Purification of large quantities of biologically active recombinant human growth hormone

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Abstract

Production and purification of human growth hormone using a simple method was studied in two recombinant Escherichia coli, D7-5 and C27-2 strains. The r-hGH was expressed in the form of inclusion body in a batch fermentation process and purified to 99% purity using a procedure based on acid precipitation of the host derived proteins and other impurities. The effect of the pH and host strain on purification of the r-hGH and efficiency of the procedure were evaluated. It was found that the optimum pH for precipitation of the host derived proteins was 4.9. The procedure was suitable for r-hGH purification from D7-5 stain but not from the other strain C27-2. The purity of > 99% and recovery of about 40% were obtained as shown by SDS-PAGE and Western blot analysis. The purified r-hGH was biologically active as judged by receptor assay with very low endotoxin content which could be suitable for therapeutic applications. This simple and cost effective production process could be useful for large scale production of recombinant hGH from specific strains.

Keywords: *Recombinant protein, hGH, E. coli, Purification.*

INTRODUCTION

Advancement in recombinant DNA technology has made possible the expression of proteins in host cells, such as *E. coli* (Wallis and Wallis, 1990; Violand *et al.*, 1994).

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Human growth hormone (hGH) is a pituitary derived protein with a wide range of biological functions including protein synthesis, cell proliferation and metabolism (Tritos and Mantzoros, 1998). hGH is well known for therapeutic applications such as treatment of dwarfism, bone fractures, skin burns, bleeding ulcers and AIDS (Roehr, 2003).

The gene encoding hGH has been cloned and expressed in the form of inclusion body within the bacterial cytoplasm (Marthal *et al.*, 1979; Cardamone *et al.*, 1990). Recombinant hGH has been obtained from the cells and purified using different purification methods (Patra *et al.*, 2000). Biologically active products for therapeutic application derived from microbial sources must be substantially free from contaminating substances such as pyrogens, native microbial proteins and nucleic acids.

Several methods based on ion exchange chromatography, affinity chromatography and gel filtration have been developed for purification of recombinant proteins (Brostedt and Roos, 1989; Wingfield *et al.*, 1987; Bonnerjea, 1986). These procedures are expensive and time consuming, but the immediate need of an hour is to develop a simple and cost effective procedure for large scale production of recombinant proteins. In fact, proteins in solution show profound changes in solubility as a function of pH, ionic strength, temperature and electrostatic properties of the solvent. Proteins in solution have different isoelectric pH values and therefore can often be separated from each other using isoelectric precipitation (Bailey and Ollis, 1986). Based on this, attempts were made to develop procedure for seperation of biologically active

r-hGH from refolded proteins by selective precipitation of host-derived impurities together with dimmer and higher oligomers of hGH leaving the desired monomers in solution in a pilot scale which may be applicable in large scale production.

MATERIALS AND METHODS

Bacterial strains and media: *E. coli* strains D7-5 carrying plasmids pGP1-2 (Tabor and Richardson, 1985) and pET23::hGH (Zomorrodipour *et al.,* 2003) and C27-2 harboring plasmid pQE30::hGH with DH5α and TG1 strains as hosts were used for cloning r-hGH gene. The plasmids constructs; pET23::hGH and pQE30::hGH contained cDNA of human growth hormone under the control of T7 and T5 promoters respectively and an ampicillin resistance gene. Plasmid pGP1-2 contained T7 RNA polymerase and a kanamycin resistance gene. The strains were stored at -20ºC and -70ºC in LB with 15% and 30% glycerol respectively. The seed and fermentation media which were optimized using Taguchi statistical method (H medium) consisted of (g/l): Na_2HPO_4, 12.8; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; $MgSO_4$, $7H_2O$, 0.24; Peptone, 15; yeast extract, 8; glycerol, 20 and 1 ml trace elements consisted of (gr) $FeSO_4$. $7H_2O$, 10; $CaCl_2$. $2H_2O$, 2; $ZnSO_4$. $7H_2O$, 2.2; $MnSO_4$. $7H_2O$, 0.5; $CuSO_4$. $5H_2O$, 1; (NH_4) Mo_7O_{24}. $4H_2O$, 0.1; $Na_2B_4O_7$. 10 H_2O, 0.02. The pH of the medium was adjusted to 6.9. $MgSO_4.7H_2O$ was sterilized separately and then added to the fermentation medium.

