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Full Length Research Paper

Intracellular expression of human calcitonin (hCT) gene in the methylotrophic yeast, *Pichia pastoris*

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This study utilized the *Pichia pastoris* expression system for expression of the synthetic human calcitonin (hCT) gene, a small peptide hormone secreted by the thyroid gland in mammals and ultimobranchial glands in lower vertebrate. The *P. pastoris* vector (pPICZB) contains the alcohol oxidase gene promoter (AOX1), which under the induction of methanol allows for the expression of heterologous protein gene inserted downstream in the vector. KM71H (mut^s) strain of *P. pastoris* was used as the host cell. Molecular analysis, including polymerase chain reaction (PCR), sequencing, restriction enzyme analysis and survival of *P. pastoris* to increase concentration of zeocin antibiotic showed that human calcitonin gene was successfully integrated into the *P. pastoris* genome. The expected peptide which had an apparent molecular mass of 5.5 kDa was detected by Tricine-SDS-PAGE analysis and confirmed by enzyme-linked immunosorbent assay (ELISA).

Key words: *Pichia pastoris,* human calcitonin, KM71H (mut^s), Tricine-SDS-PAGE.

INTRODUCTION

Calcitonin (CT) is a peptide hormone produced by specialized C-parafollicular cells of the thyroid glands in mammals or by cells of the ultimobranchial glands in fish and reptiles. CT plays an important role in regulating phosphorus and calcium metabolism, decreasing blood calcium concentrations and inhibiting bone resorption. Natural CT and synthesized analog are widely used in clinical practice for the treatment of postmenopausal osteoporosis, Paget's disease of bone, bone pain, spinal stenosis, acute pancreatitis and gastric ulcer (Li et al., 2009). Following the increase of the proportion of the elderly people in the world, osteoporosis has become a major threat to the public health due to its high morbidity and mortality (Lim et al., 2004). Low bone mass and deterioration of bone micro architecture are the major characteristics of osteoporosis, which results in increased bone brittleness and thus is associated with an increased risk of fracture. CT is one of the effective and safe agents for the treatment of osteoporosis (Munoz-Torres et al., 2004). Gills of salmon and pig thyroid glands are the main source of CT that is used in clinical practice (Tanko et al., 2004). However, these heterologous products are short of resources and thus expensive. CT activity is not species-specific which make it possible to use animal CT (porcine, salmon and eel) for treatment of human patients. However, due to immunological reactions, the prolonged application of animal CT leads to a gradual decrease or loss of activity. That is why the long term treatment of human patients with CT requires homologous human calcitonin (hCT)(Azria 1989). Thus, genetic engineering techniques with hCT gene as the target gene may provide solutions to the earlier-mentioned problem. In this study, we described the

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Abbreviations: CT, Calcitonin; **hCT,** human calcitonin; **SDS-PAGE,** sodium dodecyl sulphate poly acrylamide gel electrophoresis; **PMSF,** phenylmethylsulfonyl fluoride.

construction of a recombinant plasmid including pPICZB vector and hCT gene for intracellular expression in *Pichia pastoris* strain KM71H.

MATERIALS AND METHODS

Strains, plasmids and material

Escherichia coli TOP 10F' and *P. pastoris* KM71H (*arg4 aox1::ARG4*) strains (Invitrogen, USA) were used for plasmid construction and expression, respectively. Zeocin and pPICZB expression vector were purchased from Invitrogen. Pfu DNA polymerase, DNA ladders, T4 DNA ligase and restriction enzymes was supplied by Fermentas (Lithuania). PCR purification kit was from Roche (Germany). Plasmid extraction kit was from Bioneer (Korea). Primers were synthesized by Bioneer and low range protein molecular weight marker was from Sigma (Germany). PCR-Script plasmid (Clontech, USA) containing synthetic hCT gene was used for amplification of hCT gene. All other chemicals and media components were of analytical grade and obtained from Merck (Germany).

