

Human Calcitonin (hCT) Gene Expression and Secretion by *Pichia pastoris*

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Abstract

Recombinant human calcitonin (hCT) can be expressed by *Pichia pastoris* as a precursor peptide. Full length of synthetic hCT gene was inserted into pPICZαA vector under control of AOX1 promoter, downstream of the secretion-α-factor and electroporated into *P.pastoris* KM71H (mut^s) strain.

Molecular analysis, including polymerase chain reaction (PCR), sequencing, restriction enzyme analysis and surviving of *P. pastoris* to increasing concentration of zeocin antibiotic showed that human calcitonin gene was successfully integrated into the *P. pastoris* genome. The expected peptid which had an apparent molecular mass of 5.5 kDa, was detected by Tricine-SDS-PAGE analysis and confirmed Western blot.

Key words: *Pichia pastoris*; Human calcitonin; KM71H (mut^s); Tricine-SDS-PAGE; AOX1 promoter

Abbreviations: CT, calcitonin; hCT, human calcitonin; SDS-PAGE, sodium dodecyl sulphate poly acrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride or other protease inhibitors

Introduction

Calcitonin (CT) is a peptide hormone produced by specialized C-parafollicular cells of the thyroid glands in mammals. CT plays an important role in regulating calcium metabolism in bone resorption. Today 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 [1].

Osteoporosis has become a major threat to the public health due to its high morbidity and mortality [2]. 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 for fracture. CT is one of the effective and safe agents for the treatment of osteoporosis [3]. Gills of salmon and pig thyroid glands are the main source of CT that is used in clinical practice [4; 5]. 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)[6]. Thus, genetic engineering techniques with hCT gene as the target gene may provide solutions to the above-mentioned problem.

This paper describes the construction of a recombinant plasmid including pPICZαA vector and hCT gene for extracellular expression in *P. pastoris* strain KM71H.

Material and Method

Strains, plasmids and material

E. coli TOP 10F' and *P. pastoris* KM71H (*arg4 aox1::ARG4*) strains (INVITROGEN) were used for plasmid construction and expression, respectively. Zeocin and pPICZ α A expression vector were purchased from Invitrogen. Pfu DNA polymerase, DNA ladders, T4 DNA ligase, restriction enzymes was supplied by FERMENTAS.

PCR purification kit was from ROCHE. Plasmid extraction kit was from BIONEER. Primers were synthesized by BIONEER. Low range protein molecular weight marker was from SIGMA. PCR-Script plasmid (CLONTECH) containing synthetic hCT gene was used for amplification hCT gene. All other chemicals and media components were of analytical grade and obtained from MERCK.

Construction of the expression vector

The synthesized hCT gene was used as the template for PCR amplification with specific primers designed for cloning in pPICZ α A vector. The forward primer: 5'-CGGAATTCATGTGTGGAATCTGAGTACTTGC-3' contained an *Eco*RI restriction site at the 5'-end (underlined in the above primer sequence). The reverse primer: 5'-GCTCTAGATAAGGTGCTCCAACACCAATAGC-3' contained a *Xba*I restriction site at 5' end. The forward primer has no ATG initiation codon and is in frame with α -factor but the reverse primer does not have a stop codon. This condition led to an open reading frame (ORF) starting from α -factor ATG to C-terminal *myc* epitope tag and C-terminal polyhistidine (6xHis) tag and finally to a stop codon. hCT gene amplification was carried out through 33cycles of denaturation (60 sec at 94°C), annealing (60 sec at 65°C), and extension (60 sec at 72°C), followed by a final elongation (5 min at 72°C) in a BIO-RAD thermocycler.

Cloning and transformation

The PCR product was gel-purified and digested with *Eco*RI and *Xba*I before cloning into pPICZ α A. After transforming into *E. coli* Top10, one recombinant plasmid designated as pPICZ α A_hCT was selected on a low salt LB agar plate containing 25 μ g/ml zeocin. The insertion was checked by restriction analysis and sequencing. The enzyme for restriction analysis was *Bgl*I. The primer for DNA sequencing primer was: 5'-GACTGGTTCCAATTGACAAGC-3'.

Due to the advantages of electroporation, such as high frequency of transformation (especially possibility of multicopy insertion), this method was employed to transform yeast cells by the constructed plasmid (pPICZ α A_hCT).

For *P. pastoris* integration, 10 μ g 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). The charging voltage, capacitance, and resistance were 1.5 kV, 25 μ F, 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 μ g/ml zeocin for 2 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, clones 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 according to EasySelect Pichia Expression Kit (INVITROGEN).

