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Sialic acid patterns in *N*-linked carbohydrate chains. Structural analysis of the *N*-acetyl-/*N*-glycolyl-neuraminic-acid-containing *N*-linked carbohydrate chains of bovine fibrinogen

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Abstract. The *N*-linked carbohydrate chains of bovine fibrinogen have been released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F, fractionated by FPLC and HPLC procedures and characterized by 500/600-MHz ^{1}H NMR spectroscopy. Evidence is presented for the native sialic acid patterns, involving the occurrence of α2→6-linked *N*-acetyl- and *N*-glycolylneuraminic acid residues in the terminal position of the di-antennary carbohydrate chains. Details are included of minor oligosaccharides containing Neu5Acα2→3Galβ1→4/β1→3GlcNAc β1→structural elements, which have not been previously described in this glycoprotein.

Introduction

Interest in the amount and types of sialic acid in soluble and membrane glycoproteins has increased greatly since their important biological role has become evident[1]. In order to study the location of the different forms of sialic acid residues in the various carbohydrate chains, the glycans have to be released from the protein backbone and fractionated to purity. For the chemical liberation of the *N*-linked carbohydrate chains the hydrazinolysis procedure is usually applied[2,3]. However, the oligosaccharides (-alditols) formed do not contain the *N*- and *O*-acyl substituents originally present. This approach is not attractive when focussing on the native sialic acids occurring in carbohydrate chains from different biological origins[4]. The introduction of an enzymatic cleavage procedure, using peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (PNGase-F, E.C. 3.5.1.52)[5-9], to detach *N*-linked carbohydrate chains from glycoproteins has opened up new perspectives for detailed analysis of the native forms of sialic acid and their distribution over the individual carbohydrate chains (further referred to as sialic acid patterns). Recently, for equine fibrinogen, the *N*-acetyl-/*N*-acetyl-4-*O*-acetyl-neuraminic acid patterns in the diantennary *N*-linked carbohydrate chains have been established[10], yielding new sets of ^{1}H NMR structural-reporter groups[11]. For bovine fibrinogen, the presence of *N*-acetyl- and *N*-glycolyl-neuraminic acid has been described[12]. These studies have shown that equal amounts of both sialic acids are present, however the structural studies using ^{1}H NMR spectroscopy and methylation analysis were carried out on oligosaccharides released with hydrazine. Consequently, no conclusions could be drawn on the distribution of the two types of sialic acid over the branches in the various carbohydrate chains. In this communication, evidence is presented for the native sialic acid patterns in the diantennary *N*-linked carbohydrate chains of bovine fibrinogen. Moreover, details are included of minor oligosaccharides not previously reported for this glycoprotein.

Results

Sugar analysis of bovine fibrinogen reveals the presence of galactose (Gal), mannose (Man), *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (Neu5Ac) in the molar ratio of 2.5:3.0:3.6:1.6, forming 3.9% (w/w) of the glycoprotein. In this analysis procedure, all *N*-acylneuraminic acids are converted into Neu5Ac, and therefore no information is obtained with respect to the native substituents. However, mild acid hydrolysis of the glycoprotein (0.1 M HCl; 1 h at 80°C) and subsequent TLC on plastic-coated silica-60 plates of the isolated sialic acid constituents demonstrate the presence of both Neu5Ac (R_f 0.41) and Neu5Gc (R_f 0.35) in similar amounts. This observation is confirmed by GLC (Neu5Ac, R_f 1.00; Neu5Gc, R_f 1.25) and GLC-MS of the isolated pertrimethylsilylated sialic acids[13,14].

Bovine fibrinogen was treated with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F and the deglycosylated protein was separated from the released carbohydrate material by centrifugation. Bio-Gel P-100 fractionation of the supernatant gives a small residual protein fraction, a broad carbohydrate-positive peak and a salt-containing fraction. Medium-pressure anion-exchange chromatography[8-10] of the pooled carbohydrate fraction over Mono Q (Pharmacia FPLC-system) gives rise to three oligosaccharide-containing fractions N1, N2 and N3 (Fig. 1), having the same retention volumes as reference mono-, di- and trisialo *N*-type oligosaccharides, respectively. The additional peaks NN and N4 do not contain carbohydrate material. HPLC of fraction N3 on Lichrosorb-NH_2 reveals it to be a mixture of carbohydrate chains, but the low amount of material hampered further structural studies. HPLC on

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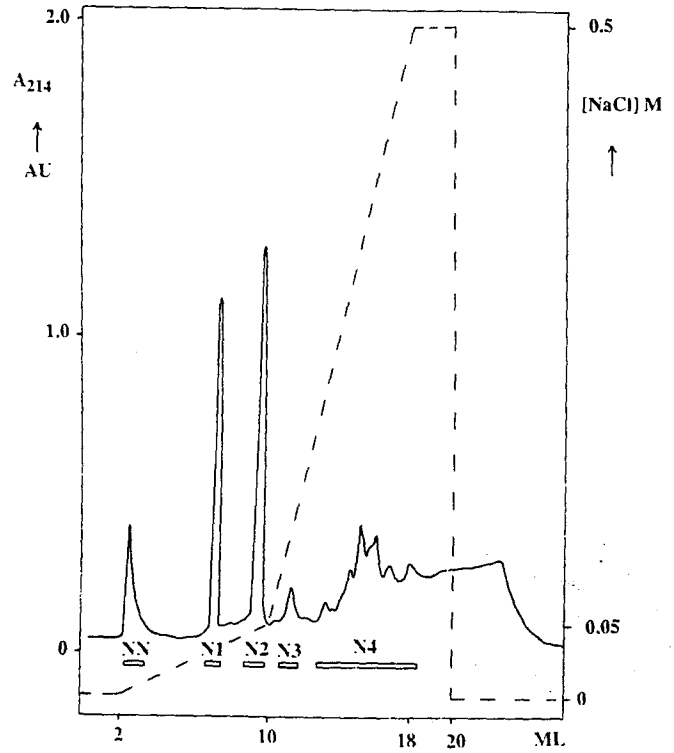


Fig. 1. Fractionation pattern at 214 nm of the carbohydrate-containing Bio-Gel P-100 fraction B, derived from bovine fibrinogen on a FPLC HR 5/5 Mono Q column.
Desalted and lyophilized fraction B was dissolved in 0.8 ml H_2O (HPLC quality). The column was eluted with a linear concentration gradient (---)from 0–50 mM NaCl in 8 ml H_2O (HPLC quality), followed by a steeper gradient from 50–500 mM NaCl in 8 ml H_2O at a flow rate of 60 ml/h. Injection volume 0.1 ml. Fractions were collected as indicated.

