Purification of a Pyrogen-Free Human Growth Hormone Antagonist

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Human growth hormone (hGH) is a polypeptide with 191 amino acids and a molecular mass of 22 kilodaltons. With the aid of computer molecular simulation, an hGH analog was created by altering an hGH gene to reflect the change of one amino acid (glycine [G] 120 to arginine [R]) within the third α-helix of the hGH molecule. This hGH analog, named hGHG120R, was found to be an hGH antagonist. It may have important implications in treating human conditions in which hGH levels are abnormally high, as found in type I diabetics. Several hundred milligrams of purified hGHG120R were needed to determine the biological activity of the antagonist in animal models. A multistep downstream process was developed to purify hGHG120R from cultured mouse L cells transfected with the hGHG120R gene. The process consisted of cell clarification, salt precipitation, membrane ultrafiltration, reversed phase high performance liquid chromatography, phase separation, and lyophilization. This work discusses the rationale for the design of the process and experimental results on the purification of hGHG120R using the process.

Key words: human growth hormone • animal cell culture • purification • serum

INTRODUCTION

Human growth hormone (hGH) is a single chain polypeptide comprised of 191 amino acids with a molecular mass of approximately 22,000 daltons.[1,4,5,10] The hGH gene has been altered and cloned into mouse L cells at Ohio University to produce hGH analogs. One of the genetically engineered hGH analogs, named hGHG120R, has one amino acid difference, namely, glycine (G) at position 120 in wild-type hGH is replaced by arginine (R). It possesses the same binding affinity to mouse liver membrane preparations as wild-type hGH. Surprisingly, transgenic mice which express this mutated hGH gene show a significant growth-suppressed phenotype.[3] The hGH analog, named hGHG120R, acts as an hGH antagonist. Figure 1 shows that wild-type hGH and hGHG120R have different retention times in a C4 reversed-phase high performance liquid chromatography (RP-HPLC) profile. Thus, there must be a significant conformation change in the hGHG120R molecule, even though it has only one amino acid difference from wild-type hGH.

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We selected animal cells to express the recombinant hGHG120R gene because hGHG120R is secreted by the cells as an active polypeptide. Other researchers have reported that when hGH analogs are produced in *Escherichia coli*, quite often the analogs are not expressed.[6] When expression does occur, the hGH molecules must be denatured and renatured before their biological activity is restored. These procedures are not required for hGH and hGH analogs secreted by mammalian cells. We are also engineering other hGH analogs, and it is impractical to establish a protein refolding protocol for each new hGH analog. However, if an analog eventually is ready for commercial production, *E. coli* may then become a feasible host organism.

To determine the biological activity of hGHG120R in animal models, several hundred milligrams of the purified peptide were needed. Because of the basic similarity between hGH and hGHG120R, existing techniques used in the purification of biosynthetic hGH may be helpful for the purification of hGHG120R. To date, hGH has been produced from recombinant *E. coli*,[9] *Bacillus subtilis*,[7] and *Saccharomyces cerevisiae*.[12]

Various separation techniques have been used to purify hGH from fermentation broths, including ultrafiltration, salt precipitation, size exclusion chromatography (SEC), ion-exchange, and RP-HPLC. Because hGHG120R is intended as an injectable drug, it requires a high purity and low pyrogen level. An HPLC step should be used near the end of the purification process to achieve a high product purity. Although HPLC has high resolution, its sample load is relatively small and it is also expensive. For an economic purification process, the product stream has to be concentrated and pretreated to remove the majority of impurities before it is applied to an HPLC column. This is especially important for the purification of proteins from serum-containing culture media, because serum proteins, such as albumin, may severely contaminate RP-HPLC columns, making column regeneration extremely difficult.

Pyrogens (bacterial endotoxins) are lipopolysaccharides that are an integral component of the outer cell surfaces of gram-negative bacteria. They are released from living or dead bacteria.[14] Pyrogens cause fever if introduced into the bloodstream of humans or other mammals. A safe parenteral (injectable) drug should be "pyrogen-free," meaning

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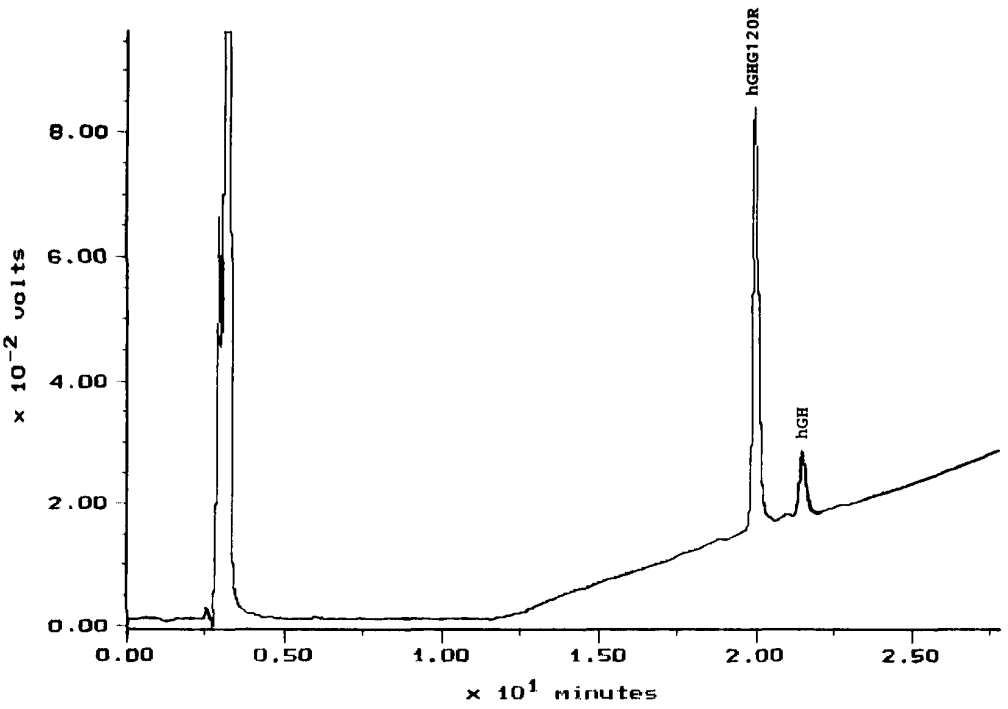

