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Primary structure of the glycans of porcine pancreatic lipase

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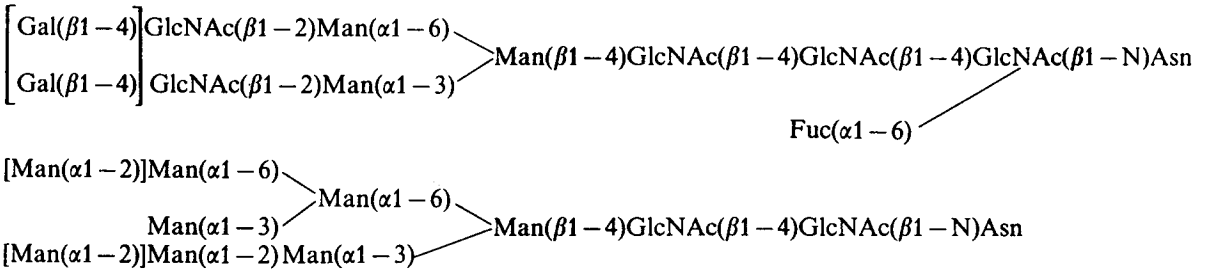
The glycan primary structure of the main glycopeptide fraction obtained by pronase and carboxypeptidase A digestions of porcine pancreatic lipase has been investigated by 500-MH_z ^{1}H-NMR spectroscopy and methylation analysis. The results demonstrate that the glycopeptide fraction was a mixture containing the following structures:



Porcine pancreatic lipase (triacylglycerol acylhydrolase) is one of the principal enzymes involved in the intraluminar digestion of fats [1]. It has been shown that porcine [2, 3] and human [4] lipases are glycoproteins whereas ovine, bovine [5] and horse [6] lipases are not glycosylated. In one of the first preparations of porcine pancreatic lipase [7], two isolipases L_A and L_B were characterized (approximate ratio 1:4). Their carbohydrate moieties were found to be made up each of a single glycan chain containing: fucose 0.83, 0.92; galactose 1.05, 0.53; glucose 0.48, 0.16; mannose 4.12, 4.81; glucosamine 3.05, 2.98 and sialic acid 0.31, 0.04 mol/mol lipases L_A and L_B, respectively [8]. The non-stoichiometric value of the sialic acid content in lipase A suggested that the preparation L_A contained more than one kind of isolipase. A few years later it was shown that lipase L_A contained several isoenzymes [9]. Since no allotypic replacement of amino acid has been noted in the course of the lipase primary structure determination [10], the variability in the isolipases could only originate from a microheterogeneity of the glycan chains.

The amino acid sequence of porcine pancreatic lipase has been reported [10]. The glycan chain (CHO) is attached to asparagine at position 166, the sequence previously found around this residue: Thr-Asn-Gly-Thr-Ile-Glu-Arg- was confirmed.
|
(CHO)

MATERIALS AND METHODS

Porcine pancreatic lipase was prepared from delipidated powder of porcine pancrei by the method of Rovery et al. [9]. The isolipase mixture was digested with *Staphylococcus aureus* protease [11] followed by pronase (twice) and carboxypeptidase treatments [12]. The glycopeptides were purified by gel-filtration on Sephadex G-25 (Superfine) with 60 mM ammonium bicarbonate buffer pH 8.5.

Qualitative and quantitative sugar analyses were carried out after methanolysis (MeOH/0.5 M HCl, 24 h, 80°C) by gas-liquid chromatography of trifluoroacetylated methyl glycosides [13].

Permethylation was performed according to Hakomori [14]. Partially methylated monosaccharides were identified as described by Fournet et al. [15].

For 500-MH_z ^{1}H-NMR spectroscopy, the glycopeptide fraction was repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich) with intermediate lyophilization. ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hF-NMR facility; Department of Biophysical Chemistry; University of Nijmegen; The Netherlands) operating at 500 MH_z in the Fourier-transform mode at a probe temperature of 27°C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [16]. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone (δ = 2.225 ppm) [17].

RESULTS AND DISCUSSION

The digestion of isolipase preparation with *Staphylococcus aureus* protease led to the isolation of two glycopeptide fractions (fractions Sta and Stb) with the same amino acid sequence [11]): Ala^{161}-Gly-Arg-Arg-Thr-Asn^{166}-Gly-Thr-Ile-Glu^{170}. Each of the fractions Sta and Stb was resolved into two subfractions, Sta A, Sta B and Stb A, Stb B, by electrophoresis on thin-layer cellulose plates [18]. The major subfractions Sta A (200 nmol) and Stb B (125 nmol) migrated

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Enzyme. Porcine pancreatic lipase, triacylglycerol acylhydrolase (EC 3.1.1.3)

Table 1. *Molar ratios of the monosaccharide methyl ethers obtained by methanolysis of the permethylated glycopeptide Sta AP followed by acetylation of free methyl ethers*
Values are calculated on the basis of 2 mol methyl-3,4,6-tri-*O*-methyl-2-mono-*O*-acetylmannoside

