



Comparison of Purification Processes for Recombinant Human Growth Hormone Produced in *E. coli*

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Abstract

In this study, purification of recombinant human growth hormone (rhGH) with FLAG tag from *E. coli* inclusion bodies was described. The step-by-step process carried out was as follows: bacterial cells were suspended in lysis buffer and disrupted in a single sonication step. Then, inclusion bodies were isolated and solubilized in different buffers to obtain the best one which was found to be Tris buffer containing 2% deoxycholate with a satisfyingly adequate capacity to dissolve inclusion bodies at pH 12.5. In the third step, the proteins in solubilizing buffer were refolded by being diluted in refolding buffer. This was carried out with lowering the pH value to 8 using a direct dilution (to five volumes) process. Following a specific enterokinase cleavage for removing the tag, the rhGH was purified by ion-exchange chromatography. Following with the research, for the first time, a comparative study was performed on two weak ion-exchangers, namely DEAE and CM-Sephacrose (two most commonly used resins), so as to investigate their pH dependence and resolution. DEAE-Sephacrose at pH 8.25 exhibited the best overall performance and efficiency for rhGH purification as measured by either yield or purity. The overall process was reproducible and easy to scale up, making it a suitable choice for large-scale production of therapeutic proteins.

Keywords Chromatography · Ion-exchange · Inclusion bodies · Recombinant proteins

1 Introduction

Human growth hormone (hGH) is a single chain peptide composed of 191 amino acid residues which is used to treat many diseases (Vance and Mauras 1999; Lebl et al. 2000; Cassidy and Driscoll 2013). Nowadays, this hormone is being produced using recombinant microorganisms such as *Escherichia coli*. However, high-level expression of recombinant proteins in *E. coli* cytoplasm often results in deposition of inclusion bodies (Shin et al. 1998; Kane and Hartley 1988).

Inclusion bodies have no biological activity and before purification process, careful work should be done upon mild solubilization and refolding of them to recover functionally active protein. Many times, the overall yield of

bioactive protein from inclusion bodies is low (Mitraki and King 1989; Schein 1989). Thus, a major bioprocess engineering challenge has been to convert, more efficiently, this inactive and insoluble protein into a soluble and correctly folded product. Solubilized proteins are then refolded by decreasing the concentration of denaturant through various procedures (mainly pulsatile dilution) (Cassidy and Driscoll 2013).

After refolding, the next critical step is the purification of protein by chromatography. Ion-exchange chromatography is one of the most commonly used techniques for the purification of proteins and peptides, and virtually all industrial purification processes involve at least one ion-exchange step. Comparison of chromatographic resins is usually performed during process development; however, only a limited number of resins are tested and compared for hGH purification (Reichert and Valge-Archer 2007; Fahrner et al. 2001). These final chromatographic steps not only remove additional host cell proteins and nucleic acids, but also contribute to removing aggregates and contaminants (Liu et al. 2010).

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The aim of this study was to purify human growth hormone from inclusion bodies of *E. coli* with solubilization, refolding and purification steps. The solubilization behavior of rhGH inclusion bodies in different buffers was analyzed for increasing the efficiency of the solubilization and refolding steps. Then, rhGH was purified by ion-exchange chromatography separation techniques at different pH values and with different buffers and comparisons were made among different methods.

2 Materials and Methods

2.1 Chemicals

Being of analytical grade, urea, acrylamide and bis-acrylamide, sodium dodecyl sulfate, BSA (Bovine Serum Albumin) and deoxycholate were obtained from Sigma Aldrich. DEAE-Sepharose (Diethylaminoethyl cellulose-Sepharose), CM-Sepharose (Carboxymethyl-Sepharose) and Sephacryl S-200 were purchased from GE Healthcare. Commercially available recombinant human growth hormone was obtained from Novo Nordisk®.

2.2 Bacterial Strain and Plasmids

To clone hGH with enterokinase tag, the PCR (Polymerase Chain Reaction) product was inserted into pET32a(+) plasmid. To clone hGH with enterokinase tag, the PCR product was inserted into pET32a(+) plasmid between NdeI and XhoI sites, resulting in pET32a(+)-hPTH84 plasmid (Pennati et al. 2014). The construct thus obtained had rhGH with one extra methionine and enterokinase tag at the N-terminus under the control of the phage T7 promoter. *Escherichia coli* origami cells containing the recombinant expression plasmid were grown in LB (Luria-Bertani) in the presence of kanamycin and ampicillin. The cultures were induced with 1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) and further grown for 4 h. Expression of rhGH in total cell extracts from both uninduced and induced cultures was checked by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

2.3 Isolation, Purification, and Estimation of rhGH from Inclusion Bodies

Induced *E. coli* cells were collected by centrifugation at 4000g for 30 min followed by suspending the cell pellet in 50 mM Tris-HCl buffer (pH 8.5) containing 5 mM EDTA (Ethylenediaminetetraacetic acid) and 1 mM PMSF (Phenylmethylsulfonyl fluoride). Cells were subsequently disrupted by sonication on ice for 7 cycles of 20 s and

centrifuged at 10,000g for 30 min to isolate inclusion bodies.