Figure 1. Retention time difference between hGH and hGHG120R in RP-HPLC. On the *x*-axis, 3.00 with a scaling factor of $\times 10^1$ means 3.00×10^1 min. The scaling factor for the *y*-axis should be interpreted similarly.

the pyrogen level should be below a set limit. According to the Food and Drug Administration (FDA), the limit is 5.0 EU/kg body weight per injection, that is, no more than 5.0 endotoxin units of pyrogen per kilogram of body weight can be introduced parenterally into a human or an animal. Because bacteria, dead or alive, are ubiquitous, a protein product often contains an unsafe level of pyrogens, unless measures are taken to avoid the introduction of pyrogens in the entire production process, or to remove pyrogens during the purification of the product. There are several common methods that can be used to remove pyrogens, which include dry heat (>250°C for 30 min), acid and base hydrolysis, and ultrafiltration. Ultrafiltration is often used to remove pyrogens from protein solutions due to its mild operating conditions. Most pyrogens can be effectively retained by an ultrafilter with a nominal molecular weight cut-off (NMWCO) of 100 kilodaltons.[14]

There have been no literature reports on preparative- or large-scale production of hGH or any hGH analog from cultured animal cells. hGH is unstable, easily losing its bioactivity.[11] We have noticed that hGHG120R is similarly unstable. Precautions must be taken to guard against the loss of bioactivity during purification. An hGH product is usually formulated into a dried powder form to preserve its potency.[11] Thus, lyophilization should be the last step in the purification process.

This work provides some details on the development of a downstream process for the purification of hGHG120R from cultured recombinant mouse L cells. A multistep process containing cell clarification, salt precipitation, membrane ultrafiltration, RP-HPLC, and lyophilization was developed for the production of several hundred milligrams of hGHG120R for animal testing. An actual production batch is used as an example to explain the process below.

EXPERIMENTAL

Animal Cell Culture

Animal cell culture was carried out using anchorage-dependent mouse L cells containing the recombinant hGHG120R gene. The cell line was engineered at Ohio University. It was found that hGHG120R is secreted by the cells in a bioactive form.[3] Twenty 850-cm^2 roller bottles (Corning Inc., Corning, NY) used for cell culture were maintained in a 5% CO_2 incubator (Model 3956, Forma Scientific, Marietta, OH) at 37°C. The cells were grown in antibiotic-free Dulbelcco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and 585 mg/L L-glutamine (Mediatech Inc., Washington, DC). Three percent Nu-Serum IV (Collaborative Research Inc., Bedford, MA) was added to the medium resulting in a 0.75% actual serum level.

The hGHG120R production protocol involved collecting 200 to 250 mL of medium from 10 roller bottles every other day. Each day's collection was divided into two pools from five roller bottles each, from which floating cells and cell debris were removed by centrifugation. The medium was

then stored at 4°C until enough was accumulated for a purification run (≈7 days worth of medium). In this manner, four pools each from five bottles were obtained every 2 days. If contamination was noted in any roller bottle, the pool with which it was associated was traced and discarded from further processing.

Downstream Process

The multistep process shown in Figure 2 was used to produce several hundred milligrams of hGHG120R. Figure 2 is a flowchart of the purification process. The process was designed to purify 10 to 15 L of culture medium containing approximately 3% to 5% serum for a single run. The initial concentration of hGHG120R in the culture medium was typically around 10 mg/L in the culture medium. The finished product was in a lyophilized form.

1. Removal of the cells from culture medium. Cell removal was carried out using centrifugation with a general-purpose refrigerated floor centrifuge (Sorvall RC5C, DuPont Sorvall, Bannockburn, IL). Two or more 500-mL plastic centrifuge bottles containing culture medium were spun at 6000 rpm for 30 min with a relative centrifugal force of 6100*g*.

2. Salt precipitation and precipitate resuspension. Ammonium sulfate was added to the clarified culture medium to precipitate hGHG120R. Typically, 350 g of ammonium sulfate was added to 1 L of culture medium. The mixture was stirred and then refrigerated overnight. After centrifugation, the precipitate was redissolved in water. Usually, 200 mL of water was needed to redissolve the precipitate obtained from 1 L of clarified culture medium.

