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Primary Structure of the Major *O*-Glycosidically Linked Carbohydrate Unit of Human Von Willebrand Factor

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A reduced tetrasaccharide chain was obtained from human von Willebrand factor (vWF) by mild alkaline borohydride treatment. The purification of this *O*-glycosidically-linked oligosaccharide was achieved by serial affinity chromatography on immobilized concanavalin A and *Lens culinaris* agglutinin and finally gel filtration. Its structure was determined by a combination of methylation studies and 500 MHz ^{1}H-NMR spectroscopy to be: NeuAc(α2-3)Gal(β1-3)[NeuAc(α2-6)]GalNAc-ol.

Human plasma von Willebrand factor (vWF) is a glycoprotein which plays an important role in haemostasis and coagulation [1-3]. It contains 15% of carbohydrate and possesses *N*- and *O*-glycosidically linked glycans with a high degree of microheterogeneity [4-6]. The primary structure of only two *N*-linked glycans has been elucidated up to now [7-8]. Recently, data obtained by sequencing both vWF cDNA [9] and amino-acids of mature vWF [10] showed that the circulating form of vWF possesses 22 potential glycosylation sites [10]. The digestion of vWF by staphylococcal protease V8 provided two main fragments : Sp III and Sp II [11] corresponding to the N- and C-terminal portions of the molecule, respectively. The distribution of the carbohydrate side chains along the sequence of vWF is not uniform : eight of the ten *O*-glycosylation sites are clustered on the amino-terminal portion of the molecule between residues 300-485 and 705-724 [10] and the two other *O*-glycosylation sites are located on threonyl residues 916 and 1535 [10]. In the present study, *N*-glycosidically linked glycopeptides and oligosaccharide-alditols released by mild alkaline reductive treatment

Abbreviations: ConA, concanavalin A ; LCA, *Lens culinaris* agglutinin ; vWF, von Willebrand factor ; NeuAc, *N*-acetylneuraminic acid ; Gal, D-galactose ; GalNAc-ol, *N*-acetyl-D-galactosaminitol ; HMW, high molecular weight ; LMW, low molecular weight.

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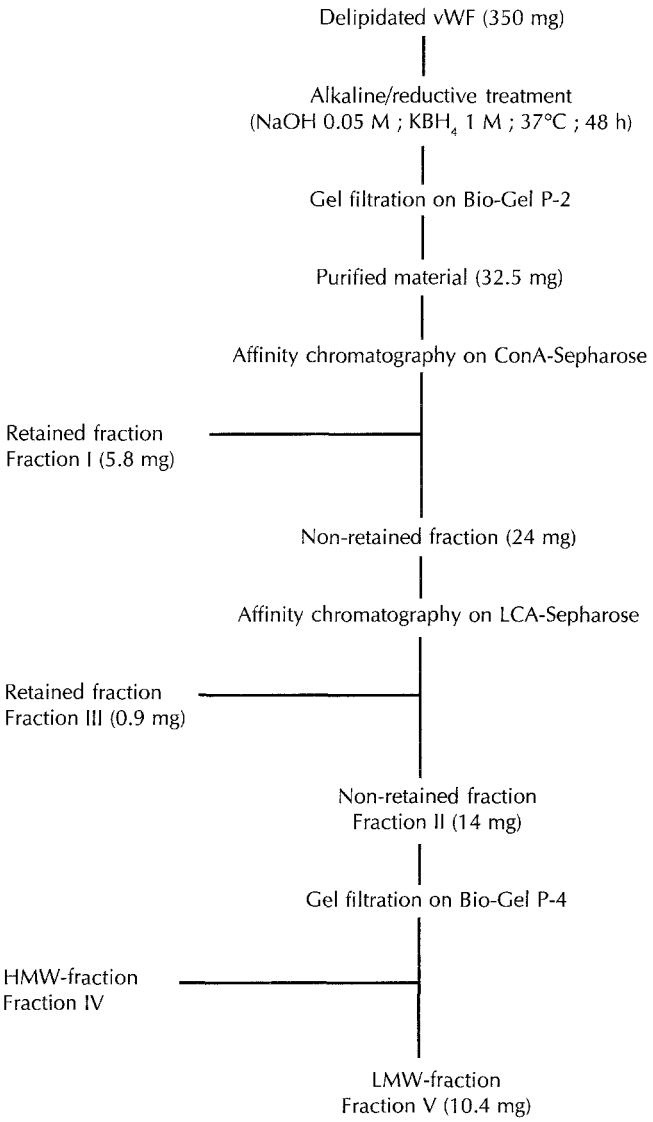

