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Primary structure of two major glycans of bovine fibrinogen

Philippe DEBEIRE [1], Jean MONTREUIL [1], Elémer MOCZAR [2], Herman VAN HALBEEK [3] and Johannes F. G. VLIEGENTHART [3]

[1] Université des Sciences et Techniques de Lille I, Laboratoire de Chimie Biologique et Laboratoire Associé au Centre National de la Recherche Scientifique 217, Villeneuve d'Ascq Cédex
[2] Université de Paris XII, Faculté de Médecine, Laboratoire du Tissu Conjonctif et Groupe de Recherche du Centre National de la Recherche Scientifique 40, Créteil
[3] Department of Bio-Organic Chemistry, University of Utrecht

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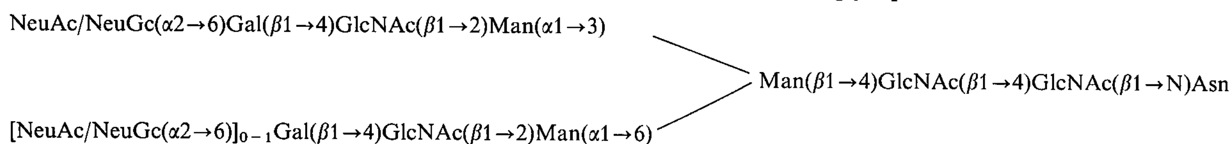
After pronase digestion of bovine fibrinogen, the asparagine-linked glycans were released from the resulting glycopeptides by hydrazinolysis, and subsequently re-*N*-acetylated. Two sialylated glycans were isolated by ion-exchange chromatography. Their primary structure has been determined by methylation analysis and 360-MHz ^{1}H-NMR spectroscopy. The structures proposed to be present in the native glycoprotein are as follows:



Fibrinogen and fibrin from a number of animal species are glycoproteins [1]. The biological role of their glycan moieties is not yet clear despite active investigations in this field. For example, it has been reported that neutral carbohydrates [2] or sialic acid [3] are released during clotting but a number of investigations were unable to confirm these results [4, 5]. The decrease in coagulability of fibrinogen after periodate oxidation of glycans also suggested a role of the latter in the clotting process [6]. This finding has since been critized, as the reduction of the clotting time may be due to oxidation of some amino acid residues [7]. Recently, it was demonstrated that the enzymatic removal of glycans does not change the clotting time of fibrinogen [8].

Study of pathological cases did not bring more information about the relationship between sugars and clotting. In fact congenitally abnormal fibrinogens [9 – 11] have in some cases a lower carbohydrate content than normal ones but is seems that the primary cause of these coagulation disturbances is an amino acid substitution. However, sialic-acid-free fibrinogen forms a gel different from the normal protein [12] and yields urea-soluble fibrin in the presence of factor XIII [13]. These findings suggest that the carbohydrate in fibrinogen could play a role in the complex polymerisation process.

Attempts to clarify the structure of the glycan of bovine fibrinogen were done earlier by Mester et al. [14, 15], but due to the poor performances of the analytical methods used at that time, the authors did not obtain exact determination of the structure. The present paper describes the primary structure of the two major glycans of bovine fibrinogen determined by methylation analysis and 360-MHz ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

Bovine fibrinogen type I (75% protein-clottable) and type IV (95% protein-clottable), pronase and *N*-glycolylneuraminic acid were purchased from Sigma (St Louis, MO, USA). Ultrogel AcA 202 was obtained from IBF (Paris, France). Anion-exchanger AG1-X2 (minus 400 mesh) and Bio-Gel P-2 (200 – 400 mesh) were furnished by Bio-Rad Laboratories (Richmond, CA, USA). Anhydrous hydrazine was from Pierce Chemical Company (Rockford, IL, USA). Silica Gel 60 thin-layer plates (0.2 mm layer) were from Merck (Darmstadt, FRG). The sialylated oligosaccharides used as standards for thin-layer chromatography were isolated from urine of a patient with sialidosis and are a gift from Dr G. Strecker. Their primary structure is as follows [16]: oligosaccharide I: NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3)[NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→6)]Man(β1→4)GlcNAc; oligosaccharide II: NeuAc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3)[Gal(β1→4)GlcNAc(β1→2)Man(α1→6)]Man(β1→4)GlcNAc.

