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Separation of recombinant human growth hormone from *Escherichia coli* cell pellet extract by capillary zone electrophoresis

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Abstract

Free zone capillary electrophoresis (FZCE) was used to resolve recombinant human growth hormone (rhGH) and its variants from very crude mixtures of *Escherichia coli (E. coli)* cell paste extract. The methodology employs a phosphate deactivated fused-silica capillary and a 250 m*M* phosphate (pH 6.8), 1% (v/v) propylene glycol buffer with a high field strength of 600 V/cm. Resolution of rhGH and its variants from very crude mixtures did not change after 80 injections for the PSC capillaries. Bare silica and PVA coated capillaries had a more limited lifetime when injected with the same crude mixtures (10 to 30 injections). This FZC method provides a very powerful tool for assessing rhGH modification during the fermentation and isolation of rhGH that is not possible with other current analytical techniques.

Keywords: *Escherichia coli*; Growth hormones

1. Introduction

Recombinant human growth hormone (rhGH) is expressed, processed and secreted into the periplasmic space of *E. coli* [1]. Production and secretion of the protein and its variants; unprocessed rhGH (rhGH with the signal peptide still attached), aggregated rhGH, deamidated rhGH, two-chain rhGH, and desPhe[1] rhGH, are strongly affected by the *E. coli* culture conditions and the initial isolation steps. Characterization of these rhGH degradation products during the fermentation and isolation process requires the use of several different analytical methods [2–5].

Formation of rhGH variants in the fermentation or isolation process occurs by a variety of processes, including proteolysis, elevated pH, and incorrect processing of the rhGH peptide. Proteolysis occurs during the fermentation or from a protease co-purifying with rhGH during the isolation process and results in two-chain rhGH formation. Prolonged exposure of asparagine (position 149) to elevated pH, produces deamidated rhGH. A spontaneous chemical reaction that results in diketopiperazine formation and cleavage of the first two N-terminal amino acids produces desPhe[1]Pro[2] rhGH. DesPhe[1]Pro[2] rhGH occurs under the same conditions that produces deamidated rhGH, but at a greatly reduced rate [6]. DesPhe[1]Pro[2] rhGH formation has only been observed upon prolonged storage of purified rhGH [7]. Unprocessed rhGH is the result of the cell's inability to transport the unprocessed protein from the cytoplasm to the periplasmic space. DesPhe[1] rhGH is the result of incorrect processing of the signal peptide or from an aminopeptidase acting during the harvest step. Aggregated rhGH is the result of incorrect processing of the protein disulphide bonds in the periplasmic space. Unprocessed

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and aggregated rhGH are insoluble in aqueous solutions. These last rhGH variants require denaturing analytical methods for characterization. Analysis of the other variants (two-chain rhGH, deamidated rhGH, desPhe^1 rhGH, and desPhe^{1}Pro^2 rhGH) requires the use of non-denaturing techniques.

Previous investigators have used capillary electrophoresis (CE) to resolve charged rhGH variants and methionyl human growth hormone (hGH) from purified rhGH [8–11]. Using hGH and its variants, Arcelloni et al. have demonstrated the exceptional resolving power of CE over liquid chromatography methods. The analysis of hGH in plasma samples required an affinity purification step to concentrate and remove interfering plasma contaminates before the sample could be analyzed by CE [10]. The design of such pre-analysis steps is important so that loss of the protein of interest is minimal and the sample is not degraded or altered. An affinity capture step increases the analysis time and lowers sample through-put. Development of a CE method that does not require such a step would minimize protein loss and degradation during sample preparation. Such a method would have higher sample throughput, very rapid analysis time and higher resolution.

In this report we describe a method using free zone capillary electrophoresis (FCZE) to resolve rhGH and its variants from very crude mixtures, such as *E. coli* cell pellet. This method does not require an affinity purification step before the CE analysis. Using this method, we were able to detect the effect of changes in fermentation conditions on rhGH production.

2. Materials and methods

2.1. Materials

Sodium phosphate (dibasic and monobasic), guanidine–HCl, and nitric acid were obtained from Mallinckrodt (Paris, KE, USA). Tris base and CHES were obtained from Research Organics (Cleveland, OH, USA). Glycine, urea, and 50% NaOH solution were obtained from J.T. Baker (Phillipsburg, NJ, USA). Propylene glycol was obtained from Dow Chemicals (Midland, MI, USA). CNBr-activated Sepharose 4B was purchased from Pharmacia (Piscataway, NJ, USA). Centricon® 10 concentrators were obtained from Amicon (Beverly, MA, USA).