Batch fermentation: Seed culture for fermentation was prepared by inoculating a single colony in 100 ml of H medium containing kanamycin (30 µg/ml) and /or ampicillin (100 µg/ml). Flasks were incubated overnight at 30ºC under shaking condition at 200 rpm on an orbital shaker. Laboratory fermentation was carried out in a 6.3 L (5 L working volume) fermentor (Bioflo. III New Brunswick Scientific, USA). The fermentor was equipped with a built-in controller for pH, temperature, agitation, DO (dissolved oxygen), and peristaltic pumps for base and acid addition. The fermentation was carried out at pH of 6.9 and temperature was set to 30ºC. Agitation speed (500-900 rpm) was automatically feed-back controlled based on dissolved oxygen (DO) at a set point of 30% of air saturation. The DO concentration was controlled automatically alley of the agitation speed up to 900 rpm followed by addition of a mixture of air and pure oxygen (when necessary) to maintain the relative dissolved oxygen above 30% of air saturation. Trace elements and salt solutions were sterilized separately and added into the fermentor before inoculation with 5% (V/V) of the seed culture.

Isolation of inclusion bodies: The cells from the fermentation medium were suspended in lysis buffer (50 mM EDTA, 50 mM Tris, 0.1% Triton X-100) and disrupted with a homogenizer (Niro Soavi S.P.A, Italy) twice at 6000 psi, in order to release the inclusion bodies and make them available for recovery by centrifugation at 11800 g for 30 min at 4ºC. The pellet was dissolved in a denaturing solution (8 M urea, pH 10.5) for 16 h. Centrifugation was carried out at 11800 g for 30 min at 4ºC and the supernatant was used for further purification (Hagei *et al.,* 1971).

Refolding: Proteins were refolded by dilution of original solution (8 M urea) to 4 M urea and the urea concentration was further decreased by ultrafiltration using a Millipore 10 kDa limiting membrane.

Isoelectric precipitation: After diafiltration, HCl (1 N) was added slowly to the solution, in order to adjust the pH was at 4.9. Suspension was centrifuged at 75000 g, at 4ºC for 45 min. Then the pH of the supernatant was adjusted at 7 by 1 N NaOH.

Assessment of r-hGH purity: The purity of r-hGH was confirmed by SDS-PAGE and Western blot technique using specific antibody against hGH produced in rabbits. SDS-PAGE was carried out as described by Lammeli, 1970. Destined gels were scanned by densitometric gel scanner (Pharmacia Ultra scan XL) and the ratio of each protein was determined by calculating the area under the peaks.

Size exclusion chromatography: Size exclusion chromatography was used for determination of the ratio of monomer, dimmer and higher oligomers (Nielsen *et al.,* 1991). A stainless steel column (300 × 7.5 mm internal diamention) packed with hydrophilic silica gel suitable for fractionation of globular proteins in the molecular mass ranging from 5 to 25 kDa was used for chromatography.

Receptor assay: The biological activity of r-hGH was evaluated using radiolabeled receptor assay (Jesse, 1976) with some modifications. For this purpose human lymphocyte cell line IM-9 (ATCC, CCL159)

was cultured in RPMI-1640 containing 10% of Fetal Calf Serum (FCS). Briefly, the cells were subcultured in a medium containing 100 mM HEPES, pH 7.0. The cells were separated from the medium by centrifugation and washed twice with assay buffer (100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM sodium acetate, 10 mM glucose, 1 mM EDTA, 10 mg/l bovine serum albumin, pH 7.0). The cells (1×10^7) were incubated with increasing concentrations of non-labeled hGH and constant concentration of I^{125} labeled hGH for 2 h at room temperature. After 3 washings of the cells with assay buffer, the radioactivity was counted using a gamma counter (Wallace, LKB).

Endotoxin content: The level of endotoxin was determined using the Limulus Amebocyte Lysate clotting Assay kit (Pyromed Co, U.S.A.). Following the procedure provided by the manufacturer.

