14

Isolation, Solubilization, Refolding, and Chromatographic Purification of Human Growth Hormone from Inclusion Bodies of *Escherichia coli* Cells

A Case Study

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1. Introduction

1.1. Isolation of Human Growth Hormone Inclusion Bodies From E. coli *Cells*

Inclusion bodies produced in *Escherichia coli* are composed of densely packed denatured protein molecules in the form of particles ***(1,2)***. In addition to the recombinant protein of interest, inclusion bodies contain small amounts of host protein, ribosomal components, and DNA/RNA fragments ***(3)***. It is advisable to purify the inclusion bodies from the cells to a high-degree purity before carrying out solubilization and purification. This will reduce the number of purification steps after solubilization and refolding, minimize the interference of other contaminating proteins during refolding, and result in a therapeutic protein free from other cellular contaminants, such as lipids, carbohydrate, and endotoxin ***(4)***. Isolation of inclusion bodies from *E. coli* occurs by cell lysis with high-pressure disruption using a French press or sonication step followed by centrifugation ***(5)***. Further purification can be achieved by washing with detergents and a low concentration of salt and/or urea ***(5,6)***. The presence of contaminants, along with the protein of interest, is mainly because of incomplete purification of the inclusion bodies following cell lysis. With proper centrifugation and washing processes, more than 95% pure inclusion bodies of recombinant proteins can be isolated from *E. coli* cells ***(7)***. As the inclusion bodies have a high density (approx 1.3 mg mL^{-1}), these are easily separated by high-speed centrifugation after cell disruption ***(8)***. Centrifugal isolation, particularly sucrose gradient centrifugation, has been found to be the best method for isolating very pure inclusion bodies from *E. coli* cell lysate ***(9)***. Expression of recombinant human growth hormone (r-hGH) in *E. coli* will illustrate

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the methods used for isolation and purification of intact inclusion bodies. The purified inclusion bodies will then be used for solubilization and refolding to obtain bioactive protein.

1.2. Solubilization and Refolding of Human Growth Hormone From Inclusion Bodies of E. coli

Inclusion bodies from *E. coli* are generally solubilized in high concentrations of denaturants, such as 8 *M* urea or 6 *M* guanidine hydrochloride with or without reducing agents ***(10–12)***. High concentrations of denaturants unfolds the protein completely, thus increasing the propensity of aggregation during refolding. Suboptimal buffer exchange during protein refolding also contributes to protein aggregation, which results in a low recovery of refolded protein from inclusion bodies. Reducing the protein aggregation improves the recovery of solubilized bioactive protein from the inclusion bodies. As the inclusion body proteins retain native-like secondary structure, it is advisable to protect this during solubilization. Partly folded protein conformations help in improved recovery of bioactive protein ***(13)***. Therefore, the use of a mild inclusion body solubilization process is the key step in successful recovery of high yields of therapeutic protein from inclusion bodies.

Recently, many new methods for solubilization of inclusion bodies without using high concentrations of denaturants have been reported, which involve using detergents ***(14)***, pressure (***15)***, pH, or a combination of these ***(7)***. Furthermore, different solubilization buffers can be used to evaluate the importance of various protein interactions that lead to the accumulation of inclusion bodies ***(16)***. Such an approach is taken to further understand the nature of the protein aggregation leading to inclusion body formation. With this information, suitable buffers can be employed to solubilize the inclusion body proteins without unfolding the protein of interest into a random coil configuration. Recombinant ovine growth hormone ***(7)*** and *zona pellucida* glycoprotein ***(17)*** have both been successfully solubilized and refolded using such mild solubilization approaches. Developing an efficient and low-denaturant solubilization step is thus necessary for achieving high-throughput purification of recombinant protein. This chapter discusses the solubilization and refolding of r-hGH from inclusion bodies. Inclusion bodies are solubilized using mild concentrations of urea while "giving" a pH shock. Solubilized hGH is subsequently refolded into a soluble bioactive form using a pulse renaturation process.

1.3. Chromatographic Purification of Recombinant Human Growth Hormone

Ion-exchange chromatography followed by gel-filtration chromatography is the most commonly used procedure for the purification of recombinant proteins ***(18,19)***. The advantages of using ion-exchange chromatography include the fact that it can be operated at different salt, pH, and buffer compositions, and, perhaps most important, diluted and refolded proteins can be concentrated during this purification step. Thus, ion-exchange chromatography is the most suitable process for protein purification from

the large volumes of dilute solution that are generated during inclusion body refolding processes. r-hGH has previously been purified using various ion-exchange chromatographic techniques ***(20)***. As the isoelectric point of human growth hormone is 4.9, operating anion-exchange chromatography processes at a pH far from the isoelectric point results in the best separation ***(21,22)***. The initial purification and concentration is undertaken using a Q-Sepharose (Amersham Pharmacia) column followed by gel-filtration chromatography using a Sephacryl S-200 (Amersham Pharmacia) matrix ***(16)***.

