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Heterologous production of hPTH in fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids (hPTH(l-84)). A gene coding for human parathyroid hormone (hPTH) was synthesized and cloned into REP1 expression vector. REP1 is an expression vector which has nmt1 strong promoter which is thiamine repressible. The DNA insert was designed for secretory production of Human Parathyroid hormone by linking cpy1 secretory signal sequence at 5' end of cDNA sequence of Human Parathyroid hormone so that the expressed protein is excreted to the growth medium, allowing for rapid and easy purification of the desired products. The REP1 vector containing the designed DNA insert was transformed into Schizosaccharomyces pombe FY12854 strain. The transformed host was grown in Essential Minimal Media and extracellular media was checked for production of hPTH. Secreted protein was concentrated and purified by passing through S Sepharose column and further purified by two phase HPTLC.

Keywords : S pombe; hPTH; Secretory production; signal sequence.

INTRODUCTION

Human parathyroid hormone (hPTH) is 84 amino acid peptide secreted from the Human parathyroid gland. Primarily translation product is a 115-amino acid preprohormone which is processed during the secretion and the prepro part is cut off, yielding the 84-amino acid mature hormone [1]. hPTH is a principal regulator of calcium and phosphate homeostasis in blood through its actions on kidney and bone [2,3]. It has been reported to be effective in the treatment of osteoporosis as it induces an anabolic or bone formation effect when injected in low to moderate doses [4,5]. Thus, hPTH is a molecule of considerable interest regarding both biological and medical aspects. However, so far studies on intact hPTH have been hampered by the limited availability and the high price of the hormone. Hence, a system for the efficient expression of hPTH in microorganisms would be very advantageous for the further progression of studies on hPTH and its role in bone biology and disease. In this report we describe the use of *Schizosaccharomyces pombe* as an expression system for secretary production of hPTH.

The fission yeast being a eukaryote, is an attractive host model for high-level protein production and functional analysis of eukaryotic proteins as it shares many molecular, genetic and biochemical pathways with higher eukaryotes such as plants and animals, and is distinguishable from other yeasts through its ability to proliferate by fission rather than budding [6-8]. Many cellular processes in higher eukaryotes, such as mRNA splicing, post-translational modification, cell-division and cell cycle control, are closely related to those in the fission yeast [9]. Furthermore, *S. pombe* has a developed Golgi apparatus and galactosyltransferase. Many types of human proteins have been successfully expressed in *S. Pombe*, such as human antithrombin III [10], human papillomavirus E7 protein [11], and human D2S dopamine receptor [12], as well as for effective production of many types of heterologous proteins [13-17].

Secretion of any heterologous protein is always advantageous as it reduces steps in downstream processing. Various signal peptides like Cpy, P3, pho1, Amy and Dpp have been used before heterologous proteins for their secretion out of *S. pombe*, Cpy signal peptide supported efficient secretion of GFP with increased yield approximating 10 mg/L. Carboxypeptidase Y(cpy1) is a peptidase of S10 family. It is 1002 AA long peptide with a 18 AA acid long potential signal peptide. Signal peptide predicted for SPAC19G12.10c by SignalP 2.0 HMM (Signal peptide probability 0.994, signal anchor probability 0.004) with cleavage site probability 0.895 between residues 18 and 19 by proteases A [18]. The main objective of this study is secretory production of hPTH in *S. pombe*.

MATERIALS AND METHODS

Strains and the construction of expression vector: *E. coli DH5α* was used for the propagation of plasmids. *S. pombe FY 12854 (h-ura4-D18leu1-32)* was used for expression and secretion of hPTH. DNA manipulations, cloning and PCR experiments were performed as standard protocols [19] or as recommended by the respective manufacturers.

The DNA segment encoding the hPTH was chemically synthesised from GeneArt, regensberg. The nucleotides in the original cDNA sequence were modified to the preferred codons for *S. pombe.* The synthetic DNA was designed to contain the Secretory signal sequence of CpY 1 upstream of human parathyroid hormone. On 5' end of synthetic DNA Nde I site was designed and on 3' end Sma I site was designed to join the nmt1 terminator.

