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Sadegh Majdi
Rowan University, majdis48@students.rowan.edu

Elahe Esfahani

Hossein Amani

Ghasem Najaf pour D

Majid Shahbazi

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Subcloning and expression of recombinant human parathyroid hormone rhPTH by fusion strategy in *E.coli*

Sadegh Majdi, Elahe Esfahani, Hossein Amani, Ghasem Najaf pour D, Majid Shahbazi*

Abstract

Osteoporosis is a progressive bone disease that over time, bone mass, and therefore bones strength, is decreased, due to depletion of calcium and bone protein. So, bones become fragile and break easily. The unique ability of human parathyroid hormone (hPTH) as a bone anabolic agent, to increase bone density makes it a promising anabolic agent in the treatment of osteoporosis. Parathyroid hormone secreted by parathyroid chief cells is composed of 84 amino acids act as one of the major hormones maintaining calcium homeostasis. Anabolic agents directly stimulate bone formation and improve the microarchitecture of bone. Hence, they have the potential to increase bone mass by promoting the growth of new bone greater extent than antiresorptives that inhibit bone resorption. N-terminal fragment (amino acids 1–34) of human parathyroid hormone was regarded to cover most of the hormonal actions of the intact human parathyroid hormone named teriparatide and is currently the only FDA approved anabolic medication for osteoporosis. According to some reports, the C-terminal region of the full-length PTH (1–84) may play an important role in its biological function. The C-terminus of PTH can regulate calcium concentration released from bone through binding to a different PTH receptor specific for the C-terminus. The aim of this study was to express and produce the recombinant PTH protein (1-84) in *E.coli*.

Keywords: *E.coli*, osteoporosis, rhPTH

Introduction

Osteoporosis is a bone silent disease characterized by reduced bone mineral density (BMD) and bone architecture alterations, due to depletion of calcium and bone protein, which makes your bones weak and enhances probability of fractures(Quattrocchi & Kourlas, 2004). The unique ability of human parathyroid hormone (hPTH) as a bone anabolic agent, to increase bone density revolutionizes treatment of osteoporosis(Neer et al., 2001). Human parathyroid hormone (hPTH) is an 84 amino acid peptide which is secreted by the parathyroid chief cells in response to low bloodstream calcium levels(Diarmuid S. O'Riordain, 1997). The original translation product is a 115 amino acid pre-pro-human parathyroid hormone , During secretion ,the pre-pro part is cleaved off to generate mature hormone, hPTH of 84 amino acid (Rokkones et al., 1994). hPTH is a major hormone which regulates the calcium and phosphate homeostasis through a variety of actions on target cells in bone

and kidney and remodels bone(Chunxiao et al., 2007) (Qin, Raggatt, & Partridge, 2004)

Although the anabolic effects of PTH(1-84) has been known , Extracellular biological function of hPTH lies in the N-terminal of the hormone hPTH(1–34) , that covers most of the biological activity of the hole hormone and was named teriparatide(Hodsman et al., 2003). it increases the mineral density of bone and stimulates bone mass formation in postmenopausal women with osteoporosis(Poole & Reeve, 2005). continuous administration (hyperparathyroidism) results in a catabolic skeletal effects that leads to stimulate osteoclast activity , cause loss of bone , accelerate bone turnover and increase the incidence of fractures, while intermittent exposure to low doses of an N-terminal fragment of PTH has anabolic skeletal effects that directly stimulates bone formation and increases bone density in each remodeling cycle , Increases number of active osteoblasts ,levels of it's activity and trabecular connectivity(Wang et al., 2010)(Poole & Reeve, 2005) . Hence, As a result of its unique mechanism of effects, parathyroid hormone (PTH), the only FDA approved anabolic medication for osteoporosis , has the potential to increase bone accrual by promoting the growth of new bone greater extent than antiresorptives agent like vitamin D , estrogens , bisphosphonates that inhibit bone resorption (Hodsman et al., 2003).

 Various lines of evidence suggest that   the   C-terminal region of the intact PTH (1–84) can play an important role in its biological activity(D'Amour & Brossard, 2005). The C-terminus of hPTH can balance calcium homeostasis that released from bone through binding to a different PTH receptor specific for the C-terminus like receptors on osteoblasts(D'Amour & Brossard, 2005).

As a result of the biological importance and multiple beneficial properties of the different parts of hPTH and necessity pharmaceutical use of this hormone in the treatment of osteoporosis and bone disorders, obviously require an efficient and functional procedure for obtaining large amounts of hPTH using genetic engineering methods must be set up. Great efforts have been made to produce high levels of hormone using recombinant DNA technology by Escherichia coli, Pichia pastoris and Saccharomyces cerevisiae, mammalian cells. Escherichia coli was the most common and the first choice prokaryotic host used to synthesis of recombinant DNA (rDNA) pharmaceutical and protein expression , initial development in commercial activities and etc .