During the refolding, high-molecular-weight aggregates are always associated with the pure monomeric protein. These aggregates constitute 2–10% of the total protein; therefore, it is necessary to remove them using gel filtration chromatography. As the hGH used as a case study in this chapter was refolded and purified in 2 *M* urea, the refolded protein is extensively dialyzed to remove the urea before gel filtration. During dialysis, the excipients and buffers required to promote protein stability are introduced. This chapter details ion-exchange and gel-filtration processes for the purification of hGH.

2. Materials

2.1. Isolation of Human Growth Hormone Inclusion Bodies From E. coli *Cells*

1. Transformed *E. coli* cells expressing hGH.
2. Luria Bertani (LB) medium: 10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl per liter of Milli-Q water. Add 5 g/L glucose in the medium for enhanced cell growth.
3. Isopropyl-β-D-thio-galactopyranoside (IPTG) filter-sterilize.
4. French press.
5. High-speed centrifuge and microfuge.
6. Ultracentrifuge.
7. Probe sonicator.
8. Phenylmethylsulfonyl fluoride (PMSF).
9. Tris-EDTA buffer: 50 m*M* Tris-HCl, 5 m*M* EDTA, and 1 m*M* PMSF, pH 8.5.
10. Tris-HCl buffer: 10 m*M* Tris-HCl, pH 8.5.
11. Sucrose solution: Prepare in phosphate buffer.
12. Sodium deoxycholate: Dissolve 1% (w/v) in Tris-HCl buffer, pH 8.
13. Sodium dodecyl sulfate (SDS) sample buffer: 0.25 *M* Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 160 m*M* dithiothreitol, and 0.04 mg/mL bromophenol blue.
14. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) equipment and buffers for running gel.
15. Molecular weight marker: 14-, 21-, 30-, 44-, 66-, and 90-kDa protein mixture markers (Amersham Pharmacia). Dissolve one vial of the marker in 1 mL SDS sample buffer and store at –20°C.
16. Coomassie blue stain: 0.1% (w/v) Phastgel Blue R (Amersham Pharmacia) in destaining solution.
17. Destaining solution: 40% (v/v) methanol and 10% (v/v) acetic acid.
18. Ampicillin and kanamycin.
19. Sodium phosphate buffer: 100 m*M*, pH 8.
20. Spectrophotometer (ultraviolet [UV]-visible range).
21. Micro-BCA protein assay kit (Pierce).

2.2. Solubilization and Refolding

1. Pure r-hGH inclusion bodies from *E. coli* cells.
2. High-speed centrifuge.
3. Spectrophotometer (UV-visible).
4. pH meter.
5. PMSF.
6. Magnetic stirrer.
7. Peristaltic pump.
8. Homogenizer.
9. Solubilization buffer: 100 m*M* Tris-HCl, 1 m*M* EDTA, and 2 *M* urea, pH 12.5.
10. Refolding buffer: 50 m*M* Tris-HCl, 1 m*M* EDTA, 2 *M* urea, 10% (w/v) sucrose, and 1 m*M* PMSF, pH 8.
11. Membrane filter (0.45 µm).
12. Micro-BCA assay kit (Pierce).

2.3. Chromatographic Purification

1. Refolded r-hGH solution.
2. AKTA purifier (Amersham Pharmacia).
3. Liquid chromatography system (Amersham Pharmacia).
4. Glass jacket column (26 × 40 cm) for ion exchange.
5. Glass jacket column (16 × 100 cm) for gel filtration.
6. Q-Sepharose matrix (Amersham-Pharmacia).
7. Sephacryl S-200 matrix (Amersham-Pharmacia) for gel filtration.
8. Dialysis bag.
9. Spectrophotometer.
10. Lyophilizer.
11. Equilibration buffer: 50 m*M* Tris-HCl, 1 m*M* EDTA, 2 *M* urea, 5% sucrose (w/v), and 1 m*M* PMSF, pH 8.5.
12. Washing buffer: 10 m*M* NaCl in equilibration buffer.
13. Elution buffer: 500 m*M* NaCl in equilibration buffer.
14. PMSF.
15. Dialysis buffers.
    a. First change: 50 m*M* Tris-HCl, 1 m*M* EDTA, 1 *M* urea, 5% (w/v) sucrose, and 1 m*M* PMSF, pH 8.5.
    b. Second change: 50 m*M* Tris-HCl, 0.5 m*M* EDTA, 0.5 *M* urea, 5% (w/v) sucrose, and 1 m*M* PMSF, pH 8.5.
    c. Third change: 10 m*M* Tris-HCl, 0.5 m*M* EDTA, 0.25 *M* urea, 1% (w/v) sucrose, and 1 m*M* PMSF, pH 8.5.
    d. Fourth change: 10 m*M* Tris-HCl and 1% (w/v) sucrose, pH 8.5.
16. Gel filtration buffer: 10 m*M* Tris-HCl and 1% (w/v) sucrose, pH 8.5.
17. Micro-BCA protein assay (Pierce).