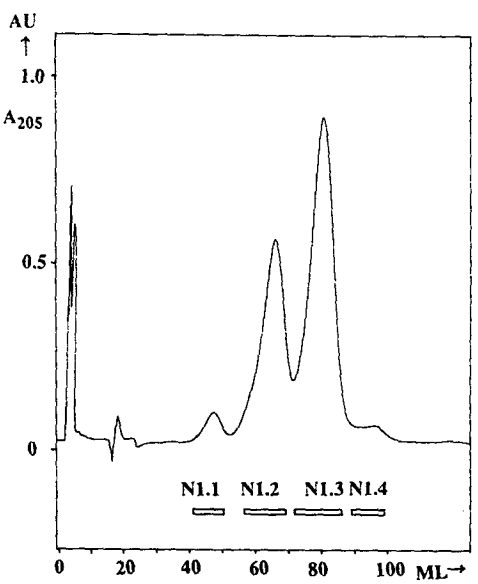


Fig. 2. Fractionation pattern at 205 nm of the bovine fibrinogen FPLC fraction N1 on a HPLC Lichrosorb-NH_2 10 μ column (25 × 0.46 cm, Chrompack).
The FPLC fraction was lyophilized, desalted and dissolved in 0.05 ml H_2O. The column was eluted isocratically with (30 mM K_2HPO_4/KH_2PO_4, pH 7.0)/acetonitrile (34:66, v/v) at a flow rate of 120 ml/h at 25.0°C. Injection volume 0.04 ml. Fractions were collected as indicated.

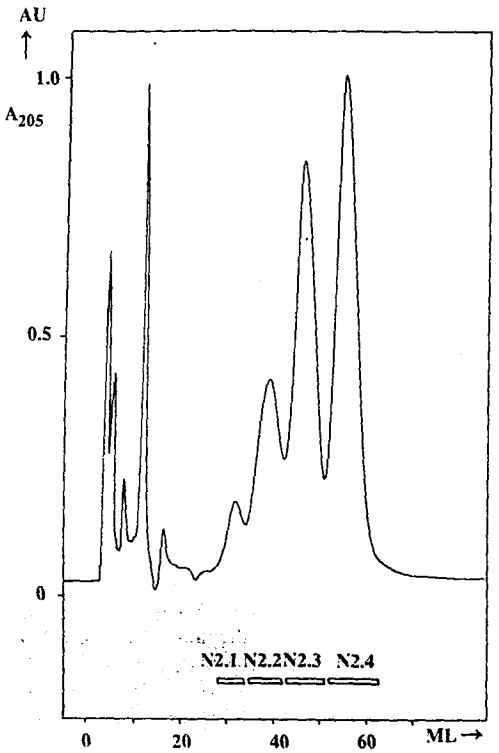
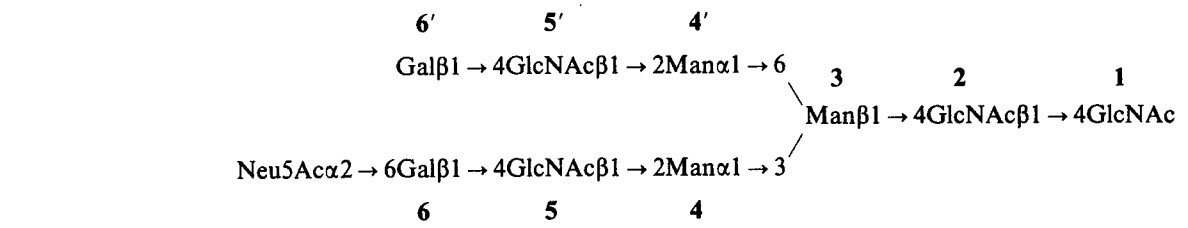


Fig. 3. Fractionation pattern at 205 nm of the bovine fibrinogen FPLC fraction N2 on a HPLC Lichrosorb-NH_2 10 μ column (25 × 0.46 cm, Chrompack).
The FPLC fraction was lyophilized, desalted and dissolved in 0.05 ml H_2O. The column was eluted isocratically with (30 mM K_2HPO_4/KH_2PO_4, pH 7.0)/acetonitrile (35:65, v/v). For further details, see Fig. 2.

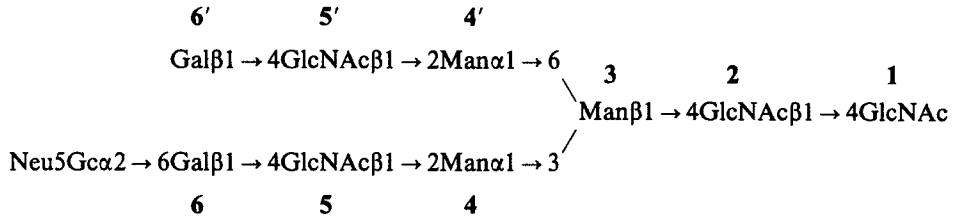
Lichrosorb-NH_2 of fraction N1 yields four subfractions, denoted N1.1–N1.4 (Fig. 2). Likewise, HPLC of fraction N2 gives four subfractions, denoted N2.1-N2.4 (Fig. 3). For the analysis of the primary structures of the oligosaccharides present in the fractions N1.1–N1.3 and N2.1–N2.4, 500- and 600-MHz ^{1}H NMR spectroscopy was applied. The amount of N1.4 was too low for further NMR investigations. The ^{1}H NMR data of the analyzed mono- and disialo carbohydrate chains are compiled in Tables I and II, respectively. In each series, structures are discussed in order of increasing complexity.

