7. High throughput purification of r-hGH using radial column chromatography

7.1 Introduction

7.2 Materials and methods

7.2.1 Expression of r-hGH using fed-batch fermentation
7.2.2 Solubilization and refolding of r-hGH from inclusion bodies
7.2.3 Purification of r-hGH
7.2.3a Radial column chromatography
7.2.3b Axial column chromatography
7.2.3c Gel filtration chromatography
7.2.4 Mass spectroscopy and circular dichroism
7.2.5 Estimation of free thiols using Ellman's reagent (DNTB)
7.2.6 Nb2 bioassay

7.3 Results and discussion

7.3.1 Expression of r-hGH in fed-batch fermentation
7.3.2 Isolation, solubilization and refolding of r-hGH from inclusion bodies of r-hGH
7.3.3 Purification of r-hGH
7.3.3a Radial column anion-exchange chromatography
7.3.3b Axial column anion-exchange chromatography
7.3.3c S-100 Gel filtration chromatography
7.3.4 Yield calculation

7.4 Characterization of purified r-hGH

7.4.1 Circular dichroism and mass spectroscopy
7.4.2 Free thiols group estimation
7.4.3 NB-2 bioassay

7.5 Conclusions

7.6 References
7. High throughput purification of r-hGH using radial column chromatography

7.1 Introduction

In general, inclusion bodies are solubilized by using high concentration of denaturants such as urea or guanidine hydrochloride, along with a reducing agent such as β-mercaptoethanol (1). Solubilized proteins are then refolded by slow removal of the denaturant in the presence of oxidizing agent (2). The interactions among the denatured protein molecules during refolding, resulting in their aggregation, are considered to be the main reason for the poor recovery of bioactive proteins from inclusion bodies (2). Many times, the overall yield of bioactive proteins from inclusion bodies is around 15-25% of the total protein and accounts for the major cost in production of recombinant proteins from E. coli cells. Solubilization of inclusion body proteins, sub-optimal refolding and final purification are three major steps in overall recovery of bioactive protein from inclusion bodies. Improvement in above three steps and a co-ordination among them is necessary for maximal recovery of bioactive proteins from inclusion bodies. Solubilization with β-mercaptoethanol, pulsatile refolding and use of radial flow column chromatography for processing huge volume of diluted refolded protein were tried for subsequent recovery of bioactive r-hGH from inclusion bodies. The purification process for r-hGH from small shaker flask culture was scaled up. Fermentation process was used to produce high amount of r-hGH as inclusion bodies. Inclusion bodies were further processed to produce bioactive protein. The objective was to recover maximum bioactive protein using minimum steps and in minimal time of operation. Another objective was to purify a minimum amount of 100 mg of pure r-hGH from inclusion bodies in a laboratory scale set up.

7.2 Materials and methods

7.2.1 Expression of r-hGH using fed-batch fermentation

A c-DNA fragment coding for was excised from pRM and recloned in pQE60 expression vector (Qiagen) under control of phage T5 promoter (3). Construct was transformed into E. coli (M15) cells and grown in LB media in the presence of kanamycin and ampicillin for expression of r-hGH. Culture was grown in a 10 litre fermenter (Chemap AG, Switzerland) using complex media
containing glucose, salts, yeast extract and minerals (4). Batch fermentation was started with an initial glucose concentration of 10% (w/v). pH was maintained at 7.0 by addition of 5 N NaOH. Air flow and stirrer speed was controlled to keep the dissolved oxygen tension more than 20%. Initial glucose and yeast extract concentration was 10 g/L. Fermentation was carried out at 37 °C with vigorous aeration and pH was maintained at 7. Fed-batch fermentation was carried out to achieve high cell density (4). The culture at OD 600 of 26 was induced with 1 mM IPTG, cultivated for another 3 hrs and harvested. Samples were collected at regular intervals during the fermentation to check cell growth and r-hGH expression. Cell density was determined by measuring the OD of culture at 600 nm with an Amersham ultrospec UV-visible spectrophotometer. Higher OD (<1) samples were diluted appropriately with fresh media to get OD in the range of 0.2-0.6. Dry cell weight was determined by centrifuging 1 ml fermentation broth at different time points at 4000 g for 20 minutes and drying the washed cells to constant weight at 110 °C. Culture broth (1 ml) at different time points was used for inclusion body preparation by detergent washing method. Final pellets of inclusion bodies were solubilized in 1 ml 2% SDS, centrifuged and supernatant was used for protein estimation using Micro BCA protein estimation kit (Pierce).