3. Methods

3.1. Isolation

hGH, an important therapeutic protein having a molecular weight of approx 22 kDa and expressed in *E. coli*, is used in this chapter as a model system for purification of inclusion bodies ***(16)***. Two methods are described with the aim of obtaining pure

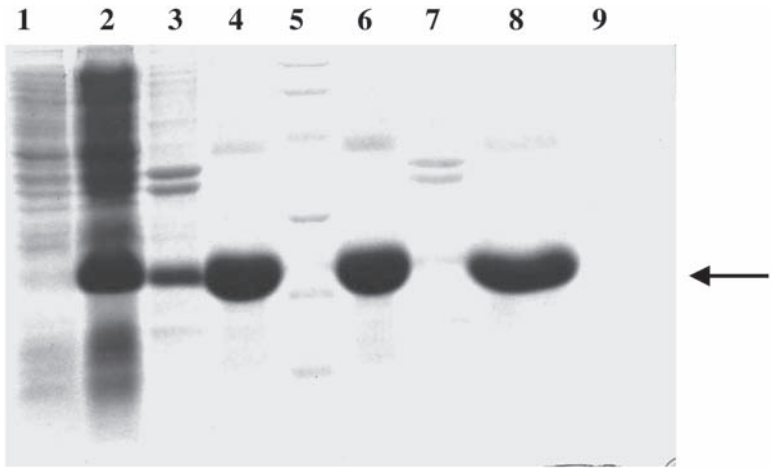

Fig. 1. SDS-PAGE analysis of r-hGH from inclusion bodies of *E. coli* cells. The arrow indicates the r-hGH band. Lane 1, uninduced cells; lane 2, induced cells; lane 3, inclusion bodies after cell lysis; lanes 4 and 6, pure inclusion body from sucrose gradient centrifugation; lane 5, molecular weight marker (14, 21, 30, 44, 66, and 90 kDa, from the bottom to top of the gel); lane 7, contaminating proteins; lane 8, pure inclusion bodies using deoxycholate treatment.

inclusion bodies of hGH from *E. coli* cells. These methods are sucrose gradient centrifugation and a sodium deoxycholate washing process. Therapeutic proteins expressed as inclusion bodies in *E. coli* can be isolated with the help of either of these methods with slight modification. Finally, an SDS-PAGE method has been described for the analysis of r-hGH expression and pure inclusion bodies. Growth and expression of r-hGH is also described, as it constitutes the starting point of the method.

3.1.1. Expression of hGH in E. coli

1. Inoculate *E. coli* strain expressing r-hGH from glycerol stock in to 10 mL sterilized LB media containing 100 µg/mL ampicillin and 25 µg/mL kanamycin. Grow the *E. coli* cells overnight at 37°C and at a shaker speed of 200 rpm in an orbital shaker.
2. Inoculate 10 mL overnight grown *E. coli* cells in to 1 L LB medium with 100 µg/mL ampicillin and 25 µg/mL kanamycin. Allow the cells to grow until the optical density (OD) of the culture at 600 nm reaches 0.5.
3. Induce *E. coli* cells with IPTG (1 m*M* final concentration) at an OD of 0.5 and grow for another 3 h (*see* **Note 1**).
4. After 3-h induction, harvest the *E. coli* cells by centrifugation at 10,000*g* for 30 min. Resuspend the *E. coli* cells in Tris-HCl buffer and centrifuge again at 10,000*g* for 30 min. Collect the cell pellet for inclusion body isolation. These cells can be directly used for inclusion body extraction or can be stored at –20°C for future processing.
5. Check *E. coli* cells for intracellular expression of r-hGH by SDS-PAGE analysis. Use both uninduced and induced cells to check the presence of r-hGH after induction with IPTG (**Fig. 1**).

