Formation of type 4 resistant starch and maltodextrins from amylose, amylopectin upon dry heating: a model study

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Abstract

Starch is one of the main components of human diet. During food processing, starch is submitted to high temperatures in the presence or absence of water. Thus, the main goal of this work was to identify structural modifications caused by dry heating in starch polysaccharides (amylose and amylopectin) and structurally related oligosaccharides, maltotetraose (M4) and glucosyl-maltotriose (GM3), simulating processing conditions. The structural modifications were evaluated by methylation analysis, electrospray mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS), and anionic chromatography after *in vitro* enzymatic digestion. Dry heating promoted dehydration, depolymerization, as well as changes in Glc glycosidic linkage positions and anomeric configuration. In oligosaccharides, it was also observed polymerization. All these changes resulted in a lower *in vitro* digestibility, suggesting that dry heating of starch

polysaccharides and related oligosaccharides may be associated with the formation of type 4 resistant starch and maltodextrins, non-digestible carbohydrates that are responsible for the beneficial effects in human intestinal tract.

Keywords: Roast; oligosaccharides; polysaccharides; polymerization; anomeric configuration; type 4 resistant starch.

1. Introduction

Starch is the major source of carbohydrate in human diet (Ratnayake & Jackson, 2008). Processing of foods rich in starch for human consumption normally involves thermal treatment, resulting in significant physical and chemical modifications (Liu, Xie, Yu, Chen, & Li, 2009). Depending on the modifications, enzymatic availability and digestion can be increased due to gelatinization and/or dextrinization. The formation of starch-lipid complexes (Guraya, Kadan, & Champagne, 1997), starch retrogradation upon cooling (Sajilata, Singhal, & Kulkarni, 2006), starch protein interactions or chemical modification of starches (Mangala, Udayasankar, & Tharanathan, 1999) are factors that reduced its digestibility. The interest in non-digestible starch fractions, called “resistant starches”, has been raised (Cummings & Englyst, 1991; Englyst, Kingman, Cummings, & Seibel, 1993). They have physiological functions similar to those of dietary fibre, and possess nutritional value (Björck & Asp, 1994; Eerlingen & Delcour, 1995). The formation of resistant starch during food processing has been mainly studied under cooking conditions that promotes starch gelatinization, amylose leaching and, upon storage, mainly amylose retrogradation. Baking has also been implicated in the formation of resistant starch due to the formation of bread crust (Baiano, Romaniello,

Lamacchia, & Notte, 2009; Liljeberg, Åkerberg, & Björck, 1996; Pizzoferrato, Rotilio, & Paci, 1999; Tovar & Melito, 1996). The decrease in available starch due to dry heating of starch is not accompanied by any increase in dietary fibre, suggesting that the fragments remaining after enzymatic digestion have low molecular weight (Björck, & Westerlund, 1989). Thermal decomposition of starch has been the aim of several studies (Liu, Xie, Yu, Chen, & Li, 2009; Liu, Yu, Liu, Chen, & Li, 2009; Tomasik, Wiejak, & Pałasiński, 1989), but many of these studies did not use reaction conditions mimicking food processing. This process engages several reactions and depends on many factors, such as temperature, presence or absence of air or any inert gas, their pressure, moisture content, and the amylose/amylopectin ratio (Aggarwal & Dollimore, 2000; Liu, Yu, Liu, Chen, & Li, 2009). Depending on these factors, different products of thermal degradation can be formed (Ciesielski & Tomasik, 1996; Tomasik, Wiejak, & Pałasiński, 1989; Zhang, Golding, & Burgar, 2002).

It has been frequently reported that starch with different amylose/amylopectin ratios have a strong influence on the formation of resistant starch, and this has been linked to the increase of amylose. Nevertheless, due to the different structural features of both polysaccharides, we hypothesised that amylose and amylopectin can have additionally different behaviour during starch dry heating, which is an issue that has not been yet addressed. Structural changes of starch that contribute to their resistance during digestion (Lehmann & Robin, 2007) include: formation of new glycosidic bonds not hydrolysed by α -amylase, like (1 $\rightarrow$ 2), (1 $\rightarrow$ 3) and new (1 $\rightarrow$ 6) bonds, formation of modified residues at the reducing end, and formation of β -anomers (Siljeström, Björck, & Westerlund, 1989). Also, the formation of maltodextrins during the initial high moisture heat treatment of starch (Hoseney, 1991), and further heating during dry heat treatment of starch can contribute to the formation of resistant maltodextrins. The

formation of resistant maltodextrins by acid catalysed pyrodextrination of starch has been used to commercially produce resistant starch by prolonged heating and enzymatic hydrolysis of starch (Cho & Samuel, 2009). If occurring during dry heating food processing, the formation of resistant maltodextrins could change the nutritional profile of foods, as well as physiological activities that include reduction of glycaemia (Brouns et al., 2007), acceleration of lipid metabolism, reducing visceral fat, and maintenance of healthy intestinal regularity (Hashizume et al., 2012).

Resistant starch is classified in 5 different types. Type I is physically inaccessible starch synthesized in the endosperm of cereal grains or seeds, where starch granules are surrounded by protein matrix and cell wall material. Granular starch with the B- or C-type polymorphism, such as uncooked potato starch and green banana starch, among others, is highly resistant to enzymatic hydrolysis and is classified as type II resistant starch. Type III is retrograded starch formed during processing when heating and subsequent cooling of starch renders the molecules of amylose and amylopectin inaccessible to enzymatic hydrolysis. Type IV is chemically modified starch resistant to enzymatic hydrolysis in consequence of the modifications. Type V is amylose-lipid complexed starch (Birt et al., 2013).

