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RESEARCH ARTICLE

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Recombinant human follicle stimulating hormone purification by a short peptide affinity chromatography

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Peptide KVPLITVSKAK was selected to design a synthetic ligand for affinity chromatography purification of recombinant human follicle stimulating hormone (rhFSH), based on the interaction of the hormone with the exoloop 3 of its receptor. The peptide was acetylated to improve its stability to degradation by exopeptidases. A cysteine was incorporated at the C-termini to facilitate its immobilization to the chromatographic activated SulfoLink agarose resin. A sample of crude rhFSH was loaded to the affinity column, using 20 mM sodium phosphate, 0.5 mM methionine, and pH 5.6 and 7.2 as adsorption and elution buffers, respectively. The dynamic capacity of the matrix was 54.6 mg rhFSH/mL matrix and the purity 94%. The percentage of oxidized rhFSH was 3.4%, and that of the free subunits was 1.2%, both in the range established by the European Pharmacopeia, as also were the sialic acid content and the isoforms profile.

KEYWORDS

affinity chromatography, biopharmaceuticals, downstream processing, peptides, solid phase peptide synthesis

1 | INTRODUCTION

The follicle stimulating hormone (FSH) is a heterodimer consisting of two subunits (α and β) that belongs to the pituitary glycoprotein hormone family.[1] Among the many FSH isoforms that circulate in blood, those with higher sialic acid content have greater lifetime and hence superior efficacy.[2] FSH is clinically used to induce ovulation in women and spermatogenesis in men, in assisted reproduction

Abbreviations: AA, amino acids; AC, affinity chromatography; Ac_2O, acetic anhydride; BSA, bovine serum albumin; CH_2Cl_2, dichloromethane; CHO, Chinese hamster ovary; DIPCDI, *N,N′*-diisopropylcarbodiimide; DIPEA, *N,N`*-diisopropylethylamine; DMAP, 4-(*N,N*-dimethylamino)pyridine; DMF, *N,N′*-dimethylformamide; DTT, dithiothreitol; EMA, European Medicines Agency; ESI MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethyloxycarbonyl; FP, fold purification; FSH, follicle-stimulating hormone; HCPs, host cell proteins; HMBA-CM, hydroxymethylbenzamide-ChemMatrix; IEF, isoelectrofocusing; IVF, in-vitro fertilization; MS/MS, tandem mass spectra; pI, isoelectric point; rhFSH, recombinant human follicle stimulating hormone; RP, reverse phase; SA-POD, streptavidin peroxidase conjugate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPPS, solid phase peptide synthesis; TBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; uFSH, urinary FSH

technologies such as in-vitro fertilization (IVF) and intracytoplasmic sperm injection.[3] There are two FSH sources for clinical treatments: urinary FSH (uFSH) and recombinant human FSH (rhFSH). Nowadays, rhFSH is the first choice for IVF because it guarantees the clinical availability of a constant and biochemically pure FSH preparation, which increases the product safety profile.[1]

As FSH is parenterally administered, its purity must be high. Current methods for rhFSH purification include several chromatographic steps to obtain the required purity, decreasing the productivity and raising the cost of the process.[4,5] Therefore, the purification step represents as much as 30% to 80% of the total direct production cost of manufacturing.[6]

Affinity chromatography (AC) consists in the specific adsorption of target biomolecules onto ligands immobilized on chromatographic supports.[7] The high selectivity between an immobilized ligand and a target protein allows the purification of that protein from complex mixtures.

Short peptides (between 5 and 12 amino acids)[8] have been described as useful ligands for AC because of their low cost, easy chemical synthesis, and higher stability in comparison to protein ligands. Furthermore, the peptide-based chromatographic supports are more robust during elution and regeneration than protein-based affinity chromatographic supports that use monoclonal antibodies as ligands.[9]

Previously, Sohn et al studied the FSH receptor and examined the interaction of its exoloop 3 with the hormone, testing each amino acid of that exoloop by Ala substitutions.[10]

In the present work, the fragment sequence with the greatest affinity (580)KVPLITVSKAK(590) was selected with the aim of designing a synthetic ligand for rhFSH AC purification.

