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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 aculeatus* and *Kluveromyces lactis*), is reported. Oligosaccharides of degree of polymerization (DP) up to 6 were detected 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 found for those obtained from the reaction with β-galactosidases from *K. lactis* (GOSLuKl) and *A. aculeatus* (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. aculeatus* and *K. lactis*, due to the presence of glycerol as enzyme stabiliser.

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 → 4)-galactosyl-fructose) is a synthetic disaccharide, produced by isomerization of lactose in basic media or enzyme-catalysed 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 aculeatus* [7] and *Kluveromyces 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-galactopyranosil-(1 → 6)-β-D-galactopyranosyl-(1→4)-fructopyranose and β-galactopyranosyl-(1 → 4)-[β-

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galactopyranosyl-(1 → 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 *Kluveromyces lactis*). Given that glycosidic linkages, monomeric composition and chain length 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 → 4)-D-fructose), lactose (β-D-galactopyranosyl-(1 → 4)-D-glucose), maltose (α-D-glucopyranosyl-(1 → 4)-D-glucose), maltotriose (α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 4)-D-glucose), maltotetraose (α-D-glucopyranosyl-(1 → 4)-(α-D-glucopyranosyl-(1 → 4))$_2$-D-glucose), maltopentaose (α-D-glucopyranosyl-(1 → 4)-(α-D-glucopyranosyl-(1 → 4))$_3$-D-glucose), maltohexaose (α-D-glucopyranosyl-(1 → 4)-(α-D-glucopyranosyl-(1 → 4))$_4$-D-glucose), 1,6-galactobiose (β-D-galactopyranosyl-(1 → 6)-D-galactose), 1,4-galactobiose (β-D-galactopyranosyl-(1 → 4)-D-galactose), 1,3-galactobiose (α-D-galactopyranosyl-(1 → 3)-D-galactose), α,α-threhalose (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside), α,β-threhalose (α-D-glucopyranosyl-(1 → 1)-β-D-glucopyranoside) and β,β-threhalose (β-D-glucopyranosyl-(1 → 1)-β-D-glucopyranoside) were obtained from Sigma (St. Louis, US); leucrose (α-D-glucopyranosyl-(1 → 5)-D-fructose), palatinose (α-D-glucopyranosyl-(1 → 6)-D-fructose), turanose (α-D-glucopyranosyl-(1 → 3)-D-fructose) were obtained from Fluka (Madrid, Spain), maltulose (α-D-glucopyranosyl-(1 → 4)-D-fructose) was from Aldrich Chem. Co. (Milwaukee, WI); trehalulose (α-D-glucopyranosyl-(1 → 1)-D-fructose) was a gift from Dr. W. Wach from Südzucker AG, Manheim; 6′-galactosyl-lactulose (β-D-galactopyranosyl-(1 → 6)-galactopyranosyl-β-D-(1 → 4)-fructose) was a gift from Dr. 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 aculeatus,* 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 μL) were injected using a Rheodyne 7725 valve and separated in a porous graphitic carbon column (Hypercarb® 100 mm × 2.1 mm; 5 μm; 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% NH_4OH 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 (N_2) 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 μL of a solution 2.5% hydroxylamine chloride in pyridine after 30 min at 75 °C. Oximes were then silylated with hexamethyldisilazane (350 μL) and trifluoroacetic acid (35 μL) at 45 °C for 30 min. After reaction, samples were centrifuged at 10,000 rpm for 4 min, and 1 μL 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 × 0.25 mm i.d. × 0.25 μm 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