Characterization of galactooligosaccharides derived from lactulose

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Abstract

Galactooligosaccharides are non-digestible carbohydrates with potential ability to modulate selectively the intestinal microbiota. In this work, a detailed characterization of oligosaccharides obtained by transgalactosylation reactions of the prebiotic lactulose, by using β -galactosidases of different fungal origin (*Aspergillus oryzae*, *Aspergillus acuelatus* and *Kluyveromyces lactis*), is reported. Oligosaccharides of degree of polymerization (DP) up to 6 were characterized and quantified by HPLC-ESI MS from a complex mixture produced by transgalactosylation reaction with *A. oryzae* (GOSLuAo), whereas only carbohydrates up to DP4 and DP5 were detected for those obtained from the reaction with β -galactosidases from *K. lactis* (GOSLuKl) and *A. acuelatus* (GOSLuAa), respectively. Disaccharides (galactosyl-galactoses and galactosyl-fructoses) and trisaccharides were characterised in the three mixtures by GC-MS as their trimethylsilyl oximes. Galactosyl- and digalactosyl-glycerols were produced during the transgalactosylation reaction of lactulose with β -galactosidases from *A. acuelatus* and *K. lactis*, due to the presence of glycerol as enzyme stabiliser.

Keywords: galactooligosaccharide; β -galactosidase; lactulose; GC-MS; HPLC-MS; prebiotic.

1. Introduction

Galactooligosaccharides (GOS) are considered non-digestible carbohydrates which are mainly constituted by galactose units and obtained from lactose by the action of β -galactosidases. These enzymes catalyse the hydrolysis of lactose into glucose and galactose, and also the transgalactosylation reactions with lactose as acceptor of galactose units giving rise to GOS of different glycosidic linkages and molecular weights [1]. A number of studies have demonstrated that hydrolysis rates and transgalactosylation pattern of GOS were different and related to the enzyme source used, substrate concentration and reaction conditions [2-8]. The beneficial effects of GOS on human gastrointestinal health have been extensively reported, being currently used as pharmaceutical as well as food ingredients [9-11].

Lactulose (β -(1 $\rightarrow$ 4)-galactosyl-fructose) is a synthetic disaccharide, produced by isomerization of lactose in basic media or enzyme-catalyzed synthesis, with a significant impact on human digestion [12,13]. Its physiological action on the colonic motility pattern [14] and their ability to promote the selective growth of healthy intestinal bacteria, mainly bifidobacteria and lactobacilli populations, in human gut has been extensively reported [15,16]. However, its use can be limited due to its laxative effects at high doses and the fact that fermentation occurs mainly in the proximal colon which results in uncomfortable gas production [17]; as a result, only a reduced percentage of lactulose is likely to reach the distal colon, where most of the digestive disorders take place, and could limit its potential beneficial effects in gut health. It has been hypothesized that non-digestible oligosaccharides of longer degree of polymerization are more slowly fermented so that their metabolism take place more distally in the colon [18]. Such longer colonic persistence has been linked to enhanced beneficial effects within the gastrointestinal tract, being one of the main current targets

in prebiotics development [19]. As a result, oligosaccharides derived from lactulose are currently attracting attention of the scientific community due to their prospective prebiotic applications [7,20,21]. Lactulose oligosaccharides have been obtained by transgalactosylation reactions catalysed by the action of β -galactosidases from different sources, including *Aspergillus acuelatus* [7] and *Kluyveromyces lactis* [21]. More recently, it has been reported that these oligosaccharides have the ability to promote the growth of bifidobacteria in human faecal cultures in a similar way of recognised prebiotic GOS [20].

Although extensive characterization of GOS derived from lactose has been reported [22,23], data regarding GOS composition derived from lactulose are scarce. Up to date, only two trisaccharides [β -D-galactopyranosyl-(1 $\rightarrow$ 6)- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-fructopyranose and β -galactopyranosyl-(1 $\rightarrow$ 4)-[β -galactopyranosyl-(1 $\rightarrow$ 1)]-fructose have been previously identified in oligosaccharide mixtures from lactulose [21].