2 | MATERIALS AND METHODS

2.1 | Materials

Bio-Rad Protein Assay Dye Reagent (Bradford reagent) was from Bio-Rad Laboratories (Philadelphia, PA, USA). Trypsin, *N*,*N*′-diisopropylethylamine (DIPEA), *N*,*N*′-diisopropylcarbodiimide (DIPCDI), 4-(*N*,*N*-dimethylamino) pyridine (DMAP), trifluoroacetic acid (TFA), triisopropylsilane (TIS), bovine serum albumin (BSA), and 4-chloro-naphtol were from Sigma-Aldrich (Saint Louis, MO, USA). Streptavidin peroxidase conjugate (SA-POD) was from Roche (Basel, Switzerland). Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, Rink-amide-MBHA resin, and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) were purchased from Peptides International Inc. (Louisville, KY, USA); 15-mL 10 MWCO centrifuge concentrators and C18 spin columns were from Merck Millipore (Billerica, MA, USA). PD-10 desalting columns were from GE Healthcare (Waukesha, WI, USA). Octylphenyl-polyethyleneglycol and SulfoLink resin were from Thermo Fisher Scientific (Waltham, MA, USA). Native rhFSH N-glycans were enzymatically released with PNGase F purchased from New England Biolabs (Ipswich, MA, USA). These glycans were derivatized with the fluorophore 2-aminobenzamide from Sigma Aldrich (Saint Louis, MO, USA). The rhFSH was kindly donated by Zelltek S.A. (Santa Fe, Argentina). The FSH standard was from the European Directorate for the Quality of Medicines (EDQM Council of Europe; Strasbourg, France). Gonal-F was from Merck-Serono (Rockland, MA, USA). *N*,*N*′-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), and piperidine were of analytical grade.

2.2 | Peptide synthesis on hydroxymethylbenzamide-ChemMatrix (HMBA-CM)

The peptide Ac-KVPLTVSKAKVAG was synthesized by solid phase peptide synthesis (SPPS) on 50 mg of HMBA-CM resin (0.63 mmol/g).[11] The C-termini Gly was incorporated as per Mellor et al[12] using DIPCDI in the presence of DMAP in 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-terminal acetylation was achieved by adding acetic anhydride (Ac_2O) (10 eq) and DIPCDI (10 eq) in CH_2Cl_2. Protecting groups removal was performed with TFA/TIS/H_2O (95:2.5:2.5 v/v/v) during 2 hours.

2.3 | rhFSH labeling

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

2.4 | Qualitative evaluation of rhFSH binding to Ac-KVPLTVSKAKVAG-HMBA-CM resin

The peptidyl-resin was evaluated in a column fitted with a polyethylene porous disk. The peptidyl-resin beads were subsequently washed five times during 1 minute with CH_2Cl_2 (5 × 1 minute), DMF (5 × 1 minute), DMF/H_2O (7:3, 5:5, 3:7), and H_2O (5 × 1 minute). Afterwards, peptidyl-resins were blocked with 10% skim milk, 2% BSA, in phosphate buffered saline (PBS), pH 6.8 and washed 5 × 1 minute with PBS-Tween (0.05% Tween 20 in PBS). Next, they were incubated during 1 hour with the rhFSH coupled with biotin in PBS-Tween. Then, after thoroughly washing with PBS-Tween (5 × 1 minute), the peptidyl-resin beads were incubated with SA-POD (1 U/mL) in PBS-Tween for 1 hour. After that, they were thoroughly washed with PBS-Tween (5 × 1 minute) and PBS (5 × 1 minute). 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 minutes, peptidyl-resin beads with rhFSH adsorbed turned violet. Portions of each peptidyl-resin were also evaluated performing the same protocol but without the addition of labeled rhFSH. All the procedure was performed at room temperature.

2.5 | Peptide stability evaluation

It was performed as per Giudicessi et al.[14] Approximately 10 mg of Ac-KVPLTVSKAKVAG-HMBA-CM resin was incubated with Chinese hamster ovary (CHO) cell culture supernatant overnight at 37°C during 24 hours, and after incubation they were thoroughly washed with H_2O, H_2O:DMF (7:3; 5:5; 3:7 v/v), DMF, and CH_2Cl_2. Peptides were detached from the resin with ammonia vapor as has been

previously described.[9] Released peptide was eluted by adding 1 mL of acetic acid:acetonitrile (CH_3CN):H_2O (3:4:3) and analyzed by electrospray ionization mass spectrometry (ESI MS) recorded in a Xevo G2S Q-TOF (Waters Corp) in positive mode.