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 16-18 hours in 250rpm) until an OD₆₀₀ of 5 was reached. To induce hCT gene production in *P. pastoris*, the cell pellet was then harvested by centrifuging at 1500-3000 g for 5 minutes 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 in a shaking incubator. Absolute methanol was added every 24 h to a final concentration of 0.5% (v/v) to maintain induction. The culture pellet was discarded after 2 days and supernatant stored at -80°C until needed for further assays according to EasySelect Pichia Expression Kit (INVITROGEN).

Protein extraction and SDS-PAGE

Supernatant was stored at -80°C, thawed quickly on ice. Supernatant was precipitated with ammonium sulfate and pellet was dissolved in 50 mM Tris-HCL buffer PH: 7.2. The solution was dialyzed overnight at 4°C and was stored at -80°C. The expression of the recombinant hCT was analyzed by Tricine-SDS-PAGE (15%). 5 µl of supernatant with 5 µl 2X SDS-PAGE Gel Loading buffer was mixed and boiled for 10 minutes and loaded per well. The bands were visualized by staining with silver nitrate[7].

Western blot analysis

After running total protein on 16% SDS-PAGE gel, Western blot analysis was performed by electroblotting of proteins from SDS-PAGE gel to Whatman nitrocellulose membranes. Tracking of the protein was achieved by employing monoclonal anti-His(C-term) antibody (INVITROGEN) as the primary and horseradish peroxidase-conjugated goat anti-mouse IgG (PROMEGA) as the secondary antibody. The bands were developed using DAB chromogenic substrate (SIGMA – ALDRICH).

Result and Discussion

Molecular cloning of hCT gene and cloning confirmation

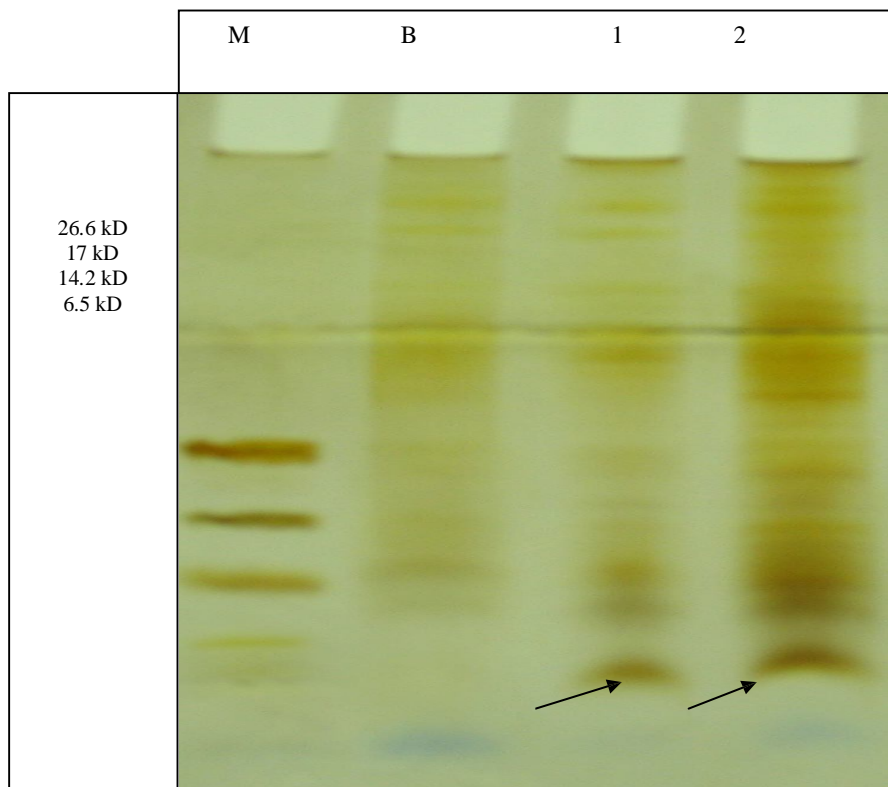
For the construction of the pPICZαA-hCT recombinant plasmid, hCT gene from PCR-Script-hCT vector was subcloned into the pPICZαA vector using forward and reverse primers. No mutations were found in the nucleotide sequence of the inserted fragment after sequencing. The hCT gene sequence was inserted in frame with α-factor, the *c-myc* epitope and polyhistidine (Figure 1). The DNA sequence of the pPICZαA-hCT vector predicts that expected molecular weight of the recombinant product after successful removing of α-factor is 5.5 kDa.

