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N- and *O*-Glycosylation of a Commercial Bovine Whey Protein Product

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Supporting Information

ABSTRACT: Bovine whey protein products are used as a base ingredient in infant formulas to optimize the amino acid pattern to a more human-like composition. Although the protein composition of bovine milk has been studied in detail, glycosylation details of commercial whey protein products are missing. To this end, both the *N*- and *O*-glycans of such a protein concentrate were sequentially released, the *N*-glycans enzymatically and the *O*-glycans chemically (reducing and nonreducing conditions). For the structural analysis of the *N*- and *O*-glycans a combination of MALDI-TOF-MS, one-dimensional ^{1}H NMR spectroscopy, *Wisteria floribunda* agglutinin affinity chromatography, HPAEC-PAD profiling, and HPLC-FD profiling (2-aminobenzamide derivatives), together with exoglycosidase treatments, were used. A mixture of over 60 *N*-glycans and 10 *O*-glycans was characterized, giving a detailed insight into the glycosylation of a bovine whey protein product, Deminal 90, which is applied as an ingredient for infant formulas.

KEYWORDS: *milk proteomics, α-galactose, N-acetylgalactosaminyl-(β1−4)-N-acetylglucosamine (LacdiNAc), N-glycolylneuraminic acid*

INTRODUCTION

Milk is a complex biological matrix, containing all nutritional components required for the healthy growth of the mammalian infant. Although the World Health Organization considers breastfeeding to be the best source of infant nutrition, modern bovine-milk-based infant formulas are good substitutes when mother's milk is not available. However, the differences in the composition of human and bovine milk are significant.[1−3] To improve the balance in protein composition for infant formulations, bovine whey protein products are used.

The *N*-glycosylation of specific human milk glycoproteins has been studied in different laboratories;[4−7] the same holds for the *O*-glycosylation.[4,8] For bovine milk, the protein *N*-glycosylation has been investigated for milk-fat-globule membrane proteins,[9] immunoglobulin G,[10] PP3 component,[11] lactophorin,[6] α-lactalbumin,[12] and lactoferrin.[4,13,14] Recently, a study on proteins of early lactation bovine milk was reported[15] using MALDI-TOF/TOF-MS approaches to monitor changes in time of *N*-glycan classes (di-, tri-, tetra-antennary chains). The *O*-glycosylation of bovine milk glycoproteins has been well studied for κ-casein[16] and milk-fat-globule membrane proteins.[17] A more comprehensive overview of all relevant literature known to the authors is presented in Table S1, Supporting Information. Besides glycosylation, proteins can be glycated as well as a result of the heat load during processing,[18] this process is also known as the Maillard reaction.

Until now a detailed analysis of whey protein N- and O-linked glycosylation in infant formulas has not been performed. In this paper, we describe the *N*- and *O*-glycosylation analysis of a commercial bovine cheese whey protein product, Deminal 90. Although the processing of Deminal 90 involves steps likely to result in glycation products, we did not study this chemical modification. The used protocols for *N*- and *O*-glycan analysis include normal-phase high-performance liquid chromatography (NP-HPLC) of 2-aminobenzamide (2AB)-labeled *N*- and *O*-glycans in combination with exoglycosidases and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), one-dimensional ^{1}H nuclear magnetic resonance (NMR) spectroscopy, weak-anion-exchange high performance liquid chromatography (WAX-HPLC), and high-pH anion-exchange chromatography (HPAEC) of *O*-glycan alditols. To reduce the complexity of the *N*-glycan pool to be analyzed, a recently developed *Wisteria floribunda* agglutinin (WFA) affinity protocol that creates subfractions based on the presence or absence of (sialylated) LacdiNAc sequences, was applied.[14]

EXPERIMENTAL PROCEDURES

Materials. A commercial bovine cheese whey protein product (Deminal 90, a 90% demineralized cheese whey protein powder with 12.5% protein) was acquired from FrieslandCampina Domo (Amersfoort, The Netherlands). 2-Aminobenzamide (2AB) was purchased from Fluka (Buchs, Switzerland) and sodium cyanoborohydride from Sigma (St. Louis, MO). Reference 2AB-labeled *N*-glycans, a 2AB-labeled dextran ladder, a 2AB-labeled fetuin *N*-glycan

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standard kit, and a nonreducing (Ludger Liberate Orela) *O*-glycan release kit were purchased from Ludger Ltd. (Oxfordshire, UK).

Isolation of Bovine Milk Serum Protein Material from Commercial Concentrate. A 5% solution of Deminal 90 was fractionated on a 5 kDa ultrafiltration membrane at 10 °C to reduce the lactose content <1%$_{wt}$ of dry matter. The demineralized whey protein concentrate was lyophilized and contained 85% protein. A sample of 200 mg was dissolved in 2 mL of Milli-Q water and prefractionated on five connected 5 mL HiTrap columns, eluted with 15 mM ammonium bicarbonate (1.5-mL fractions). Protein-containing fractions (Bradford assay) were pooled and lyophilized. The isolated bovine milk serum protein pool was dissolved in 2 mL of phosphate-buffered saline, pH 7.4, containing 10 mM EDTA and 1% SDS. For the denaturation of the proteins, the solution was mixed with 100 μL of β-mercaptoethanol in 1 mL of 1% SDS and kept for 1 h at 85 °C. Then the denatured proteins were alkylated by treatment with 100 μL of 0.5 M iodoacetamide (30 min, 55 °C, darkness). Denatured, alkylated proteins were separated from lactose, salts, and lipids on a series of 5 × 5 mL HiTrap desalting columns (GE-Healthcare Benelux, Diegem, Belgium) using an ÄKTA FPLC system (Amersham Biosciences, Roosendaal, The Netherlands), eluted with 25 mM ammonium bicarbonate.

Monosaccharide Analysis. Duplicate samples of ∼1 mg of denatured, alkylated milk serum protein material were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C), followed by re-*N*-acetylation and trimethylsilylation.[19] The trimethylsilylated (methyl ester) methyl glycosides were analyzed by gas−liquid chromatography (GLC) on an EC-1 column (30 m × 0.32 mm; Alltech, Breda, The Netherlands), using a Chrompack CP-9002 gas chromatograph (Chrompack, Middelburg, The Netherlands; temperature program 140−240 °C, 4 °C/min). Monosaccharide derivative identities were confirmed by GLC-EI-MS (EI = electron impact) analysis on a Shimadzu QP2010 Plus system (Shimadzu, 's-Hertogenbosch, The Netherlands), equipped with an EC-1 column (30 m × 0.25 mm; Grace, Breda, The Netherlands) and the same temperature program.

Sialic Acid Analysis. First, 2 mg denatured, alkylated milk serum protein samples were hydrolyzed in 200 μL of 2 M propionic acid (3 h, 80 °C). Then after centrifugation, aliquots of 50 μL of supernatant were mixed with 50 μL of DMB reagent (7 mM 1,2-diamino-4,5-methylenedioxybenzene (Sigma), 18 mM sodium hydrosulfite, 750 mM β-mercaptoethanol in 1.5 M propionic acid) and left to react in the dark (2.5 h, 50 °C). HPLC analysis of the released, and derivatized sialic acids was performed on a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands), equipped with a reversed phase Cosmosil 5C18-AR-II column (250 mm × 4.6 mm, Waters, Eschborn, Germany). The elution was carried out with acetonitrile/methanol/water (9:7:84, v/v/v) as solvent system at a flow rate of 1 mL/min and monitored by fluorescence detection (Waters 474 fluorescence detector; λ_{ex} 373 nm, λ_{em} 448 nm).[20]

***N*-Glycan Release.** For the enzymatic release of *N*-glycans, a sample of 50 mg of denatured, alkylated milk serum protein material, mixed with Nonidet P40 substitute (final concentration 1%; Sigma), was incubated with 15 U of peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase F (PNGaseF) (EC 3.5.1.52) (Roche, Mannheim, Germany) per mg of protein (48 h, 37 °C) in two batches, i.e., 10 U/mg followed by 5 U/mg after 24 h. Released *N*-glycans were separated from de-*N*-glycosylated proteins on a Superdex 75PG HiLoad 26/60 column (Pharmacia, Uppsala, Sweden), fitted in an ÄKTA FPLC system. The obtained *N*-glycan pool was treated with CalBiosorb beads (CalBiochem, La Jolla, CA) to remove Nonidet P40 and SDS, and with Dowex 50W-X8, H^+ (Sigma), and then passed across a cotton-plugged Pasteur pipet to remove solids.