Preparation of the glycopeptides

Fibrinogen type I (25 g) was digested by 0.5 g pronase as previously described [14]. The pronase was preincubated for 1 h at 49 °C to destroy glycosidase activities [17]. Pronase was removed by trichloroacetic acid at 4 °C [18] and the bulk of the acid was extracted by diethyl ether. The solution was neutralized with ammonium hydroxide, concentrated by evaporation *in vacuo* and chromatographed on Ultrogel AcA 202 column (60 × 5 cm). Elution was done with 0.1 M aqueous acetic acid. Sugars were detected by a phenol/sulfuric acid reagent [19] and the fractions containing sugars were pooled

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Correspondence to J. Montreuil, Laboratoire de Chimie Biologique et Laboratoire Associé au CNRS n° 217, Université des Sciences et Techniques, F-59655 Villeneuve d'Ascq Cédex, France

Abbreviations. Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; NeuAc, *N*-acetyl-D-neuraminic acid; NeuGc, *N*-glycolyl-D-neuraminic acid.

and lyophilized. Glycopeptides were prepared in a similar way from 1 g of fibrinogen sample Sigma type IV, using a 60 × 1 cm Ultrogel AcA 202 column for the separation of the glycopeptides. The finger-printing of the glycopeptide mixture on silica thin-layers was realised as described [20].

Hydrazinolysis

Lyophilized glycopeptides were hydrazinolysed according to Bayard et al. [21]: the sample (30 mg) was dried *in vacuo* over P_2O_5 and heated in a glass sealed tube with 500 µl anhydrous hydrazine for 20 h at 105°C. The excess of hydrazine was evaporated under nitrogen. The evaporation was repeated twice after addition of a few drops of toluene. The residual hydrazine was eliminated *in vacuo* over H_2SO_4. The liberated oligosaccharides were desalted on a column (1 × 50 cm) of Bio-Gel P-2 (200 – 400 mesh) and eluted with acetic acid (1%) in water. The carbohydrate fractions were visualized by the phenol/sulfuric acid reagent [19]. After elimination of acetic acid by evaporation *in vacuo*, the oligosaccharides were re-*N*-acetylated with acetic anhydride according to Reading et al. [22] and subsequently desalted as described above. The non-desialylation of the glycans during the hydrazinolysis/re-*N*-acetylation procedure was checked by the diphenylamine reagent [23] after each passage over Bio-Gel P-2 columns.

Separation of sialyloligosaccharides by ion-exchange chromatography

The oligosaccharide fraction obtained was submitted to ion-exchange chromatography on a column (20 × 1 cm) of AG1-X2 (minus 400 mesh, acetate form) equilibrated in water. Elution was performed with pyridine/acetic acid buffer pH 5 using a continuous gradient of ionic strength from 0 – 400 mM at a flow rate of 10 ml/h.

Thin-layer chromatography

Thin-layer chromatography of the oligosaccharides was performed on Silica Gel 60 plates during 5 h with the solvent system: pyridine/ethanol/acetic acid/*n*-butanol/water (10:100:3:10:30, by vol.) [24] and visualized with an orcinol/sulfuric acid reagent (200 mg orcinol in 100 ml of 20% sulfuric acid).

Determination of the structure of the sialyloligosaccharides

The molar carbohydrate composition of the sialyloligosaccharides was determined by gas-liquid chromatography after methanolysis and trifluoroacetylation [25]. Sialic acids were identified and determined according to Schauer [26]. Permethylation was carried out according to Finne et al. [27] and the methyl glycosides were separated by gas-liquid chromatography before (for neutral monosaccharides) and after (for neutral monosaccharides and hexosamines) peracetylation according to Fournet et al. [28]. For ^{1}H-NMR spectroscopic analysis the oligosaccharides were repeatedly exchanged in ^2H_2O (99.96 mol% ^{2}H, Aldrich, Milwaukee, WI, USA) at room temperature with intermediate lyophilization. The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer (SON NMR facility, Groningen, The Netherlands), operating in the Fourier transform mode, at a probe temperature of 25°C [29]. Chemical shifts are given relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm, with an accuracy of 0.003 ppm.