Figure 1. Purification procedure for the *O*-glycosidically linked tetrasaccharide alditol (Fraction V).

[12] of purified vWF have been fractionated using serial affinity chromatography on concanavalin A (ConA) and *Lens culinaris* A (LCA) Sepharose. From the mixture of *N*-linked glycopeptides and oligosaccharide alditols not bound to lectin columns, a pure tetrasaccharide alditol was isolated by gel filtration. The structure of this compound was studied by methylation analysis and high resolution 500 MHz ^{1}H-NMR spectroscopy.

Materials and Methods

Reagents

Human vWF was purified from therapeutic concentrates as in [13]. ConA-Sepharose was obtained from IBF (Villeneuve-la-Garenne, France). Affinity purified LCA [14] was immobilized on Sepharose 4B (Pharmacia, Uppsala, Sweden) according to [15] at a concentration of 2 mg lectin/ml gel. Bio-Gel P-2 and P-4 (mesh 200-400) were from Bio-Rad (Richmond, CA, USA), ^2H_2O was from Aldrich (Milwaukee, WI, USA), and Kieselgel 60 thin layer plates were obtained from Merck, Darmstadt, W. Germany.

Isolation and Purification of the O-Tetrasaccharide-alditol

The mixture of *N*-glycosyl glycopeptides and *O*-oligosaccharide-alditols obtained after mild alkaline reductive treatment as described in [12] was fractionated as described in Fig.1 according to [8].

Analytical Procedures

The molar ratios of hexoses, *N*-acetylhexosamines, *N*-acetylhexosaminitols and *N*-acetylneuraminic acid were determined by gas liquid chromatography (GLC) of the trifluoroacetyl derivatives [16]. The methylation analysis was performed according to the method of Finne *et al.* [17]. The partially methylated methyl glycosides released by methanolysis were acetylated (pyridine/acetic anhydride, 1/10 by vol, 0.2 ml) and the products were analysed by GLC-MS [18] with a capillary column (0.33 mm x 25 m) coated with fused CP-SIL SCB (temperature programme: 100-240°C, at 5°C/min). TLC of oligosaccharides released by alkaline cleavage was carried out on Silica gel 60 plates with *n*-butanol/ethanol/pyridine/acetic acid/water, 1/100/10/3/30 by vol, as solvent.

FAB-MS Analysis

FAB-MS of native oligosaccharide-alditols was performed using a Kratos MS-50 mass spectrometer. A 5 µg sample was applied to the target in aqueous solution; glycerol was used as a matrix. The target was bombarded with xenon atoms having a kinetic energy equivalent to 9 kev. The spectrum was recorded in negative-ion mode at 7 kv acceleration voltage in a mass controlled linear scan at a resolution of 300 ppm.

500-MHz ^{1}H-NMR

Before ^{1}H-NMR spectroscopic analysis, the oligosaccharide alditol was repeatedly treated with ^2H_2O at p^{2}H 7 at room temperature. After each exchange treatment the material was lyophilized. Finally the sample was redissolved in 400 µl ^2H_2O and ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer operating at 500 MHz in the Fourier transform mode and equipped with a Bruker Aspect 2000 computer (SON, hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). Further experimental details have been previously reported [19].

Figure 2. Thin layer chromatographic analysis of oligosaccharides released by alkaline treatment of human vWF (line 2) and of the purified tetrasaccharide alditol (line 1).