The purpose of this work was to study the mechanism of starch degradation during dry heating at conditions of low water content, approaching those of food processing. To obtain an overall view on this subject and, simultaneously, to have new insights on the structural modifications in starch polysaccharides (PS), model systems with amylose and amylopectin, and also with structurally related oligosaccharides (OS), maltotetraose (M4) and glucosyl-maltotriose (GM3), were submitted to dry thermal treatment, mimicking food processing conditions, and further analysed by methylation analysis, electrospray mass spectrometry (ESI-MS) and tandem mass spectrometry

(ESI-MS/MS), and anionic chromatography after *in vitro* enzymatic digestion. This allows studying the possibility of formation of type 4 resistant starch and maltodextrins, namely the contribution of amylose and amylopectin for their formation. The mechanism of formation of resistant maltodextrins is also addressed.

2. Material and methods

2.1. Samples

Maltotetraose (M4), amylose (from corn) and amylopectin (from potato) were purchased from Sigma-Aldrich (St. Louis, MO). Glucosyl-maltotriose (GM3) was purchased from Megazyme (County Wicklow, Ireland) and α -amylase (EC 3.2.1.1) from Fluka (St. Louis, MO, USA).

2.2. Thermal treatment of samples

The thermal treatments were performed with a thermogravimetric analyser, model TGA-50 (Shimadzu, Kyoto, Japan), operating with a controlled air flow of 20 mL/min and a heating rate of 10 °C/min. All the experiments were conducted using a platinum sample cell and an initial sample mass of 4-15 mg. As used in previous studies with arabinosyl and mannosyl oligosaccharides (OS) (Moreira, Coimbra, Nunes, & Domingues, 2013; Moreira, Coimbra, Nunes, Simões, & Domingues, 2011), three independent samples of each OS, M4 and GM3, and polysaccharide (PS), amylose and amylopectin, were heated from room temperature (RT) up to 200 °C, and maintained at 200 °C for different periods of time: 0 (200T1), 30 (200T2), and 60 (200T3) min. To study their thermal stability, a sample of each PS was heated from RT up to 600 °C. Considering their thermal stability, two samples of each PS were also heated from RT

up to 265 °C and maintained at 265 °C for different periods of time: 0 (265T1) and 30 (265T2) min. The temperatures used for dry thermal treatments resemble the baking conditions during food processing.

The OS submitted to the thermal treatments at 200 °C and PS submitted to the thermal treatments at 265 °C were recovered, weighed, suspended in ultrapure water (5 mg/mL), and vortex mixed. To facilitate the dissolution of the PS samples, they were then stirred at 50 °C for 1 h. The water-soluble and water-insoluble fractions were then separated by centrifugation and kept frozen at -20 °C for further analysis. Solutions (1 mg/mL in ultrapure water) of each untreated OS (T0) samples were also prepared and kept frozen at -20 °C for MS analysis.

2.3. Neutral sugars analysis

Neutral sugars were converted to alditol acetates, as described in Supplementary Data, and then analysed by gas chromatography (GC) on a PerkinElmer Clarus 400 chromatograph (Waltham, MA) equipped with a flame ionization detector (FID) and a DB-225 column with 30 m of length, 0.25 mm of internal diameter, and 0.15 µm of film thickness (J&W Scientific, Folsom, CA). Hydrogen was used as carrier gas with 1 mL/min of flow. The follow oven temperature program used was: initial temperature 200 °C, temperature increase of 40 °C/min to 220 °C, maintained at 220 °C for 7 min, followed by an increase of 20 °C/min up to 230 °C, holding for 1 min. The temperature of injector and detector was 220 and 230 °C, respectively. The quantification of neutral sugars was obtained using 2-deoxyglucose as an internal standard.

2.4. Methylation analysis

For determination of glycosidic linkage composition, sugars were converted to partially O-methylated alditol acetates, as described in Supplementary Data, and then analysed by gas chromatography mass spectrometry (GC-MS) on an Agilent Technologies 6890N Network (Santa Clara, CA). The GC was equipped with a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 μ m of film thickness (J&W Scientific, Folsom, CA). The samples were injected with the injector operating at 220 °C and using the following temperature program: initial temperature, 50 °C, raised at a rate of 10 °C/min until 140 °C, standing for 5 min at this temperature, followed by rate of 0.5 °C/min to 170 °C, and then followed by a rate of 15 °C/min to 280 °C, with a further 5 min at 280 °C. The helium carrier gas had a flow rate of 0.2 mL/min. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the m/z range 40-500 in a 1 s cycle in full-scan mode acquisition.

2.5. Enzymatic hydrolysis and size-exclusion chromatography (Bio-Gel P-2)

Samples (1 mg) of amylose and amylopectin (unroasted and roasted at 265 °C) were hydrolysed with 2 U of pure α -(1 $\rightarrow$ 4)-amylase (EC 3.2.1.1) during 24 h at 37 °C with continuous stirring in a 100 mM phosphate buffer, pH 7.0, containing 0.02% sodium azide. Enzymatic hydrolysis was undertaken during 24h in order to ensure the total hydrolysis of amylose, with formation of glucose. This guaranties that after thermal treatment the formation of oligosaccharides was related with structural modification of the polysaccharides that hindered the enzymatic hydrolysis. The solutions were then heated in a water bath at 80 °C for 10 min to inactivate the enzyme, and were then injected into a column GE Healthcare Life Science 16/40 containing Bio-

Gel P-2 (Bio-Rad), using a peristaltic pump with the flow set to 0.25 mL/min. The mobile phase was distilled water. The 1 mL fractions were assayed for sugars by static light scattering (Sedere, France) at 57 °C with 2.0 bar of nitrogen.