2.6 | Synthesis of the immobilized peptide affinity chromatographic support

The peptide Ac-KVPLTVSKAKVAC-NH_2 was synthesized by SPPS using as solid support the Rink-amide resin (100-200 mesh and 0.35 mmol/g) in a polypropylene column fitted with a polyethylene porous disk at room temperature. Fmoc-protected amino acids (AA) couplings were performed using 3 equivalents (equiv) of AA, 3 equiv of TBTU and 6 equiv of DIPEA in DMF and incubated for 1.5 hours. The reaction was controlled by the Kaiser test.[15] Fmoc protecting group removal was accomplished with piperidine: DMF (20:80) (1 × 1 minute and 2 × 5 minutes). The washing steps between each coupling and deprotection were performed with DMF (5 × 1 minute) and in CH_2Cl_2 (5 × 1 minute). N-terminal acetylation was achieved by adding Ac_2O (10 eq) and DIPCDI (10 eq) in CH_2Cl_2. Removal of side-chain protecting groups and peptide separation from the solid support was performed by treatment with TFA/TIS/H_2O (95:2.5:2.5) for 2 hours. Subsequently, the peptide was precipitated with cold diethyl ether and finally dissolved in water and lyophilized.[11]

The peptide was synthesized with a Cys at the C-terminal to facilitate its subsequent attachment to the chromatographic activated SulfoLink resin.

The lyophilized peptide was dissolved just before the reaction to avoid its oxidation. Coupling was performed as indicated in the Thermo Scientific protocol.[16] Briefly, a three-fold excess of peptide was dissolved in 50 mM Tris, 5 mM EDTA-Na, pH 8.5, and added to the SulfoLink resin. The suspension was agitated at room temperature for 1 hour, The unreacted iodoacetyl sites on the matrix were blocked by adding one resin-bed volume of 50 mM cysteine in coupling buffer.[9]

2.7 | Affinity chromatography runs

Chromatographic runs were carried out in a packed bed column loaded with the peptide affinity support (0.5 cm × 5 cm) at a flow rate of 0.25 mL/min. After equilibration with 10 column volumes of adsorption buffer, crude samples containing 0.727 mg of rhFSH/mL together with host cell proteins (HCPs) with a total protein concentration of 1.2 mg/mL were loaded, and a washing step with the adsorption buffer and an elution step were performed. One-milliliter fractions were collected in microtubes, and their absorbance at 280 nm was measured. Chromatography with 1 mL of crude sample was developed to evaluate the adsorption and elution steps. After AC optimization and breakthrough curve analysis, a chromatography step loading 17 mL of crude sample was done, and the eluate was used for further characterization.

2.8 | Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli.[17] To avoid rhFSH heterodimer dissociation, the loading buffer was prepared without reducing agent, and samples were not heated. The gels were stained with Coomassie Blue R-250 following the standard procedure and afterwards stained with silver. Silver staining was performed as per Schägger.[18] Gels densitometric quantification was performed with software ImageJ.

2.9 | Peptide mapping analysis

The rhFSH identification in the AC eluate was achieved by peptide mapping analysis. An aliquot of 0.5-mL eluate was reduced with 20 mM dithiothreitol (DTT) at 56°C during 45 minutes and afterwards digested with trypsin at 37°C overnight. The digested fraction was cleaned up using C18 spin columns and concentrated in a vacuum concentrator. Finally, the sample was resuspended in 10 µL of 0.1% formic acid. HPLC-electrospray ionization mass spectrometry (HPLC ESI MS) was performed with HESI-Orbitrap Q Exactive coupled to a nanoHPLC EASY-nLC 1000 from Thermo Scientific. Two microliters of sample was injected in the nanoHPLC using a C18 column (2.6 µm, 75 µm × 150 mm) thermostatted at 35°C. A linear gradient was performed from 95% A to 95% B in 120 minutes where A = H_2O (0.1% formic acid) and B = MeCN (0.1% formic acid), at a flow rate of 300 nL/min. The capillary and cone voltage of the mass spectrometry were 3.5 kV and 35 V, respectively. In each cycle, the equipment records a full mass spectrum and tandem mass spectra (MS/MS) of the selected ions with better signal/noise ratio. The data were analyzed with the program *Proteome Discoverer* v1.4 (Thermo Scientific), comparing against human proteome database for hFSH matching.