***O*-Glycan Release.** For the reductive release of *O*-glycans, a sample of 50 mg of denatured, alkylated, and de-*N*-glycosylated milk serum protein material was subjected to alkaline borohydride treatment. The protein material was dissolved in 4 mL of 0.1 M NaOH, containing 1 M $NaBH_4$, and stirred for 48 h at 37 °C. After neutralization of the solution on ice with 2 M acetic acid, boric acid was removed by repeated coevaporation with methanol under a stream of nitrogen. The generated *O*-glycan alditol pool was dissolved in 1 mL of Milli-Q water, and after centrifugation for 5 min at 10000*g*, the supernatant was desalted on a Bio-Gel P-2 column (30 cm × 1.0 cm; BioRad laboratories, Veenendaal, The Netherlands), eluted with 15 mM ammonium bicarbonate at a flow rate of 10 mL/h. The pooled carbohydrate fractions (orcinol-H_2SO_4 spottest) were lyophilized, and to remove residual salts, the sample was stirred overnight with Dowex 50W-X8, H^+ (100−200 mesh, Fluka) then passed across a cotton-plugged Pasteur pipet to remove solids. For the nonreductive release of *O*-glycans, a sample of 5 mg of denatured, alkylated, and de-*N*-glycosylated milk serum protein material was treated with a Ludger Liberate Orela *O*-glycan release kit according to the manufacturer specifications.

2AB Labeling of *N*- and *O*-Glycans. The lyophilized *N*-glycan pool (free oligosaccharides) was treated with 0.35 M 2-aminobenzamide (2AB) and 1 M sodium cyanoborohydride in dimethyl sulfoxide/acetic acid (7:3, v/v) for 2 h at 65 °C to yield 2AB-labeled products. The labeled glycans were purified by paper chromatography, using acetic-acid-treated QMA paper (Whatman) whereby the excess of 2AB was removed by washing with 8 × 500 μL acetonitrile and the glycan pool eluted with 5 × 500 μL Milli-Q water.[21,22] The lyophilized *O*-glycan pool (free oligosaccharides) was derivatized in a similar way.

Lectin Affinity Chromatography of 2AB-Labeled *N*-Glycans. The lyophilized, 2AB-labeled *N*-glycan pool was dissolved in 500 μL of 15 mM PBS, containing 0.15 M NaCl, pH 7.5 (binding buffer), and applied to an agarose-bound *Wisteria floribunda* agglutinin (WFA) column (2 mL bed volume; Vector Laboratories Ltd., Peterborough, UK). The lectin column was washed with 5 column volumes of binding buffer (fraction **I**), then eluted with 5 column volumes of 1% acetic acid in binding buffer (fraction **II**), followed by 5 column volumes of 50 mM GalNAc in binding buffer (fraction **III**). The column was reconstituted with 20 mM Tris·HCl, pH 4.5, containing 0.5 M NaCl, and stored in ethanol/20 mM Tris.HCl, pH 5.8, containing 1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ (2:8, v/v).[14] The fractions **I**, **II**, and **III** were desalted on Carbograph SPE columns, eluted with 40% acetonitrile, containing 0.05% trifluoroacetic acid.

Weak-Anion-Exchange-HPLC of 2AB-Labeled *N*-Glycans. 2AB-labeled *N*-glycan samples were analyzed by WAX-HPLC on a DEAE column (100 mm × 4.6 mm, 30 °C; Grace Vydac, Schoonebeek, The Netherlands), using a Waters 2690XE Alliance HPLC system, equipped with a Waters 474 fluorescence detector (λ_{ex} 330 nm, λ_{em} 420 nm). Separation of charge classes was achieved using a linear gradient of 0−250 mM ammonium formate in 20% acetonitrile. Identification of charged species was achieved by reference to 2AB-labeled fetuin *N*-glycan standards. Peak areas were used for quantification.

Normal Phase-HPLC Profiling of 2AB-Labeled *N*- and *O*-Glycans. 2AB-labeled *N*- and *O*-glycan samples were profiled by NP-HPLC on a TSKgel amide-80 column (150 mm × 4.6 mm, 3 μm particle size; Tosoh Bioscience GmbH, Stuttgart, Germany) at 40 °C, using a Waters 2690XE Alliance HPLC system, equipped with a Waters 474 fluorescence detector (λ_{ex} 330 nm, λ_{em} 420 nm). A 100 min gradient of 50 mM ammonium formate, pH 4.4, in acetonitrile (25−56%) was used at a flow rate of 0.8 mL/min, followed by a 3 min gradient to 100% 50 mM ammonium formate, which was kept for 5 min at 1 mL/min to clean the column, finally followed by a gradient to 25% ammonium formate, which was kept at 0.8 mL/min for 25 min to recondition the column. The column was calibrated with a 2AB-labeled external dextran ladder and reference 2AB-labeled *N*-glycans, and peak retention times of 2AB-labeled *N*-glycans were expressed in dextran-ladder-derived glucose units (GU) values. The *N*-glycan standards were used to correct GU values from the system used to values in Glycobase 2.0.[23] Peak areas were used for quantification.

Exoglycosidase Assays on 2AB-Labeled *N*- and *O*-Glycans. Digestions were performed overnight at 37 °C in 50 mM sodium acetate buffer, pH 5.5. The following enzymes were used: *Arthrobacter ureafaciens* α-sialidase (1 U/100 μL in 10 mM Na_3PO_4, 0.1% Micr-O-protect, pH 7, containing 0.25 mg/mL BSA; Roche), recombinant *Salmonella typhimurium* α-sialidase (5000 U/mL, suspension in 50 mM NaCl, 20 mM Tris.HCl, 5 mM Na_2EDTA, pH 7.5; New England Biosciences, Ipswich, MA; strong preference for cleaving Neu5Ac(α2−

3) linkages over Neu5Ac(α2−6) linkages), recombinant *Streptomyces plicatus* β-*N*-acetylhexosaminidase (500 U/100 μL; suspension in 50 mM NaCl, 5 mM Na_2EDTA, pH 7.5; New England Biosciences; affinity for cleaving GlcNAc(β1−3/4/6) and GalNAc(β1−4) linkages, leaving GlcNAc(β1−2) linkages intact), jack bean α-mannosidase (65 U/mL in 3.0 M $(NH_4)_2SO_4$, 0.1 mM zinc acetate, pH 7.5; Sigma), bovine testis β-galactosidase (0.2 U/100 μL in 3.2 M $(NH_4)_2SO_4$, pH 5.0; Sigma), green coffee bean α-galactosidase (20 U/100 μL in 3.2 M $(NH_4)_2SO_4$, pH 6.0, containing BSA; Sigma), and bovine kidney α-fucosidase (51 U/100 μL in 3.2 M $(NH_4)_2SO_4$, 10 mM NaH_2PO_4, 10 mM sodium citrate, pH 6.0; Sigma). After digestion, the enzymes were removed by 10 kDa cutoff centrifugal filters (Millipore, Amsterdam, The Netherlands).

High-pH Anion-Exchange Chromatography of *O*-Glycan Alditols. The *O*-glycan alditol pool was analyzed by HPAEC on a CarboPac PA-1 column (250 mm × 4 mm, Dionex BV, Amsterdam, The Netherlands), using an ICS3000 Ion chromatograph (Dionex BV), equipped with a pulsed-amperometric detector (PAD) with a gold working electrode (detection using a four-step potential waveform).[24] Glycans were eluted with a linear gradient of 20−200 mM sodium acetate in 0.1 M NaOH (3 mL/min). Collected fractions were immediately neutralized with 4 M acetic acid, desalted on Carbograph SPE columns (Alltech) using acetonitrile/water = 1:3 (v/v) as the eluent, and lyophilized.

Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectrometry. MALDI-TOF-MS experiments were performed on an Axima Performance mass spectrometer (Shimadzu Kratos, Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Data presented are cumulative spectra of 100−300 profiles of 10 shots averaged per profile. Positive-ion mode spectra were acquired using the reflectron mode and delayed extraction with software controlled delay time optimized and externally calibrated against malto-oligosaccharides (DP3−7) to m/z 1500 for *N*-glycan analysis and to m/z 800 for *O*-glycan analysis. The accelerating voltage was 20 kV; the acquisition range was 650−4000 Da for *N*-glycan analysis and 400−2000 Da for *O*-glycan analysis. One-μL samples were mixed with 1 μL of 2,5-dihydroxybenzoic acid (10 mg/mL) in 40% aqueous acetonitrile on the target. After drying of the droplets, the spots were recrystallized with ethanol.

^{1}H NMR Spectroscopy. *N*- and *O*-glycan(s) (pools) were lyophilized and exchanged twice with 99.9% D_2O (Cambridge Isotope Laboratories Inc., Andover, MA) and subsequently dissolved in 500 μL of D_2O. Resolution-enhanced one-dimensional 500 MHz ^{1}H NMR spectra were recorded in D_2O on a Varian Inova 500 spectrometer (GBB, NMR Center, University of Groningen) at probe temperatures of 300 K, with a spectral width of 5000 Hz and zero filled to 16k. A presaturation pulse was used to suppress the HOD signal. Spectra were processed with MestReNova 5.3 (Mestrelabs Research SL, Santiago de Compostella, Spain). ^{1}H NMR chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225).

