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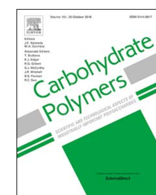

Isomalto/malto-polysaccharide structure in relation to the structural properties of starch substrates

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ABSTRACT

Isomalto/malto-polysaccharides (IMMPs) are soluble dietary fibres produced by the enzymatic modification of starch with 4,6-α-glucanotransferase (GTFB). The structure, size, and linkage distribution of these IMMPs has remained largely unknown, since most structural information has been based on indirect measurements such as total α-(1 → 6) content, iodine staining and GTFB hydrolytic activity. This study provides a deeper understanding of IMMP structure in relation to its respective starch substrate, by combining preparative fractionation with linkage compositition analysis. IMMPs were produced from a variety of amylose-rich and amylose-free starches. The extent of modification was investigated per IMMP molecular weight (M_w)-fraction, distinguishing between linear α-(1 → 6) linkages introduced by GTFB and starch's native α-(1 → 4,6) branching points. It emerged that the amount of α-(1 → 6) linkages was consistently higher in IMMP low M_w-fractions and that GTFB activity was limited by native α-(1 → 4,6) linkages. The presence of amylose turned out to be a prerequisite for the incorporation of linear α-(1 → 6) linkages in amylopectin.

1. Introduction

Starch is one of the most used polysaccharides in both food and non-food applications because of its broad functionality. The functionality of starch is the result of its molecular structure, which depends on aspects such as; amylose content, degree of branching and amylopectin chain length (Pérez & Bertoft, 2010). These aspects vary per starch source and thus, different starches are used for different applications. While starch is being used on a large scale, its unmodified form is not suited for all applications.

In order to further increase the functionality of starch, it is often modified. Most starches are further functionalized with post-harvest modification, since it is impractical to rely solely on starch origin variety. Post-harvest modification of starch is traditionally done chemically using processes such as; hydrolysis, dextrinization, cross-linking or the addition of functional groups (Tomasik & Schilling, 2004). Enzymes can also be used for the post-harvest modification of starch (Kadokawa, 2011; van der Maarel & Leemhuis, 2013). The advantages of enzymatic over chemical modification of starch include; the absence of harsh chemicals, lower energy input and an even more selective modification. Therefore enzymes are an increasingly interesting tool for the post-harvest modification of starch (van der Maarel & Leemhuis, 2013).

Enzymatic modification of starch is traditionally focussed on controlled breakdown of glucan chains using glucanohydrolases (Guzmán-Maldonado & Paredes-López, 1995; van der Maarel, van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Commonly used glucanohydrolyases in this field are; α-amylase, maltogenic amylase (Leman, Goesaert, Vandeputte, Lagrain, & Delcour, 2005), β-amylase and isoamylase (Ciric, Woortman, & Loos, 2014). Next to glucanohydrolases, glucanotransferases can also be used for the modification of starch. Glucanotransferases are capable of modifying starch and other α-glucans by altering the intrinsic linkage composition, by for example changing α-(1 → 4) glycosidic linkages into α-(1 → 6) glycosidic linkages (Kralj et al., 2011). Well documented glucanotransferases include; cyclodextrin glucanotransferase (Bissaro, Monsan, Fauré, & O'Donohue, 2015; Crini, 2014), 4,4-α-glucanotransferase (Ayudhaya, Pongsawasdi, Laohasongkram, & Chaiwanichsiri, 2016; van der Maarel & Leemhuis, 2013; Xu et al., 2014) and branching enzymes (Grimaud et al., 2013; Suzuki et al., 2015).

