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Stable expression of a human-like sialylated recombinant thyrotropin in a Chinese hamster ovary cell line expressing α2,6-sialyltransferase

Renata Damiani [a], João Ezequiel Oliveira [a], Karola Vorauer-Uhl [b], Cibele Nunes Peroni [a], Elizabeth Gimbo Vianna [a,1], Paolo Bartolini [a], Maria Teresa C.P. Ribela [a,*]

[a] Biotechnology Department, IPEN-CNEN, Av. Professor Lineu Prestes, 2242, Cidade Universitária, 05508-900 São Paulo, Brazil
[b] Department of Biotechnology, University of Natural Resources and Applied Life Sciences,Vienna, Austria

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ABSTRACT

A CHO cell line, previously genetically modified by the introduction of rat α2,6-sialyltransferase cDNA, generated for the first time a human-like sialylated recombinant hTSH (hlsr-hTSH) more similar to the native hormone, with 61% of α2,3- and 39% of α2,6-linked sialic acid residues. The best clone, when submitted to gene amplification with up to 8 μM methotrexate, presented a secretion level of ~2 μg hTSH/10^6 cells/day, useful for product purification and characterization.

The relative molecular masses (M_r) of the heterodimer and of the α- and β-subunits of purified hlsr-hTSH, determined by MALDI-TOF mass spectrometry, and the relative hydrophobicities, determined by RP-HPLC, were not remarkably different from those presented by two r-hTSH preparations secreted by normal CHO cells. Some differences were observed, though, in N-glycan composition, with more tri- and much more tetra-sialylated structures in hlsr-hTSH. When analyzed via an *in vivo* bioassay based on hTSH-induced T_4 release in mice, hlsr-hTSH was shown to be equipotent ($p > 0.05$) with the commercial preparation of r-hTSH (Thyrogen), and 1.6-fold more potent than native hTSH ($p < 0.001$).

Introduction

Chinese hamster ovary (CHO)[2] cells are widely used for the production of recombinant glycoproteins utilized as biotherapeutics, mainly because they can be easily and efficiently cultivated, can provide a high expression of the transgene and are equipped with a glycosylation machinery very similar to the human [1–4]. In these glycoproteins, sialic acid occupies the terminal position on oligosaccharide chains (glycans) and influences the biological and physical properties of several biopharmaceuticals [5]. While N-linked glycans of human origin carry terminal sialic acid residues in both α2,6- and α2,3-linkages, only α2,3-linkages are found in glycoproteins from CHO cells, since these cells lack the gene encoding α2,6-sialyltransferase [6]. In an attempt to synthesize recombinant glycoproteins with a glycosylation pattern as close as possible to that of the natural protein, several authors modified the N-glycan structure by the introduction of the α2,6-sialyltransferase (α2,6-ST) gene into the CHO host cell [6–9]. Glycoproteins such as tissue plasminogen activator (tPA) [10], erythropoietin (EPO) [11] and interferon-γ (IFN-γ) [3,12] were thus shown to acquire α2,6-sialic acid linkage when expressed in one of these modified cell lines. Only two, among these authors, investigated a possible alteration in biological activity and/or pharmacokinetics due to the type of sialic acid linkage. Zhang et al. [11] described a more modest reticulocytosis (however not statistically significant) elicited by a human-like sialylated recombinant erythropoietin containing N-glycan structures with both α2,3- and α2,6-linked sialic acid residues, compared to recombinant human erythropoietin containing only α2,3-linked sialic acid. Bragonzi et al. [3], analyzing a hlsr-interferon-γ containing 40.4% α2,6-sialic acid residues, also did not find any significant difference in biological activity. They observed, though, improved pharmacokinetics in clearance studies when compared to interferon-γ produced by normal CHO cells.

Human thyrotropin (hTSH) is a glycohormone that is clinically important for thyroid cancer management [13–15]. In the present work, with the goal of obtaining for the first time a human-like sialylated recombinant hTSH (hlsr-hTSH), the universal host (UH)

* Corresponding author. Fax: +55 11 31339694.
E-mail address: mtribela@ipen.br (M.T.C.P. Ribela).

[1] In memoriam.

[2] *Abbreviations used:* hlsr-hTSH, human-like sialylated recombinant hTSH; M_r, relative molecular masses; CHO, Chinese hamster ovary; tPA, tissue plasminogen activator; EPO, erythropoietin; IFN-γ, interferon-γ; hTSH, human thyrotropin; UH, universal host; α2,6-ST, α2, 6-sialyltransferase; dFBS, dialyzed fetal bovine serum; MTX, methotrexate; IRMA, immunoradiometric assay; RP-HPLC, reversed-phase high-performance liquid chromatography; BSA, bovine serum albumin; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HPSEC, high-performance size-exclusion chromatography; t_R, retention time.

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prepared by Bragonzi et al. [3] was utilized following a transfection and amplification procedure already described in previous work [16], thus obtaining a purified hormone that was extensively characterized with respect to N-glycan structures, relative molecular mass, hydrophobicity and *in vivo* bioactivity.

