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Enhancement of Human Thyrotropin Synthesis by Sodium Butyrate Addition to Serum-Free CHO Cell Culture

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Abstract The influence of sodium butyrate (NaBu) on the synthesis of recombinant human thyrotropin (r-hTSH) by CHO cells was investigated for the first time. A volumetric productivity of ~10 μg hTSH/mL was repeatedly obtained, with a 3.3-fold increase over a control culture carried out in the absence of NaBu. Since NaBu can induce CHO cell apoptosis and cell growth arrest, the increase in specific productivity was even higher, i.e., ca. 5-fold. Analysis of the N-glycan composition of r-hTSH obtained with the addition of NaBu to the culture medium showed an approximately 12 % increase in the amount of sialic acid, as well as in total carbohydrate, partly due to the increase in the site occupancy from 2.77 to 2.93 glycans per mole of hTSH. The two hormone preparations were characterized by N-glycan structural analysis, which showed that NaBu increased the bi-antennary structures by ca. 13 % while decreasing the tri-antennary structures by approximately the same amount. The in vivo biological activity and pharmacokinetic behavior (clearance) were found to be similar for the two hormone preparations.

Keywords hTSH · Sodium butyrate · Glycosylation · Biological activity · Pharmacokinetics

Abbreviations

NaBu	Sodium butyrate
r-hTSH	Recombinant human thyrotropin
CHO	Chinese hamster ovary cells
r-hEPO	Recombinant erythropoietin
β-IFN	β-Interferon
CMP	Cytodine monophosphate
t-PA	Plasminogen activator
GP2	Glycoprotein 2
RP-HPLC	Reversed-phase high performance liquid chromatography
HPSEC	Size exclusion high performance liquid chromatography

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SA	Sialic acid
OPD-2HCl	*o*-Phenylenediamine dihydrochloride
GlcN	*N*-acetyl-glucosamine
GalN	*N*-acetyl-galactosamine
Gal	Galactose
Fuc	Fucose
Man	Mannose
TFA	Trifluoroacetic acid
SDS	Sodium dodecyl sulfate
PNGase F	Peptide-N-glycosidase F
DHB	Dihydroxybenzoic acid
IRMA	Immunoradiometric assay
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Introduction

One of the most successful strategies for the production of a recombinant CHO cell-derived protein includes supplementation of culture medium with sodium butyrate (NaBu). This addition is a critical point to be analyzed, considering that it can potentially impact productivity and product quality.

There is a consensus in the literature that NaBu treatment can increase glycoprotein synthesis [1–11]. This increase is attributed to the arrest of cell cycle progression (G_0/G_1) provoked by NaBu, resulting in a decrease in the cell growth rate and an increase in the specific productivity of the protein of interest [12].

Glycosylation is a crucial attribute of the product that can directly affect protein quality and many different additives can influence it. Several different results have been reported for the variation of glycosylation caused by NaBu addition, including sialylation. These variations appear to be dependent on the NaBu concentration, the host cell, and the protein of interest. For example, although 5 mM NaBu reduced branching and sialylation in N-linked glycans of CHO-derived recombinant erythropoietin (r-hEPO) [2], 1 or 2 mM NaBu caused no significant changes in the glycosylation of this protein [4]. Similarly, while no modifications were found by Crowell et al. [8] utilizing 2 mM NaBu for r-hEPO expressed in another cell line (human kidney fibrosarcoma), another protein (CHO cell-derived β-interferon, β-IFN) showed a decrease in sialylation [5]. Spearman et al. [7] found an increase in the proportion of highly branched complex N-linked glycans when butyrate was utilized in β-IFN culture medium.

In this context, it should be emphasized that sialylation is of great importance for the quality of therapeutic proteins since their circulatory half-life and in vivo activity can be influenced by sialic acid content [13–15]. Hypotheses that may explain changes in the sialylation of proteins are linked to the availability of cytodine monophosphate (CMP)-sialic acid, the enzymatic activity of sialyltransferase and galactosyltransferase and the availability of galactose in the emerging glycan structure [16]. N-glycan site occupancy was also shown to be affected by the presence of NaBu. Thus, Gawlitzek et al. [17] found an increase in N-glycan site occupancy of human tissue plasminogen activator (t-PA) upon addition of just 0.75 mM NaBu. For a recombinant CHO-derived enzyme (glycoprotein 2-GP2), the same authors found a decreased site occupancy upon delaying the time of butyrate addition. Such variations play a critical role in the efficacy and pharmacokinetics of therapeutic glycoproteins [18].

