Glycobiology vol. 14 no. 1 pp. 51–64, 2004
DOI: 10.1093/glycob/cwh010

N- and O-glycans of recombinant human C1 inhibitor expressed in the milk of transgenic rabbits

Kate Koles[1,4], Patrick H. C. van Berkel[2,5], Frank R. Pieper[5], Jan H. Nuijens[5], Maurice L.M. Mannesse[5], Johannes F.G. Vliegenthart[4], and Johannis P. Kamerling[3,4]

[4]Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Section of Glycoscience and Biocatalysis, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands, and [5]Pharming Technologies BV, Archimedesweg 4, P.O. Box 451, NL-2300 AL Leiden, The Netherlands

Received on July 22, 2003; revised on September 10, 2003; accepted on September 11, 2003

Human C1 inhibitor (hC1INH) is a therapeutic N, O-glycoprotein with a growing number of clinical applications, but the current natural supplies are not likely to meet the clinical demands. Therefore, recombinant approaches are of interest, whereby specific attention has to be paid to the generated glycosylation patterns. Here, the N,O-glycoprotein was expressed in the mammary gland of transgenic rabbits and subjected to glycan analysis. After release of the N-glycans of recombinant-rabbit human C1 inhibitor (rhC1INH) by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F, the oligosaccharides were separated from the O-glycoprotein by centrifugal filtration, then fractionated by a combination of anion-exchange, normal-phase, and high-pH anion-exchange liquid chromatography. The O-glycans, released from the O-glycoprotein by alkaline borohydride treatment, were fractionated by anion-exchange high-performance liquid chromatography (HPLC). The structures of individual components were analysed by 500 MHz ^{1}H NMR spectroscopy, in most cases combined with MALDI-TOF MS. In contrast to the structural data reported for native serum hC1INH, rhC1INH contained a broad array of different N-glycans, made up of oligomannose-, hybrid-, and complex-type structures. In the case of complex-type N-glycans (partially) (α2-6)-sialylated (*N*-acetylneuraminic acid only), mono- and diantennary chains were found; part of the diantennary structures were (α1-6)-core-fucosylated or (α1-3)-fucosylated in the lower or upper antenna (Lewis x). The manno-oligosaccharide pattern of part of the hybrid- and oligomannose-type structures indicates that besides the usual N-glycan processing route, also the alternative *endo*-mannosidase pathway is followed. The small core 1-type O-glycans showed the usual (α2-3)- and (α2-6)-sialylation pattern of O-glycoproteins of nonmucinous origin.

[1]Present address: Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, TX 77843-2128
[2]Present address: GenMab BV, Jenalaan 18d, NL-3584 CK Utrecht, The Netherlands
[3]To whom correspondence should be addressed; e-mail: j.p.kamerling@chem.uu.nl

Key words: C1 inhibitor/glycosylation/transgenic rabbit milk

Introduction

Human C1 inhibitor (hC1INH), a plasma protease inhibitor belonging to the serine protease inhibitors superfamily synthesized in the liver, is involved in the inhibition of components of the complement, coagulation, fibrinolytic, and kinin-releasing systems (Carreer, 1992). The single-chain polypeptide backbone of the plasma glycoprotein (M_r ~71 kDa) consists of 478 amino acids and has six N-glycosylation sites at Asn-3, -47, -59, -216, -231, and -330, respectively, and seven O-glycosylation sites at Ser-42 and Thr-26, -49, -61, -66, -70, and -74, respectively (Bock *et al*., 1986; Perkins, 1993). Its average carbohydrate content is about 26% (Perkins *et al*., 1990). ^{1}H nuclear magnetic resonance (NMR) studies of chemically-released N- and O-glycans of native serum hC1INH revealed the presence of (α2-3)-disialylated and (α2-6)-disialylated complex-type diantennary N-glycans (molar ratio, 2:1), and the (α2-3)-monosialylated core 1-type O-glycan (Strecker *et al*., 1985). The N-glycans were (α1-6)-core-fucosylated for 30%, and the only sialic acid found was *N*-acetylneuraminic acid. Profiling studies using high-voltage paper electrophoresis followed by size-exclusion chromatography of chemically released N-glycans indicated, besides the presence of major amounts of sialylated diantennary structures, also lower amounts of sialylated tri- and tetraantennary structures (Perkins *et al*., 1990). Lectin-binding assays confirmed the presence of both (α2-3)- and (α2-6)-linked sialic acid and core 1-type O-glycans and favored the presence of complex- and hybrid-type N-glycans; no indications for oligomannose-type structures were obtained (Schoenberger, 1992).

Deficiency in the gene encoding hC1INH causes a condition called hereditary angioneurotic edema, which can be lethal when left untreated (Ebo and Stevens, 2000; Carugati *et al*., 2001). Furthermore, the lack of hC1INH leads to excessive complement activation, and this in turn to severe symptoms characterized by sudden local swellings of soft connective tissue (Ebo and Stevens, 2000). Current forms of treatment include hC1INH replacement therapy, which relies on hC1INH supplies prepared from donated human blood. However, there is a growing number of clinical conditions wherein hC1INH might be beneficial (Caliezi *et al*., 2000), hence current supplies of hC1INH are not likely to meet clinical demands.

Recombinant glycoprotein production in mammary glands of transgenic animals with milk extraction is one

of the most attractive approaches for the economic and large-scale production of therapeutic glycoproteins (Houdebine, 1995; Colman, 1996). In addition to providing eukaryotic posttranslational modifications often required for biological activity, it is also the expression platform that provides the highest recombinant protein production capacity available to date, exceeding the capacity of the more established mammalian cell lines. Typical examples so far are human lactoferrin in mice (Nuijens *et al.*, 1997) and cows (Van Berkel *et al.*, 2002), human α-glucosidase in mice (Bijvoet *et al.*, 1996) and rabbits (Visser *et al.*, 2000), human granulocyte-macrophage colony stimulating factor in mice (Uusi-Oukari *et al.*, 1997), human erythropoietin in mice and rabbits (Korhonen *et al.*, 1997), human antithrombin in goats (Edmunds *et al.*, 1998), and human tissue-type plasminogen activator in goats (Denman *et al.*, 1991).

The posttranslational glycosylation of proteins is a species-, tissue-, cell-type-, and protein-specific phenomenon. Thus recombinant proteins can have different glycosylation patterns when compared with their native counterparts. Furthermore, in cell cultures, changes in growth media composition and in growth conditions may lead to changes in glycosylation patterns. These findings are of special interest when focusing on human therapeutic recombinant glycoproteins, because specific glycosylation patterns play important roles in the secretion, antigenicity, and clearance of glycoproteins (Jenkins and Curling, 1994; Jenkins *et al.*, 1996; Kamerling, 1996; Varki *et al.*, 1999; Galet *et al.*, 2001).

Nowadays, much knowledge is available with respect to the glycosylation machineries of the two most popular mammalian cell lines for glycoprotein expression, Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells (Kamerling, 1996). However, detailed glycan data and systematic studies on the glycosylation capabilities of the mammary glands of the different species, including the effects of stage of lactation, individual variation, and seasonal variation, are scarce. For human lactoferrin from cow milk (Van Berkel *et al.*, 2002) it was concluded that besides complex-type N-glycans, as in natural human lactoferrin, also oligomannose- and/or hybrid-type glycans do occur, whereas the presence of *N*,*N*′-diacetyllactosediamine units has been suggested. It has been reported that natural bovine lactoferrin contains complex- (both partially [α2-6]-sialylated *N*-acetyllactosamine and *N*,*N*′-diacetyllactosediamine units, and Gal[α1-3]Gal[β1-4]GlcNAc units) and oligomannose-type N-glycans (Montreuil *et al.*, 1997). The occurrence of *N*,*N*′-diacetyllactosediamine units has also been suggested for human antithrombin produced in goat milk (Edmunds *et al.*, 1998). In the latter case, in contrast to plasma human antithrombin, the transgenic form also contained oligomannose-type structures, and in addition to *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid was detected.

Because the glycosylation machinery of the rabbit mammary glands is rather unexplored, it is unpredictable what kind of glycans would predominate on recombinant glycoproteins from transgenic rabbit milk. In this study, a detailed analysis of the N- and O-glycans of recombinant human C1 inhibitor expressed in the milk of transgenic rabbits (rhC1INH) is presented and discussed.

Results

General

Monosaccharide analysis (Kamerling and Vliegenthart, 1989) of rhC1INH, having a total carbohydrate content of 14% (w/w), revealed the presence of Fuc, Gal, GalNAc, GlcNAc, Man, and Neu5Ac in the molar ratio 0.2:2.4:1.7:2.6:3.0:1.3.

Release and fractionation of N-glycans of rhC1INH

The N-glycans of rhC1INH were released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) digestion, separated as a pool from the remaining O-glycoprotein, and fractionated by anion-exchange chromatography on Resource Q. Three carbohydrate-containing fast protein liquid chromatography (FPLC) fractions, eluting at positions corresponding to neutral **N0** (25%), monosialylated **N1** (67%), and disialylated **N2** (8%) structures were obtained (Figure 1). Neutral FPLC fraction **N0** was further separated into six high-performance liquid chromatography (HPLC) fractions, denoted **N0.1**–**N0.6**, by normal phase chromatography on LiChrospher-NH_2 (Figure 2). The HPLC fractions **N0.3** and **N0.4** were subfractionated by high pH anion-exhange chromatography (HPAEC) on CarboPac PA-1 (Figure 3) after 2-aminobenzamide (2AB) labeling of the components present (to minimize high pH–induced epimerization of the reducing GlcNAc residues during HPAEC; Stroop *et al.*, 2000), yielding four and five subfractions, respectively, denoted **N0.3.1$_{2AB}$**–**N0.3.4$_{2AB}$** and **N0.4.1$_{2AB}$**–**N0.4.5$_{2AB}$**. The mono- (**N1**) and dicharged (**N2**) FPLC fractions were subfractionated on CarboPac PA-1 (Figures 4 and 5), yielding fractions **N1.1**–**N1.8** and **N2.1**–**N2.3**, respectively.

Structural analysis of the various fractions was carried out by ^{1}H NMR spectroscopy in combination with

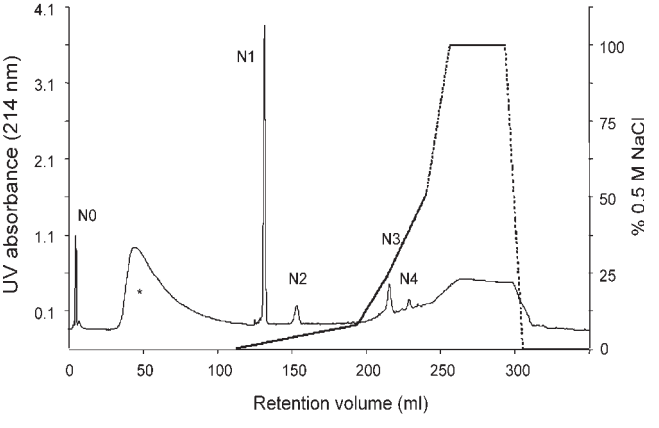


Fig. 1. Elution profile at 214 nm of the enzymically released N-glycan pool of rhC1INH on a Resource Q column. The column was first eluted with 12 ml water, followed by a linear concentration gradient of 0–8% (v/v) 0.5 M NaCl over 5 min and by a steeper linear concentration gradient of 8–50% (v/v) 0.5 M NaCl over 5 min at a flow rate of 4 ml/min. For subsequent runs, the column was washed and equilibrated to starting conditions in 30 min. The fraction marked by an asterisk and fractions **N3** and **N4** did not contain carbohydrate material.