Materials and methods

Transfection of hTSH into a modified CHO cell line

A CHO clone displaying a high rat α2,6-ST activity and FACS positivity was named UH (universal host) by Bragonzi et al. [3] and kindly donated to our laboratory by Dr. Lucia Monaco (San Raffaele Scientific Institute, Milan, Italy). This stable cell line was then co-transfected with dicistronic mRNA expression vectors pEDdc-α-hTSH and pEAdc-β-hTSH in a ratio of 1:5 (4 μg of pEDdc-α-hTSH/20 μg of pEAdc-β-hTSH) by the calcium phosphate precipitation method [16]. Post-transfection (2 days), cells were seeded into 100 mm culture dishes at 5×10^5 cells/dish, employing α-MEM medium without nucleosides supplemented with 10% dialyzed fetal bovine serum (dFBS; Gibco-BRL, Gaithersburg, MD, USA) as selective medium. After 2 weeks, the transformed colonies were isolated using cloning cylinders and, following several subculture steps, the clones were analyzed for hTSH secretion. The most productive clones were submitted to amplification with increasing concentration of methotrexate (MTX, Sigma, St. Louis, MO, USA) in the selective medium: 0.1, 0.2, 0.5, 1, 2, 4, 8, 10 and 15 μM MTX. Each amplification step lasted, in general, 2 weeks.

Cell cultivation and production

The most productive clone expressing hlsr-TSH was cultured in T-flasks (162 cm^2, from Corning Costar Corporation, Cambridge, MA) containing 20 ml of medium, at 37 °C. Transfected cells were grown until 80% confluence in α-MEM medium supplemented with 10% dFBS, in the presence of 5% CO_2. Production started when the media were changed to serum-free media, CHO-S-SFM II with nucleosides (hypoxanthine and thymidine), with the addition of 0.2 μM methotrexate, which allows a selective pressure. Every 24 h, culture media were harvested and replaced with fresh, serum-free media. Daily harvest was continued for up to 15 days. During the production period, each 162 cm^2 T-flask contained ~14×10^6 cells. hlsr-TSH concentration in CHO conditioned medium was determined by immunoradiometric assay (IRMA).

Purification process

For the purification of hlsr-TSH, we utilized a strategy developed in our laboratory consisting of an ion exchange chromatographic step followed by a reversed-phase high-performance liquid chromatography (RP-HPLC) step [17]. Briefly, conditioned medium, after adjustment to pH 5.0, was applied to a SP-Sepharose fast flow column (10 cm × 2.6 cm I.D.) previously equilibrated with 50 mM NaCl, 20 mM sodium acetate buffer, pH 5.0. After washing with five column volumes of this buffer, the proteins were eluted with a linear gradient of NaCl (from 50 mM to 250 mM) in the same buffer, at a flow-rate of 200 mL/h, and fractionated (5 mL/tube). Fractions containing hTSH, detected by IRMA, were pooled and concentrated via an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). This pool was injected onto a semi-preparative Vydac C4, 214 TP510 column (Grace-Vydac Hesperia, CA, USA) connected to a HPLC apparatus (Shimadzu Model SCL-10A). Eluent A was 0.05 M sodium phosphate buffer, pH 7.0, and eluent B was 50% acetonitrile plus 50% eluent A. The elution was carried out with a linear gradient of 25 to 100% B, over 40 min, at 25 °C. A flow-rate of 2.5 mL/min and detection by UV absorbance at a wavelength of 220 nm were used. The final pool was dialyzed against 0.02 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 5 mg/mL glycine and lyophilized.

Immunoradiometric assay (IRMA)

Human TSH IRMA was carried out by an in-house, "sandwich" format, utilizing a secondary hTSH standard calibrated against the International Standard of pituitary hTSH (WHO 80/558, 4.93 IU/mg) as described [18]. Briefly, hTSH IRMA was carried out with simultaneous addition of all reagents in a total volume of 0.5 mL: 0.05 mL of ^{125}I-monoclonal antibody (60,000 cpm), 0.10 ml of reference (0.15–100 μUI/mL) or unknown preparation and 0.05 ml of solid phase-coupled polyclonal antibody (1.25 mg/tube). Incubation was carried out on a rotary mixer, at room temperature, for 16 h, in 0.05 M phosphate buffer, pH 7.4, with 1% bovine serum albumin (BSA) and 0.5% Tween 20. The preparation was centrifuged at 5000*g* for 30 min for the separation of the ^{125}I-antibody bound hormone, after the addition of 2 mL of wash buffer (0.05 M phosphate buffer, pH 7.4, with 0.5% Tween 20). The washing procedure was repeated twice.

Protein determination

Total protein content was determined by BCA protein assay, using bicinchoninic acid (Micro BCA protein assay kit, Pierce, Rockford, IL, USA). Pure bovine serum albumin (BSA, Sigma, São Paulo, Brazil) was used as standard.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The products obtained from each purification step were analyzed by SDS–PAGE at 15% polyacrilamide, under non-reducing conditions. Proteins were visualized by staining the gels with 0.25% Coomassie Brilliant Blue G-250 [19].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

hlsr-hTSH, synthesized in our laboratory, containing excipients (sodium phosphate, NaCl and glycine), was embedded in a matrix consisting of a saturated solution of sinapinic acid in 50% acetonitrile/0.1% trifluoracetic acid. An aliquot of 10 μL (~20–30 pmol of hormone) was air-dried and analyzed by MALDI-TOF-MS using an Applied Biosystems Voyager-DESTR (Foster City, CA, USA), operated in linear positive-ion mode. The relative molecular mass of hlsr-hTSH was compared with those, previously determined [19] for a commercial recombinant hTSH preparation (Thyrogen), for another CHO-derived hTSH, also synthesized in our laboratory (r-hTSH IPEN), and for a pituitary hTSH preparation (p-hTSH NIDDK) from the National Hormone and Pituitary Program (Torrance, CA, USA).