7.2.2 Solubilization and refolding of r-hGH from inclusion bodies

Fermentation pellet was used for preparation of inclusion bodies. Inclusion bodies were solubilized in 6 M β-mercaptoethanol along with 2 M urea and subsequently refolded by pulsatile dilution method. Wet cell pellet (25 gm) was taken for isolation of inclusion bodies. Inclusion bodies were isolated and purified by detergent washing method. Final pellet of inclusion bodies were resuspended in 10 ml MQ water. Inclusion body suspension (20 μl) was used for protein estimation using micro BCA protein estimation kit (Pierce). Suspension of inclusion bodies was solubilized in solubilization buffer (50 mM Tris, 2 M Urea, 6 M β-mercaptoethanol, 10 % sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5) to a final volume of 75 ml. Protein supernatant was subjected to pulsatile refolding. Solubilized supernatant (75 ml) was diluted in refolding buffer (50 mM Tris, 2 M Urea, 10 % Sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5) to a final volume of 750 ml at a rate of 0.1 ml/minute. Refolded sample was centrifuged at 12000 rpm, 4 °C, for 30 min. Supernatant was used for further purification by chromatography.
7.2.3 Purification of r-hGH

7.2.3a Radial column chromatography

Refolded r-hGH was purified using anion exchange chromatography in a radial column (Superflow-50, Sepragen, USA) (5). DEAE-sepharose fast flow media (50 ml) was packed in the column. Column was first washed with 250 ml MQ water at a flow rate of 30 ml/min. Column was equilibrated with 250 ml of equilibration buffer (50 mM Tris, 2 M Urea, 10 % Sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5) at a flow rate of 30 ml/min. Refolded r-hGH (750 ml) was loaded onto the column at a flow rate of 30 ml/min. Column was washed with 250 ml of equilibration buffer at a flow rate of 30 ml/min. Bound protein was eluted using a NaCl gradient (0 mM to 500 mM) at a flow rate of 20 ml/min. Column was subsequently deproteinated and regenerated with 200 ml of 2 M NaCl and 200 ml of 0.5 M NaOH respectively. Homogeneity of eluted protein was checked by SDS-PAGE and yield was estimated by micro BCA (Pierce) assay. All purification experiments were carried out using AKTA explorer (Amersham Pharmacia, USA) which monitors absorbance and conductivity simultaneously.

7.2.3b Axial column chromatography

Refolded r-hGH was also purified using anion exchange chromatography in an axial column (XK-26/40). DEAE-sepharose fast flow media (50 ml) was packed in XK-26/40 with a flow rate of 4 ml/min. Column was first washed with 250 ml MQ water at a flow rate of 2 ml/min. Column was equilibrated with 250 ml of equilibration buffer (50 mM Tris, 2 M Urea, 10 % Sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5) at a flow rate of 2 ml/min. Refolded r-hGH (750 ml) was loaded onto the column at a flow rate of 2 ml/min. Column was washed with 250 ml of equilibration buffer at a flow rate of 2 ml/min. Bound protein was eluted using a NaCl gradient (0 to 500 mM) at a flow rate of 2 ml/min. Column was subsequently deproteinated and regenerated with 200 ml of 2 M NaCl and 200 ml of 0.5 M NaOH respectively. Homogeneity of eluted protein was analysed by SDS-PAGE and yield was calculated by estimating protein using micro BCA (Pierce) assay.
7.2.3c Gel filtration chromatography

