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A bicistronic expression strategy for large scale expression and purification of full-length recombinant human parathyroid hormone for osteoporosis therapy

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ABSTRACT

Parathyroid hormone (PTH) contributes to the increase of trabecular connectivity and is a candidate medication for effective treating osteoporosis. PTH is a protein of 84 amino acids and some studies have suggested that the active site lies within the range from amino acid (aa) 1 to 34. However, a few reports have indicated a causal relationship between PTH (aa 1–34) and osteogenic sarcoma in rats, while some less obvious but important roles of the carboxyl-terminus of PTH were also found. Unfortunately, it is difficult to obtain the active integrated PTH (1–84) *in vitro*, due to the instability of both the protein and its mRNA. Because an alternative translation start site is located at +25 nucleotides downstream of the true start site, a truncated PTH can be translated. We constructed a rhPTH bicistronic expression plasmid (pTrepth) that could highly express non-fusion soluble rhPTH proteins in *Escherichia coli*. The BL-21(DE3) containing pTrepth was cultured on a small scale until satisfactory expression and purification results were obtained. We then amplified the transformed cells in a 15-L fermentor and harvested 27 g/L cells (wet weight). Extensive rhPTH purification was achieved by a three step chromatography process. Activity tests demonstrated that our purified protein could dramatically increase cAMP in osteosarcoma cells *in vitro*.

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Introduction

Parathyroid hormone (PTH)[1] secreted by parathyroid chief cells is composed of 84 amino acids [1]. Importantly, endogenous PTH functions as one of the major hormones maintaining calcium and phosphate homeostasis not only by enhancing the release of calcium and phosphate from the skeleton, but also by increasing the reabsorption of calcium and slowing down the reabsorption of phosphate in the kidney [2,3]. Another effect of PTH is to transform 1,25-dihydroxyvitamin D into an active form which can promote calcium and phosphate being absorbed from the intestines [4,5]. Low extracellular calcium would stimulate the secretion of PTH, and high extracellular phosphate could also lead to elevated PTH through lowering the concentration of serum calcium; simultaneously, PTH secretion is inhibited in response to high serum calcium and decreased phosphate [6]. Interestingly, very distinctive effects are observed depending on the mode of exogenous PTH administration and the duration of exposure. Continuous injection of PTH results in a catabolic effect which accelerates bone resorption, maturates osteoclast, inhibits preosteoblast differentiation and increases the incidence of vertebral and peripheral fracture [7,8]. On the other hand, intermittent administration of PTH has shown anabolic effect on the bone, including stimulating bone formation, enhancing bone mineral density and improving the microstructure of the skeleton [9,10]. The therapeutic properties of PTH indicate its potential in clinical treatment of osteoporosis as well as other therapies for bone regeneration.

The active fragment of the human parathyroid hormone, consisting of the N-terminal amino acids (aa) 1–34, has been manufactured and marketed by Eli Lilly and Company (Indianapolis, IN) under the trade name Teriparatide. Teriparatide's treatments of post-menopausal women or glucocorticoid-induced osteoporosis in men have received satisfactory results [11,12]. However, some safety experiments on PTH (aa 1–34) indicated that when given PTH (aa 1–34) in a series of equivalent doses of 30–4500 μg/day/60 kg, adult rats with osteosarcoma were observed at all dose levels [13]. Incidentally, one case of osteogenic sarcoma related to Teriparartide was conscientiously reported by the manufacturer [14]. Safety tests were also conducted with the full-length PTH (aa 1–84) by injection of rats with doses of 10 μg/kg/day for 2 years, which was 4.6-fold greater than the equivalent human

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[1] *Abbreviations used:* PTH, parathyroid hormone; aa, amino acid; pTrepth, rhPTH bicistronic expression plasmid.

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exposure following a 100 μg dose. Surprisingly, the bone mineral density of the subjects significantly increased, and neither osteosarcoma nor other neoplastic and non-neoplastic proliferative changes were identified [15]. According to some reports, the C-terminal region of the full-length PTH (aa 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 [16]. The C-terminal regions of PTH was also shown to bind to its receptors on osteoblasts, resulting in some other less obvious effects [17,18]. PTH (aa 52–84) was found to be involved in the stimulation of collagen genes expression in chondrocytes [19]. These multiple advantageous properties of PTH (1–84) over its truncated form suggest that the full-length protein should be used for treatment of osteoporosis in the future.

However, an efficient and practical procedure for obtaining large amounts of intact rhPTH using gene engineering methods must be established. Great efforts have been made to prepare high levels of intact rhPTH from *Escherichia coli*, *Pichia pastoris* and *Saccharomyces cerevisiae* [20–22]. However, flaws exist in most of these studies related to the gene construct, expression product and efficiency. Eukaryotic expression systems are not needed due to the fact that PTH is not glycosylated. In fact, two undesired C-terminally truncated forms of PTH (aa 1–79 and aa 1–80) were observed by using *S. cerevisiae* [22]. *Escherichia coli* is the most extensively used expression system to produce intact rhPTH; however, most of them were produced using a fusion protein expression strategy to achieve high yielding efficiency and to prevent the translation of a short PTH (aa 8–84) from the alternative translational start site at +25 in the cDNA sequence [23]. Although these problems could be overcome using a tag-dependent expression strategy, additional manufacturing procedures must be established to cut the tag using enterokinase and to re-purify the product, undoubtedly increasing the costs [20].

