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Structures, properties, and digestibility of resistant starch

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ABSTRACT

This study aimed to understand effects of different treatments and modifications on the structures, properties, and digestibility of selected starches. Different lipids, including corn oil (CO), soy lecithin (SL), palmitic acid (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LA), were selected for the study, and their impacts on the properties and digestibility of normal corn (NCS), tapioca (TPS), waxy corn (WCS) and high-amylose corn (HA7) starch were investigated to elucidate mechanisms of starch-lipid interactions. After cooking with the lipids (10%, w/w, dsb), NCS, TPS and HA7 showed significant decreases in the percentage enzymatic hydrolysis, and their DSC thermograms displayed an amylose-lipid-complex dissociation peak except the one cooked with the CO. ^{13}C-NMR spectra of amylodextrin with the presence of CO showed downfield changes in the chemical shifts of carbons 1 and 4 of the anhydroglucose unit, indicating helical-complex formation. In general, free fatty acids (FFAs) reduced, but SL increased the peak viscosity of starch. FFAs and SL decreased, but CO increased the gel strength of NCS. All the lipids displayed little impacts on the digestibility and properties of WCS because it lacked amylose.

Resistant starch Type 5 (RS5) was prepared by complexing debranched HA7 with SA. Because of amylose-helical-complex formation with SA, the RS5 showed restricted swelling of starch granules at 95 °C. The RS5 displayed a larger RS-content (67.8%) than the HA7 (33.5%) and NCS (0.8%) analyzed using the AOAC Method 991.43. When the cooked RS5, HA7 and NCS were used to prepare diets for rats with 55% (w/w) starch content, RS contents of the diets were 33.7%, 15.8% and 2.6%, respectively. After feeding to the rats in Week 1, ~16% of the starch in the RS5-diet was found in the feces, substantially greater than that of the HA7-diet (~6%) and NCS-diet (0.1%). The percentage of starch not being utilized in the RS5-diet

decreased to ~5% in Week 9, which could be partially attributed to the fermentation of RS5 by gut microflora. Large proportions (68%-99%) of the SA in RS5-diet remained unabsorbed and were discharged in the rat feces.

Effects of octenyl succinic anhydride (OSA) modification of the NCS and HA7 on their digestibility were examined. After the modification with 3% and 10% OSA, RS contents of the cooked OS-NCS increased from 0.8% of the control starch to 6.8% and 13.2% (Englyst Method), respectively, whereas that of the cooked OS-HA7 decreased from 24.1% to 23.7% and 20.9%, respectively. When the cooked NCS, HA7 and OS (10%)-HA7 were used to prepare diets for rats at 55% (w/w) starch, RS contents of the diets were 1.1%, 13.2% and 14.6%, respectively. After feeding to the rats, 20%-31% of the starch in the OS (10%)-HA7-diet was not utilized *in vivo* and was found in rat feces, which was substantially larger than that of the HA7-diet (≤ 5%) and NCS-diet (≤ 0.2%).

Characteristics of starch and ethanol production of five sorghum lines (6B73, 6C21, 6C69, 7R34, and X789) were investigated and compared with that of B73 corn. Sorghum starches displayed higher gelatinization-temperatures (66.6-67.4 °C), greater gelatinization enthalpy-changes (13.0-14.0 J/g) and percentages retrogradation (60.7-69.1%), but slower enzymatic-hydrolysis rates (83.8-87.8% at 48 h) than the B73 corn starch (61.7 °C, 10.1 J/g, 51.5%, and 88.5%, respectively). These differences could result from that the sorghum amylopectins consisted of fewer short branch-chains (DP 6-12) (12.8-14.0%) than the corn amylopectin (15.0%). After 96 h fermentation, most ground sorghums exhibited lower ethanol-yields (30.5-31.8%) than the ground B73 corn (31.8%).

Structure, properties, and digestibility of starch isolated from bamboo seeds (*Bambusa textilis* species) were studied and compared with that of indica and japonica-rice starch. The

bamboo seeds also had compound starch granules, with morphology and sizes comparable to the rice starches. The amylopectin of bamboo-seed starch showed similar branch-chain-length distribution to that of the indica-rice starch, and both of them had longer branch-chains (DP = 19.1 and 19.7, respectively) than the japonica-rice amylopectin (DP = 17.1). Consequently, the bamboo-seed and indica-rice starch granules exhibited higher gelatinization-temperatures (T_o = 68.9 °C and 71.9 °C, respectively), larger enthalpy-changes (ΔH = 14.2 J/g and 15.3 J/g) and percentages of retrogradation (57.1% and 55.4%), but slower enzymatic-hydrolysis rates (50.6% and 46.3% at 24 h) than the japonica-rice starch counterpart (56.4 °C, 14.0 J/g, 11.4%, and 66.2%, respectively).

GENERAL INTRODUCTION

Starch is the major energy reserve in plants and can be found in grains, roots, tubers, stems, leaves, and fruits. Starch is present in a granular form and has different shapes and sizes depending on its botanical source. Starch consists of two major glucans: amylose and amylopectin. Amylose is an essentially linear polysaccharide with α-1, 4 linked D-glucopyranose units and a few branches of α-1, 6 linkages, whereas amylopectin is a highly-branched polysaccharide consisting of short linear-chains connected by about 5% α-1, 6 branch linkages. Starch is widely used in foods to provide various functions, including as an energy source, a gelling agent, a thickening agent, and a stabilizing agent. Starch is a major energy source for humans and animals. After being ingested, starch is hydrolyzed to glucose by amylolytic enzymes in the digestive tract. Glucose is then absorbed in the small intestine to be used for energy.

A portion of starch, known as resistant starch (RS), cannot be hydrolyzed by enzymes in the small intestine and is passed into the large intestine for fermentation by gut microflora. RS is categorized as a type of dietary fiber because it can provide similar physiological benefits to humans and animals as other dietary fibers. Health benefits of RS include: lowering postprandial plasma glucose concentration and insulin response; increasing insulin sensitivity and preventing Type-2 diabetes; improving serum lipid profile and preventing obesity; and improving colon health. RS can be classified into five different types: physically inaccessible starch (RS1); raw granular starch with the B- or C-type polymorph (RS2); retrograded starch (RS3); chemically-modified starch (RS4); and amylose-lipid complex (RS5).

When amylose forms single-helical complex with lipids, it becomes resistant to enzymatic hydrolysis. The amylose-lipid complex (ALC) also restricts the swelling of starch

granules, which further reduces the digestibility of starch. Lipids that have been shown to form helical-complex with amylose include free fatty acids (FFAs), monoglycerides, phospholipids and long-chain alcohols. However, effects of triglycerides, the major component of lipids present in foods, on the properties and digestibility of starch were not well understood. The study was conducted with the aim to examine effects of different food lipids, including triglycerides (corn oil, CO), phospholipids (soy lecithin, SL), and FFAs of different chain-lengths and numbers of double bonds, on the properties and digestibility of different starches, *i.e.*, normal corn (NCS), tapioca (TPS), waxy corn (WCS) and high-amylose corn (HA7) starch. Mechanisms of interactions between the lipids and starches were also elucidated.

A Type-5 RS (RS5), with up to 75.0% RS content (AOAC Method 991.43), has been developed by complexing debranched HA7 with FFAs. An animal-feeding study has shown that the cooked RS5 effectively inhibited the azoxymethane-induced preneoplastic lesions (colon cancer precursors) in the rat colon, suggesting that the RS5 could suppress colon carcinogenesis. *In vitro* and *in vivo* enzymatic hydrolysis of the cooked RS5 was investigated in comparison with that of the NCS and HA7 in the current study to elucidate mechanisms of the health benefits of the RS5.

Octenyl succinic anhydride (OSA) modification of starch was used to prepare RS with increased enzymatic resistance and hydrophobic property. Effects of OSA modification of the NCS and HA7 on their enzymatic-hydrolysis rates were investigated and related to the thermal and pasting properties of the modified starches. *In vivo* digestion of the OS-HA7 was also analyzed in a rat-feeding study.

In contrast, when starch is used as the feedstock to produce industrial products (*e.g.*, ethanol and high-fructose syrup), a fast rate of starch hydrolysis is desirable. Therefore, we

conducted a study to examine starch digestibility and ethanol production of five sorghum lines (6B73, 6C21, 6C69, 7R34, and X789) in comparison with that of the B73 corn and starch. The data obtained in this study can be used to predict value-added utilizations of sorghum in bio-ethanol, animal feed, and human food.

Structure, physical properties and enzymatic hydrolysis of starch isolated from bamboo seeds (*Bambusa textilis* species) were characterized and compared with that of rice starch. The study provided important information about the chemical structures and functional properties of starches produced by these two genetically-close plant species in the Poaceae family.

DISSERTATION ORGANIZATION

This dissertation is composed of five papers. The first paper, "Effects of lipids on enzymatic hydrolysis and physical properties of starch", has been published in *Carbohydrate Polymers*. The second paper, "Characterization and *in vivo* hydrolysis of amylose-stearic-acid complex", will be submitted to *Cereal Chemistry*. The third paper, "*In vitro* and *in vivo* digestion of octenyl succinic starch", has been published in *Carbohydrate Polymers*. The fourth paper, "Starch characterization and ethanol production of sorghum", has been published in the *Journal of Agricultural and Food Chemistry*. The fifth paper, "Comparison of starches from rice kernels and bamboo seeds", follows the format of *Starch – Stärke* for the submission to the journal. The five papers are preceded by a General Introduction and a Literature Review and followed by a General Conclusion and Acknowledgements. The references cited in the Literature Review are listed in the alphabetical order of the first author's last name.

CHAPTER 1. LITERATURE REVIEW

1. Structure of starch granules

Starch, one of the most abundant biomasses on earth, is the major carbohydrate for energy storage in plants. Starch can be found in grains, roots, tubers, stems, leaves, and fruits in a granular form. The morphology and structure of starch granules depend on the botanical source (Jane et al. 1994) and the development stage (Inouchi et al. 1983; Huang et al. 2006; Jiang et al. 2010c). Starch granules display many shapes, including spherical, polygonal, oval, disk, kidney and elongated shapes, and vary in size, with diameters ranging from submicron to more than 100 microns (Jane et al. 1994). Native starch granules have a semicrystalline structure and exhibit a Maltese cross observed under polarized light microscopy (Hibi et al. 1993). The semicrystalline starch granules are not soluble in water at room temperature and can be easily isolated from plant source. Thus, starch is available as one of the most economical materials in the industry.

1.1. Structures of starch molecules

Normal starch is composed of two glucans: amylose and amylopectin. Amylose is a primarily linear molecule with α-1, 4 linked D-glucose units and a few branches of α-1, 6 linkages (**Figure 1**, **A**) (Takeda et al. 1990), whereas amylopectin is a highly-branched molecule with about 5% α-1, 6 branch linkages (**Figure 1**, **B**) (Hizukuri 1986). Average molecular weights of amylose range from 8.1×10^4 to 9.7×10^5 g/mol (Jane 2004), and that of amylopectin varies between 7.0×10^7 and 5.7×10^9 g/mol for different starches (Yoo and Jane 2002). Normal starch contains around 70-80% amylopectin and 20-30% amylose; waxy starch consists of mostly amylopectin and 0-8% amylose (Hovenkamp-Hermelink et al. 1987; Nakamura et al. 1995); and high-amylose starch contains more than 40% amylose (Shi et al. 1998; Schwall et al. 2000; Li et

al. 2008). Amylose content has significant impacts on the thermal, pasting, gelling and digestive properties of starch, and they will be discussed in this chapter.

It has been proposed that the branch chains of amylopectin are organized in a cluster model, and those branch chains are designated as the A, B, and C chains (**Figure 1**, **B**) (Hizukuri 1986). The A chains carry no other chains and are attached to the B or C chains via α-1, 6 linkages. The A chains have chain length of degree of polymerization (DP) <12 and extend within one cluster. The B chains carry the A or other B chains and can be further classified into B1, B2, and B3 chains on the basis of their chain lengths: B1 with DP 13-24, B2 with DP 25-36, and B3 with DP ≥37, which extend through 1, 2, and 3 clusters, respectively (Hanashiro et al. 1996). The C chain carries the single reducing end in the amylopectin molecule.

1.2. Organization of starch molecules within granules

Amylose and amylopectin are synthesized in a radial direction in starch granules. Granule-bound starch synthase I (GBSSI) and soluble starch synthases (*e.g.*, SSI, SSII and SSIII) are responsible for the elongation of amylose and amylopectin, respectively (Smith et al. 1997). Other enzymes, including starch branching enzymes (*e.g.*, SBEI and SBEII) and starch debranching enzymes (*e.g.*, isoamylase and pullulanase), are also critical for the biosynthesis of regular amylopectin molecules (Zeeman et al. 2010; Fujita 2012). In normal starch granules, branch chains of amylopectin form double helices and contribute to the crystalline structure (Kainuma and French 1972; Sarko and Wu 1978).

The double helices of the glucan chains in starch granules are packed in two different patterns to form the A- or B-type polymorph. The A-type polymorph has a monoclinic unit cell, and the B-type polymorph has a hexagonal unit cell (**Figure 2**) (Sarko and Wu 1978; Buleon et al. 1998a). The A- and B-type polymorphs correspond to the A- and B-type X-ray diffraction

patterns of native starch granules, respectively. Some starches (*e.g.*, pea and green banana starch) display a C-type X-ray diffraction pattern, which is a combination of A- and B-type polymorphs within the granule (Bogracheva et al. 1998; Buleon et al. 1998b; Wei et al. 2010).

Structures of amylopectins from A- and B-type starch are different. The amylopectin of A-type starch has a larger proportion of short branch-chains (DP 6-12) but a smaller proportion of longer branch-chains (DP >12) than that of the B-type starch. Thus, the average branch-chain length of the amylopectin of A-type starch is shorter than that of the B-type starch (Hizukuri 1985; Jane et al. 1999). The amylopectin of C-type starch has an average branch-chain length in between that of the A- and B-type starch. Another difference in the amylopectin structure between the A- and B-type starch is that the amylopectin of the A-type starch has some branch linkages located in crystalline region but that of the B-type starch has almost all the branch linkage located in the amorphous region (**Figure 3**) (Jane et al. 1997). The differences in the amylopectin structure explain the more porous structure of the A-type starch granules than the B-type starch (Jane 2006).

For normal starch, amylose is located in the amorphous region of starch granule and interspersed among amylopectin molecules (Jane et al. 1992; Kasemsuwan and Jane 1994). The amylose molecules, particularly with the presence of lipids, can entangle with amylopectin to maintain the integrity of starch granules during heating and shearing (Jane 2006; Debet and Gidley 2007). Amylose is more concentrated at the periphery of the granule (Jane and Shen 1993; Pan and Jane 2000), which is attributed to that amylose is synthesized more actively at the later stage of starch accumulation (Morrison and Gadan 1987; Li et al. 2007).

In high-amylose corn starch with up to 90% amylose, amylose molecules also form double helices and contribute to the crystalline structure of starch granules (Jiang et al. 2010c).

The presence of long-chain double-helical crystallites of amylose in high-amylose corn starch is responsible for its high gelatinization-temperature (conclusion temperature up to 130 °C) and facilitates the formation of elongated starch granules (Jiang et al. 2010b).

2. Thermal property of starch

2.1. Gelatinization of starch

When native starch is heated in the presence of water, the granules eventually lose the semicrystalline structure and the Maltese cross disappears. This process is irreversible and known as starch gelatinization. Starch gelatinization is an endothermic transition and commonly analyzed using a differential scanning calorimeter (DSC). Starch is heated with sufficient water (at least 2× of starch weight) using the DSC to obtain consistent gelatinization temperature and enthalpy-change (Jane 2004).

The gelatinization properties of starches from different botanical sources vary and depend on the structure of amylopectin, the amylose content, and the presence of other minor components (*e.g.*, the phosphate-monoester derivatives of potato starch) in the starch. In general, starch consisting of amylopectin with longer branch-chains shows a higher gelatinization-temperature and enthalpy-change because longer branch-chains are favorable for the formation of stable double-helical crystallites (Jane et al. 1999; Srichuwong et al. 2005). Potato starch (B-type X-ray diffraction pattern) exhibits a lower gelatinization-temperature than the A-type starch (*e.g.*, normal corn and rice starch), despite the fact that the amylopectin of potato starch has much longer branch-chains (Jane et al. 1999; Cai and Shi 2010). The results can be explained by the presence of phosphate-monoester derivatives in potato amylopectin, which cause repulsion between starch molecules and destabilize the crystalline structure (Jane et al. 1999). High-

amylose starch, having amylopectin with longer branch-chains and amylose to form long-chain double-helical crystallites, usually displays a higher gelatinization-temperature than normal and waxy starch (Shi et al. 1998; Li et al. 2008; Jiang et al. 2010a).

2.2. Retrogradation of starch

When the gelatinized starch is cooled, two adjacent starch molecules tend to recrystallize to form double helices. This recyrstallization process is known as starch retrogradation (Abd Karim et al. 2000; Jane 2004). Both amylopectin and amylose molecules can retrograde. The retrogradation of amylopectin requires several days or even longer and forms crystallites with a low dissociation-temperature (40 °C-60 °C) because of the branched structure and short branch-chains of amylopectin (Abd Karim et al. 2000). On the other hand, the linear amylose molecules retrograde faster and form crystallites with a higher dissociation-temperature (130 °C-170 °C) (Sievert and Pomeranz 1989; 1990; Sievert and Wursch 1993; Jane et al. 1999).

In the DSC thermograms of retrograded waxy and normal starch, only the dissociation peak of retrograded amylopectin (dissociation temperature around 40 °C-60 °C) can be observed because the concentration of amylose is low and it is interspersed among amylopectin molecules (Sievert and Pomeranz 1990). Generally, starch having amylopectin with longer branch-chains tends to retrograde faster (Yuan et al. 1993; Jane et al. 1999). The repulsion between the negatively-charged phosphate-monoester derivates in potato starch retards starch retrogradation (Jane et al. 1999; Thygesen et al. 2003). The single-helical complex of amylose and lipids (*e.g.*, phospholipids) can restrict the swelling and prevent the dispersion of starch granules during heating, which retains starch molecules in close proximity for faster retrogradation (Jane et al. 1999). Therefore, normal corn starch shows a faster retrogradation-rate than tapioca starch because the latter has little endogenous lipids (Jacobson et al. 1997; Jane et al. 1999). The

dissociation peak of retrograded amylose (130 °C-165 °C) can be detected in some high-amylose starch (Sievert and Pomeranz 1990).

Starch retrogradation is undesirable for some food products, such as bread and pudding, because it causes staling and syneresis and, thus, shortens the shelf life of the products (Abd Karim et al. 2000; Jane 2004). Different methods, including selection of native starch with a slower retrogradation-rate (*e.g.*, waxy corn and tapioca starch), chemical modification (*e.g.*, hydroxypropylation and acetylation), and enzymatic modification (*e.g.*, controlled α-amylase hydrolysis) of starch, have been used to control starch retrogradation in starchy foods for an extended shelf-life (Miyazaki et al. 2006).

3. Pasting property of starch

When gelatinized starch is continuously heated in water with sheering, starch granules swell to develop viscosity and some starch molecules leach to the aqueous medium. The viscosity development in this process is known as the pasting property of starch. Pasting property of starch can be evaluated using an amylograph, such as Rapid Visco-Analyzer (RVA) or a Brabender Amylograph. Representative amylograms of starches from different botanical sources are shown in **Figure 4** (Debet and Gidley 2006).

In general, starch consisting of a larger amylose-content, particularly with the presence of lipids, displays a higher-pasting temperature and a lower peak-viscosity because amylose restricts the swelling of starch granules during heating and shearing (Jane 2006; Debet and Gidley 2007). Higher amylose-content is also associated with a greater setback-viscosity of starch paste, which can be attributed to the entanglement between amylose and amylopectin molecules upon cooling. The repulsion between the negatively-charged phosphate-monoester

derivates in potato starch results in remarkable swelling of the granules during heating. Consequently, potato starch exhibits the lowest pasting-temperature and the highest peak-viscosity among all the starches as shown in **Figure 4**. With a greater concentration of phospholipids to readily complex with amylose to restrict the swelling of starch granules, wheat starch shows a higher pasting-temperature and a lower peak-viscosity than the other normal starches (**Figure 4**) (Lim et al. 1994; Jane et al. 1999). Because high-amylose starch is only partially gelatinized and the granules hardly swell during heating, the starch shows very low or undetectable viscosity by RVA (**Figure 4**).

4. Gel formation of starch

After starch paste is cooled and stored for a period of time, some starch paste can develop into a starch gel. The difference between starch paste and gel lies in that starch paste has certain fluidity, whereas starch gel has a defined shape with no fluidity (Belitz et al. 2009). Gel formation is a result of the interactions between amylose and amylopectin in swollen granules to develop networks and hold water in the granules (Ott and Hester 1965). Strength of starch gel can be evaluated using a texture analyzer (Takahashi and Seib 1988; Wang et al. 1992).

Strength of starch gel generally increases with starch concentration (Whittenberger and Nutting 1948; Keetels et al. 1996a). Swollen starch granules maintaining integrity contribute to the formation of firm starch gel. It has been reported that at an equivalent concentration, amylose alone forms a much weaker gel than that from cooked starch granules (Miles et al. 1985; Ring 1985). Potato starch paste forms a substantially weaker gel than wheat starch paste because potato starch granules swell to a greater extent and lose their integrity after cooking (Keetels et al. 1996a). Cross linking and heat-moisture treatment can increase molecular entanglements of

potato starch and, thus, maintain the integrity of swollen starch granules for the formation of a stronger gel (Whittenberger and Nutting 1948; Keetels et al. 1996a). In addition, strength of starch gel increases with storage time, which is attributed to the increased stiffness of the swollen starch granules as a result of starch retrogradation (Keetels et al. 1996b).

5. Amylose-lipid complex

DSC thermograms of normal cereal starch (*e.g.*, normal corn, wheat and barley starch) show an additional endothermic peak at temperatures above 90 °C, corresponding to the dissociation of amylose-lipid complex (ALC). Amylose is known to form helical complex with lipids and other compounds (complexing agents) that possess a hydrophobic moiety. The amylose single-helical complex, also known as an inclusion complex, has the hydrophobic moiety of the complexing agent present inside the hydrophobic cavity of the helix (Putseys et al. 2010). The size of amylose-helical complex depends on the cross-section diameter of the complexing agent: six glucose units per turn for compounds with linear hydrocarbon chains (*e.g.*, free fatty acids and n-butyl alcohol) (Jane and Robyt 1984; Godet et al. 1993); seven glucose units per turn for compounds with branched chains (*e.g.*, dimethyl sulfoxide and isopropyl alcohol) (Jane and Robyt 1984; Nishiyama et al. 2010); and eight glucose units per turn for compounds with even bulkier cross-sections (*e.g.*, 1-naphthol) (Yamashit and Monobe 1971).

Lipids that have been shown to form helical-complex with amylose include free fatty acids (FFAs), monoglycerides, phospholipids, and long-chain alcohols (Putseys et al. 2010). Dissociation temperature of the ALC, in general, increases with increasing chain-length of the fatty acid, and decreases with an increasing number of double bonds (Kowblansky 1985; Tufvesson et al. 2003a; b). Among the unsaturated fatty-acids, the fatty acids with *trans*-double

bonds form ALC with a higher dissociation-temperature than that with *cis*-double bonds (Riisom et al. 1984). After heating at a temperature above the dissociation temperature, amorphous ALC (form I) can further rearrange into lamellar crystallites (form II), which display a V-type X-ray diffraction pattern and show a higher dissociation-temperature (100-125 °C) (Kowblansky 1985; Biliaderis and Galloway 1989).

The formation of ALC in starch remarkably affects its pasting, gelling and digestive properties. Compared with tapioca and waxy corn starch, normal wheat and corn starch exhibit higher pasting-temperatures and lower peak-viscosities (**Figure 4**) and they can develop into firm gel after cooking and storage. The differences result from that normal wheat and corn starch have around 1% endogenous lipids, whereas tapioca and waxy corn starch have little lipids (Debet and Gidley 2006). The lipids in normal wheat and corn starch complex with amylose to restrict the swelling of starch granules and prevent the dispersion of swollen granules (Tester and Morrison 1990; Cui and Oates 1999), which is critical for starch gel formation as discussed earlier. The ALC formation reduces enzymatic hydrolysis of starch and this will be discussed in next section of this chapter.

6. Enzymatic hydrolysis of starch

6.1. Amylolytic enzymes

Starch is hydrolyzed by amylolytic enzymes to produce glucose for energy supply in plants and animals. Major enzymes involved in the breakdown of starch molecules include α-amylase, amyloglucosidase, debranching enzyme (*e.g.*, isoamylase and pullulanase), and β-amylase (Robyt 2009). Alpha-amylase is an endo-acting enzyme that only hydrolyzes α-1, 4 linkage of starch to produce maltodextrins of various molecular sizes. Alpha-amylases from

different sources vary in the sizes of binding site and hydrolysis patterns (Robyt 2009). Amyloglucosidase is an exo-splitting enzyme that produces glucose from the non-reducing end of starch molecule. Amyloglucosidase can also hydrolyze α-1, 6 linkage but at a slower rate (Hiromi et al. 1966). Debranching enzyme only hydrolyzes α-1, 6 linkage of starch. Beta-amylase refers to a type of exo-acting enzyme that only hydrolyzes α-1, 4 linkage of starch and exclusively releases β-maltose as the product.

6.2. Enzymatic hydrolysis of raw starch

Certain amylolytic enzymes, such as porcine pancreatic α-amylase and amyloglucosidase from *Aspergillus niger*, can hydrolyze granular starch at a much faster rate than other amylolytic enzymes, such as β-amylase. Structural analyses reveal that amylolytic enzymes that can promptly hydrolyze raw starch granules possess starch-binding domain as the common feature (Christiansen et al. 2009). It has been proposed that the starch-binding domain could have the following functions to facilitate the enzyme to rapidly hydrolyze granular starch: (1) enhance the contact between the enzyme and starch granules; (2) disrupt the crystalline structure of starch granules (Christiansen et al. 2009; Guillen et al. 2010).

Many factors, including the polymorphic structure, the amylose content, and the granule size of starch, determine the enzymatic-hydrolysis rate of starch granules. In general, starch granules with the A-type crystalline structure (*e.g.*, normal corn and rice starch) are hydrolyzed faster than the B-type granules (*e.g.*, potato starch) by the enzyme, and C-type starch (*e.g.*, green banana starch) is in between (**Figure 5**) (Kimura and Robyt 1995; Jane 2003). The greater susceptibility of A-type starch could be attributed to that its amylopectin has more short branch-chains (DP 6-12) and has the branch points located in both the crystalline and amorphous regions. These structural features of the amylopectin result in less perfect crystalline structure of

A-type starch (Jane 2003; 2006). In addition, A-type starch granules have pinholes, channels and more porous internal structure (Fannon et al. 1992; Huber and BeMiller 2000; Jane 2006), which increase the susceptibility of A-type starch to enzyme attack and subsequent hydrolysis.

For starch with the same type of X-ray diffraction pattern, the enzymatic-hydrolysis rate decreases as the amylose content increases (Jane 2003; Tester et al. 2006). Amylose molecules in the granular starch intertwine and associate with amylopectin to enhance the integrity of the granules, which reduces their susceptibility to enzymatic hydrolysis (Jane 2006). Furthermore, smaller starch granules are hydrolyzed by enzyme more easily because they have a larger relative surface area for the reaction than larger ones (Tester et al. 2004).

Amylolytic enzymes also hydrolyze different granular starches following different patterns. It has been reported that most α-amylases hydrolyze A-type starch granules (*e.g.*, normal cereal starches) using an "inside-out" pathway, *i.e.*, α-amylases attack at weak points (*e.g.*, pinholes) on the granule surface, create tunnels into the granule interior, and then hydrolyze the granule from the inside out (Fuwa et al. 1979; Planchot et al. 1995). However, the proposed "inside-out" pathway is not valid for the α-amylase hydrolysis of the B- and C-type starches (*e.g.*, potato starch, sweet potato starch, green banana starch, and lotus starch). The α-amylase hydrolysis mainly takes place on the periphery of those starch granules (Fuwa et al. 1979; Planchot et al. 1995). The differences could be explained by that the B- and C-type starch granules do not possess the porous structure as the A-type starch (Fannon et al. 1992; Huber and BeMiller 2000; Jane 2006).

6.3. Enzymatic hydrolysis of gelatinized starch

When starch is gelatinized, the granules lose the crystalline structure and the molecules become amorphous. Consequently, the gelatinized starch can be easily hydrolyzed by amylolytic

enzymes. Factors affecting the enzymatic-hydrolysis rate of gelatinized starch include starch retrogradation rate, chemical derivatives of starch, the formation of ALC, and the presence of other components (*e.g.*, cellulosic material and protein). Retrograded starch becomes resistant to enzymatic hydrolysis (Jane and Robyt 1984). Chemically modified starch (*e.g.*, cross-linked and octenyl succinic starch) is also less hydrolysable (Shin et al. 2004; He et al. 2008). Single-helical complex formation with lipids and the further development of lamellar crystallites protect amylose from enzymatic hydrolysis (Jane and Robyt 1984; Seneviratne and Biliaderis 1991). Additionally, the ALC restricts the swelling of starch granules to further reduce the susceptibility of starch molecules to amylolysis (Tester and Morrison 1990; Cui and Oates 1999; Lauro et al. 2000). Surrounding of starch by cellulosic materials or protein matrix also makes it less susceptible to the enzymatic hydrolysis (Zhang and Hamaker 1998; Ezeogu et al. 2005).

Depending on the objectives, research has focused on increasing or decreasing enzymatic-hydrolysis rate of starch. When the goal is to hydrolyze starch for the production of other chemicals (*e.g.*, ethanol and high-fructose syrup), a fast rate of starch hydrolysis is desirable. Various methods, such as selecting right types of enzyme and starch, preventing starch retrogradation, and decreasing starch-protein interactions, have been applied to increase starch-hydrolysis rate for improved yield and efficiency (Crabb and Mitchinson 1997; Ezeji et al. 2005; Zhao et al. 2008). On the other hand, over-consumption of starchy foods with fast starch-hydrolysis rates has been criticized for causing metabolic syndrome, including Type-2 diabetes, obesity, and cardiovascular diseases (Ludwig 2002; Willett et al. 2002). As a result, in the past two decades research has concentrated on the development and food applications of resistant starch to improve human health.

7. Resistant starch

7.1. Definition and classification of resistant starch

Resistant starch (RS) refers to a portion of starch that is resistant to enzymatic hydrolysis in the small intestine and passed into the colon to be fermented by gut microflora (Englyst et al. 1982; Englyst and Macfarlane 1986). According to the structure and property, RS can be classified into five types:

(1) RS1: physically inaccessible starch

RS1 refers to starch entrapped in protein matrix, cell-wall materials, and other physical barriers that reduce the accessibility of starch to amylolysis (Rooney and Pflugfelder 1986; Zhang and Hamaker 1998; Ezeogu et al. 2005). A good example of RS1 is the RS in pasta, which has been shown to result in a lower postprandial plasma-glucose response in human subjects than the control bread made with the same ingredients (Granfeldt and Bjorck 1991). The compact protein matrix of pasta has been proved to be responsible for its higher RS-content than other wheat products (Fardet et al. 1998; Kim et al. 2008). Treatments that can destroy the physical barriers in foods (*e.g.*, grinding and protease hydrolysis) reduce the RS1 content (Colonna et al. 1990; Fardet et al. 1998).

(2) RS2: raw granular starch with the B- or C-type polymorph

Raw starch with the B- or C-type polymorph are more resistant to enzymatic hydrolysis than the A-type starch as discussed earlier (Kimura and Robyt 1995; Jane 2003). A disadvantage of RS2 for food applications is that its RS content decreases substantially after thermal processing (Bornet et al. 1989; Kishida et al. 2001). With the presence of long-chain double-helical crystallites, high-amylose starch can have a conclusion gelatinization-temperature up to 130 °C. The starch retains its semicrystalline structure after cooking and has a large RS-content

(up to 40%) (Jiang et al. 2010a). Consequently, high-amylose starch is a good source of RS2 for various food products (Hoebler et al. 1999; Li et al. 2008; Hasjim et al. 2010). Hydrothermal treatments, such as heat-moisture treatment and annealing, can be used to further elevate the RS content of high-amylose starch (Liu et al. 2007).

(3) RS3: retrograded starch

During retrogradation, two adjacent starch molecules recrystallize to form double helices, and the structure becomes more resistant to enzymatic hydrolysis (Jane and Robyt 1984; Sievert and Pomeranz 1989; 1990). Linear amylose molecules are preferred for the preparation of RS3 because they retrograde at a faster rate and form more resistant crystallites than amylopectin (Sievert and Pomeranz 1989; 1990; Eerlingen and Delcour 1995). Debranching, partial acid-hydrolysis, and freeze/thaw cycle enhance the retrogradation of starch and, thus, can be used to increase the content of RS3 (Sievert and Pomeranz 1989; Chiu et al. 1994; Lehmann et al. 2002; Thompson and Brumovsky 2002; Chung et al. 2003; Lehmann et al. 2003; Hasjim and Jane 2009).

(4) RS4: chemically-modified starch

Chemical modification of starch can be used to produce RS. Octenyl succinic anhydride (OSA) modification of starch introduces hydrophobic OS groups to the starch molecules, which interferes with the binding of starch by amylolytic enzymes (Zhang and Hamaker 1998; Han and BeMiller 2007; He et al. 2008). Cross-linking of starch is another effective method to prepare RS4 (Woo and Seib 2002; Xie et al. 2006; Al-Tamimi et al. 2010). Excessive cross-linking of starch increases its gelatinization temperature and restricts the swelling of granules, which enhance the enzymatic resistance of starch (Woo and Seib 2002; Xie et al. 2006).

(5) RS5: amylose-lipid complex

The single-helical complex formation between amylose and lipids has also been used to develop RS (Gelders et al. 2005; Hasjim et al. 2010). A RS5 product, containing up to 75% RS (AOAC Method 991.43), has been prepared by complexing partially-debranched high-amylose corn starch with FFAs. *In vivo* resistance of the RS5 has been demonstrated by a human-feeding study (Hasjim et al. 2010).

7.2. Health benefits of RS

RS has been included in the definition of dietary fiber given by the American Association of Cereal Chemists International and the National Academy of Sciences because it provides similar physiological benefits as other dietary fibers (Sajilata et al. 2006). Health benefits of RS have been examined extensively in human and animal subjects. Health benefits of RS include:

(1) Lowering postprandial plasma-glucose and insulin responses

Effects of different types of RS on the postprandial plasma-glucose and insulin responses of human subjects have been investigated. Studies have shown that ingestion of pasta (RS1) (Granfeldt and Bjorck 1991), high-amylose corn starch (RS2) (Behall et al. 2006), retrograded starch (RS3) (Reader et al. 1997), cross-linked starch (RS4) (Al-Tamimi et al. 2010), octenyl succinic starch (RS4) (He et al. 2008), and FFA-complexed high-amylose corn starch (RS5) (Hasjim et al. 2010) results in significantly lower postprandial plasma-glucose and/or insulin responses compared with their respective control starch. Consequently, consumption of food with a high RS-content can be used to control the postprandial blood-glucose and insulin levels of humans, and this is particularly meaningful for those with diabetes.

(2) Increasing insulin sensitivity and preventing Type-2 diabetes

Human-feeding studies have provided evidence that the intake of RS enhances the insulin sensitivity of human subjects (Robertson et al. 2003; Johnston et al. 2010; Maki et al. 2012).

Because decreased insulin sensitivity is associated with metabolic syndrome, including Type-2 diabetes and cardiovascular diseases (Caballero 2003), consumption of RS-rich food can be an effective approach for the prevention and intervention of those health problems.

(3) Improving serum lipid profile and preventing obesity

Research has demonstrated that the consumption of RS decreases the total serum-triglyceride and cholesterol levels *in vivo* (Dedeckere et al. 1993; Cheng and Lai 2000). It has also been shown that the intake of RS promotes postprandial lipid oxidation (Higgins et al. 2004; Shimotoyodome et al. 2010). Thus, long-term consumption of RS can reduce lipid accumulation and eventually prevent obesity.