3.1.2. Inclusion Body Purification Using Sucrose Gradient Centrifugation

This method is useful for the small-scale preparation of inclusion body proteins. Protein concentrations of up to 100 mg in the form of inclusion bodies can be processed using this method. As the inclusion bodies have higher densities than most membrane components, sucrose gradient centrifugation helps in separating these particles from contaminating cellular fragments/proteins. This method is useful and results in good yields of pure inclusion bodies, which can be further used for solubilization and refolding to obtain bioactive protein. This method does not require detergent treatment and can be carried out with the normal equipment available in a standard molecular biology laboratory.

1. Resuspend the *E. coli* cells expressing r-hGH from a 1-L culture (4 g wet cell pellet) in 10 mL 50 m*M* Tris- EDTA buffer containing 5 m*M* EDTA and 1 m*M* PMSF, pH 8.5.
2. Pass the cells for three cycles through a French press at 15,000–18,000 psi for cell lysis. In the absence of a French press, use sonication (10 cycles of 1 min each with a 1-min gap between cycles, 50% duty, power 28 W, on ice) for cell lysis.
3. Centrifuge the cell lysate at 20,000*g* for 20 min at 4°C. Discard the supernatant, and use the pellet for further purification of inclusion bodies.
4. Wash the pellet with Tris-HCl buffer and centrifuge once again as described in **step 3**.
5. Resuspend the inclusion body pellet in 2 mL sodium phosphate buffer (100 m*M*, pH 8).
6. Prepare sucrose step gradient in ultracentrifuge rotor tubes by dropwise addition of sucrose solution. Add 1 mL each of 72%, followed by 70%, 68%, 66%, 62%, and 60% (w/v) of sucrose solution from the bottom to the top of the tube to prepare the sucrose gradient.
7. Add 1 mL inclusion body suspension from **step 5** on top of the 60% sucrose layer in the tube, and centrifuge at 150,000*g* for 6 h in a swinging rotor at 4°C.
8. Inclusion bodies will be seen in the sucrose gradient at densities between 65% and 70% as a dense layer. Carefully remove the dense layer by pipet without disturbing the other layers, and collect the inclusion body particles in an Eppendorf tube (*see* **Note 2**).
9. Add 0.5 mL Milli-Q water to the Eppendorf tube; vortex and centrifuge in a microcentrifuge for 20 min at 10,000*g* at 4°C. Repeat **steps 6–8** to further purify inclusion bodies, or prepare gradients in two tubes to ultracentrifuge all of the inclusion bodies from **step 5**.
10. Wash the inclusion body pellet with Milli-Q water, then centrifuge at 10,000*g* for 20 min at 4°C. Discard the supernatant. The remaining pellet consists of ultra-pure inclusion bodies of r-hGH. Run an SDS-PAGE gel, and check the homogeneity of the inclusion bodies.
11. Pure inclusion bodies run at approx 22 kDa on a SDS-PAGE gel with minor contaminants. High molecular aggregates of hGH may be seen along with the pure 22-kDa r-hGH (**Fig. 1**). Inclusion bodies have regular shapes with average particles sizes about 0.5 µm as observed by a scanning electron micrograph (**Fig. 2**).
12. Dissolve a small amount of r-hGH inclusion bodies in 1% SDS solution in 10 m*M* Tris-HCl buffer (no EDTA), and prepare serial dilutions to measure the protein concentration in the inclusion bodies using a detergent-compatible Micro-BCA protein kit (*see* **Note 3**).

3.1.3. Isolation of r-hGH Inclusion Bodies by Deoxycholate Treatment

1. Suspend *E. coli* cells (4 g wet cell pellet) expressing r-hGH from a 1-L culture in 10 mL 50 m*M* Tris-EDTA buffer containing 5 m*M* EDTA and 1 m*M* PMSF, pH 8.5. Lyse the cells as described in **Subheading 3.2.** using either a French press or sonicator.

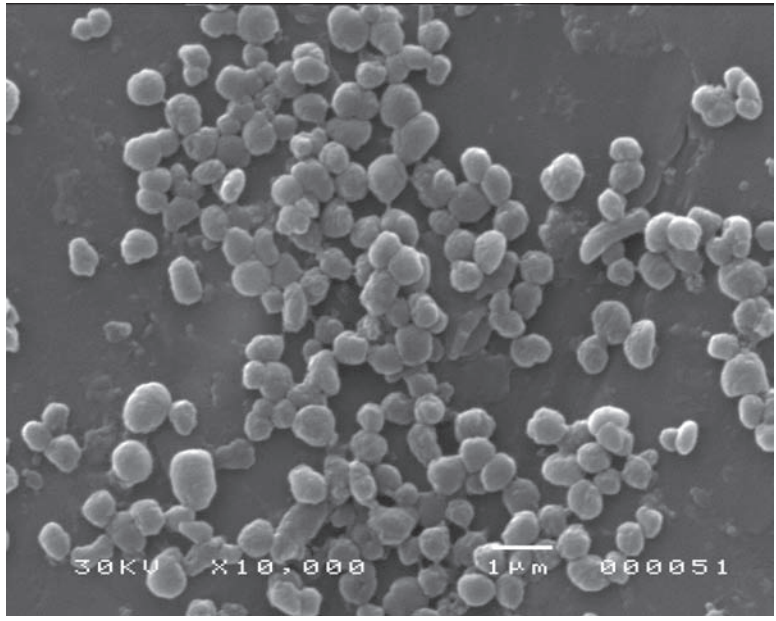

Fig. 2. Scanning electron microscopy of pure hGH inclusion bodies. The average size is approx 0.5 μm.