3. 8k membrane ultrafiltration. A Mini-ultrasette® ultrafilter (Filtron Technology Corp., Northborough, MA) containing an 8k NMWCO Omega series membrane was used to concentrate the precipitate solution. The crossflow ultrafilter was coupled with a peristaltic pump. The 8k membrane retained the 22k hGHG120R molecules effectively. Small impurity molecules, such as salts, were allowed to permeate the membrane. The ultrafiltration was carried out in a 4°C refrigerator.

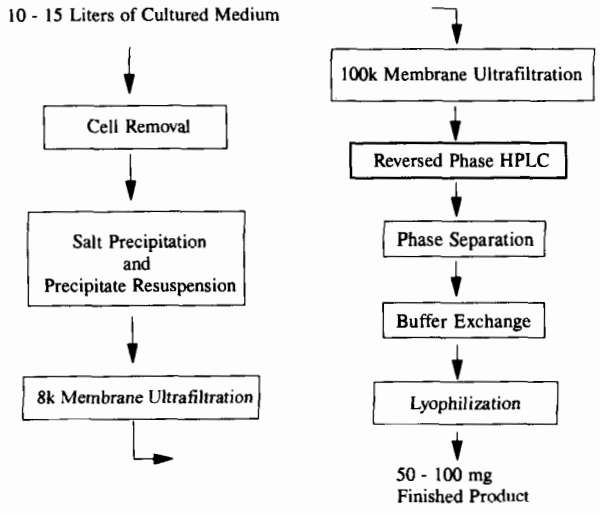

Figure 2. Process flowchart for the purification of hGHG120R.

4. 100k membrane ultrafiltration. The retentate from the 8k ultrafilter contained large amounts of serum proteins that tend to contaminate RP-HPLC columns. An Amicon (Amicon, Inc., Beverly, MA) 200-mL stirred-cell ultrafilter packed with an Amicon YM100 disk membrane (62 mm in diameter) was used to retain the serum proteins. The membrane had a NMWCO of 100k. Most hGHG120R molecules were able to permeate through the membrane. This step retained most of the pyrogens.

5. Reversed-phase HPLC. The permeate from the 100k membrane ultrafiltration was mixed with acetonitrile (ACN) and trifluoroacetic acid (TFA) to make a solution with 40% (v/v) ACN + 0.1% TFA. The solution was then applied to a preparative-scale Dynamax C4 RP-HPLC column (21.4 × 250 mm, 5 μm, pore size 300 Å, Rainin Instrument, Inc., Woburn, MA) for a final purification. A linear gradient of 40% ACN + 0.1% TFA to 80% ACN + 0.1% TFA in 60 min at 5 mL/min was used. A Waters (Millipore Corp., Bedford, MA) preparative HPLC system with a Model 486 UV-Vis detector was used to carry out the experiments. The detector wavelength was set at 220 nm. This Waters preparative system had a Model 600E quaternary gradient pump.

6. Low temperature phase separation. The hGHG120R fraction from the RP-HPLC step contained 60% to 70% ACN + 0.1% TFA. We discovered that, when the solution was stored in a −20°C freezer for several hours a phase separation occurred.[8] More than 99% of hGHG120R was in the water-rich bottom phase, probably because hGHG120R is relatively hydrophilic. The bottom phase contained some ACN and was not frozen during the process. This appears to be an easy and efficient way to remove more than half of the solvent.

7. Buffer exchange for the removal of ACN and TFA. Before the purified product was lyophilized, ACN and TFA were removed. This was achieved by diafiltration with water using a 100-mL Amicon stirred-cell ultrafilter in a 4°C refrigerator. The membrane used was an Amicon YM10 disk membrane (47 mm in diameter) with a NMWCO of 10k. The final retentate volume was around 40 mL.

8. Lyophilization. The retentate from the buffer exchange step was lyophilized using a Heto brand centrifugal evaporator (ATR Inc., Laurel, MD). This evaporator's centrifugal rotor can hold up to eight 50-mL plastic conical tubes.

HPLC Analysis of hGHG120R

A Waters (Millipore Corp.) dual-pump gradient HPLC system was used for the analysis of peptide concentrations during and after purification. This computer-controlled system had two Model 510 pumps, a Model U6K detector with

a 2.27 mL sample loop, and a Model 486 UV detector. The computer software was a Waters Baseline 810 package. A linear gradient, from 40% ACN in water + 0.1% TFA to 80% ACN in water + 0.1% TFA in 30 min at 1 mL/min, was used to achieve the separation on a Vydac C4 column (4.6 × 250 mm, 5 μm, pore size 300 Å). UV detection was set at a 220-nm wavelength. Injection volumes for dilute samples were 50 μL, and for more concentrated samples 20 μL.

To cross-check the purity of a final product, a TSKgel DEAE-5PW (7.5 × 75 mm) anion-exchange HPLC column (TosoHass, Montgomeryville, PA) and a Bio-Sil SEC 250-5 (7.8 × 300 mm) size exclusion HPLC column (Bio-Rad Laboratories, Hercules, CA) were chosen. A linear gradient of 0.1 to 0.5 *M* NaCl in glycine (pH 8) was used for the anion exchanger. Isocratic elution with 0.05 *M* NH_4HCO_3 (pH 7.8) was used for the size exclusion HPLC column.

