Mol Biotechnol (2008) 39:159–166
DOI 10.1007/s12033-008-9047-6

RESEARCH

Influence of a Reduced CO_2 Environment on the Secretion Yield, Potency and N-Glycan Structures of Recombinant Thyrotropin from CHO Cells

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Published online: 8 March 2008

Abstract A consistent increase of ~60% in the secretion yield of CHO-derived hTSH was observed by changing cell culture CO_2 conditions from 5% CO_2 to an air environment. The overall quality of the products obtained under both conditions was evaluated in comparison with a well-known biopharmaceutical (Thyrogen®). The N-glycans identified were of the complex type, presenting di-, tri- and tetra-antennary structures, sometimes fucosylated, 86–88% of the identified structures being sialylated at variable levels. The three most abundant structures were monosialylated glycans, representing ~69% of all identified forms in the three preparations. The main difference was found in terms of antennarity, with 8–10% more di-antennary structures obtained in the absence of CO_2 and 7–9% more tri-antennary structures in its presence. No remarkable difference in charge isomers was observed between the three preparations, the isoelectric focusing profiles showing six distinct bands in the 5.39–7.35 p*I* range. A considerably different distribution, with more forms in the acidic region, was observed, however, for two native pituitary preparations. All recombinant preparations showed a higher in vivo bioactivity when compared to native hTSH. Different production processes apparently do not greatly affect N-glycan structures, charge isomer distribution or bioactivity of CHO-derived hTSH.

Keywords CO_2 environment · Culture conditions · In vivo bioassay · Isoelectric focusing · N-glycan structures · Thyrotropin

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Introduction

Several approaches to enhance the productivity of CHO-derived recombinant proteins have been currently reported in the literature. Improvement of the bioprocess has been observed in response to cell-culture strategies involving culture temperature [1–5], dissolved oxygen level [6, 7], medium pH [8], dissolved carbon dioxide [9], or medium additives [10–13]. The effects of these factors have been found to be cell line- and protein-specific.

In the present work, we focused our studies on human thyroid-stimulating hormone (hTSH), which is pharmacologically important, especially for thyroid cancer management [14]. It is a heterodimeric glycoprotein, secreted by the pituitary gland, consisting of two non-covalently bound subunits, α and β, in which carbohydrates represent 15–25% of the total weight. Both subunits are glycosylated at two glycosylation sites (Asn-52 and Asn-78) in the α-subunit and one glycosylation site (Asn-23) in the β-subunit [15].

It has been reported that different cell-culture parameters can affect the glycosylation of recombinant proteins secreted by mammalian cells to various degrees, leading to products with different properties [16–19]. In fact, terminal sialic acid residues have been demonstrated to influence bioactivity of recombinant hTSH. Terminal sialylation is thought to protect the hormones from rapid renal clearance by lectins that

bind asialoglycoproteins [20]. Preparations of hTSH with a higher sialic acid content were shown to have a longer circulatory half-life compared to preparations with lower sialic acid contents and an increased in vivo activity and a reduced in vitro activity were observed [15, 21–26]. Asparagine-linked carbohydrate structures (N-glycans) of native pituitary human hTSH (p-hTSH) [27, 28] and of the recombinant hormone (r-hTSH) [29] have been described as mostly formed by biantennary complex-type structures at each glycosylation site. According to these authors, p-hTSH, unlike r-hTSH, also contains N-acetylgalactosamine sulfate at the termini of some carbohydrate chains, including a sulfated/sialylated biantennary oligosaccharide structure. Morelle et al. [29] also observed that the N-glycans of r-hTSH are different at each glycosylation site and that fucosylated glycans, which may play an important biological role, are only present in the α-subunit at Asn-52. Hiyama et al. [28] observed that, at both glycosylation sites in the α-subunit of p-hTSH, the sulfated/sialylated component was predominant, whereas the disulfated, core-fucosylated oligosaccharide was more frequent at the unique glycosylation site of the β-subunit. The carbohydrate heterogeneity of the glycoprotein hormone finally results in variability in the content of negatively charged sulfate and/or sialic acid groups, thus causing charge heterogeneity [20]. Such heterogeneity gives rise to a complex isoelectric focusing profile (IEF) [15, 30].

