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The N- and O-linked carbohydrate chains of human, bovine and porcine plasminogen

Species specificity in relation to sialylation and fucosylation patterns

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The structures of the *N*- and *O*-glycans of human, bovine and porcine plasminogen were determined by 500-MHz ^{1}H-NMR spectroscopy. The *N*-glycans of all three species proved to be of the *N*-acetyllactosamine type differing from one another with respect to the sialylation and fucosylation patterns. In the *N*-glycan of human plasminogen the two antennae are sialylated with *N*-acetylneuraminic acid (NeuAc), whereas in the bovine counterpart both branches carry significant amounts of *N*-glycolylneuraminic acid (NeuGc). In porcine plasminogen the sialic acid is mainly NeuAc; the Manα1→6 branch, however, is only partially sialylated. In addition, the porcine *N*-glycan is fucosylated to about 80% in α1→6 linkage to the GlcNAc-1 residue. The *O*-glycans of the three species possess an identical Galβ1→3GalNAc core which is α2→3 sialylated with NeuAc at Gal. The disialylated form, which is also present in all three species, has an additional NeuAc residue in α2→6 linkage to GalNAc. Mono- and disialylated forms occur in different molar ratios in the different plasminogens: 80:20 in human, 70:30 in bovine and 50:50 in porcine. This study on the carbohydrate moiety of these three plasminogens reveals species specificity in terms of various types of microheterogeneities.

Plasminogen is the inactive plasma protein precursor of the proteolytic enzyme plasmin which occupies a key position within the fibrinolytic system. Native plasminogen is a single-chain glycoprotein with a molecular mass of approximately 90 kDa [1]. Human Glu-plasminogen, whose amino acid sequence has been elucidated [2, 3], can be separated by affinity chromatography on lysine-Sepharose 4B into two variant forms, possessing identical amino acid sequences, but differing in their carbohydrate contents [4]. Variant 1 contains an N-linked diantennary *N*-acetyllactosamine-type carbohydrate chain at Asn^{288} and an O-linked mucin-type chain at Thr^{345} [5, 6]. Variant 2 is monoglycosylated and carries only the O-linked carbohydrate chain at Thr^{345} [6]. Both types of carbohydrate chains are heterogeneous with respect to the sialic acid content [5, 6]. This heterogeneity is responsible for the occurrence of several forms of glycoprotein which differ in their isoelectric points. The affinity for lysine, fibrin and α_2-antiplasmin is higher for variant 2 due to the absence of the carbohydrate chain at Asn^{288} [4, 7]. Interestingly, variant 1 is more easily activated to plasmin than variant 2 [8].

In a recent study we showed that the elution patterns of bovine and porcine plasminogen on lysine-Sepharose 4B differed from those previously reported for human, rabbit and sheep plasminogen [9]. The latter glycoproteins each contain two variants with different carbohydrate contents [10, 11]. Amino acid sequence analysis revealed that bovine and porcine plasminogen, like the human molecule, both contain two glycosylation sites. The *N*-glycosidic linkage involving Asn^{289} of BPG and PPG was localized in the corresponding position of kringle 3; however, the O-linked carbohydrate attachment site was shifted to Ser^{339} in the bovine and to Thr^{249} in the porcine protein [12, 13]. In addition, a varying degree of *N*-glycosylation was found to be responsible for differences in total carbohydrate between the three species investigated in the present study [9].

In the framework of the findings mentioned above and to complete our investigation of the primary structures of bovine and porcine plasminogen, we present here a comparison of the carbohydrate structures of human, bovine and porcine plasminogen (HPG, BPG and PPG).

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Abbreviations. HPG, human plasminogen; BPG, bovine plasminogen; PPG, porcine plasminogen; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Sia, sialic acid; GalNAc, *N*-acetyl-D-galactosamine; GalNAc-ol, *N*-acetyl-D-galactosaminitol; Fuc, L-fucose.

Enzymes. N-Acetylneuraminate monooxygenase (EC 1.14.99.18); plasmin (EC 3.4.21.7); papain (EC 3.4.22.2); pronase (EC 3.4.21.4 and 3.4.24.4).

MATERIALS AND METHODS

Isolation of plasminogens

HPG, BPG and PPG were isolated from citrated plasma by affinity chromatography on lysine-substituted Bio-Gel P-300, as described earlier [14].