Pyrogen (Endotoxin) Assay

The FDA-approved *Limulus Amebocyte* Lysate (LAL) test was used to quantify pyrogen levels in peptide solutions. The LAL test is based on an enzymatic reaction triggered by trace amount of endotoxins. The QCL-1000 chromagenic LAL test kit from BioWhittaker, Inc. (Walkersville, MD) contains a proenzyme from the LAL, a colorless substrate, and an *Escherichia coli* endotoxin standard. In a test tube, the proenzyme is converted to an active enzyme by endotoxins. This enzyme splits the colorless substrate to yield *p*-nitroaniline (pNA). The enzymatic reaction is stopped with acetic acid and the level of pNA is then measured photometrically at 405 to 410 nm. The amount of endotoxin is proportional to the level of pNA. A calibration curve was obtained using solutions with known concentrations of the *E. coli* endotoxin standard.

Radioreceptor Binding Assay

Competitive binding assays were performed using the following protocol. Microsomal membranes from transgenic or nontransgenic littermates corresponding to 1 mg protein were incubated with ^{125}I hGH (0.5 ng/mL, specific activity = 115 μCi/μg) in addition to various amounts of unlabeled hGHG120R ranging from 1 ng to 1 μg in a total volume of 0.3 mL of assay buffer (20 m*M* HEPES, 10 m*M* $CaCl_2$, 0.1% bovine serum albumin, and 0.05% $NaNO_3$, pH 8.0). After a 3-h incubation period at room temperature, the reaction was stopped by adding 1 mL of ice-cold assay buffer, followed by spinning in a microcentrifuge at 10,000 rpm for 15 min. The pellets were then assayed for radioactivity. All assays were performed in triplicate and repeated three to four times.

RESULTS AND DISCUSSION

Objectives of Individual Steps in the Purification Process

The core of the purification process is the high resolution RP-HPLC step. To reduce the sample volume and the amount of impurities, especially serum proteins, fed to the RP-HPLC column, the sample is first concentrated and prepurified. All the steps after the RP-HPLC are polishing steps. To explain the process, data obtained for one of the batches will be discussed. The objective of each step in the process is explained as follows.

Step 1: The clarification step using a centrifuge removes the cells and cell debris to obtain the supernatant that contained the hGHG120R. The volume of the clarified culture medium was 15.4 L. The analytical RP-HPLC profile of a clarified culture medium sample is shown in Figure 3. The peaks in the retention time range of 17 to 20 min represent impurities introduced by the addition of serum to the culture medium.

Step 2: The salt precipitation step concentrated the sample. Significant amounts of impurities in the retention time range of 17 to 20 min were removed as shown by the RP-HPLC profile of the precipitate resuspension in water (Fig. 4). This was also indicated by the relatively large amount of solids that could not be resuspended in water after the salt precipitation. The volume of the precipitate resuspension was 3.1 L.

Step 3: The precipitate resuspension solution was concentrated using the 8k membrane. Figure 5 is the RP-HPLC profile of the retentate. It shows that serum proteins were also retained. They were to be removed before the solution was applied to the preparative RP-HPLC column. The volume of the 8k membrane retentate was 330 mL.

Step 4: The retentate from the 8k membrane ultrafiltration was filtered through the 100k membrane ultrafilter. Water was used for diafiltration. The permeate volume was 415 mL. Figures 6 and 7 are the RP-HPLC profiles of the 100k membrane permeate and retentate samples, respectively. The two figures indicate that most, but not all, serum proteins were in the retentate. Normally, in a downstream process, a membrane with a larger NMWCO is used before a membrane with a smaller NMWCO. In our process, the sequence is the opposite. This is because our existing 8k membrane ultrafilter had a much larger processing capacity than the 100k membrane ultrafilter. Another more important reason is that the 100k membrane served as a pyrogen removal step. If the 8k membrane is placed after the 100k membrane, there will be an increased chance for pyrogen contamination.

Step 5: The 100k membrane retentate was mixed with ACN and TFA and applied to the preparative RP-HPLC column. Figure 8 shows the analytical RP-HPLC profile of

