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Purification of recombinant human growth hormone from CHO cell culture supernatant by Gradiflow preparative electrophoresis technology

Dallia Catzel,[a] Helen Lalevski,[a] Chris P. Marquis,[a] Peter P. Gray,[a] Derek Van Dyk,[b] and Stephen M. Mahler[a,*]

[a] *Bioengineering Centre, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia*
[b] *Australian Proteome Analysis Facility, Macquarie University, North Ryde, Sydney, NSW 2109, Australia*

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Abstract

Purification of recombinant human growth hormone (rhGH) from Chinese hamster ovary (CHO) cell culture supernatant by Gradiflow large-scale electrophoresis is described. Production of rhGH in CHO cells is an alternative to production in *Escherichia coli*, with the advantage that rhGH is secreted into protein-free production media, facilitating a more simple purification and avoiding resolubilization of inclusion bodies and protein refolding. As an alternative to conventional chromatography, rhGH was purified in a one-step procedure using Gradiflow technology. Clarified culture supernatant containing rhGH was passed through a Gradiflow BF200 and separations were performed over 60 min using three different buffers of varying pH. Using a 50 mM Tris/Hepes buffer at pH 7.5 together with a 50 kDa separation membrane, rhGH was purified to approximately 98% purity with a yield of 90%. This study demonstrates the ability of Gradiflow preparative electrophoresis technology to purify rhGH from mammalian cell culture supernatant in a one-step process with high purity and yield. As the Gradiflow is directly scalable, this study also illustrates the potential for the inclusion of the Gradiflow into bioprocesses for the production of clinical grade rhGH and other therapeutic proteins.

Keywords: Recombinant human growth hormone; Chinese hamster ovary cells; Purification; Preparative electrophoresis

Human growth hormone (hGH) is a small, single-chain peptide of 191 residues, with approximately 50% of the residues in α-helical conformation [28]. It is produced and secreted by the anterior pituitary gland and is responsible for many effects on growth, development, immunity, and metabolism [25]. hGH exists as a mixture of peptides with the major physical component having a molecular weight of 22 kDa. The minor hGH components differ from the 22 kDa form in terms of mass or charge. The mass variants include a 20 kDa form, with deletion of 15 amino acid residues (32–46) from the 22 kDa form, and a 45 kDa form arising from aggregation of the 22 kDa form. There is also a 24 kDa variant containing the leader sequence at the N-terminus. Another 24 kDa variant is present, which is identical to the 22 kDa form, with a nick between amino acids 139 and 140 generating a two chain form with a higher molecular weight [3]. Charge variants are formed by proteolytic cleavage of residues 135–140 and 135–146 from the 22 kDa form [14].

The first therapeutic use of exogenous hGH extracted from human pituitary glands for growth hormone deficiency was reported by [24]. Pituitary-derived hGH was subsequently administered to large numbers of patients for growth hormone deficiency. However, in 1985 the use of pituitary-derived hGH was abruptly discontinued when the association between hGH preparations and Creutzfeld–Jakob disease (CJD) was made [2,7,12,26].

As hGH is not glycosylated, rhGH was first expressed in *Escherichia coli* in 1979 [9]. An assembled, hybrid gene composed of a chemically synthesized hGH DNA

* Corresponding author. Fax: +61-2-9313-6710.
E-mail address: s.mahler@unsw.edu.au (S.M. Mahler).

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sequence and an enzymatically derived hGH DNA sequence from human pituitary DNA was inserted into an expression plasmid under the transcriptional control of the *lac* operon. Translation of messenger RNA resulted in synthesis of rhGH indistinguishable from pituitary hGH with the exception of an additional methionine at the N-terminus [20]. rhGH was subsequently approved for human therapy in the USA in 1986, and at present there are several preparations of rhGH available including Saizen and Serostim (Serono Laboratories, USA), Genotonorm (Pharmacia, USA), and Norditropin (Novo Nordisk, USA). Recombinant DNA technology has provided a safe and abundant supply of rhGH with no risk of the transfer of human pathogens, eliminating the requirement for pituitary-derived preparations.

Purification of rhGH is typically performed using combinations of adsorption chromatographies including ion exchange, hydrophobic interaction, and metal–chelate with a final gel filtration step principally for sizing and removal of aggregate. As rhGH is generally produced in *E. coli* resulting in the formation of inclusion bodies, it requires resolubilization and refolding prior to purification by chromatography. The overall rhGH recovery is influenced significantly by the efficiency of the solubilization and refolding processes [22]. In the study of rhGH production in *E. coli* by Patra et al. [23], solubilization of inclusion bodies followed by a two-step purification process using DEAE-Sepharose ion-exchange chromatography and Sephacryl S-200 gel filtration allowed purification of monomeric rhGH to a purity of 99% with an overall yield of 50%.