2.10 | Breakthrough curve dynamic capacity

A crude sample of HCPs from CHO cells with 0.73 mg/mL of rhFSH was pumped continuously at a flow rate of 0.25 mL/min through a packed column filled with the peptide AC support (0.5 cm × 5 cm) until the available capacity of the column was exhausted and the rhFSH began to appear at the column outlet. The outlet of the column was monitored by measuring the absorbance at 280 nm of each collected 1-mL fractions and also the rhFSH concentration by HPLC using a reverse phase (RP) column (C4, 5 µm, 0.25 m × 4.6 mm) thermostatted at 30°C and using as mobile phases: (1) 0.2 M phosphate buffer, pH 2.5; (2) H_2O:MeCN (40:60 v/v); and (3) H_2O Milli-Q. The gradient program was performed according to Table S1 in Supporting Information at a flow rate of 1.0 mL/min. The concentration of rhFSH at the column outlet measured by HPLC (C) divided by that of the rhFSH in the loaded sample (Co) represents the fraction of target protein (rhFSH) in the loaded sample that pass through without interacting with the affinity column (C/Co). The variation of C/Co as a function of time or volume of loaded sample is known as breakthrough curve. The dynamic capacity was defined as the mass of the rhFSH loaded to the column when its concentration at the outflow of the column (C) reached 10% respect to the initial rhFSH concentration (Co), according to Chase.[19]

2.11 | Purification table

Total protein concentration was measured with Bradford reagent and rhFSH concentration by RP-HPLC using a C4 column. Purity (%) was defined as the amount of rhFSH (mg) over the amount of total protein (mg). Fold purification (FP) was calculated as the purity after the AC step relative to the purity before the AC step. The yield (%) was defined as the amount of rhFSH after AC divided by the amount of rhFSH before AC.

2.12 | Measurement of oxidized rhFSH

The rhFSH samples were diluted to 0.3 mg/mL with ultrapure water. The percentage of oxidized rhFSH was determined by RP-HPLC with a butylsilyl silica gel analytical column (250 × 4.6 mm, Vydac 214ATP54). Mobile phases used were (1) 0.2 M phosphate buffer, pH 2.5; (2) H_2O:MeCN (40:60 v/v); and (3) H_2O Milli-Q. The gradient program was performed according to Table S1.

2.13 | Free subunits analysis

Purity was analyzed by SDS-PAGE 12% under non-reducing conditions. Fifty micrograms of rhFSH purified by AC was loaded to the gel alongside a standard curve of different amounts of rhFSH free subunits corresponding to 1%, 2%, 3%, 4%, 6%, and 8% of the total loaded mass of rhFSH sample, prepared with the reference standard Follitropin CRS dissociated at 100°C during 5 minutes. After Coomassie Blue staining, purified rhFSH free subunits band intensity should be lower than the intensity shown by the standard curve point of 1.5 μg (3%) of free subunits. A control sample was loaded to the gel (test sample with an intentional addition of free subunits) where Follitropin heterodimer and subunits were clearly separated.

2.14 | Isoelectrofocusing (IEF)

AC-purified rhFSH isoforms distribution was evaluated by IEF. This technique takes advantage of the fact that the charge of a molecule changes with the pH of its surroundings. The migration pattern within an IEF gel is predominantly dictated by the net charge of the glycans present in the glycoprotein under study.[20] Purified rhFSH was desalted, concentrated, and loaded to an IEF gel with a functional separation in the isoelectric point (pI) range of 3.5 to 5.5. The reference standard Follitropin CRS was also analyzed.

