Persian sturgeon growth hormone elaboration and purification

Nasr E.[1*]; Hovhannisyan H.G.[2]; Pourkazemi M.[3]

Received: October 2013 Accepted: June 2015

Abstract

In this study *Escherichia coli* DE3 containing expression vector (pET21a) with cloned Persian sturgeon growth hormone (psGH) gene was grown in 10 mL LB broth on a 150 rpm shaker, at the temperature of 37 °C. At the late log phase (determined by OD standard curve) 100 µL isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction of GH synthesis. Samples were taken every 2 hours and after bacterial cells lysis crude extracts with recombinant proteins inclusion bodies (IB) were loaded on 15% SDS-PAGE gel. Thenafter staining, comparative concentrations of rpsGH were measured by densitometric scanning of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel it was more than 90 %. The maximum yield of GH was observed after 4 hours of growth. To recover active psGH from inclusion bodies we used imidozole to obtain most of the total recombinant protein in the soluble fraction. Purification of 6xhisN tag recombinant psGH has been performed using affinity chromatography where nickel was bound to an agarose bead by chelation using NTA (nitrilotriacetic acid) beads. The overall yield of the purified monomeric psGH was approximately 50% of the initial IB proteins. The purification manipulations including IB isolation and solubilisation, protein refolding by dialyze and affinity chromatography ensure yields of biologically active psGH up to 30%. This study shows that, the affinity chromatography is a powerful and very specific method for recombinant proteins purification of psGH.

Keywords: Persian sturgeon, Growth hormone, Purification, Affinity chromatography

1-Islamic Azad University, Meshgin Shahr Iran

2-S&P Center "Armbiotechnology" of NAS, Yerevan, Armenia

3-Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran.

*Corresponding author's email: nasr_ehsan1357@yahoo.com

Introduction

Growth hormone is a small single-chain globular polypeptide, synthesized, stored, and secreted by somatotropic cells within the body. The growth hormone has many effects on the body such as, stimulating growth, increasing muscle mass through-sarcomere hypertrophy, and increasing protein synthesis. Thus, GH has proven to be of great importance in farm animals, as its exogenous supply has a positive impact on the production of milk and meat with an increase in growth rate (Lipinski *et al*., 2003; Jamil Sami, 2006; Kim *et al*., 2013).

In biotechnology, most of products are proteins which must be prepared in large quantities in purified form, through an intracellular reaction mechanism based on sequential transfer of information from the genes in the DNA to RNA and then to protein. The result of research in the 1960s demonstrated that enzymes could be purified using a specific competitor inhibitor immobilized on solid matrices covalently attached.

The fish recombinant GH (rGH) can be synthesized by *E. coli* cells, although it exists in denatured and devoid of biological activity form in inclusion bodies (IB). Usually *E.coli* was used for the rapid and economical production of recombinant proteins. However, overexpression of recombinant proteins (r-protein) results in aggregation and accumulation in IB (Lilie *et al*., 1998). IBs are insoluble that are often observed with many other eukaryotic proteins. A high scale of purification of the r-protein can be attained by inclusion body isolation (Vallejo and Rinas, 2004).

Affinity chromatography, or more precisely biospecific interaction chromatography, comprises a number of highly selective purification techniques in which chemical molecules are bonded to a solid support and undergo specific and reversible interactions with a biomolecule to be purified. In the process, the biomolecule forms a stable, reversible association complex with the support-bound biospecific ligands (Gupta *et al*., 2003; Kaur and Reinhardt, 2012).

Thus, the accumulated r-protein requires solubilization and refolding steps prior to purification by chromatography, and the overall protein recovery is significantly affected by the efficiency of these pre-purification steps. Therefore, the accumulated proteins need to be solubilized using high concentrations of denaturants such as urea or guanidine hydrochloride (GnHCl), followed by removal of denaturants for protein refolding. In many cases, however, the overall yield of biologically active protein from inclusion bodies is very low, and these purification procedures are often costly and time–consuming. Therefore, considerable efforts have been made to increase the efficiency of solubilization and refolding as a means of improving the overall recovery of biologically active r-protein from inclusion bodies.