For production of clinical grade rhGH, high levels of purity are required, with strict guidelines limiting the amounts of host cell protein (HCP) contaminants, host-derived DNA, endotoxin, and product variants. To achieve the required product specifications, a number of purification processes need to be incorporated into the downstream process. For example, a purification protocol for rhGH produced in *E. coli* as inclusion bodies consisted of six purification steps incorporating ammonium sulfate fractionation, Sephacryl S-100 gel filtration, DEAE-Sepharose Fast Flow ion-exchange, a further Sephacryl S-100 gel filtration, Q-Sepharose Fast Flow ion-exchange, and phenyl-Sepharose CL4B hydrophobic interaction chromatographies, resulting in a 40% overall recovery [6]. In another study, a 28–48% cumulative yield of rhGH was obtained from a culture medium of transformed monkey kidney cells, depending on the use of either octyl- or phenyl-Sepharose hydrophobic interaction chromatography in the first of three chromatographic steps [13]. Other purification procedures for rhGH from both bacterial and mammalian systems have resulted in sub-optimal recoveries [17].

To improve the yields for producing clinical grade rhGH, it is necessary to improve the efficiency of purification processes in the downstream process. Affinity chromatography approaches such as immunoaffinity are high efficiency purification processes with respect to target protein binding; however, there are associated problems including leaching of the antibody affinity ligand from the chromatographic matrix, and the regulatory requirement to produce the monoclonal antibody to clinical grade. In another example of an affinity-based chromatographic separation, fusion of a C-terminal His tag to rhGH has allowed the one-step, affinity purification of *E. coli*-derived rhGH via metal chelate affinity chromatography [21]. rhGH has also been purified from *Bacillus subtilis* using metal chelate affinity chromatography by exploiting the histidine residues and their location on the rhGH molecule. In a hydrophilic environment, residues His-18 and His-21 are exposed to solvents and therefore more readily accessible by metal ligands on an affinity column. In another example, rhGH showed high affinity for a column adsorbent charged with copper (II) [18]. Following on from these principles, immobilized metal affinity chromatography has been used to purify rhGH from *E. coli* [17].

This study describes a non-affinity, high yielding method for the purification of rhGH from CHO cell supernatant using Gradiflow preparative electrophoresis. Gradiflow technology relies on the electrophoretic movement of proteins in an electric field through thin polyacrylamide membranes with defined pore sizes to separate proteins on the basis of size and/or charge under native conditions [4,5,19,11]. This study also demonstrates the potential for incorporation of the Gradiflow in a bioprocess for the production of clinical grade rhGH and highlights the possibilities for Gradiflow technology to be integrated into other bioprocesses for the production of recombinant proteins for therapeutic use. Today there are a variety of recombinant DNA-derived protein biopharmaceuticals available for human therapy including cytokines, monoclonal antibodies, growth factors, therapeutic enzymes, and other classes of biopharmaceuticals. As the downstream processing is a major cost in production of protein biopharmaceuticals, innovative methods for protein purification that result in higher yields will allow the development of more cost-effective bioprocesses resulting in competitive pricing and potentially greater availability.

Materials and methods

Production of rhGH in CB515 recombinant CHO cell line

The recombinant CHO cell line CB515, containing an expression vector with the inducible human metallothionein IIA promoter region upstream of the structural gene for hGH, was used to produce rhGH [8].

The rhGH was produced by culturing CB515 cells in 10% fetal bovine serum (FBS) until 90% cell confluence was attained in 1700 cm^2 ribbed roller bottles. The culture supernatant was removed by aspiration and attached cells were washed with warm basal DMEM/Coon's F12 protein-free media (PFM) (BDH, Australia). The washings were discarded and the cells were incubated in 300 ml of warm PFM for 2 days prior to induction to reduce carryover of bovine-derived proteins from the FBS supplemented growth media into the production media. The PFM were then removed and replaced with 300 ml of warm PFM containing 80 μM $ZnSO_4$ to induce the promoter. The cells were gassed with 5% CO_2 and incubated at 37 °C for 2–3 day intervals prior to harvesting of the supernatant. The process was repeated until 3 L of supernatant was collected. After collection, the culture supernatants were combined and filtered through a 0.22 μm filter to remove cellular debris. Aliquots (50 ml) of the supernatant were prepared and stored at −20 °C for later purification.

SDS–PAGE, Western blot, and IEF

Samples were analyzed by SDS–PAGE and Western blot. All samples were concentrated 5 times by TCA precipitation prior to gel loading. This ensured that all proteins in the sample could be visualized. Samples in SDS loading buffer were boiled and loaded onto 4–20% NuPage (Novex) precast gels. Visualization of the SDS–PAGE gels was by silver-stain. For Western blot analysis, proteins were transferred to PVDF membranes using a semi-dry graphite transfer system. The membrane was blocked with 5% skim milk powder/PBST buffer (0.1% Tween 20) overnight at 4 °C. Subsequently membranes were incubated for 1 h at room temperature with biotin-conjugated polyclonal rabbit anti-hGH preparation (Dako) diluted (1:1000) in PBS. Membranes were then washed in PBST followed by another 1 h incubation with ExtrAvidin-AP (1:5000), a streptavidin-alkaline phosphatase (AP) conjugate (Sigma). The blot was visualized using WesternBlue substrate for AP (Promega).