(4) Improving colon health

Research has provided evidence that RS reaching the large intestine can be fermented by gut microflora to generate short-chain fatty acids (SCFA), including acetic acid, propionic acid and butyric acid (Martin et al. 1998; Ferguson et al. 2000; Zhao et al. 2011). In addition, intake of RS increases the fecal bulk, which can have a dilution effect on potential carcinogens and reduce their exposure to the colon (Phillips et al. 1995; Hylla et al. 1998; Ferguson et al. 2000). These physiological effects of RS have been related to its improvement on colon health and prevention of colon cancer (Topping and Clifton 2001; Le Leu et al. 2007; Zhao et al. 2011).

Compared with other dietary fibers, RS has certain advantages for food applications. For instance, RS has a small particle size, bland flavor, and white appearance, making it desirable for a variety of food products. RS has been incorporated in bakery goods, pasta products, and extruded cereals and snacks to increase the dietary-fiber contents and improve the quality of the final products (Fuentes-Zaragoza et al. 2010). More research is needed to expand the application spectrum of different types of RS in the food industry to improve the health of people.

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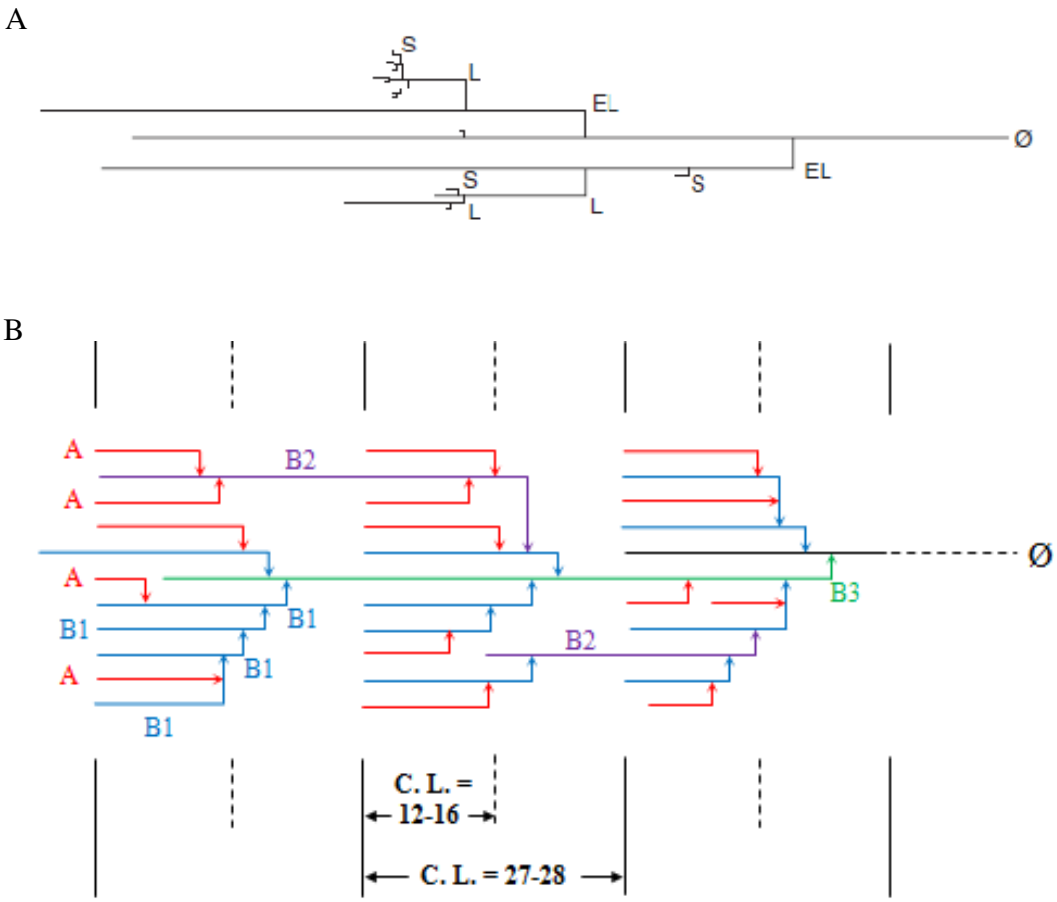

Figure 1. (A) Proposed structure of the branched amylose molecule comprising immature clusters. EL, extremely long; L, long; and S, short chains; Ø , reducing end (Takeda et al. 1990). (B) A proposed cluster model of amylopectin with A (—), B1 (—), B2 (—), and B3 (—) chains. The chain carrying the reducing end (Ø) is the C chain. —, α-1, 4-glucan chain; →, α-1, 6 linkage (Hizukuri 1986).

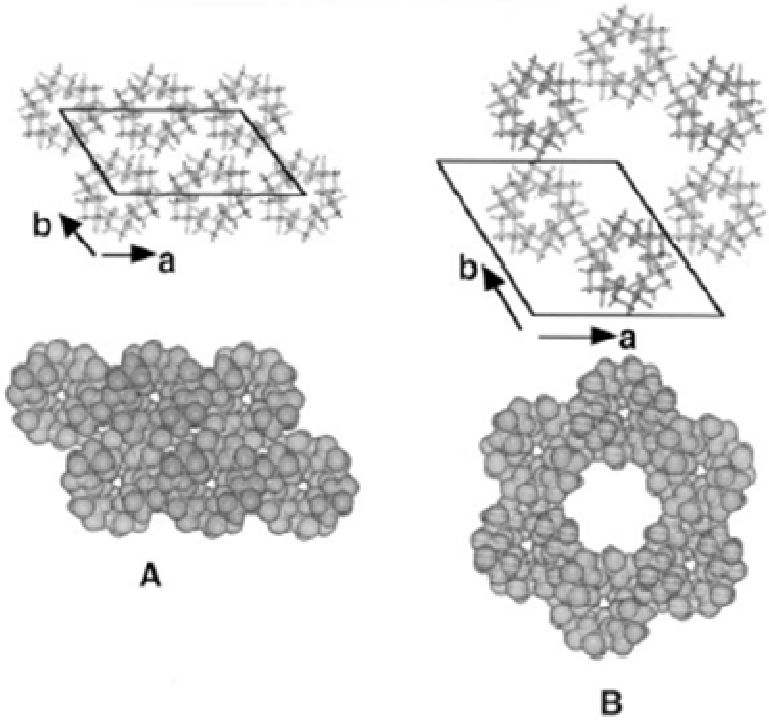

Figure 2. Crystalline packing of double helices in A-type and B-type polymorph (Buleon et al. 1998a).

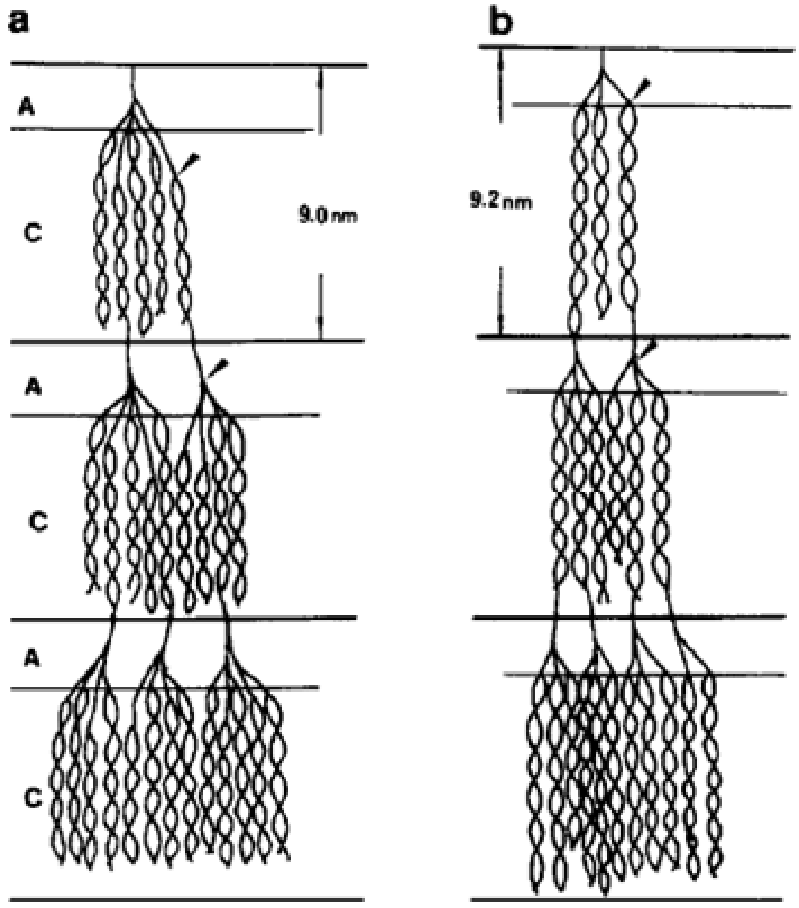

Figure 3. Proposed models for branching patterns of amylopectin of waxy corn (a, A-type) and potato (b, B-type) starch (Jane et al. 1997). 'A' and 'C' stand for the amorphous and crystalline regions, respectively; 9.0 nm and 9.2 nm are the repeating distances of waxy corn and potato starches, respectively. The chain length between the arrows stands for the internal long B chain.

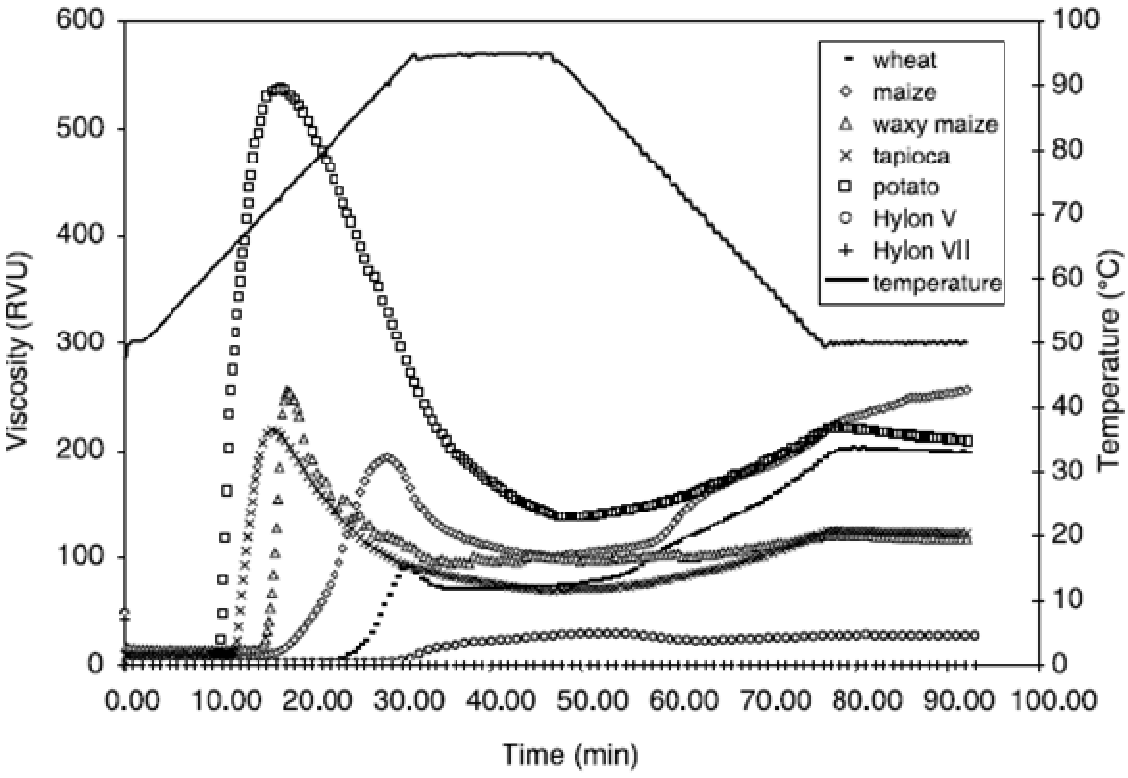

Figure 4. Pasting profiles of wheat, corn ("maize"), waxy corn ("waxy maize"), tapioca, potato and high-amylose corn ("Hylon V" and "Hylon VII") starches analyzed using a Rapid Visco-Analyzer with 10% (w/w) starch suspension (Debet and Gidley 2006).

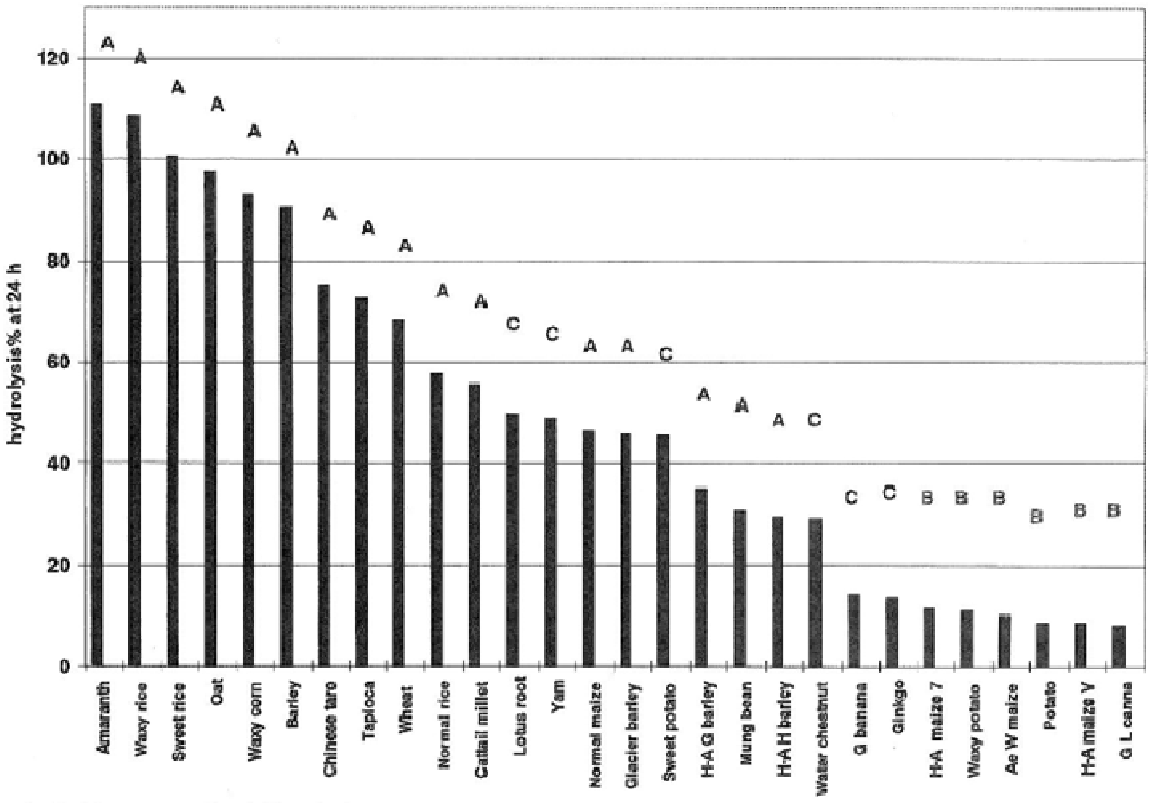

Figure 5. Relative enzyme digestibility of selected granular starches of different crystalline structures (Jane 2003). The letter above each bar indicates the type of crystallinity of the starch. Porcine pancreatic α-amylase (120 U/20 mg starch in 9 mL phosphate buffer, pH 6.9) was used to hydrolyze starch at 37 °C for 24 h.

CHAPTER 2. EFFECTS OF LIPIDS ON ENZYMATIC HYDROLYSIS AND PHYSICAL PROPERTIES OF STARCH

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Abstract

This study aimed to understand effects of lipids, including corn oil (CO), soy lecithin (SL), palmitic acid (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LA), on the enzymatic hydrolysis and physical properties of normal corn (NCS), tapioca (TPS), waxy corn (WCS) and high-amylose corn (HA7) starch, and to elucidate mechanisms of interactions between the starches and lipids. After cooking with the lipids (10%, w/w, dsb), NCS, TPS and HA7 showed significant decreases in enzymatic hydrolysis, and their DSC thermograms displayed amylose-lipid-complex dissociation peaks except with the CO. ^{13}C-NMR spectra of amylodextrin with CO showed downfield changes in the chemical shifts of carbons 1 and 4 of the anhydroglucose unit, indicating helical complex formation. Generally, free fatty acids (FFAs) reduced, but SL increased the peak viscosities of starches. FFAs and SL decreased, but CO

increased the gel strength of NCS. These lipids displayed little impacts on the enzymatic hydrolysis and physical properties of WCS because it lacked amylose.

Key words: starch enzymatic hydrolysis; starch pasting property; starch gel strength; amylose-lipid complex

1. Introduction

Starch and lipids are major components of foods, and they play important roles in the caloric density, texture, and flavor of foods. Starch consists of two major components: amylose and amylopectin. Amylose is an essentially linear molecule with α-1, 4 linked D-glucose units and a few branches of α-1, 6 linkages, whereas amylopectin is a highly branched molecule with about 5% α-1, 6 linkages (Hizukuri et al., 1981). Lipids are broadly defined as a group of compounds that are soluble in organic solvents. They can be further divided into three groups: simple lipids, such as monoglycerides, diglycerides and triglycerides; compound lipids, such as phospholipids; and derived lipids, such as free fatty acids (FFAs) and long-chain alcohols (Duncan, 2000; McClements and Decker, 2008).

It is well known that amylose forms single-helical complexes with lipids and other guest compounds, such as iodine to give a blue color (Putseys et al., 2010). Structures and physical properties of the amylose-helical complexes vary with the structures of the guest compounds. Studies have shown that compounds with linear hydrocarbon chains (*e.g.*, *n*-butyl alcohol and FFAs) form amylose-helical complexes with 6 glucose-units per turn (Rappenecker and Zugenmaier, 1981; Jane and Robyt, 1984; Godet et al., 1993), compounds with branched chains (*e.g.*, isopropyl alcohol and dimethyl sulfoxide) form amylose-helical complexes with 7 glucose-

units per turn (Simpson et al., 1972; Jane and Robyt, 1984; Nishiyama et al., 2010), and compounds with even bulkier cross-sections (*e.g.*, 1-naphthol) form amylose-helical complexes with 8 glucose-units per turn (Yamashita and Monobe, 1971). Lipids that have been reported to form helical complexes with amylose include FFAs (Raphaelides and Karkalas, 1988; Fanta et al., 1999; Tufvesson et al., 2003 b), monoglycerides (Krog, 1971; Tufvesson and Eliasson, 2000; Tufvesson et al., 2003 a), and alcohols (Jane and Robyt, 1984; Kowblansky, 1985). Dissociation temperatures of amylose-lipid complexes (ALC), in general, increase with increasing length of hydrocarbon chains of the lipids, and decrease with increasing number of double bonds in the hydrocarbon chains (Kowblansky, 1985; Eliasson and Krog, 1985; Raphaelides and Karkalas, 1988; Karkalas et al., 1995; Tufvesson et al., 2003 a, b). After heating at a temperature above the dissociation temperature, amorphous ALC (form I) can further rearrange into lamellar crystallites (form II) (Kowblansky, 1985; Biliaderis and Galloway, 1989). The complex formation between amylose and lipids has been used to prepare starch products with improved properties for food application (Yuan, 2000; Yuan, 2001; Morgan, 2003). In addition, after it is complexed with lipids and other compounds, amylose shows resistance to amylase hydrolysis (Jane and Robyt, 1984; Seneviratne and Biliaderis, 1991).

As a major energy source for humans and animals, starch is hydrolyzed to glucose by amylolytic enzymes present in the gastrointestinal tract. Glucose is then absorbed in the small intestine and increases the blood glucose concentration. The starch digestive rate of starchy foods is expressed as glycemic index (Jenkins et al., 1981). Because of the growing population suffering with insulin resistance, diabetes, overweight, obesity and other related metabolic syndromes, there are increasing demands for starchy foods that have reduced glycemic-index (Livesey et al., 2008; Barclay et al., 2008). A novel Type 5 resistant starch (RS5) has been

developed via processing high-amylose corn starch with FFAs. Compared with control bread made with wheat flour, ingestion of bread with palmitic-acid-complexed RS5 resulted in substantially less postprandial plasma-glucose and insulin responses in human subjects (Hasjim et al., 2010). Drum-drying has also been used to enhance the ALC formation in wheat flour to reduce its *in vivo* starch digestive rate (Björck et al., 1984).

How food lipids of different structures, such as triglycerides, lecithins and FFAs, affect enzymatic hydrolysis and physical properties of starches with different structures are not well understood. Triglyceride is the major component of lipids present in foods. Lecithin, a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol present in soybeans and egg yolk, is commonly used as an emulsifier in foods. Palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1) and linoleic acid (LA, C18:2) are the most common FFAs found in food lipids. The saturated FFAs (*e.g.*, C16:0 and C18:0) have straight-chain structures, whereas the unsaturated FFAs with double bonds (*e.g.*, C18:1 and C18:2) have bent-chain structures. Objectives of this study were to examine effects of different food lipids, including triglycerides (corn oil, CO), phospholipids (soy lecithin, SL), and FFAs of different chain-lengths and numbers of double bonds, on the enzymatic hydrolysis, pasting properties and gel formation of starches of different structures, *i.e.*, normal corn (NCS), tapioca (TPS), waxy corn (WCS) and high-amylose corn (HA7) starch. We also elucidated mechanisms of interactions between the lipids and starches.

2. Materials and methods

2.1. Materials

Normal corn starch (NCS, Cargill Gel ™) and high-amylose corn starch (HA7, AmyloGel ™) were purchased from Cargill Inc. (Minneapolis, MN). Tapioca starch (TPS) was a gift from Miwon Vietnam Co., Ltd. (Viet Tri City, Vietnam). Waxy corn starch (WCS) was a gift from Daesang Co. (Seoul, South Korea). Corn oil (CO) was purchased from a local grocery store. Soy lecithin (SL) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Palmitic acid (PA), stearic acid (SA) and porcine pancreatic α-amylase (PPA, Type VI-B, 21.6 units/mg solid) were purchased from Sigma Chemical Co. (St. Louis, MO). Oleic acid (OA) and linoleic acid (LA) were purchased from Acros Organics (Geel, Belgium). Amyloglucosidase of *Aspergillus niger* (200 U/mL) and D-Glucose Assay Kit were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Amylodextrin (average DP 25) was prepared following the method of Jane et al. (1985).

2.2. Amylose content of starch

The amylose content of starch was determined by measuring the iodine affinity of defatted starch using a potentiometric autotitrator (702 SM Titrino, Metrohm, Herisau, Switzerland) (Song and Jane, 2000). The amylose content of the starch was calculated by dividing the iodine affinity by 0.2 (Takeda et al., 1987). The analysis was done in duplicate.

2.3. Thermal properties of starch and effects of adding lipids

Native starch (~6 mg, dsb) with deionized water (3X, w/w, dsb) was scanned from 10 °C to 150 °C at a rate of 10 °C/min in a sealed stainless steel pan using a differential scanning calorimeter (DSC, Diamond, Perkin-Elmer, Norwalk, CT) (Li et al., 2008). Effects of adding lipids to starch on its thermal properties were analyzed by scanning the starch that was

thoroughly mixed with each lipid (10%, w/w, dsb) using the same procedure. The analysis was done in duplicate.

2.4. Preparation of cooked starch with or without added lipids

Each starch (4.0 g, dsb) with or without 10% added lipids (0.4 g) was cooked with deionized water (3X, w/w, dsb) in a boiling water-bath (~95 °C) for 8 min under constant manual stirring to fully gelatinize starch.

2.5. Content of the amylose-lipid complex (ALC) of the cooked starch with or without added lipids

The content of ALC of the starch sample that was cooked with or without 10% (w/w, dsb) added lipids (described in Section 2.4.) was analyzed using the DSC. The cooked starch samples with or without added lipids were dried at 45 °C in a convection oven, and then ground into powder using a Coffee Grinder (ID S77, Sunbeam Products Inc., Boca Raton, FL). The ground sample (~6 mg, dsb) was heated using the DSC, following the same procedure as described in Section 2.3. (first scan), cooled to 10 °C at 40 °C/min, and then heated again to 150 °C at 10 °C/min (rescan) to confirm the dissociation peak of ALC (Hasjim et al., 2010). The thermal transition was analyzed using a Pyris Software (Perkin-Elmer, Norwalk, CT), and the enthalpy change was calculated on the dry starch basis. The analysis was done in duplicate.

2.6. Enzymatic hydrolysis of the cooked starch with or without added lipids

The cooked starch sample (described in Section 2.4.) containing 300 mg starch (db) was weighed and dispersed in a phosphate buffer solution (15.0 mL, 0.1 M, pH 6.9, containing 0.25 mM calcium chloride) using a homogenizer (T25 Digital Ultra-Turrax® Homogenizer, IKA® Works Inc., Wilmington, NC) at 10,000 rpm for 20 sec. The dispersion was pre-incubated in a shaker water-bath (37 °C and 80 rpm) for 30 min. PPA (32 units) in the same phosphate buffer

solution (5.0 mL) was then added to the starch dispersion to start the starch hydrolysis. An aliquot (0.4 mL) of the hydrolysate was withdrawn at time intervals of 10, 20, 30, 60, 90 and 120 min, and mixed with 0.6 mL 100% ethanol to stop the enzyme reaction. After centrifuging at 5,200 *g* for 5 min, the supernatant was collected, and soluble sugars in the supernatant were hydrolyzed to glucose and quantified using the GOPOD method (Setiawan et al., 2010). The percentage of starch hydrolysis was calculated using the equation: % starch hydrolysis = 100 × total mass of glucose released / initial dry mass of starch × (162 / 180). The analysis was done in duplicate.

2.7. Pasting properties of starch with or without added lipids

Each starch (2.24 g, dsb), alone or thoroughly mixed with 10% of each lipid (0.224 g), was suspended in deionized water to make a total weight of 28.0 g (8% dry starch, w/w) and then analyzed using a Rapid Visco-Analyzer (RVA, Newport Scientific, Sydney, Australia) following the program reported by Ai et al. (2011). The analysis was done in duplicate.

2.8. Gel strength of starch gel with or without added lipids

Each starch (9.60 g, dsb), alone or thoroughly mixed with 10% of each lipid (0.96 g), was suspended in deionized water to make a total weight of 120.0 g (8% dry starch, w/w) and then cooked under constant stirring (250 rpm) using a Micro Visco-Amylograph (C.W. Bradender Instruments, South Hackensack, NJ) from 30 °C to 95 °C at a rate of 5 °C/min and held at 95 °C for 5 min. The hot paste was poured into a petridish-shaped container (internal diameter = 45.0 mm, height = 25.0 mm). The height of the container was extended (5 mm) by taping a piece of aluminum foil around the outside wall (Takahashi and Seib, 1988). The sample was covered with a lid and stored at 4 °C for 72 h. A fresh surface of the starch gel was obtained right before the gel-strength analysis by removing the excess gel above the rim of the container using a wire

cheese cutter. The gel strength of the starch gel was analyzed using a Texture Analyzer TA-XT2i (Texture Technologies, Scarsdale, NY) with Probe TA-11 (diameter = 25.4 mm) at a test speed 0.2 mm/sec and a compression depth 4.0 mm. The peak force at 4.0 mm compression was defined as the gel strength. The analysis was done in five replicates.

2.9. Detection of ALC formation using ^{13}C-nuclear magnetic resonance (NMR) spectroscopy

^{13}C-NMR spectra of amylodextrin (average DP 25) in an aqueous solution (50 mg/mL) with or without the addition of CO, SL or OA (25%, w/w, dry amylodextrin basis) were obtained after acquiring 2,500 scans at 25 °C following the method of Jane et al. (1985). The changes in chemical shifts of carbons of the anhydroglucose unit were obtained using the equation: chemical shift change = chemical shift of amylodextrin with lipid - chemical shift of amylodextrin control.

2.10. Statistical Analysis

Statistical significance was analyzed using one-way ANOVA and multiple comparison test with Tukey's adjustment at p value <0.05. Correlations between the thermal properties of ALC and their effects on the enzymatic hydrolysis and physical properties of starches were analyzed using the Pearson correlation test. The statistical analyses were conducted in SAS (Version 9.2, SAS Institute, Inc., Cary, NC).

3. Results and discussion

Amylose contents and thermal properties of the NCS, TPS, WCS and HA7 are shown in **Table 1**. The HA7 had the largest amylose content (68.4%), followed by the NCS (34.3%), TPS (29.0%) and WCS (1.9%). The HA7 displayed the highest conclusion gelatinization temperature (109.3 °C), which was above the temperature used for starch cooking (95 °C), whereas the others

were similar, between 80.2 °C and 81.9 °C. The DSC results showed that gelatinization temperatures and enthalpy changes (ΔH) of all the starches were not substantially affected by physically mixing with most of the lipids.

The thermogram of the NCS displayed an ALC dissociation peak (T_p =101.3 °C), which was separate from the starch gelatinization peak. The HA7, however, showed an ALC dissociation peak as the second peak (T_{p2} = 100.2 °C) (Jiang et al., 2010 a), which partially overlapped with the starch gelatinization peak (T_{p1} = 75.7 °C) (**Table 1**). There was no ALC dissociation peak for the TPS and WCS because of lacking endogenous lipids and little amylose present in the WCS (Swinkels, 1985; Lim et al., 1994; Kasemsuwan and Jane, 1996; Debet and Gidley, 2006). With the addition of FFAs (PA, SA, OA and LA) to the starch, ΔH of the ALC dissociation peak of the NCS increased, indicating increases in ALC. For the TPS, OA was the only added lipid to produce an ALC dissociation peak (ΔH = 0.6 J/g). This feature could be attributed to that OA was in a liquid form at room temperature (melting temperature = 13.3 °C) and it was an effective complexing agent for having only one double bond in the hydrocarbon chain. The liquid form of OA facilitated its penetration into starch granules after mixing with TPS, and, thus, OA could readily complex with amylose in the granule when the starch was gelatinized.

After the addition of SL, the peak temperature (T_p) and ΔH of the ALC dissociation peak of NCS decreased from 101.3 °C to 94.5 °C and from 0.9 J/g to 0.1 J/g, respectively, and the T_{p2} of HA7 decreased from 100.2 °C to 93.0 °C (**Table 1**) and the peak became smaller, indicating a decrease in ALC. These features could be attributed to that SL, having an amphiphilic characteristic, functioned as a detergent to remove endogenous lipids from the NCS and HA7

granules and, thus, resulted in reduced ΔH. The lower dissociation-temperatures corresponded to amylose-SL complex, which will be discussed later.

DSC thermograms of starch samples that were pre-cooked with lipids displayed no starch gelatinization peak, indicating complete gelatinization of starch (except HA7), but showed prominent ALC dissociation peaks, except for the WCS (**Table 2**). All the samples were rescanned to confirm the presence of ALC (Hasjim et al., 2010). Compared with the respective control, ΔH of the ALC dissociation peak increased in the NCS, TPS and HA7 after cooking with SL and FFAs. For the FFAs, the amylose-SA (C18:0) complex displayed the highest dissociation-temperature, followed by PA (C16:0), OA (C18:1) and LA (C18:2) (**Table 2**). The different ALC dissociation temperatures were attributed to that SA (C18:0), with a straight and the longest hydrocarbon chain, had the strongest interaction with the hydrophobic cavity of the amylose helix, whereas PA with a straight but shorter hydrocarbon chain, OA with one double bond, and LA with two double bonds had weaker interactions with amylose.

Although the SL had an ability to remove endogenous lipids from the starch granules when physically mixed with starch (**Table 1**), it also formed complexes with amylose after cooking with the NCS, TPS and HA7 as shown by the increases in ΔH of the ALC dissociation peaks (**Table 2**). The amylose-SL complex had dissociation temperatures substantially lower than the control but similar to the amylose-LA complex. The low dissociation-temperatures of amylose-SL complex could be attributed to steric hindrance caused by the two fatty acids on the SL molecule for the complex formation. Compared with that of physically-mixed starch and lipids (**Table 1**), the substantially larger ALC contents of the starch samples previously cooked with lipids (**Table 2**) could result from better mixing and longer reaction time during cooking and subsequent drying. There was, however, no increase in ΔH of the ALC dissociation peak

after starch was cooked with CO. Because of little amylose in the WCS, there was no ALC dissociation peak for the WCS with or without added lipids (**Table 1** and **2**).

Enzymatic-hydrolysis rates of starches previously cooked with or without added lipids are shown in **Figure 1**. After incubation with PPA for 120 min, the percentage hydrolysis of cooked TPS alone was 77.5%, followed by WCS (76.3%), NCS (72.0%) and HA7 (35.0%). The very low enzymatic-hydrolysis of cooked HA7 was a result of the starch not being fully gelatinized because of its very high gelatinization temperature (T_c = 109.3 °C, **Table 1**). After cooking with lipids, the TPS showed significant decreases ($p < 0.05$) in the percentage enzymatic-hydrolysis at different time intervals. The percentage hydrolysis of the cooked TPS at 120 min was reduced from 77.5% to 65.5% with the addition of SA, followed by PA (66.1%), CO (66.4%), OA (66.8%), SL (69.5%) and LA (72.0%) (**Figure 1**). Enzymatic-hydrolysis rates of the NCS and HA7 decreased in similar orders with the addition of those lipids, but the ranges of reductions were smaller than that of the TPS.

The reductions in enzymatic-hydrolysis rates of the NCS, TPS and HA7 after cooking with SA, PA, OA, SL and LA could be attributed to complex formation between amylose and the lipids as shown in **Table 2**. The amylose-helical complex is known to be resistant to amylase hydrolysis (Jane and Robyt, 1984; Seneviratne and Biliaderis, 1991). The ALC also restricted the swelling of starch granules, which further reduced the starch hydrolysis (Tester and Morrison, 1990; Cui and Oates 1999; Hasjim et al., 2010). The decreases in percentages enzymatic-hydrolysis (at 120 min) of the NCS, TPS and HA7 after cooking with the SL and FFAs positively correlated with T_o of ALC as shown in **Table 2** (r = 0.88, p = 0.047; r = 0.94, p = 0.02; r = 0.85, p = 0.071 for the NCS, TPS and HA7, respectively). These results were consistent with previously reported data showing that ALC with higher dissociation-temperatures were more

resistant to amylase hydrolysis (Eliasson and Krog, 1985). Although the DSC results showed no ALC formation after the starches were cooked with CO (**Table 2**), enzymatic hydrolyses of NCS, TPS and HA7 were significantly reduced (p <0.05) by CO (**Figure 1**). The enzymatic-hydrolysis rate of WCS was not affected by the addition of those lipids (**Figure 1**), which correlated well with the results of no ALC dissociation peak detected after cooking the WCS with any of the lipids (**Table 2**).

Pasting properties of starch heated with or without 10% (w/w, dsb) added lipids are shown in **Figure 2**. The NCS displayed a higher pasting-temperature (80.1 °C) but lower peak- and breakdown-viscosities (185.7 RVU and 61.6 RVU, respectively) than the TPS (69.1 °C, 269.8 RVU and 172.7 RVU, respectively) and WCS (70.0 °C, 277.3 RVU and 177.6 RVU, respectively). The differences were attributed to the lack of endogenous lipids in the TPS and WCS (Swinkels, 1985; Lim et al., 1994; Kasemsuwan and Jane, 1996; Debet and Gidley, 2006) and little amylose in the WCS. The ALC present in the NCS granules restricted the swelling of starch granules (Swinkels, 1985; Tester and Morrison, 1990; Debet and Gidley, 2006) and resulted in its higher pasting-temperature and lower peak-viscosity. Without endogenous lipids and ALC, the TPS and WCS granules swelled freely during cooking and eventually dispersed. Thus, the TPS and WCS displayed large breakdown-viscosities but small setback-viscosities. The less swelling of the cooked NCS could result in its slower enzymatic-hydrolysis rate (72.0 % at 120 min) than the cooked TPS (77.5%) and WCS (76.3%) (**Figure 1**).

With the addition of SL, the NCS displayed a decrease in the pasting temperature, from 80.1 °C to 72.3 °C, and an increase in the peak viscosity, from 185.7 RVU to 231.0 RVU, and the HA7 displayed detectable viscosity (final viscosity = 26.9 RVU) (**Figure 2**). These results were consistent with the mechanism that the SL removed the endogenous lipids from the NCS

and HA7 as evidenced by the reduction in ΔH of the ALC dissociation peak (**Table 1**). The results also agreed with that the removal of endogenous lipids from starch using detergents facilitated the swelling of starch granules during heating (Debet and Gidley, 2006). With the addition of SL, the NCS displayed a pasting temperature and peak viscosity close to the TPS (**Figure 2**).

