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O-Glycosylated 24-kDa human growth hormone (hGH) has a mucin-like biantennary disialylated tetrasaccharide attached at Thr-60

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Abstract

Mass spectrometry was used to characterize the 24-kDa human growth hormone (hGH) glycoprotein isoform and determine the locus of *O*-linked oligosaccharide attachment, the oligosaccharide branching topology, and the monosaccharide sequence. MALDI-TOF/MS and ESI-MS/MS analyses of glycosylated 24-kDa hGH tryptic peptides showed that this hGH isoform is a product of the hGH normal gene (hGH-N). Analysis of the glycoprotein hydrolysate by high-performance anion-exchange chromatography with pulsed amperometric detection and HPLC with fluorescent detection for NeuAc, yielded the oligosaccharide composition (NeuAc₂, GalNAc₁, Gal₁). After β -elimination to release the oligosaccharide from glycosylated 24-kDa hGH, collision-induced dissociation of tryptic glycopeptide T6 indicated that there had been an *O*-linked oligosaccharide attached to Thr-60. The sequence and branching structure of the oligosaccharide were determined by ESI-MS/MS analysis of tryptic glycopeptide T6. The mucin-like *O*-oligosaccharide sequence linked to Thr-60 begins with GalNAc and branches in a bifurcated topology with one appendage consisting of Gal followed by NeuAc and the other consisting of a single NeuAc. The oligosaccharide moiety lies in the high-affinity binding site 1 structural epitope of hGH that interfaces with both the GH and prolactin receptors and is predicted to sterically affect receptor interactions and alter the biological actions of hGH.

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Keywords

human growth hormone; *O*-linked oligosaccharide; glycosylated; structural analysis; mass spectrometry

1 Introduction

Human growth hormone (hGH) is part of the cytokine family that also includes prolactin and placental lactogen [1–7]. The hGHs derived from the hGH-normal gene (hGH-N) and those derived from the hGH-variant gene (hGH-V) differ by 13 amino acids [8]. The hGH-N gene is expressed primarily in the anterior pituitary while the hGH-V gene is expressed in the placenta [9–11].

Pituitary hGH is a heterogeneous mixture of structural isoforms. The pituitary hGH isoforms that have been identified include those with apparent molecular weights by SDS polyacrylamide gel electrophoresis of 5-kDa, 12-kDa, 17-kDa, 20-kDa, 22-kDa, 24-kDa, 35-kDa and 45-kDa [2,3,6,10,12–18]. [It is important to note that in this report, all hGH isoforms are referred to by their apparent molecular weight; the designation does not indicate the actual molecular weight of the isoform.] The pituitary 22-kDa hGH isoform is the most extensively characterized and most abundant hGH isoform [19]. The molecular polymorphism of pituitary hGH stems from alternative splicing and post-translational modifications such as proteolysis, deamidation, phosphorylation, acetylation, glycosylation and aggregation [2,3,6,10,12–17,19–21].

Individual molecular variants of pituitary hGH can exhibit a wide range of biological potencies that can be greater than, equal to, less than, or limited to a subset of biological actions compared to the predominant 22-kDa hGH isoform [22,23]. For example, the alternatively-spliced 20-kDa hGH promotes growth but is devoid of the insulin-like properties of 22-kDa hGH [24]. On the other hand, 5-kDa hGH does not promote growth but has a greater insulin potentiating activity than the 22-kDa hGH [25,26]. With respect to hyperglycemic actions, the 17-kDa hGH is more potent than the 22-kDa hGH isoform; however, it lacks the growth-promoting and insulin-like properties of 22-kDa hGH [3,27]. Ascertaining the structures of the hGH isoforms is a necessary step in gaining an accurate understanding of the differences in their biological properties.

In this report, our work is focused on ascertaining the structure of an hGH isoform previously identified by our laboratory that has an apparent molecular weight in an SDS polyacrylamide gel of 24-kDa and is glycosylated [14]. In our earlier work, *N*-terminal amino acid sequencing of the first 30 residues of 24-kDa hGH suggested that it was a product of the hGH-N gene. Deglycosylation of 24-kDa hGH by chemical and enzymatic methods resulted in a reduction in its apparent molecular weight to 22-kDa in an SDS polyacrylamide gel [14]. Since products of the hGH-N gene lack a consensus sequence for *N*-linked glycosylation [8–10], it was anticipated that any carbohydrates attached to glycosylated 24-kDa hGH must be *O*-linked.

In this study we sought to determine the structure of the glycosylated 24-kDa hGH in order to gain insights into its structure-function relationships, because the size and location of carbohydrate moieties attached to 24-kDa hGH may alter interactions with GH or PRL receptors, thereby influencing the scope and potency of the biological actions of this isoform. To accomplish this, we utilized a number of mass spectrometry-based techniques together with carbohydrate analysis via high-performance anion-exchange chromatography with pulsed amperometric detection and HPLC with fluorescent detection to obtain

information about the primary amino acid sequence of the glycosylated 24-kDa hGH, the site of oligosaccharide attachment as well as the oligosaccharide sequence and branching topology.

2 Materials and Methods

2.1 Materials

Glycerol, methanol, and acetic acid were purchased from EM Science (Gibbstown, NJ). Ammonium bicarbonate, sodium azide, acrylamide, DM-VII molecular weight markers, mercaptoethanol, Tris, glycine, SDS, sodium hydroxide, DTT, 2,5-dihydroxybenzoic acid, acetonitrile (ACN), trifluoroacetic acid (TFA) and Coomassie Brilliant blue (CBB) R250 were obtained from Sigma Chemical Co. (St. Louis, MO).

