2. Cloning, expression and purification of rhPTH (1-34)

2.1. INTRODUCTION:

Para thyroid hormone (PTH) is a naturally occurring peptide hormone involved in bone morphogenesis and remodeling. Calcium ion homeostasis in vertebrates is regulated primarily through the action of parathyroid hormone.

In humans, PTH is synthesized as a 115-amino acid precursor polypeptide, which is processed by the endoplasmic reticulum, golgi apparatus and secreted as an 84-amino acid peptide (Potts et al., 1980). Interestingly, the fragment consisting of the 1-34 amino acid sequence appears to contain all of the information necessary for full biological activity. In the original chemical synthesis of PTH, the phenylalanine residue at position 34 was chosen as the amino acid to couple to the solid support resin (Potts et al., 1971), virtually all studies for the past three decades have used this synthetic fragment or analog of it.

The hormone acts on bone and kidney cells which express high affinity surface membrane receptors. PTH binding results in receptor activation and stimulation of the adenylatecyclase complex, leading to accumulation of intracellular cAMP. Cyclic AMP activates protein kinaseA to phosphorylate key regulatory proteins that presumably mediate the biological actions of PTH in its target tissues.

The expression in Escherichia coli of biologically active peptides has met with limited success due to both the low yield, presumably related to the rapid intra cellular degradation of the peptide as well as the difficulty in purification of the peptide away from endogenous contaminating proteins and peptides.
Several groups have recently reported the expression of fusion proteins containing either 1-84 or 1-34th form of PTH in E.coli. Expression from E.coli lac promoter yielded not more than 500ug of immunoreactive /liter of culture (Rabbani et al. 1988; Morella et al 1988, Mayer,1988). Gardella et al have expressed human PTH(1-84) as a factor –Xa cleavable fusion protein with human growth hormone. Intact hPTH could be purified following factor-Xa cleavage at levels of 1.5-3mg/lt of original cell culture. Kareem et al describe similar results using protein A as a fusion protein partner. Wingerder et al (1989) reported improved yields of PTH using a acid cleavable fusion protein expression strategy. This system produced upto 250 mg of fusion protein /lt of culture, which after treatment with formic acid yielded 3-5 mg of [pro’] PTH. Olstad et al and Gantrik et al reported expression of hPTH as a resulted in the secretion of the fusion to yeast malting protein. The expression system resulted in the secretion of hPTH into the media and peptide was found to be o-glycosylated. An overall yield for this system was not given.

Oldenburg et al (1994) reported high yield production of hPTH (1-34) by using a gene polymerization strategy. The PTH gene polymerization contains upto 8 copies of the gene, each separated by a cleavable linker. The monomeric rPTH (1-34) is released from the polymer by chemical cleavage with cyanogen bromide. Peptides produced through this methodology will also contain a homoserine/homoserine lactone residue at the carboxyl terminal.

The purpose of the present work is to describe a methodology that results in very high expression and rapid purification of intact rhPTH (1-34) in E.coli.
2.2. Materials and methods:

2.2.1 cDNA Synthesis, Cloning and Expression of rhPTH(1-34):

2.2.1.1 mRNA isolation from human parathyroid adenomas:

Poly(A) selected RNA was isolated from human parathyroid adenomas immediately after surgery. The tissue was homogenized in RNA extraction buffer (500µl) containing 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sacrosyl, 0.1 M 2-mercaptoethanol at 4°C and disrupted for 1 min with a Polytron tissue homogenizer. 50 µl of 2M Sodium acetate, 500µl water saturated phenol and 200µl Chloroform:Isoamyl alcohol (49:1) were added to the lysate. Aqueous phase was collected after centrifugation at 10,000 rpm for 10 min. RNA was precipitated by the addition of 2.5 volumes of isopropanol and incubated at -70°C for 1 h. The RNA pellet was washed with 70% ethanol and resuspended in 20µl of water and stored at -70°C until use. All the solutions were made in DEPC treated distilled water. RNA was isolated, and poly(A) mRNA was prepared by oligo(dT) chromatography as described by Maniatis et al.

2.2.1.2 cDNA synthesis:

hPTH(1-34) cDNA was synthesized from mRNA by using the following gene specific reverse primer.

Reverse primer: 5'-AAA ATT GTG CAC ATC CTG -3'.

