Single Step Recombinant Human Growth Hormone (rhGH) Purification from Milk by Peptide Affinity Chromatography

Soledad L. Saavedra
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956, Buenos Aires 1113 Argentina

CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113 Argentina

María C. Martínez Ceron
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956, Buenos Aires 1113 Argentina

CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113 Argentina

Silvana L. Giudicessi
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956, Buenos Aires 1113 Argentina

CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113 Argentina

Guillermina Forno
R&D Zelltek S.A., UNL, FBCB, Ciudad Universitaria, Paraje el Pozo, CC 242, Santa Fe, Argentina

María Belén Bosco
R&D Zelltek S.A., UNL, FBCB, Ciudad Universitaria, Paraje el Pozo, CC 242, Santa Fe, Argentina

Mariela M. Marani
IPEEC-CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas, Bvd. Brown 2915, 9120, Puerto Madryn, ChubutArgentina

Rosa Erra-Balsells
Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Pabellón II, 3er P., Ciudad Universitaria, Buenos Aires 1428 Argentina

CONICET, Universidad de Buenos Aires, Centro de Investigación en Hidratos de Carbono (CIHIDECAR), Facultad de Ciencias Exactas y Naturales, Pabellón II, 3er P. Ciudad Universitaria, Buenos Aires 1428 Argentina

Fernando Albericio
CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, Barcelona 08028 Spain

School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001 South Africa

Osvaldo Cascone
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956, Buenos Aires 1113 Argentina

CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113 Argentina

Silvia A. Camperi
Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología, Junín 956, Buenos Aires 1113 Argentina

CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires 1113 Argentina

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Recombinant human growth hormone (rhGH) is used for the treatment of several pathologies, most of them related to growth. Although different expression systems can be used for its production, the milk from transgenic cows is one of the most interesting due to the high rhGH level achieved (5 g/L). We have designed and synthesized short peptides (9 or 10

Correspondence concerning this article should be addressed to Silvia A. Camperi at scamperi@ffyb.uba.ar.

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amino acid long) using Fmoc chemistry and studied their ability to purify rhGH from milk once immobilized on an agarose support. Using spiked milk with the hormone as a sample, rhGH was purified with 88% yield and 92% purity in a single step with a fold purification of 4.5. © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 34:999–1005, 2018

Keywords: recombinant biopharmaceuticals, downstream processing, peptide ligands, transgenic cow milk, biologics, solid-phase peptide synthesis

Introduction

Although growth hormone has been therapeutically used since late 1950s.[1] its recombinant production has increased dramatically its application, especially as replacement therapy for the treatment of growth hormone deficiency disorders.[2]

The rhGH production in the milk of cloned transgenic cows at levels of up to 5 g/L has been reported over 10 years ago.[3] As other recombinant therapeutic proteins, its parenteral administration requires a high degree of purification. However, as milk is a very complex medium, rhGH purification from this starting material requires many steps and, until today, prokaryote expression systems are preferred for its production.[4,5]

Affinity chromatography (AC) allow the reduction in the number of purification steps due to the specific interaction of the target protein with the immobilized ligand. However, to be cost-effective, the ligand must be inexpensive. Small peptides of 5–12 amino acids are ideal affinity ligand candidates for industrial separations as they can be synthesized by standard chemistry at low cost[6] and they are more chemically and physically stable than protein ligands.[7] Immobilized metal ions are also inexpensive ligands and it has been previously demonstrated that Cu(II)-iminodiacetic acid adsorbed hGH.[8] However, chelating ligands lack selectivity and contaminant proteins are also adsorbed. To increase selectivity a His-tag has been added to rhGH,[9] but additional steps are needed in the bioprocess to cleave the tag and separate the protein from the byproducts before its pharmaceutical use.

The aim of this work was to design a suitable peptide ligand for rhGH purification from milk by AC.

