Carbohydrate Research 425 (2016) 48–58

Contents lists available at ScienceDirect

Carbohydrate Research

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

Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products

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ARTICLE INFO

Article history:
Received 4 February 2016
Accepted 11 March 2016
Available online 16 March 2016

Keywords:
Galacto-oligosaccharides
NMR spectroscopy
Prebiotics
High-pH anion-exchange chromatography profiling

ABSTRACT

Many β-galactosidase enzymes convert lactose into a mixture of galacto-oligosaccharides (GOS) when incubated under the right conditions. Recently, the composition of commercial Vivinal GOS produced by *Bacillus circulans* β-galactosidase was studied in much detail in another study by van Leeuwen et al. As a spin-off of this study, we used the developed analytical strategy for the evaluation of 6 anonymous commercial GOS products, in comparison with Vivinal GOS. These GOS products were first subjected to HPLC-SEC, calibrated HPAEC-PAD profiling (glucose units in relation to a malto-oligosaccharide ladder), and 1D ^{1}H NMR spectroscopy. For a more detailed analysis and support of the conclusions based on the initial analysis, the GOS products were separated into DP-pure subpools on Bio-Gel P-2 (MALDI-TOF-MS analysis), which were subjected to calibrated HPAEC-PAD profiling and ^{1}H NMR analysis. Unidentified peaks from different GOS products, not present in Vivinal GOS, were isolated for detailed structural characterization. In this way, the differences between the various GOS products in terms of DP distribution and type of glycosidic linkages were established. A total of 13 new GOS structures were characterized, adding structural-reporter-group signals and HPAEC-PAD based glucose unit G.U. values to the analytical toolbox. The newly characterized products enhance the quality of the database with GOS structures up to DP4. The combined data provide a firm basis for the rapid profiling of the GOS products of microbial β-galactosidase enzymes.

1. Introduction

Galacto-oligosaccharides (GOS) are mixtures of oligosaccharides with a degree of polymerization (DP) up to 9, produced by the *trans*-galactosylation activity of β-galactosidase enzymes (EC 3.2.1.23), using lactose as both donor and acceptor substrate.[1]

Following the discovery of the *trans*-glycosylation potential of β-galactosidases, many of these enzymes have been studied,[2] especially those of *Lactobacillus reuteri*,[3,4] *Bacillus circulans*,[5–9] *Aspergillus oryzae*,[10,11] *Kluyveromyces lactis*,[12] *Sporobolomyces singularis*,[13] *Kluyveromyces fragilis*,[14] and *Escherichia coli*.[10] With respect to the characterization of GOS components, the ensemble produced by the *B. circulans* enzyme has been studied in most detail and up to the DP5 level.[6–8,15–17] Structural studies of GOS mixtures produced by other β-galactosidases (mixtures) have focused in general only on the main DP2 and DP3 structures.[7,18]

Previously, we have reported the detailed structural characterization of commercial Vivinal GOS produced by *B. circulans* β-galactosidase, making use of our analytical toolbox, which includes HPLC-SEC, HPAEC-PAD, LPLC-SEC, NMR spectroscopy, and MALDI-TOF-MS.[8,17] Here, we describe the structural analysis of 6 other commercial GOS products, in comparison with Vivinal GOS. The detected differences between the various GOS products in terms of DP distribution and type of glycosidic linkages [(1→2), (1→3), (1→4), (1→6)], provide a firm basis for comparison of the structure/function relationships of (commercial) β-galactosidase enzymes. Knowing these structural differences in GOS products will also provide strong support in studies aiming to understand the prebiotic potential of their different structural elements.

2. Results and discussion

2.1. DP analysis of GOS I-VI

The commercial GOS **I-VI** products were analyzed by HPLC size-exclusion chromatography on a Rezex RSO-oligosaccharide Ag^+ column, and the percentages of DP1-DP6 are summarized in Table 1. The DP1-DP6 data of the previously studied Vivinal GOS have been

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http://dx.doi.org/10.1016/j.carres.2016.03.006
0008-6215/

Table 1
Percentages of Gal, Glc and GOS DP2-DP6 in Vivinal GOS and in GOS samples **I-VI**, as determined by high-pressure liquid chromatography—size-exclusion chromatography (Rezex RSO-01), monitored by refractive-index detection. Values are averages of triplicate measurements

