**GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC ANALYSIS OF
GALACTOSYL DERIVATIVES OBTAINED BY THE ACTION OF TWO
DIFFERENT β -GALACTOSIDASES**

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Abstract

Di- and trisaccharides obtained by transgalactosylation reaction of lactose using two different commercial enzyme preparations (Pectinex Ultra SP-L and Lactozym 3000 L HP G) have been characterized by gas chromatography-mass spectrometry as their trimethylsilyl oximes. Although mass fragments are common for most of the glycosidic linkages, their relative abundances can be used to determine carbohydrate identity. Moreover, formation of galactosyl- and digalactosyl-glycerols from the reaction mixture of Pectinex Ultra SP-L with lactose has been described by first time. Evolution of these compounds during transgalactosylation reactions and their influence on galactooligosaccharides (GOS) synthesis has been also evaluated.

Keywords: galactooligosaccharides, GC-MS, trimethylsilyl-oximes, galactosyl-glycerol, Pectinex Ultra SP-L, Lactozym 3000 L HP-G

1. Introduction

Galactooligosaccharides (GOS) are mainly di-, tri-, and tetrasaccharides synthesized from lactose via enzymatic transgalactosylation catalyzed by β -galactosidases (Prenosil, Stuker, & Bourne, 1987). Oligosaccharides of higher molecular weights can also be present when high lactose levels are used (Mahoney, 1998).

Direct or intramolecular transgalactosylation leads to the formation of isomers of lactose such as allolactose (β -1,6-Gal-Glc), while intermolecular or indirect transgalactosylation give rise to new di-, tri- and tetrasaccharides such as 6'-galactobiose (β -1,6-Gal-Gal), 4'-galactobiose (β -1,4-Gal-Gal), 6'-galactosyl-lactose (β -1,6-Gal-Lac). The importance of one or other route depends on the enzymatic source and reaction conditions, such as time, pH or temperature (Mahoney, 1998; Boon, Janssen, & Van't Riet, 2000; Splechtna, Nguyen, Steinböck, Kulbe, Lorenz, & Haltrich, 2006).

Currently, GOS have gained great interest for research and industrial applications since they are recognized as prebiotics (Sako, Matsumoto, & Tanaka, 1999; Vulevic, Rastall, & Gibson, 2004; Gaur, Pant, Jain, & Khare, 2006). Prebiotics are non-digestible oligosaccharides that reach the human colon without being hydrolysed and are selectively metabolized by benign or health-positive species, such as bifidobacteria and lactobacilli (Gibson & Roberfroid, 1995). Since composition of the oligosaccharide mixture (glycosidic linkages, monomeric composition and molecular weight) obtained during lactose hydrolysis may affect to its prebiotic properties (Sanz, Gibson, & Rastall, 2005), determination of factors affecting composition is necessary for selecting the appropriate experimental conditions.

Many studies have been focused on the enzymatic synthesis of GOS, however, the identification of the individual carbohydrates was in most of them omitted (Smart, 1991; Gaur et al., 2006; Aslan & Tanriseven, 2007) and there are only few references about the

study of the obtained disaccharides (Maugard, Gaunt, Legoy, & Besson, 2003; Chen, Ou-Yang, & Yeh, 2003). At present, various sophisticated and advance analytical techniques are available for oligosaccharide analysis such as high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Corradini, Bianchi, Matteuzzi, Amoretti, Rossi, & Zanoni, 2004, Splechtna et al., 2006), high performance capillary electrophoresis with laser-induced fluorescence detection (HPCE-LIF) (Khandurina, Blum, Stege, & Guttman. 2004) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Stikarovska & Chmelik, 2004). We have recently studied the formation of di- and trisaccharides during the enzymatic synthesis of GOS using Lactozym 3000 L HP G and Pectinex Ultra SP-L by HPAEC-PAD (Cardelle-Cobas, Villamiel, Olano, & Corzo, 2008; Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano & Villamiel, 2008); nevertheless, only 3 disaccharides different from lactose and 2 trisaccharides could be identified while the detection of other carbohydrates could not be achieved with this technique.