RESULTS AND DISCUSSION

Glycan Composition of the Commercial Bovine Whey Protein Product Deminal 90. Monosaccharide analysis of the protein material (denatured and alkylated), isolated from Deminal 90, showed the presence of Fuc, Man, Gal, GlcNAc, GalNAc, and Neu5Ac, with a total carbohydrate content of 6.1 ± 0.4%$_{wt}$ (Table 1). Note that in the applied methanolysis protocol *N*-glycolylneuraminic acid (Neu5Gc) is analyzed as *N*-acetylneuraminic acid (Neu5Ac). Sialic acid analysis using DMB (1,2-diamino-4,5-methylenedioxybenzene) derivatives and HPLC with fluorescence detection (not shown) revealed the presence of both Neu5Ac and Neu5Gc and that 1.1 ± 0.2% of the sialic acid seen as Neu5Ac in the monosaccharide analysis is Neu5Gc, which is comparable to previous findings on whey protein isolates.[25] Recently, higher levels of Neu5Gc (8.5 ± 0.3% of the total amount of sialic acid) were observed in bovine lactoferrin,[14] whereas low levels for κ-casein (0.6 ± 0.2%) are known (data from the author's laboratory), indicating a nonhomogeneous distribution of this type of sialic acid on the milk proteome of Deminal 90.

Table 1. Relative Abundances of Monosaccharides in the Bovine Whey Protein Material, Isolated from Deminal 90, Released *N*-Glycan Pool, De-*N*-glycosylated Whey Protein, and Released *O*-Glycan Pool[a]

	whey protein	*N*-glycan pool	de-*N*-glycosylated whey protein	*O*-glycan pool
Fuc	0.5	0.5		
Man	3.0	3.0	tr	tr
Glc	tr[b]	tr	tr	tr
Gal	7.2	1.4	1.1	1.2
GlcNAc	2.7[c]	2.8	0.1	0.2
GalNAc	5.7	1.0	1.0	1.0
Neu5Ac[d]	5.7	1.0	1.3	1.4
Carb (%)	6.1 ± 0.4	nd[b]	2.8 ± 0.2	nd

[a]Whey protein and *N*-glycan pool are standardized to Man = 3.0; de-*N*-glycosylated whey protein and *O*-glycan pool are standardized to GalNAc = 1.0. For the protein fractions, the carbohydrate content is included. [b]tr = trace; nd = not determined. Environmental contamination of Glc is commonly observed in this type of analysis. [c]Asn-linked GlcNAc is not cleaved by methanolysis, leading to an underestimation of GlcNAc from N-linked oligosaccharides. [d]The methanolysis protocol shows all sialic acids as Neu5Ac.

After enzymatic release of the *N*-glycans with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGaseF), the de-*N*-glycosylated glycoprotein material was shown to contain Gal, GlcNAc, GalNAc, and sialic acid, with a total carbohydrate content of 2.8 ± 0.2%$_{wt}$.

Monosaccharide analysis of the released *N*-glycan pool revealed the presence of Fuc, Man, Gal, GlcNAc, GalNAc, and sialic acid (Table 1). The ratio of the monosaccharides supports the occurrence of both oligomannose- and (fucosylated) complex-type *N*-glycans, whereas the detection of both Gal and GalNAc suggests the presence of both Gal(β1−4)GlcNAc (LacNAc) and GalNAc(β1−4)GlcNAc (LacdiNAc) epitopes in the *N*-glycan pool. Monosaccharide analysis of the released *O*-glycan pool demonstrated the presence of Gal, GlcNAc, GalNAc, and sialic acid. The data of the de-*N*-glycosylated glycoprotein material and the *O*-glycan pool agree perfectly with each other, indicating nonfucosylated structures in the *O*-glycan pool.

MALDI-TOF-MS and ^{1}H NMR Analysis of the Released *N*-Glycan Pool. The *N*-glycan pool, generated by PNGaseF digestion of the denatured and alkylated whey protein material, was analyzed by MALDI-TOF-MS and one-dimensional ^{1}H NMR spectroscopy (Figure 1).

Among the many pseudomolecular ion peaks in the MALDI-TOF mass spectrum (not shown), clear evidence was obtained for the presence of $Man_{5-9}GlcNAc_2$ structures (sodium adducts at m/z 1257.4, 1419.5, 1582.1, 1743.6, and 1905.8).

The ^{1}H NMR spectrum of the *N*-glycan pool showed a great variety of characteristic structural-reporter group signals, in agreement with the occurrence of both oligomannose- and (fucosylated) complex-type *N*-glycans;[26,27] the coding system is visualized for a $Man_9GlcNAc_2$ and a fucosylated disialylated diantennary structure in Scheme 1.

The chemical shifts for Neu5Ac H-3a (δ 1.721) and H-3e (δ 2.663) agreed with (α2−6)-linked Neu5Ac residues to both Gal or GalNAc; no signals corresponding with (α2−3)-linked Neu5Ac were observed [Neu5Ac(α2−3)Gal: H-3a, δ ∼1.80; H-

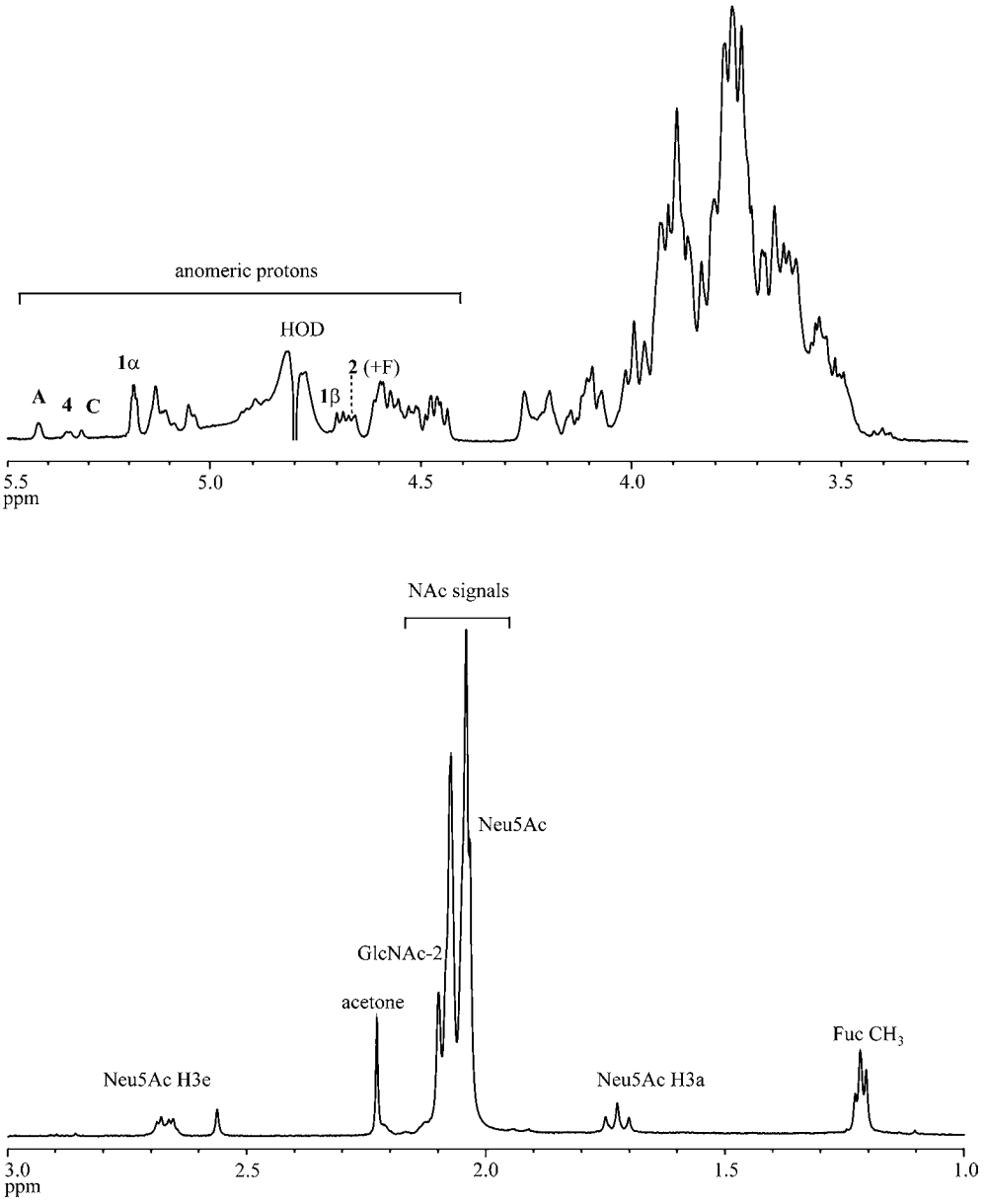
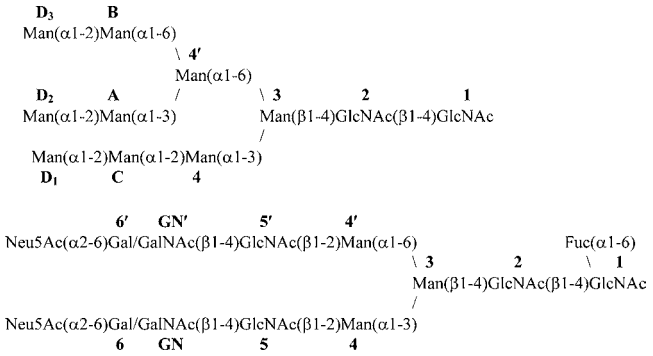


