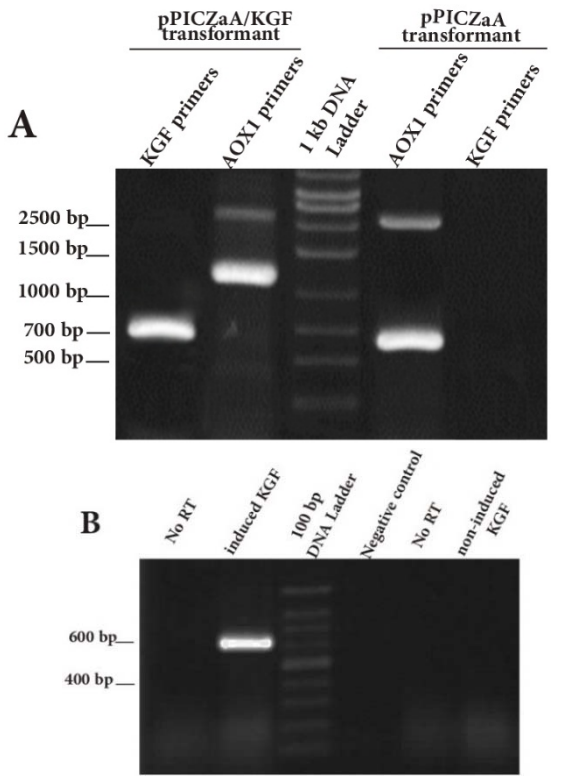

Figure 2. PCR analysis of *pichia* transformants. Panel A, PCR analysis for confirming the genomic integration of the expression construct. Lane 1 and 2: pPICZαA/full-length KGF transformant (using KGF special and AOX1 universal primers, respectively); Lane 3: DNA Ladder; Lane 4 and 5: pPICZαA transformant (using AOX1 universal and KGF special primers, respectively). Panel B, RT-PCR for detection of KGF expression at mRNA level. Lane 1 and 2: Methanol-induced transformant expressing full-length KGF recombinant protein and NoRT. Lane 3: 100 bp DNA Ladder. Lane 4: negative control. Lane 5 and 6: non-induced transformants expressing KGF recombinant protein and No RT.

Detection of human KGF protein expression in transformant *P. pastoris* cells

In order to examine KGF protein expression, Western blot analysis was performed using specific antibody against KGF. It was expected that the recombinant KGF protein to be secreted into the medium, because of the existence of signal peptide in KGF amino acid sequence. Therefore, concentrated media of the transformants were analyzed by Western blot technique. As it is shown in Figure 3A, no protein band was detected in media of different transformants expressing $rhKGF_{194}$. $RhKGF_{140}$ was used as a positive control with a molecular size of approximately 17 kD. On the other hand, media of transformants containing pPICZαAwas used as a negative control, and no band was observed for pPICZαA transformant media.

In order to check if the recombinant protein is trapped inside the cell, the cell lyses were examined using Western blotting. As showen in figure 3B, the recombinant protein was detected in cell lysis of the transformants with a molecular size of 17 kD (Figure 3B). The results indicated that rhKGF$_{194}$was not secreted into medium, but signal peptide and 23 N-terminal amino acidshad been removed from the precursor protein.

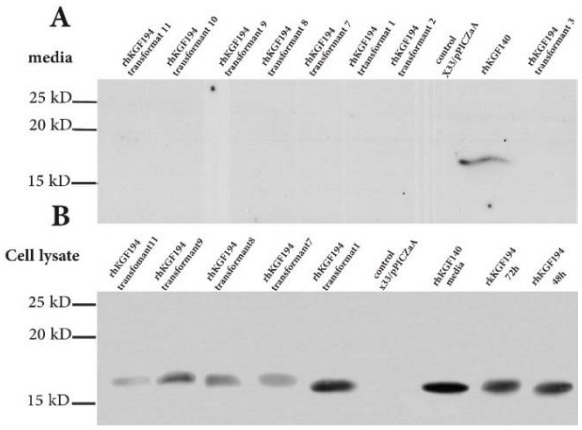

Figure 3. Western blot analysis for detection of recombinant proteins. Panel A. screening of different pPICZαA/ RhKGF$_{194}$ transformant media for detection of RhKGF$_{194}$. Media of pPICZαA/ RhKGF$_{140}$ transformant was used as a positive control. Media of pPICZα transformantwas used as a negative control. Panel B. screening of different pPICZαA/ RhKGF$_{194}$ transformant cells for detection of rhKGF$_{194}$ expression in cell lysis.