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was utilized for testing the purity of hlsr-hTSH and for comparison of the hydrophobicity of six different hTSH preparations, three of them CHO-derived (hlsr-hTSH, r-hTSH IPEN, Thyrogen) and three pituitary-derived: p-hTSH NIDDK, p-hTSH NOR from Aker University Hospital of Oslo, Norway, and p-hTSH WHO 68/38 from the National Institute for Biological Standards and Control (London, UK). RP-HPLC was carried out on a Vydac C4 column (300 Å pore size, 5 μm particle size, 25 cm × 4.6 mm I.D.) purchased from Grace-Vydac (Hesperia, CA, USA), connected to a Shimadzu Model SCL-10A HPLC apparatus. Chromatography was carried out at 25 °C with detection by UV absorbance at a

wavelength of 220 nm. The running conditions utilized 0.05 M sodium phosphate buffer, pH 7.0, as eluent A and acetonitrile as eluent B. The elution was carried out with a flow-rate of 0.5 mL/min and a linear gradient of 12.5 to 50% B, over 40 min, was used.

Analytical high-performance size-exclusion chromatography (HPSEC)

HPSEC was utilized for testing the purity of hlsr-hTSH and was carried out on a G2000 SW column (60 cm × 7.5 mm I.D., particle size of 10 μm and pore size of 125 Å), purchased from Tosohaas (Montgomeryville, PA, USA), connected to the same Shimadzu apparatus. Detection was by UV absorbance at 220 nm with a flow-rate of 1.0 mL/min, employing 0.15 M NaCl in 0.02 M sodium phosphate buffer, pH 7.0, as the mobile phase.

In vivo bioassay

The potency (biological activity) relative to a reference preparation (p-hTSH NIDDK) of the two r-hTSH preparations produced in our laboratory and of Thyrogen was evaluated by an *in vivo* bioassay, in which TSH-induced T_4 is measured after suppression of endogenous TSH by T_3 administration, in BALB/c mice. We employed a modification of the *in vivo* East-Palmer method [20], utilizing a single dose (10 μg hTSH/mouse) and 10 mice for each preparation, as previously described [17]. The response parameter "μg T_4/dl of mouse serum" and related standard deviation were used for calculating the relative potencies and for carrying out significance tests.

Carbohydrate structure analysis

The analysis of the N-glycan structures was performed after cleaving the glycans from hlsr-hTSH preparation by PNGase F from *F. meningosepticum* (New England Biolabs, Hitchin, UK), separating the glycans from the peptides and labeling them with aminobenzoic acid (4-ABA) fluorescent dye. After labeling, the glycans were analyzed by normal-phase HPLC using a TSK-Gel Amide-80 column (4.6 × 250 mm, 5 μm; TOSOH Bioscience, Stuttgart, Germany). The retention times of the detected peaks were compared to the retention times of known commercial glycan standards from Dextra Laboratories (Reading, UK) and Glyko (Novato, CA, USA) [21].

Sialic acid linkage analysis

The type of sialic acid linkage in N-glycans of hlsr-hTSH and of p-hTSH NIDDK was determined by Proteodynamics and Glycodiag (Saint Beauzire/Chevilly, France) using Neu5Ac α2-3 (*Maackia amurensis* I) and Neu5Ac α2-6 (*Sambucus nigra*) specific lectins. Their interaction with hTSH glycans was detected by using an anti-TSH ($f(ab')_2$ form) biotinylated monoclonal antibody and peroxidase-conjugated streptavidin. In order to follow only the level of α2,3- or α2,6-terminally sialylated structures, the background level of interaction obtained after neuraminidase digestion was subtracted from the results obtained without digestion. Moreover, digestion with α2,3 neuraminidase (from *Salmonella typhimurium* LT2), which catalyzes the hydrolysis of α2,3- and at a much lower rate of α2,6-linked N-acetyl-neuraminic acid, was carried out in order to confirm that the results were in good agreement with the expected specificities of the lectins. These experiments were conducted simultaneously on a control glycoprotein (bovine fetuin) with known levels of α2,3- and α2,6-terminally sialylated glycans.

Results and discussion

The hlsr-hTSH secretion levels of the chosen cell line, determined by IRMA, are shown in Fig. 1. The highest expression

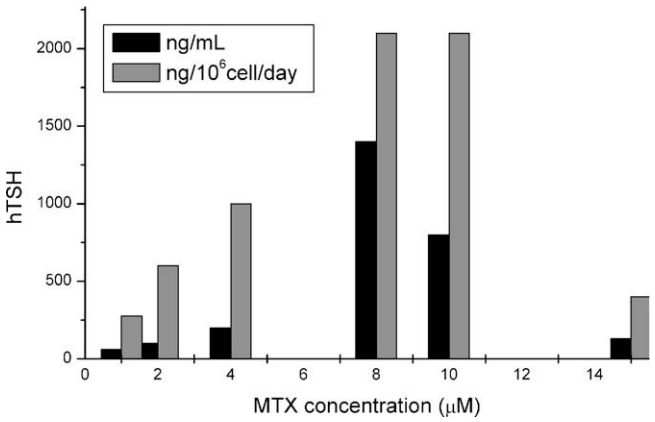

Fig. 1. hTSH expression levels, determined by IRMA, after MTX amplification (steps from 1 to 15 μM) of a hlsr-hTSH-secreting selected clone.