The addition of FFAs, however, increased the pasting temperature of NCS and decreased its peak viscosity (except for LA), resulting from ALC formation (**Table 1**). The addition of CO slightly decreased the pasting temperature of NCS and decreased the peak viscosity from 185.7 RVU to 161.3 RVU. The PA, LA, SL and OA increased the final viscosity of NCS from 202.0 RVU to 420.9 RVU, 279.6 RVU, 253.5 RVU and 252.1 RVU, respectively. The onset of a remarkably higher final-viscosity of the NCS at around 65 °C with the presence of PA could be related to the melting temperature of PA (62.1 °C). The amylose-PA complex in the swollen granule and the free PA could solidify and become rigid at around that temperature, and therefore drastically increased the final viscosity of the NCS-PA mixture.

In contrast to the NCS, the TPS showed no difference in its pasting temperature with the presence of lipids. The differences could be attributed to that the TPS granules lacked endogenous lipids and swelled promptly to reach its peak viscosity and then dispersed. At the temperature above the pasting temperature (69.1 °C), amylose of TPS could complex with the added lipids in the aqueous suspension. The complex formation with FFAs decreased the peak viscosity of TPS, resulting from restricted swelling. The SL slightly increased the peak viscosity of TPS, but CO decreased it. The PA slightly increased the final viscosity of TPS, suggesting little amylose-PA complex present in swollen granules. Addition of lipids showed less impacts on the pasting properties of WCS (**Table 1**).

Results of starch-gel strength with or without added lipids are shown in **Table 3**. Without added lipids, the NCS was the only starch to form a gel at 8% (w/w, dsb) concentration after storage at 4 °C for 72 h. Gel formation is a result of the interaction between amylose and amylopectin in the granule to develop networks and hold water in swollen granules (Ott and Hester, 1965). The swollen starch-granules maintaining integrity greatly contribute to the gel strength of starch (Ring, 1985; Miles et al., 1985). The ALC present in the native NCS interacted with amylopectin to form networks, held water, and maintained integrity of swollen starch-granules and, therefore, facilitated its gel formation. The native TPS and WCS granules did not have ALC (**Table 1**) and, thus, were easily dispersed during cooking (**Figure 2**) and could not form a gel.

The gel strength of NCS increased with the addition of CO, but decreased with the addition of other lipids (**Table 3**). As the results in **Table 2** suggested, after cooking the NCS with the SL and FFAs, there were increasing ALC formed. ALC are known to develop lamellar crystallites at a high concentration and lose water-binding and gel-forming ability (Takahashi and Seib, 1988; Putseys et al., 2010). The decreases in the gel strength of NCS with the addition of SL and FFAs positively correlated ($r = 0.92$, $p = 0.03$) with T_o of the ALC dissociation peaks shown in **Table 2**.

After the TPS was thoroughly mixed with the SL, OA and LA as dry mixtures and then cooked with water to form pastes, the pastes developed gel, but not with the PA and SA. The differences could be attributed to that the liquid-form OA and LA and water-soluble SL could readily penetrate into the TPS granules, form complexes with amylose, and develop networks with amylopectin in the granules during cooking. PA and SA, however, had high melting-temperatures, 62.1 °C and 70.6 °C, respectively, which were close to or above the pasting

temperature of the TPS (69.1 °C). The complex formation with these two FFAs likely occurred after TPS granules were already swollen or dispersed. Thus, ALC with SA and PA failed to interact with amylopectin within the granule to maintain the integrity of swollen granules for the subsequent gel formation. The explanation was further supported by the results of no gel formation when the SL, OA and LA were added after the TPS was cooked (data not shown). The WCS did not develop a gel with or without added lipids because of lacking amylose (**Table 1** and **3**). HA7 was only partially gelatinized after cooking in the amylograph, and the starch granules hardly swelled (**Table 1** and **Figure 2**) (Jiang et al., 2010 b; Hasjim et al., 2010). Therefore, it failed to form a gel.

The addition of CO reduced the enzymatic-hydrolysis rates and viscosities of the NCS, TPS and HA7 (**Figure 1** and **2**) and increased the gel strength of the NCS (**Table 3**). DSC thermograms, however, did not show an ALC dissociation peak with the added CO (**Table 1** and **2**). To reveal whether CO formed helical complexes with amylose, ^{13}C-NMR was used to examine the conformational change of amylodextrin (short-chain amylose, average DP 25) with the presence of CO.

To form a helical complex with lipids, the conformation of the amylose molecule is changed from a random coil to a helix, which alters the torsion angles (φ and ψ) of the glycosidic bonds (French and Murphy, 1977). The changes in the torsion angles affect the patterns of electron distribution on the carbons 1 and 4 of the bonds and, thus, cause downfield changes in the chemical shifts of carbons 1 and 4 of the anhydroglucose unit in the NMR spectrum (Jane et al., 1985; Gidley and Bociek, 1988). The downfield changes in ^{13}C-chemical shifts of carbons 1 and 4 of amylodextrin with the presence of CO, SL and OA demonstrated that the three lipids formed helical complexes with linear starch molecules with the extent of SL > OA > CO (**Table**

4) (Jane et al., 1985). It was plausible that one or more fatty acids of the CO formed complexes with amylose, which restricted granule swelling and, consequently, decreased the enzymatic-hydrolysis rates and viscosities of the NCS, TPS and HA7. Because of steric hindrance for the three fatty acids of the triglyceride molecule to form helical complex with amylose, interactions between the fatty acids of CO and amylose could be heterogeneous and weak. Consequently, the ALC with CO did not show a dissociation peak in the DSC thermograms (**Table 1** and **2**).

4. Conclusions

Lipids of different structures showed different effects on the enzymatic-hydrolysis rates and physical properties of starches. The SL and FFAs formed ALC with NCS, TPS and HA7 and showed well-defined ALC dissociation peaks in the DSC thermograms. The ALC with FFAs decreased the peak viscosities of NCS and TPS during cooking. The addition of SL to the NCS and HA7, however, facilitated the swelling of the starches, resulting from that the SL removed the endogenous lipids from the starch granules. Adding SL, OA and LA to the TPS as dry mixtures prior to cooking introduced ALC formation within the granules, which allowed gel formation. Adding SL and FFAs to the NCS, however, decreased the gel strength of NCS, resulting from lamellar-crystallite formation and loss of water-binding capacity.

Adding CO to starches decreased their viscosities and enhanced the gel strength of NCS, but did not show an ALC dissociation peak in the DSC thermograms. ^{13}C-NMR spectra of amylodextrin showed downfield changes in the chemical shifts of carbons 1 and 4 of anhydroglucose unit with the presence of CO, indicating that the CO did form helical with amylose, but to a lesser extent.

All the lipids, after cooking with the starches, significantly decreased the starch-hydrolysis rates except WCS because it lacked amylose. The interactions between starch and lipids could be useful to produce starchy foods with reduced glycemic-index.

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Table 1. Amylose contents [a] and thermal properties of native starches and starches with addition of 10% (w/w, dsb) different lipids [b]

Sample [c]		Amylose	Starch gelatinization [d]					Dissociation of amylose-lipid complex [d]			
		(%)	T_o (ºC)	T_{p1} (ºC)	T_{p2} (ºC)	T_c (ºC)	ΔH (J/g)	T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)
NCS	Control	34.3 ± 0.4	69.1 ± 0.5	74.0 ± 0.4	-	80.2 ± 0.5	13.4 ± 0.1	90.1 ± 0.6	101.3 ± 0.7	108.2 ± 1.5	0.9 ± 0.0
	+ CO	-	69.7 ± 0.2	75.0 ± 0.1	-	80.6 ± 0.2	14.0 ± 0.5	90.4 ± 0.1	101.3 ± 0.7	109.1 ± 0.2	0.9 ± 0.1
	+ SL	-	68.5 ± 0.1	74.3 ± 0.1	-	81.2 ± 0.1	14.0 ± 0.2	91.9 ± 0.0	94.5 ± 1.7	101.8 ± 0.6	0.1 ± 0.0
	+ PA	-	N.A. [e]	74.8 ± 0.6	-	80.8 ± 0.5	N.A.	89.8 ± 0.3	103.8 ± 0.0	109.6 ± 0.3	1.8 ± 0.1
	+ SA	-	N.A.	N.A.	-	N.A.	N.A.	89.8 ± 0.0	101.5 ± 0.1	109.0 ± 0.4	1.2 ± 0.1
	+ OA	-	68.6 ± 0.2	73.7 ± 0.1	-	80.5 ± 0.0	15.3 ± 0.7	90.9 ± 0.4	102.2 ± 0.0	110.2 ± 0.2	1.9 ± 0.1
	+ LA		68.8 ± 0.6	73.5 ± 0.7	-	79.9 ± 0.6	14.2 ± 0.3	90.5 ± 0.9	96.7 ± 0.0	103.0 ± 0.2	1.7 ± 0.4
TPS	Control	29.0 ± 0.1	67.4 ± 0.5	73.9 ± 0.5	-	81.9 ± 0.6	14.7 ± 0.2	N.D. [f]	N.D.	N.D.	N.D.
	+ CO	-	68.0 ± 0.6	73.9 ± 0.0	-	82.2 ± 0.0	14.2 ± 0.3	N.D.	N.D.	N.D.	N.D.
	+ SL	-	67.6 ± 0.3	74.2 ± 0.4	-	86.0 ± 0.7	14.5 ± 0.1	N.D.	N.D.	N.D.	N.D.
	+ PA	-	N.A. [e]	73.6 ± 0.2	-	81.4 ± 0.5	N.A.	N.D.	N.D.	N.D.	N.D.
	+ SA	-	N.A.	N.A.	-	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
	+ OA	-	68.0 ± 1.0	72.5 ± 1.6	-	81.3 ± 0.3	14.2 ± 0.7	90.4 ± 0.7	100.7 ± 0.1	108.4 ± 0.0	0.6 ± 0.1
	+ LA	-	67.6 ± 0.3	73.5 ± 0.1	-	81.3 ± 0.2	14.8 ± 1.1	N.D. [f]	N.D.	N.D.	N.D.
WCS	Control	1.9 ± 0.0	66.3 ± 0.1	73.7 ± 0.4	-	81.5 ± 0.4	15.3 ± 0.1	N.D. [f]	N.D.	N.D.	N.D.
	+ CO	-	66.6 ± 0.4	74.3 ± 0.4	-	83.0 ± 1.1	15.3 ± 0.2	N.D.	N.D.	N.D.	N.D.
	+ SL	-	67.4 ± 0.1	75.1 ± 0.5	-	81.2 ± 0.0	15.1 ± 0.7	N.D.	N.D.	N.D.	N.D.
	+ PA	-	N.A. [e]	73.6 ± 0.2	-	81.1 ± 0.3	N.A.	N.D.	N.D.	N.D.	N.D.
	+ SA	-	N.A.	N.A.	-	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.
	+ OA	-	66.2 ± 0.1	73.9 ± 0.0	-	81.9 ± 0.1	15.9 ± 0.4	N.D.	N.D.	N.D.	N.D.
	+ LA	-	66.3 ± 0.5	73.2 ± 0.6	-	81.3 ± 0.0	16.1 ± 1.2	N.D.	N.D.	N.D.	N.D.
HA7	Control	68.4 ± 1.4	70.6 ± 0.0	75.7 ± 0.1	100.2 ± 0.1	109.3 ± 0.6	12.1 ± 1.2	N.A. [g]	N.A.	N.A.	N.A.
	+ CO	-	70.9 ± 0.1	76.7 ± 0.3	100.5 ± 0.4	109.7 ± 1.6	12.5 ± 0.4	N.A.	N.A.	N.A.	N.A.
	+ SL	-	71.4 ± 0.2	76.5 ± 0.7	93.0 ± 1.7	105.3 ± 0.4	11.5 ± 1.9	N.A.	N.A.	N.A.	N.A.
	+ PA	-	N.A. [e]	N.A.	101.8 ± 0.0	109.7 ± 0.1	N.A.	N.A.	N.A.	N.A.	N.A.
	+ SA	-	N.A.	N.A.	99.6 ± 0.1	109.6 ± 0.3	N.A.	N.A.	N.A.	N.A.	N.A.
	+ OA	-	70.6 ± 0.3	75.7 ± 0.2	100.6 ± 0.1	109.6 ± 0.0	12.6 ± 0.0	N.A.	N.A.	N.A.	N.A.
	+ LA	-	69.8 ± 0.8	74.0 ± 0.0	98.4 ± 0.5	107.0 ± 0.0	14.4 ± 0.6	N.A.	N.A.	N.A.	N.A.

[a] Measured using iodine potentiometric titration method.
[b] Measured using differential scanning calorimetry.
[c] NCS: normal corn starch, TPS: tapioca starch, WCS: waxy corn starch, HA7: high-amylose corn starch. CO: corn oil, SL: soy lecithin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid.
[d] T_o: onset temperature, T_p: peak temperature, T_c: conclusion temperature, ΔH: enthalpy change.
[e] N.A.: not available because of the overlapping between the melting peak of free fatty acid and starch gelatinization peak.
[f] N.D.: not detected.
[g] N.A.: not available because of the overlapping between the starch gelatinization peak and amylose-lipid complex dissociation peak of HA7.

Table 2. Dissociation of amylose-lipid complex of pre-cooked starch alone (control) and starch pre-cooked with 10% (w/w, dsb) different lipids [a, b, c]

Sample [d]		First scan [e, f]				Rescan [e]			
		T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)	T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)
NCS	Control	89.2 ± 0.2 a	99.6 ± 0.2	107.0 ± 0.1	2.1 ± 0.1	83.4 ± 0.2 d	92.3 ± 0.2	101.3 ± 1.8	0.5 ± 0.2
	+ CO	88.0 ± 0.1 ab	100.1 ± 0.6	107.1 ± 0.1	2.1 ± 0.0	83.0 ± 0.0 d	92.3 ± 0.1	103.4 ± 0.6	0.9 ± 0.0
	+ SL	75.1 ± 0.0 c	91.4 ± 0.1	99.5 ± 0.1	3.3 ± 0.2	76.2 ± 0.1 e	88.7 ± 0.4	96.5 ± 0.2	2.2 ± 0.0
	+ PA	88.2 ± 0.0 ab	100.7 ± 0.2	109.0 ± 0.3	3.9 ± 0.0	88.4 ± 0.1 b	102.5 ± 0.1	109.3 ± 0.1	5.0 ± 0.0
	+ SA	89.1 ± 0.8 a	101.2 ± 0.2	109.9 ± 0.2	4.3 ± 0.1	92.5 ± 1.0 a	100.0 ± 0.1	109.9 ± 0.0	3.7 ± 0.1
	+ OA	87.4 ± 0.0 b	96.4 ± 0.5	108.1 ± 0.5	4.1 ± 0.1	86.8 ± 0.0 c	95.6 ± 0.4	108.3 ± 0.4	6.7 ± 0.1
	+ LA	72.8 ± 0.3 d	91.7 ± 0.2	103.6 ± 0.4	6.8 ± 0.0	75.8 ± 0.2 e	86.5 ± 0.2	102.8 ± 0.3	5.4 ± 0.3
TPS	Control	N.D. [g]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ CO	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ SL	76.1 ± 0.1 c	89.3 ± 0.5	98.6 ± 0.4	2.9 ± 0.2	76.9 ± 0.0 d	86.9 ± 0.0	94.9 ± 0.9	1.2 ± 0.1
	+ PA	90.2 ± 0.4 a	102.5 ± 0.5	109.0 ± 0.0	3.8 ± 0.0	89.7 ± 0.2 b	103.1 ± 0.0	109.1 ± 0.1	3.4 ± 0.1
	+ SA	91.7 ± 0.5 a	103.4 ± 0.5	109.9 ± 0.6	2.1 ± 0.3	94.3 ± 0.5 a	101.0 ± 0.1	110.1 ± 0.0	1.7 ± 0.0
	+ OA	88.0 ± 0.8 b	100.2 ± 0.2	108.2 ± 0.2	3.9 ± 0.0	87.8 ± 0.1 c	99.9 ± 0.1	108.1 ± 0.1	5.0 ± 0.0
	+ LA	76.7 ± 0.3 c	92.1 ± 0.1	100.6 ± 0.1	4.1 ± 0.1	77.7 ± 0.3 d	92.0 ± 0.0	100.1 ± 0.1	3.9 ± 0.1
WCS	Control	N.D. [g]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ CO	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ SL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ PA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ SA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ OA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	+ LA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
HA7	Control	90.2 ± 0.2	94.7 ± 0.2	109.2 ± 0.1	6.5 ± 0.2	85.3 ± 0.5 bc	94.7 ± 0.2	106.4 ± 0.1	4.1 ± 0.1
	+ CO	93.6 ± 0.2	92.1 ± 0.1	111.3 ± 0.4	5.3 ± 0.4	86.7 ± 0.4 b	92.1 ± 0.1	103.8 ± 0.1	2.2 ± 0.1
	+ SL	N.A. [h]	N.A.	N.A.	N.A.	75.1 ± 0.5 d	86.9 ± 0.1	96.2 ± 0.4	3.0 ± 0.3
	+ PA	92.9 ± 0.3	101.6 ± 0.4	109.2 ± 0.3	7.7 ± 0.1	85.4 ± 0.3 bc	95.8 ± 0.2	108.6 ± 0.1	9.4 ± 0.6
	+ SA	94.4 ± 0.1	103.0 ± 0.0	109.9 ± 0.0	7.4 ± 0.1	88.6 ± 0.3 a	98.3 ± 0.2	109.9 ± 0.0	7.6 ± 0.5
	+ OA	90.7 ± 0.0	100.3 ± 0.6	108.8 ± 1.1	7.6 ± 0.5	84.8 ± 0.1 c	94.6 ± 0.8	107.9 ± 0.7	8.7 ± 0.1
	+ LA	N.A. [h]	N.A.	N.A.	N.A.	74.4 ± 0.1d	86.7 ± 0.1	102.7 ± 0.0	6.4 ± 0.1

[a] The samples were pre-cooked in a boiling water-bath, dried at 45 °C, and ground before the scanning with water (3X, w/w, dsb) to 150 °C.
[b] Measured using differential scanning calorimetry.
[c] Values of the same starch with the same letter in a column are not significantly different at $p < 0.05$.
[d] NCS: normal corn starch, TPS: tapioca starch, WCS: waxy corn starch, HA7: high-amylose corn starch. CO: corn oil, SL: soy lecithin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid.

Table 2. continued

[e] T_o: onset temperature, T_p: peak temperature, T_c: conclusion temperature, ΔH: enthalpy change.
[f] For HA7, the peak in the first scan was a combination of starch gelatinization and amylose-lipid complex dissociation.
[g] N.D.: not detected.
[h] N.A.: not available because of the overlapping between the melting peak of retrograded starch and amylose-lipid complex dissociation peak.

Table 3. Gel strength of starch gels (8%, w/w, dsb) prepared with or without added lipids (10%, w/w, dsb) [a, b, c]

Sample [d]	Gel strength (*g*)						
	Control	+ CO	+ SL	+ PA	+ SA	+ OA	+ LA
NCS	403.4 ± 11.2 b	523.7 ± 14.4 a	283.6 ± 8.8 c	165.0 ± 5.6 f	172.6 ± 5.5 f	192.2 ± 5.6 e	246.1 ± 8.0 d
TPS	N.A. [e]	N.A.	92.2 ± 4.8	N.A.	N.A.	85.6 ± 4.0	92.6 ± 4.6
WCS	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HA7	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

[a] The samples were prepared by cooking starch suspensions using an amylograph followed by storage at 4 °C for 72 h.
[b] The gel strength was analyzed using a Texture Analyzer TA-XT2i.
[c] Values of the NCS with the same letter are not significantly different at $p < 0.05$.
[d] NCS: normal corn starch, TPS: tapioca starch, WCS: waxy corn starch, HA7: high-amylose corn starch. CO: corn oil, SL: soy lecithin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid.
[e] N.A.: not available because the sample did not form starch gel.

Table 4. Downfield changes in ^{13}C-chemical shifts of amylodextrin [a]

Sample [b]	Chemical shift change (p.p.m.) [c]					
	C 1 [d]	C 4	C 2	C 3	C 5	C 6
+ CO	0.01	0.02	0.00	0.00	0.00	0.00
+ SL	0.03	0.06	0.00	0.00	0.00	0.00
+ OA	0.03	0.05	0.00	0.00	0.00	0.00

[a] ^{13}C-NMR spectra were obtained after 2,500 scans of amylodextrin (average DP 25) with or without added lipids (25%, w/w, dry amylodextrin basis) in aqueous solutions at 25ºC.
[b] CO: corn oil, SL: soy lecithin, OA: oleic acid.
[c] Chemical shift change = chemical shift of amylodextrin with lipid - chemical shift of amylodextrin control.
[d] Different carbons in the anhydroglucose unit of the amylodextrin.

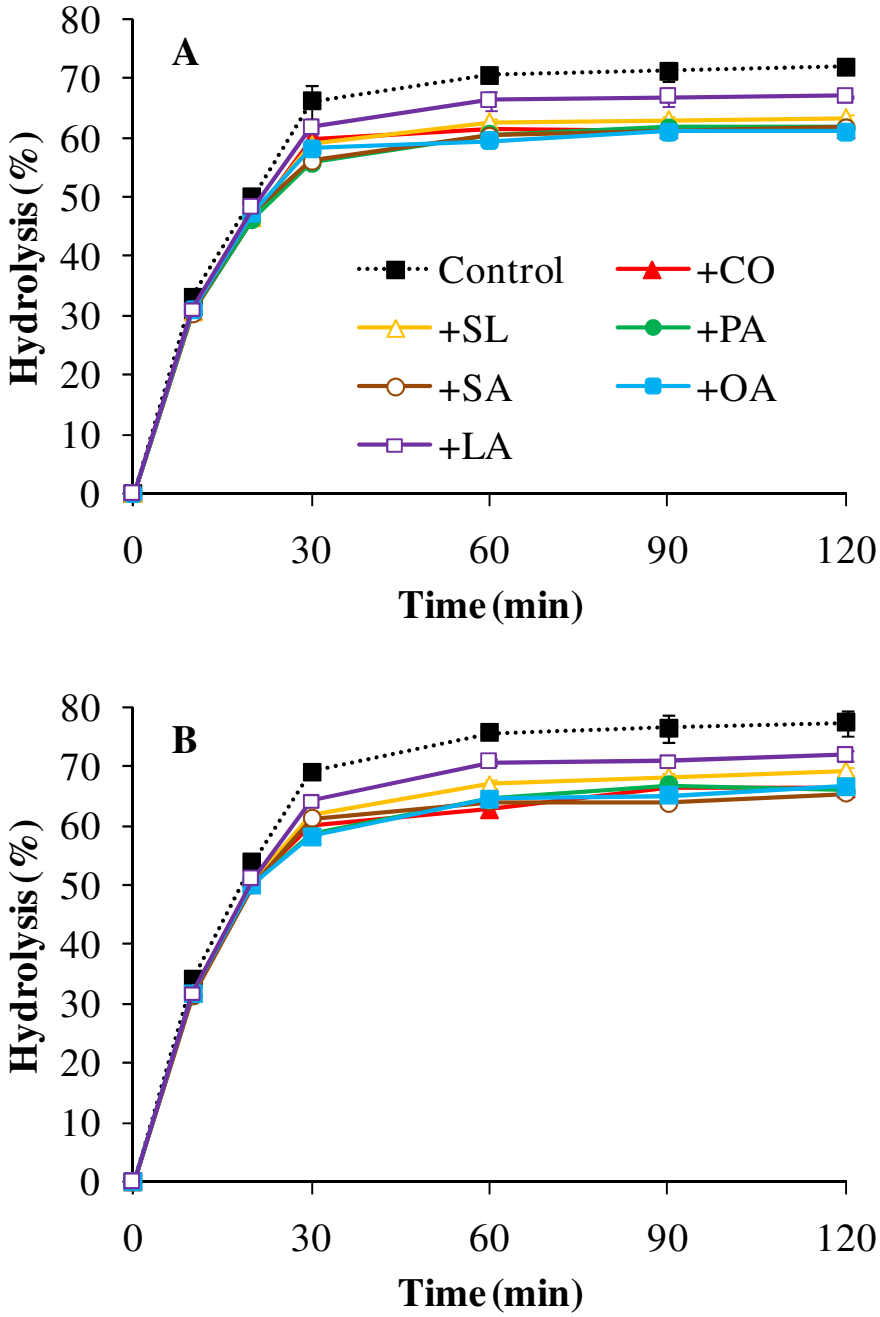
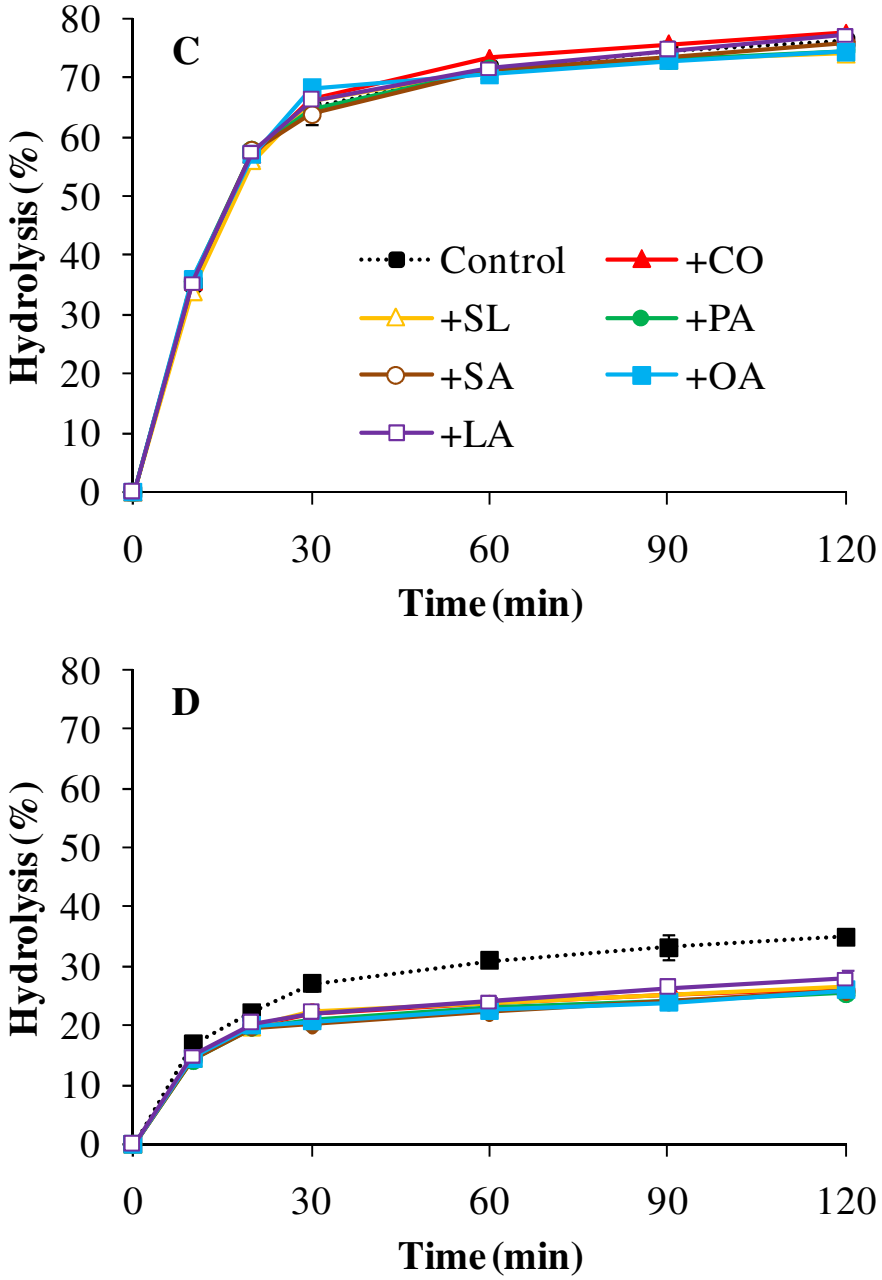


Figure 1. Enzymatic-hydrolysis rates of cooked starches and starches cooked with 10% (w/w, dsb) different lipids. PPA was used for the hydrolysis at 37 °C, pH 6.9. % starch hydrolysis = 100% × total mass of glucose released from soluble sugars / initial dry mass of starch × (162 / 180). A: normal corn starch, B: tapioca starch, C: waxy corn starch, D: high-amylose corn starch. CO: corn oil, SL: soy lecithin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid.

Figure 1. continued



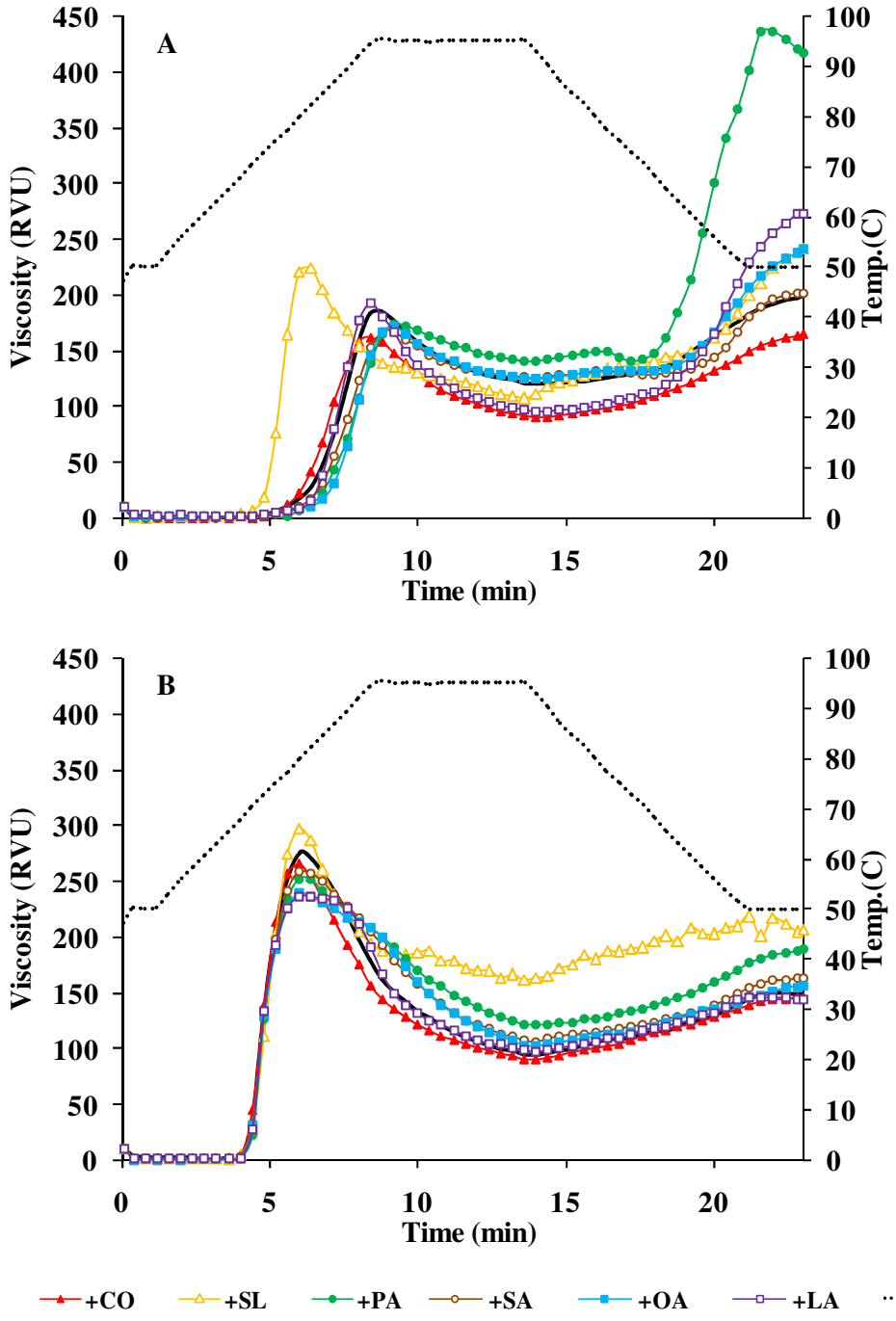
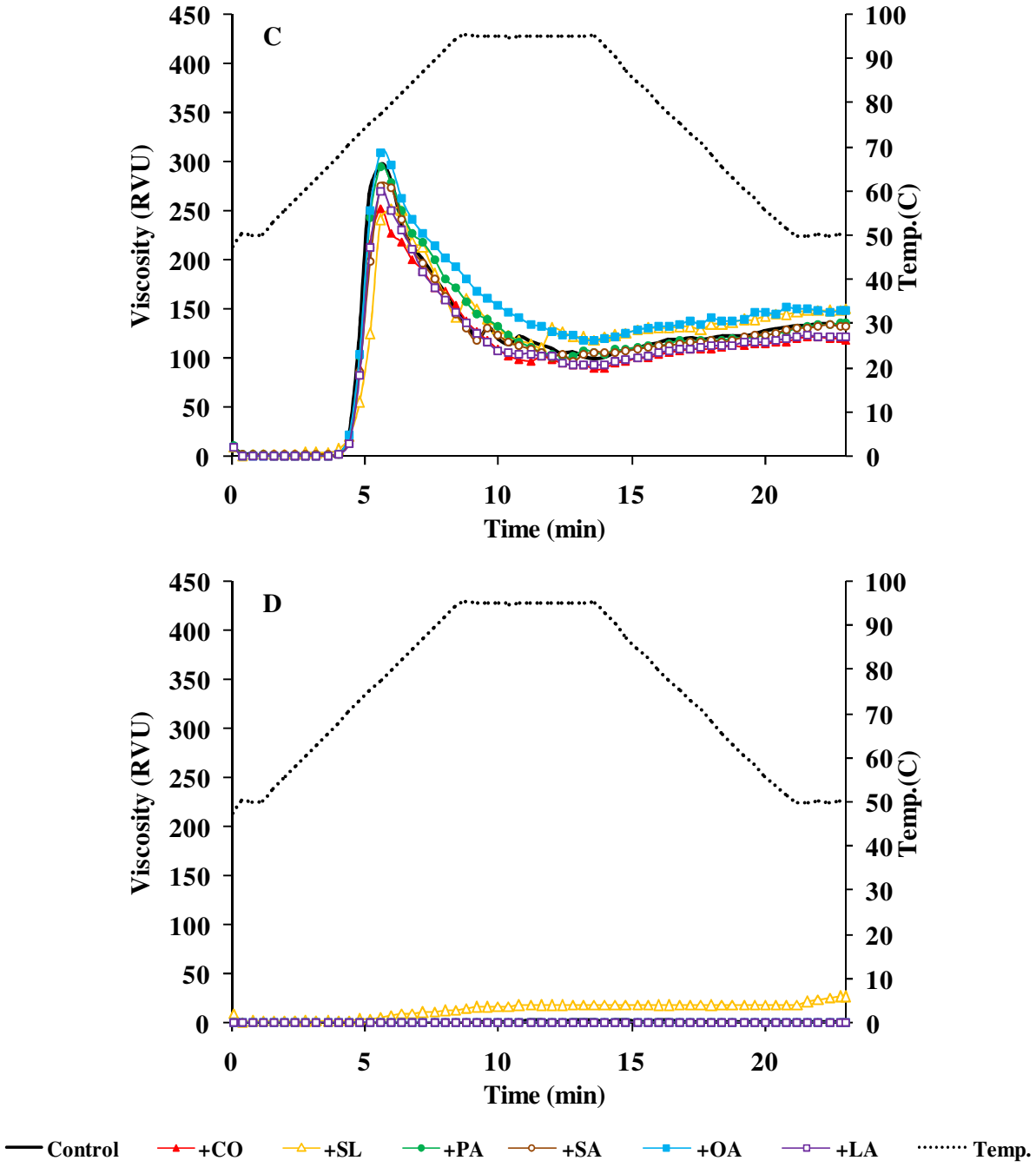


Figure 2. Pasting profiles of starches and starch-lipid mixtures (starch:lipid = 10:1, w/w, dsb) measured using a Rapid Visco-Analyzer. Starch suspension (28.0 g total weight) with 8% starch (w/w, db) was used for the analysis. A: normal corn starch, B: tapioca starch, C: waxy corn starch, D: high-amylose corn starch. CO: corn oil, SL: soy lecithin, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid.