Construction of the expression vector

The synthesized hCT gene with this sequence 5´-ATGTGTGGGAATCTGAGTACTTGCATGCTTGGCACATACACCC AAGATTTCAACAAGTTTCATACTTTTCCACAGACAGCTATTGGT GTTGGAGCACCTTAA-3´ was used as the template for PCR amplification with specific primers designed for cloning in pPICZB vector. The forward primer: 5´-CGGAATT**C ATAATGTGT**GGGAATCTGAG-3´ contained an *Eco*RI restriction site at the 5´-end (underlined in the primer sequence) and a yeast consensus sequence (bolded) (Romanos et al., 1992).

The reverse primer: 5´-GCTCTAGATAAGGTGCTCCAACACCAATAGC-3´ contained an XbaI restriction site at 5´ end (underlined in the primer sequence). The forward primer has an ATG initiation codon but the reverse primer does not have a stop codon. This condition led to an open reading frame (ORF) starting from ATG to C-terminal *myc* epitope tag and C-terminal polyhistidine (6xHis) tag and finally to a stop codon. After initial denaturation at 94°C for 5 min, hCT gene amplification was carried out through 33 cycles of denaturation (60 s at 94°C), annealing (60 s at 62°C) and extension (60 s at 72°C), followed by a final elongation (5 min at 72°C) in a Bio Rad (USA) thermocycler.

Cloning and transformation

The PCR product was gel-purified and digested with *EcoR*I and *Xba*I before cloning into pPICZB. After transforming into *E. coli* Top10, one recombinant plasmid designated as pPICZB_hCT was selected on a low salt LB agar plate containing 25 µg/ml zeocin. The insertion was checked by restriction enzyme analysis and sequencing. The enzyme for restriction analysis was BglI. The DNA sequencing primer was designed according to 5' AOX1 priming site on the pPICZB vector. The sequence was: 5´-GACTGGTTCCAATTGACAAGC-3´. DNA sequencing was performed by MWG (Germany).

For *P. pastoris* integration, 10 µg of recombinant plasmid was linearized with *Sac*I, and transformed into *P. pastoris* by electroporation. For electroporation, linearized recombinant plasmid was mixed with competent KM71H cells. The mixture was immediately transferred to a pre-chilled 0.2 cm electroporation cuvette and incubated on ice for 5 min. About 1 ml of ice-cold 1 M sorbitol was immediately added to the cuvette after electroporation on a Gene Pulser (Bio-Rad, USA). The charging voltage, capacitance and resistance were 1.5 kV, 25 µF and 200 Ω, respectively. The transformants were selected at 28°C on the YPDS (1% (w/v) yeast extract, 1 M sorbitol, 2% (w/v) peptone and 2% (w/v) D-glucose) agar plates containing 100 µg/ml zeocin for 3 days. The integration of the hCT gene into the genome of *P. pastoris* was confirmed by PCR using 5'AOX1 and 3'AOX1 primers. DNA extraction from *P. pastoris* for PCR was done following a standard protocol. The sequence of 3'AOX1 primers was: 5´-GCAAATGGCATTCTGACATCC-3´. For screening of multicopy integration of hCT gene, colonies were grown on 100 µg/ml zeocin YPDS medium and were transferred to 200 µg/ml, then 500 µg/ml and finally to 1000 µg/ml zeocin YPDS medium. The clones grown on 1000 µg/ml zeocin YPDS medium were the multicopy integrants and selected for expression in KM71H.

Expression of hCT gene in KM71H

P. pastoris transformants were grown on 50 ml of fresh buffered minimal glycerol complex medium, BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 0.0004% (w/v) biotin and 1% (v/v) glycerol) at 30°C (approximately 16 to 18 ho in 250 rpm) until an OD600 of 2 to 6 was reached. To induce hCT gene expression in *P. pastoris*, the cell pellet was harvested by centrifuging at 1500 to 3000 g for 5 min at room temperature and was resuspended in buffered minimal methanol medium, BMMY (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34% (w/v) YNB, 0.0004% (w/v) biotin and 0.5% methanol) using 1/5 volume of the original culture (10 ml) in a shaking incubator (250 rpm). Absolute methanol was added every 24 h to a final concentration of 0.5% (v/v) to maintain induction. The culture pellet was collected after 3 days and stored at -80°C until ready to assay.