Considering, as mentioned above, that culture conditions have been recognized as a critical factor influencing the expression level of a protein, we examined in this work the effect of the level of carbon dioxide (CO_2) on the productivity and quality of recombinant human thyrotropin. A related study analyzing the effects of different CO_2 levels on the production of CHO-derived human erythropoietin (r-EPO) was previously developed by Yoon et al. [9] who noted a remarkable increase (~2 fold), by immunoassay, in the productivity of r-EPO. No apparent alteration in EPO quality was observed in the in vivo bioactivity as assessed by the reticulocyte measurement assay and in the molecular weight distribution pattern determined by Western blot analysis. The authors suggested that the increased EPO production could be the result of a cell cycle arrest in the G_1 phase, caused by changing from 5% CO_2 to an almost CO_2-free condition [9].

In the present work, two recombinant hTSH preparations were evaluated and compared with a well-known commercial preparation (Thyrogen) [31] with respect to their biological activity, charge isomer distribution and carbohydrate structures: one of these preparations was produced under standard conditions [32, 33] and the other by switching from 5% CO_2 to an air (0.03% CO_2) environment when the culture reached ~ 80% of confluence.

Material and Methods

Cell Cultivation

A clone, obtained in our laboratory, expressing human TSH [32], was cultured in T-flasks (225 cm^2, from Corning Costar Corporation, Cambridge, MA) containing 40 ml of medium, at 37°C. Transfected cells were grown until 80% confluence in α-MEM medium supplemented with 10% dialyzed foetal bovine serum, in the presence of 5% CO_2. The media were then changed to serum-free medium, CHO-S-SFM II with nucleosides (hypoxanthine and thymidine) and with the addition of 0.1 μM methotrexate, which allows a selective pressure. After 24 h with this medium in the presence of 5% CO_2, the level of CO_2 was reduced to 0.03% CO_2 (air environment) in one of the cultivation conditions ($-CO_2$) and maintained at 5% CO_2 in the second condition ($+CO_2$). Every 24 h, culture media were harvested and replaced with fresh, serum-free media. Daily harvest was continued for up to 30 days. Human hTSH concentration in CHO conditioned medium was determined by immunoradiometric assay (IRMA).

Purification Process

For the purification of r-hTSH obtained under the two different cultivation conditions, we utilized a strategy developed in our laboratory consisting of an ion exchange chromatographic step with a subsequent reversed-phase high performance liquid chromatography (RP-HPLC) step [34]. Briefly, conditioned medium, after adjustment to pH 5.0, was applied to a SP-Sepharose fast flow column 26/10 (GE Healthcare) equilibrated with 50 mM NaCl, 20 mM sodium acetate buffer, pH 5.0. After the loading and washing of the column with this buffer, the proteins were eluted with a linear gradient of NaCl (0.05–0.25 M) in the same buffer 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 on a semi-preparative Vydac C4, 214 TP510 column, 25 cm × 10 mm i.d., (Grace-Vydac) 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 25–100% 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.

Analytical Reversed-phase High Performance Liquid Chromatography

For RP-HPLC, a C4-Vydac 214 TP 54 column (25 cm × 4.6 mm i.d., pore diameter of 300 Å and particle diameter of 5 μm) coupled to a guard column (Vydac 214 FSK 54) was utilized. The running conditions were the same as described for the semi-preparative column utilized in the purification process, except for the flow-rate (0.5 ml/min). Thyrogen, a well-known commercial preparation from Genzyme Corporation (Framingham, MA, USA), was utilized as reference preparation for qualitative and quantitative purposes.

Immunoradiometric Assay

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 [35]. Briefly, hTSH IRMAs were 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 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 5,000*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.

Isoelectric Focusing

Separation of hTSH charge isomers was carried out on a pH 3.5–9.5 isoelectric focusing gel (6% polyacrylamide). The gel was focused for 1 h at 500 V and 1 h at 1,500 V. Protein bands were transferred to polyvinylidene-fluoride (PVDF) membranes for 90 min at 25 V using an Hoefer TE 70 semi-dry transfer apparatus (GE Healthcare). Following the transfer, membranes were stained with Coomassie brilliant blue R-250. Five preparations were analyzed: three of recombinant origin (Thyrogen, r-hTSH-IPEN (+CO_2), r-hTSH-IPEN (−CO_2) and two of pituitary origin (p-hTSH-NIDDK and p-hTSH-NOR). The last preparation was kindly provided by Dr. P.A. Torjesen (Aker University Hospital, Oslo, Norway).

