Analytical Biochemistry 421 (2012) 680–690

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Elucidation of a novel lacto-*N*-decaose core structure in human milk using nonlinear analytical technique combinations

Dennis Blank [a], Hildegard Geyer [a], Kai Maass [a,1], Katsuko Yamashita [b], Rudolf Geyer [a,*]

[a] Institute of Biochemistry, Faculty of Medicine, Justus-Liebig-University Giessen, Friedrichstrasse 24, 35392 Giessen, Germany
[b] Innovative Research Initiatives, Tokyo Institute of Technology, Yokohama 226-85-1, Japan

ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 21 November 2011
Accepted 22 November 2011
Available online 1 December 2011

Keywords:
Human milk oligosaccharides
Carbohydrate structure analysis
Mass spectrometry
GC/MS linkage analysis

ABSTRACT

Detailed structural analysis of high molecular weight human milk oligosaccharides (HMOs) is still a challenging task. Here we present a modular strategy for a flexible *de novo* structural characterization of this class of molecules. The protocol combines established techniques such as separation by two-dimensional high-performance liquid chromatography with different types of mass spectrometry, exoglycosidase digestion, and linkage analysis in an individual glycan-based manner. As a proof of principle, this approach was applied to two distinct HMO isomers representing a difucosylated octaose core and a trifucosylated decaose core. Obtained data revealed the presence of one terminal Lewis A and one internal Lewis X epitope in the case of the octaose and led to the identification of this molecule as a difucosylated *iso*-lacto-*N*-octaose. The trifucosylated, doubly branched lacto-*N*-*neo*-decaose was shown to represent a new type of HMO core structure in which the branched antenna is linked to carbon atom 3 of the innermost galactosyl residue. Hence, using this analytical protocol a novel HMO structure could be defined. Our results further demonstrate that a combination of different techniques may be required for *de novo* structural analysis of these molecules.

Structural characterization of complex human milk oligosaccharides (HMOs)[2] is still far away from lab routine. So far, more than 115 neutral and acidic HMOs have been described [1–4]. However, this may just represent the tip of the iceberg of expressed oligosaccharides as, in particular with increasing molecular weight and complexity as well as decreasing amounts, their analysis becomes more and more challenging. Due to the fact that accurate structural information is essential for further research on biological functions, e.g., the interaction of HMOs with pathogens, their prebiotic as well as immunomodulatory functions and the ability of HMOs to act as soluble receptor analogs [5–10], highly sensitive methods are indispensable for precise structural elucidation of these high molecular weight oligosaccharides.

HMOs consist of five different monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and, in part, *N*-acetylneuraminic acid (sialic acid). The disaccharide lactose (Gal(β1–4)Glc) always represents the reducing end of these glycans [11]. Chain elongation occurs by attachment of two different types of *N*-acetyllactosamine (LacNAc) units, which are distinguished in type I (Gal(β1–3)GlcNAc) and type II (Gal(β1–4)GlcNAc) LacNAc chains. These units can be either attached in (β1–3) linkage to an internal galactose resulting in linear, so-called *para* type of structure or may be linked in both (β1–3) and (β1–6) position to an internal Gal leading to branched glycans. So far, 13 different core structures have been elucidated [1]. Core oligosaccharides are further decorated by three different types of fucosyl transferases which transfer fucose residues either in (α1–2) position to terminal Gal residues, in (α1–3) position to the Glc unit at the reducing end or in (α1–3) or (α1–4) position to GlcNAc moieties of type II or type I LacNAc units, respectively. Acidic HMOs may be formed by attachment of *N*-acetylneuraminic acid in (α2–3) position to terminal Gal residues of both type I LacNAc chains as well as free lactose. Moreover, the attachment of sialic acid in (α2–6) linkage is found at the terminal Gal of type II structures, free lactose and subterminal GlcNAc units of type I chains [12].

Based on the published structural data, rules have been established delimiting the total number of possibly expressed HMOs. According to these rules, for example, only type II LacNAc chains

* Corresponding author. Fax: +49 641 99 47409.
E-mail address: Rudolf.Geyer@biochemie.med.uni-giessen.de (R. Geyer).