RESULTS

The D7-5 strain is a heat inducible strain and expression of the r-hGH is under the control of the phage T7 RNA polymerase/promoter system. In C27-2 strain the hGH gene is under the control of the T5 promoter and could be induced by Isopropyl-β-D- thiogalactosidase (IPTG). Both strains have a high expression rate at 30ºC without inducer (Zomorrodipour *et al.*, 2003). Accordingly, production of r-hGH in these clones was performed in batch fermentation. Fermentation was carried out in H medium at 30ºC, pH 6.9 and dissolved oxygen (DO) of 30% of air saturation for 12-13 h. Under these conditions a cell dry weight of 15 g/l (OD_{550} = 30) was obtained.

Extraction and purification of the r-hGH from these strains was carried out in a pilot plant. The purification was based on the acid precipitation of the host derived proteins in the isoelectric point (PI) of the r-hGH. The PI of the recombinant growth hormone was determined by isoelectric focusing electrophoresis which was 4.9-5 (data not shown). Based on the PI of the r-hGH, a series of experiments were conducted to evaluate the effect of the pH on the removal of the host derived proteins. The inclusion body was dissolved and the proteins in the solution were refolded at pH 8.5 overnight. Decreasing the pH of the solution to a pH end point less than 5 resulted in precipitation of the host proteins leaving the r-hGH in solution. The optimum pH for the purification of the r-hGH was achieved by examination of a range of pH from 4.7 to 5.2 (Fig. 1).

The electrophoretic pattern of the recovered r-hGH in different pH (4.7 to 5.2) as shown in figure 1. Part of the r-hGH remained in the supernatant as soluble protein and the rest of the proteins was precipitate as insoluble. Evaluation of the soluble and insoluble phases in each pH, showed that the highest purity (99%) and recovery was obtained at pH 4.9 as judged by SDS-PAGE after scanning the gel by densitometry (Table 1). The purified solution from D7-5 strain comprises at least 97% hGH monomer and about 2% of oligomers and bacterial residues (Table 2). Figure 2

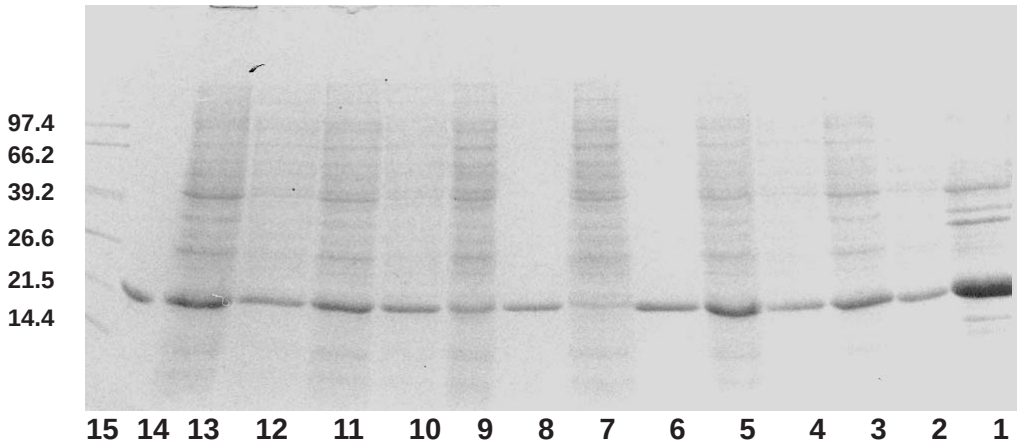

Figure 1. SDS-PAGE analysis of the purified r-hGH based on pH precipitation using different pHs. Lane 1- Inclusion body after refolding, Lane 2- Supernatant in pH 4.7, Lane 3- Pellet in pH 4.7, Lane 4- Supernatant in pH 4.8, Lane 5- Pellet in pH 4.8, Lane 6- supernatant in pH 4.9, Lane 7- Pellet in pH 4.9, Lane 8- supernatant in pH 5, Lane 9- Pellet in pH 5, Lane 10- Supernatant in pH 5.1, Lane 11- pellet in pH 5.1, Lane 12- Supernatant in pH 5.2 Lane 13- pellet in pH 5.2, Lane 14- hGH standard and Lane 15- MW-Standard.

Table 1: The product yield in different feed stock.

pH	4.7	4.8	4.9	5.0	5.1	5.2
Purified r-hGH monomer (%)	67	72	99	98	85	76

Table 2: Purification efficiency of r-hGH produced in two E. coli strains..