Monosialo carbohydrate chains

The ^{1}H NMR spectrum of fraction N1.2 ($R_{N1.2}$ 1.00) indicates the presence of a conventional diantennary structure terminated with an α2→6-linked Neu5Ac residue in the Manα1→3 branch (Table I). The chemical-shift values of the structural-reporter groups match exactly those of reference compound eF.N1.3 isolated from equine fibrinogen (eF)[10], having an identical retention volume on HPLC. As demonstrated by ^{1}H NMR spectroscopy, fraction N1.3 ($R_{N1.3}$ = 1.21 × $R_{N1.2}$) contains the same basic structure as shown for N1.2 but, in N1.3, the α2→6-linked Neu5Ac is replaced by an α2→6-linked Neu5Gc residue. The occurrence of the α2→6-linked Neu5Gc is reflected by its set of structural-reporter-group signals, namely, H-3a at δ 1.735 ppm, H-3e at δ 2.685 ppm and NGc at δ 4.118 ppm[15]. Comparison of the structural-reporter groups of N1.2 and N1.3 (Table I) shows that the replacement of Neu5Ac by Neu5Gc leads to significant downfield shifts for the sialic acid H-3a (Δδ +0.017 ppm) and H-3e (Δδ +0.017 ppm)



(N1.2)



(N1.3)

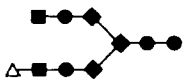
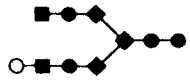
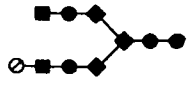
signals. In addition, minor shifts are observable for the Gal-**6** H-1 (Δδ +0.004 ppm) and the GlcNAc-**5** NAc (Δδ +0.003 ppm) signals.

The ^{1}H NMR spectrum of the minor fraction N1.1 ($R_{N1.1}$ = 0.74 × $R_{N1.2}$) shows the presence of the following compound (Table I).

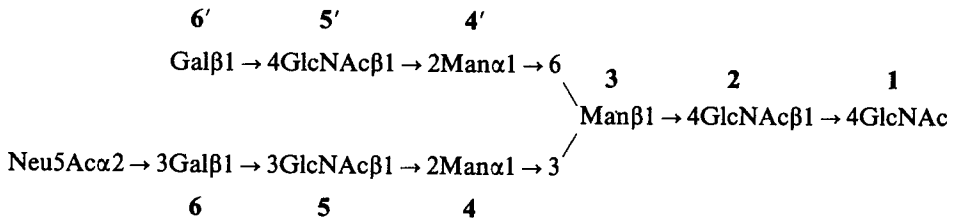
The structural reporters of the *N,N′*-diacetylchitobiosyl-trimannosyl element, together with the Manα1→6 branch, completely match those of N1.2. The occurrence of Neu5Ac

Table I ^{1}*H chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N1.1, N1.2 and N1.3 derived from bovine fibrinogen. Chemical shifts are given at 300 K in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate in* ^2H_2O*, but were actually measured by reference to internal acetone (δ 2.225 ppm in* ^2H_2O *at 300 K). Compounds are represented by short-hand symbolic notation*[11].

●, *GlcNAc;* ◆, *Man;* ■, *Gal;* ○, *Neu5Acα2→6;* ∅, *Neu5Gcα2→6;* △, *Neu5Acα2→3.*

Reporter group	Residue[a]	 N1.1	 N1.2	 N1.3
H-1	GlcNAc-**1**	α5.188, β n.d.[b]	α5.190, β n.d.	α5.189, β4.698
	GlcNAc-**2**	α4.614, β4.606	α4.614, β4.606	α4.614, β4.607
	Man-**3**	n.d.	n.d.	n.d.
	Man-**4**	5.123	5.136	5.136
	Man-**4′**[c]	4.930	4.931	4.929
	GlcNAc-**5**	4.606	4.606	4.607
	GlcNAc-**5′**	4.582	4.582	4.582
	^{4}Gal-**6**[d]	–	4.446	4.450
	^{3}Gal-**6**	4.515	–	–
	^{4}Gal-**6′**	4.473	4.473	4.473
H-2	Man-**3**	4.250	4.252	4.254
	Man-**4**	4.195	4.197	4.196
	Man-**4′**	4.112	4.113	4.113
H-3	Gal-**6**	4.086	n.d.	n.d.
H-3a	Neu5Ac	1.783	1.718	–
	Neu5Gc	–	–	1.735
H-3e	Neu5Ac	2.759	2.668	–
	Neu5Gc	–	–	2.685
NAc	GlcNAc-**1**	2.038	2.038	2.038
	GlcNAc-**2**	2 081	2.082	2.081
	GlcNAc-**5**	2.043	2.069	2.072
	GlcNAc-**5′**	2.047	2.047	2.047
	Neu5Ac	2.030	2.030	–
NGc	Neu5Gc	–	–	4.118

[a] For numbering of the monosaccharide residues, see text. [b] n.d. = not determined. [c] A prime (′) at the monosaccharide number indicates that the specified sugar residue is localized in the Manα1 → 6 branch. [d] A superscript preceding the name of a sugar residue indicates to which position of the adjacent monosaccharide it is linked.



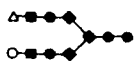
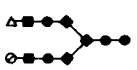
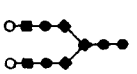
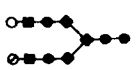
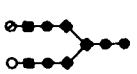
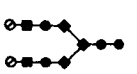
(N1.1)

in α2 → 3 linkage to β1 → 3-linked Gal-**6** is indicated by the set of chemical-shift values of the Neu5Ac H-3a and H-3e resonances (δ 1.783 ppm and δ 2.759 ppm, respectively). This set is identical to that found for α2 → 3-linked Neu5Ac H-3a and H-3e in the Neu5Acα2 → 3Galβ1 → 3(Neu5Acα2 → 6)GlcNAcβ1 → 2Manα1 → 3 element of compound GP-II obtained from rat plasma hemopexin[16] and the Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → 4Manα1 → 3 element in compound N-3/T* (N) obtained from bovine fetuin[17]. It clearly differs from the set of structural-reporter groups known for Neu5Ac H-3a/H-3e in the Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 element[11] (δ 1.797/2.757 ppm). Going from the Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 element, as present in the diantennary compound hCG.N2B obtained from human chorionic gonadotropin (hCG)[8], to the Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → 2Manα1 → 3 element in N1.1 the following typical shift increments are observed: Neu5Ac H-3a, δ 1.797 ppm → δ 1.783 ppm; Gal-**6** H-1, δ 4.543 ppm → δ 4.515 ppm; Gal-**6** H-3, δ 4.112 ppm → δ 4.086 ppm; GlcNAc-**5** H-1, δ 4.572 ppm → δ 4.606 ppm; and GlcNAc-**5** NAc, δ 2.048 ppm → δ 2.043 ppm.