2.1 Human GH preparations

Isolation of 1) a mixture of non-glycosylated 22-kDa hGH and glycosylated 24-kDa hGH (as a 22-kDa/24-kDa hGH protein pool) and 2) a purified, non-glycosylated 22-kDa hGH were accomplished as previously described [28]. Human pituitaries (850) of unknown sex, age, or disease state were provided as a batch by the National Hormone and Pituitary Program. Briefly, GHs extracted from human pituitaries were chromatographed on a 10 × 100 cm Sephadex G-100 column (Pharmacia, particle size 40–120 μm; Piscataway, NJ, USA) in 10 mM ammonium bicarbonate. In all chromatographic and preparative electrophoresis procedures protein elution was monitored at 280 nm. Chromatographic and preparative electrophoresis fractions were monitored for purity and protein content by first precipitating proteins from aliquots of each fraction using the Pyrogallol Red Molybdate reagent [29] followed by analysis of precipitates by SDS-PAGE (12.5% T, 2.7% C_{BIS}) [30] and silver-staining [31]. Sephadex G-100 fractions containing 24-kDa hGH, were pooled and lyophilized (Virtis Freezemobile 12SL; Gardiner, NY, USA). Twenty-five milligrams of the hGH mixture containing 24-kDa hGH were dissolved in loading buffer-I (12.5 mM Bis-Tris, 0.5 mM EDTA, and 0.5 mM EGTA, pH 7.5) then chromatographed on a 1 × 40 cm DEAE column (Toyopearl DEAE 650 M ion-exchange resin, 65 μm particle size; Supelco, Bellefonte, PA, USA) using a medium-pressure LC BioLogic Workstation system (Bio-Rad, Hercules, CA, USA). Bound proteins were eluted from the column at 25 °C using a flow rate of 1.0 mL/min and a linear sodium chloride gradient in loading buffer-I of 0 mM to 250 mM over 300 min. DEAE fractions containing 24-kDa hGH were pooled and further separated by cation-exchange chromatography. To accomplish this, the pooled DEAE sample was resuspended in loading buffer-II (10 mM ammonium acetate, 1 mM EGTA, 1 mM EDTA, pH 5.0) using Centricon Plus-20 ultrafiltration centrifuge tubes (8000 NMWL; Millipore, Bedford, MA, USA) to exchange the buffers. The sample was then chromatographed on a 0.46 × 20 cm PolyCat A column (5 μm particle size, 1000 Å pore size; PolyLC, Columbia, MD, USA) using a high-pressure HPLC system (Waters 626 pump, 600S controller, 996 PDA absorbance detector, fraction collector, and Millennium Software; Milford, MA, USA). Bound proteins were eluted from the PolyCat A cation-exchange column at 25 °C by employing two consecutive linear ammonium acetate gradients—10 to 33 mM over 16 min followed by 33 to 300 mM over 140 min. The flow rate for both gradients was 0.5 mL/min; 2-min fractions were collected. The PolyCat A fractions 34 thru 38 (132 mM thru 147 mM ammonium acetate) containing both 22-kDa hGH and 24-kDa hGH were pooled and concentrated. This preparation constituted the 22-kDa/24-kDa hGH protein pool. PolyCat A fractions 44 thru 55 (170 mM thru 212 mM ammonium acetate) containing only 22-kDa hGH were pooled and concentrated. This preparation constituted the non-glycosylated 22-kDa hGH protein pool. (See Supplementary Figure 1)

2.3 SDS-PAGE separation of the 22-kDa/24-kDa hGH protein pool, in-gel tryptic digestion of excised protein bands, and MALDI-TOF/MS analysis of tryptic peptides

Proteins in the 22-kDa/24-kDa hGH pool were separated on an SDS polyacrylamide gel (13% T, 2.7% C_{BIS}) [30] after loading 15.5 µg into each well of the gel. The separated proteins were visualized by staining with CBB R250 [32]. Excised gel bands containing 22-kDa hGH or glycosylated 24-kDa hGH were digested *in situ* with trypsin according to standard protocols based on the initial work of Mann and co-workers [33]. Briefly, gel bands were treated with 5–10 ng/µL of trypsin (Promega, modified; Madison, WI) in 40 mM ammonium bicarbonate and incubated overnight at 37 °C. The tryptic peptides were analyzed by MALDI-TOF MS on an Applied Biosystems Voyager-DE STR mass spectrometer. Some samples were reduced by incubation of the tryptic digest in 100 mM DTT at 45 °C for 1 hr. The MALDI-TOF mass spectra of the tryptic peptides were acquired in reflectron mode using delayed extraction. The tryptic digest was mixed with matrix (a saturated solution of 2,5-dihydroxybenzoic acid in 50% ACN/0.1% TFA), and 1 µl was applied to the target plate and allowed to dry at room temperature. The instrument was calibrated with Calibration Mixture I (des-Arg1-bradykinin, angiotensin I, glu1-fibrinopeptide B, neurotensin; Applied Biosystems) prior to each set of analyses. When possible, each spectrum was internally calibrated based on the trypsin autolysis peaks at *m/z* 842.51 and *m/z* 2211.11. With this approach, mass accuracies of ≤ 10 ppm were routinely achieved. Spectra were generated from the average of 100 laser shots. Spectral processing included the following: advanced baseline correction (peak width, 32; flexibility, 0.5; degree, 0.1), noise filter (correlation factor, 0.7), noise removal (std. dev. to remove, 2), Gaussian smooth (filter width, 5 points). Monoisotopic masses were assigned by the instrument software (Data Explorer) and were verified by visual inspection. Peak lists were compared to the predicted tryptic peptides generated *in silico* by GPMaw (Lighthouse Data, Odense, Denmark).

2.4 Comparative HPLC analysis of the tryptic peptides of 22-kDa hGH versus the 22-kDa/24-kDa hGH protein pool

HPLC chromatograms of the tryptic peptides were generated according to the method described by Singh et al. [34] for both the 22-kDa hGH preparation and the 22-kDa/24-kDa hGH mixture. Briefly, the non-reduced 22-kDa hGH (753 µg) and the non-reduced 22-kDa/24-kDa hGH (836 µg) preparations were separately digested with Immobilized TPCK Trypsin (Pierce, Rockford, IL) in 0.1 M ammonium bicarbonate, pH 8.0, for 3 hr at 37 °C. After removal of the immobilized trypsin by centrifugation at 1,000 × g, the peptides were analyzed by HPLC using a C18 column (Waters, µBondapak C18, 10µm, 0.39 × 30 cm) at 25 °C. The column was equilibrated with 0.1% TFA. After loading the tryptic digests, the column was washed for 10 min with 0.1% TFA. Tryptic peptides were then eluted by applying a linear gradient of 0% to 45% ACN in 0.1% TFA over a period of 140 min. The elution profile was monitored at 210 nm, and peaks were manually collected.

2.5 Electrospray ionization-tandem mass spectrometry analysis of the peptides eluting at 100 min from the HPLC separation of the 22-kDa/24-kDa hGH tryptic digest

An aliquot of the HPLC fraction of the tryptic digest of the 22-kDa/24-kDa hGH pool that eluted at 100 min was reduced as described above and then analyzed by ESI-MS/MS on a Thermo Fisher LCQ Classic ion trap mass spectrometer. The sample was infused into the mass spectrometer in 50% methanol containing 0.5% acetic acid. Acquisition of full-scan and tandem mass spectra was controlled manually. The precursor isolation window was 2.5 and the relative collision energy was 35%. Five spectra were acquired and averaged for each analysis. Mass assignments were made by comparison of observed fragments to those predicted by GPMaw.