CB515 culture supernatant and an rhGH standard were analyzed by IEF using the PhastSystem (Amersham–Pharmacia). Prior to application to the gel, the supernatant sample was dialyzed overnight at 4 °C against reverse osmosis-purified water using dialysis tubing with M_r cut-off of 10 kDa (Pierce). Dialyzed samples were loaded onto a p*I* 3–9 Phastgel and bands were visualized by silver-stain according to Phastsystem specifications.

Reverse-phase HPLC

The rhGH concentrations in the culture supernatant were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Hamilton polystyrene analytical HPLC column (USA) (4 μm, 30 mm × 3.1 mm). The column was equilibrated to achieve steady baseline at the initial flow conditions and a blank gradient was run. Standards and samples were filtered through 0.45 μm filters prior to analysis (50 μl injections). Sample running time was 6 min with an injection delay of 1 min. A step gradient was performed and the amount of rhGH, measured by the detector at 210 nm, was related to the peak area eluted at approximately 3.0–3.1 min. The rhGH peak area was calculated by Millennium 2010 V2.0 chromatography software (Millipore Waters).

Determination of rhGH purity and yield by densitometry

Scanning densitometry has been reported as a reliable method for the quantification of protein bands on Coomassie blue-stained polyacrylamide gels [27]. Determination of purities and yields for rhGH, purified using the Gradiflow, was performed by scanning densitometric analysis of Coomassie blue-stained gels of serially diluted samples (gels not shown) using the Bio-Imager System (Bio-Rad). Yield was determined by comparing the rhGH monomer band scores of final S2 samples with that of the initial starting material. Purity was determined by single lane analysis of rhGH monomer and contaminant band scores.

2D gel electrophoresis

CB515 supernatant, pre- and post-induction were analyzed by 2D gel electrophoresis (2-DE). Supernatant proteins were precipitated by trichloroacetic acid (TCA) and deoxycholic acid (DOC), and resolubilized in loading buffer (7 M urea, 2 M thiourea, 65 mM DTT, 2% w/v Chaps, and 1% Ampholytes, pH 3–10, Bio-Rad) prior to 2-DE analysis. The proteins were precipitated by adding 2 ml of 2% DOC (Sigma) to 200 ml of supernatant and incubating for 15 min at room temperature. TCA (24%, 33.7 ml) was then added to the sample, which was incubated on ice for 1 h before centrifuging for 10 min at 12,500 rpm. The supernatant was discarded and 200 ml of ice-cold acetone was added to the pellet and incubated on ice for 15 min. Samples were again centrifuged, the supernatant was discarded, and the pellet was resuspended in Milli Q water ready for analysis by 2-DE. The gels were fixed for 2 h in 10% methanol, 7% acetic acid and stained overnight in Sypro-Ruby. Following destaining in 10% methanol with 7% acetic acid for 4 h, images were acquired using a Bio-Rad FX fluorescent scanner.

Identification of major proteins in 2-DE gels

Protein spots of interest were excised from Coomassie blue-stained gels and digested using sequencing grade

modified trypsin (Promega, Madison, WI). Gel pieces were destained by washing twice in 200 µl of 0.2 M NH_4HCO_3, 50% acetonitrile for 30 min and dried in a Speedvac. They were subsequently rehydrated in 10 µl trypsin solution (0.1 mg/ml trypsin in 0.2 M NH_4HCO_3, 0.5 mM $CaCl_2$) at room temperature for 30 min. Sufficient buffer (0.2 M NH_4HCO_3, 0.5 mM $CaCl_2$) to cover the gel fragment was added and the digest was incubated for 16 h at 37 °C. Digested supernatant was collected and the gel piece was sonicated for 30 min in 200 µl of 1% TFA. Supernatant was collected and the extraction was repeated in 200 µl of 0.1% TFA with 60% acetonitrile. Extracts were pooled and dried by Speedvac.

MALDI mass spectrometry

Digested samples were solubilized in 3 µl of 0.08% TFA, 80% acetonitrile. Sample (1 µl) was spotted onto the sample plate followed by 0.5 µl matrix [5 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) in 0.08% TFA, 80% acetonitrile]. The sample was allowed to dry prior to MALDI-mass spectrometry.

Peptide mass spectra were generated on a Voyager DE-STR mass spectrometer (Perseptive Biosystems, Framingham, MA). The instrument was used with delayed extraction in reflectron mode and accelerating voltage was 20,000 V. Laser power varied between 1600 and 2500 over 256 shots. Mass spectra were acquired for peptides in the range 800–3500 Da. Peptide mass assignment was done following internal calibration against trypsin peptides occurring at 842.51, 1045.56 or 2211.10 Da. Peptide mass data were used to search mammalian entries in the SwissProt protein database accessible from the Australian ExPasy mirror site (http://au.expasy.org/tools/peptident.html).

Growth hormone standard

An rhGH standard was obtained by a chromatographic purification protocol from CB515 cell culture supernatant yielding rhGH of >98% purity.

