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Molecular rearrangement of waxy and normal maize starch granules during *in vitro* digestion

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ABSTRACT

The objective of the present study is to understand the changes in starch structures during digestion and the structures contributing to slow digestion properties. The molecular, crystalline, and granular structures of native waxy maize, normal maize, high-amylose maize, and normal potato starch granules were monitored using SEC, XRD, DSC, and SEM. The amylose and amylopectin molecules of all four starches were hydrolyzed to smaller dextrins, with some having linear molecular structure. Neither the A- nor B-type crystallinity was resistant to enzyme hydrolysis. Starch crystallites with melting temperature above 120 °C appeared in waxy and normal maize starches after digestion, suggesting that the linear dextrins retrograded into thermally stable crystalline structure. These crystallites were also observed for high-amylose maize starch before and after digestion, contributing to its low enzyme digestibility. On the contrary, the enzyme-resistant granular structure of native normal potato starch was responsible for its low susceptibility to enzyme hydrolysis.

1. Introduction

Starch is the major energy source in most staple foods. Its digestion rate and extent of digestion in the small intestine have a large impact on human health and nutrition. According to Englyst, Kingman, and Cummings (1992), starch can be categorized into three groups, which are rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). It has been proposed that RDS is completely digested within 20 min, causing high postprandial glycemic and insulinemic responses and therefore increasing the risks of metabolic disorders such as insulin resistance, diabetes, and obesity (Byrnes, Br& Miller, & Denyer, 1995; Ludwig, 2002; Willett, Manson, & Liu, 2002). SDS is digested between 20 and 120 min after consumption and RS is the remnants that are not digested after 120 min. Both SDS and RS can lower postprandial glycemic and insulinemic responses with SDS prolonging the supply of glucose to the body, especially to the brain, which is particularly beneficial for maintaining cognitive functions. Similar to other dietary fibers, RS escapes the digestion in the small intestine and reaches the colon, where it serves as an important substrate for gut fermentation producing short-chain fatty acids with the potential to prevent the development of colon cancer cells (Ferguson, Tasman-Jones, Englyst, & Harris, 2000; Zhao et al., 2011).

The structures of native starch granules can be simplified into five levels (Dona, Pages, Gilbert, & Kuchel, 2010; Tran et al., 2011). Linear branches of starch molecules (Level 1 structure) are made of glucose monomers linked together by α-(1 → 4) glycosidic linkages, and these linear branches are connected together by α-(1 → 6) glycosidic linkages forming individual fully branched molecules (Level 2 structure). These lowest two levels are molecular levels composed of mainly amylose and amylopectin. Amylose is primarily linear with a few long branches, whereas amylopectin is highly branched with a vast number of short branches and a molecular size two orders of magnitude larger than amylose. Some branches of amylopectin are arranged in a double helical conformation, packing into crystallites that form alternating crystalline and amorphous layers (semi-crystalline structure, Level 3). In contrast, amylose is typically amorphous or in a single helical complex with lipid molecules. The semi-crystalline structures are arranged into

Abbreviations: CLD, chain length distribution; DP, degree of polymerization; DSC, differential scanning calorimetry/calorimeter; RDS, rapidly digestible starch; RS, resistant starch; SDS, slowly digestible starch; SEC, size-exclusion chromatography; SEM, scanning electron microscopy; T_c, conclusion temperature; T_o, onset temperature; T_p, peak temperature; XRD, X-ray diffractometry.

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concentric growth rings, which alternate with amorphous growth rings around the amorphous hilum (Level 4 structure) in a single starch granule (Level 5 structure).

The rate and extent of starch digestion are largely influenced by starch structures, including the branch-chain length of amylose and amylopectin molecules (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005; Syahariza, Sar, Hasjim, Tizzotti, & Gilbert, 2013), molecular size (Li et al., 2015a), amylose content (Li, Jiang, Campbell, Blanco, & Jane, 2008; Syahariza et al., 2013), amylose–lipid complexes (Ai, Hasjim, & Jane, 2013; Hasjim et al., 2010b), crystalline structure (Hasjim & Jane, 2009; Jane et al., 2003; Sievert & Pomeranz, 1990), granule size and surface structure (Dhital, Shrestha, & Gidley, 2010; Hasjim, Srichuwong, Scott, & Jane, 2009). The changes in starch structure during *in vitro* and *in vivo* digestions have recently been investigated. The rearrangement of starch molecules during *in vitro* digestion of extruded high-amylose maize starches to form enzyme-resistant crystalline structures was reported by several authors (Htoon et al., 2009; Li et al., 2015a; Lopez-Rubio, Flanagan, Shrestha, Gidley, & Gilbert, 2008b; Shrestha et al., 2010). The dextrins produced after prolonged *in vitro* digestion of either extruded maize starches with various amylose contents or native normal maize starch granules were both found to be predominately linear with degree of polymerization (DP) $X \sim 50$ (Hasjim, Cesbron Lavau, Gidley, & Gilbert, 2010a; Witt, Gidley, & Gilbert, 2010). Similarly, the digesta collected from the lower-half small intestine of the pigs fed with native normal maize starch granules contained substantial amounts of linear dextrins with DP $X \sim 50$ (Hasjim et al., 2010a). Combining the results from these studies, it can be assumed that the linear dextrins produced during starch digestion are able to rearrange themselves into highly ordered crystalline structures that are less susceptible to enzyme hydrolysis. However, this relationship has not been explicitly proven in a single study before.