About 1µg (10µl) of RNA, 1µl of 10µM gene specific reverse primer and 2µl of sterile de-ionized water were taken for the first strand cDNA synthesis in a 0.2 ml PCR tube. Contents were mixed and the tube was spun briefly. The sample was incubated at 70°C for 5 min for removing the secondary structures of RNA and cooled on ice for 2 min to anneal the primer. 4 µl of 5X first strand synthesis buffer, 2µl of 10mM dNTP and 1µl
(200 units) of MMLV reverse transcriptase devoid of RNaseH activity were added to the tube and mixed by pipetting gently for final reaction volume of 20μl. The tubes were incubated at 42°C for 1h. Termination of the first strand reaction was achieved by incubating at 70°C for 10 min.

2.2.1.3 PCR amplification with cDNA:
Specific primers were designed for hPTH (1-34) having the Prolyl endopeptidase cleavage site bearing the sequence 5' - CC GGA TCC CCG TCT GTT TCT GAA ATC -3' as a forward primer and 5' - CC GAA TTC TCA AAA ATT GTG CAC -3' as a reverse primer. The final concentration of synthesized primers were adjusted to 10μM. The PCR reaction was setup by adding 8μl of synthesized single stranded c-DNA, 31.5μl of sterile MilliQ water, 5μl of 10X PCR buffer containing 1.5mM MgCl₂, 1.5 μl of 10mM dNTP mix, 1.5μl of each of the forward and reverse primers (10μM) and 1μl of Taq polymerase for a 50μl final reaction volume. The reaction mix was mixed well and kept in thermal cycler for amplification of desired product with a program of initial denaturation of 95°C/2min, and 40 cycles of 94°C / 30Sec, 52°C/30sec, 72°C /45sec followed by a final extension step of 72°C/5min.

2.2.1.4 Agarose gel electrophoresis:
PCR products were resolved on 1.5% agarose gel. Agarose gel electrophoresis was carried out as described (Sambrook et.al.,1989). Agarose gel was cast by melting 0.6gm of agarose in 40ml of 1XTAE buffer (40mM Tris-acetate and 1mM EDTA pH 8.0). The solution was cooled to 60°C and ethidium bromide was added to a final concentration of 0.5μg/ml. Warm agarose solution was poured into mould (sealed with tape) and comb
was inserted. Comb was removed from complete set gel after 30 min. DNA samples mixed with 6X sample buffer (0.25% Bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) were loaded on to gel and electrophoresis was carried out in 1X TAE buffer.

**2.2.1.5 Isolation of DNA fragment from agarose gel:**

The portion of the agarose gel containing the amplified DNA fragment excised and equal volume of T\textsubscript{10}E\textsubscript{0.1} (10mM Tris and 0.1mM EDTA), pH 8.0 was added followed by an equal volume of Tris-saturated phenol and Chloroform:Isoamylalcohol(24:1). The entire mix was vortexed vigorously for 2 min, frozen at -70°C for 15 min and centrifuged at 12,000 g for 15 min at 4°C. The aqueous layer was transferred to a fresh tube and chilled at -70°C for 15 min. The tube was centrifuged at 12,000 rpm for 15 min at 4°C to remove agarose. The supernatant was collected and the DNA was precipitated by addition of sodium acetate to the final concentration of 0.3M and 2 volumes of ethyl alcohol and incubated at -70°C for 1h followed by centrifugation at 12,000 g for 15 min at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 10 μl of T\textsubscript{10}E\textsubscript{0.1} pH 8.0. The purified product was visually quantified on agarose gel.

**2.2.1.6 Cloning of rhPTH(1-34):**

The PCR product and the vector p-VB (in house constructed vector containing GST tag) (Fig 4A) was restriction digested with BamHI and EcoRI. After restriction digestion, ligation reaction was carried out at 16°C for overnight in a 10μl volume containing ligation buffer (50mM Tris-HCl pH 7.6, 10mM MgCl\textsubscript{2}, 10mM DTT and 5mM ATP), 50ng of the plasmid vector p-VB, 12ng of insert DNA and 10 Units of T4 DNA ligase.
Fig 4: Expression plasmid construction:
A) The rhPTH(1-34) was spliced into pVB vector as BamH1 and EcoR1 fragment. The expression was driven by Tac promoter regulated by IPTG.
B) Agarose gel electrophoresis for checking the clone. Lane M, marker. Lane 1, rhPTH(1-34) PCR product. Lane 2, pVB alone. Lane 3, vector containing the insert (clone). Lane 4, plasmid digested with BamH1 and EcoR1 enzymes.
The ligation mix was transformed into E.coli competent cells.