[1] Present address: Institute of Inorganic and Analytical Chemistry, Justus-Liebig-University of Giessen, Schubertstrasse 60, Haus 16, 35392 Giessen, Germany.

[2] *Abbreviations used:* HMOs, human milk oligosaccharides; 2AB, 2-aminobenzamide; ACN, acetonitrile; ATT, 6-aza-2-thiothymine; CID, collision-induced dissociation; EIC, extracted ion chromatogram; ESI, electrospray ionization; GC/MS, gas–liquid chromatography/mass spectrometry; HCCA, α-cyano-4-hydroxycinnamic acid; HF, hydrofluoric acid; HPLC, high-performance liquid chromatography; LacNAc, *N*-acetyllactosamine; LID, laser-induced dissociation; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS/MS, tandem mass spectrometry; TFA, trifluoroacetic acid.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.11.030

are further elongated by additional LacNAc units. Based on published structure databases, it can be further predicted that attachment of LacNAc in (β1–6) linkage only occurs when the (β1–3) position of the respective Gal unit is already occupied. Moreover, the first branch point of the molecules appears to be always located at the galactose of the lactose unit and extended, branched antennae are linked to carbon 6 of this Gal residue. Finally, it has been predicted that HMOs do not comprise internal (α1–2)-linked Fuc residues [13–15].

As HMOs are usually characterized by pronounced structural isomerism, multidimensional chromatographic separation of these compounds is often a prerequisite for subsequent structural analysis. Moreover, due to the fact that high molecular weight HMOs are only expressed in low abundance, analytical techniques must be as material saving as possible. Recently, several new and highly sensitive methods have been published, introducing different types of mass spectrometric approaches, such as, offline nano-electrospray ionization (nano-ESI) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) in the negative-ion mode [16,17], matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) after improved on-target derivatization of the glycans with 3-aminoquinoline [18,19], or HMO labeling with pyrene butanoic acid hydrazide [14,20] or 1-phenyl-3-methyl-5-pyrazolone [21] as well as ESI-ion trap-MS/MS of permethylated HMOs using electron transfer dissociation [22]. Valuable structural information has been also achieved by hydrophilic interaction chromatography of 2-aminobenzamide-labeled glycans in conjunction with exoglycosidase digestion and offline negative-ion mode MS/MS [23] or online porous graphitic carbon–high-performance liquid chromatography (HPLC)–Chip/TOF–MS of reduced HMOs before and after exoglycosidase treatment [3,4].

All these techniques reveal important structural information, although a complete *de novo* structure elucidation of HMOs is still challenging. For HPLC-based approaches, for example, reference substances are usually needed for retention time comparison. If accessible, however, these techniques can be combined with exoglycosidase digestion and may deliver highly reproducible information about single isomers [23]. The bottleneck of MS-based methods is that a prediction of the fragmentation pathways of complex HMO structures is often demanding and, therefore, standard substances may be again essential for correct annotation of the obtained fragment ions. Furthermore, MS/MS spectra of these glycans are widely characterized by a limited number of cross-ring cleavage products [14,16–18,20] which are needed for linkage assignment, thus increasing the risk of misinterpretation of data with respect to internal monosaccharide linkage positions. Though online MS techniques belong to the most powerful strategies for an accurate structural determination, drawbacks like the short time period, during which single isomers are accessible for further MS/MS analysis, make these methods still amendable. Most complete structural information including linkage positions and anomeric status of HMO monosaccharides can be achieved by NMR analysis [24–26]. However, though massive improvements have been made especially with regard to a reduction of the required amounts of sample, the development of 2D phase-sensitive techniques, and the development of powerful software tools enabling an annotation of even complex carbohydrate structures, NMR could not be applied in this particular case due to low amounts of material available.