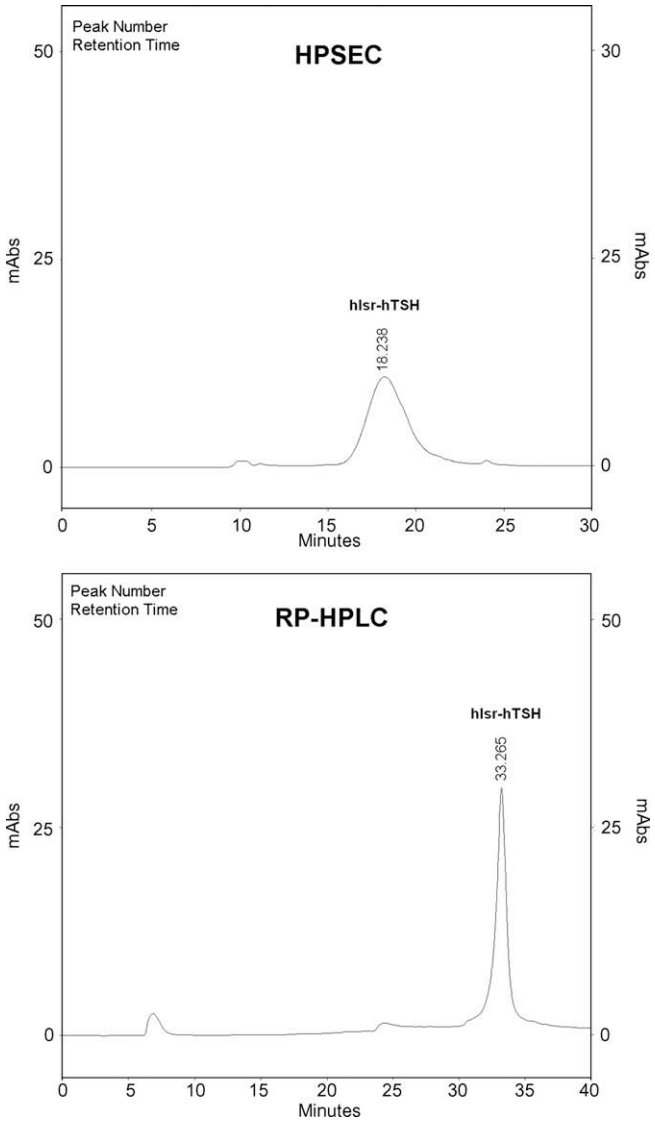

Fig. 2. HPSEC and RP-HPLC profiles of purified hlsr-hTSH after a two-step purification strategy. In a parallel run, the r-hTSH reference preparation (Thyrogen) presented a t_R = 18.50 min by HPSEC and a t_R = 33.45 min by RP-HPLC.

Table 1
Purification table of hlsr-hTSH.

Purification step	Total protein[a] (mg)	hTSH (mg)	Step yield (%)	Mass fraction (%)
Conditioned medium	665.0	4.52[b]	100	0.68
Cationic exchanger eluate	9.47	2.15[c]	48	22.7
RP-HPLC eluate	1.25	1.28[c]	60	102

[a] Estimated by BCA assay.
[b] Estimated by RP-HPLC.
[c] Estimated by HPSEC.

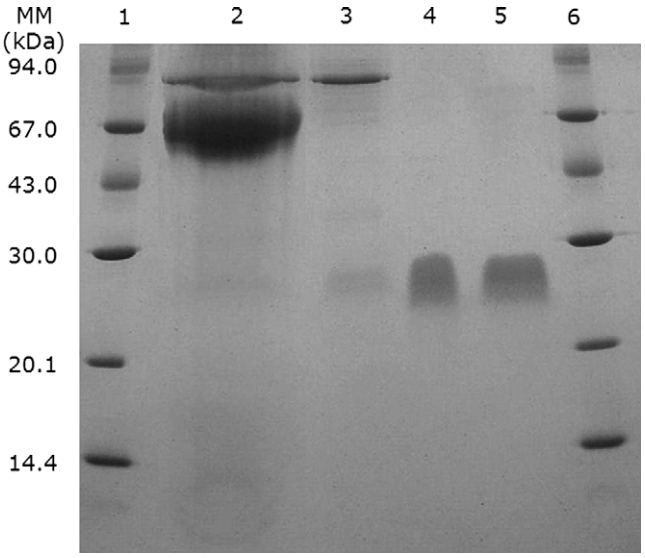

Fig. 3. SDS–PAGE under non-reducing conditions of hlrs-hTSH purification steps. Lanes 1 and 6, molecular weight markers; lane 2, CHO conditioned medium; lane 3, cationic exchange eluate; lane 4, RP-HPLC eluate; lane 5, Thyrogen, utilized as a reference preparation for recombinant hTSH.

(2.1 μg hTSH/10^6 cells/day), with a concentration of 1.4 μg/mL was obtained after seven amplification steps. Significantly, before MTX treatment, the best clone derived from UH secreted 1.3 ng hTSH/10^6 cells/day, thus undergoing a ~1600-fold amplification process by using up to 8 μM MTX. During the amplification of the hTSH gene, carried out on a non-previously modified CHO cell line [16], the best clone increased its expression from 33 ng hTSH/10^6 cells/day to 7.2 μg hTSH/10^6 cells/day (i.e., with ~218-fold amplification), using up to 1 μM MTX. Although higher secretion rates have been reported in the literature for hTSH-secreting CHO cells, both by our research group, as mentioned above, or by Cole et al. [13] (4.5 μg/10^6 cells/day), the productivity obtained with this human-like CHO cell line is also suitable for setting up a production and purification process.

