University of Groningen

Use of Wisteria floribunda agglutinin affinity chromatography in the structural analysis of the bovine lactoferrin N-linked glycosylation

van Leeuwen, Sander S.; Schoemaker, Ruud J. W.; Timmer, Christel J. A. M.; Kamerling, Johannes P.; Dijkhuizen, Lubbert

Published in:
Biochimica et Biophysica Acta-General Subjects

DOI:
[10.1016/j.bbagen.2011.12.014](https://doi.org/10.1016/j.bbagen.2011.12.014)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Leeuwen, S. S., Schoemaker, R. J. W., Timmer, C. J. A. M., Kamerling, J. P., & Dijkhuizen, L. (2012). Use of Wisteria floribunda agglutinin affinity chromatography in the structural analysis of the bovine lactoferrin N-linked glycosylation. *Biochimica et Biophysica Acta-General Subjects*, 1820(9), 1444-1455. <https://doi.org/10.1016/j.bbagen.2011.12.014>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Use of *Wisteria floribunda* agglutinin affinity chromatography in the structural analysis of the bovine lactoferrin N-linked glycosylation[☆]

Sander S. van Leeuwen^a, Ruud J.W. Schoemaker^b, Christel J.A.M. Timmer^b,
Johannis P. Kamerling^a, Lubbert Dijkhuizen^{a,*}

^a Department of Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, NL-9747 AG Groningen, The Netherlands

^b FrieslandCampina Research and FrieslandCampina Domo, Stationsplein 4, NL-3818 LE Amersfoort, The Netherlands

ARTICLE INFO

Article history:

Received 2 November 2011

Received in revised form 21 December 2011

Accepted 22 December 2011

Available online 2 January 2012

Keywords:

Wisteria floribunda agglutinin

Lectin affinity

Bovine lactoferrin N-glycosylation

LacdiNAc

αGal

Neu5Gc

ABSTRACT

Background: Over the years, the N-glycosylation of both human and bovine lactoferrin (LF) has been studied extensively, however not all aspects have been studied in as much detail. Typically, the bovine LF complex-type N-glycans include certain epitopes, not found in human LF N-glycans, i.e. Gal(α1-3)Gal(β1-4)GlcNAc (αGal), GalNAc(β1-4)GlcNAc (LacdiNAc), and N-glycolylneuraminic acid (Neu5Gc). The combined presence of complex-type N-glycans, with αGal, LacdiNAc, LacNAc [Gal(β1-4)GlcNAc], Neu5Ac (N-acetylneuraminic acid), and Neu5Gc epitopes, and oligomannose-type N-glycans complicates the high-throughput analysis of such N-glycoproteins highly.

Methods: For the structural analysis of enzymatically released N-glycan pools, containing both LacNAc and LacdiNAc epitopes, a prefractionation protocol based on *Wisteria floribunda* agglutinin affinity chromatography was developed. The sub pools were analysed by MALDI-TOF-MS and HPLC-FD profiling, including sequential exoglycosidase treatments.

Results: This protocol separates the N-glycan pool into three sub pools, with (1) free of LacdiNAc epitopes, (2) containing LacdiNAc epitopes, partially shielded by sialic acid, and (3) containing LacdiNAc epitopes, without shielding by sialic acid. Structural analysis by MALDI-TOF-MS and HPLC-FD showed a complex pattern of oligomannose-, hybrid-, and complex-type di-antennary structures, both with, and without LacdiNAc, αGal and sialic acid.

Conclusions: Applying the approach to bovine LF has led to a more detailed N-glycome pattern, including LacdiNAc, αGal, and Neu5Gc epitopes, than was shown in previous studies.

General significance: Bovine milk proteins contain glycosylation patterns that are absent in human milk proteins; particularly, the LacdiNAc epitope is abundant. Analysis of bovine milk serum proteins is therefore excessively complicated. The presented sub fractionation protocol allows a thorough analysis of the full scope of bovine milk protein glycosylation. This article is part of a Special Issue entitled Glycoproteomics.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Lactoferrin (LF) is an 80 kDa, single subunit N-glycoprotein with multiple functions and involved in many processes, such as activation of the immune system [1–4] and inflammatory regulation [5–7], but also iron absorption in the intestinal tract [8], or in prevention of

reactive oxygen species formation [9], and growth promotion in certain tissues [10,11]. Furthermore, LF has been shown to have inhibitory effects on tumourigenesis [12,13] and anti-metastatic activity [14–16]. LFs contain between 1 and 5 potential glycosylation sites, depending on the species [17–19]. In human milk, LF is a highly abundant protein, with 5.3 mg/ml in colostrum and around 1 mg/ml in later stages of the lactation [20]. Bovine milk contains a significantly lower amount of LF, with 1.5 mg/ml in colostrum and 20–200 µg/ml in mature milk [21]. There is a sequence homology of 69% between human and bovine LF, and although their 3D structures can be largely superimposed [22], there are some structural differences [23,24]. Human LF consists of 691 amino acids and contains 3 potential glycosylation sites at Asn138, Asn479, and Asn624; Asn138 and Asn479 are occupied with complex-type N-glycans, whereas Asn624 is usually unoccupied [25,26]. Bovine LF consists of 689 amino acids and has 5 potential glycosylation sites at Asn233, Asn288, Asn368, Asn476, and Asn545; Asn233, Asn368, Asn467, and Asn545 are always occupied, and Asn288 is occupied for about 30% [18,19,27].

Abbreviations: 2AB, 2-aminobenzamide; BSA, bovine serum albumin; DMB, 1,2-diamino-4,5-methylenedioxybenzene; FD, fluorescence detection; GRAS, generally recognised as safe; GU, glucose units; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; LF, lactoferrin; MALDI-TOF-MS, matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry; NP, normal phase; PAD, pulsed amperometric detection; PNGaseF, peptide-N⁴-(N-acetyl-β-glucosaminyl)asparagine amidase F; SDS, sodium dodecyl sulfonate; WFA, *Wisteria floribunda* agglutinin; WAX, weak-anion-exchange

[☆] This article is part of a Special Issue entitled Glycoproteomics.

* Corresponding author. Tel.: +31 50 3632150; fax: +31 50 3632154.

E-mail address: l.dijkhuizen@rug.nl (L. Dijkhuizen).

