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Studies on mechanisms of resistant starch analytical methods

by

Samuel Ahlias Moore

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

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Ames, Iowa

2013

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ABSTRACT

This study aimed to quantify resistant starch (RS) contents in native and modified starches from various botanical sources using standard methods for RS analysis (AOAC Methods 991.43, 2002.02, and the Englyst method) to better understand differences in RS contents of starchy foods analyzed using these methods. RS contents of octenyl succinic (OS) normal corn starch increased with increasing wt% substitution, whereas RS contents of OS-modified high-amylose maize starch decreased. Differences in RS contents of RS3 were within 10% regardless of the method used. The largest differences were observed in cross-linked wheat distarch phosphate and debranched high-amylose maize stearic-acid complex (RS5) analyzed using AOAC Method 991.43 and other methods. Differences in RS contents were partially attributed to binding-site differences between *Bacillus licheniformis* (BL) and porcine pancreas (PPA) α-amylase. Starch granules treated with BL and PPA at equal activity showed distinct morphological changes after 84 h of hydrolysis. Starches hydrolyzed by BL were mainly eroded on the surface of the granules, whereas PPA showed pitting on granule surfaces. Banana starch granules were hydrolyzed mainly in distal regions of the granule. These results suggest that mechanistic differences in enzyme action are responsible for differences in measured RS contents of native and modified starches.

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INTRODUCTION

Starch is the major energy storage of higher plants. Starch is a mixture of two glucans: amylose and amylopectin. Amylose is a primarily linear polymer of α-1,4 linked D-glucose units, whereas amylopectin is a highly branched molecule with linear chains of α-1,4 links connected by α-1,6 branch linkages (ca. 5%, Pérez and others 2009). Starchy foods are major calorie sources in human diets. Fast rates of starch digestion characteristic of foods made with finely milled flours instead of whole grains have been associated with high postprandial serum glucose (Jenkins and others 1981) and insulin concentrations (Holt and others 1997). Chronic repetition of these hyperglycemic and hyperinsulinemic responses contributes to conditions such as diabetes, obesity, and the metabolic syndrome (Finley and others 2010). Thus, starches that resist digestion and are digested slowly may prevent the development of these conditions.

Resistant starch (RS) is defined as a portion of starch that survives digestion in the small intestine of healthy humans (Asp 1992). RS thus functions as a prebiotic and can be fermented in the colon to yield products beneficial to the host, such as short-chain fatty acids (Cummings and Macfarlane 1991). Additionally, RS acts as dietary fiber (DF) and contributes to fecal bulking (Phillips and others 1995), shortens intestinal transit time of food bolus (Mathers, Smith, and Carter 1997), and reduces development of cancerous lesions of the bowel in animal models (Hinnebusch and others 2002, Le Leu and others 2009, Zhao and others 2011). RS is classified according to its structural characteristics. RS1 is physically inaccessible starch such as in whole or coarsely milled grains (Englyst, Kingman, and Cummings 1992). RS2 is enzyme-resistant native, raw starch granules with the B or C-type polymorph such as that of potato, green banana, and high-amylose maize (Englyst, Kingman, and Cummings 1992). RS3 is retrograded starch formed by recrystallization of starch chains during storage after cooking (Englyst, Kingman, and

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Cummings 1992). RS4 is chemically modified starch such as starch cross-linked with phosphate diesters (Shin, Song, and Seib 2004) or derivatized using octenyl succinic anhydride (Wang and others 2011). RS5 is the helical inclusion-complex formed between amylose and lipids (Hasjim and others 2010).

Various *in vitro* methods for determination of RS contents in foods have been proposed and applied in the food industry. Some of these methods are mainly for analysis of DF contents in foods (Englyst and others 2013). RS contents of foods analyzed using those standard methods, however, have shown discordant results for the same food sample depending on the method used (Kontrazsti, Hudson, and Englyst 1999, Englyst and others 2013). Because of the health benefits of RS in the human diet, accurate quantification of RS in foods is of critical importance.

This study aimed to quantify RS contents in native and modified starches using three standard analytical methods, AOAC International Official Methods 991.43, 2002.02 (AOAC International 2013), and the method developed by Englyst and others (1992, Englyst method) to better understand causes of apparent differences in RS contents using these methods. Standard methods of RS analysis used in this study were selected based on commonality of use and differences in enzymes and sample treatment conditions. To the best of our knowledge, no papers describing a wide variety of different RS types analyzed using these standard methods have been reported in the literature.

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LITERATURE REVIEW

General Starch Structure and Chemistry

Starch is the major storage polysaccharide in higher plants. Starch is largely synthesized in storage organs such as tubers, roots, and seeds, but stored polysaccharide as starch may also be found in leaves, stems, and to varying degrees in fruits (e.g. green banana, Shannon and others 2009). The role of starch is to provide a stable source of energy as glucose to the plant for normal metabolic function during germination and times of scarcity (Fettke and others 2012). Starch molecules are homopolymers of D-glucose and are present in two structures within starch granules, amylose and amylopectin, which differ in molecular size and degree of branching (Pérez and others 2009). Amylose is a linear molecule consisting mainly of α-1,4 glucosidic linkages with a few α-1,6 branches (Pérez and others 2009). In contrast, amylopectin is a highly branched molecule with α-1.4 glucosidic linkages along the main chains and about 5% α-1,6 branch linkages (Pérez and others 2009). Depending on the botanical source and genetic background of the parent plant, amylose and amylopectin content of the starch granule may vary, but generally amylose content in normal starches ranges from approximately 20 – 30% (Jane 2009). Waxy starches may contain from 0% (e.g. waxy maize) up to approximately 9% amylose (e.g. waxy barley, Jane and others 1999, Song and Jane 2000). High-amylose starches are enriched in amylose content due to a mutation of the amylose extender (*ae*) gene locus and generally range from 50 – 70% amylose depending on botanical source (Li and others 2008). Amylose content has been shown to influence starch granule morphology and rate of starch digestion (Li and others 2008). Average molecular weight and degree of polymerization (DP) of amylose and amylopectin are dependent on botanical source of starch. Takeda and others (1987) found that amylose molecules in cereal starches were generally smaller than those in starch of

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other plant storage organs. Degree of polymerization (DP) of amylose from common normal botanical sources ranges from about 960 (normal maize) to 3280 (sweet potato, Takeda and others 1987).

Starch granules have semicrystalline structure containing both crystalline and amorphous regions that render whole starch granules insoluble in water (Imberty and others 1991). Nikuni (1978) described a model of amylopectin superstructure wherein amylopectin branches are chiefly responsible for granular crystallinity *via* local, helical, crystalline interactions. The attribution of starch granule crystallinity to the amylopectin component was later supported by solid-state ^{13}C nuclear magnetic resonance spectroscopy studies by Veregin and others (1986). Starch granules may assemble into one of four X-ray crystalline diffraction patters based on amylopectin fine structure characteristics. The A-type crystalline pattern is characteristic of cereal starches such as maize and wheat and is a result of associations of short amylopectin branch-chains (DP 10 – 12) without inclusion of water in the unit cell (Wu and Sarko 1978). The B-type crystalline pattern is characteristic of potato, some fruits such as winter squash, or high-amylose cereal varieties and results from looser packing of long amylopectin branch chains (DP > 12) associated with 36 molecules of water in the unit cell (Pfannemüller 1987). The C-type crystalline pattern results from a mixture of A-type and B-type regions within a single starch granule and is characteristic of starches from pulses (e.g. pea), lotus root, water chestnut, and certain fruits such as some varieties of banana and apple (Bogracheva and others 1998, Jane and others 1999, Stevenson and others 2006). The V-type crystalline pattern arises from single helices of amylose or single helical complexes of amylose with lipid and is observed in cooked dough (Zobel 1988).

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Starch crystallites composed of parallel amylopectin branch-chains are arranged in crystalline growth rings up to 10000 times larger than individual crystallites alternating with amorphous regions extending radially from the organic center of granule growth, which is known as the hilum (Badenhuizen and Dutton 1956). The location of amylopectin branch junctions is determined by granule crystalline pattern: branch points occur in amorphous regions in B-type starches, whereas branch points are partially localized in crystalline regions in A-type starches (Jane and others 1997). Average thickness of the alternating amorphous-crystalline region structural repeat was determined by Jenkins (1993) as 9.0 and 9.2 nm for A-type and B-type starches, respectively. Amylose molecules are synthesized by apposition from the granule hilum (Badenhuizen and Dutton 1956) and exist in amorphous form in a radial arrangement and may span one or more crystalline lamellae (Jane and others 2003). The proportion of amylose molecules concentrated at the periphery of starch granules is increased, whereas average DP of amylose is decreased at the periphery instead (Pan and Jane 2000).

Starch granules are hygroscopic and exhibit limited swelling in the presence of water (Jane 2001). Heating in excess water disrupts the granule crystalline structure and is known as gelatinization (Jane 2001). Gelatinization temperature is not a discrete entity but a range due to the association of starch chains of varying DP that make up granule crystalline regions (Colonna and others 1992). The temperature at which the native granule crystalline structure begins to be lost is known as the onset gelatinization temperature (T_o, Jane 2001). T_o is also increased when the ratio of water to starch in the system is less than 2:1 (Stevens and Elton 1971, Yu and Christie 2001). Energy required to break noncovalent interactions in starch crystallites is quantified using enthalpy change of gelatinization (ΔH). Both ΔH and range of gelatinization temperature are generally increased in high-amylose starches (Kibar and others 2010).

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Application of mechanical shear to swollen, gelatinized starch results in breakdown of granular structures and is known as pasting (Jane 2001).