Strain	hGH expression (%)	Total protein, in IB (mg/l)	hGH purity (%)	Final purified protein (mg/l)	Efficiency (%)	Monomer (%)	Dimmer and polymer (%)
D7-5	30±1.5	310±7	98±1.9	124±6.9	40±1.9	97±2.7	2±1.1
C27-2	20±1.02	300±9.7	30±1.02	31±1.02	10±0.3	45±2.6	34±6.2

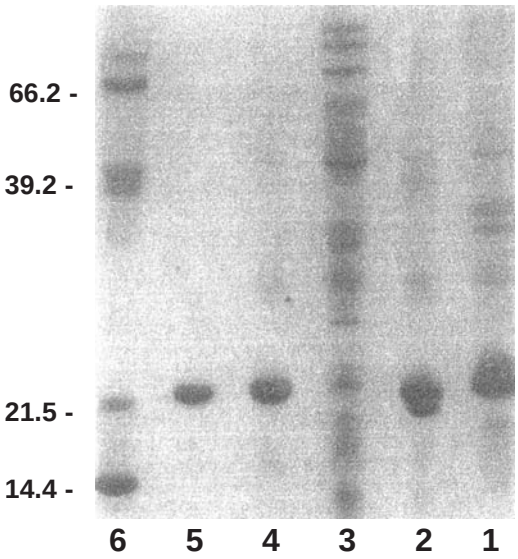

Figure 2. SDS-PAGE analysis of the samples of sequential purification steps. Lane 1- Crude *E. coli* (D7-5) lysate after homogenization, Lane 2- Inclusion body after solubilization and refolding. Lane, 3- Pellet after pH changing, Lane 4- Supernatant after pH changing, Lane 5- Standard growth hormone, Lane 6- Standard M.W. Marker.

shows the SDS-PAGE analysis of the purification step of the r-hGH from D7-5 strain. In the primary step of the purification, the solution resulting from the and refolding of the IB contains a mixture of r-hGH and bacterial proteins.

However similar results were not obtained for C27-2 strain and the oligomers content of the final product was also higher (34% versus 2% in case of D7-5).

Purified hormone from D7-5 strain was identified by r-hGH specific polyclonal antibody. Western blot analysis showed a related band similar to the commercial r-hGH (Fig. 3). Bioactivity assay of the purified hormone and its comparison with standard r-hGH (NOVO nordisk, Demark) showed that the two proteins are identical. Biological activity of the purified hormone, after 6 months storage at 4ºC remained unchanged (Fig. 4) which indicates the stability of the protein. The level of the endotoxin was less than 5 IU/mg of protein which is acceptable for therapeutic applications.

DISCUSSION

Most of the biotechnology products are proteins which must be prepared in large quantities in purified form. The degree of purity required for injectable proteins is very important. The desirable protein must be separated from other proteins, nucleic acids, carbohydrates, lipids or any other material in the sample. In addition to purity, the protein product must retain its biological activity. This forces the use of very robust purification processes that may be not the very best methods that could be used to purify the protein. Method that works exquisitely in a research laboratory may fail miserably on the production floor where it must be scaled up and reproduced exactly each time. Therefore to purify proteins we made use of their inherent similarities and differences. Protein similarity is used to purify them away from the other non-protein contaminants and differences are used to purify one protein from another. Different methods are used to separate the proteins in a complex solution such as those produced by bursting cells of *E. coli* to get a recombinant pro-

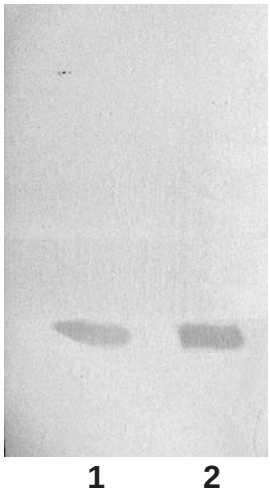

Figure 3. Western blot analysis of purified hGH. 1- hGH standard, 2- hGH purified by pH precipitation.

