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# Prokaryotic overexpression of TEV–rhGH and characterization of its polyclonal antibody

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ABSTRACT

Recombinant protein technology represents one of the best solutions to achieve rapid, efficient, and cost-effective protein expression and purification of therapeutic proteins. Growth hormone (GH) is an excellent example of these proteins used in the therapy of hormone deficiencies. In this work, a plasmid, pRSET–TEV–rhGH, has been constructed to overexpress recombinant human GH (rhGH) by cloning its gene downstream of an N-terminal 6× His-tagged polypeptide (43 aa) in the T7 promoter-plasmid pRSET. This polypeptide was cleavable by means of the integrated recognition site for the tobaccos etch virus (TEV) protease, resulting in an rhGH protein at an exact length and sequence. After IPTG induction, this plasmid effectively expressed TEV–rhGH protein (27 kDa) in the cytoplasm of *Escherichia coli*, which accumulated in the form of inclusion bodies. The 6× His-tagged protein, with a yield of ~150 mg/L of culture, was purified from the cell extract using metal affinity chromatography, as shown after SDS-PAGE blue staining, and was confirmed by immunoblotting using specific commercial monoclonal antibodies. In order to detect TEV–rhGH, in ELISA and immunoblotting, specific polyclonal antibody, with high titer (~$10^{-5}$ fold dilution), was produced in a rabbit and purified using affinity chromatography. Preliminary tests have proved that TEV–rhGH protein and its specific purified IgG antibody could provide valuable tools for rhGH productive and diagnostic purposes.



## 1. Introduction

Human growth hormone (hGH) is a single-chain polypeptide hormone mainly synthesized by the acidophilic somatotrophs of the anterior pituitary gland (Baumann, 2009). GH is encoded by the *GH* gene in a gene cluster, with five closely related genes, located at the chromosome 17q22–24 (Zhan et al., 2005). GH exists in several forms, but the predominant one contains 191 aa residues and two disulfide bonds and has a molecular mass of about 22 kDa (Baumann, 2009). Somatotropin is the common name of the GH produced naturally in animals, whereas the term somatropin (or rhGH) refers to the recombinant form of hGH (Bowen, 2006). By binding to its receptor on target cells, GH can induce direct and indirect effects. Fat cells (adipocytes), for example, have GH receptors, and GH stimulates them to break down triglyceride and suppresses their ability to take up and accumulate circulating lipids. Indirect effects of GH are mediated primarily by insulin-like growth factor I (IGF-I), a hormone that is mainly secreted from the liver. A majority of the growth promoting effects of GH is actually due to IGF-I acting on its target cells (Keating, 2008). Regular doses of rhGH are administered to patients whose pituitary glands generate insufficient quantities to support normal growth and development (Vance and Mauras, 1999). Although, the physiological effects of rhGH have been studied in a variety of medical conditions and genetic syndromes such as children with Down's syndrome, Noonan's syndrome (Carrel et al., 2002; Sanchez-Ortiga et al., 2012; Vance and Mauras, 1999) and Prader–Willi syndrome (Ghasemi et al., 2004). In addition, applications for rhGH in the treatment of bone fractures, skin burns and bleeding ulcers and AIDS have been suggested (Roehr, 2003; Tritos and Mantzoros, 1998). Besides its licensed medical application, rhGH is widely abused by many athletes for its anabolic and lipolitic properties. Therefore, it is on the list of substances issued by World Anti-Doping Agency (WADA) as banned for competitive sports. However until recently, a standard test was lacking to detect rhGH abuse (McHugh et al., 2005).

Before rhGH became available, hGH for therapeutic use was obtained from pituitary glands of cadavers. This unsafe practice led to some patients developing Creutzfeldt–Jacob disease (Abrams et al., 2011). Recombinant DNA technology has facilitated a safe and abundant production of rhGH in various heterologous systems, like *Escherichia coli*

*Abbreviations:* BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; NHS, N-hydroxysuccinimide ester; Ni-NTA, nickel-charged nitrilotriacetic acid; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine; TEV, tobacco etch virus; WADA, world anti-doping agency.