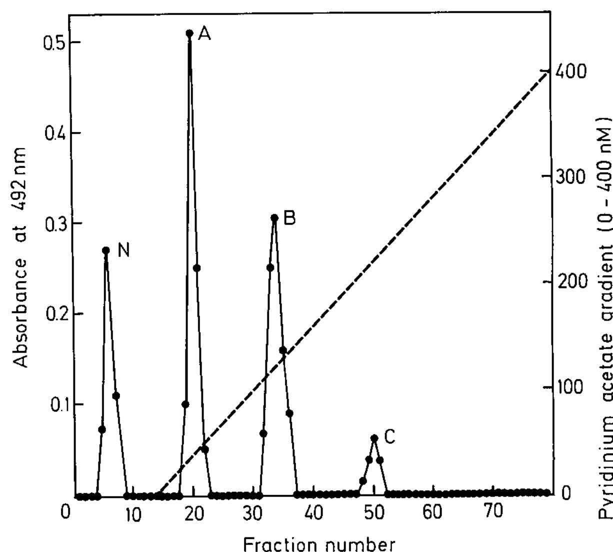

Fig. 1. *Ion-exchange chromatography on AG 1-X2 (minus 400 mesh) of oligosaccharides liberated from fibrinogen glycopeptides by hydrazinolysis and re-*N*-acetylation.* For details, see Materials and Methods

Preparation of glycopeptides

25 g of bovine fibrinogen type I yielded 450 mg of a purified glycopeptide mixture. The patterns obtained by chromatography on silica thin-layers [20] of the glycopeptides from fibrinogen samples with 75% (type I) or of 95% (type IV) clottable protein were similar. It was decided to carry out the structural studies on the glycopeptide mixture prepared from the type I sample.

RESULTS

Sialic acid characterization

N-Acetyl and *N*-glycolyl neuraminic acids were found to be present in the glycopeptides in equal proportions. As hydrazinolysis removes all *N*-acyl groups (acetyl and glycolyl) and since re-*N*-acetylation is achieved with acetic acid anhydride, the native heterogeneity is lost during this treatment and the liberated glycans contain only *N*-acetylneuraminic acid [30].

Isolation of glycans

The ion-exchanger chromatography of glycans obtained by hydrazinolysis and re-*N*-acetylation afforded two major fractions (fractions A and B), which were eluted with 40 and 120 mM pyridine/acetic acid buffer, respectively, and two minor fractions, a non-retained neutral one (fraction N) and one eluted with 220 mM pyridine/acetic acid buffer (fraction C) (Fig. 1). The fractions N, A, B and C represented 16%, 38%, 39% and 7% of the total oligosaccharide content, respectively. Fractions A and B were homogeneous on thin-layer chromatography (Fig. 2) and migrates as the monosialylated (oligosaccharide II) and the disialylated (oligosaccharide I) standards, respectively.

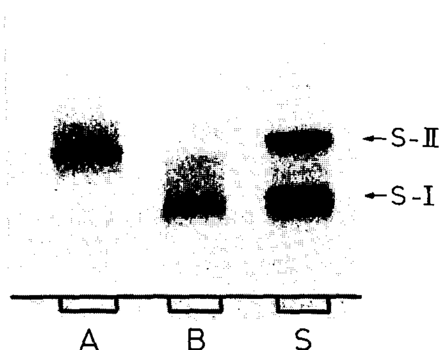

Fig. 2. *Thin-layer chromatography of the mixtures (S) of standard oligosaccharides I (S-I) and II (S-II) and of fractions A and B isolated by ion-exchange chromatography.* For details, see Materials and Methods

Table 1. *Molar carbohydrate composition of the fibrinogen glycopeptides and of the oligosaccharides obtained by hydrazinolysis and separated by ion-exchange chromatography (fractions A and B)*
The molar ratios were calculated on the basis of three mannose residues

Monosaccharide	Glycopeptides	Fraction A	Fraction B
Gal	2.2	2.1	2.1
Man	3.0	3.0	3.0
Fuc	0.0	0.0	0.0
GlcNAc	3.6 (4)	3.6 (4)	3.6 (4)
NeuAc	0.85	1.1[a]	2.1[a]
NeuGc	0.85	—[a]	—[a]

[a] Only *N*-acetylneuraminic acid is present since hydrazinolysis removed acetyl and glycolyl groups and re-*N*-acetylation fully re-*N*-acetylated the neuraminic acid residues.