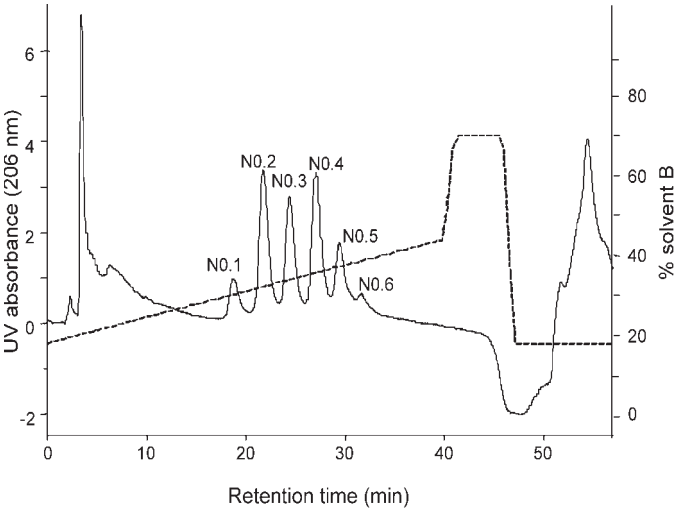
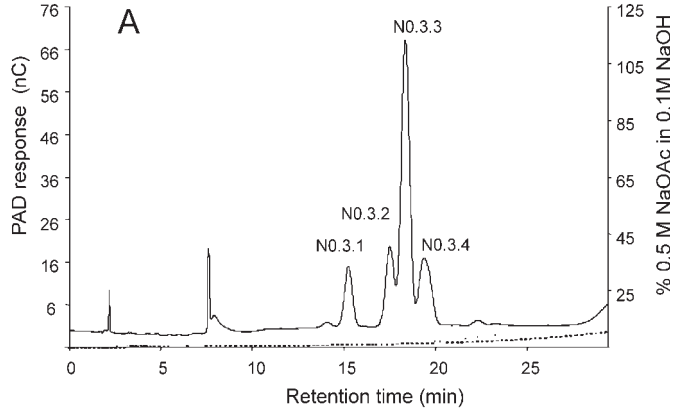


Fig. 2. Elution profile at 206 nm of neutral FPLC fraction **N0** on a LiChrospher-NH_2 column. The column was eluted with a linear gradient of 18–42% (v/v) solvent B [10% (v/v) acetonitrile in water] in solvent A [10% (v/v) water in acetonitrile].



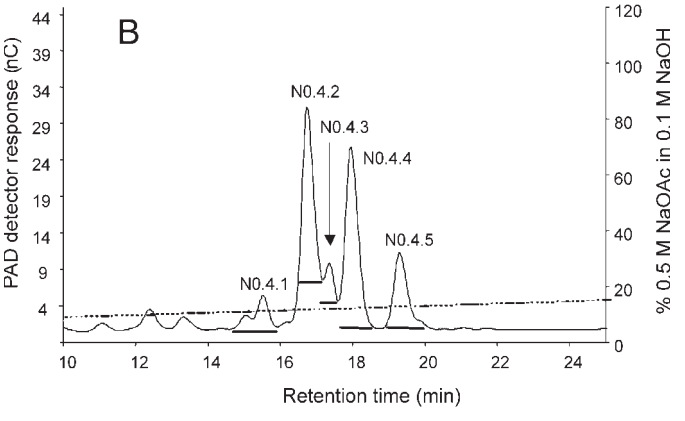


Fig. 3. Elution profiles using PAD of the neutral HPLC fractions **N0.3** (**A**) and **N0.4** (**B**) on a CarboPac PA-1 column. The column was eluted with a linear concentration gradient of 5–18% (v/v) solvent B (0.5 M NaOAc in 0.1 M NaOH) in solvent A (0.1 M NaOH) over 32 min, at a flow rate of 4 ml/min.

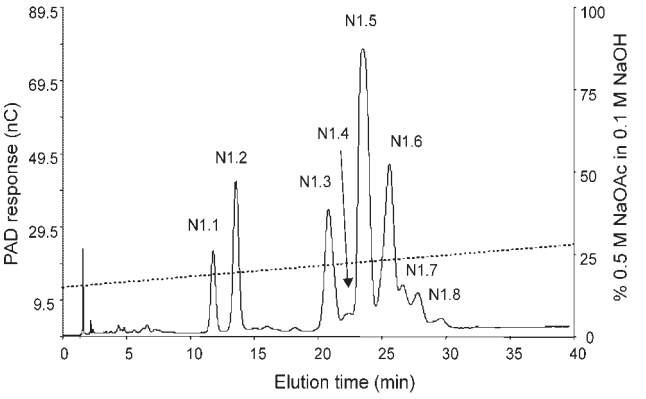


Fig. 4. Elution profile using PAD of the monocharged FPLC fraction **N1** on a CarboPac PA-1 column. The column was eluted with a linear concentration gradient of 15–28% (v/v) solvent B (0.5 M NaOAc in 0.1 M NaOH) in solvent A (0.1 M NaOH) over 40 min at a flow rate of 4 ml/min.

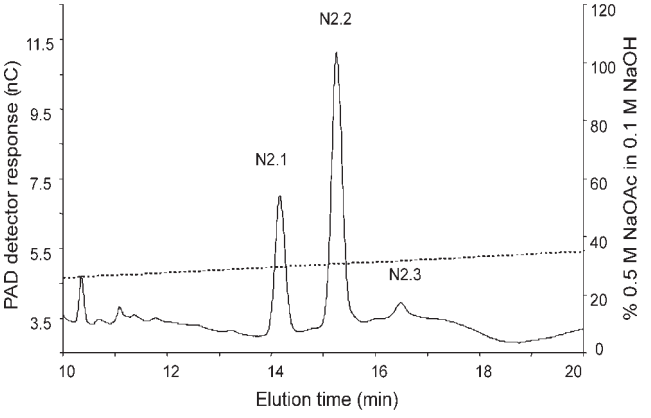
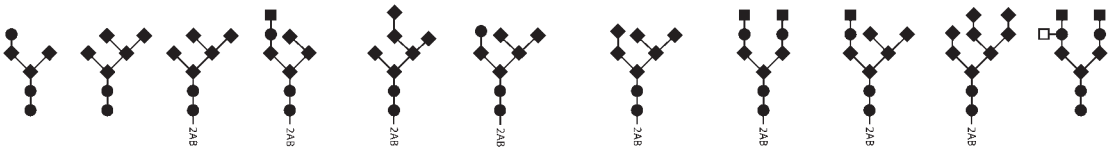


Fig. 5. Elution profile using PAD of the dicharged FPLC fraction **N2** on a CarboPac PA-1 column. The column was eluted with a linear concentration gradient of 20–25% (v/v) solvent B (0.5 M NaOAc in 0.1 M NaOH) in solvent A (0.1 M NaOH) over 9 min, followed by a linear concentration gradient of 25–35% (v/v) solvent B in solvent A over 11 min at a flow rate of 4 ml/min.

matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For complex-type N-glycans holds that an (α1-6)-fucosylated *N*,*N*′-diacetylchitobiose core element is recognized from the anomeric signals of αGlcNAc-**1** at δ 5.18, GlcNAc-**2** at δ 4.66–4.67, and Fuc at δ 4.89–4.91, together with the *N*-acetyl methyl signals of GlcNAc-**1** at ~δ 2.039 and GlcNAc-**2** at δ 2.09–2.10, as well as from the Fuc CH_3 signals at δ 1.21 (αGlcNAc-**1**) and δ 1.22 (βGlcNAc-**1**), and the Fuc H-5 multiplets at δ 4.10 (αGlcNAc-**1**) and δ 4.13 (βGlcNAc-**1**). The nonfucosylated *N*,*N*′-diacetylchitobiose unit is recognized from the anomeric signals of αGlcNAc-**1** at δ 5.19 and GlcNAc-**2** at δ 4.60–4.61, together with the *N*-acetyl methyl signals of GlcNAc-**1** at ~δ 2.039 and GlcNAc-**2** at ~δ 2.081 (Hård *et al.*, 1992). In case of hybrid- and oligomannose-type N-glycans, the *N*-acetyl methyl signals of GlcNAc-**2** are generally observed at ~δ 2.064 (Stroop *et al.*, 2000; Tseneklidou-Stoeter *et al.*, 1995).

Table I. ^{1}H-chemical shifts of the structural-reporter-group protons of the constituent monosaccharides of neutral N-glycans, derived from rhC1INH

Reporter group	Residue	Chemical shift in										
												
		N0.1	**N0.2**	**N0.2$_{2AB}$**	**N0.3.2$_{2AB}$**	**N0.3.3$_{2AB}$**	**N0.3.4A$_{2AB}$**	**N0.3.4B$_{2AB}$**	**N0.4.2$_{2AB}$****	**N0.4.4$_{2AB}$**	**N0.4.5$_{2AB}$***	**N0.5**
		ppm										
H-1	GlcNAc-**1**α	5.191	5.189	-	-	-	-	-	-	-	-	5.191
	GlcNAc-**1**β	4.698	4.696	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	GlcNAc-**2**$^{\alpha}$	4.613	4.601	-	-	-	-	-	-	-	-	n.d.
	GlcNAc-**2**$^{\beta}$	4.609	4.593	4.624	4.624	4.628	4.624	4.624	4.635	4.624	4.627	n.d.
	Man-**3**	4.782	4.783	4.762	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Man-**4**	5.120	5.096	5.089	5.111	5.088	5.106	5.341	5.110	5.108	5.338	5.109
	Man-**4**′	4.920	4.872	4.87	4.892	4.876	4.87	4.87	4.925	4.87	4.867	4.93
	Man-**A**	-	5.096	5.089	5.104	5.404	5.090	5.090	-	5.089	5.402	-
	Man-**B**	-	4.908	4.904	-	4.909	4.907	4.907	-	4.906	5.141	-
	Man-**C**	-	-	-	-	-	-	5.051	-	-	5.054	-
	Man-**D$_2$**	-	-	-	-	5.058	-	-	-	-	5.054	-
	Man-**D$_3$**	-	-	-	-	-	-	-	-	-	5.040	-
	GlcNAc-**5**	4.554	-	-	4.574	-	4.551	-	4.578	4.577	-	4.59
	GlcNAc-**5**′	-	-	-	-	-	-	-	4.578	-	-	4.59
	Gal-**6**	-	-	-	4.466	-	-	-	4.466	4.466	-	4.445
	Gal-**6**′	-	-	-	-	-	-	-	4.473	-	-	4.474
	Fuc(α1-3)	-	-	-	-	-	-	-	-	-	-	5.126
H-2	Man-**3**	4.253	4.253	4.219	4.209	4.218	4.211	4.198	4.216	4.214	4.197	4.249
	Man-**4**	4.189	4.077	4.071	4.189	4.088	4.194	4.111	4.185	4.197	4.124	4.192
	Man-**4**′	3.977	4.146	4.143	4.133	4.148	4.146	4.146	4.108	4.145	4.151	4.108
	Man-**A**	-	4.065	4.061	4.061	4.096	4.064	4.064	-	4.063	4.092	-
	Man-**B**	-	3.982	3.977	-	3.978	3.984	3.984	-	3.98	n.d.	-
	Man-**C**	-	-	-	-	-	-	4.064	-	-	4.07	-
	Man-**D$_2$**	-	-	-	-	4.074	-	-	-	-	4.07	-
	Man-**D$_3$**	-	-	-	-	-	-	-	-	-	4.07	-
CH_3 NAc	Fuc(α1-3)	-	-	-	-	-	-	-	-	-	-	1.174
	GlcNAc-**1**	2.040	2.039	1.986	1.988	1.987	1.988	1.988	1.989	1.989	1.988	2.040
	GlcNAc-**2**	2.080	2.065	2.055	2.056	2.060	2.056	2.056	2.072	2.056	2.061	2.083
	GlcNAc-**5**	2.054	-	-	2.045	-	2.049	-	2.045	2.045	-	2.040
	GlcNAc-**5**′	-	-	-	-	-	-	-	2.045	-	-	2.046