Gradiflow technology

The Gradiflow (Gradipore) is an electrophoresis-based technology that separates macromolecules on the basis of both size and charge. The core of the technology is the separation cartridge, which consists of three polyacrylamide membranes that form two streams, stream 1 (S1) and stream 2 (S2), by sandwiching a separation membrane of defined pore size between two restriction membranes (Fig. 1). The cathode and the anode are located on each side of S1 and S2, respectively, and buffer circulates around the cartridge to provide cooling and pH control. The anode and cathode may be configured in the reverse orientation to that depicted in Fig. 1.

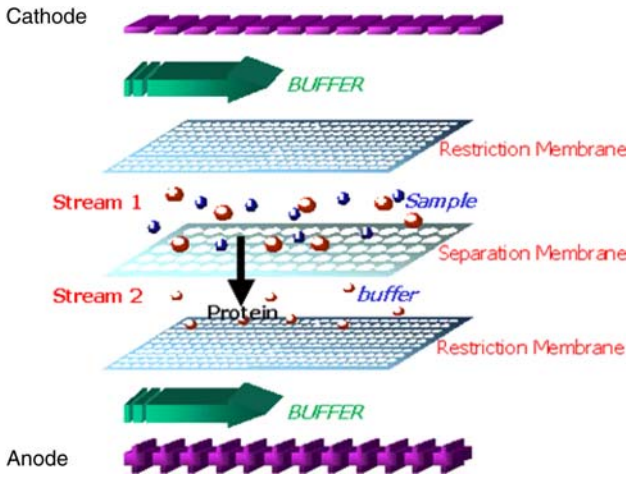

Fig. 1. Gradiflow separation unit. Diagram shows configuration for simultaneous charge-based and size-based separation.

The restriction membranes allow the movement of small molecules and ions up to M_r 5 kDa into the bulk buffer while keeping the protein of interest within S1 and S2. The separation membrane, which has a defined pore size, allows protein separation to take place depending on the size and charge of the protein of interest. The charge on the protein is determined by the pH of the bulk buffer, which is selected to impart a negative or positive charge on the protein of interest. As the sample enters the electric field, proteins migrate towards the electrode of opposite charge, and thus move from one stream to the other unless their size and/or shape prohibit their movement through the separation membrane. Hence, it is possible to separate samples by both size and charge simultaneously. Fig. 1 shows the movement of proteins within the separation cartridge in an electric field.

The BF200 Gradiflow instrument used in this research has recently been superseded by the BF400. These bench-top instruments allow for minimum load volumes of 8 up to 250 ml. Custom-made disposable separation cartridges (50 kDa) were obtained from Gradipore (Australia). Pore sizes of the membranes vary from 1000 to 3 kDa and are custom-made depending on the application. In these experiments, the restriction membranes were composed of a 5 kDa membrane.

Gradiflow is a fully scalable technology, making it suitable for industrial scale separations. Processing capacity in the Gradiflow is proportional to membrane surface area. In a single separation cartridge, increased membrane area is obtained by using larger membranes, and then by placing repeating sets of separation and restriction membranes in a stacked plate and frame configuration. The current prototype instrument that embodies these scale-up principles is capable of conducting Gradiflow separations up to 250 times the capacity of the standard laboratory scale instrument [16].

Combined size and charge separation

Three combined size and charge separations were conducted to separate the rhGH from contaminants within the supernatant. These separations were conducted over 1 h using a separation cartridge with M_r cut-off of 50 kDa and separation buffers at pH 8.5, 7.5, and 6.5. Three separation buffers were used; 50 mM Tris (Univar)/Borate (Univar), pH 8.5; 50 mM Tris/Hepes (ICN Biomedical), pH 7.5; and 50 mM Mes (Sigma)/Bis-Tris (Sigma), pH 6.5. The Gradiflow apparatus was set up prior to the run by placing 10 ml of the supernatant (with pH adjusted to the running conditions) in S1 and 10 ml of the appropriate running buffer in S2. All streams were allowed to circulate prior to application of the electric field. The separation run was started once the sample buffer and bulk buffer had cooled to approximately 10 °C. Samples (200 μl) were collected from both S1 and S2 at regular intervals throughout the separation run for subsequent analysis.

At the completion of the separation run, 10 ml of appropriate buffer was circulated through S1 and S2 to wash any adsorbed proteins from the separation and restriction membranes. Samples collected during the separation run were analyzed by non-reducing SDS–PAGE and rhGH recoveries were determined by densitometry analysis.