The purification methods of r-proteins depending to the same class and therefore exhibiting close structural

and functional relationships may require a very different methodology.

Affinity chromatography is a powerful and very specific method for purification of r-proteins (Gupta *et al*., 2003). Six histidine aa at the end of a protein usually is known as a 6X His tag (either N or C terminus). Nickel is bound to an agarose bead by chelation using NTA (nitroloacetic acid) beads. It is a general method to batch absorb the protein onto the column, by mixing the beads with the sample, then pouring the slurry of NTA beads and protein into a column, where low concentrations of phosphate and imidazole areused to remove low affinity bound proteins. Then, higher concentrations of imidazole are used to elute the protein from the NTA-beads.

The aim of this paper was optimization of separation process for recombinant PS growth hormone (r-psGH) from the Inclusion bodies and purification by affinity chromatography based on the affinity of histidine to nickel ions and solubilization of the accumulated proteins by using high concentrations of denaturants with urea. This is followed by the renaturation of r-psGH to help facilitate the production of a large amount of biologically active r-psGH.

Materials and methods

The strain *E. coli* DE3 containing cloned psGH cDNA in vector PET 21a constructed by us was used for biosynthesis of recombinant hormone.

Tris, Trypton, Dodecyl sulfat were from Merck (Germany), Imidazole, Urea, Guanidine hydrochloride were from Qiagene (USA), Isopropyl b-D-thiogalacto-pyranoside (IPTG) was from Sinagene (Iran). Other chemicals and reagents were commercial preparations of analytical grade or of the highest purity available.

Induction, biosynthesis and release of the rpsGH

Recombinant PS growth hormone (r-psGH) was expressed in *E.coli*. Then, a freshly streaked colony with recombinant plasmid of psGH was used to inoculate a preculture grown overnight in L-broth medium (Luria-Bertani, 10 g Bacto tryptone, 5 g yeast extract and 10 g NaCl per liter of solution) with 50 µg/mL ampicillin. An aliquot (1 ml) of this pre-culture was used to inoculate 10mL of L-broth medium containing 50 µg/mL ampicillin and put in a shaker incubator. The temperature was 37 °C and the agitation was 150 rpm.

After 2h of growth 100 µL IPTG was added into media. Sampling was carried out every 2h to 8h.

For GH purification we used the Ni-NTA Fast Start Kit (USA) including everything needed for fast, efficient purification of His-tagged proteins from cleared *E.coli* lysates and prefilled Ni-NTA columns. Buffers supplied in the kit enable proteins to be purified either under native or denaturing conditions. The kit also contains an Anti-His antibody for detection of expressed His-tagged proteins.

Inclusion body preparation

E.coli cells containing rpsGH inclusion bodies were harvested from 100 mL of broth, suspended in 20 ml of 20mM phosphate buffer (pH 7.2), and lysed by lysocim. The resulting homogenate was centrifuged at 8000rpm for 40min at 4°C, and the pellet was washed with 20 m1 of 1 m sucrose and then with 20 ml of 4% Triton X-100, 20mM phosphate buffer, and 1mL ethylenediaminetetraacetic acid (EDTA) (pH 7.2) to purify inclusion bodies of rpsGH. Soluble components, bacterial cell wall and cell membrane, and lipid components were removed by these processes.

The inclusion bodies were solubilized by stirring for 2h at 4°C in 40 m1 of 5m guanidine hydrochloride (GuHCl), 50mM Tris-HCl, 0.005% Tween 80 (pH 8.0) and the solution was centrifuged at 8000rpm for 40 min at 4°C. To refold rpsGH molecules, the supernatant was dialyzed 3 times against 24 litre of l0 mM Tris-HCl (pH 8.0) at 4°C, and the dialyzate was centrifuged as above. The supernatant was put onto EDTA Toyopearl 650 Mcolumn (7x25cm) equilibrated with l0 mM Tris-HCl (pH 8.0) and eluted with a linear gradient from 0 to 25mM.NaCl in 3,1.of 10mM Tris-HCl (pH 8.0).