Following a two-step chromatographic purification strategy based on a cationic ion exchanger and a reversed-phase semi-preparative HPLC column, highly purified (>95% purity) hlsr-hTSH was obtained, as determined by analytical HPSEC and RP-HPLC

Table 2
Average relative molecular mass (M_r) × 10^{-3} of the heterodimer and related subunits of different hTSH preparations, determined by MALDI-TOF mass spectrometry (n = 2).

Preparation	Heterodimer (experimental)	α-Subunit	β-Subunit	(α + β)	(α + β)/ Exp.
p-hTSH NIDDK[a]	27.8	13.1	14.5	27.6	0.99
r-hTSH IPEN[a]	30.0	13.9	15.4	29.3	0.98
hlsr-TSH IPEN	29.2	14.0	14.9	28.9	0.99
r-hTSH Thyrogen[a]	29.6	13.8	15.6	29.4	0.99

[a] Data from Mendonça et al. [19].

Table 3
Relative hydrophobicity of hTSH preparations (CHO- and pituitary-derived).

Preparation	RP-HPLC t_R (min)
r-hTSH IPEN	33.37
hlsr-hTSH IPEN	33.27
r-hTSH (Thyrogen)	33.44
p-hTSH NIDDK	33.75
p-hTSH NOR	33.68
p-hTSH WHO	33.78

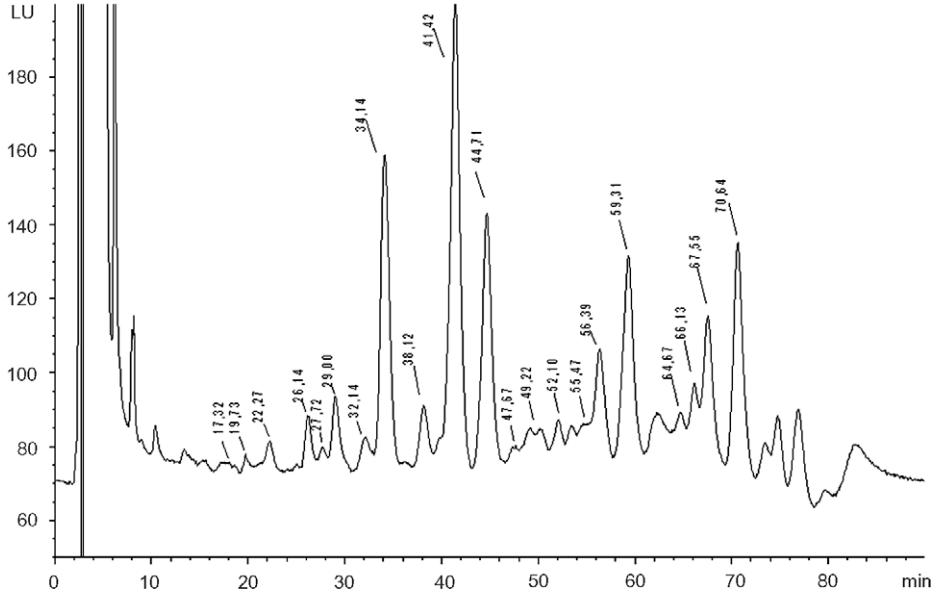

Fig. 4. Normal-phase HPLC separation of hlsr-hTSH N-glycans using a TSK-Gel Amide-80 column (4.6 × 250 mm, 5 μm). The retention times of the detected peaks were compared to the retention times of known commercial glycan standards.

Table 4
HPLC qualitative and quantitative analysis data of hlsr-hTSH N-glycans.

Retention time	Area	N-glycans	%
17.32	326	N2G0	0.78
19.73	277	N2G0F	0.66
22.27	563	N2G1	1.35
26.14	810	N2G1F	1.94
27.72/29.00	1595	N2G2	3.83
32.14	669	N2G2F	1.60
34.14	5135	N2G1S1	12.32
38.12	1005	N3G3	2.41
41.42	8921	N2G2S1	21.40
44.71	4598	N2G2S1F	11.03
47.67	270	N2G2S2	0.65
49.22	632	N2G2S2F	1.52
52.10	412	N4G4F	0.99
55.47	773	N3G3S2	1.85
56.39	2081	N3G3S3	4.99
59.31	3961	N3G3S3F	9.50
64.67	1091	N4G4S2	2.62
66.13	1330	N4G4S3	3.19
67.55	2787	N4G4S4	6.69
70.64	4451	N4G4S4F	10.68

[17] (Fig. 2). From Table 1, on the basis of mass fractions, it is possible to calculate an overall purification factor of ca. 150-fold, with a cumulative recovery of 28%. Product purity was also confirmed by SDS–PAGE analysis (Fig. 3), which showed that hlsr-hTSH apparently has the same purity as Thyrogen. Some contaminants that were present in the conditioned medium were found to be progressively eliminated. The electrophoretic bands of the purified products are quite broad and show subunit dissociation, particularly noticeable in lane 3. This seems to be due to an effect of boiling of heterodimeric glycoproteins in the presence of SDS, as mentioned by Perlman et al. [22] for the case of hFSH. Unlike hFSH, however, we observed that, without boiling, hTSH has very limited mobility inside the gel, which hampered successful analysis (data not shown).