Preparation of glycopeptides

Plasminogen was reduced with tri-*n*-butylphosphine [15]. After carboxamidomethylation [16] the protein was dialyzed against 0.05 M NH_4HCO_3 and lyophilized. Reduced and alkylated plasminogen (100 mg) was dissolved in 5 ml activating

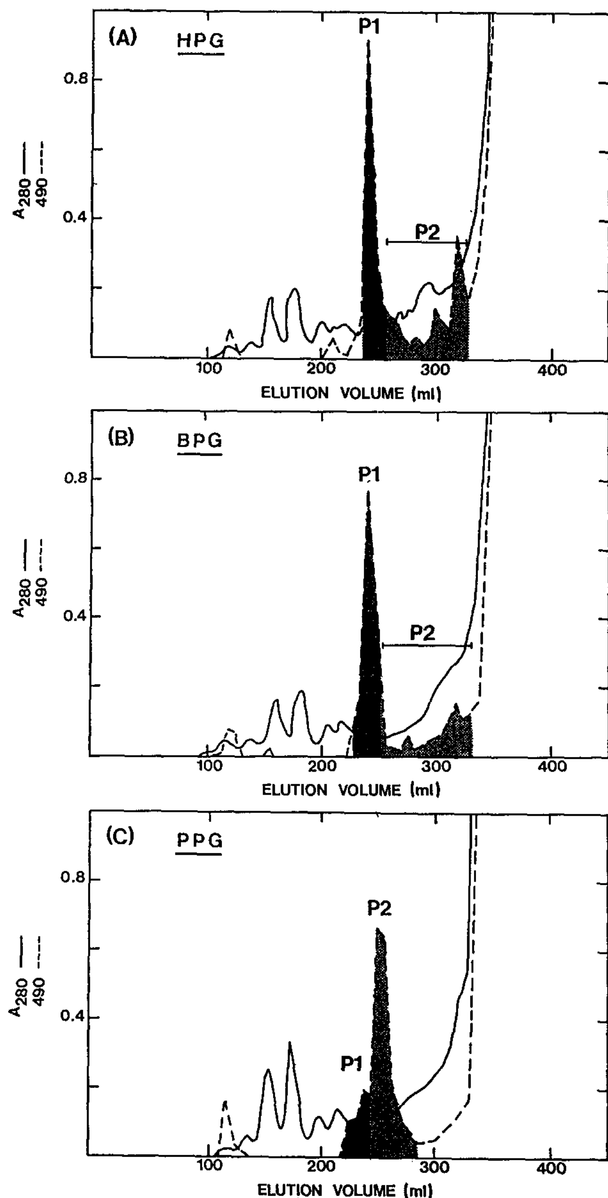


Fig. 1. *Isolation of the glycopeptide fractions P1 and P2 by gel filtration on Sephadex G-50.* (A) Human; (B) bovine; (C) porcine plasminogen

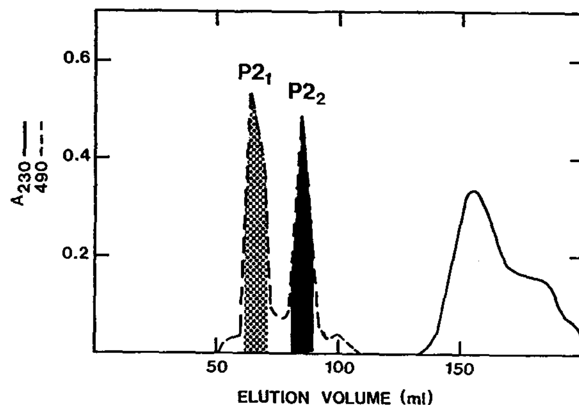


Fig. 2. *Isolation of* O*-glycans.* The oligosaccharide-alditols formed by β-elimination of the P2 fractions (see Fig. 1) were isolated by chromatography on Bio-Gel P-6. The chromatogram is representative for the three species investigated