Figure 1. Relevant parts of the one-dimensional 500 MHz ^{1}H NMR spectrum of the *N*-glycan pool, enzymatically released from denatured and alkylated glycoproteins of a commercial bovine whey protein product, Deminal 90, in D_2O at 300 K. Several structural-reporter group signals are discussed in the text, and some of them have been indicated in the spectrum.

Scheme 1. Structures Depicting the Coding of Residues in a $Man_9GlcNAc_2$ and a Fucosylated Disialylated Diantennary Structure



3e, δ ~2.75−2.76. Neu5Ac(α2−3)GalNAc: H-3a, δ ~1.59; H-3e, δ ~2.70]. The Neu5Ac NAc signal is reflected by the signal at δ 2.031. The partial presence of (α1−6)-linked core Fuc residues is reflected by the Fuc CH_3 signals at δ 1.208 (linkage to GlcNAc-**1**$\boldsymbol{\alpha}$) and 1.221 (linkage to GlcNAc-**1**$\boldsymbol{\beta}$), combined with the GlcNAc-**1** H-1α, GlcNAc-**2** H-1, and GlcNAc-**2** NAc signals at δ 5.182, 4.666, and 2.096, respectively. In the case of nonfucosylation, these parameters are found at δ 5.188, ~4.61, and 2.081.[28−30] In the 4.4−4.7 ppm region, several signals are observed corresponding with the H-1 atoms of GlcNAc-**2** and the antennary Gal, GlcNAc, and GalNAc residues. In the 4.9−5.5 ppm region, several signals are detected belonging to the αMan H-1 atoms of oligomannose segments,[27] e.g., Man-**A**, when substituted by Man-$\mathbf{D_2}$, δ 5.411; terminal Man-**A**, δ 5.089; Man-**4**, when substituted by Man-**C**, δ 5.345/5.336; Man-**C**, when substituted by Man-$\mathbf{D_1}$, δ 5.310; terminal Man-$\mathbf{D_2}$/Man-**C**, δ 5.054; terminal Man-$\mathbf{D_1}$/Man-$\mathbf{D_3}$, δ 5.041; Man-

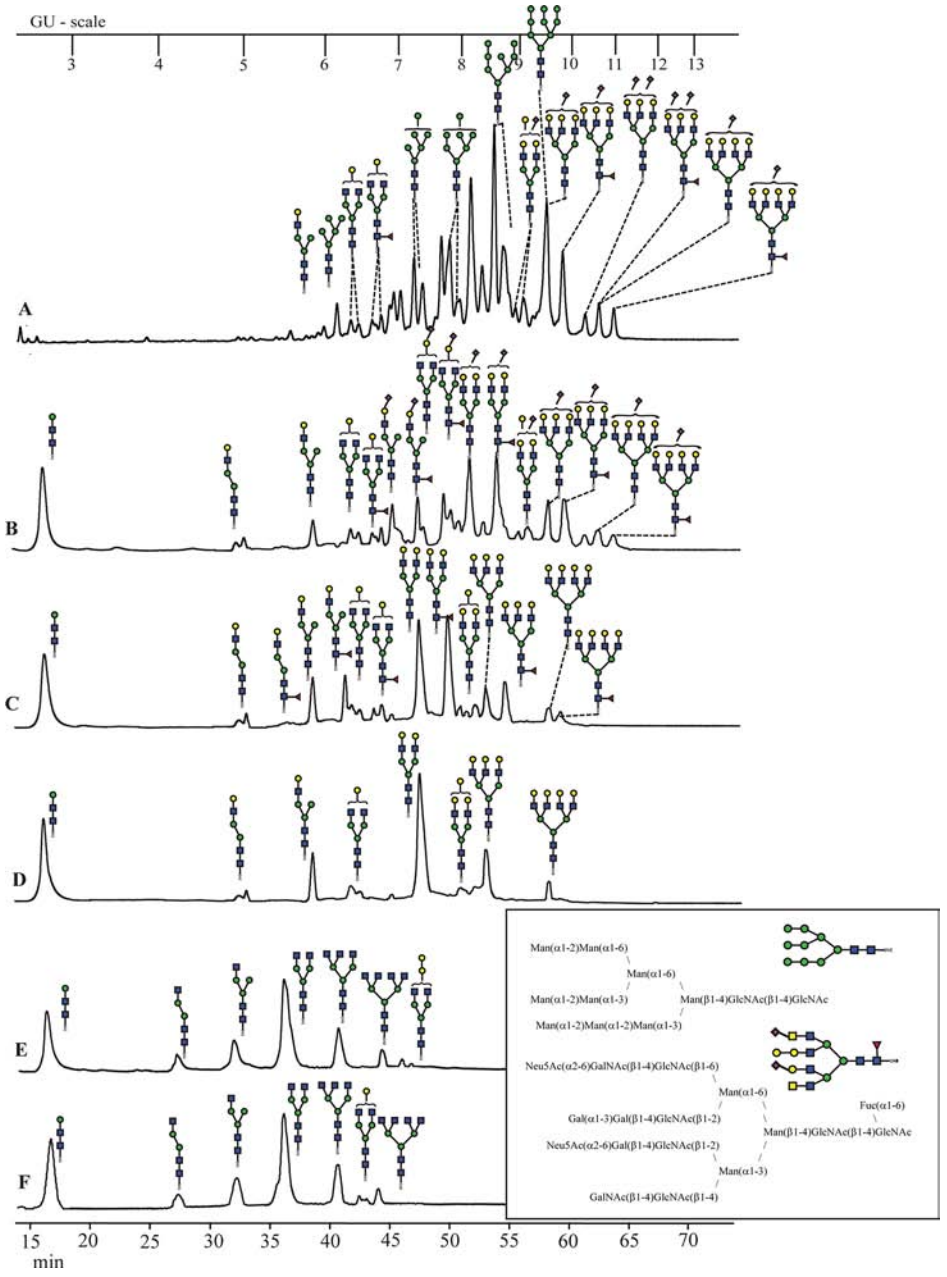


Figure 2. NP-HPLC profiles of 2AB-labeled *N*-glycans of WFA-fraction **I**: (A) without exoglycosidase treatment, and after sequential treatment with (B) α-mannosidase, (C) α-sialidase, (D) α-fucosidase, (E) β-galactosidase, and (F) α-galactosidase. For exact GU values, see Table S2, Supporting Information. The caption shows a key to how structures are constructed.

B, when substituted by Man-$\mathbf{D_3}$, δ 5.143 (Man-**4** of complex-type system, δ 5.134). The NAc signals in the range δ 2.073–2.039 are derived from the nondiscussed GlcNAc and GalNAc residues in different microenvironments.