2. Centrifuge the cell lysate at 20,000*g* for 20 min at 4°C. Use the pellet for inclusion body purification.
3. Suspend the inclusion body pellet in 10 mL Tris-EDTA buffer and sonicate (10 cycles of 1 min each with a 2-min gap between cycles, 40–50% duty, power 28 W, on ice).
4. Centrifuge the inclusion body suspension from **step 3** at 20,000*g* for 20 min at 4°C. Decant the supernatant, and use the pellet for further processing and purification of inclusion bodies. Suspend the pellet in 10 mL 1% deoxycholate (sodium salt) solution.
5. Mix the solution thoroughly and sonicate (six cycles of 1 min each with 2-min gap, 40–50% duty, power 28 W, on ice). Centrifuge the suspension at 20,000*g* for 20 min at 4°C. Use the pellet for further processing to obtain ultra-pure inclusion bodies.
6. Dissolve the pellet again in 10 mL 1% deoxycholate solution and sonicate (six cycles of 1 min each with 2-min gap, 40–50% duty, power 28 W, on ice). Keep the inclusion body suspension in deoxycholate solution at room temperature overnight. This step helps in removing contaminating membrane proteins (*see* **Note 4**).
7. Vortex the inclusion body suspension thoroughly and centrifuge at 20,000*g* for 20 min at 4°C. Resuspend the inclusion body pellet after the second deoxycholate wash in 20 mL Tris-HCl buffer, pH 8.5. Mix thoroughly and centrifuge at 20,000*g* for 20 min at 4°C. Collect and process the pellet for further removal of detergent.
8. Wash the inclusion body pellet with 20 mL Milli-Q water to remove residual detergent and salts; centrifuge at 20,000*g* for 20 min at 4°C. The pellet obtained after centrifugation contains the pure inclusion bodies of r-hGH and looks similar when analyzed by SDS-PAGE to that described for sucrose gradient purification (**Fig. 1**).

3.1.4. SDS-PAGE Procedure for Protein Analysis

3.1.4.1. Casting of the Gels

Prepare 12% resolving/separating gel and 5% stacking gel. The following describes the composition of the gels.

Preparation of a 12% resolving/separating gel (for one gel)	
29.2% Acrylamide + 0.8% bisacrylamide	4 mL
1.5 *M* Tris-HCl, pH 8.8	2.5 mL
Distilled water	3.3 mL
10% SDS	100 µL
10% Ammonium persulfate (APS)	100 µL
TEMED	4 µL
Preparation of a 5% stacking gel (for one gel)	
29.2% Acrylamide + 0.8% bisacrylamide	0.5 mL
1 *M* Tris-HCl, pH 6.8	0.38 mL
10% SDS	30 µL
Distilled water	2.1 mL
10% APS	30 µL
TEMED	3 µL

3.1.4.2. Preparation of Samples

Use *E. coli* cells expressing r-hGH for SDS-PAGE analysis. One milliliter of both uninduced and induced *E. coli* cells should be used and centrifuged at 10,000*g* for 10 min for cell isolation. Dissolve the cell pellet in 50 µL SDS sample buffer, and boil for 5 min. Vortex the solution, centrifuge at 10,000*g*, and load 20 µL supernatant onto a SDS-PAGE gel. Dissolve pure inclusion bodies in SDS sample buffer, boil, centrifuge as described previously, and load the clear soup onto the SDS-PAGE gel (*see* **Note 5**). Load 10 µL low-molecular-weight marker in a separate well in the SDS-PAGE gel along with samples.

3.1.4.3. Running SDS-PAGE Gel

1. Load the SDS-PAGE gel well with 20 µL *E. coli* cell extract and pure inclusion body solubilized and processed in sample dye and 10 µL molecular weight marker as described previously. Run the gel at a constant current of 30 mA. Stop electrophoresis when the tracker dye is approx 1 cm from the bottom of the glass plate.
2. Remove the gel gently, and place it in the container containing Coomassie blue stain. Stain the gel for 1 h by slowly mixing on a rocker. Place the gel in destaining solution (40% methanol and 10% acetic acid) and leave until the protein bands are visualized (*see* **Fig. 1**).