Alternatively, gas chromatography-mass spectrometry (GC-MS) is a suitable technique for the analysis of carbohydrates due to its simplicity, resolution, sensibility and low cost (Sanz, Sanz, & Martinez-Castro, 2002). In a previous work, we carefully studied the mass spectra of twenty three commercial disaccharides with different linkages and monomeric composition after conversion to their trimethylsilyl (TMS) oximes (Sanz et al., 2002). Here, we show the usefulness of this GC-MS method to characterize the carbohydrates obtained during lactose transgalactosylation using two commercial enzyme preparations (Pectinex Ultra SP-L and Lactozym 3000 L HP G). The identity of some galactosyl derivatives obtained from these reactions has been assessed by first time.

2. Material and methods

2.1. Reagents

Lactose and glycerol were acquired from Scharlau (Barcelona, Spain), D-galactose, D-glucose and D-melezitose from Fluka (Steinheim, Germany). 6'galactobiose, 4'galactobiose and phenyl- β -glucoside were purchased from Sigma (St. Louis, MO, USA). The commercial enzyme preparation, Pectinex Ultra SP-L produced by *Aspergillus aculeatus* and Lactozym 3000 L HP G, a soluble preparation of β -galactosidase from *Kluyveromyces lactis* were a generous gift from Novozymes (Dittingen, Switzerland and Bagsvaerd, Denmark respectively). Vivinal®GOS was kindly provided by Borculo Domo Ingredients (Zwolle, The Netherlands). High-purity water was produced in-house using a Milli-Q Synthesis A10 system (Millipore, Bellerica, Mass., USA) and was used throughout.

2.2. Enzymatic synthesis of GOS

Enzymatic syntheses of galactosyl derivatives from lactose using two commercial enzyme preparations (Pectinex and Lactozym) at different conditions were assayed. For incubations with Pectinex: lactose solutions (285 g/L) were prepared in 0.1 M phosphate buffer at pH 6.5, enzyme concentrations 16 U/mL, corresponding to 190 μ L/mL, and the assayed temperature was 60 °C. For Lactozym assay mixtures containing lactose (250 g/L) and glycerol (0, 184 and 368 g/L) or inactivated Pectinex (190 g/L) (at 100 °C for 10 minutes) were prepared with 3 U/mL of enzyme in 50 mM potassium phosphate buffer containing 1 mM MgCl₂ at pH 6.5 and the assays were carried out at 40 °C. The samples were taken out at different times between 1 and 8 hours.

Reactions were carried out in duplicate with individual tubes of 2 mL, incubated in an orbital shaker at 600 rpm and stopped by heating at 100 °C for 5 min. The samples were stored at -18° C for subsequent analysis.

The amount of lactose remaining and the yield of galactosyl derivatives were expressed as percentage by weight of the total carbohydrate content in the reaction mixtures.

2.3. Gas chromatographic analysis

A volume of 15 µL of sample, corresponding to approximately 4 mg of saccharides, was added to 0.4 mL of internal standard (IS) solution containing 0.5 mg/mL of phenyl-β-glucoside.

The mixture was dried at 38-40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 350 µL hydroxylamine chloride (2.5 %) in pyridine and heating the mixture at 75 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) and kept at 45 °C for 30 min (Brobs & Lott, 1966). Reaction mixtures were centrifuged at 7000 x g for 5 min at 5 °C (Baños, Olano, & Corzo, 2000). Supernatants were injected in the GC or stored at 4 °C prior to analysis.

GC analyses were performed with a Hewlett-Packard gas chromatograph (HP6890) equipped with a flame ionisation detector (FID). The trimethylsilyl oximes were separated using a 25 m x 0.25 mm x 0.25 µm film fused silica capillary column coated with CP-SIL 5CB (methyl silicone from Chrompack, Middelburg, The Netherlands) giving rise to two peaks corresponding to the syn (E) and anti (Z) isomers. The carrier gas (nitrogen) flow rate was 0.8 mL/min. Injector and detector temperatures were 280 and 300 °C, respectively. The oven temperature was programmed as follow: 200 °C hold for 15 min,

ramp to 280 °C at 15 °C min⁻¹, ramp to 290 °C at 1 °C min⁻¹, ramp to 300 °C at 15 °C min⁻¹ and keeping this temperature for 35 min. Injections were made in the split mode (1:10). Data acquisition and integration was done using HP ChemStations software (Hewlett-Packard, Wilmington, USA).