2.6. Electrospray ionization mass spectrometry

ESI-MS and ESI-MS/MS spectra were acquired in the positive-ion mode on a Q-TOF2 hybrid tandem mass spectrometer (Micromass, Manchester, U.K.). For ESI analysis, 2 µL of each untreated and thermally treated sample, previously dissolved in water, was diluted in methanol with 1% of formic acid to a final volume of 200 µL. In order to obtain lithium adduct ions, $[M+Li]^+$, 2 µL of lithium acetate (1 mg/mL in ultrapure water) were added to 10 µL of each sample. Before MS analysis, 2 µL of the solutions containing lithium acetate were further diluted in methanol with 1% of formic acid, to a final volume of 200 µL.

For the acquisition of ESI-MS and ESI-MS/MS spectra, samples were introduced at a flow rate of 10 µL/min into the ESI source. Nitrogen was used as nebulizing and drying gas. The cone voltage was set at 30 V and the capillary voltage was maintained at 3 kV. The source temperature was 80 °C, and the desolvation temperature was 150 °C. The ESI-MS spectra were acquired scanning the m/z range from 100 to 1500. In the MS/MS experiments, the collision energy was set between 25 and 52 eV. The raw data were processed using the MassLynx software (version 4.0).

2.7. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

MALDI mass spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyser (Applied Biosystems, Framingham, MA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in a

reflectron mode. Full-scan mass spectra ranging from m/z 500 to 4000 were acquired in the positive mode. Sample preparation for MALDI analysis was performed by deposition of 0.5 μ L of each sample dissolved in water on top of a layer of crystals of 2,5-dihydroxybenzoic acid (DHB) formed by deposition of 0.5 μ L of DHB solution on the MALDI plate and letting dry at room temperature. The matrix solution was prepared by dissolving 5 mg of DHB in a 1 mL mixture of acetonitrile/methanol/aqueous trifluoroacetic acid (1%, v/v) (1:1:1, v/v/v).

2.8. Determination of non-resistant starch and maltodextrins and composition of the enzymatic hydrolysate

For the determination of non-resistant starch and maltodextrins, a modification of the AOAC method 2002.02 using the Megazyme Resistant Starch kit (K-RSTART) was used. Samples (4-10 mg) were incubated in a shaking water bath with pancreatic α -amylase and amyloglucosidase for 16 h at 37 °C, during which time non-resistant starch and maltodextrin is solubilized and hydrolysed to D-glucose by the combined action of the two enzymes. The amount of glucose was determined by high-performance anion-exchange chromatography (HPAEC, ICS-3000, Dionex) with a CarboPac PA-100 (Dionex), using lactose as internal standard. The non-resistant starch and maltodextrins were quantified by the amount of glucose expressed as polymers. The qualitative composition of the hydrolysates was analysed by comparison of the retention time of pure carbohydrate standards (fructose, trehalose, maltose, cellobiose, sophorose, nigerose, laminariobiose, isomaltose, and gentiobiose) with those present in the hydrolysate.

3. Results and discussion

3.1. Thermal treatment of OS and PS samples

The thermograms of the malto-oligosaccharides (OS) **structurally related to amylose and amylopectin, maltotetraose (M4) and glucosyl-maltotriose (GM3)**, and glucan polysaccharides (PS), **amylose and amylopectin**, were dry heated from room temperature (RT) up to 200 °C and maintained at 200 °C during 0 (200T1), 30 (200T2) and 60 (200T3) min are shown in Fig. 1 A and B. After thermal processing, OS showed changes in colour from light yellow (200T1) to dark brown (200T3), similar to what was observed when arabinosyl and mannosyl OS were submitted to the same treatments (Moreira, Coimbra, Nunes, & Domingues, 2013; Moreira, Coimbra, Nunes, Simões, & Domingues, 2011). However, no changes were observed in the colour of PS, suggesting that under these conditions the OS (M4 and GM3) were more heat labile than the correspondent amylose and amylopectin. Differences were also observed in the percentages of mass loss, when the initial mass loss due to the loss of adsorbed water molecules was excluded, ranging from 1.7-11.5% for OS and 0-0.4% for PS (Supplementary Tables S1-S2, showing mass loss from 150 °C). Considering that the formation of maltodextrins occurs in the initial stage of baking, where the amount of water is still high, this step should be critical to the formation of Glc degradation compounds.

Further thermogravimetric analyses of amylose and amylopectin were performed from RT to 600 °C. Based on the respective thermogravimetric curves (Fig. 1C), the temperature of 265 °C was selected as the optimal temperature for further roasting of both PS as this corresponds to the temperature immediately before of the first derivative inflexion, as was also described in the literature (Liu, Xie, Yu, Chen, & Li, 2009). The

alterations of the colour observed when amylopectin and amylose were heated to 265 °C and maintained at this temperature during 0 (265T1) and 30 (265T2) min were analogous to those observed when the OS were subjected to the thermal treatments T2 and T3 at 200 °C.

In order to exclude the presence of type 3 resistant starch, i.e. retrograded starch (Birt et al., 2013), the materials resulting from thermal treatments of OS at 200 °C and POS at 265 °C were suspended in water. In the case of OS roasted at 200 °C, they were 100% soluble in water in every condition. However, in the case of PS roasted at 265 °C in condition T2, the percentage of soluble material was only 34.5% for amylose and 27.3% for amylopectin (Table S2). Each water-soluble fraction was further characterised by sugar and methylation analyses and by mass spectrometry (MS). The water-insoluble fractions were only analysed by methylation analysis.