3.2. Solubilization and Refolding

3.2.1. Solubilization of r-hGH Inclusion Bodies at High pH

For hGH, ionic and hydrophobic interactions are the main forces causing protein aggregation in inclusion bodies ***(16)***. High-alkaline pH (12.5) in the presence of 2 *M* urea

promotes the solubilization of hGH from inclusion bodies. Use of 2 *M* urea does not completely unfold the protein and preserves the existing native-like secondary structure. Using high pH also helps in better solubilization, as the buffer pH is far from the isoelectric point of hGH, which is 4.9. Therefore, a combination of alkaline pH and 2 *M* urea helps in destabilizing both the ionic and hydrophobic interactions—the major cause of protein aggregation in inclusion bodies of r-hGH.

1. Suspend the pure inclusion body pellet of r-hGH (50 mg) in 10 mL 100 m*M* Tris-EDTA buffer containing 1 m*M* EDTA and 2 *M* urea, pH 12.5 (*see* **Note 6**).
2. Homogenize the suspension thoroughly at 5000 rpm using a homogenizer for 5 min on ice, then leave for 15 min at room temperature. Following this, centrifuge the solution at 20,000*g* for 20 min at 4°C (*see* **Note 7**).
3. Carefully aspirate off the supernatant obtained after centrifugation, and check the protein concentration in the supernatant by reading the OD at 280 nm. This will provide an indication of the protein solubility in the solubilizing buffer.
4. Check for the presence of hGH both in the pellet and supernatant by running a SDS-PAGE gel.
5. Repeat **steps 1–4** if a large amount of unsolubilized pellet remains.
6. Pool the solubilized hGH solution (total of ~40 mg r-hGH solubilized in 10 mL of solubilization buffer), and use immediately for refolding by the pulsatile renaturation process (*see* **Subheading 3.2.2.** and **Note 8**). Carry out the solubilization and refolding on the same day.

3.2.2. Refolding by Pulsatile Renaturation

Protein aggregation occurs due to the intermolecular interactions between partially folded intermediates well before the formation of either stable intermediate or fully folded protein ***(23)***. However, it has been reported that partially folded intermediates of protein molecules do not interact with fully folded protein, as described for the P22 tail spike protein ***(23)***. This supports using a pulse renaturation process for high-throughput recovery of denatured protein ***(24,25)***. This process essentially involves the addition of small amounts of solubilized inclusion body proteins in batches to the same volume of refolding buffer. After a few minutes, further unfolded protein is added, assuming that the first batch of protein has been refolded into its native-like structure. As the unfolded protein does not interact with the folded protein, pulse addition keeps on refolding protein in the same buffer tank. This helps in reducing the volume of buffer required for refolding and results in high concentrations of refolded protein. Solubilized hGH is refolded using a pulse renaturation process as described here.

1. Take 90 mL refolding buffer (50 m*M* Tris-HCl, 0.5 m*M* EDTA, 2 *M* urea, 10% glycerol, 5% sucrose, and 1 m*M* PMSF, pH 8), and maintain it at 4–6°C with constant stirring (*see* **Note 9**).
2. Add solubulized r-hGH solution (~4 mg/mL) dropwise either using a peristaltic pump (keeping the flow rate at the 0.5 mL/min minimum), or use a micropipet to add small protein amounts at regular intervals.
3. Continue adding. It will take approx 1 h for the pulsatile renaturation of 10 mL of solubilized r-hGH into 100 mL refolding buffer. Refolding will also result in reducing the pH of the solution from a high-alkaline value to a pH of 8–8.5 (*see* **Note 10**).

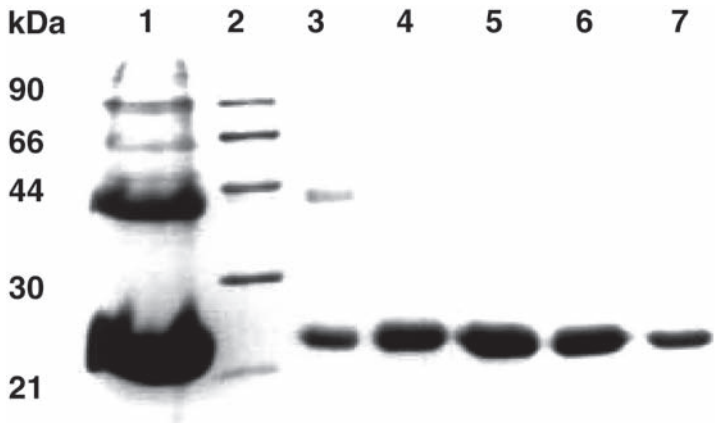

Fig. 3. SDS-PAGE analysis of the described r-hGH purification process using Q-Sepharose ion-exchange chromatography. Lane 1, solubilized inclusion body proteins; lane 2, molecular weight marker; lane 3, load on to Q-Sepharose column; lanes 4–7, purified fractions of hGH.