Recombinant human growth hormone (rhGH) is a Genentech, product. rhGH variants (desPhe^1-rhGH, two-chain rhGH, desPhe^{1}Pro^2-rhGH, deamidated and dideamidated rhGH) and rhGH binding protein were prepared according to the procedure of Roswall et al. [6]. *E. coli* cells containing 95% unprocessed rhGH were prepared according to the procedure of Chang et al. [2].

2.2. Apparatus

Separations were performed using a HP^{3D} capillary electrophoresis system (Hewlett-Packard, Palo Alto, CA, USA). The system is equipped with a built-in diode-array detector and HP^{3D} CE ChemStation software for system control, data collection, and data analysis. Detection was carried out at 214 nm and 280 nm, with a band width of 2 nm. Sample injections were performed by pressure at 50 mbar (5 kPa) for 50 s. Capillary temperature was maintained at 15°C. All CZE analysis used field strengths of 500 or 600 V/cm.

Polyvinyl alcohol coated (PVA) capillaries and bare silica capillaries, using a bubble cell light path, were used in all experiments. PVA capillaries (Hewlett-Packard) had a total length of 40 cm (effective length, 32.5 cm) and an inner diameter of 50 μm. Bare silica capillaries (Hewlett-Packard) had a total length of 48.5 cm (effective length, 40 cm) and an inner diameter of 25 or 50 μm.

Phosphate deactivated fused-silica capillary run buffer was 0.25 *M* sodium phosphate (pH 6.8) and 1% (v/v) propylene glycol. Bare-silica and PVA coated capillaries used 0.1 *M* Tris, 0.1 *M* CHES (pH 8.8) for run buffer.

After each analysis, PVA and phosphate deactivated bare-silica capillaries were flushed with 0.2 *M* sodium phosphate (pH 7.0), 3 *M* guanidine–HCl for 5 min. Bare silica capillaries were flushed for 5 min with 0.1 *M* NaOH after each run. All capillaries were stored in run buffer.

2.3. Phosphate deactivated fused-silica capillary protocol

The phosphate deactivation of a fused-silica capillary is a modification of the procedure from Swed-

berg and Miller [12]. All capillary conditioning treatments were performed at 50°C. A new 25 μm I.D. bare, fused-silica capillary was treated as follows:

1. Capillary flushed with 0.1 *M* nitric acid for 30 min.
2. Capillary flushed with 0.1 *M* NaOH for 30 min.
3. Capillary flushed with 0.1 *M* nitric acid for 30 min.
4. Capillary flushed with deionized water for 15 min.
5. Capillary flushed with phosphate run buffer for 1 h.

The capillary was then incubated for up to 16 h at 50°C in phosphate run buffer, to complete the phosphate deactivation of the fused-silica.

2.4. Bare silica capillary conditioning

CE analysis performed with bare-silica capillaries was as described below. All capillary conditioning treatments were performed at room temperature. A new 50 μm I.D. bare, fused-silica capillary was treated as follows:

1. Capillary flushed with 0.1 *M* NaOH for 30 min.
2. Capillary flushed with deionized water for 15 min.
3. Capillary flushed with running buffer for 15 min.

The capillary was flushed for 5 min with 0.1 *M* NaOH after each run.

2.5. Preparation of E. coli extracts

Frozen *E. coli* cells from rhGH fermentations, were resuspended in PI (protease inhibitor) buffer (25 m*M* Tris, 5 m*M* EDTA, 5 m*M* EGTA, and 1 m*M* benzamidine–HCl, pH 7.5) or Tris–glycine buffer (25 m*M* Tris, 200 m*M* glycine, 5 m*M* EDTA, 5 m*M* EGTA, and 1 m*M* benzamidine–HCl, pH 8.3) at a concentration of 1 g of cell pellet per 10 ml. The resuspended cells were then sonicated for 20 s at 4°C. Sonicated cell extract was centrifuged to remove cell debris before analysis.