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(Ikehara et al., 1984; Patra et al., 2000), yeast (Apte-Deshpande et al., 2009; Calik et al., 2008), *Bacillus* (Ozdamar et al., 2009) and eukaryotic alga (Hawkins and Nakamura, 1999), without the risk of transferring human pathogens (Bell et al., 2010). Although, rGH from different species (Ho et al., 1998; Leung et al., 1984, 1986; Li et al., 2009; Nyholt de Prada et al., 2010; Seeburg et al., 1977; Sereikaite et al., 2007) was successfully obtained using several elaborated production systems, like transgenic plants (Cunha et al., 2011; Shigemitsu et al., 2012), animals (Han et al., 2009; Lipinski et al., 2012; Salamone et al., 2006), and insects (Cholin et al., 1996; Ho et al., 1998; Sumathy et al., 1996), and was mainly applied for forage purposes (Ho et al., 1998). Most studies reported on recombinant protein production have described the use of N- or C-terminal 6× His tag for subsequent steps of purification and detection. Ligands that bind 6× His protein tags are commercially available in pre-immobilized forms ready to use for affinity chromatography purification (Lewis, 1984). However, rhGH and just like most other medical recombinant proteins need to be tag-free before administration in humans, thus further steps for tag removal need to be accomplished. The presence of an extra tag in the protein native structure could affect its biological activity, or even worse, could stimulate the immune system, reducing the recombinant protein half-life in the blood circulation. Before the final step of affinity purification, the removal of the N-terminal fusion tag from the recombinant protein could be achieved by a cleavage with site-specific proteases, such as the tobacco etch virus (TEV) protease (Miladi et al., 2011).

An alternative solution for producing untagged GH implies the presence of more specialized ligands, such as specific antibodies that can be immobilized using one of several commercially available activated affinity supports.

Since hGH is a heterogeneous protein consisting of several isoforms, disparity among GH assay results from different laboratories. This is related mainly to the different specificities of anti-GH (monoclonal and polyclonal) antibodies and the use of different calibrators (reference GH) (Rigamonti et al., 2012). Beside their role as indispensable reagents in basic research and diagnostics, conventional poly- and mono-clonal antibodies are still used as trapping molecules in affinity chromatography. For this purpose, an accurate, reproducible and universally valid GH measurement would be highly desirable.

In the present study, *GH* gene was isolated and cloned into T7-promoter plasmid pRSET in order to obtain a high level of protein expression in *E. coli*. Purified TEV–rhGH could easily be obtained after metal affinity chromatography via the 6× His tag at its N-terminal, which is although removable by a cleavage reaction using TEV protease. For purification and detection purposes, polyclonal anti-GH antibody was produced in rabbit and characterized for its reactivity against GH in ELISA and Western blot. Recombinant GH, with its specific polyclonal antibody, provides a valuable tool for developing of GH measurement assay.

## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions and plasmid

*E. coli* strains TOP10 (Invitrogen) and BL21 (DE3) Rosetta (Novagen) were used in cloning and protein expression after transformation by electroporation with the plasmid pRSET (Invitrogen). For general maintenance and protein expression, *E. coli* was grown in Luria Broth (LB; 1% Tryptone, 0.5% yeast extract, 171 mM NaCl) (Bio Basic INC) with ampicillin antibiotic (Sigma; 100 μg/ml) in an orbit-rotating 37 °C incubator.

