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Separation of recombinant porcine growth hormone monomer from dimer and other oligomers in the production process from *E. coli* inclusion bodies

Jolanta Sereikaitė,

Vladas Algirdas Bumelis

Department of Chemistry and Bioengineering, Vilnius Gediminas Technical University, Saulėtekio al. 11, LT-10223 Vilnius, Lithuania

Porcine growth hormone expressed in *E. coli* accumulates as inclusion bodies. After the refolding process, besides the monomer, also a dimer and higher oligomers of this protein were found. A successful application of hydrophobic chromatography for porcine growth hormone monomer separation from other forms in the production process is described. The results of target protein purification from 4 g of biomass are presented.

Key words: porcine growth hormone, hydrophobic chromatography, inclusion bodies, aggregation

INTRODUCTION

Porcine growth hormone (pGH) is a single-chain polypeptide hormone synthesized and secreted by the pituitary gland of pigs. The molecular mass of pGH is 22 kDa, and a molecule has two disulphide bridges. The first attempt to isolate the hormone was made in 1951 [1]. Since then, during the following thirty years, various procedures for its preparation from pituitary gland had been reported by different laboratories [2–4]. The first publication on pGH cloning appeared in 1983 [5]. Later, numerous articles were published on pGH cloning and expression in bacteria, yeast and insect cells [6–10]. On the one hand, the recombinant DNA technology allows producing large amounts of this hormone; on the other hand, some new problems arise, especially in case of inactive protein synthesis and inclusion bodies formation in host *E. coli* strains. Thus, efforts focused on developing a new refolding technique and the subsequent effective purification steps of the target protein are still of great importance. Here we report the applicability of hydrophobic chromatography for pGH monomer separation from higher oligomeric forms in the production process from *E. coli* inclusion bodies.

MATERIALS AND METHODS

Q-Sepharose Fast Flow, Phenyl-Sepharose 6 Fast Flow (low sub) and Sephadex G-25 were from Amersham Biosciences. Reagents for SDS-PAGE were purchased from Bio-Rad, and protein molecular weight markers were obtained from Pharmacia. In the text, the pH values of Tris-HCl buffer solutions are indicated at 25 ˚C.

For large scale production of pGH, *E. coli* BL21 (DE3) harbouring the plasmid pET21a+/pGH was cultivated as described by Baranauskaite et al. [11]. Four grams of frozen biomass was homogenized in 40 ml of 0.1 M Tris-HCl buffer, pH 7.2, containing 5 mM EDTA. The cells were sonicated on ice, five times for 30 s. PMSF to a final concentration of 1 mM was added just before the sonication. The suspension of disrupted cells was centrifuged at 13000*g* for 25 min at 4 ˚C. The collected pellets of inclusion bodies were washed with an equal volume of water, then resuspended in 40 ml of cleaning solution (2M urea, 1 M NaCl, 5 mM EDTA, 1 mM PMSF in 0.1 M Tris-HCl buffer, pH 9.0) and centrifuged at 16000*g* for 25 min at 4 ˚C. Subsequently, the pellets were washed with water. Purified inclusion bodies were solubilized in 40 ml of 0.1 M Tris-HCl buffer pH 9.0) containing 8 M urea and 20 mM reduced glutathione with gentle stirring at 4 ˚C for 15 min. The solution was centrifuged at 16000*g* for 25 min at 4 ˚C and the resulting supernatant diluted with 0.1 M Tris-HCl buffer pH 9.0 containing 6 mM oxidized glutathione in order to reduce the urea concentration to 3 M. The renaturation reaction was carried out for 20 h at 4 ˚C with gentle stirring. Finally, the solution was centrifuged at 16000*g* for 25 min at 4 ˚C, and the chaotropic agent and thiol-compounds were removed by size exclusion chromatography using a Sephadex G-25 gel filtration column (2.5 × 80.5 cm), equilibrated with 0.025 M Tris-HCl buffer, pH 8.5, at a flow rate of 1.5 ml/min. Fractions containing pGH were pooled and loaded on a Q-Sepharose column (2.5 × 12 cm) previously equilibrated with 0.025 M Tris-HCl buffer, pH 8.5, at a flow rate of 2 ml/min. Unbound proteins were washed out with the same buffer, and

bound proteins were eluted with a linear salt gradient as follows: 1 bed volume of 0 M NaCl to 0.15 M NaCl, 2 bed volumes of 0.15 M NaCl to 0.3 M NaCl and two bed volumes of 0.3 M NaCl to 1 M NaCl in 0.025 M Tris-HCl buffer pH 8.5. Peak which appeared in the range of 0.3–1 M of NaCl and contained pGH was pooled. The salt concentration was adjusted approximately to 2 M, and the protein solution was applied on a Phenyl-Sepharose column (1.5 × 13.5 cm) previously equilibrated with 0.025 M Tris-HCl buffer, pH 8.5, containing 2 M NaCl at a flow rate of 1 ml/min. Unbound proteins were washed out with the same buffer, and bound proteins were eluted with 10 bed volumes of the linear NaCl gradient (0.8 M–0.2 M) in 0.025 M Tris-HCl buffer, pH 8.5. Fractions containing pGH were pooled, and protein solution was obtained for purity analysis.