The competent cells were prepared according to Cohen (1972) with minor modifications. A single colony of E.coli Top10 was inoculated in 5ml of LB (Luria Bertini) medium (10gm of Peptone, 5gm of Yeast extract and 10gm of NaCl /L) and incubated at 37°C overnight. From the overnight grown culture, 1ml was inoculated in 100 ml of LB (1:100 dilution) medium and incubated at 37°C with vigorous shaking till optical density at 600nm reached 0.4. The culture was chilled on ice for 15 min and the cells were pelleted by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was drained thoroughly and cells were resuspended in 20ml of ice cold 0.1M CaCl₂. After chilling on ice for 20min, cells were pelleted as before, resuspended in 4ml of 0.1M CaCl₂ and left on ice for another 15 min. Aliquots of 200μl of these cells with 30% glycerol were flash frozen in liquid nitrogen and stored at -70°C till needed.

The frozen competent cells were thawed on ice slowly just before use for transformation. Ligation mix of 10 μl was mixed with competent cells and incubated on ice for 30 min. The cells were subjected to heat shock for 90 seconds at 42°C in water bath, followed by rapid chilling on ice for 5 min. An aliquot of 800μl of LB medium was added to the cells and the cells were incubated for 1h at 37°C with gentle shaking. The entire mix was pelleted at 4000 rpm for 2 min and suspended in 100μl of LB medium. For blue white selection, cells were plated on an LB plate (LB medium with 1.5% agar) containing ampicillin of final concentration of 70μg/ml overlaid with 4μl of 200mg/ml Isopropyl thio-β-D galactoside (IPTG) and 40μl of 20mg/ml 5-Bromo 4-Chloro 3-indolyI-β-D galactoside (X-Gal) and incubated overnight at 37°C.

White colonies from plate after transformation were inoculated for isolation of plasmid.
An *E. coli* colony was grown overnight in LB medium containing ampicillin 70 μg/ml. Cells of 1.5ml culture were harvested by centrifugation. The DNA was purified by using the Qiagen mini prep kit.

For checking the presence of insert, restriction digestion of DNA was carried out in the volume of 20μl containing ~2 (g of isolated plasmid DNA, 2 μl of 10X Buffer 2 of NEB(New England Biolabs, USA), 1 μl of EcoRI( 10units), 1 μl of BamH1 and incubated at 37°C for 3h. Enzyme was inactivated by incubating the reaction mix at 65°C for 15min. The reaction mix was resolved on 1.5% agarose gel as described earlier.(FIG 4B). Once the presence of insert is confirmed the isolated recombinant plasmid is subjected to automated sequencing.

2.2.2 Expression of GST fusion protein:

E.coli competent cells were transformed with recombinant plasmid (pVB-PTH (1-34)) and grown on LB plate containing 70μg/ml ampicillin. A single colony was inoculated in 10ml of LB medium containing 70μg/ml ampicillin and grown overnight in a 50ml flask at 37°C. 100ml of pre-warmed media containing antibiotic was inoculated with 1ml overnight grown culture and incubated at 37°C with vigorous shaking till OD600 reached 0.6. Prior to induction, a 1ml aliquot of culture was collected; cells were pelleted and stored at -20°C for further analysis. Adding IPTG to a final concentration of 1mM induced expression of recombinant protein. The samples were collected after 3h of induction and cells were pelleted.

2.2.2.1 SDS-PAGE:

SDS-PAGE (Sodium dodecyl Sulphate-Poly acrylamide gel electrophoresis) analysis was
done using induced and uninduced samples to determine optimum expression time. SDS-PAGE was carried out by the method described by Lamemli (1970) using a discontinuous buffer system. A solution containing 29% acrylamide and 1% N, N'-methyl bis acrylamide (w/v) was used. The gels were cast on a vertical mini protein gel apparatus (Bangalore Genei, India) with 1mm spacers. For 12% resolving gel (5ml), 1.6ml sterile water, 2ml of 30% Acrylamide, 1.3ml of 1.5M tris pH 8.8, 50 µl of 10% SDS, 50 µl of 10% Ammonium persulphate (APS), and 4 µl of N,N,N',N' tetramethylethylenediamine (TEMED) were added in clean glass vial and mixed well before pouring between sealed glass plates leaving one-fourth of plate size for staking gel. Butanol was overlaid on the resolving gel mixture. After complete polymerization (30min) the overlaid butanol was poured off and the top of the gel was washed several times with deionized water to remove any unpolymerized acrylamide. For 2ml of 5% stacking gel solution, sterile water 1.4ml, 330 µl of 30% Acrylamide:bisacrylamide, 250 µl of 1M Tris pH 6.8, 20 µl of 10% SDS, 20 µl of 10% APS and 2 µl TEMED were added and mixed. Stacking gel solution was poured directly onto the surface of polymerized resolving gel and teflon comb was immediately inserted into the stacking gel solution.