Purification psGH with Affinity chromatogarphy (Ni-NTA)

For purification r-protein was extracted from PsGH using reagent Ni-NTA kit (Qiagen, USA) according to the manufacturer's instruction.

5 mL of culture medium, the cell pellet is prepared, then the pellet was added to 630 µL lysis buffer, 70 µL Lysosomal (10mg/mL) and 15 µL Benzanas nucleuse (3 unit/ml), then incubated on ice for 30 min. Centrifuge of empty Ni-NTA columns was at 2900 rpm for 2 min, then 600 µL was put into the empty columns, and centrifuged at 16000 rpm for 5min.

The columns were put into new microtube and 600 µL wash buffer (5.8 mL H_2O+200 µL Imidozole+3 mL NaCl + 1 ml NaH_2PO_4) was added and centrifuged at 2900 rpm for 2 min. This step was repeated twice.

At the end; the columns were loaded into new microtubes and 300 µL elution buffer (1 mL H_2O +5 µL Imidozole + 3 mL NaCl + 1 mL NaH_2PO_4) was added and centrifuged at 2900 rpm for 2 min. This step was repeated twice.

In every step 20 µL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was loaded for essessment.

SDS-PAGE analysis

Electrophoresis on 15 % (w/v) SDS-PAGE in the presence of SDS was performed according to the method of Laemmli.

The qualitative measurement of the protein was done using 15% SDS-PAGE gel, run according to the method of Laemmli. Total cell lysates and purified protein were resolved on SDS-PAGE and stained with $NaNO_3$. The loading volume in SDS-PAGE was always 20µL.

Results

E.coli DE3 containing expression vector (pET21a) with Persian sturgeon GH gene was grown in 10 ml LB broth on a 150 rpm shaker, at the temperature of 37 °C. At the late log phase (determined by OD measure and using standard curve)100 µl IPTG was added for induction of GH synthesis. Samples are taken every 2 hours and after bacterial cells lysis crude extracts with recombinant proteins IB was loaded on 15% SDS-PAGE gel and after staining comparative concentration of rpsGH was measured by densitometric scanning of SDS-PAGE gel (Fig. 1).

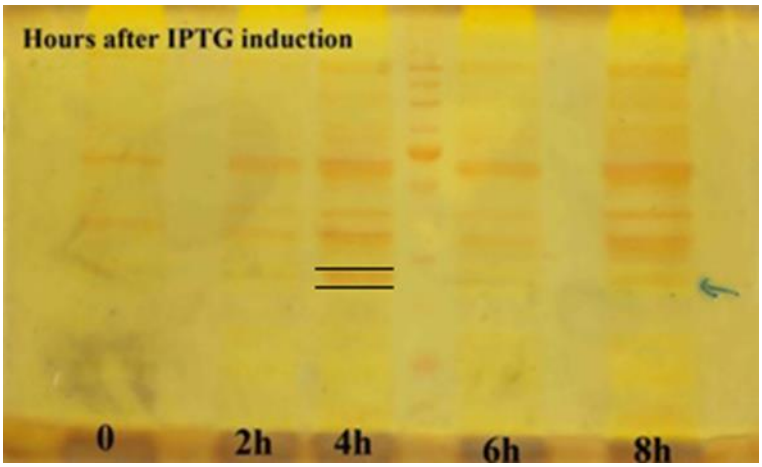

Figure 1: IBs accumulation in *Escherichia coli* cells.

In this condition high-level expression of rpsGH in *E. coli* was achieved at the 4h after adding IPTG.

Soluble components, bacterial cell wall and cell membrane, and lipid components were removed after lysis of *E.coli* cells containing rps GH inclusion bodies. The purity of rps GH in the inclusion bodies was about 90% in total protein, judging from SDS-PAGE under reducing conditions.

