

Sulfated *N*-linked carbohydrate chains in porcine thyroglobulin

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N-linked carbohydrate chains of porcine thyroglobulin were released by the hydrazinolysis procedure. The resulting mixture of oligosaccharide-alditols was fractionated by high-voltage paper electrophoresis, the acidic fractions were further separated by high-performance liquid chromatography on Lichrosorb-NH₂, and analyzed by 500-MHz ¹H-NMR spectroscopy and, partially, by permethylation analysis. Of the acidic oligosaccharide-alditols, the following sulfated carbohydrate chains could be identified: NeuAc α 2 \rightarrow 6Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3((SO₃Na \rightarrow 3)Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2-Man α 1 \rightarrow 6]Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4[Fuca1 \rightarrow 6]GlcNAc-ol and NeuAc α 2 \rightarrow 6Gal β 1 \rightarrow 4(SO₃Na \rightarrow 6)₀₋₁GlcNAc β 1 \rightarrow 2-Man α 1 \rightarrow 3[NeuAc α 2 \rightarrow 6Gal β 1 \rightarrow 4(SO₃Na \rightarrow 6)₁₋₀GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6]Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4[Fuca1 \rightarrow 6]GlcNAc-ol. The sulfated structural elements for porcine thyroglobulin form novel details of *N*-linked carbohydrate chains. They contribute to the fine structure of these oligosaccharides and are another type of expression of microheterogeneity.

Thyroglobulin; Sulfated *N*-linked carbohydrate; (Porcine)

1. INTRODUCTION

Thyroglobulin, the major iodinated glycoprotein synthesized in the thyroid gland, is the polypeptide precursor of the thyroid hormones. Analysis of the carbohydrate chains of thyroglobulin (molecular mass ~660 kDa) from several species has demonstrated that mainly two types of chains occur, generally called unit-A type (oligomannose type) and unit-B type (*N*-acetyllactosamine type) [1–10]. For human thyroglobulin also unit-C type (mucin type) [4] and unit-D type (proteoglycan type) [11] chains have been indicated. In many cases α 1 \rightarrow 3Gal residues can form part of the unit-B type chains [12–14].

Concerning the *N*-linked oligosaccharide chains of porcine thyroglobulin [5,6], the literature data indicate the presence of Man₅₋₉GlcNAc₂ structures, whereby the trimming of Man residues

seems to take place randomly [7]. For the *N*-acetyllactosamine type a series of partially sialylated di- (75%) and tri- (25%) antennary structures with an α 1 \rightarrow 6-linked Fuc residue at the Asn-bound GlcNAc unit have been reported [8]. In addition, a terminal Gal α 1 \rightarrow 3Gal β 1 \rightarrow 4 element in the Man α 1 \rightarrow 6 branch of a monosialylated (α 2 \rightarrow 6-linked NeuAc; Man- α 1 \rightarrow 3 branch) diantennary structure has been shown to occur [14].

The recent finding of phosphate [10,15–17] and, especially, sulfate [18] groups in thyroglobulins from different biological origins prompted us to report data on the structural identification of sulfated *N*-linked unit-B type carbohydrate chains in porcine thyroglobulin.

2. MATERIALS AND METHODS

2.1. Porcine thyroglobulin

Porcine thyroglobulin was obtained from Sigma. SDS-polyacrylamide gel electrophoresis gave the usual pattern of two main bands, together with three faint bands of lower molecular mass [19,20]. Its sugar composition (Fuc/Man/Gal/GlcNAc/

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NeuAc = 0.4:3.0:1.2:2.6:0.4) and carbohydrate content (7%) are in accordance with literature data [1,2,21].

2.2. Preparation of oligosaccharide-alditols

The hydrazinolysis procedure on porcine thyroglobulin (six 125-mg portions) followed by high-voltage paper electrophoresis was carried out essentially as described [22,23]. The oligosaccharide-alditols were recovered from the paper by elution with water, yielding one neutral and three acidic fractions. The acidic fractions were further subfractionated by HPLC on Lichrosorb-NH₂ using elution systems of acetonitrile/15–30 mM KH₂PO₄-K₂HPO₄, pH 5.2 [24], and monitored at 205 nm. Fractions were desalted on Bio-Gel P-2 (200–400 mesh) using water as eluent and refractive index detection.