Figure 2. continued



CHAPTER 3. CHARACTERIZATION AND *IN VIVO* HYDROLYSIS OF AMYLOSE-STEARIC-ACID COMPLEX

A paper to be submitted to *Cereal Chemistry*

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Abstract

Objectives of this study were to compare thermal properties, swelling power, and enzymatic hydrolysis of a Type 5 resistant starch (RS5) with that of normal corn (NCS) and high-amylose corn starch (HA7). The RS5 was prepared by complexing debranched HA7 with stearic acid (SA). Because of amylose-helical-complex formation with SA, the RS5 starch granules showed restricted swelling at 95 °C. The RS5 displayed a larger RS-content (67.8%) than the HA7 (33.5%) and NCS (0.8%) analyzed using the AOAC Method 991.43. When the cooked RS5, HA7 and NCS were used to prepare diets for rats with 55% (w/w) starch content, RS contents of the diets were 33.7%, 15.8% and 2.6%, respectively. After feeding to the rats in Week 1, ~16% of the starch in the RS5-diet was found in the feces, substantially greater than that of the HA7-diet (~6%) and NCS-diet (0.1%). The percentage of starch not being utilized in the RS5-diet decreased to ~5% in Week 9, which could be partially attributed to fermentation of RS5

by gut microflora. Large proportions (68%-99%) of the SA in RS5-diet remained unabsorbed and were discharged in the rat feces.

1. Introduction

Starch is widely used in foods to provide various functions, including as an energy source, a gelling agent, a thickening agent, and a stabilizing agent. Starch consists of two major glucans: amylose and amylopectin. Amylose is an essentially linear polysaccharide with α-1, 4 linked D-glucopyranose units and a few branches of α-1, 6 linkages, whereas amylopectin is a highly-branched polysaccharide consisting of short linear-chains connected by about 5% α-1, 6 branch linkages (Hizukuri et al. 1981). Starch is a major energy source for humans and animals. After being ingested, starch is hydrolyzed to glucose by amylolytic enzymes in the digestive tract. Glucose is then absorbed in the small intestine to be used for energy source.

A fraction of starch in food escapes the digestion in the small intestine, which is known as resistant starch (RS) (Englyst et al. 1982; Englyst and Macfarlane 1986). RS is passed into the colon to be available for fermentation by gut microflora to produce short-chain fatty acids, e.g., acetic acid, propionic acid and butyric acid, which can prevent colon cancer (Phillips et al. 1995; Kleessen et al. 1997; Martin et al. 1998; Silvi et al. 1999; Henningsson et al. 2003). Some RS is present in the feces and increases fecal bulk (Phillips et al. 1995; Hylla et al. 1998; Jenkins et al. 1998; Kishida et al. 2001). Other potential health benefits of RS include: (1) lowering postprandial plasma-glucose concentration and insulin response (Carroll 1958; Yamada et al. 2005; He et al. 2008; Hasjim et al. 2010); (2) improving postprandial insulin sensitivity and preventing Type II diabetes (Robertson et al. 2003; Johnston et al. 2010; Maki et al. 2012); and (3) increasing lipid metabolism and preventing obesity (Kabir et al. 1998; Cheng and Lai 2000;

Higgins et al. 2005; Shimotoyodome et al. 2010). According to the definition of dietary fiber given by the American Association of Cereal Chemists International and the National Academy of Sciences, RS is a type of dietary fiber because it provides similar physiological benefits as other dietary fibers (Sajilata et al. 2006).

RS has been classified into five different types: physically inaccessible starch (RS1), native granular starch with the B- or C-type crystalline structure (RS2), retrograded starch (RS3), chemically-modified starch (RS4), and amylose-lipid complex (RS5) (Englyst et al. 1992; Muir et al. 1994; Hasjim et al. 2010). The single-helical-complex formation between debranched high-amylose corn starch and free fatty acids (FFAs) has been used to prepare RS5 with up to 75.0% RS content (AOAC Method 991.43) (Hasjim et al. 2010). A human-feeding study has shown that ingestion of bread made with the RS5 resulted in substantially lower postprandial plasma-glucose concentration and insulin response than the control of white bread. An animal-feeding study has shown that the cooked RS5 effectively reduced the azoxymethane-induced preneoplastic lesions (colon cancer precursors) in the rat colon, suggesting that the RS5 could suppress colon carcinogenesis (Zhao et al. 2011). The mechanisms for the inhibitory effects of the RS5, however, are not fully understood.

Objectives of this study were to investigate *in vitro* and *in vivo* enzymatic hydrolysis of cooked RS5 in comparison with that of cooked high-amylose corn starch (HA7) and normal corn starch (NCS). Structures and physical properties of the starches were characterized and related to their enzymatic hydrolysis. The data obtained from this study could help us better understand mechanisms of the health benefits of RS5. The knowledge will also be valuable for the applications of this RS in food products.

2. Materials and methods

2.1. Materials

Normal corn starch (NCS, Cargill Gel ™, 34.3% apparent amylose) and high-amylose corn starch (HA7, AmyloGel ™, 68.4% apparent amylose) were purchased from Cargill Inc. (Minneapolis, MN). Stearic acid (SA), pullulanase from *Bacillus acidopullulyticus*, thermostable α-amylase and protease from *Bacillus licheniformis*, amyloglucosidase from *Aspergillus niger*, pyrogallol and heptadecanoic acid (C17:0) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Casein, glucose, mineral mix (AIN-93), choline, methionine, vitamin mix (AIN-93) and corn oil used to prepare diets for rats were purchased from Harlan Laboratories (Madison, WI). Total Starch Assay Kit was purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Fifty Fisher-344 rats (five-week-old) were purchased from Charles River Laboratory (Wilmington, MA).

2.2. Preparation of RS5

RS5 was prepared using HA7 following the method of Hasjim et al. (2010) with modifications. A HA7 suspension (10%, w/w, dry basis, db) was pre-heated at 80 °C for 1 h with stirring, followed by debranching using pullulanase (5 units/g starch, dry starch basis, dsb) at 60 °C for 24 h. SA (10%, w/w, dsb) was added to the debranched-starch suspension, and the mixture was maintained at 80 °C for additional 1 h with vigorous stirring. The RS5 was recovered by centrifugation, washed using 50% (v/v) ethanol aqueous solution, dried at 37 °C, and then ground to fine powder.

2.3. Thermal properties of starch

Thermal properties of the NCS, HA7 and RS5 were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT) (Hasjim et al. 2010). The

starch sample (~10 mg) was mixed with 3× (w/w) water and heated from 10 °C to 150 °C at a rate of 10 °C/min. After the first scan, the sample was immediately cooled to 10 °C at 40 °C/min and rescanned to confirm the dissociation peak of amylose-lipid complex (ALC). The analysis was done in duplicate.

2.4. Swelling power of starch

Swelling power of the NCS, HA7 and RS5 in 1% (w/v, db) aqueous suspension at 70 °C, 80 °C and 95 °C (boiling-water temperature) was analyzed following the method of Srichuwong et al. (2005). The analysis was done in duplicate.

2.5. RS content of starch

The RS content of starch was analyzed following the AOAC Method 991.43 (2000) with modifications (Li et al. 2008). The starch sample (1.0 g, dsb) was suspended in a Mes-Tris buffer solution (pH 8.2, 0.05 M, 40.0 mL) and hydrolyzed by *B. licheniformis* α-amylase (500 units) in a boiling water-bath (~95 °C) for 30 min with stirring. The starch sample was then incubated with 5.0 mg protease at 60 °C for 30 min with shaking (100 rpm). After adjusting the pH to 4.4-4.6 using 0.5 M hydrochloric acid, the starch was hydrolyzed by amyloglucosidase (300 U) at 60 °C for 30 min with shaking (100 rpm). The RS was collected by filtration through a celite layer in a crucible and dried at 110 °C overnight. The RS content was calculated using the equation: % RS = 100% × total RS residue weight (db) / initial starch weight (db). The analysis was done in duplicate.

2.6. Preparation of rat diets

The NCS, HA7 and RS5 were boiled and used to prepare rat diets following the method of Zhao et al. (2011). Each starch was boiled with 3× (w/w) distilled water and then thoroughly mixed with other ingredients as shown in **Table 1** to prepare diet with 55% (w/w) starch content.

The dry starch content of each diet and the SA content of the RS5-diet were calculated and shown in **Table 1**. RS contents of the water-boiled starches and prepared diets were analyzed in duplicate as described above.

2.7. Rat feeding and feces collection

The rat-feeding study was performed following the method reported by Ai et al. (2013b). After arriving at the facility, all the 50 rats were fed with the NCS-diet for the first two weeks (Week 0), and then fed with respective diets for additional nine weeks: 20 rats with the NCS-diet, 10 rats with the HA7-diet, and 20 rats with the RS5-diet. "Week 1" referred to the first week when the rats were fed with different diets. To examine the utilization of starch by gut microflora, 10 rats eating the NCS- or RS5-diet were randomly assigned to consume water containing vancomycin and imipenem (antibiotics, 50 μg/mL of each, ~13 mL/rat/day) during Week 1, and the other 30 rats consumed water with no antibiotics. From Week 2 to 9, all the 50 rats had water with no antibiotics. The daily feed-disappearance weight, the body weight, and the daily feces-weight of each rat were measured as described by Ai et al. (2013b). The animal study was carried out in compliance with the guidelines of the Institutional Animal Care and Use Committee.

2.8. Starch content of feces

Feces collected weekly from the bottom of rat cages with the same treatment were mixed, dried and ground before starch and lipid content analyses. Starch content of the feces was determined using a Total Starch Assay Kit following the AACC method 76-13 (2000). The feces sample (~100 mg) was wet with aqueous ethanol solution (0.2 mL, 80%, v/v) and then dispersed in dimethyl sulfoxide (2.0 mL) with vigorous boiling and stirring for 15 min. After adding sodium acetate buffer (3.0 mL, 100 mM, pH 5.0), the dispersed starch was hydrolyzed to glucose

using *B. licheniformis* α-amylase and amyloglucosidase and then quantified using the glucose oxidase/peroxidase (GOPOD) method. The analysis was done in duplicate. The percentage of starch not being utilized by the rats and their gut microflora was calculated as: % starch not being utilized = 100% × [starch content of feces (db) × average daily feces weight (db)] / [starch content of diet (db) × average daily feed-disappearance weight (db)].

2.9. Lipid content of feces

Lipid was extracted from the feces following the AOAC Method 996.06 (2000). The lipid content of the feces was calculated using the equation: % lipid content = 100% × weight of extracted lipid / initial feces weight (db). The analysis was done in duplicate.

2.10. Free-fatty-acid compositions of feces lipid

Free fatty acids (FFAs) and neutral lipids extracted from the rat feces were separated using a thin layer chromatography following the method of Majoni and Wang (2010). The FFA fraction was collected and esterified with methanol by using sulfuric acid (3%) as the catalyst at 60 °C for 24 h. Heptadecanoic acid (C17:0) was added as an internal standard for the quantification. After adding deionized water to stop the reaction, the fatty acid methyl esters (FAMEs) were extracted twice using hexane. The FAMEs were analyzed and quantified using a capillary gas chromatography (GC) equipped with Hewlett-Packard model 5890 series II gas chromatograph with flame ionizer detector (Majoni and Wang 2010). The analysis was done in duplicate. The percentage of SA in the RS5-diet not being utilized by the rats and their gut microflora was calculated as: % SA not being utilized = 100% × [SA content of feces lipid × lipid content of feces (db) × average daily feces weight (db)] / [SA content of RS5-diet (db) × average daily feed-disappearance weight (db)].

2.11. Cecum tissue weight, cecal content weight and pH

The rats were sacrificed at the end of the 9-week feeding study. The cecum tissue weight, cecal content weight and pH were determined for each rat (Kishida et al. 2001; Zhao et al. 2011).

2.12. Statistical analysis

Statistical significance was analyzed using one-way ANOVA and multiple comparison test with Tukey's adjustment at *p* value = 0.05. Correlation analysis of the results was conducted using the Pearson correlation test. The statistical analyses were conducted in SAS (Version 9.2, SAS Institute, Inc., Cary, NC).

3. Results and discussion

DSC thermograms of the NCS, HA7 and RS5 are shown in **Figure 1**, and the results are summarized in **Table 2**. The first scan of native NCS showed two separate peaks. The first peak (T_p = 72.1 °C) was attributed to the gelatinization of amylopectin crystallites. The second peak (T_p = 96.8 °C) corresponded to the ALC dissociation, which was confirmed by the re-appearance of the peak (T_p = 93.6 °C) in the rescan. In the first scan of HA7, the gelatinization peak of amylopectin crystallites (T_{p1} = 74.8 °C) partially overlapped with the ALC dissociation peak (T_{p2} = 99.5 °C), and the thermogram displayed a very broad thermal transition from 70.1 °C to 108.1 °C. The first scan of the RS5 showed two separate peaks: the first peak at 70.7 °C was the melting peak of crystalline free SA; the second peak (T_p = 103.8 °C) was the ALC dissociation peak. The ALC peak of the RS5 showed a higher dissociation-temperature and larger enthalpy-change than that of the NCS and HA7 because of the presence of thermally more stable amylose-SA complex (**Figure 1**) (Kowblansky 1985; Ai et al. 2013a). The gelatinization peak of

amylopectin crystallites (T_{p1} = 74.8 °C in the HA7) was not detected in the RS5, indicating the loss of amylopectin crystallites during the preparation process at 80 °C.

Results of swelling power of the starches are shown in **Figure 2**. At the selected temperatures, the NCS showed significantly greater swelling-power than the HA7 and RS5, which could be attributed to the lower gelatinization-temperature, larger amylopectin-content, and less ALC in the NCS (**Table 2** and **Figure 1**). At 70 °C and 80 °C, no significant differences were observed between the swelling power of the HA7 and RS5. When the starches were heated at a boiling-water temperature (~95 °C), the RS5 showed significantly (p <0.05) lower swelling-power than the HA7, resulting from the amylose-SA complex present in the RS5 (**Table 2** and **Figure 1**) (Tester and Morrison 1990; Ai et al. 2013a).

RS contents of the starch samples and rat diets analyzed using the AOAC Method 991.43 are shown in **Table 3**. Without prior cooking, the RS content of the NCS (0.8%) was substantially lower than that of the HA7 (33.5%) because the NCS was gelatinized and easily hydrolyzed during the incubation (30 min) with thermostable α-amylase at a boiling-water temperature (~95 °C) in the analysis, whereas the HA7 was only partially gelatinized (**Table 2** and **Figure 1**). The RS content of the RS5 (67.8%) was much larger than that of the HA7 (33.5%) because of the presence of amylose-SA complex (ALC) in the RS5 (**Table 2** and **Figure 1**). It is known that ALC is resistant to enzymatic hydrolysis (Jane and Robyt 1984). The ALC also restricted the swelling of starch granules (**Figure 2**) and some free SA could coat on the surface of starch granules, which further reduced the susceptibility of the RS5 to enzymatic hydrolysis (Tester and Morrison 1990; Hasjim et al. 2010; Ai et al. 2013a). After being water-boiled, the RS content of the HA7 decreased from 33.5% to 27.3%, indicating the loss of some RS in the HA7 after prior boiling. The RS content of the RS5 slightly decreased from 67.8% to

64.8%. The results showed that the RS5 had better stability during thermal processing. After mixing with other ingredients at 55% starch content (w/w, as-is basis) to prepare diets, the RS5-, HA7- and NCS-diet showed RS contents of 33.7%, 15.8% and 2.6%, respectively, which were slightly higher than its respective calculated RS contents (31.8%, 14.6% and 0.5%, respectively).

After feeding the rats with these diets, average daily feed-disappearance weights, body weights of the rats, daily feces weights, and starch and lipid contents of rat feces were analyzed and shown in **Table 4**. Some feed randomly dropped to the bottom of the cage and could not be recovered. Thus, the daily feed-disappearance weight could be larger than the amount of feed consumed by the rats and the differences might not be consistent between diets and with time. When all the rats were fed with the NCS-diet in Week 0, there were no significant differences in the feces weight between different groups and less than 1% starch was found in the feces in Week 0 (**Table 4**). These results indicated that almost all the cooked NCS was digested *in vivo*, which were consistent with previously reported data (Ai et al. 2013b).

Starting from Week 1, the rats were fed with different diets for nine weeks. The feces weight of the rats fed with the RS5-diet was 2-3 times larger than that of the rats fed with the other two diets (**Table 4**). The feces collected from the rats fed with the RS5-diet also had larger starch-contents (45.7% in Week 1 - 20.0% in Week 9) than that with the HA7-diet (43.0% in Week 1 - 14.2% in Week 9) and the NCS-diet (0.8% in Week 1 - 0.6% in Week 9). The decreases in the starch contents of feces samples showed that increased proportions of RS5 and HA7 in the diets were utilized *in vivo* as the rats grew older and bigger. The results could be partially attributed to that more RS5 and HA7 were fermented by the gut microflora after a longer feeding-period because of the increase in the population of the microflora. The fermentation of RS was supported by the significantly lower (p <0.05) cecal content pH of the

RS5- and HA7-fed groups (5.55 and 5.53, respectively) than that of the NCS group (7.47) (**Table 5**) (Kishida et al. 2001; Zhao et al. 2011; Ai et al. 2013b). The fermentation of RS5 was also supported by the data that the average feces weight of the rats fed with the RS5-diet increased from 1.17 g (db) to 2.90 g (db) and the starch content of the feces increased from 45.7% to 55.4% when the rats were treated with antibiotics in Week 1 (**Table 4**). The antibiotics, however, did not show significant effects on the weight and starch content of the rat feces of the NCS-fed group because all the NCS was metabolized before reaching the cecum. Furthermore, when the rats stopped drinking water containing antibiotics after Week 1, the weight and starch content of the feces from the two groups of RS5-fed rats gradually became similar, suggesting that the gut microflora might have recovered after the antibiotic treatment was discontinued (**Table 4**). Another factor likely contributing to the increased proportions of RS5 and HA7 utilized *in vivo* could be that the digestive tract of the rats became longer and produced more amylolytic enzymes as the rats grew bigger (Marounek et al. 1995).

The lipid content of the feces from the rats fed with the RS5-diet in Week 1 (26.5%) was significantly greater than that fed with the HA7-diet (12.0%) and NCS-diet (13.1%) (**Table 4**). The lipid content of the feces from the rats fed with the RS5-diet further increased to 52.3% in Week 9, whereas those of the other two groups were consistently low throughout the whole feeding period. Analysis of the FFA compositions of the lipids extracted from the feces by GC showed that the feces lipids from the rats fed with the RS5-diet consisted of mainly SA (C18:0), which ranged from 65.9% to 73.0% during the feeding period (**Table 6**). The lipids extracted from the rat feces of the other two groups, however, contained negligible FFA contents. The results indicated that most of SA in the RS5 was not absorbed and was discharged in the feces, despite the fact that a larger proportion starch of the RS5 was utilized *in vivo* after a longer

feeding period. The low absorption of SA was a result of SA being in a solid, crystalline structure because of its melting temperature of 70.7 °C (**Figure 1**), which was substantially higher than the temperature of the digestive tract of the rats (~37 °C) (Kritchevsky 1994; Berry and Sanders 2005). The palmitic acid (PA, C16:0) and arachidic acid (AA, C20:0) detected in the feces lipids of the RS5-fed rats were contaminants of the SA (≤ 5% PA and ≤ 3% AA, reported by the manufacturer) used for the RS5 preparation.

The percentages of starch not being utilized in the NCS-, HA7- and RS5-diets and the percentage of SA not being utilized in the RS5-diet are summarized in **Table 4** and **6**, respectively. In Week 1, 16.1% of the starch in the RS5-diet was not utilized *in vivo* and was discharged in the rat feces, which was greater than the HA7-diet (6.4%) and the NCS-diet (0.1%). The greater *in vivo* enzymatic resistance of the RS5 was consistent with its larger RS-content analyzed using the AOAC Method 991.43. The percentages of starches not being utilized in Week 1 positively correlated with the RS contents of the diets ($r = 0.999$, $p = 0.02$). With a prolonged feeding-period, increased proportions of starch in the RS5- and HA7-diet were utilized *in vivo*, and the percentages of starch not being utilized decreased to 4.7% and 1.3% in Week 9, respectively (**Table 4**). Most of the SA (68.2%-99.0%) in the RS5, however, remained unabsorbed and was discharged in the feces (**Table 6**). The presence of RS and SA contributed to the largest fecal weight of the rats fed with the RS5-diet (**Table 4**) and increased the hydrophobic property of the feces, which could have enhanced physical interactions with hydrophobic components, including toxic chemicals, and carry them out of the digestive tract of the rats. These features of RS5 could contribute to reduction in toxic constituents in the digestive tract during proliferation of colon lesion cells, although in the study described here the azoxymethane treatment was completed before the rats were fed with different diets and, thus,

the RS5 did not affect the initiation of colon lesions by azoxymethane (Zhao et al. 2011). Different *in vivo* digestive rate of the starches and the absorption of small amount of SA in the RS5, however, did not significantly affect the body-weight gain of the rats (**Table 4**).

4. Conclusions

SA formed single-helical complex with amylose in the RS5 and restricted the swelling of starch granules during cooking. Consequently, the cooked RS5 displayed greater resistance to *in vitro* and *in vivo* enzymatic hydrolysis than the cooked HA7 and NCS. After feeding to the rats for a prolonged period, an increased proportion of the RS5 was utilized *in vivo*. Most of the SA (68%-99%) remained unabsorbed through the feeding period and was discharged in the feces. Large quantities of RS and SA present in the feces contributed to the large fecal bulk of the rats fed with the RS5. Differences in the *in vivo* digestion of the starches, however, did not significantly affect the body-weight gain of the rats.

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Table 1. Composition of rat diet

Ingredient [a]	Weight percentage (%)		
	NCS-diet	HA7-diet	RS5-diet
NCS [b]	55.0	-	-
HA7	-	55.0	-
RS5	-	-	55.0
Casein	20.0	20.0	20.0
Glucose	15.0	15.0	15.0
Mineral mix (AIN-93)	3.5	3.5	3.5
Choline	0.2	0.2	0.2
Methionine	0.3	0.3	0.3
Vitamin mix (AIN-93)	1.0	1.0	1.0
Corn oil	5.0	5.0	5.0
Total	100.0	100.0	100.0
Dry starch content of diet [c]	54.2	53.6	49.1
Stearic acid content of diet [c]	-	-	4.9

[a] The starches and other ingredients were weighed on as-is weight basis.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[c] On the dry basis.

Table 2. Thermal properties of starches used to prepare rat diets [a]

Starch	Gelatinization of starch [b, c]					Dissociation of amylose-lipid complex [b, c]			
	T_o (ºC)	T_{p1} (ºC)	T_{p2} (ºC)	T_c (ºC)	ΔH (J/g)	T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)
First scan									
NCS [d]	67.6 ± 0.3	72.1 ± 0.2	-	77.7 ± 0.2	13.7 ± 0.2	88.8 ± 0.0	96.8 ± 0.0	104.9 ± 0.1	1.0 ± 0.1
HA7	70.1 ± 0.3	74.8 ± 0.4	99.5 ± 0.6	108.1 ± 0.3	11.2 ± 0.1	N.A. [e]	N.A.	N.A.	N.A.
RS5	-	-	-	-	-	95.6 ± 0.5	103.8 ± 0.2	110.8 ± 0.3	9.0 ± 0.2
Rescan									
NCS	-	-	-	-	-	88.3 ± 0.7	93.6 ± 0.1	103.9 ± 0.1	0.9 ± 0.0
HA7	-	-	-	-	-	86.4 ± 0.5	96.3 ± 0.3	106.4 ± 0.5	4.1 ± 0.1
RS5	-	-	-	-	-	86.7 ± 0.0	96.5 ± 0.1	109.4 ± 0.0	8.5 ± 0.5

[a] Measured using differential scanning calorimetry.
[b] T_o: onset temperature, T_p: peak temperature, T_c: conclusion temperature, ΔH: enthalpy change.
[c] Mean ± standard deviation from duplicate.
[d] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[e] Not available because the dissociation peak of amylose-lipid complex (T_{p2}) overlapped with starch gelatinization peak (T_{p1}).

Table 3. Resistant starch (RS) contents of starch varieties and rat diets [a]

Sample [b]	RS content (%, db) [c, d]			
	Uncooked starch	Water-boiled starch	Rat diet [e]	Calculated RS content of diet [f]
NCS	0.8 ± 0.1 c	1.0 ± 0.2 c	2.6 ± 0.2 c	0.5
HA7	33.5 ± 0.3 b	27.3 ± 0.5 b	15.8 ± 0.2 b	14.6
RS5	67.8 ± 0.1 a	64.8 ± 0.7 a	33.7 ± 0.5 a	31.8

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[c] Mean ± standard deviation from duplicate.
[d] Analyzed following AOAC Method 991.43.
[e] Diet was prepared following the formulae shown in Table 1 containing 55% (w/w, as is) cooked starch.
[f] Calculated RS content = RS content of water-boiled starch × starch content of diet, db.

Table 4. Average daily feed-disappearance weight (db), rat body weight, daily feces weight (db), starch and lipid content of rat feces, and percentages of starch not being utilized *in vivo* in different diets [a]

Group [b]		Week 0 [c, d]	Week 1 [c]	Week 3 [c]	Week 6 [c]	Week 9 [c]
Daily feed-disappearance weight (g/rat, db)	NCS	6.04 ± 1.33 a	6.59 ± 0.94 a	7.80 ± 0.66 b	12.50 ± 1.63 a	13.86 ± 1.74 a
	NCS, antibiotic [e]	5.98 ± 0.40 a	6.46 ± 0.42 a	8.18 ± 0.93 ab	12.84 ± 1.09 a	14.17 ± 0.87 a
	HA7	5.95 ± 1.55 a	6.69 ± 1.13 a	8.42 ± 0.58 ab	12.11 ± 1.62 a	14.64 ± 2.51 a
	RS5	5.80 ± 2.61 a	6.77 ± 1.66 a	9.06 ± 1.02 a	12.06 ± 1.00 a	13.94 ± 2.30 a
	RS5, antibiotic [e]	5.29 ± 1.23 a	6.64 ± 0.86 a	8.77 ± 0.46 a	11.82 ± 0.86 a	14.28 ± 0.93 a
Body weight (g/rat)	NCS	106.7 ± 9.7 a	129.6 ± 7.1 a	184.0 ± 6.5 a	235.7 ± 15.4 a	271.8 ± 17.8 a
	HA7	110.1 ± 9.6 a	128.3 ± 9.0 a	178.9 ± 9.3 a	225.2 ± 8.1 a	257.5 ± 12.1 a
	RS5	106.5 ± 12.0 a	122.8 ± 6.8 a	180.3 ± 12.5 a	226.7 ± 7.5 a	258.7 ± 7.1 a
Daily feces weight (g/rat, db)	NCS	0.36 ± 0.17 a	0.40 ± 0.22 c	0.38 ± 0.15 b	0.45 ± 0.13 b	0.53 ± 0.13 b
	NCS, antibiotic [e]	0.32 ± 0.16 a	0.22 ± 0.10 c	0.30 ± 0.14 b	0.51 ± 0.15 b	0.56 ± 0.19 b
	HA7	0.38 ± 0.18 a	0.53 ± 0.12 c	0.75 ± 0.68 b	0.63 ± 0.16 b	0.70 ± 0.13 b
	RS5	0.38 ± 0.13 a	1.17 ± 0.22 b	1.75 ± 0.70 a	1.82 ± 0.28 a	1.62 ± 0.07 a
	RS5, antibiotic [e]	0.37 ± 0.13 a	2.90 ± 0.65 a	1.91 ± 0.33 a	1.73 ± 0.42 a	1.84 ± 0.30 a
Starch content of feces (%, db) [f]	NCS	0.9 ± 0.3 a	0.6 ± 0.0 d	0.7 ± 0.1 d	0.7 ± 0.2 c	0.8 ± 0.1 d
	NCS, antibiotic [e]	0.7 ± 0.2 a	1.1 ± 0.2 d	1.0 ± 0.1 d	0.6 ± 0.1 c	1.2 ± 0.2 d
	HA7	0.5 ± 0.2 a	43.0 ± 0.2 c	35.4 ± 0.2 c	25.0 ± 0.2 b	14.2 ± 0.9 c
	RS5	0.6 ± 0.1 a	45.7 ± 0.3 b	40.1 ± 0.1 b	30.0 ± 0.7 a	20.0 ± 0.2 b
	RS5, antibiotic [e]	0.5 ± 0.1 a	55.4 ± 0.6 a	45.1 ± 0.7 a	31.3 ± 0.6 a	23.2 ± 0.4 a
Lipid content of feces (%, db) [g]	NCS	13.4 ± 0.2 a	13.1 ± 0.0 b	12.6 ± 0.7 b	11.2 ± 0.8 b	12.6 ± 0.1 b
	HA7	13.3 ± 0.4 a	12.0 ± 0.4 b	12.4 ± 0.6 b	12.1 ± 0.9 b	13.6 ± 1.2 b
	RS5	12.8 ± 0.2 a	26.5 ± 0.8 a	29.7 ± 0.2 a	48.7 ± 2.2 a	52.3 ± 2.1 a
Starch not being utilized (%) [h]	NCS	0.1	0.1	0.1	0.0	0.1
	NCS, antibiotic [e]	0.1	0.1	0.1	0.0	0.1
	HA7	0.1	6.4	5.9	2.4	1.3
	RS5	0.1	16.1	15.8	9.2	4.7
	RS5, antibiotic [e]	0.1	49.3	20.0	9.3	6.1

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[c] Mean ± standard deviation.
[d] All rats were fed with NCS-diet in Week 0.
[e] The rats drank water containing vancomycin and imipenem (50 μg/ml of each, ~13 mL/rat/day) in Week 1 only.
[f] Analyzed using a Total Starch Assay Kit following the AACC method 76-13.
[g] Analyzed following the AOAC Method 996.06.
[h] % Starch not being utilized = 100% × [starch content of feces (db) × average daily feces weight (db)] / [starch content of diet (db) × average daily feed-disappearance weight (db)].

Table 5. Average cecum tissue weights, cecal content weights and pH values of different rat groups [a]

Group	Cecum tissue weight (g/rat) [c]	Cecal content weight (g/rat, db) [c]	Cecal content pH [c]
NCS [b]	1.01 ± 0.36 b	0.93 ± 0.18 b	7.47 ± 0.19 a
HA7	2.18 ± 0.53 a	3.42 ± 0.67 a	5.53 ± 0.24 b
RS5	2.65 ± 0.58 a	3.78 ± 0.45 a	5.55 ± 0.16 b

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[c] Mean ± standard deviation from ten rats.

Table 6. Free-fatty-acid contents of lipids extracted from rat feces, and percentages of stearic acid in RS5 not being utilized *in vivo* [a]

Free fatty acid [b]	Content (%) [c]				
	Week 0	Week 1	Week 3	Week 6	Week 9
NCS [d]					
Palmitic acid (C16:0)	0.9 ± 0.1	1.1 ± 0.1 c	0.9 ± 0.1 c	1.1 ± 0.1 c	1.0 ± 0.0 c
Stearic acid (C18:0)	0.6 ± 0.1	1.3 ± 0.1 c	0.9 ± 0.1 c	1.3 ± 0.1 c	1.1 ± 0.0 c
Arachidic acid (C20:0)	0.2 ± 0.0	0.2 ± 0.1 c	0.4 ± 0.1 d	0.5 ± 0.1 d	0.4 ± 0.0 d
HA7 [d]					
Palmitic acid (C16:0)	0.8 ± 0.1	1.1 ± 0.0 c	1.2 ± 0.1 c	0.9 ± 0.1 cd	1.4 ± 0.1 c
Stearic acid (C18:0)	1.0 ± 0.1	0.8 ± 0.1 c	0.9 ± 0.1 c	1.0 ± 0.3 c	1.0 ± 0.0 c
Arachidic acid (C20:0)	0.1 ± 0.0	0.3 ± 0.1 c	0.3 ± 0.0 d	0.4 ± 0.1 d	0.6 ± 0.1 d
RS5 [d]					
Palmitic acid (C16:0)	1.0 ± 0.1	3.4 ± 0.2 b	4.0 ± 0.1 b	4.1 ± 0.1 b	3.8 ± 0.1 b
Stearic acid (C18:0)	0.9 ± 0.1	73.0 ± 1.3 a	71.3 ± 0.1 a	66.0 ± 0.0 a	65.9 ± 0.3 a
Arachidic acid (C20:0)	0.3 ± 0.1	1.1 ± 0.0 c	1.1 ± 0.1 c	1.1 ± 0.1 c	1.1 ± 0.0 c
Stearic acid (C18:0) in RS5 not being utilized (%) [e]	-	68.2	83.5	99.0	81.7

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Analyzed using a capillary gas chromatography.
[c] Mean ± standard deviation from duplicate.
[d] NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.
[e] % Stearic acid not being utilized = 100% × [SA content of feces lipid × lipid content of feces (db) × average daily feces weight (db)] / [SA content of RS5 diet (db) × average daily feed-disappearance weight (db)].

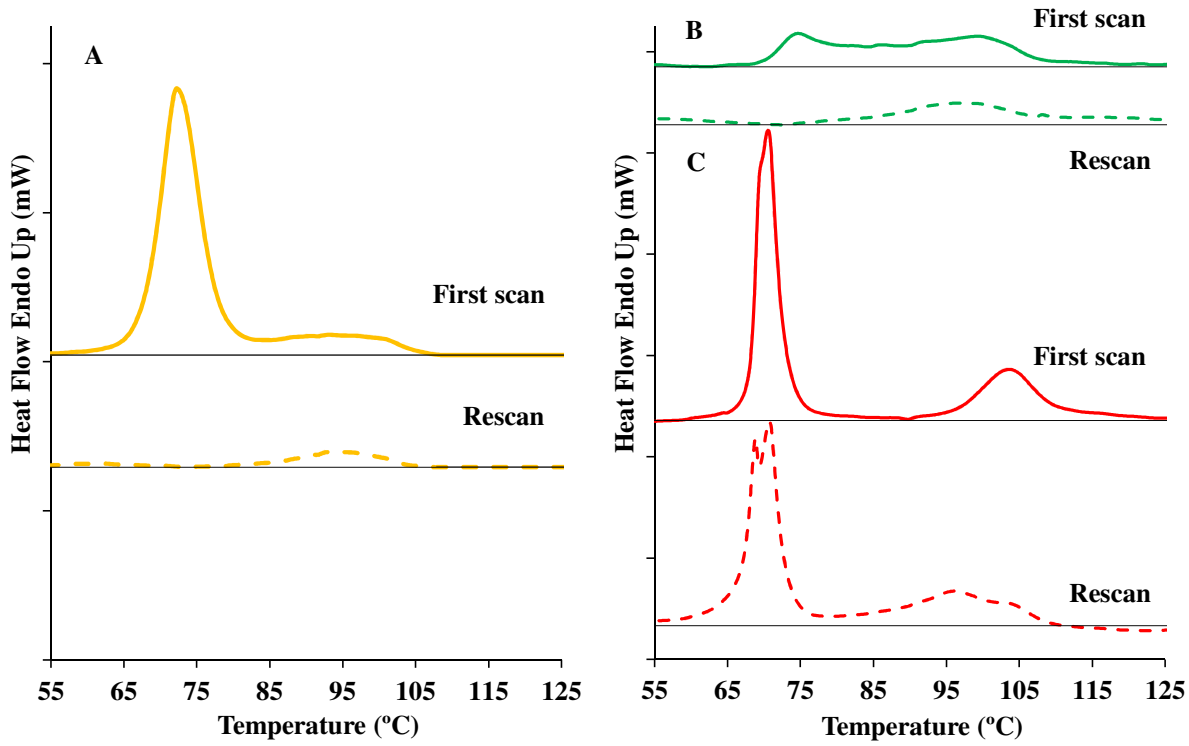

Figure 1. DSC thermograms of different starches: (A) normal corn starch (NCS); (B) high-amylose corn starch (HA7); (C) resistant starch Type 5 (RS5). First scan: the starch sample (~10 mg) was heated with 3× (w/w) water from 10 °C to 150 °C at a rate of 10 °C/min in a stainless steel pan. Rescan: after the first scan, the sample was immediately cooled to 10 °C at 40 °C/min, and then rescanned to 150 °C at a rate of 10 °C/min. The baseline was drawn under each curve.