2.6. Preparation of rhGH and rhGH variant standards

2.6.1. rhGH and rhGH Variant standard mixture

rhGH, deamidated rhGH, dideamidated rhGH, two-chain rhGH, desPhe^{1} rhGH, and desPhe^{1}Pro^{2} rhGH were combined in PI buffer. The final concentration was 200 μg/ml for rhGH and 25 μg/ml for each of the variants

2.6.2. rhGH standards

rhGH was diluted to a range of 5 to 500 μg/ml in PI buffer.

2.7. Affinity chromatography

Recombinant human growth hormone binding protein (rhGH binding protein) was immobilized to activated CNBr Sepharose 4B according to the procedure of Battersby et al. [13]. A similar procedure was used for coupling polyclonal goat rhGH antibody to activated CNBr Sepharose 4B.

The capacities of both affnity matrices (rhGH binding protein Sepharose and rhGH antibody Sepharose) were determined using rhGH standards. Both affnity matrices had an rhGH capacity of 40 μg per ml of resin.

E. coli cell pellet extract for affnity chromatography, was prepared as described above using Tris–glycine buffer. The volume (approximately 100 μl) of cell pellet extract loaded onto the affinity matrix did not exceed 20 μg of bound rhGH per ml of affinity resin. Nonspecifically bound proteins were removed by washing with PBS containing 0.85 *M* NaCl (5 column volumes). Elution of specifically bound rhGH was carried out using 0.25 *M* glycine–HCl, pH 2.5 (5 column volumes). When not in use, the affinity columns were stored at 4°C in PBS containing 0.01% sodium azide. The load-flow-through and the eluted rhGH fractions were pooled separately. The load-flow-through pool was concentrated to the original load volume level using a Centricon 10 concentrator. The eluted bound rhGH pool was buffer exchanged into PI buffer containing 4 *M* urea, and then concentrated to the original load volume level using a Centricon 10 concentrator.

3. Results and discussion

Using standards and the phosphate deactivated fused-silica capillary method, we separated the charged (deamidated, dideamidated, and two-chain) and uncharged (desPhe[1] and desPhe[1]Pro[2]) rhGH variants (Fig. 1a). In contrast, bare-silica and PVA capillaries were only able to resolve deamidated rhGH, dideamidated rhGH, and rhGH (Fig. 1b and 1c). Using the phosphate deactivated fused-silica capillary run buffer with bare-silica and PVA capillaries, the rhGH variants had longer migration times with no improvement in resolution of charged and uncharged variants over conditions in Fig. 1b and c. Analysis of rhGH using the deactivated fused-silica deactivated capillary, had a linear range from 5 to 500 μg/ml for purified rhGH, with a correlation coefficient of 1.00. R.S.D. values at each concentration were less than 5% (five separate injections per concentration). The limit of detection for purified rhGH was 2 μg/ml.

As found previously [12,14,15], the phosphate concentration of the deactivation/run buffer was important for the phosphate deactivation of fused-silica and for the separation of rhGH and its variants. We found that the addition of a strong acid and