Therefore, we hypothesized that a bicistronic expression strategy would be a promising solution for full-length expression of PTH. We synthesized a chimeric translation unit which could produce two polypeptides independently, including a short leader peptide and a mature PTH protein. To utilize the translation of the short leader peptide, the ribosome involved in this early process can open the secondary structure of the subsequent PTH mRNA [24]. The genuine translational start site then could be exposed in the linear PTH mRNA and translation can initiate at the correct start codon rather than the downstream alternative start site.

Materials and methods

Materials

The expression vector pThioHisA and *E. coli* strains including DH5α and BL-21(DE3) were obtained from Invitrogen (CA, USA), and Novagen (CA, USA), respectively. All primers, LA Taq polymerase, restriction enzymes, T4 DNA ligase and DNA the subcloning vector pMD18-T simple were purchased from TaKaRa (Dalian, China). All chemical reagents and antibiotics used in the cell culture media and buffers were analytical reagents obtained from Sigma (MO, USA) or a domestic provider in China. SP-Sepharose Fast Flow, Butyl Sepharose 4B, Superdex 75 pre grade and BPG columns, AKTA Pilot were purchased from GE Healthcare (Switzerland). Ultra filtration devices were from PALL (NY, USA). Cyclic AMP Assay Kit was purchased from R&D (MN, USA).

Construction of bicistronic expression vector

The gene sequences for the leader peptide and mature parathyroid hormone were synthesized by overlapping extension PCR, and the codons for PTH (GenBank Accession No. NM000315) were optimized for *E. coli* expression. Primers involved in the bicistron synthesis are listed in Table 1. The leader peptide included 18aa: NH_2-MIVSEIQLMHNLGKHLTS coded by the primer F1. The artificial bicistron with NdeI and SalI restriction enzyme cutting sites was inserted into the TA cloning vector pMD18-T simple. The recombinant clone was confirmed through sequencing on the ABI 377 (Applied Biosystems, CA, USA) then subcloned into pThioHisA between the NdeI and SalI restriction sites. As a result, the region from the HP-thioredioxin to the SalI cut site in the multiple cloning site was eliminated and occupied by the artificial gene (Fig. 1). The confirmed bicistron containing vector pTrepth was amplified in *E. coli* DH5α and then transformed into *E. coli* BL-21(DE3) to express the integrated rhPTH *in vitro*. We also synthesized and inserted only the gene sequence of the PTH mature protein into the pThioHisA as a negative control in our experiments.

Expression of PTH in small scale synthesis and fermentation

For the purpose of detecting the expression of rhPTH and selecting the highest-producing bacteria strain, six or more antibiotic-screened clones were cultured in tubes containing 3 ml LB broth (1% bacto-trypone, 0.5% bacto-yeast extract, 1% NaCl 100 μg/ml ampicillin) at 37 °C. As soon as the OD_{600} of each tube reach 0.6, 200 μl of bacteria culture medium was removed from each tube to serve as baseline controls. Subsequently, IPTG (1 mol/L) was added in each tube to induce the protein expression and ensure the final IPTG concentrations were 1 mmol/L. After the inductions were initiated, times were recorded and 200 μl of the cultures were sampled at 2, 4, 6, 8 h. Finally, the samples were analyzed by glycine SDS–PAGE (5–18% gel) and Coomassie blue R-250 staining. The stock which could highly express rhPTH was preserved as fermentation seeds. The control vector containing only the gene of the PTH mature peptide was also delivered into BL-21(DE3) and expressed in tubes.

A high rhPTH expressing bacterial stock was used to produce large amounts of the protein in a 15-L fermentor (Sartorius, Germany). First, a single clone was cultured in 10 ml LB broth containing 100 μg/ml ampicillin overnight. The 10 ml overnight culture was transferred to 800 ml of LB broth containing ampicillin and the medium was cultivated at 37 °C for 10 h. Then, the 800 ml LB medium was inoculated into the 15 L fermentor which was already filled with 14 L sterilized fermentation medium. Fermentation was initiated with the following maintenance parameters. Oxygen (pO_2) was maintained at 35% with a 300 rpm stirring speed; pH and temperature were fixed at 7.0 and 30 °C, respectively. Feeding

Table 1
Names and sequences of primers used in the study.

Primer name	Sequence (5′–3′)
F1	CATATGATCGTAAGTGAAATACAGCTAATGCACAACCTGGGAAAACATCTGACTTCATAATAAAGGAGGA
F2	CACTTCATAATAAAGGAGGAAACAGCTATGTCAGTAGCAGAAATACAATTAATGCATAATTTAGGTAAGC
F3	AATGCATAATTTAGGTAAGCATTGAACTCTATGGAAAGAGTTGAATGGTTGAGAAAGAAGCTGCAGGAC
R1	GTCGACTTACTGGGATTTAGCTTTAGTGAGTACATTCACATCGGCCTTGTCAGCCTCGCCA
R2	CGGCCTTGTCAGCCTCGCCAAGGGATTTTTCATGGCTCTCAACTAAGACATTGTCTTCCT
R3	AACTAAGACATTGTCTTCCTTTTTACGTGGTCTTTGGGAACCAGCATCACGAGGCGCCA
R4	CCAGCATCACGAGGCGCCAACGGAGCGCCCAGCGCAACGAAATTGTGAACGTCCTGCAGCTTCTTTCTCA

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