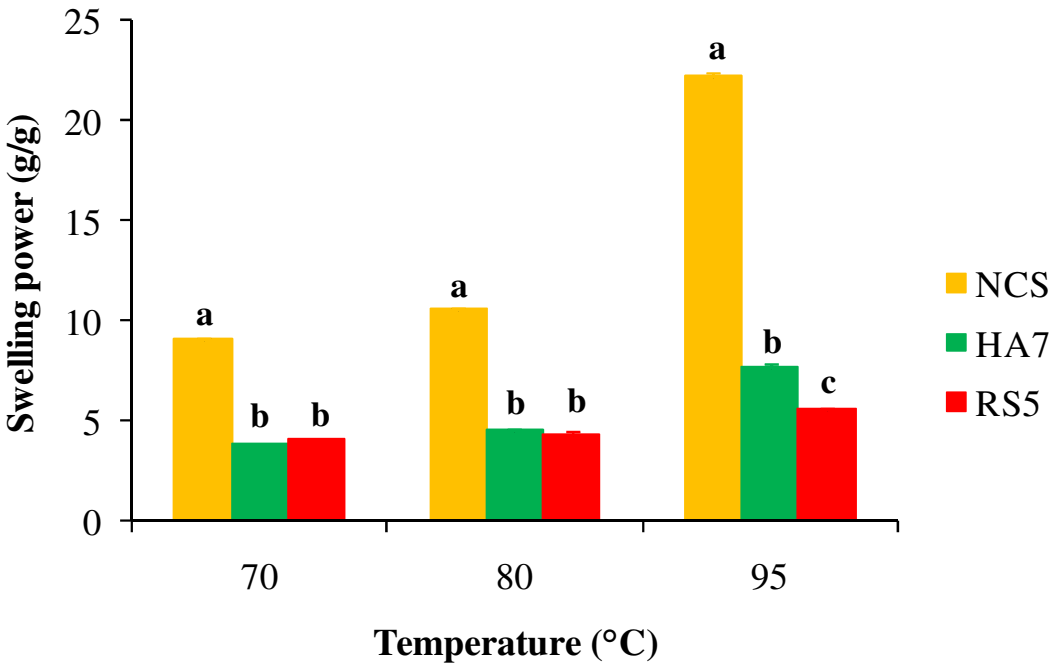

Figure 2. Swelling power of starch in 1% (w/v, db) suspension at different temperatures. Values with the same letter at the same temperature are not significantly different at p <0.05. NCS: normal corn starch, HA7: high-amylose corn starch, RS5: resistant starch Type 5.

CHAPTER 4. *IN VITRO* AND *IN VIVO* DIGESTION OF OCTENYL SUCCINIC STARCH

A paper published in *Carbohydrate Polymers*

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Abstract

This study aimed to understand effects of octenyl succinic anhydride (OSA) modification of normal corn (NCS) and high-amylose corn (HA7) starch on their enzymatic hydrolysis rates. After modification with 3% and 10% OSA, resistant starch (RS) contents of the cooked OS-NCS increased from 0.8% of the control starch to 6.8% and 13.2% (Englyst Method), respectively, whereas that of the cooked OS-HA7 decreased from 24.1% to 23.7% and 20.9%, respectively. When the cooked NCS, HA7 and OS (10%)-HA7 were used to prepare diets for rats at 55% (w/w) starch, RS contents of the diets were 1.1%, 13.2% and 14.6%, respectively. After feeding to the rats, 20.2%-31.1% of the starch in the OS (10%)-HA7-diet was not utilized *in viv*o and was found in rat feces, which was substantially larger than that of the HA7-diet (≤ 4.9%) and NCS-diet (≤ 0.2%). The body weights of the rats, however, remained similar between different groups.

Key words: octenyl succinic starch; resistant starch; *in vivo* digestion of starch; rat body weight

1. Introduction

Starch, one of the most abundant biomasses on earth, is a major energy source for humans and animals. Starch is composed of two types of glucans: amylose and amylopectin. Amylose is an essentially linear molecule with α-1, 4 linked D-glucose units and a few branches of α-1, 6 linkages, whereas amylopectin is a highly-branched molecule with about 5% α-1, 6 branch linkages (Hizukuri, Takeda, Yasuda & Suzuki, 1981). Native starch is commonly modified using chemical, physical, and enzymatic methods to improve its functional properties for different applications. One of the common modified starches used in the food industry is octenyl succinic (OS) starch, which is prepared using a chemical reaction of starch with octenyl succinic anhydride (OSA) (Caldwell & Wurzburg, 1953; Bai & Shi, 2011). The starch derivatized with the OS groups has amphiphilic properties and, thus, is suitable for various applications, including emulsification (Chiu, 1990; Woo, Maningat & Bassi, 2004; Nilsson & Bergenstahl, 2007), encapsulation (Morehouse, 1994; Drusch & Schwarz, 2006; Murua-Pagola, Beristain-Guevara & Martinez-Bustos, 2009), and as a fat replacement (Sarneel, Peremans & Jonckers, 2008; Chung, Lee, Han & Lim, 2010).

OSA modification of starch has been used to produce resistant starch (RS) (Han & BeMiller, 2007; He, Liu & Zhang, 2008). RS refers to a portion of starch that is resistant to the enzymatic hydrolysis in the small intestine and passed into the colon to be fermented by the gut microflora (Englyst, Wiggins & Cummings, 1982; Englyst & Macfarlane, 1986). RS has been classified into five different types: physically inaccessible starch (RS1), native granular starch with the B- or C-type crystalline structure (RS2), retrograded starch (RS3), chemically-modified starch (RS4) and amylose-lipid complex (RS5) (Englyst, Kingman & Cummings, 1992; Muir,

Young & Odea, 1994; Hasjim, Lee, Hendrich, Setiawan, Ai & Jane, 2010). The RS derived from OSA modification is categorized as the RS4. Waxy corn starch modified with OSA (3%, dry starch basis, dsb) followed by a heat-moisture treatment resulted in a substantially less postprandial plasma-glucose response than the unmodified starch after being ingested by human subjects (He, Liu & Zhang, 2008).

A recent human-feeding study showed that ingestion of bread made with RS5, a free-fatty-acid complexed high-amylose corn starch (HA7), resulted in significantly less postprandial plasma-glucose and insulin responses than ingestion of bread made with normal wheat flour, suggesting that the RS5 was more resistant to *in vivo* digestion than starch in the wheat flour (Hasjim, Lee, Hendrich, Setiawan, Ai & Jane, 2010). In addition, the cooked RS5 diet effectively inhibited azoxymethane (AOM)-induced preneoplastic lesions (colon cancer precursors) in rat colon, suggesting that the RS5 suppressed colon carcinogenesis (Zhao, Hasjim, Li, Jane, Hendrich & Birt, 2011). The inhibitory effects of the RS5 on the development of preneoplastic lesions could be attributed to both the enzymatic resistance and hydrophobic property of the RS5. The RS5 with hydrophobic property could physically interact with other hydrophobic chemicals, including toxic compounds, and carry them out of the digestive tract of rats.

To test this hypothesis, we selected OS starch for a rat-feeding study because of its enzymatic resistance and hydrophobic property. The objective of the present study was to understand effects of OSA modification of normal corn (NCS) and high-amylose corn (HA7) starch on their enzymatic hydrolysis rates. The *in vivo* digestion of the OS-HA7 was compared with that of the NCS and HA7 in a rat-feeding study. Thermal and pasting properties of the modified starches were also characterized, and their relationships with the enzymatic hydrolysis of the starches were elucidated.

2. Materials and methods

2.1. Materials

Normal corn starch (NCS, Cargill Gel ™, 34.3% amylose) and high-amylose corn starch (HA7, AmyloGel ™, 68.4% amylose) were purchased from Cargill Inc. (Minneapolis, MN). 2-Octen-1-ylsuccinic anhydride (OSA), porcine pancreatin and amyloglucosidase from *Aspergillus Niger* were purchased from Sigma-Aldrich Co. (St. Louis, MO). Casein, glucose, mineral mix (AIN-93), choline, methionine, vitamin mix (AIN-93) and corn oil used to prepare diets for rats were purchased from Harlan Laboratories (Madison, WI). Five-week-old male Fisher-344 rats were purchased from Charles River Laboratories (Wilmington, MA). D-Glucose Assay Kit and Total Starch Assay Kit were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland).

2.2. Preparation of OS-starch

NCS and HA7 were modified with 3% and 10% (w/w, dry starch basis, dsb) OSA following the method of Zhang et al. (2011). The starch was suspended in distilled water at 35% (w/w, dsb). The pH of the starch suspension was adjusted to 8.0 using a sodium hydroxide aqueous solution (3%, w/w), and the temperature was maintained at 35 °C. OSA was added dropwise to the starch suspension while maintaining the pH at 8.0 and 35 °C. After the reaction was completed, the pH of the starch suspension became stable and was then adjusted to 6.5 using hydrochloric acid (1.0 M). The starch was recovered using centrifugation, washed twice with distilled water and twice with 100% ethanol, dried at 37 °C and then ground. Each of the starches subjected to the same processing without addition of OSA was used as the control.

2.3. Degree of substitution and reaction efficiency

The degree of substitution (DS) of the OS-starch was determined using a titration method (Bai & Shi, 2011). Reaction efficiency (RE) of the starch modification was calculated as: % RE = 100% × (mass percentage of OS group in the OS-starch / mass percentage of OSA added to the starch for modification). The analysis was done in duplicate. The mass of the OS group was excluded from the mass of the OS-starch when the OS-starch was used on the dry starch basis for other analyses in this study.

2.4. Thermal properties of starch

Thermal properties of the OS-starch and the control starch were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT) (Hasjim, Lee, Hendrich, Setiawan, Ai & Jane, 2010). The starch (~10 mg, dsb) was mixed with distilled-deionized water (3×, w/w, dsb) and heated from 10 °C to 150 °C at a rate of 10 °C/min. After the first thermal scan, the sample was cooled down at 40 ℃/min and immediately rescanned to confirm the dissociation peak of amylose-lipid complex (ALC). The enthalpy change (ΔH) of the thermal transition was calculated on the dry starch basis. The analysis was done in duplicate.

2.5. Pasting properties of starch

Pasting properties of the OS-starch and the control starch were analyzed using a Rapid Visco-Analyzer (RVA, Newport Scientific, Sydney, Australia). Each starch sample (2.24 g, dsb; equivalent to dry weight of 2.24 g, 2.29 g, 2.37 g, 2.30 g and 2.41g for the control starch, OS (3%)-NCS, OS (10%)-NCS, OS (3%)-HA7 and OS (10%)-HA7, respectively) was suspended in distilled-deionized water or 0.5% (w/w) sodium chloride solution to reach a total weight of 28.0 g and analyzed following the program reported by Ai et al. (2011). The analysis was done in duplicate.

2.6. RS content of starch

RS contents of the raw and cooked OS-starch and the control starch were analyzed using the Englyst Method (Englyst, Kingman & Cummings, 1992) with modifications (Li, Jiang, Campbell, Blanco & Jane, 2008). The starch sample (1.0 g, dsb; equivalent to dry weight of 1.0 g, 1.02 g, 1.06 g, 1.03 g and 1.07 g for the control starch, OS (3%)-NCS, OS (10%)-NCS, OS (3%)-HA7 and OS (10%)-HA7, respectively), with or without cooking, was hydrolyzed *in vitro* using porcine pancreatin extract and amyloglucosidase in a shaker water-bath (37 °C and 80 rpm). The concentrations of glucose released from the starch at time intervals of 20 min and 120 min were quantified using a D-Glucose Assay Kit containing glucose oxidase and peroxidase (GOPOD). Rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS contents of the starch samples were calculated on the dry starch basis (Englyst, Kingman & Cummings, 1992). The analysis was done in duplicate.

2.7. Diet preparation for rats

The NCS, HA7 and OS (10%)-HA7 were used to prepare rat diets with 55% (w/w, as-is weight basis) starch, 20% casein, 5% corn oil, and other ingredients as shown in **Table 1**, following the method of Zhao et al. (2011). Each starch sample was boiled with distilled water (3×, w/w, dsb) under constant manual stirring. After cooling to room temperature, the cooked starch paste was thoroughly mixed with other ingredients to prepare rat diet (**Table 1**). Starch contents of the NCS, HA7 and OS (10%)-HA7-diet on the dry basis (db) were 54.2%, 53.6% and 51.2%, respectively (**Table 1**). The mass of the OS group was excluded from the mass of the OS (10%)-HA7 for the dry starch content calculation. RDS, SDS and RS contents of each diet were analyzed as described earlier. The analysis was done in triplicate.

2.8. Rat feeding and feces collection

Thirty male Fisher-344 rats (five-week-old) were received and housed as described by Zhao et al. (2011). All the rats were fed with the NCS-diet for the first two weeks after arriving at the facility (Week 0), and then fed with respective diets (10 rats/diet) for additional nine weeks. The first week when the rats were fed with selected diets was designated as "Week 1." The diets were fed to the rats with high moisture contents, which ranged from 49.3% to 59.7% of the freshly prepared diets, and from 35.4% to 52.3% of the diets after 2 days of feeding on the cage top rack. The daily feed-disappearance weights were calculated on the dry diet basis: daily feed-disappearance weight (db) = (weight of diet given to the rat, db – weight of diet recovered after 2 days, db) / 2. On the last day of each week, the body weight of each rat was recorded, and the feces discharged by each rat was collected from the bottom of the cage, weighed, and stored at -20 °C before analysis. The animal study was performed in compliance with the guidelines of the Institutional Animal Care and Use Committee.

2.9. Starch content of feces

The feces collected in the same week from the rats fed with the same diet was combined, dried at 45 °C, and ground before analysis. The starch content of the feces was determined using a Total Starch Assay Kit following the AACC Method 76-13 (2000) with modifications. Because the OS derivatives of the modified starch interfered with the enzymatic hydrolysis of starch, it produced less glucose and resulted in a less starch content using the standard method. Thus, the feces (~100 mg) from the rats fed with the OS (10%)-HA7-diet was dispersed in 2.0 mL sodium hydroxide aqueous solution (0.5 M) for 0.5 h to completely remove the OS derivatives of the starch before the analysis of starch content. The alkali-treated feces dispersion was neutralized using hydrochloric acid (0.5 M), and the starch was hydrolyzed to glucose using thermostable α-

amylase and amyloglucosidase and then quantified using the GOPOD method. To prevent any discrepancy caused by different methods used for the analysis, starch contents of the feces from rats fed with the other two diets were also measured using the same method. The analysis was done in duplicate. The percentage of starch not being utilized by the rats and their gut microflora was calculated as: % starch not being utilized = 100% × (starch content of feces, db × average daily feces weight, db) / (starch content of diet, db × average daily feed-disappearance weight, db).

2.10. Lipid content of feces

Lipid was extracted from the feces and quantified following the AOAC Method 996.06 (2000). The analysis was done in duplicate.

2.11. Cecum tissue weight, cecal content weight and pH

The cecum tissue weight, cecal content weight and pH were measured for each rat after the rats were sacrificed at the end of the feeding study (Kishida, Nogami, Himeno & Ebihara, 2001; Zhao, Hasjim, Li, Jane, Hendrich & Birt, 2011).

2.12. Statistical analysis

Statistical significance was analyzed using one-way ANOVA and multiple comparison test with Tukey's adjustment at p value <0.05. The statistical analyses were conducted in SAS (Version 9.2, SAS Institute, Inc., Cary, NC).

3. Results and discussion

The NCS and HA7 were modified with 3% and 10% (w/w, dsb) OSA to assess suitable OS-starch for the rat-feeding study. The DS and RE of the NCS and HA7 modified with 3% and 10% (w/w, dsb) OSA are shown in **Table 2**. With 3% OSA, the DS of OS-NCS and OS-HA7

were 0.019 and 0.022, respectively, and the RE were 78.9% and 92.2%, respectively. With 10% OSA, the DS of OS-NCS and OS-HA7 increased to 0.045 and 0.058, respectively, but the RE decreased to 55.0% and 69.4%, respectively. The decreased RE results with increased OSA concentrations were consistent with previously reported data because there was more OSA hydrolyzed into OS acid at a higher reagent concentration (Song, He, Ruan & Chen, 2006; Bai & Shi, 2011). With the same concentration of OSA, the DS and RE of the OS-HA7 were greater than that of the OS-NCS. The reaction with OSA primarily occurred in the amorphous structures of starch granules, and there was a larger proportion of amorphous amylose present in the HA7 than the NCS (He, Song, Ruan & Chen, 2006; Kuakpetoon & Wang, 2006).

Thermal properties of the control and the OS-starches are shown in **Table 3**. After modification with 3% and 10% OSA, onset gelatinization temperatures (T_o) and ΔH of the modified NCS and HA7 substantially decreased, indicating that the OS derivatives destabilized the double helices of starch molecules (Bao, Xing, Phillips & Corke, 2003; Han & BeMiller, 2007). In addition, the second peak temperature of HA7 (T_{p2}), which corresponded to the dissociation of amylose-lipid complex (ALC) (Jiang, Lio, Blanco, Campbell & Jane, 2010; Ai, Hasjim & Jane, 2013), decreased from 99.8 °C to 91.9 °C with 3% OSA modification and disappeared with 10% OSA modification. When the starch samples were rescanned using the DSC, the ΔH of the ALC dissociation peak of the OS-NCS decreased from 1.1 J/g of the control starch to 0.7 J/g and 0.6 J/g with 3% and 10% OSA modification, respectively, and that of the OS-HA7 decreased from 4.0 J/g to 3.4 J/g and 1.2 J/g, respectively. These results indicated that the OS derivatives interfered with the ALC formation, which were consistent with the results reported for acetyl and hydroxypropyl starches (Eliasson, 1994; Liu, Arntfield, Holley & Aime, 1997).

Pasting properties of the control and the OS-starches suspended in distilled-deionized-water medium are shown in **Figure 1**. After the OSA modification, the pasting temperatures of the modified NCS and HA7 decreased, but their peak and final viscosities increased. When a NaCl solution (0.5%) was used as the medium, the viscosities of the unmodified NCS and HA7 control samples remained the same, whereas that of the OS-NCS and OS-HA7 decreased. The pasting temperature and peak viscosity of the 3% OSA modified starch became similar to that of the respective control starch. The results indicated that the remarkably higher viscosities of the OS-starches were caused by the repelling between negative charges of the OS groups.

RDS, SDS and RS contents of the raw and cooked control and OS-starches analyzed using the Englyst Method are shown in **Table 4**. Compared with the respective control starch, the RDS contents of the raw OS-NCS, raw OS-HA7 and cooked OS-NCS decreased, but their RS contents increased, suggesting that the OS derivatives interfered with enzymatic hydrolysis of starch (Han & BeMiller, 2007; He, Liu & Zhang, 2008; Zhang, Huang, Luo, Fu, Jiang & Jane, 2011). After cooking, the 3% and 10% OS-HA7, however, displayed increased RDS contents from 71.7% of the control starch to 75.7% and 78.0%, respectively, but decreased RS contents from 24.1% to 23.7% and 20.9%, respectively. The reduced RS contents of the OS-HA7 samples were attributed to that the modified HA7 had lower gelatinization temperatures (T_c decreased from 108.1 °C to 105.2 °C and 95.3 °C, respectively, **Table 3**) and less ALC formation (**Table 3**). The OS-HA7 samples were gelatinized to a greater extent and more swollen after cooking (**Figure 1**). Consequently, the OS-HA7 became more susceptible to enzymatic hydrolysis than the HA7 control.

The RS contents of the diets containing boiled starch (51.2%-54.2%, dsb) (**Table 1**), mixed with casein and corn oil, are shown in **Table 4**. Among the diets, the determined RS

content of the OS (10%)-HA7-diet (14.6%) was remarkably larger than the RS content (10.7%) calculated on the basis of weight percentage of starch in the diet (**Table 4**). This substantial difference could be attributed to that the OS (10%)-HA7 carried hydrophobic octenyl groups, which physically interacted with the hydrophobic moiety of casein and corn oil present in the diet and became glomerate to further reduce the susceptibility of the starch to enzymatic hydrolysis.

Average daily feed-disappearance weights, body weights, daily feces weights, starch and lipid contents of the feces of rats fed with different diets are shown in **Table 5**. The daily feed-disappearance weights could be larger than the amount of feed ingested by the rats because some feed fell to the bottom of the cage and could not be recovered. In Week 0, all the rats were fed with the NCS-diet, and, thus, there were no significant differences in the feces weight between different groups. Little starch (0.3%-1.4%) was detected in the feces in Week 0, indicating that almost all the cooked NCS was digested *in vivo*. The results were in agreement with the very small RS content of the NCS-diet (1.1%, **Table 4**) determined using *in vitro* analysis.

Starting from Week 1, the rats were fed with different diets for a total of nine weeks. During the feeding period, it was observed that the feces weight of the rats fed with the OS-HA7-diet was 3-5 times larger than that of the rats fed with the other two diets (**Table 5**). The feces from the OS-HA7-fed rats also had consistently larger starch contents (48.9%-57.5%, w/w, db). These data indicated that the OS-HA7 was substantially more resistant to *in vivo* digestion compared with the NCS and HA7. On the basis of the feces weight and the starch content, it was calculated that 20.2% -31.1% starch in the OS-HA7-diet was not utilized *in vivo* (**Table 5**). After the rats were sacrificed, it was observed that the rats fed with the OS-HA7-diet had larger cecum tissue weight (3.00 g/rat) and cecal content weight (4.25 g/rat, db) than that fed with the NCS-

diet (0.92 g/rat and 1.05 g/rat, respectively) and HA7-diet (1.90 g/rat and 3.55 g/rat, respectively) (**Table 6**). Despite the large quantity of OS-HA7 starch not being utilized during the feeding period, the body-weight gain of the rats fed with the OS-HA7 was not significantly different from that of the rats fed with the other diets (**Table 5**).

The starch content of the feces from the rats fed with the HA7-diet decreased from 44.1% in Week 1 to 23.2% in Week 9 (**Table 5**), indicating increased proportion of the HA7 starch was utilized *in vivo* as the rats grew older and bigger. The results could be attributed to two factors: (1) the digestive tract grew longer and produced more amylolytic enzymes as the rats grew bigger and more mature (Marounek, Vovk & Skrivanova, 1995); (2) the population of the gut microflora that could utilize the HA7 increased with the increasing feeding time and was able to ferment HA7 more efficiently. The fermentation of the HA7 was evidenced by the significantly lower (p <0.05) cecal content pH (5.33) than that of the NCS-fed group (7.53) (**Table 6**) (Kishida, Nogami, Himeno & Ebihara, 2001; Zhao, Hasjim, Li, Jane, Hendrich & Birt, 2011). The slightly higher pH of cecal content (5.79) and consistently larger starch contents of the feces from the rats fed with the OS-HA7-diet suggested that the OS-HA7 was less fermentable than the HA7 (**Table 5** and **6**).

The calculated percentage of the OS-HA7 starch that was not utilized by the rats and their gut microflora (20.2%-31.1%, dsb, excluding the weight of OS group) on the basis of feed-disappearance weight was substantially larger than that of the HA7-diet (≤ 4.9%) and NCS-diet (≤ 0.2%) (**Table 5**). The percentage of starch in the OS-HA7-diet not being utilized *in vivo* was much larger than the RS content analyzed *in vitro* using the Englyst Method (14.6%, **Table 4**). The differences between the *in vivo* and *in vitro* results could be attributed to the glomeration formed between the hydrophobic octenyl groups and other hydrophobic components in a

concentrated matrix in the digestive tract (~20% solid content compared with 4% used for *in vitro* analysis, the Englyst Method), which made the starch physically less accessible to enzymatic hydrolysis. This was supported by the substantially larger lipid content of the feces from the OS-HA7 group (**Table 5**). It was also plausible that the octenyl groups of the OS-HA7 had a greater affinity interacting with the hydrophobic surface of the digestive tract and further hampered its susceptibility to enzymatic hydrolysis. The hydrophobic interactions between the octenyl groups of the OS-HA7 and other hydrophobic compounds in the digestive tract could facilitate the removal of those compounds, including toxins, out of the digestive tract and improve the gut health. It was intriguing that the rats fed with OS-HA7-diet maintained similar body-weight gain, despite 20.2%-31.1% starch not being utilized. The rats fed with the OS-HA7-diet appeared to be calmer by casual observation than rats fed with the other two diets. It has been reported that other types of dietary fiber (*e.g.*, sugar beet pulp and wheat bran) also reduce physical activities of animals (Ramonet, Robert, Aumaitre, Dourmad & Meunier-Salaun, 2000; Rijnen, Verstegen, Heetkamp, Haaksma & Schrama, 2003). More studies are needed to understand what caused the metabolic change for the rats fed with the OS-HA7-diet.

4. Conclusions

In this study, effects of OSA modification of the NCS and HA7 on their enzymatic hydrolysis were investigated. After the reaction with 3% and 10% OSA, RS content of the cooked OS-NCS increased, whereas that of the cooked OS-HA7 decreased. In contrast to the RS contents obtained by the *in vitro* analysis, the rat-feeding study showed that 20.2%-31.1% of the cooked OS (10%)-HA7 was not utilized *in vivo* and was found in the feces, which was

substantially larger than that of the cooked HA7 (≤ 4.9%) and NCS (≤ 0.2%). Different *in vivo* digestion of the starches, however, did not significantly affect the body-weight gain of the rats.

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Table 1. Compositions of rat diets

Ingredient	Weight percentage (%)		
	NCS-diet	HA7-diet	OS (10%)-HA7-diet
NCS [a, b]	55.0	-	-
HA7	-	55.0	-
OS (10%)-HA7	-	-	55.0
Casein	20.0	20.0	20.0
Glucose	15.0	15.0	15.0
Mineral mix (AIN-93)	3.5	3.5	3.5
Choline	0.2	0.2	0.2
Methionine	0.3	0.3	0.3
Vitamin mix (AIN-93)	1.0	1.0	1.0
Corn oil	5.0	5.0	5.0
Total	100.0	100.0	100.0
Starch content of dry diet [c]	54.2	53.6	51.2

[a] The ingredients were all weighed on as-is weight basis.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, OS (10%)-HA7: HA7 modified with 10% (w/w, dsb) OSA.
[c] Dry starch basis, excluding the mass of chemical derivatives, OS group.

Table 2. Degree of substitution (DS) and reaction efficiency (RE) of normal corn (NCS) and high-amylose corn (HA7) starch modified with 3% or 10% (w/w, dsb) octenyl succinic anhydride (OSA)

Starch	DS [a]	RE (%) [b]
OS (3%)-NCS	0.019 ± 0.000	78.9 ± 0.6
OS (10%)-NCS	0.045 ± 0.001	55.0 ± 1.6
OS (3%)-HA7	0.022 ± 0.000	92.2 ± 0.6
OS (10%)-HA7	0.058 ± 0.002	69.4 ± 1.9

[a] Determined using a titration method (Bai & Shi, 2011).
[b] % RE = 100% × (mass percentage of OS group in the OS-starch / mass percentage of OSA added to the starch for modification).

Table 3. Thermal property of normal corn (NCS) and high-amylose corn (HA7) starch modified with 3% or 10% (w/w, dsb) octenyl succinic anhydride (OSA) [a]

Starch	Gelatinization of starch [b]					Dissociation of amylose-lipid complex in rescan [b]			
	T_o (ºC)	T_{p1} (ºC)	T_{p2} (ºC)	T_c (ºC)	ΔH (J/g)	T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)
NCS, control	65.5 ± 0.2	70.3 ± 0.1	-	74.8 ± 0.1	12.9 ± 0.1	88.9 ± 0.2	95.5 ± 0.0	101.3 ± 0.2	1.1 ± 0.2
OS (3%)-NCS	62.6 ± 0.2	68.9 ± 0.1	-	74.2 ± 0.6	12.4 ± 0.8	89.5 ± 0.0	95.3 ± 0.2	100.7 ± 0.1	0.7 ± 0.1
OS (10%)-NCS	60.7 ± 1.1	69.4 ± 0.1	-	77.2 ± 0.1	11.6 ± 0.5	88.1 ± 0.3	95.4 ± 0.1	100.5 ± 0.8	0.6 ± 0.2
HA7, control	70.1 ± 0.0	74.8 ± 0.4	99.8±0.1	108.1 ± 0.0	11.8 ± 0.2	86.2 ± 0.1	95.2 ± 0.2	106.5 ± 0.7	4.0 ± 0.1
OS (3%)-HA7	67.1 ± 0.2	74.0 ± 0.1	91.9±0.1	105.2 ± 0.5	11.5 ± 0.1	78.6 ± 0.6	91.6 ± 0.6	102.9 ± 0.8	3.4 ± 0.0
OS (10%)-HA7	61.7 ± 0.8	71.3 ± 0.8	-	95.3 ± 2.8	10.5 ± 0.5	73.0 ± 0.1	82.4 ± 0.0	96.6 ± 1.0	1.2 ± 0.1

[a] Measured using differential scanning calorimetry.
[b] T_o: onset temperature, T_p: peak temperature, T_c: conclusion temperature, ΔH: enthalpy change.

Table 4. Rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) contents of different starches and diets analyzed using the Englyst Method

Sample [a]	Raw starch			Cooked starch			Diet [b]			
	RDS (%)	SDS (%)	RS (%)	RDS (%)	SDS (%)	RS (%)	RDS (%)	SDS (%)	RS (%)	Calculated RS (%) [c]
NCS, control	20.7 ± 0.3	42.3 ± 1.0	36.9 ± 0.8	97.2 ± 0.4	2.0 ± 0.2	0.8 ± 0.2	51.8 ± 0.5	1.3 ± 0.5	1.1 ± 0.5	0.4
OS (3%)-NCS	12.2 ± 0.4	37.3 ± 0.9	50.5 ± 1.3	90.1 ± 1.3	3.1 ± 1.1	6.8 ± 0.2	-	-	-	-
OS (10%)-NCS	9.0 ± 0.1	22.9 ± 0.2	68.1 ± 0.3	83.9 ± 1.0	3.0 ± 0.4	13.2 ± 1.4	-	-	-	-
HA7, control	14.9 ± 0.4	13.3 ± 0.6	71.7 ± 0.2	71.7 ± 1.4	4.2 ± 0.0	24.1 ± 1.4	35.1 ± 0.1	5.2 ± 0.3	13.2 ± 0.4	12.9
OS (3%)-HA7	13.5 ± 0.2	11.8 ± 0.6	74.6 ± 0.4	75.7 ± 0.3	0.6 ± 0.0	23.7 ± 0.3	-	-	-	-
OS (10%)-HA7	10.2 ± 0.2	9.9 ± 0.0	79.8 ± 0.2	78.0 ± 0.8	1.1 ± 0.1	20.9 ± 0.7	34.2 ± 0.4	2.4 ± 0.5	14.6 ± 0.6	10.7

[a] NCS: normal corn starch, HA7: high-amylose corn starch, OS: octenyl succinic.
[b] Diet was prepared following the formulae shown in Table 1. The NCS, HA7 and OS (10%)-HA7-diet contained 54.2%, 53.6% and 51.2% starch on the dry basis, respectively.
[c] Calculated RS content = RS content of cooked starch, dsb × starch content of diet, db.

Table 5. Average daily feed-disappearance weights, body weights, daily feces weights, starch and lipid contents of feces of rats fed with different diets [a]

Group		Week 0 [c]	Week 1	Week 3	Week 6	Week 9
Daily feed-disappearance weight (g/rat, db)	NCS [b]	5.58 ± 1.00 a	6.31 ± 1.09 a	7.60 ± 0.13 b	13.79 ± 0.90 a	15.00 ± 1.25 ab
	HA7	6.47 ± 1.28 a	7.21 ± 1.26 a	8.84 ± 0.23 a	13.52 ± 0.59 a	16.68 ± 1.56 a
	OS (10%)-HA7	6.92 ± 0.68 a	7.51 ± 0.67 a	8.56 ± 0.87 a	10.56 ± 1.66 b	14.51 ± 0.59 b
Body weight (g/rat)	NCS	107.7 ± 13.1 a	129.1 ± 11.2 a	183.6 ± 7.2 a	239.6 ± 7.4 a	276.8 ± 15.5 a
	HA7	108.3 ± 10.3 a	127.3 ± 10.2 a	178.6 ± 10.5 a	227.4 ± 12.8 b	263.8 ± 11.7 a
	OS (10%)-HA7	106.2 ± 12.6 a	128.2 ± 12.1 a	177.7 ± 12.4 a	228.8 ± 11.8 ab	265.7 ± 10.2 a
Daily feces weight (g/rat, db)	NCS	0.43 ± 0.22 a	0.52 ± 0.22 b	0.49 ± 0.12 b	0.55 ± 0.09 b	0.56 ± 0.11 b
	HA7	0.42 ± 0.30 a	0.43 ± 0.07 b	0.44 ± 0.10 b	0.68 ± 0.07 b	0.67 ± 0.12 b
	OS (10%)-HA7	0.55 ± 0.32 a	1.77 ± 0.26 a	2.46 ± 0.33 a	2.77 ± 0.51 a	2.75 ± 0.59 a
Starch content of feces (%, db) [d]	NCS	1.1 ± 0.2	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.2	0.8 ± 0.1
	HA7	1.4 ± 0.7	44.1 ± 0.5	40.5 ± 0.5	32.5 ± 0.5	23.2 ± 0.5
	OS (10%)-HA7	0.3 ± 0.1	48.9 ± 1.3	55.4 ± 0.5	57.5 ± 0.1	54.6 ± 0.5
Starch not being utilized (%) [e]	NCS	0.2	0.1	0.1	0.1	0.1
	HA7	0.2	4.9	3.8	3.0	1.7
	OS (10%)-HA7	0.0	22.5	31.1	29.5	20.2
Lipid content of feces (%, db) [f]	NCS	6.2 ± 0.4	6.3 ± 0.4	9.9 ± 0.5	10.4 ± 0.2	12.5 ± 0.6
	HA7	7.3 ± 0.2	4.7 ± 0.8	5.2 ± 0.1	6.8 ± 0.4	6.4 ± 0.6
	OS (10%)-HA7	6.5 ± 0.4	13.8 ± 0.2	11.3 ± 0.4	11.9 ± 1.0	12.7 ± 1.1

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, OS (10%)-HA7: HA7 modified with 10% (w/w, dsb) OSA.
[c] Rats in different groups were all fed with NCS-diet in Week 0.
[d] Analyzed using a Total Starch Assay Kit with modifications.
[e] % Starch not being utilized = 100% × (starch content of feces, db × average daily feces weight, db) / (starch content of diet, db × average daily feed-disappearance weight, db).
[f] Analyzed following the AOAC Method 996.06.

Table 6. Cecum tissue weights, cecal content weights and pH values of rats fed with different diets [a]

Group	Cecum tissue weight (g/rat)	Cecal content weight (g/rat, db)	Cecal content pH
NCS [b]	0.92 ± 0.08 c	1.05 ± 0.15 b	7.53 ± 0.22 a
HA7	1.90 ± 0.33 b	3.55 ± 0.75 a	5.33 ± 0.10 c
OS (10%)-HA7	3.00 ± 0.60 a	4.25 ± 0.20 a	5.79 ± 0.09 b

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] NCS: normal corn starch, HA7: high-amylose corn starch, OS (10%)-HA7: HA7 modified with 10% (w/w, dsb) OSA.