For food-related applications, bovine LF, isolated from milk or whey and having the GRAS (Generally Recognised As Safe) status, has widely found use as a humanising protein component for infant formulae. Differences in the glycosylation pattern of human and bovine LF may be a source of concern, considering several structural epitopes present in the bovine LF glycans that do not occur commonly in human glycoproteins, and in particular in the human LF glycans. The Gal(α 1-3)Gal epitope (α Gal epitope), absent in human glycoproteins, e.g. human LF, but found in bovine glycolipids and glycoproteins, e.g. bovine LF, is an immunogenic structure and has been indicated in allergies [28,29], possibly induced by tick bites [28]. A second epitope that is uncommon in human glycosylation, absent in human LF, but present in bovine LF, is the GalNAc(β 1-4)GlcNAc (LacdiNAc) sequence, next to the Gal(β 1-4)GlcNAc (LacNAc) epitope (known human glycoprotein examples are urokinase [30], lutropin [31], glycodelin [32], human protein C [33], Tamm-Horsfall glycoprotein [34], and kallidinogenase [35]). Immunogenicity of this epitope has been shown in parasite infections [36], but not in relation to food allergies. Finally, *N*-glycolylneuraminic acid (Neu5Gc), a variant of *N*-acetylneuraminic acid (Neu5Ac), is synthesised by hydroxylation of Neu5Ac at the nucleotide sugar level in all known mammals, except in humans, where a mutation has inactivated the responsible enzyme [37]. However, dietary Neu5Gc can be taken up and used in human glycosylations; it has been found in several human tissues at different levels [38,39].

Glycosylation of bovine lactoferrin was studied previously [40,41], however, no specific focus on LacdiNAc epitopes was made. More sensitive equipment allows for more detailed analysis, as has been done for recombinant human lactoferrin, expressed in the milk of transgenic cows [42]. In this paper we use lectin-affinity chromatography to isolate sub pools of *N*-glycans, released from bovine LF. By reducing the complexity of glycan mixtures, more detailed analysis of minor compounds is made possible. Using NP-HPLC profiling of 2AB-labelled *N*-glycans, as well as MALDI-TOF-MS, the lectin-separated sub pools were analysed in native form and after sequential exoglycosidase treatments.

2. Materials and methods

2.1. Materials

Commercial bovine LF was provided by FrieslandCampina DOMO (Amersfoort, The Netherlands). Reference 2AB-labelled *N*-glycans were obtained from Ludger Ltd (Oxfordshire, UK).

2.2. Monosaccharide analysis

Duplicate samples of ~1 mg bovine LF were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by re-*N*-acetylation and trimethylsilylation. The trimethylsilylated (methyl ester) methyl glycosides were analysed by GLC on an EC-1 column (30 m $\times$ 0.32 mm; Alltech, Breda, The Netherlands), using a Chrompack CP9002 gas chromatograph (Chrompack, Middelburg, The Netherlands; temperature program 140–240 °C, 4 °C/min). Monosaccharide derivative identities were confirmed by GLC-MS analysis on a Shimadzu QP2010 Plus system (Shimadzu, 's-Hertogenbosch, The Netherlands), using an EC-1 column

Table 1

GU values of monosaccharide constituents determined from glycan standards in this study, compared to GU values of *N*-glycans from Glycobase 2.0.

	This study	Glycobase 2.0
Core-linked Fuc(α 1-6)	0.3–0.5	0.5
Man	0.75–0.95	0.7–0.9
Gal anywhere	0.85–0.95	0.8–0.9
Neu5Ac(α 2-3) anywhere	~0.7	~0.7
Neu5Ac(α 2-6) anywhere	~1.2	~1.15
GlcNAc (non-bisecting)	~0.7	0.6–0.8

Fig. 1. NP-HPLC profile of 2AB-labelled *N*-glycans released from bovine LF.

(30 m $\times$ 0.25 mm, Grace, Breda, The Netherlands) and the same temperature program [43].

2.3. Sialic acid determination

For HPAEC-PAD analysis, 10 mg bovine LF samples were hydrolysed with 1.0 ml 0.1 M HCl (1 h, 80 °C). Samples were cooled to RT and neutralised with 50 μ l 2 M NaOH. Cleaved sialic acid was separated from glycoprotein by 5 kDa centrifugal filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and subsequently analysed on an ICS3000 system (Dionex BV, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (4 mm $\times$ 250 mm) and an ICS3000 ED pulsed amperometric detector (pulse potentials and durations: E_1 +0.1 V, 410 ms; E_2 –2.0 V, 20 ms; E_3 +0.6 V, 10 ms, E_4 –0.1 V, 60 ms), using a linear gradient of 30–360 mM NaOAc in 0.1 M NaOH. Quantitations of Neu5Ac and Neu5Gc were done by reference to Neu5Ac and Neu5Gc calibration curves, respectively [44].

Fig. 2. WAX-HPLC profiles of 2AB-labelled *N*-glycans of bovine LF. A. total *N*-glycan pool, B. WFA fraction I (unbound), C. WFA fraction II (intermediate), D. WFA fraction III (bound). N0, neutral glycans; N1, monocharged glycans; N2, dicharged glycans.

For analysis by HPLC-FD, 2 mg bovine LF samples were hydrolysed in 200 μ l 2 M propionic acid (3 h, 80 °C). After centrifugation, aliquots of 50 μ l supernatant were mixed with 50 μ l DMB reagent (7 mM 1,2-diamino-4,5-methylenedioxybenzene, 18 mM sodium hydrosulfite, and 750 mM β -mercaptoethanol in 1.5 M propionic acid) and left to react in

the dark for 2.5 h at 50 °C. Analyses were performed on a Waters 2690XE Alliance HPLC system (Waters, Etten-Leur, The Netherlands), equipped with a Waters 474 fluorescence detector (λ_{ex} 373 nm, λ_{em} 448 nm) on a reversed phase Cosmosil 5C18-AR-II column (4.6 mm $\times$ 250 mm, Waters, Eschborn, Germany). Elutions were carried

Fig. 3. NP-HPLC profiles of 2AB-labelled N-glycans of WFA fraction I: A. without exoglycosidase treatment, and after sequential treatment with B. α -mannosidase, C. α -sialidase, D. β -galactosidase, E. α -fucosidase, F. α -galactosidase, G. β -galactosidase.

out with acetonitrile/methanol/water (9:7:84, v/v/v) as solvent system at a flow rate of 1 ml/min [45].