The relative molecular mass (M_r) of purified hlsr-hTSH and of its subunits was determined via two independent MALDI-TOF-MS assays, always considering the average of the m/z values obtained in the central part of each peak, which frequently presented some microheterogeneity, suggesting the presence of different glycosylated forms (Table 2). While the data for r-hTSH IPEN, Thyrogen and p-hTSH are from previous work [19], those related to hlsr-hTSH were determined in the present study. The experimental value for the heterodimer agrees perfectly with that calculated by summation of the molecular masses of the two subunits. As already observed for other recombinant preparations of this hormone [19], hlsr-hTSH and its subunits also present a similarly increased molecular mass compared to p-hTSH (5–8% in this case).

Comparison of the relative hydrophobicities of six hTSH preparations (three pituitary and three recombinant) by intra-day RP-HPLC determination (Table 3) indicates that hlsr-hTSH is the least hydrophobic, exhibiting a retention time (t_R) that is 1.4% lower than the average t_R derived from the three pituitary preparations: p-hTSH NIDDK, NOR and WHO. From the same table, an inter-pituitary preparation average t_R (33.74 ± 0.051 min, RSD = 0.15%; $n = 3$) that is significantly different ($p < 0.005$) from the inter-recombinant preparation average t_R (33.36 ± 0.085 min, RSD = 0.26%; $n = 3$) can also be calculated, confirming previous data obtained by comparing only one recombinant with one pituitary hTSH preparation [23].

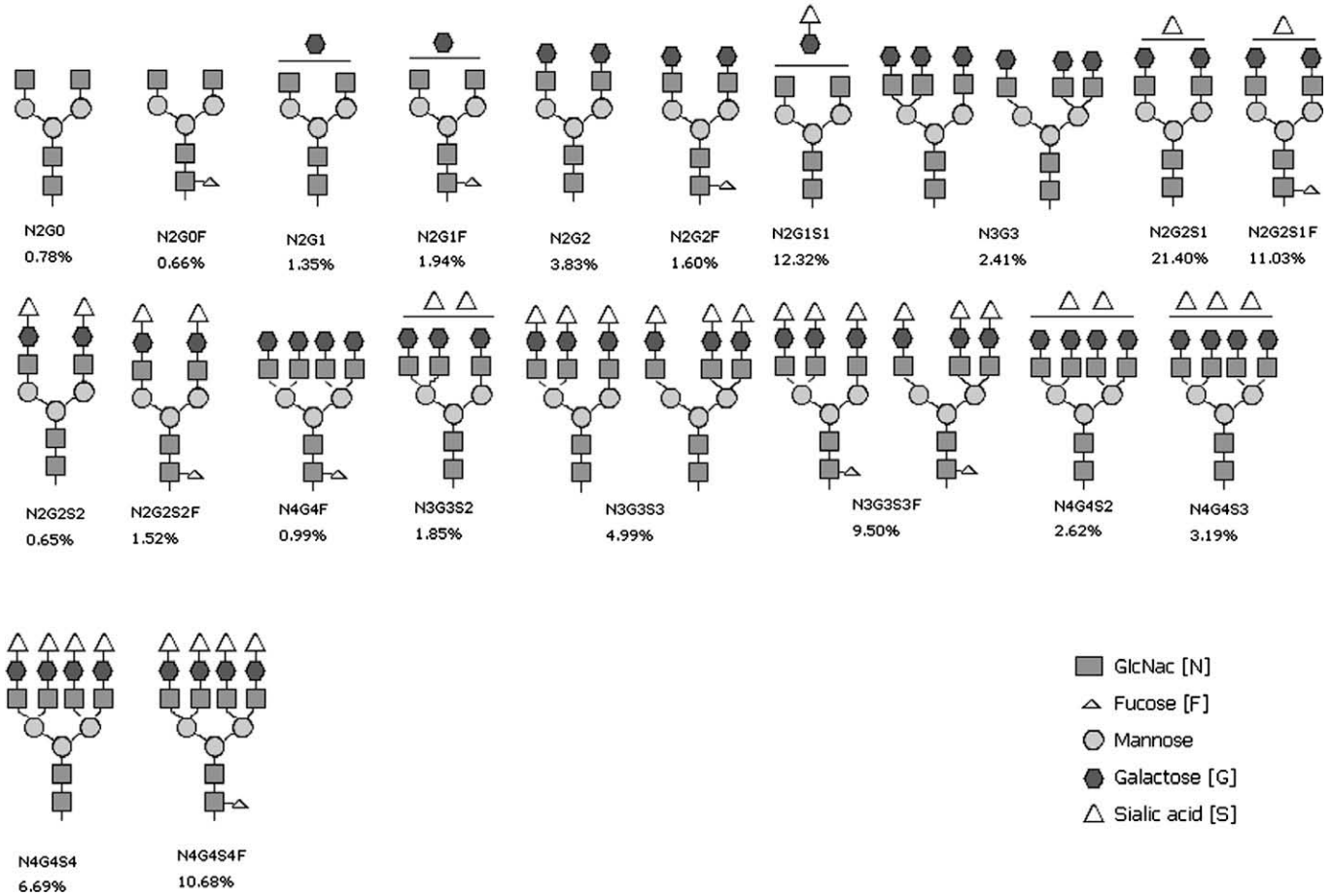

Fig. 5. N-glycan structures of hlsr-hTSH.