2.3. Monosaccharide analysis

Carbohydrate samples were subjected to the methanolysis procedure and analyzed by GLC on CP Sil 5, as described [25].

2.4. Methylation analysis

Methylation analysis was carried out essentially as described [26]. Permethylated material was hydrolyzed with 4 M trifluoroacetic acid, the obtained mixtures of partially methylated monosaccharides were reduced with NaB²H₄ in water, and the partially methylated alditol acetates analyzed by GLC-MS.

2.5. 500-MHz ¹H-NMR spectroscopy

Oligosaccharide-alditols were repeatedly exchanged in ²H₂O (99.96 atom% ²H) with intermediate lyophilization. Resolution-enhanced 500 MHz ¹H-NMR spectra were recorded in ²H₂O at 27°C on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone ($\delta = 2.225$ ppm) [27].

3. RESULTS

In the framework of our NMR studies on sulfated mono- [28] and oligo- [29,30] saccharides, porcine thyroglobulin was investigated for the presence of sulfated *N*-linked carbohydrate chains. The hydrazinolysis procedure in combination with high-voltage paper electrophoresis yielded one neutral (N) and three acidic (A1, A2, A3) fractions in the molar ratio of 27:35:28:10, respectively. As A2 and A3 turned out to contain the searched material, attention will only be paid to these fractions in this paper.

Subfractionation of A2, isolated from the 'double-negatively-charged' region of the paper electropherogram, on Lichrosorb-NH₂ yielded a series of peaks, of which A2b is of interest. The ¹H-NMR spectrum of A2b shows the presence of

one major compound. The NMR features of this oligosaccharide-alditol, presented in table 1, resemble those of reference compound IgM, which is a classical diantennary structure terminated with β 1 \rightarrow 4-linked Gal in the Man α 1 \rightarrow 6 branch and α 2 \rightarrow 6-linked NeuAc in the Man α 1 \rightarrow 3 branch and having an α 1 \rightarrow 6-linked L-fucose residue at the Asn-bound *N*-acetylglucosamine [31]. However, of the structural-reporter-group signals the Gal-6' H-1 doublet had shifted downfield from $\delta = 4.470$ ppm to $\delta = 4.587$ ppm. Furthermore, in contrast to the spectrum of IgM, in that of A2b two additional signals can be observed in the structural-reporter-group region, namely, a doublet of doublets at $\delta = 4.339$ ppm (*J* values of 3.2 Hz and 10.0 Hz) and a doublet of doublets at $\delta = 4.294$ ppm (*J* values of 3.2 Hz and \sim 1 Hz). The latter resonance has the typical shape of a Gal H-4 signal. By selective ¹H-decoupling of the signal at $\delta = 4.339$ ppm, connected signals at $\delta = 4.294$ ppm and $\delta = 3.684$ ppm are found by difference spectroscopy. In the same way, selective ¹H-decoupling of the H-1 signal of Gal-6' ($\delta = 4.587$ ppm) and difference spectroscopy identified the resonance at $\delta = 3.684$ ppm as Gal-6' H-2. When a similar experiment is carried out for the H-1 signal of Gal-6, the corresponding H-2 resonance is found at $\delta = 3.534$ ppm. In conclusion, the signals at $\delta = 4.339$ ppm and $\delta = 4.294$ ppm can be attributed to Gal-6' H-3 and H-4, respectively.

Since the major compound in subfraction A2b is a monosialylated structure, an additional acidic substitution has to be present. In view of the literature data on acidic carbohydrate chains, it is obvious to suppose that either a phosphate or a sulfate group is involved [10,15–18]. The most obvious positions of attachment are Gal-6' C-3 or C-4, as can be concluded from the downfield positions of Gal-6' H-3 and H-4. Because of the absence of ³¹P couplings on these signals, the occurrence of phosphate can be excluded. In the case of sulfate, the chemical shift values at $\delta = 4.339$ ppm and $\delta = 4.294$ ppm were compared to those of sulfated monosaccharide references. In table 2, the ¹H-NMR data of the methyl glycosides of α -D-galactopyranose, β -D-galactopyranose, 3-*O*-SO₃Na- α -D-galactopyranose and 4-*O*-SO₃Na- α -D-galactopyranose [28] are summarized. Going from methyl α -D-galactopyranoside to methyl