Final DNA construct as depicted in pREP1 map (Fig. 1) is 5'-CA^TATG-
TTAATGAAACAAACTTTTTTATATTTTTTATTAACTTGTGTTGTTTCTGCTTCTGTTTCTGAAATTCAATT
AATGCATAATTTAGGTAAACATTTAAATTCTATGGAACGTGTTGAATGGTTACGTAAAAAATTACAAG
ATGTTCATAATTTTGTTGCTTTAGGTGCTCCTTTAGCTCCTCGTGATGCTGGTTCTCAACGTCCTCGTAA
AAAAGAAGATAATGTTTTAGTTGAATCTCATGAAAAATCTTTAGGTGAAGCTGATAAAGCTGATGTTA
ATGTTTTAACTAAAGCTAAATCTCAATAATGA-CCC^GGG-3'

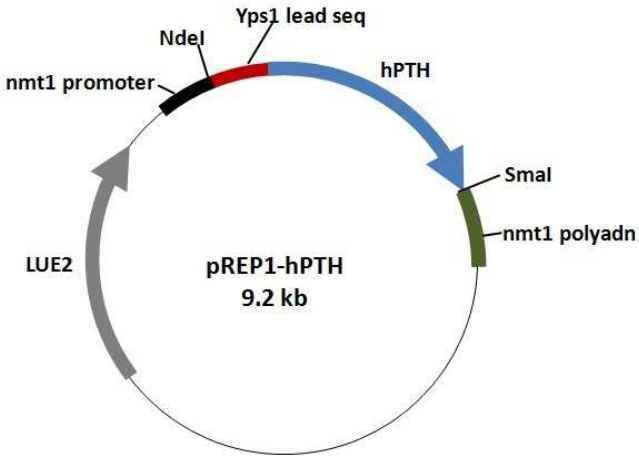

Fig.1 Structure of plasmid REP1-hPTH

The designed construct was synthesized from GENEART, Germany. Construct was received in a 2.7 kb vector. Vector was multiplied in *E coli DH5α* from where it was isolated and stored at -20^{o}C for further experiments.Construct was cut out of vector using Nde1 and Sma1 restriction enzymes and ligated into 8.9 kb Rep1 vector of *S. pombe.* Ligation of gene to Rep1 vector was confirmed through PCR. Primers for hPTH DNA were designed and analysed using generunner. PCR was run to amplify the hPTH DNA from both Geneart and Rep1 vector with thermal profile as Table1. The samples were analyzed on 10% PAGE run at 100V for 3 hours in TBE buffer, Gel was silver stained [20].

Table 1: Thermal profile for PCR

S. No.	PCR phases	Temperature	Time	No. of cycles
1.	Initial Denaturation	95 ºC	5 min	1
2.	Denaturation	95 ºC	1	30
3.	Annealing	50.7 ºC	1	
4.	Extension	72 ºC	1.5	
5.	Final Extension	72 ºC	10 min	1
Stored at 4 ºC for 20 min				

***S. pombe* transformation with Rep1 plasmid containing hPTH gene:** YES medium (1% yeast extract+supplements) was used for the cultivation of host. MS media was used for culturing host before transformation. *S. pombe FY 12854 (h-ura4-D18leu1-32)* was transformed with Rep1 plasmid containing hPTH gene using lithium acetate technique [21]. An EMM medium (Edinburgh minimal media; without LUE) was used for the selection of yeast transformants. Transformed cells were plated on EMM (without LUE) and incubated at 30 oC for 4days.Composition of EMM medium was as follows (*g/l*): Potassium hydrogen phthalate, 3; Na2HPO4, 2.2; NH4Cl, 5; Glucose, 20; Salts stock solution, 20ml; Vitamins stock solution,1ml;Minerals, 0.1ml;Uracil 150mg, for ura4- strains;Leucine 150mg, for leu1- strains.

Expression of the hPTH Single colony of fresh transformants of *S. pombe* FY 12854 harbouring the expression vectors was inoculated and cultured in 50 ml of EMM broth (Edinburgh minimal media; without LUE) and were incubated at 30 ^{o}C for 36 hours under shaking conditions at 150rpm . After 36 hours the culture was harvested and whole cells were removed from the culture by centrifugation (6000 g, 10 min at 4 °C).