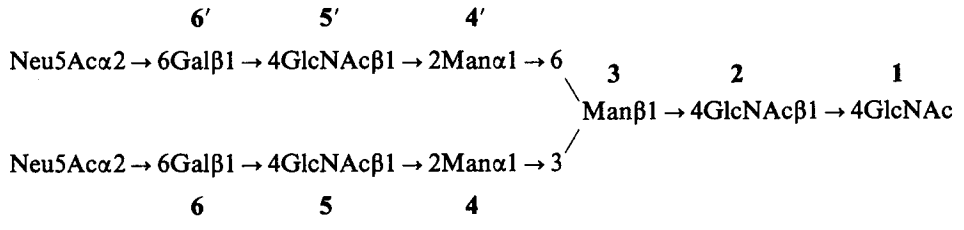
Disialo carbohydrate chains

The ^{1}H NMR spectrum of fraction N2.1 ($R_{N2.1}$ = 0.81 × $R_{N2.2}$) indicates the major constituent to be the disialo diantennary compound N2.1.1.

Table II ^{1}*H chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides N2.1.1, N2.2.1, N2.2.2, N2.3A, N2.3B and N2.4 derived from bovine fibrinogen. For further details, see Table I.*

Reporter group	Residue[a]	 N2.1.1	 N2.2.2	 N2.2.1	 N2.3A	 N2.3B	 N2.4
H-1	GlcNAc-**1**	α5.190, β n.d.[b]	α5.190, β n.d.	α5.189, β n.d.	α5.190, β n.d.	α5.190, β n.d.	α5.190, β4.696
	GlcNAc-**2**	α4.613, β4.605	α4.614, β4.605	α4.614, β4.605	α4.615, β4.606	α4.615, β4.606	α4.614, β4.606
	Man-**3**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Man-**4**	5.134	5.134	5.133	5.134	5.134	5.135
	Man-**4′**[c]	4.926	4.924	4.947	4.947	4.947	4.946
	GlcNAc-**5**	4.605	4.605	4.605	4.606	4.606	4.606
	GlcNAc-**5′**	4.573	4.573	4.605	4.606	4.606	4.606
	Gal-**6**	4.444	4.448	4.444	4.448	4.444	4.448
	Gal-**6′**	4.550	4.550	4.447	4.448	4.453	4.452
H-2	Man-**3**	4.253	4.253	4.253	4.255	4.255	4.255
	Man-**4**	4.197	4.197	4.196	4.198	4.198	4.197
	Man-**4′**	4.116	4.118	4.116	4.116	4.116	4.116
H-3	Gal-**6′**	4.118	n.d.	n.d.	n.d.	n.d.	n.d.
H-3a	Neu5Ac	1.718	–	1.718	–	1.718	–
	Neu5Ac′	1.801	1.800	1.718	1.718	–	–
	Neu5Gc	–	1.734	–	1.735	–	1.735
	Neu5Gc′	–	–	–	–	1.735	1.735
H-3e	Neu5Ac	2.668	–	2.669	–	2.667	–
	Neu5Ac′	2.758	2.758	2.675	2.674	–	–
	Neu5Gc	–	2.685	–	2.686	–	2.686
	Neu5Gc′	–	–	–	–	2.691	2.691
NAc	GlcNAc-**1**	2.038	2.039	2.037	2.038	2.038	α2.039, β2.037
	GlcNAc-**2**	2.081	2.082	2.082	2.084	2.084	α2.084, β2.083
	GlcNAc-**5**	2.068	2.071	2.069	2.072	2.069	2.072
	GlcNAc-**5′**	2.043	2.043	2.066	2.066	2.069	2.069
	Neu5Ac	2.030	–	2.030	–	2.030	–
	Neu5Ac′	2.030	2.031	2.030	2.030	–	–
NGc	Neu5Gc	–	4.118	–	4.119	–	4.119
	Neu5Gc′	–	–	–	–	4.119	4.119

[a] For numbering of the monosaccharide residues, see text.



(N2.2.1)

The usual *N,N′*-diacetylchitobiosyl-trimannosyl core, as part of a diantennary type of carbohydrate chain, is reflected by the characteristic set of H-1 and NAc signals of GlcNAc-**1** and GlcNAc-**2** and the H-1 and H-2 structural-reporter-group signals of Man-**3**, Man-**4** and Man-**4′** (Table II). The chemical-shift values of the structural-reporter groups of both antennae are in accordance with those of reference compound **39** isolated from sialidosis urine but missing GlcNAc-**1**[11]. High-performance anion-exchange chromatography, coupled with pulsed-amperometric detection (HPAE-PAD) of fraction N2.1, yields two peaks having identical PAD-response areas. The first-eluting peak contains N2.1.1 as demonstrated by 600-MHz ^{1}H-NMR spectroscopic analysis. The amount of material in the second eluting peak N2.1.2 was too low for further ^{1}H NMR analysis but, based on the ^{1}H NMR data of the original HPLC fraction N2.1, it must still contain a mixture of compounds. It is suggested that these compounds contain the Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → 2 sequence at Man-**4** or Man-**4′**, as indicated by the ^{1}H NMR spectrum of N2.1.

^{1}H NMR spectroscopy of fraction N2.2 ($R_{N2.2}$ 1.00) shows a mixture of compounds of which the major component N2.2.1 is a conventional diantennary structure terminated with α2 → 6-linked Neu5Ac residues only[11]. The structural reporters are in accordance with those reported for reference compound eF.N2.3 isolated from equine fibrinogen (eF)[10], having an identical retention volume on HPLC.