* **D$_3$** **B**
Man(α1-2)Man(α1-6)
\ **4**′
Man(α1-6)
D$_2$ **A** / \ **3** **2** **1**
Man(α1-2)Man(α1-3) Man(β1-4)GlcNAc(β1-4)GlcNAc
/
Man(α1-2)Man(α1-3)
C **4**

** **6**′ **5**′ **4**′
Gal(β1-4)GlcNAc(β1-2)Man(α1-6)
\ **3** **2** **1**
Man(β1-4)GlcNAc(β1-4)GlcNAc
/
Gal(β1-4)GlcNAc(β1-2)Man(α1-3)
6 **5** **4**

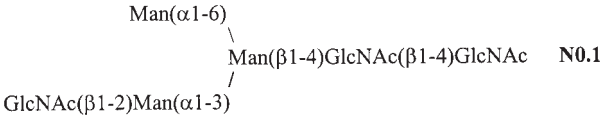
Chemical shifts are given at 300K and were measured in ^2H_2O at p^{2}H7 relative to internal acetone (δ 2.225; Vliegenthart *et al.*, 1983). Compounds are represented by short-hand symbolic notation: □, Fuc; ■, Gal; ●, GlcNAc; ◆, Man; 2AB, 2-aminobenzamide. For numbering of the monosaccharide residues, see ∗ and ∗∗. n.d., not determined; α and β stand for the anomeric configuration of GlcNAc-**1**.

Structural analysis of neutral N-glycans

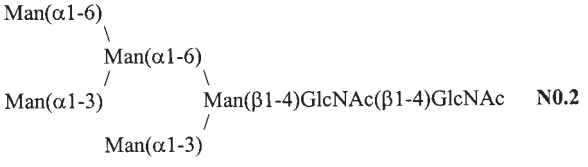
The ^{1}H NMR data of the identified neutral oligomannose-, hybrid-, and complex-type N-glycans are presented in Table I. The table includes also the coding system for the different monosaccharide constitutents. In the HPAEC subfraction **N0.3.1$_{2AB}$** no carbohydrate material could be detected, whereas HPAEC subfractions **N0.4.1$_{2AB}$** and **N0.4.3$_{2AB}$** did not contain sufficient material for NMR assignments. The same held for HPLC fraction **N0.6**.

MALDI-TOF-MS analysis (positive-ion mode) of HPLC fraction **N0.1** showed a sodiated molecular ion at *m/z* 1136 [$Hex_3HexNAc_3 + Na$]. Its ^{1}H NMR spectrum indicated a monoantennary N-glycan whereby Man-**4** is

extended with GlcNAc-**5**, and Man-**4′** is terminal. The structural-reporter-group protons of Man-**3**, Man-**4**, Man-**4′**, and GlcNAc-**5** match the ^{1}H NMR data of the (α1-6)-fucosylated analogue (compare with compound 5 in Bendiak *et al*. [1988]).

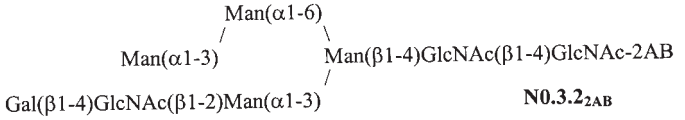


HPLC fraction **N0.2** was identified by ^{1}H NMR spectroscopy as $Man_5GlcNAc_2$ (compare with compound U8 in Hård *et al*. [1991]). MALDI-TOF-MS analysis (positive-ion mode) supported this finding with the sodiated molecular ion at *m/z* 1257 [$Hex_5HexNAc_2$ + Na]. This oligosaccharide was used as a model substance to evaluate the impact of the 2AB labeling (→ **N0.2$_{2AB}$**) on the structural reporters of the nonlabeled counterpart. Typical up- or downfield shifts were found for Man-**3** H-2 (δ 4.253 → δ_{2AB} 4.219), Man-**4** H-1 (δ 5.096 → δ_{2AB} 5.089), Man-**4** H-2 (δ 4.077 → δ_{2AB} 4.071), GlcNAc-**1** NAc (δ 2.039 → δ_{2AB} 1.986), GlcNAc-**2** H-1 (δ 4.593 → δ_{2AB} 4.624), and GlcNAc-**2** NAc (δ 2.065 → δ_{2AB} 2.055).

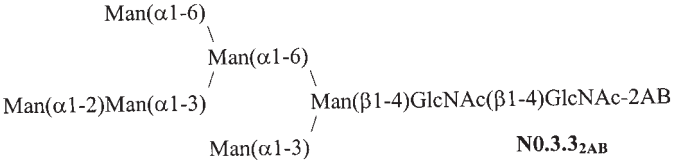


MALDI-TOF-MS analysis (positive-ion mode) of HPLC fraction **N0.3** showed a major sodiated molecular ion at *m/z* 1419 ($Hex_6HexNAc_2$ + Na), and minor ones at *m/z* 1257 ($Hex_5HexNAc_2$ + Na) and 1460 ($Hex_5HexNAc_3$ + Na).

The ^{1}H NMR spectrum of HPAEC subfraction **N0.3.2$_{2AB}$** contained the typical signals for a Gal(β1-4)GlcNAc(β1-2)Man(α1-3) sequence attached to Man-**3** (Man-**4**, H-1/H-2, δ 5.111/4.189; GlcNAc-**5**, H-1, δ 4.574; Gal-**6**, H-1, δ 4.466). These structural-reporter-group signals and those of terminal Man-**A** (H-1/H-2, δ 5.103/4.061) and Man-**4′** (H-1/H-2, δ 4.892/4.133) occur in equimolar proportions. In the *N*-acetyl methyl region only three signals were present, assigned to GlcNAc-**5** (NAc, δ 2.045), GlcNAc-**2** (NAc, δ 2.056), and GlcNAc-**1** (NAc, δ 1.988). Such a combination of structural-reporter groups indicates the presence of a hybrid-type glycan, with Man-**A** extending Man-**4′** on the α1-6 branch, whereas Man-**4** on the α1-3 branch carries an *N*-acetyllactosamine unit. ^{1}H NMR data of a similar structure, but α(2-3)-sialylated and with GlcNAc-**2** as reducing unit have been reported (compare with compound EH-1 in Spellman *et al*. [1991]).

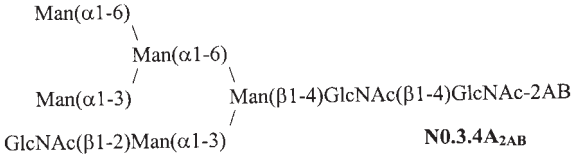


^{1}H NMR analysis of the major component of HPLC fraction **N0.3**, HPAEC subfraction **N0.3.3$_{2AB}$**, revealed the presence of only two *N*-acetyl signals corresponding to GlcNAc-**1** (δ 1.987) and GlcNAc-**2** (δ 2.060). Taking into account the MALDI-TOF MS data of fraction **N0.3**, an oligomannose-type N-glycan is indicated. The anomeric region of the ^{1}H NMR spectrum showed a H-1 signal at δ 5.404, in accordance with the presence of a substituted Man-**A** residue (Vliegenthart *et al*., 1983). The reporter signals at δ 5.058/4.074 for Man-**D$_2$** H-1/H-2 confirm this substitution. Furthermore, the occurrence of a terminal Man-**B** residue and an unsubstituted Man-**4** residue are reflected by the presence of the H-1/H-2 signals at δ 4.909/3.978 and δ 5.088/4.088, respectively. An oligomannose-type glycan with the same arrangement of Man residues has been described earlier by Michalski *et al*. (1990), and its Man H-1/H-2 δ values are in good agreement with those of **N0.3.3$_{2AB}$** (compare with compound $Man_6GlcNAc_2$ II in Michalski *et al*., 1990). The difference between the Man-**4** H-1 values (δ_{2AB} 5.088 versus δ 5.094) is due to the 2AB labeling (compare **N0.2** and **N0.2$_{2AB}$**, Table I). The assignments of the various Man H-1 and H-2 signals were verified by 2D total correlation spectroscopy (TOCSY) experiments. The structural assignment was further supported by 2D rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY), showing cross-peaks between Man-**D$_2$** H-1 and Man-**A** H-2 and between Man-**4** H-1 and Man-**3** H-3.

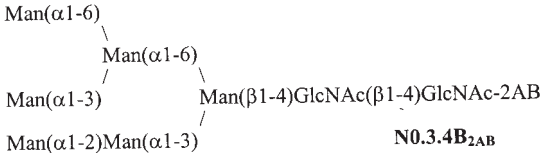


HPAEC subfraction **N0.3.4$_{2AB}$** turned out to contain a mixture of a hybrid- and an oligomannose-type N-glycan. Its ^{1}H NMR spectrum showed H-1 signals for terminal GlcNAc-**5** (δ 4.551), terminal Man-**A** (δ 5.090), terminal Man-**B** (δ 4.907), and terminal Man-**C** (δ 5.051) with dominating signal intensities for H-1 of Man-**B** and Man-**A**. The peak intensity ratio of GlcNAc-**5** H-1:Man-**C** H-1 is similar to that of Man-**4** H-1 (δ 5.106):Man-**4** H-1 (δ 5.341), whereby the first δ value is indicative for Man-**4** substituted

with GlcNAc-**5** (**N0.3.4A$_{2AB}$**) and the second one for Man-**4** substituted with Man-**C** (**N0.3.4B$_{2AB}$**) (Vliegenthart *et al.*, 1983). Therefore the structural reporters clearly indicate two structures with the same upper arm and variations in the substitution of Man-**4** in the lower arm (molar ratio A:B, 5:8). The downfield shifts of Man-**4** H-1/H-2, caused by the attachment of GlcNAc-**5**, going from **N0.2$_{2AB}$** to **N0.3.4A$_{2AB}$**, is comparable with such shifts reported in the literature (Mulder *et al.*, 1995). Compound **N0.3.4B$_{2AB}$** is the conventional $Man_6GlcNAc_2$ structure, and taking into account the influence of the 2AB labeling, the structural reporters are in agreement with those of the nonlabeled compound (Tseneklidou-Stoeter *et al.*, 1995).