2.15 | N-glycans content determination

The rhFSH has 4 N-glycosylation sites, with two N-linked glycans in each subunit. Due to extensive post-translational glycosylation, the hormone exists in a variety of isoforms. For the release of native N-glycans, rhFSH samples were subjected to enzymatic digestion with PNGase F. These glycans were purified and labeled with the fluorophore 2-aminobenzamide (2-AB) as indicated in Gugliotta et al.[21] Follitropin CRS standard was processed at the same time and in the same way that the sample solution.

The separation according to the sialylation degree was performed by weak anion exchange chromatography (Shodex Asahipak ES-502 N 7C column) using a Waters Alliance equipment with fluorescence detector (λexc = 330 nm; λemis = 420 nm). The mobile phases used were (1) acetonitrile; (2) 0.5 M ammonium acetate buffer, pH 4.5; and (3) ultrapure water. The gradient program was performed according to Table S2 in Supporting Information. The Z number was calculated as:

$$\text{Z number} = (A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4),$$

where A_0, A_1, A_2, A_3, and A_4 are the relative peaks area of the neutral, mono-, bi-, tri-, and tetra-sialylated glycans, respectively.

3 | RESULTS AND DISCUSSION

In a previous work,[10] Sohn et al studied the interaction of the FSH receptor exoloop 3 with the hormone, testing each amino acid of that exoloop by Ala substitutions. The fragment sequence with the highest affinity (580)KVPLITVSKAK(590) was selected in the present work to design a synthetic ligand for AC. First, to evaluate the capability of the peptide to bind rhFSH and its chemical stability, the peptide Ac-KVPLTVSKAKVAG was synthesized on HMBA-CM resin. HMBA-CM resin is water compatible, what allowed qualitative evaluation of rhFSH binding to Ac-KVPLTVSKAKVAG-HMBA-CM resin carried out in aqueous solution. Also, it is compatible with the organic solvents used for solid-phase peptide synthesis. The bond formed between the first amino acid and the linker is stable to all synthetic elongation reactions as well as to the conditions required for the removal of the side-chain protecting groups. The linker HMBA allows to release the peptide from the solid support before its MS analysis.[9]

3.1 | Qualitative evaluation of rhFSH binding to the peptidyl-ChemMatrix resin

A small amount of Ac-KVPLTVSKAKVAG-ChemMatrix resin was synthesized. The peptide was acetylated to increase its stability against possible attack by proteases present in the crude FSH sample. Ac-KVPLTVSKAKVAG-ChemMatrix resin and a Gly-ChemMatrix resin were incubated with rhFSH-biotin, and the adsorption of the hormone was qualitatively detected with SA-POD and 4-chloro-naphtol. Ac-KVPLTVSKAKVAG-ChemMatrix resin beads turned violet due to rhFSH-biotin adsorption, while the Gly-ChemMatrix beads without the protein adsorbed remained colorless (Figure S1 in Supporting Information).

3.2 | Peptide stability evaluation

Stability evaluation was necessary to determinate useful life of the affinity support. Crude samples usually have proteases and peptidases. These enzymes can cleave peptide ligands from the support. To evaluate ligand stability, Ac-KVPLTVSKAKVAG-HMBA-CM resin beads were incubated with CHO cell supernatants during 24 hours. After washing the beads, the peptide was detached from the resin to evaluate its integrity by ESI MS. Figure S2 in Supporting Information shows the mass spectrum obtained, where only signals corresponding to the entire peptide were observed. On the other hand, no signals corresponding to C-terminal degradation product were detected.

3.3 | Peptide affinity support design and synthesis

The peptide was synthesized as amide and was acetylated to increase the stability of the resin against possible attack by proteases present in the crude sample. A Cys was incorporated at the C-termini to facilitate its subsequent attachment to the chromatographic activated SulfoLink resin. A ligand density of 10 μmol per mL of matrix was achieved.