Amylose molecules may be leached from swollen starch granules during pasting associated with cooking, which results in a gel upon cooling (Jane 2001). Retrogradation is the term used to describe reassociation of starch chains in double-helical conformation after cooking and is known to negatively impact quality attributes of starchy food products, most notably through staling of bakery products (Aguirre and others 2011). Amylose molecules are more mobile and retrograde rapidly after removing heat from the system to form a gel network that gives cooked starchy products a gel-like texture, whereas amylopectin side-chains retrograde over a longer time-scale (days to weeks) and are responsible for adverse effects on product quality attributes due to staling (Aguirre and others 2011). Retrogradation induces formation of a thermostable crystalline double-helix that does not readily dissociate upon reheating (Farhat and others 1997). Formation of retrograded double helices of starch chains is maximized at refrigeration temperatures (0 – 4 °C, Farhat and others 1997) and is enhanced in foods containing predominantly B-type starch due to the presence of long amylopectin branch-chains that are readily reassociated (Lee and others 2012). Notably, the double-helical, crystalline conformation of retrograded starch is resistant to hydrolysis by digestive enzymes and is considered a type of resistant starch (Jane and Robyt 1984).

Resistant starch (RS) is the fraction of starch polysaccharide that survives digestion in the stomach and small intestine of healthy humans (Asp 1992).The presence of a starch fraction resistant to digestion by amylases was first reported by Englyst and colleagues (1982). Five classes of RS are currently known: resistant starch type 1 (RS1), or physically inaccessible starch such as that found in whole or coarsely ground cereal grains (Englyst and others 1992); resistant

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starch type 2 (RS2), or uncooked starch granules that resist digestion on the basis of native granule structure such as those of potato and green banana (Englyst and others 1992); resistant starch type 3 (RS3), or retrograded amylose double helices formed following gelatinization of the starch granule (Brumovsky and others 2009); resistant starch type 4 (RS4), or chemically-modified starch such as cross-linked starch (Sang and Seib 2006) or octenyl succinic starch (Zhang and others 2011); and resistant starch type 5 (RS5), or amylose-lipid V-type complex (Cui and Oates 1999, Hasjim and others 2010). RS content in starchy foods may be influenced by processing such as thermal treatments or by mechanical forces acting on the granule, as in milling. RS content from RS1 is decreased in proportion to the degree of destruction of the whole grain in milling processes (Alsaffar 2011). RS3 is increased in cooked starches with cooling and storage due to retrogradation associated with labile amylose chains leached from gelatinized starch granules. Long amylopectin side chains may also complex with amylose or neighboring long chains to form RS3. The double-helical amylose complexes comprising RS3 are generally thermostable and are not lost upon reheating.

Structural Features of Enzyme-Resistant Native Banana Starch (RS2)

Starch derived from green bananas is one of the few means of introducing RS2 into the human diet using whole foods and has attracted singular attention in the field of nutrition research for this reason (Sugimoto and others 1980, Silvester and others 1995, Faisant and others 1995, Langkilde and others 2002). Banana starch granules have usually been described as having a C-type crystalline structure (Jane and others 1997, Peroni-Okita and others 2010), but other reports indicate that growing conditions and cultivar may predispose the banana starch granule to the A-type or C-type allomorph (Bello-Pérez and others 2000). Banana starch granules are described as elongated, irregularly shaped structures with an eccentric birefringence pattern

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bearing the hilum at the narrow end of the granule (Li and others 1982). The granules have a smooth surface without evidence of pores or pinholes characteristic of cereal starches (Peroni-Okita and others 2010, Soares and others 2011). The enzymatic degradation of banana starch during transit through the small intestine of humans (Faisant and others 1995, Langkilde and others 2002) and rats (Sugimoto and others 1980) as well as during ripening of the banana fruit (Peroni-Okita and others 2010) has been previously described. Endogenous amylase action on the banana starch granule during ripening was shown to wear away the smooth surface of the granules to expose deep, canal-like structures between growth rings (Peroni-Okita and others 2010). In contrast, *in vivo* hydrolysis of banana starch granules in rats (Sugimoto and others 1980) and humans (Faisant and others 1995, Langkilde and others 2002) showed mixed results. Sugimoto and colleagues (1980) reported that banana starch granules recovered from stomachs of sacrificed rats showed only surface erosion due to acid hydrolysis, whereas banana starch granules recovered from the small and large intestines of these animals showed similar surface striations and an erosion of the outer layer of the banana starch granule. This pattern was in distinct contrast to hydrolysis of maize starch granules, which showed characteristic enlargement of surface pores and inside-out hydrolysis following enzyme attack (Sugimoto and others 1980). More extensive damage to starch granules after transit through the small intestine of humans was reported by Faisant (1995) and Langkilde (2002). Faisant (1995) observed the presence of surface pinholes in banana starch granules recovered from ileal effluent of humans using transmission electron microscopy, whereas surface pinholes were not observed in banana starch granules recovered from rats (Sugimototo and others 1980) or in starch granules from ripened bananas (Peroni-Okita and others 2010). Sugimoto (1980) suggested that physical damage due to chewing, acid hydrolysis in the stomach, and interaction with bile acids were all factors

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contributing to damage of banana starch granules in addition to attack by α-amylases. Specific formation of irregular scratches and crevasses on banana starch granules, in contrast with exocorrosion of the surface to reveal surface striations, were attributed to the motions of chewing and/or peristalsis (Faisant and others 1995).

Physicochemical properties of banana and plantain starches have been reported by various authors (Ling and others 1982, Soares and others 2011, Bezerra and others 2013). Starch of green bananas of the Cavendish cultivar contained 19.5% amylose and showed a gelatinization temperature range of 70.1 – 74.6 °C (Ling and others 1982), which is notably higher than the range (60.8 – 70.6 °C) observed in normal potato starch (McPherson and Jane 1999). The lower gelatinization temperature range observed in normal potato starch is attributed to the presence of a relatively high concentration (0.075%) of phosphorous as phosphate monoesters of amylopectin molecules (McPherson and Jane 1999). An exhaustive report of phosphorous content and form in banana starches has not appeared in the literature; however, phosphorous content in plantain starches was reported by Eggleston and others (1992) and ranged from 0.020 – 0.031% of dry mass of starch, depending on cultivar.

A model for organization of starch structures in RS2 such as banana and potato starch contributing to amylolytic enzyme resistance have been proposed by Gallant (1992) and Faisant (1995). Starch crystallite blocklets were found to form a dense layer approximately 15 µm thick in potato starch granules, which was proposed by Gallant (1992) to be the causative mechanism behind the superficial enzyme hydrolysis and erosion by enzymes in potato and banana starch granules. Faisant and others (1995) observed a similar pattern of concentric, spherical blocklets in the exterior of banana starch granules and found that endocorrosion of banana starch granules occurred only after this surface layer was removed by acid and enzyme hydrolysis processes of

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human digestion. Atomic-force microscopy studies of starch granules isolated from bananas of the Nanicão cultivar showed that exterior regions of the granule consisted of harder material than the interior (Peroni-Okita and others 2010). The proportion of B-type to A-type crystallinity was found to increase from 1.22 to 1.56 after 0 and 18 d post-harvest in this same cultivar, indicating that the A-type polymorph is preferentially hydrolyzed by endogenous amylases during ripening (Peroni-Okita and others 2010). However, degree of crystallinity simultaneously decreased from 23.6% to 21.1% during ripening (Peroni-Okita and others 2010). These results appeared to indicate that enzyme resistance of banana starch granules may be due to an interior region of densely-packed, largely B-type crystalline blocklets, while the exterior of the granule may consist of a hard shell of largely A-type crystalline material. However, no reports of localized ultrastructural features of banana starch granules such as crystalline polymorph have yet appeared in the literature.

Analysis of Dietary Fiber and Resistant Starch Contents of Foods

Dietary fiber (DF) refers to non-digestible polysaccharide constituents of plant foods that survive hydrolysis by human digestive enzymes (Phillips 2011). This definition includes RS, resistant maltoligosaccharides (e.g. resistant maltodextrins), lignocellulosic materials, hemicelluloses, and food gums (Mongeau and Brooks 2011). Retrograded starch (RS3) is included in the insoluble DF fractions of starchy foods analyzed using methods for determination of DF and/or RS contents (Englyst and Hudson 1996). Some authors have proposed an expanded definition of DF that includes all food components that survive digestion and pass on to the large intestine and may become substrates for fermentation by the colonic microbiota, including lignin, amylase-resistant oligosaccharides, RS, and undigested protein fragments (Asp and others 1996, Prosky 1999, Saura-Calixto and others 2000, Saura-Calixto and Goñi 2004). This latter

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definition would expand the traditional understanding of DF to include all components of an insoluble fraction instead of only carbohydrate components. Under both of these definitions, RS represents a contribution to total DF content of a food. However, common analysis methods for DF content have been known to yield discordant results for the same food (Kontrazsti and others 1999, Englyst and others 2013). Presently, the reasons for these differences are only partially understood.