The ease of growth, easy handling, high concentration of product, the physiological, biochemical and genetically knowledge respect to other microorganisms are some of it's advantages. but observation showed that there is no Uncertainty that expression level of recombinant gene be high , because of the instability of mRNA and protein due to digestion by intracellular proteases, in *E.coli* (Murby et al., 1991). To overcome these problems, considerable amount of effort has been used and expression hPTH by fusion protein in E. coli had been suggested(Rabbani et al., 1988).Fusion protein is an important strategy that has been successfully used for producing soluble proteins with high yielding efficiency in *E.coli*, as the dominant aim of

recombinant protein expression is often to reach a high degree of aggregation of soluble product in the bacterial cell(Morelle & Mayer, 1988).

More solubility of the expressed protein is one of benefits of using Thioredoxin. In addition, it is possible to obtain high level of expression by using fusion partner and it can be purified using IMAC regarding His6 tag binding affinity to N terminal of the expression cassette(Fu, Tong, & Wei, 2005).

In this study, we indicated an effective procedure for production of intact hPTH in E. coli using a soluble fusion protein method. We constructed a pET32a(+) expression plasmids containing a newly designed synthetic gene allowing synthesis of a His6-thioredoxin-hPTH (1–84) fusion protein under the control of the T7 promoter in *E. coli*(Liu, 2007).

Material & method

Material

DH5α and BL21 (DE3) (Novagen, USA), strain of Escherichia coli, were used as the hosts for subcloning and gene expression, respectively. The plasmid pUC57, containing a 298 bp, His6-thioredoxin-hPTH (1–84) in MscI/BamHI cDNA fragment, was provided from GenScript (USA). The pET32a (+) expression vector was obtained from Novagen, USA. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (UK). Cells were cultured in TB and LB medium(Sambrook J, 2001).

Construction of expression vector

The pUC57-PTH84 transformed into DH5α for amplification target gene. After subcloning, pUC57-PTH84 extracted by QIAGEN plasmid extract kits. The optical density of plasmid solution measured. For digesting His$_6$-thioredoxin-hPTH (1–84) cDNA fragment from pUC57 ,18 μl plasmid solution (30 ng/μl), 1 μl BamHI (20,000 unit/ml) and 1.5 μl MscI (5,000 unit/ml) mixed and put in benmary at 37°C for 2h by 25 μl total volume.(Fig 1) 20 μl of pET32a (+) vector (50.4 ng/μl) was digested by same condition. Both of them loaded in 1% agaros gel. His$_6$-thioredoxin-hPTH (1–84) cDNA fragment and digested pET32a (+), extracted by QIAGEN gel extract kits. Ligation in 10 μl total volume with 2.5 μl His$_6$-thioredoxin-hPTH (1–84) cDNA, 2.5 μl digested pET32a (+) and 5 μl master mix ligase was done .The product of ligation was used for transformation into BL21 (DE3) competent cells.so we transformed pET32a (+)-PTH84 vector, in to BL21 (DE3).Appearing single colonies in LB agar medium containing Ampicillin (100 μg/ml) during overnight, confirmed transformation cells containing pET32a (+)-PTH84 vector. Colony-PCR verified accuracy of transformation expression vector into expression host. (Fig 2)

Fermentation

Fermentation was carried out in a BioFlo 110 bioreactor (New Brunswick). Temperatures were monitored and kept on $37^\circ c \pm 0.1$. The pH was measured by a glass electrode (Ingold) and maintained at 6.8 through addition of either 1 mol/l H_3PO_4 or NaOH. Filter sterilized air and oxygen was supplied to the medium. The impeller speed was regulated to keep the dO_2 (oxygen partial pressure) at 43% air saturation in the medium .foam was removed by silicon anti-foam.

Fusion protein expression in bioreactor

100 µl of 2o% glycerol stock that preserved as fermentation seeds, cultured in LB agar medium overnight. Eight colonies were grown in 5 ml LB medium containing 100 µg/ml ampicillin. Best colony was selected by OD & densitometry. Best single colony was grown in 8 ml LB medium containing 100 µg/ml ampicillin. Subculture for scaling up was done in 50 ml and 500 ml, respectively. 3 liter of TB medium inoculated with 500ml seed was cultivated at $37^\circ c$, pH 7.0. Feeding 1 (table 1) was initiated when OD_{595} of the culture reached 20. Protein expression was induced when OD_{595} of the culture reached 100 by feeding 2 (table 1) and IPTG to a final concentration of 0.5 mM. After 4h induction, cells were harvested by centrifugation (10000 rpm, $4^\circ c$, 30 min). (Fig3).

