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Two-step chromatographic purification of recombinant human thyrotrophin and its immunological, biological, physico-chemical and mass spectral characterization

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Abstract

A purification strategy for rapidly obtaining recombinant human thyrotropin (rhTSH) was designed based on size exclusion and reversed-phase high-performance liquid chromatographic (HPLC) analysis, carried out on hTSH-secreting CHO cell conditioned medium. These analyses permitted the identification of the main contaminants to be eliminated. Considering that hTSH is highly hydrophobic and elutes only with the addition of organic solvents, hydrophobic interaction chromatography was adopted as the first purification step; this resulted in the elimination of, among others, the major contaminant. A second purification step, based on size exclusion chromatography, was then utilized, being effective in the elimination of other previously identified contaminating proteins. Useful purity, as high as 99% at the chemical reagent level, and recoveries (37%) were obtained by adopting this two step strategy, which also provided adequate material for physico-chemical, immunological and biological characterization. This included matrix-assisted laser desorption ionization time-of-flight mass spectral analysis (MALDI-TOF-MS), Western blotting analysis, in vivo biological assay, size-exclusion HPLC (HPSEC) and reversed-phase HPLC (RP-HPLC) analysis, which confirmed the integrity and bioactivity of our rhTSH in comparison with the only two reference preparations available at the milligram level of native (hTSH-NIDDK) and recombinant (Thyrogen) hTSH. Thyrogen and rhTSH-IPEN, when compared to pit-hTSH-NIDDK, presented more than twice as much biological activity and about 7% increased molecular mass by MALDI-TOF-MS analysis, an accurate heterodimer mass determination providing the M_r values of 29 611, 29 839 and 27 829, respectively. The increased molecular mass of the two recombinant preparations was also confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and HPSEC analysis. Comparing the two recombinant preparations, minor though interesting physico-chemical and biological differences were also observed.

Keywords: Thyrotropin; Purification; HPLC; MALDI-TOF; Biological assay; SDS–PAGE

1. Introduction

Recombinant thyrotropin (rTSH) is a glycoprotein hormone used as a pharmaceutical with high clinical and aggregate value. Recent reports have demonstrated the successful use of this hormone in the follow-up and treatment of patients with thyroid cancer [1–6], as well as in several other medical applications [7,8]. Besides the clinical applications, important laboratorial use has also been described [9,10].

We have previously described the synthesis of rhTSH expressed in mammalian hosts (Chinese hamster ovary, CHO cells) after co-transfection with two dicistronic expression vectors [11]. Given the importance of this product, our goal in the present work was the purification and physico-chemical, immunological and biological characterization of this hormone in comparison with the only recombinant hTSH commercially available (Thyrogen) and with native pituitary human TSH (hTSH) from the National Hormone and Pituitary Program (USA).

Purification protocols described in the literature for rhTSH, and recently reviewed [12], generally consist of

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Table 1
Literature values of relative molecular mass (M_r) obtained via MALDI-TOF-MS analysis for human pituitary or placental glycoprotein hormones

Hormone	Reference	α-Subunit	β-Subunit	Heterodimer		
				Experimental	α + β	Calc/exp
plac-hCG	[25]	13 408	21 446	35 140	34 854	0.992
rhFSH	[26,27]	∼14 000	∼17 000		∼31 000	
pit-hFSH	[28]	14 377.1	13 450		27 827.1	