Methodologies for analysis of DF contents in foods have undergone significant evolution since their first use. The earliest published protocol for determination of DF is attributed to Henneberg and Stohmann (1860) as crude fiber extract. Crude fiber is obtained by first extracting a sample with an appropriate organic solvent followed by treatment with dilute strong acid (H_2SO_4) and neutralization with strong base (NaOH) and is measured gravimetrically (Soest 1994). The crude fiber method is today mainly applied to analysis of animal feeds (Soest 1994). Later analytical methods for DF employed enzyme mixtures of varying purity to mimic the action of human digestion more accurately than acid hydrolysis alone. Common analytical methods for DF contents of foods include AOAC CODEX Methods 991.43 (Total, Soluble, and Insoluble Dietary Fiber in Foods, 1994), Method 2001.03 (Dietary Fiber Containing Supplemented Resistant Maltodextrin, 2004), Method 2009.01 (Total Dietary Fiber in Foods, 2009) and Method 2011.25 (Insoluble, Soluble, and Total Dietary Fiber in Foods, 2011). Methods specifically designed for determination of RS content of foods include AOAC Method 2002.02 (Resistant Starch in Starch and Plant Materials, 2002), which has been commercialized in an assay kit sold by Megazyme (Wicklow, Ireland), and the Englyst method (Englyst and others 1992). AOAC Method 991.43 is widely used for reporting of DF content of foods in the United States and European Union. The Englyst method was formerly used for analysis of DF in

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foods in the United Kingdom prior to 1999, but use of AOAC Method 991.43 in the United Kingdom is now standard (Institute of Food Science & Technology 2013). These methods represent distinct methodological approaches to the analysis of DF content in foods, and most methods for analysis of DF content are generally similar to one of the above. A brief summary and inherent limitations of each method are described below:

AOAC Method 991.43 (Official Methods of AOAC International) measures total dietary fiber (TDF) as the filtrate residue of a food sample following digestion with a thermostable α-amylase from *Bacillus licheniformis* in 2-(*N*-Morpholino) ethanesulfonic acid – tris(hydroxymethyl)aminomethane buffer (MES-Tris, 0.05 M MES, 0.05 M Tris, pH = 8.2) in a boiling water bath for 30 min. The pH is then adjusted to 4.4 – 4.6, amyloglucosidase from *Aspergillus niger* is added, and a secondary digestion in a 60 °C water bath follows. An intermediate protease digestion step may be added for foods containing protein in addition to starch. The residue representing insoluble DF is filtered through diatomaceous earth, and the weight of this residue represents the RS content in a starch sample. Because of the boiling water bath treatment, only thermostable RS is determined using this procedure, and thus some types of RS, such as raw potato starch, are lost during sample processing. In addition, the reaction conditions do not accurately emulate the mechanics of human digestion, meaning that DF content measured using AOAC Method 991.43 chiefly reflects chemical characteristics of the sample rather than the functional-based definitions of both DF and RS. The method, however, is among the most rapid for DF/RS analysis, and an automated instrument for the procedure has recently been commercialized (Komarek 2012). AOAC Method 991.43 was an evolution of an earlier standard method, AOAC Method 985.29 (Official Methods of AOAC International) allowing for optional fractionation of soluble and insoluble fractions of DF. Because this aspect

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of the method is not relevant to the analysis of RS and because AOAC Method 991.43 is now the industry standard, discussion will refer to AOAC Method 991.43 instead of 985.29.

AOAC Method 2002.02 (Official Methods of AOAC International) is intended for use with raw starch samples and gives a direct measure of resistant starch in the raw starch granule. The method is briefly described as follows: starch sample (0.1 g) is added to a sealed tube with 0.1 M sodium acetate buffer (pH 4.5), porcine pancreatic α-amylase, and amyloglucosidase from *Aspergillus niger*, mixed using a vortex mixer, and digested in a 37 °C water bath for 16 h. Following the digestion step, 4 mL 100% ethanol is added to stop the reaction, samples are centrifuged, and the supernatants are collected in a volumetric flask. Each sample is washed twice with 8 mL 50% ethanol using a vortex mixer, centrifuged, and the supernatant is decanted and added to the collected supernatant. The pooled sample supernatants representing digestible starch (DS) fractions are diluted to 100 mL, centrifuged, and used for glucose oxidase-peroxidase (GOPOD) assay for quantification of glucose in solution without further processing. The residue is dried in air, solubilized over an ice bath in 2 M KOH to solubilize starch residue, and pH 3.8 sodium acetate buffer is added to adjust the pH to approximately 4.5. The dispersed starch residue representing RS fractions is digested using amyloglucosidase in a 37 °C water bath for 30 min, diluted to 100 mL, and used for GOPOD assay. Total starch content of the sample is taken as the sum of DS and RS fractions. AOAC Method 2002.02 has some distinct advantages for RS analysis in that it is specifically designed for starch analysis instead of gross DF content of a food. However, the use of purified porcine pancreatic α-amylase and prolonged 16 h digestion period used may not accurately mimic the conditions of human digestion. In addition, the procedure is lengthy and has numerous opportunities for human error during the decanting steps, rendering it impractical for rapid analysis of RS-containing samples.

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The Englyst method for DF and RS content analysis (Englyst and others 1992) is intended to mimic the process of human digestion as accurately as can be achieved *in vitro*. This method includes a determination of rapidly-digestible starch (RDS) and slowly-digestible starch (SDS) fractions in addition to RS. The Englyst method uses an aqueous extract of crude porcine pancreatin mixed with amyloglucosidase for hydrolysis of starch up to 2 h. Samples are generally used as-is for whole foods or cooked with a boiling treatment for raw food ingredients prior to hydrolysis. Aliquots are withdrawn after 20 and 120 min for determination of RDS and SDS fractions, respectively; RS is taken as the remaining undigested starch out of the total starch content of the sample. A trypsin proteolysis and/or acid hydrolysis pre-treatment option is given as a potential option to better simulate physiological digestion.

Other contemporary methods for analysis of DF contents in foods are typically elaborations of one of the above three methods. AOAC Method 2011.25 (Official Methods of Analysis of AOAC International) conforms to the general principle of AOAC Method 2002.02 but separates DF into ethanol-soluble and ethanol-insoluble fractions following precipitation with absolute ethanol and includes a final high-pressure liquid chromatography (HPLC) step to quantify ethanol-soluble DF. AOAC Method 2001.03 (Official Methods of Analysis of AOAC International) is an extension of AOAC Method 985.29/991.43 employing an HPLC finish to quantify supplemented resistant maltodextrins in foods not precipitated by addition of ethanol. AOAC Method 2009.01 (Official Methods of Analysis of AOAC International) is a new method intended to integrate the 16 h digestion time of AOAC Method 2002.02 with the gravimetric determination of DF content used in AOAC Methods 985.29/991.43, fractionation of soluble and insoluble DF by ethanol precipitation used in AOAC Method 991.43, and chromatographic workup for determination of resistant maltodextrins used in AOAC Method 2001.03.

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Limitations of and Sources of Differences in DF/RS Analysis Methods

Factors affecting the apparent DF values obtained by AOAC standard methods have been identified by Mañas (1993) and Saura-Calixto (1994). Apparent lignin content in non-starchy plant materials is increased by omission of the protease digestion step in AOAC Method 991.43 (Mañas and others 1994). Soluble DF may be under-estimated and insoluble DF over-estimated by absorption of soluble components retained in the gravimetric filtration step, such as plant polyphenols. Inherent errors in protein and ash content estimations may be propagated to DF content determinations when these corrections are applied, which may be particularly problematic for starchy foods containing low RS content (Mañas and others 1994). The ethanol precipitation step may also systematically over-estimate DF content by: (1) co-precipitation of non-fiber, or (2) incomplete precipitation of SDF components (e.g. pectin, Mañas and others 1993). AOAC Method 991.43 systematically under-estimates non-thermostable RS2 in starchy food samples due to boiling treatment (Englyst and others 2013).

Evans (2007) evaluated DF content of high-amylose maize starch and two RS-containing food ingredients containing retrograded amylose using two commercial kits for RS content analysis obtained from Megazyme and Sigma-Aldrich (St. Louis, MO) designed for gravimetric determination of DF residue after digestion with a thermostable α-amylase. RS content of high-amylose maize starch was 37.8% using the α-amylase from the Megazyme test kit and 24.4% using the α-amylase from the Sigma-Aldrich test kit (Evans 2007). Activity of the α-amylase included in test kits was determined to be the main causative factor for differences in RS contents of high-amylose maize starch and commercial RS ingredients when reaction conditions were held constant. Prescribed buffer conditions (MES-Tris buffer, pH 8.2 *versus* phosphate

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buffer, pH 6.0) also differed between test kits evaluated and was proposed as a secondary factor underlying differences in RS contents (Evans 2007).

Kontraszti and colleagues (1999) compared DF contents as non-starch polysaccharide in representative foods in the typical Hungarian diet analyzed using the Englyst method (Englyst and others 1992) and the Prosky procedure (Prosky and others 1988), which was used as an officially adopted method for DF analysis prior to the adoption of the current AOAC Method 991.43. However, the operating principles are essentially similar to those of the 991.43 standard method. Starchy foods analyzed in this study include puffed rice, potato, and rye bread, along with ten homogenized mixed meals. DF contents of starchy foods were 50 – 500% higher when analyzed using the Prosky method compared with the Englyst method procedure, and the same general trend was observed in mixed meals. Differences were most pronounced in puffed rice (6.1 and 0.9% DF for Prosky and Englyst methods, respectively) and rye bread (7.4 and 3.1% DF, respectively); differences were less pronounced in raw potatoes (1.6 and 1.1% DF, respectively). These differences were attributed to insensitivity of the Prosky procedure to retrograded starch (RS3) in baked products not present in freshly-cooked potatoes.

The resistance to digestion of phosphate cross-linked wheat starches (RS4) Fibersym™ and FiberRite™ (MGP Ingredients, Atchison, KS) analyzed for RS content using AOAC standard methods 985.29 and 2009.01 was recently reported by McCleary and others (2013). AOAC Method 985.29 is functionally similar to AOAC Method 991.43 but determines total DF only, and AOAC Method 2009.01 uses a 16 h digestion with PPA and amyloglucosidase followed by a brief heat treatment (ca. 95 °C) and pH change from 6.0 to 8.2 to denature amylolytic enzymes. Greater RS contents (as total DF) were found for both types of cross-linked starch using AOAC Method 985.29 (84.0 and 70.7% RS, Fibersym and FiberRite, respective) than 2009.01 (28.7 and

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8.8% RS, Fibersym and FiberRite, respectively). Additionally, these authors report that digestibility of these starches to bacterial α-amylase from *Bacillus licheniformis* was lower at 100 °C than at lower temperatures. This phenomenon was believed to be due to swelling of starch granules in the presence of surface-reinforcing cross-links under high heat, creating a physical barrier to enzyme penetration.