Protein extraction and SDS-PAGE

Cell pellets was stored at -80°C, thawed quickly on ice. The following reagents including 100 µl breaking buffer (50 mM sodium phosphate (pH 7.4), 1 mM PMSF (phenylmethylsulfonyl fluoride or other protease inhibitors), 1 mM EDTA and 5% glycerol were added to 1 ml cell pellet and resuspended. An equal volume of acid-washed glass beads (size 0.5 mm) was added. The sample was vortexed for 30 s, and was then incubated on ice for 30 s. This step was repeated for a total of 8 cycles. The sample was centrifuged at 14000 rpm for 10 min at 4°C. The clear supernatant was transferred to a new microtube. Electrophoresis was performed using Bio-Rad's Mini Protean II Redi-Gel System. The expression of the recombinant hCT was analyzed by Tricine–SDS-PAGE (15%) according to the method of Schagger (2006). 5 µl of supernatant (cell lysate) with 5 µl 2X SDS-PAGE Gel Loading buffer (sample buffer). was mixed and boiled for 10 min and loaded per well. The bands were visualized by staining with silver nitrate.

ELISA

ELISA was performed with commercial hCT detection kit (Diasorin, Italy). Principle of the procedure is two-site immunoluminometric assay (sandwich principle). Two different highly specific monoclonal antibodies are used for the coating of the solid phase (magnetic particles) and for the tracer. This kit is suitable for the quantitative determination of hCT and have measurement that range from 1.0 to 2000 pg/ml. 75 µl of sample and 75 µl of control were added to 100 µl of tracer in separate vials. The vials were incubated for 10 min at room temperature and then 20 µl of magnetic particles was added.

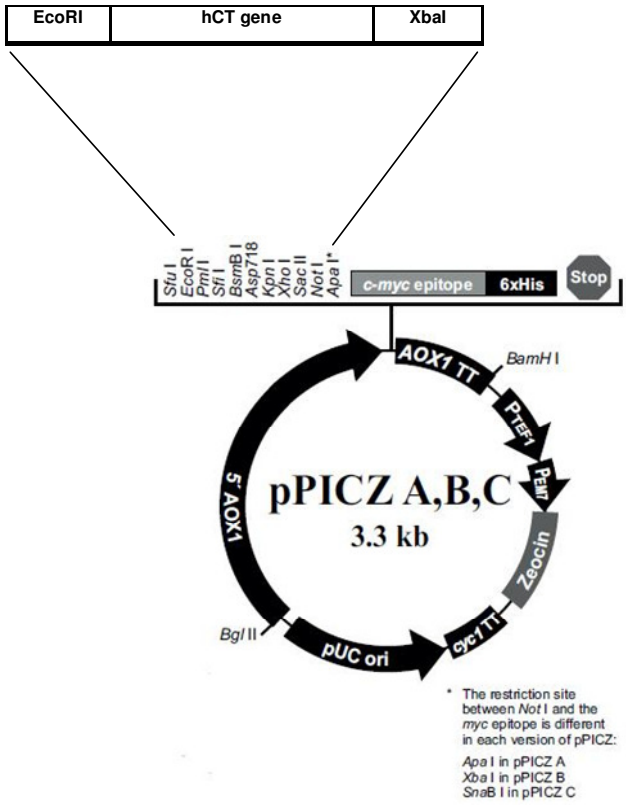

Figure 1. Schematic representation of hCT gene and pPICZB vector. hCT gene was cloned between EcoRI and XbaI sites and is in frame with c- myc epitope and 6XHis tag. The vector has a strong promoter (AOX1) and a gene for resistance in zeocin antibiotic.

The vials were incubated for 10 min at room temperature and incubation, followed by a wash cycle. The LIAISON® Analyser (USA) automatically calculated the hCT concentration in each sample by means of a calibration curve which is generated by a 2-point calibration master curve procedure. The results are expressed in pg/ml.

RESULTS

Cloning of hCT gene in pPICZB

For the construction of the pPICZB-hCT recombinant plasmid, hCT gene from PCR-Script-hCT vector was subcloned into the pPICZB vector using forward and reverse primers. No mutations were found in the nucleotide sequence of the inserted fragment after sequencing. The mature hCT peptide sequence had an initiation consensus sequence for expression in *P. pastoris* and was inserted in frame with the *c-myc* epitope and polyhistidine (Figure 1). The DNA sequence of the pPICZB-hCT vector predicts that the recombinant protein will contain 55 amino acids including 32 amino acids in the mature hCT peptide and the remaining 23 amino acids comprising the *c-myc* epitope, and 6XHis tag. The expected molecular weight of the recombinant product is 5.5 kDa.