2.4. N-glycan release

A sample of 50 mg bovine LF was denatured with 100 μ l β -mercaptoethanol in 1 ml 1% SDS for 1 h at 85 °C, then 100 μ l 1 M

iodoacetamide was added, and the mixture was kept for 30 min at 55 °C in darkness. N-glycans were released enzymatically, using peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (PNGaseF) (EC 3.5.1.52) (Roche Applied Science, Mannheim, Germany). First Nonidet P40 substitute (Sigma, St. Louis, MO) was added to a final concentration of 1%. Digestions were performed by adding 15 units of enzyme activity per mg of protein (48 h, 37 °C) in two batches, i.e. 10 U/mg

Table 2

Structures observed in WFA fraction I and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A–G are based on HPLC peak integrations), MALDI-TOF-MS confirmation was acquired (*m/z* values are theoretical masses, observed values lie within 1.5 *m/z* range from this value).

Structure	<i>m/z</i>	GU	A ^a	B ^a	C ^a	D ^a	E ^a	F ^a	G ^a
	2025.7	9.45	19	0	0	0	0	0	0
	n d ^b	9.12	n d ^c	1	0	0	0	0	0
	1945.8	7.98/8.06	n d	1	1	0	0	0	0
	n d	9.96	n d	1	0	0	0	0	0
	1929.7	7.70	n d	1	1	0	0	0	0
	1863.7	8.80	8	0	0	0	0	0	0
	1863.7	8.71	16	0	0	0	0	0	0
	n d	9.47	n d	7	0	0	0	0	0
	n d	8.34	n d	2	0	0	0	0	0
	1783.7	7.30	7	6	14	0	0	0	0
	1783.7	7.21	0	0	0			0	0
	1783.7	7.08	0	0	0	1	1	0	0
	1701.6	7.91	13	0	0	0	0	0	0
	1701.6	7.85	3	0	0	0	0	0	0
	1621.6	6.42	0	0	0	0	0		0
	1621.6	6.32	0	0	0	0	0	1	0
	1564.6	5.97	0	0	0	1	0	0	0
	1539.5	7.18	5	0	0	0	0	0	0
	1539.5	7.05	4	0	0	0	0	0	0
	1459.5	5.50	0	0	0	15	15	14	15
	1418.5	5.94	n d	7	8	0	0	0	0
	1377.5	6.18	5	0	0	0	0	0	0
	1256.5	5.07	n d	1	1	0	0	0	0
	1256.5	4.95	n d	1	2	9	9	10	9

^aExoglycosidase treatments are in sequence: A. Untreated, B. α -mannosidase, C. α -sialidase, D. β -galactosidase, E. α -fucosidase, F. α -galactosidase, G. β -galactosidase.

^bIt should be noted that sialic acids are commonly lost prior to analysis by the MALDI-TOF-MS detector and are therefore not observed.

^cPrior to treatment with α -mannosidase many of the minor components are not observed, due to overlap with the large oligomannose-derived peaks.

followed by 5 U/mg after 24 h. Released N-glycans were separated from de-N-glycosylated protein on a Carbohydrate SPE column (300 mg graphitised carbon, Alltech), using 40% aqueous acetonitrile, containing 0.05% TFA as eluent. Then, the obtained N-glycan pool was treated with CalBio-sorb beads (CalBiochem, La Jolla, CA), to remove Nonidet P40 and SDS, and Dowex H⁺ 50WX8 (Sigma), and passed across a cotton wool plug to remove solids.

2.5. 2AB labelling of N-glycans

The purified and lyophilised N-glycan pool was treated with 0.35 M 2-aminobenzamide (2AB, Sigma), 1 M sodium cyanoborohy-dride in dimethyl sulfoxide/acetic acid (7:3, v/v) for 2 h at 65 °C. La-belled glycans were purified by paper chromatography, using acetic acid-treated QMA paper (Whatman). Excess 2AB was removed by washing with 8 times 500 µl acetonitrile. Labelled glycans were eluted with 5 times 500 µl Milli-Q water [46,47].

2.6. Lectin affinity chromatography

The lyophilised, 2AB-labelled N-glycan pool was dissolved in 500 µl binding buffer (15 mM PBS, 0.15 M NaCl, pH 7.5) and applied to an agarose-bound *Wisteria floribunda* agglutinin (WFA) column (2 ml bed volume, Vector Laboratories Ltd., Peterborough, UK). After washing with 5 column volumes binding buffer, the lectin column was eluted with 5 column volumes binding buffer/acetic acid (pH 5.5), followed by 5 column volumes 100 mM GalNAc in binding buffer/acetic acid (pH 5.5). The column was reconstituted with 20 mM Tris·HCl, pH 4.5, containing 0.5 M NaCl, and stored in 20% EtOH, 80% 20 mM Tris·HCl, pH 5.8, containing 1 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂.

2.7. Mass spectrometry

Matrix-assisted laser-desorption ionisation time-of-flight mass spec-trometry (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). Positive-ion mode spectra were acquired using the reflectron mode and delayed ex-traction with software controlled delay time optimised for *m/z* 1500. The accelerating voltage was 20 kV and the acquisition range was 800–

4000 Da. 1-µl samples were mixed on the target plate with 1 µl 2,5-dihy-droxybenzoic acid (10 mg/ml) as matrix in 40% aqueous acetonitrile. After drying of the droplets, the spots were re-crystallised with ethanol.

2.8. Normal phase-HPLC profiling

2AB-labelled N-glycan samples were profiled on a TSKgel amide-80 column (4.6 mm × 150 mm, 3 µm particle size; Tosoh Bioscience GmbH, Stuttgart, Germany) at 40 °C, using a Waters 2690XE Alliance HPLC sys-tem (Waters, Etten-Leur, The Netherlands), 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-labelled external dextran ladder and reference N-glycans (Ludger Ltd., Oxfordshire, UK), and peak retention times of 2AB-labelled N-glycans were expressed in dextran-ladder-derived glucose units (GU) values. In Table 1 GU values for monosaccharide residues, as determined from our standards, and compared to GU values found in Glycobase 2.0 [48], are presented.