The inclusion body pellets were washed twice with 50 mM Tris-HCl buffer (pH 8.5) containing 5 mM EDTA and 2% deoxycholate or 2 M urea. Finally, the inclusion bodies were washed with distilled water to remove remaining salt and detergent, and centrifuged at 10,000g for 30 min (Singh et al. 2015). At this stage, inclusion bodies contained mostly rhGH (90%), majority of which was in monomer form of around 22 kDa in SDS-PAGE along with some high molecular aggregates. The inclusion bodies were completely soluble in 50 mM Tris-HCl buffer (pH 8.5) containing 1% SDS; the solubilized r-hGH samples were diluted appropriately and estimated using Bradford protein assay. SDS-PAGE was carried out using the method described by Laemmli (1970).

2.4 Solubilization of rhGH from Inclusion Bodies

Buffers of different pH values along with different denaturants and ionic detergents were used to solubilize the rhGH inclusion bodies. The different buffers used to solubilize rhGH from the inclusion bodies included (a) 100 mM Tris buffer at pH 12.5, (b) 100 mM Tris buffer at pH 12.5 containing 2 M urea, (c) 100 mM Tris buffer at pH 12.5 containing 2 M urea and 1% deoxycholate, (d) 100 mM Tris buffer at pH 12.5 containing 2 M urea and 2% deoxycholate, (e) 100 mM Tris buffer at pH 12.5 containing 2 M urea and 1% Triton X-100, (f) 100 mM Tris buffer at pH 12.5 containing 2 M urea and 2% Triton X-100, (g) 100 mM Tris buffer at pH 8 containing 8 M urea, (h) 100 mM Tris buffer at pH 8 containing 8 M urea and 1% Triton X-100, and (i) 100 mM Tris buffer at pH 8 containing 8 M urea and 1% deoxycholate. Purified inclusion bodies (7 mg/ml) in 50 mM Tris buffer at pH 8.5 were centrifuged. The supernatant was discarded and 1 ml of each of the above solubilizing buffers was added to the pellets. The suspension was shaken followed by being left at room temperature for 10 min after which time it had its turbidity measured at 450 nm. Subsequently, the samples were again centrifuged at 10,000 rpm for 10 min. The supernatant absorbance was recorded at 280 nm to estimate its protein content. The extent of solubilization was further confirmed by SDS-PAGE.

2.5 Refolding and Enterokinase Cleavage

The solubilized rhGH was diluted five times with refolding buffer (50 mM Tris-HCl, 0.5 mM urea, 1 mM EDTA, at pH 8.5). A recombinant bovine enterokinase (bEK) produced in *E. coli* was used to separate FLAG tag from native rhGH molecules. The reaction was carried out in 10 mM Tris-HCl at pH 8.5 at ambient laboratory temperature by

adding 5 U of bEK to 1 mg of rhGH diluted to 3 mg/ml to prevent the aggregation from occurring at higher concentrations.

The reactions were stopped by adding PMSF to a final concentration of 2 mM, and cleavage efficiencies were analyzed by SDS-PAGE.

2.6 Purification by Ion-Exchange Chromatography

Refolded digested proteins were purified by two different ion-exchange resins commonly used for purification purposes. A 2.5×10 cm column (Pharmacia) packed with either of CM-Sepharose or DEAE-Sepharose was used for purification, with the corresponding results to either of the resins compared. First, each column was equilibrated by being washed with 5 column volumes (CV) of an equilibration buffer composed of 100 mM Tris-HCl, 0.5 mM urea, 1 mM EDTA, 5% sucrose and 1 mM PMSF. Each resin was examined at different pH values (4, 6.5, 7.5, 8.25 and 8.5). 5 ml of the rhGH (3 mg/ml) from enterokinase assay was spilled into a column at a flow rate of 0.5 ml/min. The proteins were eluted from the column by 50 ml of NaCl solution in equilibration buffer at a linear gradient of 0–500 mM at room temperature. Fractions (1.5 ml) were collected at a flow rate of 1 ml/min. Proteins were determined by the Bradford protein assay method. SDS-PAGE was employed to analyze selected fractions. Once finished with finding optimal resin and pH value, the experiments were carried out in PBS (Phosphate buffered saline) buffer in order to compare the buffering systems.

2.7 Gel Filtration

Peak fractions showing rhGH were pooled, checked in SDS-PAGE, and lyophilized. The lyophilized rhGH solubilized in 5 ml of 20 mM Tris buffer containing 5 mM EDTA, 5% sucrose and 1 mM PMSF was loaded onto a Sephacryl S-200 column (bed volume 5×60 cm) operating at a flow rate of 0.5 ml/min for further purification. The fractions showing a single rhGH protein band on SDS-PAGE were pooled.

2.8 Spectroscopic Analysis

The rhGH sample was re-suspended in deionized water to a final concentration of 0.1 mg/ml. A circular dichroism spectrum was obtained at 25 °C in the wavelength range of 190–250 nm using a Circular Dichroism Spectrometer Model-215 (Aviv; USA) in 20 mM Tris buffer. The sample was scanned 10 times for data accumulation and the average spectrum was plotted.