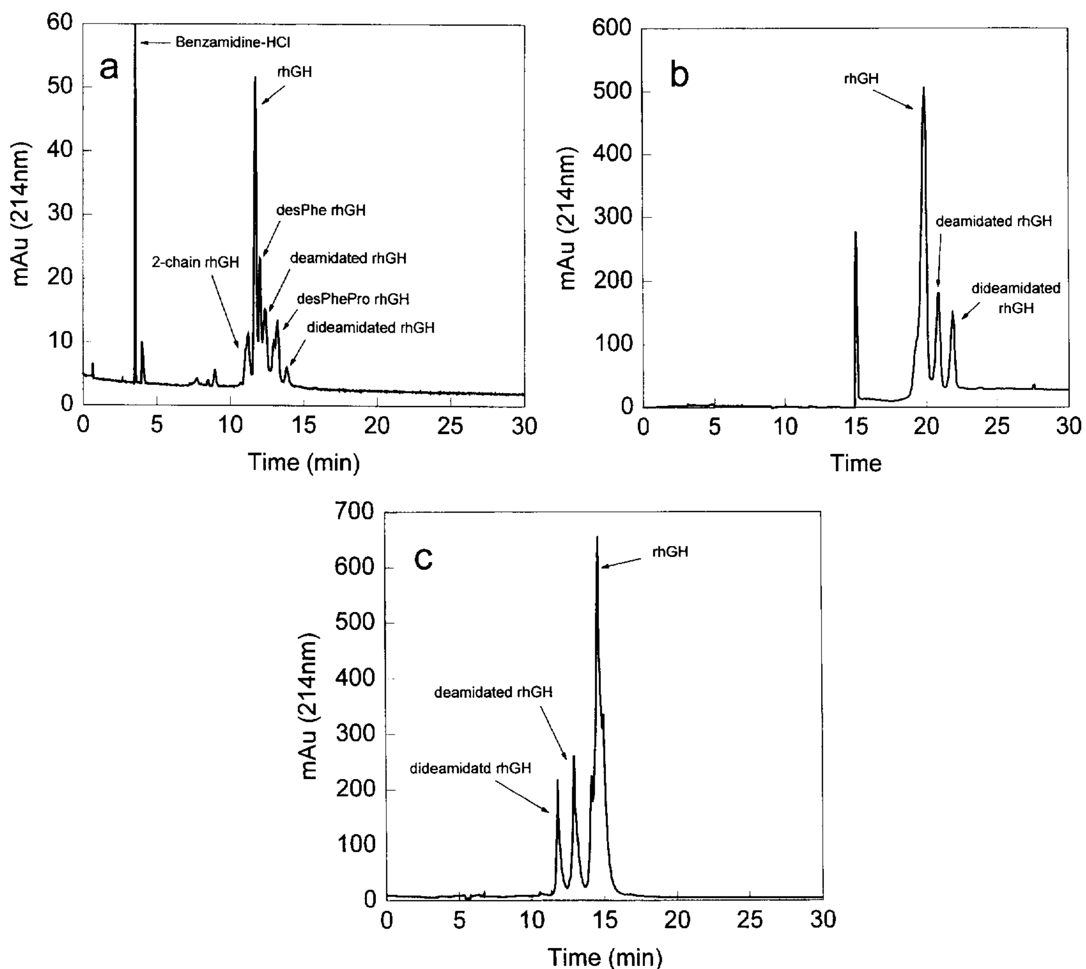

Fig. 1. Separation of rhGH and rhGH variant standards using (a) a phosphate deactivated fused-silica capillary, (b) a bare-silica capillary, and (c) a PVA coated capillary. Bare-silica and PVA coated capillaries were not able to resolve rhGH from two-chain rhGH, desPhe[1] and desPhe[1]Pro[2] rhGH. These rhGH variants co-migrated with rhGH. Analyses were performed at a field strength of 600 V/cm.

NaOH wash before the deactivation step, removed contaminants that prevented phosphate from binding to the capillary's silica surface [16]. This extensive capillary conditioning protocol gave better separation reproducibility between capillaries.

Using different types of capillaries and run buffers we resolved rhGH from *E. coli* pellet extract components by CZE without employing an affinity purification step. The best separation of rhGH and its variants from *E. coli* cell pellet extract was achieved with a phosphate deactivated fused-silica capillary and a phosphate run buffer (Fig. 2b). During the CZE analysis, cell pellet extract material accumulates on the phosphate deactivated fused-silica surface If these deposits are not removed, rhGH migration time increases in the next analysis. Flushing the capillary with 3 *M* guanidine–HCl, 0.2 *M* sodium phosphate (pH 7.0) after each run, produced an acceptable rhGH migration time precision of 2.3% R.S.D. for run to run, and 5% R.S.D. for day-to-day. Storage of the phosphate deactivated fused-silica capillary in a buffer other than the run buffer, reduced the resolution of rhGH and variants from cell pellet extract, and the migration time varied with each analysis. The loss of resolution and the inconsistent migration times, are due to alterations in the phosphate–silicate chemistry on the capillary surface. These changes were caused by changes in pH or phosphate concentration of the run or storage buffer [17].