In view of these divergent results and the fact that the alterations caused by sodium butyrate depend on the protein being synthesized, we have analyzed in this work the effects of the addition of NaBu on the productivity, carbohydrate quality, and bioactivity of recombinant human thyrotropin (hTSH). This hormone is a heterodimer formed by noncovalently linked α- and β-subunits. Both subunits are glycosylated, the α-subunit being glycosylated at two sites (Asn-52 and Asn-78) in the C-terminal half of the molecule, while the β-subunit is glycosylated at one position in the amino terminal portion, i.e., at Asn-23. Human thyrotropin is pharmacologically very important, especially for thyroid cancer management, both in the diagnostic follow-up of differentiated thyroid carcinoma and in post-surgical thyroid remnant ablation with radioiodine-131. It is also used to evaluate thyroid reserve capacity and to enhance radiodine uptake in patients with multinodular goiter [19, 20]. Clearly, then, investigation of the influence of NaBu addition on productivity must be accompanied by an analysis of the accompanying effects on the carbohydrate moiety, which can be very important for in vivo use of the recombinant hormone. We believe more work must be done for its quality control, possibly in comparison with the native, pituitary forms.

Materials and Methods

Hormone Preparations

Four hTSH preparations were utilized in this work, three of them recombinant CHO-derived and one pituitary-derived. Among the recombinant preparations, two of them were synthesized, purified, and characterized in our laboratory (r-hTSH IPEN) and one was a well-known commercial recombinant product (Thyrogen) from Genzyme Corporation (Framingham, MA, USA). The pituitary-derived preparation (p-hTSH), used as a reference, was obtained from Dr. A. F. Parlow of the National Hormone and Pituitary Program (NHPP, Torrance, CA, USA).

Cell Cultivation

A clone expressing human TSH [21], obtained in our laboratory, was cultured at 37 °C in T-flasks (75 cm^2, from Corning Costar Corporation, Cambridge, MA) containing 10 mL of medium. Transfected cells were grown until 80 % confluence in α-MEM medium supplemented with 10 % dialyzed fetal bovine serum in the presence of 5 % CO_2. For production, the medium was then changed to serum-free medium, CHO-S-SFM II with nucleosides (hypoxanthine and thymidine), with or without 1 mM sodium butyrate. The culture was maintained for 10 days, the conditioned culture medium being harvested daily and replaced with fresh serum-free medium. The harvested conditioned medium pool was then concentrated in a tangential flow filtration system (Millipore, Bedford, MA, USA) with a Pellicon membrane (*M*r 5,000 cutoff). The hTSH content of this concentrated pool was determined by reversed-phase high performance liquid chromatography (RP-HPLC) [22] against an internal reference preparation of r-hTSH (Thyrogen).

Purification Process

The two-step purification strategy developed in our laboratory via cation exchange chromatography on SP-Sepharose FF (GE Healthcare Life Sciences, São Paulo, Brazil) and reversed-phase high performance liquid chromatography on a 214 TP 510 C4-Vydac semi-preparative column (Grace-Vydac, The Nest Group, Southborough, MA, USA) was

adopted, employing the conditions previously described by Oliveira et al. [23]. For each hTSH preparation, the purification process started with 3 L conditioned medium. Final product purity was evaluated by high-performance size exclusion liquid chromatography (HPSEC) and by RP-HPLC [24, 25]. Four lots of r-hTSH IPEN were prepared, two of them derived from CHO cell cultures without NaBu addition (r-hTSH IPEN control) and two from CHO cell cultures with NaBu addition (r-hTSH IPEN+NaBu).

High-Performance Size Exclusion Liquid Chromatography (HPSEC)

HPSEC 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 a Shimadzu Model SCL-10A HPLC apparatus. Detection was by UV absorbance at 220 nm with a flow rate of 1.0 mL/min, employing 0.15 M NaCl in sodium phosphate buffer (pH 7.0; 0.02 M) as the mobile phase.

Analytical Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

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, connected to a Shimadzu HPLC apparatus. Chromatography was carried out at 25 °C with detection by UV absorbance at a wavelength of 220 nm. Gradient solutions A and B were utilized, solution A being sodium phosphate buffer (pH 7.0; 0.05 M) and solution B acetonitrile. For hTSH elution, a linear gradient from A/B (87.5:12.5, *v*/*v*) to A/B (50:50, *v*/*v*) over 40 min was used.

Sialic Acid Determination

Sialic acid (SA) was released from hTSH purified preparations (down to 5 μg of protein) by mild acid hydrolysis with sodium bisulfate (0.5 M $NaHSO_4$) during 20 min at 80 °C, followed by derivatization with *o*-phenylenediamine dihydrochloride, OPD-2HCl (20 mg/mL in 0.25 M $NaHSO_4$), during 40 min at 80 °C. The derivatives were then separated by reversed-phase high performance liquid chromatography with fluorescence detection (λ_{ex}=230 nm, λ_{em}=425 nm) using a Supelcosil LC-318 column (4.6 I.D.×250 mm, 5 μm particle size) purchased from Supelco (Bellefonte, PA, USA) and following the conditions previously described by Anula et al. [26]. Quantitation was carried out by reference to known amounts of *N*-acetylneuramic acid derivatized and injected in parallel. Sialic acid contents are reported as the ratio of moles per mole of recombinant protein.