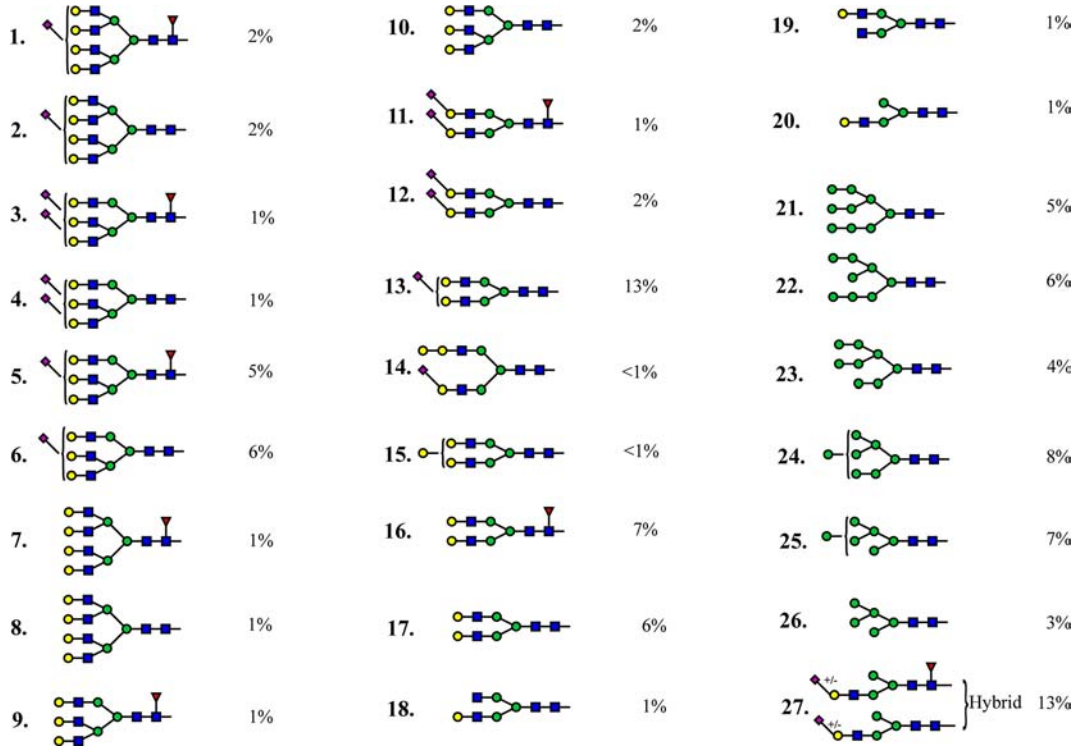
The *N*-glycan-pool-derived structural information, based on the initial ^{1}H NMR analysis, is further used in judging the chromatographic profiling data.

Analysis by HPLC Profiling Combined with Exoglycosidase Treatments and MALDI-TOF-MS of the 2AB-Labeled *N*-Glycan Pool. Analysis of the charge distribution of the 2AB-labeled *N*-glycan pool by WAX-HPLC profiling (combined with fluorescence detection, FD) (Figure S1A, Supporting information) showed evidence for the presence of neutral (N0, 32%), monocharged (N1, 61%), and dicharged (N2, 7%) *N*-glycans.

In view of the occurrence of both LacNAc and LacdiNAc sequences in the *N*-glycan pool (see monosaccharide analysis), the 2AB-labeled *N*-glycan pool was subfractionated by *Wisteria floribunda* agglutinin (WFA) affinity chromatography, as recently developed,[14] yielding three *N*-glycan fractions: fraction **I**, an unbound fraction eluted with binding buffer; fraction **II**, an intermediate fraction eluted with binding buffer calibrated to pH 5.5 with acetic acid; and fraction **III**, a bound fraction eluted with binding buffer calibrated to pH 5.5 with acetic acid, containing 100 mM GalNAc. Due to slightly different work-ups of the WFA-fractions **I**, **II**, and **III**, a quantitation in relation to the total *N*-glycan pool is difficult to make, but a rough estimation suggests a ratio of **I**:**II**:**III** = 6.5:2.5:1.0.

WAX-HPLC analysis of WFA-fraction **I** (Figure S1B, Supporting Information) showed neutral (N0, 37%), mono-

Scheme 2. Overview of Bovine Whey Protein *N*-Glycan Structures (Deminal 90) Found in WFA-Fraction I



charged (N1, 58%), and dicharged (N2, 5%) structures. The normal phase (NP)-HPLC data are expressed in glucose unit (GU) values and correlated with values in Glycobase 2.0.[23] The NP-HPLC profile (FD detection) of WFA-fraction **I**, depicted in Figure 2A, contained major peaks corresponding with $Man_{5-9}GlcNAc_2$, as well as complex type di-, tri- and tetra-antennary structures, both with and without core (α1−6)-fucosylation, and up to two Neu5Ac residues. In Table S2, Supporting Information, an overview of all structures observed in the NP-HPLC profile of WFA-fraction **I**, as well as in the sequential exoglycosidase assays, are shown. Relative peak areas of the HPLC profiles are used, in combination with MALDI-TOF-MS peak intensities, for a rough estimation of the relative abundance of the different structures. Scheme 2 shows all *N*-glycans found, together with their estimated abundances. It should be noted that overlap of structures, like for example $Man_9GlcNAc_2$ with fucosylated disialo diantennary structures, as well as monosialo triantennary structures, complicates the quantitation; their quantities are derived from observed peak decreases after exoglycosidase treatments (with respect to $Man_9GlcNAc_2$, compare for instance Figure 2A with 2B for the digestion with α-mannosidase). In the final exoglycosidase profile, a peak is observed at 2.77 GU, representing oligomannose-type (29%), and two peaks corresponding with hybrid-type structures (4.20 GU, 5%; 4.95 GU, 10%), furthermore, one peak for diantennary (5.50 GU, 39%), one peak for triantennary (6.18 GU, 13%), and one peak for tetra-antennary (6.72 GU, 5%) structures, and finally, two small peaks, corresponding with remains of αGal containing structures (6.32/6.42 GU, <1%). This amounts to a molar ratio of oligomannose-, hybrid-, and complex-type structures of 2.9:1.0:3.8 in WFA-fraction **I**. Moreover, among the complex-type structures, it was found that approximately a 1:1 molar ratio of core-fucosylated vs nonfucosylated structures occurs. This finding is supported by the observation of a significant amount of fucose by monosaccharide analysis and peaks in the ^{1}H NMR spectrum of the *N*-glycan pool.

In the WAX-HPLC profile of WFA-fraction **II** (Figure S1C, Supporting Information), a small amount of neutral structures (N0, 5%), a majority of single-charged structures (N1, 89%), and a minor amount of dicharged structures (N2, 6%) was observed. NP-HPLC analysis of WFA-fraction **II** (Figure 3A) showed a range of peaks between 5.5 and 11.0 GU. Similar to WFA-fraction **I**, all HPLC peaks in Figure 3A were followed in a series of sequential exoglycosidase treatments (Table S3, Supporting Information). After treatment with α-sialidase (Figure 3B), all major peaks shift downward by 1.0−1.2 GU, corresponding with the loss of a single (α2−6)-linked Neu5Ac residue. The minor peaks below 6.5 GU in Figure 3A do not shift and belong to the small amount of neutral glycans observed by WAX-HPLC analysis. Glycans observed in the NP-HPLC analysis showed a range of hybrid-type and complex-type structures containing one or two LacdiNAc epitopes, partially shielded from the WFA-lectin by Neu5Ac, as observed before.[14] The ratio of core (α1−6)-fucosylated structures vs nonfucosylated structures is approximately 1:1, similar to observations in WFA-fraction **I**, and suggested already by the monosaccharide analysis and ^{1}H NMR data. The observed structures could be confirmed by MALDI-TOF-MS, with the exception of the sialylated structures, due to in-source decay of the labile glycosidic bond under the conditions used. In Table S3, Supporting Information, a survey of all structures detected in the NP-HPLC profile of WFA-fraction **II**, as well as in the sequential exoglycosidase assays, is presented, whereby relative intensities are based on HPLC profiles, supported by MALDI-TOF-MS intensities. All identified compounds in WFA-fraction **II**, together with their estimated amounts, are presented in Scheme 3. The relative molar ratio of the hybrid-, di-, tri-, and tetra-antennary structures (3.4:13.2:2.4:1.0) compared to that observed in WFA-fraction **I** (3.0:7.8:2.6:1.0). It shows a

