Enzyme and Microbial Technology 111 (2018) 12–20

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Producing functional recombinant human keratinocyte growth factor in *Pichia pastoris* and investigating its protective role against irradiation

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ARTICLE INFO

Keywords:
Keratinocyte growth factor
Recombinant protein
Pichia pastoris
Cytoprotection
Irradiation
KGF

ABSTRACT

Keratinocyte Growth Factor (KGF) is a paracrine-acting, epithelial mitogen that plays a prominent role in the regeneration of damaged epithelial tissues. In spite of different attempts to produce recombinant human KGF in many organisms, including bacteria, mammalian cells, plant cells and insect cells; production of recombinant form suffers from lower yields and recovery relative to other recombinant proteins of similar size and properties. Due to many advantages of *Pichia pastoris* expression systems for producing industrial enzymes and pharmaceutical proteins, in this study *P. pastoris* was chosen as a host for KGF expression. For preparing human KGF coding sequence, MCF-7 cell line was treated with 1,25-Dihydroxy vitamin D3 for inducing the expression of KGF. The coding sequence of 23N-terminal truncated KGF form was amplified using RT-PCR technique and then cloned into the yeast expression vector in frame with the yeast α-factor secretion signal. The recombinant plasmid was integrated into *Pichia pastoris* strain X-33 genome. Western blotting and Mass Spectrometry demonstrated that recombinant human KGF (rhKGF) was correctly expressed after methanol induction and secreted into the media. The recombinant protein was purified from the media by heparin affinity chromatography. MTT assay showed that the purified rhKGF had a proliferative effect on NIH3T3 and A549 cell lines. In addition, protective effect of recombinant KGF was assessed in A549 cell line after irradiation. The results showed that the recombinant protein was biologically active. Finally, the effect of recombinant KGF was investigated on proliferation of MCF-7 cell line and its response to radiation. The results showed that pre-treatment of KGF have a protective effect on MCF-7 cell line after irradiation.

1. Introduction

Keratinocyte Growth Factor (KGF), a member of the fibroblast growth factor family (FGF-7), functions as a locally acting mitogen exclusively through fibroblast growth factor receptor2b (FGFR2b) isoforms that are expressed specifically by epithelial cells [1]. KGF is produced by cells of mesenchymal origin and promote proliferation and differentiation of different types of epithelial cells and protects them from the toxic effects of various insults under stress conditions. KGF has been shown to play an important role in the repair of the injured epithelium and wound healing in various tissues and organs [2]. Besides its specificity for epithelial cells, the restoration is achieved by KGF without adverse inflammatory responses, fibrosis, or angiogenesis [1]. A recombinant truncated KGF, known as Palifermin, has been approved by the 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.

KGF was originally isolated from conditioned medium of the human embryonic lung fibroblast cell line, M426, with a molecular mass of 26–28 kDa [3]. The KGF cDNA encodes 194-amino acid protein containing a signal peptide for secretion and both N- and O- linked glycosylation sites near the N-terminus [1,4]. Biologically active KGF protein was expressed in bacteria with an apparent molecular size of 21 kDa and a specific activity of KGF protein was 10-fold greater than isolated native KGF from fibroblast-conditioned medium. A number of previous studies revealed that elimination of the first 23 amino acid residues of KGF downstream from the signal sequence, didn't reduced mitogenic activity of the truncated protein, but increased its stability [3,5]. Because of the low production level of recombinant human KGF (rhKGF) in *E. coli* expression system and instability of the protein, pharmaceutical applications of KGF have been limited even though the protein has been produced in mammalian cells, plant cells, and insect cells.

Pichia pastoris is one of the most cost-effective eukaryotic protein expression systems, exploiting strong promoters in the *P. pastoris* system

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https://doi.org/10.1016/j.enzmictec.2018.01.003
Received 31 July 2017; Received in revised form 12 December 2017; Accepted 5 January 2018
Available online 05 January 2018
0141-0229/

for the expression of a foreign gene, enabling large-scale production of the target protein at a lower cost as compared to other eukaryotic systems [6]. The ability to secrete high titers of properly folded proteins with eukaryotic post-translational modifications into the culture media is one of the main advantages of *P. pastoris* as a protein production host. Moreover, due to the relatively low levels of endogenous proteins in the extracellular medium of the yeast, purification processes of secreted proteins are feasible and simple [7]. In this work, truncated form of KGF protein (commercial form, $rhKGF_{140}$) was expressed and produced in the recombinant *P. pastoris* by using yeast integrative vector. To the best of our knowledge, this is the first report for expression of KGF in the yeast system. Our results showed that the recombinant protein was successfully secreted into the medium, and subsequently the secreted KGF was purified in one-step using heparin affinity chromatography. The activity assay showed that rhKGF could significantly promote the proliferation of NIH3T3 and A549b cell lines and possessed a statistically significant effect on surviving rate of irradiated A549 cell line. Moreover, our data showed that rhKGF had a strong and significant proliferative effect on MCF7 cell line and protected these cells against irradiation significantly.

2. Materials and methods

2.1. Cell culturing and RNA extraction

MCF-7 human breast cancer cell lines were grown in RPMI 1640 medium supplemented with 10% FBS, streptomycin (100 mg/ml) at 37 °C and 5% CO_2. Cells were treated with 1, 25-Di hydroxyl vitamin D3 (Sigma, St. Louis, MO) at 300 nM concentration. 24 h before treatment, medium was replaced with 1.5% FBS to minimize effects of exogenous growth factors. Using trypsin/EDTA solution cells were detached from the six-well Plates 36 h after treatment, and total RNA was extracted using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. For removing any trace of DNA contamination, the extracted RNA was treated by RNase-free DNaseI (Takara, Japan).