Materials and Methods

Materials

Pure rhGH obtained from recombinant *Escherichia coli* expression was from Zelltek S.A. (Santa Fe, Argentina). Skimmed milk was from SanCor (Córdoba, Argentina). 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (TBTU), and Fluorenylmethyloxycarbonyl (Fmoc) protected amino acid were acquired from Peptides International Inc. (Louisville, KY, USA). α-Cyano-4-hydroxycinnamic acid (CHCA), *N,N*-diisopropylethylamine (DIPEA), 4-(*N,N*-dimethylamino)pyridine (DMAP), 1,3-diisopropylcarbodiimide (DIPCDI), triisopropyl silane (TIS), bovine serum albumin (BSA), and 4-chloro-naphtol were from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin peroxidase conjugate (SA-POD) was from Roche (Basel, Switzerland). *N*-Hydroxysuccinimide EZ-Link® Sulfo-*N*-hydroxysuccinimide (NHS)-LC-Biotin and SulfoLink™ coupling Resin were from ThermoFisher Scientific Inc. (Rockford, IL, USA). RPMI-1640 medium and fetal calf serum (FCS) were from Gibco™, ThermoFisher Scientific (Rockford, IL, USA). Sephadex G-25 PD-10 desalting columns were from GE Healthcare (Waukesha, WI, USA). Bio-Rad Protein Assay Dye Reagent (Bradford reagent) was from Bio-Rad Laboratories (Philadelphia, PA, USA). All other reagents were AR grade.

Fmoc peptide synthesis on hydroxymethylbenzamide-ChemMatrix (HMBA-CM) resin

Sequences involved in the interaction of human growth hormone (hGH) with its receptor were evaluated.[10] Using the Fmoc strategy, the peptides ligands: Ac-Asp-Ile-Arg-Gly-Trp-Glu-Val-Ala-Gly (L_A), Ac-Glu-Trp-Gly-Arg-Ile-Asp-Val-Ala-Gly (L_B), Ac-Pro-Ile-Trp-Ile-Arg-Trp-Gly-Val-Ala-Gly (L_C), and Ac-Gly-Trp-Arg-Ile-Trp-Ile-Pro-Val-Ala-Gly (L_D) were synthesized on 50 mg of HMBA-CM resin (0.63 mmol/g).[11] Val-Ala-Gly was introduced as a spacer arm to facilitate rhGH interaction with the peptide ligand. The C-termini Gly was incorporated as per Mellor et al.[12] using DIPCDI in the presence of DMAP in *N,N*-dimethylformamide (DMF). The other protected amino acids were incorporated with TBTU/DIPEA. Piperidine 20% in DMF was used for Fmoc removal. Washings between each coupling and deprotection step were performed with DMF and dichloromethane (CH_2Cl_2). *N*-terminus acetylation was performed after peptide elongation by adding acetic anhydride (Ac_2O) (10 eq) and DIPCDI (10 eq) in CH_2Cl_2. Protecting groups removal was performed with trifluoroacetic acid (TFA)/TIS/H_2O (95:2.5:2.5 v/v/v) during 2 h.

rhGH labeling

EZ-Link® Sulfo-NHS-LC-Biotin was coupled to rhGH as indicated in the manufacturer's protocol.[13]

Evaluation of rhGH binding to each peptidyl-ChemMatrix resin

Each peptidyl-resin was evaluated in a separated column fitted with a polyethylene porous disk. The peptidyl-resin beads were subsequently washed five times during 1 min with CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), DMF/H_2O (7:3, 5:5, 3:7), and H_2O (5 × 1 min). Afterwards, peptidyl-resins were blocked with 10% skim milk, 2% BSA, in phosphate buffered saline (PBS), pH 6.8 and washed 5 × 1 min with PBS-Tween (0.05% Tween 20 in PBS). Next, they were incubated during 1 h with the rhGH coupled with biotin in PBS-Tween. Then, after thoroughly washing with PBS-Tween (5 × 1 min), the peptidyl-resin beads were incubated with SA-POD (1 U/mL) in PBS-Tween for 1 h. After that, they were thoroughly washed with PBS-Tween (5 × 1 min) and PBS (5 × 1 min). Finally, they were revealed with a mixture of 1 mL solution A (3 mg 4-chloro-naphtol in 1 mL CH_3OH) and 4 mL solution B (4 mL PBS containing

20 μL of 30 vol H_2O_2). After 5 min, peptidyl-resin beads with rhGH adsorbed turned violet. Portions of each peptidyl-resin were also evaluated performing the same protocol but without the addition of labeled rhGH. All the procedure was performed at room temperature.