Samples of pGH were analyzed by RP-HPLC using a reversed-phase column Protein C4 (250 × 4.6 mm, Vydac); solvent A, 0.1% trifluoracetic acid in water; solvent B, 0.1% trifluoracetic acid in acetonitrile (HPLC gradient grade). The column was initially equilibrated with 19% B at a flow rate of 1 ml/min. After injection, a linear gradient of 19% B to 51% B for 3 min, 51% B to 59% B for 10 min, 59% B to 68% B for 40 min, 68% B to 78% B for 10 min, 78% B to 81% B for 1 min was applied. The absorbance was monitored at 215 nm using a Waters 2487 Dual λ absorbance detector. Data analysis was performed using Waters Breeze chromatography software.

Protein concentration was estimated using the Bradford method [12]. SDS-PAGE was carried out under reducing and non-reducing conditions according to the method of Laemmli [13]. The acrylamide concentration of the resolving gel was 15%. Proteins in gel were visualized with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

High-level expression of recombinant proteins in *E. coli* often results in protein aggregation and formation of inclusion bodies. It needs to be solubilized by strong denaturants, and the proteins have to be refolded to their native biologically active conformation [14–18] However, the refolding of target protein is in competition with its aggregation. In the case of disulphide bonded proteins, covalently bonded aggregates of different size are formed. The recombinant porcine growth hormone also faces common problems of protein aggregation.

The washed pellets of inclusion bodies were found to contain nearly 70% of pGH. Solubilization of inclusion bodies and protein refolding conditions were discussed earlier by Baranauskaite et al. [11]. As shown by SDS-PAGE under non-reducing conditions (Fig. 1, lane 4), after renaturation and removing of the chaotropic reagent (in our case 3 M urea) and thiol-compounds (reduced glutathione / oxidized glutathione pair) the formation of aggregates of high molecular mass (even higher than 100 kDa which cannot be fractionated by 15% acrylamide gel) was observed. Most of them were removed by ion-exchange chromatography using Q-Sepharose, and pGH seemed to be of very high purity (Fig. 1, lane 3). However, SDS-PAGE under non-reducing conditions shows the presence of pGH dimer and a slight amount of higher oligomers formed via disulphide bonds. Those forms have to be removed producing highly purified pGH. It is widely known that size exclusion chromatog-

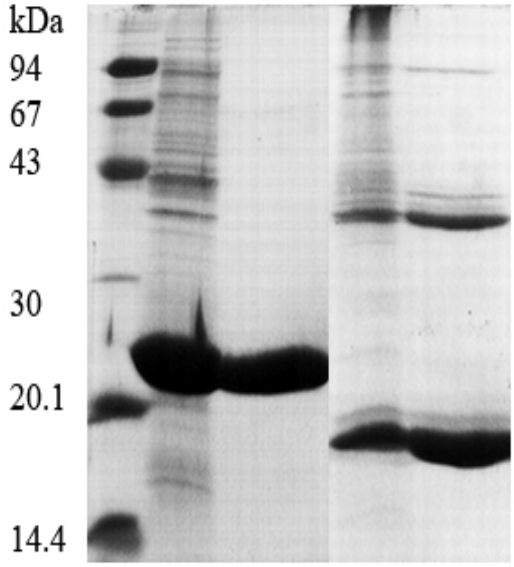

Fig. 1. SDS-PAGE (15%) analysis of recombinant porcine growth hormone at different stages of purification. Lane 1 – protein markers; lane 2 and 4 – protein solution after gel filtration on Sephadex G-25, lanes 3 and 5 – protein solution after chromatography on Q-Sepharose. SDS-PAGE analysis was done under reducing (lanes 2 and 3) and non-reducing (lanes 4 and 5) conditions.