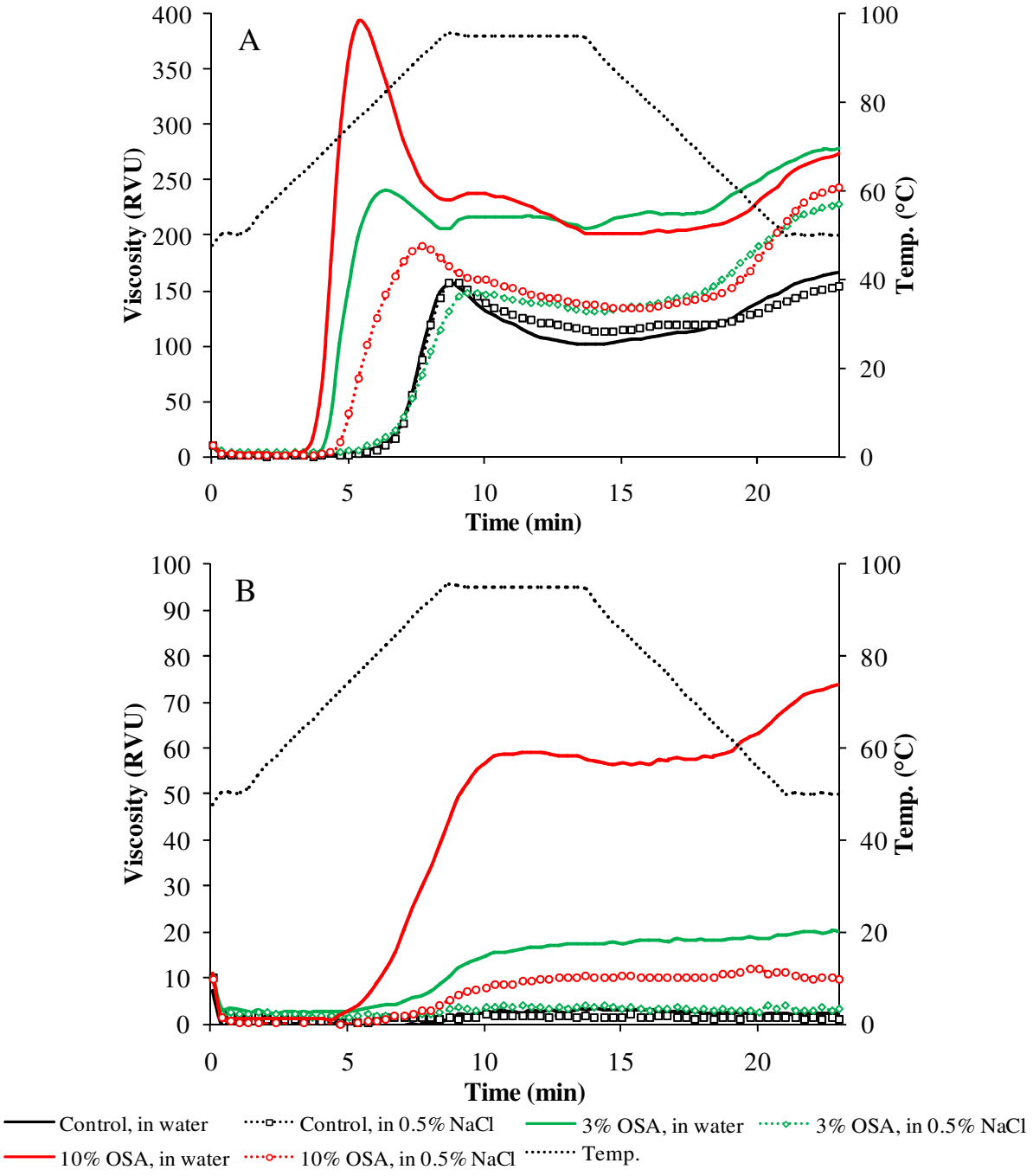

Figure 1. Pasting properties of normal corn (A) and high-amylose corn starch (B) modified with 3% or 10% (w/w, dsb) octenyl succinic anhydride (OSA). Pasting profiles of the starches were analyzed using a Rapid Visco-Analyzer with 8% (w/w, dsb) starch suspension (28.0 g total weight) in distilled-deionized water or 0.5% (w/w) sodium chloride solution.

CHAPTER 5. STARCH CHARACTERIZATION AND ETHANOL PRODUCTION OF SORGHUM

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Abstract

This study aimed to characterize and compare the chemical structure, physical property, and enzymatic hydrolysis rate of five sorghum starches (6B73, 6C21, 6C69, 7R34, and X789) with that of corn starch (B73). Sorghum kernels consisted of 68.7-70.6% starch, more than the B73 corn (67.4%). Sorghum starches displayed higher gelatinization temperatures (66.6-67.4 °C), greater gelatinization enthalpy changes (13.0-14.0 J/g) and percentages retrogradation (60.7-69.1%), but slower enzymatic hydrolysis rates (83.8-87.8% at 48 h) than the B73 corn starch (61.7 °C, 10.1 J/g, 51.5%, and 88.5%, respectively). These differences could result from that the sorghum amylopectins consisted of fewer short branch-chains (DP 6-12) (12.8-14.0%) than the corn amylopectin (15.0%). The sorghum starches showed greater peak- and breakdown-viscosities but lower setback-viscosities than the B73 corn starch, resulting from less amylose content of the sorghum starches. After 96 h fermentation, most ground sorghums exhibited less ethanol yields (30.5-31.8%) than the ground B73 corn (31.8%).

Key words: sorghum starch; corn starch; starch structure; starch property; starch enzymatic hydrolysis; ethanol yield

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important cereal crops in the world. Sorghum can replace corn as the main crop in regions where the annual rainfall is less than 900 mm (*1*). Because of its adaptability to a dry climate, sorghum is primarily grown in semiarid and dry areas around the world. Sorghum is consumed as the staple in parts of Asia and Africa, and provides protein and energy needed for their population. In the United States, most of the sorghum is used for animal feed, and a small proportion is used for ethanol production (*2*).

Similar to other cereal crops, sorghum kernels have large starch contents, ranging from 72.3 to 75.1% among different varieties (*3*). Animal feeding studies have showed that sorghum had a lower starch digestibility than other cereal crops (*4-6*). The low starch digestibility of sorghum has been attributed to the highly cross-linked prolamine protein matrices surrounding starch granules and the presence of tannin in sorghum kernels (*7-10*).

The entrapment of starch granules in the protein matrices also results in lower starch yields from wet-milling of sorghum (*11*). In addition, phenolic pigments of sorghum leach out from the pericarp, testa, and aleurone tissues and cause off color of the isolated sorghum starch (*12*). The poor yield and off color of the sorghum starch are the reasons for no commercial wet-milling production of sorghum starch in the United States (*13*).

In recent years, commercial production of ethanol in the United States has expanded rapidly. Following this trend, there is a growing interest in using sorghum as an alternative

feedstock for ethanol production besides corn. Annual ethanol production from sorghum fermentation has grown steadily since 2004 (*14*). The ethanol yield has been reported positively correlated with the starch content of the sorghum, and negatively correlated with the protein content (*1*, *15*). Various methods, including decortication, sonication, protease hydrolysis, and steam-flaking, have been applied to increase the ethanol yield by reducing the interaction between starch and protein in sorghum kernels (*2*, *14*, *16*, *17*).

In this study, the composition of sorghum kernels from five lines (6B73, 6C21, 6C69, 7R34, and X789), and the structure, thermal property, pasting property, and enzymatic hydrolysis rate of the sorghum starches were analyzed and compared with that of the B73 corn and starch. Ethanol production using a cold fermentation process with uncooked sorghums and corn was also conducted and compared. The data obtained in this study can be used to predict value-added utilizations of sorghum in bio-ethanol, animal feed, and human food.

2. Materials and methods

2.1. Materials

Sorghum kernels of five lines, 6B73, 6C21, 6C69, 7R34, and X789, used in this study were harvested in Mt. Hope (KS) by Sorghum Division of Monsanto in 2004. The B73 corn kernels were provided by Dr. Michael Blanco, Germplasm Enhancement of Maize Project, ARS, USDA. The corn was grown at the North Central Regional Plant Introduction Station Farm (Ames, IA) in 2008.

Porcine pancreatic α-amylase (Type VI-B, 21.6 units/mg solid), maltohexaose, and maltoheptaose were purchased from Sigma Chemical Co. (St. Louis, MO). Amyloglucosidase from *Aspergillus niger* (200 U/mL), isoamylase from *Pseudomonas* sp. (1000 U/mL), Total

Starch Assay Kit, Starch Damage Assay Kit, and D-Glucose Assay Kit were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Catechin monohydrate was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Raw starch hydrolyzing enzyme (Distillase® SSF, 380 GAU/g) was a gift from Genencor International Inc. (Palo Alto, CA). One GAU was defined as the amount of enzyme that would release one gram of reducing sugars calculated as glucose per hour from soluble starch substrate under the assay condition. Yeast (Ethanol Red®) was purchased from Lesaffre Yeast Co. (Milwaukee, WI). Lactrol® (virginiamycin) was purchased from Phibro Animal Health Co. (Ridgefield, NJ). IsoStab™ (hop acid) was purchased from Beta Tec Hop Products (Washington, DC).

2.2. Starch isolation

Starch was isolated from sorghum and corn kernels using a wet-milling method (*18*).

2.3. Dry-grinding of kernel

The sorghum and corn kernels were dry-ground using a Cyclone Mill (UDY Corp., Fort Collins, CO) and passed through a 0.5 mm sieve to prepare ground sorghum and corn samples.

2.4. Kernel composition

The ground kernels were used for the analysis of sorghum and corn kernel compositions. The starch content of the kernels was determined using a Total Starch Assay Kit following the AACC method 76-13 (*19*). The nitrogen content of the kernels was determined using a Vario MAX CN Analyzer (Elementar Analysensysteme, Hanau, Germany). The protein content of the kernels was calculated by multiplying the nitrogen content with a conversion factor of 6.25. The lipid content of the kernels was determined using hexanes and Goldfisch Fat Extractors (Labconco Corp., Kansas City, MO) following the AACC Method 30-25 (*19*). The tannin content of the kernels was determined following the method of Price *et al*. with catechin

monohydrate as the standard (*20*). The starch, protein, lipid, and tannin contents were analyzed in duplicate.

2.5. Amylose content of starch

The amylose content of starch was determined using an iodine potentiometric titration method (*21*). The starch was defatted using an aqueous solution of 85% (v/v) methanol in a Soxhlet extractor for 24 h. The iodine affinity of the defatted starch was determined using an automatic potentiometric titrator (702 SM Titrino, Metrohm, Herisau, Switzerland). The amylose content of the starch was calculated by dividing the iodine affinity by 20.0% (*22*). The amylose content of the starch sample was analyzed in duplicate.

2.6. Branch-chain length distribution of amylopectin

Amylopectin was fractionated from the isolated starch and purified by repeating the 1-butanol complex method (*23*). The purified amylopectin was then debranched using isoamylase (*24*). The debranched chains were labeled with 8-amino-1, 3, 6-pyrenetrisulphonic acid, and the chain-length distribution was analyzed using a fluorophore-assisted capillary electrophoresis (P/ACEMDQ, Beckman Courter, Fullerton, CA) (*25*). Maltohexaose and maltoheptaose were used as the reference standards for the analysis. The sample was analyzed in duplicate.

2.7. Starch damage

Damaged-starch contents of the isolated starch and ground kernels were determined using a Starch Damage Assay Kit following the AACC Method 76-31 (*19*).

2.8. Starch granule morphology

Granule morphology of the isolated starch was studied using a scanning electron microscope (SEM, JEOL JAM-5800LV, Tokyo, Japan) following the method of Jane *et al*. (*26*).

2.9. Starch crystallinity

X-ray diffraction patterns of isolated starches were obtained using a diffractometer (D-500, Siemens, Madison, WI) with copper Kα radiation (*27*). The percentage crystallinity was calculated as: % crystallinity = 100% × A_c / (A_c+A_a), where A_c and A_a are the crystalline and amorphous area in the X-ray diffractogram, respectively (*28*).

2.10. Thermal property of starch

Gelatinization and retrogradation properties of the isolated starch were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT). The sample was prepared and analyzed following the method of Song and Jane (*21*). The thermal transition parameters were determined using a Pyris Software (Perkin-Elmer, Norwalk, CT). The percentage starch retrogradation was calculated as: % retrogradation = 100% × ΔH of dissociation of retrograded starch / ΔH of starch gelatinization, where ΔH is the enthalpy change of the thermal transition. The analysis was done in duplicate.

2.11. Pasting property of starch

The pasting property of isolated starch was analyzed using a Rapid Visco-Analyzer (RVA, Newport Scientific, Sydney, Australia). A suspension (28.0 g) containing 8% starch (w/w, dry base, db) was equilibrated at 50 ºC for 1 min, heated to 95 ºC at a rate of 6 ºC/min, held at 95 ºC for 5 min, and then cooled to 50 ºC at a rate of 6 ºC/min. The rotating speed for the paddle was 160 rpm except that 960 rpm was used for the first 10 s. The analysis was done in duplicate.

2.12. Enzymatic hydrolysis of starch

Enzymatic hydrolysis of the isolated starch and that of starch in the ground kernels (≤ 0.5 mm) were carried out following the method of Setiawan *et al*. (*29*) with modification. Starch

(1%, w/v) was hydrolyzed into soluble sugars using porcine pancreatic α-amylase (PPA, 1000 units/g starch, db) at 37 °C with shaking (100 rpm). The supernatant containing soluble sugars was separated by centrifugation and was collected and further digested into glucose using amyloglucosidase. The concentration of glucose released was quantified using a D-Glucose Assay Kit containing glucose oxidase and peroxidase. The percentage of starch hydrolysis was calculated as: % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180). The analysis was done in duplicate.

2.13. Ethanol production

A cold fermentation process was used to produce ethanol from ground kernels. A ground sample (35 g, db) was placed in a polypropylene bottle. An aqueous solution (5 mL), containing 30 mg urea, 0.2 mg Lactrol®, and 4 mg IsoStab™, and an acetate buffer solution (5 mL, 200 mM, pH 4.2) was added to the sample. Deionized water was added to make a total mass of 100 g. The mash was stirred for 0.5 h at 27 °C before adding raw starch hydrolyzing enzyme (72.4 GAU) and yeast (0.50 g), and then incubated at 27 ºC with shaking (160 rpm). Aliquots (8.0 mL) were removed from the fermentation broth at time intervals of 24 h, 48 h, 72 h, and 96 h and centrifuged at 7200 *g* for 10 min to collect supernatant. After filtration through a membrane filter (0.2 µm), ethanol concentration of the supernatant was analyzed using an HPLC system consisting of a Prostar 210 pump (Varian, Walnut Creek, CA), an injection valve (5 µL sample loop, Model 7725i, Rheodyne), and a Prostar 355 refractive index detector (Varian, Walnut Creek, CA). A Shodex SH-G guard colum (WAT034243) and an ion exclusion column (WAT010295, Waters, Milford, MA) were used to separate ethanol from other components. The columns were maintained at 75 ºC using a Prostar 510 column oven (Varian, Walnut Creek, CA), and the detector was set at 30 ºC. The mobile phase was a sulfuric acid solution (0.5 mM)

at 1.0 mL/min. Ethanol was quantified using a standard curve. Ethanol yield was calculated as: % ethanol yield = 100% × total mass of ethanol produced / initial dry mass of ground kernels. Ethanol conversion efficiency was calculated as: % ethanol conversion efficiency = 100% × actual yield of ethanol / theoretical yield of ethanol, where theoretical yield of ethanol is 56.73 g ethanol / 100 g starch, on the basis of that 1 g starch is hydrolyzed into 1.11 g of glucose, and 1 mol glucose is fermented to produce 2 mol of ethanol. The analysis was done in duplicate.

2.14. Statistical analysis

Statistical significance was evaluated using one-way ANOVA and multiple comparison using Tukey's adjustment with a 5% significance level. Correlations between the kernel compositions, structure and properties of the sorghum starches were analyzed using the Pearson correlation test. The statistical analyses were conducted in SAS (Version 9.1, SAS Institute, Inc., Cary, NC).

3. Results and discussion

Starch, protein, lipid and tannin contents of sorghum and corn kernels and amylose contents of the starches are shown in **Table 1**. Kernels of all the sorghum lines consisted of more starch (68.7-70.6%) than that of the B73 corn (67.4%). Sorghum line 6C69 had the largest starch content (70.6%), whereas line 6C21 had the least (68.7%) (**Table 1**). Protein contents of the sorghum samples ranged from 9.7 to 11.0%, which were comparable with that of the B73 corn (10.2%) (**Table 1**). Lipid contents of most sorghum samples ranged from 3.7 to 3.9% with an exception of 6C69 (2.7%), compared with 3.1% for the B73 corn (**Table 1**). Tannin contents of sorghum samples ranged from 2.9 to 6.6 mg/g (catechin equivalent), whereas the B73 corn had

no tannin (**Table 1**). The sorghum starches had less amylose (28.6-31.3%) than the B73 corn starch (32.2%) (**Table 1**).

Branch-chain length distributions of amylopectin molecules fractionated from the sorghum and corn starches are shown in **Figure 1**, and the results are summarized in **Table 2**. Amylopectins of the sorghum starches had smaller proportions (12.8-14.0%) of short branch-chains (DP 6-12), but larger proportions (41.3-43.3%) of branch-chains of DP 13-24 than that of the B73 corn starch (15.0% and 41.3%, respectively) in general. Average branch-chain lengths of the sorghum amylopectins varied between DP 20.3 and 20.8, which were longer than that of the B73 corn starch amylopectin (DP 20.2) (**Table 2**).

Damaged-starch contents of the isolated starches and the ground samples are shown in **Table 3**. For all the tested samples, ground kernels displayed substantially larger contents of damaged starch (2.0-8.7%) than the starches isolated by wet-milling (0-0.9%) (**Table 3**). This difference was attributed to that dried kernels with a low moisture content (about 11%) were at a glassy state during dry-grinding and thus required large force to break apart. Consequently, some weak starch granules yielded to the force and became damaged (*30*). Among the ground samples, X789 and 6B73, with the least amylose contents of their starches (28.6% and 28.7%, respectively), displayed the greatest percentages damaged starch (8.7% and 6.2%, respectively), whereas 6C69 sorghum and B73 corn, having the largest amylose contents of their starches (31.3% and 32.2%, respectively), showed the least damaged starch (2.0% and 2.4%, respectively) (**Table 1**, **3**). Damaged-starch contents of the ground sorghums negatively correlated with the amylose contents of starches ($r = -0.83$, $p = 0.08$), although the correlation was not significant. Among the wet-milled starches, X789 and 6C21samples displayed the largest damaged-starch content (0.9%) (**Table 3**). Both starches consisted of low amylose

contents (28.6% and 29.8%, respectively) (**Table 1**). Also, starch of line 6C21 consisted of amylopectin with the largest proportion of short branch-chains (DP 6-12) and the shortest average branch-chain length among the sorghums (**Table 2**). These results suggested that amylose in the starch granules played a role of holding the integrity of starch granules (*31*). Starch granules consisting of less amylose and amylopectin with more short branch-chains were more fragile and easily damaged (*32*, *33*).

Morphology and surface structures of isolated sorghum and corn starch granules studied using SEM are shown in **Figure 2**. Both sorghum and corn starch granules displayed polygonal and irregular shapes with diameters of 4-35 μm, but the B73 corn starch had more granules with diameter smaller than 10 μm. The sorghum starch showed more indentations on the surface of starch granules than the B73 corn starch, resulting from the presence of protein bodies located between starch granules (**Figure 2, A-F**) (*34*, *35*). Another distinct feature of the sorghum starch was that more granules displayed large pinholes than the B73 corn starch, indicating more severe endogenous amylase hydrolysis of the sorghum starch (**Figure 2, G-H**). Similar characteristic pinholes of sorghum starch granules have been reported by Huber and BeMiller (*36*).

X-ray diffraction patterns of the sorghum and corn starches are shown in **Figure 3**. All starch samples displayed the A-type diffraction pattern. Percentages crystallinity of the sorghum starches ranged from 25.8 to 29.6%, which were greater than that of the B73 corn starch (25.0%).

Gelatinization and retrogradation properties of the sorghum and corn starches are shown in **Table 4**. The sorghum starches had significantly higher onset (66.6-67.4 °C) and peak (69.9-71.1 °C) gelatinization temperatures, greater gelatinization enthalpy changes (13.0-14.0 J/g) and

percentages retrogradation (60.7-69.1%) than the B73 corn starch (61.7 °C, 68.9 °C, 10.1 J/g, and 51.5%, respectively) (**Table 4**).

The differences in the percentage crystallinity and the thermal property between sorghum and corn starches resulted from the different branch-chain length distributions of their amylopectins (**Table 2**). The large proportion of the short branch-chains (DP 6-12) of the amylopectin of B73 corn starch led to a defective crystalline structure, which resulted in a smaller percentage crystallinity, a lower gelatinization temperature, and a smaller gelatinization enthalpy change (*31*, *37*). During retrogradation, the starch molecules reassociate to form double helices. Having fewer short branch-chains of DP 6-12 and longer average branch-chain lengths of the amylopectins, sorghum starch retrograded faster. The percentages retrogradation of the sorghum starches positively correlated with their average amylopectin branch-chain lengths ($r = 0.92$, $p = 0.03$). The results agreed with previously reported data showing that long branch-chains of amylopectin reassociated more promptly to form double helices, whereas short branch-chains (DP 6-12) retarded the retrogradation (*31*, *38*, *39*).

Pasting properties of the sorghum and corn starches are shown in **Figure 4**. Although the pasting temperatures of the sorghum and corn starches were similar, peak temperatures of the sorghum starches were lower than that of the B73 corn starch. The sorghum starches generally displayed greater peak- and breakdown-viscosities but smaller setback-viscosities than the B73 corn starch. These differences could be attributed to the greater amylose content of the B73 corn starch (**Table 1**), which restricted the swelling of starch granules during heating but facilitated gel network formation when the starch paste cooled down (*31*, *40*, *41*).

Enzymatic hydrolysis of the isolated starches and that of starch in the ground kernels without cooking are shown in **Figure 5**, and the results are summarized in **Table 5**. There were

soluble sugars present in both the isolated starch and ground kernels. All the sorghum starches and most ground sorghum kernels (except 6C69) displayed greater soluble-sugar contents than the B73 corn counterparts. The soluble sugars were likely produced by endogenous enzyme hydrolysis (*29*), which was evidenced by pinholes on the surface of starch granules (**Figure 2**).

For the isolated starch samples, B73 corn starch and 6C21sorghum starch showed the greatest hydrolysis rates, which could be attributed to the larger proportions of short branch-chains (DP 6-12) of their amylopectins (**Table 2**). Amylopectin with greater proportions of short branch-chains results in more porous starch granules, which are more susceptible to enzymatic hydrolysis (*37*, *42*, *43*). Percentages hydrolysis of the isolated sorghum starches by PPA for 48 h positively correlated with the proportions of short branch-chains (DP 6-12) of amylopectins ($r = 0.89$, $p = 0.04$), and negatively with the average amylopectin branch-chain lengths ($r = -0.91$, $p = 0.03$).

Contrary to the view proposed in the literature that the highly cross-linked prolamine protein matrices in sorghum kernels limited the enzymatic hydrolysis of starch (*7*, *8*), in this study the ground kernels displayed greater percentages starch hydrolysis (91.7-95.9%) after 48 h incubation with PPA than the isolated starches (83.8-88.5%) except 6C69. The differences could be attributed to the presence of more damaged starch (**Table 3**) and endogenous amylases in the ground kernels (*29*). When starch granules became damaged, they were more easily hydrolyzed by enzymes (*44*).

Among the ground sorghum samples, the X789 sorghum exhibited the greatest starch hydrolysis rate by PPA despite that its isolated starch displayed a relatively slower hydrolysis rate, whereas the 6C69 sorghum, with the least damaged-starch content, showed the slowest starch hydrolysis rate (**Figure 5**, **Table 3, 5**). Percentages hydrolysis of starch in the ground

sorghum kernels after 48 h incubation positively correlated with the percentages damaged starch (r = 0.93, p = 0.02), but did not show significant correlation with the tannin contents of the kernels (r = 0.79, p = 0.11).

Ethanol yields obtained from ground sorghum and corn samples are shown in **Figure 6**, and the results are summarized in **Table 6**. The production of ethanol from ground sorghums was slower than that of the B73 corn. The ground sorghum 6C21 and 6C69, with the least tannin in the kernels, displayed the greatest ethanol production rates of the sorghum samples (**Figure 6**, **Table 1**, **6**). These results suggested that the slower ethanol production rates of ground sorghum samples could be partially attributed to the presence of tannin in their kernels (**Table 1**). In the fermentation broth with a high solid content (35%, w/w, db), tannin that leached into the solution could interfere with the starch enzymatic hydrolysis and yeast growth, and therefore slowed down the ethanol production rates of the ground sorghum samples. The delaying effects of tannin on enzymatic hydrolysis of starch and yeast growth have been reported before (*9*, *10*, *45*). After 96 h fermentation, the ethanol yields of sorghum samples (30.5-31.8%) were less than or equal to that of the B73 corn (31.8%), despite that sorghum kernels had greater starch contents than the B73 corn (**Table 1**, **6**). The ethanol conversion efficiency of sorghums (78.3-80.9%) was also substantially lower than that of the B73 corn (83.3%) (**Table 6**).

Among the sorghum lines, 6B73 and 6C69, consisting of the greatest starch contents, showed the greatest ethanol yields at 96 h (31.8% and 31.5%, respectively), and 6C21 that had the least starch content, displayed the smallest ethanol yield (30.5%) (**Table 1**, **6**). There was no significant difference in ethanol conversion efficiency between sorghum samples.

In conclusion, the isolated sorghum starches had higher gelatinization temperatures, greater gelatinization enthalpy changes and percentages retrogradation, but slower hydrolysis

rates using PPA than the B73 corn starch. The differences were attributed to the fewer short branch-chains (DP 6-12) of the sorghum amylopectins. Most of the ground sorghum and corn kernels displayed greater percentages starch hydrolysis than their respective isolated starches after hydrolyzing for 48 h. This could be attributed to the presence of more damaged starch and endogenous amylases in the ground kernels. After being subjected to cold fermentation for 96 h using raw starch hydrolyzing enzyme, most of the ground sorghum samples exhibited smaller ethanol yields and conversion efficiency than the ground B73 corn, despite the greater starch contents of the sorghum kernels. The data obtained in this study are useful for the utilization of sorghum in ethanol production as well as in feed and food processing, particularly for those regions where sorghum is grown as the major crop.

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Table 1. Compositions of sorghum and corn kernels (db), and amylose contents of starches [a]

Sample	Kernel				Amylose content of starch (%)
	Starch (%)	Protein (%)	Lipid (%)	Tannin (C.E., mg/g) [b]	
6B73	69.2 ± 0.4 ab	10.9 ± 0.1 a	3.8 ± 0.0 a	4.6 ± 0.2 b	28.7 ± 0.5 c
6C21	68.7 ± 0.0 ab	10.4 ± 0.2 b	3.9 ± 0.0 a	3.1 ± 0.0 c	29.8 ± 0.5 bc
6C69	70.6 ± 0.8 a	9.7 ± 0.0 c	2.7 ± 0.2 c	2.9 ± 0.1 c	31.3 ± 0.4 ab
7R34	69.2 ± 0.3 ab	11.0 ± 0.0 a	3.7 ± 0.0 a	4.3 ± 0.5 b	31.0 ± 0.3 ab
X789	69.0 ± 0.2 ab	10.6 ± 0.0 ab	3.7 ± 0.1 a	6.6 ± 0.5 a	28.6 ± 0.4 c
B73 (corn)	67.4 ± 0.7 b	10.2 ± 0.1 b	3.1 ± 0.1 b	0.0 ± 0.0 d	32.2 ± 0.3 a

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] C.E. = catechin equivalent.

Table 2. Branch-chain length distributions of amylopectins of starches [a, b]

Sample	Branch-chain length distribution				Average DP
	DP 6-12 (%) [c]	DP 13-24 (%)	DP 25-36 (%)	DP >36 (%)	
6B73	12.8 ± 0.5 b	42.7 ± 1.0	13.1 ± 0.0	31.4 ± 1.5	20.8 ± 0.4
6C21	14.0 ± 0.1 ab	42.0 ± 0.5	13.1 ± 0.0	30.9 ± 0.5	20.3 ± 0.2
6C69	13.1 ± 0.3 b	43.3 ± 1.5	13.6 ± 0.4	30.0 ± 1.6	20.5 ± 0.2
7R34	13.7 ± 0.4 ab	42.8 ± 0.4	13.3 ± 0.0	30.1 ± 0.8	20.4 ± 0.2
X789	13.3 ± 0.5 b	41.3 ± 0.6	13.6 ± 0.2	31.9 ± 1.3	20.8 ± 0.3
B73 (corn)	15.0 ± 0.3 a	41.3 ± 0.4	13.1 ± 0.1	30.7 ± 0.9	20.2 ± 0.4

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Analyzed using a fluorophore-assisted capillary electrophoresis.
[c] Mass basis.

Table 3. Damaged-starch contents of isolated starches and ground kernels [a, b]

Sample	Damaged starch (%)	
	Isolated starch	Ground kernel
6B73	0.3 ± 0.0 a	6.2 ± 0.2 ab
6C21	0.9 ± 0.3 a	4.4 ± 0.1 bc
6C69	0.0 ± 0.0 a	2.0 ± 0.7 c
7R34	0.4 ± 0.0 a	5.1 ± 1.0 b
X789	0.9 ± 0.3 a	8.7 ± 0.8 a
B73 (corn)	0.7 ± 0.2 a	2.4 ± 0.6 c

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Determined using a Starch Damage Assay Kit.

Table 4. Thermal properties of isolated starches [a, b]

Sample	Gelatinization of starch				Dissociation of retrograded starch				Retrogradation (%) [d]
	T_o (ºC) [c]	T_p (ºC)	T_c (ºC)	ΔH (J/g)	T_o (ºC)	T_p (ºC)	T_c (ºC)	ΔH (J/g)	
6B73	66.7 ± 0.6 a	69.9 ± 0.3 a	74.4 ± 0.6	13.6 ± 0.1 a	39.3 ± 0.5	51.1 ± 1.2	62.5 ± 0.1	9.1 ± 0.5 a	66.9
6C21	67.1 ± 0.0 a	70.7 ± 0.1 a	75.5 ± 0.2	13.8 ± 0.4 a	39.6 ± 0.3	51.7 ± 0.0	62.6 ± 0.1	8.4 ± 0.2 a	60.7
6C69	67.4 ± 0.7 a	71.1 ± 0.7 a	75.3 ± 1.2	13.1 ± 0.6 a	41.0 ± 0.7	52.3 ± 0.1	63.4 ± 0.1	8.1 ± 0.9 a	61.8
7R34	66.6 ± 0.0 a	70.3 ± 0.1 a	74.4 ± 0.3	14.0 ± 0.1 a	40.1 ± 1.1	52.4 ± 0.0	64.2 ± 0.1	8.9 ± 0.5 a	63.7
X789	66.6 ± 0.1 a	70.5 ± 0.1 a	74.6 ± 0.2	13.0 ± 0.0 a	38.1 ± 0.7	51.0 ± 0.3	62.8 ± 0.7	9.0 ± 0.5 a	69.1
B73 (corn)	61.7 ± 0.0 b	68.9 ± 0.7 b	74.3 ± 0.1	10.1 ± 0.2 b	44.2 ± 0.9	53.4 ± 0.0	62.4 ± 0.1	5.1 ± 0.7 b	51.5

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Measured using a differential scanning calorimeter.
[c] T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, and ΔH = enthalpy change.
[d] % retrogradation = 100% × ΔH of dissociation of retrograded starch / ΔH of starch gelatinization.

Table 5. Enzymatic hydrolysis of isolated starches and that of starches in ground kernels [a, b]

Sample	Starch hydrolysis (%) [c]					
	Isolated starch			Ground kernel		
	0 h [d]	24 h	48 h	0 h [d]	24 h	48 h
6B73	3.3 ± 0.0 ab	80.8 ± 0.9 b	83.8 ± 0.2 c	7.2 ± 0.1 a	84.0 ± 0.3 b	95.4 ± 0.7 ab
6C21	2.2 ± 0.0 ab	85.0 ± 0.3 a	87.8 ± 1.0 ab	5.4 ± 0.1 b	80.6 ± 0.6 b	91.7 ± 0.2 c
6C69	2.6 ± 0.3 ab	81.7 ± 1.0 b	86.1 ± 1.4 abc	2.0 ± 0.2 d	74.5 ± 0.9 c	85.5 ± 1.3 d
7R34	3.1 ± 1.5 ab	80.9 ± 0.3 b	86.2 ± 0.6 abc	6.4 ± 0.2 a	81.3 ± 0.6 b	92.6 ± 0.2 bc
X789	4.3 ± 0.5 a	81.6 ± 0.4 b	85.2 ± 0.2 bc	7.4 ± 0.0 a	88.6 ± 1.2 a	95.9 ± 0.8 a
B73 (corn)	0.8 ± 0.5 b	84.4 ± 0.4 a	88.5 ± 0.1 a	4.2 ± 0.5 c	90.3 ± 1.1 a	95.9 ± 1.0 a

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] PPA was used for the hydrolysis of uncooked starch and ground kernels at 37°C, pH6.9, with 100 rpm shaking.
[c] % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180).
[d] Soluble sugar determined using a D-glucose Assay Kit before adding PPA.

Table 6. Ethanol yields and conversion efficiency of ground kernels using cold fermentation [a]

Sample	Ethanol yield (%) [b]				Ethanol conversion efficiency at 96 h (%) [c]
	24 h	48 h	72 h	96 h	
6B73	15.3 ± 0.1 b	24.2 ± 0.5 bc	29.1 ± 0.7 ab	31.8 ± 0.4 a	80.9 ± 1.0 ab
6C21	16.6 ± 0.1 b	25.3 ± 0.0 b	29.5 ± 0.1 ab	30.5 ± 0.1 b	78.3 ± 0.1 b
6C69	17.1 ± 0.0 b	25.3 ± 0.2 b	29.6 ± 0.0 ab	31.5 ± 0.0 ab	78.6 ± 0.0 b
7R34	15.3 ± 0.7 b	23.3 ± 0.5 c	28.4 ± 0.3 b	30.8 ± 0.2 ab	78.5 ± 0.5 b
X789	16.2 ± 0.9 b	24.2 ± 0.8 bc	29.2 ± 0.7 ab	31.2 ± 0.4 ab	79.8 ± 0.9 b
B73 (corn)	20.2 ± 0.6 a	27.7 ± 0.3 a	30.8 ± 0.2 a	31.8 ± 0.3 a	83.3 ± 0.7 a

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] % ethanol yield = 100% × total mass of ethanol produced / initial dry mass of ground kernels.
[c] % ethanol conversion efficiency = 100% × actual yield of ethanol / theoretical yield of ethanol.

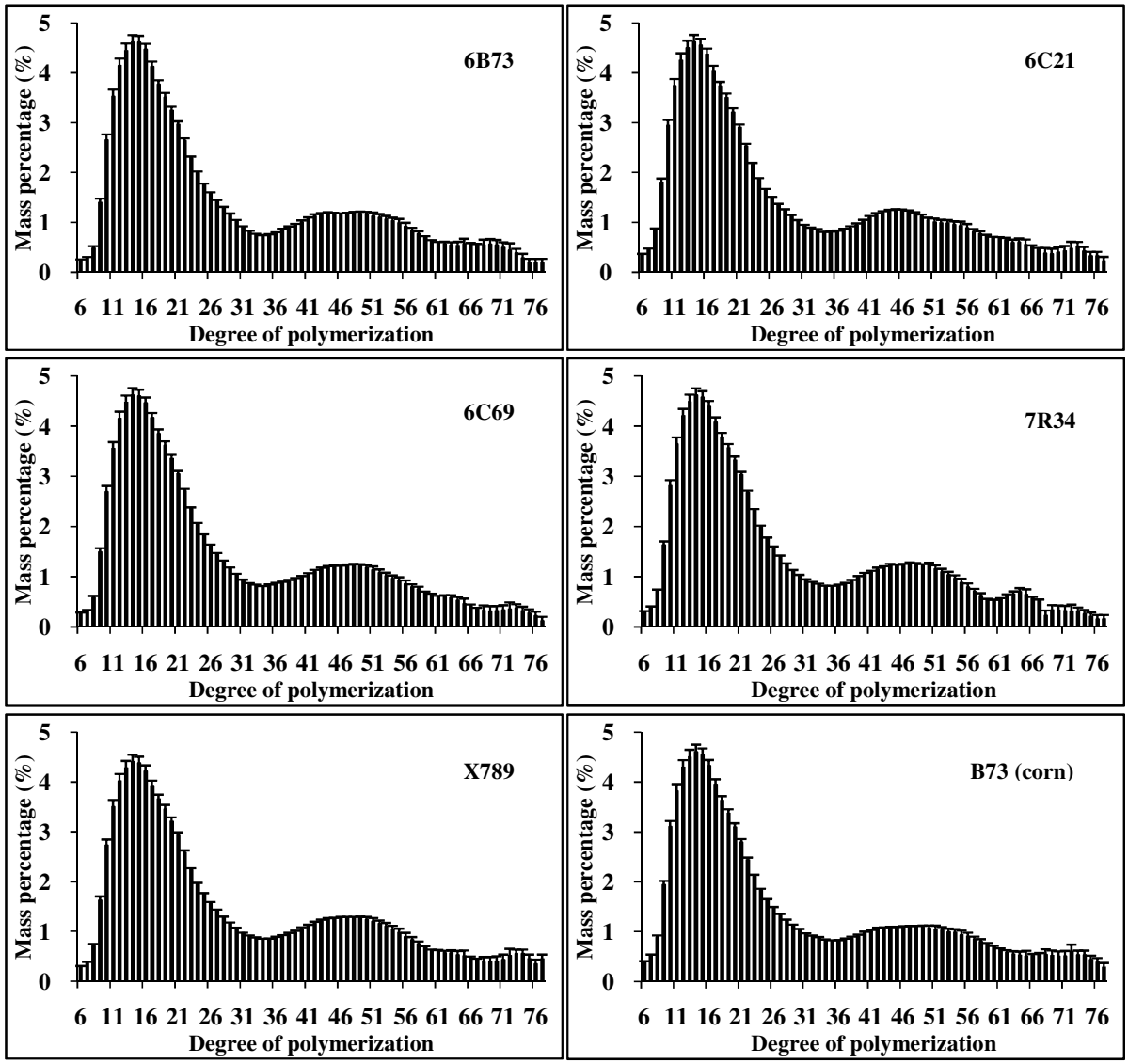


Figure 1. Branch-chain length distributions of amylopectins of starches analyzed using a fluorophore-assisted capillary electrophoresis.