Preparation of AC matrices

Solid phase synthesis of the peptide ligands Ac-Glu-Trp-Gly-Arg-Ile-Asp-Val-Ala-Gly-Cys-NH_2 (L_B) and Ac-Gly-Trp-Arg-Ile-Trp-Ile-Pro-Val-Ala-Cys-NH_2 (L_D) was performed in polypropylene columns fitted with a polyethylene porous disk by the Fmoc chemistry as has been previously described.[11] The peptides were synthesized on solid phase as amides using the Rink-amide resin to avoid polymerization and other side-reactions during its attachment to the chromatographic support. Three-fold excess of the amino acids with TBTU/DIPEA were added at each coupling step and Fmoc was removed with piperidine 20% in DMF. Washings between each step of coupling and deprotection was performed with DMF and CH_2Cl_2. Peptide ligands were separated from the solid support and their side-chain protecting groups were removed by treatment with TFA/TIS/H_2O (95:2.5:2.5) for 2 h. Next, peptides were precipitated with cold diethyl ether and subsequently dissolved in water and lyophilized. To ensure site-directed immobilization of the peptides on the chromatographic support, a Cys was added at the C-terminus and the amino terminal was acetylated. Each peptide was immobilized on SulfoLink™ Coupling Resin according to the manufacturer's protocol.[14]

Mass spectrometry analysis

Peptide analysis was performed by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) using a Ultraflex II TOF/TOF (Bruker Daltonics GmbH, Leipzig, Germany). A 1 μL aliquot of peptide solution was loaded onto the sample plate, air-dried at room temperature and then 1 μL of CHCA 4 mg/mL in CH_3CN/H_2O (1:1) with 0.1% TFA was added on the dry sample layer. Mass spectra were obtained using the MS reflector positive-ion mode and the LIFT device in the MS/MS mode. The parameters used for MS/MS spectra acquisition were: ion source voltage 1 = 8.0 kV, ion source voltage 2 = 7.2 kV, lens voltage 3.6 kV, LIFT voltage 1 = 19 kV, and LIFT voltage 2 = 3.4 kV.

AC with pure rhGH

Samples of 200 μL of pure rhGH 2.4 mg/mL (Zelltek SA) in adsorption buffer were loaded to peptidyl-columns (0.5 × 5 cm) filled with L_B-agarose or L_D-agarose. Each column was washed with adsorption buffer at a flow rate of 0.5 mL/min until the absorbance at 280 nm achieves baseline value. Afterwards, the elution of the bound protein was accomplished by addition of 100 mM sodium acetate buffer, pH 3.8, 250 mM NaCl, at the same flow rate. Different adsorption buffers were evaluated: (a) 100 mM sodium acetate buffer pH 5.6; (b) 20 mM sodium phosphate buffer pH 5.6, 7.0 and 8.0; (c) 50 mM Tris–HCl buffer pH 9.0; (d) 25 mM glycine pH 9.7.

Protein analysis

Total protein concentration was measured with Bradford reagent. rhGH concentration was measured by Dot Blot at room temperature. A calibration curve was done using successive dilutions of a 5 μg/mL standard of rhGH in Tris-buffered saline (TBS) and spotted using 50 μL on a previously TBS-immersed nitrocellulose membrane. The same procedure was followed with the samples to analyze. Afterwards, the membrane was immersed in a BSA-TBS blocking solution (1% bovine serum albumin in buffer Tris–HCl saline) pH 7 during 1 h, washed 2 × 3 min with TBA and incubated 1 h with anti-rhGH rabbit mAb in BSA-TBS. The membrane was subsequently washed 2 × 3 min with TBA and incubated with a 1:1000 peroxidase-conjugated anti-rabbit IgG (DAKO, P0448) in TBS-BSA 0.1% (p/v) for 1 h. A final 2 × 3 min wash with TBS was done and the samples were developed using a mixture of 1 mL solution A (3 mg 4-chloro-naphtol in 1 mL CH_3OH) and 4 mL of solution B (4 mL PBS containing 20 μL of 30 vol H_2O_2).