Molecular analysis of positive clones

After plasmid extraction from *E. coli*, PCR and restriction analysis was done for the confirmation of insert orientation. PCR was done by primers used for cloning. PCR product was approximately 120 bp equal to hCT gene size in the PCR-Script-hCT. Since two BglI restriction sites are present in the MCS of uncloned pPICZB, restriction analysis of pPICZB-hCT was done by BglI enzyme. The pPICZB control vector produced four fragment including 1403, 1211, 682 and 32 bp, while the pPICZB-hCT produced two fragments of 1972 and 1403 bp, which coincided with expectation (Figure 2).

Screening of elecroporated clones and expression of hCT gene

Approximately, 60 transformants of the KM71 strain were generated. Forty (40) clones were isolated and screened

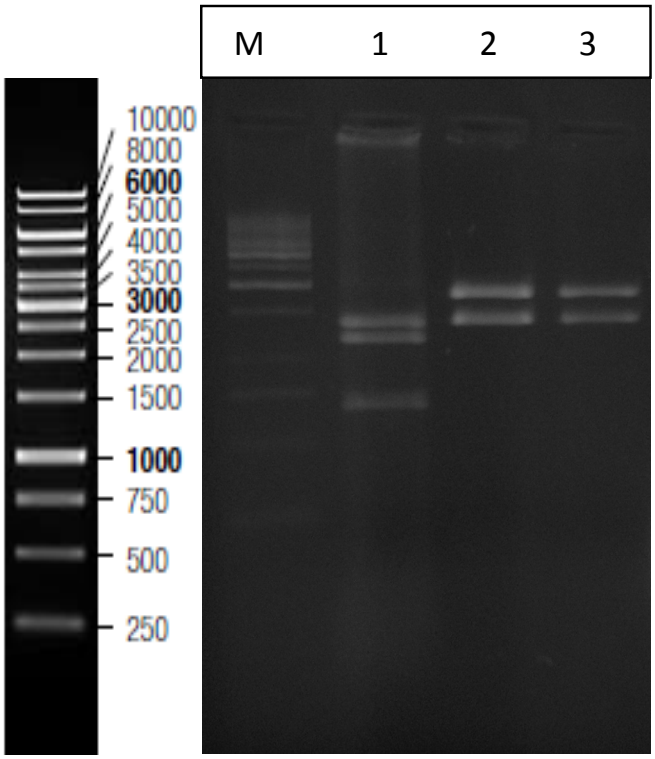

Figure 2. The restriction analysis of pPICZB vector on 1% agarose gel. M, 1 Kb ladder; 1, pPICZB control; lanes 2 and 3 are positive clone including pPICZB-hCT vector.

by PCR with 5'AOX1 and 3'AOX1 primers. Some of the clones contained the expected 364 bp DNA fragment, indicating that the hCT gene was integrated into the *P. pastoris* genome. These forty clones also were screened on YPDS medium including 200, 500 and 1000 µg/ml zeocin. Six clones which were positive in PCR and grown on 1000 µg/ml zeocin-YPDS medium were selected for expression on BMMY medium and one was used for expression. After 3 days, KM71H was harvested and protein extraction was performed. The recombinant hCT produced intracellular KM71H. The peptide was analyzed by Tricine-SDS-PAGE and the band corresponding to the expected size (5.5 kDa) was visible on the gel. This protein band was not detected in the control KM71H sample (Figure 3). The amount of recombinant hCT which was analyzed by ELISA was 1100 pg/ml.

DISCUSSION

In order to produce recombinant pharmaceutical peptides and proteins, there is a need to have a set of different expression systems. Bacteria offer the advantage of high space-time yields and are favorable with respect to cultivation costs. However, as the major drawback, post-translational modification of peptides or proteins, needed for human applications, does not occur in bacteria. In the last decade, *P. pastoris* became one of the favorite expression systems for the production of various proteins of interest (Macauley et al., 2005). This report describes the production of hCT in the methylothropic yeast *P. pastoris* strains KM71H (Mut^s). The benefits of *P. pastoris* for expression of hCT and other protein are abundant. When compared with mammalian cells, *P. pastoris* does not require a complex growth medium or culture con-ditions.