3 Results and Discussion

3.1 Isolation of the Inclusion Bodies

E. coli cells expressing rhGH were cultivated in a shake-flask. The cultures at a cell OD of 40 were induced with 1 mM IPTG (optimum IPTG for induction was 0.01 mM/OD culture) and grown for another 4 h. The expression of rhGH plateaued after 4 h of IPTG induction and the level of rhGH expression was measured at about 30% of total cellular protein (Fig. 1). The specific cellular rhGH yield was found to be 26.6 mg/l/OD.

Inclusion bodies of rhGH from *E. coli* cells were isolated with extensive washing once with deoxycholate-containing buffer and another time with urea-containing buffer. The buffers helped remove *E. coli* membrane and cell wall contaminants. This step resulted in relatively pure inclusion bodies containing 90% monomeric rhGH (Fig. 1). The percentage of inclusion bodies was estimated by densitometry using imageJ software. Most of the purified inclusion body proteins visualized in SDS-PAGE had reacted with polyclonal hGH antibody (data not shown), indicating the purity of the preparation. Dimers as well as oligomers of rhGH were also present in the prepared pure inclusion bodies. Dimer formation was also observed at the early stages of recombinant protein synthesis. The purity and homogeneity of the prepared rhGH inclusion bodies were in agreement with the proposed composition for the inclusion bodies formed by specific aggregation of single protein intermediates (Schródel and de Marco 2005; Upadhyay et al. 2012). In this stage, as the level of contaminating proteins was low, it could not affect the

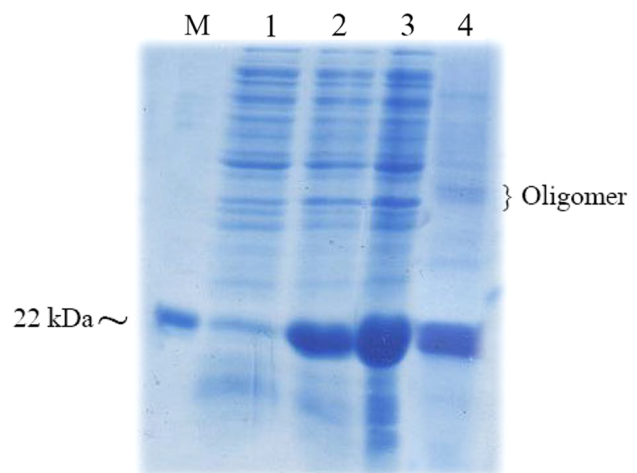


Fig. 1 SDS-PAGE analysis of rhGH expression during shake-flask fermentation. Lanes 1, uninduced cells. Lanes 2, 3, induced cells after 1 and 4 h of IPTG induction, respectively. Lane 4, rhGH inclusion bodies isolated from cells after extensive washing. Lane M, hGH marker

refolding yield of the proteins (Yamaguchi and Miyazaki 2014), and the relatively purified inclusion bodies containing mostly rhGH were used for subsequent solubilization and refolding before further purification.

3.2 Solubilization of rhGH from Inclusion Bodies

It has been widely reported that the growth hormone inclusion bodies of different species expressed in *E. coli* can be solubilized in buffers containing high concentration of chaotropes or those in alkaline pH range when supplemented with detergents (Singh and Panda 2005). Thus, the purified rhGH inclusion bodies were solubilized at 9 different buffers (as mentioned in the Sect. 2), monitoring solubilization percentage of rhGH as measured by absorbance at 280 and 450 nm (Fig. 2). The results have shown that the solubilization of rhGH from the inclusion bodies proceeds better at an alkaline pH value (12.5), rather than in high concentration of urea (8 M) at a pH value of 8. The high alkaline pH (12.5) did not result in the degradation of rhGH (Fig. 2b). The solubilizing effect of 100 mM Tris buffer at a pH value of 12.5 containing 2 M urea and 2% deoxycholate on rhGH inclusion bodies was comparable to that of 100 mM Tris buffer at a pH value of 12.5 containing 2 M urea and 1% deoxycholate (Fig. 2a, b). A maximum of 7 mg/ml of rhGH was solubilized in 100 mM Tris buffer at pH 12.5 with 2 M urea and 2% deoxycholate. Other buffers used for solubilization were not as effective

as buffers C and D. Expression of rhGH in *E. coli* as inclusion bodies was always associated with the formation of dimers and oligomers which were also observed at the early stage of protein synthesis. Oligomers and dimers were observed in the SDS-PAGE (Fig. 2b) in all buffers, despite the highly reducing and denaturing environment established by the gel. Dissociation of these aggregates to monomeric rhGH was also not observed even in 8 M urea solution. Analyzing, in CD, the solubilized rhGH in 100 mM Tris buffer at pH 12.5 containing 2 M urea, it was found to have native-like structures. But, high concentration of urea resulted in random coil formation of inclusion body proteins which were of higher propensity to aggregate (data not shown).