rhGH purification from milk

A 1/10 dilution of skimmed milk in 50 mM Tris–HCl buffer, pH 9.0, was spiked with rhGH to obtain a concentration of 0.5 mg rhGH/mL. The chromatography was performed by loading 1 mL of the sample on the L_D-agarose column (0.5 × 5 cm) previously equilibrated with 50 mM Tris–HCl buffer, pH 9.0. The column was washed with equilibrating buffer at a flow rate of 0.5 mL/min until the absorbance at 280 nm achieves baseline value. Next, bound protein elution was accomplished by application of 100 mM sodium acetate buffer, pH 3.8, 250 mM NaCl, at the same flow rate. The chromatographic samples were desalted with Sephadex G-25 resin and lyophilized for further analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide) was performed as per Laemmli[15] and stained with Coomassie Blue following the standard procedure.

Bioassay to measure the activity of rhGH

Recombinant human growth hormone biological activity was measured on International Units (IU) of biological activity per volume unit (IU/mL) and obtained by comparison of dose–response curves of samples and an international standard of known activity (NIBSC 98/574), by applying a parallel line model. Rat lymphoma cell line Nb2 was cultured at 37°C in plastic flasks in RPMI-1640 medium supplemented with 10% FCS. Cells were washed with FCS-free growth medium and maintained in washing media during 24 h. The next day, serial dilutions of hGH standard from 9.76×10^{-6} to 3.05×10^{-7} IU/mL or serial dilutions of rhGH test samples in assay medium (RPMI-1640 medium supplemented with 10% horse serum), were added in 96-well plates. Then, cells were seeded in the plates at a density of 5×10^3 cells/well in a final volume of 100 mL of assay medium. After 72 h incubation at 37°C, bioactive responses were determined with a kinetic microplate reader, reading optical densities at the test wavelength of 550 nm and a reference wavelength of 650 nm to correct for differential scattering.

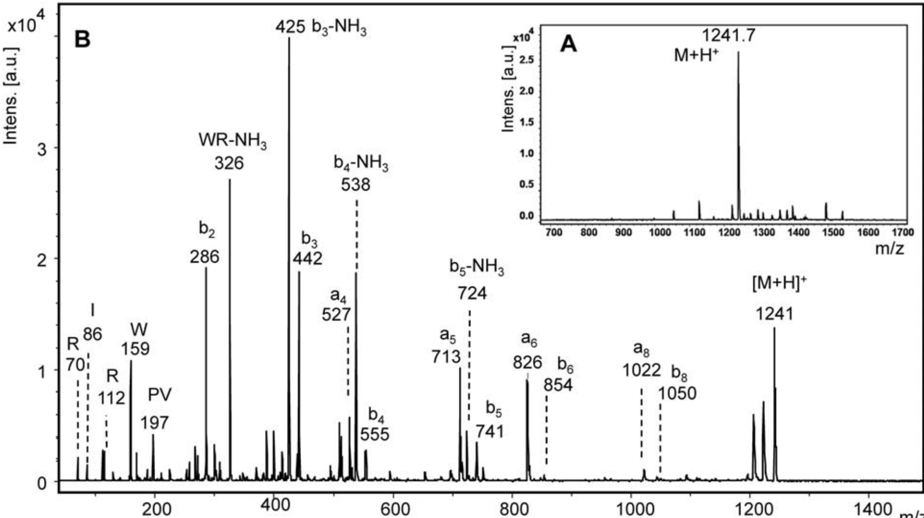

Figure 1. (A) MALDI mass spectrum and (B) MALDI MS/MS mass spectrum of the peptide Ac-GWRIWIPVAC-NH$_2$ ([M + H]$^+$, m/z = 1,241.7).

A total of 1 μL sample was loaded onto the sample plate, air dried at room temperature and then 1 μL matrix solution was added to the spot (sandwich method).