Neutral Monosaccharide Content Determination

Free *N*-acetyl-glucosamine (GlcN), *N*-acetyl-galactosamine (GalN), galactose (Gal), fucose (Fuc), and mannose (Man) were obtained by hydrolysis of the samples with trifluoroacetic acid (2 M TFA) for 4 h at 100 °C, followed by analysis via high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a PA1 column with an aminotrap pre-column (Dionex system).

N-glycan Structure Analysis

N-glycoprofiling was performed after glycosidase digestion and permethylation of N-glycans. Samples were denatured in 0.5 % sodium dodecyl sulfate (SDS) and 1 % β-

mercaptoethanol (90 °C, 10 min) and deglycosylated with 20 U of peptide-N-glycosidase F (PNGase F, Roche Diagnostics, Mannheim, Germany) (37 °C, 15 h). N-glycans were purified on an Ultra Clean SPE Carbograph column (Alltech, Deerfield, IL, USA), and after elution with 25 % acetonitrile containing 0.1 % TFA, they were lyophilized. Permethylation was then performed using sodium hydroxide and dimethyl sulfate. The reaction products were purified on C18 Sep Pak Plus (Waters) and lyophilized. The purified permethylated glycans were solubilized with 50:50 water/methanol mixed with 2,5-dihydroxybenzoic acid (DHB) matrix solution (10 mg/mL) (LaserBiolabs, Sophia-Antipolis, France). Positive ion reflectron MALDI mass spectra were acquired on a VOYAGER DE PRO mass spectrometer (AB Sciex, Framingham, MA, USA).

Animal Studies

All assays were conducted in accordance with the national protection laws on animal welfare.

Pharmacokinetic Studies

Single doses (2 μg) of the different hTSH preparations were administrated to BALB/c mice (five males per group) by intraperitoneal injection. Serum samples were obtained at 30, 60, 90, 120, 180, 240, and 300 min post injection. Serum hTSH concentrations were determined by immunoradiometric assay (IRMA) and expressed as the percentage of the maximum concentration.

Bioassay

The biological activity of the different hTSH preparations was determined by an in vivo bioassay in BALB/c mice in which TSH-induced T_4 is measured after a 5-day suppression of endogenous TSH by T_3 administration. A single dose (10 μg hTSH/mouse) and 10 mice were utilized for each preparation. Serum samples were obtained at 6 h post injection. The serum T_4 concentrations were determined using human T4 RIA kits (Siemens, Los Angeles, USA), according to the manufacturer's instructions.

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 [27]. 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, 0.05 mL of solid phase-coupled polyclonal antibody (1.25 mg/tube) and 0.30 mL of 0.05 M phosphate buffer (pH 7.4, with 1 % bovine serum albumin and 0.5 % Tween 20). Incubation was carried out on a rotary mixer at room temperature for 16 h. 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 phosphate buffer (0.05 M; pH 7.4) with 0.5 % Tween 20. The washing procedure was repeated twice.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)

For mass determinations, the preparations of r-hTSH obtained with or without NaBu addition to the culture medium (1 μL of each sample with a concentration of 1 mg/mL) were mixed with sinapinic acid matrix (1 μL) and analyzed on a Voyager-DE MALDI-TOF mass spectrometer from Applied Biosystems (Foster City, CA, USA), operated in the linear positive ion mode and scanned over the *m*/*z* range from 1,000 to 40,000.

Results

The growth capacity of hTSH-secreting CHO cells was tested in the presence of different NaBu concentrations (0.2, 0.5, 1.0, 2.0, and 4.0 mM) and the hTSH productivity determined. A concentration of 1 mM was chosen for NaBu in the present study on the basis of an appropriate balance between cell growth and hTSH volumetric productivity. Figure 1 shows the cell growth, analyzed during 6 days in the presence and absence of 1 mM NaBu. The maximum viable cell concentration (at day 4) was 54 % lower when 1 mM NaBu was utilized. With NaBu, cell proliferation was ca. 3-fold slower in the first 4 days, the slopes of the growth curves being 1.14 versus 3.85 cells/day in the presence or absence of NaBu, respectively. Despite the decrease in cell growth due to NaBu addition, the final volumetric productivity of hTSH in the 3-L production pool increased 3.3±0.53-fold (n=3 independent experiments) compared to that obtained in the absence of NaBu (Fig. 2). This corresponds to a 5.2-fold higher specific productivity (microgram/10^6 cells/day) in the presence of NaBu.

Purified hTSH preparations (r-hTSH IPEN) were obtained as described, starting from conditioned culture medium with (r-hTSH IPEN+NaBu) or without (r-hTSH IPEN control) NaBu addition. These products were ~98 % pure when analyzed by HPSEC and RP-HPLC (Fig. 3). Their identity and integrity were also confirmed by t_R comparison with an internal reference preparation (Thyrogen).

The total sialic acid content of these purified preparations, of Thyrogen and of p-hTSH was determined by C_{18} RP-HPLC after hydrolysis and derivatization (Table 1). Samples treated with butyrate showed a significant sialic acid increase of ~12 % relative to the control (p<0.001). As expected, the sialic acid content of the pituitary preparation was 3–4-fold lower than that of the three recombinant preparations.