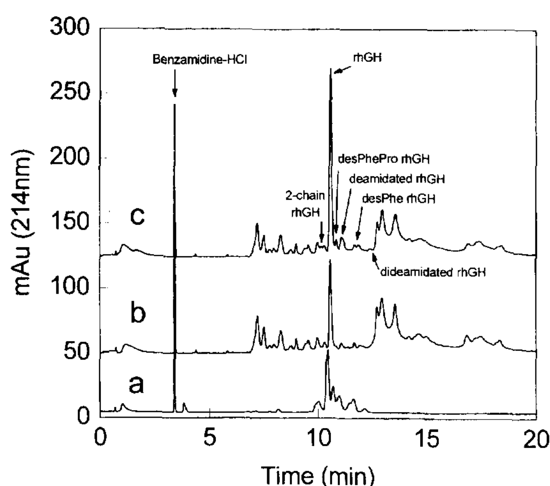

Fig. 2. Using the phosphate deactivated fused-silica capillary method, we separated (a) rhGH and rhGH variant standards, (b) rhGH from cell pellet extract, and (c) cell pellet extract spiked with rhGH and rhGH variant standards. Analyses were performed at a field strength of 600 V/cm.

Using the phosphate deactivated fused-silica capillary CZE method, rhGH and its variants were identified by spiking in known standards in to the cell pellet extract (Fig. 2). Intra-run reproducibility for determining rhGH concentration in the cell pellet extract was less than 2.5% R.S.D.. Different extractions of the same cell pellet lot varied by 6.6% R.S.D. for determining rhGH concentration. This method has comparable precision and accuracy to HPLC analytical methods in determining hGH from cell pellet extract [18].

The phosphate deactivated fused-silica capillary had a greater life span than the PVA capillary when injected with cell pellet extract. Injecting cell pellet extract into a phosphate deactivated fused-silica capillary, did not clog the capillary after 80 injections at a field strength of 600 V/cm (over 200 injections at a field strength of 500 V/cm). At a field strength of 600 V/cm, PVA capillaries clogged after 2–30 injections. Capillary life span for bare-silica capillaries was similar to PVA capillaries. The shorter life of PVA and bare-silica capillaries may be due to the irreversible accumulation of cell pellet extract material on the capillary surface that was not removed by 3 *M* guanidine–HCl or NaOH. Sufficient accumulation of these deposits will cause capillary blockage.

Changes in fermentation conditions that favor the accumulation of unprocessed rhGH in the *E. coli* cells were detected by this method by a reduction in the measured concentration of rhGH (Fig. 3). As unprocessed rhGH (rhGH with the leader peptide still attached) and rhGH inclusion bodies are not released into the extraction buffer, denaturing conditions would be required to solubilize these variants before analysis.

Affnity chromatography, using immobilized rhGH antibody and immobilized rhGH binding protein, was used to determine the purity of rhGH and its variants resolved from cell pellet extract by CZE. This capture technique also determined if other rhGH variants were present in the cell pellet extract that were not identified by spiking with known standards. Immobilized rhGH antibody has been used by previous investigators to capture rhGH from plasma

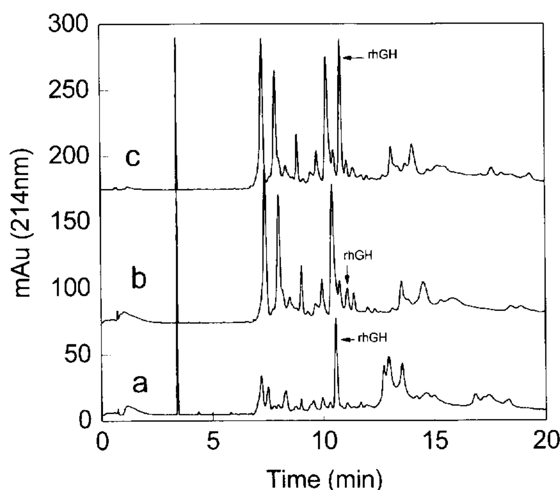

Fig. 3. CZE analysis of different fermentation conditions and their affect on rhGH production. (a) Conditions that favor rhGH production. (b) Conditions that favor unprocessed rhGH production. (c) rhGH standard spiked into the sample from (b). Analysis was performed using the phosphate deactivated fused-silica capillary at a field strength of 600 V/cm.