Enzyme Hydrolysis of Native Starch Granules

Differences in the hydrolyzing action of the amylase family based on specific morphological features and starch structures in native starch granules have been widely studied (Sugimoto and others 1980, Gallant and others 1992, Colonna and others 1992, Kimura and Robyt 1995). Hydrolysis by acids and enzymes occurs preferentially in the amorphous lamellae of the starch granule as opposed to double-helical crystalline regions (Jane and Robyt 1984). Enzyme hydrolysis is a diffusion-limited process, the rate of which is increased in the presence of surface pores that serve as openings to interior, radial, serpentine channels characteristic of cereal starches and others showing the A-type polymorph (Colonna and others 1992, Fannon and others 1992, Huber and BeMiller 1997). Hydrolysis of raw starch granules is limited compared with that of starch pastes due to destruction of the native crystalline order (Demirkan and others 2005). Amylose content is strongly correlated with lower starch hydrolysis rates and resistant starch contents of native starch granules (Regmi and others 2011, Syahariza and others 2013). Amylose is enriched at the periphery of normal maize starch granules (Pan and Jane 2000) and normal potato starch granules (Seng and Jane 1993) due to synthesis by apposition (Badenhuizen and Dutton 1956). Starch granules derived from high-amylose botanical sources contain a larger proportion of amylose chains and long amylopectin branch-chains in double-helical associations than normal starch granules and as a result display lower enzyme hydrolysis rates and higher RS

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contents than normal starch granules of similar botanical species (Li and others 2004, Jiang and others 2010). Starch granules from waxy maize are more loosely packed at the periphery than normal granules due to the radial arrangement of amylopectin molecules without intervening amylose chains (Atichokudomchai and others 2004). This loose packing results in greater permeability to hydrolytic enzymes, faster starch hydrolysis rates, and lower RS content than that of comparable normal starch granules of similar botanical species (Li and others 2004).

In addition to starch granule fine structure, extent of amylase binding to raw starch granules is another factor limiting raw starch hydrolysis (Sumitani and others 2000, Goyal and others 2006). Raw starch binding domains are most commonly expressed in fungal organisms, most notably from the genus *Aspergillus* (Goto and others 1994, Ashok and others 2000). Chimeric α-amylase enzymes incorporating starch binding domains from similar biological origin have been shown to improve raw starch hydrolyzing ability above that of wild type α-amylases (Ohdan and others 2000).

Structural Characteristics and Reaction Patterns of α-Amylase

Amylolytic enzymes catalyze the hydrolysis of glycosidic linkages in starch chains and are present in all biological classes of organisms (Robyt 2009). Amylolytic enzymes may be generally classified as *endo*- or *exo*-acting enzymes by their mode of action in starch pastes (Robyt 2009). *Endo*-acting amylolytic enzymes hydrolyze starch chains at interior linkages within the molecule to yield a rapid decrease in viscosity in starch paste and are thus sometimes referred to as liquefying amylases, whereas *exo*-acting enzymes liberate oligosaccharide units from non-reducing ends of starch molecules and do not significantly impact viscosity (Robyt 2009).

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α-Amylases catalyze the hydrolysis of α-1,4 linkages in amylose and amylopectin molecules of starch and produce oligosaccharide and polysaccharide products having the α-configuration on the reducing end (Robyt 2009). The α-amylase enzyme family shares certain common structural features and hydrolysis mechanisms (Kuriki and Imanaka 1999). Three-dimensional crystalline structures of α-amylases have been studied after complex formation with amylase inhibitors such as tendamistat (Wiegand and others 1995, Machius and others 1996) and acarbose (Brzozowski, and Davies 1997). Primary sequence and X-ray crystallographic analysis of crystalline α-amylases from different biological sources reveals that: (1) α-amylases are comprised of three distinct structural domains (Domains A, B, C), and; (2) α-amylases and related enzymes contain four highly conserved amino acid sequences that often contain the key catalytic residues of the enzyme, but may differ in location within the primary amino acid structure (Kuriki and Imanaka 1999). Domain A consists of a circular array of eight alternating *β*-strands and *α*-helices called the $(\alpha/\beta)_8$ barrel. The catalytic and glucose chain binding sites are located within the $(\alpha/\beta)_8$ barrel of Domain A. Domain B is comprised of three parallel and one antiparallel *β*-sheet and usually comprises around 70 residues in the primary sequence. Domain C is comprised of ten antiparallel *β*-strands. α-Amylases are also associated with a tightly-bound Ca^{2+} anion that links the A and B subunits and lends stability to the protein tertiary structure (Janeček 1994).

Although α-amylases from different biological sources differ in the number of glucose-binding subsites and reaction patterns to yield specific products, a common catalytic mechanism and organization of the catalytic subsite has been observed in numerous *α*-amylases and related enzymes (e.g. isoamylase, pullulanase, transferases, etc.,Janeček 1994, Kuriki and Imanaka 1999). The *α*-amylase catalytic site is comprised of a single Glu and two Asp residues in close proximity (Mishra and others 2002). Structural homology of this active site has been confirmed

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in a variety of amylolytic enzymes (e.g. pullanase from *Klebsiella aerogenes*, isoamylase from *Pseudomonas amyloderamosa*, glycogen debranching enzyme from human hepatic tissue, etc.) using X-ray crystallography and site-directed mutagenesis (Kuriki and Imanaka 1999). Because reducing end stereochemistry of products liberated by amylolysis is retained, two possible mechanisms for the hydrolytic action of *α*-amylase on starch chains have been proposed: one, a S_N1 substitution proceeding through a secondary carbonium ion intermediate at C-1 of the reactive glucose unit, or; two, a S_N2 substitution proceeding *via* a covalently-bound Asp-glucose complex. More recently, the S_N2 double-displacement mechanism was definitively demonstrated by Kuriki and Imanaka (1999).

α-Amylase enzymes from different biological sources have been shown to exhibit different action patterns based on the number of binding sites per enzyme active site (Robyt and French 1963, Robyt and French 1964, Robyt and French 1967, MacGregor and MacGregor 1985). Action patterns of amylolytic enzymes describe the product specificity resulting from hydrolysis of linear amylose and give an indication of the mode of binding and hydrolytic attack (MacGregor and MacGregor 1985). The catalytic site of PPA randomly binds five glucose units in starch chains in the initial stage of each productive encounter followed by multiple attack and liberation of multiple maltose and maltotriose products before dissociating from the substrate (Robyt and French 1967, Thoma 1976). In contrast, α-amylase from *Bacillus licheniformis* and *Bacillus amyloliquifaciens* (also known as *Bacillus subtilis*) have binding sites requiring nine glucose units and exhibits a multiple-chain, random attack mechanism (Robyt and French 1963, Kandra and others 2002). Random attack does not require complete dissociation of the enzyme-substrate complex after each hydrolytic cleavage; however, enzymes that exhibit an apparent random attack mechanism have a low probability of multiple attack for each enzyme-substrate

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complex formed (Thoma 1976). Because the binding site of *B. licheniformis* α-amylase is larger than that that of PPA, the chief oligosaccharide hydrolysis products associated with this enzyme are maltopentose (about 33%) and oligosaccharides from G1 – G12 (Morgan and Priest 1981). The hydrolysis rate of *endo*-acting α-amylases on gelatinized starches ranges from 100 – 1000 times that of raw starches (Manners 1971, Chung and others 2006). Gelatinization facilitates interaction of starch chains with amylases by disrupting crystalline order in the starch granule, which restricts enzyme binding and diffusion into the granule (Chung and others 2006).

Structural Characteristics and Reaction Patterns of Amyloglucosidase (Glucoamylase)

Amyloglucosidase (AMG, glucan 1,4-α-glucosidase) is an *exo*-acting member of the α-amylase family of starch-hydrolyzing enzymes (Kuriki and Imanaka 1999). AMG enzymes act on the non-reducing ends of starch and oligosaccharide molecules to yield glucose and low molecular weight dextrins (Robyt 2009). For this reason, AMG is often used as a secondary enzyme for quantification of starch hydrolysis by α-amylases, as in the Englyst method for RS analysis (Englyst and others 1992). Commercial AMG preparations are typically isolated from fungal species, notably of the genus *Aspergillus*, *Rhizopus*, and *Endomyces*, some bacterial cultures including *Lactobacillus brevis* and *Bacillus coagulans*, and yeast cultures including *Saccharomyces cerevisae, Saccharomycopsis fibuligera,* and *Endymycopsis fibuligera* (Manners 1971, Ashok and others 2000).

AMG from *Aspergillus niger* consists of a catalytic and starch binding domain joined by a linking domain (Belshaw 1990, Stoffer 1993). Activity of these individual domains was demonstrated to be independent following site-specific cleavage of the linking domain using the proteolytic enzyme subtilisin (Belshaw 1990, Stoffer 1993). Goh and colleagues (2012) demonstrated that activity of AMG was largely retained following covalent affixation of AMG to

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iron oxide filled single-walled carbon nanotubes, thereby allowing for simple recovery of AMG from starch slurry such as that used in biofuel production. Physical absorption and covalent bonding of AMG to carbon nanotubes was found to largely convert α-helical structures to a less active β-sheet conformation, indicating that catalytic site conformation was largely unchanged by enzyme interaction with carbon nanotubes (Goh and others 2012). Historically, AMG action on raw starch granules has been shown to be limited, necessitating a pre-treatment of starch by acid hydrolysis with heating or co-treatment with α-amylase for efficient glucose production (Manners 1971). However, limited raw starch hydrolyzing activity has been observed in some AMG isozymes derived from *Aspergillus niger* (Ashok and others 2000).