Table 1

¹H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of fractions A2b and A3c obtained from porcine thyroglobulin, together with those of reference compounds IgM and PT

Reporter group	Residue ^a	Chemical shifts ^b in ^c			
		A2b	IgM	A3c ^d	PT
H1	GlcNAc-2	4.713	4.714	n.d.	4.715
	Man-3	4.778	4.760	n.d.	4.786
	Man-4	5.136	5.136	5.139	5.135
	Man-4'	4.924	4.924	4.942	4.942
	GlcNAc-5	4.607	4.605	4.602	4.605
	GlcNAc-5'	4.580 ^e	4.581	4.643	4.605
	Gal-6	4.444	4.445	4.445	4.443
	Gal-6'	4.587 ^e	4.470	4.486	4.443
	Fuc	4.898	4.896	4.896	4.896
H-2	GlcNAc-1-ol	4.213	4.220	4.212	4.219
	Man-3	4.255	4.258	4.258	4.260
	Man-4	4.198	4.197	4.205	4.198
	Man-4'	4.108	4.110	4.112	4.114
H-3a	NeuAc	1.718	1.720	1.719 ^f	1.718 ^f
H-3e	NeuAc	2.669	2.668	2.671 ^f	2.669/2.673
H-3	Gal-6'	4.339	n.d.	n.d.	n.d.
H-4	Gal-6'	4.294	n.d.	n.d.	n.d.
H-5	Fuc	4.072	4.071	4.073	4.072
H-6	GlcNAc-5'	n.d.	n.d.	4.434	n.d.
H-6'	GlcNAc-5'	n.d.	n.d.	4.314	n.d.
CH ₃	Fuc	1.223	1.224	1.225	1.224
NAc	GlcNAc-1-ol	2.056	2.057	2.056	2.056
	GlcNAc-2	2.089	2.088	2.089	2.090
	GlcNAc-5	2.070	2.071	2.072	2.071
	GlcNAc-5'	2.048	2.048	2.066	2.065
	NeuAc	2.030	2.031	2.030 ^f	2.030 ^f

^a For numbering of the monosaccharide residues, see text

^b Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ²H₂O, acquired at 500 MHz (but were actually measured relative to internal acetone: $\delta = 2.225$ ppm)

^c Structures are represented by short-hand notation [27]: ●, GlcNAc; ◆, Man; ■, Gal; ○, NeuAc α 2 \rightarrow 6; □, Fuc; S, sulfate

^d For reasons of simplicity, the δ values for GlcNAc-5/5' H-1 and Gal-6/6' H-1 have been grouped for a sulfate group at GlcNAc-5'

^e Assignments may have to be interchanged

^f Signal stems from two NeuAc residues

3-O-SO₃Na- α -D-galactopyranoside, similar downfield shifts for H-3 and H-4 ($\Delta\delta = +0.666$ ppm and $\Delta\delta = +0.356$ ppm, respectively) were observed, as going from methyl β -D-galactopyranoside to Gal-6' in subfraction A2b (H-3, $\Delta\delta = +0.695$ ppm; H-4, $\Delta\delta = +0.372$ ppm). In the case of the 4-O-sulfated analog quite a different NMR

peak pattern was observed. It has to be noted that similar positions for Gal H-3 and H-4 have recently been reported for a terminal 3-sulfated Gal- β 1 \rightarrow 4 residue in an O-linked chain [32]. Summarizing, the terminal Gal residue in the Man α 1 \rightarrow 6 antenna (Gal-6') in A2b is sulfated at position C-3, leading to the following structure:

Also the occurrence of a mixture of both structures cannot be excluded so far. The recently reported δ values of GlcNAc H-6 and H-6' in the Gal β 1 \rightarrow 4(SO₃Na \rightarrow 6)GlcNAc element, present in an O-linked chain, are in the same range as the values included here [34]. Recent studies on carbohydrate chains liberated from various ³⁵S-labeled mammalian cell lines suggested the occurrence of a NeuAc α 2 \rightarrow 6/3Gal β 1 \rightarrow 4(SO₃Na \rightarrow 6)GlcNAc element in N-linked oligosaccharides [35].

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