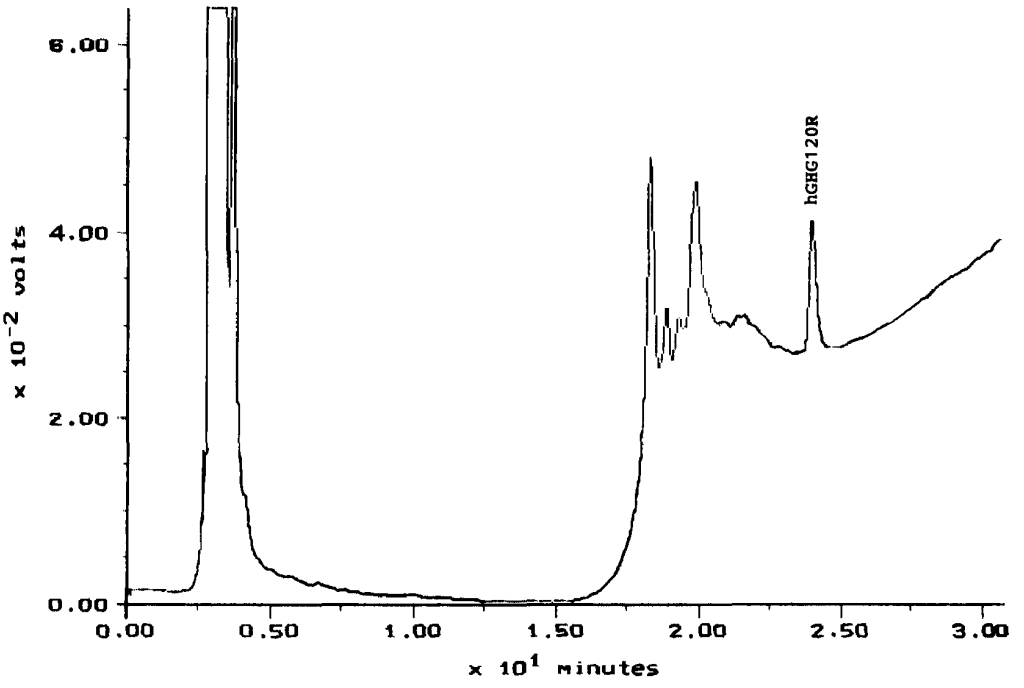

Figure 3. RP-HPLC profile of clarified culture medium before purification.

the hGHG120R fraction. This step achieved the needed purity. The volume of the hGHG120R fraction was 108 mL.

Step 6: The hGHG120R fraction from the RP-HPLC step was in a solution containing 60% to 70% ACN + 0.1% TFA. It was found that direct lyophilization using a centrifugal solvent evaporator damaged hGHG120R, possibly due to increased TFA concentration toward the end of the lyophilization, because TFA is less volatile than ACN. The

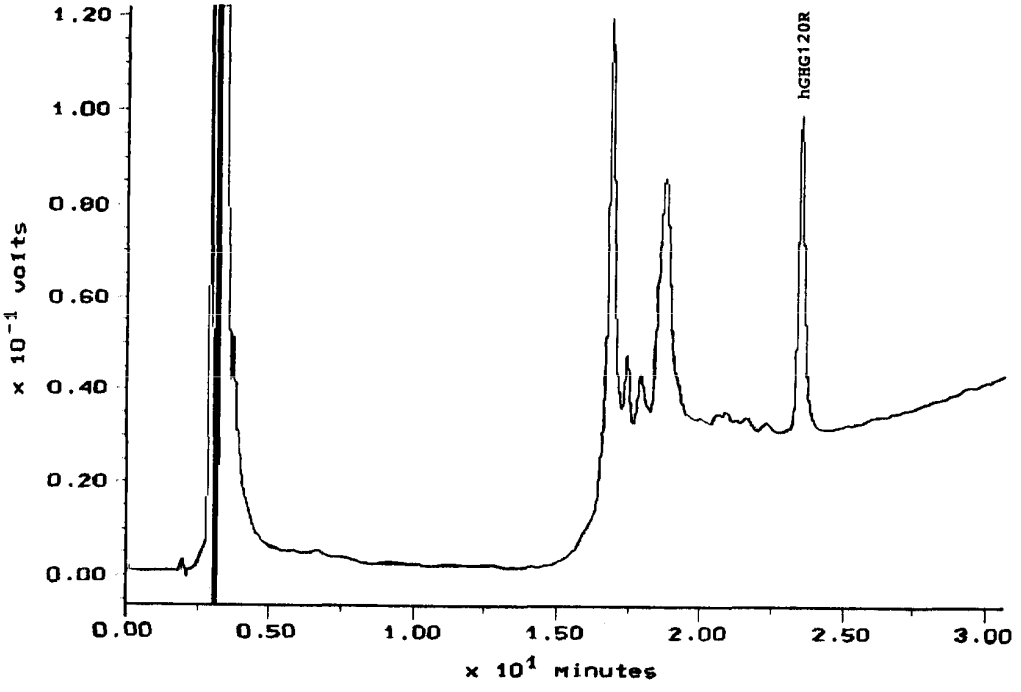

Figure 4. RP-HPLC profile of precipitate resuspension in water.

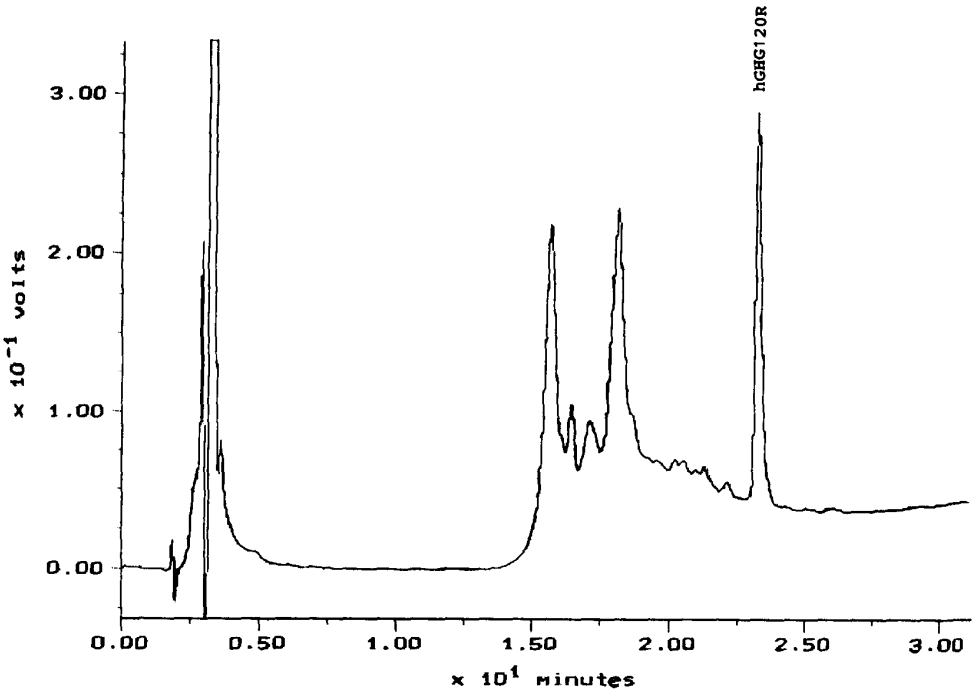

Figure 5. RP-HPLC profile of 8k membrane retentate.