***In Vivo* Studies of Starch Digestibility**

In addition to *in vitro* measurements of DF and RS contents of foods, direct and indirect *in vivo* measures of starch digestibility have been investigated. Direct measurements of starch digestibility in the small intestine have been made in animal models (Gidenne and Perez 1993, Mathers and others 1997, Giubert and others 2012, Jarret and others 2012) and human ileostomy patients without active disease (Faisant and others 1995, Englyst and Cummings 1987). Because DF and RS, by definition, are not digested in the small intestine of humans, ileostomy patients may therefore be considered as an ideal system for the study of indigestible food fractions such as RS.

The digestibility of RS2 and RS3 have been measured in human ileostomates. Digestibility of green banana starch (RS2) in the diet of ileostomy patients was reported by Faisant (1995). A portion of intact granular starch and resistant oligosaccharides (83.7%) was found to reach the ileal terminus of these subjects; this value was lower than RS content of banana starch obtained using a modified Englyst method procedure (70% RS). Suggested reasons for the discrepancy

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between *in vivo* and *in vitro* results included potential differences in banana ripeness at time of starch isolation, kinetic motion of the stomach and GI tract *via* peristalsis not accurately replicated by the *in vitro* Englyst method, and/or failure of the *in vitro* method to account for resistant oligosaccharides (Faisant 1995).

Bezerra and others (2013) determined hydrolysis characteristics and RS contents in pulp and flour from peeled and unpeeled green bananas of the Cavendish cultivar with and without a spouted bed drying treatment. RS contents were determined using minor modifications of AACC Method no 76-30A (1999), which is similar in operating principle to AOAC Method 2002.02. RS contents of the peeled banana flour dried at 80 and 90 °C were 42.02% and 38.24%, respectively. Although these temperatures are above the T_g of banana starch (about 70 °C, Ling and others 1982), processing conditions during spouted bed drying did not permit gelatinization to occur (Bezerra and others 2013). These RS content values were substantially lower than those obtained using *in vivo* analysis in ileostomy patients (Englyst and others 1986, Faisant and others 1995, Langkilde and others 2002).

Digestibility of retrograded starch in cooked and cooled potatoes (RS3) was compared to that of freshly-cooked potatoes in ileostomy patients by Englyst and Cummings (1987). Cooked and cooled potatoes contained 1.48% RS, whereas freshly-cooked potatoes contained only 0.54% RS (Englyst and Cummings 1987). These results were in good agreement with *in vitro* Englyst method results (Englyst and Cummings 1987).

Concentrations of $^{13}CO_2$ in breath were reported as an indirect measurement of gut fermentation of starch by Vonk (2000). Participants in this study were fed normal maize starch, Hylon VII (Ingredion, IL), a high-amylose maize starch containing about 70% amylose, or Novelose 330 (Ingredion, IL), a commercial RS3 prepared from cooked and retrograded high-

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amylose maize starch, at a test meal, and breath $^{13}CO_2$ measurements were recorded up to 6 h post-prandium (Vonk and others 2000). Total fermentation of RS substrates measured using pooled $^{13}CO_2$ were in good agreement with general trends of RS contents measured in ileal effluents of subjects fed whole diets containing similar proportions of normal and high-amylose maize starches (Muir 1993, Silvester and others 1995).

Clarke and colleagues (2007) reported the use of acetate, propionate, and butyrate high-amylose maize starch derivatives for potential delivery of short-chain fatty acids to the colon. Short-chain fatty acids are readily absorbed in the colon for use in cell metabolism (Ruppin and others 1980) and may reduce development of precancerous lesions in the bowel (Hinnebusch and others 2002). Modified high-amylose maize starches were incorporated into a cooked custard for better acceptance among ileostomy patients recruited as study participants (Clarke and others 2007). Approximately 75% of short-chain fatty acids were recovered in ileal effluents, indicating that modified high-amylose starches were a good means of delivery of SCFA to the colon and were substantially resistant to digestion (Clarke and others 2007).

Digestibility of resistant starches in rats (Mathers and others 1997), pigs (Regmi and others 2011, Giuberti and others 2012, Jarret and others 2012), and rabbits (Gidenne and Perez 1993) have been investigated. Animal models are typically used to investigate effects of RS supplementation on health conditions of concern to human populations, including obesity (Belobrajdic and others 2012), diabetes (Shen and others 2011), and colorectal cancer (Le Leu and others 2009) because animal subjects must generally be sacrificed to determine physiological effects of RS on underlying health conditions. Consequently, methods to non-destructively determine starch digestibility in animal models are therefore desirable.

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An empirical method to determine starch contents in rat cecum using Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) was recently described by Anderson and others (2013). The Total Starch Kit from Megazyme was used to calibrate the empirical model through partial least squares (PLS) regression analysis and had a cross-validation correlation coefficient (R^2) of 0.997, showing good agreement between spectral and wet method results for RS contents in cecal contents (Anderson and others 2013). Empirical methods like FTIR-PAS may therefore be a viable means to reduce cost associated with enzyme assays and sacrifice of study animals.

Porcine pancreatic α-amylase is commonly used in RS analytical methods (AOAC Method 2002.02, Englyst and others 1992) because it has similar product specificity and activity as the human enzyme (Abdullah and others 1966, Hägele and others 1982). However, amylolytic activities of enzymes from animal sources have been shown to differ slightly from those of humans (Sugimoto and others 1980). Although α-amylase from rat pancreas shares 83% primary sequence homology with α-amylase from porcine pancreas (Pasero and others 1986), the rat enzyme has been shown to have higher initial hydrolytic activity on raw starches, including maize, green banana, and potato (Sugimoto and others 1980).

Effects of RS as a subclass of DF on postprandial serum glucose concentrations have been investigated (Englyst and others 1999, Eelderink and others 2012). A commonly used measure of starch digestibility is the glycemic index (GI, Jenkins and others 1981), which directly measures the rise in postprandial blood glucose concentrations following ingestion, and less commonly the insulinemic index (II), which measures postprandial serum insulin concentrations following consumption of different foods (Holt and others 1997). Englyst and colleagues (1999) have described an *in vitro* measurement of available glucose after 20 min (rapidly available glucose, RAG) and 120 min (slowly available glucose, SAG) that derives from the Englyst method

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previously described (Englyst and others 1992) and that correlates well with the *in vivo* glycemic response in humans. The RDS fraction determined using the Englyst method is most closely correlated with higher RAG and thus GI response (Englyst and others 1999). By definition, alteration in proportions of starch fractions in a foodstuff using the Englyst method procedure is a zero-sum calculation: a decrease in one starch fraction must yield a proportional increase in one or both of the others. Thus, a decrease in the RDS fraction, such as by substitution of normal starches with a resistant starch in a product formulation, must yield increased SDS, RS, or both, and results in decreased GI values (Englyst and others 1999).

Eelderink and colleagues (2012) report that supplementation of breads with ^{13}C-labeled wheat bran and purple wheat bran grown in an environment enriched in $^{13}CO_2$ to determine the source of glucose in the blood produced no discernible difference in the rate of appearance of exogenous glucose in healthy male volunteers. Surprisingly, lower *in vitro* starch digestibility rates using the Englyst method were observed in bran-supplemented samples compared with controls, suggesting that a secondary mechanism limiting glucose absorption into the bloodstream was present (Eelderink and others 2012). Al-Mssallem and colleagues (2011) showed that a significant difference in RAG and SAG contents of Hassawi and parboiled long-grain white rice was not reflected in the overall glycemic response. However, SAG-rich Hassawi rice elicited a lower postprandial insulin response in serum than parboiled white rice (Al-Mssallem and others 2011).

Health Benefits of Resistant Starches

The benefits of DF consumption have been well documented and reviewed elsewhere (Buttriss and Stokes 2008). Studies of RS as DF subclass have generally focused on short-term metabolic effects postprandium rather than long-term longitudinal studies across populations

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(Nugent 2005, Storey and others 2007, Ble-Castillo and others 2010). Limited data on estimated resistant starch intakes of distinct populations are available (Brighenti and others 1998, Elmståhl 2002, Murphy and others 2008), but these data may be of limited utility in assessing benefits of dietary resistant starch due to dissimilarities of *in vitro* resistant starch quantification methods.

Health benefits of RS intakes in humans have been recently reviewed (Nugent 2005). Resistant starch has been shown to have a variety of benefits to the digestive tract of humans and other mammals, including increased faecal bulking (Phillips and others 1995), attenuation or prevention of cancerous lesions in the colon (Zhao and others 2011), and promotion of a beneficial profile of colonic microflora (Silvi and others 1999). The chief products of RS fermentation in the colon include short-chain fatty acids (SCFAs, e.g. butyrate, propionate) that may be used as an energy source after being re-absorbed by colonocytes, and gases (e.g. hydrogen, methane), the measurement of which in the breath may be used as an indicator of the extent of gut fermentation processes (Cummings and Macfarlane 1991).

The energy value of RS in foods was calculated as 2 kcal / g, whereas that of fully digestible starch has been estimated as 4.2 kcal / g (Liversey 1994). This reduced calorie value has resulted in RS being proposed as a dietary component that may aid weight loss (Tapell 2004, Ble-Castillo and others 2010). Studies comparing treatment groups in animal models fed diets containing RS and nonresistant starch show reduced glycemia and reduced food intake (Bodinham and others 2010, Klosterbuer and others 2012). However, limited data on outcomes of satiety and appetite regulation in humans has appeared in the literature. Bodinham (2010) investigated effects of acute dosages of high-amylose maize starch (60% RS by AOAC Method 991.43) on appetite and postprandial serum glucose and insulin concentrations compared with normal maize starch given to adult male volunteers (n = 20) as 24 g supplements prior to test meals over the course of a

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single day. Fasting insulin sensitivity was not significantly different among study participants at the start of the study. Postprandial serum glucose and insulin concentrations were significantly reduced in the RS treatment group, which was in agreement with animal studies in rat models (Higgins and others 2011, Belobrajdic and others 2012). Total energy intake at test meals and over a 24 h period following test meals, based on food diary recall, was significantly lower in the RS treatment group (5241 and 12603 kJ, respectively) compared with the placebo group (5606 and 13949 kJ, respectively). However, the RS supplement did not significantly impact subjective self-reported ratings of satiety in this study.