High Performance Liquid Chromatography (HPLC) have been commonly used for the analysis of prebiotic oligosaccharides; different stationary phases such as alkyl-bonded silica, aminoalkyl-bonded silica, graphitized carbon, cation and anion exchange etc. are commercially available [24]. The use of mass spectrometric (MS) detectors coupled to HPLC systems has considerably enriched the field of carbohydrate analysis.

Gas chromatography-mass spectrometry (GC-MS) is also a suitable technique to determine di- and trisaccharide structures. Trimethylsilyl oximes (TMSO) are commonly used for oligosaccharide analyses considering their volatility, simplicity of preparation and easy data interpretation [25,26]. Nevertheless, characterization of oligosaccharides of the same DP in complex mixtures is not an easy task mainly due to the non-availability of commercial standards and the similarity of their structures.

In this study, we report for the first time an extensive GC-MS and HPLC-MS characterization of oligosaccharides obtained from lactulose by transgalactosylation reactions catalysed by β -galactosidases from different fungal species (*Aspergillus oryzae*, *Aspergillus aculeatus* and *Kluyveromyces fragilis*). Given that glycosidic linkages, monomeric composition and chain length ~~can~~could affect to their prebiotic properties, this study will reveal critical information to support the relationships between structure and potential prebiotic properties of these novel GOS.

2. Materials and methods

2.1. Standards

Analytical standards of fructose, galactose, lactulose (β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-fructose), lactose (β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose), maltose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose), maltotriose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose), maltotetraose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)-(α -D-glucopyranosyl-(1 $\rightarrow$ 4))₂-D-glucose), maltopentaose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)-(α -D-glucopyranosyl-(1 $\rightarrow$ 4))₃-D-glucose), maltohexaose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)-(α -D-glucopyranosyl-(1 $\rightarrow$ 4))₄-D-glucose), 1,6-galactobiose (β -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-galactose), 1,4-galactobiose (β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-galactose), 1,3-galactobiose (α -D-galactopyranosyl-(1 $\rightarrow$ 3)-D-galactose), α,α -threhalose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside), α,β -threhalose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- β -D-glucopyranoside) and β,β -threhalose (β -D-glucopyranosyl-(1 $\rightarrow$ 1)- β -D-glucopyranoside) were obtained from Sigma (St. Louis, US); leucrose (α -D-glucopyranosyl-(1 $\rightarrow$ 5)-D-fructose), palatinose (α -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-fructose), turanose (α -D-glucopyranosyl-(1 $\rightarrow$ 3)-D-fructose) were obtained from Fluka

(Madrid, Spain), maltulose (α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-fructose) was from Aldrich Chem. Co. (Milwaukee, WI); trehalulose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-fructose) was a gift from Dr. W. Wach from Südzucker AG, Mannheim; 6'-galactosyl-lactulose (β -D-galactopyranosyl-(1 $\rightarrow$ 6)- galactopyranosyl- β -D-(1 $\rightarrow$ 4)-fructose) was a gift from Dra. Corzo from CIAL-CSIC, Madrid, Spain.