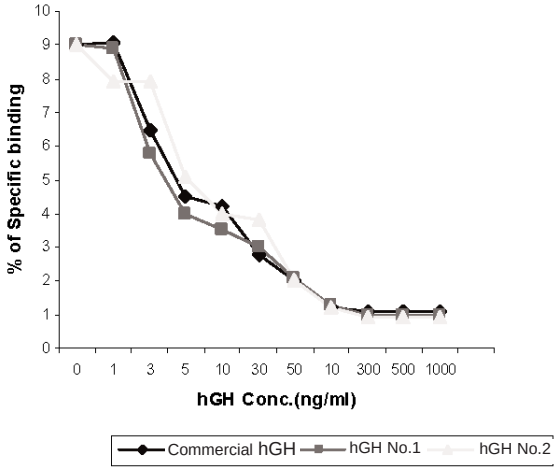

Figure 4. Radio Receptor Assay of purified hGH with IM-9 cell lines. Different concentrations of non-labeled hGH and 50 ng/ml of I^{125} hGH incubated with IM-9 cells for 2 h at 30ºC.

tein. In some cases we can make our job easier by attaching a fusion "tag" to the protein, but this is not allowed for injectable proteins.

In this study two recombinant *E. coli* expressing r-hGH with different genetic structure were used to evaluate a simple purification procedure in pilot scale. The r-hGH was accumulated as inclusion bodies in *E. coli* and was obtained to more than 50% purity by homogenizing the cells. The purity and homogeneity of the r-hGH inclusion body preparation was in agreement with the proposed composition of the inclusion bodies that they are found due to specific aggregation of single protein (Speed *et al.*, 1996).

As the inclusion bodies consisted of mostly r-hGH, solubilization and refolding were carried out before further purification. High concentration of urea, being strong denaturant and solubilizing agent, enhanced solubilization of the r-hGH from the inclusion bodies indicating that the hydrophobic interaction is probably the most dominant force for aggregation of proteins during high level expression.

The use of *E. coli* expression system has some limitations such as the difficulties associated with purification of recombinant protein from several host proteins. To improve purification and yield of the recombinant proteins, different procedures including fusion systems have been employed and some proteins such as r-hGH were purified by a single step affinity chromatography (Neidhardt and Luther, 1992). But in these systems the fusion partner contributes part of the total mass of the fusion protein and could be highly immunogenic. So a simple procedure for purification of high quality r-hGH in large scale is needed. In present work we have developed a practical procedure which employs a single step purification of r-hGH expressed in *E. coli*. This purification process is based on the finding that hGH monomers and oligomers posses overlapped isoelectric points (Brostedt and Roos, 1988) and may nevertheless be separated by selective precipitation over a narrow pH range. It has been shown that effective separation of hGH monomers from oligomers by precipitation is possible even though the PI of these proteins lying in overlapping ranges (Scopes, 1987). The optimum pH endpoint value must be determined individually for each protein batch which depends on many factors such as the ratio of the monomers to the oligomers of the protein and the amount of host proteins in different strains (Puri and Cardamone, 1992).

Previous works indicated that the quality and concentration of feed stock are very important. High concentration of proteins in the solution affects the subsequent purification steps of hGH monomers which may be due to higher levels of oligomers or other impurities leading to lower purification yield (Frazier and Konishi, 1990). Feed stock quality and the efficiency of the precipitation process may be improved, in some cases, by additional washings of inclusion bodies prior to the solubilization and refolding of the proteins. Solubilization and folding steps should be carried out under conditions that maximize monomer yield and minimize the oligomers and other undesirable components (Charman *et al.*, 1993). The most important objective in production of recombinant proteins is to

lower the production cost and also to simplify the purification process which may be achieved by elimination of the time consuming and expensive steps such as chromatography. In our study purified r-hGH with the yield of 124 mg/l of culture medium and 98% purity was obtained which is more preferable than the yield (30 mg/l) and purity reported by Mukhija and coworkers (1995) and Ettori *et al.* (1992). The purification procedure used in the present study is strain dependent and relatively more efficient as compared to other methods. It is also simple and cost effective for large scale r-hGH production and may be useful for purification other proteins.

References

Bailey J, Ollis E (1986). In: *Biochemical Engineering fundamentals* 2nd ed, pp: 745-749.

Brostedt P, Roos P (1989). Isolation of dimeric forms of human pituitary growth hormone. *Prep Biochem* 19: 217-29.