Results

Analysis of CB515 cell culture supernatants pre- and post-induction

To calculate the amount of rhGH produced and the purity of rhGH in cell culture supernatant harvests, total protein and rhGH were estimated by total protein estimation (Pierce) and reverse phase HPLC, respectively (Table 1). The results show a high level of CB515 productivity, with 70–80% rhGH purity in supernatant incubated for 2–3 days in PFM with 80 μM $ZnSO_4$,

Table 1
Total protein and rhGH concentration in culture supernatant post-induction

Harvest	Total protein concentration (μg/ml)	rhGH concentration (μg/ml)	Purity (%)
1	92	79.1	69
2	132	105.5	80
3	131	107.5	82

Cells were cultured for 2 days in 1700 cm^2 ribbed roller bottles in growth media containing 10% FBS, washed, and incubated in PFM for 2 days to remove residual FBS proteins. PFM (300 ml) containing 80 μM $ZnSO_4$ was then added and harvested after 2–3 day intervals. The procedure was repeated 2–3 times in sufficient numbers of flasks to accumulate 3 L of cell culture supernatant.

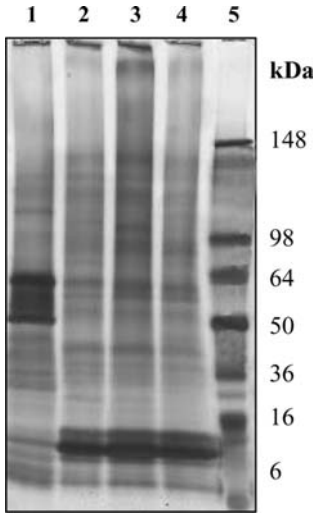

Fig. 2. Silver-stained SDS–PAGE gel of CB515 culture supernatant. Proteins were separated according to size under denatured conditions. Lane 1, culture supernatant from CB515 cells after 2 days in protein-free media (PFM); lanes 2, 3, and 4, samples of CB515 culture supernatant after 2–3 days in PFM with 80 μM $ZnSO_4$; and lane 5, SeeBlue Plus2 M_r marker.

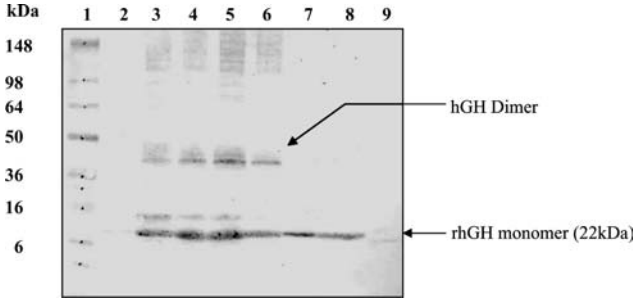

Fig. 3. Western blot of CB515 culture supernatant. Lane 1, size marker; lane 2, culture supernatant harvested from CB515 in PFM; lanes 3–6, culture supernatant harvested from CB515 in PFM with 80 μM $ZnSO_4$; and lanes 7–8, standard rhGH, CHO-K1 cell culture supernatant (negative control).

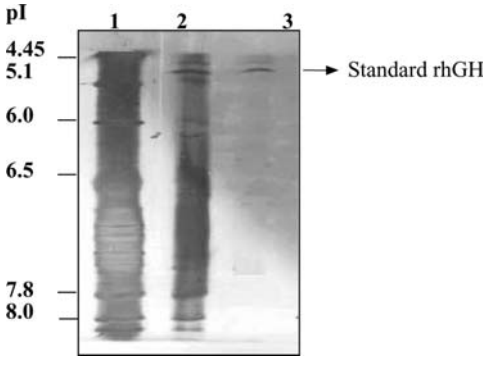

Fig. 4. IEF silver-stained PhastGel (Pharmacia) of hGH. Lane 1, p*I* markers and CB515 culture supernatant; lane 3, standard hGH.

consistent with productivity data for the CB515 cell line of Friedman et al. [8]. CB515 cell culture supernatants pre- and post-induction were also analyzed by SDS–PAGE, Western blot, and IEF. SDS–PAGE (Fig. 2) and Western blot analyses (Fig. 3) of CB515 cell culture supernatant post-induction show the major 22 kDa form of rhGH, with bands of 20 and 45 kDa (Fig. 3) representing the minor variant of hGH and hGH dimers, respectively.

IEF analysis of CB515 culture supernatant pre-induction (Fig. 4) shows CHO host cell protein contaminant over the range of the IEF Phastgel (3–9). The p*I* of standard rhGH was estimated to be 5, correlating with the reported p*I* for hGH of 4.9 [1,15]. Recent data from protein databases have assigned a theoretical p*I* for hGH of 5.16 (ANGIS) and 5.27 (ExPASy), respectively. 2-DE analyses of culture supernatants from both CHO-K1 control and induced CB515 cell cultures were performed in order to characterize the background protein profile in conditioned medium, and are shown in Figs. 5A and B (24 h post-induction), and C (72 h