In the presence of NaBu, an increase of neutral monosaccharides (ranging from 6 to 43 %; Table 2), together with an increase of ~13 % in overall glycosylation and of ~6 % in site occupancy was also observed (Table 3).

Figure 4a shows the N-glycan structures of CHO-derived hTSH obtained with or without NaBu and their relative abundances. While 18 different complex type N-glycan structures have been identified in r-hTSH obtained without NaBu, only 13 structures were detected in r-hTSH obtained with 1 mM NaBu. In both preparations, disialylated bi-antennary N-glycans (2,792*m*/*z*) were observed as the major forms followed by monosialylated bi-antennary (2,431*m*/*z*) and by disialylated fucosylated bi-antennary (2,966*m*/*z*) N-glycans (Fig. 4b). Although the three dominant structures were qualitatively the same for the two preparations, they were quantitatively different, being

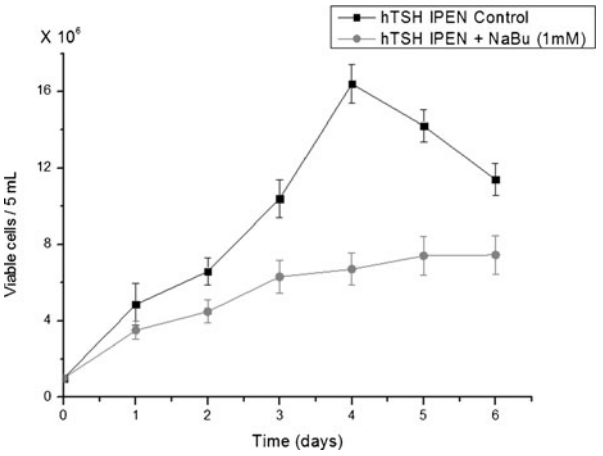

Fig. 1 Growth profile of hTSH-secreting CHO cells in culture medium with and without 1 mM NaBu. Data are the mean ±standard error of two independent determinations

16.5 % more frequent in r-hTSH obtained with NaBu (Table 4). The hormone obtained in the presence of NaBu also presented a 12.9 % higher content of bi-antennary and a 12.7 % lower content of tri-antennary structures. Tetra-antennary N-glycan structures, present in very low abundance, were only identified in r-hTSH obtained in the absence of NaBu.

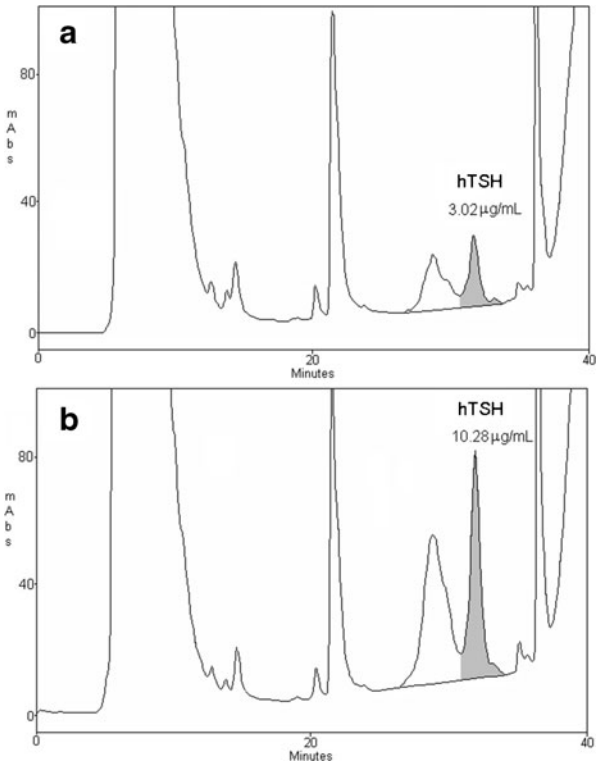

Fig. 2 Qualitative and quantitative RP-HPLC analysis of a production pool of r-hTSH-secreting CHO cell conditioned medium. **a** Conventional culture medium; **b** culture medium+1 mM NaBu

