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High throughput purification of recombinant human growth hormone using radial flow chromatography

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ABSTRACT

Recombinant human growth hormone (r-hGH) was expressed in *Escherichia coli* as inclusion bodies. Using fed-batch fermentation process, around 670 mg/L of r-hGH was produced at a cell OD600 of 35. Cell lysis followed by detergent washing resulted in semi-purified inclusion bodies with more than 80% purity. Purified inclusion bodies were homogenous in preparation having an average size of 0.6 μm. Inclusion bodies were solubilized at pH 12 in presence of 2 M urea and refolded by pulsatile dilution. Refolded protein was purified with DEAE-anion exchange chromatography using both radial and axial flow column (50 ml bed volume each). Higher buffer flow rate (30 ml/min) in radial flow column helped in reducing the batch processing time for purification of refolded r-hGH. Radial column based purification resulted in high throughput recovery of diluted refolded r-hGH in comparison to axial column. More than 40% of inclusion body protein could be refolded into bioactive form using the above method in a single batch. Purified r-hGH was analyzed by mass spectroscopy and found to be bioactive by Nb2 cell line proliferation assay. Inclusion body enrichment, mild solubilization, pulsatile refolding and radial flow chromatography worked co-operatively to improve the overall recovery of bioactive protein from inclusion bodies.

Introduction

Since the advent of recombinant DNA technology, *Escherichia coli* has been widely used for recombinant protein expression at laboratory scale as well as for industrial production [1,2]. However, most of the time, over-expression of a recombinant protein in *E. coli* leads to accumulation of partially folded protein intermediates into intracellular aggregates called as inclusion bodies [3,4]. Inclusion body formation has been a major hurdle in production of recombinant proteins in soluble and bioactive form using *E. coli* as host [5]. In general, inclusion body proteins are solubilized in high concentration of denaturants such as urea or guanidine hydrochloride, along with or without a reducing agent, e.g., β-mercaptoethanol [6–8]. Loss of secondary structure during solubilization at high concentration of denaturant (8 M urea or 6 M guanidine HCl) and the interaction among the denatured protein molecules leading to their aggregation are considered to be main reasons for low recovery of bioactive proteins from the inclusion bodies [9]. In order to circumvent this, mild denaturation has been widely used (pH 12.5 along with 2 M urea) for solubilization and refolding of inclusion body proteins [10,11]. Mild solubilization essentially preserves the existing native-like secondary structure of the inclusion body protein and thereby helps in reducing aggregation during refolding [12]. Buffers having high or low pH has also been reported for solubilization of inclusion bodies of recombinant zona pellucida and LHRH-T multimer protein [13,14].

Once inclusion body proteins are solubilized, the next hurdle is to refold at suitable condition. To avoid aggregation, proteins are generally refolded at very low concentration. This not only results in larger volumes of liquids but also increases the process time for purification of the diluted refolded protein. In fact, refolding at low protein concentration has been the major cost for overall refolding of inclusion bodies protein [15]. To reduce the problem, different novel methods have been explored for the refolding and purification of solubilized proteins [16]. Radial flow column offers advantage of higher efficiency and low back pressure exerted by packed matrix [17]. Operation at higher flow rates help in reducing the process time for protein purification during chromatographic steps and thus helps in achieving high throughput protein recovery. Radial flow column has been used for purification of recombinant protein [18,19], however very little information is available on its suitability for purification inclusion body protein. It is expected that for purification of diluted refolded protein solution; it will offer greater advantages in comparison to axial flow column.

Present work describes a high throughput process, for production of pure bioactive r-hGH[1] (≈100 mg/batch), by coupling high

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[1] *Abbreviations used*: r-hGH, recombinant human growth hormone; c-hGH, commercially available recombinant human growth hormone; DOC, deoxycholate; SDS, sodium dodecylsulfate; DEAE, diethyl aminoethane; RPMI, Roswell Park Memorial Institute; LHRH, luteinizing hormone releasing hormone.

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cell density fermentation, mild denaturation, pulsatile refolding in combination with radial flow column chromatography. Purification of dilute refolded r-hGH using radial flow column was compared to that of axial column. Finally, the bioactivity of the refolded purified protein has been evaluated to substantiate the suitability of radial flow column based chromatography for purification of inclusion body proteins.

Materials and methods

Materials

Axial column, DEAE-Sepharose Fast Flow matrix, Sephacryl S-100 were from Amersham Pharmacia, USA. Micro BCA protein estimation kit was from Pierce, USA. Deoxycholate was from Amresco chemicals, USA. Radial flow column was from Sepragen, USA. RPMI, horse serum albumin, & fetal bovine serum were from Gibco BRL, USA. All other chemicals were of analytical grade.