Quantitative analysis was carried out through the internal standard method. Response factors were calculated after the triplicate analysis of 4 standard solutions (galactose, glucose, lactose and melezitose) at different concentrations ranging from 0.02 to 1.6 mg/ml for galactose, glucose and melezitose and from 0.03 to 2.0 mg/mL for lactose. Phenyl- β -glucoside was used as IS at a concentration of 0.2 mg/mL.

Repeatability of the method was determined by the preparation, derivatisation, and injection of the same reaction mixture 5 times during one day. Examination of the reproducibility was conducted by injecting one reaction mixture prepared, derivatised and injected in five days. Relative standard deviations (RSD) lower than 8% were observed in both assays.

2.4. Gas chromatographic-Mass spectrometric analysis

A HP-5890 chromatograph coupled to a MD 5971 quadrupole mass detector (Hewlett-Packard, Palo Alto, CA, USA) was employed. Sugars separation was performed under the same chromatographic conditions described above substituting the carrier gas by helium. Samples were injected in split mode (40 mL/min), except for analyses of trisaccharides which were carried out in splitless mode (oven temperature: 100 °C for 1 min increased at 30°C/min to 200°C, programmed temperature continued as above). Mass spectrometer was operated in EI mode at 70 eV. Mass spectra were acquired using HP-G1034C MS ChemStation software (Hewlett-Packard, Palo Alto, CA, USA).

3. Results and Discussion

3.1. Identification of GOS

GC-MS profiles of the TMS oximes of GOS synthesised from lactose by β -galactosidase from (a) *K. lactis* and (b) *A. aculeatus* are shown in Figure 1. The optimal reaction conditions chosen for GOS production were those previously established by our investigation group (Cardelle-Cobas et al., 2008; Martínez-Villaluenga et al., 2008). A good separation of mono-, di- and trisaccharides was obtained. Although the two enzymes gave rise to different profiles, the main common carbohydrates were obtained. Monosaccharides, galactose and glucose and disaccharides such as lactose, 1,4-galactobiose and 1,6-galactobiose were identified by comparison of their retention times and mass spectra with those from commercial standards. Other di- and trisaccharides were identified based on the study of the characteristic mass fragments of each chromatographic peak; literature data previously reported by Sanz et al. (2002) were also taken into account.

GC profiles of the TMS oximes of disaccharides synthesized from lactose by Lactozym (a) and Pectinex (b) are shown in Figure 2. Mass spectra of TMS oxime disaccharides were characterized by m/z 73, 147, 191, 204, 217, 307, 319 and 361 ions and only differed from each other by the relative abundances of these ions (Table 1). As previously indicated by Sanz et al. (2002), characteristic abundances of some linkages could be distinguished. 2' Galactosyl glucoses (peaks 9 and 11) could be identified by the high intensity of m/z 319 ion corresponding to the lost of a TMSOH group from C3-C4-C5-C6 of the glucose residue. Peaks 5, 7, 8 and 11 showed m/z 244 ion relatively high and they could be assigned to disaccharides with 1,3-linkage. Relationships between m/z 319 and 305 ions for peaks 12, 13, 16 and 17 were close to 1 and m/z 205 relative abundance was lower than that found in the other compounds. These peaks could be assigned to 1,6-linked carbohydrates (Sanz et al., 2002). Moreover, it is known that carbohydrates with