Hull and others (2010) report a similar reduction in voluntary energy intake in subjects (n = 34) fed a mid-morning snack containing a commercial polydextrose preparation (Litesse®, DuPont, Wilmington, DE), which is functionally similar to RS in terms of enzyme resistance in the small intestine. Subjects were administered a drinkable yoghurt snack containing 0, 6.25, or 12.5 g polydextrose prior to two *ad libitum* test meals. Total energy intake at test meals was decreased in a dose-dependent fashion for treatments containing 0, 6.25, and 12.5 g at both lunch and supper test meals. Average reduction in energy intake at the lunch meal was 218.8 kJ for the 12.5 g group.

Hasjim and others (2010) reported one of the few studies on the effects of RS5 on health biomarkers in human subjects. RS5 used in this study was high-amylose maize starch complexed with palmitic acid after partial gelatinization at 95 °C for 1 h with mechanical agitation followed by incubation with isoamylase to debranch amylose chains. RS content of this complex was 52.7% when analyzed using AOAC Method 991.43. Breads containing 60% RS5 by weight and white bread as control were administered to healthy male subjects (n = 20) 19 – 38 years of age with body mass index of 21.0 – 42.8. Breads containing wheat flour as the only source of starch

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were used as control. Subjects were administered 50 g of starch in the form of bread, and postprandial serum glucose and insulin concentrations were monitored every 15 min up to 2 h. Total RS contents of RS5 and control breads measured using AOAC Method 991.43 were 34.4% and 3.3%, respectively. Postprandial plasma glucose and insulin concentrations were lower at all time points for the RS bread treatment, and total plasma glucose and insulin responses determined using area under each curve were significantly reduced in the RS bread treatment. These results appear to indicate that RS5 functions similarly to other forms of RS studied in humans in terms of attenuating plasma glucose and insulin responses.

Ble-Castillo and others (2010) evaluated the effect of supplementing native green banana starch from the Cavendish cultivar on weight loss and insulin sensitivity in obese Type 2 diabetics (n = 30) compared with those given soy milk supplement as control in a 4 week, two-phase, blind, within-subject crossover study. 24 g of native banana starch or soy milk were dispersed in 240 mL water and were administered to patients daily; no other changes to individual diets or exercise habits were made or recommended. *In vitro* RS contents (34% RS) were determined according to the method described by Goñi and others (1996), which is methodologically similar to AOAC Method 2002.02 using an initial treatment with pepsin to remove surface proteins of banana starch granules. Body weight, serum fasting insulin concentrations, and insulin resistance determined using the Homeostasis Model Assessment, defined as the product of fasting glucose and insulin concentrations divided by 405, were significantly reduced when measured after the banana starch supplementation period compared with the control period. This study showed that RS2 supplementation using native green banana starch was sufficient to effect positive changes in biomarkers related to diabetes and insulin resistance in the absence of additional dietary and lifestyle changes. However, serum lipoprotein

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biomarkers (triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and total cholesterol) were not significantly influenced by RS supplementation.

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MATERIALS AND METHODS

Starches

Normal corn starch (NC, Cargill Gel™) was from Cargill, Inc (Minneapolis, MN). High-amylose corn starch (HC, RS2) and Novelose 330© (resistant corn maltodextrin, RS3) were from Ingredion (Westchester, IL). Raw green banana starch (BS) was a gift from Professor A. R. Bonilla, University of Costa Rica. Octenyl-succinic (OS) NC and HC starches (OS-NC and OS-HC, respectively, 3 and 10 wt%, RS4) were prepared by Ai and others (2013). Potato starch (PS) and normal wheat starch (WS) were from Sigma Chemical (St. Louis, MO). Fibersym™ (FS) cross-linked wheat distarch phosphate (RS4) was from MGP Ingredients (Atchison, KS). High-amylose stearic acid complex (RS5) was prepared as described by Hasjim and others (2010).

Reagents and Enzymes

All reagents were purchased from Fisher Scientific (Pittsburgh, PA), Sigma-Aldrich (St. Louis, MO), or J. T. Baker Chemical Co and were used as received. (Phillipsburg, NJ). *Bacillus licheniformis* α-amylase (BL), protease from *Bacillus licheniformis*, pancreatin from porcine pancreas, and amyloglucosidase from *Aspergillus niger* were from Sigma-Aldrich. Porcine pancreatic α-amylase (PPA), glucose-oxidase peroxidase (GOPOD) assay kit, and resistant starch assay kit were from Megazyme International Ireland, Ltd. (Wicklow, Ireland).

RS Content Analyses

Starch samples were analyzed using AOAC Method 991.43 omitting the precipitation step with 95% ethanol for determination of soluble dietary fiber. Samples were treated in a boiling water bath with thermostable BL for 30 min in 2-(*N*-morpholino)ethanesulfonic acid-trisaminomethane buffer (MES-Tris, pH 8.2) followed by treatment with protease (30 min, 60 °C) to remove proteins. Samples were cooled to 25 °C, the pH was adjusted to 4.4 – 4.6 to

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inactivate protease, and amyloglucosidase was added to hydrolyze soluble starch (30 min, 60 °C). The RS content was calculated as:

$$RS = \frac{R}{W}(100\%) \qquad (1)$$

where R is the weight of residue remaining after enzyme hydrolysis and W is the weight of starch sample on the dry-starch basis (dsb).

AOAC Method 2002.02 and the Englyst method are characterized as glucogenic methods, in which starch is hydrolyzed to glucose and quantified using GOPOD assay. Starch samples were analyzed using AOAC Method 2002.02 (Megazyme Resistant Starch kit) without modification. In brief, starch samples were hydrolyzed without prior cooking using PPA and amyloglucosidase at 37 °C for 16 h with shaking. Resistant residue was dispersed in 2 M potassium hydroxide solution and hydrolyzed using amyloglucosidase to determine RS content, which was calculated as:

$$RS = \left(\frac{A_t}{(A_s W)}\right)\left(\frac{162}{180}\right)(100\%) \qquad (2)$$

where A_t and A_s are the absorbances of the sample and glucose standard solution (100 µg / mL) measured at 540 nm, respectively, W is the weight of starch sample (dsb), and (162 / 180) is a correction factor accounting for the addition of water to anhydroglucose (AHG) units to starch during the hydrolysis reaction. FS, however, could not be dispersed in potassium hydroxide solution, and the RS content of this starch was determined gravimetrically using Equation 1.

Starch samples were also analyzed according to the method described by Englyst, Kingman, and Cummings (1992) omitting the addition of guar gum. Starch samples (1.0 g) were analyzed with and without prior cooking in 15 mL distilled water with stirring for 30 min. Starch samples were incubated with an enzyme mixture containing porcine pancreatin and amyloglucosidase,

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and sample aliquots were removed after 20 and 120 min to a tube containing 66% ethanol (aq) to stop the enzyme reaction. The percentage of starch converted to glucose during enzyme hydrolysis was quantified using GOPOD colorimetric measurement at 540 nm against a reagent blank as:

$$G_t = \frac{(A_t \times V \times C \times D)}{(A_s \times W)}(100\%) \qquad (3)$$

where G_t is percent hydrolysis of starch sample at time t, A_t and A_s are the absorbances of the sample and glucose standard solution (1 mg / mL), V is the volume of solution containing starch, C is the glucose standard concentration, D is the sample dilution factor from dilution with 66% ethanol, and W is the weight of starch sample (dsb). RS content was calculated based on the rapidly-digestible starch (RDS) and slowly-digestible starch (SDS) contents measured after 20 and 120 min, respectively, as:

$$RDS = (G_{20} - G_0)\left(\frac{162}{180}\right) \qquad (4)$$

$$SDS = (G_{120} - G_{20})\left(\frac{162}{180}\right) \qquad (5)$$

$$RS = 100\% - (RDS + SDS) \qquad (6)$$

where G_0, G_{20}, and G_{120} are free glucose and glucose produced from starch hydrolysis after 20 and 120 min, respectively. All sample absorbances were measured using a Beckman DU® 520 UV/Vis Spectrophotometer (Beckman Coulter Inc., Brea, CA).

Thermal Property Analysis

Thermal properties of starch samples were analyzed using a differential scanning calorimeter (DSC, Diamond Perkin-Elmer, Shelton, CT). Starches were precisely weighed into an aluminum or stainless steel pan (2.5 or 6.0 mg, dsb, respectively) with 3x deionized, distilled water (w/w). Pans were sealed and allowed to equilibrate for 2h to fully hydrate starch prior to

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analysis. Samples were heated at 10 °C / min from 10 °C to 110 °C (aluminum pans) or 150 °C (steel pans). NC, OS-NC (3 and 10 wt%), WC, PS, BS, and WS were analyzed using aluminum pans, and HC, OS-HC (3 and 10 wt%), Novelose 330, FS, and RS5 were analyzed using stainless steel pans.

Amylose Content Analysis

Amylose contents of starches were obtained using an autotitrator (Metrohm 702 SM Titrino, Herisau, Switzerland) according to the method of Song and Jane (2000). Starches were defatted by dispersing in 90% dimethyl sulfoxide solution and precipitating with five volumes of absolute ethanol prior to analysis. The amylose content of the sample was calculated using the iodine affinity value divided by 0.2 (Takeda and Hizukuri 1987).