2.6 Monosaccharide analysis of the hydrolyzed 22-kDa/24-kDa hGH protein preparation by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and HPLC with fluorescent detection for NeuAc

HPAEC-PAD was used for analysis of monosaccharides released from 480- μ g of the 22-kDa/24-kDa hGH pool. Acid hydrolysis for release of monosaccharides was conducted as described [35]. The sample (100 μ L) was placed in a Vari-Clean vial (Pierce, Rockford, IL), an equal volume of 4 M TFA was added, the vial was flushed with N₂ then capped and the sample hydrolyzed for 4 hr at 100 °C on a heating block. The sample was then dried by vacuum centrifugation, rinsed twice with 99% methanol and re-evaporated. The sample was then resuspended in 100 μ L of water and aliquots of 10 μ L and 25 μ L were used for monosaccharide composition analysis by HPAEC-PAD (Dionex, Houston, TX). Control samples consisted of either water (blank) or a mixture of 1 nmol each of fucose, galactosamine, glucosamine, galactose, glucose and mannose. The HPAEC-PAD analysis was performed as described [36] with the following exceptions. Separation of monosaccharides was accomplished using a CarboPac PA10 column fitted with an aminotrap guard column (Dionex). Isocratic elution of monosaccharides was achieved with 18 mM NaOH at a flow rate of 1 mL/min. Quantification of monosaccharides was based on their elution peak areas relative to 1 nmol of a mixture of monosaccharides that had been similarly treated.

Since *O*-acetylated sialic acids are detected with reduced sensitivity and may exhibit reduced release from glycoconjugates via acid hydrolysis or sialidases, chemical de-*O*-acetylation with base treatment prior to acid hydrolysis is necessary for accurate quantitation [37]. Base treatment to remove *O*-acetyl groups was carried out by diluting 5 μ L of sample to 25 μ L with deionized water (18 M Ω -cm; MilliQ, Millipore) and then adding 25 μ L of 0.2 N NaOH. The sample was incubated at 37 °C for 30 min and then neutralized with 25 μ L of 0.2 N HCl. Acid hydrolysis of oligosaccharides [35] was accomplished by adding 75 μ L of 4 M acetic acid to the neutralized sample to give a final concentration of 2 M and the mixture was incubated at 80 °C for 5 hr. The sample was then filtered through a Microcon 3 (3,000 MW cutoff; Millipore) filter and dried by vacuum centrifugation. Acetic acid hydrolysis liberated sialic acids without destroying the rest of the oligosaccharides and minimized degradation of the released sialic acid. The 1,2-diamino-4,5-methylenedioxymethylene (DMB) HPLC assay for sialic acids was performed as described [37]. The dried sample was dissolved in 50 μ L of deionized water (18 M Ω -cm) and derivatized by adding 50 μ L of DMB reagent (1.6 g DMB, 80.4 μ L acetic acid, 52.8 μ L 2-mercaptoethanol, 72 μ L 0.25 M sodium hydrosulfite) and incubating the sample at 50 °C for 2.5 hr. Next, 20 μ L of derivatized sample was injected onto an HPLC column (TOSOHAS ODS-120T Reverse Phase, 4.6 $\times$ 25 cm; Montgomeryville, PA); component separation was accomplished under isocratic conditions using an aqueous mobile phase containing 9% (v/v) ACN and 7% (v/v) methanol. Fluorescent derivatives of the monosaccharides were detected with an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

2.7 Deteremination of the oligosaccharide attachment site to hGH polypeptide by HPLC-electrospray ionization-trandem mass spectrometry

The oligosaccharide linked to the tryptic glycopeptide having a retention time of 100 min was subjected to β -elimination [38]. Briefly, a 50 μ L aliquot of the fraction containing approximately 12 μ g 24-kDa hGH (500 pmol) was mixed with 50 μ L 200 mM NaOH and the mixture was incubated for 8 hr at 45 °C. The reaction was terminated by addition of 100 μ L of 300 mM acetic acid. The sample was stored at -80 °C prior to analysis by capillary HPLC-ESI-MS/MS on a Thermo Fisher LCQ Classic ion trap mass spectrometer in conjunction with a Michrom BioResources MAGIC 2002 micro HPLC and a home-built microspray interface. On-line separation of tryptic peptides was conducted using the

following conditions: column, New Objective PicoFrit, 75 μ m ID, packed to 10 cm with C18 Vydac 218MSB5 resin; mobile phase A, 0.5% acetic acid/0.005% TFA in water; mobile phase B, 90% ACN/0.5% acetic acid/0.005% TFA in water; gradient, 2% B to 72% B over 30 min; flow rate, 0.4 μ L/min. Data-dependent acquisition was employed in which a survey scan was acquired followed by collision-induced dissociation spectra of the four most abundant ions in the survey scan. Tandem MS fragments were assigned by comparison with theoretical patterns generated by GPMW.

2.8 Prediction of O-glycosylation sites in glycosylated 24-kDa hGH

The bioinformatics program NetOGlyc 3.1 [39,40] was employed to predict the loci in hGH polypeptide sequences with the greatest potential to be sites of mucin-like O-glycosylation after *a posteriori* evidence indicating the presence of mucin-like sites. The predicted protein sequence derived from the pituitary hGH-N gene sequence [8] was submitted to the NetOGlyc 3.1 Server (www.cbs.dtu.dk/services/NetOGlyc) to produce neural network predictions of the loci for mucin type GalNAc O-glycosylation sites in pituitary hGH.

2.9 Construction of theoretical 3D model of O-glycosylated 24-kDa hGH

The molecular components used to construct a 3D model of O-glycosylated 24-kDa hGH were the crystallographic coordinates for hGH (PDB ID 1HGU) [41] and the 3D coordinates for an O-linked oligosaccharide corresponding to the one elucidated in this work. An oligosaccharide structure search using the monosaccharide composition data (2 NeuAc, 1 Hex, 1 HexNAc) was submitted to the GLYCOSCIENCES sweet database server (http://www.glycosciences.de/sweetdb/start.php?action=form_structure_composition) [42]. The search resulted in an identical match (LinucsID 5335) [43] to our final deduced Thr-linked oligosaccharide, and the glycan structure was used in constructing a model of O-glycosylated 24-kDa hGH. The Brookhaven PDB file (2670.pdb) containing the 3D coordinates for LinucsID 5335 (http://www.glycosciences.de/sweetdb/start.php?action=view_coordinates&linucs_id=5335) was downloaded from the GLYCOSCIENCES sweet database.