3.2. Structural modification during dry heating: changes in sugar content and glycosidic linkages

In order to access the structural modifications and their extent induced by the thermal treatments applied, neutral sugar and methylation analyses were performed in both untreated and thermal treated oligosaccharide (OS) and polysaccharide (PS) samples. The results obtained are shown in Table 1.

3.2.1 Oligosaccharide (OS)

According to the neutral sugar analysis, the dry heating of the tested OS up to 200 °C (200T1) resulted in a loss of 2.7% and 6.1% of the initial carbohydrates for maltotetraose (M4) and glucosyl-maltotriose (GM3), respectively. When the OS were heated to 200 °C and maintained at 200 °C for 30 min (200T2), a higher carbohydrate

loss was observed, 23% in average for both OS. In comparison to the treatment 200T2, the loss of carbohydrates increased by additional 2% and 6%, respectively, when the OS were heated to 200 °C and maintained at 200 °C for 60 min (200T3). These results are in accordance with the thermogravimetric data, where it was observed that M4 was more resistant to thermal degradation when compared to GM3. The percentage of mass loss observed after 150 °C when M4 and GM3 were submitted to the treatment 200T3 was 6.5 and 11.5%, respectively (Table S1).

When comparing the OS samples submitted to the treatment 200T3 with the respective untreated one (T0), the percentage of α -linked Glcp, the α -amylase susceptible linkage, decreased by 25.2 for M4 and 23.7 for GM3. Despite their different thermal stability of M4 and GM3, the glycosidic linkage composition after the thermal treatments at 200 °C (Table 1), for both OS studied, showed a decrease of (1 $\rightarrow$ 4)-linked glucopyranose (Glcp) residues. This decrease was even higher for the longer the time at 200 °C. Contrarily, it was observed an increase of (1 $\rightarrow$ 6) and (1 $\rightarrow$ 4,6) Glc *p* linkages that are α -amylase resistant. For both OS, it was also observed an increase of terminally linked Glcp for the longer thermal treatments (200T2 and 200T3). Also, new Glcp linkages, absent in the untreated OS samples (T0), were identified, namely (1 $\rightarrow$ 2) and (1 $\rightarrow$ 3). In the case of GM3, other new Glcp linkages were also formed during thermal processing, namely (1 $\rightarrow$ 3,4), (1 $\rightarrow$ 2,4), (1 $\rightarrow$ 2,6), (1 $\rightarrow$ 3,6), (1 $\rightarrow$ 2,4,6) and (1 $\rightarrow$ 2,3,6) linkages. The identification of new glycosidic linkages, absent in the untreated samples, corroborates the occurrence of non-enzymatic transglycosylation reactions induced by dry thermal processing (Moreira et al., 2011, 2013, 2014).

The (1 $\rightarrow$ 4)-linked mannopyranose (Manp) residues were also identified in the thermal treated GM3 samples, and were absent in the untreated sample. As Glcp can be formed from Manp via isomerization at C2 when mannosyl OS are submitted to the

dry heating (Moreira et al., 2014), it can be inferred that the (1→4)-linked Manp identified in the treated GM3 samples should derived from (1→4)-Glc_p by isomerization. In the M4 samples submitted to the longer treatments (200T2 and 200T3), it was observed an increase of terminally linked Manp, which can be derived from (1→4) -Manp via transglycosylation reactions, as described by Moreira *et al.* (2014) for pentose transglycosylation.

3.2.2 Polysaccharides (PS)

The percentage of sugars in the PS submitted to dry heating processing at 265 °C was lower for amylopectin when compared to amylose (Table 1), which is in accordance with the difference in the mass losses determined from thermogravimetric data (Supplementary Table S2). For both PS, the highest mass loss occurred during the thermal treatment 265T2, yielding a major fraction insoluble in water.

Considering both water-soluble and water-insoluble fractions recovered after thermal processing of the PS, it was observed, in comparison to the respective untreated samples, a decrease in the percentage of (1→4)-linked Glc_p and an increase of terminally and (1→6) -linked Glc_p, as observed for the OS samples. Also, new Glc_p linkages, like (1→2), (1→3), (1→2,3) , (1→2,6), (1→3,6), (1→3,4,6), (1→2,4,6) and (1→2,3,6), absent in the untreated samples, were identified after thermal processing of amylose and amylopectin. The thermal processing of amylose, under the condition 265T1 or 265T2, also formed (1→4,6)-linked Glc_p (%area of 3.0-8.4), absent in the untreated sample. When amylopectin was submitted to the treatment 265T1, it was observed an increase of (4,6) -linked Glc_p. However, the area percentage of (1→4,6)-linked Glc_p in the water-soluble and water-insoluble fractions recovered from

the amylopectin sample submitted to the longer treatment (265T2) was similar to that obtained in the untreated sample (2.1-2.7%). In the water-insoluble fractions recovered from the POS after thermal treatment 265T2, specially that from amylopectin, it was observed an abundant peak (%area of 42.8) assigned to (1→2,3,4,6)-linked Glcp. The occurrence of deep structural modifications was supported by the very dark brown colour of the water-insoluble fractions, as well as the difficulty of dissolving them even using DMSO and stirring overnight.

The decrease of (1→4)-linked Glcp detected by methylation analysis after thermal processing of both OS and PS samples can have a contribution of 1,6-anhydroglucose residues formed at the reducing end (Nunes, Reis, Domingues, & Coimbra, 2006; Simões, Maricato, Nunes, Domingues, & Coimbra, 2014).