The inclusion bodies were solubilized by stirring for 2h at 4°C in 40 m1 of 5m guanidine hydrochloride (GuHCl), 50mM Tris-HCl, 0.005% Tween80 (pH 8.0) and the solution was centrifuged at 8000 rpm for 40 min at 4°C. To refold rps GH molecules, the supernatant was dialyzed 3 times against 24 liter of l0 mM Tris-HCl (pH 8.0) at 4°C, and the dialyzate was centrifuged as above. Refolding was monitored by SDS-PAGE under non-reducing conditions. PsGH shows a single 22-kDa hand in SDS-PAGE under non-reducing conditions. The recovery of rps GH in the refolding process was 92%. The supernatant was put onto EDTA Toyopearl 650 M column (7x25cm) equilibrated with l0mMTris-HCl (pH 8.0) and eluted with a linear gradient from 0 to 25mM NaCl in 3.1 of 10mM Tris-HCl (pH 8.0).

Using this method of preparation, about 50 mg of rps GH was obtained from 100 ml of broth, yielding an overall recovery of about 90% of the rps GH originally present in the cells

(Table 1). The purity of rps GH, as measured by densitometric scanning of SDS-PAGE gels, was more than 90%. Further purification of soluble his-tagged rpsGH (22kDa) from impurities derived from *E. coli* cells as carried out byNi-NTA affinity chromatography. Some of the *E.coli* proteins which also have histidine oramino acids like cysteine, tryptophan etc., also can bind to the affinity matrix. Imidazole was uesd for elution bound protein other than psGH in laysis buffer, washing buffer and elution buffer. It was seen that bount proteins other than r-protein eulted out with a 10% concentration of imidozole in washing buffer.

Table 1: Steps of refolding and purification of recombinant ps GH.

Step	Volume (ml)	Whole rpsGH (mg)	Yield (%)
Broth	100	53	100
Homogenate	20	53	100
Sucrose suspension	20	53	100
Triton X-100 suspension	10	53	100
Solubilizate	25	50	92
Ni-NTA Affinity chromatography	25	50	90

Concentration of rpsGH was measured by densitometric scanning of SDS-PAGE gel.

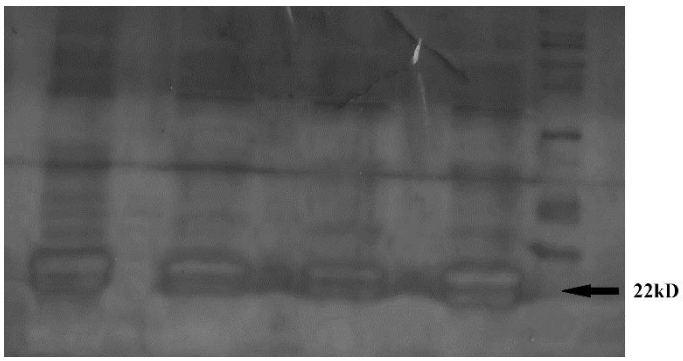

Figure 2: SDS-PAGE gel of recombinant protein of Persian sturgeon growth hormone with 22KD weight. The GH of *Huso huso* was used as control (1 line).

Discussion

Evidence to confirm that, the growth hormone gene is very important in the regulation of development and somatic growth in vertebrates (Gu *et al*., 1995; Lipiński *et al*., 2003; Yom Din *et al*., 2008; Kim *et al*., 2013).

Recombinant protein of GH in many species has already been expressed, purified then elucidated. For instance, Human (Igout *et al*., 1993; Gu *et al*., 1995), Domestic Animal Species (Davis *et al*., 1992) ovine (Gupta *et al*., 2003), Water Buffalo (Sami, 2006), miss as well as various species of teloest fish (Hsih *et al*., 1997) And r-protein of GH in some species of Acipenseridea family such as Beluga (*H. huso*) Russian sturgeon (*Acipenser gueldenstaedtii*) (Yom Din *et al*., 2008)

has been expressed and purified but it is the first time that the r-protein of Persian sturgeon (*A. persicus*) was expressed and purified.