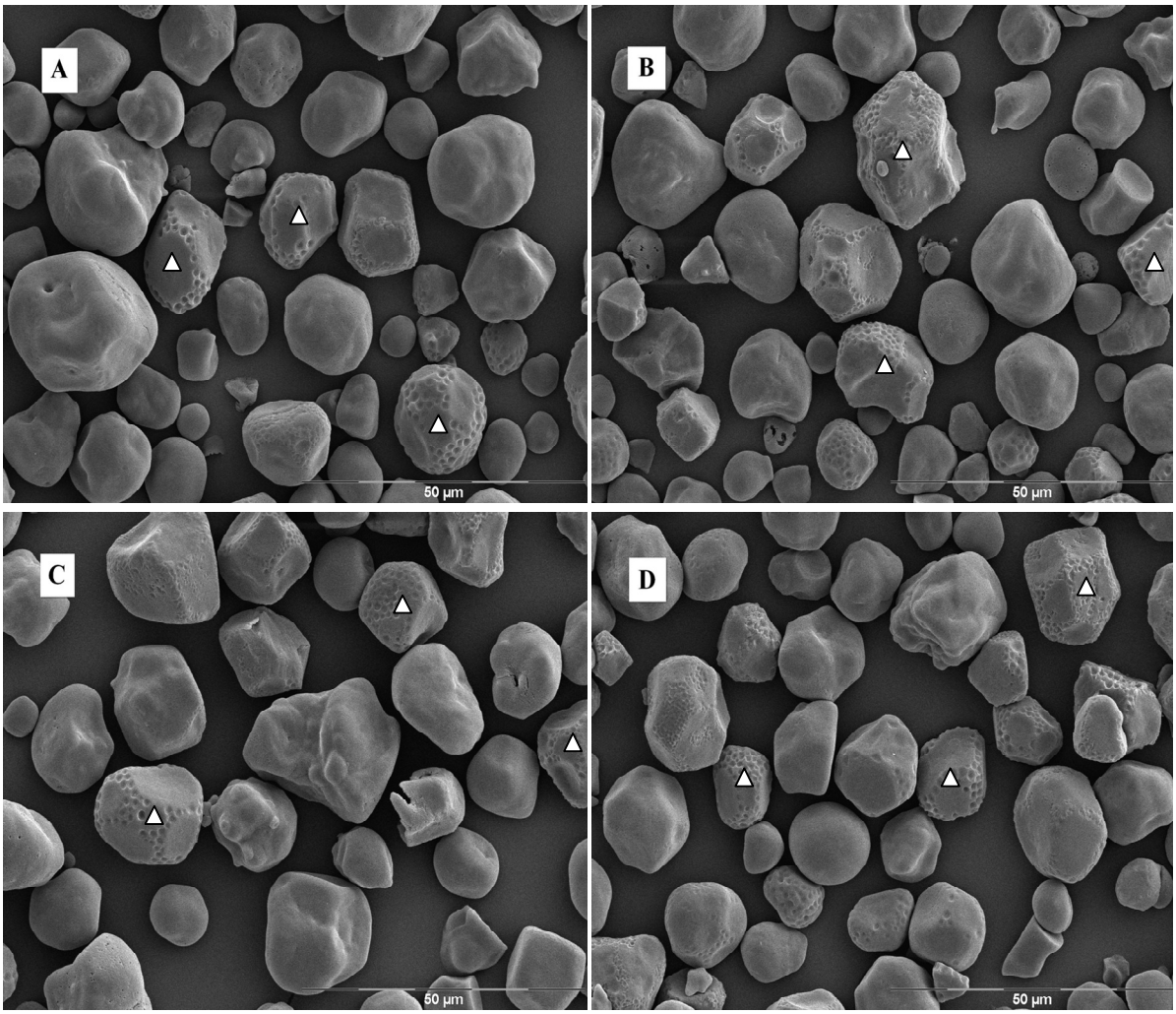
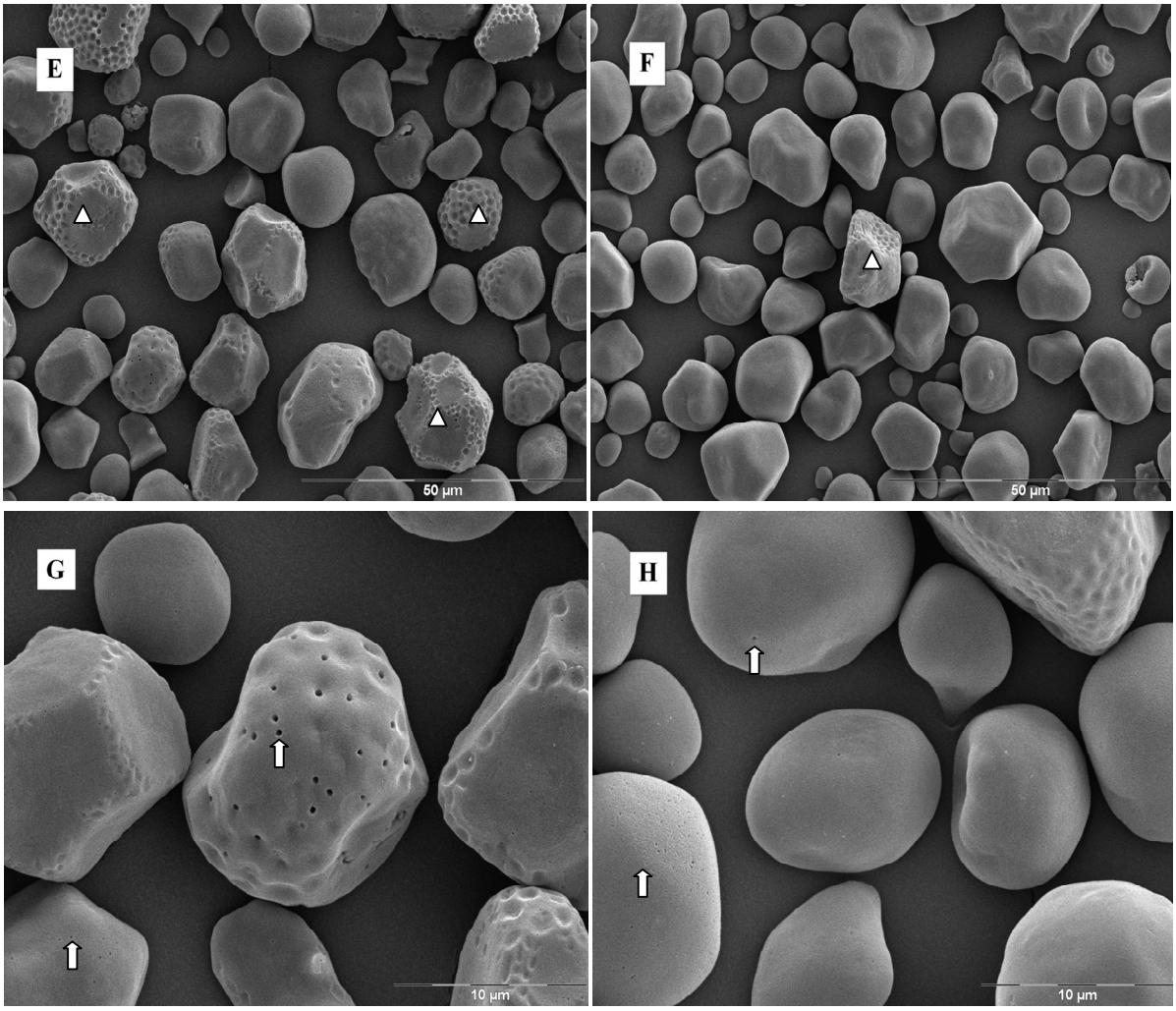


Figure 2. Scanning electronic micrographs of sorghum and corn starch granules. A: 6B73, B: 6C21, C: 6C69, D: 7R34, E: X789, and F: B73 (corn) at 1500 × magnification. G: 6B73 and H: B73 (corn) at 5000 × magnification. △ marks starch granules with indentations on the surface. ⇧ marks pinholes observed on the granule surface.

Figure 2. continued



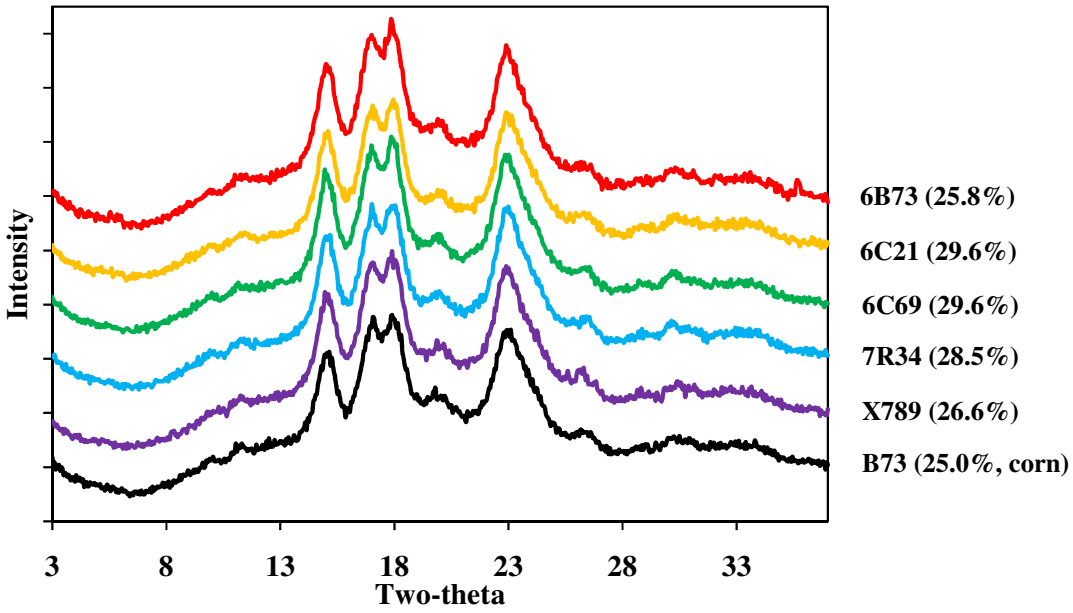


Figure 3. X-ray diffraction patterns of isolated starches. Percentage crystallinity is given in parentheses.

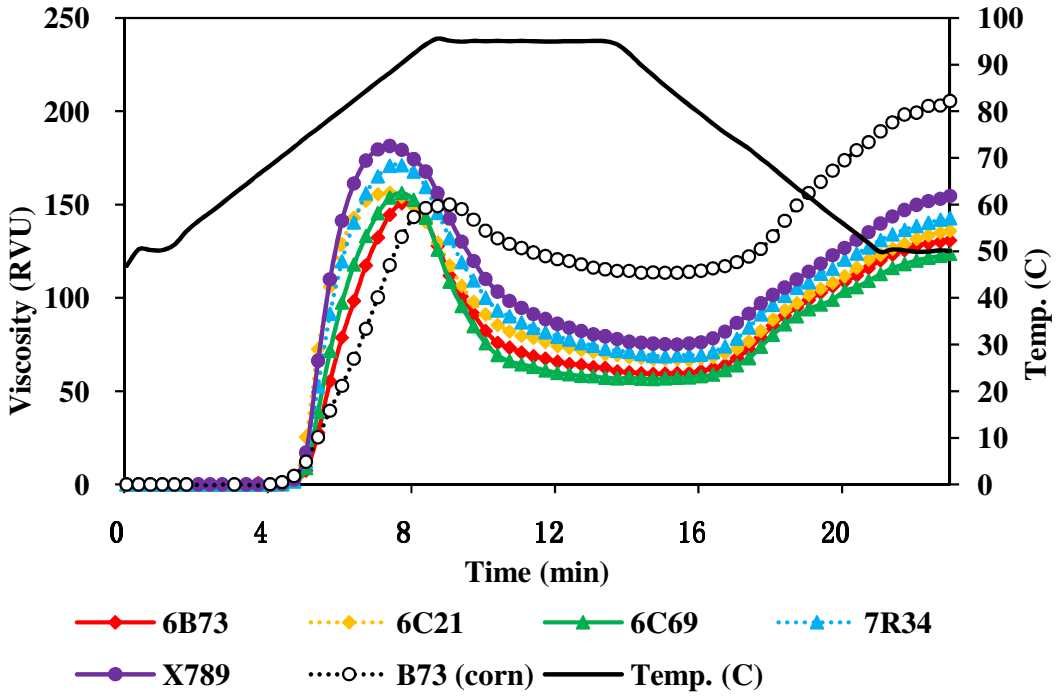


Figure 4. Pasting profiles of isolated starches measured using a Rapid Visco-Analyzer with 8% (dsb, w/w) starch suspension.

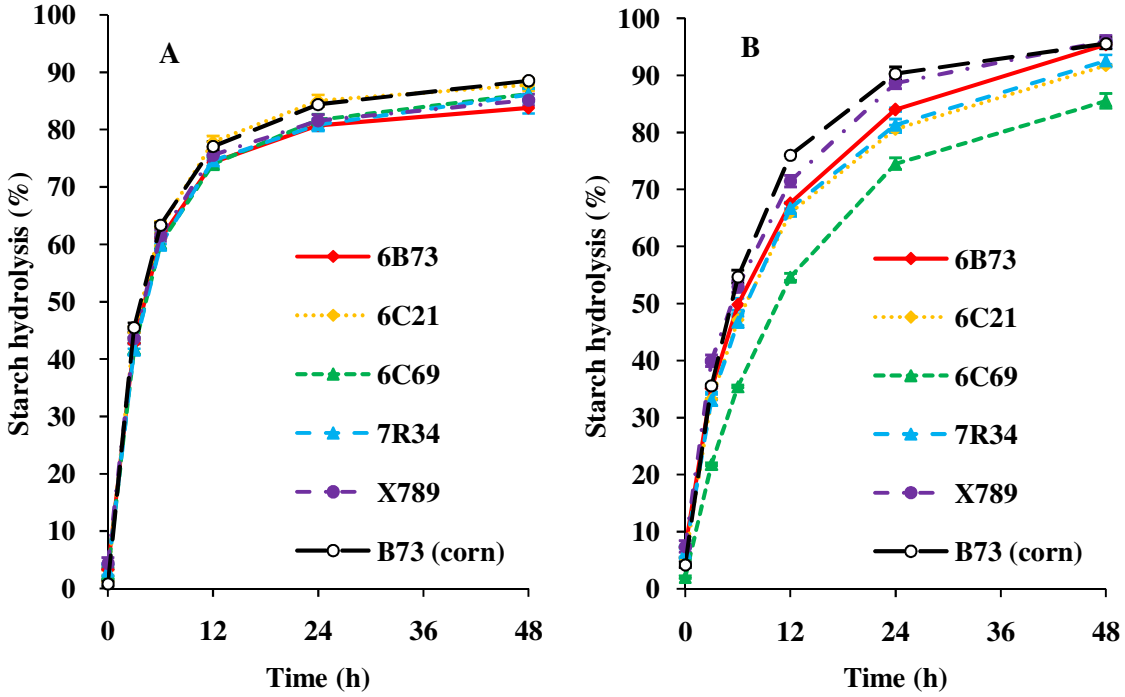


Figure 5. Enzymatic hydrolysis of isolated starches (A), and that of starches in ground kernels (B). PPA was used for the hydrolysis of uncooked starch and ground kernels at 37 °C, pH 6.9, with 100 rpm shaking. % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180).

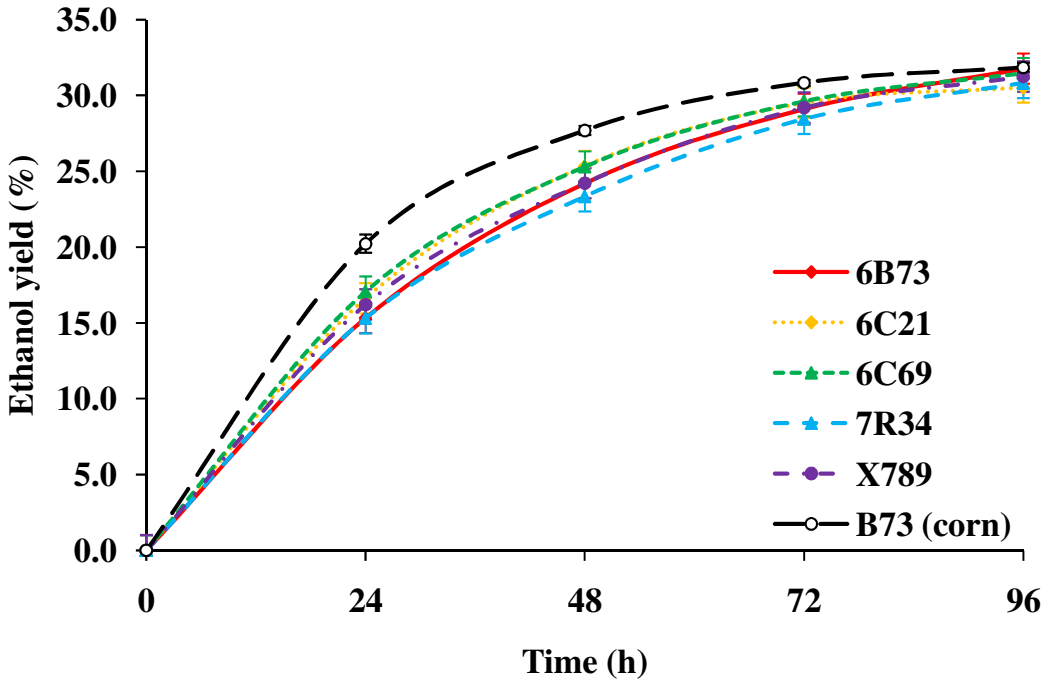


Figure 6. Ethanol yields of ground kernels using cold fermentation. % ethanol yield = 100% × total mass of ethanol produced / initial dry mass of ground kernels.

CHAPTER 6. COMPARISON OF STARCHES FROM RICE KERNELS AND BAMBOO SEEDS

A paper to be submitted to *Starch – Stärke*

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Abstract

This study aimed to characterize structure, physical properties, and enzymatic-hydrolysis rate of starch isolated from bamboo seeds (*Bambusa textilis* species) in comparison with that of indica and japonica-rice starch. The bamboo seeds consisted of 68.2% starch, which was lower than the indica and japonica-rice kernels (85.1% and 87.1%, respectively). The bamboo seeds also had compound starch granules, with morphology and sizes comparable to the rice starches. The amylopectin of bamboo-seed starch showed similar branch-chain-length distribution to that of the indica-rice starch, and both of them had longer branch-chains (DP = 19.1 and 19.7, respectively) than the japonica-rice amylopectin (DP = 17.1). Consequently, the bamboo-seed and indica-rice starch granules exhibited higher gelatinization-temperatures (T_o = 68.9 °C and 71.9 °C, respectively), larger enthalpy-changes (ΔH = 14.2 J/g and 15.3 J/g) and percentages of

retrogradation (57.1% and 55.4%), but slower enzymatic-hydrolysis rates (50.6% and 46.3% at 24 h) than the japonica-rice starch (56.4 °C, 14.0 J/g, 11.4%, and 66.2%, respectively). The bamboo-seed starch displayed a higher pasting-temperature but substantially lower viscosity than the rice starches, which could be attributed to its larger amylose-content and smaller molecular-weight and gyration-radius of the amylopectin.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world, and more than 90% of the world's rice is produced in Asia [1]. Rice is primarily consumed as a staple food worldwide, providing nutrients and energy for more than half of the global population [2]. Rice is also consumed in the form of processed foods, such as rice noodles and puffed rice cakes [3, 4]. Because rice grains contain no gluten, there are increasing interests in replacing wheat flour with rice flour to prepare gluten-free food products for those individuals suffering from celiac disease [5].

Similar to other cereal grains, starch is the major component of rice kernels. Removal of the hull after milling produces brown rice, which can have starch content up to 77% (dry basis, db). Further removal of the embryo and bran layer produces milled rice, which consists of up to 90% starch (db) [6, 7]. The granular size of rice starch range from 3-8 μm, and they are relatively small compared with starch from other botanical sources [8, 9]. Rice kernels are known to have compound starch granules [10, 11]. During starch biosynthesis, more than one granule is synthesized simultaneously within an individual amyloplast. The compound starch granules of rice display a polyhedral shape, which could be a result of space constraint within the amyloplast [12].

Rice cultivated worldwide can be classified into japonica or indica type. One important difference between these two types of rice lies in different branch-chain lengths of their amylopectins. Amylopectin of japonica-type rice generally has a larger proportion of short branch-chains (degree of polymerization, DP ≤12), but a smaller proportion of intermediate branch-chains (DP 13-24) than that of indica-type rice. The structural differences are attributed to that japonica-type rice has lower activity of starch synthase IIa (SS IIa), which is responsible for the elongation of short branch-chains (DP 8-12) to longer branch-chains (DP 13-24) during starch biosynthesis [13, 14]. Because of the structural differences in their amylopectins, starch from indica-type rice usually has higher gelatinization-temperatures and faster retrogradation-rates than that from japonica-type rice [13, 15, 16]. In addition, the texture of cooked indica-type rice is hard and not sticky, whereas that of cooked japonica-type rice is more elastic and sticky [15].

In recent decades, research has focused on the development of new rice varieties with improved rice quality, increased yield, but less water consumption [17-19]. Following this trend, a novel rice variety, bamboo rice, has been developed in China through distant hybridization between rice and bamboo (*Bambusa textilis* species) [20]. The breeding of bamboo rice took the advantage of that rice is genetically closer to bamboo than other plants in the Poaceae family (*e.g.*, maize, wheat and barley) [21]. The bamboo rice has stronger root and stalk than regular rice varieties, which might be attributed to the incorporation of bamboo genes into the rice plants. The bamboo rice has enhanced lodging-resistance, improved drought-tolerance, and increased yield [20]. These desirable agronomic traits make the bamboo rice a popular rice variety for commercial cultivation.

Objectives of this study were to characterize structure, physical properties, and enzymatic hydrolysis of starch from bamboo seeds (*Bambusa textilis* species) in comparison with that of rice starch. The study will provide valuable information about the chemical structures and functional properties of starches from these two species of plants in the same family. The data will also be useful for us to understand the development of bamboo rice.

2. Materials and methods

2.1. Materials

The seeds of bamboo (*Bambusa textilis* species) were obtained from Meizhou, Guangdong Province, China. The bamboo seeds were harvested in 2011 and milled in 2012. Kernels of milled indica (Extra Long Grain Rice, Riceland Foods Inc., Stuttgart, AR) and japonica (Extra Fancy US #1, Koda Farms Inc., South Dos Palos, CA) rice were purchased from a local grocery store.

Amyloglucosidase from *Aspergillus niger* (200 U/mL), isoamylase from *Pseudomonas* sp. (1000 U/mL), Total Starch Assay Kit, Starch Damage Assay Kit, and D-Glucose Assay Kit were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). Porcine pancreatic α-amylase (PPA, Type VI-B, 21.6 units/mg solid), maltohexaose, and maltoheptaose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

2.2. Dry grinding of kernels

Bamboo seeds and rice kernels were dry ground using a Cyclone Mill (UDY Corp., Fort Collins, CO) and passed through a sieve (0.5 mm) to prepare ground samples.

2.3. Chemical composition of kernels

The ground sample was used for the analyses of chemical compositions of bamboo seeds and rice kernels. The starch content of the sample was measured using a Total Starch Assay Kit (AACC method 76-13) [22]. The protein content was determined using Vario MAX CN Analyzer (Elementar Analysensysteme, Hanau, Germany) and calculated by multiplying the nitrogen content with a conversion factor of 5.95 (AACC Method 46-13.01) [22]. The lipid content was measured using Goldfisch Fat Extractors (Labconco Corp., Kansas City, MO) with hexanes as the solvent (AACC Method 30-25) [22]. The above analyses were done in duplicate.

2.4. Isolation of starch

Starch was isolated from bamboo seeds and rice kernels following the wet-milling method reported by Bao et al. [23].

2.5. Damage-starch content

Damaged-starch contents of the isolated starch and ground sample were determined using a Starch Damage Assay Kit following the AACC Method 76-31 [22].

2.6. Morphology of starch granule

Granule morphology of the isolated starch was studied using a scanning electron microscope (SEM, JEOL JAM-5800LV, Tokyo, Japan) [9]. The micrographs were captured at magnifications of 1500 × and 5000 ×, respectively.

2.7. Starch crystallinity

X-ray diffraction pattern of isolated starch was analyzed using a diffractometer (D-500, Siemens, Madison, WI) with copper Kα radiation following the method of Yoo and Jane [24]. The percentage crystallinity was calculated as: % crystallinity = 100% × A_c / (A_c+A_a), where A_c and A_a are the crystalline and amorphous area in the X-ray diffractogram, respectively [25].

2.8. Amylose content of starch

The amylose content of isolated starch was determined using an iodine potentiometric titration method [26]. After defatting the starch using a methanol aqueous solution (85%, v/v) in a Soxhlet extractor for 24 h [27], the iodine affinity of the starch was measured using an automatic potentiometric titrator (702 SM Titrino, Metrohm, Herisau, Switzerland). The amylose content of the starch was calculated by dividing the iodine affinity by 0.2 [28]. The analysis was done in duplicate.

2.9. Branch-chain-length distribution of amylopectin

Amylopectin was fractionated from the isolated starch using a gel permeation chromatography method and then debranched using isoamylase in an acetate buffer solution (10.0 mM, pH 4.5, containing 0.02% NaN_3) [29]. After labeling the debranched chains with 8-amino-1, 3, 6-pyrenetrisulphonic acid, the branch-chain-length (BCL) distribution was analyzed using a fluorophore-assisted capillary electrophoresis (P/ACEMDQ, Beckman Courter, Fullerton, CA) [30]. Maltohexaose and maltoheptaose were used as the standards for the calibration. The analysis was done in duplicate.

2.10. Molecular weight and gyration radius of amylopectin

Weight-average molecular weight (M_w) and *z*-average radius of gyration (R_z) of starch amylopectin were analyzed using a high-performance size-exclusion chromatography equipped with a multi-angle laser-light scattering detector and a refractive index detector (HPSEC-MALLS-RI) as described by Yoo and Jane [31].

2.11. Thermal property of starch

Gelatinization and retrogradation properties of the isolated starch were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT) [26]. The starch

(~2 mg, dry starch basis, dsb) was mixed with deionized water (3×, w/w, dsb) and heated from 10 °C to 110 °C at a rate of 10 °C/min. The gelatinized starch sample was stored at 4 °C for 7 days and then scanned using the same method to examine the retrogradation property. The thermal transition parameters were measured using a Pyris Software (Perkin-Elmer, Norwalk, CT). The percentage starch retrogradation was calculated as: % retrogradation = 100% × ΔH of dissociation of retrograded starch / ΔH of starch gelatinization, where ΔH is the enthalpy change of the thermal transition. The analysis was done in duplicate.

2.12. Pasting property of starch

Pasting property of isolated starch was measured using a Rapid Visco-Analyzer (RVA, Newport Scientific, Sydney, Australia). The starch sample (2.24 g, dsb) was suspended in deionized water to reach a total weight of 28.0 g (8% dry starch) and analyzed following the program reported by Ai et al. [32]. The analysis was done in duplicate.

2.13. Enzymatic hydrolysis of starch

Enzymatic hydrolysis of the isolated starch and that of starch in the ground samples (≤ 0.5mm) were performed following the method of Setiawan et al. [33] with modifications. The sample containing 200.0 mg of starch (dsb) was suspended in 20.0 mL of phosphate buffer solution (10.0 mM, pH 6.9, with 0.25 mM $CaCl_2$ and 0.02% NaN_3) and then hydrolyzed into soluble sugars using PPA (750 units/g starch, dsb) at 37 °C with shaking (80 rpm). The hydrolyzate (0.5 mL) was collected at 0 h, 2 h, 4 h, 8 h and 24 h, and immediately centrifuged at 5,200 *g* for 5 min. The supernatant containing soluble sugars was further digested into glucose using amyloglucosidase. The concentration of released glucose was quantified using a D-Glucose Assay Kit containing glucose oxidase and peroxidase (GOPOD). The percentage of

starch hydrolysis was calculated as: % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180). The analysis was done in duplicate.

2.14. Statistical analysis

Statistical significance was evaluated using one-way ANOVA and multiple comparison with Tukey's adjustment at a significance level of 5%. The statistical analyses were performed in SAS (version 9.1, SAS Institute, Inc., Cary, NC).

3. Results

3.1. Chemical composition of kernels and amylose content of starch

Starch, protein and lipid contents of the bamboo seeds and rice kernels, and amylose contents of the isolated starches are shown in **Table 1**. The starch content of bamboo seeds (68.2%) was much smaller than that of the indica (85.1%) and japonica-rice kernels (87.1%), whereas the protein and lipid contents of the bamboo seeds (18.6% and 1.1%, respectively) were larger than that of the rice kernels (8.7% and 0.4% for indica, 7.3% and 0.4% for japonica, respectively). The starch isolated from the bamboo seeds displayed the largest amylose-content (24.1%), followed by the indica (18.1%) and japonica-rice starch (14.0%).

3.2. Damaged-starch content

Damaged-starch contents of the isolated starches and ground kernels are shown in **Table 2**. For both the isolated starch and ground kernel, the bamboo seeds exhibited comparable damaged-starch contents to the indica rice, and the values were significantly lower than that of the japonica rice. The ground samples had substantially larger damaged-starch contents (8.9%-11.4%) than its respective isolated starch counterparts (2.2%-2.7%), and the results were consistent with previously reported data [32, 34].

3.3. Morphology of starch granule

SEM images of isolated starches are shown in **Figure 1**. Starches from the bamboo seeds and rice kernels all showed compound granules with a polyhedral shape. Some of the compound starch granules were not separated after the isolation by wet-milling. Bamboo-seed starch, having some granules with diameters up to 10 μm (**Figure 1**, **A1**), showed slightly larger granule-sizes than the indica and japonica-rice starch (diameters 2-7 μm, **Figure 1**, **B1** and **C1**).

3.4. Starch crystallinity

X-ray diffraction patterns of the isolated starch are shown in **Figure 2**. All the starch samples displayed the A-type crystalline structure. The indica-rice starch had the largest percentage crystallinity (37.7%), followed by the japonica-rice (34.3%) and bamboo-seed starch (32.1%).

3.5. BCL distribution of amylopectin

BCL distributions of starch amylopectins are summarized in **Table 3**. The amylopectin of bamboo seeds showed similar BCL distributions to that of the indica rice. Both of them consisted of a smaller proportion of short branch-chains (DP 6-12; 29.5% and 27.2%, respectively), but larger proportions of intermediate (DP13-24; 53.7% and 53.9%, respectively) and long (DP >36; 10.5% and 11.2%, respectively) branch-chains than that of the japonica rice (35.9%, 50.5%, and 6.3%, respectively). The average branch-chain lengths of bamboo-seed and indica amylopectins (DP = 19.1 and 19.7, respectively) were longer than that of the japonica rice (DP = 17.1). The differences in the amylopectin BCL distribution between the indica and japonica rice were consistent with the data reported previously [13, 14].

3.6. M_w and R_z of amylopectin

M_w and R_z of starch amylopectins are shown in **Table 4**. The amylopectin of japonica rice

showed the largest M_w (16.3×10^8 g/mol) and R_z (499.8 nm), followed by that of the indica rice (11.9×10^8 g/mol and 437.4 nm, respectively), and the amylopectin of bamboo seeds had the smallest M_w (5.4×10^8 g/mol) and R_z (369.9 nm).

3.7. Thermal property of starch

Thermal properties of the isolated starches are shown in **Table 5**. Thermal properties of the bamboo-seed and indica-rice starches were comparable, and both of them displayed higher gelatinization-temperatures (T_o = 68.9 °C and 71.9 °C, respectively) and larger gelatinization enthalpy-change (ΔH = 14.2 J/g and 15.3 J/g, respectively) than the japonica-rice starch (56.4 °C and 14.0 J/g, respectively). The differences in the gelatinization properties between the indica and japonica-rice starch were in good agreement with the data reported in the literature [13, 16]. After storing at 4 °C for 7 days, the gelatinized bamboo-seed and indica-rice starches displayed greater percentages of retrogradation (57.1% and 55.4%, respectively) than the japonica-rice starch (11.4%).

3.8. Pasting property of starch

Pasting properties of the isolated starches are shown in **Figure 3**. The bamboo-seed starch exhibited a higher pasting-temperature (84.1 °C) but lower peak (107.5 RVU) and final-viscosity (120.4 RVU) than the indica-rice (81.1 °C, 163.0 RVU and 201.3 RVU, respectively) and japonica-rice starch (76.5 °C, 153.4 RVU and 180.1 RVU, respectively).

3.9. Enzymatic hydrolysis of starch

Enzymatic hydrolysis of isolated starches and that of starches in ground kernels are shown in **Figure 4**. There were soluble sugars found in the isolated starches (3.0% - 3.3%) and ground kernels (5.1% - 5.7%) at 0 h. For the isolated starch, the japonica-rice starch was hydrolyzed by PPA at the fastest rate (66.2% at 24 h), followed by the bamboo-seed (50.6%) and

indica-rice starch (46.3%) (**Figure 4**, **A**). The ground japonica-rice also showed a faster starch-hydrolysis rate (75.8% at 24 h) than the ground indica-rice (58.5%) and bamboo-seeds (52.2%) (**Figure 4**, **B**). The ground samples were hydrolyzed to greater extents (52.2%-75.8%) by PPA than the isolated starch counterparts (46.3%-66.2%). The ground bamboo-seeds showed a slower starch-hydrolysis rate than the ground indica-rice, despite the result that the isolated bamboo-seed starch was more susceptible to PPA hydrolysis.

4. Discussion

The bamboo seeds had a lower starch-content than the rice kernels, and the starch from bamboo seeds also had compound granules, with morphology and sizes similar to rice starches (**Figure 1**), which was in good agreement with that bamboo is genetically closer to rice than other plants in the Poaceae family [21]. Thus, genes from bamboo could potentially be introduced and stabilized in the bamboo rice via distant hybridization [20].

Structural analyses revealed that the amylopectin from the bamboo-seed starch had comparable BCL distribution to that of the indica-rice starch, and their amylopectins consisted of less short branch-chains (DP 6-12) but more intermediate (DP 13-24) and long (DP > 37) branch-chains than the japonica-rice amylopectin (**Table 3**). Branch chains of amylopectin form double helices and contribute to the crystalline structure of starch granules [35]. Starch consisting of amylopectin with longer branch-chains tends to have more stable double-helical crystallites and less defect crystalline structure [36, 37]. Therefore, the bamboo-seed and indica-rice starches displayed larger percentages of crystallinity (not the bamboo-seed starch), higher gelatinization-temperatures and enthalpy-changes, larger percentages of retrogradation, but slower enzymatic-hydrolysis rates than the japonica-rice starch (**Table 5**; **Figure 2** and **4**). In

addition, the bamboo-seed starch, with slightly shorter branch-chains in the amylopectin (**Table 3**), had a lower gelatinization-temperature and enthalpy-change, and a faster enzymatic-hydrolysis rate than the indica-rice starch (**Table 5**; **Figure 4**, **A**).