buffer (6.1 mg NaCN, 43.9 mg cysteine · HCl and 73.1 mg Na_2EDTA per 25 ml, adjusted to pH 8.0) and digested for 24 h at 60°C with 10 mg papain (3.5 mAnson-E/mg; Merck), added in two 5-mg portions at zero time and 12 h. Bacterial degradation was prevented by the addition of one drop of toluene. The pH was kept constant between 7.8 and 8.0. Subsequently, the digest was mixed with 5 mg pronase (type XIV, 5.8 units/mg; Sigma), and the reaction mixture was kept at 40°C for 72 h. Five additional 1-mg portions of pronase were added every 12 h. The proteolytic digest was then lyophilized, dissolved in 0.05 M NH_4HCO_3 and applied to a column (150 × 1.8 cm) of Sephadex G-50 sf (Pharmacia) equilibrated with the same solvent. The elution was carried out with 0.05 M NH_4HCO_3 (15 ml/h; 3-ml fractions), monitoring the absorbance at 280 nm and 490 nm (phenol/H_2SO_4 assay) [17]. The carbohydrate-positive fractions were also analyzed for hexosamines (hydrolysis: 4 M HCl, 6 h, 110°C) employing a Liquimat III amino acid analyzer (Kontron). Based on these analyses two glycopeptide fractions were obtained from the plasminogen of all three species (Fig. 1A, B, C). The glycopeptide fraction P1 containing mainly N-linked carbohydrate chains was redigested with pronase at an enzyme/substrate mass ratio of 1:10 and purified by gel filtration as described above. The effluent was monitored at 230 nm and 490 nm.

Alkaline borohydride treatment

The glycopeptide fraction P2 containing mainly O-linked carbohydrate chains was treated with 4 ml 0.1 M NaOH containing 1 M $NaBH_4$. After incubation for 24 h at 40°C the solution was acidified to pH 5.0 with 4 M acetic acid and applied to a column (19 × 1.6 cm) of Dowex 50 WX8, H^+ form (100 – 200 mesh; Fluka) [18]. The column was washed with 150 ml water and the eluate lyophilized. Boric acid was removed by co-evaporation with methanol under reduced pressure. The residue was fractionated on a column (95 × 1.6 cm) of Bio-Gel P-6 (200 – 400 mesh; Bio-Rad) using water as eluent (7 ml/h, 1.6-ml fractions). Oligosaccharide-alditols were monitored at 490 nm (Fig. 2). If necessary, oligosaccharide-alditol fractions were further purified by HPLC on a Lichrosorb-NH_2 column (5 µm; 250 × 4.6 mm; Merck). The column was eluted with a linear gradient of 80:20 to 20:80 (by vol.) acetonitrile/water containing 2.5 mM NH_4HCO_3 for 35 min at room temperature at a flow rate of 1 ml/min.

Sugar analysis

Monosaccharide analysis on 3 – 4 mg glycoprotein was carried out by GLC on a capillary CPsil 5 CB WCOT fused silica column (25 m × 0.32 mm; Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis (1 M methanolic HCl, 24 h, 85°C), *N*-(re)acetylation and trimethylsilylation [19]. Mannitol was used as an internal standard.

Determination of NeuAc/NeuGc ratios

Carbohydrate samples (50 – 200 µg) were subjected to methanolysis under mild conditions (0.05 M methanolic HCl, 1 h, 80°C) to split off sialic acid without removing the N-

Table 1. *Carbohydrate content and molar composition of native human, bovine and porcine plasminogen*
The content of Man was assumed to be 3 mol/mol. The values of GlcNAc were corrected for the amount of Asn-linked GlcNAc that is not cleaved under the methanolysis conditions used [19]

Monosaccharide	Molar ratio in		
	HPG	BPG	PPG
Fuc	0.1	0.3	0.4
Man	3.0	3.0	3.0
Gal	4.3	1.8	3.4
GlcNAc	3.4	3.0	2.5
GalNAc	1.9	1.8	3.4
Sia	4.0	2.5	5.3
Carbohydrate content (by mass)	1.9%	1.3%	0.8%

substituent. Then, GLC analysis of the trimethylsilylated methyl ester methyl glycosides of the sialic acids present was performed on a capillary CPsil 5 CB WCOT fused silica column [19, 20]. Combined GLC-MS was carried out using a Carlo Erba GC/Kratos MS 80/Kratos DS 55 system (electron energy 70 eV; accelerating voltage 2.7 kV; ionizing current 100 µA; ion-source temperature 225°C; capillary CPsil 5 CB WCOT fused silica column; oven temperature program 180°C during 2 min, 180–260°C at 5°C/min).

500-MHz ¹H-NMR spectroscopy

Glycopeptides and oligosaccharide-alditols were repeatedly exchanged in ${}^2H_2O$ (99.96 atom % ${}^2H$, Aldrich) with intermediate lyophilization. ¹H-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 MHz in the Fourier-transform mode at a probe temperature of 27°C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [21]. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane 1-sulfonate, but were actually measured indirectly to acetone in ${}^2H_2O$ (δ = 2.225 ppm) [22].