Expression of r-hGH using fed-batch fermentation

A c-DNA fragment coding for r-hGH was cloned in pQE60 expression vector (Qiagen) under control of phage T5 promoter [10]. Transformed *E. coli* (M15) cells were grown in Luria–Bertani (LB) media in the presence of kanamycin and ampicillin and induced with IPTG for expression of r-hGH. Culture was grown in a 10-L fermentor (Chemap AG, Switzerland) using complex media containing glucose, salts, yeast extract and minerals using high cell density fed-batch process [20]. 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. Fed-batch fermentation was carried out at 37 °C with vigorous aeration and pH was maintained at 7 [20]. Culture was induced at OD600 of 26 with 1 mM IPTG, cultivated for another 3 h and harvested. Samples were collected at regular intervals during the fermentation to evaluate cell growth and r-hGH expression. Cell density was determined by measuring the OD600 of the culture at 600 nm with an Amersham Pharmacia Ultrospec UV–visible spectrophotometer. Higher OD600 (>1) samples were diluted appropriately with fresh media to get OD600 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 min 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 [12]. Final pellets of inclusion bodies were solubilized in 1 ml 2% SDS, and centrifuged to get clear supernatant. Micro BCA protein estimation kit (Pierce, USA) was used for estimation of solubilized r-hGH using bovine serum albumin (BSA) as standard.

Isolation and enrichment of inclusion bodies of r-hGH

Induced *E. coli* cells harvested from the fermentor batch was used for preparation of inclusion bodies. Cell pellet (70 g) was resuspended in 200 ml re-suspension buffer (50 mM Tris, pH 8.5) and was divided into batches of 100 ml each. Each batch was sonicated (Branson 450, USA) for 15 cycles of 20 W for 1 min each with 1 min gap on ice. Lysed bacterial suspension was centrifuged at 12,000 rpm for 30 min at 4 °C ($Evolution_{RC}$, Sorvall USA, SA-300 rotor, Oakridge tubes). Supernatant was decanted and pellet was resuspended in 50 mM Tris buffers containing 2% (w/v) sodium deoxycholate (DOC). Sonication was repeated and pellet was washed twice with 40 ml of re-suspension buffer having 2% (w/v) DOC. Residual DOC was removed by washing inclusion bodies with 40 ml re-suspension buffer and twice with MilliQ (MQ) water at 4 °C. Final pellets of inclusion bodies were resuspended in 10 ml MQ and 20 μl of this suspension was used for protein estimation using micro BCA protein estimation kit (Pierce, USA). Purified inclusion bodies of r-hGH were analyzed by transmission and scanning electron microscopy. Inclusion body suspension (10 μl) was smeared over a carbon grid on aluminum stub individually and dried in desiccators. Stub loaded with inclusion bodies was coated with a thin layer of gold with gold spray gun and observed under Jeol JSM scanning electron microscope (Jeol Tokyo, Japan). Pure inclusion body pellets were also analyzed by Transmission electron microscopy (Philips CM10, Holland). Composition of the purified inclusion bodies was checked by using 12% SDS–PAGE.

Inclusion body solubilization and pulsatile refolding of solubilized r-hGH

Inclusion body suspension (10 ml) was mixed into 65 ml solubilization buffer (50 mM Tris, 2 M urea, 0.5 mM EDTA, 1 mM PMSF at pH 12). After incubation of 1 h at room temperature, the suspension was centrifuged at 16,000 rpm for 30 min at 10 °C (Oakridge tubes, SA-300 rotor, Sorvall, USA). Solubilized supernatant (75 ml, ~5 mg/ml) was diluted in refolding buffer (50 mM Tris, 2 M urea, 10% sucrose, 0.5 mM EDTA, 1 mM PMSF at pH 8.5) to a final volume of 750 ml at a rate of 0.1 ml/min. Refolded sample was incubated at 4 °C for 1 h. Refolded r-hGH was centrifuged at 12,000 rpm, 4 °C, for 30 min. Supernatant was collected and used for purification using ion exchange chromatography.

Purification of refolded r-hGH

Radial column chromatography

Half of refolded r-hGH (375 ml) was purified by anion exchange chromatography in a radial column (Superflow-50, Sepragen, USA) using AKTA-FPLC (Amersham Biosciences, Sweden). 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 (375 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 (0–500 mM, 0–100% in 15 min) at a flow rate of 20 ml/min. Column was subsequently de-proteinated 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 calculated by estimating protein using micro BCA assay.

Axial column chromatography

The other half of refolded r-hGH (375 ml) was purified using anion exchange chromatography in an axial column (XK-26/40) using AKTA-FPLC (Amersham Biosciences, Sweden). 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 (375 ml) was loaded onto the column and was washed with 250 ml of equilibration buffer at a flow rate of 2 ml/min. Bound protein was eluted using a NaCl gradient (0–500 mM, 0–100% in 150 min) at a flow rate of 2 ml/min. Column was subsequently de-proteinated and regenerated with 200 ml of 2 M NaCl and 200 ml of 0.5 M NaOH, respectively. Homogeneity of eluted protein