Assembly of the 3D model of O-glycosylated 24-kDa hGH was carried out by coupling the hGH and the Thr-linked oligosaccharide. The LinucsID 5335 oligosaccharide was oriented relative to the structure of hGH (PDB ID 1HGU) [41] in SYBYL (SYBYL 7.2, The Tripos Associates, Inc.: 2006) by superimposing the Thr moiety attached to the carbohydrate with the backbone atoms of Thr-60 in the hGH structure. The carbohydrate structure was then merged into the hGH structure, the redundant Thr moiety was removed and an appropriate bond was specified between the carbohydrate and the gamma oxygen of Thr-60. In order to avoid severe steric clashes between the carbohydrate and the protein, torsional flips of 180 degrees were performed between the beta carbon and gamma oxygen of Thr-60 and on the carbohydrate between atoms C5 and C6 (LinucsID 5335 numbering). The resulting structure was then protonated in SYBYL under the assumption of a normal biological pH of 7.4.

Molecular mechanics was employed to find the lowest energy conformation for the assembled O-glycosylated 24-kDa hGH. Hence, the carbohydrate structure was permitted to relax in the presence of the protein environment via molecular dynamics simulations. These simulations entailed a 1.0-psec equilibration period followed by a run of 10.0 psec for analysis. Forces and energetics were measured according to the Tripos Force Field [44] and Gasteiger Marsili electrostatics [45] (using an 8.0-Å nonbonding cutoff threshold), with the effect of an aqueous environment simulated by applying a linear dielectric field with a constant of 78.2. The 10.0-psec analysis run was analyzed by scanning snapshots every 100 fsec for local potential energy minima. Eighteen such minima were identified and were subjected to 100 molecular mechanics minimization steps in SYBYL using the same

energetic construct as specified for the dynamics simulations. The single lowest-energy conformer arising from step 7700 in the dynamics simulation was then optimized to completion in SYBYL using default energetic convergence settings, and the resulting atomic coordinates for the structure were stored in the format of Protein Data Bank file (m7700.pdb).

Drawings of the 3D molecular model of *O*-glycosylated 24-kDa hGH were made with the open-source molecular graphics visualization program MacPyMOL version 1.0r1 (<http://pymol.sourceforge.net/>) (DeLano Scientific LLC, Palo Alto, CA). The 3D atomic coordinates of the m7700.pdb file were used to render images of the predicted molecular model of *O*-glycosylated 24-kDa hGH. Three graphics with various rendering effects were drawn to visualize the 3D shape of glycosylated 24-kDa hGH. In the first graphic, a Richardson Diagram [46], the most common method of 3D protein depiction, was used to render the visual basics and overall organization (coils, turns, helicies) of the molecular structure of *O*-glycosylated 24-kDa hGH with the alpha helices displayed as colored cylinders and the rest of the polypeptide chain depicted as thin tubes for random coils and turns. The amino and carboxyl terminal residues are shown as space-filling residues while Thr-60 is indicated by black sticks. The monosaccharides of the oligosaccharide are depicted as colored sticks as follows: GalNAc (blue), Gal (red) and NeuAc (yellow). In the second graphic, a space-filling model of *O*-glycosylated 24-kDa hGH is rendered as a Lee-Richards/Connolly molecular surface [47,48] showing the surface area that is accessible to solvent, with the protein component depicted as an opaque, light grey isosurface and the oligosaccharide as an opaque yellow isosurface. In the third graphic, *O*-glycosylated 24-kDa hGH is again rendered as a Lee-Richards/Connolly molecular surface with the protein depicted as a light grey isosurface; amino acids comprising the high-affinity binding site 1 structural epitope which interacts with the hGH receptor [49,50] are colored according to their contribution to binding energy [4,51,52], while the monosaccharides of the oligosaccharide are depicted in various colors as sticks, identical to those in the first graphic.

3 Results

3.1 MALDI-TOF/MS analyses of tryptic peptides from 22-kDa hGH and glycosylated 24-kDa hGH after in-gel digestion

In order to gain insight into the differences between 22-kDa hGH and glycosylated 24-kDa hGH, the proteins were separated by SDS-PAGE, as shown in Fig. 1. Gel bands of interest were excised, the proteins were digested with trypsin (with and without reduction) and the digests analyzed by MALDI-TOF/MS, as summarized in Supplementary Table 1. The results show that many of the same tryptic peptides were detected in the digests of both 22-kDa hGH and 24-kDa hGH and were in agreement with the published sequence for hGH [9]. The primary sequence of 22-kDa hGH is shown in Fig. 2, with the residues in peptides detected by MALDI-TOF/MS analysis of the corresponding tryptic digests indicated by black circles with white letters (panel A, 22-kDa hGH band; panel B, 24-kDa hGH band). In the tryptic digest of 24-kDa hGH (Fig. 2) peptides T6, T7 and T14 were missing. These peptides contain Ser/Thr residues and if those peptides were glycosylated at any of those sites the peptides would have a different mass and hence account for their absence in the tryptic digest of 24-kDa hGH. Since there was evidence for the presence of oligosaccharide modification on 24-kDa hGH [14], efforts next focused on obtaining structural information about any oligosaccharides attached to 24-kDa hGH (see below). In view of the fact that hGH does not contain a consensus sequence for *N*-linked glycosylation, potential sites of *O*-linked glycosylation on Ser or Thr were considered.

3.2 Monosaccharide analysis via HPAEC-PAD and HPLC with fluorescent detection for NeuAc of the hydrolyzed 22-kDa/24-kDa hGH protein preparation

Monosaccharides in a hydrolysate of the 22-kDa/24-kDa hGH protein pool were analyzed by HPAEC-PAD (CarboPac PA10), verifying the presence of a glycoprotein in the preparation. The quantities and types of monosaccharides released via acid hydrolysis of the 22-kDa/24-kDa hGH protein pool are shown in Table 1, revealing that the oligosaccharide is comprised of GalNAc, Gal, and NeuAc in relative abundances of ~1:1:2 (0.37:0.32:0.56 nmol/25 μ L). Since there were 0.37 nmol of GalNAc in a 25 μ L aliquot, the ratio of oligosaccharide chains/protein chains obtained to the nearest integer is 1, as shown in the following calculation: 0.37 nmol GalNAc / 0.31 nmol 24-kDa hGH = 1.19 ~1. Thus there is one GalNAc-linked oligosaccharide chain per 24-kDa hGH polypeptide chain.