2.9. Exoglycosidase assays

Digestions were performed overnight at 37 °C in 50 mM sodium acetate buffer, pH 5.5. The following enzymes were used: *Arthrobac-ter ureafaciens* α -sialidase (1 U/100 µl in 10 mM Na₃PO₄, 0.1% Micr-O-protect, pH 7, containing 0.25 mg/ml BSA; Roche, Mannheim, Ger-many), recombinant *Streptomyces plicatus* β -N-acetylhexosaminidase (500 U/100 µl; suspension in 50 mM NaCl, 5 mM Na₂EDTA, pH 7.5; New England BioLabs Inc., Ipswich, MA), jack bean α -mannosidase (65 U/ml in 3.0 M (NH₄)₂SO₄, 0.1 mM zinc acetate, pH 7.5, Sigma), bo-vine testis β -galactosidase (0.2 U/100 µl in 3.2 M (NH₄)₂SO₄, pH 5.0; Sigma), green coffee bean α -galactosidase (20 U/100 µl in 3.2 M (NH₄)₂SO₄, pH 6.0, containing BSA; Sigma), and bovine kidney α -fucosidase (51 U/100 µl in 3.2 M (NH₄)₂SO₄, 10 mM NaH₂PO₄, 10 mM sodium citrate, pH 6.0; Sigma). After digestion the enzymes were removed by 10 kDa cut-off centrifugal filters (Millipore).

Scheme 1. Overview of bovine lactoferrin N-glycan structures found in WFA fraction I.

2.10. Weak-anion-exchange chromatography

2AB-labelled N-glycans were analysed by weak-anion-exchange chromatography (WAX) on a DEAE column (Grace Vydac, 4.6 mm × 100 mm, 30 °C), 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 a fetuin N-glycan standard (Ludger Ltd.). Peak areas were used for quantitation.

Fig. 4. NP-HPLC profiles of 2AB-labelled N-glycans of WFA fraction II; A. without exoglycosidase treatment, and after sequential treatment with B. α -sialidase, C. β -N-acetylhexosaminidase, D. α -fucosidase, E. β -galactosidase, F. α -mannosidase.

Structures observed in WFA fraction **II** and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A-F are based on peak integrations), MALDI-TOF-MS confirmation was acquired (m/z values are theoretical masses, observed values lie within 1.5 m/z range from this value).

Structure	m/z	GU	A ^a

n d	9.58	1	0	0	0	0	0
n d	9.34	1	0	0	0	0	0
n d	9.07	8	0	0	0	0	0
n d	8.49	6	0	0	0	0	0
n d	8.41	15	0	0	0	0	0
n d	8.89	2	0	0	0	0	0
n d	8.80	2	0	0	0	0	0
n d	8.54	7	0	0	0	0	0
n d	8.15	6	0	0	0	0	0
n d	8.01	19	0	0	0	0	0
n d	7.93	4	0	0	0	0	0
n d	7.38	18	0	0	0	0	0
n d	7.13	3	0	0	0	0	0
n d	7.05	5	0	0	0	0	0
n d	6.68	5	0	0	0	0	0
1970.7	7.61/7.56	0	5	0	0	0	0
	1945.8	8.16	0	1	0	0	0	0
	2011.8	7.36	0	8	0	0	0	0
	1824.7	7.17/7.10	0	16	0	0	0	0
	1783.7	7.04	0	6	0	0	0	0
	1865.7	6.92	0	27	0	0	0	0
	1621.6	6.83	0	18	0	0	0	0
	1662.6	6.08	0	5	0	0	0	0
	1662.6	6.02	0	3	0	0	0	0
	1459.5	5.56	0	5	0	0	0	0
	1767.7	6.83	0	4	0	0	0	0
	1742.6	7.48	0	0	2	3	3	0
	1767.7	6.89	0	0	5	0	0	0
	1767.7	6.81	0	0	4	0	0	0
	1580.6	6.67	0	0	6	5	6	0
	1621.6	6.49	0	0	7	11	0	0
	1621.6	6.37	0	0	9	14	0	0
	1564.6	6.21	0	0	3	0	0	0
	1418.5	5.76	0	0	16	18	17	0
	1605.6	5.98	0	0	8	0	0	0
	1459.5	5.55	0	0	31	40	65	66
	1256.5	5.03	0	0	9	9	9	23
	1093.4	4.23	0	0	0	0	0	11

^bIt should be noted that sialic acids are commonly lost prior to analysis by the MALDI-TOF-MS detector and are therefore not observed.

3. Results and discussion

3.1. Glycan composition

Monosaccharide analysis [43] of bovine LF showed the presence of Fuc (trace), Man (3.0), Gal (0.4), GalNAc (0.3), GlcNAc (0.7), and Neu5Ac (0.3). The low relative abundances of Gal and GlcNAc in reference to Man, standardised at 3.0 units, are in accordance with large quantities of oligomannose-type structures [40]. Taking into account that O-glycans are absent [40–42], the presence of GalNAc in approximately equal amounts as Gal, indicates a significant occurrence of GalNAc(β1-4)GlcNAc (LacdiNAc) epitopes. It should be noted that the Asn-linked GlcNAc is not cleaved by methanolysis, resulting in an underestimation of GlcNAc. The carbohydrate content of bovine LF was found to be $6.7 \pm 0.2\%$ wt. Sialic acid analysis using both HPLC-FD (DMB-labelling; two analyses of duplicate samples) [45] and HPAEC-PAD analysis (triple analysis of duplicate samples) [44] revealed that $8.5 \pm 0.3\%$ of released sialic acid is Neu5Gc. It should be noted that in a pool of bovine milk serum proteins $\sim 1.9\%$ of sialic acid is Neu5Gc [49].

3.2. N-glycan release

In order to develop analytical procedures with one uniform batch of N-glycans, considering the findings of an inter-laboratory study [50,51], a large quantity of bovine LF (100 mg) was chosen for digestion with PNGaseF. The released glycans were isolated by Carbohydrate SPE chromatography, and screened by MALDI-TOF-MS (data not shown), showing major peaks at m/z 1257.4, 1419.5, 1582.1, 1743.6, and 1905.8, corresponding with the sodium adduct masses of Hex₅₋₉HexNAc₂. Taking into account the N-glycan biosynthetic pathways in mammals, these peaks indicate a major presence of Man₅GlcNAc₂–Man₉GlcNAc₂ structures, respectively. Note that for presentation of glycan structures in this report the CFG colour format is used in figures and schemes [52].