In addition, low-intensity signals, indicating the occurrence of small amounts of oligosaccharides having Neu5Acα2 → 3Galβ1 → 3/4 or Neu5Gcα2 → 6 elements, are visible in the spectrum. To obtain further information on these compounds, fraction N2.2 was subfractionated by HPAE-PAD chromatography, yielding three subfractions denoted N2.2.1, N2.2.2 and N2.2.3 (Fig. 4). Subfraction N2.2.1 coelutes with reference compound eF.N2.3 and represents the compound N2.2.1 already discussed.

Subfraction N2.2.2 contains oligosaccharide N2.2.2 as the single constituent (Table II). The α2 → 3-linked Neu5Ac residue (H-3a, δ 1.800 ppm; H-3e, δ 2.758 ppm; NAc, δ 2.031 ppm) is located on the Manα1 → 6 branch, which is deduced from the H-1 signal of Man-**4′** (δ 4.924 ppm), in combination with the H-1 signals of GlcNAc-**5′** (δ 4.573 ppm) and Gal-**6′** (δ 4.550 ppm). The α2 → 6-linked Neu5Gc residue (H-3a, δ 1.734 ppm; H-3e, δ 2.685 ppm; NAc, δ

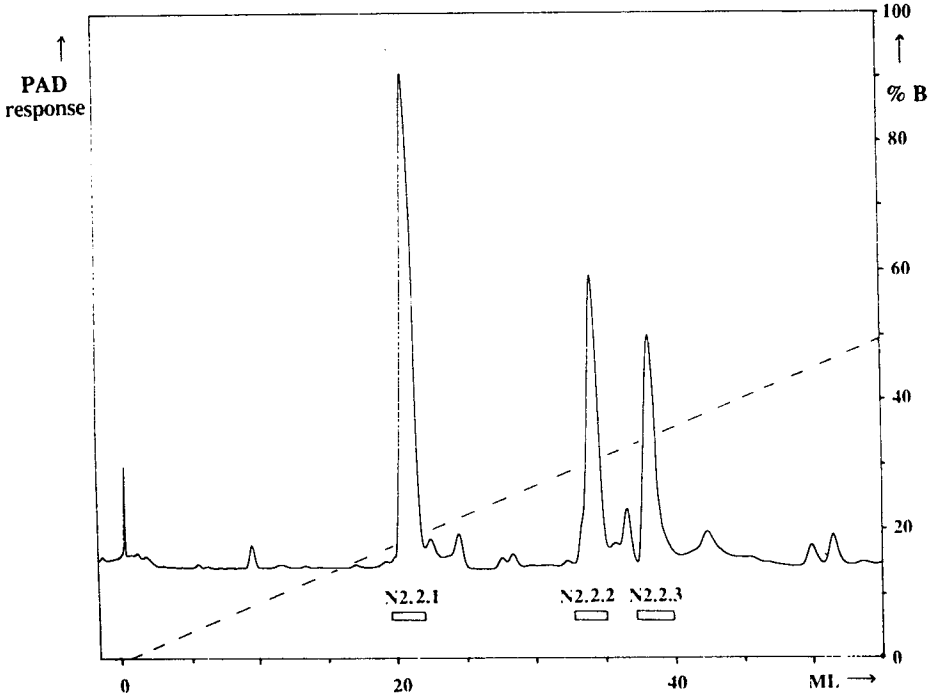
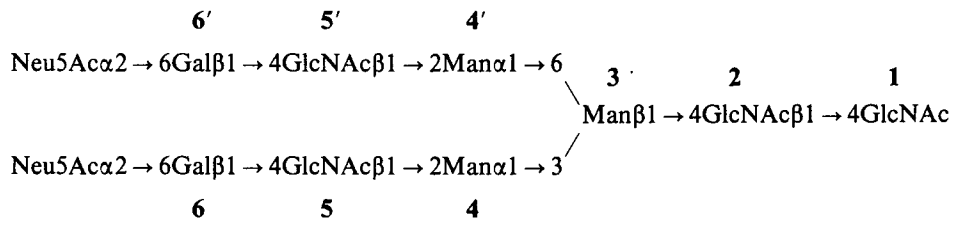
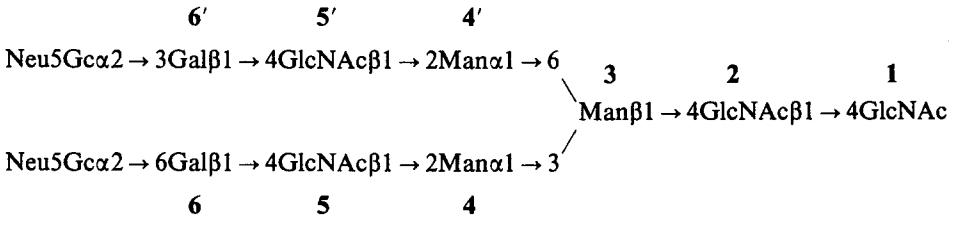


Fig. 4. Fractionation pattern of HPLC fraction N2.2 on a CarboPac PA-1 column (25 × 0.46 cm, Dionex). N2.2 was desalted and dissolved in 25 µl H_2O. The column was eluted isocratically for 0.3 min with a mixture of 90% (v/v) eluent A/10% (v/v) eluent B, followed by a linear increase of eluent B to 90% over 120 min (dashed line) at a flow rate of 1 ml/min at ambient temperature. Eluent A: 0.1 M NaOH; eluent B: 0.1 M NaOH, containing 0.5 M NaAc. Injection volume 20 µl, detection by PAD at 1000 nA full scale. Fractions were collected as indicated.