Lysis of the cell

Harvested cells were resuspended in DTT (Dithiothreitol) buffer and passed thrice through a homogenizer at 1200 bar. Then suspension was centrifuged (10000 rpm,$4^\circ c$, 30min) to remove cellular debris. Supernatant was used for analyzing the amount of fusion protein with Glycine-SDS-PAGE. Protein concentration was measured according to method of Bradford With bovine serum albumin as standard (BSA).

Analysis by SDS-PAGE

Each sample diluted by appropriate sample buffer. All of them remained 5 min in boiling water and then 5 min on ice.2o µl of each one slowly loaded on wells for 45min and 250 V. (Fig 4)

Result

We first transformed BL-21(DE3) containing pET32a(+)-PTH84 from the LB agar pellet and cultivated six randomly selected colonies in LB broth. The expression levels of the six colonies were quantified by densitometry. We decided a strain whose expression ability was relatively strongest among the colonies as the fermentation seed(Wang, 2010). For achieving high cell density in manual fed batch, we try to following (specific growth rate) constant µ strategy for feeding. For this reason, increasing the feeding must be exponentially as was shown in Fig3.

Before feeding 1, for better agitation and respiration of *E.coli* increased rate of impeller and flow of air. Reduction of pH showed growth of *E.coli*, feeding 1 consist of glucose is Source of carbon. As OD was measured continuously, we saw that it's constant and maybe decreases (end of the growth phase).at this point, we achieved high cell density and it was a good time for inducing with feeding 2, containing lactose, and IPTG. We saw that after induction with lactose, the T7 promoter led a highly effective expression of fusion protein.

 Essentially, induction of PTH expression was started when the OD_{595} of the cultures achieved 100, after which the cultivated mediums were sampled at 4 h intervals to detect the expression level by glycine SDS–PAGE. (Fig 4)

 With 18 h cultivation, about 81 g/l cell (wet weight) was achieved. Fusion protein was in a soluble form and could reach 30% of the total protein. The harvested cells (680 g in 8 L) were suspended in 3 L buffer DTT and homogenized. There were about 13.5 g/l total soluble proteins in the supernatant after centrifugation measured by method of Bradford.

This new production method is potentially a useful strategy for the large scale preparation of bioactive peptides due to its high yield, low cost and easy handling(Fang et al., 2009).

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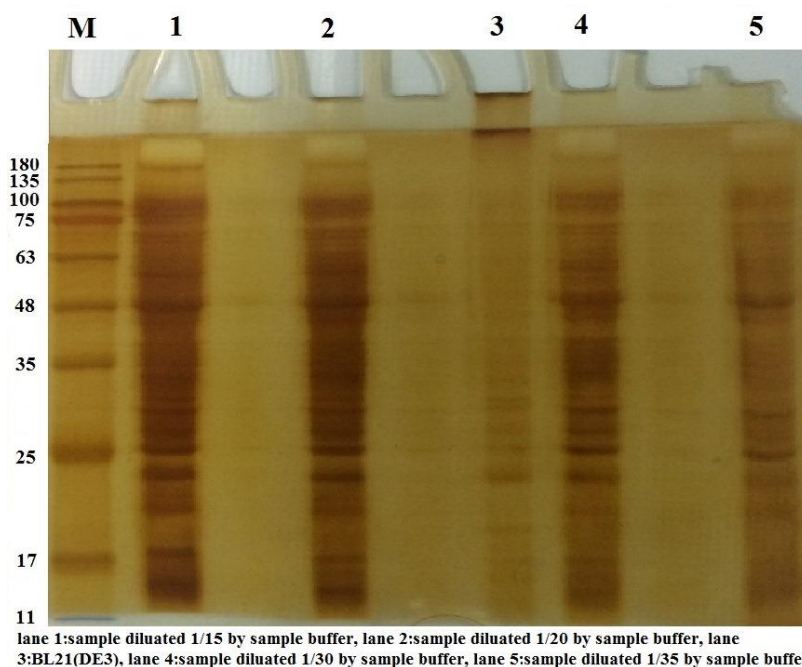
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tabel 1