RESULTS AND DISCUSSION

Isolation of N- *and* O-*glycans and carbohydrate compositions*

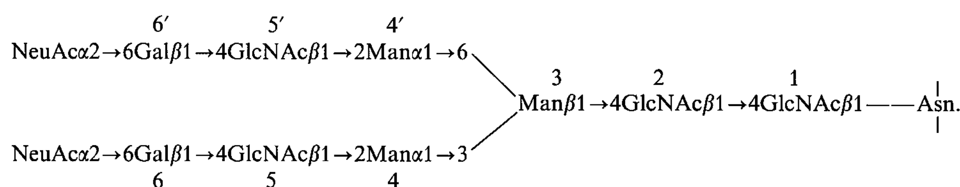
The sugar compositions of native HPG, BPG and PPG are presented in Table 1. The carbohydrate contents vary between approximately 1.9% (HPG) and 0.8% (PPG). In general, the presence of Man indicates the occurrence of N-linked carbohydrate chains, whereas the presence of GalNAc points to O-linked chains. Although it has been shown that these rules hold true for HPG [5, 6], they ought to be used with considerable caution [23–27].

For structural analysis, the proteolytic digest derived from the reduced and alkylated plasminogens was fractionated on Sephadex G-50 (Fig. 1). Judging from the neutral hexose and hexosamine patterns, each plasminogen afforded two glycopeptide fractions. Fractions P1 contained mainly N-linked carbohydrate material as evidenced by the essential absence of GalNAc, and were redigested with pronase. Fractions P2 contained primarily *O*-glycans as judged by the presence of GalNAc, and were subjected to β-elimination followed by Bio-Gel P-6 chromatography. The elution patterns of the resulting oligosaccharide-alditols of the three species were very similar. A typical chromatogram is shown in Fig. 2, demonstrating the presence of two components (P2_1 and P2_2). The HPG-P2_1, BPG-P2_1 and BPG-P2_2 fractions were further purified by HPLC (elution patterns not shown); the other samples did not require additional purification. The carbohydrate compositions of these oligosaccharide-alditols are listed in Table 2. For BPG-P2_1, PPG-P2_1 and PPG-P2_2 low molar percentages of NeuGc were found, the remaining fractions lacking this acidic sugar.

The carbohydrate compositions of the N-linked glycopeptides HPG-P1, BPG-P1 and PPG-P1 are summarized in Table 3. As the routine procedure for sugar analysis is known to lead to *N*-deacylation [19], this technique could not be used to distinguish between NeuAc and NeuGc. To quantify these two sialic acids, the P1 fractions were treated with mild methanolysis that did not lead to *N*-deacylation. Subsequent GLC-MS analysis of the resulting derivatives showed the absence of NeuGc in HPG-P1 and the presence of NeuGc in both BPG-P1 and PPG-P1 (Table 3).

Structures of the N-*glycans*

Fractions HPG-P1, BPG-P1 and PPG-P1 were analyzed by 500-MHz ¹H-NMR spectroscopy. The pertinent NMR parameters together with those of some reference compounds are compiled in Table 4 (spectra not shown). Fraction HPG-P1 was demonstrated to possess a major *N*-glycan with the following structure:



The various structural-reporter group signals completely agree with those of reference compound 33 [22]. Heterogeneity in the peptide backbone is reflected by the presence of more than one set of signals for the GlcNAc-1 H-1 and NAc groups (Table 4). The established structure is in accordance with that reported previously [5]. Microheterogeneity occurs to a small extent (≈5%) with respect to the lack of NeuAc at the Man α1→6 branch (δ = 4.931 ppm, Man-4′ H-1; δ = 4.479 ppm, Gal-6′ H-1; δ = 2.047 ppm, GlcNAc-5′ NAc; see reference compound 31) and the presence of α-Fuc at C-6 of GlcNAc-1 (δ = 1.200 ppm, Fuc CH_3; δ = 2.094 ppm, GlcNAc-2 NAc; see reference compound 43).

Fraction BPG-P1 showed the various structural-reporter group signals that are characteristic for the mannotriose *N,N′*-diacetylchitobiosyl core and the GlcNAc-5/Gal-6 and GlcNAc-5′/Gal-6′ of α2→6-sialylated *N*-acetyllactosamine antennae, and thus indicated a disialo diantennary *N*-linked glycopeptide structure (Table 4). However, compared to the ¹H-NMR spectrum of HPG-P1 (and reference compound 33), the Man H-2 region showed an intense signal at δ = 4.118 ppm, overlapping with the Man-4′ H-2 signal. In view