Carbohydrate Structure Analysis

The analysis of the N-glycan structures was performed after cleaving the glycans from r-hTSH 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) [36].

In Vivo Bioassay

The potency (biological activity) relative to a Reference Preparation (pituitary hTSH, NIDDK) of the two r-hTSH preparations produced in our laboratory under different culture conditions and of a commercial product (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 [37], utilizing a single dose (10 μg/mouse) and 6 mice for each preparation, as described [34].

Results and Discussion

An improvement of hTSH yield was demonstrated when genetically modified CHO cells were cultivated in an air environment (0.03% CO_2) instead of under standard conditions (5% CO_2). Qualitative and quantitative RP-HPLC analysis of hTSH present in the CHO conditioned medium showed a higher peak of hTSH in air environment, while all other protein peaks apparently did not show a remarkable variation; a mean production increase of 61% and an identical chromatographic behavior for both products was observed (Table 1 and Fig. 1). This type of analysis, carried out directly on CHO-conditioned medium, was possible thanks to this particular RP-HPLC set up [38]. Our data confirm the validity of this approach, with an improvement that is only slightly lower than that determined, via immunoassay, by Yoon et al. [9] for the production of CHO-derived EPO under reduced CO_2 levels.

When changing culture conditions, attention was paid to possible alterations in the carbohydrate moiety of r-hTSH. N-glycan structures, identified in both hTSH–IPEN

Table 1 r-hTSH content in 1 l of conditioned CHO medium, obtained under standard conditions ($+CO_2$) and in an air atmosphere ($-CO_2$)

Assay number	Total hTSH ($+CO_2$) yield (μg/l)	Total hTSH ($-CO_2$) yield (μg/l)	Production increase (%)
1	895	1270	42
2	909	1570	73
3	546	925	69
			Mean ± SD = 61.3 ± 16.9; RSD (%) = 27.5