Selection of high expression strains

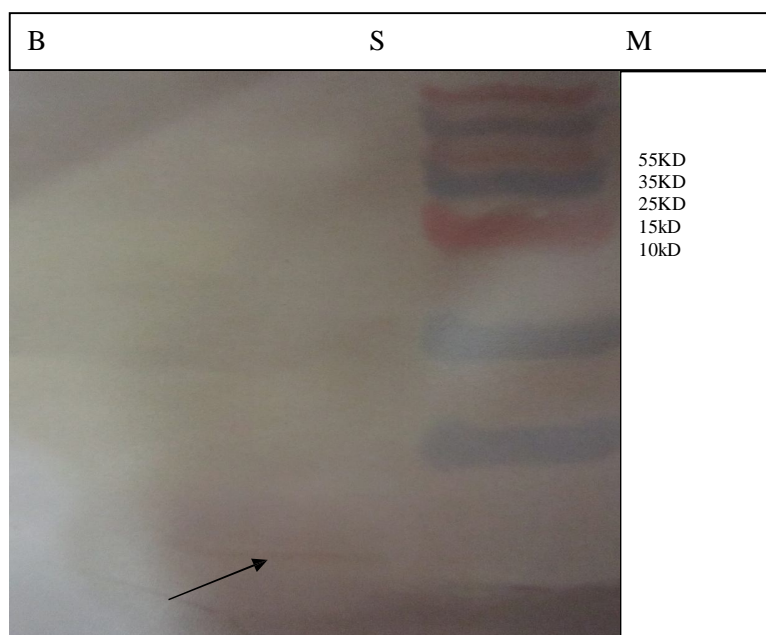
To reach the highest possible expression levels for our gene of interest, we primarily tried to select transformants that contain multiple copies of the integrated vector. Approximately 80 transformants of the KM71 strain were generated. Fifty clones were isolated and screened by PCR with 5'AOX1 and 3'AOX1 primers. Some of the clones contained the expected 680 bp DNA fragment, indicating that the hCT gene was integrated into the *P. pastoris* genome. These fifty clones also were screened on YPDS medium including 200, 500 and 1000 µg/ml zeocin. Moreover, the highest expression clone for scale-up experiment was screened from 20 randomly selected Zeocin resistant transformants (1mg/ml) by culturing them in small scale (10 ml) for comparatively appraising the level of expression among them.

SDS-PAGE and Western blot analysis

After 2 days, KM71H was harvested and protein extraction was performed. The recombinant hCT was produced extracellularly in 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 strain (Figure 3). In western blotting also this band was detected (Figure 4).



(Fig 3) SDS-PAGE (15%) of protein extracted from KM71H. M, protein marker; B, KM71 H control; lane 1 and 2 are induced samples, the arrow show expressed hCT gene in KM71H.



(Fig 4) Western-blot analysis of recombinant human calcitonin expressed in *P. pastoris*. Lane M, prestained molecular-weight marker. Lane B is control and lane S is induced sample and arrow shows hCT expressed in *P. pastoris*.

For produce recombinant 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 [8]. This report describes the production of hCT in the methylotrophic yeast *P. pastoris* strains KM71H (Mut^s) as a secretory small peptide. The advantages of *P. pastoris* for expression of hCT and other protein are abundant. *P. pastoris* does not require a complex growth medium.

Furthermore, it is particularly suited to foreign protein expression due to ease of genetic manipulation, e.g. 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 [9]. The glycosylated gene products generally have much shorter glycosyl chains than those expressed in *S. cerevisiae*, thus making *P. pastoris* a much more attractive host for the expression of human recombinant proteins [10]. *Pichia* can be grown to very high cell densities using minimal media [11]. Therefore, the powerful genetic techniques available, together with its economy of use, make *P. pastoris* a system of choice for heterologous protein expression. Some proteins that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae* or the insect cell/baculovirus system, have been successfully produced in functionally active form in *P. pastoris* [10].

hCT has been previously expressed in *E. coli* [12], potato[13], silkworm [14], insect cells[15], *Staphylococcus Carnosus* [16] and NIH3T3 Cells [1].

Osteoporosis is characterized with low bone mass and deterioration of bone microarchitecture which can cause decreased bone strength and an increased risk for fracture [2]. Calcitonin in the market use for the treatment of osteoporosis extracted from porcine, salmon and eel or synthetic form of above molecules. It is believed that salmon calcitonin can inhibit bone

resorption, reduce bone mass loss and relieve bone pain [17]. However, due to immunological reactions the long application of animal calcitonin leads to a gradual decrease or loss of activity and acute allergic reaction. 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 [18]. Human recombinant calcitonin may provide solution to above mentioned Solution. This objective of research is highly recommended and is under study worldwide.

Conclusions

We have established an expression system based on recombinant DNA for extracellular expression of recombinant hCT in *P. pastoris*. To our knowledge, this is the first study describing expression of hCT gene in *P. pastoris* as methylotrophic yeast. In summary, we successfully expressed hCT gene in *P. pastoris*. The expressed hCT gene was detected by SDS-PAGE and Western blotting. This recombinant hCT can be made available on a scale which could meet the high demand of its use as a potential therapeutic agent for treating osteoporosis.

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