Cell pellets collected before and after induction were suspended in SDS protein loading dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue and 5% β-mercaptoethanol) and heated in a boiling water bath for 10 min. These boiled samples were spun briefly and supernatant was loaded on to the gel. Electrophoresis was carried out in a constant current mode at 20mA till the samples crossed the stacking gel and then at 35mA through the resolving gel in Tris-Glycine Buffer (25mM Tris, 250mM Glycine (pH 8.3) and 0.1%SDS).
2.2.2.2 Staining with Coomassie brilliant blue

The gels were stained with 0.2% Coomassie brilliant blue R250 in methanol:acetic acid:water (45:10:45 v/v). The gels were de-stained with the same solution without dye for 6 h on the rocking platform with 3 changes.

2.2.3 High Density Fermentation:

The high-density fermentation of recombinant bacterial E.coli (pVB/PTH (1-34)) was carried out with 5 litre fermentor by fed-batch cultivation. 4.2lit of sterile LB media was inoculated with 100ml of seed inoculum and supplemented with 2% dextrose. During the batch fermentation, the temperature and pH were maintained at 37°C and 7.0 respectively. The dissolved oxygen level was maintained at 30% to 40% by using air or pure oxygen and maintain the speed at 600 rpm. After OD600 reaches to 60, a final concentration of 1mM IPTG was added to induce the expression of fusion protein for 3h. After 3hrs, harvest the cells by centrifuging at 4000rpm for 10 min at 4°C and frozen at -70. The expression was checked by running the samples on SDS-PAGE gel and stained with CBB staining. (Fig 5B)

2.2.4 Cell lysis and fusion protein isolation:

The frozen pellet (91gms) was thawed on ice and resuspended in 100ml of 1XPBS. The pellet was homogenized to get the suspension. The homogenate was lysed by using the bead-beater (Bio-Spec). The beads of 0.1mm size was washed with 1XPBS and added to the homogenate. The beads were removed by setting and separating the suspension.
Triton-X-100 (1% final) was added and mixed gently by stirring for 30 min at room temperature to aid solubilization of fusion protein. The solution was centrifuged at 18,000 rpm for 30 min at 4°C in Sorval RC-6 with SV-800 rotor.

The clear solution was loaded on to the 200ml Glutathione-sepharose 4B affinity column pre-equilibrated with 1XPBS (column Bed height-8cm and diameter-6cm). Wash the column with 4bed volumes of 1XPBS to remove the unbound proteins. The bound fusion protein (GST-PTH (1-34)) was eluted with the 600ml of 5mM reduced glutathione in 50mM Tris/Hcl, pH 8.0. Collect the 100ml fractions. The purity of Fusion protein was analyzed by SDS-PAGE gels. The yield of fusion protein was calculated by taking the O.D of pure fractions at O.D_{280} (O.D 1 = 0.5 mg/ml).

### 2.2.5 Cleavage of Fusion protein:

The pure fraction of fusion protein was pooled and dialyzed into 50mM Tris/Hcl, pH 8.0, 150 mM NaCl and 2mM CaCl, to remove the glutathione reduced. The dialyzed fused GST-hPTH protein was cleaved with thrombin (1:200 w/w enzyme to fusion protein ratio) at 22°C for 2 hrs. Thrombin was purified from the bovine blood in our laboratory only. To collect the Gly-Ser-Pro-hPTH (1-34), the digested sample was reloaded onto the GST affinity column and collect the unbound protein.

The Gly-Ser-Pro-hPTH (1-34) was further cleaved by Prolyl endo-peptidase(PEP) to remove the Gly-Ser-Pro amino acids. The protein was mixed with PEP in the ration of 1:50 (w/w) and the mixture was incubated at 25°C for O/N.
2.2.6 Purification of hPTH(1-34) by HPLC:

The cleaved hPTH (1-34) was further purified by using the reversed phase column chromatography. To the sample add 0.1% TFA followed by loading onto the source 30 RP matrix packed into a 50 ml column. The column was pre-equilibrated with 0.1% TFA. The pure peptide was eluted with a linear gradient of Acetonitrile (acetonitrile 0%/0.1% TFA - acetonitrile 70%/0.1% TFA). The pure fractions were analysed by SDS-PAGE. The peptide was then lyophilized to remove the acetonitrile. The purity of the peptide was analyzed by running the sample on analytical C18 column. The molecular weight and amino acid sequence was confirmed by Mass spectra and N-terminal sequencing.