Feed 1	
glucose(Merck)	0.58 g/ml
peptone(Merck)	0.25 g/ml
yeast extract(Merck)	0.215 g/ml
$MgSO_4.7H_2O$(Merck)	0.01 g/ml
total volume	2500 ml
Feed 2	
lactose(Merck)	0.37 g/ml
peptone	0.18 g/ml
yeast extract	0.27 g/ml
$MgSO_4.7H_2O$	0.01 g/ml
total volume	1700 ml

lane 1:sample diluated 1/15 by sample buffer, lane 2:sample diluated 1/20 by sample buffer, lane 3:BL21(DE3), lane 4:sample diluated 1/30 by sample buffer, lane 5:sample diluated 1/35 by sample buffer. lane M: marker[*]
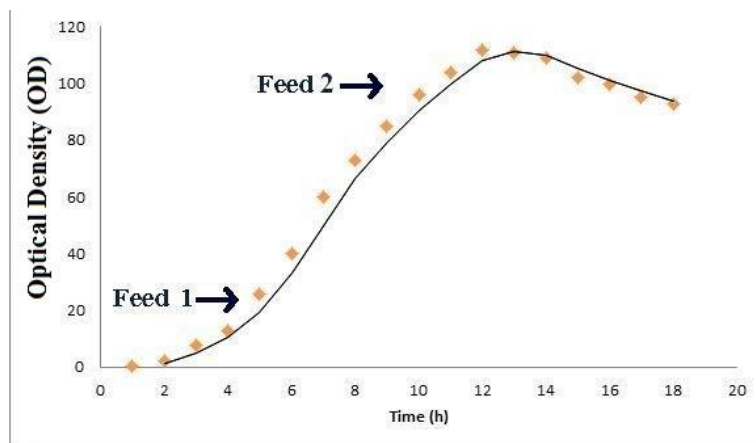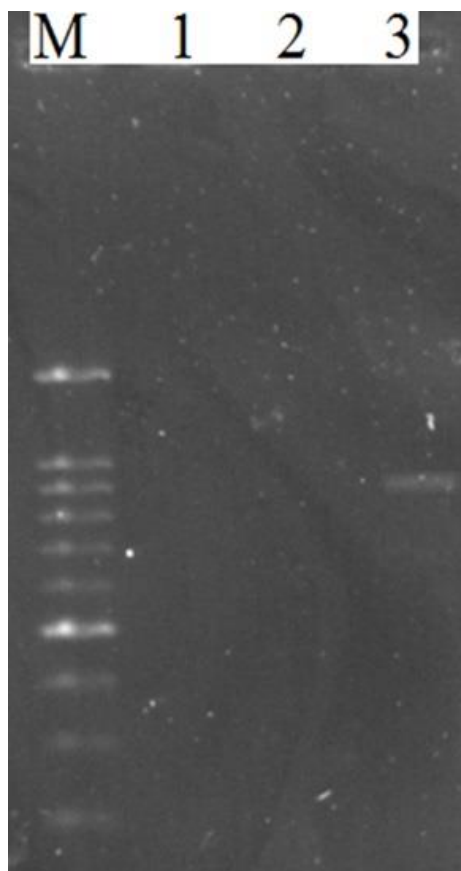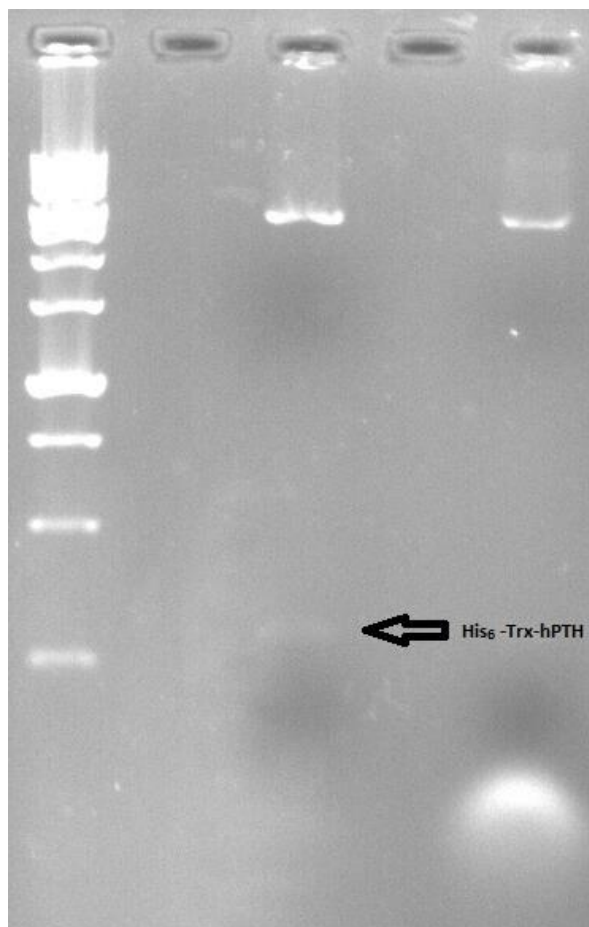[*]suntakrous (Cat. No:PR911654)

Fig. 4

Fig. 3

lane M: marker
lane 1:negative control PCR
lane 2:negative control E.coli
lane 3: colony- PCR
Fig 2

His$_6$ -Trx-hPTH

lane M: marker, lane 1: 216 ng digested
pUC57-hPTH

Fig 1