Purified r-hGH after anion exchange chromatography was further purified by gel filtration chromatography. Pooled elutes from ion exchange chromatography were dialyzed against 10 mM Tris, 0.5 % sucrose. Dialyzed sample was lyophilized and again solubilized in MQ water (10 ml final volume). Solubilized sample was centrifuged at 12000 rpm, 4 °C for 10 minutes. Supernatant was subjected to gel filtration chromatography. 450 ml Sephacryl-100 was packed in Pharmacia K 2.6/100 column to a height of 90 cm with a flow rate of 2 ml/min. 50 mM Tris, pH 8.5, 200 mM NaCl was used as elution buffer. Eluted protein was dialyzed against 10 mM phosphate buffer, pH 7.5. Homogeneity and concentration of dialyzed elute was analysed by SDS-PAGE and micro BCA assay (Pierce). Dialyzed sample was lyophilized and used for further characterization and bioactivity assay.

7.2.4 Mass spectroscopy and circular dichroism

Molecular weight of purified r-hGH (10 µg) was characterized by mass spectroscopy. Purified desalted r-hGH was solubilized in 1 ml MQ water with 0.1 % formic acid and 20 µl of this solution was used for mass spectroscopy (LCMS-Waters). r-hGH was solubilized and secondary structure of purified r-hGH was characterized by circular dichroism spectroscopy. Circular dichroism spectrum of purified r-hGH was obtained at 25 °C in the wavelength range of 190-250 nm using a JASCO-Spectropolarimeter (6). Concentration of r-hGH was 200 µg/ml in 20 mM Tris buffer for CD spectra analysis. The sample was scanned 10 times for data accumulation and average spectrum was plotted. Similarly, a near UV circular dichroism spectrum was obtained at 25 °C in the wavelength range of 250-300 nm using a JASCO-Spectropolarimeter.

7.2.5 Estimation of free thiols using Ellman's reagent (DNTB)

Number of free thiols in enriched inclusion bodies of r-hGH and purified r-hGH were determined using Ellman's reagent (5,5'-dithiobis(2-nitro-5-thiobenzoic acid), DNTB) (7). The procedure is based on the reaction of thiol (-SH) group with DNTB to produce mixed disulfide and anion of TNB which is quantified by the absorbance at 412 nm. Purified inclusion bodies (1 mg/ml) of r-hGH and refolded r-hGH protein in 100 mM phosphate buffer of pH 8.0 containing 6 M GdmCl was used for free thiols estimation. 10 mM DNTB in 100 mM phosphate buffer was used as stock. 100 µl of
phosphate buffer with GdmCl was added to cuvette. To this, 100 μl of DNTB stock solution was added. Absorbance was measured at 412 nm. Thereafter, 100 μl of sample (r-hGH inclusion bodies or pure r-hGH) was added and Afinal at 412 was measured. Concentration of free thiols was calculated by molar absorbance of TNB anion by using the following equation.

\[ \Delta A_{412} = E_{412} \text{TNB}^2 [\text{RSH}] \]

Where \[ \Delta A_{412} = A_{\text{final}} - (3.1/3.2) (A_{\text{DNTB}} - A_{\text{buffer}}) \]

and \[ E_{412} \text{TNB}^2 = 1.37 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1} \]

### 7.2.6 Nb2 Bioassay

The biological activity of purified r-hGH was determined by its growth promoting action on rat Nb2 lymphoma cell line (3). Nb2 lymphoma cell line was maintained in complete RPMI-1640 media containing 10 % horse serum (HS) and 10 % foetal calf serum (FCS). Cells were grown in CO2 incubator at 37 °C. Nb2 cells have prolactin receptor. Nb2 cells start proliferation in presence of lactogenic hormone like prolactin. 0.5X10^5 cells per well were taken in 96 well plate to study the growth promoting action of purified r-hGH and commercially obtained human growth hormone from Boehringer Mannheim (Standard). Cells growth was arrested in G0-G1 phase by reducing FCS to a final concentration of 1 % and 10 % horse serum. Different concentration of prolactin and r-hGH was used for cell proliferation. Growth promoting activity of r-hGH was compared with that of RPMI only, RPMI + 10 ng/ml BSA, RPMI + 10 ng/ml standard r-hGH and RPMI + purified r-hGH. Cell number was counted at different time points (0 hr-hGH120 hrs) using haemocytometer. All experiments were carried out in triplicates.
7.3 Results and discussion