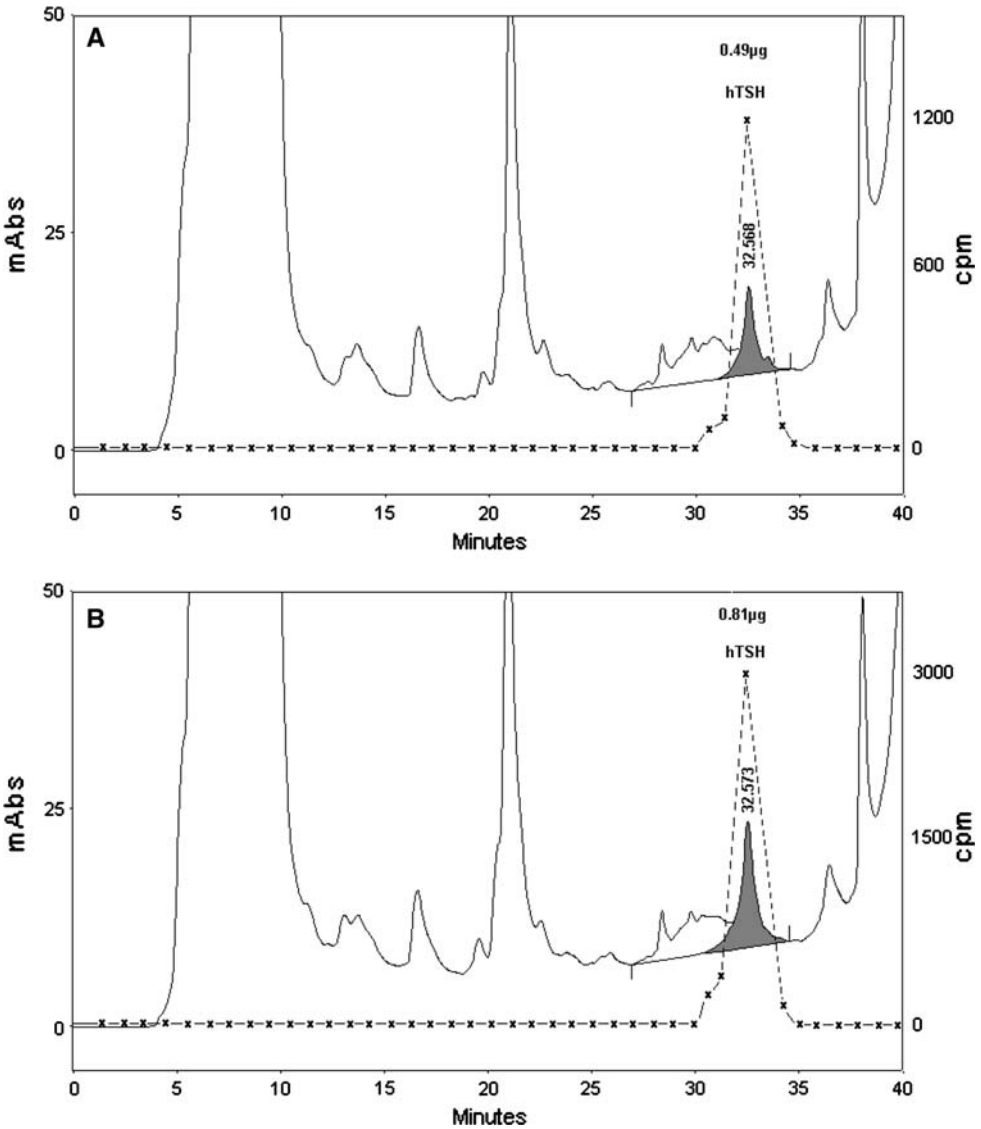

Fig. 1 RP-HPLC profile of CHO-conditioned medium from different bioprocess conditions: (**a**) 5% CO_2; (**b**) 0.03% CO_2. (—) A_{220}; (×–×) immunoactivity determined via hTSH IRMA. In a parallel run, the hTSH reference preparation (Thyrogen) presented a $t_R = 32.57$ min

preparations and also in a commercial one (Thyrogen), are shown in Fig. 2 and Table 2. The N-glycans identified were of the complex type, presenting di-, tri-, and tetra-antennary structures, sometimes fucosylated and with variable levels of sialylation. The most abundant structures were the monosialylated biantennary N-linked sugar chains (N2G2S1, N2G1S1, and N2G2S1F), representing ~69% of all identified forms in the three preparations. About 86–88% of the total oligosaccharides in the three preparations were sialylated, with 3.61, 3.37, and 3.57 mol sialic acid/mol protein in r-hTSH IPEN ($+CO_2$), r-hTSH IPEN ($-CO_2$), and Thyrogen, respectively. The sialic acid:galactose molar ratio (S/G) was found practically identical for the three preparations, i.e. 0.61, 0.61, and 0.59 respectively, confirming Cole et al.'s observation [31]. From these authors' data we can in fact calculate S/G ratios of 0.66