Results

Fractionation of Glycopeptides and Oligosaccharide-alditols

The carbohydrate-containing material obtained by mild alkaline reductive treatment of human vWF which represents 9.3% of the starting material was sub-fractionated as illustrated in Fig.1. *N*-Glycosylpeptides retained on ConA and LCA-Sepharose columns (fractions I and III, respectively) have been previously characterized [7, 8]. Fraction II (14 mg) non-retained on immobilized lectins was further fractionated on a Bio-Gel P-4 column in low and high molecular mass subfractions. The low molecular mass fraction (fraction V), represents 32% of the carbohydrate-containing material obtained after β-elimination and 72.3% of the non-retained fraction II. It contains only one oligosaccharide-alditol as proved by thin layer chromatography (Fig.2). Its composition and primary structure was elucidated by chemical and spectrometry analysis.

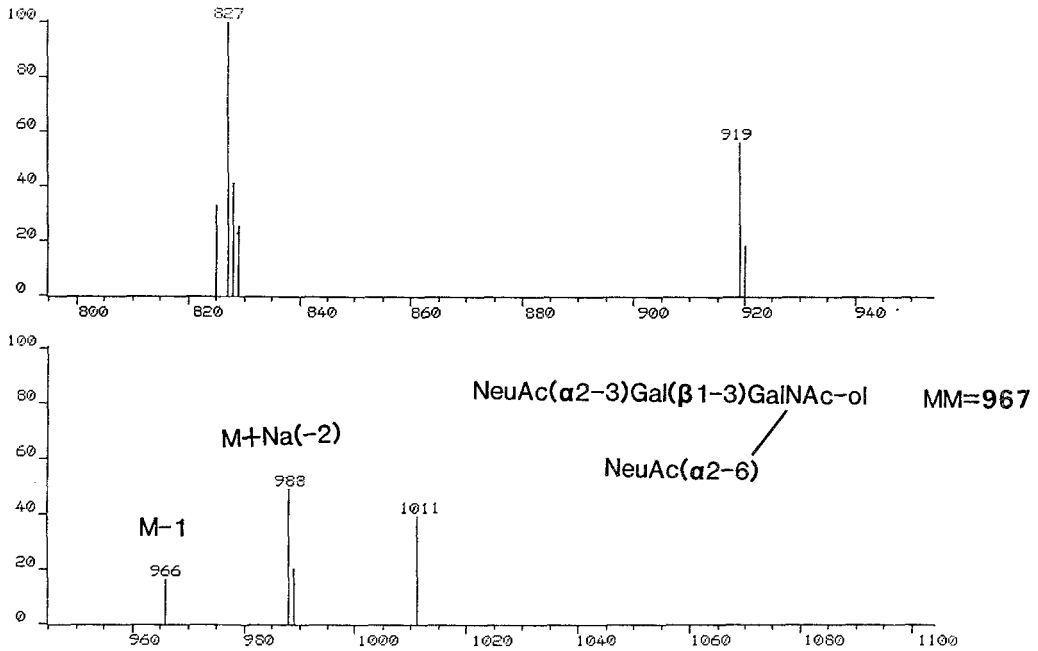

Figure 3. FAB MS of the tetrasaccharide alditol (negative mode, glycerol matrix). G_9 to G_{11} polymers - $(Glycerol)_9$ to $(Glycerol)_{11}$ from the glycerol matrix.

Studies on the Oligosaccharide-alditol

The FAB-mass spectrum (Fig. 3) of the compound revealed a pseudo molecular ion (M-H) with a m/z value of 966 and an ion with m/z value of 988 corresponding to (M + Na) - 2H, giving rise to an M value of 967. This value in combination with the carbohydrate composition (Table 1) proves the oligosaccharide-alditol to be a tetrasaccharide consisting of *N*-acetylneuraminic acid, galactose and *N*-acetylgalactosaminitol in the ratio 2:1:1.