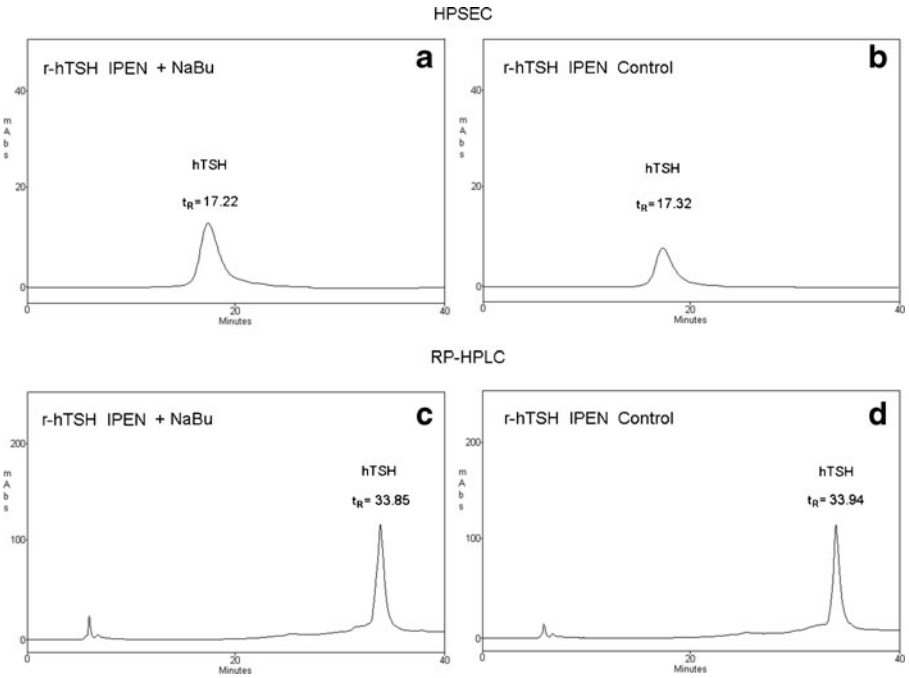

Fig. 3 RP-HPLC and HPSEC analysis of purified r-hTSH IPEN, with (**a, c**) and without (**b, d**) NaBu addition to the culture medium; t_R=retention time. In a parallel run, the r-hTSH reference preparation (Thyrogen) presented t_R=17.29 min by HPSEC and t_R=34.05 min by RP-HPLC

Table 1 Sialic acid quantification in the different hTSH preparations

Preparation	No. of assays	Sialic acid content (mol SA/mol hTSH)	Difference from control (%)
r-hTSH IPEN control	6	6.71±0.36	–
r-hTSH IPEN+NaBu	6	7.52±0.09	+12.1
Thyrogen	6	6.25±0.54	−6.9
p-hTSH	2	1.88±0.14	−72

Table 2 Neutral monosaccharide quantification in r-hTSH obtained with or without NaBu addition to the culture medium

Monosaccharide	r-hTSH IPEN control[a] (mol/mol hTSH)	r-hTSH IPEN+NaBu[a] (mol/mol hTSH)	Difference from control (%)
Fucose	0.7±0.02	1.0±0.08	+42.8
Galactose	6.6±0.67	7.9±0.09	+19.7
Glucosamine	12.8±0.82	15.6±0.04	+21.9
Mannose	8.3±0.51	8.8±0.08	+6.0
Galactosamine	n.d.	n.d.	

n.d. not detected

[a] Average±standard deviation of two analyses of independently hydrolyzed aliquots of each sample

Table 3 Total carbohydrate (percent) and occupancy (percent) of hTSH samples obtained with or without NaBu addition to the culture medium

Preparation	Carbohydrate[a] (%)	N-glycans/mol hTSH[a]	Occupancy (%)
r-hTSH IPEN control	21.4±2.62	2.77±0.17	92.3
r-hTSH IPEN+NaBu	24.1±0.12	2.93±0.03	97.7