(N2.2.1)



(N2.2.2)

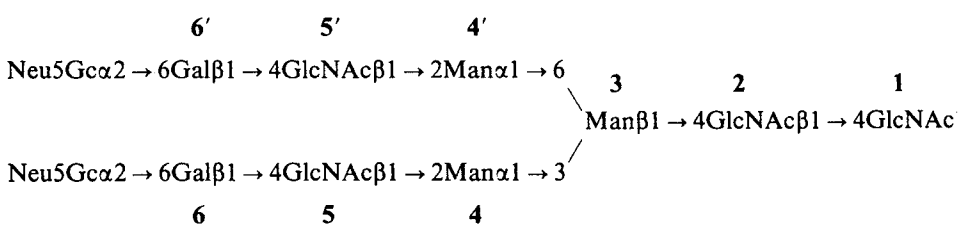
4.118 ppm) occurs in terminal position of the Manα1 → 3 branch, reflected by the H-1 signals of Man-**4** at δ 5.134 ppm, GlcNAc-**5** at δ 4.605 ppm and Gal-**6** at δ 4.448 ppm. The GlcNAc-**5** and GlcNAc-**5′** NAc singlets resonate at δ 2.071 ppm and δ 2.043 ppm, respectively, in accordance with the proposed structure. On going from N2.1.1 to N2.2.2, which corresponds with the replacement of a Neu5Ac residue by a Neu5Gc residue, similar downfield shift effects are observed for Gal-**6** H-1 (δ 4.448 ppm), Neu5Gc H-3a (δ 1.734 ppm), Neu5Gc H-3e (δ 2.685 ppm) and GlcNAc-**5** NAc (δ 2.071 ppm) as mentioned for the step N1.2 → N1.3.

Fraction N2.2.3 consists of a mixture of oligosaccharides in which the Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → 2 and Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2 branches can be recognized. However, for a complete structural elucidation of the oligosaccharides present in fraction N2.2.3, more material and further purification are required.

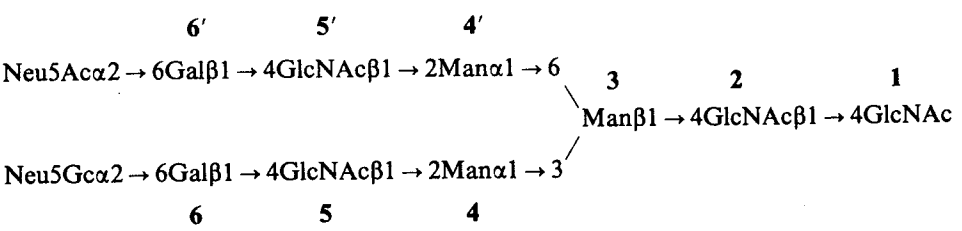
The ^{1}H NMR spectrum of fraction N2.4 ($R_{N2.4}$ = 1.42 × $R_{N2.2}$) indicates a diantennary structure with two α2 → 6-linked Neu5Gc residues (Table II).

The occurrence of the α2 → 6-linked Neu5Gc residue in terminal position of the Manα1 → 3 branch is reflected by its H-3a, H-3e and NGc signals at δ 1.735 ppm, δ 2.686 ppm and δ 4.119 ppm, the Man-**4** H-1 signal at δ 5.135 ppm, the Gal-**6** H-1 signal at δ 4.448 ppm and the GlcNAc-**5** NAc singlet at δ 2.072 ppm (*cf.* N1.3). Replacement of α2 → 6-linked Neu5Ac in the Manα1 → 6 branch by α2 → 6-linked Neu5Gc (N2.2.1 → N2.4) shows downfield shifts for sialic acid H-3a (Δδ + 0.017 ppm), sialic acid H-3e (Δδ + 0.016 ppm), Gal-**6′** H-1 (Δδ + 0.005 ppm) and GlcNAc-**5′** NAc (Δδ + 0.003 ppm). Apparently, the effects on the structural-reporter-group signals, on going from α2 → 6-linked Neu5Ac to α2 → 6-linked Neu5Gc, are independent of whether the sialic acid substitution is occurring at the Manα1 → 3 or the Manα1 → 6 branch.

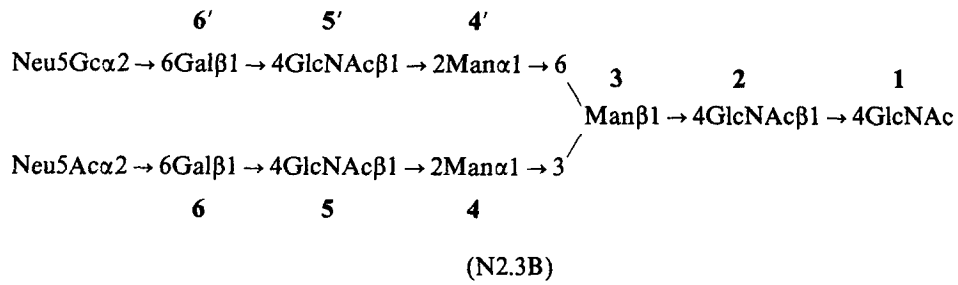
The ^{1}H NMR spectrum of fraction N2.3 ($R_{N2.3}$ = 1.19 × $R_{N2.2}$) demonstrates that it is composed of almost equal amounts of N2.3A and N2.3B (Table II).



(N2.4)



(N2.3A)



(N2.3B)

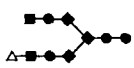
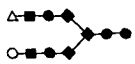
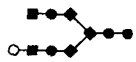
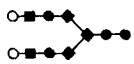
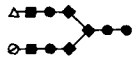
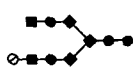
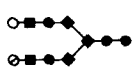
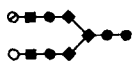
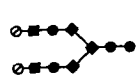
The occurrence of Neu5Ac and Neu5Gc in almost equal amounts is deduced from the intensities of the NAc (δ 2.030 ppm) and NGc (δ 4.119 ppm) singlets, respectively. Furthermore, similar intensities are observed for the sialic acid H-3a signals at δ 1.718 ppm (Neu5Ac) and δ 1.735 ppm (Neu5Gc). The specific assignments of the H-3e signals at δ 2.667 ppm (Neu5Ac at Manα1→3 branch), δ 2.674 ppm (Neu5Ac at Manα1→6 branch), δ 2.686 ppm (Neu5Gc at Manα1→3 branch) and δ 2.691 ppm (Neu5Gc at Manα1→6 branch) are based on a comparison with the H-3e signals in N2.2.1 and N2.4 (see also ref. 11).