3.3. Analysis by mass spectrometry

3.3.1. ESI-MS of GM3 and M4 after thermal treatment

In order to have a deeper insight in the chemical transformations occurring during the dry heat processing of glucosyl-maltotriose (GM3) and maltotetraose (M4), both unroasted (T0) and roasted (200T1, 200T2 and 200T3) oligosaccharides (OS) were analysed directly by ESI-MS. As polysaccharides are not suitable to be directly analysed by ESI-MS, unroasted (T0) and roasted samples (265T1 and 265T2) of amylose and amylopectin were selectively hydrolysed with an α -amylase and further fractioned by size-exclusion chromatography on a Bio-Gel P-2 in order to obtain fractions suitable to be analysed by ESI-MS.

The ESI-MS spectra of unroasted GM3 and M4 (Fig. 2A-B) showed the ion at m/z 689, attributed to the sodium adduct ions of the non-modified tetrasaccharides

351 ([Hex₄+Na]⁺). After thermal treatments at 200 °C (ESI-MS spectra of treatment T3 in
352 Fig. 2C and D), new ions were identified with higher and lower *m/z* values in
353 comparison with the non-modified tetrasaccharides. The higher the time of roasting the
354 higher the number ions observed and higher their relative abundance (Fig. 3).

355 For both roasted OS, it was possible to identify ions at *m/z* 203, 365 and 527,
356 attributed, respectively, to the sodium adduct ions of Hex, Hex₂, and Hex₃, which should
357 have been formed during dry thermal processing by depolymerization of the original
358 tetrasaccharides. It was also possible to observe ions at *m/z* 851, 1013 and 1175,
359 attributed to sodium adduct ions of Hex₅₋₇, showing that also polymerization occurred
360 during the dry thermal processing. The presence of OS and derivatives with a higher
361 degree of polymerization (DP) than those observed by ESI-MS was investigated by
362 MALDI-MS analysis. MALDI-MS spectra of OS submitted to the thermal treatments at
363 200 °C (Supplementary Fig. S1) showed the ion at *m/z* 689, corresponding to
364 [Hex₄+Na]⁺, and two series of ions corresponding to [Hex₅₋₁₂+Na]⁺ and [Hex₅₋₁₃-
365 H₂O+Na]⁺, confirming the formation of OS and dehydrated derivatives with a DP
366 higher than 7, the highest DP observed by ESI-MS.

367 The ions observed at *m/z* 185, 347, 509, 671, 833, 995 were attributed to the
368 sodium adduct ions of derivatives resulting from the loss of one water molecule ([Hex₁-
369 H₂O+Na]⁺). While ions at *m/z* 149, 311, 473, 635, attributed to the sodium adduct ions
370 of derivatives formed by the loss of three water molecules ([Hex₁₋₄-3H₂O+Na]⁺) were
371 observed in the case of GM3, in the case of M4 only the ion at *m/z* 311, attributed to
372 [Hex₂-3H₂O+Na]⁺, was observed. Also, it was observed the ion at *m/z* 257, attributed to
373 [Hex₂-6H₂O+Na]⁺, in the ESI-MS spectra of GM3 submitted to the treatments 200T2
374 and 200T3. Depolymerization, polymerization via non-enzymatic transglycosylation),
375 and derivatives resulting from the loss of one and three water molecules have already

been described to be formed during dry heating of mannosyl and arabinosyl OS (Moreira et al., 2011, 2012, 2013, 2014).

3.3.2. ESI-MS of PS after thermal treatment

Thermally treated amylose and amylopectin samples were submitted to selective hydrolysis using an α -amylase, followed by size-exclusion chromatography on a Bio-Gel P-2 column prior to ESI-MS analysis. Considering the peak areas in the Bio-Gel P-2 chromatograms, it was also possible to determine the resistance to α -amylase digestion of the thermal treated POS.

As can be seen in Fig. 4 (A-B), the native PS (T0) were extensively hydrolysed by α -amylase, with an extent of 93% for amylose and 81% for amylopectin. The major peak (fractions 33-36) observed in the Bio-Gel P-2 chromatograms corresponds to glucose and a small amount of a disaccharide, as inferred by the respective ESI-MS spectra that showed as base peak the ion at m/z 203 ($[\text{Hex}+\text{Na}]^+$) and a very low abundant ion at m/z 365 ($[\text{Hex}_2+\text{Na}]^+$)(data not shown).

After thermal processing of PS at 265° C, the Bio-Gel P-2 chromatogram changed, becoming visible new bands corresponding to compounds with higher molecular weight than mono and disaccharides (Fig. 4 A-B). As only 39% were recovered in the low molecular weight fraction, of the dry heated of amylose until 265 °C (265T1), 34% where recovered in the intermediate fraction and 27% of the material eluted in the high molecular weight material, it can be inferred that the digestibility of the thermal processed product decreased. The digestibility of amylopectin after the treatment 265T1 decreased to 65%. The decrease of the α -amylase digestibility of

amylose and amylopectin even after the light treatment (265T1) suggests changes in the glycosidic linkages induced by dry heating, which did not allow a complete and effective enzymatic digestion by the α -amylase. In fact, this is in agreement with the results of methylation analysis where new type of linkages, not hydrolysed by the α -amylase, like (1 $\rightarrow$ 3) and (1 $\rightarrow$ 6) Glcp linkages, were identified after thermal treatments of PS at 265 °C.