3.4 | Affinity chromatography runs

The AC support with the peptide ligand immobilized was evaluated by loading crude samples composed of HCPs from the CHO and rhFSH. When working at low pH (pH 5.6), the rhFSH bound to the peptide, but most HCPs also bound to the chromatographic support. On the other hand, at pH 7.2, the rhFSH did not bind to the peptide while many HCPs did. Thus, the AC was performed using (1) adsorption buffer: 20 mM sodium phosphate, pH 5.6; (2) elution buffer: 20 mM sodium phosphate, pH 7.2; and (3) regeneration buffer: 20 mM sodium phosphate, pH 7.2, 2 M NaCl (Figure 1). A non-reducing SDS-PAGE was done to check rhFSH purification (Figure 2). The identity of the protein band obtained in the SDS-PAGE was checked by peptide mapping analysis using LC-MS/MS. The fragments obtained were compared with a proteome human database and a *crisetulus griseus* database for the identification of the hFSH and contaminant proteins from the CHO cells, respectively (Proteome discover v1.4). High scores were obtained for both polypeptide chains of the heterodimeric hormone, with high coverage rates as well. Fourteen peptides of high confidence were found for β-subunit, and six for α-chain (Tables S3-S5 in Supporting Information). These results confirmed the presence of rhFSH in the eluate. On the other hand, the scores for the possible contaminant proteins were much lower, as well as their coverage, compared with hFSH. From the list of those proteins,

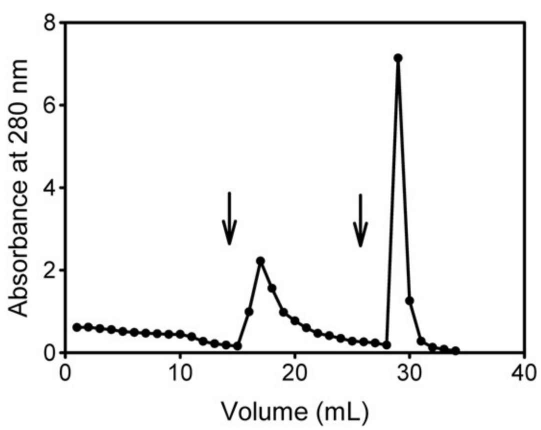

FIGURE 1 Chromatography of crude samples of rhFSH with host cell proteins (HCPs) using the affinity matrix with the peptide Ac-KVPLTVSKAKVAC immobilized. The mobile phases were as follows: A, adsorption buffer (20 mM sodium phosphate, pH 5.6); B, elution buffer (20 mM sodium phosphate, pH 7.2); and C, regeneration buffer (20 mM sodium phosphate, pH 7.2, 2 M NaCl). The arrows indicate the buffer change. The rhFSH eluted when the elution buffer was added. Some HCP proteins passed through the column. Most HCPs eluted after applying the regeneration buffer

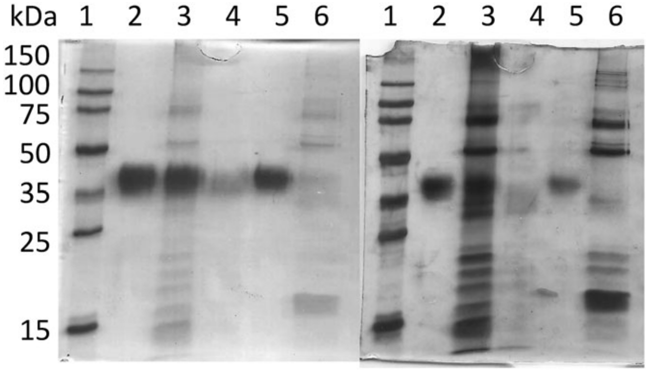

FIGURE 2 Non-reducing SDS-PAGE of the chromatographic step to check rhFSH purification. Left: Coomassie Blue staining. Right: Silver staining. Lane 1) protein molecular weight marker; Lane 2) standard of pure rhFSH; Lane 3) crude sample of rhFSH; Lane 4) washing fractions; Lane 5) elution fraction; Lane 6) regeneration fraction

the ones with higher score were a peroxidasin-like protein, complement C3, basement membrane-specific heparan sulfate proteoglycan core protein, and a sulphydryl oxidase (Table S6 in Supporting Information). To obtain a good yield, 17 mL of the crude sample (concentration = 0.727 mg/mL) was recirculated to the column to finally obtain 6 mL of eluate (concentration = 0.845 mg/mL). As the purification chart shows (Table 1), the AC allowed to obtain a high purity product (94%) with a yield of 41%. Due to the peptide stability, column performance did not change after repeating more than 10 times the chromatographic process.