7.3.1 Expression of r-hGH in fed-batch fermentation

A high cell density fed-batch fermentation profile of *E. coli* cells expressing r-hGH is shown in Fig. 7.1. Fed–batch fermentation resulted in increased cell mass in the fermenter per unit volume. Throughout the fermentation process, the residual glucose concentration was <1 g/L. Apparently at such low concentration of glucose, due to less accumulation of acetic acid, no inhibitory effect on growth of *E. coli* was observed. At cell OD of 26, the culture was induced with 1 mM IPTG. Till IPTG induction, aggregated protein concentration in cell lysate was low, around 125 mg/L. However after IPTG induction, the protein concentration in insoluble aggregates increased rapidly around 670 mg/L in 3 hours. The inclusion body preparation used for estimation was around 75% pure, thus around 500 mg/L of r-hGH was expressed as inclusion bodies in fed-batch fermentation. The expression of r-hGH after IPTG induction was associated with reduction in cell growth. Maximum cell OD achieved during 10 hours of fed-batch fermentation was 35. Recombinant r-hGH yield in shaker flask culture was around 60 mg/L at a cell OD of 2. Fed-batch fermentation thus improved the volumetric concentration of r-hGH by 9 times. At the end of fed-batch fermentation, total biomass achieved from 10 L batch was 670 gm wet weight containing ~7 gm of crude inclusion bodies protein as r-hGH. The yield achieved was 1 gm of crude r-hGH per 100 gm of wet cell weight. After 10 hrs of fed-batch fermentation, 140 g dry cell weight and 6.7 g crude r-hGH as inclusion bodies was produced (Table 7.1). The yield of expressed r-hGH was around ~8% of total cellular protein. High level expression of the foreign protein had an effect on cell growth as shown in Fig. 7.1 and Table 7.1. This indicated that after induction, the growth declined due to metabolic burden associated with over expression of r-hGH. High cell density fed-batch fermentation improved the volumetric yield of r-hGH around 9 times in comparison to shake flask culture.
Table 7.1 Growth of *E. coli* and expression of r-hGH during fed-batch fermentation.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Time in hours</th>
<th>Absorbance at 600 nm</th>
<th>Protein in IBs in mg/L of broth</th>
<th>Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.19</td>
<td></td>
<td>Seeding</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>4.50</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>7.80</td>
<td>50</td>
<td>Feeding</td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>12.84</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>19.50</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.0</td>
<td>26.00</td>
<td>125</td>
<td>Induction</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>31.15</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9.0</td>
<td>34.35</td>
<td>655</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.0</td>
<td>34.45</td>
<td>670</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7.1 Growth kinetics of *E. coli* expressing r-hGH as inclusion bodies during fed-batch fermentation.
7.3.2 Isolation, estimation, solubilization and refolding of r-hGH from inclusion bodies of r-hGH

*E. coli* cells (25 g wet weight) containing around 220 mg of r-hGH as inclusion bodies were used for high throughput purification of r-hGH. Cells were lysed by sonication and inclusion bodies were prepared using deoxycholate (DOC) washing method as described in chapter 3. The inclusion bodies were solubilized in 75 ml solubilization buffer (50 mM Tris, 2 M Urea, 6 M β-mercaptoethanol, 10 % sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5). Amount of protein solubilized was around 175 mg in 75 ml of solubilization buffer. The solubilized protein was refolded using pulsatile dilution in refolding buffer (50 mM Tris, 2 M Urea, 10 % Sucrose, 0.5 mM EDTA, 1 mM PMSF, pH 8.5) to a final volume of 750 ml. The refolded protein was around 160 mg in 750 ml of refolding buffer. This refolded r-hGH was purified using radial as well as axial flow anion exchange chromatography.