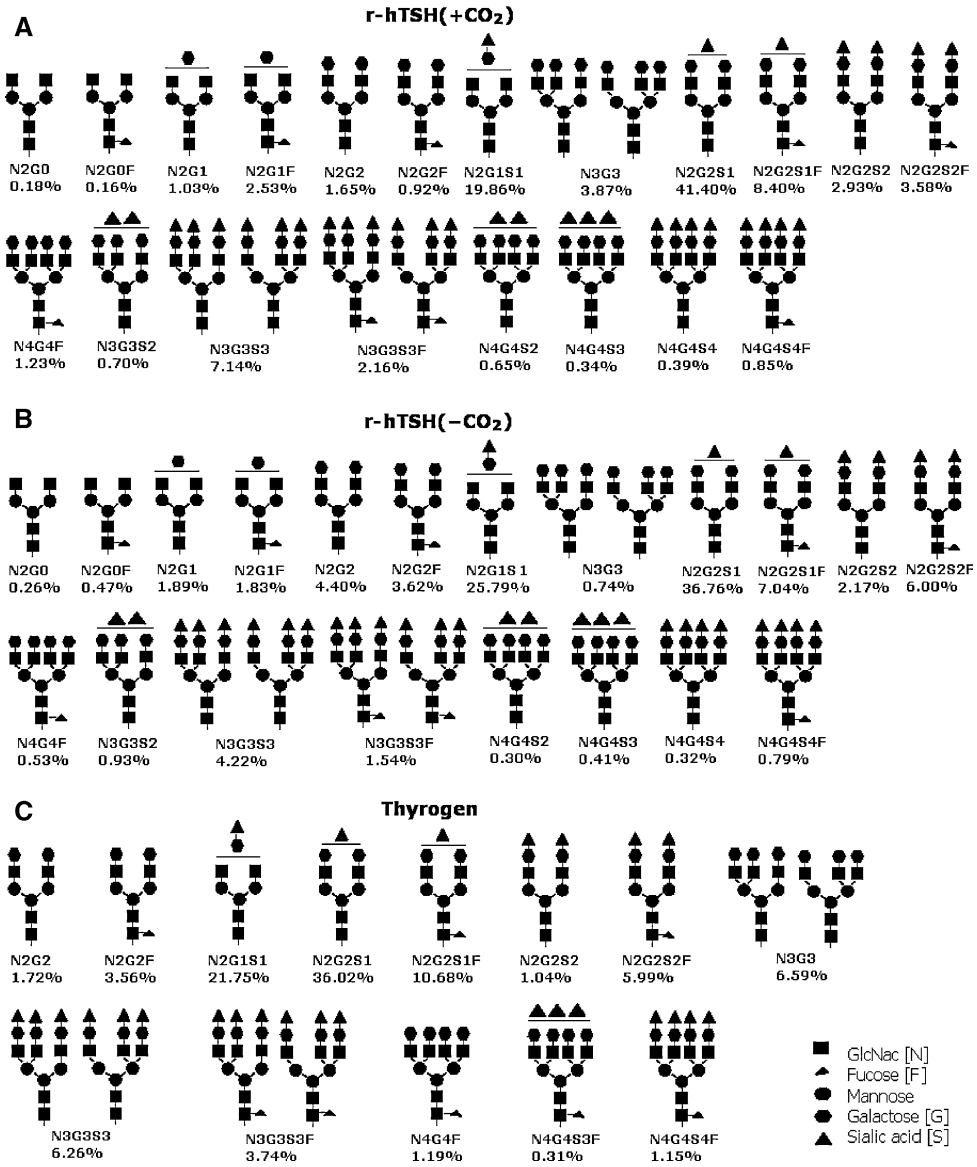

Fig. 2 N-glycan analyses of the different hTSH preparations

Table 2 N-glycan distribution in the three r-hTSH preparations, according to antennarity and sialylation level

Sialylation level	Di-antennary (%)			Tri-antennary (%)			Tetra-antennary (%)			Total per sialylation level (%)		
	IPEN ($-CO_2$)	IPEN ($+CO_2$)	THY	IPEN ($-CO_2$)	IPEN ($+CO_2$)	THY	IPEN ($-CO_2$)	IPEN ($+CO_2$)	THY	IPEN ($-CO_2$)	IPEN ($+CO_2$)	THY
0	12.47	6.47	5.28	0.74	3.87	6.59	0.53	1.23	1.19	13.74	11.57	13.06
1	69.59	69.66	68.45	_			_			69.59	69.66	68.65
2	8.17	6.51	7.03	0.93	0.70	n.d.	0.30	0.65	n.d.	9.40	7.86	7.03
3	–			5.76	9.30	10.0	0.41	0.34	0.31	6.17	9.64	10.31
4	–			_			1.11	1.24	1.15	1.11	1.24	1.15
Total per antennae	90.2	82.64	80.76	7.43	13.87	16.59	2.35	3.46	2.65			
Total sialylated glycans										86.27	88.40	87.14