The methylation analysis points to a 3,6-disubstitution of N-acetylgalactosaminitol. The methanolysis of methylated oligosaccharide-alditol leads to the formation of 1,4,5-Me_3-3,6 anhydro-GalNAc(Me)-ol as has previously been described [20]. The set of chemical shifts values of the H-3 protons of the *N*-acetylneuraminic acid residues (δ H-3ax = 1.799 and 1.694 ppm; δ H-3eq = 2.771 and 2.723 ppm) shows that the *N*-acetylneuraminic acid residues are (α2-3) and (α2-6) linked. The chemical shifts of H-1 (δ = 4.540 ppm) and H-3 (δ = 4.116 ppm) of galactose are characteristic for the NeuAc(α2-3)Gal(β1-3)GalNAc-ol sequence.

Table 1. Molar carbohydrate composition and molar ratios of partially methylated monosaccharides present in the methanolysates of the permethylated oligosaccharide-alditol (fraction V).

	Molar ratio	Methyl ester molar ratio[b] in oligosaccharide alditol	
GalNAc-ol[a]	1.0	2,4,6-Me_3-Gal	1.0
Gal	0.9	1,4,5-Me_3-3,6-anhydro-GalNAc(Me)-ol	0.8
NeuAc	1.8	4,7,8,9-Me_4-NeuAc(Me)	1.8

[a] Including anhydro derivative.
[b] Calculated relative to 2,4,6-Me_3-Gal = 1.0.

These NMR data, largely described in the literature [21], clearly prove the structure of the oligosaccharide-alditol to be :

NeuAcα2
6
GalNAc-ol
3
NeuAcα2-3Galβ1

Discussion

The monosaccharide analysis of the carbohydrate moiety of vWF suggested the simultaneous presence of *N*- and *O*-linked glycans [5, 6]. Until now the structure of two *N*-linked glycans has been elucidated [7, 8]. The present study describes the first structural determination of an *O*-linked oligosaccharide of human vWF. β-Elimination of vWF under relatively mild conditions resulted in the recovery of the *N*-linked glycans as glycopeptides and of the *O*-linked glycans as peptide-free oligosaccharide-alditols [12]. By a combination of affinity chromatography on immobilized lectins [8, 14] and gel filtration, we obtained an *O*-linked glycan of vWF in a pure state. This oligosaccharide is predominant among vWF *O*-linked glycans, representing more than 70% of the mixture of reduced *O*-linked glycans and *N*-glycosylpeptides not bound on ConA and LCA. The structure of this tetrasaccharide (Fig. 2) was determined on the basis of methylation analysis, direct probe mass spectrometry and ^{1}H-NMR spectroscopy. This tetrasaccharide, in which two sialic acid residues are linked to C-3 of galactosyl and C-6 of *N*-acetylgalactosaminitol residues, has been isolated from numerous glycoproteins [22]. Although vWF *O*-glycosidically linked carbohydrate chains have been located in regions possessing biological activities [23, 24], the role of the carbohydrate moiety in the multimeric re-partition and biological function of vWF remains controversial [25, 27]. Despite the fact that vWF *O*-linked glycans are heterogeneous [5, 6],

Table 2. ^{1}H-NMR Chemical shifts of the characteristic protons of constituent monosaccharides for the oligosaccharide-alditol.

Residue	Reporter group	Chemical shift
GalNAc-ol	H-2	4.378
	H-3	4.075
	H-4	3.522
	H-5	4.237
	H-6	n.d.
	H-6'	3.471
	NAc	2.041
Gal	H-1	4.540
	H-3	4.116
NeuAc^3	H-3 ax	1.799
	H-3 eq	2.771
	NAc	2.031
NeuAc^6	H-3 ax	1.694
	H-3 eq	2.723
	NAc	2.031

this study led to the purification and to the determination of the primary structure of an *O*-linked tetrasaccharide of human vWF. Its localization on the different vWF functional domains requires further studies which could be useful to elucidate the relationship between structure and biological activities of this glycoprotein.

Acknowledgments

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