3.2.1. NP-HPLC profiling of 2AB-labelled N-glycans

NP-HPLC profiling of the derivatised N-glycan pool (Fig. 1) showed major peaks at GU values corresponding with Man₅GlcNAc₂ (GU 6.18), Man₆GlcNAc₂ (GU 7.06, 7.18), Man₇GlcNAc₂ (GU 7.85, 7.91),

Man₈GlcNAc₂ (GU 8.71, 8.80), and Man₉GlcNAc₂ (GU 9.45), respectively. To eliminate these compounds in the NP-HPLC profile, the N-glycan pool was treated twice with jack bean α -mannosidase, whereby the N,N'-diacetylchitobiose core with a single (β1-4)-linked mannose residue attached (GU 2.77) is formed. Integration of the peak at GU 2.77 in relation to the remaining peaks after α -mannosidase treatment, shows a relative abundance of $\sim 65\%$ oligomannose-type structures and $\sim 35\%$ hybrid- and complex-type structures in the N-glycan pool. This observation is in accordance with the relatively high amount of mannose found by monosaccharide analysis, as well as the major MALDI-TOF-MS peaks corresponding with Man₅₋₉GlcNAc₂ structures. These findings fit very well with previous reports on bovine LF, showing the occurrence of mainly oligomannose-type glycans [41]. However, so far the presence of relatively large amounts of hybrid-type structures has not been reported.

3.2.2. WAX-HPLC profiling of 2AB-labelled N-glycans

Analysis of the charge distribution of the N-glycan pool by weak-anion-exchange chromatography (Fig. 2A) showed a majority of neutral glycans (76%), a significant amount of di-charged structures (15%), with a lower amount of mono-charged structures (9%).

3.2.3. WFA-lectin chromatography combined with NP-HPLC and WAX-HPLC

By using a WFA-agarose column, the N-glycan pool could be separated into three fractions. The non-binding fraction I was eluted with binding buffer (PBS/NaCl, pH 7.5), the intermediate fraction II with binding buffer, calibrated to pH 5.5 with acetic acid, and the bound fraction III with 100 mM N-acetylgalactosamine in binding buffer, calibrated to pH 5.5 with acetic acid.

3.2.3.1. WFA fraction I. The NP-HPLC profile of WFA fraction I showed major peaks corresponding with Man₅₋₉GlcNAc₂ (Fig. 3A), together with some minor peaks. WAX-HPLC analysis (Fig. 2B) revealed a large peak for neutral glycans (87%), a peak for mono-charged glycans (3%), and a peak for di-charged glycans (10%). In order to analyse the non-oligomannose-type glycans, all oligomannose-type structures were digested with α -mannosidase (Fig. 3B), resulting in one major

Scheme 2. Overview of bovine lactoferrin N-glycan structures found in WFA fraction II.

peak at 2.77 GU (~70%), three peaks corresponding with the remains of hybrid-type structures (~12%), and peaks corresponding with di-antennary complex-type structures (~18%). For a further unravelling of the profile, a series of sequential exoglycosidase treatments was performed. After each exoglycosidase step, a NP-HPLC profile (Fig. 3C to G) and a MALDI-TOF mass spectrum were recorded. In Table 2 a survey of the relative surface area data in the changing NP-HPLC profiles is presented. All included structures, found after each exoglycosidase step, were confirmed by MALDI-TOF-MS. In this way for each HPLC peak in Fig. 3A and B the exoglycosidase steps were followed, yielding the information for elucidating the starting structures.

Using the combined HPLC/exoglycosidase/MS/Glycobase data, the various N-glycan structures in WFA fraction I were assigned. A

survey of these glycans, together with a rough quantitation within this fraction, based on Fig. 3A and B, is presented in Scheme 1. It has to be noted that many of the structures overlap. Two notable glycans are the minor compounds containing the Gal(α 1-3)Gal epitope, one of which, i.e. structure 11 in Scheme 1, was previously found as a major component in a ConA-Sepharose intermediate fraction [41]. It should also be noted that the α -mannosidase treatment at the start of the analysis also degrades all hybrid-type structures (structures 6–8, Scheme 1), thereby limiting the information on hybrid-type structures in this fraction. Considering the abundance of oligomannose-type structures in the N-glycan pool prior to WFA-lectin separation (~65%) and the amount in fraction I (~71%), fraction I consists of approximately 91% of the LF N-glycans. For the location of Gal(α 1-3)- and Neu5Ac(α 2-6)- residues,

Fig. 5. NP-HPLC profiles of 2AB-labelled 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, E. α -mannosidase.

Structures observed in WFA fraction **III** and after sequential exoglycosidase treatments. For each assigned NP-HPLC peak (percentages for each profile A-E are based on peak integrations), MALDI-TOF-MS confirmation was acquired (m/z values are theoretical masses, observed values lie within 1.5 m/z range from this value).

^aExoglycosidase treatments are in sequence: A. Untreated, B. α -fucosidase, C. β -N-acetylhexosaminidase, D. β -galactosidase, E. α -mannosidase.

Scheme 3. Overview of bovine lactoferrin N-glycan structures found in WFA fraction III.

1.24), respectively, corresponding with the loss of a single (α 2-6)-linked Neu5Ac residue. Treatment with *Streptomyces plicatus* β -N-acetylhexosaminidase the NP-HPLC profile (Fig. 4C) caused again shifts in all peaks. The peaks at 6.34, 6.92, and 7.10 GU shifted to 5.76 (Δ GU 0.58), 5.55 (Δ GU 1.37), and 6.37 (Δ GU 0.73), corresponding with the loss of one, two and one HexNAc residues, respectively. The enzyme that was selected (*Streptomyces plicatus*) has affinity for GlcNAc(β 1-3/4/6)- and GalNAc(β 1-4)-linked, leaving the (β 1-2)-linked GlcNAc residues of hybrid-type and di-antennary complex-type structures intact. So far, no tri- or tetra-antennary complex-type structures have been reported for LF [40–42], nor have we observed any evidence for such structures in WFA fraction I. In view of this absence, the selected enzyme will only cleave GalNAc residues in LacdiNAc epitopes. Combining the NP-HPLC and MALDI-TOF-MS data, collected after each exoglycosidase treatment, and taking into account the Glycibase library data, the structures in WFA fraction II were assigned, and are presented in Scheme 2. The quantification of the assigned compounds is based on peaks in NP-HPLC. It should be noted that the intensity of the peaks in NP-HPLC of WFA fraction II, taken up in the same amount of Milli-Q water after WFA-separation, is much higher than for fraction III, indicating fraction II is the major WFA-bound fraction.