3.3 Comparative HPLC analysis of tryptic peptides of the 22-kDa hGH and the 22-kDa/24-kDa hGH mixture

Comparative HPLC analysis under non-reducing conditions of tryptic peptides of purified 22-kDa hGH and of 22-kDa/24-kDa hGH mixture was carried out to look for novel peaks that might indicate a modified hGH peptide. In Fig. 3 is a comparison of the HPLC chromatograms of tryptic peptides of purified 22-kDa hGH (A) and of the 22-kDa/24-kDa hGH mixture (B). Additional peaks can be seen in the trace for the 22-kDa/24-kDa hGH protein preparation compared to that of 22-kDa hGH (35 min and 100 min).

3.4 MALDI-TOF/MS analysis of the 22-kDa/24-kDa hGH tryptic peptides in HPLC peaks eluting at 35 min, 100 min and 102 min

In the MALDI-TOF/MS analysis of the additional 22-kDa/24-kDa hGH tryptic digest peak eluting at 35 min, there was a predominant ion at m/z of 694.50 that corresponds to $[M + H]^+$ for deamidated hGH tryptic peptide T13 ($^{135}\text{TGQIFK}^{140}$; m/z 693.4; data not shown), indicating that this extra tryptic HPLC peptide did not correspond to a novel hGH sequence. The MALDI-TOF mass spectra of the peptides in the tryptic digest of the 22-kDa/24-kDa hGH mixture that eluted at 100 min and 102 min in the HPLC analysis are shown in Fig. 4, panels A and B, respectively. In the spectrum in panel B of the peptides eluting at 102 min, ions assigned to peptides T16 (m/z 1148.7), T6 (m/z 2616.5) and disulfide linked T6-T16 (m/z 3762.2) were clearly detected. The presence of peptides T6 and T16 in this fraction was presumably due to reduction of the S-S bond that took place during preparation of the sample for MS analysis. An HPLC tryptic peptide with the same retention time (102 min) is seen in the digest of the purified 22-kDa hGH (Figure 3, panel A). The mass spectrum of the HPLC tryptic peptide having a retention time of 100 min, shown in Fig. 4A, shows an ion at m/z 1148.7 that corresponds to $[M + H]^+$ of hGH peptide T16. In view of the fact that peptides T6 and T16 are disulfide linked in hGH, in the HPLC fraction of a non-reduced tryptic digest that contains T6-T16, both T6 and T16 (either disulfide-linked or liberated by reduction during isolation) should be present. Since an ion corresponding to peptide T16 was observed but no ions for peptides T6 or T6-T16 were found, it was postulated that T6 contained the site for oligosaccharide modification and that the corresponding modified peptides would, therefore, be detected at a higher molecular mass than the unmodified analogs. From the HPAEC-PAD analyses, the oligosaccharide was found to contain one GalNAc, one Gal, and two NeuAc. Accordingly, an ion corresponding to peptide T6-T16 covalently modified with a tetrasaccharide of that composition can be seen in Fig. 4A at m/z 4709.6 (m/z 4709.1 calculated). In order to determine the nature of the peptides eluting in the 100-min peak, the fraction was subsequently analyzed by ESI-MS/MS.

3.5 Characterization of glycosylated peptide T6 eluting at 100 min in the HPLC analysis of the 22-kDa/24-kDa hGH tryptic digest

An aliquot of the 22-kDa/24-kDa hGH HPLC fraction eluting at 100 min was reduced and then analyzed by ESI-MS/MS. In the full-scan mass spectrum (not shown) it could be seen that the major component exhibited 2+ and 3+ ions that were consistent with the mass of peptide T6 (hGH^{42–64}; YSFLQNPQTSLCFSESIPTPSNR) that was modified by attachment of a GalNAc₁Gal₁NeuAc₂ tetrasaccharide—the composition indicated by the HPAEC-PAD monosaccharide composition analysis. The tandem mass spectrum of the 2+ ion (m/z 1782.0) is shown in Fig. 5. Although the spectrum is dominated by fragments that result from loss of sugar moieties, the masses of b_{17} and $b_{17}-H_2O$ provide evidence that the oligosaccharide is attached to one of the six C-terminal residues. Support for this conclusion was obtained from the ions at m/z 671.2, 874.3, and 1036.2 which correspond to y_6 PTPSNR or PTPSNR with GalNAc and GalNAc-Gal attached, respectively. Information about the complete tetrasaccharide can be seen from the sequential losses of sugars from the doubly-charged precursor: m/z 1636.8, $[M+2H-NeuAc]^{2+}$; m/z 1555.3, $[M+2H-(NeuAc+Gal)]^{2+}$; m/z 1490.9, $[M+2H-2NeuAc]^{2+}$; m/z 1409.9, $[M+2H-(2NeuAc+Gal)]^{2+}$; and m/z 1308.5, $[M+2H-(2NeuAc+Gal+GalNAc)]^{2+}$. Detection of an ion, m/z 1555.3, generated by the combined loss of NeuAc and Gal indicates that the tetrasaccharide is branched at GalNAc, as shown in Fig. 6, and is not linear. It was predicted at this stage that the oligosaccharide is attached at Ser or Thr because the hGH tryptic peptide T6 lacks a consensus sequence for *N*-linked glycosylation. The glycan structure shown in Fig. 6 was, therefore, deduced from the following: the monosaccharide composition data shown in Table 2 that showed the presence of NeuAc, GalNAc and Gal; the MS data that indicated the oligosaccharide branched topology; and the monosaccharide sequence of the oligosaccharide attached to hGH tryptic glycopeptide T6.

The difference in apparent molecular weights between glycosylated 24-kDa hGH and 22-kDa hGH as determined by SDS-PAGE is 2 kDa [14] and does not directly correspond to the 948 Da associated with the tetrasaccharide modification. It is known that glycoproteins bind less SDS than non-glycosylated proteins which leads to reduced mobility during SDS-PAGE [53,54]. This, in turn, resulted in an artificially high molecular weight estimate for the glycoprotein.