Hydrolysis of Raw Starches

Enzyme activities of BL and PPA were assayed using a NC dispersion (1%, w/w) cooked in a phosphate buffer solution (100 mM, pH 6.9). Enzyme activity was determined as reducing sugars liberated from starch detected using dinitrosalicylic acid reagent following the method of Miller (1959) and measuring absorbance at 575 nm. BL and PPA with the same enzyme activity were used to hydrolyze raw starch suspensions (1%, w/w) up to 84 h. Sample aliquots were removed after 2, 4, 6, 8, 12, 24, 48, and 84 h and analyzed for reducing sugar content as above. After 84 h, absolute ethanol (2x, v/v) was added to stop the enzyme reaction, and samples were centrifuged at 1500 *g* for 10 min. The supernatant was decanted, and residues were dried overnight in a 37 °C oven prior to analysis using an optical microscope and an electron microscope.

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Thin-Layer Chromatography

Decanted supernatant containing enzyme-hydrolysis products of raw starches was analyzed according to the method of Robyt and White (1987). Sample hydrolysate (10 µL) was spotted onto a TLC plate and dried using a hair dryer. This spotting and drying was repeated twice. Oligosaccharide products up to G9 were separated using a mixture of 3:1:1isopropyl alcohol:ethyl acetate:water (v/v/v) using a single ascent. Hydrolysis products were visualized by dipping the plate in a solution of 3:1 methanol-sulfuric acid (v/v) and drying in a 110 °C oven for 30 min.

Light Microscopy

Bright-field optical micrographs of starch samples were obtained by suspending untreated or enzyme-hydrolyzed starch in 50% glycerol and spreading on a microscope slide covered with a glass cover slip. Samples were observed within 30 min of preparation. Images were obtained using a Nikon optical microscope (Tokyo, Japan) fitted with an Infinity microscope camera (Lumenera Corp., Ottawa, Canada).

Scanning Electron Microscopy

Untreated and hydrolyzed starch granules were gently spread onto silver adhesive tape using a wooden applicator stick and sputter-coated with palladium-gold alloy (60:40). Samples were imaged at an accelerator potential of 10 kV using a scanning electron microscope (SEM, Japan Electron Optics Laboratory, Peabody, MA)

Statistical Analysis

Homogeneous groups of RS and amylose contents of starches were determined according to the Tukey-Kramer method using the Microsoft Excel software package. Pearson correlation coefficients of linear univariate associations between RS contents of starches determined using

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standard analytical methods and their physicochemical properties were also determined in this way.

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RESULTS AND DISCUSSION

RS contents of starch samples are shown in Table 1. After starch samples were cooked, as in AOAC Method 991.43 and the Englyst method with cooking, native normal starches (NC, WS, BS, and PS) were almost completely hydrolyzed using AOAC Method 991.43, but about 8 – 9% RS remained using the Englyst method. Cooked HC showed significantly higher RS content than cooked normal starches. Without cooking, the A-type polymorphic starches, NC and WS, were completely hydrolyzed using AOAC Method 2002.02, but had 42% and 32% RS, respectively, using the Englyst method without cooking. The B-type polymorphic PS and the C-type polymorphic BS, however, showed significantly greater RS contents, 57% and 32% RS using AOAC Method 2002.02 and 84% and 91% RS using the Englyst method without cooking, than cooked counterparts, 1% RS using AOAC Method 991.43 and 9.3% and 9.7% RS using the Englyst method with cooking. RS contents of OS-NC and OS-HC (3 and 10 wt% OS) derivatives were generally increased and decreased with increasing wt% OS-modification, respectively, except using the Englyst method without cooking. RS contents of RS5 and FS were significantly greater when analyzed using AOAC Method 991.43 than other methods. The RS contents of Novelose 330©, an RS3, were very similar using all methods. Additionally, light micrographs of cooked starch granules show that starches retaining granular character after 30 min of cooking corresponded with the highest observed RS contents using AOAC Method 991.43 and the Englyst method with cooking (Figure 1).

Starches rich in amylose, such as HC (Table 2), are known to contain relatively higher RS contents than normal counterparts determined using AOAC Method 991.43. This is attributed to the presence of resistant amylose double-helices that have conclusion gelatinization-temperature (T_c) above the boiling temperature of water (Table 3, Li and others 2004, Li and others 2008,

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Jiang and others 2010). Addition of OS groups disrupts these stable amylose helices and lowers the gelatinization temperature (Table 3) with a net effect of decreasing RS contents (Table 1). Conversely, OS-modification reduced the available substrate binding-sites for modified NC, resulting in increased RS contents (Table 1).

To determine whether any significant associations between RS contents of starches determined using standard analytical methods and starch physicochemical properties existed, we performed univariate linear correlations comparing these outputs, and results are shown in Table 4. One significant association was between RS contents determined using AOAC Method 2002.02 and the Englyst method without cooking (r = 0.70). Other significant associations existed between RS contents determined using the Englyst method with cooking and the amylose contents (r = 0.98) and T_o of starches (r = 0.78). The lack of other significant associations between RS contents and physicochemical properties of starches may imply that factors other than amylose content and thermal properties, such as chemical modification, are responsible for enzyme-resistance of some starches in this study.

To better understand whether the different RS contents determined using AOAC 991.43 and other methods were due to cooking or whether these were related to the enzymes, BL and PPA, used for the analyses, we hydrolyzed raw starches using BL and PPA at equal activity. Because native BS and PS were hydrolyzed very slowly, we also used a higher (20x) enzyme activity to hydrolyze these starches. Enzyme-hydrolysis rates of raw starches using PPA were greater than those using BL in the early stage, up to about 8 – 12 h with the exception of WS (Figure 2 A). Native PS, a B-type polymorphic starch, showed a gradual, approximately linear increase in hydrolysis up to 84 h by BL and was hydrolyzed more quickly by PPA in early stages at both the low and high enzyme doses (Figure 2 B). Native BS, a C-type polymorphic starch, was

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hydrolyzed slowly by BL in early stages but showed a drastic increase in hydrolysis after 6 h using the high dose. BS was also hydrolyzed more rapidly by PPA in early stages, similar to PS. After 24 – 48 h, extents of starch hydrolysis by PPA, reflected as soluble sugar content, either remained the same, as shown in HC and WS, or decreased as shown in FS, BS, and PS. The decrease in the hydrolysis rate by PPA could be attributed to recrystallization and precipitation of liberated soluble starch. PPA is known to exhibit a multiple-attack reaction mechanism, which may liberate longer starch chains prone to crystallization (Robyt and French 1967).

Oligosaccharide hydrolysis end-products of BL and PPA enzyme action on raw starches were determined using TLC. No differences in hydrolysis end-products based on the starch hydrolyzed were apparent (Figure 3). Products observed following BL hydrolysis were primarily maltose (G2), maltotriose (G3), and maltopentose (G5) with maltotetraose (G4) as a minor product. Products observed following PPA hydrolysis were mostly G2 and G3. No oligosaccharide products were observed after BL hydrolysis of FS, which agreed with the negligible extent of hydrolysis observed for this starch by BL. Because no qualitative differences in hydrolysis end-products were observed between starches treated with the same enzyme, the amount of oligosaccharide end-products liberated from starch chains is unlikely to be a major factor underlying differences in starch hydrolysis rates.

Light micrographs of raw starches hydrolyzed by BL and PPA up to 84 h revealed that morphological changes of starch granules differed depending on the enzyme used. Damage to starch granules hydrolyzed by BL was generally localized to surfaces of starch granules, whereas pits were generally formed on the surface of granules hydrolyzed by PPA. The hydrolysis patterns of NC (Figure 4 A – C) hydrolyzed by BL and PPA were largely similar, but some faint pitting is observed in PPA-hydrolyzed NC (Figure 4 C). Minimal surface damage to HC (Figure

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4 D – F) and RS5 granules (Figure 4 P – R) due to hydrolysis by BL and PPA was observed. The surface of large WS A-granules was noticeably pitted by PPA hydrolysis, and a portion of broken granules was present after BL hydrolysis (Figure 4 G – I). FS granules were pitted by PPA hydrolysis (Figure 4 L) but were not noticeably affected by BL hydrolysis (Figure 4 K), which was consistent with the negligible hydrolysis by BL in this starch (Figure 2 A). Hydrolysis of BS revealed striations, which agreed with studies of morphological changes in BS granules during ripening (Peroni-Okita and others 2010), and disproportionate damage to starch granules at distal regions from the hilum (Figure 4 M – O). Similar striations were revealed in raw BS granules recovered from ileal effluents of rats (Sugimoto and others 1980) and humans (Faisant and others 1995, Langkilde and others 2002). This hydrolysis pattern could be due to less tight packing of starch molecules at farther regions from the hilum, which was synthesized later than the proximal region closer to the hilum. Damage to PS granules is localized at the hilum after PPA hydrolysis (Figure 4 U), which is the most loosely packed region of the granule and the most susceptible to enzyme hydrolysis (Pan and Jane 2000).

Starches with distinctive features following hydrolysis using BL or PPA, including BS, WS, and FS, were selected for further study using SEM. SEM images (Figure 5) of enzyme-hydrolyzed starches showed morphological changes in good agreement with those observed using optical microscopy. Asymmetric removal of surface starch from growth rings was observed in BL-hydrolyzed BS granules (Figure 5 B), whereas pitting (Figure 5 C) and inside-out hydrolysis (Figure 5 D), leaving an intact, enzyme-resistant outer shell, was observed in PPA-hydrolyzed BS granules. BL-hydrolyzed WS A-granules were in some cases “opened” following attack at the equatorial groove (Figure 5 F), whereas PPA-hydrolyzed WS showed a clear preference for pitting and inside-out hydrolysis (Figure 5 G). Only PPA hydrolyzed the FS

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granules (Figure 5 J), which appeared to occur only at points of imperfection in the granule surface of FS present before enzyme hydrolysis (Figure 5 H).