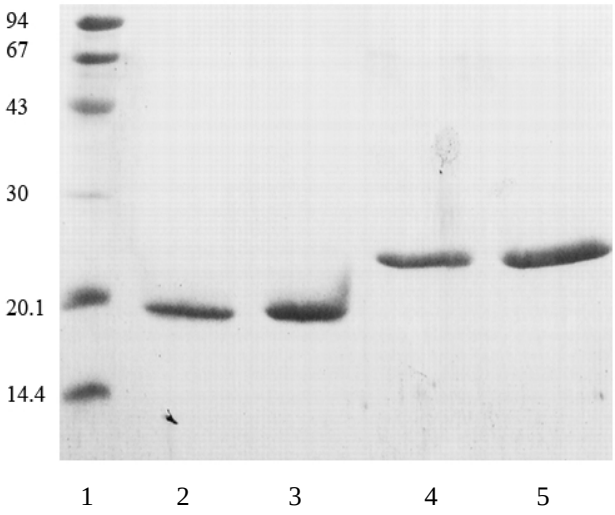

Fig. 2. SDS-PAGE (15%) of highly purified recombinant porcine growth hormone after chromatography on Phenyl-Sepharose. Lane 1 – protein markers, lanes 2 and 4 – 6.5 μg of protein were loaded under non-reducing and reducing conditions, respectively; lanes 3 and 5 –13 μg of protein were loaded under non-reducing and reducing conditions, respectively

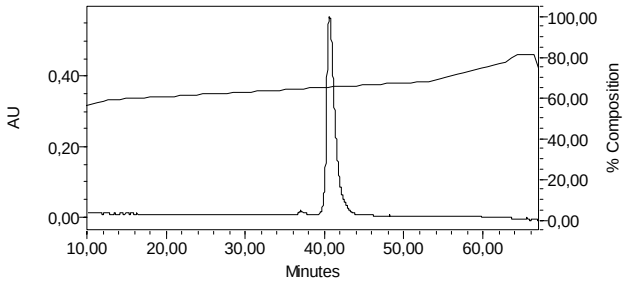

Fig. 3. RP-HPLC analysis of highly purified pGH using reversed-phase column Protein C4 (250 × 4.6 mm, Vydac).

Table. **Results of chromatographic cycle from 4 g of biomass**

No	Stage	Amount of protein, mg*
1	Solution of solubilized inclusion bodies	179 ± 11**
2	pGH refolding	136 ± 11
3	Gel filtration on Sephadex G-25	129 ± 11
4	Ion exchange chromatography on Q-Sepharose	29 ± 5
5	Hydrophobic chromatography on Phenyl-Sepharose	16 ± 3

*The amount of protein is given after chromatographic stage. **Standard deviations for three chromatographic cycles are presented.

raphy separates proteins on the basis of differences in their mass or shape. We consider that this kind of chromatography could be applicable for future purification of pGH. However, in size exclusion chromatography for protein separation, but not for desalting, small sample volumes (usually 2–5 per cent of the column volume) must be applied in order to achieve effective resolution. Moreover, column flow rates are also considerably lower than those used in other chromatographic techniques. The above-mentioned properties render size exclusion chromatography not very attractive, especially in large-scale purification process. Therefore, our attempt was aimed to avoid size exclusion chromatography and find a more convenient chromatographic step. The best result was achieved using hydrophobic chromatography on Phenyl-Sepharose. Under the above chromatographic conditions (see Materials and Methods), recombinant porcine growth hormone monomer was eluted as a peak whose maximum appeared at ~0.45 M NaCl. In pooled fractions pGH was pure as could be judged by SDS-PAGE (Fig. 2). The purity of protein was also checked by RP-HPLC and was found to be ≥95% (Fig. 3). The results of a chromatographic cycle from 4 g of biomass are presented in Table. Therefore, successful application of hydrophobic chromatography allowed producing protein with sufficient purity for biological activity and structural investigations. Our further investigation and yet unpublished results show that this approach could be applicable for purification of other animal hormones.

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J. Sereikaitė, V. A. Bumelis

REKOMBINANTINIO KIAULĖS AUGIMO HORMONO MONOMERINĖS FORMOS ATSKYRIMAS NUO DIMERINĖS IR KITŲ OLIGOMERŲ IŠGAVIMO IŠ *E. COLI* INTARPINIŲ KŪNELIŲ

Santrauka

Kiaulės augimo hormonas, ekspresuotas *E. coli*, kaupiasi intarpinių kūnelių pavidalu. Po renatūracijos, be monomero, taip pat randama šio baltymo dimero ir didesnio svorio oligomerų. Šioms formoms atskirti ir monomerinei formai išgryninti sėkmingai pritaikyta hidrofobinė chromatografija. Taip pat pateikti tikslinio baltymo gryninimo iš 4 g biomasės rezultatai.