[a] Mean±standard deviation

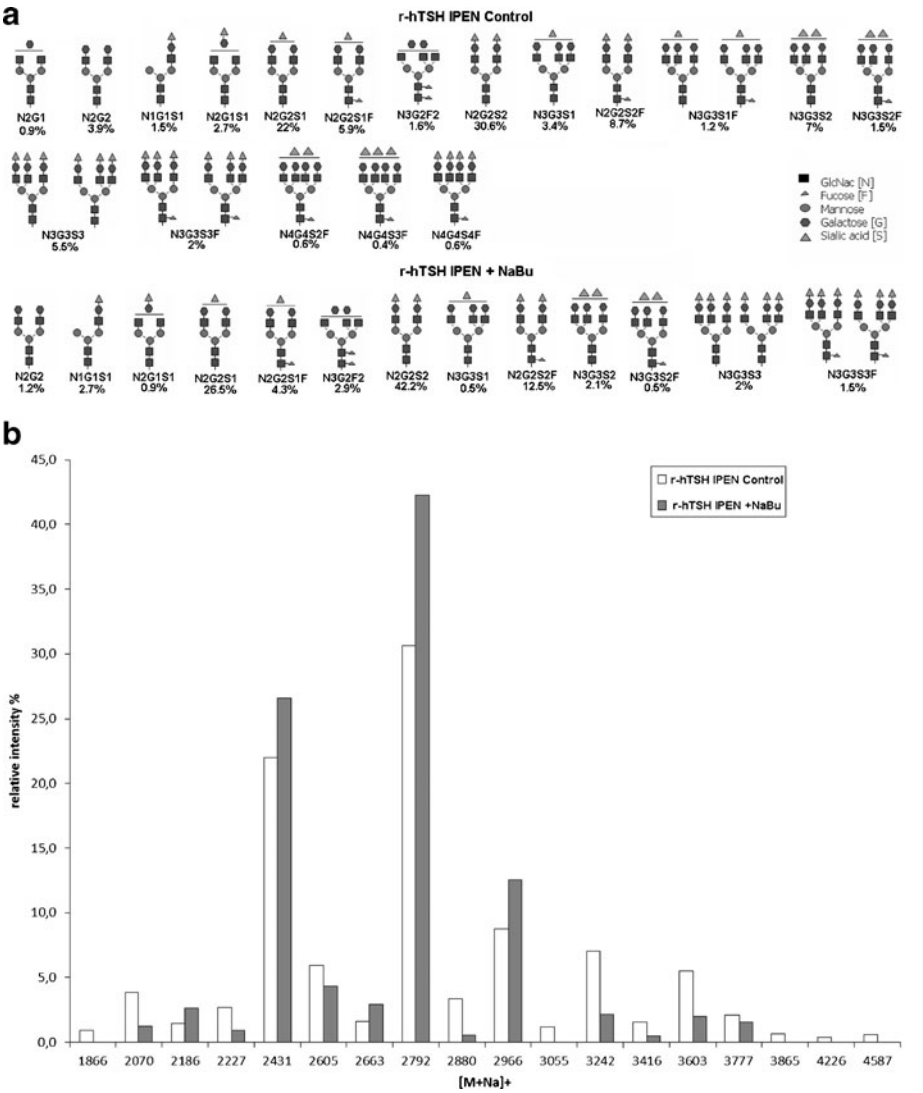

Fig. 4 **a** N-glycan structures of r-hTSH obtained with and without NaBu addition to the culture medium; **b** frequency profile for the different N-glycan structures present in r-hTSH obtained with and without NaBu addition to the culture medium

Table 4 N-glycan distribution, according to antennarity and sialylation level, of hTSH samples obtained with or without NaBu addition to the culture medium

Sialylation level	% mono-antennary		% bi-antennary		% tri-antennary		% tetra-antennary		% N-glycans per sialylation level	
	− NaBu	+ NaBu	− NaBu	+ NaBu	− NaBu	+ NaBu	− NaBu	+ NaBu	− NaBu	+ NaBu
0	n.d.		4.8	1.2	1.6	2.9	n.d.		6.4	4.1
1	1.5	2.7	30.6	31.7	4.6	0.5	n.d.		36.7	34.9
2	n.d.		39.3	54.7	8.5	2.6	0.6	n.d.	48.4	57.3
3	n.d.		n.d.		7.5	3.5	0.4	n.d.	7.9	3.5
4	n.d.		n.d.		n.d.		0.6	n.d.	0.6	n.d.
Total per antennae	1.5	2.7	74.7	87.6	22.2	9.5	1.6			

n.d. not detected

Mass spectral analysis of the r-hTSH preparations obtained in the absence and presence of NaBu revealed experimental masses of, respectively, 29,390 and 29,459 for the heterodimeric form and values ranging from 13,436 to 14,316 and from 14,570 to 15,485, respectively, for the distinct isoforms of the α- and β-subunits (Fig. 5). These masses are in agreement with the results of previous studies [24, 28], where the α-subunits ranged from 13,749 to 14,093 and the β-subunits from 15,277 to 15,544.

The biological activities of the four mentioned hTSH preparations were also assessed and no significant differences ($p>0.05$) were observed between the T_4 levels obtained except upon pituitary hTSH administration (Table 5).

Concentration–time profiles of the different hTSH preparations after intraperitoneal administration in mice are shown in Fig. 6 and the corresponding estimates of pharmacokinetic parameters presented in Table 6. The use of NaBu did not produce significant changes ($p>0.05$) in hTSH pharmacokinetics. For all recombinant preparations, the maximum hTSH serum concentration occurred at 90 min after administration, while this maximum was reached somewhat earlier (at 60 min) for the pituitary preparation. This is confirmed by the ~2-fold higher absorption half-life ($p<0.05$) and by the ~30 % higher exposure ($p<0.05$), measured as the area under the serum concentration curve, for the recombinant preparations in comparison to the pituitary preparation.

Discussion

The findings of the present study demonstrate that the addition of NaBu to the culture medium of hTSH-secreting CHO cells is an effective way to highly enhance hTSH productivity without compromising the biological activity or plasmatic clearance of the recombinant hormone, despite some alterations in the glycosylation. As far as we know, this is the first report analyzing these aspects of the production of hTSH by CHO cells.