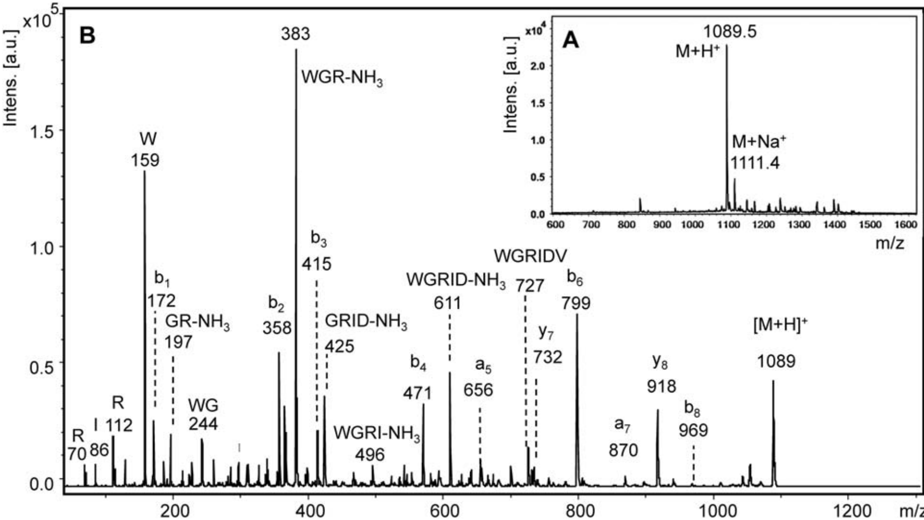

Figure 2. (A) MALDI mass spectrum and (B) MALDI MS/MS mass spectrum of the peptide Ac-EWGRIDVAC-NH$_2$ ([M + H]$^+$, m/z = 1,089.5, [M + Na]$^+$, m/z = 1,111.4).

A total of 1 μL sample was loaded onto the sample plate, air dried at room temperature and then 1 μL matrix solution was added to the spot (sandwich method).

Results and Discussion

Peptide ligand design

The peptides were designed from previous studies of the interaction of hGH with its receptor, reported by Clackson et al.,[10] in which the interaction site in the binding protein had been described with full detail of the amino acids side-chains involved. Four peptides containing the amino acids involved in the affinity site were evaluated: Asp-Ile-Arg-Gly-Trp-Glu, Glu-Trp-Gly-Arg-Ile-Asp, Pro-Ile-Trp-Ile-Arg-Trp-Gly and Gly-Trp-Arg-Ile-Trp-Ile-Pro.

Evaluation of rhGH binding to each peptidyl-ChemMatrix resin

The peptidyl-resin beads were incubated with rhGH-biotin and the adsorption of the hormone to each peptide was qualitatively detected with streptavidin-peroxidase conjugate (SA-POD) and 4-chloro-naphtol. Positive beads with the rhGH adsorbed turned violet while those without the protein adsorbed remained colorless. Only the peptidyl resin beads with immobilized L_B and L_D turned violet, showing a positive reaction, while beads with immobilized L_A and L_C showed no color change (negative reaction).

Ligand peptides mass spectra

Positive peptides were synthesized in larger quantities for their immobilization on agarose. Figures 1 and 2 show their MS and MS/MS spectra. The main peak corresponded to the m/z of the peptides synthesized. Other significant signals were not observed, demonstrating the purity of the peptides (Figures 1A and 2A). Peptide sequences could be deduced from the tandem mass spectra demonstrating the identity of each peptide (Figure 1B and 2B).[16]

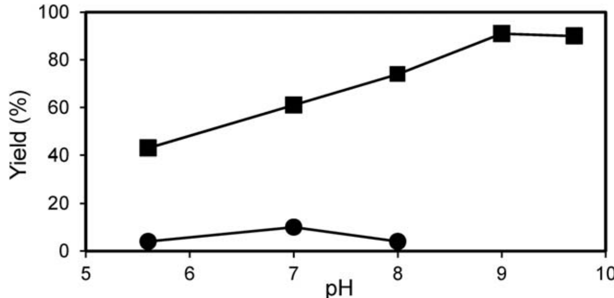

Figure 3. Influence of pH on rhGH chromatographic performance.

Samples of rhGH in adsorption buffer, with a range of pHs from 5.6 to 9.7, were loaded on columns filled with L_B-agarose (•) or L_D-agarose (■). The columns were washed with equilibrating buffer at a flow rate of 0.5 mL/min until the absorbance at 280 nm achieves baseline value. Thereafter, the elution of the bound protein was accomplished by applying 100 mM sodium acetate buffer, pH 3.8, 250 mM NaCl, at the same flow rate.