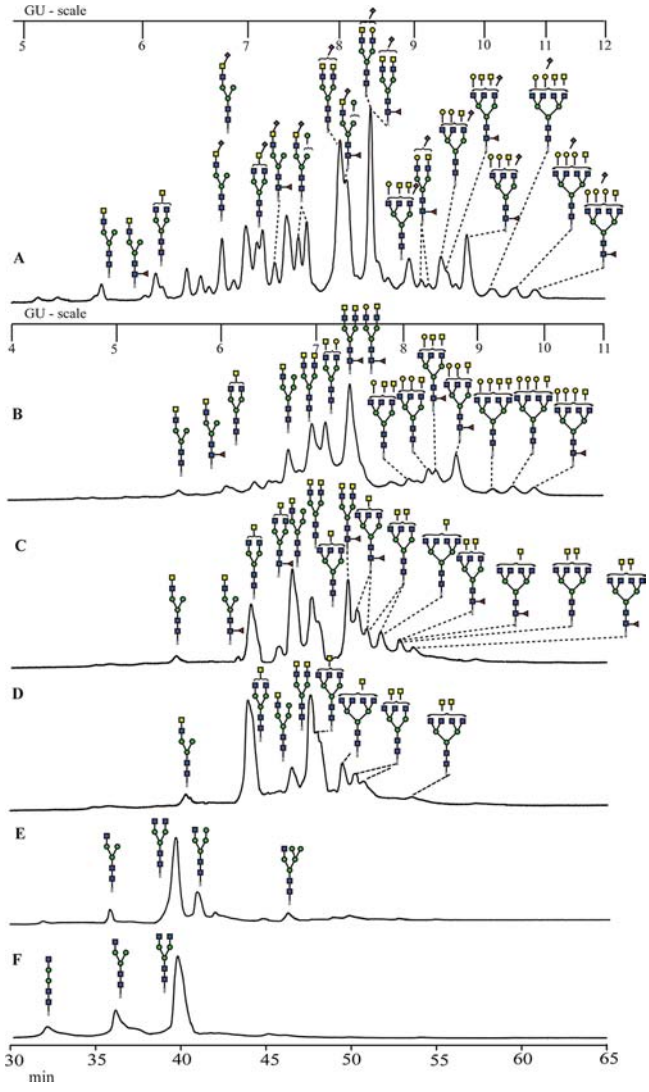


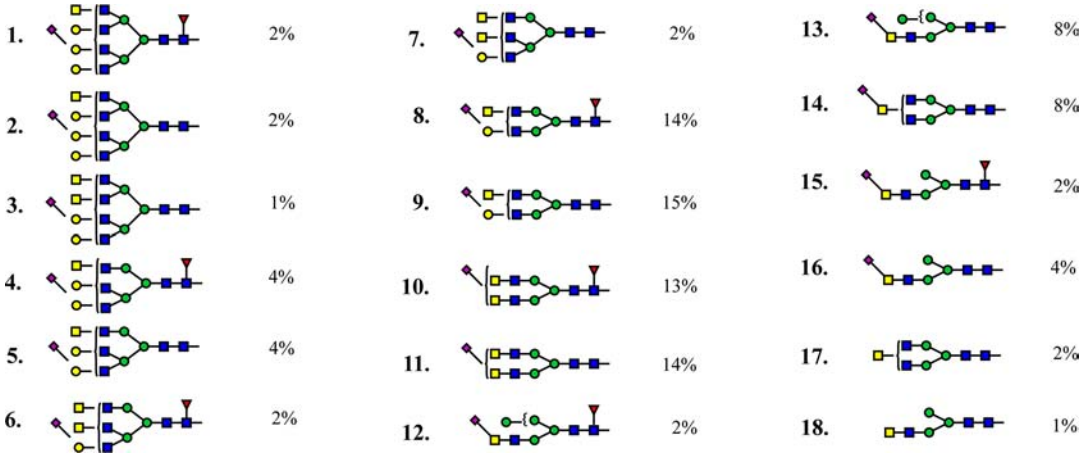
Figure 3. NP-HPLC profiles of 2AB-labeled *N*-glycans of WFA-fraction **II**: (A) without exoglycosidase treatment, and after sequential treatment with (B) α-sialidase, (C) β-galactosidase, (D) α-fucosidase, (E) β-*N*-acetylhexosaminidase, and (F) α-mannosidase. For exact GU values, see Table S3, Supporting Information. A key to the graphical depiction of the structures is shown in Figure 2.

stronger presence of diantennary structures, whereas the other species are in similar balance. The relative ratio of the sialylated LacdiNAc containing hybrid-type and complex-type structures amounted to 1.0:3.8. Note that the α-mannosidase digestion, going from part E to part F of Figure 3, was not complete.

The WAX-HPLC analysis of WFA-fraction **III** (Figure S1D, Supporting Information) showed only evidence of the presence of neutral structures (N0). In the NP-HPLC profile of WFA-fraction **III** (Figure 4A), the two major peaks at 6.95 and 7.28 GU indicate the presence of di-LacdiNAc-containing diantennary structures, without and with core (α1−6)-linked fucose, respectively. Minor peaks corresponding with LacdiNAc-containing diantennary structures, as well as double and triple LacdiNAc-containing tri- and tetra-antennary structures, were also observed, both with and without core (α1−6)-linked fucose. The ratio of fucosylated versus nonfucosylated structures is approximately 1:1 for all observed structures, similar to the observations in WFA-fractions **I** and **II**. This observation is also reflected in the significant presence of fucose observed in both the monosaccharide analysis, as well as in the ^{1}H NMR spectrum of the *N*-glycan pool. Table S4, Supporting Information, presents all structures observed in the NP-HPLC profile of WFA-fraction **III**, as well as in the sequential exoglyosidase assays, whereby relative intensities are derived from HPLC peak surfaces. The various structures are confirmed by MALDI-TOF-MS. An overview of the identified compounds is presented in Scheme 4. Here, the molar ratio of di-, tri-, and tetra-antennary structures (28.7:3.7:1.0) shows a very strong presence of diantennary structures, as compared to that observed in WFA-fractions **I** and **II**. More notably, no peaks relating to hybrid-type structures were observed in the HPLC profiles. Possibly due to relatively weaker interaction with the WFA-lectin, these were already eluted in the intermediate fraction, together with part of the single LacdiNAc-containing diantennary structures without Neu5Ac.

The total amount of LacdiNAc-containing structures in the *N*-glycan pool is significant. Although the human milk glycome does not show the LacdiNAc epitope, several examples of LacdiNAc in human protein glycosylation have been reported (urokinase,[30] lutropin,[31] glycodelin,[32] human protein C,[33] Tamm−Horsfall glycoprotein,[29,34] and kallidinogenase[35]). Over 60 *N*-glycan structures have been identified for the *N*-glycosylation of Deminal 90. The majority of these structures overlaps with the list of structures recently reported for the

Scheme 3. Overview of Bovine Whey Protein *N*-Glycan Structures (Deminal 90) Found in WFA-Fraction II



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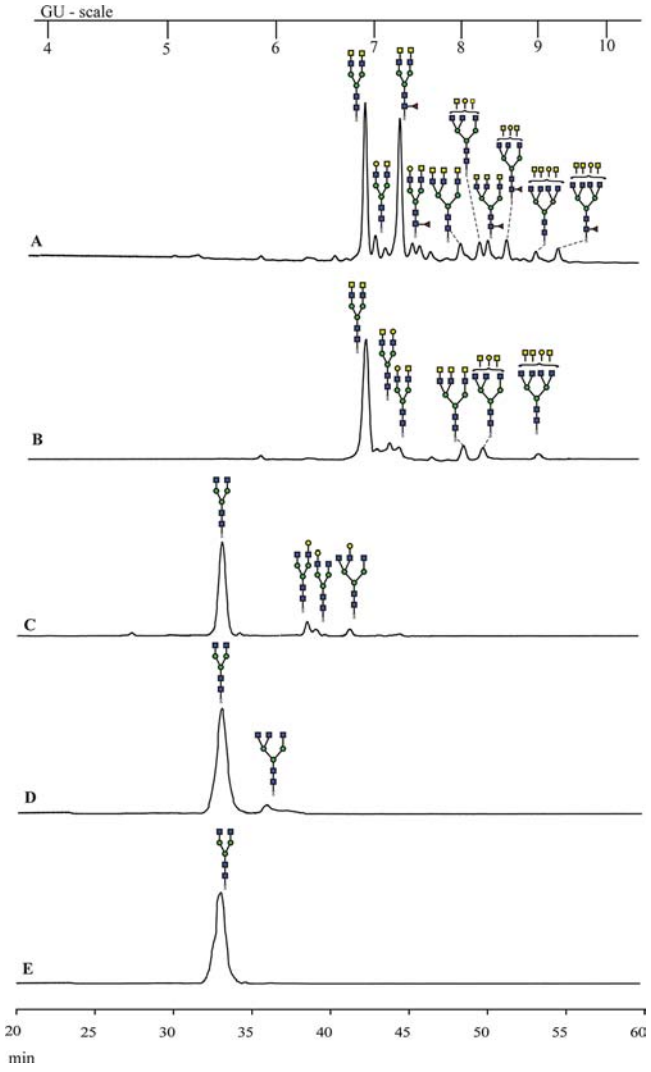

Figure 4. NP-HPLC profiles of 2AB-labeled *N*-glycans of WFA-fraction **III**: (A) without exoglycosidase treatment, and after sequential treatment with (B) α-fucosidase, (C) β-*N*-acetylhexosaminidase, (D) β-galactosidase, and (E) β-*N*-acetylhexosaminidase. For exact GU values, see Table S4, Supporting Information. A key to the graphical depiction of the structures is shown in Figure 2.

total bovine milk *N*-glycome;[15] however, a minority of the structures found here does not occur in the bovine milk *N*-glycome list and vice versa. In view of the small amounts of Neu5Gc, it is understandable that definite Neu5Gc-containing compounds could not be traced in the MS, HPLC, and NMR studies.