Inclusion body proteins have been reported to have native conformation. As such, when the proteins are solubilized while retaining their native-like structure, one can end up with a high recovery of the bioactive proteins (Oberger et al. 1994). To protect the native-like secondary structure, rhGH inclusion bodies were solubilized in a buffer at alkaline pH containing mild concentrations of chaotropic agents (2 M urea). High pH changes the charge distribution along the protein chain thereby causing higher solubilization of the rhGH obtained from inclusion bodies. High pH helped impart negative charges onto the partially folded protein intermediate, and enabled 2 M urea to break hydrogen bonds among the aggregated molecules (Oberger et al. 1994). This suggested that pH plays a crucial role in destabilizing the inclusion body aggregation. Although alkaline pH makes the protein to be charged negatively, this has no contribution into the protein stability, i.e., it cannot unfold the protein (Nall et al. 1988). However, for bovine growth hormone, the pH-induced unfolding has been reported to be reversible (Lee et al. 2006).

Diminishing hydrophobic interaction between water and protein molecules is another function of urea; this effect can be seen even at low concentrations of this chaotrope. However, such low concentration of urea cannot denature the proteins; it rather probably contributes only into physical separation of the molecules by disrupting the hydrophobic interactions and hydrogen bonds. Urea not only helped solubilize inclusion bodies, but also served as a stabilizer for proteins (Holzman et al. 1990). Thus, in conclusion, rhGH was solubilized from the inclusion bodies without its existing native-like structure being disturbed. A combination of alkaline pH and 2 M urea destabilized major causes of protein aggregation in inclusion bodies of human growth hormone, namely the hydrogen bonds and ionic and hydrophobic interactions.

Being highly reducing in nature, bacterial cytosol does not allow disulfide bonds to form, rejecting the hypothesis that the aggregates could be due to intermolecular disulfide bonds. According to previous studies (Patra et al. 2000),

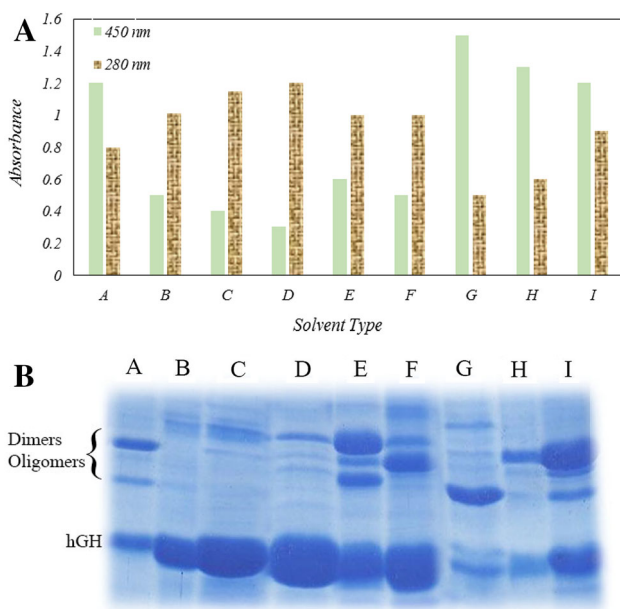


Fig. 2 a Effect of different solvents A–I on the solubility of r-hGH inclusion bodies. A fixed amount of r-hGH inclusion bodies was used and the turbidity and solubility were measured at 450 and 280 nm, respectively. b SDS-PAGE of solubilized sample in different buffers (A–I). Buffers are described in Sect. 2

the rhGH oligomers could not be dissociated into monomers in the presence of a strong reducing agent such as β -mercaptoethanol in solubilization buffer, and disulfide bonds were not critical for rhGH inclusion bodies solubilization, so in this report, no disulfide reducing agent was used when preparing the solubilization buffer.

Deoxycholate is an ionic detergent that loosely binds to the proteins and can be removed by simply washing or dialysis. In this study, this bile salts improved solubilization significantly. According to the CD results, it did not either denature or distort the function and structure of the protein, but rather significantly improved the protein solubilization. Enhanced solubilization of the rhGH from the inclusion bodies in all of the above-mentioned buffers indicated that the hydrophobic interaction is probably the most important driving force for aggregation of proteins during inclusion body formation. However, the presence of hydrogen bonds and ionic interactions in the formation and stabilization of inclusion bodies cannot be ignored. As the extent of the aggregation of partially denatured solubilized rhGH was low during buffer exchange, purification and refolding of the rhGH were carried out at high initial protein concentrations.