1,6-linkages show the highest retention times in methylsilicone columns (Sanz, Diez-Barrio, Sanz, & Martínez-Castro, 2003). Peaks 12 and 16 with relative retention times (RRT) of 3.43 and 3.55 were not obtained using both enzymes and galactose as the only source of carbohydrate (data not shown), therefore, these peaks could be assigned to allolactose (β -1,6-Gal-Glc). This disaccharide was the most abundant in reactions with Lactozym, reaching the 11.3 % of the total carbohydrates present in the reaction. Peaks 13 and 17 were identified as 1,6-galactobiose by comparison with the commercial standard. The disaccharides allolactose and 6' galactobiose were previously identified in transgalactosylation reactions of lactose with β -galactosidase from *K. lactis* (Asp, Burvall, Dahlqvist, Hallaren, & Lundblad, 1980 and Martínez-Villaluenga et al., 2008) and with β -galactosidase from *A. aculeatus* (Cardelle-Cobas et al., 2008); also Chockchaisawasdee, Athanasopoulos, Niranjana and Rastall (2005) indicated that the enzyme of *K. lactis* produces mainly GOS with linkages β -1,6, although galactobioses with β -1,3 and β -1,4 linkages are present at 7 and 6% respectively.

Six main peaks corresponding to trisaccharides were detected in both Lactozym and Pectinex reactions (Figures 3a and 3b), the most abundant peaks being 21 and 23. 6' Galactosyl lactose was previously isolated and identified by RMN by Martínez-Villaluenga et al. (2008) and Cardelle-Cobas et al. (2008) from similar reaction mixtures obtained with the same commercial enzyme preparations. This compound was derivatised and analysed by GC-MS showing two peaks with the same retention time and mass spectra to those of peaks 21 and 23 (RRT 6.05 and 6.12 min. respectively). 6'Galactosyl-lactose has been described by other authors as the most important trisaccharide formed with both enzymes (Asp et al., 1980; del Val, Hill, Jimenez-Barbero, & Otero, 2001; Bridiau, Taboubi, Marzouki, Legoy, & Maugard, 2006; Aslan & Tanriseven, 2007). Peaks 20 and 22 (RRT 5.98 and 6.07), only present in the sample with Pectinex, were assigned to 4'galactosyl-

lactose by comparison with the main trisaccharide of Vivinal® (data not shown), where it had been previously identified (Chockchaisawasdee, *et al.* 2005). Peaks 24 and 25 were found in both chromatographic profiles, although they were more abundant in that from Lactozym. Taking into account their relative retention times (RRT 6.27 and 6.37) and mass spectra, and considering that it was the unique trisaccharide formed from reactions using both enzymes and galactose as the only source of carbohydrate (data not shown), these peaks could correspond to 6'galactosyl-galactobiose. Identity of peaks 18 and 19 could not be determined.

GC profiles of reaction mixtures obtained with Pectinex (Figure 1b) also showed the presence of some unknown compounds with relative retention times between mono and disaccharides (RRT 0.93 and 1.07) and di- and trisaccharides (RRT between 4.05 and 4.37). However, these peaks were absent in the reaction mixture with Lactozym. Since glycerol is a constituent of Pectinex used as stabilizer, galactosyl-glycerol derivatives could be formed in reactions with lactose. This is explained because β -galactosidases can exhibit a wide specificity with regards to acceptor groups, thus, apart from carbohydrates, other compounds such as alcohols, polyalcohols like glycerol, hydroxyl-amino acids and nucleosides, can act as acceptor of galactosyl groups (Stevenson, Stanley & Furneaux, 1993; Rantwijk, Woudenberg-van Oosteron, & Sheldon, 1999; Bridiau *et al.*, 2006; Lang, Kamrat, & Nidetzky, 2006). Nevertheless, their presence has not been considered before in those studies obtaining GOS from Pectinex without purification (del Val *et al.*, 2001; Aslan & Tanriseven, 2007).