2.2. Synthesis of galactooligosaccharides from lactulose

The synthesis of GOS from lactulose was carried out by using optimal conditions previously reported for Lactozym 6500 L (*Kluveromyces lactis*, GOSLuKl) [21], for Pectinex Ultra (*Aspergillus acuelatus*, GOSLuAa) [7] and for *Aspergillus oryzae* (GOSLuAO) [27]. Summarily, lactulose was incubated at 50-60 °C for 2, 7 and 20 h, depending on the enzymatic source. Mixtures were immediately immersed in boiling water for 5 min to inactivate the enzymes. Subsequently, GOS mixtures were treated with activated charcoal to remove monosaccharides following the method of Morales et al. [28] with some modifications. Briefly, GOS mixtures (4 mL) were diluted with water (200 mL) and stirred with 2.4 g of Darco G-60 100 mesh activated charcoal (Sigma Chemical Co., St. Louis, MO) for 30 min. This mixture was filtered under vacuum and the activated charcoal was further washed with 50 mL of water. Oligosaccharides adsorbed onto the activated charcoal were then extracted by stirring for 30 min in 50 mL of 50:50 (v/v) ethanol:water. Activated charcoal was washed with 5 mL of this ethanol:water solution and subsequently eliminated by filtering through paper as previously described. The sample was evaporated under vacuum at 30°C.

2.3. Analyses of galactooligosaccharides from lactulose

2.3.1. HPLC-ESI MS

Oligosaccharide analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (20 μ L) were injected using a Rheodyne 7725 valve and separated in a porous graphitic carbon column (Hypercarb® 100 x 2.1 mm; 5 μ m; Thermo Fisher Scientific, Barcelona, Spain) at a flow rate of 0.4 mL min⁻¹ at 30 °C. Elution gradient using Milli-Q water: methanol both having 0.1 % NH₄OH was changed from 70:30 (v:v) to 33:67 (v:v) in 27 min, then to 0:100 (v:v) in 7 min and kept for 6 min. Initial conditions were resumed in 2 min and were maintained for 15 min for conditioning. The electrospray ionization was operated under positive polarity using the following MS parameters: nebulizing gas (N₂) pressure 276 KPa, nitrogen drying gas at a flow rate of 12 L min⁻¹ and 300 °C and capillary voltage of 4000 V. Mass spectra were acquired in SIM mode using a variable fragmentator voltage by registering the ions corresponding to sodium adducts of oligosaccharides under analysis: m/z 203 (monosaccharide), 365 (disaccharide), 527 (trisaccharides), 689 (tetrasaccharides), 851 (pentasaccharides), 1013 (hexasaccharides) and 1175 (heptasaccharides). Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Quantitative analysis was carried out using calibration curves of glucose and maltooligosaccharides (DP2-DP6) as standards in the range 0.001 – 0.01 mg mL⁻¹. Trace m/z [M+Na]⁺ ions of mono-, di-, etc. were independently extracted for their quantification.

2.3.2. GC-MS

GC analysis was carried out using a two-step derivatization procedure (oximation and trimethylsilylation). Oximes were obtained by addition of 350 μ L of a solution 2.5% hydroxylamine chloride in pyridine after 30 min at 75 °C. Oximes were then silylated with hexamethyldisilazane (350 μ L) and trifluoroacetic acid (35 μ L) at 45° C for 30 min. After reaction, samples were centrifuged at 10,000 rpm for 4 min, and 1 μ L of supernatants was injected into the GC injection port.

GC-MS analyses were carried out in a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium as carrier gas. A 22 m x 0.25 mm i.d. x 0.25 μ m film thickness fused silica column coated with SPB-1 (crosslinked methyl silicone) from Quadrex Corporation (Woodbridge,US) was used. Oven temperature was held at 200 °C for 15 min, then programmed to 270 °C at 15 °C min⁻¹ and programmed to 290 °C at 1 °C min⁻¹, and finally programmed to 300 °C at 15 °C min⁻¹ and kept for 15 min. Injector temperature was 300 °C and injections were made in the split mode with a split ratio 1:20. Mass spectrometer was operating in electronic impact (EI) mode at 70 eV, scanning the 35-700 m/z range. Interface and source temperature were 280 °C and 230 °C, respectively. Acquisition was done using an HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA).

Identification of trimethylsilyloximes (TMSO) derivatives of carbohydrates was carried out by comparison of their retention indices (I^T) and mass spectra with those of standard compounds previously derivatized. Characteristic mass spectra and data reported in the literature [23,29] were used for identification of those carbohydrates non available as commercial standards. These identifications were considered as tentative.