3.6 The oligosaccharide moiety is O-linked to the hGH polypeptide backbone at Thr-60

Based on the sequence of peptide T6 hGH^{42–64} (YSFLQNPQTSLCFSESIPTPSNR), there are seven possible sites of attachment for an *O*-linked oligosaccharide (five Ser and two Thr). As noted above, examination of the tandem mass spectrum shown in Fig. 5 provided insight into the general location of oligosaccharide attachment to the peptide. To determine the specific amino acid linked to the oligosaccharide, the 22-kDa/24-kDa hGH tryptic peptide that eluted at 100 min was subjected to mild alkaline β -elimination. Under these conditions, β -elimination would convert glycosylserine to 2-aminopropenoic acid and glycosylthreonine to 2-amino-2-butenic acid [38,55]. The ESI tandem mass spectrum of the peptide that resulted from β -elimination of the oligosaccharide from peptide T6 (Fig. 7) revealed the presence of an ion at m/z 924.0 ($[M+2H]^{2+}$) that corresponds to deglycosylated hGH peptide T6 with deamidation of Asn-63 and loss of residues ⁴²YSFLQN⁴⁷ (hGH^{48–64}; PQTSLCFSESIPTPSNR). The missing residues are the result of peptide cleavage that can take place during the β -elimination reaction [38]. The spectrum is consistent with deglycosylation at Thr-60. In particular, the mass difference (m/z 83.1) between y_4 at m/z 474.2 and y_5 at m/z 557.3, corresponding to 2-amino-2-butenic acid, showed that the oligosaccharide had been linked to Thr-60.

A bioinformatics approach confirmed the conclusions reached based on our experimental data regarding the locus in hGH with greatest potential to be *O*-glycosylated. Results from the NetOGlyc 3.1 Server (www.cbs.dtu.dk/services/NetOGlyc) [39,40] for the potential of each Ser/Thr residue in hGH to be *O*-glycosylated are shown in Table 2. In this analysis, the G-score is the best general predictor, with a G-score > 0.5 predicting that a residue is glycosylated; the higher the score the more confident the prediction. However, based on G-scores, none of the Ser/Thr residues in hGH were predicted to be glycosylated because none had a G-score > 0.5. The I-score predicts the best isolated site. If the G-score is < 0.5 but the I-score > 0.5 and there are no predicted neighboring sites within < 10 residues, the residue is also predicted as glycosylated. Hence, an I score for Thr-60 of > 0.5 predicts that it is the only residue in hGH that is likely to be a site of *O*-glycosylation—in agreement with our experimental data.

Taken together, our results support the presence of a tetrasaccharide attached to Thr-60 of hGH with the monosaccharide sequence and branching topology shown in Fig. 8. There is a GalNAc attached to Thr-60 via an *O*-glycosidic linkage and a biantennary fork in which a Gal-NeuAc forms one branch and a terminal NeuAc is the other. All Ser/Thr-linked oligosaccharides with this sequence and branching motif in the GLYCOSCIENCES structural glycomics sweetdatabase [42,43] have identical monosaccharide linkages and anomeric configurations (LinucsID 676, LinucsID 4350, LinucsID 5335, LinucsID 5336, LinucsID 16225, LinucsID 17672, LinucsID 19945, LinucsID 19946). This finding strongly suggests that the hGH oligosaccharide has the same anomeric configurations and monosaccharide linkages as the database entries (depicted in Fig. 8). The Gal β 1–3GalNAc structure is that of a core 1 *O*-oligosaccharide [56–61]. In the hGH mucin-like oligosaccharide there is an elongation of the core 1 consisting of a NeuAc addition to each monosaccharide of the core to produce a biantennary glycan. The depicted GalNAc- α -Thr linkage appears to be limited to eukaryotes while the β -configuration (GalNAc- β -Thr) is confined to archaebacterium [57].

3.7 Molecular model of *O*-glycosylated 24-kDa hGH

A 3D molecular model of *O*-glycosylated 24-kDa hGH based on the crystallographic coordinates of hGH (PDB ID 1HGU) [41] with an attached oligosaccharide having monosaccharide linkages and anomeric configurations corresponding to the spatial coordinates of the Thr-linked oligosaccharide LinucsID 5335 is shown in Fig. 9. The model indicates that *O*-glycosylated 24-kDa hGH forms a globular protein with four interfaced α -helices connected by random coil loops and possessing two intra-chain disulfide bonds, as shown in panel A. In this model, the amino-terminus is shown with blue spheres and the carboxy-terminus with red spheres. The α -helical structures of hGH beginning at the amino terminus and proceeding towards the carboxy terminus are represented as follows: solid cylinders for helix 1 (blue); a minihelix (light grey); helix 2 (green); helix 3 (yellow); and helix 4 (red). These helices are connected by random loops of the polypeptide chain. Disulfide bonds are shown as dark grey sticks. There is a discontinuity in a section of the loop connecting helix 1 and the minihelix and another in a section of the loop connecting helix 3 and helix 4 due to missing crystallographic data. Thr-60 (black sticks) and its attached oligosaccharide comprised of GalNAc (blue sticks), Gal (red sticks) and two residues of NeuAc (yellow sticks) is located in the loop connecting the minihelix and helix 2. The predicted molecular model of *O*-glycosylated 24-kDa hGH exhibits secondary structural features (the conformation of helix 3 and in the absence of minihelix 2 and minihelix 3) that are similar to apo-hGH [41] and porcine GH [62], with only slight differences from the receptor-complexed hGH [50]. Crystallographic differences between hGH and receptor-bound hGH reflect induced conformational changes that occur in hGH after it combines with the receptor [4,50]. The surface of hGH (shown in Fig. 9B) contains