The ESI-MS spectra obtained from each PS sample submitted to the condition 265T1 after combination of the collected Bio-Gel P-2 fractions 33-36 (Fig. 4 C-D) showed the ions at m/z 203 ($[\text{Hex}+\text{Na}]^+$) and 347 ($[\text{Hex}_2-\text{H}_2\text{O}+\text{Na}]^+$) as the most abundant identified ions. The same was found in the amylopectin sample submitted to the longer treatment (265T2) (Fig. 4E). However, in the amylose sample submitted to the condition 265T2, it is clearly visible the presence of ions with m/z values corresponding to $[\text{M}+\text{Na}]^+$ ions of Hex_{2-4} (Fig. 4F), suggesting that the heat treatment 265T2 caused changes in the structure of amylose, which difficult the α -amylase action. This is also supported by the MALDI-MS spectra obtained from each POS sample submitted to the condition 265T1 after combination of the collected Bio-Gel P-2 fractions 18-21, where two ion series, $[\text{Hex}_{5-12}+\text{Na}]^+$ and $[\text{Hex}_{5-12}-\text{H}_2\text{O}+\text{Na}]^+$ were identified. Table 2 summarizes the m/z values of all the ions found throughout the study in the various samples and the respective identification.

In resume, the MS data showed that the dry heating of OS and PS samples caused not only depolymerization but also dehydration, effects which are described in the literature for similar compounds (Chiku, Nishimoto, & Kitaoka, 2010; Čmelík & Chmelík, 2010; Golon, González, Dávalos, & Kuhnert, 2013). In the case of OS samples, it was also able to induce polymerization (Moreira et al., 2011) and confirmed by Golon & Kuhnert (2012). In the case of thermal treated PS samples, evidences were

also gathered proving the existence of structural changes affecting the activity of the enzyme α -amylase, could be will be clarified by ESI-CID-MS/MS data.

3.3.3. ESI-CID-MS/MS

In order to confirm the assignments of sodium adduct ions identified in the ESI-MS spectra of OS and PS samples and to find additional details about their structures, ESI-CID-MS/MS spectra of the ions attributed to oligomeric compounds were acquired.

In respect to OS samples, selected ESI-MS/MS spectra of the most representative OS acquired from GM3 samples are presented as example in Fig. 5. According to the typical fragmentation pathways of neutral and reducing OS (Da Costa et al., 2012), the ESI-MS/MS spectrum (A) of the original OS (m/z 689) shows product ions resulting from glycosidic cleavages, namely the product ion at m/z 203 ($[\text{Hex}+\text{Na}]^+$) formed by loss 3 hexose residues (Hex_{res}) (3×162 Da), confirming the presence of a Hex tetrasaccharide. As typical of ($\alpha 1 \rightarrow 4$) Glcp linkages (Simões et al., 2007), a product ion resulting from cross-ring cleavage at the reducing end sugar residue with loss of $\text{C}_2\text{H}_4\text{O}_2$ (60 Da) is also observed in this spectrum. As observed for the ion at m/z 689, the ESI-MS/MS spectrum of the ion at m/z 851, attributed to $[\text{Hex}_5+\text{Na}]^+$ (B), shows the product ion at m/z 203 formed by loss of 4 Hex_{res} , confirming the presence of a Hex pentasaccharide. The MS/MS fragmentation of mono- and tri-dehydrated derivatives (Fig. 5C-D) yielded, respectively, a product ion at m/z 185 and 149, attributed to $[\text{Hex}-\text{H}_2\text{O}+\text{Na}]^+$ and $[\text{Hex}-3\text{H}_2\text{O}+\text{Na}]^+$, resulting from loss of one or more Hex_{res} . These product ions suggest that the loss of one and three water molecules occurred at the reducing end sugar residue. However, in the case of tri-

dehydrated derivatives, as illustrated in Fig. 5D, the presence of other structures with a non-modified Hex located at the reducing end cannot be excluded.

In relation to the Bio-Gel P-2 fractions recovered from enzymatically hydrolysed thermal treated POS, the MS/MS spectra acquired for the ions with m/z 365 ($[\text{Hex}_2+\text{Na}]^+$) showed an inversion in the ratio between the relative abundances of product ions with m/z 203 (-162 Da) and 185 (-180 Da) in the amylose sample submitted to the condition 265T2 (Supplementary Fig. S2). As observed with glucosyl disaccharides (Asam & Glish, 1997), this ratio is much superior to 1 for α anomers. However, the differentiation of glucosyl disaccharides with different type of linkage or anomeric configuration was bestly achieved by the fragmentation of lithium adduct ions, $[\text{M}+\text{Li}]^+$, rather than the corresponding $[\text{M}+\text{Na}]^+$ ions (Azenha, Coimbra, Moreira, Domingues, & Domingues, 2013; Simões et al., 2007). For this reason, in this work, the formation of $[\text{Hex}_2+\text{Li}]^+$ ions (m/z 349) was induced by addition of lithium acetate to the Bio-Gel P-2 fractions recovered from enzymatically hydrolysed thermal treated PS prior MS analysis.

In Fig. 6 are shown the MS/MS spectra acquired from the thermal treated POS for the ion with m/z 349 ($[\text{Hex}_2+\text{Li}]^+$). The inversion between the relative abundances of the product ions at m/z 187 (-162 Da) and 169 (-180 Da), formed respectively by the loss of a Hex_{res} and a Hex unit, remained in the amylose sample submitted to the condition 265T2. This observation suggests the formation of bonds with β anomeric configuration, considering that the MS/MS fragmentation of $[\text{M}+\text{Li}]^+$ ions of α anomers of glucosyl disaccharides yielded as base peak the product ion with m/z 187, distinctly of the corresponding β anomers (Simões et al., 2007).