phase separation step removed more than half of the ACN. This step also made it possible to use the Amicon stirred-cell ultrafilter for the buffer exchange step, since the device takes a maximum of around 30% ACN. Without the phase separation step, the sample would have to be diluted with water before using the Amicon ultrafilter. The bottom phase from the phase separation, which contained hGHG120R, had a volume of 72 mL.

Step 7: The buffer exchange step removed all the ACN and TFA through diafiltration with water using the 10k

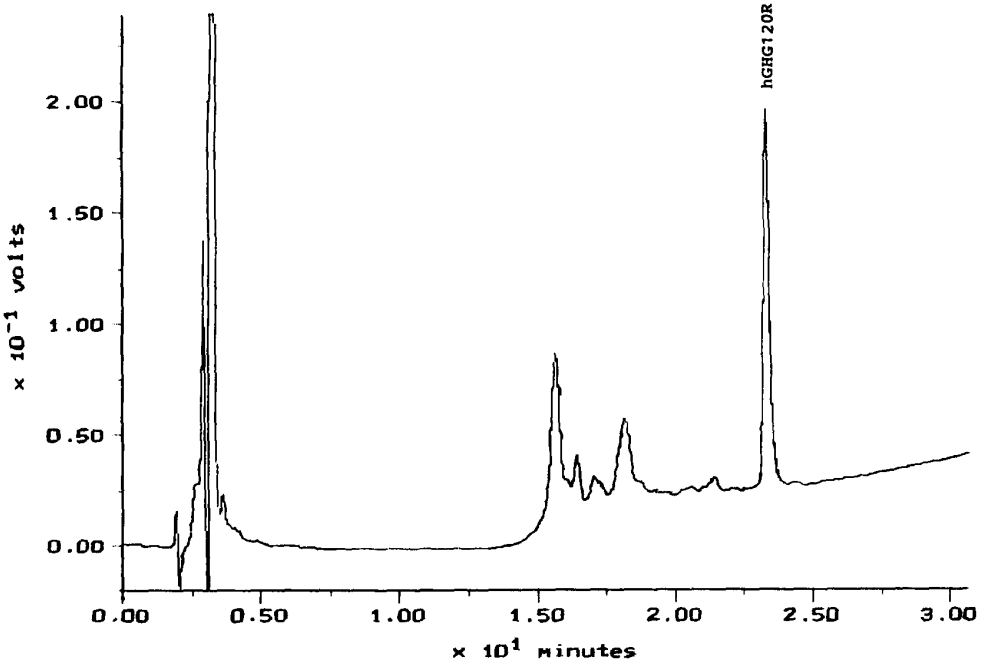

Figure 6. RP-HPLC profile of 100k membrane permeate.

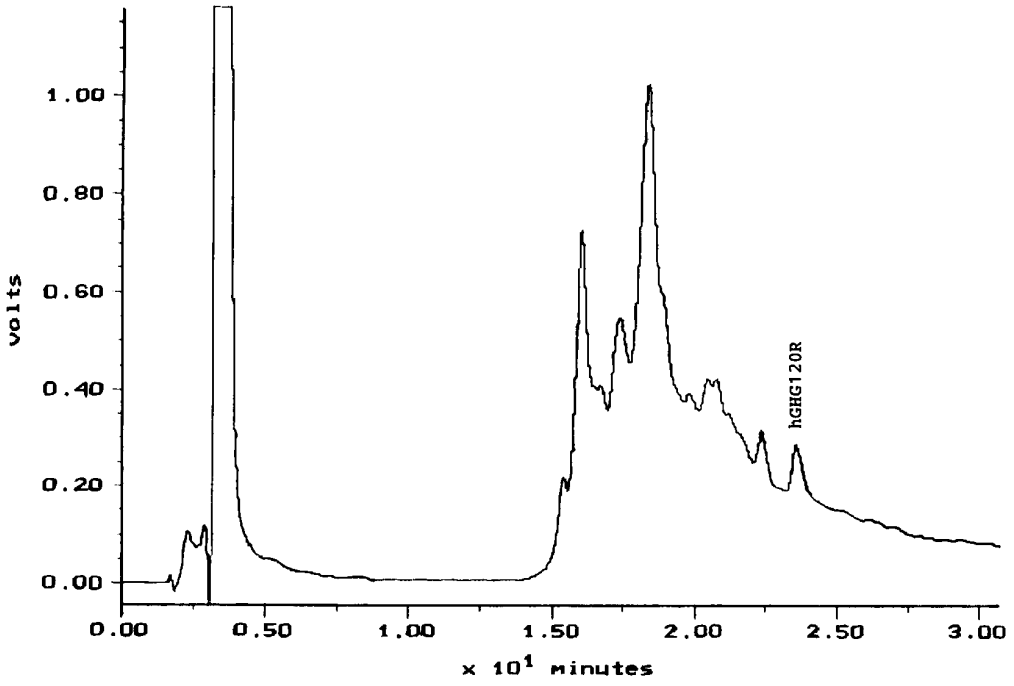

Figure 7. RP-HPLC profile of 100k membrane retentate.

membrane. After this step, the sample, which had a volume of 73 mL, was ready to be lyophilized.