3.3 Proteolytic Cleavage of FLAG-hGH Fusion Protein by Enterokinase

Enterokinase is a highly specific serine protease which is recognized as the sequence [(Asp)₄Lys] and cleaves the fusion proteins after the lysine residue in vitro (Shirokova et al. 2011). The solubilized rhGH was diluted five times by refolding buffer in a pulsatile manner and at a very slow rate, with the pH of the buffer adjusted to 8.5. No aggregation of the solubilized rhGH was observed during the time when Bradford dilution assay was carried out to determine approximate concentration of proteins and calculate required amount of hEK for the cleavage of fusion protein. Reducing the amount of enterokinase in the reaction could decrease non-specific cleavage. 10 mg of the fusion protein was cleaved by 0.1 U enterokinase under slight stirring at 37 °C for 16 h. About 80% of the fusion protein was cleaved to release rhGH from FLAG tag (analyzed by imageJ software). The efficiency of proteolysis was then analyzed by SDS-PAGE. The estimated molecular weight of EK via SDS-PAGE was approximately 30 kDa. Further degradation of the protein in cleavage reaction was probably due to an intrinsic broad specificity of the enzyme (Fig. 3). Thus, fast removal or inhibition of the enzyme is highly recommended to prevent further degradation of the target protein after cleavage of fusion protein. This issue is easily addressed by removing the enzyme by one chromatographic step or adding serine protease inhibitors; however, as this study was aimed at

proposing a production process with as low cost and complexity as possible, no inhibitor was used and the evaluated degree of degradation was considered as acceptable after the optimization of cleavage conditions.

In spite of its strong inhibitory effect on enterokinase activity, urea had its final concentration in renaturation buffer below 0.05 M; at higher concentrations, the enzyme activity exhibited a sharp decrease. The optimal concentration of urea was found to be 0.2 M. Other renaturation buffer components were of insignificant influences.

To the best of our knowledge, a report on the production of methionine-free hGH via procedures such as using enterokinase is yet to be released into scientific literature. Recently, Shirokov et al. (2011) described production of IFN α without N-terminal formyl-Met using enteropeptidase. Semikhin et al. (2009) also obtained methionines-free IFN β . However, the cleavage of the recombinant proteins obtained with enteropeptidase was not described in detail. This approach appears more suitable for the digestion of therapeutically applicable proteins, as compared to the study by Nguyen et al. where TEV protease was used to separate Trx from hGH leaving a single Gly residue on the target molecule after digestion, potentially increasing the chances of immunogenicity (Nguyen et al. 2014).

3.4 Purification with Cation-Exchange Chromatography

Isoelectric focusing revealed an isoelectric point (pI) for rhGH at 4.8 (well in agreement with the theoretical value of 4.9) based on the primary sequence. In order to optimize the purification performance of this hormone (as measured by resolution), we examined the efficiency of two most commonly used ion-exchange resins (CM-Sepharose and DEAE-Sepharose) at various pH values (4, 7.5 and 8.5).

pH values below 4 contribute to the precipitation of the hormone, making it impossible to work with the protein at such pH values. As such, the minimum (i.e., starting) pH value was set to 4. At this pH, the protein had a net positive charge, so that it was expected to bind with the resin and the contaminants passing through the flow. Digested rhGH with enterokinase enzyme was loaded on to the column and the proteins were eluted with NaCl at a linear gradient (0–500 mM). The eluted proteins were analyzed via SDS-PAGE and subjected to spectrometry measurements at 280 nm. The results showed that, at this pH, the corresponding peaks to the protein were not sufficiently resolved, so that in no case the sharp and isolated peaks were disappeared (Fig. 4a). On SDS-PAGE results (Fig. 4b), bands with the electrophoretic mobility of rhGH were seen along with those of the contaminants throughout the range of 150–400 mM NaCl.

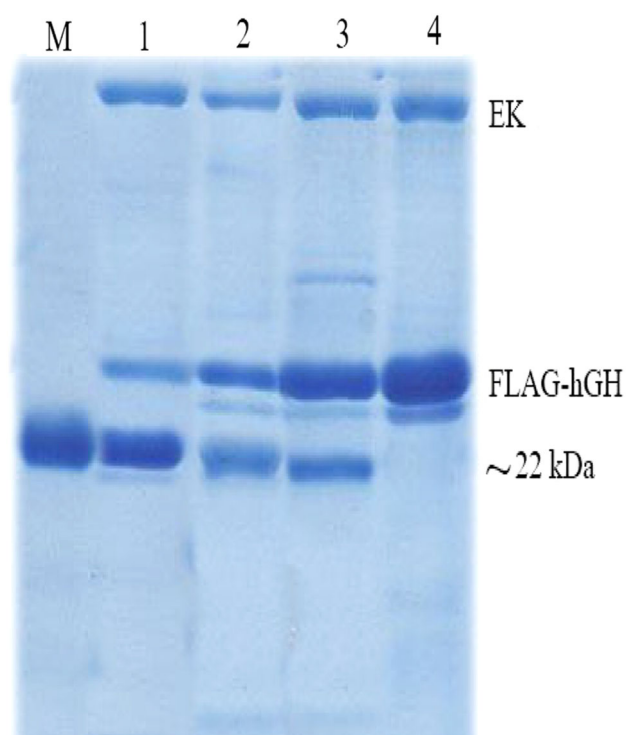


Fig. 3 The fusion protein was cleaved with purified EK for 4, 8 and 24 h at 30 °C. The cleaved products were applied on 12% SDS-PAGE and stained with Coomassie Blue. M: hGH marker. Lane 1: fusion protein only; Lane 2: 4 h; Lane 3: 8 h; Lane 4: 24 h; Lane 5: protein standard. *EK* Enterokinase