Discussion

One of the critical issues in biotechnological processes and drug production for human therapy is avoiding aggregation of recombinant protein during production. Because aggregation of the heterologous protein results in restricted solubility and biological activity and low yields of the products.Protein engineering strategies is a valuable approach to reduce aggregation and improve stability and folding of a protein drug during production (Vazquez et al., 2011). KGFhas been proposed as a protein drug for treatment of pathologies associated with dermal adnexae, liver, lung, and gastrointestinal tract diseases, particularly wound healing in various tissues and organs (Finch et al., 2013; Yen et al., 2014). However, the production of the fairly unstable growth factor have been limited by its low level of expression in different expression systems. Therefore, it is of significant importance to design a more cost-effective and simpler expression system that improves the yield of recombinant KGF protein production with correctly folded and appropriately post-translational modifications. In this study, *P.pastoris* expression system has been used for expression and secretion of a full-length human KGF protein containing yeast α-factor and native human signal sequences to elucidate some details of its biosynthesis and secretion in pichia expression system. This would open an avenue to improve the production yield and stability of the growth factor in yeast expression system.

Pichia pastoris expression system has been widely and successfully used to produce recombinant medical and industrial proteins. As *P. pastoris* combines the advantages of unicellular organisms (i.e. rapid growth and being easy to genetic manipulation) with the ability to produce proteins with eukaryotic post-translational modifications, the expression system is easier, faster and less expensive than other eukaryotic expression systems such as baculovirus or mammalian cell/tissue cultures (Ahmad et al., 2014; Daly and Hearn, 2005).

The most popular secretion signal that widely used in budding yeasts is α-factor signal peptide. The α-factor signal sequence contains pre- and pro-region. Pre-region is a 19-residue signal sequence and directs co-translational translocation of the recombinant protein into the ER. The pro-rergion contains 66 amino acids and is a hydrophobic peptide ended by short stretches of charged amino acids. Pro-region improves the efficiency of post-translational translocation across the ER membrane and is removed in the late-Golgi by the endo-protease kex2p (Ahmad et al., 2014; Fitzgerald and Glick, 2014) .It is thought that the pro-peptide ensures proper folding of the nascent protein and can function as a molecular chaperon for secreted proteins. Some proteins can only be secreted in the presence of pro-peptide in the leader sequence (Lin-Cereghino et al., 2013). Based on previous reports and molecular dynamics simulation, protein engineering to make fusion protein of KGF with a chaperon molecule, like GST, is necessary for improving the stability of the protein and avoiding some conformational changes that proceed aggregation.

For these reasons, we used an internal second signal peptide to study the effect of early removal of pro-peptide in ER on the secretion of KGF protein.We hypothesized that the pro-peptide have a role as a chaperon for for ER exit and secretion of KGF protein. In general, internal signal peptide could play two different roles; one role in directing translocation of the protein as a conventional signal peptide, and the other role in anchoring the protein on the membrane and creating a single or multipassintergralprotein.

Function of internal signal depends on the distance between the two signal peptides and post- or co-translational function of the first signal peptide(Coleman, Inukai et al. 1985). Since the internal signal peptide was removed in ER by signal peptidase, pro-region α-factor was cleaved in ER as it located before second signal sequence.

In **conclusion**, our data demonstrated that rhKGF$_{194}$ protein was expressed in the *P. pastoris* cell lysis but not secreted into the conditioned medum. Molecular weight of the produced recombinant KGF protein was estimated to be around 17 kDa. This molecular size suggested that the pre-pro-α factor, native human signal peptide, and its N-terminal 23-amino acids had been removed from rhKGF$_{194}$ protein. On the other hand, using different KGF constructs containing only a single N-terminal signal peptide did not interfer with the secretion of mature KGF (Bahadori et al., submitted). Therefore, we can conclude that pro-peptide-α-factor region in KGF expression construct has a vital role in proper production and secretion of KGF.

Acknowledgments

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