3.4. Effect of the thermal treatment of amylose, amylopectin and glucosyl-maltotriose on the *in vitro* digestibility

In order to evaluate the effect of the thermal treatments T1 and T2 of amylose and amylopectin at 265 °C and glucosyl-maltotriose (GM3) at 200 °C in the production of carbohydrates resistant to digestion, the amount of non-resistant starch and maltodextrins, measured as the amount of glucose released after simulated *in vitro* digestion, with α -amylase and amyloglucosidase, was analysed and compared with the results obtained for total glucose after acid hydrolysis (Table 1). This approach enables also to study the carbohydrate structures formed during thermal treatment and resistant to digestion, by comparison of their retention time on HPAEC chromatograms with that of pure carbohydrate standards (Fig. 7). Though the lowest thermal treatment (T1) resulted in structures resistant to α -amylase, the amount of glucose recovered after simulated “*in vitro*” digestion of amylose, amylopectin, and GM3 resulted in the recovery of a similar amount of glucose as that determined after acid hydrolysis (Table 1). This means that although some structures formed are resistant to the α -amylase hydrolysis, ultimately they are broken down to glucose by amyloglucosidase. For GM3, there was also observed an abundant peak of fructose (Fig. 7), possibly due to the isomerization of the reducing terminal residue of GM3 during the thermal treatment (Kroh, Jalyschko, & Häsel, 1996). This peak is not detected in the *in vitro* digests of amylose and amylopectin for T1, although being present in low abundance in T2 (Fig. 7), probably due to the increase in reducing ends caused by the heat induced depolymerization. With increasing thermal treatment there is a significant decrease in the amount of free glucose recovered after enzymatic digestion (only 7 to 8%), when compared to the amount of free glucose determined by acid hydrolysis (Table 1). These results show that a significant proportion of the glucose present in the amylose and

amylopectin samples after T2 is resistant to the *in vitro* digestion. Besides the formation of fructose at the highest thermal treatment (T2), where observed also disaccharides corresponding to gentiobiose, cellobiose and laminariobiose, showing the formation of β -linked glucose residues as inferred by ESI-MS/MS. Also observed were a set of oligosaccharides eluting at higher retention times, than the void volume, also resistant to the *in vitro* digestion (Fig. 7). These results demonstrate that during the dry heat treatment of amylose and amylopectin but also of GM3 oligosaccharides that resist the *in vitro* digestion. There are resistant maltodextrins formed during the dry heat treatment of these starch polysaccharides and related oligosaccharides. These results allows to associate the dry heat treatment to the formation of type 4 resistant starch, which is not able to be digested in human gastrointestinal tract and thus is accumulated in the intestinal tract and exert several beneficial effects (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010; Higgins & Brown, 2013; Rodríguez-Cabezas et al., 2010).

4. Concluding remarks

The data presented showed that the dry heat treatment of starch polysaccharides, amylose and amylopectin, result in the formation of resistant starch and resistant maltodextrins. The resistance to *in vitro* digestion seems to be due to the formation of new glycosidic linkages, different from the characteristics α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) of these polysaccharides. Amylopectin was more susceptible to heat treatment than amylose. The heat treatment of α -glucan oligosaccharides resulted in the formation of resistant maltodextrins, as observed for starch polysaccharides. These results show that the dry heat treatment of starch-rich foods can result in complex structural changes of

amylose, amylopectin and maltodextrins. The maltodextrins can be naturally present or formed during heat treatment by depolymerization of amylose and amylopectin, contributing to the formation of resistant starch.

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Figure captions

Fig. 1. Thermogravimetric curves of A) glucosyl-maltotriose and maltotetraose and heated from room temperature (RT) up to 200 °C, remaining at this temperature for 60 min (200T3); B) amylopectin and amylose also submitted to the treatment 200T3; C) amylose and amylopectin heated from RT up to 600 °C; D) amylopectin and amylose heated from RT up to 265 °C, remaining at this temperature for 30 min (265T2). Different thermal treatments (T1, T2 and T3) performed at 200 and 265 °C are illustrated.

Fig. 2. ESI-MS spectra of glucosyl-maltotriose (GM3) and maltotetraose (M4), A-B) before (T0) and C-D) after thermal treatment at 200 °C, remaining at 200 °C for 60 min (200T3). *Solvent impurities.

Fig. 3. Relative abundances of $[M+Na]^+$ ions observed in the ESI-MS spectra of A) glucosyl-maltotriose and B) maltotetraose obtained after thermal treatments at 200 °C (200T1, 200T2 and 200T3). Values are given as mean $\pm$ standard deviation of three replicate spectra acquisitions made in different days. Ions with a mean relative abundance less than 3 % were not considered.

Fig. 4. Size-exclusion chromatograms (Bio-Gel P-2) of the material obtained after enzymatic hydrolysis with an α -amylase of A) amylopectin and B) amylose, before (T0) and after thermal treatments at 265 °C (265T1 and 265T2), and C-F) respective ESI-MS spectra obtained from each enzymatically hydrolysed thermal treated sample after combination of the collected fractions 33-36. *Solvent impurities.

Fig. 5. ESI-MS/MS spectra acquired from glucosyl-maltotriose samples for the ions observed at m/z A) 689 ($[\text{Hex}_4+\text{Na}]^+$); B) 851 ($[\text{Hex}_5+\text{Na}]^+$); C) 671 ($[\text{Hex}_4-\text{H}_2\text{O}+\text{Na}]^+$); and D) 635 ($[\text{Hex}_4-3\text{H}_2\text{O}+\text{Na}]^+$).

Fig. 6. ESI-MS/MS spectra of $[\text{M}+\text{Li}]^+$ ions of Hex_2 (m/z 349) observed in the MS spectra after addition of lithium acetate to Bio-Gel P-2 fractions recovered from enzymatically hydrolysed thermal treated samples of amylopectin, A) 265T1 and C) 265T2, and of amylose, B) 265T1 and D) 265T2.