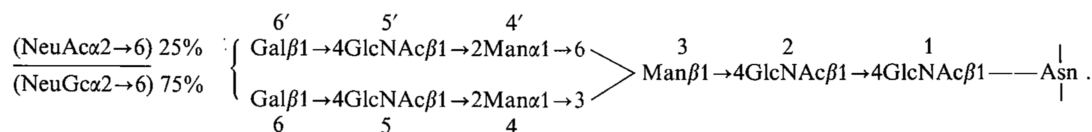
Table 2. *Molar carbohydrate composition of oligosaccharide-alditol fractions $P2_1$ and $P2_2$ derived from human, bovine and porcine plasminogen*
All preparations contained traces of Xyl and Glc. The presence of Man and GlcNAc is due to minor amounts of *N*-glycopeptide. Gal-ol is present as a result of the peeling reaction. Values of GalNAc-ol were corrected for anhydro analogues [28]. The molar ratios were calculated relative to 1 or 2 mol NeuAc/mol

Monosaccharide	Molar ratio in					
	HPG-$P2_1$	HPG-$P2_2$	BPG-$P2_1$	BPG-$P2_2$	PPG-$P2_1$	PPG-$P2_2$
Fuc	n.d.	–	–	–	+	–
Man	n.d.	+	0.8	0.3	0.1	+
Gal	n.d.	0.6	1.7	0.8	0.6	0.5
Gal-ol	n.d.	+	+	+	–	0.1
GlcNAc	n.d.	–	0.3	+	0.1	–
GalNAc	n.d.	+	–	–	–	–
GalNAc-ol	n.d.	0.7	0.6	0.4	0.7	0.5
Sia	n.d.	1.0	2.0	1.0	2.0	1.0
NeuAc:NeuGc	100:0	100:0	93:7	100:0	99:1	99:1

of the presence of NeuGc (see Table 3), this signal can be attributed to the NGc group of NeuGc. With regard to this finding it should be noted that the intensity of the characteristic NAc singlet of NeuAc at $\delta = 2.030$ ppm is considerably reduced, as compared to that of HPG-P1. Comparison of the ^{1}H-NMR data of the NeuGc-containing disialo diantennary glycopeptide CN5a [29] with those of BPG-P1 demonstrated clearly that in BPG-P1, besides the NeuAc residues, the NeuGc residues also occur as $\alpha2\rightarrow6$-linked substituents at Gal-6 and Gal-6′ (Table 4). In the Sia H-3a region the triplet-like signal at $\delta = 1.717$ ppm is due to NeuAc H-3a, whereas that at $\delta = 1.734$ ppm belongs to NeuGc H-3a. Integration of the two Sia H-3a signals shows a ratio of NeuAc to NeuGc similar to that found by GLC analysis (22:78). The Sia H-3e region reveals characteristic main doublets of doublets at $\delta = 2.686$ ppm and $\delta = 2.690$ ppm, stemming from H-3e signals of NeuGc residues at terminal positions of the Man $\alpha1\rightarrow6$ and Man$\alpha1\rightarrow3$ branches. Assigning these resonances in a similar way as reported for the NeuAc H-3 signals in reference compounds 33 and 43 ($\delta_{NeuAc'} > \delta_{NeuAc}$), there seems to exist a slight preference of NeuGc for the Man $\alpha1\rightarrow3$ branch. Owing to certain (peptide) overlapping resonances, the H-3e signals at $\delta \approx 2.67$ ppm typical for $\alpha2\rightarrow6$-linked NeuAc residues could not be traced exactly. Summarizing, in fraction BPG-P1 the main *N*-glycan has the following structure:

Table 3. *Molar carbohydrate composition of glycopeptide fractions P1 derived from human, bovine and porcine plasminogen*
The content of Man was assumed to be 3 mol/mol. The GlcNAc values were corrected for the amount of Asn-linked GlcNAc that is not cleaved under the methanolysis conditions used [19]. The presence of GalNAc is due to contamination with *O*-glycans

Monosaccharide	Molar ratio in		
	HPG-P1	BPG-P1	PPG-P1
Fuc	0.2	0.3	1.3
Man	3.0	3.0	3.0
Gal	2.2	2.2	2.5
GlcNAc	4.0	4.3	4.0
GalNAc	0.3	0.4	0.5
Sia	2.7	2.9	2.2
NeuAc:NeuGc	100:0	28:72	88:12



Low-intensity signals at $\delta = 4.922$ ppm (Man-4′ H-1), $\delta = 4.471$ ppm (Gal-6′ H-1) and $\delta = 2.046$ ppm (GlcNAc-5′ NAc) suggest the presence of small amounts ($\approx 5\%$) of a monosialo diantennary carobohydrate chain, lacking a Sia residue in the Man$\alpha1\rightarrow6$ branch. Further, another minor component ($\approx 5\%$) is $\alpha1\rightarrow6$-fucosylated as indicated by the low-intensity signals at $\delta = 1.191$ (Fuc CH_3) and $\delta = 2.092$ ppm (GlcNAc-2 NAc).