Abbreviations: DP, degree of polymerisation; GC–MS, gas chromatography–mass spectroscopy; GPC-MALLS, gel permeation chromatography- multi-angle laser light scattering; GTFB-ΔN, N-terminal truncated 4,6-α -glucanotransferase; ^{1}H NMR, proton nuclear magnetic resonance; IMMP, isomalto/malto-polysaccharide; LMW,MMW,HMW, low- medium- high- molecular weight; PMAA, partially methylated alditol acetate; SEC-RI, size exclusion chromatography & refractive index detector

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Innovative α-glucan structures can be produced by modifying starch with a combination of glucanohydrolases and glucanotransferases. The resulting α-glucan is a combination of its natural structure and structural elements introduced by the action of the enzymes. Examples of α-glucans produced with synergistic enzyme action are cycloisomalto-oligosaccharides (Funane et al., 2014), enzymatically synthesized glycogen (Kajiura, Takata, Kuriki, & Kitamura, 2010), isomalto-oligosaccharides (IMOs) (Kaulpiboon, Rudeekulthamrong, Watanasatitarpa, Ito, & Pongsawasdi, 2015), highly branched maltodextrins (Lee et al., 2013) and the alternating elongation and branching of amylopectin using amylomaltase and branching enzymes (Sorndech et al., 2015). As shown above, a wide array of structures can be obtained with the enzymatic modification of α-glucans. In this paper we investigate the unexplored structure of starches modified with the 4,6-α-glucanotransferase (GTFB) enzyme, an enzyme that is capable of converting α-(1 → 4) glycosidic linkages into α-(1 → 6) glycosidic linkages.

The modification of starch with the GTFB enzyme results in the formation of isomalto/malto-polysaccharides (IMMPs). IMMPs are not to be confused with IMOs covering a DP range from 2 to ∼10 (Chockchaisawasdee & Poosaran, 2013; Goffin et al., 2011; Hu, Ketabi, Buchko, & Gänzle, 2013; Kaulpiboon et al., 2015) since IMMPs are considerably larger, IMMPs up to DP 35 have already been identified (Leemhuis et al., 2014). The functionality of most novel α-glucans is directed towards slow digestibility, prebiotic functionality and application as a dietary fibre, this is usually done by increasing the amount of branching points, increasing crystallinity or by chemical modification (Lee et al., 2013; Raigond, Ezekiel, & Raigond, 2015). The GTFB enzyme is able to decrease the digestibility of starch by reducing the amount of easily digestible α-(1 → 4) linkages and introducing linear α-(1 → 6) linkages that are not degradable by α-amylase. Therefore, IMMPs have potential applications in food as slow-digestible fibres with prebiotic potential (Dijkhuizen et al., 2010; Leemhuis et al., 2014).

The activity of GTFB (Bai, van der Kaaij, Leemhuis et al., 2015), its crystal structure (Bai, Böger et al., 2016; Bai, Gangoiti, Dijkstra, Dijkhuizen, & Pijning, 2016) and action on different substrates (Bai, Böger et al., 2016; Bai, Gangoiti et al., 2016; Dobruchowska et al., 2012; Leemhuis et al., 2014) has been studied. To date, it has been demonstrated that GTFB is able to partially convert starch to IMMPs and it was proposed that the extent of GTFB modification is related to the amount of amylose in the substrate (Leemhuis et al., 2014). Although some research has been carried out on IMMP structure, most structural information on IMMPs so far, is based on indirect measurements such as total α-(1 → 6) content, iodine staining and GTFB hydrolytic activity. Until now, the structure, size and linkage distribution of starch-based IMMPs has remained largely unknown.

This study is the first to fractionate starch-based IMMPs, and the first to differentiate between starch's native α-(1 → 4,6) branching points and the linear α-(1 → 6) linkages introduced by the GTFB-ΔN enzyme, instead of solely relying on the total α-(1 → 6) content measured with ^{1}H NMR spectroscopy. IMMPs were produced from a selection of starches and subsequently fractionated on a preparative scale. The linkage content was analysed with ^{1}H NMR and permethylation analysis, the molecular weight was determined with GPC-MALLS. Combining fractionation with linkage compositition analysis makes it possible to determine the extent of GTFB-ΔN modification in relation to the molecular weight of the IMMP fractions. This in-depth characterization also provides more information on the relation between the GTFB-ΔN reaction pathway and the molecular structure of the starch substrate.