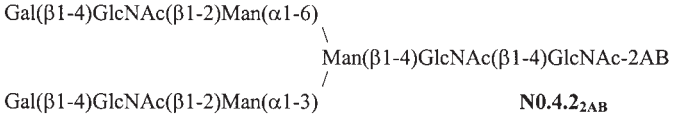
N0.3.4A$_{2AB}$



N0.3.4B$_{2AB}$

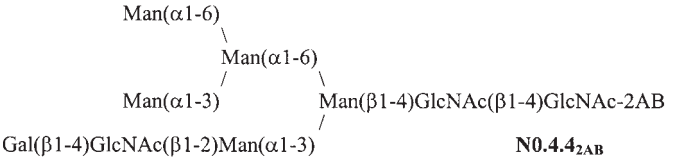
MALDI-TOF-MS analysis (positive-ion mode) of HLPC fraction **N0.4** revealed *m/z* values of 1580, 1619, 1660 (major), and 1742, in accordance within 3 mass units with the sodiated adducts of $Hex_7HexNAc_2$, $Hex_6HexNAc_3$, $Hex_5HexNAc_4$, and $Hex_8HexNAc_2$.

HPAEC subfraction **N0.4.2$_{2AB}$** contains a digalactosylated diantennary complex-type N-glycan. The ^{1}H NMR structural-reporter groups are in accordance with earlier published data for such a glycan (compare with compound 11 in Bendiak *et al.* [1988]), except for the values affected by the 2AB labeling. Note the difference in δ value between GlcNAc-**2** NAc for complex-type (δ 2.072) and hybrid-/oligomannose-type (δ 2.055–2.062) 2-AB-labeled N-glycans (Table I).



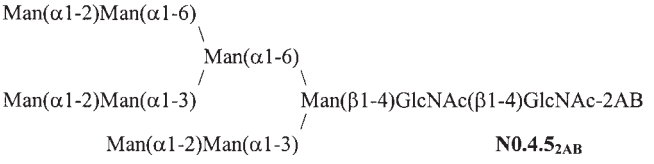
N0.4.2$_{2AB}$

The ^{1}H NMR spectrum of HPAEC subfraction **N0.4.4$_{2AB}$** showed the occurrence of one major compound in a mixture of hybrid and/or oligomannose-type N-glycans, a hybrid-type N-glycan (>85% as judged from the ratio of the intensities of the *N*-acetyl methyl signals of GlcNAc-**5** and GlcNAc-**1**). The compound is an extension of structure **N0.3.4A$_{2AB}$** with a Gal-**6** residue. The structural-reporter-group signals of the lower arm match those of the lower arm of **N0.4.2$_{2AB}$**, the ones of the upper arm those of the upper arm of **N0.3.4A$_{2AB}$**.



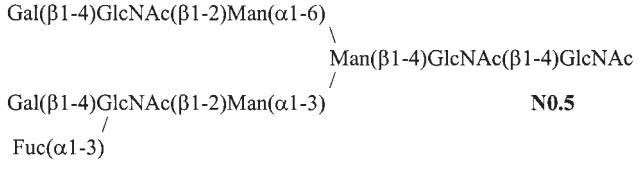
N0.4.4$_{2AB}$

The ^{1}H NMR spectrum of HPAEC subfraction **N0.4.5$_{2AB}$** indicated the presence of one major compound in a mixture of oligomannose-type N-glycans, a $Man_8GlcNAc_2$ structure. For this structure holds that Man-**4** is extended with a terminal Man-**C** residue (H-1/H-2, δ 5.054/4.07), and Man-**4′** with α(1-2)-substituted Man-**B** (H-1, δ 5.141) and Man-**A** (H-1, δ 5.402) residues, as evidenced by the presence of Man-**D$_3$** and Man-**D$_2$** H-1 signals at δ 5.040 and 5.054, respectively (compare with compound 71 in Vliegenthart *et al.* [1983]; see also Van Halbeek *et al.* [1981]).



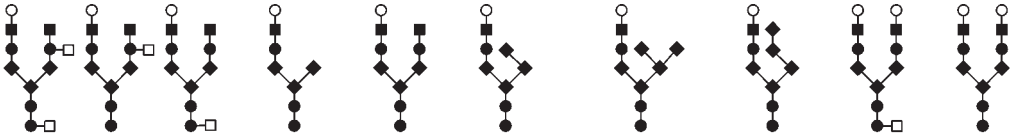
N0.4.5$_{2AB}$

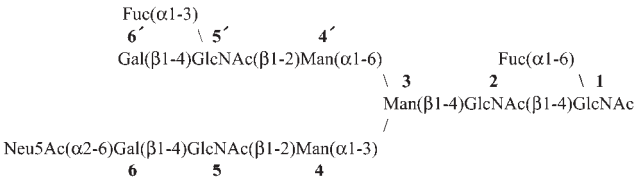
MALDI-TOF-MS analysis (positive-ion mode) of HLPC fraction **N0.5** showed a major *m/z* value of 1812, in accordance within 3 mass units with the sodiated adduct of $Hex_5HexNAc_4dHex$. ^{1}H NMR analysis revealed as the major compound a fucosylated complex-type diantennary N-glycan, but the fucosylation does not occur at the Asn-bound GlcNAc-**1** (GlcNAc-**1** H-1, δ 5.191; GlcNAc-**2** NAc, δ 2.083). The structural-reporter-group signals indicate that the Man-**4′** residue (H-1, δ 4.93) is extended with an *N*-acetyllactosamine unit, and the Man-**4** residue (H-1, δ 5.109) with a Lewis x epitope (compare with Qd1.5A and Qd1.5C in Stroop *et al.*, 2000; see also asialo-afuco diantennary structure Q0-F in Spellman *et al.* [1991]). The (α1-3)-fucosylated GlcNAc-**5** is reflected by the typical Fuc signals at δ 5.126 (Fuc H-1) and 1.174 (Fuc CH_3) (Kamerling and Vliegenthart, 1992).



N0.5

Table II. ^{1}H-chemical shifts of the structural-reporter-group protons of the constituent monosaccharides of sialylated N-glycans, derived from rhC1INH

Reporter group	Residue	Chemical shift in									
											
		N1.1*	**N1.2**	**N1.3**	**N1.4**	**N1.5A**	**N1.5B**	**N1.6A**	**N1.6B**	**N2.1**	**N2.2**
		ppm									
H-1	GlcNAc-**1**α	5.181	5.190	5.181	5.190	5.190	5.190	5.190	5.190	5.181	5.190
	GlcNAc-**1**β	n.d.	4.693	4.693	n.d.	4.696	4.696	4.694	4.694	n.d.	n.d.
	GlcNAc-**2**	4.667	4.611	4.667	4.610	4.614	4.614	n.d.	n.d.	n.d.	n.d.
	Man-**3**	n.d.	4.777	4.771	4.783	4.770	4.783	4.785	4.785	n.d.	4.775
	Man-**4**	5.135	5.137	5.135	5.135	5.136	5.136	5.132	5.132	5.134	5.135
	Man-**4´**	4.912	4.918	4.927	4.919	4.931	4.897	4.875	4.898	4.942	4.948
	Man-**A**	-	-	-	-	-	5.108	5.091	5.410	-	-
	Man-**B**	-	-	-	-	-	-	4.910	-	-	-
	Man-**D$_2$**	-	-	-	-	-	-	-	5.056	-	-
	GlcNAc-**5**	4.606	4.608	4.605	4.603	4.606	4.606	4.606	4.606	4.605	4.609
	GlcNAc-**5´**	4.588	4.588	4.582	-	4.581	-	-	-	4.605	4.609
	Gal-**6**	4.447	4.446	4.445	4.445	4.445	4.445	4.445	4.445	4.444	4.444
	Gal-**6'**	4.447	4.449	4.473	-	4.472	-	-	-	4.447	4.447
	Fuc(α1-6)$^{\alpha}$	4.888	-	4.889	-	-	-	-	-	4.889	-
	Fuc(α1-6)$^{\beta}$	4.902	-	4.896	-	-	-	-	-	4.897	-
	Fuc(α1-3)	5.132	5.133	-	-	-	-	-	-	-	-
H-2	Man-**3**	4.257	4.256	4.255	4.255	4.251	4.251	4.251	4.251	4.258	4.257
	Man-**4**	4.194	4.195	4.196	4.195	4.196	4.196	4.207	4.207	4.198	4.197
	Man-**4´**	4.095	4.101	4.104	n.d.	4.111	4.136	4.146	n.d.	4.115	4.118
	Man-**A**	-	-	-	-	-	4.065	4.066	-	-	-
H-3a	Neu5Ac	1.717	1.718	1.718	1.717	1.718	1.718	1.718	1.718	1.717 (2x)	1.718 (2x)
H-3e	Neu5Ac	2.668	2.669	2.668	2.668	2.669	2.669	2.668	2.668	2.671 (2x)	2.669 (2x)
H-5	Fuc(α1-3)	4.827	4.834	-	-	-	-	-	-	-	-
CH_3	Fuc(α1-6)$^{\alpha}$	1.216	-	1.209	-	-	-	-	-	1.216	-
	Fuc(α1-6)$^{\beta}$	1.216	-	1.221	-	-	-	-	-	1.216	-
	Fuc(α1-3)	1.178	1.180	-	-	-	-	-	-	-	-
NAc	GlcNAc-**1**	2.039	2.039	2.039	2.038	2.038	2.038	2.037	2.037	2.039	2.038
	GlcNAc-**2**	2.097	2.083	2.095	2.078	2.082	2.064	2.064	2.064	2.097	2.084
	GlcNAc-**5**	2.069	2.069	2.069	2.068	2.069	2.069	2.069	2.069	2.069	2.069
	GlcNAc-**5´**	2.042	2.041	2.048	-	2.047	-	-	-	2.066	2.066
	Neu5Ac	2.030	2.030	2.030	2.029	2.030	2.030	2.030	2.030	2.030 (2x)	2.030 (2x)

* 

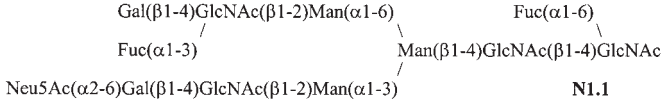
Chemical shifts are given at 300K and were measured in $^{2}H_2O$ at p^{2}H7 relative to internal acetone (δ 2.225; Vliegenthart *et al.*, 1983). Compounds are represented by short-hand symbolic notation: ○, Neu5Ac(α2-6); □, Fuc; ■, Gal; ●, GlcNAc; ◆, Man. For numbering of the monosaccharide residues, see ∗. n.d., not determined; α and β stand for the anomeric configuration of GlcNAc-**1**.