Amylose content is another important factor affecting the physical properties and enzymatic hydrolysis of starch. Bamboo-seed starch had the largest amylose-content among all the starches (**Table 1**). Amylose is present in the amorphous regions of starch granules and does not contribute to the crystalline structure of starch granules [38]. Thus, the bamboo-seed starch has a smaller percentage of crystallinity than the japonica-rice starch (**Figure 2**), even though the branch chains of bamboo-seed amylopectin were longer (**Table 3**). The presence of amylose restricted the swelling of starch granules during heating [39, 40], which partially explained that the bamboo-seed starch had a higher pasting-temperature but much lower peak and final-viscosity than the rice starches (**Figure 3**). The lower peak and final-viscosity of the bamboo-seed starch could also result from the smaller M_w and R_z of its amylopectin than the rice amylopectins (**Table 4**). Amylopectin with a smaller molecular-weight and size could lead to a lower viscosity of the starch [41, 42].

After the bamboo seeds and rice kernels were subjected to dry grinding, their damaged-starch contents and starch-hydrolysis rates were also analyzed. The ground samples showed remarkably greater damaged-starch contents than their respective isolated starch counterparts (**Table 2**). The results could be explained by that the dry kernels, with a lower moisture-content (~13%), were at a glassy state during dry grinding and required more mechanical force to break apart. Therefore, a larger proportion of starch yielded to the mechanical force and became damaged in the ground kernels [32, 34]. For both the isolated starch and ground sample, the bamboo seeds and indica rice displayed lower damaged-starch contents than the japonica rice

(**Table 2**), suggesting that the starches in the bamboo seeds and indica-rice kernels were less fragile to the mechanical force. The results could be attributed to greater amylose-contents and longer branch-chains of the amylopectins of the bamboo-seed and indica-rice starches (**Table 1** and **3**). Amylose molecules helped to maintain the integrity of starch granules, and amylopectins with longer branch-chains contributed to more perfect crystalline structure [32, 36].

All the ground kernels were hydrolyzed to greater extents than their isolated starches after the incubation with PPA for 24 h, which could be attributed to the existence of more damaged starch (**Table 2**) and endogenous α-amylase in the ground samples [32, 34]. Compared with the ground bamboo-seeds and indica-rice, the ground japonica-rice showed a greater starch-hydrolysis rate because its starch was hydrolyzed more easily by the enzyme and there was more damaged starch in the sample (**Table 2**; **Figure 4**). The starch-hydrolysis rate of the ground bamboo-seeds was slower than that of the ground indica-rice (**Figure 4**, **B**), despite the greater susceptibility of bamboo-seed starch to the enzymatic hydrolysis (**Figure 4**, **A**). The differences could result from that the bamboo seeds had a significantly larger protein-content than the indica rice (**Table 1**). The proteins present in the ground bamboo-seeds could act as a physical barrier and reduce the accessibility of starch to the enzymatic hydrolysis, and this could also explain that the ground bamboos-seeds exhibited a slower starch-hydrolysis rate (32.4% at 8 h) than its isolated starch counterpart (37.3%) at the early incubation with PPA [43-45].

5. Conclusions

Chemical structure, physical properties, and enzymatic-hydrolysis rate of starch from bamboo seeds were examined and compared with that of indica and japonica rice. Bamboo-seed starch also had compound starch granules, and their morphology and sizes were similar to the

rice starch granules. Bamboo-seed starch consisted of amylopectin with comparable BCL distribution to that of the indica-rice starch, and their amylopectins had longer branch-chains than that of the japonica-rice starch. Therefore, bamboo-seed and indica-rice starches exhibited higher gelatinization-temperatures and enthalpy-changes, larger percentages of retrogradation, and greater resistance to PPA hydrolysis than the japonica-rice starch. The bamboo-seed starch showed a higher pasting-temperature but lower peak and final-viscosity than the rice starches, which could result from that the bamboo-seed starch contained more amylose and amylopectin with smaller M_w and R_z.

6. Acknowledgements

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Table 1. Chemical compositions of bamboo seeds and rice kernels (db) and amylose contents of isolated starches [a]

Sample	Chemical composition (%)			Amylose content of starch (%) [b]
	Starch	Protein	Lipid	
Bamboo seed	68.2 ± 0.5 c	18.6 ±0.9 a	1.1 ± 0.0 a	24.1 ± 0.2 a
Indica	85.1 ± 0.6 b	8.7 ± 0.0 b	0.4 ± 0.1 b	18.1 ± 0.1 b
Japonica	87.1 ± 0.0 a	7.3 ± 0.1 b	0.4 ± 0.1 b	14.0 ± 0.7 c

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Determined using an iodine potentiometric titration method.

Table 2. Damaged-starch contents of isolated starches and ground kernels [a, b]

Sample	Damaged starch (%)	
	Isolated starch	Ground kernel
Bamboo seed	2.4 ± 0.1 b	8.9 ± 0.1 b
Indica	2.2 ± 0.1 b	9.0 ± 0.0 b
Japonica	2.7 ± 0.0 a	11.4 ± 0.2 a

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Determined using a Starch Damage Assay Kit.

Table 3. Branch-chain-length distributions of amylopectins [a, b]

Sample	Branch-chain-length distribution [c]				Average DP
	DP 6-12 (%)	DP 13-24 (%)	DP 25-36 (%)	DP >36 (%)	
Bamboo seed	29.5 ± 0.4 b	53.7 ± 0.4 a	6.4 ± 0.2 b	10.5 ± 0.6 a	19.1 ± 0.8 a
Indica	27.2 ± 0.2 b	53.9 ± 0.1 a	7.7 ± 0.4 a	11.2 ± 0.3 a	19.7 ± 0.1 a
Japonica	35.9 ± 0.9 a	50.5 ± 0.9 b	7.2 ± 0.2 ab	6.3 ± 0.1 b	17.1 ± 0.1 b

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Analyzed using fluorophore-assisted capillary electrophoresis.
[c] Molar basis.

Table 4. Molecular weights (M_w) and radii of gyration (R_z) of starch amylopectins [a, b]

Sample	M_w ($\times 10^8$, g/mol) [c]	R_z (nm) [d]
Bamboo seed	5.4 ± 0.6 c	369.9 ± 6.0 b
Indica	11.9 ± 0.1 b	437.4 ± 34.9 ab
Japonica	16.3 ± 1.4 a	499.8 ± 0.4 a

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Measured using a high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors [31].
[c] Weight-average molecular weight.
[d] *z*-average radius of gyration.

Table 5. Thermal properties of isolated starches [a, b]

Sample	Gelatinization of starch				Dissociation of retrograded starch				Retrogradation (%) [d]
	T_o (°C) [c]	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	
Bamboo seed	68.9 ± 0.1 b	73.4 ± 0.3 b	78.1 ± 0.4 b	14.2 ± 0.2 b	40.6 ± 0.5 b	53.4 ± 1.2 a	63.5 ± 1.1 a	8.1 ± 0.1 a	57.1 ± 1.3 a
Indica	71.9 ± 0.2 a	77.8 ± 0.1 a	83.0 ± 0.2 a	15.3 ± 0.2 a	41.6 ± 0.1 ab	55.3 ± 0.0 a	63.1 ± 0.1 a	8.5 ± 0.3 a	55.4 ± 1.4 a
Japonica	56.4 ± 0.0 c	63.5 ± 0.1 c	70.2 ± 0.1 c	14.0 ± 0.1 b	42.1 ± 0.1 a	52.5 ± 0.0 a	60.1 ± 0.0 b	1.6 ± 0.1 b	11.4 ± 0.4 b

[a] Values with the same letter in a column are not significantly different at $p < 0.05$.
[b] Measured using a differential scanning calorimeter.
[c] T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, and ΔH = enthalpy change.
[d] % retrogradation = 100% × ΔH of dissociation of retrograded starch / ΔH of starch gelatinization.

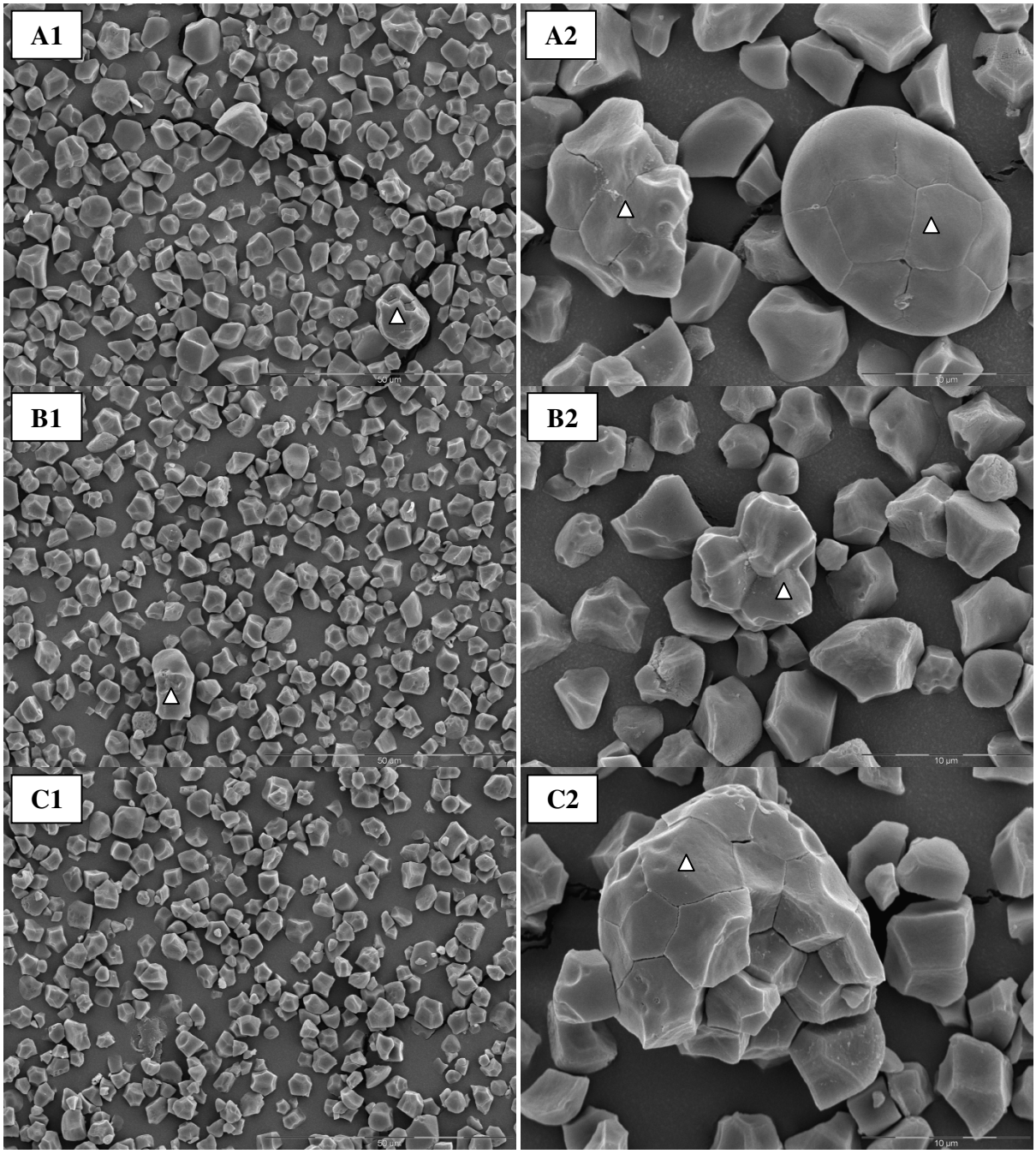

Figure 1. Scanning electronic micrographs of isolated starch granules. A: Bamboo seed, B: indica, C: Japonica. 1: 1500 × magnification, 2: 5000 × magnification. △ marks compound starch granules not separated after starch isolation.

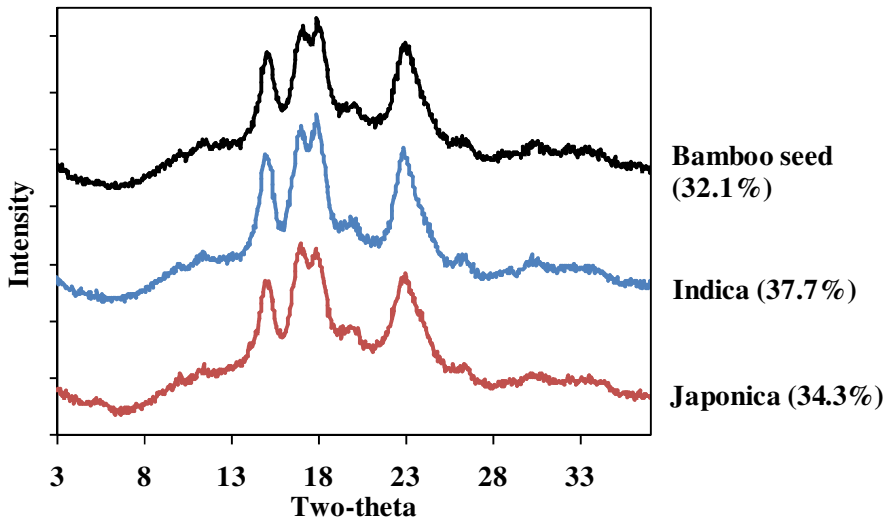

Figure 2. X-ray diffraction patterns of isolated starches. Percentage crystallinity is given in parentheses.

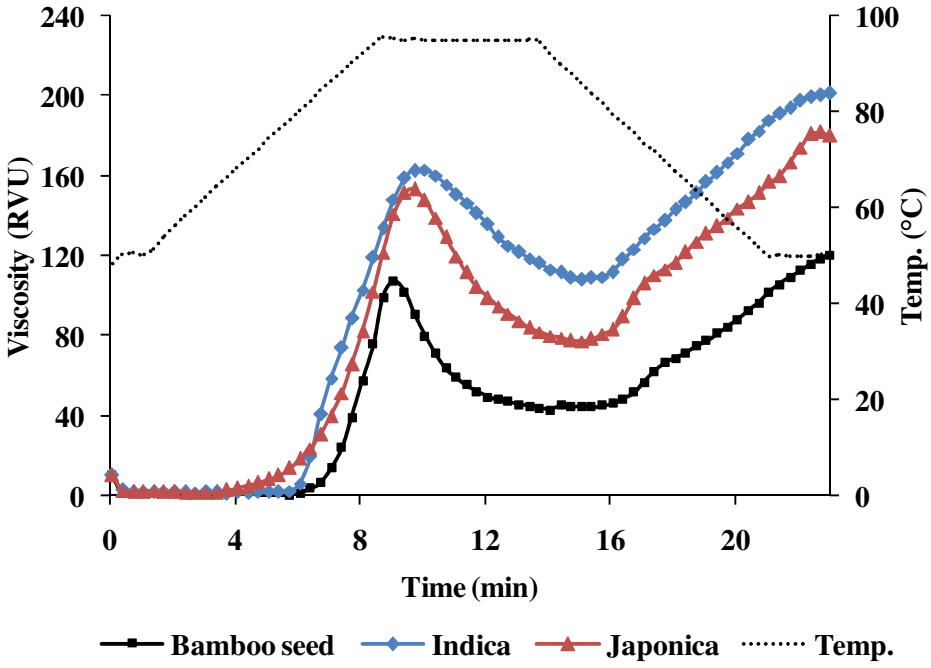

Figure 3. Pasting profiles of isolated starches analyzed using a Rapid Visco-Analyzer with 8% (dsb, w/w) starch suspension.

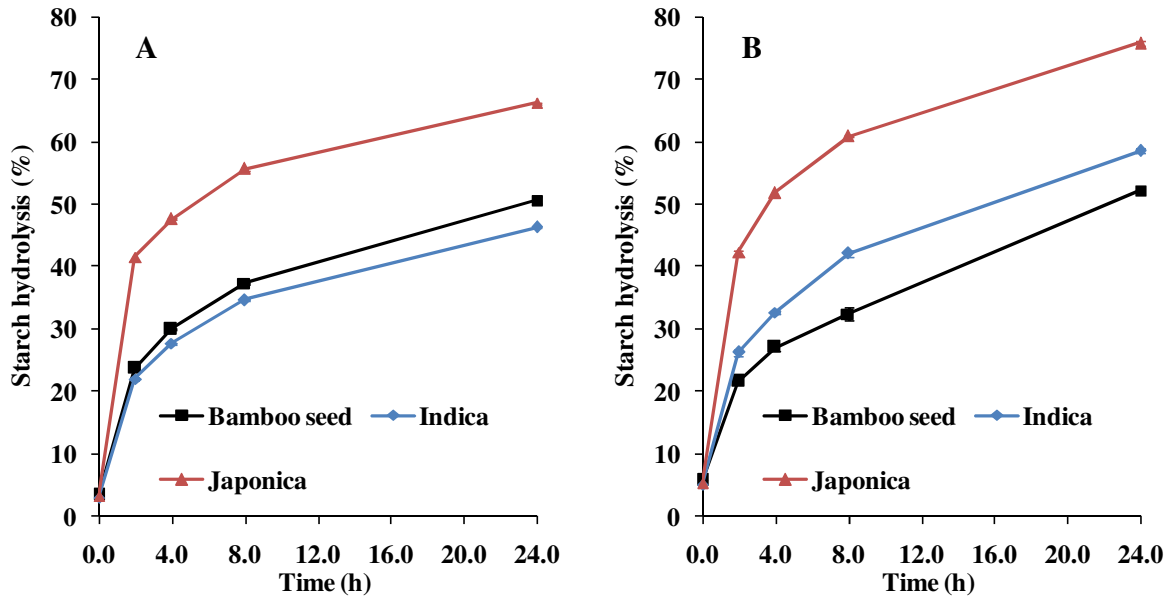

Figure 4. Enzymatic hydrolysis of isolated starches (A) and that of starch in ground kernels (B). PPA was used for the hydrolysis of uncooked starch and ground kernels at 37 °C, pH 6.9, with 80 rpm shaking. % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180).

Appendix of Chapter 6. Characterization of bamboo-rice starch

Milled rice kernels of two bamboo-rice varieties, Zhuxiang and Zhufeng, were obtained from Bamboo-rice Agriculture and Technology Co., Ltd (Renhua, China). The rice was harvested and milled in 2012. The bamboo-rice kernels were also subjected to dry grinding and starch isolation as described in **Chapter 6**. Structures, physical properties and enzymatic hydrolysis of starches isolated from Zhuxiang and Zhufeng bamboo rice were analyzed following the same methods reported in **Chapter 6**. The results are summarized below.

Table 1. Chemical compositions of bamboo-rice kernels (db) and amylose contents of isolated starches

Sample	Composition (%)			Amylose content of starch (%) [a]
	Starch	Protein	Lipid	
Zhuxiang	84.7 ± 0.7	8.8 ± 0.0	0.4 ± 0.0	14.4 ± 0.1
Zhufeng	82.8 ± 0.6	7.3 ± 0.0	0.3 ± 0.0	13.8 ± 0.4

[a] Determined using an iodine potentiometric titration method.

Table 2. Damaged-starch contents of isolated starches and ground kernels [a]

Sample	Damaged starch (%)	
	Isolated starch	Ground kernel
Zhuxiang	2.5 ± 0.0	11.6 ± 0.4
Zhufeng	2.8 ± 0.1	11.4 ± 0.9

[a] Determined using a Starch Damage Assay Kit.

Table 3. Branch-chain-length distributions of amylopectins [a]

Sample	Branch-chain-length distribution [b]				Average DP
	DP 6-12 (%)	DP 13-24 (%)	DP 25-36 (%)	DP >36 (%)	
Zhuxiang	34.6 ± 1.9	51.1 ± 0.9	6.1 ± 0.9	8.1 ± 0.3	17.8 ± 0.3
Zhufeng	34.7 ± 0.2	50.2 ± 0.7	6.9 ± 0.6	8.2 ± 0.2	17.9 ± 0.3

[a] Analyzed using fluorophore-assisted capillary electrophoresis.
[b] Molar basis.

Table 4. Molecular weights (M_w) and radii of gyration (R_z) of starch amylopectins [a]

Sample	M_w ($\times 10^8$, g/mol) [b]	R_z (nm) [c]
Zhuxiang	18.6 ± 4.8	492.5 ± 29.8
Zhufeng	17.7 ± 0.4	513.3 ± 2.0

[a] Measured using a high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors [31].
[b] Weight-average molecular weight.
[c] *z*-average radius of gyration.

Table 5. Thermal properties of isolated starches [a]

Sample	Gelatinization of starch				Dissociation of retrograded starch				Retrogradation
	T_o (°C) [b]	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	(%) [c]
Zhuxiang	59.3 ± 0.0	64.3 ± 0.1	69.4 ± 0.0	12.6 ± 0.4	41.4 ± 0.1	52.1 ± 0.1	61.4 ± 1.2	1.7 ± 0.1	13.3 ± 0.6
Zhufeng	59.2 ± 0.1	64.4 ± 0.3	69.3 ± 0.1	11.6 ± 0.0	41.1 ± 0.5	52.3 ± 0.2	62.3 ± 0.9	2.3 ± 0.1	20.1 ± 1.2

[a] Measured using a differential scanning calorimeter.
[b] T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, and ΔH = enthalpy change.
[c] % retrogradation = 100% × ΔH of dissociation of retrograded starch / ΔH of starch gelatinization.

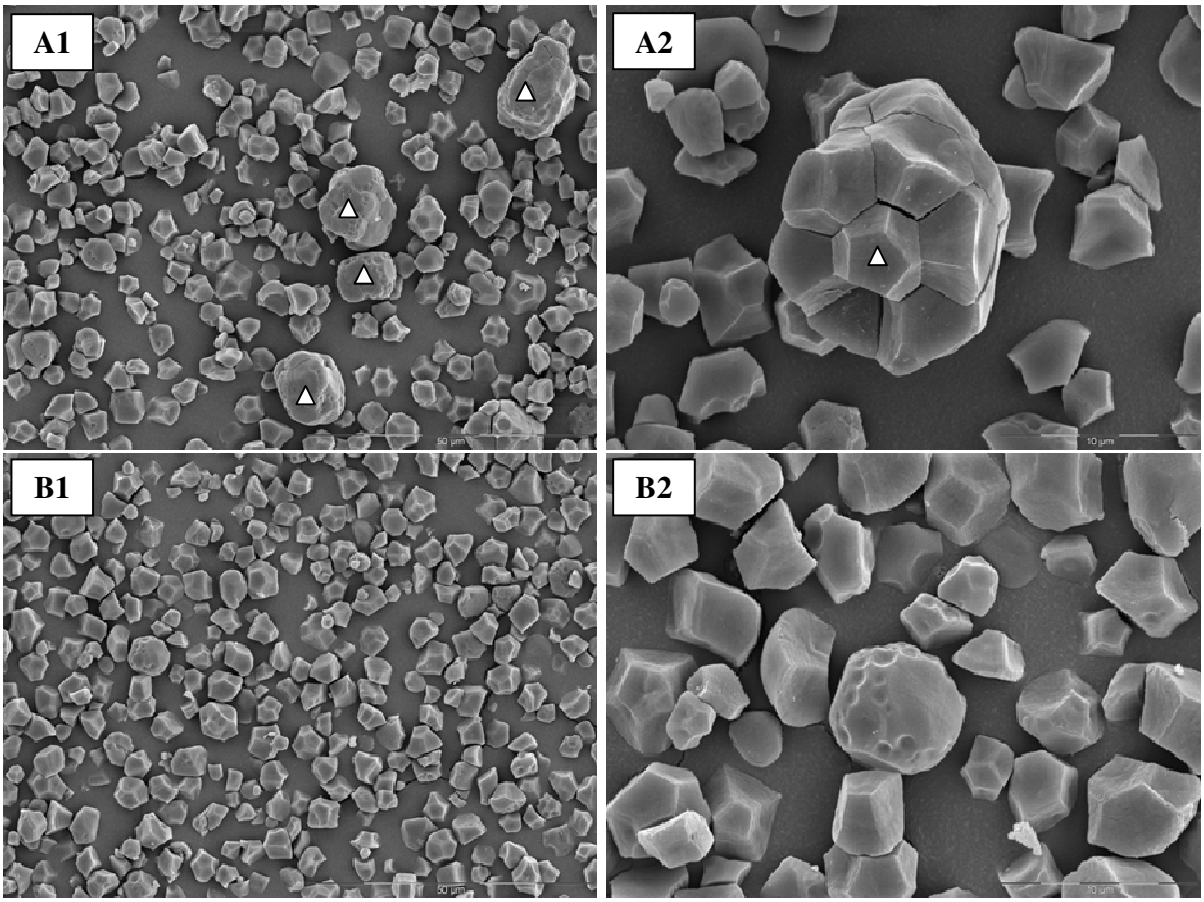

Figure 1. Scanning electronic micrographs of isolated starch granules. A: Zhuxiang, B: Zhufeng. 1: 1500 × magnification, 2: 5000 × magnification. △ marks compound starch granules not separated after starch isolation.

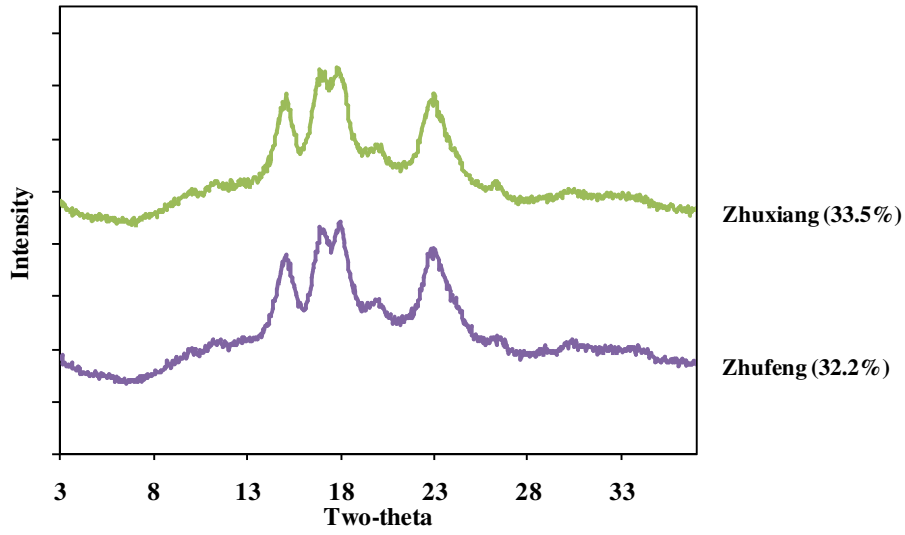

Figure 2. X-ray diffraction patterns of isolated starches. Percentage crystallinity is given in parentheses.

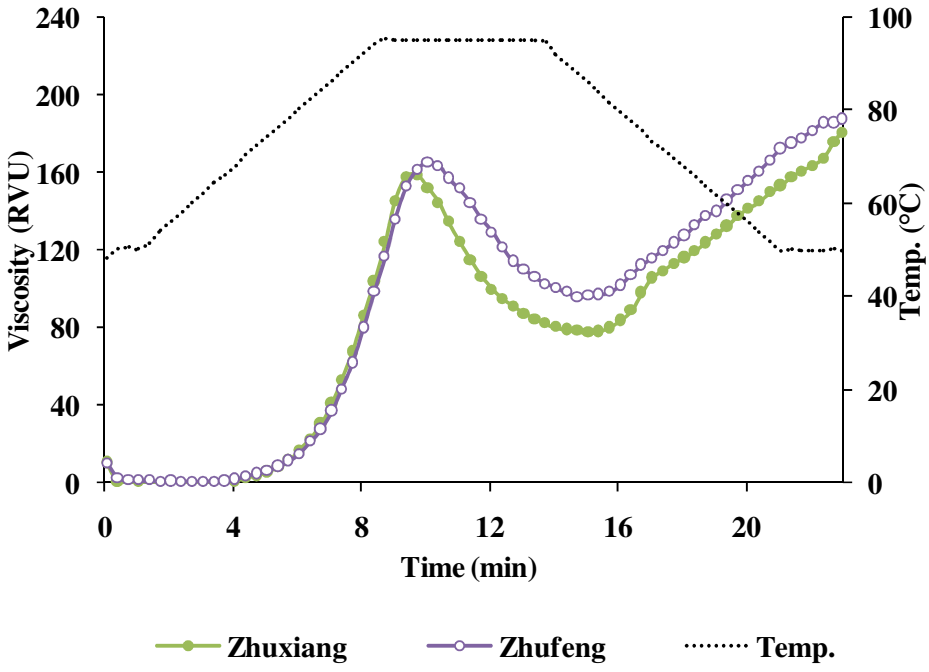

Figure 3. Pasting profiles of isolated starches analyzed using a Rapid Visco-Analyzer with 8% (dsb, w/w) starch suspension.

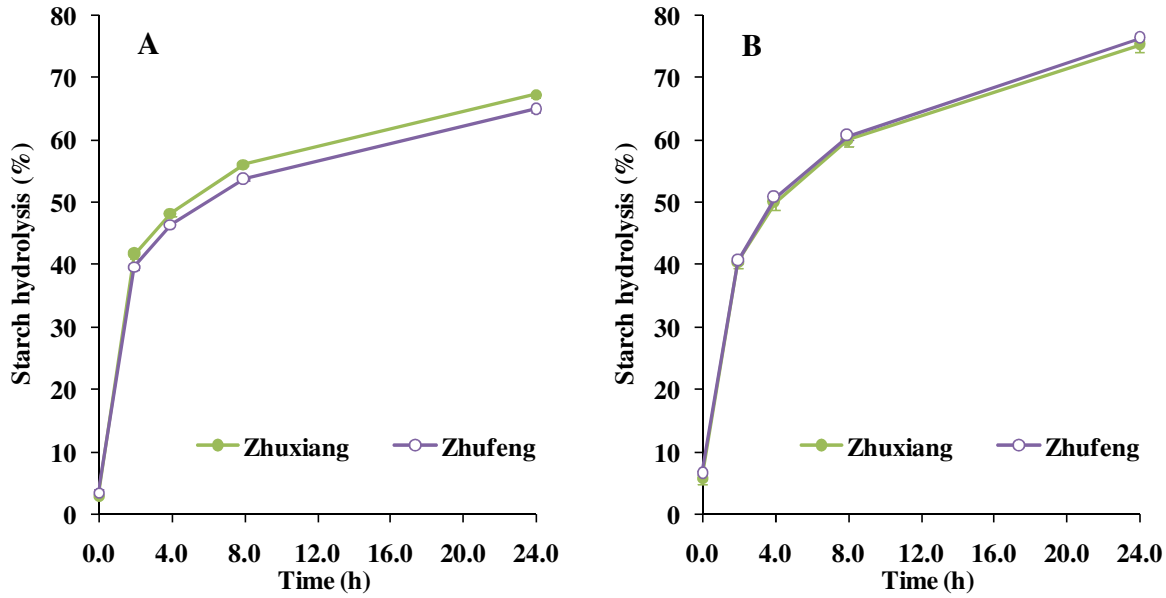

Figure 4. Enzymatic hydrolysis of isolated starches (A) and that of starch in ground kernels (B). PPA was used for the hydrolysis of uncooked starch and ground kernels at 37 °C, pH 6.9, with 80 rpm shaking. % starch hydrolysis = 100% × total mass of glucose released / initial dry mass of starch × (162 / 180).

Conclusion:

The starches from the bamboo rice, Zhuxiang and Zhufeng, exhibited characteristics similar to the japonica-rice starch analyzed in **Chapter 6**, suggesting the two bamboo-rice varieties were japonica type.

GENERAL CONCLUSIONS

The selected lipids, corn oil (CO), soy lecithin (SL), palmitic acid (PA), stearic acid (SA), oleic acid (OA), and linoleic acid (LA), reduced enzymatic-hydrolysis rates of normal corn (NCS), tapioca (TPS), and high-amylose corn (HA7) starch after cooking with the starches at 10% level (w/w, dsb). The DSC thermograms of the cooked starch-lipid mixtures displayed amylose-lipid-complex dissociation peaks for SL and free fatty acids (FFAs), but not with CO. The amylose-helical-complex formation with CO was confirmed by ^{13}C-NMR spectra. In general, FFAs decreased, but SL increased the peak viscosities of the starches. FFAs and SL reduced, but CO increased the gel strength of NCS. Because of the absence of amylose in WCS, these lipids showed little effects on the enzymatic hydrolysis and physical properties of the starch.

Because of amylose-SA-complex formation, the RS5 showed restricted swelling of starch granules at 95 °C. The RS5 showed a larger RS-content (67.8%) than the HA7 (33.5%) and NCS (0.8%) analyzed using the AOAC Method 991.43. When the cooked RS5, HA7 and NCS were used to prepare diets for rats with 55% (w/w) starch content, RS contents of the diets were 33.7%, 15.8% and 2.6%, respectively. The rat-feeding study showed that in Week 1, 16.1% of the starch in the RS5-diet was found in the feces, substantially greater than that of the HA7-diet (6.4%) and NCS-diet (0.1%). The percentage of starch not being utilized in the RS5-diet decreased to 4.7% in Week 9, which could be partially attributed to the fermentation of RS5 by gut microflora. Large proportions (68.2%-99.0%) of the SA in RS5-diet remained unabsorbed and were discharged in the rat feces.

After the modification with 3% and 10% octenyl succinic anhydride (OSA), RS contents of the cooked OS-NCS increased from 0.8% of the control starch to 6.8% and 13.2% (Englyst

Method), respectively, whereas that of the cooked OS-HA7 decreased from 24.1% to 23.7% and 20.9%, respectively. When the cooked NCS, HA7 and OS (10%)-HA7 were used to prepare diets for rats at 55% (w/w) starch, RS contents of the diets were 1.1%, 13.2% and 14.6%, respectively. The rat-feeding study showed that 20.2%-31.1% of the starch in the OS (10%)-HA7-diet was not utilized *in viv*o and was found in rat feces from Week 1 to 9, which was substantially larger than that of the HA7-diet (≤ 4.9%) and NCS-diet (≤ 0.2%).

Sorghum kernels from the five lines (6B73, 6C21, 6C69, 7R34, and X789) consisted of 68.7-70.6% starch, more than the B73 corn (67.4%). Sorghum starches exhibited higher gelatinization-temperatures (66.6-67.4 °C), greater gelatinization enthalpy-changes (13.0-14.0 J/g) and percentages retrogradation (60.7-69.1%), but slower enzymatic-hydrolysis rates (83.8-87.8% at 48 h) than the B73 corn starch (61.7 °C, 10.1 J/g, 51.5%, and 88.5%, respectively). The results could be attributed to that the sorghum amylopectins consisted of fewer short branch-chains (DP 6-12) (12.8-14.0%) than the corn amylopectin (15.0%). After being subjected to cold fermentation for 96 h using raw starch hydrolyzing enzyme, most of the ground sorghum samples exhibited smaller ethanol-yields (30.5-31.8%) and conversion-efficiencies (78.3%-80.9%) than the ground B73 corn (31.8% and 83.3%, respectively).

The bamboo seeds (*Bambusa textilis* species) also had compound starch granules, with morphology and sizes similar to the rice starches. The amylopectin of bamboo-seed starch displayed similar branch-chain-length distribution to that of the indica-rice starch, and both of them had longer branch-chains (DP = 19.1 and 19.7, respectively) than the japonica-rice amylopectin (DP = 17.1). The differences in the amylopectin structure resulted in higher gelatinization-temperatures (T_o = 68.9 °C and 71.9 °C, respectively), larger enthalpy-changes (ΔH = 14.2 J/g and 15.3 J/g) and percentages of retrogradation (57.1% and 55.4%), but slower

enzymatic-hydrolysis rates (50.6% and 46.3% at 24 h) of the bamboo-seed and indica-rice starches than the japonica-rice starch (56.4 °C, 14.0 J/g, 11.4%, and 66.2%, respectively). The bamboo-seed starch exhibited a higher pasting-temperature but substantially lower viscosity than the rice starches, which could be attributed to its larger amylose-content and smaller molecular-weight and gyration-radius of the amylopectin. In addition, the starches from the bamboo rice, Zhuxiang and Zhufeng, had characteristics comparable to the japonica-rice starch, suggesting the two bamboo-rice varieties were japonica type.

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