two structural regions which independently interface with separate GH receptors [49,50] and are referred to as high-affinity binding site 1 and low-affinity binding site 2. These binding sites have been further investigated to identify those residues that are most important in comprising the functional epitopes as defined by alanine scanning mutagenesis [4,51,52]. The spatial relationships between the surface polypeptide (depicted in light grey), Thr-60 (black), GalNAc (blue), Gal (red), and the two residues of NeuAc (yellow) show that the oligosaccharide moiety covers a portion of the hGH surface. Crystallographic analysis has shown that a 31-residue structural epitope becomes buried when hGH interacts with the extracellular binding domain of its receptor [50]. This structural epitope constitutes the high-affinity binding site 1 of hGH. The spatial relationships between the *O*-linked oligosaccharide of 24-kDa hGH and the residues that define the structural epitope of high-affinity binding site 1 complexed to the extracellular domain of the hGH receptor are shown in Fig. 9C using the following colors: grey, the surface topology of hGH; red, orange, green and blue, the amino acid residues that constitute the structural epitope of high-affinity binding site 1 colored according to their contributions to free energy of binding to the GH-receptor as described below; Thr-60 and the oligosaccharide are shown as sticks in the same colors used in Fig. 9A. The amino acids on the surface of hGH that interface with the hGH-receptor form a structural epitope that defines the high-affinity binding site 1 of hGH, however, a subset of these amino acids are the major contributors to the binding free energy and comprise a smaller domain referred to as the functional epitope [51]. The functional map of the binding surface reveals to what extent each amino acid in the structural epitope contributes to the overall free energy of binding. To map the high-affinity binding site 1 functional epitope, residues of the structural epitope were mutated to Ala and then changes in the free energy of the binding of hGH to the receptor were assessed by measurement of gain or loss in binding affinity. Similar studies employing the shotgun scanning mutagenesis approach confirmed that the same surface residues form the hGH functional epitope for binding to the hGH receptor [63–65]. In Fig. 9C, the degree to which an individual residue alters the free energy of hGH binding to the hGH receptor when converted to Ala [51] is designated with the following color scheme. Eight of the residues, shown in red, (Lys-41, Leu-45, Pro-61, Arg-64, Lys-172, Thr-175, Phe-176, and Arg-178) account for approximately 85% of the binding free energy. When these residues are mutated to Ala, there is a substantial reduction in binding affinity (increase in binding free energy). The remaining portion of the binding free energy is accounted for by six more residues, shown in orange, (Pro-48, Glu-56, Gln-68, Asp-171, Ile-179, and Arg-183). Eleven of the structural epitope residues (Met-14, His-21, Gln-22, Asp-26, Gln-46, Ser-62, Asn-63, Tyr-164, Arg-167, Lys-168, and Glu-186, shown in green) have essentially no effect on overall binding free energy when converted to Ala. Five residues, shown in blue, (His-18, Phe-25, Gln-29, Glu-65 and Glu-174) normally obstruct binding because mutation of these residues to Ala increases binding affinity 2 to 5-fold with associated decreases in binding free energy. The model indicates that the oligosaccharide moiety of *O*-glycosylated 24-kDa hGH is physically situated within both the structural epitope and the functional epitope that comprise high-affinity binding site 1.

4 Concluding Remarks

This research has provided insight into the structure of glycosylated 24-kDa hGH. The glycoprotein is *O*-glycosylated at a single locus with a mucin-like tetrasaccharide attached through GalNAc to Thr-60 of the hGH polypeptide chain. The GalNAc has two glycosidic linkages that form a biantennary branched oligosaccharide. The first branch is comprised of a sole NeuAc and the second a disaccharide is attached to GalNAc through a glycosidic bond to Gal that is, in turn, linked to a terminal NeuAc.

It is apparent from the molecular model of hGH that the oligosaccharide attached at Thr-60 occupies a position within the region of the hGH surface that contains the receptor-binding epitopes for structural and functional high-affinity binding site 1. The location of the oligosaccharide in the binding site 1 epitope suggests that *O*-glycosylated 24-kDa hGH will have a decreased binding affinity for the hGH receptor compared to non-glycosylated 22-kDa hGH due to steric hindrance. The interaction between *O*-glycosylated 24-kDa hGH and the hGH receptor is also likely to result in a different conformation than is found when the receptor binds non-glycosylated 22-kDa hGH [66–69]. Likewise, the binding of *O*-glycosylated 24-kDa hGH to the GH receptor is likely to change the degree of GH-dependent recruitment of the transcriptional regulator Coactivator Activator to the GH receptor [70] compared to non-glycosylated 22-kDa hGH. GH-induced conformational changes in the GH receptor that determine the choice of signaling pathway [71] may be different from those conformational changes induced by *O*-glycosylated 24-kDa hGH. Consequently, it is predicted that *O*-glycosylated 24-kDa hGH will have attenuated biological actions and possibly possess properties of an hGH antagonist. Since hGH interacts with the hPRL receptor using essentially the same structural epitope that it uses to bind to the GH receptor [72], the binding affinity of *O*-glycosylated 24-kDa-hGH to the hPRL receptor is also expected to be decreased compared to non-glycosylated 22-kDa hGH, possibly leading to altered hPRL-like bioactivities.

Interestingly, although the pituitary hGH-N gene lacks a consensus sequence for *N*-linked glycosylation, *N*-glycosylated hGHs of 12 kDa, 22kDa and 34 kDa have been detected in pituitary extracts [13,73]. These glycosylated GHs may have resulted from pituitary expression of the hGH-V gene or are derived from an unidentified member of the hGH gene family. The placental hGH derived from the hGH-V gene [74,75] is *N*-glycosylated at residue 140 [19,74–76]. It exhibits high somatogenic activity and low lactogenic activity compared to pituitary hGH; during gestation, hGH-V replaces circulating maternal pituitary hGH [76].

Glycosylated GHs have been detected in human, chicken, rat, mouse, pig, buffalo and shark pituitaries [13,14,77–85]. Glycosylated chicken GH has a longer half-life than non-glycosylated GH [84,86], stimulates deiodination of thyroid hormone in the chick embryo [78], has a lower affinity than non-glycosylated GH for liver receptors [87], and stimulates Nb2 cell proliferation [87]. Moreover, the release of glycosylated GH from pituitary explants and primary cell cultures is up-regulated by growth hormone releasing hormone [84], and its levels in the pituitary gland fluctuate during development [87,88] as do its levels in tissues of the immune system [89]. In pigs, the levels of glycosylated porcine GH increased in the pituitary during progression of fetal life [90], and obese pigs had less glycosylated GH than lean pigs [80]. In rodent pituitaries, glycosylated GH was lower in females than males, lower in lactating than in virgin females, increased after ovariectomy, and decreased following estradiol administration [79]. Thus, glycosylated GH is present in various species, exhibits a variety of biological activities and undergoes physiological fluctuations.

Glycosylation is one of the most important post-translational modifications found in nature. In this work we have determined the site of attachment and glycan structure of *O*-glycosylated 24-kDa hGH. This information will be important in understanding structure-function relationships of 24-kDa hGH because the influence of *O*-glycosylation on the biological activity of this hGH isoform is as yet unknown. Purification of *O*-glycosylated 24-kDa hGH in a native state will ultimately be required to examine its GH-like and PRL-like activities in established bioassays and receptor assays [22,23]. Studies of that kind will allow us to determine if *O*-glycosylated 24-kDa hGH functions as a full or partial agonist or antagonist of non-glycosylated 22-kDa hGH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ACN	acetonitrile
Bis-Tris	bis(2-hydroxyethyl)amino-Tris
CBB R250	Coomassie brilliant blue R250
C_{BIS}	percent of total acrylamide that is bis-acrylamide
DEAE	diethylaminoethyl
DMB	1,2-diamino-4,5-methylenedioxymethylene
Gal	galactose
GalNac	<i>N</i> -acetyl galactosamine
Hex	hexose
HexNac	<i>N</i> -acetyl hexosesamine
hGH	human growth hormone
HPAEC-PAD	high performance anion-exchange chromatography with pulsed amperometric detection
NeuAc	<i>N</i> -acetyl neuraminic acid
T	total acrylamide
Tris-HCl	Tris hydrochloride

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Figure 1. SDS-PAGE separation of the 22-kDa/24-kDa hGH protein pool. Aliquots of the 22-kDa/24-kDa hGH protein pool (15.5 µg/well) were separated in an SDS polyacrylamide gel (13% T, 2.7% C_{BIS}) and the proteins visualized via CBB R250 staining.