The aim of this study is to understand the molecular rearrangement of starch during digestion and the structures contributing to slow digestion properties by investigating the changes in the molecular, crystalline, and granular structures at different digestion times. Native starch granules (Level 5 structure) were used as the substrates for an *in vitro* digestion. Although native starch granules are less common in human food than gelatinized starch, they are present in animal feed, fresh produce (fruits and vegetables), and low-moisture foods (such as biscuits and some breakfast cereals), and the lack of retrograded starch in native starch granules allows the distinct observation of starch molecular rearrangement (retrogradation) during digestion. The molecular structures (Levels 1 and 2) of the starch digesta collected during the *in vitro* digestion were characterized using size-exclusion chromatography (SEC), while their crystalline structures (Level 3) were characterized using X-ray diffractometry (XRD) and differential scanning calorimetry (DSC). The changes on the granular structure (Level 5) were identified using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

Four types of native starch granules were used: waxy maize, normal maize, high-amylose maize (Gelose 50), and normal potato starches. The maize starches were obtained from Penford Australia Ltd. (Lane Cove, NSW, Australia). Normal potato starch, pancreatin from porcine pancreas, and LiBr (ReagentPlus) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Amyloglucosidase from *Aspergillus niger*, isoamylase from *Pseudomonas* sp., and D-glucose (GOPOD Format) kit were purchased from Megazyme International Ltd. (Bray, Co. Wicklow, Ireland). Dimethyl sulfoxide (DMSO, GR for analysis ACS) was from Merck and Co., Inc. (Kilsyth, VIC, Australia). Other chemicals were reagent grade and used as received.

2.2. In vitro *starch digestion*

Native starch granules (1.5 g, dry weight basis) were suspended in sodium acetate buffer (5.0 mL, 0.2 M, pH 6.0) containing 200 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 0.02% NaN_3. An enzyme solution (5.0 mL) containing 60 mg pancreatin and 300 μL amyloglucosidase in sodium acetate buffer solution was added to the starch suspension after being equilibrated at 37 °C with stirring for 5 min. The mixture was incubated at 37 °C with stirring. The digestion was stopped by adding 30 mL absolute ethanol to the mixture at 0, 2, 4, 8, and 16 h for waxy maize starch; 0, 2, 4, 8, 16, and 24 h for normal maize starch; and 0, 16, 24, and 48 h for high-amylose maize and normal potato starches. The starch digesta (precipitate) was collected by centrifugation at 4000 × *g* for 10 min and dried in a fume hood at room temperature. The structure of starch digesta was characterized using SEC, DSC, XRD, and SEM. The amount of glucose in the supernatant was determined using the Megazyme D-glucose kit following the instructions from the manufacturer and converted to the amount of digested starch using the factor of 0.9 (the conversion factor for glucose to anhydroglucose unit in starch). The degree of starch hydrolysis was calculated as follows:

$$\%\ \text{hydrolysis} = \frac{\text{total weight of glucose in supernatant} \times 0.9}{\text{dry weight of starch}} \times 100\%$$

The concentrations of pancreatin and amyloglucosidase (dry starch weight basis) were higher than previous studies (Hasjim et al., 2010a; Sopade & Gidley, 2009; Witt et al., 2010) in order to produce substantial digestion of the native high-amylose maize and normal potato starch granules, which contain large amounts of RS (Dhital et al., 2010; Jane et al., 2003; Tester, Qi, & Karkalas, 2006). The *in vitro* digestion steps simulating the digestion in the mouth and the stomach were not used in the present study as the starch granules entering the small intestine from a previous *in vivo* study were not significantly digested in the mouth or the stomach (Hasjim et al., 2010a). The digestion times were selected based on the digestibility of the starches. Because the native high-amylose maize and normal potato starch granules are more resistant to enzyme hydrolysis than the native waxy and normal maize starch granules, the digestions of the native high-amylose maize and normal potato starch granules were carried out for longer times.

2.3. Size-exclusion chromatography and amylose content

Starch digesta (~2 mg) were dissolved in 1 mL DMSO solution containing 0.5% w/w LiBr for 24 h at 80 °C and 350 rpm for the characterization of whole molecular structure (Level 2 structure) following the method of Syahariza, Li, and Hasjim (2010). The preparation of the individual branches of starch molecules (Level 1 structure) for characterization involved dissolving ~6 mg starch digesta in 1 mL DMSO solution containing 0.5% w/w LiBr for 24 h at 80 °C and 350 rpm, precipitated using 5 mL absolute ethanol, dissolved in 0.9 mL distilled water in a boiling water bath, and then debranched using 2.5 μL isoamylase at 37 °C for 3 h (pH was adjusted using 0.1 mL 0.1 M acetate buffer of pH 3.5) following the method described elsewhere (Hasjim et al., 2010a; Tran et al., 2011). The debranched starch was neutralized to pH ~ 7 with 100 μL 0.1 M NaOH, heated at 80 °C for 2 h, and freeze dried. The dried debranched starch was redissolved in 1 mL DMSO solution containing 0.5% w/w LiBr at 80 °C and 350 rpm for 24 h.

The structures of whole and debranched starch molecules were characterized in duplicate using SEC, also known as gel permeation