3.2.3.3. WFA fraction III. The NP-HPLC profile of WFA fraction III (Fig. 5A) showed one major peak at 6.95 GU, corresponding with the GU value found in WFA fraction II for [GalNAcGlcNAc]₂-Man₃-GlcNAc₂ (*m/z* 1865.7); its relationship was confirmed by MALDI-TOF-MS (not shown). WAX-HPLC profiling (Fig. 2D) indicated the presence of only a neutral fraction. In the NP-HPLC profile several smaller peaks occurred, corresponding with structures containing a single LacdiNAc epitope, and minor peaks for core fucosylated structures. In a similar fashion as carried out for WFA fractions I and II, WFA fraction III (start profile in Fig. 5A) was subjected to a series of sequential exoglycosidase treatments (Table 4), monitored by NP-HPLC (Fig. 5B–E) and MALDI-TOF-MS. Combining the HPLC/exoglycosidase/MS data and taking into account the biosynthetic pathways, structures were assigned, and are presented in Scheme 3. Again, relative abundances of compounds are based on NP-HPLC profiles.

4. Conclusions

Although the N-glycosylation of bovine LF has been studied extensively before [41], not all aspects were studied in as much detail. First, the report shows a methodology to simplify the analysis of N-glycans in case that besides LacNAc also LacdiNAc units are present in the hybrid- and complex-type oligosaccharides. The inclusion of WFA-lectin affinity chromatography, applied to the released and 2AB-labelled N-glycans, in the usual analysis protocol based on NP-HPLC/WAX-HPLC/exoglycosidases/MS, makes a first unravelling of complex glycan mixtures containing both LacNAc and LacdiNAc elements possible, thereby simplifying the assignment procedures. Well-separated fractions, containing mixtures of glycans with specific structural elements, are obtained, of which the strongly bound fraction contains structures with the LacdiNAc unit, and the weakly bound fraction structures with LacdiNAc units shielded by sialic acid. Secondly, previous studies had not focused on the quantity of Neu5Gc present in this glycoprotein. In the present report it is shown that bovine LF contained $8.5 \pm 0.3\%$ Neu5Gc. This is a relatively high amount, compared to bovine milk and whey protein concentrates ($\sim 2.6\%$ and $\sim 1.9\%$, respectively), however, still lower than reported for ewe and goat milk [49].

In this research a total of 42 N-glycan structures have been assigned for bovine LF; in previous work on bovine LF, only 26 N-glycan structures were reported [41]. For recombinant human LF,

expressed in bovine milk, 17 N-glycan structures were published [42]. Structures containing LacdiNAc were observed in all possible forms, with up to two sialylated LacdiNAc units, and even previously unreported LacdiNAc-containing hybrid-type structures. The Gal(α 1-3)Gal- epitope was observed in minor amounts in different structures of the unbound bulk fraction; previously, only a single structure as a major component in a ConA-Sepharose intermediate fraction was found [41].

Acknowledgements

This work was financially supported by a grant from the Northern Netherlands collaboration initiative (SNN EZ/KOMPAS RM119) and by FrieslandCampina Domo.

References

- [1] M. Gahr, C.P. Speer, B. Damerou, G. Sawatzki, Influence of lactoferrin on the function of human polymorphonuclear leukocytes and monocytes, *J. Leukoc. Biol.* 49 (1991) 427–433.
- [2] K. Sorimachi, K. Akimoto, Y. Hattori, T. Ieiri, A. Niwa, Activation of macrophages by lactoferrin: secretion of TNF- α , IL-8 and NO, *IUBMB Life* 43 (1997) 79–87.
- [3] C.S. Curran, K.P. Demick, J.M. Mansfield, Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways, *Cell. Immunol.* 242 (2006) 23–30.
- [4] K.-N. Son, J. Park, C.-K. Chung, D.K. Chung, D.-Y. Yu, K.-K. Lee, J. Kim, Human lactoferrin activates transcription of IL-1 β gene in mammalian cells, *Biochem. Biophys. Res. Commun.* 290 (2002) 236–241.
- [5] B.J. Appelmelk, Y.-Q. An, M. Geerts, B.G. Thijs, H.A. de Boer, D.M. MacLaren, J. de Graaff, J.H. Nuijens, Lactoferrin is a lipid A-binding protein, *Infect. Immun.* 62 (1994) 2628–2632.
- [6] E. Elaiss-Rochard, A. Roseanu, D. Legrand, M. Trif, V. Salmon, C. Motas, J. Montreuil, G. Spik, Lactoferrin-lipopolysaccharide interaction: involvement of the 28–34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide, *Biochem. J.* 312 (1995) 839–845.
- [7] Y.A. Suzuki, V. Lopez, B. Lönnnerdal, Mammalian lactoferrin receptors: structure and function, *Cell. Mol. Life Sci.* 62 (2005) 2560–2575.
- [8] B. Lönnnerdal, A. Bryant, Absorption of iron from recombinant human lactoferrin in young US women, *Am. J. Clin. Nutr.* 83 (2006) 305–309.
- [9] M. Konishi, M. Iwasa, K. Yamauchi, R. Sugimoto, N. Fujita, Y. Kobayashi, S. Watanabe, S. Taraguchi, Y. Adachi, M. Kaito, Lactoferrin inhibits lipid peroxidation in patients with chronic hepatitis C, *Hepatol. Res.* 36 (2006) 27–32.
- [10] J.H. Nuijens, P.H.C. van Berkel, F.L. Schanbacher, Structure and biological actions of lactoferrin, *J. Mammary Gland Biol. Neoplasia* 1 (1996) 285–295.
- [11] D. Naot, A. Grey, I.R. Reid, J. Cornish, Lactoferrin – a novel bone growth factor, *Clin. Med. Res.* 3 (2005) 93–101.
- [12] H. Tsuda, K. Sekine, K. Fujita, M. Iigo, Cancer prevention by bovine lactoferrin and underlying mechanisms – a review of experimental and clinical studies, *Biochem. Cell Biol.* 80 (2002) 131–136.
- [13] H. Tsuda, Y. Ohshima, H. Nomoto, K. Fujita, E. Matsuda, M. Iigo, N. Takasuka, M.A. Moore, Cancer prevention by natural compounds, *Drug Metab. Pharmacokinet.* 19 (2004) 245–263.
- [14] A. Varadhachary, J.S. Wolf, K. Petrak, B.W. O'Malley Jr., M. Spadaro, C. Curcio, G. Forni, F. Pericle, Oral lactoferrin inhibits growth of established tumors and potentiates conventional chemotherapy, *Int. J. Cancer* 111 (2004) 398–403.
- [15] M. Benaïssa, J.-P. Peyrat, L. Hornez, C. Mariller, J. Mazurier, A. Pierce, Expression and prognostic value of lactoferrin mRNA isoforms in human breast cancer, *Int. J. Cancer* 114 (2005) 299–306.
- [16] M. Iigo, T. Kuhara, Y. Ushida, K. Sekine, M.A. Moore, H. Tsuda, Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice, *Clin. Exp. Metastasis* 17 (1999) 35–40.
- [17] G. Spik, B. Coddeville, J. Mazurier, Y. Bourne, C. Camillaut, J. Montreuil, Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans, *Adv. Exp. Med. Biol.* 357 (1994) 21–32.
- [18] Z. Wei, T. Nishimura, S. Yoshida, Characterization of glycans in a lactoferrin isoform, lactoferrin-a, *J. Dairy Sci.* 84 (2001) 2584–2590.
- [19] Z. Wei, T. Nishimura, S. Yoshida, Presence of a glycan at a potential N-glycosylation site, Asn-281, of bovine lactoferrin, *J. Dairy Sci.* 83 (2000) 683–689.
- [20] M. Hamosh, Bioactive factors in human milk, *Pediatr. Clin. North Am.* 48 (2001) 69–86.
- [21] J.M. Steijns, A.C.M. van Hooijdonk, Occurrence, structure, biochemical properties and technological characteristics of lactoferrin, *Br. J. Nutr.* 84 (Suppl. 1) (2000) S11–S17.
- [22] A. Pierce, D. Colavizza, M. Benaïssa, P. Maes, A. Tartar, J. Montreuil, G. Spik, Molecular cloning and sequence analysis of bovine lactotransferrin, *Eur. J. Biochem.* 196 (1991) 177–184.
- [23] S.A. Moore, B.F. Anderson, C.R. Groom, M. Haridas, E.N. Baker, Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution, *J. Mol. Biol.* 274 (1997) 222–236.