7.3.3 Purification of r-hGH

7.3.3a Radial column anion-exchange chromatography

In radial column (Fig. 7.2a and 7.2b), liquid flows radially inside, as compared with axial column where direction of liquid flow is along the axis of the column as depicted in Fig. 7.2c (5). Working principle of radial column is described in Fig. 7.2d. It is clear from the figure that keeping the bed volume constant, liquid has to travel lesser distance through matrix in radial column as compared with axial column. This is simply because of more surface area in case of radial column as compared to axial column. In axial column, due to higher back pressure, buffers are passed at very low flow rates (2-4 ml/minute). In radial column, back pressure exerted by matrix is quite low. Therefore buffer flow rate can be as high as 50 ml/minute. Radial column, thus, can be used to process huge volumes of diluted refolded protein in less time period. It is easy to scale up and results in high resolution separation of proteins. Refolded r-hGH (750 ml) was loaded onto DEAE-sepharose packed radial column. The protein was eluted using 0 to 500 mM NaCl gradient.
Fig 7.2a Radial flow column (Sepragen, USA).

Fig 7.2b Inner section of radial flow column.

Fig 7.2c Liquid flow in axial vs radial column.

Fig 7.2d Mechanism of radial flow column.
Two major peaks were observed in chromatogram as shown in Fig. 7.3. First peak corresponds to β-mercaptoethanol in flow through whereas second peak corresponds to elutes of r-hGH. r-hGH was eluted in between conductivity range of 14 to 16 mS/cm. There were two minor peaks, one at the beginning of elution and another at the end of the elution. These peaks might be because of impurities eluted from the matrix.

Fig. 7.3 Chromatogram of radial column anion exchange chromatography for purification of r-hGH.

Fig. 7.4 SDS-PAGE analysis of r-hGH purified by radial column chromatography. Lane 1 to 5 are r-hGH inclusion bodies, solubilized, refolded, flow through, wash and lane 6-9 are elutes respectively. Lane M is LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa).
Elutes of radial anion exchange chromatography were analysed by SDS-PAGE and is shown in Fig. 7.4. Purification level of r-hGH after radial column anion exchange chromatography was more than 90%. No band corresponding to dimer of r-hGH was observed in SDS-PAGE. Use of radial column decreased processing time from 850 minutes to 62.5 minutes as described in Table 7.2. Thus, use of radial column helped in high throughput purification of recombinant human growth hormone. Elutes were dialyzed, lyophilized and further purified by gel filtration chromatography.

**7.3.3b Axial column anion-exchange chromatography**

Same amount of protein was loaded on axial column packed with DEAE-sepharose. Flow rates of buffers were very low (2 ml/min) as compared with flow rates in radial column. Elutes of r-hGH from axial column were analysed by SDS-PAGE and is shown in Fig. 7.5.

![SDS-PAGE analysis of r-hGH purified by axial column chromatography](image)

**Fig. 7.5** SDS-PAGE analysis of r-hGH purified by axial column chromatography. Lane 1 to 5 are r-hGH inclusion bodies, solubilized, refolded, flow through, wash respectively and lane 6-8 are elutes respectively. Lane M is LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa).

Purification level of r-hGH after axial anion exchange chromatography was more than 90%. No band corresponding to dimer of r-hGH was observed in SDS-PAGE. Elutes were pooled together, dialyzed, lyophilized and further purified by gel filtration chromatography.

**7.3.3c S-100 Gel filtration chromatography**

Lyophilized r-hGH after DEAE-anion exchange chromatography was further purified by S-100 gel filtration chromatography (3). r-hGH eluted as a single peak, precluding the presence of
structural isoforms of folded r-hGH as shown in Fig. 7.6. No peak corresponding to dimer of r-hGH was observed. Elutes from gel filtration chromatography were analysed by SDS-PAGE and is shown in Fig. 7.7. Purification level of r-hGH after S-100 gel filtration was more than 98%. No bands corresponding to impurities were observed in SDS-PAGE even after loading higher amount of r-hGH. Elutes of gel filtration were pooled, dialyzed and lyophilized. Total amount of protein in lyophilized powder was estimated by micro BCA kit (Pierce). Purified r-hGH was further used for characterization and biological activity assays.