3.5 | Breakthrough curve

Figure 3 shows the breakthrough curve obtained after pumping continuously a crude sample of HCPs and rhFSH through the packed peptide column and analyzing by HPLC the rhFSH concentration of each 1-mL sample collected. The dynamic capacity of the affinity media, defined as the amount of rhFSH the matrix adsorbed under flow conditions before 10% of the initial rhFSH concentration was detected at the column outlet ($C/Co = 0.1$), was 54.6 mg (1.5 μmol) of rhFSH per mL of chromatographic resin. Therefore, 10 μmol of immobilized peptide was needed to purify 1.5 μmol of rhFSH.

Each 1-mL sample of the breakthrough curve collected after (1) overloading the column, (2) during the washing with adsorption buffer, and (3) elution with elution buffer was analyzed by isoelectric focusing (Figure 4). The isoforms lost when overloading the AC column were the less acidic, which are the ones that have less sialic acid content and hence those with lower biological activity.

3.6 | Purified product characterization

The quality of the purified rhFSH obtained by AC with the peptide immobilized was analyzed by determining the presence of oxidized forms of rhFSH and the sialic acid and free subunits content.

3.6.1 | Oxidized forms of rhFSH

The oxidation of rhFSH occurs at the superficial Met residue present in both subunits. When performing the AC without Met in the buffer,

TABLE 1 Purification chart obtained after chromatography of a crude sample of rhFSH with the host cell proteins using the immobilized Ac-KVPLTVSKAKVAC-NH_2 affinity matrix

Sample	Volume, mL	Conc. rhFSH, mg/mL	Total Protein, mg/mL	Purity %, mg/mg	Fold Purification	Yield, %
Crude sample	17	0.727	1.203	60.4	-	-
Eluate	6	0.845	0.899	94.0	1.56	41

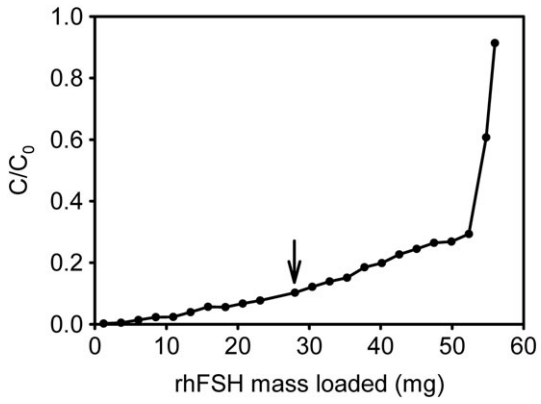

FIGURE 3 Breakthrough curve of a crude sample of rhFSH. Sample was conditioned in 20 mM phosphate buffer pH 5.6, with 0.5 mM of methionine. The column was 0.5 cm × 5 cm with 0.5 mL of chromatographic matrix. The flow was 0.25 mL/min. The arrow indicates C/Co = 0.1

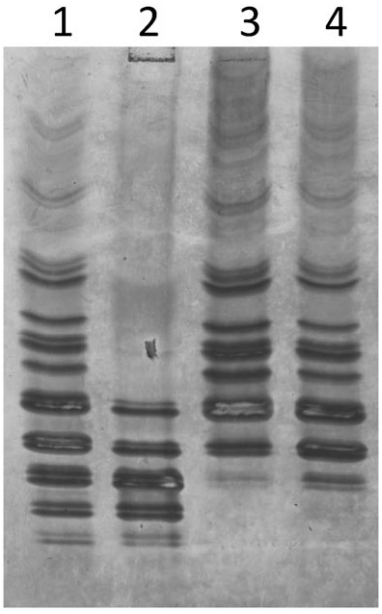

FIGURE 4 IEF gel of breakthrough curve fractions. Lane 1) rhFSH standard. Lane 2) Eluate. Lane 3) Pool of fractions 17 to 33. Lane 4) Pool of fractions 34 to 46