The Neu5Gc and Neu5Ac residues are evenly distributed over the Manα1→3 branch, reflected by two sets of signals of identical intensity, namely, for Neu5Gc (N2.3A): Gal-**6** H-1 at δ 4.448 ppm, GlcNAc-**5** NAc at δ 2.072 ppm and Neu5Gc H-3e at δ 2.686 ppm (*cf.* N1.3) and for Neu5Ac (N2.3B): Gal-**6** H-1 at δ 4.444 ppm, GlcNAc-**5** NAc at δ 2.069 ppm and Neu5Ac H-3e at δ 2.667 ppm (*cf.* N1.2). Similarly, it can be deduced that Neu5Gc and Neu5Ac are evenly distributed over the Manα1→6 branch. In this case, Neu5Ac is evidenced by the following signals (N2.3A): Gal-**6**′ H-1 at δ 4.448 ppm (coinciding with the Gal-**6** H-1 signal of N2.3A), GlcNAc-**5**′ NAc at 2.066 ppm and Neu5Ac H-3e at δ 2.674 ppm, whereas Neu5Gc (N2.3B) is clear from the signals of Gal-**6**′ H-1 at δ 4.453 ppm, GlcNAc-**5**′ NAc at δ 2.069 ppm (coinciding with the GlcNAc-**5** NAc singlet of N2.3B) and Neu5Gc H-3e at δ 2.691 ppm. Because the HPLC of fraction N2.3 gives rise to a single peak having a retention volume intermediate between that of N2.2 and N2.4, the possibility that N2.3 represents an equimolar mixture of N2.2.1 and N2.4 can be eliminated.

Discussion

In Table III, a compilation is given of the carbohydrate structures occurring in bovine fibrinogen in relation to their relative and absolute molar amounts. As can be deduced from the respective peak areas in Fig. 1 (for quantification procedure, see Table III), N1 contains 424 nmol monosialo diantennary oligosaccharides and N2 490 nmol disialo diantennary oligosaccharides, representing a molar ratio of 1:1.2, which is in agreement with literature data[12]. The degree of *N*-glycolylation of the mono- and disialo glycans is 60% and 64%, respectively, in accordance with the overall value determined for the isolated sialic constituents by GLC (60%). Using the enzymatic cleavage procedure as part of the structural analysis methodology, it has now been possible to determine the sialic acid patterns for the oligosaccharides reported previously as glycans A[12], namely, N1.2 + N1.3, and B[12], namely, N2.2.1 + N2.3A + N2.3B

Table III Carbohydrate chains occurring in bovine fibrinogen. The oligosaccharides were released by treatment with PNGase-F, fractionated by subsequent FPLC and HPLC and analyzed by 500/600-MHz ^1H NMR spectroscopy. The molar ratios of oligosaccharides are given as percentages (total amount 100%). For quantification of the oligosaccharides the following procedure holds: (1) The molar ratio of oligosaccharides present in the FPLC fractions N1 and N2 is determined on the basis of the number of C=O groups (absorption at 214 nm) being known after structural identification. (2) The molar ratio of the constituent oligosaccharides within each FPLC fraction is determined on the basis of HPLC peak areas (corrected for the number of C=O groups) at 205 nm.

Monosialo		Mol%	nmol	Disialo		Mol%	nmol
	N1.1	4	16		N2.1.1	2	11
	N1.2	35	148		N2.2.1	8	42
					N2.2.2	4	20
	N1.3	58	245		N2.3A	18	94
					N2.3B	18	94
					N2.4	44	220
Total[a]		97	409			94	461

[a] Definite proof for some of the minor oligosaccharides could not be obtained (see text). These compounds constitute 3% and 6% (on the basis of HPLC peak area, assuming similar molar absorptions) of the total mono- and disialo carbohydrate chains, respectively.

+ N2.4, demonstrating the power of this approach. It has to be noted that the determined degree of *N*-glycolylation reflects a mean value, obtained for fibrinogen from pooled bovine serum. This value might change from batch to batch because of the age-dependent increase of *N*-glycolylneuraminic acid in the serum of the donor animals[18]. The carbohydrate chains having Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → (N1.1 and the glycans in fractions N2.1.2 and N2.2.3) or Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 sequences (N2.1.1 and N2.2.2) as structural elements have not been previously reported for bovine fibrinogen. The Neu5Acα2 → 3Galβ1 → 3GlcNAcβ1 → element is more generally found in bovine glycoproteins, frequently in conjunction with an α2 → 6-linked Neu5Ac at the GlcNAc residue of this unit (*e.g.* bovine prothrombin[19], the bovine blood coagulation factors X[20], IX[21] and II[2], and bovine fetuin[17]). It is noteworthy that α2 → 3-linked Neu5Gc has not been found among the various carbohydrate chains reported in this study, which is in line with the observation[18] that Neu5Gc residues occur preferentially in α2 → 6 linkage.

Experimental

Bovine fibrinogen (96% clottable) was obtained from Sigma. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (PNGase-F) from *Flavobacterium meningosepticum* (E.C. 3.5.1.52) was obtained from Boehringer.

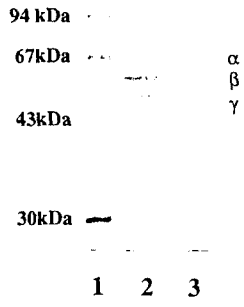


Fig. 5. SDS-PAGE of bovine fibrinogen on a 10% slab gel before and after treatment with peptide-N^4*-(N-acetyl-β-glucosaminyl)asparagine amidase-F. Sample size 5–15 μg, staining with Coomassie brilliant blue. Lane 1, molecular weight-markers (phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa). Lane 2, bovine fibrinogen, native (α, α-chain; β, β-chain; γ, γ-chain). Lane 3, bovine fibrinogen, treated with peptide-*N^4*-(N-acetyl-β-glucosaminyl)asparagine amidase-F.*