Fig. 7.7 SDS-PAGE analysis of r-hGH purified by S-100 gel filtration chromatography. Lane 1 is pooled r-hGH purified by radial column chromatography. Lane 2 to 4 are elutes from S-100 gel filtration chromatography. Lane M is LMW marker (97, 66, 45, 30, 20.1, 14.4 kDa).
7.3.4 Yield calculation

r-hGH was solubilized using 6 M β-mercaptoethanol as a solubilizing agent and purified to its homogeneity using radial column chromatography. Radial column was equilibrated, loaded and washed at a flow rate of 30 ml/min. Bound r-hGH was eluted with NaCl gradient at a flow rate of 20 ml/min, whereas in axial column buffer flow rate was 2 ml/min throughout the process. This required more than 6 hours for loading the refolded protein into axial columns. Similarly, washing and elution of protein from axial column took more time in comparison to radial column. To purify 100 mg of r-hGH it took around 62.5 min in radial column whereas it took 850 min in axial column. Thus, radial column decreased the process time from 850 min to 62.5 min as described in Table 7.2. This helped in high throughput purification of r-hGH. However the actual recovery of bioactive protein from inclusion bodies was around 45 % (Table 7.3). Use of radial column resulted in high throughput purification of r-hGH from inclusion bodies. From 220 mg of r-hGH inclusion bodies around 100 mg of ultra pure refolded r-hGH could be recovered.

<table>
<thead>
<tr>
<th>Process</th>
<th>Volume passed</th>
<th>Axial column (Time in minutes)</th>
<th>Radial column (Time in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>250 ml</td>
<td>150</td>
<td>9</td>
</tr>
<tr>
<td>Loading</td>
<td>750 ml</td>
<td>375</td>
<td>25</td>
</tr>
<tr>
<td>Washing</td>
<td>250 ml</td>
<td>125</td>
<td>9</td>
</tr>
<tr>
<td>Elution</td>
<td>250 ml</td>
<td>125</td>
<td>12.5</td>
</tr>
<tr>
<td>Deproteination</td>
<td>100 ml</td>
<td>50</td>
<td>3.5</td>
</tr>
<tr>
<td>Regeneration</td>
<td>100 ml</td>
<td>50</td>
<td>3.5</td>
</tr>
<tr>
<td>r-hGH recovery</td>
<td>109 mg</td>
<td></td>
<td>120 mg</td>
</tr>
<tr>
<td>Total time</td>
<td>850 minutes</td>
<td></td>
<td>62.5 minutes</td>
</tr>
</tbody>
</table>

Table 7.2 Comparative process time analysis of axial versus radial flow column chromatography for purification of r-hGH.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Total protein in mg</th>
<th>Step yield</th>
<th>Overall yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude inclusion bodies</td>
<td>550</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Pure inclusion bodies</td>
<td>220</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Solubilization</td>
<td>175</td>
<td>79.5 %</td>
<td>79.5 %</td>
</tr>
<tr>
<td>Refolding</td>
<td>161</td>
<td>92 %</td>
<td>73.2 %</td>
</tr>
<tr>
<td>Anion exchange chromatography</td>
<td>120</td>
<td>68.5 %</td>
<td>54.5 %</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>100</td>
<td>83.3 %</td>
<td>45.4 %</td>
</tr>
</tbody>
</table>

Table 7.3 Overall recovery of r-hGH from inclusion bodies of E. coli.
7.4 Characterization of purified r-hGH

7.4.1 Circular dichroism and mass spectroscopy

Far and near UV circular dichroism spectrum of purified r-hGH was similar to commercially available r-hGH from Boehringer Mannheim as shown in Fig 7.8 and 7.9 (6). Purified r-hGH had characteristics of CD spectrum with peaks at 210 and 222 nm indicating presence of α-helical structure. Molecular weight of purified r-hGH was confirmed with mass spectroscopy.

Fig. 7.8 Near UV circular dichroism spectrum of purified r-hGH.

Fig. 7.9 Far UV circular dichroism spectrum of purified r-hGH.