Here we report on a strategy for the *de novo* structural characterization of complex HMOs using two-dimensional preparative HPLC separation, MALDI-TOF-MS (/MS) of native and permethylated glycans, and highly sensitive gas–liquid chromatography/mass spectrometry (GC/MS) linkage analyses in conjunction with exoglycosidase digestion. This combination of widely used analytical techniques enables both determination and structural affirmation in an individual manner. As exemplified for a distinct difucosylated HMO octaose isomer, the outlined panel of chemical, enzymatic, and mass spectrometric methods allowed the complete assignment of the HMO structure. Furthermore, using an individually adapted strategy a novel decaose core structure was characterized as a trifucosylated *inverse*-lacto-*N*-*neo*-decaose. Intriguingly, less than 3 nmol of each isomer was sufficient for these analyses.

Materials and methods

Reagents

2-Aminobenzamide (2AB), ammonium acetate, acetonitrile (ACN), and methanol were acquired from Merck (Darmstadt, Germany). 6-Aza-2-thiothymine (ATT) was purchased from Sigma Aldrich (Hamburg, Germany), α-cyano-4-hydroxycinnamic acid (HCCA) from Bruker Daltonics (Bremen, Germany), hydrofluoric acid (HF; 48%) from Carl Roth (Karlsruhe, Germany), and trifluoroacetic acid (TFA) from Promochem (Wesel, Germany). Water was filtered by a MilliQ system (Millipore, Billerica, MA, USA). All other chemicals were ordered from Sigma Aldrich and used without further purification. (α1–3,4)-Fucosidase from *Xanthomonas manihotis*, (β1–4)-galactosidase from *Diplococcus pneumoniae*, (β1–3,4)-galactosidase from bovine testis, and β-*N*-acetylhexosaminidase from jack beans were purchased from Sigma Aldrich.

Isolation of human milk oligosaccharides

Human milk was centrifuged at 2 °C. After solidified lipid was removed, ethanol was added to 68% at 0 °C. After removal of most lactose and proteins by centrifugation, the supernatant was used as crude human milk oligosaccharides [27].

Human milk oligosaccharides from a nonsecretor, Lewis (a+b−) individual were fractionated by Bio-Gel P-4 (<45 μm) column chromatography (100 × 2 cm) at 55 °C with distilled water. The oligosaccharides were grouped into seven groups (1–7) as previously reported [28]. Groups 1, 3, 4, 5, 6, and 7 involved all sialylated oligosaccharides (group 1), $(Fuc\alpha)_{0-4}$ decaoses (group 3), $(Fuc\alpha)_{0-3}$ octaoses (group 4), $(Fuc\alpha)_{0-2}$ hexaoses (group 5), $(Fuc\alpha)_{0-2}$ tetraoses (group 6), and $(Fuc\alpha)_{1-2}$ lactoses (group 7), since *N*-acetylglucosamine and (α1–3,4) fucose residues behave as approximately 2 glucose units and 0.5 glucose units, respectively [28,29]. Group 4 was further separated by paper chromatography as described previously [30,31]. In the present study, the difucosylated octaose fraction [30] was further separated by reversed-phase HPLC, whereas group 3 glycans were fractionated by normal-phase HPLC and sequential reversed-phase HPLC. The total amount of carbohydrate was determined by HPLC monosaccharide analysis [32]. About 1 mg of each HMO fraction was fluorescently tagged with 2AB according to the protocol described by Wuhrer et al. [33].

HPLC fractionation

Normal-phase HPLC separation was performed using a TSK gel Amide-80 column (4.6 × 250 mm; Tosoh Bioscience, Stuttgart, Germany) with fluorescence detection at 330/420 nm. ACN was used as solvent A and 0.5% ammonium acetate (pH 4.4) as solvent B. A stepwise gradient was applied from 38% B to 50% B within 28 min.

For separation of isomeric glycan mixtures, reversed-phase HPLC was performed using a LiChroCART RP-18 end-capped column (4 × 250 mm; Merck). Solvent A was 0.1% TFA; solvent B was methanol:ACN:water (4:4:92; v/v/v) including 0.1% TFA. A linear gradient from 20% to 45% solvent B was pumped in 23 min. Fluorescence was monitored at 330/420 nm.