3. Results and discussion

3.1. LC-MS analysis

HPLC-ESI MS profiles of oligosaccharides obtained by transgalactosylation reactions catalysed by β -galactosidases of *Aspergillus acuelatus* (GOSLuAa), *Kluveromyces lactis* (GOSLuKl) and *Aspergillus oryzae* (GOSLuAo) are shown in Figure 1. Notable differences in the oligosaccharide pattern of the three mixtures can be observed. A more complex chromatogram was obtained for GOSLuAo (Figure 1C), whereas two major peaks, corresponding to di- and trisaccharides respectively, can be mainly distinguished for GOSLuAa and GOSLuKl (Figures 1A and 1B).

Table 1 shows the percentages of oligosaccharides of different DPs found in these complex mixtures. Oligosaccharides up to DP6 were detected for GOSLuAo, whereas oligosaccharides up to DP4 and DP5 were detected for GOSLuKl and GOSLuAa, respectively. After charcoal treatment, 1% of monosaccharides were still remaining in the three oligosaccharide mixtures. Disaccharides were the most abundant carbohydrates followed by tri- and tetrasaccharides.

3.2. GC-MS analysis

GC-MS analyses (retention indices and characteristic m/z ratios) were used to determine the chemical structures of the main carbohydrates (di- and trisaccharides) present in the oligosaccharide reaction mixtures (Table 2). As an example, Figure 2 shows the GC-MS profile of the TMS oximes of GOSLuAo. In contrast to HPLC method, mono-, di- and trisaccharides were clearly separated. Similar GC profiles were observed for GOSLuAa and GOSLuKl, although relative proportions of individual

carbohydrates were different (Table 3). It is worth noting that reducing carbohydrates gave rise to two peaks corresponding to the TMS oximes of syn (*E*) and anti (*Z*) isomers after derivatization. As previously described, abundances of these peaks are generally found in a ratio close to 1 for those carbohydrates with a free ketose group, whereas this ratio varied from 3 to 7 for those carbohydrates with a free aldose group [30,31]. Non-reducing carbohydrates showed a single peak corresponding to the octakis-TMS ether. It is also worthy to point out that all the carbohydrates produced in the reactions catalysed by β -galactosidases showed β -glycosidic linkages. The human gastrointestinal tract lacks enzymes with the ability to hydrolyse these glycosidic bonds, with the exception of that corresponding to lactose. Thus, the β -linked galactose is one of the key structural elements that protect the galactooligosaccharides from digestion, having them available for the beneficial bacteria of the human intestine [32].

As previously observed by HPLC analyses, monosaccharides were still detected in the three samples after charcoal treatment. These peaks were identified as fructose (peaks 1 and 2) and galactose (peaks 3 and 4) (Tables 2 and 3).

3.2.1. Disaccharide fraction characterization

Figure 2B shows the disaccharide profile of GOSLuAo. Twelve peaks with I^T values from 2878 to 3094 were detected (Table 2). Lactulose (peaks 7 and 8) was identified by comparison of their I^T values and mass spectra with those of the corresponding standard.

Peak 9a showed a relationship of m/z 191: 204: 217 ions of 1.3: 1: 1.3 similar to that of trehaloses and characteristic of non-reducing sugars with 1 $\rightarrow$ 1 glycosidic linkages. Therefore, this peak could be assigned to 1,1-galactobiose. Peak 9b was identified as 1,4-galactobiose *E* by comparison with the commercial standard.

Peak 10 showed a small abundance and it could not be identified by its mass spectra. However, this peak was not present in the oligosaccharide mixtures of lactose with β -galactosidases [23] and it could correspond to an isomer of a galactosyl-fructofuranose with a 1 $\rightarrow$ 5 glycosidic linkage.