- [24] M. Haridas, B.F. Anderson, E.N. Baker, Structure of human diferric lactoferrin refined at 2.2 Å resolution, *Acta Crystallogr. D: Biol. Crystallogr.* 51 (1995) 629–646.
- [25] G. Spik, G. Strecker, B. Fournet, S. Bouqulet, J. Montreuil, L. Dorland, H. van Halbeek, J.F.G. Vliegthart, Primary structure of the glycans from human lactotransferrin, *Eur. J. Biochem.* 121 (1982) 413–419.
- [26] P.H.C. van Berkel, H.A. van Veen, M.E.J. Geerts, H.A. de Boer, J.H. Nuijens, Heterogeneity in utilization of N-glycosylation sites Asn₆₂₄ and Asn₁₃₈ in human lactoferrin: a study with glycosylation-site mutants, *Biochem. J.* 319 (1996) 117–122.
- [27] S. Yoshida, Z. Wei, Y. Shinmura, N. Fukunaga, Separation of lactoferrin-a and -b from bovine colostrum, *J. Dairy Sci.* 83 (2000) 2211–2215.
- [28] S.P. Commins, H.R. James, L.A. Kelly, S.L. Pochan, L.J. Workman, M.S. Perzanowski, K.M. Kocan, J.V. Fahy, L.W. Nganga, E. Ronmark, P.J. Cooper, T.A.E. Platts-Mills, The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose- α -1,3-galactose, *J. Allergy Clin. Immunol.* 127 (2011) 1286–1293.
- [29] U. Galili, C.B. Basbaum, S.B. Shohet, J. Beuhler, B.A. Macher, Identification of erythrocyte Gal α 1-3Gal glycosphingolipids with a mouse monoclonal antibody, Gal-13, *J. Biol. Chem.* 262 (1987) 4683–4688.
- [30] A.A. Bergwerff, J. van Oostrum, J.P. Kamerling, J.F.G. Vliegthart, The major N-linked carbohydrate chains from human urokinase. The occurrence of 4-O-sulfated, (α 2-6)-sialylated or (α 1-3)-fucosylated N-acetylglucosamine(β 1-4)-N-acetylglucosamine elements, *Eur. J. Biochem.* 228 (1995) 1009–1019.
- [31] G. Weisshaar, J. Hiyama, A.G.C. Renwick, M. Nimtz, NMR investigations of the N-linked oligosaccharides at individual glycosylation sites of human lutropin, *Eur. J. Biochem.* 195 (1991) 257–268.
- [32] A. Dell, H.R. Morris, R.L. Easton, M. Panico, M. Pantakar, S. Oehninger, R. Koistinen, H. Koistinen, M. Seppala, G.F. Clark, Structural analysis of the oligosaccharides derived from glycodefin, a human glycoprotein with potent immunosuppressive and contraceptive activities, *J. Biol. Chem.* 270 (1995) 24116–24126.
- [33] B.W. Grinnell, R.B. Hermann, S.B. Yan, Human protein C inhibits selectin-mediated cell adhesion: role of unique fucosylated oligosaccharide, *Glycobiology* 4 (1994) 221–225.
- [34] J.J.M. van Rooijen, J.P. Kamerling, J.F.G. Vliegthart, Sulfated di-, tri- and tetraantennary N-glycans in human Tamm-Horsfall glycoprotein, *Eur. J. Biochem.* 256 (1998) 471–487.
- [35] N. Tomiya, J. Awaya, M. Kurono, H. Hanzawa, I. Shimada, Y. Arata, T. Yoshida, N. Takahashi, Structural elucidation of a variety of GalNAc-containing N-linked oligosaccharides from human urinary kallidinogenase, *J. Biol. Chem.* 268 (1993) 113–126.
- [36] A. Dell, S.M. Haslam, H.R. Morris, K.-H. Khoo, Immunogenic glycoconjugates implicated in parasitic nematode disease, *Biochim. Biophys. Acta* 1455 (1999) 353–362.
- [37] R. Schauer, J.P. Kamerling, Chemistry, biochemistry and biology of sialic acids, in: J. Montreuil, J.F.G. Vliegthart, H. Schachter (Eds.), *Glycoproteins II*, New Comprehensive Biochem., 29b, Elsevier Science BV, Amsterdam, 1997, pp. 243–402.
- [38] V. Padler-Karavani, H. Yu, H. Cao, H. Chokhawala, F. Karp, N. Varki, X. Chen, A. Varki, Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: potential implications for disease, *Glycobiology* 18 (2008) 818–830.
- [39] M. Bardor, D.H. Nguyen, S. Diaz, A. Varki, Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells, *J. Biol. Chem.* 280 (2005) 4228–4237.
- [40] G. Spik, B. Coddeville, J. Montreuil, Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species, *Biochimie* 70 (1988) 1459–1469.
- [41] B. Coddeville, G. Strecker, J.-M. Wieruszkeski, J.F.G. Vliegthart, H. van Halbeek, J. Peter-Katalinić, H. Egge, G. Spik, Heterogeneity of bovine lactotransferrin glycans. Characterization of α -D-Galp-(1 $\rightarrow$ 3)- β -D-Gal- and α -NeuAc-(2 $\rightarrow$ 6)- β -D-GalpNAc-(1 $\rightarrow$ 4)- β -D-GlcNAc-substituted N-linked glycans, *Carbohydr. Res.* 236 (1992) 145–164.