Fig. 7.10 Mass spectrogram of purified r-hGH.
Molecular weight was found to be 22.2 kDa as shown in Fig. 7.10. r-hGH had 191 amino acids along with one N terminal methionine during expression in E. coli. The total molecular mass became 22.2 and was confirmed by mass spectroscopy (Fig. 7.10)

### 7.4.2 Free thiols group estimation

Number of free thiols present in purified r-hGH were compared with inclusion bodies of r-hGH. Due to the presence of partially folded intermediates in inclusion bodies, more number of free thiols were expected in inclusion bodies as compared with pure r-hGH. Same was corroborated by experimental results. Numbers of free thiols were almost 10 times more in inclusion bodies of r-hGH as compared with pure r-hGH as described in Table 7.4. This indicated the oxidation of sulphydryl group (-SH) to disulfide bond (S-S) during refolding.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( A_{412} ) Buffer/Auto zero</th>
<th>( A_{412} ) DNTB</th>
<th>( A_{412} ) DNTB +Protein</th>
<th>[RSH]</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBs of r-hGH</td>
<td>0</td>
<td>0.096</td>
<td>0.102</td>
<td>6.56*10^-7</td>
</tr>
<tr>
<td>Pure r-hGH</td>
<td>0</td>
<td>0.097</td>
<td>0.096</td>
<td>8.02*10^-8</td>
</tr>
</tbody>
</table>

Table 7.4 Comparative analysis of disulfide bonds in inclusion bodies and pure r-hGH.

### 7.4.3 Nb-2 bioassay

Growth kinetics of the prolactin-dependent Nb2 lymphoma cell line was monitored to evaluate the bioactivity of purified r-hGH (3). The addition of prolactin, commercial r-hGH and r-hGH promoted growth of Nb2 cells arrested at G0-G1 phases by serum deprivation. Different concentrations (1-25 ng/ml) of r-hGH resulted in increased growth of Nb2 cells (Fig. 7.11). Dose dependent growth stimulations by r-hGH demonstrated the homogeneity of purified refolded protein. However, prolactin growth promoting action was highest at 10 ng/ml. High concentration of prolactin resulted in growth inhibition (Fig. 7.12). Growth of Nb2 cells in the presence of 10 ng/ml r-hGH was found to be comparable to that observed for the commercial and prolactin as shown in Fig. 7.13.
Fig. 7.11 Growth kinetics of Nb2 cells in different concentrations of r-hGH.

Fig. 7.12 Growth kinetics of Nb2 cells in different concentrations of prolactin (PRL).
Fig. 7.13 Growth promoting activity of r-hGH in comparison to c-hGH and prolactin (PRL).

No growth stimulation was observed in presence of BSA which was a negative control. Dose dependent promotion of Nb2 cells growth indicated the bioactive conformation of refolded r-hGH solubilized by β-mercaptoethanol. This indicated that the inclusion body proteins can be solubilized in 6 M β-mercaptoethanol and refolded into its bioactive form.

7.5 Conclusions

1. High cell density fed batch fermentation resulted in improved volumetric yield of crude inclusion bodies. Recombinant hGH production was almost 9 times more than shake flask culture.

2. Solubilization in 6 M β-mercaptoethanol and subsequent refolding by pulsatile dilution could be scaled up to process few hundreds mg of r-hGH inclusion bodies.
3. Anion exchange chromatography helped in removal of β-mercaptoethanol from the refolding buffer as flow through and helped in purification of r-hGH in single step.

4. Use of radial column decreased the processing time substantially and helped in high throughput recovery of purified r-hGH. Process time for purification of 100 mg of r-hGH was decreased from 850 minutes in axial column chromatography to 62.5 minutes in radial column chromatography.

5. Primary and secondary structure of purified r-hGH was similar to commercially available hGH. Far and near UV CD spectra of purified r-hGH gave similar characteristic peaks. Molecular weight was confirmed by mass spectroscopy.

6. Large amount of bioactive r-hGH was purified using radial column chromatography. r-hGH (100 mg) was purified in a single batch from 25 g wet biomass pellet.

7. Bioactivity of purified r-hGH was comparable to commercially available as evaluated by Nb2 rat lymphoma cell line growth. Inclusion body enrichment, solubilization in β-mercaptoethanol, pulsatile refolding and use of radial flow column chromatography helped in higher recovery of bioactive r-hGH (45.4 %).

7.6 References