Table 5
N-glycan distribution in three r-hTSH preparations, according to antennarity and sialylation level.

Sialylation level	Bi-antennary (%)			Tri-antennary (%)			Tetra-antennary (%)			Total per sialylation level (%)		
	hlsr	IPEN[a]	THY[a]	hlsr	IPEN[a]	THY[a]	hlsr	IPEN[a]	THY[a]	hlsr	IPEN[a]	THY[a]
0	10.16	6.47	5.28	2.41	3.87	6.59	0.99	1.23	1.19	13.56	11.57	13.06
1	44.75	69.66	68.45	—			—			44.75	69.66	68.65
2	2.17	6.51	7.03	1.85	0.70	n.d.	2.62	0.65	n.d.	6.64	7.86	7.03
3	—			14.49	9.30	10.0	3.19	0.34	0.31	17.68	9.64	10.31
4	—			—			17.37	1.24	1.15	17.37	1.24	1.15
Total per antennae	57.08	82.64	80.76	18.75	13.87	16.59	24.17	3.46	2.65			
Total sialylated glycans										86.44	88.40	87.14

[a] Data from Oliveira et al. [23].

The interaction of the N-glycan structures of hlsr-hTSH with the polar groups of an Amide-80 normal-phase HPLC column (Fig. 4 and Table 4), permitted their separation, identification and quantification by comparison with glycan standards. These structures were found to be of the complex type, presenting bi- (57.1%), tri- (18.8%) and tetra-antennary (24.2%) forms, sometimes fucosylated and with variable levels of sialylation (Fig. 5 and Table 5). The most abundant structures were the monosialylated bi-antennary N-linked sugar chains (N2G2S1, N2G1S1 and N2G2S1F), representing ~45% of all identified forms. About 86% of oligosaccharides were sialylated, with 5.47 mol sialic acid/mole hTSH. The sialic acid: galactose molar ratio (S/G) was 0.73, still in relative agreement with reported data for pituitary and recombinant hTSH preparations [13,24]. The most remarkable difference, however, was the presence of ~16% more tetra- and ~8% more tri-sialylated structures than in the other two recombinant preparations reported in Table 5. These data suggest molecular masses systematically somewhat higher than the relative molecular mass data determined by MALDI-TOF-MS. Thus, when the theoretical molecular mass was estimated from the mass due to hTSH primary aminoacid sequence (24330 Da) plus the mass of the carbohydrate moiety calculated on the basis of each specific N-glycan contribution, we obtained a value 8.3% higher for hlsr-hTSH, but only 2.1% and 3.1% higher for r-hTSH IPEN and Thyrogen, respectively. Although the motive for this difference is not yet clear, there might be a lower level of glycosylation site occupancy in the case of hlsr-hTSH or perhaps a selective loss of the heaviest N-linked glycans during the MALDI-TOF process.

An analysis of N-linked hTSH glycans has also been reported by Morelle and coworkers [25] who could identify 16 structures, three of which, including a tetra-sialylated penta-antennary structure (N5G5S4F), were not found in our study. An interesting comparison can be made between glycan structures identified in our recombinant hTSH preparations and those reported by Loumaye et al. [26] for CHO-derived r-hFSH. In both cases, 20 different structures were identified (excluding enantiomeric forms), 14 of which were common to the two hormones.

Sialic acid linkage analysis of hlsr-hTSH, carried out by using two α2-3 and α2-6 specific lectins, in comparison with a control glycoprotein (Figs. 6–8) revealed values that are relatively close

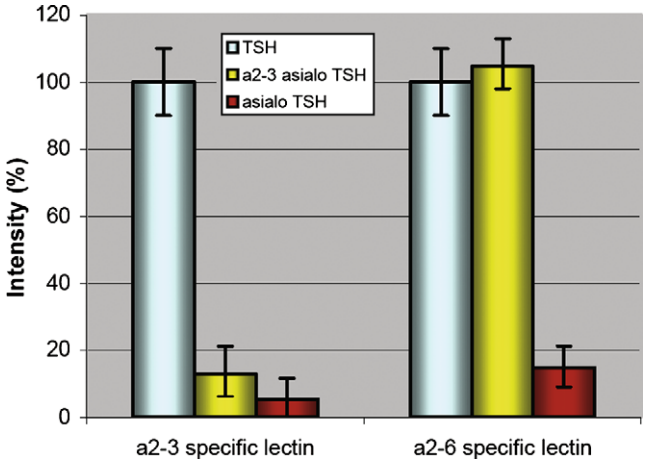

Fig. 6. Interaction of NeuAcα2,3 and NeuAcα2,6 specific lectins with hlsr-hTSH glycans. The intensity of interaction of each lectin was studied before and after hTSH degradation by neuraminidase (asialo TSH) or neuraminidase specific for α2,3-linkage (α2,3 asialo TSH). Two independent assays (each with $n = 4$ replicates) were carried out.