Fig. 7. High performance anion exchange chromatograms of the *in vitro* digests from A) glucosyl-maltotriose after thermal treatment 200T1, B) amylose after thermal treatment 265T2 and C) amylopectin after thermal treatment 265T2.

Fig. 1. Thermogravimetric curves of A) glucosyl-maltotriose and maltotetraose and heated from room temperature (RT) up to 200 °C, remaining at this temperature for 60 min (200T3); B) amylopectin and amylose also submitted to the treatment 200T3; C) amylose and amylopectin heated from RT up to 600 °C; D) amylopectin and amylose heated from RT up to 265 °C, remaining at this temperature for 30 min (265T2). Different thermal treatments (T1, T2 and T3) performed at 200 and 265 °C are illustrated.

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Fig. 3. (Grayscale version for print)

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Fig. 5. ESI-MS/MS spectra acquired from glucosyl-maltotriose samples for the ions observed at m/z A) 689 ($[Hex_4+Na]^+$); B) 851 ($[Hex_5+Na]^+$); C) 671 ($[Hex_4-H_2O+Na]^+$); and D) 635 ($[Hex_4-3H_2O+Na]^+$).

Fig. 6. ESI-MS/MS spectra of $[M+Li]^+$ ions of Hex₂ (m/z 349) observed in the MS spectra after addition of lithium acetate to Bio-Gel P-2 fractions recovered from enzymatically hydrolysed thermal treated samples of amylopectin, A) 265T1 and C) 265T2, and of amylose, B) 265T1 and D) 265T2.

Fig. 6. (Grayscale version for print)

Fig. 7. High performance anion exchange chromatograms of the *in vitro* digests from A) glucosyl-maltotriose after thermal treatment 200T1, B) amylose after thermal treatment 265T2 and C) amylopectin after thermal treatment 265T2.

Table 1. Sugar and glycosidic-linkage compositions and *in vitro* digestibility of maltotetraose and glucosyl-maltotriose, before (T0) and after thermal treatments at 200 °C, and of amylose and amylopectin, before (T0) and after thermal treatments at 265 °C.

Linkage	Maltotetraose				Glucosyl-maltotriose				Amylose				Amylopectin			
	T0	200T1	200T2	200T3	T0	200T1	200T2	200T3	T0	265T1	265T2	265T2INS ^b	T0	265T1	265T2	265T2INS ^b
T-Glcp	20.3	27.8	26.2	31.8	27.1	25.4	27.4	29.1	1.5	6.5	40.1	31.7	5.9	10.3	44.4	23.9
2-Glcp		0.1	0.5	1		0.1	0.9	1.5		0.2	6.9	5.3		0.3	5.5	3.6
3-Glcp		0.2	0.3	0.5		0.1	0.4	0.6		0.2	3.2	3.7		0.4	1.7	1.9
4-Glcp	78.6	69.8	62.6	53.3	44.1	40.4	25.7	20.2	98.5	85.9	6.9	9.1	91.4	80	6	5.5
6-Glcp	0.1	0.7	2.9	4.9	28.7	31.9	37.4	36.4		1.8	26.5	16.9	0.2	1.9	28	9.5
3,4-Glcp	0.1	0.1	0.2	0.3		0.1	0.3	0.3		0.3	0.7	1.2	0.1	0.6	1.2	1.4
2,3-Glcp											0.5	0.7			0.4	0.3
2,4-Glcp	0.1	0.1	0.5	0.4		0.1	0.5	0.5		0.3	0.5	0.7	0.2	0.4	0.3	0.4
4,6-Glcp	0.6	1	6.3	6.3		0.8	4.9	7.7		4.6	3	8.4	2.2	5.8	2.7	2.1
2,6-Glcp						0.1	0.8	1.4			0.8	0.6			0.5	0.3
3,6-Glcp						0.1	0.9	1			4.4	4.7			5	3.1
3,4,6-Glcp											1	2.5			0.7	2.4
2,4,6-Glcp							<i>t</i>	0.1			0.8	1.2			0.2	0.9
2,3,6-Glcp							0.1	0.1			0.5	0.9			0.3	0.8
2,3,4,6-Glcp	0.1	0.1	0.1	0.2	0.1	0.6	0.4	0.9		0.2	0.5	9.3			0.7	42.8
T-Manp	0.1	0.1	0.4	1.3							3.2	3			2.4	1.1
4-Manp						0.3	0.3	0.2			0.5	0.1		0.3		
% of sugars ^a	99.5	96.8	76.4	74.7	99.1	93.0	76.3	70.7	96.2	64.4	45.2		97.4	49.0	20	
<i>in vitro</i> digestion	-	-	-	-	-	93.8	74.4	-	-	42.0		^c 3.1	-	54.1		^c 1.6

-Not determined. *t* for traces (<0.1%) ^aTotal sugar percentage obtained by neutral sugar analysis. ^bWater-insoluble fraction. ^cDetermined using whole material resulting from the thermal treatment, and not water-soluble and water-insoluble fractions.

Table 2. Summary of the $[M+Na]^+$ ions identified in the ESI-MS and MALDI-MS spectra of thermal treated samples with indication of m/z values and proposed assignments.

n	1	2	3	4	5	6	7	8	9	10	11	12
$[Hex_n+Na]^+$	203	365	527	689	851	1013	1175	1337	1499	1661	1823	1985
$[Hex_n-H_2O+Na]^+$	185	347	509	671	833	995	1157	1319	1481	1643	1805	1967
$[Hex_n-3H_2O+Na]^+$	149	311	473	635	797							
$[Hex_n-6H_2O+Na]^+$		257										