Step 9: The lyophilization step produced a white flaky substance. The lyophilized form is desirable for storage and formulation of the GH family of peptides. Figure 9 shows the RP-HPLC profile of purified hGHG120R redissolved in a buffer solution.

Recovery Yields

Table I shows recovery yields of individual steps for the batch discussed above. All the recovery yields were calculated based on the hGHG120R weight obtained using analytical RP-HPLC. The average yield for each step was 95.5%, which was quite high. The overall recovery yield

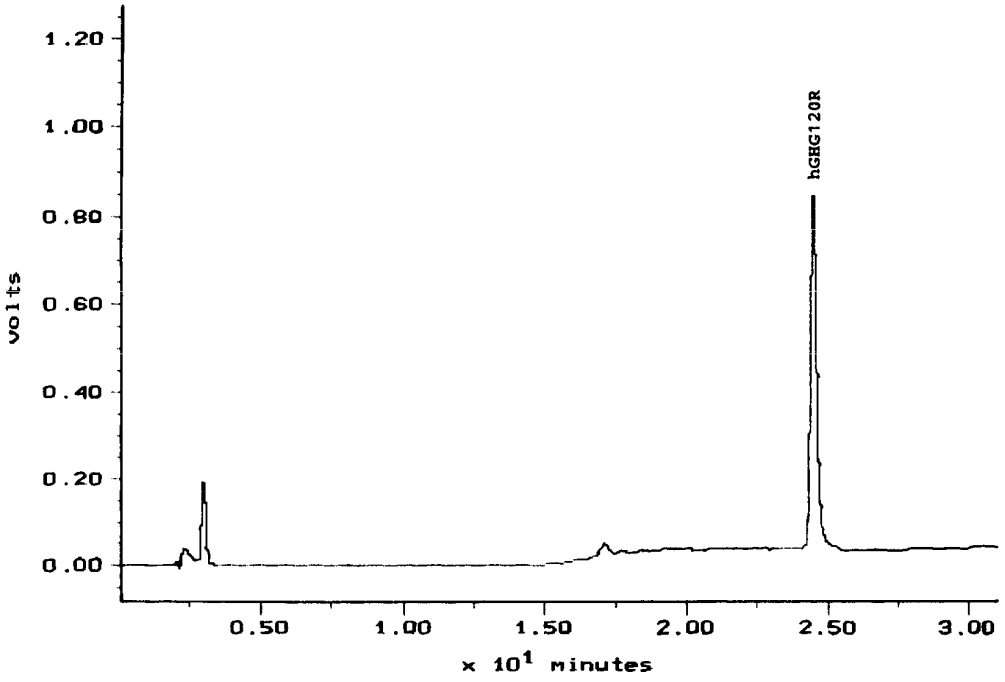

Figure 8. RP-HPLC profile of hGHG120R fraction after preparative RP-HPLC.

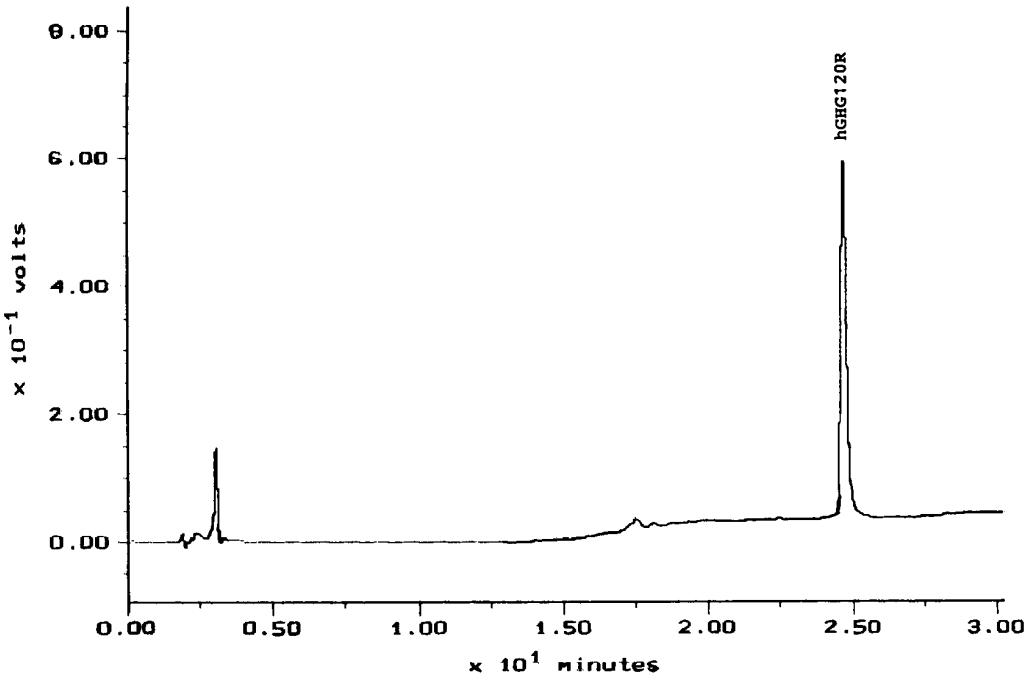

Figure 9. RP-HPLC profile of lyophilized hGHG120R redissolved in water.

