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# Engineering of a *Pichia pastoris* expression system for secretion of high amounts of intact human parathyroid hormone

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**Abstract**

Human parathyroid hormone (hPTH) is involved in calcium metabolism, and the unique ability of this hormone to stimulate bone growth makes it a promising agent in the treatment of osteoporosis. We have engineered the methylotrophic yeast *Pichia pastoris* for the production of over 300 mg intact hPTH per liter growth medium. The presence of 10 mM EDTA in the culture medium was essential to obtain this high hormone yield, indicating that metallopeptidases are mainly responsible for the otherwise instability of hPTH. Furthermore, the secretion process of hPTH was considerably improved by coexpression of *Saccharomyces cerevisiae* protein disulphide isomerase (*Sc*PDI). Since hPTH does not contain any cystein residues, this effect may be indirect or ascribed to the chaperone activity of PDI. Contrary to the situation in *S. cerevisiae*, use of a protease-deficient host strain provided no additional advantage. The hormone secreted by *P. pastoris* was not subjected to proteolytic processing by Kex2p in the two internal tribasic sites, nor were any C-terminal truncated hPTH forms detected. However, the *P. pastoris* hPTH producing transformants cosecreted ubiquitin to the culture medium, possibly as a result of a stress-related response.


*Keywords:* *Pichia pastoris*; Human parathyroid hormone; Heterologous protein expression; Protein disulphide isomerase; EDTA; Ubiquitin

## 1. Introduction

Human parathyroid hormone (hPTH) is a peptide hormone of 84 amino acids that serves as the principal regulator of calcium homeostasis and bone turnover in vertebrates by activating specific receptors located on osteoblastic and renal tubular cells (Potts et al., 1995). The unique ability of this hormone to increase bone density makes it a promising agent in the treat-

*Abbreviations:* *AOX1*, alcohol oxidase gene; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(beta-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; hPTH, human parathyroid hormone; PDI, protein disulphide isomerase; PpPDI and ScPDI, the coding region of the protein disulphide isomerase gene from *P. pastoris* and *S. cerevisiae*, respectively

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ment of osteoporosis (Neer et al., 2001; Whitfield et al., 2002; Rubin and Bilezikian, 2003). The anabolic and classical cAMP-stimulating activities of PTH have primarily been assigned to the N-terminal portion, PTH(1–34), which is sufficient for complete activation of the G-protein-coupled parathyroid hormone 1 receptor (PTH1R). The 1–14 portion of PTH is a determinant of receptor activation for second-messenger signaling (Luck et al., 1999), and the 15–34 portion controlling receptor binding affinity (Caulfield et al., 1990; Bergwitz et al., 1996). The signal transduction mechanism of PTH1R has been described as a two-site model, in which the C-terminal part of the ligand interacts with the large extracellular N-terminal domain of the receptor, and the N-terminal region of the ligand interacts with the juxtamembrane domain comprising the transmembrane α-helices and interconnecting loops. This model has later been extended to take into account the effect of different receptor conformational states arising from receptor-G-protein interaction on the molecular mechanism of ligand binding to the PTH1 receptor (Hoare et al., 2001; Chorev, 2002).

Various lines of evidence suggest that the mid- and C-terminal regions of PTH have more physiological relevance than previously thought. The mid-region, PTH(28–48), had a strong in vivo anabolic effect on neonatal mouse bones, possibly in interplay with IGF-I (Rihani-Bisharat et al., 1998). C-terminal PTH fragments stimulated gene expression of the two bone formation markers type-1 procollagen and IGFBP-5 in osteoblastic UMR-106 cells (Nasu et al., 1998), while residues 73–76 were essential for activation of a nickel-insensitive $Ca^{2+}$ influx pathway in growth plate chondrocytes (Erdmann et al., 1998). Receptors that specifically recognize the C-terminal region of intact PTH and circulating C-terminal fragments were highly expressed in osteocytic cells, where they may play a role in regulating cell survival and intercellular communication (Divieti et al., 2001). Furthermore, human PTH(7–84) influenced the $Ca^{2+}$ concentration negatively in thyroparathyroidectomized rats, thereby limiting the calcemic responses to hPTH(1–84) and hPTH(1–34), and inhibited bone resorption in vitro by acting on receptors distinct from PTH1R (Nguyen-Yamamoto et al., 2001; Divieti et al., 2002).

Elucidation of the biological activities of hPTH related to the structure of the hormone is of considerable medical importance, as more information concerning regulation of bone formation and characterization of molecular interactions between the ligand and receptor should make it possible to create more potent therapeutic agonists. Such studies obviously require access to large amounts of pure hormone, which can be achieved by heterologous production in yeast or *Escherichia coli*. The most effective bacterial expression system reported produced a fusion protein, where hPTH was obtained as [$Pro^{-1}$]-hPTH(1–84) after acid cleavage (Paulsen et al., 1995). Another strategy including expression of a synthetic hPTH gene under the control of the T7 promoter in *E. coli* resulted in N-terminally correct hormone, but a lower hormone yield (Oshika et al., 1994). One of the advantages with yeast as host organism is the effective secretion apparatus, which facilitates the purification procedure of the foreign protein. The combination of a growth medium rich in amino acids and a protease-deficient host strain was essential to obtain intact hPTH in *Saccharomyces cerevisiae* (Gabrielsen et al., 1990). The expression system was further improved by elimination of the spacer peptide in the MFα fusion (Gabrielsen et al., 1990) and one of two internal potential Kex2p processing sites by K26Q mutation, which resulted in a more degradation-resistant hPTH agonist, $hPTH^{Q}$ (Reppe et al., 1991). Two biologically active O-glycosylated hPTH forms were identified among minor products (Olstad et al., 1992). Finally, the level of intact K26Q mutated hormone was enhanced 30-fold to 16 mg $l^{-1}$ by changing from the constitutive MFα promoter to the inducible CUP1 promoter in the expression cassettes, use of another host strain, and optimization of fermentation conditions (Vad et al., 1998).

In the present work, we have explored the potential of the methylotrophic yeast *Pichia pastoris* to secrete intact hPTH. This yeast has the ability to express and secrete foreign proteins at high levels, and perform complex post-translational modifications (Cereghino and Cregg, 2000). Changing from the *S. cerevisiae* hPTH expression system to *P. pastoris* considerably increased the yield of intact non-mutated hormone, which was as stable as $hPTH^{Q}$ in this expression system. Coexpression of PDI from *S. cerevisiae* (*Sc*PDI) in the hPTH expressing cells and addition of EDTA to a final concentration of 10 mM in the culture medium contributed to a maximum level of over 300 mg hPTH per liter growth medium.