Methyl ether	Molar ratio
Methyl 2,3,4-tri-*O*-methylfucoside	0.9
Methyl 2,3,4,6-tetra-*O*-methylmannoside	0.4
Methyl 2,3,4,6-tetra-*O*-methylgalactoside	0.4
Methyl 3,4,6-tri-*O*-methyl-2-mono-*O*-acetylmannoside	2
Methyl 2,4-di-*O*-methyl-3,6-di-*O*-acetylmannoside	1.3
Methyl 3,4,6-tri-*O*-methyl-*N*-methyl-acetylglucosaminide	1.5
Methyl 3,6-di-*O*-methyl-4-mono-*O*-acetyl-*N*-methyl-acetylglucosaminide	1.4
Methyl 3-mono-*O*-methyl-4,6-di-*O*-acetyl-*N*-methyl-acetylglucosaminide	0.6

8.5 cm and 7.5 cm, respectively, towards the cathode and the minor subfractions Sta B and Stb A migrated 7.5 and 8.5 cm, respectively, towards the cathode. In order to shorten the peptide chain, the major component Sta A (280 nmol) was digested with pronase (twice) and caboxypeptidase A. The glycopeptide Sta AP Thr-Asn(CHO)-Gly was isolated.

Determination of the sugar composition of glycopeptide Sta AP by gas-liquid chromatography yielded fucose, galactose, mannose and *N*-acetylglucosamine in a molar ratio 0.7:0.3:3.3:2.9, respectively.

The molar ratios of the partially methylated and acetylated methylglycosides obtained by methanolysis of the permethylated glycopeptide Sta AP followed by acetylation were determined by GLC-MS [15] and are given in Table 1. The identification of 3,4,6-tri-*O*-methyl and 2,4-di-*O*-methyl mannosides is in favour of the presence of a mannotriosido branching core with a biantennary structure. The presence of 0.6 mol 3-mono-*O*-methyl glucosamine indicates that one residue of *N*-acetylglucosamine was substituted by one fucose residue (0.9 mol 2,3,4-tri-*O*-methylfucose) in $\alpha(1-6)$ linkage.

The ^{1}H-NMR spectrum of Sta AP reveals a mixture of asparagine-linked carbohydrate chains. Relevant NMR parameters are listed in Table 2. The major component (70%, estimated from the relative intensity of the anomeric proton signals) of this mixture is an incomplete biantennary structure with GlcNAc-$\underline{5}$ and -$\underline{5}'$ in terminal position and with Fuc in $\alpha(1-6)$ linkage to GlcNAc-$\underline{1}$. For the numbering of the monosaccharide residues, see Fig. 1. The biantennary core structure is evident from the characteristic set of chemical shifts of Man H-2 atoms (Man-$\underline{3}$, δ = 4.249 ppm; Man-$\underline{4}$, δ = 4.189 ppm; Man-$\underline{4}'$, δ = 4.118 ppm). The Fuc residue gave rise to a well known set of structural-reporter groups (H-1, δ = 4.872 ppm; H-5, δ = 4.12 ppm; CH_3, δ = 1.21 ppm) which reveals Fuc in $\alpha(1-6)$ linkage to GlcNAc-$\underline{1}$. This is corroborated by GlcNAc-$\underline{2}$ H-1, which is resonating at 4.684 ppm, and NAc, which is found at 2.089 ppm [17, 19]. The absence of an *N*-acetyl signal at $\approx$ 2.079 ppm reflects the absence of an afuco analogue of the biantennary structure. The diversity of GlcNAc-$\underline{1}$ NAc signals is due to heterogeneity in the peptide moiety. The GlcNAc-$\underline{5}$ and -$\underline{5}'$ anomeric protons are resonating mainly at δ = 4.555 ppm, which is typical for terminal GlcNAc residues [17]. However, a small part (10%) of the GlcNAc-$\underline{5}$/$\underline{5}'$ H-1 signals is shifted downfield to δ = 4.580 ppm. In combination with a signal of equal intensity

Table 2. *Relevant ^{1}H-chemical shifts of structural-reporter group constituent monosaccharides for glycopeptide Sta AP from por pancreatic lipase*
Chemical shifts are given in ppm downfield from sodium dimethyl-4-silapentane-1-sulfonate in ^2H_2O at 27°C acquir 500 MHz (but were actually measured relative to internal acetone δ = 2.225 ppm). For the numbering of the monosaccharide residue and complete structures, see Fig. 1
n.d. = not determined