The peptide was formulated into formulation buffer. Formulation Buffer contains Sodium succinate, glycerol and M-cresol.

2.2.7 Biological activity:

PTH regulates mineral ion metabolism and bone turnover by activating specific receptors located on osteoblastic and renal tubular cells. In these tissues PTH stimulates multiple intracellular signals that include cAMP (Chase et al. 1968; Rodan et al. 1974; Hunt et al. 1976; Goltzman et al. 1976; Nissenson et al. 1979) and Calcium (Farese et al. 1981; Meltzer et al. 1982; Rappaport et al. 1986; Hruska et al. 1987; Yamaguchi et al. 1987; Babich et al. 1989; Cosman et al. 1989).

The Biological activity of the purified rhPTH (1-34) was assayed by it's ability to stimulate renal adenylate cyclase. Renal adenylate cyclase was assessed by measuring the stimulation and accumulation of cAMP in the Rat osteosarcoma cells UMR-106.
2.2.7.1 Cell culture:

The Rat Osteosarcoma cells UMR-106 (ATCC# CRL-1661) was obtained from American type culture collection. The cells were maintained in a humidified 5% CO2 atmosphere. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. For assay, cells were sub-cultured into 24 wells plate in regular medium to a confluence of 70-80%. For the Assay, cells were treated with different concentrations of rhPTH (1-34) and standard PTH for 1 hr at 37°C in absence of serum. After incubation with PTH, cells were washed with 1XPBS and the cells were lysed in 1ml of 0.1N HCL on ice. The accumulated cAMP was determined by indirect cAMP enzyme immunoassay kit from ASSAY DESIGNS.

The principle of the immunoassay kit briefly, Assay Designs' Correlate™ EIA Direct cyclic AMP kit is a competitive immunoassay for the quantitative determination of cyclic AMP in samples treated with 0.1M HCl. The kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the standard or sample or an alkaline phosphatase molecule which has cAMP covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of cAMP in either standards or samples. The measured optical density is used to calculate the concentration of cAMP.
2.3. Results:

2.3.1 Expression plasmid construction:
p-VB vector was used to construct an expression plasmid. The cDNA was placed downstream from E.coli Tac Promoter. An extra amino acid was placed at N-terminal to PTH (1-34) to cleave with PEP. The hPTH cDNA coding sequence was inserted into BamH1 and EcoR1 site of the plasmid.

The GST-hPTH fusion protein was overproduced in E.coli cells transformed with pVB-hPTH plasmid in an inducer dependent manner. Induction of Tac promoter by addition of IPTG to the cell culture resulted in accumulation of fusion protein to the extent that it was the most abundant protein detected by SDS gel analysis of whole cell lysates. In the induced cells a predominant band migrating with a molecular mass of approximately 30KDa was observed which was not detected in the uninduced cells. (Fig5). The percentage of the expressed protein versus total protein was detected approximately 25% when quantified by densitometry. The total wet weight of the cell pellet was 30gms/L culture.

2.3.2 Purification of Fusion protein and cleavage:
Like many other proteins that are overproduced in E.coli cells, the GST-PTH fusion protein secreted into the cytosol. The fusion protein was solubilized and extracted by lysing and solubilizing with 1% Triton-X-100. The fusion protein was purified over immobilized glutathione which yielded over 85% pure fusion protein (Fig 6). The yield of the fusion protein was 600mg/lit culture. After dialysis against thrombin cleavage buffer, the fusion protein was cleaved with Thrombin followed by Prolyl endopeptidase.
Fig 5: Expression analysis of pVB-PTH(1-34):
The cells were induced with 0.5mM IPTG for 3hrs. The samples were
Collected every hr and Lysed in lysis buffer and subjected to SDS-PAGE
analysis. A) Expression analysis of shake flask samples, Lane M-marker,
1-4 is 0hr to 3rd hr expression samples respectively. lane 5 is vector alone.
B) Expression levels in fermentation batch. Lane1, ohr. Lane 2-4 is 1-3rd hr
expression samples respectively.
Fig 6: SDS-PAGE analysis of processed samples:
The cell pellet was lysed by using bead beater and solubilized with Triton-X-100 and fusion protein was purified over GST matrix. The bound fusion protein was eluted with 5mm reduced glutathione. Lane 1. pellet after solubilization. Lane 2, Supernatent. Lane 3, unbound. Lane 4&5 are washes. Lane 6-9 are eluted fractions.
(PEP). This yielded two cleavage products which migrated on SDS gels with a molecular masses of 26 and 4KDa corresponding to GST and PTH (1-34) fragment, respectively (Fig7). The cleaved product was purified over RPC Source-30 matrix. The peptide was eluted with a linear gradient of 24-28% Acetonitrile. (Fig8). The eluted peptide was lyophilized and analysed on the Analytical HPLC C18 column and on the SDS gel. (Fig9 and Fig 10). The yield of the pure peptide was 25mg/L culture pellet.

