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Full Length Research Paper

Purification of recombinant C-terminus polyhistidine tagged human calcitonin with cobalt-based and nickel-based affinity chromatography: A comparative study

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Human calcitonin (hCT) is a 32 amino acid peptide that participates in the regulation of calcium and phosphorus metabolism in human. It is used in clinics for the treatment of diseases related to bone decalcification, such as Paget disease, osteoporosis imperfecta and parathyroid gland carcinoma. In this research, C-terminus polyhistidine tagged human recombinant calcitonin which was expressed by *Pichia pastoris* strain KM71H was purified with cobalt-based IMAC resin and Nickel-based IMAC column. The result shows that the yield of purification was higher by cobalt-based IMAC resin in comparison with nickel-based IMAC column.

Key words: Human calcitonin, *Pichia pastoris*, cobalt-based IMAC resin, nickel-based IMAC column.

INTRODUCTION

Calcitonin (CT) hormone is a peptide 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 (Li et al., 2009).

Osteoporosis has become a major threat to the public health due to its high morbidity and mortality (Lim and Takahashi, 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 used in clinical practice (Karsdal et al., 2009; 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 (Merli et al., 1996). That is why the long term treatment of human patients with CT requires homologous human calcitonin (hCT) (Azria and Kiger, 1974). Thus, genetic engineering techniques with hCT gene as the target gene may provide solutions to the above-mentioned problem.

This paper describes purification of hCT from *Pichia pastoris* strain KM71H which expressed this hormone with cobalt-based IMC resin and nickel-based IMAC column.

MATERIALS AND METHODS

Strains, plasmids and material

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

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Abbreviations: **CT**, Calcitonin; **hCT**, human calcitonin; **IMAC**, immobilized metal ion affinity chromatography.

TAS.

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 of hCT gene. Cobalt based IMAC resin was from CLONTECH and Nickel-NTA (Ni-NTA) resins, column was from QIAGEN. 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 pPICZαA vector. The forward primer: 5'-CGGAATTCATGTGTGGGAATCTGAGTACTTGC-3' contained an *Eco*RI restriction site at the 5´end. The reverse primer: 5'-GCTCTAGATAAGGTGCTCCAACACCAATAGC-3' contained an *Xba*I restriction site at 5´ end. The forward primer has no ATG initiation codon and was in frame with α-factor but the reverse primer did 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 33 cycles of denaturation (60 s at 94°C), annealing (60 s at 65°C) and extension (60 s 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 pPICZαA. After transforming into *E. coli* Top10, several recombinant plasmids designated as pPICZαA_hCT were selected on a low salt LB agar plate containing 25 µg/ml zeocin. The insertion was checked by sequencing. The primer for DNA sequencing primer was: 5´-GACTGGTTCCAATTGACAAGC-3´.

For *P. pastoris* electroporation, 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). 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 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 according to INVITROGEN manual. 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.

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 h at 250 rpm) 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 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 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 was stored at -80°C until needed for further assays.

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.

Purification of C-Terminus Polyhistidine Tagged hCT with cobalt-based IMAC resin

TALON resin was centrifuged at 700 xg for 2 min to pellet. Supernatant was discarded. Ten bed volumes of 1X Equilibration/Wash buffer was added and mixed briefly to pre-equilibrate the resin. Tube was re-centrifuged at 700 xg for 2 min to pellet the resin. Supernatant was discarded. Clarified hCT was added to the resin. Tube was agitated for 20 min on a platform shaker to allow the polyhistidine-tagged hCT to bind the resin. Tube was centrifuged at 700 xg for 5 min. Supernatant was carefully removed as much as possible without disturbing the resin pellet. Resin was washed by adding 10 to 20 bed volumes of 1X Equilibration/ Wash buffer. Suspension was gently agitated for 10 min on a platform shaker to promote thorough washing and centrifuged at 700 xg for 5 min. Supernatant was removed and discarded. One bed volume of the 1X equilibration/wash buffer was added to the resin and resuspended by vortexing. Resin was transferred to a 2 ml gravity-flow column with an end-cap in place, and the resin was allowed to settle in the suspension. End-cap was removed and the buffer was allowed to drain until it reaches the top of the resin bed. Column was washed once with 5 bed volumes of 1X equilibration/wash buffer. The polyhistidine-tagged protein was eluted by adding 5 bed volumes of Elution Buffer to the column. Fractions were collected in 500 µl elutions. Bradford method was used to determine which fraction contains the bulk of the polyhistidine-tagged protein.

Purification of C-terminus polyhistidine tagged hCT with nickel-based IMAC column

C-Terminus Polyhistidine Tagged hCT was added to 1 ml Ni-NTA resin and was shaken in room temperature for 30 min. Resin was poured on column and allowed to settle. Column was eluted by 2 ml of washing buffer (pH: 6.3) for 3 times. In the next step, column was eluted by 500 µl of elution buffer (pH: 4.5) for 5 times. Bradford method was used to determine which fraction contains the bulk of the polyhistidine-tagged protein.