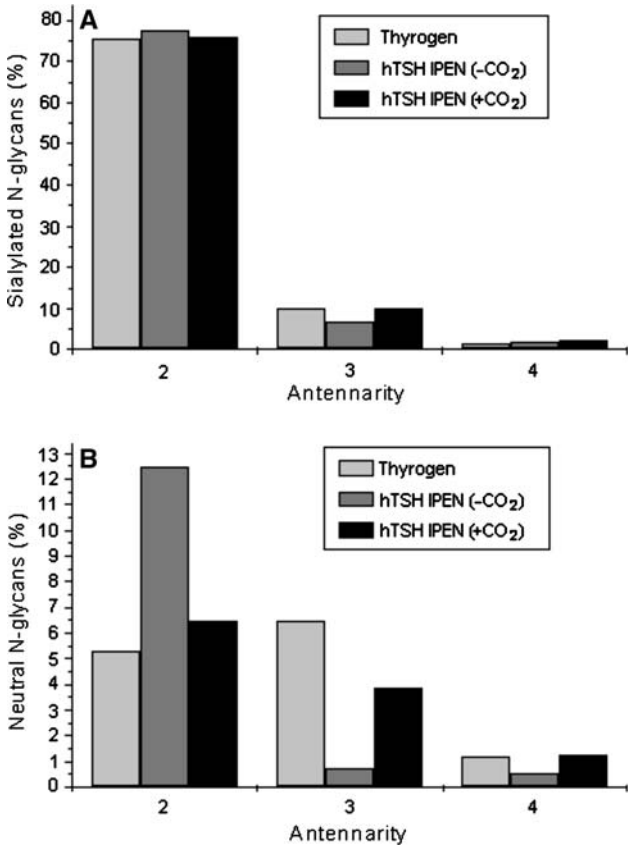

Fig. 3 Frequency of N-glycan structures in three recombinant hTSH preparations. (**a**) Sialylated N-glycans; (**b**) neutral N-glycans

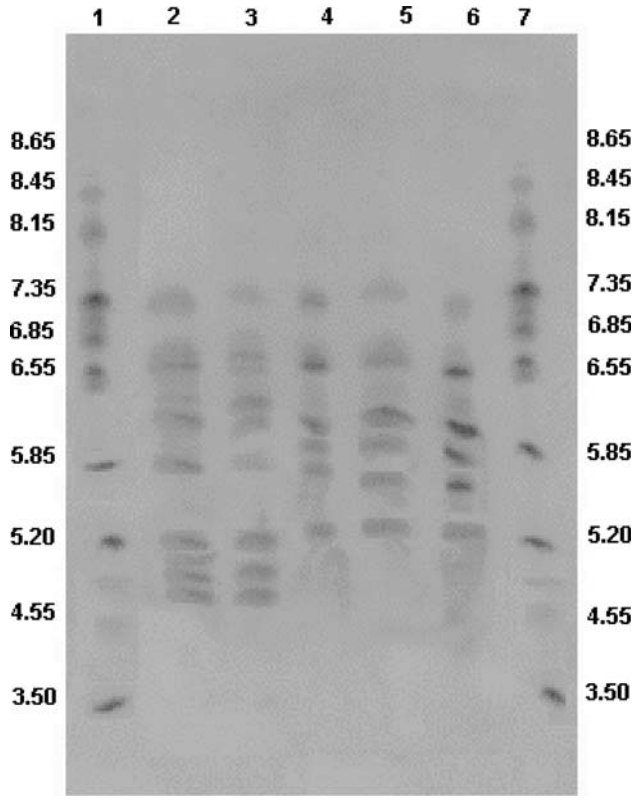

Fig. 4 Eletroblotting of different hTSH preparations. 1: isoelectric point marker; 2: pituitary hTSH (NOR); 3: pituitary hTSH (NIDDK); 4: Thyrogen; 5: hTSH IPEN (+CO_2); 6: hTSH IPEN (−CO_2); 7: isoelectric point marker

(r-hTSH) and 0.68 (p-hTSH), thus estimating an excellent interlaboratory precision (% SD) of ±6.0% for this parameter, considering the 5 values. Some differences in the percentage of di- and tri- antennary glycans were found (Table 2). For hTSH-IPEN produced in the absence of CO_2 (−CO_2), 8–10% more N2 structures were found, while for hTSH-IPEN (+CO_2) and Thyrogen, 7–9% more N3 structures were observed. This difference in antennarity is mainly observed for neutral glycans, whose total percentage (12–14%) remains, however, practically the same for the three preparations (Table 2 and Fig. 3). Some differences were also observed in fucosylation, fucosyl residues being present in the core of about 27% of N-glycans in Thyrogen and 20–22% of N-glycans in the IPEN preparations (Fig. 2). An analogous analysis of N-linked hTSH glycans has been reported by Morelle et al. [29]. These authors also released all N-glycans from recombinant hTSH by peptide N-glycosidase F digestion, but analyzed them by RP-HPLC, Concanavalin A affinity chromatography and mass spectrometric techniques. While in our case we could identify and quantify up to 23 different structures, Morelle et al. identified 16 structures, one of which, a tetrasialylated pentaantennary structure (N5G5S4F), was not found in our study. In their case, moreover, the three major glycans were