Analysis of *O*-Glycan. The pool of *O*-glycan alditols, obtained via alkaline borohydride treatment of the de-*N*-glycosylated material, was investigated by MALDI-TOF-MS, one-dimensional ^{1}H NMR spectroscopy, and HPAEC (combined with pulsed amperometric detection, PAD) profiling.

The MALDI-TOF mass spectrum showed seven distinct peaks, corresponding with m/z values fitting up with more than seven possible *O*-glycan compositions (Table 2). The ^{1}H NMR spectrum of the *O*-glycan alditol pool (Figure S2A, Supporting Information) is quite similar to the spectrum reported for Neu5Ac(α2−3)Gal(β1−3)[Neu5Ac(α2−6)]GalNAc-ol,[27,36] demonstrating that this is the main compound. The relevant chemical shifts are presented in Table 3. The substitution pattern of GalNAc-ol is reflected by the GalNAc-ol H-2 and H-5 resonances at δ 4.379 and 4.240, respectively. The two Neu5Ac residues show their H-3a/H-3e signals at δ 1.799/2.774 [(α2−3)-linked Neu5Ac] and 1.691/2.724 [(α2−6)-linked Neu5Ac], respectively. Other typical signals are found at δ 4.543 (Gal^3 H-1), 4.116 (Gal^3 H-3), 4.069 (GalNAc-ol H-3), and 3.927 (Gal^3 H-4). In the *N*-acetyl region, besides signals for GalNAc-ol NAc (δ 2.042) and Neu5Ac NAc (δ 2.031), additional minor NAc signals are observable at δ 2.056 and δ 2.071, indicating the possible presence of minor compounds containing HexNAc residues. Integration of the Neu5Ac NAc and GalNAc-ol NAc signals yielded a Neu5Ac:GalNAc-ol molar ratio of 1.6:1.0.

Further separation of the *O*-glycan alditol pool by HPAEC (Figure S3, Supporting Information) yielded six fractions, i.e., fractions **1**, **2**, and **a**−**d**. The major fractions **1** and **2** contained

Table 2. MALDI-TOF-MS Peaks Observed in the *O*-Glycan Alditol Pool[a]

m/z obsd	m/z theor	HPAEC fraction	glycan composition
699.1	699.6	**1**, **d**	$Neu5Ac_1Hex_1HexNAc$-ol
990.3	990.6	**2**	$Neu5Ac_2Hex_1HexNAc$-ol
408.7	408.3	**a**	$Hex_1HexNAc$-ol
772.9	773.3	**b**	$Hex_2HexNAc_1HexNAc$-ol
1064.3	1064.9	**c**	$Neu5Ac_1Hex_2HexNAc_1HexNAc$-ol
449.6	449.3	nd	$HexNAc_1HexNAc$-ol
862.1	861.7	nd	$Neu5Ac_1Hex_2HexNAc$-ol

[a]Fractions found by HPAEC and checked by MALDI-TOF-MS are indicated; peaks marked nd are not observed by HPAEC, probably due to low abundance.

Scheme 4. Overview of Bovine Whey Protein *N*-Glycan Structures (Deminal 90) Found in WFA-Fraction III

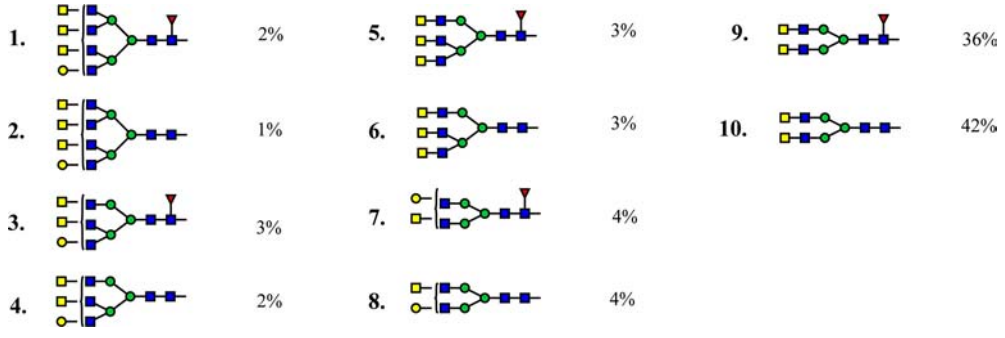

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Table 3. ^{1}H NMR Structural-Reporter Group Signals[b] for the Major Glycan in the *O*-Glycan Alditol Pool, Together with the *O*-Glycan Alditols Isolated by HPAEC from the Pool[a]

residue	proton	pool[c]	fraction 1[d]	fraction 2[c]
GalNAc-ol	H-2	4.379	4.390	4.380
	H-3	4.069	4.071	4.068
	H-5	4.240	4.189	4.243
	NAc	2.042	2.045	2.041
Gal^3	H-1	4.543	4.546	4.544
	H-3	4.116	4.120	4.118
	H-4	3.927	3.926	3.927
Neu5Ac^3	H-3a	1.799	1.801	1.802
	H-3e	2.774	2.770	2.774
	NAc	2.031	2.033	2.031
Neu5Ac^6	H-3a	1.691		1.691
	H-3e	2.724		2.724
	NAc	2.031		2.031

[a]NMR spectra are depicted in Figure S2, Supporting Information. [b]The superscript after the abbreviated name of galactose and *N*-acetylneuraminic acid indicates to which position of the adjacent monosaccharide it is glycosidically linked. [c]Pool and fraction 2: Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α2-6)]GalNAc-ol. [d]Fraction 1: Neu5Ac(α2-3)Gal(β1-3)GalNAc-ol.

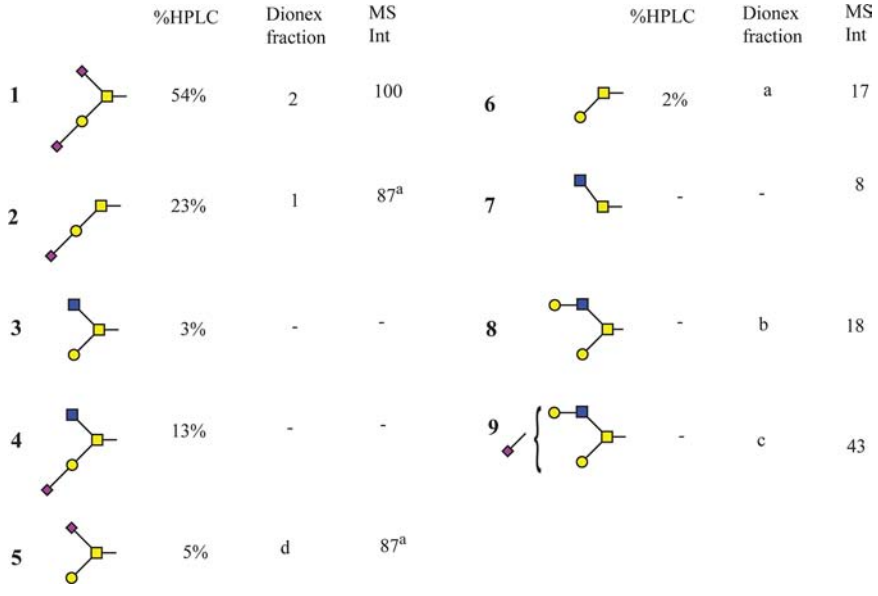
sufficient material for ^{1}H NMR analysis and were identified as Neu5Ac(α2−3)Gal(β1−3)GalNAc-ol (Figure S2B, Supporting Information) and Neu5Ac(α2−3)Gal(β1−3)[Neu5Ac(α2−6)]GalNAc-ol (Figure S2C, Supporting Information), respectively (Table 3, Scheme 5).[36] Taking into account the monosaccharide analysis of the *O*-glycans (see above), MALDI-TOF-MS analysis of the fractions **a**−**d** supported the formula GalGalNAc-ol (**a**), Gal_2GlcNAcGalNAc-ol (**b**), Neu5AcGal_2GlcNAcGalNAc-ol (**c**), and Neu5AcGalGalNAc-ol (**d**) (Table 2). The suggested GlcNAc(β1−6)GalNAc *O*-glycan has so far only been found in human κ-casein.[37] For Neu5Ac$_1$Hex$_2$HexNAc-ol, no structure is proposed.