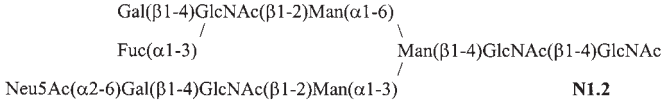
Structural analysis of monosialylated N-glycans

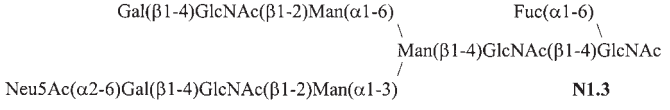
The ^{1}H NMR data of the identified monosialylated hybrid- and complex-type N-glycans are presented in Table II. The table includes also the coding system for the different monosaccharide constituents. HPAEC fractions **N1.7** and **N1.8** did not contain sufficient material for NMR assignments. In all presented structures the lower, (α1-3) arm is Neu5Ac(α2-6)Gal (β1-4) GlcNAc(β1-2)Man(α1-3): Neu5Ac H-3e, δ 2.667–2.671; Neu5Ac H-3a, δ 1.717–1.718; Gal-**6** H-1, δ 4.444–4.446; GlcNAc-**5** H-1, δ 4.603–4.609; Man-**4** H-1, δ 5.132–5.137; Man-**4** H-2, δ 4.194–4.207

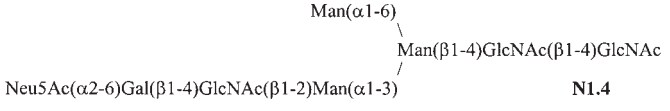
(slightly influenced by the type of upper arm) (compare with compound HST in Damm *et al.* [1987]).

The ^{1}H NMR spectrum of HPAEC fraction **N1.1** showed the presence of a diantennary N-glycan, (α2-6)-sialylated at the lower arm and an (α1-6)-fucosylated GlcNAc-**1** residue. The upper arm contains a Lewis x epitope as indicated by the typical structural reporters of the monosaccharide constituents (compare with compound Qd1.6A in Stroop *et al.*, 2000). As indicated by ^{1}H NMR analysis, HPAEC fraction **N1.2** contains the noncore-fucosylated analog of **N1.1**. The ^{1}H NMR spectrum of HPAEC fraction **N1.3** reflects the presence of a convential (α1-6)-core-fucosylated diantennary N-glycan, (α2-6)-sialylated at the (α1-3) arm (compare with compound N1.5 in Hård *et al.* [1992]). ^{1}H NMR analysis of HPAEC fraction **N1.4** showed the presence of a monoantennary N-glycan, (α2-6)-sialylated at the (α1-3) arm. The ^{1}H NMR data of a similar structure, but missing GlcNAc-**1** (compare with compound 526 in Van Pelt *et al.* [1989]) as well as of the (α1-6)-fucosylated variant (compare compound 1-1 in De Waard *et al.* [1991]) have been reported.

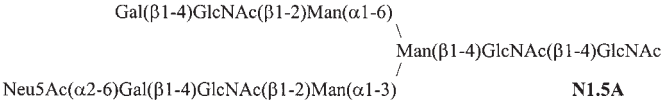


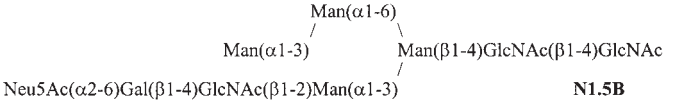




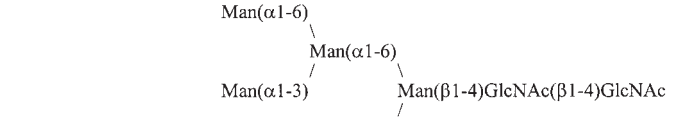


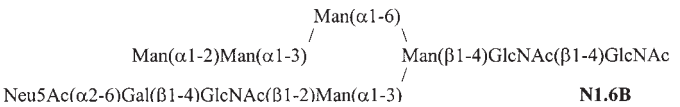
MALDI-TOF-MS analysis (negative-ion mode) of HPAEC fraction **N1.5** revealed two pseudomolecular ions, one at *m/z* 1931 [$Hex_5HexNAc_4Neu5Ac$ – H], denoted **N1.5A**, and one at *m/z* 1727 [$Hex_5HexNAc_3Neu5Ac$ – H], denoted **N1.5B**. From the ^{1}H NMR spectrum of **N1.5** it could be deduced that the major (α2-6)-sialylated nonfucosylated N-glycan **N1.5A** has an *N*-acetyllactosaminylated upper arm, being the nonfucosylated analogue of **N1.3** (compare with compound N1.4 in Hård *et al.* [1992]). Component **N1.5B** is an (α2-6)-sialylated analog of **N0.3.2$_{2AB}$**, a hybrid-type N-glycan with Man-**4′** extended with a Man-**A** residue. The molar ratio A:B amounts 3.6:1, as calculated from the two Man-**4′** H-1 signal intensities.





MALDI-TOF-MS analysis (negative-ion mode) of HPAEC fraction **N1.6** showed a pseudomolecular ion at *m/z* 1890 [$Hex_6HexNAc_3Neu5Ac$ – H]. However, the ^{1}H NMR spectrum gave evidence for the occurrence of two components, major **N1.6A** and minor **N1.6B**. The major component was indicated to be an (α2-6)-sialylated hybrid-type N-glycan with terminal Man-**A** (H-1/H-2, δ 5.091/4.066) and terminal Man-**B** (H-1, δ 4.910) residues attached to Man-**4′** (H-1/H-2, δ 4.875/4.146) (compare with **N0.4.4$_{2AB}$**). A second set of H-1 signals of lower intensity observed at δ 4.898 (H-1 of monosubstituted Man-**4′**; compare with **N1.5B**), 5.410 (H-1 of substituted Man-**A**), and 5.056 (H-1 of terminal Man-**D$_2$**) supports the presence of isoform **N1.6B**, with a different arrangement of Man-**4′**-linked Man residues. The ratio of terminal to substituted Man-**A** is approximately 8:3, reflecting the relative abundance of these structures.





Structural analysis of disialylated N-glycans

^{1}H NMR analysis of HPAEC fraction **N2.1** revealed the presence of an (α2-6)-disialylated, (α1-6)-fucosylated diantennary glycan (compare with compound Q2.2 in Van Rooijen *et al.* [1998]). Its nonfucosylated analog turned out to be present in HPAEC fraction **N2.2** (compare with compound HST in Damm *et al.* [1987]). The ^{1}H NMR

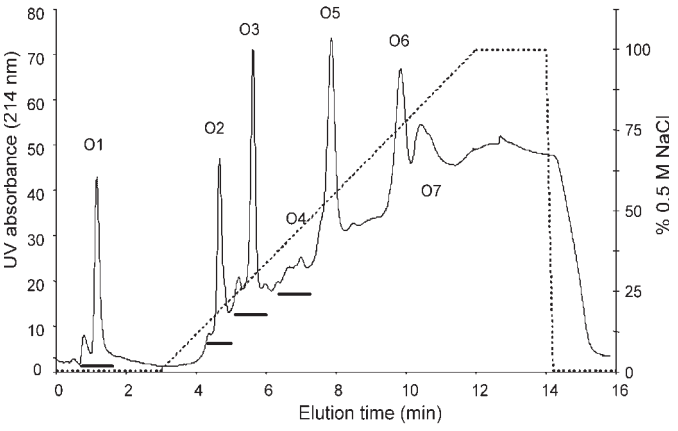


Fig. 6. Elution profile at 214 nm of the chemically released O-glycan pool from N-deglycosylated rhC1INH on a Resource Q column. The column was first eluted with 12 ml water, followed by a linear concentration gradient of 0–100% (v/v) 0.5 M NaCl over 9 min at a flow rate of 4 ml/min. Fractions **O.4**, **O.5**, **O.6**, and **O.7** did not contain carbohydrate material.

data of both compounds are included in Table II. HPAEC fraction **N2.3** did not contain sufficient material for NMR assignments.

Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Fuc(α1-6)
\ \
Man(β1-4)GlcNAc(β1-4)GlcNAc
/
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) **N2.1**

Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)
\
Man(β1-4)GlcNAc(β1-4)GlcNAc
/
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) **N2.2**

Release, fractionation, and structural analysis of O-glycans of rhC1INH

Resource Q fractionation of the O-linked oligosaccharide-alditols, obtained after alkaline borohydride treatment of the N-deglycosylated rhC1INH, yielded three carbohydrate-containing fractions, denoted **O.1** (45%), **O.2** (33%), and **O.3** (22%) (Figure 6). ^{1}H NMR analysis of the neutral FPLC fraction **O.1** showed the presence of the core 1 structure Gal(β1-3)GalNAc-ol (compare with compound 2 in Kamerling and Vliegenthart [1992]) (Table III). The mono-charged FPLC fraction **O.2** contained a mixture of two monosialylated alditols, Neu5Ac(α2-3)Gal(β1-3)GalNAc-ol **(O.2A)** (compare with compound 78 in Kamerling and Vliegenthart [1992]) and Gal(β1-3)[Neu5Ac(α2-6)]GalNAc-ol (**O.2B**) (compare with compound 9 in Kamerling and Vliegenthart [1992]) in an approximate molar ratio of 1:2, as judged from the intensities of the H-1 signals of substituted (δ 4.546) and terminal (δ 4.474) Gal. The dicharged FPLC fraction **O.3** contained Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α2-6)]GalNAc-ol (compare with compound 85 in Kamerling and Vliegenthart [1992]).

Table III. ^{1}H-chemical shifts of the structural-reporter-group protons of the constituent monosaccharides of O-glycans, derived from rhC1INH

Residue	Reporter group	Chemical shift in			
					
		O.1	**O.2A**	**O.2B**	**O.3***
		ppm			
GalNAc-ol	H-2	4.392	4.389	4.379	4.379
	H-3	4.063	4.073	4.058	4.068
	H-4	3.508	3.498	n.d.	3.524
	H-5	4.195	4.189	4.242	4.238
	H-6b	n.d.	n.d.	n.d.	3.472
	NAc	2.050	2.046	2.046	2.042
Gal	H-1	4.477	4.546	4.474	4.542
	H-2	3.564	n.d.	n.d.	n.d.
	H-3	n.d.	4.125	n.d.	4.118
	H-4	3.901	3.931	n.d.	3.926
Neu5Ac(α2-6)	H-3a	-	-	1.692	1.691
	H-3e	-	-	2.730	2.724
	NAc	-	-	2.034	2.031
Neu5Ac(α2-3)	H-3a	-	1.809	-	1.799
	H-3e	-	2.773	-	2.774
	NAc	-	2.034	-	2.031

* Neu5Ac(α2-6)
\
GalNAc-ol
/
Neu5Ac(α2-3)Gal(β1-3)

Chemical shifts are given at 300K and were measured in ^2H_2O at p^{2}H7 relative to internal acetone (δ 2.225; Vliegenthart *et al.*, 1983). Compounds are represented by short-hand symbolic notation: △, Neu5Ac(α2-3); ○, Neu5Ac(α2-6); ◇, GalNAc; ■, Gal. n.d., not determined.