Table 3 Bioactivity and relative potency of different preparations based on T_4 determination by RIA after hTSH administration to T_3-treated mice

Preparation	T4 (μg/dl)[a]	Potency (relative to pit hTSH)
Saline	n.d.[b]	–
pit-hTSH NIDDK	2.0 ± 0.31	1.00
Thyrogen	3.2 ± 0.40	1.60
r-hTSH IPEN (+CO_2)	3.6 ± 0.78	1.80
r-hTSH IPEN (−CO_2)	2.5 ± 0.40	1.25

[a] each value is the average of 6 mouse sera determinations, each serum being analyzed in duplicate

[b] non-detectable

reported to be mono- or disialylated biantennary structures, while our three major forms were all monosialylated. These authors provided a semiquantitative structural analysis, determining in addition what types of glycans were linked to each glycosylation site. A quite interesting comparison can be carried out between glycan structures identified in our recombinant preparation of hTSH and those reported by Loumaye et al. [39] for CHO-derived r-hFSH. In both cases, up to 20 different structures were identified (excluding

enantiomeric forms), 14 of which were common to the two hormones.

Charge heterogeneity of recombinant and native pituitary hTSH preparations was evaluated by isoelectric focusing (IEF). Figure 4 shows about six components with p*I* between 5.20 and 7.35, for the three recombinant preparations (lanes 4, 5, and 6), the major isoforms being located between p*I* 5.85 and 6.55. No remarkable differences were observed between the three recombinant preparations. Approximately the same number of bands were found in a Thyrogen preparation by Zhou et al. [30]. These were, however, distributed in a slightly more acidic region, with p*I*s approximately between 4 and 7. Moreover, these authors identified 10 different glycan structures, assigning them to one of 5 different IEF bands. In contrast, a band distribution in a more basic region (p*I* between 6.2 and 8.5) was reported by Szkudlinski et al. [15] for different preparations of r-hTSH. A quite different IEF pattern was obtained in the present work for two native pituitary preparations: p-hTSH-NIDDK and p-hTSH-NOR (lanes 2 and 3), with 8–9 components distributed between p*I* 4.55 and 7.35, the major isoforms being focused in the acidic region, between p*I* 4.55 and 5.20.

The in vivo bioassay, based on hTSH-induced T_4, showed that r-hTSH-IPEN ($-CO_2$) was slightly less active ($P < 0.02$) than r-hTSH-IPEN ($+CO_2$), which was equipotent ($P > 0.05$) with Thyrogen (Table 3). The three preparations were 1.25-, 1.6-, and 1.8-fold more potent than p-hTSH-NIDDK, with significance levels of $P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively.

Conclusions

Recombinant hTSH secretion yields were considerably increased under reduced CO_2 conditions. Different production processes apparently do not greatly influence N-glycan structures, charge isomer distribution or biological activity. It is noteworthy that while our product was obtained in T-flasks, as far as we know Genzyme is carrying out Thyrogen production in pilot scale stirred tank, oxygen sparged bioreactors. Even though the bioactivity of the product obtained in an air environment appears somehow lower, it is still significantly higher than the pituitary reference preparation of hTSH. We can anticipate that a considerable difference in these parameters was found between native-pituitary and recombinant hTSH, as expected. This is probably a consequence of the well-known difference in the composition of the carbohydrate moiety existing between human and CHO-derived hTSH.

Acknowledgments This work was supported by FAPESP, São Paulo, Brazil (03/11023-0 and 04/08904-7) and by the National Research Council (CNPq), Brasilia, Brazil (PQ 305108/2005-0 and PQ 301103/2006-2).

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