### 2.2. Construction of pRSET–TEV–rhGH plasmid

Total RNA was isolated from blood lymphocytes of leukemia patient using (Invitrap® Spin Blood RNA Mini Kit Invisorb®). The quality of total RNA was checked in agarose gel electrophoresis. From the total RNA (2 μg), first strand cDNA was synthesized following the standard method using 0.5 μg oligo $dT_{18}$ primer (Invitrogen). *GH* gene was amplified from the cDNA by PCR using a pair of specific primers F1/R1 designed on the GH sequence (Table 1). Nested PCR amplification was achieved on the first PCR product using a pair of forward and reverse cloning primers containing sites for *Bam*HI and *Hind*III restriction enzymes, respectively (Table 1). AccuPrim™ Taq Polymerase High fidelity (Invitrogen) was used in all PCR reactions and the amplification program consisted of 2 min of denaturation at 94 °C followed by 35 cycles of short denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1.5 min. PCR-amplified *GH* gene fragment was purified by PCR purification kit (Qiagen) and digested, as well as the plasmid pRSET, with the restriction enzymes *Bam*HI, *Hind*III. Digested products were then ligated using ready-To-Go™ T4 DNA Ligase Kit (GE Lifescience), and used to transform a freshly prepared electro-competent *E. coli* TOP10 cells by electroporation. Colony PCR screening for positive GH clones was performed using pRSET-specific primers T7F/T7R (Table 1) resulting in a distinct fragment of 853 bp. Plasmid constructs were isolated from positive clones by Plasmid Miniprep Kit (Qiagen) and used to confirm the sequences of the cloning either by digestion with restriction enzymes or by sequencing using specific primers (Table 1), which are distributed along the inserted gene to give overlapping sequencing fragments covering the full-length of the cloned GH.

### 2.3. Expression and purification of GH

Freshly prepared electro-competent *E. coli* BL21(DE3) cells were transformed with the confirmed plasmid construct pRSET–TEV–rhGH by electroporation, and grown on LB/Amp Plate. A single positive colony from the plate was inoculated into LB medium (10 ml, preculture) containing ampicillin. This pre-culture was grown overnight at 30 °C with vigorous shaking and about 10 ml of the above culture was inoculated into 1 l LB/Amp medium. The culture was grown at 37 °C until the $OD_{600}$ reached 0.5–0.7 and isopropyl β-D-thiogalactoside (IPTG, 0.5 mM, Promega) was added to the culture for the induction of TEV–rhGH protein expression and the culture was grown for O/N at 19 °C. Cells from induced cultures were pelleted by centrifugation and suspended in binding buffer (20 mM imidazole, 20 mM tris-base, 300 mM NaCl, pH 7.4) and lysed by French-Press (pressure 1.4 bar), centrifuged 8000 rpm for 8 min, and supernatant was saved. This supernatant contains TEV–rhGH protein. Using fast protein liquid chromatography (FPLC) AKTAprime plus system (GE lifescience), TEV–rhGH was purified from this cytoplasmic extract using a 5 ml column of nickel charged nitrilotriacetic acid (NTA) superflow sepharose (Qiagen). After washing, the bound proteins were eluted from the column with elution buffer (500 mM imidazole, 20 mM tris-base, 300 mM NaCl). The eluted fraction was concentrated on Vivaspin concentrators with a molecular weight cut-off 10 kDa (Vivascience). The purity of the TEV–rhGH was evaluated in a Coomassie-stained SDS-PAGE. The concentration of the purified protein was determined by Bradford (1976) method.

### 2.4. Production of polyclonal anti-GH antibody

For the first immunization, purified TEV–rhGH protein (500 μg) in PBS (1 ml) was mixed with an equal volume of Freund's complete adjuvant (Bio Basic Inc.) to form a stable emulsion. One white rabbit was injected subcutaneously at 2 to 4 different sites. Three booster injections were given with 250 μg recombinant protein mixed with incomplete Freund's adjuvant at 15 day intervals. To evaluate the immune response, blood samples (0.5 ml) were collected before each injection and bleeding (30 ml) was done 10 days after the last boost. Polyclonal antibodies (IgGs) were purified from rabbit serum by affinity chromatography on a 5 ml HiTrap Protein A column (GE Lifescience) according to the manufacturer's instructions. Binding was performed in 0.02 M sodium phosphate, pH 7, with elution 0.1 mM citric acid, pH 3. Eluted IgGs were collected and immediately neutralized to physiological pH by