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

Specific primers for amplification of KGF (Gen Bank accession number NM_002009.3) with RT-PCR- technique were designed using Oligo (version 6.54), Gene runner (version 3.02; Hastings Software), and PerlPrimer (version 1.1.16) software. Approximately 1.0 μg of DNase-treated RNA was reverse transcribed by employing Revert AID cDNA synthesis kit (Fermentas, Canada) and KGF reverse specific primer: 5′-CACTTAAAGAAATCTCCCTGCTG-3′, according to the manual. The synthesized cDNA was used for amplification of fragment coding truncated form of KGF (140aa) using PCR-technique. PCR primers were the following: Forward primer: 5′-CCGCTCGAGAAAAGAAT GCACAAATGGATACTG-3′, Reverse primer: 5′-TGCTCTAGATTAAGTT ATTGCCATAGGAAGAAAGTG-3′. Marked letters showed XhoI and XbaI restriction sites in Forward and Reverse primers, respectively. PCR was carried out for 2 min at 94 °C followed by 30 cycles of 40 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, followed by a 5 min extension at 72 °C. The PCR product was visualized by electrophoresis on 1% agarose gel and ethidium bromide staining. Digested PCR product with XhoI and XbaI restriction enzymes was ligated into the expression pPICZαA vector, in frame with the yeast α-factor secretion signal and transformed in *DH5α E. coli*. True insertion of the coding fragment was confirmed by DNA sequencing (Microgen, South Korea).

2.3. P. pastoris transformation

P. pastoris strain X-33 was prepared for electro-transformation according to the method as described in manufacturer's recommendations (Invitrogen, UK). Recombinant (pPICZαA/KGF140) and control pPICZαA plasmids were linearized with SacI restriction enzyme and transformed into 80 μl of electro-competent X-33 using a gene-Pulser apparatus (BIO-RAD, USA) with the following settings: 1.5 KV voltage, 25 μF capacitance, and 400 Ω resistance. The cells were selected by growing for 2–3 days at 30 °C on YPDS plates containing 100 μg/ml zeocin. The genomic DNA of a number of *X-33* transformant colonies were used as templates for PCR analysis to verify chromosomal integration of the plasmids. AOX1 universal primers (5′-GACTGGTTCCA ATTGACAAGC-3′ as Forward and 5′-GCAAATGGCATTCTGACATCC-3′ as Reverse primer) and KGF specific primers (mentioned before) were used in PCR reaction. The PCR parameters were 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C.

2.4. Expression of recombinant KGF in P. pastoris

Selected transformants were picked up from the plates and grown in Buffered Glycerol Complex Medium (BMGY) medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (YNB), 1% glycerol, 4 × 10-5% biotin, and 100 mM potassium phosphate pH 6.0) at 30 °C and 300 rpm. After reaching OD600 of 3, the cells were centrifuged (3000 × *g*, 10 min, 4 °C) and re-suspended to an OD600 of 1.5 in Buffered Methanol-complex Medium (BMMY) medium containing 0.5% (v/v) methanol. Methanol was added once a day to a final concentration of 0.5% (v/v) to induce the expression of recombinant protein. For mRNA expression analysis, two cultures of selected transformants grew up as explained above, but only one of them induced with methanol. After incubating at 30 °C for 48 h, the cells were harvested by centrifugation at 1500 × *g* for 10 min at room temperature. Total RNA was extracted by phenol/chloroform method based on Invitrogen manual. cDNA synthesis was carried out using Revert AID cDNA synthesis kit (Fermentas, Canada) and random hexamer primer from 1.0 μg of DNase-treated RNA. PCR with KGF specific primer was used for analysis the KGF expression. For study the recombinant KGF protein expression and secretion, 96 h after induction with methanol, cells were removed and the supernatant was transferred to a separate tube. The supernatant was concentrated about 10-fold through a 10-kDa molecular weight cut-off concentrator (Amicon). Concentrated media containing 5 μg protein were run on a 12% SDS-PAGE gel and stained with silver nitrate.

2.5. Mass spectrometry analysis

To determine KGF protein expression, LC/MS-MS technique was used to analyze the corresponding band on SDS-PAGE. After excision of protein band from SDS-gel, chopped gel was digested with 500 ng trypsin in 50 mM ammonium bicarbonate for overnight. Using DionexUltiMate 3000 RSLC equipped with an analytical column, digested peptides were separated by nano-liquid chromatography. After injection of separated peptides into a high capacity trap (HCT) Ultra Ion Trap mass spectrometer (Bruker Daltonics) by electrospray ionization (ESI), MS/MS spectra were searched in the human UniProt database.

2.6. Western blotting analysis

Bradford protein assay was used for quantification of Protein concentration in the supernatants. Equal amounts of protein (about 10 μg) were separated on 12% SDS-PAGE gel, and then the protein transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) at 100 V for 1 h. After incubating of the membrane in blocking solution for 1 h, Immunoblotting was performed with a poly clonal goat KGF antibody (C-19) (1:300 dilution, Santa Cruz biotechnology, Santa Cruz, CA). After being washed in PBS-T, the membrane was incubated with the rabbit anti-goat IgG horseradish peroxidase- conjugated (1:3000 dilution, Santa Cruz biotechnology, Santa Cruz, CA). The Peroxidase activity was detected using a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) on X-ray film.