4. 10 mL r-hGH (solubilized in a buffer at pH 12.5 containing 2 *M* urea) is refolded in a pulsatile manner to give a final concentration of 400 µg/mL r-hGH.
5. After refolding, filter the protein solution using a 0.45-µm membrane filter to remove any aggregates. Obtain an estimate of the protein concentration using a Micro-BCA protein assay. The protein solution is now ready for chromatographic separation to obtain ultrapure hGH. Check for the presence of r-hGH in the refolding buffer by SDS-PAGE.

3.3. Chromatographic Purification

3.3.1. Purification by Ion-Exchange Chromatography

1. Pack 25 mL Q-Sepharose into the glass column (~5-cm bed height). Equilibrate the column at flow rate of 2 mL/min with equilibration buffer. Use the AKTA purifier for ion-exchange chromatography.
2. Filter the refolded protein through a 0.45-µm filter, and load onto the pre-equilibrated Q-Sepharose ion-exchange column at a flow rate of 2 mL/min. Wash the column using three-column volumes of equilibration buffer containing 10 m*M* NaCl to remove nonspecific contaminants.
3. Elute the bound r-hGH using a linear continuous gradient from 10 to 500 m*M* NaCl in equilibration buffer.
4. Homogeneous r-hGH in the form of monomer elute at concentrations of between 150 and 250 m*M* NaCl, whereas protein aggregates elute at higher ionic strength (*see* **Note 11**).
5. The samples containing homogeneous r-hGH bands on SDS-PAGE (**Fig. 3**) should be pooled (approx 20 mL) and dialyzed against a lower urea gradient in each dialysis change, changing after every 8 h as described in the following buffers.
   a. First change: 250 mL 50 m*M* Tris-HCl, 1 m*M* EDTA, 1 *M* urea, 5% (w/v) sucrose, and 1 m*M* PMSF, pH 8.5.
   b. Second change: 250 mL 50 m*M* Tris-HCl, 0.5 m*M* EDTA, 0.5 *M* urea, and 2.5% (w/v) sucrose, pH 8.5.

c. Third change: 250 mL 10 m*M* Tris-HCl, 0.5 m*M* EDTA, 0.25 *M* urea, and 1% (w/v) sucrose, pH 8.5.
d. Fourth change: 250 mL 10 m*M* Tris-HCl, and 1% (w/v) sucrose, pH 8.5.

6. When the dialysis step is complete, lyophilize the r-hGH and use for the subsequent gel filtration step (*see* **Note 12**).

3.3.2. Purification by Gel Filtration

1. Pack the gel filtration column with Sephacryl S-200 resin up to 96 cm. Use the liquid chromatography system for gel filtration.
2. Dissolve the lyophilized hGH (approx 14 mg) in 3–4 mL 10 m*M* Tris-HCl buffer containing 1% (w/v) sucrose. Filter the solution through a 0.45-µm filter to remove any aggregates (*see* **Note 13**).
3. Load the protein solution in the form of a layer on top of the gel filtration matrix.
4. Run the column at a flow rate of 20 mL/h using a peristaltic pump, and collect fractions.
5. Check each fraction by SDS-PAGE for the presence of hGH, which will elute after approximately half a column volume of buffer.
6. Pool all the protein fractions containing pure hGH, and dialyze against 10 m*M* Tris-HCl buffer containing 1% sucrose. Check by SDS-PAGE for purity.
7. Lyophilize the protein and store for physicochemical and biological assay. Determine the protein concentration using the Micro-BCA assay procedure (*see* **Notes 14** and **15**).