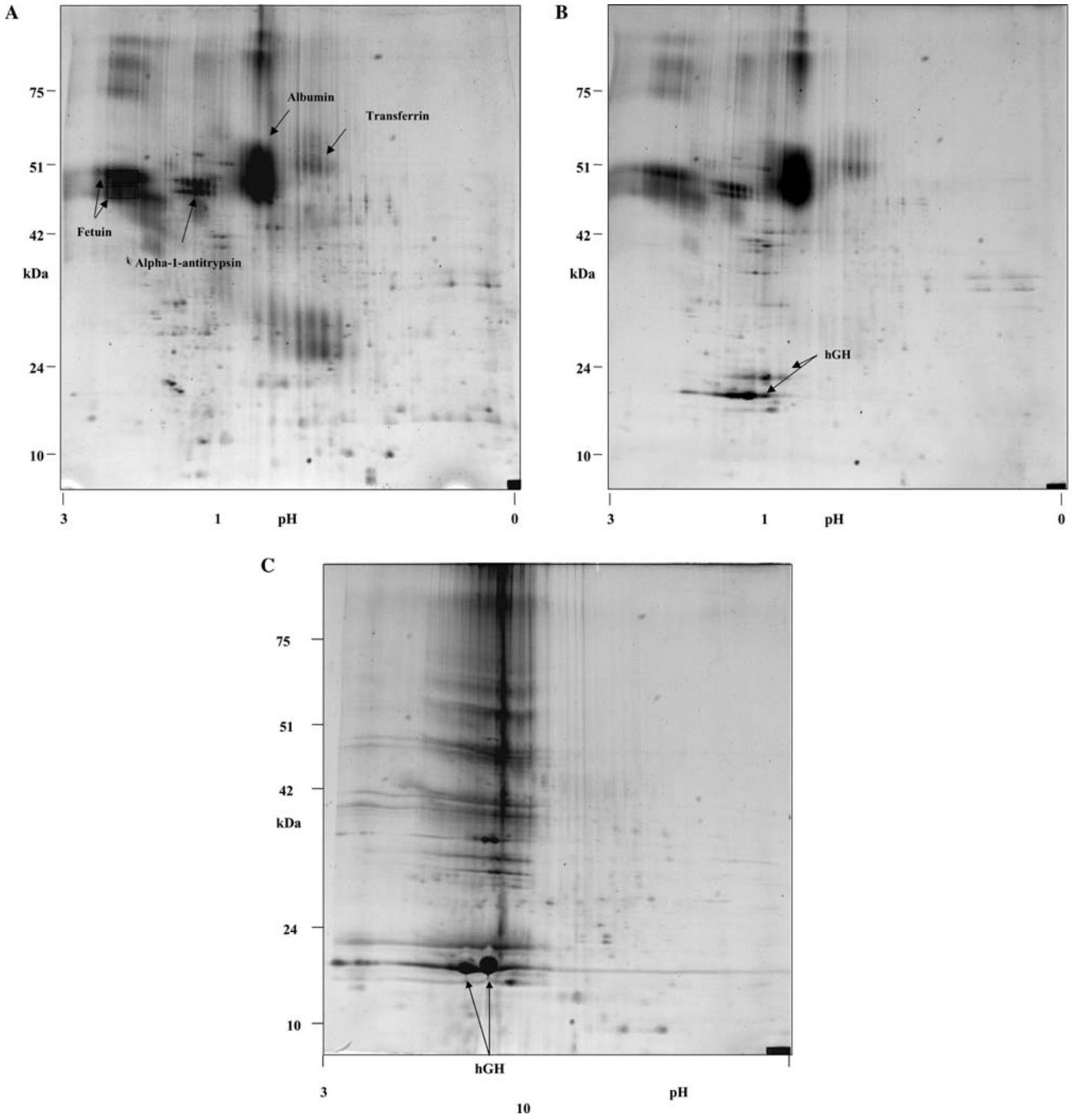

Fig. 5. Denatured Sypro Ruby-stained 2D-E Gels of proteins in CHO-K1 culture supernatant (A), induced CB515 culture after 24 h (B) and 72 h (C) in protein free production media. First dimension: IEF, pH 3–10, Second dimension: SDS–PAGE 10–200 kDa (scale not linear).

post-induction), respectively. Although 2-DE analysis shows the denatured protein profile, it is nevertheless a useful guide for determining the distribution of M_r and isolectric points of host cell and media carryover contaminant proteins. A comparison of the 2-DE images shows that there are common proteins present in both the control and induced cultures. Major proteins were identified by MALDI-MS as transferrin, albumin, α-1-antitrypsin, and fetuin, all of bovine origin. Although 2-DE samples were taken from a culture in protein-free production media, these major contaminant proteins are a result of carryover from the FBS supplemented growth media. The induced cultures contain additional proteins with approximate pI and M_r of 5 and 20 kDa, respectively, consistent with the M_r for rhGH.

Gradiflow size- and charge-based separation

Separations were conducted over a pH range 6.5–8.5 using three different buffers and 50 kDa separation cartridges. As shown in Fig. 5, the majority of CHO-K1 host cell proteins are acidic, thus most host cell proteins in CHO cell culture supernatant at pH 6.5–8.5 are negatively charged. Contaminant proteins with M_r of greater than 50 kDa would be expected to be retained in S1 (anode), while the rhGH would transfer to S2. The procedure for the running of the Gradiflow was as follows: (1) cell culture supernatant (10 ml) and buffer (10 ml) were added to S1 and S2, respectively; (2) pumps were switched on allowing circulation of S1 and S2, the voltage was applied (250 V, 500 mA), and sampling was performed at different time points during the separation (circulating samples); (3) after 60 min, the apparatus (pumps and voltage) was switched off, S1 and S2 were harvested, and final samples taken.