3.2. Identification of galactosyl-glycerol derivatives

The two unknown peaks eluted before disaccharide fraction (Figure 4a) were characterized by ions at m/z 204, 217 and 337. The most abundant peak could be assigned

to galactosyl-glycerol substituted in the primary hydroxyl groups of glycerol (1-O- β -D-galactopyranosyl glycerol; RRT of 1.07) and the less abundant peak to galactosyl-glycerol substituted in the secondary hydroxyl groups (2-O- β -D-galactopyranosyl glycerol; RRT of 0.93). The higher formation of 1-O- β -D-galactopyranosyl glycerol may be attributed to the fact that primary alcohols are better acceptors than secondary alcohols (Rantwijk, et al., 1999). Peaks eluting between di- and trisaccharides (Figure 4b) with main ions at m/z 204 and 597, could be identified as compounds with one molecule of glycerol and two molecules of galactose with different glycosidic linkages. Stevenson et al. (1993) detected by GC the formation of two compounds during the enzymatic hydrolysis of lactose in presence of glycerol and identified by NMR a major peak as 1-O- β -D-galactopyranosyl glycerol and another minor peak as 2-O- β -D-galactopyranosyl glycerol, also was identified the 1-O- β -D-digalactopyranosyl glycerol compound.

3.3. Formation of galactosyl-glycerol derivatives

To verify the nature of galactosyl and digalactosyl-glycerols, solution of lactose (25%) alone (control) or with added glycerol (18.4% and 36.8%) or added inactivated Pectinex (19.0%) were incubated with Lactozym. Galactosyl-glycerols were formed during the incubations with added glycerol or inactivated Pectinex; while were absent in the control.

Figure 5 shows the time course of galactosyl-glycerol derivatives production during the enzymatic incubation of lactose added with different glycerol concentration (18.4, 36.8% or inactivated Pectinex, 19.0%). The rate of galactosyl-glycerol formation was higher with 18.4% glycerol than when 36.8% glycerol or inactivated Pectinex were used. The maximum yield (30.2%) was achieved after 8 hours of reaction when 18.4% glycerol was used. The enzyme activity decreased with increasing glycerol content, this could be

due to the higher polarity of glycerol and the decrease of the water activity (Maugard et al., 2003).

3.4. Influence of glycerol on GOS formation

GOS formation was affected by the presence of glycerol. The synthesis of GOS (Figure 6a) in absence of glycerol reached a maximum of 50.5% after 3 h of reaction, however, the presence of glycerol markedly inhibited their formation. This may be due to the fact that glycerol is a more powerful nucleophile acceptor (Nu) than lactose, and its selectivity constant, defined as $k_{\text{Nu}}/k_{\text{water}}$ is higher than that of lactose (Lang et al., 2006). This effect was more pronounced for trisaccharide than for disaccharide synthesis (Figures 6b and 6c) possibly due to the formation of trisaccharides occurs via intermolecular transglycosylation while certain disaccharides as allolactose can be formed by intramolecular galactosylation (Petzelbauer, Zeleny, Reiter, Kulbe, & Nidetzky, 2000). During the intermolecular transgalactosylation mechanism, the glycosidic β -(1-4) linkage of lactose is cleaved and immediately galactose joins with glucose at a different position before it diffuses out the active site so that the glycerol molecule may not compete for the galactose moiety.

Monosaccharides were also affected by the presence of glycerol. Figure 6d shows the ratio glucose/galactose during the time course of different reaction. The value of this relation was higher in presence of glycerol; the amount of galactose present in the medium was lower due to galactosyl-glyceride derivatives formed. The greater relationship glucose/galactose in presence of glycerol is in agreement with the formation of galactosyl-glycerols. The disaccharides profile also changed in presence of glycerol, probably due to the competition of glycerol with glucose as galactosyl acceptor.

4. Conclusions

In this work, the characteristic mass fragments of di- and trisaccharides produced in the reaction mixture of lactose with Pectinex Ultra SP-L and Lactozym 3000 L HP G and the formation of galactosyl- and digalactosyl glycerols during lactose hydrolysis by Pectinex Ultra SP-L has been reported by first time. The presence of these compounds is due to the fact that the commercial enzyme is supplied in aqueous solution and stabilized with glycerol, so that for a selective synthesis of GOS, a previous purification of the enzyme should be necessary.

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Table 1.- Characteristic mass spectra of TMS oximes of galactosyl disaccharides.