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated oligosaccharides obtained by hydrazinolysis of the fibrinogen glycopeptides (fractions A and B)*
The molar ratios were calculated on the basis of two residues of 3,4,6-Me_3-Man

Monosaccharide methyl ether	Fraction A	Fraction B
2,3,4,6-Me_4-Gal	1.0	0.0
2,3,4-Me_3-Gal	1.0	1.9
3,4,6-Me_3-Man	2.0	2.0
2,4-Me_2-Man	1.0	1.1
3,6-Me_2-GlcNAc(NMe)	3.5	3.4
4,7,8,9-Me_4-NeuAc(Me)	1.0	1.9

Table 3. *^1H chemical shifts of structural-reporter-group protons of constituent monosaccharides for two major sialyloligosaccharides derived from bovine fibrinogen*
Chemical shifts were acquired at 360 MHz, for solutions of the oligosaccharides in ^2H_2O, at $p^2H \approx 2$ and at 25 °C

Reporter group	Residue	Chemical shift in oligosaccharide A	Chemical shift in oligosaccharide B
		ppm	
H-1 of	GlcNAc-1	5.189 (α) / 4.72 (β)	5.187 (α) / 4.72 (β)
	GlcNAc-2	4.608	4.605
	Man-3	4.773	4.776
	Man-4	5.137	5.133
	Man-4′	4.932	4.947
	GlcNAc-5	4.608	4.605
	GlcNAc-5′	4.583	4.605
	Gal-6	4.449	4.447
	Gal-6′	4.473	4.447
H-2 of	Man-3	4.257	4.257
	Man-4	4.196	4.197
	Man-4′	4.115	4.117
H-3ax of	NeuAc(α2→6)	1.774	1.763[a]
H-3eq of	NeuAc(α2→6)	2.659	2.663[a]
NAc of	GlcNAc-1	2.037	2.037
	GlcNAc-2	2.083	2.082
	GlcNAc-5	2.070	2.070
	GlcNAc-5′	2.047	2.066
	NeuAc	2.031	2.031[b]

[a] Signal of two protons.
[b] Signal of two methyl groups.

Primary structure of oligosaccharides A and B

The primary structure of the two major glycans A and B of bovine fibrinogen was established by 360-MHz ^1H-NMR spectroscopy in combination with sugar analysis and permethylation studies. The carbohydrate compositions of A and B are compiled in Table 1, the results of permethylation analysis in Table 2 and the pertinent ^1H-NMR data in Table 3.

The 360-MHz ^1H-NMR spectra of ^2H_2O solutions of oligosaccharides A and B are very similar to those of the mono-(α2−6)-sialylated and di-(α2−6)-sialylated oligosaccharides A3a and A4 from human meconium [31], respectively. It should be mentioned, that the structures of A3a and A4 are identical to those of oligosaccharides II and I, respectively, from sialidosis urine which were used as standards for thin-layer chromatography in this study. The above spectral resemblances indicate the branching pattern of both A and B glycans to be biantennary, and the NeuAc residues glycan in A to be located in the (α1−3)-linked, upper branch [29, 31].

It should be noted that the spectra of fibrinogen oligosaccharides A and B were recorded for ^2H_2O solutions at $p^2H \approx 2$. At this relatively low p^2H, a small amount of sialic acid had been split off. Concomitantly, the structural-reporter-group signals of the desialylated analogue of A and the partially desialylated counterpart of oligosaccharide B are

GLYCAN A

NeuAc or NeuGc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3)

Man(β1→4)GlcNAc(β1→4)GlcNAc(β1→N)Asn

Gal(β1→4)GlcNAc(β1→2)Man(α1→6)

GLYCAN B

6 5 4

NeuAc or NeuGc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→3)

Man(β1→4)GlcNAc (β1→4)GlcNAc(β1→N)Asn

3 2 1

NeuAc or NeuGc(α2→6)Gal(β1→4)GlcNAc(β1→2)Man(α1→6)

6' 5' 4'

Fig. 3. *Primary structure of the two major glycans from bovine fibrinogen*

observable. The structural-reporter-group signals of free sialic acid, which occurs mainly (≈ 92%) in the β-anomeric form under the applied conditions, at p^2H ≈ 2 are observed at δ = 1.872 ppm (H-3ax, doublet of doublets), δ = 2.304 ppm (H-3eq, doublet of doublets) and at δ = 2.050 ppm (NAc, singlet) (for p^2H influences on these chemical shifts, see [32, 33]).