2. Materials & methods

2.1. Materials

Potato starch, waxy potato starch (Eliane 100) and wheat starch (Excelsior) were provided by AVEBE (Veendam, the Netherlands). Maize starch (C-Gel) (Cargill, Wayzata, MN, USA) waxy maize starch (Amioca power TF, National Starch), rice starch (S7260, Sigma-Aldrich, St. Louis, MO, USA) and waxy rice starch (Remyline XS, Beneo, Mannheim, Germany) were purchased from their respective supplier. Sweet potato starch (SuShu2) was provided by the laboratory of Food Chemistry, Wageningen University & Research (Wageningen, the Netherlands) (Zhao et al., 2015).

2.2. Production of GTFB-ΔN

The GTFB-ΔN enzyme was produced in cooperation with Dr. Y. Bai, who kindly provided the *E. coli* BL21 DE3 cells carrying the pET15b-ΔNGTFB plasmid as described and produced in Bai, van der Kaaij, Leemhuis et al., 2015;. The *E. coli* cells were grown at 37 °C for 16 h shaking at 220 rpm in flasks containing LB medium supplemented with 100 μg/mL ampicillin. The culture was transferred into 600 mL flasks and kept at 37 °C for 2–3 h at 220 rpm until OD_{600} = 0.4. The flasks were cooled on ice, 0.1 mM isopropyl β-D-1-thiogalactopyranoside was added and the flasks were incubated at 18 °C for 22 h at 160 rpm. Cells were centrifuged at 4000 x *g* at 10 °C for 30 min. The pellets were washed with washing buffer (20 mM Tris-HCl, pH = 8.0) and centrifuged at 3000 x *g* at 10 °C for 30 min. Each pellet was suspended in 15 mL lysis buffer (20 mM Tris-HCl, pH = 8.0, 250 mM NaCl, 1 mM $CaCl_2$), 100 μL Lysozyme (50 mg/mL) and 20 μL DNase (20 mg/mL) was added and left to rotate at room temperature for 2 h. The suspension was lysed three times with a French press and centrifuged at 10,000 × *g* at 10 °C for 30 min. The supernatant was retained and mixed with Ni-NTA beads (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C overnight, GTFB-ΔN was then purified using His-tag affinity column chromatography. After washing steps high purity GTFB-ΔN was eluted with a 300 mM imidazole elution buffer. The final GTFB-ΔN concentration was determined using a Nanodrop 2000 spectrophotometer (Isogen Life Science, De Meern, The Netherlands).

2.3. GTFB-ΔN activity

GTFB-ΔN hydrolytic activity on maltoheptaose (Sigma-Aldrich, St. Louis, MO, USA) was measured with a GOPOD assay (Megazyme, Bray, Wicklow, Ireland). The hydrolytic activity of the GTFB-ΔN enzyme used in this paper is comparable to previous research (Bai, van der Kaaij, Leemhuis et al., 2015; Bai, van der Kaaij, Woortman et al., 2015).

2.4. IMMP synthesis

Starch substrate was suspended at 2.5% (w/v) in 20 mM acetate buffer, pH = 4.9 containing 5 mM $CaCl_2$. The starch suspension was gelatinized by autoclaving at 121 °C for 15 min. GTFB-ΔN was added after this sterilization step as soon as the reaction mixture was cooled down to 37 °C, to avoid excessive retrogradation. IMMP synthesis was carried out by adding 0.3 mg GTFB-ΔN/g substrate and incubating the solution at 37 °C for 24 h. Possible acidification was monitored by checking the pH before and after modification. The pH increased by an average of 0.1 in each sample, which indicates that the samples were not contaminated. After reaction, GTFB-ΔN was inactivated by heating the reaction mixture to 95 °C for 15 min in a waterbath. Next, the solution was cooled to 50 °C, Amberlite MB 20-resin (DOW, Midland, MI, USA) was added and the mixture was incubated at 50 °C for 2 h. The MB20-resin was sieved out. The IMMP solution was stored at −20 °C overnight and subsequently freeze-dried. IMMP yield (w/w) was determined by comparing the freeze-dried IMMP weight to the amount of starch substrate used, supplementary information (7.1).

2.5. Fractionation with size exclusion chromatography

Preparative fractionation was executed on an Akta Explorer (GE Healthcare, Uppsala, Sweden) with a Sephacryl S-500, 4.3 L (r = 5 cm,

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