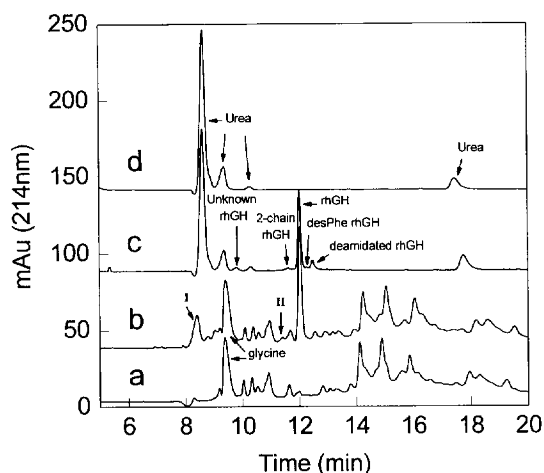

Fig. 4. CZE analysis of the affinity purified rhGH from cell pellet extract using an immobilized rhGHbp Sepharose column. (a) Flow-through fraction of the cell pellet extract loaded onto the immobilized rhGHbp Sepharose. (b) Cell pellet extract containing rhGH load material. (c) Affinity-purified rhGH, buffer exchanged into PI buffer containing 4 *M* urea, prior to CE analysis. (d) PI buffer containing 4 *M* urea. Peaks I and II in (b) are present in the cell pellet extract, but not in the rhGHbp affinity columns load flow though or elution pool. When cell pellet extract is buffer exchanged using a Centricon 10 concentrator, these peaks disappeared upon analysis by CZE. These peaks have low molecular masses which allow them to easily pass through a $10 \cdot 10^3$ molecular mass cut off membrane. The rhGH antibody affinity column had similar results to the rhGHbp affinity column. Analyses were performed at a field strength of 600 V/cm.

[10,13]. The rhGH binding protein which corresponds to the extracellular domain of the growth hormone receptor [19], has been characterized and shown to bind with high affinity to a variety of growth hormone variants [6]. In addition, by using the immobilized binding protein instead of the antibody for capture, we could determine which peaks had rhGH biological activity. With this approach, rhGH, $desPhe^1$ rhGH, deamidated rhGH, and an unknown rhGH variant peak were purified from *E. coli* cell pellet extract using either rhGH binding protein or rhGH antibody affinity chromatography methods.

Removal of rhGH, $desPhe^1$ rhGH, and deamidated rhGH from cell pellet extract by rhGH binding protein affnity chromatography, established that the phosphate deactivated fused-silica capillary CZE method resolved these proteins from cell pellet extract to a level of greater than 98% homogeneity (Fig. 4). The detection limit for rhGH and these variants was 5 μg/ml. The detection limit for two-chain rhGH was higher at 25 μg/ml as this variant was only partially resolved from other *E. coli* extract components. The unknown rhGH variant peak shown in Fig. 4, was also only partially resolved from the other *E. coli* contaminants, and glycine. Similar results were obtained using rhGH antibody affnity chromatography. The rhGH variants $desPhe^1Pro^2$ rhGH and dideamidated rhGH were not found in the cell pellet extract using either of the affinity chromatography techniques. This is in an agreement, with previous investigators [2,5]. The linearity and precision of the determination of rhGH concentration in the cell pellet extract by rhGH binding protein affinity chromatography was similar to the results obtained by the phosphate deactivated fused-silica capillary CZE method.

4. Conclusions

A capillary electrophoresis method, using a phosphate deactivated fused-silica capillary and a phosphate buffer, resolved rhGH and its variants from *E.*

coli cell pellet extract without requiring a pre-affinity purification step. Similar results should be obtained for less complex sample matrices such as osmotic-shocked *E. coli* cells or partially purified rhGH. Using affinity chromatography, we showed that rhGH, desPhe[1] rhGH, and deamidated rhGH were resolved from other cell pellet extract components to a level of greater than 98% homogeneity, with a detection limit of 5 μg/ml. The detection limits of other rhGH variants improved from 25 to 5 μg/ml as the co-migrating contaminants were removed. Changes in fermentation conditions that affected rhGH production were detected by this method. Currently, three different HPLC methods are required to detect rhGH, desPhe[1] rhGH, two-chain rhGH, and deamidated rhGH from cell pellet extract. Using the CZE method described in this paper, all of these variants were resolved from rhGH and cell pellet extract components in less than 20 min. Consequently, this method provides a very powerful tool to assess the impact of modified fermentation and isolation parameters on the production of rhGH.

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