	Gal	Glc	DP2	DP3	DP4	DP5	DP6
Vivinal GOS	1.70 ± 0.08	18.52 ± 0.12	42.55 ± 0.22	23.63 ± 0.20	10.21 ± 0.23	3.00 ± 0.17	0.39 ± 0.17
GOS **I**	5.70 ± 0.11	18.04 ± 0.15	32.70 ± 0.24	34.98 ± 0.17	7.37 ± 0.23	1.21 ± 0.17	0
GOS **II**	0.69 ± 0.04	6.03 ± 0.07	39.30 ± 0.14	32.35 ± 0.13	14.34 ± 0.11	5.49 ± 0.11	1.80 ± 0.11
GOS **III**	3.30 ± 0.07	5.73 ± 0.12	45.69 ± 0.13	32.34 ± 0.13	11.40 ± 0.11	1.54 ± 0.11	0
GOS **IV**	0.12 ± 0.02	0.17 ± 0.05	20.04 ± 0.13	46.26 ± 0.16	21.94 ± 0.11	8.48 ± 0.15	2.99 ± 0.08
GOS **V**	12.48 ± 0.14	25.88 ± 0.11	36.24 ± 0.12	16.56 ± 0.11	6.70 ± 0.15	2.14 ± 0.17	0
GOS **VI**	1.16 ± 0.07	1.62 ± 0.04	47.21 ± 0.09	36.15 ± 0.08	12.40 ± 0.04	1.46 ± 0.02	0

included for comparison.[8] It should be noted that data about the production protocols were not available to us. All products showed monosaccharides Gal and Glc, together with DP2 up to DP5 oligosaccharides in detectable levels. Vivinal GOS, GOS **II** and GOS **IV** showed also clear amounts of DP6 oligosaccharides. Except for GOS **IV**, the DP2-DP4 fractions of the various products contain >90% of all GOS components. Most products contain a majority of DP2 components, except for GOS **I** and **IV**, where DP3 is the predominant fraction. GOS **IV** stands out, since it has the highest DP4, the highest DP5, and the highest DP6 content of all GOS products; here the very low amounts of Gal and Glc suggest a DP-based purification step. Also GOS **VI** has minor amounts of Gal and Glc, which may reflect a DP-based purification step.

2.2. Structural analysis of GOS I-VI

The HPAEC CarboPac PA-1 profiles and 1D ^{1}H NMR spectra of Vivinal GOS and GOS **I-VI** are collected in Figs. 1–3. The GOS **I-V** products were separated into DP subpools by preparative size-exclusion chromatography on Bio-Gel P-2. The isolated fractions were screened by MALDI-TOF-MS, and DP-pure fractions of DP2, DP3, DP4, and DP5 were pooled for further analysis. The HPAEC CarboPac PA-1 profiles and 1D ^{1}H NMR spectra of these subpools, together with those of Vivinal GOS for comparison,[8] are collected in Supplementary Information Figs. S3–S14. Scheme 1 summarizes the structures of the different GOS components, assigned in the various HPAEC profiles. Of these components, compounds **1**–**24** and **xa**-**zc** were earlier isolated and identified from Vivinal GOS.[8,17] Compounds **25**–**27**, **28**–**33**, and **34**–**37**, isolated from relevant GOS **I**, GOS **II**, and GOS **III** DP subpools, respectively, were characterized in the present study and their NMR data are presented in Table 2. Descriptions of their structural characterizations and their 1D ^{1}H NMR spectra (Figs. S1 and S2) are provided as Supplementary Information. GOS **VI** was only investigated as total GOS. Table 3 gives an overview of the components found in Vivinal GOS and GOS **I-VI**.

2.2.1. GOS I preparation

HPAEC-PAD analysis of GOS **I** (Fig. 1B) yielded a profile of which peaks **1**–**11**, **13**, **15**, **17**, **18**, and **22**, but not **25**, **26**, and **27**, could be annotated by comparison with Vivinal GOS G.U. (glucose units) values (Fig. 1A and Scheme 1).[8,17] Comparison of the relative percentages of Gal (**1**) and Glc (**2**) with those of Vivinal GOS showed a higher percentage for Gal. Applying the earlier developed structural-reporter-group concept for GOS components,[8,17] evaluation of the 1D ^{1}H NMR spectrum of GOS **I** (Fig. 2B) gave insight into most basic structural elements of the components present (Table 4). In the α-anomeric region, these are the H-1 signals **a** of →2)-α-D-Glc*p*, **b** of →2)-α-D-Glc*p* of the →4)-β-D-Gal*p*-(1→2)-α-D-Glc*p* sequence, **h** of free α-D-Gal*p*, **c** of →3)-α-D-Glc*p*, and **d** of →4, 6 and/or 1)-α-D-Glc*p* and of free α-D-Glc*p*. The β-anomeric region shows a quite heterogeneous signal pattern. Then, in the region 4.25-4.00 ppm the H-6a **e** and **f** signals of →6)-D-Glc*p* (βα), the H-4 signal **g** of →3 and/or 4)-β-D-Gal*p*, and the H-6a signal **i** of →6)-β-D-Gal*p* are present. Signal **b** is very minor. The clear presence of signal **h** in GOS **I** reflects the higher amounts of free Gal, when compared with Vivinal GOS (Fig. 2A, B and Table 1). To support the various assignments based on G.U. values and NMR data, the five GOS **I** DP-pure subpools were further analyzed by HPAEC-PAD and ^{1}H NMR spectroscopy.