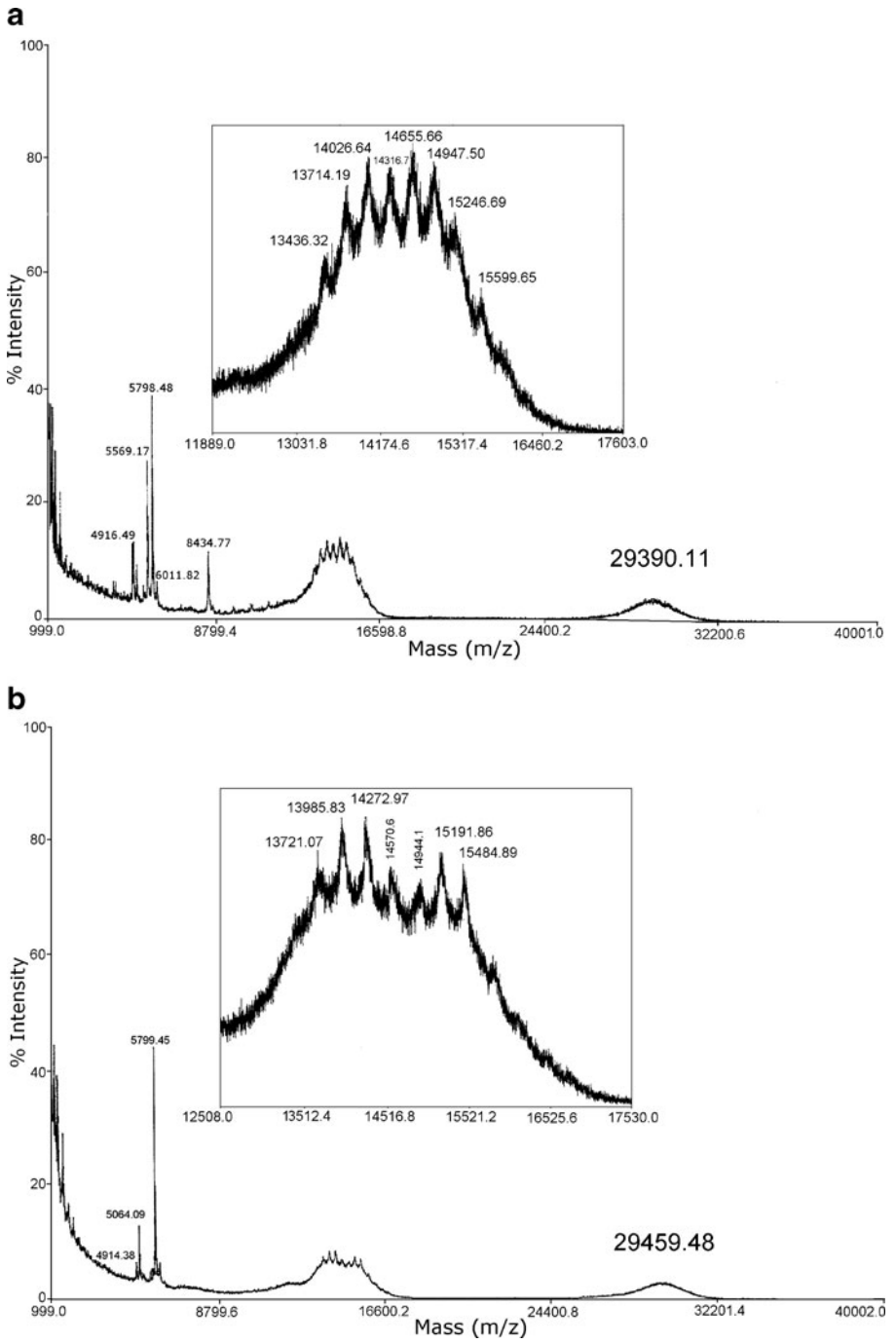

Fig. 5 MALDI-TOF mass spectra of r-hTSH IPEN with (**a**) and without (**b**) NaBu addition to the culture medium. Expanded views of the regions corresponding to the α- and β-subunits are shown in the *inserts*

Table 5 T_4 levels obtained in in vivo bioassays (n=10 mice) after administration of the different hTSH preparations

Preparation	T_4 (μg/dL)
r-hTSH IPEN-A control	2.57±0.58
r-hTSH IPEN-A (+ NaBu)	2.27±0.37
r-hTSH IPEN-B control	2.70±0.46
r-hTSH IPEN-B (+ NaBu)	3.14±0.72
Thyrogen	2.40±0.34
p-hTSH	0.75±0.18

The significant increases in volumetric and specific hTSH productivity (~3- and ~5-fold, respectively) reported here upon the addition of 1 mM NaBu to the culture medium are among the highest reported in the literature for different CHO-derived proteins, including antibodies, as can be seen in the comparison of eight different reports in Table 7.

It has already been demonstrated for hTSH that, depending on the magnitude of the change, a variation in the level of sialylation can influence its biological properties, a higher degree of sialylation corresponding to a longer in vivo half-life [31–33]. The increased levels of sialic acid observed upon butyrate treatment (~12 %, Table 1) did not cause any significant modifications of the quality attributes, as indicated by the lack of an effect on either the biological properties or the pharmacokinetics of hTSH (Tables 5 and 6). However, the higher sialylation level (~3–4-fold) found for the recombinant preparations compared to the native pituitary hormone (Table 1) produced highly significant differences in the biological activity (3–4-fold higher) (Table 5) and in the circulatory half-life (~2-fold higher) (Table 6).