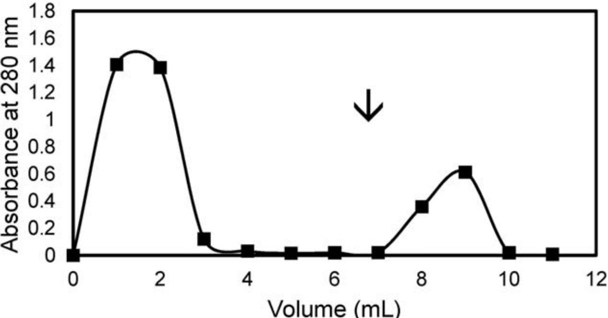

Figure 4. Chromatogram obtained by loading a sample of skimmed milk diluted 1/10 spiked with 0.5 mg/mL rhGH, on a L_D-agarose column (0.5 × 5 cm).

The column was washed with 50 mM Tris–HCl buffer, pH 9.0, at a flow rate of 0.5 mL/min until the absorbance at 280 nm achieves baseline value. Thereafter, elution of the bound protein was accomplished with 100 mM sodium acetate buffer, pH 3.8, 0.25 M NaCl, at the same flow rate. The arrow indicates the buffer change.

rhGH adsorption evaluation on peptide chromatography supports

Samples of rhGH were loaded on chromatographic columns with L_B-agarose or L_D-agarose at different pHs between 5.6 and 9.7. All the mass of rhGH loaded was recovered in the pass-through, wash and elution fractions. Figure 3 shows the yield of the elution peak of rhGH at these pHs. L_B-agarose showed a poor performance under all the assayed conditions, in which most of the rhGH was recovered in the pass-through and wash fractions. The addition of salt to the buffer did not improve the adsorption degree. On the other hand, the protein was highly adsorbed to L_D-agarose, in which most of the rhGH was recovered in the elution fraction. The yield increased with the pH, showing its better performance when using 50 mM Tris–HCl, pH 9.0, buffer.

Although structural analysis of the interaction of the hGH with its receptor helped to design the peptides, empirical evaluation confirmed that only one of them was useful as affinity ligand probably because the conformation of the peptide sequence on the chromatographic matrix differs from the conformation of the same sequence as part of the whole protein.

rhGH AC purification from milk

The use of milk as a vehicle for the expression of recombinant protein has the advantage of high concentrations of soluble recombinant protein. However, the milk caseins form aggregates with the calcium which behaves as micelles causing chromatographic matrix fouling. Considering the high concentration of rhGH production in the milk of cloned transgenic cows (5 g/L)[3] an easy way to avoid matrix pores blockage is to dilute the milk 1/10 obtaining a final concentration of rhGH of 0.5 g/L. Figure 4 shows the chromatogram obtained after loading the milk sample, spiked with rhGH, on the L_D-agarose column. SDS-PAGE analysis (Figure 5) shows the high effectiveness of the peptide AC for rhGH purification.

According to Dot-Blot analysis, rhGH was adsorbed and eluted with the elution buffer, while most milk proteins were recovered in the pass-through and wash fractions. The purity (%) defined as the amount of target protein (mg) over the

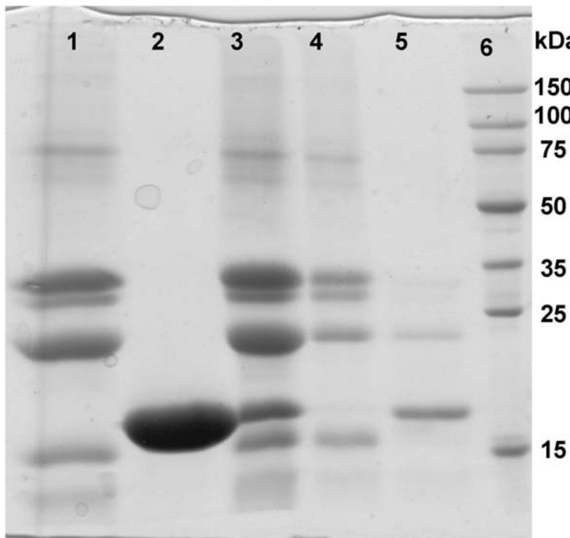

Figure 5. SDS-PAGE of the chromatographic fractions of milk spiked with rhGH.