was around 69.0%. The batch produced 91.4 mg of finished hGHG120R product. Because of the multistep nature, a very high recovery yield was extremely difficult to achieve. On the other hand, cutting down steps would compromise product purity, or affect the sustainability of the process.

Pyrogen Level

LAL tests showed that the relative pyrogen level for the retentate of the 8k membrane was 1.37 EU/mg hGHG120R. After the 100k membrane ultrafiltration, the pyrogen level was reduced to 0.07 EU/mg hGHG120R. The lyophilized end product, after being redissolved in pyrogen-free water, was found to have a relative pyrogen level of 0.3 EU/mg hGHG120R. If the dosage for a 20-g mouse is 0.2 mg hGHG120R, the pyrogen injection level will be 3.0 EU/kg

Table I. Recovery yield of each step.

	Recovery (%)
Cell removal	100%
Salt precipitation and resuspension	99.0%
8k Membrane ultrafiltration	94.4%
100k Membrane ultrafiltration	90.0%
RP-HPLC	96.2%
Phase separation	97.3%
Buffer exchange	97.2%
Lyophilization	90.2%
Average yield for each step	95.5%
Overall yield	*69.0%*

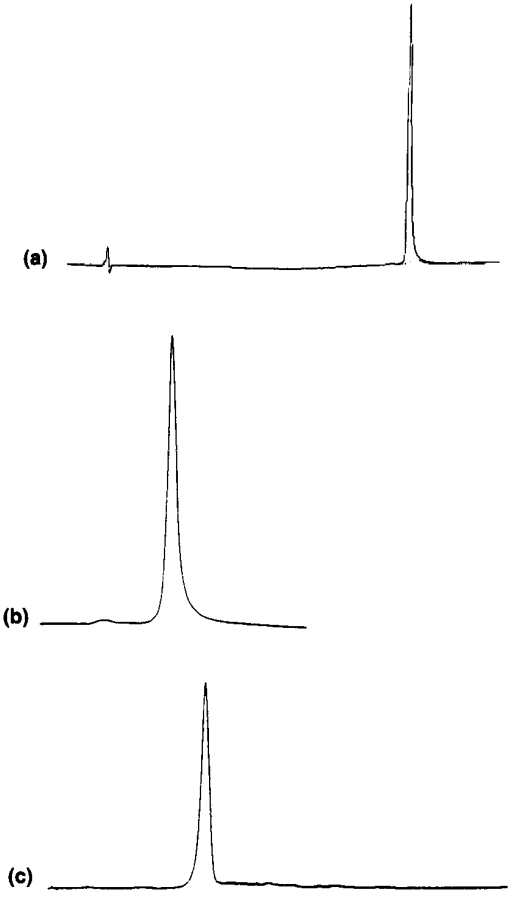

Figure 10. RP-HPLC, size exclusion HPLC, and ion-exchange HPLC profiles for a purified hGHG120R sample. (a) RP, (b) SEC, (c) IEC.

body weight, below the FDA limit of 5.0 EU/kg body weight.

Product Purity Analysis

Figure 9 shows that the product was relatively pure with only a small amount of impurity at the retention time of 17.4 min. The peak purity of the product was around 98%, which meets the preset technical specification of 95% purity. Previously, we adopted a size exclusion chromatography (SEC) step after the 8k membrane step to remove the serum proteins. SEC usually has a higher resolution than membrane ultrafiltration. A low pressure 44 × 1000 mm glass column packed with 1.2 L of Bio-Gel P-60 (Bio-Rad Laboratories) gel was used. The mobile phase was 0.05 *M* NH_4HCO_3 with a flow rate of 0.5 mL/min. Figure 10 shows the HPLC analyses of a product purified with a SEC step in the process. No significant impurity was detected by RP-HPLC, size exclusion HPLC, or ion-exchange HPLC. The SEC step enhances purity, but this step is time consuming.

Bioactivity Assays

Our radioreceptor binding assay showed that the purification process produced bioactive hGHG120R. It acted as an antagonist using a GH-dependent pp95 tyrosine phosphorylation induction assay.[13] Additionally, the product has been administered to diabetic lab mice via intraperitoneal injection. Initial results indicate that hGHG120R reduces the level of insulinlike growth factors in the test animals as predicted.

CONCLUSIONS AND RECOMMENDATIONS

A downstream process has been developed for the purification of hGHG120R from serum-containing culture media. The process is capable of producing multimilligrams of bioactive hGHG120R. This process can be further improved with some modifications. We are looking into the possibility of replacing the salt precipitation step with a crossflow membrane ultrafiltration system to increase the process volume. We are also considering use of a hydrophobic interaction chromatography step to remove serum proteins after the salt precipitation step. The centrifugation step may be replaced with a microfiltration step to reduce process time.

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