Figure 2.

Peptides detected in MALDI-TOF/MS analysis of in-gel tryptic digests of 22-kDa hGH and 24-kDa hGH. Analyses of reduced and non-reduced in-gel tryptic digests of 22-kDa hGH and 24-kDa hGH were conducted on an Applied Biosystems Voyager-DE STR as described in Methods. The predicted tryptic peptides (T1 thru T21) of hGH are labeled in order starting from the amino terminus. Residues in matched peptides are indicated by black circles with white lettering for 22-kDa hGH (A) and for 24-kDa hGH (B). Tryptic peptides T6, T7 and T14 were not observed in the MALDI-TOF/MS analysis of 24-kDa hGH possibly due to their O-glycosylation at Ser/Thr residues.

Figure 3.

Comparison of reversed-phase HPLC chromatograms of tryptic peptide of purified 22-kDa hGH and of the 22-kDa/24-kDa hGH mixture. The tryptic peptides were generated from either a purified non-reduced 22-kDa hGH preparation (A) or a non-reduced 22-kDa/24-kDa hGH mixture (B). Arrows indicate the retention times of peaks that are unique to the 22-kDa/24-kDa hGH mixture.

Figure 4.

MALDI-TOF mass spectra of peptides eluting at 100 min and 102 min in the HPLC analysis of the 22-kDa/24-kDa hGH mixture. The peptides eluting at 100 min (A) and 102 min (B) were analyzed by MALDI-TOF/MS as described in Methods. The monoisotopic ion at m/z 4709.6 in panel A is in close agreement with the calculated value of m/z 4709.1 for disulfide-linked T6_{sugar}-T16. For the spectrum in panel B, when internal calibration was conducted based on T16 (m/z 1148.6, calculated) and T6 (m/z 2616.2, calculated), the monoisotopic ion for T6-T16 was detected at m/z 3762.2, matching the expected value. The ion at m/z 1882.7 in panel B corresponds to doubly-charged T6-T16.

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Figure 6.
Branched topology and monosaccharide sequence of the oligosaccharide of hGH tryptic glycopeptide T6 inferred by mass spectrometry and monosaccharide composition analysis.

Figure 7.

Identification of Thr-60 as the site of attachment for *O*-linked glycosylation in 24-kDa hGH.

The tandem mass spectrum was obtained by collision-induced dissociation of the 2+ ion corresponding to the hGH peptide produced by mild alkaline β -elimination of peptide T6 (hGH^{48–64}; NPQTSLCFSEIPTPSNR) that was partially cleaved during treatment.

Assigned b- and y-ions are indicated on the spectrum and on the peptide sequence. The results are consistent with deglycosylation at Thr-60 (dT) and deamidation of N (*). Note the mass difference between the ions y₅ and y₄ (83.1 amu), corresponding to the conversion of a glycosylthreonine residue to 2-amino-2-butenic acid. The loss of water from the adjacent b-ion is indicated by “-w.”

Figure 8.

Oligosaccharide branching topology, monosaccharide sequence and site of attachment to hGH. The monosaccharide linkages and anomeric configurations are those of the oligosaccharide LinucsID 5335 from the sweetdatabase where all Ser/Thr-linked oligosaccharides of this sequence and branching motif had the same monosaccharide linkages and anomeric configurations.

Figure 9.

Molecular model of *O*-glycosylated 24-kDa hGH. The Brookhaven PDB file (2670.pdb; LinusID 5335) containing 3D Coordinates of an oligosaccharide structure matching the Thr-linked oligosaccharide of Figure 8 were obtained from the GLYCOSCIENCES sweet database server. The carbohydrate was attached to T⁶⁰ of hGH (PDB ID 1HGU) in SYBYL and the single lowest energy conformer identified by molecular mechanics minimization. The resulting structure was then rendered with the molecular graphics visualization program MacPyMOL 1.0r1 to generate the image of *O*-glycosylated 24-kDa hGH.

Table 1

Composition of the oligosaccharides in the 22-kDa /24-kDa hGH mixture

Monosaccharide	nmol/25 μ L ¹	Ratio
GalNAc	0.37	1.2
Gal	0.32	1.0
NeuAc	0.56 ²	1.8

¹ Quantified by HPAEC-PAD (CarboPac PA10) in a hydrolysate of the 22-kDa/24-kDa hGH protein pool.² Quantified by HPLC and fluorescent detection in a hydrolysate of the 22-kDa/24-kDa hGH protein pool.

Table 2
Bioinformatics prediction of likelihood of *O*-glycosylation at each S/T residue in the sequence of hGH

Bioinformatics prediction results were obtained from the NetOGlyc 3.1 Server. A G-score > 0.5 predicts a residue as glycosylated; the higher the score the more confident the prediction. A G-score < 0.5 for Thr but with an I-score > 0.5 and no predicted neighboring sites (distance < 10 residues) forecasts glycosylation at that site.

S/T	Position	G-Score	I-Score	(Y/N)
T	3	0.453	0.289	-
S	7	0.254	0.071	-
T	27	0.191	0.029	-
S	43	0.209	0.045	-
T	50	0.445	0.049	-
S	51	0.345	0.034	-
S	55	0.318	0.077	-
S	57	0.378	0.041	-
T	60	0.481	0.569	Y
S	62	0.358	0.101	-
T	67	0.374	0.023	-
S	71	0.249	0.017	-
S	79	0.116	0.044	-
S	85	0.101	0.032	-
S	95	0.155	0.039	-
S	100	0.152	0.022	-
S	106	0.131	0.026	-
S	108	0.151	0.026	-
T	123	0.273	0.049	-
S	132	0.193	0.034	-
T	135	0.327	0.056	-
T	142	0.310	0.021	-
S	144	0.196	0.040	-
T	148	0.219	0.051	-
S	150	0.117	0.028	-
T	175	0.162	0.044	-
S	184	0.231	0.030	-
S	188	0.326	0.033	-