Lane 1: skimmed milk; Lane 2: pure rhGH; Lane 3: skimmed milk spiked with rhGH; Lane 4: pool of pass-through and wash fractions; Lane 5: elution fraction; Lane 6: molecular weight marker.

amount of total protein (mg) was 92%. To analyze the impact of the purification process on the hormone, the activity of rhGH in the milk spiked with rhGH and in the eluted fraction were measured. Total rhGH activity in the spiked sample loaded to the column, defined as the biological activity in IU/mL multiplied by the volume, was 1.57 IU while in the elution fraction was 1.38 IU, corresponding to the 88% of the rhGH loaded to the column. The specific activity defined as the total rhGH activity (IU) divided by the total protein (mg) before and after the AC step were 0.61 and 2.76 (IU/mg), respectively. Fold purification defined as the specific activity after the AC step relative to the specific activity before the AC step, was 4.5. These results are resumed in the purification table (Table 1).

Column performance did not change after repeating the chromatography process. Although there are many proteases

Table 1. Purification Table of a Sample of Milk Spiked with rhGH Loaded on Agarose with Ac-Gly-Trp-Arg-Ile-Trp-Ile-Pro-Val-Ala-Cys-NH_2 (L_D) Immobilized

	Total protein (mg)	rhGH (mg)	Purity %*	Total activity (IU)	Specific activity (IU/mg)†	FP‡	Yield (%)§
Spiked milk	2.56	0.51	20	1.57	0.61	1	100
Elution fraction	0.50	0.46	92	1.38	2.76	4.5	88

*Purity (%) = (amount of target protein/amount of total protein) × 100.
†Specific activity = total rhGH activity/amount of total protein.
‡Fold purification (FP) = specific activity in the elution fraction relative to the specific activity in the milk sample.
§Yield (%) = (total rhGH activity in the elution fraction/total rhGH activity in the milk sample) × 100.

in milk, they bound predominately to milk proteins, such as caseins, which are preferred substrates for many proteases. Thus, high concentration of milk proteins, such as caseins, competitively inhibit proteolysis of recombinant proteins in the milk. In the same way, short peptide ligands immobilized on the chromatographic matrix are not degraded either.[17]

In the previous report from Melo et al.,[18] the rhGH purification form milk comprises many chromatographic steps: two ionic exchange chromatographies, reverse phase chromatography and molecular exclusion chromatography. Although there are reported methods to achieve a high purification degree, they consisted on increasing IMAC selectivity using a His-tag fused to the rhGH, but additional steps are needed in the bioprocess to cleave the tag and separate the protein from the byproducts before its pharmaceutical use.

Conclusions

These results show that AC with the small peptide designed immobilized as ligand is a very good strategy for the purification of rhGH from the milk of transgenic cows.

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Notation

Ac_2O = acetic anhydride
AC = affinity chromatography
BSA = bovine serum albumin
CHCA = α-cyano-4-hydroxycinnamic acid
DIPCDI = 1,3-diisopropylcarbodiimide
DIPEA = *N,N*-diisopropylethylamine
DMAP = 4-(*N,N*-dimethylamino)pyridine
DMF = *N,N*-dimethylformamide
FCS = fetal calf serum
Fmoc = fluorenylmethyloxycarbonyl
HMBA-CM = hydroxymethylbenzamide-ChemMatrix
HOBt = 1-hydroxybenzotriazole
mAb = monoclonal antibody
MALDI = matrix-assisted laser desorption/ionization
MS = mass spectrometry
NHS = N-hydroxysuccinimide
L_A = Ac-Asp-Ile-Arg-Gly-Trp-Glu-Val-Ala-Gly
L_B = Ac-Glu-Trp-Gly-Arg-Ile-Asp-Val-Ala-Gly
L_C = Ac-Pro-Ile-Trp-Ile-Arg-Trp-Gly-Val-Ala-Gly
L_D = Ac-Gly-Trp-Arg-Ile-Trp-Ile-Pro-Val-Ala-Gly
PBS = phosphate buffered saline
rhGH = recombinant human growth hormone
SA = spacer arm
SA-POD = streptavidin peroxidase conjugate
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBTU = 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide
TFA = trifluoroacetic acid
TIS = triisopropyl silane
TOF = time of flight
TSB = Tris-buffered saline

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