Concentration and purification of hPTH The supernatant was first chromatographed on S Sepharose fast-flow column (5.0 cm x 19.0 cm), (Qiagen USA, Valencia, CA). The column was equilibrated in a buffer containing 0.3 M glycine, pH 3.0. The pH of supernatant was adjusted to 3.0 with 1 M HCI before application. After application of the supernatant, the column was washed with 0.1 M HAc, pH 6.0 and hPTH was eluted with 0.1 M Na2HP04, pH 8.5 at a flow rate of 300 ml/h. The eluted fractions were monitored spectrophotometrically by reading absorbance at 280 nm. Parathyroid hormone was eluted together with the fraction showing maximum absorbance at 280 nm.

Analysis of hPTH using analytical gel electrophoresis

The hPTH material obtained from the different purification steps was collected and analyzed on a 15% polyacrylamide gel in the presence of 0.1% SDS [19]. The gel was Silver stained [22].

Reverse-phase HPLC

The concentrated medium from the S Sepharose column was subjected to further purification by a two-step reverse phase HPLC procedure. In both steps, the following eluents were used: eluent A, 0.3% trifluoroacetic acid in MilliQ water; eluant B, 0.3% trifluoroacetic acid in 70% aqueous acetonitrile. The flow rate was maintained at 0.05 ml/min. The first step employed a Shimadzu protein/peptide CI8 column (4.6 mm x 250 mm). A linear gradient of 40-60 % B was run over 35 min. The second HPLC step used a Shimadzu C18 column (0.8 cm x 10 cm). The column was subjected to isocratic elution with 47% B. The eluted fractions were monitored by ultraviolet detection at 220 nm.

RESULTS

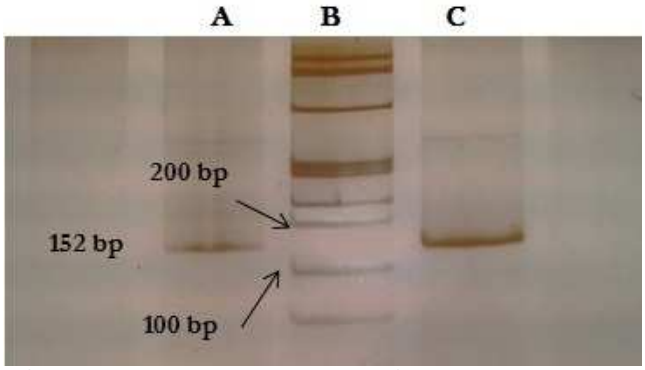

Fig. 2: A-152 bp PCR product from Geneart vector ; B-Ø×175 Marker from Genei ; C-152 bp PCR product from Rep1-hPTH.

Construction of expression vector: hPTH gene Construct was excised out of GENEART vector using Nde1 and Sma1 restriction enzymes and ligated into 8.9 kb Rep1 vector of S. pombe. Ligation of gene to Rep1 vector was

confirmed through PCR. PCR was run to amplify the hPTH DNA from both Geneart and Rep1 vector. A single 152 bp band confirmed ligation of DNA construct to Rep1 vector (Fig. 2).

Expression and secretion of hPTH(1-84) in *S. pombe*

The hPTH was expressed in S pombe and the products were secreted to the growth medium were analyzed by SDS-PAGE(Fig. 3). The supernatant of Transformed culture (lane3) showed an extra band of molecular mass 9 kD as that for PTH(1-84)