Reporter group	Residue	Chemical shift in (6) -5 -4 \ 3-2-1-Asn / (6′)-5′-4′ F		(D$_1$)-C-4 \ A 3-2-1-Asn / 4′ (D$_3$)-B	
		A galacto component	galacto component	Man 6 component	extended Man 6 component
		ppm			
H-1	$\underline{1}$	5.032		5.032	
	$\underline{2}$	4.684		n.d.	
	$\underline{4}$	5.116		5.345	
	$\underline{4}'$	4.914		4.87[a]	
	$\underline{5}$	4.555	4.580		
	$\underline{5}'$	4.555	4.580		
	$\underline{6}$		4.451		
	$\underline{6}'$		4.451		
	Fuc	4.872			
	$\underline{A}$			5.090	
	$\underline{B}$			4.91[a]	5.146
	$\underline{C}$			5.051	5.304
	$\underline{D}_1$				5.042
	$\underline{D}_3$				5.042
H-2	$\underline{3}$	4.249		4.235	
	$\underline{4}$	4.189		4.107	
	$\underline{4}'$	4.118		4.142	
	$\underline{A}$			4.064	
	$\underline{B}$			4.00[a]	4.02[a]
	$\underline{C}$			4.07[a]	4.10[a]
	$\underline{D}_1$			4.064	
	$\underline{D}_3$			4.064	
H-5	Fuc	4.12[a]			
CH_3	Fuc	1.21[a]			
NAc	$\underline{1}$	2.000[b]		2.000[b]	
		2.005[b]		2.005[b]	
		2.012[b]		2.012[b]	
	$\underline{2}$	2.089		2.060	
	$\underline{5}$	2.051			
	$\underline{5}'$	2.051			

[a] Values could not be determined more accurately ($\pm$0.01 pp due to the complexity of the mixture.
[b] Signals indicate stemming from the main components with r spect to the heterogeneity of the peptide moiety

at δ = 4.451 ppm, which is attributed to Gal-$\underline{6}$/$\underline{6}'$ H-1, th indicates that 10% of the mixture has Gal attached in β(1 4) linkage to the biantennary structure. A set of lower-intensi signals in the region 5.4 > δ > 4.8 ppm is indicative for min components of the oligomannoside type. It was estimat from the relative intensities of the α-anomeric protons th 30% of the mixture consists of three minor components, whi all share the Man6 moiety consisting of Man-$\underline{3}$, -$\underline{4}$, -$\underline{4}'$, -$\underline{B}$ and -$\underline{C}$ [20]. This can be concluded from Man-$\underline{4}$ H-1 (

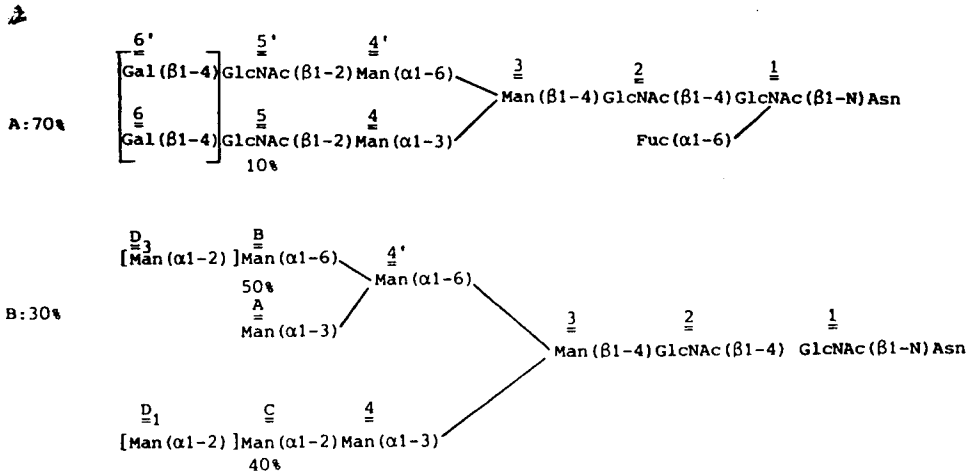

Fig. 1. *Primary structures of glycans of glycopeptide Sta AP from pancreatic lipase*

5.345 ppm) which is characteristic for Man-C attachment, and Man-4′ H-1 (δ = 4.87 ppm), which is indicative for the presence of Man-A and -B, Man-A H-1 is detected at δ = 5.090 ppm. For H-1 of Man-B two signals are observed, 1 ly at δ = 4.91 ppm, pointing to Man-B in terminal position, and at δ = 5.146 ppm, which is typical for Man-B substituted with Man-D_3. From the intensity ratio of these two signals it was estimated that Man-B is substituted for 50% with Man-D_3. Furthermore two signals, of equal intensity, for Man-C H-1 are present. A signal at δ = 5.051 ppm is indicative for terminal Man-C, and a signal at δ = 5.304 ppm shows (40%) substitution of Man-C with Man-D_1. Both Man-D_1 and Man-D_3 H-1 atoms resonate at δ = 5.042 ppm.

On the basis of carbohydrate composition, permethylation analysis and 500-MH_z ^{1}H-NMR spectroscopy, the primary structure of glycans from glycopeptide fraction Sta AP as given in Fig. 1 was deduced. The fraction is heterogenous and consists of a mixture of two glycans (glycans A and B). On the basis of molar ratios of monosaccharides and monosaccharide methyl ethers and the relative intensity of the anomeric proton signals, the mixture contains 70% glycan A and 30% glycan B. The presence of two different structures, the first one of the *N*-acetyllactosamine type and the other of the oligomannoside type on the same protein is not unusual and has been previously described in various glycoproteins like thyroglobulin [21], 'Ashwell receptor' of rabbit liver [22] and in bovine transferrin [23].

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