GalNAc-ol
/
Gal(β1-3) **O.1**

GalNAc-ol
/
Neu5Ac(α2-3)Gal(β1-3) **O.2A**

Neu5Ac(α2-6)
\
GalNAc-ol
/
Gal(β1-3) **O.2B**

Neu5Ac(α2-6)
\
GalNAc-ol
/
Neu5Ac(α2-3)Gal(β1-3) **O.3**

Discussion

In 1985 an ^{1}H NMR analysis of the carbohydrate chains of native hC1INH, purified from normal serum, was reported (Strecker *et al.*, 1985), and, following our coding system, compounds **N2.1**, **N2.2**, and **O.2A**, and the (α2-3)-disialylated isomers of **N2.1** and **N2.2** were shown to occur. In view of the followed isolation protocol it is tempting to assume that only major components were identified.

Additional profiling studies (Perkins *et al.*, 1990) indicated that besides sialylated complex-type diantennary, also low amounts of tri- and tetraantennary N-glycans do occur. Lectin-based assays revealed the occurrence of (α2-6)/(α2-3)-sialylated complex- and hybrid-type N-glycans (Schoenberger, 1992). Recently, biologically active rhC1INH with mainly oligomannose-type N-glycans has been expressed in a baculovirus expression system (Wolff *et al.*, 2001).

Here, we focus on the N,O-glycosylation pattern of rhC1INH, the transgenic material expressed in the mammary gland of transgenic rabbits, excreted in the milk. A first inspection of the results learns that oligomannose-, hybrid-, and diantennary complex-type N-glycans do occur. When sialylated, only Neu5Ac is present, and only (α2-6) linkages are found. Part of the complex-type N-glycans contains the Lewis x epitope. The small core 1-type O-glycans show the usual (α2-3)- and (α2-6)-sialylation pattern of O-glycoproteins of nonmucinous origin. Neu5Gc, found in low amounts in CHO- and BHK-expressed recombinant glycoproteins (Hokke *et al.*, 1990; Nimtz *et al.*, 1993) and suggested to occur in transgenic human antithrombin produced in goat milk (Edmunds *et al.*, 1998), was not observed on the rhC1INH glycans. This sialic acid is not expressed in normal adult human cells; higher levels of Neu5Gc may lead to immune reactions in humans. In this context, it should be noted that N-glycans of CHO- and BHK-derived recombinant glycoproteins contain (α2-3)-linked sialic acid only. Another well-documented immunogenic carbohydrate antigen, Gal(α1-3)Gal(β1-4)GlcNAc(β1- (Hamadeh *et al.*, 1992), was also not detected in rhC1INH.

In Table IV a survey is presented of the amounts in molar percentages of the N- and O-glycans established. The neutral N-glycans in rhC1INH make up about 25% of the total N-glycan pool. The majority of these glycans were of the oligomannose- and hybrid-type, with $Man_5GlcNAc_2$ being the most abundant structure, accounting for about 10% of the total N-glycan pool. About 75% of the N-glycans were sialylated. The monocharged N-glycans (about 67% of the total N-glycan pool) were of the hybrid- and mono- or diantennary complex type. Here, the major structures were the monosialylated, partially (α1-6)-fucosylated diantennary N-glycans (about 30% of the total N-glycan pool). The dicharged N-glycans (about 8% of the total N-glycan pool) comprised disialylated, partially (α1-6)-fucosylated diantennary N-glycans. In this context it is of interest to mention that with increasing expression levels of rhC1INH, the ratio of oligomannose-type:hybrid-type N-glycans increases and that a decrease in the extent of sialylation occurs with the progress of lactation (Koles *et al.*, unpublished data).

An evaluation of the N-glycosylation pattern found for rhC1INH indicates that several of the oligomannose- and hybrid-type glycans contained a Man-**D_2** residue, which is rather unusual for mature glycoproteins. Following the major pathway in the trimming of glycoprotein N-glycans from $Glc_3Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, the three Glc residues are removed by the endoplasmic reticulum (ER)–resident glucosidases I and II and Man-**D_2** by a Golgi-localized α-mannosidase I, yielding a specific $Man_8GlcNAc_2$ isomer. Two other alternate early processing

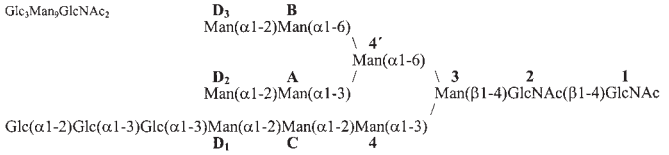
Table IV. Survey of N- and O-glycans obtained from rhC1INH, together with their relative amounts

Structure N-glycans	Code	Amount (%)
	**N0.1**	2
	**N0.2**	9
	**N0.3.2**	1
	**N0.3.3**	4
	**N0.3.4A**	<1
	**N0.3.4B**	1
	**N0.4.2**	2
	**N0.4.4**	2
	**N0.4.5**	1
	**N0.5**	3
	**N1.1**	4
	**N1.2**	8
	**N1.3**	10
	**N1.4**	1
	**N1.5A**	21
	**N1.5B**	7
	**N1.6A**	12
	**N1.6B**	4
	**N2.1**	3
	**N2.2**	5

Structure O-glycans	Code	Amount (%)
	**O.1**	28
	**O.2A**	10
	**O.2B**	21
	**O.3**	41

Compounds are represented by shorthand symbolic notation (see Tables I–III).

routes to $Man_8GlcNAc_2$ isomers comprise the removal of Man-**D_2** by ER-localized α-mannosidase I or of Man-**D_3** by ER-localized α-mannosidase II. In the Golgi (or partially in the ER), specific α-mannosidases release the remaining Man-**D** residues and Man-**C** (Weng and Spiro, 1993, 1996; Verbert, 1995).



The finding of $Man_8GlcNAc_2$ isomer **N0.4.5**, containing Man-**D_2** and Man-**D_3** indicate that in the mammary gland of the rabbit the alternative *endo*-mannosidase pathway (More and Spiro, 1990) is competetively active. Here a Glc(α1-3)Man disaccharide is released by a Golgi-localized *endo*-α-mannosidase yielding $Man_8GlcNAc_2$ missing Man-**D_1**, followed by a sequential cleavage of Man-**D_3** and Man-**C** by Golgi-localized α-mannosidases (Verbert, 1995), yielding the $Man_6GlcNAc_2$ isomer **N0.3.3**, the second most abundant oligomannose-type structure in this study (a product that also could arise from the ER-α-mannosidase II processing). Interestingly, the *endo*-mannosidase pathway seems to be an active pathway in the mammary glands of cow, goat, sheep, and rhesus monkey, too, as lactoferrins from these animals contain, in addition to $Man_9GlcNAc_2$, the $Man_8GlcNAc_2$ structure **N0.4.5** (Van Halbeek *et al*., 1981; Coddeville *et al*., 1992; Montreuil *et al*., 1997). Usually the *endo*-mannosidase pathway, a processing with one exception so far only observed in vertebrates (Dairaku and Spiro, 1997), is followed for circumventing α-glucosidase blockades (Moore and Spiro, 1990; De Praeter *et al*., 2000) but can also be induced by inhibiting ER/Golgi-localized α-mannosidases I, thereby blocking the formation of $Man_8GlcNAc_2$ missing Man-**D_2** (Weng and Spiro, 1996). The reason why the mammary tissue is using partly the *endo*-mannosidase pathway remains to be clarified.

Comparing the N-glycosylation patterns of native serum hC1INH and rhC1INH (Table IV), it can be concluded that the total processing in native serum hC1INH (only **N2.1/N2.2** and the [α2-3]-sialylated isomers) is much more complete than in rhC1INH (**N2.1/N2.2** make up about 10% of the total N-glycan pool). Comparing the O-glycosylation patterns of native serum hC1INH and rhC1INH (Table IV), it is remarkable that the only structure (**O.2A**) present in native material is a minor one (about 10% of the total O-glycan pool) in the recombinant material. In rhC1-INH the (α2-6)-sialylated extensions of **O.1** and **O.2A** are the major products (about 60% of the total O-glycan pool). Taking together, when compared with native serum hC1-INH, in terms of sialylation the N-glycans of rhC1INH are (α2-6)-undersialylated [Gal(β1-4)GlcNAc α-2,6-sialyltransferase/ST6Gal I], whereas the O-glycans are (α2-6)-oversialylated (GalNAc α-2,6-sialyltransferase/ST6GalNAc II]. Biological activity studies of rhC1INH are in progress and will be published elsewhere.

The detailed analysis of the N,O-glycosylation pattern of rhC1INH, revealing remarkable differences in glycosylation pattern between native serum hC1INH and rhC1INH, makes clear the importance of such studies for therapeutic glycoproteins when expressed in new biological systems. Earlier research has demonstrated the usefulness of studying the glycosylation machinery of CHO and BHK cells, and similar research has to be set up to explore the glycosylation machinery of the mammary gland of animals used for transgenic purposes.

Materials and methods

Release and isolation of N-glycans

Purified rhC1INH, isolated from pooled milk of line 2972p, was obtained from Pharming Technologies BV (Leiden, Netherlands). The N-glycans of rhC1INH were enzymically released with PNGase F (EC 3.5.1.52) (Roche Molecular Biochemicals, Indianapolis, IN), using incubation conditions optimized for rhC1INH. Briefly, 100 mg rhC1INH, dissolved in 5 ml 20 mM PBS pH 7.2 (Fluka, Buchs, Switzerland) containing 10 mM ethylenediamine tetraacetic acid, was denatured in the presence of 10% sodium dodecyl sulfate (w/v) and 10 mM 2-mercaptoethanol for 5 min at 100°C, then digested with 5 U/mg PNGase F in the presence of 6.4 mg/ml 3-[(3-cholamidodopropyl)dimethylammonio]-1-propanesulfonate (Fluka) for 24 h at 37°C. Portions of the digest were filtered through 30 kDa cut-off centrifugal concentrators and the filtrates, containing the released N-glycans, desalted on graphitized carbon columns (Alltech, Breda, the Netherlands) (Packer *et al*., 1998) after removal of detergents by Calbiosorb (Calbiochem, San Diego, CA) beads. The concentrated N-deglycosylated glycoprotein (~0.4 ml) was then extensively dialyzed and subjected to de-O-glycosylation.