4. Notes

1. The postinduction time required after IPTG addition for maximum gene expression is different for varying proteins and depends on the host vector relationship. Most often, optimal induction time is 3–6-hours postinduction for inclusion body accumulation. Do not grow *E. coli* cells overnight after induction, as cell lysis may expose the inclusion bodies to the culture medium and create problems during purification. Use freshly grown, induced cells for the isolation of inclusion bodies and subsequent protein refolding.
2. Remove the dense inclusion body layer very carefully without disturbing the other layers. If the homogeneity of the inclusion bodies is not sufficient, repeat the ultracentrifugation sucrose gradient again using the pellet of the first ultracentrifugation step. Depending on the density of the inclusion bodies, they will be deposited at different places in the sucrose gradient after ultracentrifugation.
3. Do not freeze the purified inclusion bodies to be used at a later date for solubilization. It is always advisable to isolate, purify, and refold the protein into a soluble form without freezing the protein. For quantification of the amount of protein in inclusion bodies, completely solubilize them in detergent solution (1% SDS in the case of r-hGH), and undertake a protein estimation using the detergent-compatible protein assay kit as described. Add detergent to the protein standard solution to minimize error. The presence of EDTA interferes with the Micro-BCA protein assay.
4. There is some loss of r-hGH in the supernatant during deoxycholate treatment. However, this step helps in eliminating contaminating membrane proteins. Depending on the nature of the therapeutic protein, deoxycholate concentrations may need to be optimized. Some proteins will be solubilized in 1% deoxycholate solution, whereas for others, this will not be the case. Additional use of 0.5–1 *M* NaCl and/or 2 *M* urea sometimes helps in solubilization of contaminating cellular proteins and may help improve the purity of inclusion bodies.

5. Use gloves while performing SDS-PAGE (acrylamide is a neurotoxin). Do not pipet the pellet at the bottom of the microfuge tube during sample loading for SDS-PAGE. Rinse the syringe a few times with distilled water after loading each individual well. If *E. coli* cells or the inclusion body pellet does not dissolve in sample dye, use 10–20 μL 10% SDS solution to dissolve them, then add SDS sample buffer for processing before loading onto the SDS-PAGE gel.
6. To determine the best buffer for solubilization of inclusion bodies of a particular protein, it is necessary to know the dominant forces that cause protein aggregation that lead to inclusion body formation. This can be determined by solubilization of aliquots of pure inclusion bodies in different buffers and monitoring the percent solubility either by protein assay or reduction in solution turbidity ***(16)***. Alternatively, a sparse matrix approach can be used to design the solubilization protocol for a particular protein ***(26)***. If disulfide bonds have an important role in the protein structure and stability, use β-mercaptoethanol in the solubilization buffer. If the protein of interest degrades at pH 12.5, adjust the pH of the buffer to pH 12.
7. Never use frozen inclusion body pellets for solubilization or keep the leftover pellet for solubilization at a later date. It is advisable to undertake the inclusion body purification, solubilization, and refolding in one attempt without storing the protein between any step.
8. Do not keep the solubilized protein at a high pH for any longer than absolutely necessary. Thiolate ion formation may result, leading to protein amidation and ultimately resulting in poor-quality bioactive protein. Use freshly prepared buffers, particularly adding the urea to the buffer just before the experiments to reduce cyanate ion formation. Carry out refolding at a low temperature to reduce the extent of protein aggregation during refolding.
9. The use of glycerol, sucrose, and 2 *M* urea helps to prevent protein aggregation during refolding and, more importantly, during gel filtration and lyophilization of the refolded protein. It is advisable to add these excipients to improve the protein stability during the different stages of processing.
10. Check for turbidity during the refolding process; otherwise, carry out the pulsatile-refolding process in large volumes, or dilute the solubilized protein to a concentration of 1 mg/mL before refolding. This helps to lower the aggregation of proteins. A different refolding process may be used instead of pulse dilution if this approach does not give a good recovery of soluble protein ***(24)***.
11. The recombinant protein aggregates will usually elute at a higher ionic strength than the monomer during ion-exchange chromatography. However, sometimes the aggregates will coelute with the monomer. If significant amounts of protein coelute with the monomer, collect the mixture, dialyze, and run the gel filtration step as described to recover the monomeric protein.
12. Carry out dialysis with decreasing concentrations of urea at each step. The risk of protein aggregation is high during the removal of urea. Depending on the nature of a protein, the dialysis time and stepwise decreases of urea can be optimized to reduce the extent of any protein aggregation.
13. Refolded protein should be soluble in aqueous buffer. If the lyophilized r-hGH does not dissolve in aqueous buffer, this indicates that the protein is not refolded correctly. Try different excipients or concentrations of sucrose during lyophilization to achieve a better recovery. Filter the sample, and only use the soluble protein for further purification by gel filtration.

14. Calculate the total amount of r-hGH recovered at the end of the gel filtration process. The recovery should be around 40–50% (considering that the starting amount of r-hGH was 50 mg). The total recovery after gel filtration should be approx 20 mg of pure hGH.
15. If the recovery of protein is very low using the gel filtration process, it may be necessary to use different methods of refolding ***(25)*** as described in previous chapters of this book. Refolding by column chromatography ***(27)***, reverse micelles ***(28)***, or microfiltration ***(29)*** can be used to improve the recovery of protein.

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