The pool of *O*-glycans, released by the nonreductive Orela kit and labeled with 2-aminobenzamide, was further studied by WAX-HPLC and NP-HPLC. WAX-HPLC showed evidence for neutral (O0, 8%), monocharged (O1, 34%), and dicharged (O2, 58%) structures (Figure S1E, Supporting Information). NP-HPLC analysis (Figure 5A) revealed two major peaks (2.94 and 4.46 GU) and four minor peaks (1.83, 2.71, 3.20, and 3.69 GU). The six peaks were followed in sequential exoglycosidase treatments (Table S5, Supporting Information), first with *Salmonella typhimurium* α-sialidase (Figure 5B), having a strong preference for (α2−3)-linked Neu5Ac over (α2−6)-linked Neu5Ac (260 fold),[38] followed by the nonspecific *Arthrobacter ureafaciens* α-sialidase (Figure 5C).

Combining the various analytical data from the *O*-glycan alditol and 2AB-labeled *O*-glycan pools, taking also into account the bovine biosynthetic *O*-glycan pathways and the *O*-glycan structures reported for bovine κ-casein,[16] the various compounds identified are presented in Scheme 5. Combined with the Neu5Ac$_1$Hex$_2$HexNAc-ol, it means that 10 *O*-glycan structures have been found. The observation of differences in the *O*-glycan profiles, when different release chemistries and workup protocols are followed, especially with respect to the minor components, illustrates again the usefulness of following different approaches in carbohydrate structural analysis. Notably, neither method of release resulted in any detectable levels of peeling products, such as Neu5Ac(α2−3)Gal.

It should be noted that in this study a bovine cheese whey protein product was analyzed. In the renneting process, casein macro peptide (CMP), a C-terminal part of κ-casein, is formed. The major constituents found in the *O*-glycan pool were also previously found for κ-casein.[39] Interestingly, the second most abundant *O*-glycan structure in Deminal 90 is the sialylated T-antigen (Neu5Ac(α2−3)Gal(β1−3)GalNAc), which also oc-

Scheme 5. Overview of Bovine Whey Protein *O*-Glycan Structures (Deminal 90)[a]



[a]For each structure indications are given if they were observed by NP-HPLC (%HPLC), HPAEC-PAD (Dionex fraction), or mass spectrometry (MS Int). − Indicates that this structure was not found by this technique. It should be noted that the relative MS-intensity of 87% for structures **2** and **5** is a shared value.

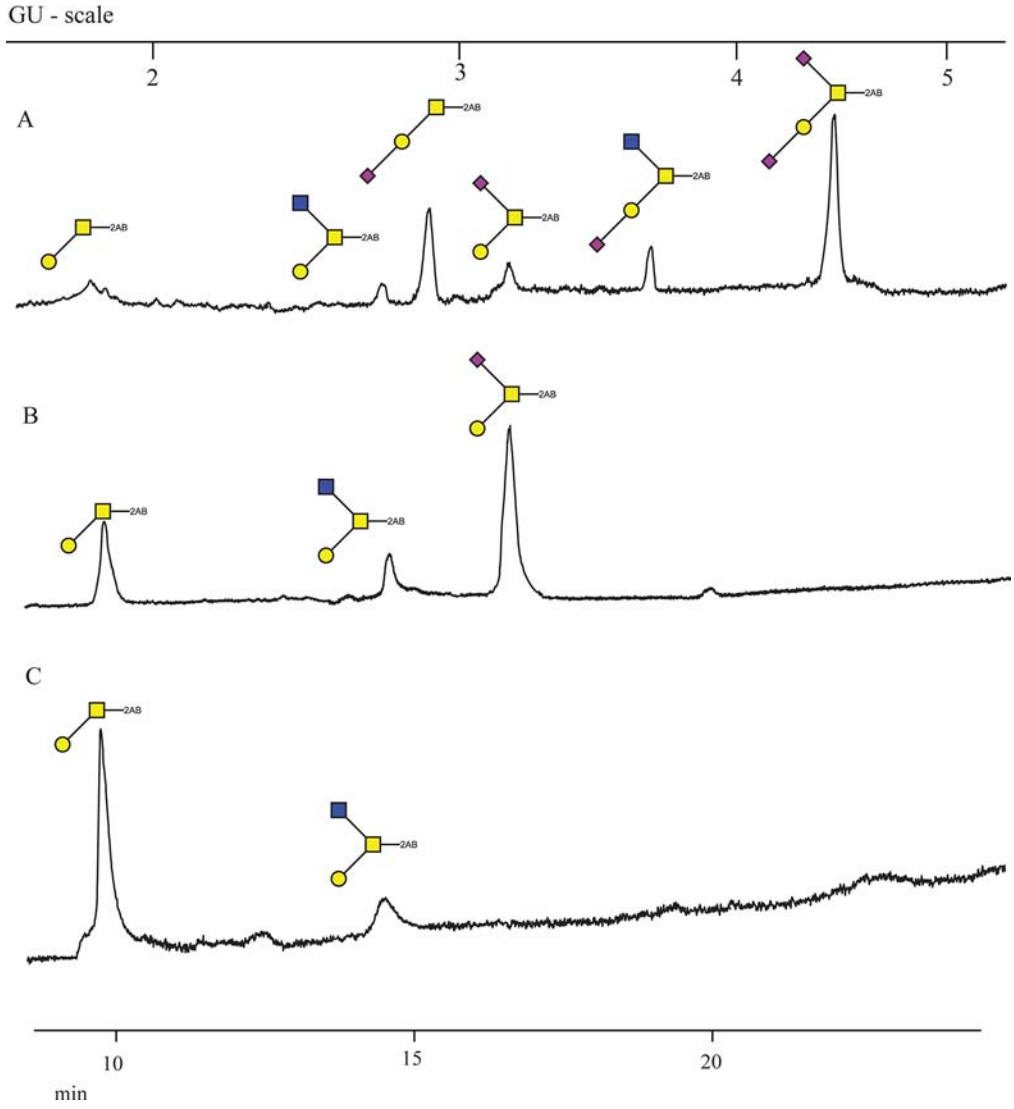


Figure 5. NP-HPLC profiles of 2AB-labeled *O*-glycans: (A) without exoglycosidase treatment, and after sequential treatment with (B) *Salmonella typhimurium* α-sialidase, and (C) *Arthrobacter ureafaciens* α-sialidase. For exact GU values, see Table S5, Supporting Information.

curs on κ-casein in human milk,[37] as well as on human IgA_1 found in milk.[40]

Final Remarks. In total, three epitopes were observed in the glycosylation of bovine whey proteins that do not occur in human milk-protein glycosylation: the Gal(α1−3)Gal epitope (αGal), the GalNAc(β1−4)GlcNAc sequence (LacdiNAc), and *N*-glycolylneuraminic acid (Neu5Gc).

Taking into account the roughly estimated ratios of fractions **I**, **II**, and **III**, there are approximately 0.3% αGal-containing structures in the total *N*-glycan pool of Deminal 90. To our knowledge, there have been no reports of αGal-related allergic reactions in infants and toddlers. Allergies related to the αGal epitope have been found in some cases related to delayed-onset allergy to red meat,[41,42] however, individuals suffering from this allergy rarely respond to consumption of dairy products.[42] There are indications that there is a cross-reactivity with tick and chigger bites and this delayed-onset allergy for the αGal epitope,[41,42] suggesting the presence of αGal might not be the cause of this allergy. It should be noted that recently a monoclonal antibody, Cetuximab, was approved by the FDA for cancer treatment. Cetuximab contains high amounts of αGal (approximately 30% of the glycans) and Neu5Gc (12% of all glycans are sialylated, 100% of all sialic acids was Neu5Gc).[43]

The LacdiNAc levels in Deminal 90 are significant, however, this epitope does occur naturally in human glycosylation patterns. Also, there are no reports of food allergy related to LacdiNAc, although this epitope has been indicated as immunogenic factor in parasite infections.[44]

Neu5Gc is a variant of *N*-acetylneuraminic acid (Neu5Ac) and is synthesized by hydroxylation of Neu5Ac at the nucleotide sugar level in all known mammals, except in humans, where a mutation has inactivated the responsible enzyme.[45] Dietary Neu5Gc is shown to be taken up and used in human glycosylations.[46,47] Despite the occurrence of antibodies against Neu5Gc, there have been no reports of allergies related to Neu5Gc. The observed levels of Neu5Gc in Deminal 90 are small (0.003%) and in line with findings on other dairy products and infant formulas.[25]

■ ASSOCIATED CONTENT

Supporting Information

A detailed overview of milk protein *N*- and *O*-glycosylation research; extensive tables displaying the exoglycosidase

digestions for WFA fractions **I**–**III** as well as for the *O*-glycan fraction; the WAX-HPLC profiles of 2AB-labeled *N*- and *O*-glycans; the HPAEC-PAD profile of the *O*-glycan alditol pool; relevant parts of the 1D ^{1}H NMR spectra of the *O*-glycan alditol pool and the HPAEC *O*-glycan fractions **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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