Fraction PPG-P1, based on a comparison of its ^{1}H-NMR spectrum with those of fractions HPG-P1 and BPG-P1 dicussed above, was shown to possess also the basic structure of a diantennary *N*-glycan, bearing $\alpha2\rightarrow6$-linked Sia residues in terminal positions. Close inspection of the ^{1}H-NMR data shows the following detailed properties. Firstly, the carbohydrate chain is highly fucosylated (80%) via an $\alpha1\rightarrow6$-linkage to GlcNAc-1. This is concluded from the characteristic Fuc parameters at $\delta = 4.866$ ppm (Fuc H-1) and at $\delta = 1.201/1.190$ ppm (Fuc CH_3). The attachment of α-Fuc at C-6 of GlcNAc-1 has a characteristic influence on the positions of the GlcNAc-2 H-1 and NAc signals [22], but the GlcNAc-2 H-1 of the fucosylated structure could not be evaluated because of the broad HO^2H line. However, in the NAc region two singlets occur for GlcNAc-2 NAc, namely at $\delta = 2.093$ ppm (with Fuc) and at $\delta = 2.077$ ppm (without Fuc). Secondly, the carbohydrate chain is undersialylated at the Man$\alpha1\rightarrow6$ branch (55% is devoid of Sia). This heterogeneity is reflected by the occurrence of two Man-4′ H-1 signals at $\delta = 4.942$ ppm (with NeuAc) and $\delta = 4.921$ ppm (without NeuAc). Signals for GlcNAc-5′ H-1/NAc and Gal-6′ were also observed in the spectrum, supporting this conclusion. Owing to its low percentage (see Table 3), NeuGc could not be

Table 4. ^{1}*H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the* N-*glycan fractions HPG-P1, BPG-P1 and PPG-P1 derived from human, bovine and porcine plasminogen, respectively, together with those of the reference compounds 31, 33, 42, 43 [22] and CN5a [29]*

Chemical shifts are given at 27°C, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate in $^{2}H_2O$. Compounds are represented by short-hand symbolic notation [22]: ●, GlcNAc; ◆, Man; □, Fuc; ■, Gal; ○, α2→6NeuAc; ◐, α2→6NeuGc

Reporter group	Residue	33	43	CN5a	31	42	HPG-P1	BPG-P1	PPG-P1
H-1	GlcNAc-1	5.088	5.045	5.052	5.073	5.102[a]	5.052/5.015[b]	5.034	5.043[f]
	GlcNAc-2	4.616	4.684	4.679	4.620	4.682[a]	4.616	4.612	n.d.
	Man-3	4.773	≈4.77	n.d.	4.769	4.771	n.d.	4.773	n.d.
	Man-4	5.133	5.131	5.136	5.138	5.134	5.132	5.134	5.132
	Man-4′	4.949	4.946	4.938	4.928	4.927	4.947	4.943	4.942;4.921(-NeuAc)
	GlcNAc-5	4.603	4.607	4.606	4.599	4.605	4.601	4.605	4.604
	GlcNAc-5′	4.603	4.607	4.606	4.577	4.579	4.601	4.605	4.604;4.57(-NeuAc)
	Gal-6	4.442	4.444	4.448	4.445	4.445	4.444	4.446	4.442
	Gal-6′	4.447	4.446	4.448	4.472	4.472	4.444	4.446	4.442:4.468(-NeuAc)
	Fuc	–	4.873	4.872	–	4.877	–	–	4.866
H-2	Man-3	4.254	4.257	4.254	4.251	4.256	4.254	4.252	4.246
	Man-4	4.195	4.200	4.194	4.192	4.191	4.196	4.196	4.196
	Man-4′	4.116	4.112	~4.11	4.112	4.112	4.114	~4.11	4.118
H-3a	NeuAc	1.716	1.717	–	1.716	1.720	1.718	1.717	1.719
	NeuAc′	1.716	1.717	–	–	–	1.718	1.717	1.719
	NeuGc	–	–	1.738	–	–	–	1.734	n.d.
	NeuGc′	–	–	1.738	–	–	–	1.734	n.d.
H-3e	NeuAc	2.666	2.670	–	2.670	2.667	2.671	n.d.[c]	2.672
	NeuAc′	2.672	2.673	–	–	–	2.671	n.d.[c]	2.672
	NeuGc	–	–	2.697	–	–	–	2.686[d]	n.d.
	NeuGc′	–	–	2.697	–	–	–	2.690[d]	n.d.
H-5	Fuc	–	≈4.12	≈4.11	–	4.125	–	–	≈4.11
CH_3	Fuc	–	1.200	1.201	–	1.206/1.210[a]	–	–	1.201/1.190[f]
NAc	GlcNAc-1	2.002	2.020	2.007	2.005	2.006/2.016[a]	2.009/2.001/1.993[b]	2.009/2.002[e]	2.011/2.005/1.999
	GlcNAc-2	2.081	2.094	2.092	2.078	2.094	2.081	2.079	2.093;2.077(-Fuc)
	GlcNAc-5	2.067	2.065	2.070	2.069	2.069	2.069	2.071	2.069
	GlcNAc-5′	2.063	2.065	2.070	2.046	2.048	2.065	2.069	2.065;2.047(-NeuAc)
	NeuAc	2.029	2.030	–	2.030	2.030	2.030	2.030	2.030
	NeuAc′	2.028	2.030	–	–	–	2.030	2.030	2.030
NGc	NeuGc	–	–	4.118	–	–	–	4.118	n.d.
	NeuGc′	–	–	4.118	–	–	–	4.118	n.d.