Besides this contamination, an important difference between the spectra of the fibrinogen oligosaccharides and the corresponding ones from meconium/sialidosis urine originates from this low p^2H value. The chemical shifts of the H-3ax and H-3eq signals of the glycosidically linked sialic acid residues are influenced by the p^2H value of the solution. For glycans A and B, δH-3ax ≈ 1.77 ppm and δH-3eq ≈ 2.66 ppm are observed (see Table 3), while for neutral solutions δH-3ax ≈ 1.72 ppm and δH-3eq ≈ 2.67 ppm are found [29, 33]. Nonetheless, it can be concluded that the NeuAc residues in glycans A and B are (α2 – 6)-linked to Gal [33].

Another deviation between the NMR spectra of the fibrinogen oligosaccharides and the meconium/sialidosis oligosaccharides stems from the presence of an additional GlcNAc-1 residue at the reducing end of the molecules. For this GlcNAc-1, the NAc signal is found at δ = 2.037 ppm, while the chemical shift of the NAc signal of GlcNAc-2 (δ = 2.083 ppm) points to (β1 – 4)-glycosidic linkage of this residue [29]. A similar reasoning holds for the anomeric signals. The chemical shift of H-1 of GlcNAc-1 in the α-anomer of oligosaccharides A and B (δ = 5.187 ppm) differs slightly from that for the reducing GlcNAc-2 in the meconium analogues (δ = 5.212 ppm), while the H-1 signal of GlcNAc-2 for oligosaccharides A and B is observed at a position (δ = 4.605 ppm) which is in accord with its (β1 – 4)-linkage to GlcNAc-1 [29]. These parameters for the reducing chitobiose core unit in these fibrinogen oligosaccharides are in good agreement with those found for similar asialyl-oligosaccharides isolated from urine of beagle dogs with GM_1-gangliosidosis [34].

Based on the aforementioned NMR results and those of carbohydrate (Table 1) and methylation (Table 2) analysis, the primary structure of the major fibrinogen oligosaccharide chains is established to be as depicted in Fig. 3. The given structure takes into account the loss of *N*-glycolyl groups from *N*-glycolylneuraminic acid during hydrazinolysis.

DISCUSSION

On the basis of the carbohydrate composition, methylation analysis and 360-MHz ^{1}H-NMR spoectroscopy, the primary structure of the two major glycans of bovine fibrinogen has been determined. The structures are identical to those of the two major glycans A-1 and A-2 of human fibrinogen, except for the presence of *N*-glycolyl nueraminic acid and the ratio of 2 for mono/disialylated glycans in human compared to 1 in bovine fibrinogen [11, 35]. The latter investigators applied 300-MHz ^{1}H-NMR spectroscopy of fibrinogen glycopeptides, in combination with exoglycosidase treatment, as the method of structural analysis. However, they assigned the difference in chemical shift ($\Delta\delta$ = 0.007 ppm) observed between H-1 of Man-4′ for their GP VII versus GP IV – VI, as a result of removal of NeuAc from the (α1 – 6)-branch by neuraminidase treatment. As can be concluded from [29], the chemical shift of H-1 of Man-4′ is influenced slightly but significantly by the presence of NeuAc in the (α1 – 3)-branch. This might be the explanation of the effect described by Townsend et al. [35]. The 300-MHz ^{1}H-NMR spectrum published in [35] suggests that these authors dealt mainly with the monosialylated glycopeptide, corresponding to oligosaccharide A in the present study.

Considering the amino acid sequences of the glycopeptides obtained from bovine fibrinogen [15, 36] it seems that the

glycans are located in peptide sequences of the β and γ chains analogous to those of human fibrinogen [37]. On the basis of previous results [15], it can be assumed that, like in human fibrinogen, the monosialylated glycans are preferentially located on the Asn-Lys-Thr sequence of the γ chain, while the disialylated ones are mainly located on the Val-Gly-Glu-Asn-Arg sequence of the β chain.

The monosialylated and disialylated biantennary structure of the *N*-acetyllactosamine type found in fibrinogens is widely distributed and has been found in numerous glycoproteins from various animal sources, playing very different roles (see the recent review of Montreuil [38]). Thus, the biological significance of these common structures remains to be investigated.

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