multi-step procedures involving conventional chromatographic methods such as affinity, ion-exchange (cationic or anionic) and gel filtration chromatography [13–15]. While there is a certain emphasis on dye affinity chromatography, none describe the utilization of hydrophobic interaction chromatography. In the present work, we combined the utilization of hydrophobic and gel filtration chromatography for the selective detection, identification and efficient purification of rhTSH expressed in CHO cells. This scheme was designed taking into account the physico-chemical properties of the proteins generally present in CHO culture supernatant, relying on literature information [16] and our analysis of the conditioned medium from hTSH-secreting CHO cells by size-exclusion HPLC (HPSEC) and by a RP-HPLC method developed in our laboratory [17]. Very few workers have utilized hydrophobic interaction chromatography in their protocols for hTSH purification. Torjesen [18] used Phenyl Sepharose after gel filtration and anion exchange chromatography for separating pituitary hTSH from hFSH, thus obtaining higher yields and purity when compared to previously described procedures [19]. Hiyama et al. [20] adopted Phenyl Sepharose chromatography to separate hTSH and hLH from hFSH still in pituitary extracts. Gadkari et al. [21] used hydrophobic interaction procedures for purifying recombinant glycoprotein hormones of the same family, hLH and hCG, obtained from Pichia pastoris. The elimination of an affinity chromatography step from the hTSH purification scheme is interesting for therapeutic applications because of the potential toxicity and safety problems related with the possible release of the immobilized ligand, that might contaminate the product [22].

Several physico-chemical, immunological and biological assays (in vitro or in vivo) have been described in the literature for recombinant hTSH characterization [7,14,23,24]. We used analogous physico-chemical and immunological methods, with the important addition of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), an extremely sensitive and accurate methodology whose application to molecular mass determination of pituitary and placental glycoprotein hormones has up to now been very limited. As indicated in Table 1 , as far as we know, only three other research groups have carried out a similar determination on these heterodimeric human glycoprotein hormones, namely pituitary and recombinant hFSH, placental chorionic gonadotropin and their α- and β-subunits. Mention should be made, however, of other extensive MALDI-TOF-MS analyses carried out by Bousfield et al. [29] for equine LH, FSH and CG α- and β-subunits structural characterization. For biological activity determination, rather than use one of the available in vitro bioassays, we chose instead to employ a modification [30] of the classical in vivo McKenzie method [31]. This assay has the advantage that it determines a potency parameter that takes into account all the integrated in vivo physiological responses, namely circulating half-life ($t_{1/2}$), receptor affinity and activation of intracellular signal transduction pathways, all of which are apparently influenced by glycosylation [32]. This is particularly important if we want to include effects due to the increased $t_{1/2}$ of highly sialylated CHO-derived hTSH molecules [15,33]. Besides the methodologies described, our work also emphasizes the important comparison between our product and two reference preparations of pituitary and recombinant hTSH available at the milligram level, as well as the frequently overlooked comparison between the native and the synthetic hormones.

2. Materials and methods

2.1. Chemicals and reagents

Water was obtained from a “Millipore Milli-Q plus” water purification system (Bedford, MA, USA). Acetonitrile (HPLC-grade), Mallinckrodt Baker S.A., was purchased from Satelit (Araraquara, Brasil). All other chemicals were analytical reagent grade, purchased from Merck (São Paulo, Brazil) and Sigma (St. Louis, MO, USA). Antibody anti hTSH (MAb TC 14) was kindly provided by the National Institute for Biological Standards and Control (Hertforshire, UK). Chromatographic resins were purchased from Amersham Biosciences (São Paulo, Brazil). CHO cultivation medium (CHO-S-SFM II) was provided by Gibco-BRL (Gaithersburg, MD, USA). Recombinant hTSH (Thyrogen) from Genzyme (Framingham, MA, USA), lot no. 2750598, was purchased from Biobras (Montes Claros, Brasil). Pituitary hTSH (pit-hTSH, NIDDK-hTSH SIAFP-B-2) was kindly provided by Dr. A.F. Parlow of the National Hormone and Pituitary Program (Torrance, CA, USA). Solid-phase hTSH immunoradiometric assay (IRMA) was purchased from Skybio (Wyboston, Bedford, UK).

2.2. Cell cultivation

A clone, obtained in our laboratory (IPEN), derived from CHO DHFR$^-$ cells (mutant line DXB11) cotransfected with