At pH values of 7.5 and 8.5, the protein had a net negative charge (according to pI), so that it was expected to get eluted in the flow through the void volume without being retained by either the sorbent or the contaminant eluting off the column by NaCl gradient. The fractions were collected and analyzed by UV spectrometry (the peaks are not shown). The peak areas from rhGH eluted shortly after the void peak even though the rhGH had a large deal of impurities and the peaks were not resolved. Changing the column size, buffering system (PBS instead of Tris), flow rate, gradient slope, sample loading and other factors gave the same results with no improvement in the resolution. Accordingly, one can conclude that the cation-exchange chromatography does not represent a good choice when it came to the purification of growth hormone at this step. Neither the acidic pH nor the neutral pH resulted in high selectivity or resolution.

These results are in contrast to those reported by Russell et al. (2005) who reported that CM-Sepharose is a good resin when the isolation of rhGH from maize cells at a pH value of 5 is concerned. Such a contrast may stem from the different buffering system (acetate) used in their study, as compared to that in the present investigation (Tris and PBS). Accordingly, it can be inferred that anionic buffers like acetate or citrate exhibit better performance for cation-

exchange resins, while it is better to use cationic buffers such as Tris for anion-exchange resins. Based on their physical properties such as pKa and counter ions which offer a high resolution, especially for high molecular mass compounds such as proteins, acetate or citrate buffers usually represent the preferred buffering system for CM resins. In contrast, Tris and PBS buffers result in peak-broadening and aggregation of proteins in cation-exchange resins. In addition, there are reports indicating differences in binding strength of proteins in different buffers. Indeed, binding strength has contributions from the types of buffer and counter ions.

Another possibility regarding the observed results is related to resin-induced protein conformational changes that occur gradually throughout the time and affect, principally, the conformation of protein. Although, IEX resins are inherently less likely to cause unfolding, a few recent studies have suggested that the protein conformational changes can also occur with these resins. Guo and Carta (2015) reported that some cation-exchange resins can cause unfolding and aggregation of proteins, making the proteins impossible to purify leading to the appearance of multiple peaks during the elution and on-column formation of aggregates.

3.5 The Purification of rhGH with Anion-Exchange Chromatography

In the next step, rhGH was purified by DEAE-Sepharose FF anion-exchange chromatography to remove the cleaved FLAG fusion partner and residual uncleaved hormones. Separations were conducted using Tris buffer at pH values of 6.5, 7.5, 8.25 and 8.5. Contaminant proteins, most of which were positively charged at pH above 6 with their molecular weights greater than 30 kDa, would be expected to be in flow through, while the rhGH would be absorbed to the resin. The abundant negative charges of D₄K sequence provides the uncleaved FLAG-hGH with higher binding affinity toward the anion-exchange resin rather than rhGH thus making the separation easily accomplished. Spectrometric analysis of the samples obtained from the separation runs at pH values of 6.5, 7.5 and 8.25 are shown in Fig. 5a–c, respectively. Separation at pH 6.5 resulted in low yields of rhGH. At this pH, the rhGH was eluted over a broad range of NaCl concentration (150–300 mM), indicating tight adherence of proteins to the resin, i.e. somewhat aggregation. Additionally, low amounts of contaminants were seen at these fractions. Separations at pH values of 7.5, 8.25 and 8.5 gave similar results in terms of the yield of rhGH monomer. However, co-transfer of minimal contaminant proteins were seen at pH values of 7.5 and 8.5 (because of the similarity shown by SDS-PAGE analysis at pH 7.5). Quantitative analysis by scanning densitometry of

Fig. 4 SDS-PAGE analysis of the fractions collected from CM-Sepharose chromatography at pH 6.5. Lane M, molecular weight standards; lane 1, 150 mM NaCl-eluted fraction; lane 2, 200 mM NaCl-eluted fraction; lane 3, 250 mM NaCl-eluted fraction; lane 4, 300 mM NaCl-eluted fraction; lane 5, 400 mM NaCl-eluted fraction