Bonnerjea J (1986). *Protein purification*. The right step at the right time. 4: 955-958.

Brostedt P, Roos P (1988). Isolation of four isomers of the 20000 Dalton variant of human pituitary growth hormone. *Prep Biochem* 18: 277-91.

Cardamone M, Puri NK, Sawyer WH, Capon RJ, Brandon MRA (1990). Spectroscopic and equilibrium binding analysis of cationic detergent protein interactions using soluble and insoluble recombinant porcine growth hormone. *Biochem Biophys Acta* 18: 71-82.

Charman SA, Mason KL, Charman WN (1993). Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth hormone. *Pharmacol Res* 10: 954-62.

Ettori C, Righetti PG, Chiesa C, Frigerio F, Galli G, Grandi G (1992). Purification of recombinant human growth hormone by isoelectric focusing. *J Biotechnol* 25: 307-18.

Frazier PB, Konishi Y (1990). Method of folding somatotropine. *US patent 4975529.*

Hagei P, Gerding JJT, Fieggen W, Bloemendal H (1971). Cyanate formation in solutions with urea. *Biochem. Biophys Acta* 243: 366-372.

Jessee R (1976). Radio receptor assay of human growth hormone. *Method Enzymol* 37: 70-81.

Lammeli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophages. *Nature* 227: 680.

Marthal JA, Hallewall RA, Baxter JD (1979). Human growth hormone complementary DNA cloning and expression in bacteria. *Science* 205: 602-607.

Mukhija R, Rupa P, Pillai D, Garg LC (1995). High-level production and one step purification of biologically active hGH in *E. coli*. *Gene* 165: 303-306.

Neidhardt EA. Luther MA (1992). Rapid two steps purification process for the preparation of pyrogen–free murine immunolgobulin G1 monoclonal antibodies, *J Chromatogr* 590: 255-261.

Nielsen RG, Rickard EC, Santa PF, Sharknas DA, Sittampalam GS (1991). Separation of antibody–antigen complexes by capillary zone electrophoresis, isoelectric focusing and high-performance size exclusion chromatography. *J Chromatogr* 539: 177-85.

Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK (2000). Optimization of inclusion body solubilization and renaturation of r-hGH from *E. coli. Protein Expr Purif* 18: 82-192.

Puri NK and Cardamone M (1992). A relationship between the starting secondary structure of recombinant porcine growth hormone solubilized from inclusion bodies and the yield of native (monomeric) protein. *FEBS Lett* 305: 177-180.

Roehr B (2003). The many faces of human growth hormone. *B Exper Treat AIDS* 4: 12-16.

Scopes RK. (1987) *Protein Purification:* Principles and Practices, 3rd ed, Springer Verleg, pp: 41-71.

Speed MM, Wang DIC, King J (1996). Specific aggregation of partially folded polypeptide chains. The molecular basis of inclusion body composition. *Nature Biotech* 14: 1283-87

Tabor S, Richardson CC (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci* 82: 1074-1078,

Tritos NA, Mantzoros CS (1998). Recombinant human growth hormone: old and novel uses. *The American J Medicine* 105: 44-57.

Violand BN, Schlittler MR, Lawson CQ, Kane JF, Siegel NR, Smith CE, Kolodziej EW, Duffin KL (1994). Isolation of *E. coli* synthesized recombinant eukaryotic proteins that contain epsilon–N-acetyllysine. *Protein Sci* 3: 1089-97.

Wallis OC, Wallis M (1990). Purification and properties of recombinant DNA-derived bovine growth hormone analogue expressed in *E. coli. J Mol Endocrinol* 4: 61-9.

Wingfield PT, Graber P, Buell G, Rose k, Simona MG, Burleigh BD (1987). Preparation and characterization of bovine growth hormones produced in recombinant *E. coli. Biochem J* 1: 829-39.

Zomorrodipour A, Yakhchali B, Khodabandeh M, Deezagi A, Hosseini Mazinani M, Valian Borujeni S, Ahmadzadeh Ragi M, Rahimi M, Rahbar R, Sanati MH (2004). The over-expression of biologically active human growth hormone in a T5-based system in *Escherichia coli*. *J Sci I. R. Iran*, 15: 27-32.