Release and isolation of O-glycans

N-deglycosylated rhC1INH was subjected to alkaline borohydride treatment according to Piller and Piller (1993). Briefly, the concentrated N-deglycosylated glycoprotein solution (~0.4 ml) was mixed with 10 ml 0.1 M NaOH containing 1 M $NaBH_4$, and kept for 16 h at 45°C, then cooled on ice and neutralized with 2 M aqueous HOAc. Boric acid was removed by repetitive coevaporation with MeOH, containing 1% (v/v) HOAc. Finally, the material was applied to a Dowex 50W-X8, H^+ column (100–200 mesh, Pharmacia), and O-glycans (oligosaccharide-alditols) were eluted with water and lyophilized. When required, samples were further purified on graphitized carbon columns (Packer *et al*., 1998).

FPLC fractionation

The pools of N- and O-glycans were fractionated into neutral and charged species on a Resource Q column (6 ml, Pharmacia, Uppsala, Sweden) using a Pharmacia FPLC system. Elutions were performed with water followed by a linear concentration gradient of NaCl in water at a

flow rate of 4 ml/min; for gradient details, see relevant figure captions. Fractionations were monitored by UV absorbance at 214 nm and conductivity measurements. Individual fractions were lyophilized, desalted on 5 connected HiTrap columns (5 ml, Amersham, Little Chalfont, U.K.) using 5 mM NH_4HCO_3 as eluent, and lyophilized again.

HPLC fractionation

The FPLC fraction of neutral N-glycans was further fractionated on a LiChrospher-NH_2 5 μm (250 mm × 4.6 mm, Alltech) column equipped with a LiChrospher Amino 5 μm guard column (7.5 × 4.6 mm), using a Waters 600 HPLC system. Elutions were carried out with a linear gradient of water in acetonitrile at a flow rate of 1 ml/min; for gradient details, see relevant figure captions. The fractionation was monitored by UV absorbance at 206 nm. Individual fractions were lyophilized.

Labeling of N-glycans with 2AB

Neutral N-glycans from the HPLC fractionation were treated with 0.35 M 2AB (Sigma)/1 M $NaCNBH_3$ in Me_2SO–HOAc (7:3, v/v) for 2 h at 65°C (Bigge *et al.*, 1995; Stroop *et al.*, 2000). The 2AB fluorescently labeled glycans were purified via paper chromatography on acid-pretreated QMA (Whatman) filter paper strips using acetonitrile (three times) as a mobile phase. Glycans (remaining at the base line) were eluted from the dried paper strips with water, concentrated, and subfractionated by HPAEC.

HPAEC fractionation

HPAEC was performed on a Dionex DX 500 workstation equipped with a pulsed amperometric detection (PAD) system. A series of N-glycan fractions were subfractionated on a CarboPac PA-1 (250 × 9 mm) column using linear gradients of 0.5 M NaOAc in 0.1 M NaOH at a flow rate of 4 ml/min; for gradient details, see relevant figure captions. The following pulse potentials and durations were used during the triple-pulse amperometric detection with a gold electrode at 300 mA: E_1 0.05 V, 480 ms; E_2 0.60 V, 120 ms; E_3 −0.60 V, 60 ms. Fractions were immediately neutralized with 0.1 M HCl, then lyophilized, desalted on five connected HiTrap columns (5 ml, Amersham Pharmacia) using 5 mM NH_4HCO_3 as eluent, and lyophilized again.

Quantification of oligosaccharides

The molar ratio of the FPLC fractions was calculated from the FPLC peak areas on the basis of the weighted average number of C=O groups (responsible for UV absorption at 214 nm) being known after structural identification and determination of the relative amounts of each individual component. The molar ratio of the constituent oligosaccharides within each FPLC fraction was determined from the HPLC peak areas (corrected for the number of C=O groups) at 206 nm. When HPAEC/PAD was used for further fractionation of HPLC fractions, the PAD response was assumed to be equal for each oligosaccharide present within one HPLC fraction. For mixtures, molar ratios were determined on the basis of the ^{1}H NMR spectra.

^{1}H NMR spectroscopy

Prior to ^{1}H NMR spectroscopy, samples were lyophilized twice from 99.9% ^2H_2O (Cambridge Isotope Laboratories, Andover, MA), then dissolved in 99.96% ^2H_2O (Cambridge Isotope Laboratories). ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 300K and p^{2}H 7. 1D spectra of 5000 Hz spectral width were recorded in 16K complex data sets using a water eliminated Fourier transform pulse sequence as described by Hård *et al.* (1992). Chemical shifts are expressed in ppm relative to internal acetone (δ 2.225 in ^2H_2O) or acetate (δ 1.908 in ^2H_2O) (Vliegenthart *et al.*, 1983). 2D-TOCSY spectra at 500 MHz were recorded using Bruker software with MLEV-17 mixing sequence cycles between 20 and 100 ms. Data matrices of 512 × 2048 points were collected, representing a spectral width of 4800 Hz in each dimension. The $^2HO^1H$ signal was suppressed by presaturation for 1 s during the relaxation delay. 2D-ROESY spectra were recorded with a mixing time of 300 ms. Phase-sensitive handling of data was performed by the time-proportional phase increment method implemented by the Bruker software. NMR data were processed using a locally developed software package (J. A. van Kuik, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

MS

For MALDI-TOF-MS in the positive-ion mode, samples (0.5–1 μl) were mixed in a 1:1 ratio with a mixture of 5 mg/ml 2,5-dihydroxybenzoic acid and 0.25 mg/ml 5-methoxysalicylic acid in 1 ml ethanol/10 mM NaCl (1:1, v/v) as a matrix. For the negative-ion mode, 2 mg/ml 2′,4′, 6′-trihydroxyacetophenone monohydrate in acetonitrile/13.3 mM ammonium citrate (1:3, v/v) was used as a matrix. In this case the sample matrix mixture was dried under reduced pressure (Papac *et al.*, 1998). Measurements were performed on a PerSeptive Biosystems Voyager-DE MALDI-TOF mass spectrometer with implemented delayed extraction technique using an N_2 laser (337 nm) with 3 ns pulse width. Spectra were recorded in a linear mode at an accelerating voltage of 24.5 kV using an extraction delay of 90 ns.

Acknowledgments

This study was financially supported by the Technology Foundation STW of the Netherlands Organization for Scientific Research NWO and by Pharming Technologies BV (Leiden, Netherlands).

Abbreviations

2AB, 2-aminobenzamide; BHK, baby hamster kidney; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; hC1INH, human C1 inhibitor; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry;

NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; rhC1INH, recombinant-rabbit human C1 inhibitor; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy.

References

Bendiak, B., Orr, J., Brockhausen, I., Vella, G., and Phoebe, C. (1988) Separation of neutral reducing oligosaccharides derived from glycoproteins by HPLC on a hydroxylated polymeric support. *Anal. Biochem.*, **175**, 96–105.

Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.N., Charles, S.M., and Parekh, R.B. (1995) Nonselective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. *Anal. Biochem.*, **230**, 229–238.

Bijvoet, A.G.A., Kroos, M.A., Pieper, F.R., de Boer, H.A., Reuser, A.J.J., van der Ploeg, A.T., and Verbeet, M.P. (1996) Expression of cDNA-encoded human acid α-glucosidase in milk of transgenic mice. *Biochim. Biophys. Acta*, **1308**, 93–96.

Bock, S.C., Skriver, K., Nielsen, E., Thøgersen, H.-C., Wiman, B., Donaldson, V.H., Eddy, R.L., Marrinan, J., Radziejewska, E., Huber, R., and others. (1986) Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. *Biochemistry*, **25**, 4292–4301.

Caliezi, C., Wuillemin, W.A., Zeerleder, S., Redondo, M., Eisele, B., and Hack, C.E. (2000) C1-esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol. Rev.*, **52**, 91–112.

Carreer, F.M. (1992) The C1 inhibitor deficiency. A review. *Eur. J. Clin. Chem. Clin. Biochem.*, **30**, 793–807.

Carugati, A., Pappalardo, E., Zingale, L.C., and Cicardi, M. (2001) C1-inhibitor deficiency and angioedema. *Mol. Immunol.*, **38**, 161–173.

Coddeville, B., Strecker, G., Wieruszeski, J.-M., Vliegenthart, J.F.G., van Halbeek, H., Peter-Katalinic, J., Egge, H., and Spik, G. (1992) Heterogeneity of bovine lactotransferrin glycans. Characterization of α-D-Gal*p*-(1 → 3)-β-D-Gal- and α-NeuAc-(2 → 6)-β-D-Gal*p*NAc-(1 → 4)-β-D-GlcNAc-substituted N-linked glycans. *Carbohydr. Res.*, **236**, 145–164.

Colman, A. (1996) Production of proteins in the milk of transgenic livestock: problems, solutions, and successes. *Am. J. Clin. Nutr.*, **63**, 639S–645S.

Dairaku, K., and Spiro, R.G. (1997) Phylogenetic survey of endomannosidase indicates late evolutionary appearance of this N-linked oligosaccharide processing enzyme. *Glycobiology*, **7**, 579–586.

Damm, J.B.L., Kamerling, J.P., van Dedem, G.W.K., and Vliegenthart, J.F.G. (1987) A general strategy for the isolation of carbohydrate chains from *N*-,*O*-glycoproteins and its application to human chorionic gonadotropin. *Glycoconj. J.*, **4**, 129–144.

Denman, J., Hayes, M., O'Day, C., Edmunds, T., Bartlett, C., Hirani, S., Ebert, K.M., Gordon, K., and McPherson, J.M. (1991) Transgenic expression of a variant of human tissue-type plasminogen activator in goat milk: purification and characterization of the recombinant enzyme. *Bio/Technology*, **9**, 839–843.

De Praeter, C.M., Gerwig, G.J., Bause, E., Nuytinck, L.K., Vliegenthart, J.F.G., Breuer, W., Kamerling, J.P., Espeel, M.F., Martin, J.-J.R., De Paepe, A.M., Shows, T.B., and Magnusson, S. (2000) A novel disorder caused by defective biosynthesis of N-linked oligosaccharides due to glucosidase I deficiency. *Am. J. Hum. Genet.*, **66**, 1744–1756.

De Waard, P., Koorevaar, A., Kamerling, J.P., and Vliegenthart, J.F.G. (1991) Structure determination by ^{1}H NMR spectroscopy of (sulfated) sialylated *N*-linked carbohydrate chains released from porcine thyroglobulin by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F. *J. Biol. Chem.*, **266**, 4237–4243.

Ebo, D.G. and Stevens, W.J. (2000) Hereditary angioneurotic edema: review of the literature. *Acta Clin. Belg.*, **55**, 22–29.

Edmunds, T., van Patten, S.M., Pollock, J., Hanson, E., Bernasconi, R., Higgins, E., Manavalan, P., Ziomek, C., Meade, H., McPherson, J.M., and Cole, E.S. (1998) Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. *Blood*, **91**, 4561–4571.

Galet, C., Menck Le Bourhis, C., Chopineau, M., Le Griec, G., Perrin, A., Magallon, T., Attal, J., Viglietta, C., Houdebine, L.M., and Guillou, F. (2001) Expression of a single βα chain protein of equine LH/CG in milk of transgenic rabbits and its biological activity. *Mol. Cell. Endocrinol.*, **174**, 31–40.