- [42] T. Yu, C. Guo, J. Wang, P. Hao, S. Sui, X. Cheng, R. Zhang, P. Wang, G. Yu, L. Zhang, Y. Dai, N. Li, Comprehensive characterization of the site-specific N-glycosylation of wild-type and recombinant lactoferrin expressed in the milk of transgenic cloned cattle, *Glycobiology* 21 (2011) 206–224.
- [43] J.P. Kamerling, J.F.G. Vliegthart, Carbohydrates, in: A.M. Lawson (Ed.), *Clinical Biochemistry – Principles, Methods, Applications, Mass Spectrometry*, Vol. 1, Walter de Gruyter, Berlin, 1989, pp. 176–263.
- [44] J.S. Rohrer, J. Thayer, M. Weitzhandler, N. Avdalovic, Analysis of the N-acetylneuraminic acid and N-glycolylneuraminic acid contents of glycoproteins by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD), *Glycobiology* 8 (1998) 35–43.
- [45] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Fluorometric high-performance liquid chromatography of N-acetyl- and N-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids, *Anal. Biochem.* 164 (1987) 138–145.
- [46] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid, *Anal. Biochem.* 230 (1995) 229–238.
- [47] A. Kinoshita, K. Sugahara, Microanalysis of glycosaminoglycan-derived oligosaccharides labeled with a fluorophore 2-aminobenzamide by high-performance liquid chromatography: application to disaccharide composition analysis and exosequencing of oligosaccharides, *Anal. Biochem.* 269 (1999) 367–378.
- [48] M.P. Campbell, L. Royle, C.M. Radcliffe, R.A. Dwek, P.M. Rudd, GlycoBase and autoGU: tools for HPLC-based glycan analysis, *Bioinformatics* 24 (2008) 1214–1216.
- [49] V. Spichtig, J. Michaud, S. Austin, Determination of sialic acids in milks and milk-based products, *Anal. Biochem.* 405 (2010) 28–40.
- [50] Y. Wada, P. Azadi, C.E. Costello, A. Dell, R.A. Dwek, H. Geyer, R. Geyer, K. Kakehi, N.G. Karlsson, K. Kato, N. Kawasaki, K.-H. Khoo, S. Kim, A. Kondo, E. Lattova, Y. Mechref, E. Miyoshi, K. Nakamura, H. Narimatsu, M.V. Novotny, N.H. Packer, H. Perreault, J. Peter-Katalinić, G. Pohlentz, V.N. Reinhold, P.M. Rudd, A. Suzuki, N. Taniguchi, Comparison of the methods for profiling glycoprotein glycans – HUPO human disease glycomics/proteome initiative multi-institutional study, *Glycobiology* 17 (2007) 411–422.
- [51] S. Thobhani, C.-T. Yuen, M.J.A. Bailey, C. Jones, Identification and quantification of N-linked oligosaccharides released from glycoproteins: an inter-laboratory study, *Glycobiology* 19 (2009) 201–211.
- [52] A.G. McDonald, K.F. Tipton, C.J.M. Stroop, G.P. Davey, GlycoForm and Glycologue: two software applications for the rapid construction and display of N-glycans from mammalian sources, *BMC Res. Notes* 3 (2010) 173.
- [53] D.H. van den Eijnden, D.H. Joziase, L. Dorland, H. van Halbeek, J.F.G. Vliegthart, K. Schmid, Specificity in the enzymic transfer of sialic acid to the oligosaccharide branches of bi- and triantennary glycopeptides of α -acid glycoprotein, *Biochem. Biophys. Res. Commun.* 92 (1980) 839–845.
- [54] H. van Halbeek, J.F.G. Vliegthart, H. Winterwerp, W.M. Blanken, D.H. van den Eijnden, α -D-Galactosyltransferase activity in calf thymus: a high-resolution ^1H -NMR study, *Biochem. Biophys. Res. Commun.* 110 (1983) 124–131.
- [55] L. Dorland, H. van Halbeek, J.F.G. Vliegthart, The identification of terminal α (1 $\rightarrow$ 3)-linked galactose in N-acetylglucosamine type of glycopeptides by means of 500-MHz ^1H -NMR spectroscopy, *Biochem. Biophys. Res. Commun.* 122 (1984) 859–866.
- [56] D.H. Joziase, M.L.E. Bergh, H.G.J. ter Hart, P.L. Koppen, G.J.M. Hooghwinkel, D.H. van den Eijnden, Purification and enzymatic characterization of CMP-sialic acid: β -galactosyl 1 $\rightarrow$ 3-N-acetylglucosamine α 2 $\rightarrow$ 3-sialyltransferase from human placenta, *J. Biol. Chem.* 260 (1985) 4941–4951.
- [57] D.H. Joziase, W.E.C.M. Schiphorst, D.H. van den Eijnden, J.A. van Kuik, H. van Halbeek, J.F.G. Vliegthart, Branch specificity of bovine colostrum CMP-sialic acid: Gal β 1 $\rightarrow$ 4GlcNAc-R α 2 $\rightarrow$ 6-sialyltransferase: sialylation of bi-, tri-, and tetraantennary oligosaccharides and glycopeptides of the N-acetylglucosamine type, *J. Biol. Chem.* 262 (1987) 2025–2033.