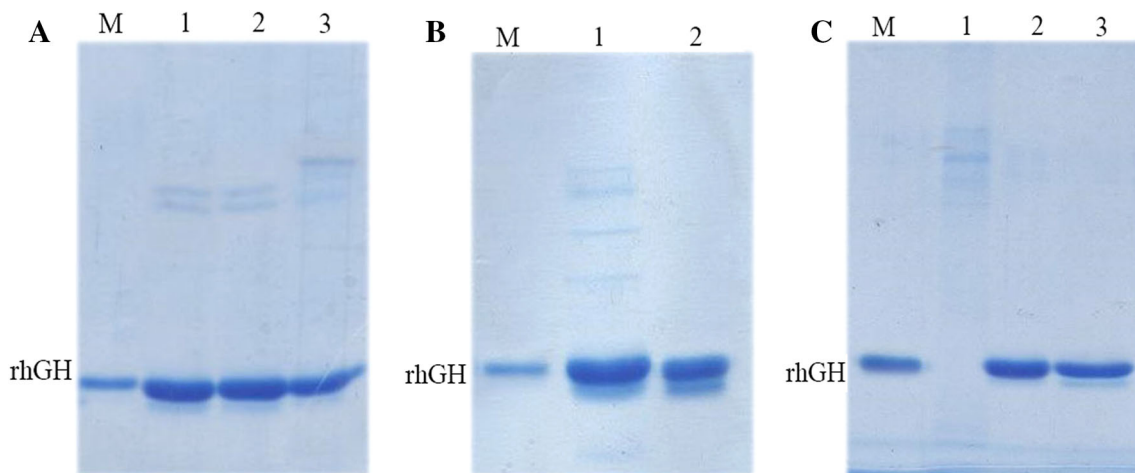
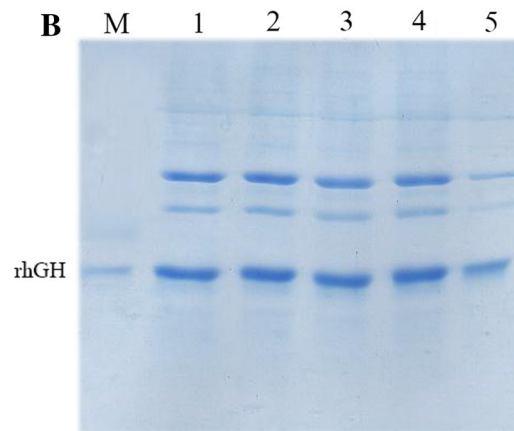
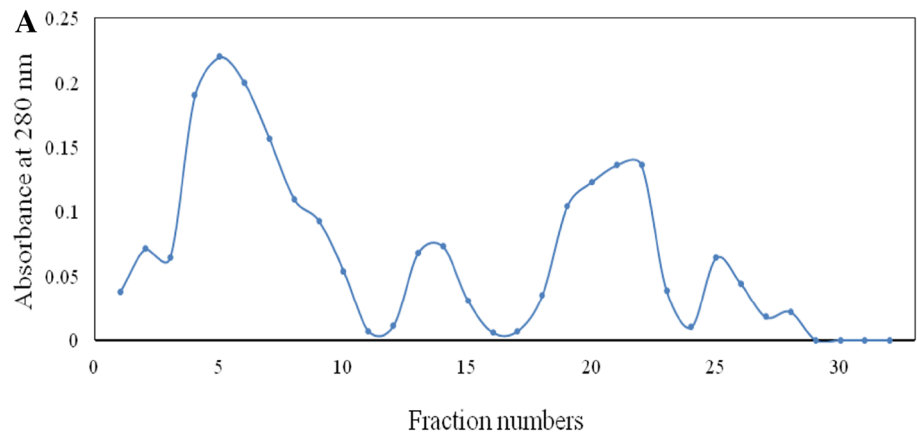


Fig. 5 Coomassie blue-stained SDS-PAGE of rhGH purification using DEAE-Sepharose at different pH. **a** pH 6.5 Lane M, molecular weight standards; lane 1, 150 mM NaCl-eluted fraction; lane 2, 200 mM NaCl-eluted fraction; lane 3, 300 mM NaCl-eluted fraction;

b pH 7.5 Lane M, molecular weight standards; lane 1, 150 mM NaCl-eluted fraction; lane 2, 200 mM NaCl-eluted fraction; **c** pH 8.25 Lane M, molecular weight standards; lane 1, flow through; lane 2, 150 mM NaCl-eluted fraction; lane 3, 200 mM NaCl-eluted fraction

a gel for the separation run at pH 8.25 resulted in rhGH monomer purity and yield of approximately 95% after enterokinase cleavage. At this pH, rhGH eluted within the NaCl concentration range of 150–200 mM (fractions 23–26); the product was found to be homogeneous and

measured to be 40% of total protein. Enterokinase was eluted at 100 mM NaCl. However, some amount of rhGH was co-eluted along with rhGH dimer within the NaCl concentration range of 300–350 mM (fractions 33–43); the eluted rhGH constituted about 25–30% of total protein. The

overall recovery of rhGH from ion-exchange matrix was found to be about 85%.

After setting up the column with Tris buffer at a pH value of 8.25, PBS with 0.5 mM urea, 1 mM EDTA, 5% sucrose and 1 mM PMSF was examined as an alternative buffer. The results showed that, the use of the Tris buffering system resulted in superior selectivity and resolution, as compared to PBS, giving as much purity as 95% in limited fractions. This suggests that cationic buffers such as Tris are preferred for anion-exchange chromatography. However, anionic buffers (such as phosphate or MES) should be considered as preferred for resins such as hydroxylapatite, because the buffer should have the same charge as that of the ion-exchange material, so as to prevent it from being bound to the ion-exchanger.

Thus, DEAE-Sephacrose was proved to be an efficient anion-exchange resin when it comes to protein purification process. The separation run at the pH value of 6.5 resulted in a low yield of rhGH, presumably due to a lower net charge on the protein. Thus, optimization of the purification procedure involves finding the optimal balance between the separation resin and the choice of buffer.