Peak 11 was constituted by a mixture of three carbohydrates, as it could be deduced from the traces corresponding to several characteristic ions. Peak 11a could be assigned to 1,3-galactobiose *E* previously identified in the transgalactosylation reaction products of lactose with β -galactosidases [23]. This carbohydrate showed a relatively high ratio of m/z 205/204 ions and relative high abundance of m/z 244 ion. Presence of m/z 307 ion was also distinguished. This spectrum was also similar to that of α -D-galactopyranosyl-(1 $\rightarrow$ 3)-D-galactose standard (as well as to those of laminaribiose and nigerose) and can be considered characteristic of glycosidic linkage 1 $\rightarrow$ 3. Peak 11b was identified as 1,2-galactobiose *E* considering the high abundance of m/z 319 ion corresponding to the loss of a TMSOH group from the chain C3-C4-C5-C6 of a hexose residue and therefore, characteristic of 1 $\rightarrow$ 2 glycosidic linkages [33]. Trailing edge (peak 11c) displayed a spectrum without characteristic ions which was compatible with the second isomer of the 1 $\rightarrow$ 5 galactosyl fructofuranose.

Peak 12 corresponded to the second peak of 1,4-galactobiose (isomer *Z*), whereas peak 13 was a mixture of 1,2-galactobiose *Z* and 1,3-galactobiose *Z* identified in a similar way to their *E* isomers.

Peaks 14 and 15 showed a similar mass spectrum and an abundance ratio of 0.81 characteristic of carbohydrates with a free ketose group. Therefore they could correspond to a galactosyl-fructose; m/z ion 422 indicate that these peaks could be assigned to 1,6-galactosyl-fructose. This is also supported by their high I^T values [33].

Peak 16 and 17b showed similar mass spectra characterised by a high m/z 307 ion. This fragment of relatively high intensity has been previously detected in di- and trisaccharides with a reducing fructose unit substituted in C1 or C3 [31,33] and correspond to the C4-C5-C6 chain. Considering the equivalent glucosyl-fructose standards (trehalulose and turanose), the disaccharide with 1→1 linkage was most retained than the 1→3, therefore these peaks with high I^T values (3029 and 3046) could correspond to 1,1-galactosyl-fructose. Moreover, m/z 334 ion characteristic of turanose did not appear in the mass spectrum of peaks 16 and 17b, whereas the m/z ratios 191/204/207 were closer to those showed by trehalulose.

Peaks 17a and 18 were assigned to 1,6-galactobiose by comparison with the commercial standard and characterised by its high I^T value and mass spectra with relatively high intensity of m/z 422 ion (peak 17a) which correspond to C1-C2-C3-C4 of the oxime chain [34] and typical of 1→6 linkages.

Traces of two peaks at retention times around 24.1 min with m/z 307 ions were only found for oligosaccharides from *Aspergillus oryzae* (GOSLuAo). These peaks could correspond to 1,3-galactosyl-fructose.

The non reducing compound β -galactopyranosyl (1→2) fructoside which should be formed in this reaction was not detected. A possible explanation for this may be the overlapping of this compound with the peaks of lactulose, which make very difficult to recognise its characteristic ion traces.

3.2.2. Trisaccharide fraction characterization

Eight peaks corresponding to trisaccharides with I^T values between 3734-3945 were detected in GOSLuAo (Figure 2C), six of them also appeared in GOSLuAa and

GOSLuKI (Table 2). Peaks 31 and 32 were assigned to 6'-galactosyl-lactulose by comparison with the standard purified by Martínez-Villaluenga *et al.* [21].

Peak 33b was assigned to the reducing trisaccharide β -galactopyranosyl-(1 $\rightarrow$ 4)-[β -galactopyranosyl-(1 $\rightarrow$ 1)]-fructose by comparison with GOS from lactulose using Lactozym 3000 L HP where it was identified as a major trisaccharide by Cardelle-Cobas *et al.* [7]

A m/z relatively low 307 ion was detected in peaks 34 and 35. As mentioned above, this ion is indicative of a carbohydrate with a reducing ketose substituted in position C1 or C3, although in this case its relative abundance makes this assignation doubtful. Moreover, m/z 422 ion was also distinguished at very low a level which is characteristic of 1 $\rightarrow$ 6 linkages. Peaks 28 and 29 did not show significant fragments and could not be characterised.