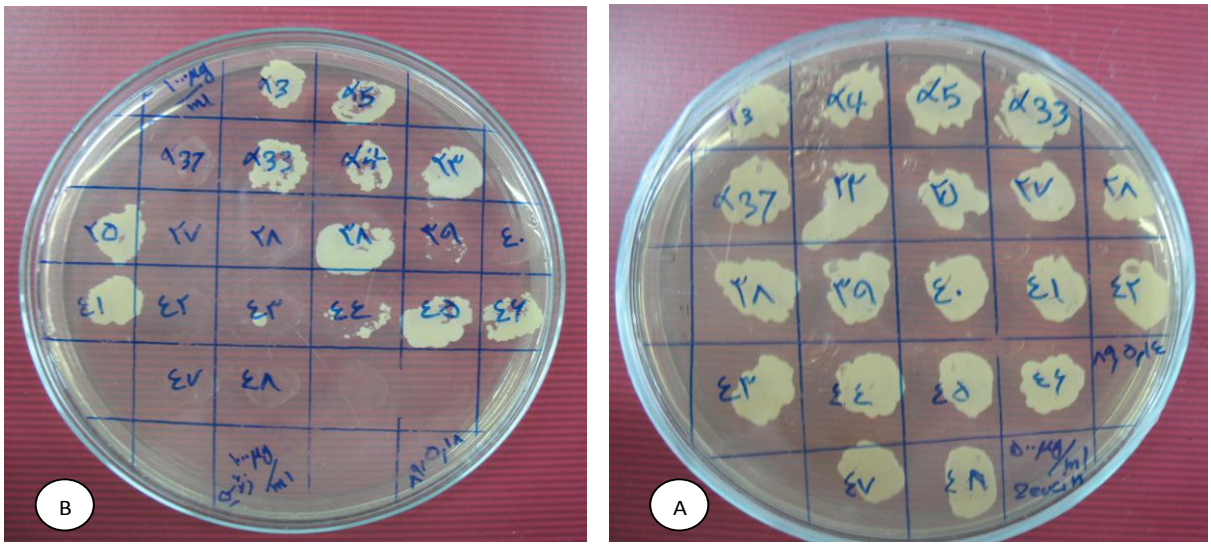

Figure 1. Selection of multicopy integrant clones. A, YPDS medium with concentration of 500 µg/ml zeocin. In this medium, all the clones were grown. B, YPDS medium with concentration of 1000 µg/ml zeocin. In this medium (1000 µg/ml zeocin), 10 clones were grown. These clones were selected for induction.

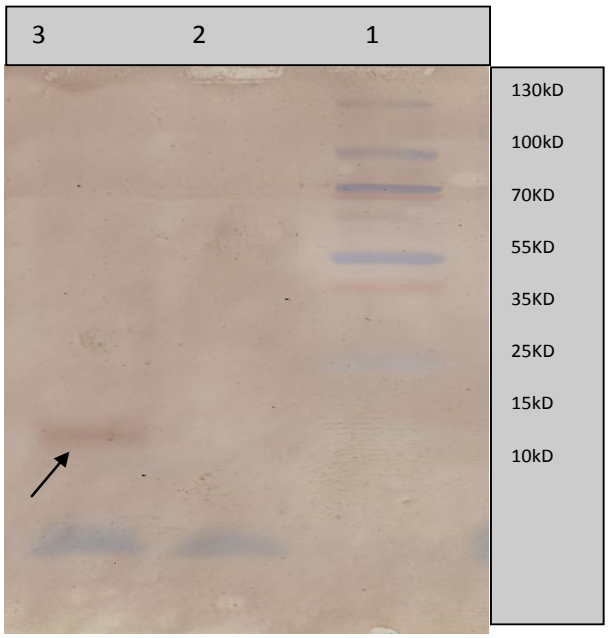

Figure 2. Western-blot analysis of recombinant human calcitonin expressed in *P. pastoris*. Lane 1, Prestained molecular-weight marker. Lane 2 is the control, lane 3 is induced sample and arrow shows hCT expressed in *P. pastoris*.

RESULTS

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 mutation was 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. The DNA sequence of the pPICZαA-hCT vector predicted that expected molecular weight of the recombinant product after successful removing of α-factor was 5.5 kDa.

Selection of multicopy integrant clones and western blot analysis

After electroporation clones were transferred to 100 µg/ml zeocin, about 50 clones were grown in this concentration. Then clones were transferred to 500 µg/ml zeocin in YPDS medium. In this medium, all clones transferred from 100 µg/ml zeocin in YPDS medium, were grown (Figure 1A). The clones observed in this medium were transferred to 1000 µg/ml zeocin in YPDS medium and about ten clones were observed (Figure 1B). One of these clones was used for induction. Recombinant calcitonin was detected by western blot. The calculated molecular mass of expressed human calcitonin hormone was approximately 5.5 kDa (Figure 2).