3.6 Gel Filtration

Pure rhGH containing dimers as well as oligomers was passed through a gel filtration chromatography column for final purification step to remove dimeric or higher forms of the proteins. The overall yield of the purified refolded rhGH from the inclusion bodies of *E. coli* was 50%. Fractions of 5 ml in volume were collected and the corresponding peaks were pooled separately. Gel filtration chromatography revealed a single peak between fractions 25 and 35, which corresponded to the protein size of 22 kDa (Fig. 6). SDS-PAGE analysis of the purified rhGH

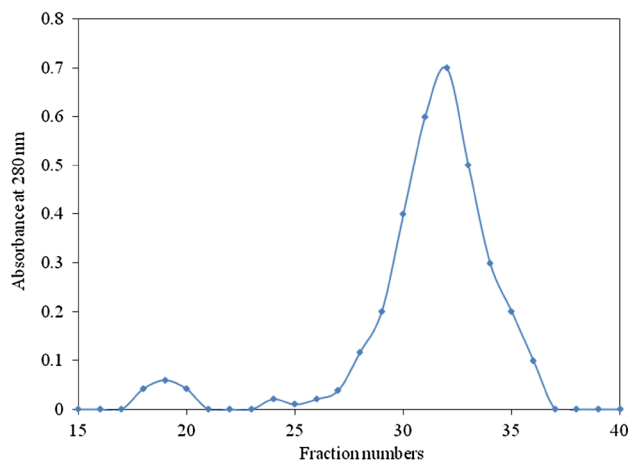


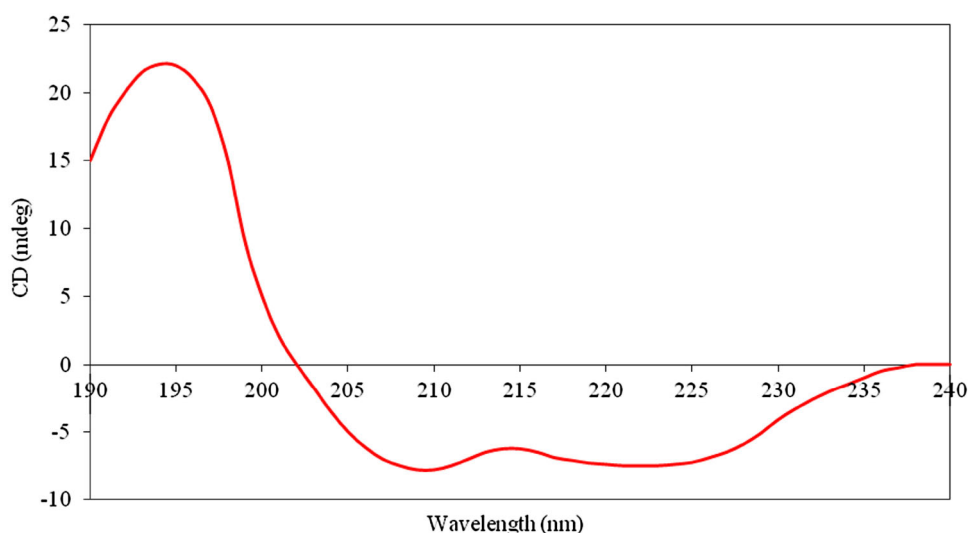
Fig. 6 Final purification of rhGH through gel filtration chromatography. Monomeric pure rhGH analyzed on a Sephacryl S-200 gel filtration column at 280 nm showing a single peak

showed a single band, thus indicating the high purity of the rhGH (nearly 99%, data not shown). The CD spectrum of the purified rhGH was found to be similar to that of native hGH [as reported in literature (Kim et al. 2013)] (Fig. 7).

4 Conclusion

In this study, rhGH inclusion bodies were dissolved in different solubilization buffers containing low concentration of urea at alkaline pH, and refolded through pulsatile dilution. Solubilization of the rhGH from inclusion bodies was done with the proteins retaining their native-like secondary structures. This could facilitate the refolding of the proteins in subsequent steps. Diluted refolded protein was subjected to anion-exchange as well as gel filtration chromatography. Comparing two ion-exchange resins at

Fig. 7 Far-UV CD analysis of purified rhGH. The CD spectra of purified rhGH were scanned in the UV range 190–250 nm. The spectra were recorded at the protein concentration of 0.1 mg/ml



different pH values, optimum results were obtained with DEAE at a pH value of 8.25. The final purified protein had the native structure of human growth hormone.

So far, many purification processes have been developed for the production of rhGH; these involved several alternating chromatographic steps. However, in the present research, mild solubilization combined with DEAE column resulted in high-throughput purification of rhGH from inclusion bodies. Considering the data presented herein, it can be suggested that the proposed system offers a viable alternative for the production of rhGH with lower number of unit processes. The protein produced in this manner had a purity of ~ 99%. Being associated with lower production costs, this production process is potentially interesting for the production of this therapeutic protein (rhGH) in less-developed markets.

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