2.3.3 Biological activity:

The biological activity of the recombinant hormone was evaluated in cAMP stimulation assay using the Rat osteosarcoma cell line UMR-106. Chemically synthesized and Forteo was used as standards. Recombinant hPTH and synthetic peptides showed similar dose response curves in the cAMP stimulation assay. The activity of the rhPTH(1-34) was estimated as 1.04X10^4 IU/mg protein (Fig 11).
**Fig 7: analysis of digested samples:**
The eluted fusion protein was dialysed into thrombin cleavage buffer. The fusion protein was then cleaved with thrombin for 2 and 4 hrs followed by PEP. U.C-uncut sample, C-cut sample. Samples were subjected to SDS-PAGE to see the percentage of cleavage.
Fig 8: Reversed phase HPLC purification of PTH(1-34):
The unbound collected from the GST matrix was fractionated on Source-30 column equilibrated with 0.1%TFA and eluted with linear gradient of acetonitrile. The pure peptide peak (peak 1) which eluted at 25-28% acetonitrile. The pure peptide fractiones Were collected and analysed by SDS gel.
Fig 9: Analytical HPLC profile of purified rhPTH(1-34):
The purity of the PTH peptide was analysed by passing the Sample on C\textsubscript{18} RP-HPLC column and eluted with a linear gradient of Acetonitrile. The peptide was eluted at 23% acetonitrile. The purity of the peptide was more than 98%.
**Fig 10: SDS-PAGE analysis of pure PTH(1-34):**
The purified peptide was run on SDS-PAGE and stained with Silver staining method. Both the standard peptide and rhPTH(1-34) were migrating at same size and above 98% pure.
Fig 11: **Biological activity of rhPTH(1-34):**
The Biological activity of the peptide was assessed against a Comercially available standard Forteo. The activity of the peptide was calculated by measuring the intracellular cAMP production after treating with the peptides. The calculated specific activity of the Prepared Peptide was found as $1.04 \times 10^4$ IU/mg protein.
2.4. Discussion:

We have described a high yield system for producing hPTH (1-34) from bacteria. The hormone is expressed from the plasmid pVB as a cleavable fusion protein. In the preparation of polypeptides by genetic engineering, expression via fusion protein has been used often. The advantages are: (i) prevention of degradation of the polypeptides by proteases in the host, (ii) an increase in the expression yield, (iii) and the lead sequence in the fusion protein can be designed as a tag for the use in affinity purification.

The high level of GST/PTH fusion protein obtained may also be partly due to increased mRNA or protein stability of the hybrid sequences relative to the native PTH sequence alone. Cleaving of the fusion protein with Thrombin produced two extra amino acids at the N-terminus of the hPTH (1-34). PEP can cleave the peptides at proline residue. Hence, we used PEP to cleave those two extra amino acids by incorporating Proline residue. After reverse phase chromatography, the rhPTH (1-34) is estimated to be greater than 99% pure. The results of SDS gel electrophoresis, analytical run and partial N-terminal amino acid sequence analyses indicate that the recombinant hPTH product had the expected structure and that no major contaminating proteins were present. The recombinant hormone eluted on HPLC as a single peak and had the same retention time as synthetic hPTH (1-34). In vitro biological activity studies substantiated the biological activity of the recombinant hormone.

The method described here is a rapid and efficient producer for high-level expression and subsequent purification of not only PTH, but presumably a wide variety of other peptides in E.coli. Since numerous peptides of size comparable to that of hPTH(1-34) are
becoming an increasing focus for biotechnology and pharmaceutical development.