[a,b,e,f] The peptide moiety is heterogeneous; the indicated signals stem from the main components.
[c] Not determined because of disturbing signals.
[d] Assignments may have to be interchanged.
n.d. not determined.

Table 5. ^{1}*H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the oligosaccharide-alditols HPG-P2$_1$/P2$_2$, BPG-P2$_1$/P2$_2$ and PPG-P2$_1$/P2$_2$ derived from human, bovine and porcine plasminogen, respectively, together with those of the reference oligosaccharide-alditols obtained from κ-casein [30]*
Chemical shifts are given at 27°C, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate in ^2H_2O. Compounds are represented by short-hand symbolic notation [22]: ◇, GalNAc-ol; ■, Gal; ○, α2→6NeuAc; △, α2→3NeuAc

Reporter group	Residue	△3■3◇	HPG-P2$_2$	BPG-P2$_2$	PPG-P2$_2$	○6 △3■3◇	HPG-P2$_1$	BPG-P2$_1$	PPG-P2$_1$
H-2	GalNAc-ol	4.390	4.386	4.388	4.388	4.378	4.378	4.380	4.378
H-3		4.074	4.071	4.073	4.069	4.067	4.067	4.068	4.067
H-4		3.498	3.495	3.504	3.495	3.524	3.523	3.522	3.521
H-5		4.187	4.185	4.189	4.185	4.240	4.239	4.235	4.239
H-6		3.68	n.d.	n.d.	n.d.	3.84	n.d.	n.d.	n.d.
H-6′		3.65	n.d.	n.d.	n.d.	3.475	3.471	3.472	3.471
NAc		2.046	2.045	2.046	2.045	2.042	2.042	2.042	2.042
H-1	Gal	4.547	4.544	4.544	4.544	4.541	4.542	4.545	4.540
H-3		4.122	4.119	4.122	4.119	4.117	4.116	4.113	4.117
H-4		3.931	3.929	3.926	3.929	3.927	3.925	3.926	3.925
H-3a	NeuAcα2→3	1.800	1.799	1.799	1.799	1.800	1.799	1.800	1.799
H-3e		2.774	2.771	2.774	2.771	2.774	2.774	2.777	2.773
NAc		2.034	2.033	2.034	2.033	2.032	2.031	2.031	2.031
H-3a	NeuAcα2→6	–	–	–	–	1.692	1.691	1.69	1.691
H-3e		–	–	–	–	2.723	2.722	2.724	2.722
NAc		–	–	–	–	2.032	2.031	2.031	2.031

traced unambiguously in the NMR spectrum. The *N*-glycolyl signal at $\delta = 4.118$ ppm coincides with the Man-4′ H-2 and Fuc H-5 resonances, whereas the Sia H-3e and H-3a regions contain contaminating (peptide) signals. In conclusion, the GLC and ^{1}H-NMR data lead to the following structure of the *N*-glycan present in fraction PPG-P1:

```
{NeuAcα2→6}        6′        5′           4′                                  (Fucα1→6) 80%
{NeuGcα2→6} 45%  Galβ1→4GlcNAcβ1→2Manα1→6                                              \         |
                                          >Manβ1→4GlcNAcβ1→4GlcNAcβ1——Asn .
 NeuAcα2→6       Galβ1→4GlcNAcβ1→2Manα1→3    3          2           1              |
 NeuGcα2→6         6        5           4
```