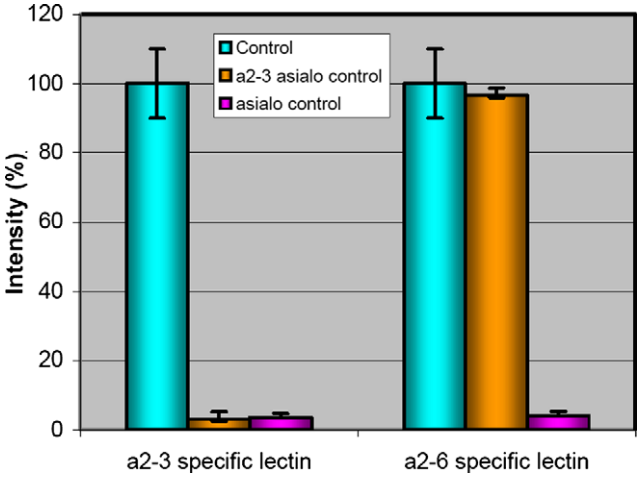

Fig. 7. Interaction of NeuAcα2,3 and NeuAcα2,6 specific lectins with control glycoprotein. The intensities of interaction of each lectin was studied before and after degradation by neuraminidase (asialo control) or neuraminidase specific for α2,3-linkage (α2,3 asialo control). Two independent assays (each with $n = 4$ replicates) were carried out.

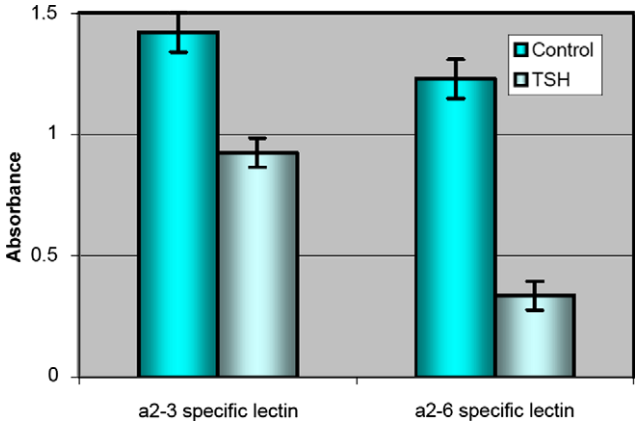

Fig. 8. Interaction of NeuAcα2,3 and NeuAcα2,6 specific lectins with control glycoprotein and hlsr-hTSH: comparison of the absolute absorbance values. Two independent assays (each with $n = 4$ replicates) were carried out.

Table 6
Relative potency of different hTSH preparations determined by *in vivo* bioassay in comparison with some basic N-glycan parameters.

Preparation	Potency (relative to p-hTSH)	Sialic acid (mol)/hTSH (mol)	Galactose (mol)/hTSH (mol)	Sialic acid (mol)/ galactose (mol)	Exposed galactose (mol)/TSH (mol)	α2,3-linkages (%)	α2,6-linkages (%)
hlsr-hTSH	1.58	5.47	7.53	0.73	2.06	61	39
r-hTSH IPEN	1.51	3.61	5.95	0.61	2.34		
Thyrogen	1.59	3.57	6.06	0.59	2.49		
p-hTSH	1.00[a]	0.90[b]	1.33[b]	0.68[b]	0.43[c]	68[a]	32[a]

[a] Pituitary hTSH from NIDDK.
[b] Data from Cole et al. [13]: monosaccharide analysis of p-hTSH, Research Standard B from WHO.
[c] From Cole et al. [13] considering approximately that moles of exposed Gal = moles of Gal − moles of SA.

to those, also determined in the present work, for pituitary hTSH-NIDDK (Table 6). It is of interest that values of 72% for the α2-3 and of 28% for the α2-6 linkage can also be calculated for hTSH on the basis of the pioneering works of Green and Baenziger [27] and Baenziger and Green [28].

The *in vivo* bioassay in mice ($n = 10$), based on the stimulation of thyroxin (T_4) by TSH, showed that hlsr-hTSH is equipotent to Thyrogen ($p > 0.05$), apparently slightly more active than r-hTSH IPEN and 1.6-fold more potent than pit-hTSH NIDDK ($p < 0.001$) (Table 6). According to literature data the higher activities of the recombinant versus the pituitary preparation seem to be due basically to the higher sialylation level or to a different level of exposed galactose [19,29–31], which determines a more prolonged plasma half-life. In Table 6, besides those already mentioned, we can observe other important glycosylation parameters comparing hlsr-hTSH and other two recombinant preparations to the reference pituitary preparation from NIDDK. It is noteworthy that the moles of sialic acid, galactose and exposed galactose of the latter are constantly ~5-fold lower than the average values presented by the three recombinant preparations, while the sialic acid: galactose ratio is essentially identical for the four preparations ($\bar{X}$=0.65 ± 0.064; RSD = 9.9%; $n = 4$) confirming Cole and coworkers observation [13]. The fact that our preparation of hlsr-hTSH presented an "*in vivo*" bioactivity in mice that is not different from that of a regular r-hTSH preparation does not necessarily say much about its potential pharmaceutical interest. Although the *in vivo* bioassay based on hTSH-induced T_4 in mice is extremely useful, it is certainly a very limited model for TSH action in humans.

In conclusion, for the first time, recombinant hTSH with sialic acid bound to galactose by both α2-3 and α2-6 linkages, similar to the native preparation, has been obtained in CHO cells modified to produce α2,6-sialyltransferase. This modification does not seem to influence significantly the molar mass, hydrophobicity or bioactivity of the recombinant glycoprotein, although differences were observed in N-glycan structures. A possible influence on its pharmacokinetic and/or physiological behavior is expected, as was found, for example, for human-like sialylated interferon-γ obtained utilizing the same universal host [3]. We believe that hlsr-glycoprotein hormones need to be considered into the promising new generation of biopharmaceuticals based on genetic modification of their carbohydrate moieties.

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