3.2.3. Galactosyl- and digalactosyl-glycerols characterization

GOSLuAa and GOSLuKI also showed two peaks eluting before and after internal standard (peaks 5 and 6 with I^T values of 2347 and 2397, respectively, Figure 3a) corresponded to galactosyl-glycerols (1-O- β -galactosyl-glycerol and 2-O- β -galactosyl-glycerol), previously detected in transgalactosylation reactions of lactose using β -galactosidase from Pectinex (*A. acuelatus*) [7]. In the present study, both peaks were detected in transgalactosylation reactions of lactulose with both Pectinex and Lactozym (*K. lactis*). Both enzymatic preparations use glycerol as stabiliser which can act as acceptor of galactosyl groups. These compounds showed a mass spectrum with m/z 204, 217 and 337 as characteristic ions. It should be noted that 1- β -galactosyl-glycerol was one of the most abundant compounds of these mixtures (Table 2).

Chromatographic peaks with I^T values between 3135 and 3347 were identified as digalactosyl-glycerols (Figure 3b). Peaks 19-21, 23-26 showed the m/z 337 characteristic ion which corresponds to the fragment $\text{TMSiO-C}^+\text{HOCH}_2\text{-CHOTMSi-CH}_2\text{OTMSi}$ or to the equivalent $\text{TMSiO-C}^+\text{HOCH-(CH}_2\text{OTMSi)}_2$ and include the glycosidic carbon and an OTMSi from the sugar ring. Thus, all these compounds contained the glycerol chain with two free hydroxyls, being the most probable structure galactopyranosyl-galactopyranosyl-glycerol, formed by addition of a galactosyl group to both 1-O- β -galactosyl-glycerol and 2-O- β -galactosyl-glycerol at different positions.

However, peaks 22 and 27 showed a similar mass spectrum, but m/z 337 ion was lacking and a fragment at m/z 175 was clearly visible. Thus, these products could contain two units of galactose linked to different hydroxyl groups of glycerol, i.e 1,2-digalactosyl-glycerol and 1,3-digalactosyl-glycerol. However, this last compound should be present in higher concentration than the 1,2 isomer and that was not detected in both GOSLuAa and GOSLuKl products.

Interestingly, the formation of galactosyl-glycerols and digalactosyl-glycerols could be the cause of the lower yields of oligosaccharides (DP4, DP5 and DP6) obtained from transgalactosylation reactions from lactulose using *A. acuelatus* and *K. lactis* compared to those of *A. oryzae* (Figure 1). These findings may reveal the drawback of using glycerol as a stabilizer in β -galactosidase preparations addressed to produce oligosaccharides of DP above 3.

4. Conclusions

Oligosaccharides obtained by transgalactosylation reactions from lactulose using β -galactosidases from different fungal sources (*A. acuelatus*, *A. oryzae* and *K. lactis*) has been characterised by first time. Oligosaccharides of DP up to 6 were quantified in

GOSLuAo by HPLC-ESI MS, whereas only carbohydrates up to DP4 and DP5 were detected for GOSLuKl and GOSLuAa, respectively. Disaccharides, either galactosyl-galactoses or galactosyl-fructoses, and trisaccharides were characterised in the three mixtures by GC-MS. Galactosyl- and digalactosyl-glycerols were also formed during the transgalactosylation reaction of lactulose with Pectinex and Lactozym.

Considering the substantial influence of the chemical structure (type of linkage, nature of the monosaccharide, and degree of polymerization) on the prebiotic activity of oligosaccharides [35-38], the knowledge of the carbohydrate composition of these novel oligosaccharides is crucial for providing new evidences for their potential prebiotic activity.

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