Structures of the O-glycans

The 500-MHz ^{1}H-NMR spectroscopy data of the oligosaccharide-alditols (HPG-P2$_1$, HPG-P2$_2$, BPG-P2$_1$, BPG-P2$_2$, PPG-P2$_1$ and PPG-P2$_2$), together with those of the reference monosialo-trisaccharide-alditol and disialo-tetrasaccharide-alditol obtained from κ-casein [30] are presented in Table 5. The ^{1}H-NMR spectra of the three P2$_2$ fractions (spectra not shown) were found to be essentially identical to those of the monosialo-trisaccharide-alditol reference compound, demonstrating that the smaller *O*-glycosidic chain possesses the following structure:

```
                          GalNAc-ol
                         /
NeuAcα2→3Galβ1→3
```

The structural-reporter signals of the three P2$_1$ fractions were in accordance with those of the disialo-tetrasaccharide-alditol reference compound. Thus the following structure could be deduced:

```
         NeuAcα2→6
                   \
                    GalNAc-ol .
                   /
NeuAcα2→3Galβ1→3
```

Both structures were reported earlier for HPG [6]. The ratio of tri- to tetra-saccharide-alditols varies for the three species as follows: in HPG the ratio of P2$_2$ to P2$_1$ proved to be 80:20, in BPG 70:30 and in PPG 50:50. The fact that no linear disaccharide-alditol Galβ1→3GalNAc-ol or branched trisaccharide-alditol Galβ1→3(NeuAcα2→6)GalNAc-ol were present suggests that the monosialo-trisaccharide compound NeuAcα2→3Galβ1→3GalNAc-ol is not produced by release of sialic acid from the disialo-tetrasaccharides during isolation. The amount of NeuGc was too low to be detected in the ^{1}H-NMR spectra.

Concluding remarks

The present comparative study on the carbohydrate moiety of human, bovine and porcine plasminogen revealed several unique species-specificity characteristics. Although the basic structures of the *N*- and *O*-glycans were found to be identical, several types of microheterogeneity regarding the α2→6 sialylation and/or α1→6 fucosylation patterns were noted. In HPG the N-linked structure is only sialylated with NeuAc, whereas in BPG the dominant sialic acid is NeuGc in both branches. The disialo-diantennary complex-type chain of the two species is nearly free of α1→6-linked Fuc. In PPG the sialic acid present is mainly NeuAc (90%); however, the Manα1→6 branch is evidently partially sialylated (45%) and the Asn-bound GlcNAc partially fucosylated (80%). It should be noted that both NeuAc and NeuGc were present only in α2→6 linkage, whereas many other glycoproteins possess Sia in α2→3 and α2→6 linkage to Gal [22]. The O-linked carbohydrate chains of the three species are identical trisaccharide and tetrasaccharide structures, occurring in different ratios and having mainly or only NeuAc as sialic acid constituent.

An uneven distribution of sialic acids in N- and O-linked oligosaccharides is unusual and therefore of particular interest [31]. In the bovine blood coagulation factor X for instance, the *N*-glycans contain only NeuAc, whereas in the mucin-type structures both NeuAc and NeuGc are present [32]. This heterogeneity in the sialic acid composition of carbohydrate chains of the bovine and porcine species is most likely to arise from a different action of the *N*-acetylneuraminate monooxygenase, which converts NeuAc to NeuGc [33].

Amino acid sequence analysis in our laboratory has recently established that the *N*-glycosidic carbohydrate moiety of bovine and porcine plasminogen, in accordance with comparable studies of HPG, is only partially present in a corresponding position of kringle 3 [12, 13]. Asn^{288} of HPG and Asn^{289} of BPG are glycosylated to approximately 35%, whereas in PPG 20% of the molecules carry the carbohydrate unit at Asn^{289}. All three species, however, are completely *O*-glycosylated, although the sites of attachment to the peptide backbone are remarkably different (HPG, Thr^{345}; BPG, Ser^{339}; PPG, Thr^{249}) [13]. Whether the observed heterogeneities in structure are of biological significance regarding the activity of plasminogen in the fribrinolytic system remains to be elucidated.

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