The HPAEC-PAD profile of GOS **I** DP2 (Supplementary Information Fig. S4A and Scheme 1) revealed peaks at G.U. values corresponding with β-D-Gal*p*-(1→6)-D-Gal*p* (**3**), β-D-Gal*p*-(1→6)-D-Glc*p* (**4**; allolactose), β-D-Gal*p*-(1→4)-D-Glc*p* (**5**; lactose), β-D-Gal*p*-(1→4)-D-Gal*p* (**7**), β-D-Gal*p*-(1→2)-D-Glc*p* (**8a**), and β-D-Gal*p*-(1→3)-D-Glc*p* (**8b**).[8] Comparison of the profile with that of Vivinal GOS DP2 (Supplementary Information Fig. S3A) shows that peak **4** has a higher relative intensity and peak **8** a lower relative intensity. The various fractions were isolated by semi-preparative HPAEC on CarboPac PA-1 and the identity of the compounds was verified by 1D ^{1}H NMR spectroscopy. Inspection of the 1D ^{1}H NMR spectrum of GOS **I** DP2 (Supplementary Information Fig. S10B) revealed the different structural-reporter-group signals expected for the different disaccharides,[8,17] as summarized in Table 4.

The most significant peaks in the HPAEC-PAD profile of GOS **I** DP3 (Supplementary Information Fig. S4B and Scheme 1) are **6** corresponding with 4,6-digalactosyl-glucose/β-D-Gal*p*-(1→4)-[β-D-Gal*p*-(1→6)-]D-Glc*p* (**6a**) and 6′-galactosyl-lactose/β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc*p* (**6b**), **11** corresponding with 4′-galactosyl-lactose/β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p*, and **13**, corresponding with β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→2)-D-Glc*p* (**13a**) and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→3)-D-Glc*p* (**13b**), like previously observed for Vivinal GOS (Supplementary Information Fig. S3B).[8] Inspection of the 1D ^{1}H NMR spectrum (Supplementary Information Fig. S10C), however, showed the absence of structural-reporter-group signal **p**, corresponding with →4,6)-β-D-Glc*p* H-6a, indicating the absence of structure **6a**. The structural-reporter-group signals for both 2- (signal **b**) and 3- (signal **c**) substituted Glc are present, verifying the presence of both **13a** and **13b**. Other assigned HPAEC peaks are **9** (branched trisaccharide), **10** (branched trisaccharides), and **12**; HPAEC peak **25** is absent in Vivinal GOS. The various fractions were isolated by HPAEC and the identity of the compounds, except **25**, was verified by 1D ^{1}H NMR spectroscopy. Detailed NMR analysis of fraction **25** showed its structure to be β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→3)-D-Glc*p*/**C**1→6**B**1→3**A** (Table 2; see Supplementary Information). Inspection of the 1D ^{1}H NMR spectrum of GOS **I** DP3 (Supplementary Information Fig. S10C) revealed the structural-reporter-group signals that indicate typical fragments of the identified trisaccharides,[8,17] as summarized in Table 4. Component **25** can be considered as a β-D-Gal*p*-(1→6)- elongation of the terminal Gal residue of disaccharide **8b**. The same holds for **6b**, being a similar extension of **5**. No β-D-Gal*p*-(1→6)- elongations of disaccharides **4** and **8a** were observed. Compounds **11**, **13a**, and **13b** are β-D-Gal*p*-(1→4)- elongations of the terminal Gal residue of disaccharides **5**, **8a**, and **8b**, respectively, and the minor compound **12** is a β-D-Gal*p*-(1→3)- elongation of the terminal Gal residue of disaccharide **5**. Analysis by ^{1}H NMR indicates that HPAEC peak **10** represents both compounds **10a** and **10b** (Scheme 1).