SDS–PAGE analyses of samples from the Gradiflow separation runs at pH 6.5 (50 mM Mes/Bis-Tris buffer), pH 7.5 (50 mM Tris/Hepes buffer), and pH 8.5 (50 mM Tris/Borate buffer) are shown in Figs. 6A, B, and C, respectively. Separations at pH 7.5 and 8.5 gave similar

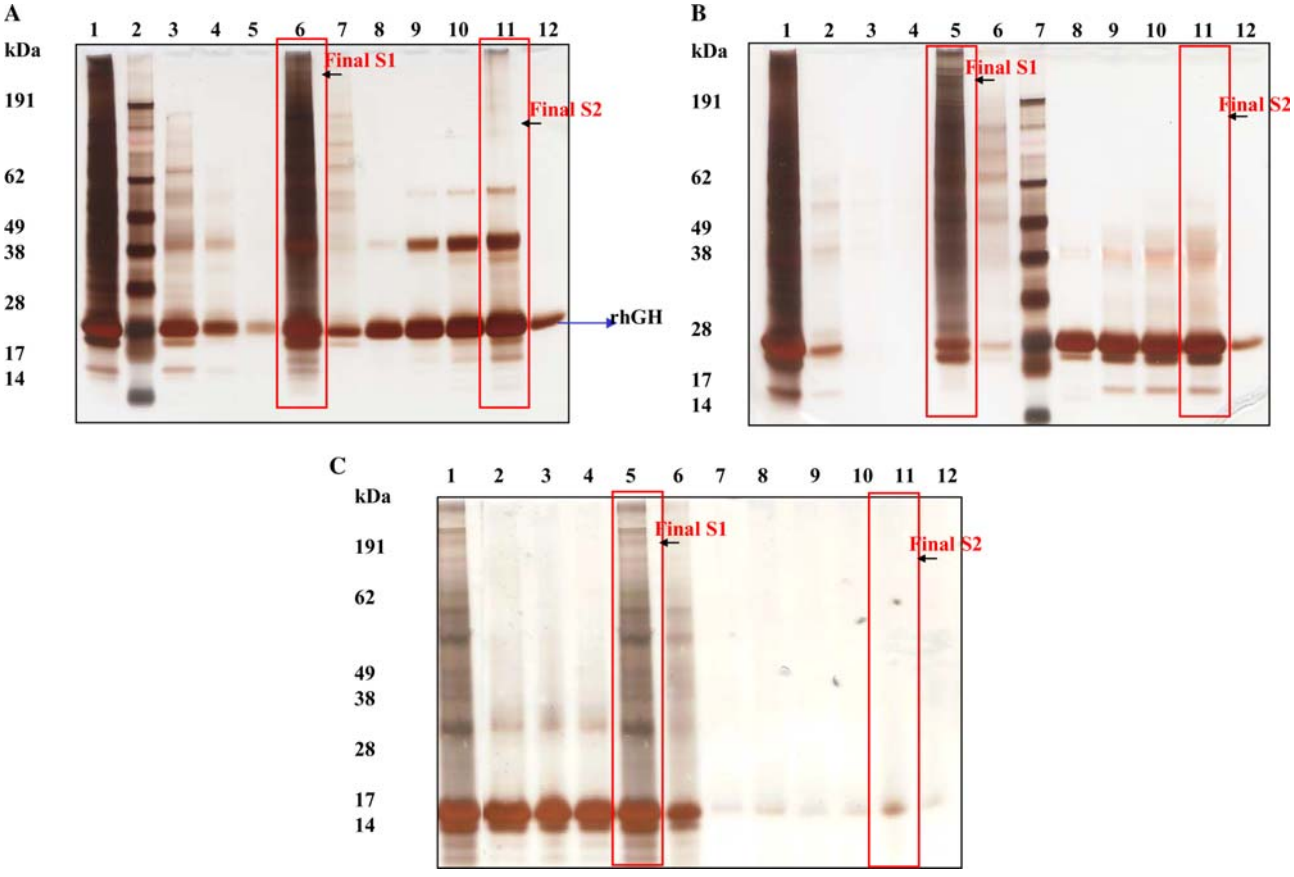

Fig. 6. Silver-stained SDS–PAGE of rhGH purification using Tris/Borate, pH 8.5 (A), Tris/Hepes, pH 7.5 (B), and Mes/Bis-Tris, pH 6.5 (C). (A) Lane 1, stream 1, starting material; lane 2, SeeBlue Plus2 marker; lanes 3–5, stream 1, 30, 45, and 60 min circulating samples, respectively; lane 6, stream 1, 60 min final; lane 7, stream 1, wash; lanes 8–10, Stream 2, 30, 45, and 60 min circulating samples, respectively; lane 11, stream 2, 60 min final; and lane 12, stream 2, wash. (B) Lane 1, stream 1, starting material; lanes 2–4, stream 1, 30, 45, and 60 min circulating samples, respectively; lane 5, stream 1, 60 min final; lane 6, stream 1, wash; lane 7, SeeBlue plus2 marker; lanes 8–10, stream 2, 30, 45, and 60 min circulating samples, respectively; lane 11, stream 2, 60 min final; and lane 12, stream 2, wash. (C) Lanes 1, stream 1, starting material, lanes 2–4, stream 1, 30, 45, and 60 min circulating samples, respectively; lanes 5, stream 1, 60 min final; lane 6, stream 1, wash; lanes 7–10, stream 2, 15, 30, 45, and 60 min circulating samples, respectively; lane 11, stream 2, 60 min final; and lane 12, stream 2, wash.

results with respect to yield and purity of rhGH monomer, while separation at pH 6.5 resulted in low yields of rhGH. Separations at pH 7.5 and 8.5 show that there was co-transfer of minimal contaminant proteins into S2 after 60 min, and quantitative analysis by scanning densitometry of a Coomassie blue-stained gel (results not shown) of S1 and S2 samples for the Gradiflow separation run at pH 7.5 resulted in a rhGH monomer purity and yield of approximately 98 and 90%, respectively. The buffer washes of S1 after completion of the separation run show contaminant proteins over a broad range of M_r including a small quantity of rhGH, indicating some adherence of proteins to the membrane.

Discussion

While rhGH preparations have been produced and purified predominantly from bacterial sources, mammalian cells have also proven capable of successfully producing rhGH. For example, rhGH has been produced in monkey kidney cells using an SV40 vector containing the entire genomic hGH gene [10]. Monkey cells recognize the signals for messenger RNA processing and transport hGH out of the cell into the medium. Since the secretion of proteins into the fermentation medium often results in large volumes of culture supernatant with the product at low concentrations, initial filtration of culture supernatant for elimination of insoluble particulates, and concentration of product are required. Hydrophobic interaction chromatography has been used as the initial step to concentrate and partially purify rhGH from the fermentation medium of a transformed monkey kidney cell line. Further purification of the concentrated rhGH solution was obtained by DEAE-Sepharose ion-exchange chromatography and Ultrogel gel filtration chromatography [13].