Glycoprotein quality, evaluated with respect to the degree of branching of the sugar chain, showed a reduction in the proportion of N-glycans with tri- and tetra-sialylated oligosaccharides (Table 4). This was also observed by Chung et al. [2] upon the addition of 5 mM NaBu to EPO-secreting CHO cells in culture. In contrast, Spearman

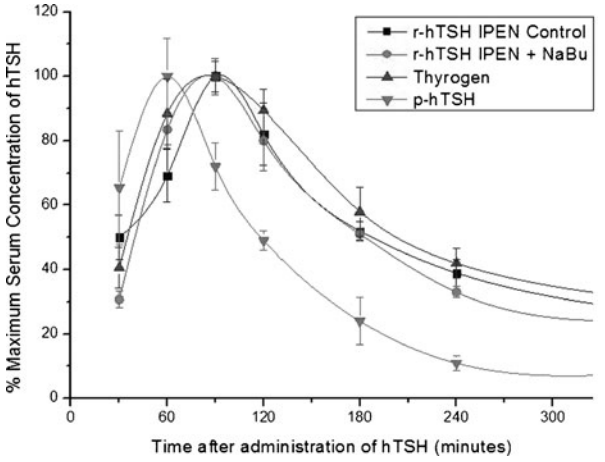

Fig. 6 Plasmatic concentrations of the different hTSH preparations in a 5-h pharmacokinetic test. Data are the mean±standard error relative to n=5 mice

Table 6 Pharmacokinetic parameters for the different hTSH preparations

Preparation	No. of assays	$t_{1/2}$ (min)	Significance level	AUC (μg min/mL)	Significance level
r-hTSH IPEN control	6	105.5±18.14	–	15,555±1,197.8	–
r-hTSH IPEN+NaBu	6	98.6±20.54	NS	15,298±2,484.7	NS
Thyrogen	3	115.1±3.12	NS	16,155±1,398.1	NS
p-hTSH	3	56.5±15.7	$p<0.05$	11,463±682.0	$p<0.05$

et al. [7] found an increase of more highly branched glycans for β-IFN upon butyrate treatment. On the other hand, in the present system, we found a marked increase (~17 %) in the predominant bi-antennary structures (especially disialylated) in the presence of NaBu. In contrast, a decrease of the same order was reported for these same structures in the case of β-IFN [5]. Specifically considering antennarity, it is noteworthy that NaBu treatment resulted in a 12.9 % increase in bi-antennary structures at the expense of a 12.7 % reduction in tri-antennary structures. This suggests that, at least in the case of r-hTSH, NaBu might limit in some way the formation of an additional antenna. In this context, the NaBu-induced cell cycle arrest, which favors the synthesis of more hormone, might somehow disfavor the formation of the third antenna from bi-antennary glycans and of the fourth antenna from the now very reduced fraction of tri-antennary glycans.

An increase in occupancy (~6 %) of the three potential N-linked glycosylation sites was also observed. This confirms the findings of Andersen et al. [34] of an increased occupancy for t-PA when NaBu was utilized.

The slight mass increase (+0.24 %) observed via MALDI-TOF-MS for r-hTSH obtained upon NaBu addition is comparable to that (+0.61 %) obtained by stoichiometric calculation based on each N-glycan contribution and occupancy. Even considering the good inter-assay reproducibility for heterodimeric hTSH determination [24, 28], we believe that the values reported in Fig. 5 (M_R=29,390 and M_R=29,459) may have a lower precision than usual due to the lack of sharpness of these two peaks.

Table 7 Volumetric and specific productivity of different recombinant proteins upon addition of 1–3 mM NaBu to CHO cell culture medium

Reference	Protein	Volumetric productivity increase (−fold)	Specific productivity increase (−fold)
Lamotte et al. [1]	Γ-IFN	1.5	3
Hendrick et al. [3]	t-PA	2	3
Yoon et al. [4]	EPO	–	2
Sung and Lee [6]	Thrombopoietin (TPO)	3	9
Rodriguez et al. [5]	β-IFN	1.2	4
Goulart et al. [10]	Prolactin (PRL)	2	3
Mimura et al. [29]	Chimeric IgG	3.5	15
Hong et al. [30]	Antibody	1.5	4
Present work	TSH	3	5

Conclusion

The present results emphasize the importance of weighing the analysis of the modifications in glycosylation due to NaBu in terms of the effects on the biological properties of the resultant glycoprotein. Thus, although butyrate greatly increased hTSH production and influenced its glycosylation by altering sialylation, site occupancy and, to a lesser extent, branching, there was no evidence of alterations in the bioavailability that might have produced an undesired influence on hormone efficacy.

As exemplified for hTSH in the present work, the systematic investigation of all aspects involved in the production strategy of a bioactive glycoprotein is extremely important not only for biotechnological development, but also for the quality control of biopharmaceuticals in general.

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