Furthermore, it is particularly suited to foreign protein expression due to ease of genetic manipulation, example gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of protein expression at the intra- or extracellular level, and the ability to perform higher eukaryotic protein modifications, such as glycosylation, disulphide bond formation and proteolytic processing (Cregg et al., 1985). The glycosylated gene products generally have much shorter glycosyl chains than those expressed in *Saccharomyces cerevisiae*, thus making *P. pastoris* a much more attractive host for the expression of human recombinant proteins (Cereghino et al., 2002). *Pichia* can be grown to very high cell densities using minimal media (Wegner et al., 1990) and integrated vectors contribute to the genetic stability of the recombinant elements, even in continuous and large scale fermentation processes (Romanos, 1995). Therefore, the powerful genetic techniques available, together with its economic use,

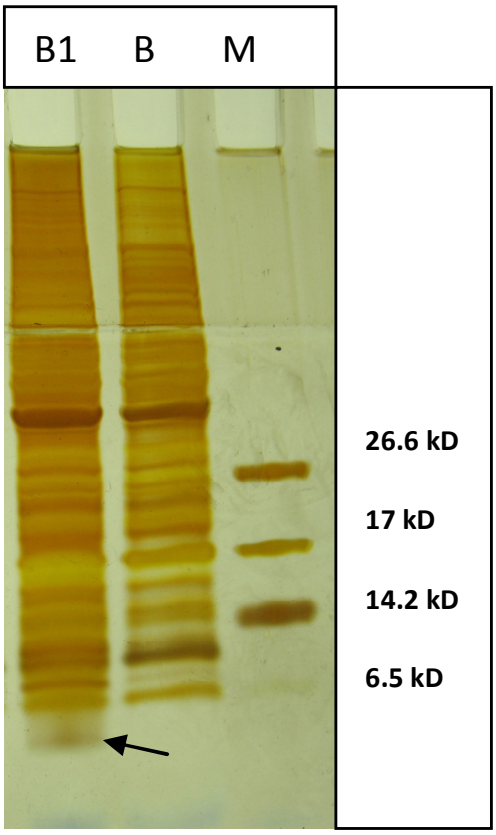

Figure 3. SDS-PAGE of proteins extracted from KM71H strain. M, Protein marker; B, KM71H control; B1, induced sample, the arrow show expressed hCT gene in KM71H.

make *P. pastoris* a system of choice for heterologous protein expression. Some proteins that cannot be expressed efficiently in bacteria, *S. cerevisiae* or the insect cell/baculovirus system, have been successfully produced in functionally active form in *P. pastoris* (Cereghino et al., 2002).

hCT has been previously expressed in *E. coli* (Yabuta et al., 1995), potato (Ofoghi et al., 2000), silkworm (Yang et al., 2002), insect cells (Yang, 2002), *Staphylococcus carnosus* (Dilsen et al., 2000) and NIH3T3 cells (Li et al., 2009).

Osteoporosis is characterized with low bone mass and deterioration of bone microarchitecture which can cause decreased bone strength and an increased risk of fracture (Lim et al., 2004). Calcitonin is one of the most effective reagents for osteoporosis with antalgic activities (Munos et al., 2004). It is believed that salmon calcitonin can inhibit bone resorption, reduce bone mass loss and relieve bone pain (Patel et al., 1993). But oral or nasal administration of salmon calcitonin can cause many side effects in osteoporosis patients. Otherwise, long-term application of animal calcitonins leads to a sharp activity decrease in clinical use of osteoporosis due to the accumulation of antibodies against these heterologous calcitonins (Merli et al., 1996). With the problems in using salmon calcitonin, production of hCT in a suitable host can overcome these problems.

In summary, to our knowledge, for the first time, we successfully expressed hCT gene in *P. pastoris.* The expressed hCT gene was detected by SDS-PAGE and ELISA but the amount was low and need optimization.

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