Purification of expressed hCT

The result of purification with cobalt-based IMAC resin showed 0.0526 mg/ml of hCT and result of purification with nickel-based IMAC column was 0.0320 mg/ml. The difference between methods was 0.0206 mg/ml

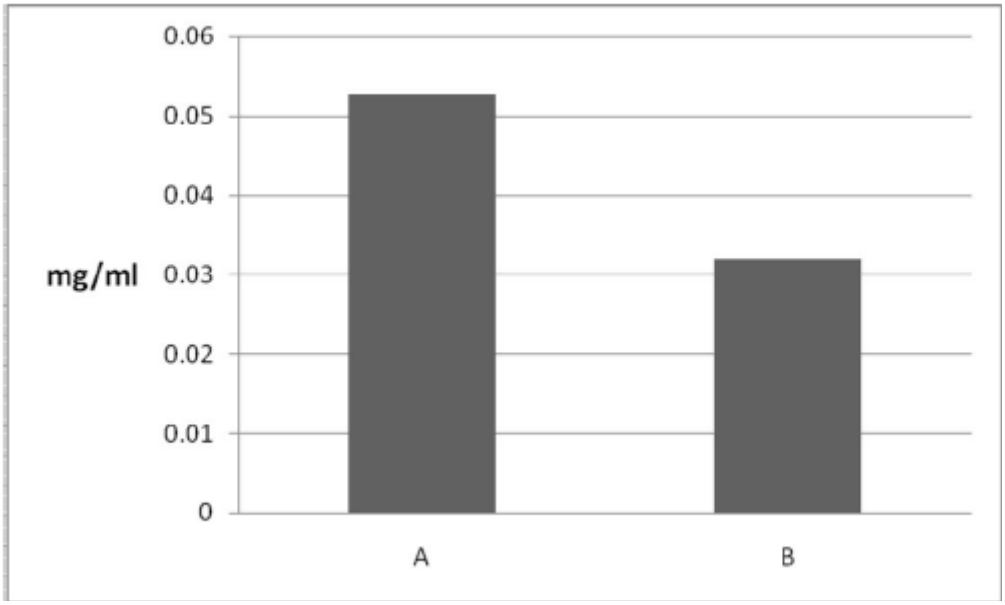

Figure 3. The bar chart shows the concentration of calcitonin after purification with IMAC resins. A column is the concentration after purification with cobalt-based IMAC resin and B column is the concentration after purification with nickel-based IMAC column. The difference between methods was 0.0206 mg/ml.

(Figure 3). This result did not change after three replicates.

DISCUSSION

Isolation and study of a purified protein lies in the heart of modern biochemistry. If a protein can be isolated and purified, it can be studied in isolation from other proteins and its enzymology or signaling capability can be studied in detail. Beyond this, there are a number of important reasons for purifying proteins. For example purified proteins serve as extremely valuable biochemical reagents. It is remarkably valuable to be able to obtain things like purified growth factors or hormones (Knutson et al., 2010).

Proteins have evolved very complex structures in order to perform a diverse array of functions. As a result, their physicochemical properties vary greatly, posing difficulties in developing versatile purification protocols. One way to circumvent this problem is to incorporate a purification tag into the primary amino acid sequence of a target protein, thus constructing a recombinant protein with a binding site that allows purification under well-defined, generic conditions.

IMAC was introduced in 1975 as a group-specific affinity technique for separating proteins (Porath et al., 1975). The principle is based on the reversible interaction between various amino acid side chains and immobilized metal ions. Depending on the immobilized metal ion, different side chains can be involved in the adsorption process. Most notably, histidine, cysteine and tryptophan side chains have been implicated in protein binding to immobilized transition metal ions and zinc (Porath et al., 1985).

Cobalt-based IMAC resin exhibits subtle yet important differences in binding of polyhistidine-tagged proteins when compared with nickel-based IMAC resins. For example, nickel-based IMAC resins often exhibit an undesirable tendency to bind unwanted host proteins containing exposed histidine residues (Kasher et al. 1993). While cobalt-based IMAC resin binds polyhistidine-tagged proteins with enhanced selectivity than nickel-based resins, it exhibits a significantly reduced affinity for host proteins.

For patient applications, a scalable and efficient purification process is an essential step in providing clinical-grade human calcitonin. The objective of this research was to test the feasibility and comparison of utilizing nickel-based IMAC and cobalt-based IMAC resin.

In this study, hCT gene was cloned in pPICZαA vector and expressed. Expressed hCT had a polyhistidine tag in C-terminus. In comparison, purification by cobalt-based IMAC resin was more than that of nickel-based IMAC resin.

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