Glycosidic linkage	Mass spectrum ^a
1,1	73 (79), 103 (22), 129 (23), 147 (48), 191 (99), 204 (56), 205 (18), 217 (65), 361 (100)
1,2	73 (100), 103 (47), 147 (65), 191 (44), 204 (75), 205 (61), 217 (63), 307 (26), 319 (76), 361 (23)
1,3	73 (85), 103 (23), 147 (57), 169 (12), 191 (17), 204 (100), 205 (42), 217 (65), 244 (12), 319 (17), 361 (64)
1,4	73 (85), 103 (19), 147 (74), 191 (20), 204 (100), 205 (52), 217 (72), 319 (34), 361 (92)
1,6	73 (55), 103 (12), 147 (34), 191 (14), 204 (100), 205 (23), 217 (41), 305 (8), 319 (10), 361 (57)

^a m/z values (relative abundance in brackets).

Figure 1.- Gas chromatographic profile of the TMS oximes of mono- (A), di- (B) and trisaccharides (C) of GOS and galactosyl-glycerol derivatives I and II obtained from the reaction of lactose with a) Lactozym 3000 L HP G (pH 6.5, 40 °C, 2 hours, using 250 g/L of lactose and 3 U/mL of enzyme) and b) Pectinex Ultra SP-L (pH 6.5, 60 °C, 5 hours, using 285 g/L of lactose and 16 U/mL of enzyme). Internal standard (IS) (phenyl- β -glucoside).

Figure 2.- Gas chromatographic profile of the TMS oximes of disaccharides of GOS obtained from the reaction of lactose with a) Lactozym and b) Pectinex. 1: Unknown; 2 and 3: Disaccharides with 1,1 linkage; 4: 1'-Galactosyl-galactose; 5: Lactose E and 3'-Galactosyl-glucose E; 6: Lactose Z and 4'-Galactobiose; 7: 3'-Galactosyl-glucose Z; 8: 3'-Galactobiose E; 9: 2'-Galactosyl-glucose E and unknown; 10: 4'-Galactobiose and unknown; 11: 2'-Galactosyl-glucose Z and 3'-Galactobiose Z; 12: 6'-Galactosyl-glucose E; 13: 6'-Galactobiose E; 14 and 15: unknown; 16: 6'-Galactosyl-glucose Z; 17: 6'-Galactobiose Z.

Figure 3.- Gas chromatographic profile of the TMS oximes of trisaccharides of GOS obtained from the reaction of lactose with a) Lactozym and b) Pectinex. 18-19: unknown trisaccharides, 20: 4'-Galactosyl-lactose 1; 21: 6'-Galactosyl-lactose 1; 22: 4'-Galactosyl-lactose 2; 23: 6'-Galactosyl-lactose 2; 24: 6'-Galactosyl-galactobiose 1; 25: 6'-Galactosyl-galactobiose 2 .

Figure 4.- Gas chromatographic profile of the TMS oximes of galactosyl glycerol derivatives obtained from the reaction of lactose with Pectinex. a) galactosyl-glycerol; b): digalactosyl-glycerol. 1: 2-O- β -D-galactopyranosyl glycerol; 2: 1-O- β -D-galactopyranosyl glycerol. Internal standard (phenyl- β -glucoside): IS.

Figure 5.- Effect of glycerol concentration (18.4% Gly and 36.8% Gly) or inactivated Pectinex (19.0% Px) on galactosyl-glycerol (solid lines) and digalactosyl-glycerol (dotted lines) during the time course of reaction performed with Lactozym (3 U/mL) at 40 °C, initial lactose concentration 250 g/L and pH 6.5.

Figure 6.- Effect of glycerol concentration (18.4% Gly and 36.8% Gly) or inactivated Pectinex (19.0% Px) on galactooligosaccharides (GOS) (a), disaccharides (b) and trisaccharides (c) formed and over ratio of glucose/galactose (d) during the time course of reaction performed with Lactozym (3 U/mL) at 40 °C, initial lactose concentration 250 g/L and pH 6.5.