Several strain of *E. coli* has been efficiently used for the high-yield production of many r-proteins. Moreover, high-level expression often leads to insoluble protein aggregates such as inclusion bodies, and thus requires additional steps including solubilization and refolding prior to purification. Although the formation of inclusion bodies has certain advantages such as convenient isolation and protection from proteolysis, the recovery of biological activity has often been unsatisfactory. Furthermore, the resolubilization of protein aggregates can be problematic, requires the use of high concentrations of denaturants, and the subsequent refolding process generally requires extensive optimization. Several methods were used for purification of recombinant protein such as: Ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, and isoelectric precipitation which are some of the separation methods performed.

This paper describes the purification and optimization protein of Persian sturgeon growth hormone gene with recombinant plasmid in *E. coli*. Our studies show that, affinity chromatography is a powerful and very specific method for r-proteins purification of PS GH.

In conclusion, we found that IPTG is a valuable alternative inducer for PS rGH production by *E. coli* cells. Affinity chromatography was a powerful and very specific method for r-protein purification of PS GH.

Moreover, a relatively high yield of renatured, biologically active rGH can be obtained from the denatured rGH extracted from inclusion bodies using the optimal conditions detailed in this study.

Acknowledgements

This study was supported by the International Sturgeon Research Institute Rasht, Iran.

References

Davis, S.L., Graf, M., Morrison, C.A., Hall, T.R. and Swift, P.J., 1992. Identification and partial purification of serum growth hormone binding protein in domestic animal species. *Journal of Animal Science*, 70, 773–780.

Gu, T., Zheng, Y., Gu, Y., Haldankar, R., Bhalerao, N., Ridgway, D., Wiehl, P. E., Chen, W. Y. and Kopchick, J.J., 1995. Purification of a pyrogen-free human growth hormone antagonist biotechnology and bioengineering. *Biotechnology and Bioengineering*, 48, 520-528.

Gupta, V., Eshwari, A.N., Panda, A.K. and Agarwal, G.P., 2003. Optimization of immobilized metal ion affinity chromatography for single-step purification of

recombinant ovine growth hormone expressed *Escherchia coli*. *Journal of Chromatography A*, 998(**1-2**), 93-101.

Hsih, M.H., 1997. Optimization of the solubilization and renaturation of fish growth hormone produced by *Escherichia coli*. *Applied Microbiology and Biotechnology*, 48, 66-72.

Igout, A., Van Beeumen, J., Frankenne, F., Scippo, M.L., DeVreese, B. and Hennen, G., 1993. Purification and biochemical characterization of recombinant human placental growth hormone produced in *E. coli*. *Biochemical Journal*, 295, 719–724.

Kim, M.J., Park, H.S., Seo K.H., Yang, H.J., Kim, S.K. and Choi, J.H., 2013. Complete solubilization and purification of recombinant human growth hormone produced in *Escherichia coli. PLoS One*, 8(**2**), e56168.

Kaur, J. and Reinhardt, D.P., 2012. Immobilized metal affinity chromatography co-purifies TGF-b1 with histidine-tagged recombinant extracellular proteins. *PLoS One*, 7(**10**), e48629.

Lilie, H., Schwarz, E. and Rudolph, R., 1998. Advances in refolding of proteins produced in *E. coli. Current Opinion in Biotechnology*, 9, 497–501.

Lipiński, D., Jura, J., Kalak, R., Pławski, A., Kala, M., Szalata, M., Jarmuz, M., Korcz, A., Słomska, K., Jura, J., Gronek, P., Smorag, Z., Pieńkowski, M. and Słomski, R., 2003. Transgenic rabbit producing human growth hormone in milk. *Journal of Applied Genetics*, 44, 165–174.

Sami, A.J., 2006. Purification and characterization of growth hormone of water buffalo, *Bubalus bubalis* (Neeli Ravi) from Pakistan. *Pakistan Journal of Zoology*, 38(**4**), 279-282.

Vallejo, L.F. and Rinas, U., 2004. Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. *Microbial Cell Factories*, 3, 11

Yom Din, S., Hurvitz, A., Goldberg, D., Jachson, K., Levavi, S.B. and Degani, G., 2008. Cloning of Russian sturgeon (*Acipenser gueldenstaedtii*) growth hormone and insulin like growth factor I and their expression in male and female fish during the first period of growth. *Journal of Endocrinology Investigation*, 31, 201-210.