In this study, we have devised a one-step purification protocol for rhGH from CB515 cell culture supernatant using a preparative electrophoresis instrument, the Gradiflow. CB515 is a CHO cell line expressing rhGH in protein-free medium [8]. The cell line was produced by transfection of the CHO K1 host with a plasmid vector harbouring the heavy metal-inducible human metallothionein IIA promoter. The 2.3 kb hGH genomic DNA complete with introns was cloned downstream of the inducible promoter for high yield expression without deliberate gene amplification.

A purification process has previously been developed for the production of clinical grade rhGH derived from CB515 and involved several steps alternating between chromatographic methods of various chemistries (unpublished data). Diluted culture supernatant was passed through an anion-exchange column under conditions for adsorbing rhGH to the column. Upon elution of rhGH followed by ammonium sulfate precipitation, centrifugation, and 0.22 μM filtration, the emerging concentrated solution containing rhGH was purified further through four chromatographic stages. These included two phenyl-Sepharose hydrophobic interaction chromatography steps followed by anion-exchange and finally Sephacryl S-200 size-exclusion chromatography, integrated within a closed computer controlled system. In the quest to decrease the number of unit processes in the downstream processing of rhGH, the Gradiflow was evaluated as a unit process with the potential to be incorporated into a bioprocess for rhGH production.

For Gradiflow separations performed at pH 7.5 and 8.5, rhGH and the majority of host cell contaminants are negatively charged, so that the rhGH moves through the separation membrane into S1 while the majority of contaminants are restricted from moving through the separation membrane and therefore remain in S1. This results in the purification and concentration of rhGH, as the circulating volume of S2 is less than S1. Qualitative analysis by silver-stain/SDS–PAGE showed separations at pH 7.5 and 8.5 resulted in similar yields and purities of rhGH monomer; however, there was more rhGH dimer present in the separation run at pH 8.5. Thus, the Gradiflow separation has proved to be an efficient, single-step purification process comparable to an affinity separation.

The separation run at pH 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 the correct balance between the separation membrane pore size and the buffer choice. Studies using extended running times and membranes with larger pore size did not show significant improvement in either the yield or purity of rhGH (data not shown).

In summary, we have demonstrated a simple, one-step purification protocol for the production of highly purified hGH preparation from mammalian cell culture supernatants, suitable for incorporation into diagnostic-based assays and bioassays. The study also shows the potential for Gradiflow to supplement and/or replace traditional methods of protein purification. As Gradiflow is directly scalable, it has the potential to be incorporated into bioprocesses for production of clinical grade rhGH, which may include other purification processes such as ion exchange, hydrophobic interaction, and gel filtration.

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