the percentage of oxidized isoforms in the eluate was 8%, which is outside the limits allowed by the European Pharmacopeia (6%) (Figure S3A in Supporting Information). To avoid oxidation, free Met at a final concentration of 0.5 mM was added to the adsorption and elution buffers. Free Met competed with the exposed rhFSH Met residues, and the oxidation was reduced to a level of 3.4% (Figure S3B in Supporting Information). Figure S3B shows the chromatographic analysis of the oxidation of the isoforms, with peaks corresponding to the α (retention time; 12.67 minutes) and β subunit (retention time: 6.47 minutes). Just before those elution peaks, their oxidized isoforms peaks appear in the chromatogram. Only exposed Met of α subunit got oxidized. This finding agrees with the peptide mapping analysis of the rhFSH α subunit, where it could also be observed the oxidation of Met 29 in peptide APDVQDcPEcTLQENPFFSQPGAPILQc**m**GccFSR, and oxidation of Met 71 in peptide VTV**m**GGFK (Table S4 in Supporting Information).

3.6.2 | Free subunits assay

The therapeutic rhFSH heterodimer dissociation gives rise to the presence of free subunits that bind to the receptor but do not activate it, acting as competitive inhibitors. Therefore, the percentage of free subunits must be below 3%, as requested by European Pharmacopeia. A standard of free subunit rhFSH was obtained by heating Follitropin CRS at 100°C during 5 minutes. A calibration curve of the subunit standard was performed in SDS-PAGE to determine the level of free rhFSH subunit in the sample eluted from the peptide AC column (Figure S4 in Supporting Information). The mass of free subunits in the sample was calculated by extrapolating the densitometric value of the sample in the calibration curve. The mass value obtained was 0.585 μg, which represented 1.2% of the total mass of rhFSH.

3.6.3 | Isoforms profile

The rhFSH isoform profile influences its half-life, biopotency, number of matured oocytes, and treatment efficacy. Figure 5 shows the IEF

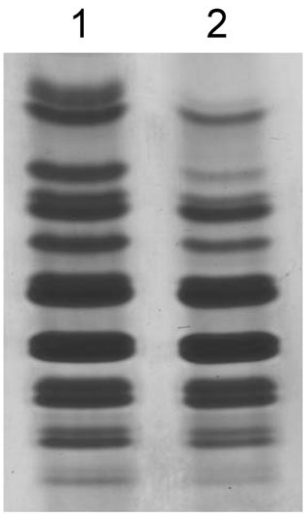

FIGURE 5 IEF gel for isoforms profile. Lane 1) rhFSH standard; Lane 2) sample of rhFSH purified by AC

TABLE 2 Densitometric values of IEF gel of purified rhFSH using immobilized Ac-KVPLTVSKAKVAC-NH_2 affinity matrix

Isoforms	Reference Values	Standard	Sample
1	0-4	1.69	0.23
2	2-16	15.99	6.89
3	0-10	7.14	1.77
4	8-19	10.85	12.72
5	1-8	6.73	6.14
6	13-28	15.19	21.38
7	10-27	16.78	21.49
8	10-22	14.45	18.44
9	2-17	8.44	8.79
10	0-13	2.73	2.16
Others	≤ 5	---	---

gel, while Table 2 shows the percentage of each isoform determined by densitometry in comparison to the CRS standard and internal reference values. Isoforms 1, 2, and 3 are low compared with the standard. These isoforms are the less acidic and have the lowest pharmacological activity.

3.6.4 | Sialic acid content

The sialic acid content (Z number) was determined by weak anion exchange HPLC analysis of the glycans removed from the rhFSH (Figure S5 in Supporting Information). The neutral, mono-, bi-, tri-, and tetra-sialylated glycans were separated by HPLC, and the areas of the chromatographic peaks were calculated resulting 0.51%, 11.94%, 63.18%, 19.32%, and 5.05%. The Z number obtained (217) was within the range suggested by European Pharmacopeia (177-233).

4 | CONCLUSION

The method here designed allows obtaining a high quality rhFSH using a low-cost affinity matrix based on a short peptide ligand. The yield is consistent with results previously published,[22] and it is noteworthy that isoforms with high content of sialic acid were recovered by this new chromatographic process. These isoforms have higher plasma half-life and hence higher in-vivo potency. Furthermore, the physico-chemical characterization achieved all standards of European Medicines Agency (EMA) biosimilar FSH guideline. These results evidence that AC with the ligand peptide Ac-KVPLTVSKAKVAC-NH_2 is a suitable strategy for rhFSH purification from CHO crude extracts.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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