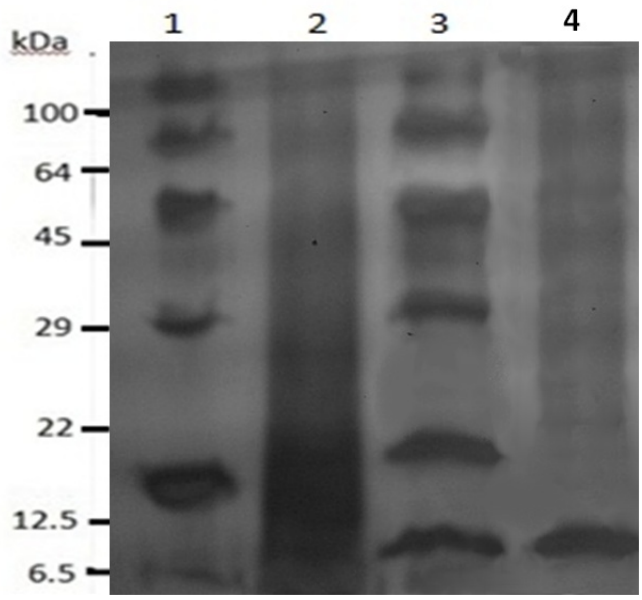

Fig.3 SDS-PAGE Profile. Protein was analysed by SDS-PAGE. lane1: Extracellular protein from host *S. pombe FY 12854 (h-ura4-D18leu1-32)*; Lane 3: extracellular protein from *S. pombe* transformed with Rep1; lane4: purified Hormone after RP-HPLC. Gel was silver stained. Molecular weights (kDa) are indicated on the left.

Purification of hPTH from the Growth Medium

hPTH was concentrated from the growth medium by chromatography on S-Sepharose (showing 70% pure hPTH), and the fraction was then subjected to reverse phase HPLC. As shown in Fig. 4 a major peak with the same retention time as Standard hPTH could be identified. SDS-Page of the peak fraction showed one band at 9 kD, lane 4 Fig. 3.

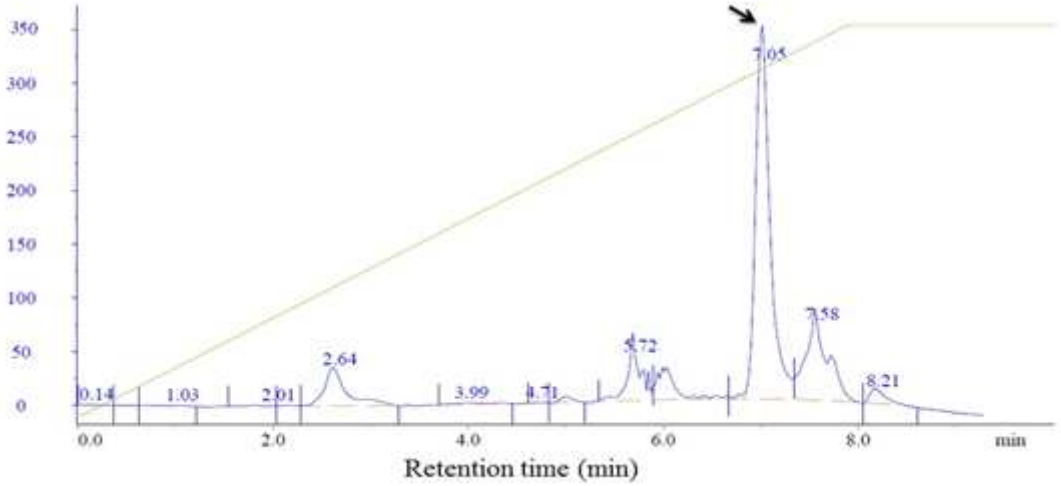

Fig.4 RP-HPLC purification of hPTH(1-86). Supernatant concentrated on S-Sepharose was further purified as described in Materials and Methods. The elution profile from the first HPLC step, a Shimadzu C-18 column, is shown. The peak containing hPTH(1-84) is marked.

DISCUSSION

hPTH is a hormone with immense medical significance with limited availability and high costs. In this study, we describe here the successful construction of an expression plasmid which efficiently directs the synthesis of hPTH

and its secretion out of the yeast cell. This construct was under the control of a pREP1 strong promoter. The highest level of expression achieved was approximately 100 µg/litre. The reason for low level of expression is probably due to proteolytic degradation of the secreted protein by extracellular proteases of the host. Increased level of expression would be anticipated by reducing or masking the effect of extracellular proteases on the secreted product and we are presently constructing such a strain which would be deficient in proteolytic activity and having higher expression of hPTH.

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