Liberation of N-*linked carbohydrate chains*

The purity of the fibrinogen sample was checked by sodiumdodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE)[22] on a 10% slab gel, giving rise to three major bands (Fig. 5), belonging to the subunits α, β and γ. The molecular masses were determined (Ferguson plot) to be 64 kDa, 57 kDa and 50 kDa, respectively. The *N*-linked carbohydrate chains were released from the protein moiety essentially as described earlier[8], the major difference being that the free enzyme was used. Briefly, 125 mg bovine fibrinogen were dissolved in 12 ml 50 mM Tris, adjusted with 12.4 M HCl to pH 7.2 and containing 50 mM EDTA. Subsequently, 0.67 ml 25% (w/v) SDS and 8 μl 2-mercaptoethanol were added and the mixture was kept for 1 h at 40°C. After the addition of 360 mg Non-idet P-40 (NP-40), the sample was incubated with 24 U PNGase-F for 24 h at room temperature in an end-over-end mixer. SDS-PAGE and Coomassie brilliant blue staining shows (Fig. 5) that treatment of denatured bovine fibrinogen with PNGase-F leads to a complete shift of the bands originally present at M_r 57 kDa and M_r 50 kDa to positions corresponding to apparent molecular masses of M_r 54 kDa and M_r 47 kDa, respectively, indicating virtually quantitative deglycosylation. The band belonging to the α-chain shows identical mobility before and after PNGase-F treatment. The differences in apparent molecular mass before and after PNGase-F treatment for the β- and γ-subunits (ΔM_r 3 kDa) suggest that each subunit contains only one glycoslylation site, which is in agreement with the determined carbohydrate content of 3.9% (w/w). Since fibrinogen formed a suspension after deglycosylation, the major part of the deglycosylated fibrinogen could be removed by centrifugation for 5 min at 2000 *g*. The supernatant was saved and the pellet was washed with 4 ml ice-cold ethanol and centrifuged at 0°C for 5 min at 2000 *g*. The combined supernatants were lyophilized, redissolved in 2 ml 0.05 M NH_4HCO_3 and fractionated on a Bio-Gel P-100 column (1.9 × 50 cm, 200–400 mesh, Bio-Rad) using 0.05 M NH_4HCO_3, adjusted to pH 7 with HCl, as eluent. Carbohydrate-positive material (orcinol/H_2SO_4) was pooled and lyophilized.

Analytical methods

Fractionation of the enzymatically released carbohydrate chains according to charge was carried out on a Mono Q HR 5/5 anion-exchange column using a Pharmacia FPLC system, as previously described[8]. The carbohydrate fractions were collected, desalted and lyophilized.

Subfractionation of the carbohydrate-containing FPLC fractions was carried out using a Kratos Spectroflow 400 HPLC system equipped with a Lichrosorb-NH_2 10μ column (25 × 0.46 cm, Chrompack), essentially as reported earlier[10]. Elutions were carried out isocratically with 30 mM K_2HPO_4/KH_2PO_4, pH 7.0: acetonitrile (34:66 or 35:65, v/v) at a flow rate of 120 ml/h at 25.0°C.

Prior to ^{1}H NMR-spectroscopic analysis, the desalted samples were repeatedly treated with ^2H_2O, finally using 99.96 atom % ^2H_2O (Aldrich) at p^{2}H 7 and room temperature. Resolution-enhanced 500- and 600-MHz ^{1}H NMR spectra were recorded using Bruker AM-500 (Department of Chemistry, Utrecht University) and AM-600 spectrometers (SON Hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University), respectively, as previously described[10,11].

Monosaccharide analysis was carried out by GLC on a capillary CP-Sil 5 WCOT fused-silica column (25 m × 0.34 mm, Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation[13].

Identification of Neu5Ac and Neu5Gc

Bovine fibrinogen (100 mg) was dissolved in 10 ml H_2O and dialyzed exhaustively against H_2O. Subsequently, the suspension was lyophilized and the residue was resuspended in 10 ml 0.1 M HCl and incubated for 1 h at 80°C. Released Neu5Ac and Neu5Gc were collected by dialysis against H_2O and lyophilization of the diffusate. The sialic acids were extracted with methanol and, after evaporation, purified by anion-exchange chromatography on Mono Q HR 5/5 (Pharmacia FPLC system) applying a linear concentration gradient from 0–50 mM NaCl in 8 ml H_2O, followed by a gradient from 50–500 mM NaCl in 8 ml H_2O[8]. The sialic acid-containing fraction was collected and, after lyophilization, the sialic acids were dissolved in methanol and analyzed by TLC on plastic-coated (0.2 mm) Silica-60 sheets, using 1-propanol/H_2O (7:3, v/v) as eluent[23]. Sialic acid-containing spots were visualized by spraying with orcinol/Fe^{3+}/HCl and subsequent heating of the plates for 15 min at 120°C[23]. GLC analysis of pertrimethylsilylated derivatives was carried out on a capillary CP-Sil 5 WCOT fused-silica column (25 m × 0.34 mm, Chrompack) using a temperature program from 120–220°C at 4°C/min. Combined GLC-MS of the same derivatives was performed on 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; BP1 capillary WCOT fused-silica column (25 m × 0.33 mm, Scientific Glass Engineering); oven temperature program, 140–180°C at 2°C/min, 180–300°C at 4°C/min.

HPAE-PAD chromatography

Subfractionation of the HPLC fractions N2.1 and N2.2 was carried out by HPAE-PAD chromatography[24] on a Dionex LC system consisting of a Dionex Bio LC quaternary gradient module and a model PAD 2 detector using a Dionex CarboPac PA-1 pellicular anion-exchange column (25 × 0.46 cm). Samples were dissolved in 25 µl H_2O (HPLC grade) and applied in two subsequent injections of 5 µl and 20 µl, respectively. Elutions were carried out starting with 90% (v/v) 0.1 M NaOH (eluent A)/10% 0.1 M NaOH, containing 0.5 M NaAc (eluent B) for 0.3 min, and going to 10% (v/v) eluent A/90% (v/v) eluent B in 120 min at a flow rate of 1 ml/min at ambient temperature. Subsequent equilibration was accomplished by elution with 100% eluent A for 15 min at 1 ml/min. Detection was made by PAD with a gold working electrode and triple-pulse amperometry[25] comprising the following pulse potentials and durations: E_1 0.05 V, 360 ms; E_2 0.80 V, 120 ms; E_3 −0.60 V, 420 ms. Data were collected and plotted using a Spectra Physics SP 4290 integrator. Fractions were immediately neutralized by addition of 1 M HCl and lyophilized. Subsequently, they were desalted on a Bio-Gel P-2 column (1 × 40 cm, 200–400 mesh). It should be noted that, under the conditions applied, epimerization of terminal reducing GlcNAc residues to ManNAc was not detected.

Acknowledgements

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