Hamadeh, R.M., Jarvis, G.A., Galili, U., Mandrell, R.E., Zhou, P., and Griffiss, J.M. (1992) Human natural anti-Gal IgG regulates alternative complement pathway activation on bacterial surfaces. *J. Clin. Invest.*, **89**, 1223–1235.

Hård, K., Mekking, A., Kamerling, J.P., Dacremont, G.A.A., and Vliegenthart, J.F.G. (1991) Different oligosaccharides accumulate in the brain and urine of a cat with α-mannosidosis: structure determination of five brain-derived and seventeen urinary oligosaccharides. *Glycoconj. J.*, **8**, 17–28.

Hård, K., van Zadelhoff, G., Moonen, P., Kamerling, J.P., and Vliegenthart, J.F.G. (1992) The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel *N*-acetylgalactosamine-containing N-linked carbohydrate chains. *Eur. J. Biochem.*, **209**, 895–915.

Hokke, C.H., Bergwerff, A.A., van Dedem, G.W.K., van Oostrum, J., Kamerling, J.P., and Vliegenthart, J.F.G. (1990) Sialylated carbohydrate chains of recombinant human glycoproteins expressed in Chinese hamster ovary cells contain traces of *N*-glycolylneuramic acid. *FEBS Lett.*, **275**, 9–14.

Houdebine, L.M. (1995) The production of pharmaceutical proteins from the milk of transgenic animals. *Reprod. Nutr. Dev.*, **35**, 609–617.

Jenkins, N. and Curling, E.M.A. (1994) Glycosylation of recombinant proteins: problems and prospects. *Enzyme Microbial Technol.*, **16**, 354–364.

Jenkins, N., Parekh, R.B., and James, D.C. (1996) Getting the glycosylation right: Implications for the biotechnology industry. *Nature Biotechnol.*, **14**, 975–981.

Kamerling, J.P. (1996) Carbohydrate features of recombinant human glycoproteins. *Biotecnologia Aplicada*, **13**, 167–180.

Kamerling, J.P. and Vliegenthart, J.F.G. (1989) Carbohydrates. In Lawson, A.M. (Ed.) *Clinical biochemistry; principles, methods, applications, vol. 1, Mass spectrometry*. Walter de Gruyter, Berlin, pp. 175–263.

Kamerling, J.P. and Vliegenthart, J.F.G. (1992) High-resolution ^{1}H-nuclear magnetic resonance spectroscopy of oligosaccharide-alditols released from mucin-type *O*-glycoproteins. *Biol. Magn. Reson.*, **10**, 1–194.

Korhonen, V.-P., Tolvanen, M., Hyttinen, J.-M., Uusi-Oukari, M., Sinervirta, R., Alhonen, L., Jauhiainen, M., Jänne, O.A., and Jänne, J. (1997) Expression of bovine β-lactoglobulin/human erythropoietin fusion protein in the milk of transgenic mice and rabbits. *Eur. J. Biochem.*, **245**, 482–489.

Michalski, J.-C., Haeuw, J.-F., Wieruszeski, J.-M., Montreuil, J., and Strecker, G. (1990) In vitro hydrolysis of oligomannosyl oligosaccharides by the lysosomal α-D-mannosidases. *Eur. J. Biochem.*, **189**, 369–379.

Montreuil, J., Spik, G., and Mazurier, J. (1997) Transferrin superfamily. An outstanding model for studying biochemical evolution. In Montreuil, J., Vliegenthart, J.F.G., and Schachter, H. (Eds.), *Glycoproteins II*. Elsevier Science, Amsterdam, pp. 203–242.

Moore, S.E.H. and Spiro, R.G. (1990) Demonstration that Golgi endo-α-D-mannosidase provides a glucosidase-independent pathway for the formation of complex *N*-linked oligosaccharides of glycoproteins. *J. Biol. Chem.*, **265**, 13104–13112.

Mulder, H., Dideberg, F., Schachter, H., Spronk, B.A., de Jong-Brink, M., Kamerling, J.P., and Vliegenthart, J.F.G. (1995) In the biosynthesis of N-glycans in connective tissue of the snail *Lymnaea stagnalis* incorporation of GlcNAc by β2GlcNAc-transferase I is an essential prerequisite for the action of β2GlcNAc-transferase II and β2Xyl-transferase. *Eur. J. Biochem.*, **232**, 272–283.

Nimtz, M., Martin, W., Wray, V., Klöppel, K.-D., Augustin, J., and Conradt, H.S. (1993) Structures of sialylated oligosaccharides of human erythropoietin expressed in recombinant BHK-21 cells. *Eur. J. Biochem.*, **213**, 39–56.

Nuijens, J.H., van Berkel, P.H.C., Geerts, M.E.J., Hartevelt, P.P., de Boer, H.A., van Veen, H.A., and Pieper, F.R. (1997) Characterization of recombinant human lactoferrin secreted in milk of transgenic mice. *J. Biol. Chem.*, **272**, 8802–8807.

Packer, N.H., Lawson, M.A., Jardine, D.R., and Redmond, J.W. (1998) A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconj. J.*, **15**, 737–747.

Papac, D.I., Briggs, J.B., Chin, E.T., and Jones, A.J.S. (1998) A high-throughput microscale method to release N-linked oligosaccharides from glycoproteins for matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis. *Glycobiology*, **8**, 445–454.

Perkins, S.J. (1993) Three-dimensional structure and molecular modelling of C1 inhibitor. *Behring Inst. Mitt.*, **93**, 63–80.

Perkins, S.J., Smith, K.F., Amatayakul, S., Ashford, D., Rademacher, T.W., Dwek, R.A., Lachmann, P.J., and Harrison, R.A. (1990) Two-domain structure of the native and reactive centre cleaved forms of C1 inhibitor of human complement by neutron scattering. *J. Mol. Biol.*, **214**, 751–763.

Piller, F. and Piller, V. (1993). Structural characterization of mucin-type *O*-linked oligosaccharides. In Fukuda, M. and Kobata, A. (Eds.), *Glycobiology, a practical approach*. Oxford University Press, New York, pp. 291–328.

Schoenberger, O.L. (1992) Characterization of carbohydrate chains of C1-inhibitor and of desialylated C1-inhibitor. *FEBS Lett.*, **314**, 430–434.

Spellman, M.W., Leonard, C.K., Basa, L.J., Gelineo, I., and van Halbeek, H. (1991) Carbohydrate structures of recombinant soluble human CD4 expressed in Chinese hamster ovary cells. *Biochemistry*, **30**, 2395–2406.

Strecker, G., Ollier-Hartmann, M.-P., van Halbeek, H., Vliegenthart, J.F.G., Montreuil, J., and Hartmann, L. (1985) Primary structure elucidation of carbohydrate chains of normal C1-esterase inhibitor (C1-INH) by 400-MHz ^{1}H-NMR study. *CR. Acad. Sci. Paris*, **301**, 571–576.

Stroop, C.J.M., Weber, W., Gerwig, G.J., Nimtz, M., Kamerling, J.P., and Vliegenthart, J.F.G. (2000) Characterization of the carbohydrate chains of the secreted form of the human epidermal growth factor receptor. *Glycobiology*, **10**, 901–917.

Tseneklidou-Stoeter, D., Gerwig, G.J., Kamerling, J.P., and Spindler, K.-D. (1995) Characterization of N-linked carbohydrate chains of the crayfish, *Astacus leptodactylus* hemocyanin. *Biol. Chem. Hoppe-Seyler*, **376**, 531–537.

Uusi-Oukari, M., Hyttinen, J.-M., Korhonen, V.-P., Västi, A., Alhonen, L., Jänne, O.A., and Jänne, J. (1997) Bovine α_{S1}-casein gene sequences direct high level expression of human granulocyte-macrophage colony-stimulating factor in the milk of transgenic mice. *Transgenic Res.*, **6**, 75–84.

Van Berkel, P.H.C., Welling, M.M., Geerts, M., van Veen, H.A., Ravensbergen, B., Salaheddine, M., Pauwels, E.K.J., Pieper, F., Nuijens, J.H., and Nibbering, P.H. (2002) Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nature Biotechnol.*, **20**, 484–487.

Van Halbeek, H., Dorland, L., Vliegenthart, J.F.G., Spik, G., Cheron, A., and Montreuil, J. (1981) Structure determination of two oligomannoside-type glycopeptides obtained from bovine lactotransferrin, by 500 MHz ^{1}H-NMR spectroscopy. *Biochim. Biophys. Acta*, **675**, 293–296.

Van Pelt, J., Hård, K., Kamerling, J.P., Vliegenthart, J.F.G., Reuser, A.J.J., and Galjaard, H. (1989) Isolation and structural characterization of twenty-one sialyloligosaccharides from galactosialidosis urine. An intact *N*,*N*′-diacetylchitobiose unit at the reducing end of a diantennary structure. *Biol. Chem. Hoppe-Seyler*, **370**, 191–203.

Van Rooijen, J.J.M., Jeschke, U., Kamerling, J.P., and Vliegenthart, J.F.G. (1998) Expression of N-linked sialyl Le^x determinants and O-glycans in the carbohydrate moiety of human amniotic fluid transferrin during pregnancy. *Glycobiology*, **8**, 1053–1064.

Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J. (1999) *Essentials of glycobiology.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Verbert, A. (1995). Biosynthesis. 2b. From $Glc_3Man_9GlcNAc_2$-protein to $Man_5GlcNAc_2$-protein: transfer "en bloc" and processing. In Montreuil, J., Vliegenthart, J.F.G., and Schachter, H. (Eds.), *Glycoproteins*. Elsevier Science, Amsterdam, pp. 145–152.

Visser, E.C., Dijkstra, R., Brakenhoff, J.P.J., van Corven, E.J.J.M., and van Berkel, P.H.C. (2000) Analysis of the glycosylation of recombinant human acid α-glucosidase produced in the milk of transgenic rabbits. *Glycobiology*, **10**, 1133.

Vliegenthart, J.F.G., Dorland, L., and van Halbeek, H. (1983) High-resolution, ^{1}H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.*, **41**, 209–374.

Weng, S. and Spiro, R.G. (1993) Demonstration that a kifunensine-resistant α-mannosidase with a unique processing action on *N*-linked oligosaccharides occurs in rat liver endoplasmic reticulum and various cultured cells. *J. Biol. Chem.*, **268**, 25656–25663.

Weng, S. and Spiro, R.G. (1996) Evaluation of the early processing routes of N-linked oligosaccharides of glycoproteins through the characterization of $Man_8GlcNAc_2$ isomers: evidence that endomannosidase functions *in vivo* in the absence of a glucosidase blockade. *Glycobiology*, **6**, 861–868.

Wolff, M.W., Zhang, F., Roberg, J.J., Caldwell, E.E.O., Kaul, P.R., Serrahn, J.N., Murhammer, D.W., Linhardt, R.J., and Weiler, J.M. (2001) Expression of C1 esterase inhibitor by the baculovirus expression vector system: preparation, purification, and characterization. *Protein Expr. Purif.*, **22**, 414–421.