Hydrolysis rates of raw starches may provide insight into the mechanisms underlying differences in RS contents when analyzed using different standard methods. Simultaneous heat treatment with hydrolysis using BL in AOAC Method 991.43 may totally or partially gelatinize starch and thus decrease measured RS contents (Englyst and others 2013). The native B-type polymorphic structure of PS is known to have a homogeneous internal structure without pores (Jane 2006), which is responsible for enzyme resistance of this starch. (Gallant and others 1992). PPA exhibits a multiple-attack mechanism (Robyt and French 1967, Thoma 1976), indicating a high probability of consecutive scissile action on bound starch chains prior to dissociation of the enzyme-substrate complex (Butterworth, Warren, and Ellis 2011). In contrast, BL exhibits a random attack mechanism (Robyt and French 1963, Kandra and others 2002) requiring dissociation of the bound enzyme from starch chains before further hydrolysis can occur. The multiple attack mechanism of PPA likely facilitates the binding and progressive hydrolysis of starch chains and accounts for the formation of pits on the surface of starch granules and the more rapid extent of hydrolysis observed in most starches treated with PPA. These mechanistic differences likely account for the formation of pits on the surface of starch granules and the more rapid extent of hydrolysis observed in most starches treated with PPA. NC and WS are of the A-polymorph (Jane and others 1999) and have shown great internal porosity (Jane 2006), which facilitates enzyme penetration into the granule interior and increased rates of enzyme hydrolysis (Kim and others 2008, Kim and Huber 2010).

The large A-granules of WS have an equatorial groove that has a loose structure and is highly susceptible to enzyme attack (Fannon and others 1992). Loosely-packed starch in the

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equatorial groove of WS granules may facilitate hydrolysis by BL and account for the faster initial hydrolysis of WS using this enzyme. The linear increase in extent of hydrolysis of B-type polymorphic PS by BL likely corresponds to a surface-hydrolysis process. A similar linear increase was observed in extent of hydrolysis of C-type polymorphic BS by BL up to 12 h in the low dose and 6 h in the high dose, after which the rate of hydrolysis rapidly increased. It is plausible that granules of BS have an enzyme-resistant B-polymorph shell similar to that of PS, whereas the granule interior may consist primarily of the A-polymorph, which is hydrolyzed rapidly.

Cross-linking modifications of starch granules are concentrated on the granule surface (Huber and BeMiller 2001). Cross-linking may therefore represent a reinforcing of the starch granule surface and reduction in available length of starch chains without cross-linkages for enzyme binding (Butterworth, Warren, and Ellis 2011). BL and PPA differ in the size of glucose-binding sub-sites for enzyme hydrolysis: BL has a binding-site of nine glucose-units (Robyt and French 1963, Kandra and others 2002), whereas PPA has that of five glucose-units (Robyt and French 1967). Reduction in starch chain-length that is free of heavy cross-linking for enzyme hydrolysis may explain the lack of hydrolysis of FS by BL compared with PPA. The moderate increase in RS content of OS-NC with increasing wt% OS is also attributed to reduction in the length of starch chains free of OS-derivatives available to BL. OS-modifications, however, facilitate swelling of the granules (Bhosale and Singhal 2007). Consequently, phosphate cross-links inhibit α-amylolysis of the starch to a greater extent than the OS-modification. The helical complex of debranched high-amylose maize starch with stearic acid (RS5) also displayed restricted swelling and had excess stearic acid on the surface of the granule (Tester and Morrison

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1990). Therefore, RS5 was substantially less hydrolyzed by BL using AOAC 991.43 than by PPA.

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Table 1 - RS contents (%) of selected starches analyzed using standard methods of RS content analysis.

Starch	AOAC Methods		Englyst Method	
	991.43	*2002.02*	*Cooked*	*Raw*
NC	0.65 ± 0.45[a]	1.45 ± 0.19[b]	8.50 ± 1.68[a]	41.95 ± 1.31[d]
OS-NC (3%)	1.44 ± 0.16[b]	1.35 ± 0.22[b]	9.59 ± 5.50[c]	50.49 ± 2.40[e]
OS-NC (10%)	1.85 ± 0.15[bc]	1.58 ± 0.13[b]	17.64 ± 1.63[d]	69.63 ± 0.29[h]
HC	32.92 ± 1.97[d]	39.84 ± 0.24[g]	34.45 ± 1.39[h]	67.19 ± 0.83[g]
OS-HC (3%)	16.07 ± 1.91[e]	36.29 ± 2.36[f]	22.77 ± 3.44[f]	73.91 ± 0.87[i]
OS-HC (10%)	10.22 ± 0.31[f]	31.46 ± 1.22[f]	18.15 ± 4.90[e]	78.75 ± 1.95[j]
WS	0.44 ± 0.09[a]	0.32 ± 0.51[a]	12.38 ± 4.18[c]	31.53 ± 1.91[b]
FS	90.57 ± 1.54[j]	0.32 ± 0.21	21.24 ± 1.42[f]	89.60 ± 0.95[l]
	-	49.80* ± 0.10[a]	-	-
BS	1.00 ± 0.47[ab]	27.17 ± 3.83[e]	9.74 ± 0.76[ab]	84.39 ± 0.30[k]
PS	1.09 ± 0.78[b]	57.39 ± 3.45[c]	9.27 ± 0.54[ab]	90.92 ± 0.41[m]
RS5	65.34 ± 0.76[g]	27.85 ± 0.21[d]	28.33 ± 2.19[g]	41.09 ± 0.21[c]
Novelose 330	46.58 ± 0.79[h]	45.23 ± 2.43[h]	58.60 ± 2.21[i]	60.89 ± 0.25[f]

NC = normal corn starch; HC = high-amylose corn starch; OS-NC/OS-HC = octenyl succinic normal / high-amylose corn starch (wt%); WS = normal wheat starch; BS = green banana starch; PS = potato starch; FS = Fibersym™ cross-linked wheat starch; RS5 = debranched high-amylose corn-stearic acid complex.

*Indicates that the RS content given is total undigested residue not dispersible in 2 M KOH after 16 h digestion with PPA and amyloglucosidase.

Values with the same letter within a column were not significantly different.

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Table 2 – Amylose contents of native starches analyzed using standard methods of RS content analysis.

Starch	Amylose Content (%)
NC	28.58 ± 0.20[b]
HC	70.54 ± 0.08[d]
WS	27.13 ± 0.55[a]
BS	27.01 ± 0.75[a]
PS	31.25 ± 0.11[c]

NC = normal corn starch; HC = high-amylose corn starch; WS = normal wheat starch; BS = green banana starch; PS = potato starch. Values with the same letter within the column were not significantly different ($\alpha = 0.05$)

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Table 3 - Thermal properties of selected starches.

Starch	T_o (°C)	T_p (°C)	T_{p2} (°C)	T_c (°C)	ΔH (J/g)
NC	65.28 ± 0.28	70.10 ± 0.27	-	74.55 ± 0.22	11.28 ± 1.06
OS-NC(3%)	63.60 ± 0.16	68.95 ± 0.11	-	73.94 ± 0.18	10.91 ± 0.11
OS-NC(10%)	58.58 ± 0.88	69.11 ± 0.35	-	75.93 ± 0.40	9.37 ± 0.39
HC	69.47 ± 0.33	74.09 ± 0.00	97.99 ± 1.26	108.17 ± 2.69	16.61 ± 1.19
OS-HC(3%)	67.76 ± 1.53	73.52 ± 0.1	91.35 ± 0.21	101.59 ± 2.35	14.94 ± 0.83
OS-HC(10%)	63.52 ± 1.05	71.23 ± 0.24	-	78.31 ± 1.82	11.26 ± 0.82
WS	59.00 ± 0.14	63.20 ± 0.36	-	68.91 ± 2.26	8.76 ± 0.07
FS	73.16 ± 0.08	76.70 ± 0.00	-	80.25 ± 0.21	10.76 ± 0.42
BS	71.37 ± 0.07	74.91 ± 0.07	-	80.43 ± 0.40	17.99 ± 2.86
PS	61.88 ± 0.08	65.69 ± 0.23	-	72.99 ± 0.08	18.65 ± 0.45
Novelose	109.16 ± 0.18	117.73 ± 0.62	-	124.97 ± 1.11	25.21 ± 3.54
RS5	68.46 ± 0.24 (1) 96.43 ± 0.59 (2)	70.63 ± 0.36 (1) 103.94 ± 0.47 (2)	-	73.07 ± 0.23 (1) 109.74 ± 0.30 (2)	20.51 ± 2.63 (1) 9.43 ± 3.47 (2)

T_o/T_p/T_c, Onset, peak, and conclusion gelatinization temperatures; ΔH, enthalpy change of gelatinization; NC, normal corn starch; HC, high-amylose corn starch; OS-NC/OS-HC, octenyl succinate normal/high-amylose corn starch (wt%); WS, normal wheat starch; BS, green banana starch; PS, potato starch; FS, Fibersym™ (wheat distarch phosphate)

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Table 4 – Pearson correlation coefficients of linear univariate associations of RS contents of starches determined using standard methods of analysis, amylose content, and starch thermal properties.

	RS Contents						
	AOAC 991.43	AOAC 2002.02	Englyst (Cooked)	Englyst (Raw)	Amylose Content	T_o	ΔH
AOAC 991.43	1.00						
AOAC 2002.02	0.52	1.00					
Englyst (Cooked)	0.50	0.45	1.00				
Englyst (Raw)	0.12	**0.70**	0.03	1.00			
Amylose Content	1.00	0.39	**0.98**	0.13	1.00		
T_o	0.46	0.44	**0.78**	0.06	0.42	1.00	
ΔH	0.42	0.49	0.51	-0.04	0.29	0.57	1.00

T_o, Onset gelatinization temperature of starch (°C); ΔH, enthalpy change of gelatinization of starch (J/g). Significant associations ($|r| \geq 0.70$) are given in **bold**.

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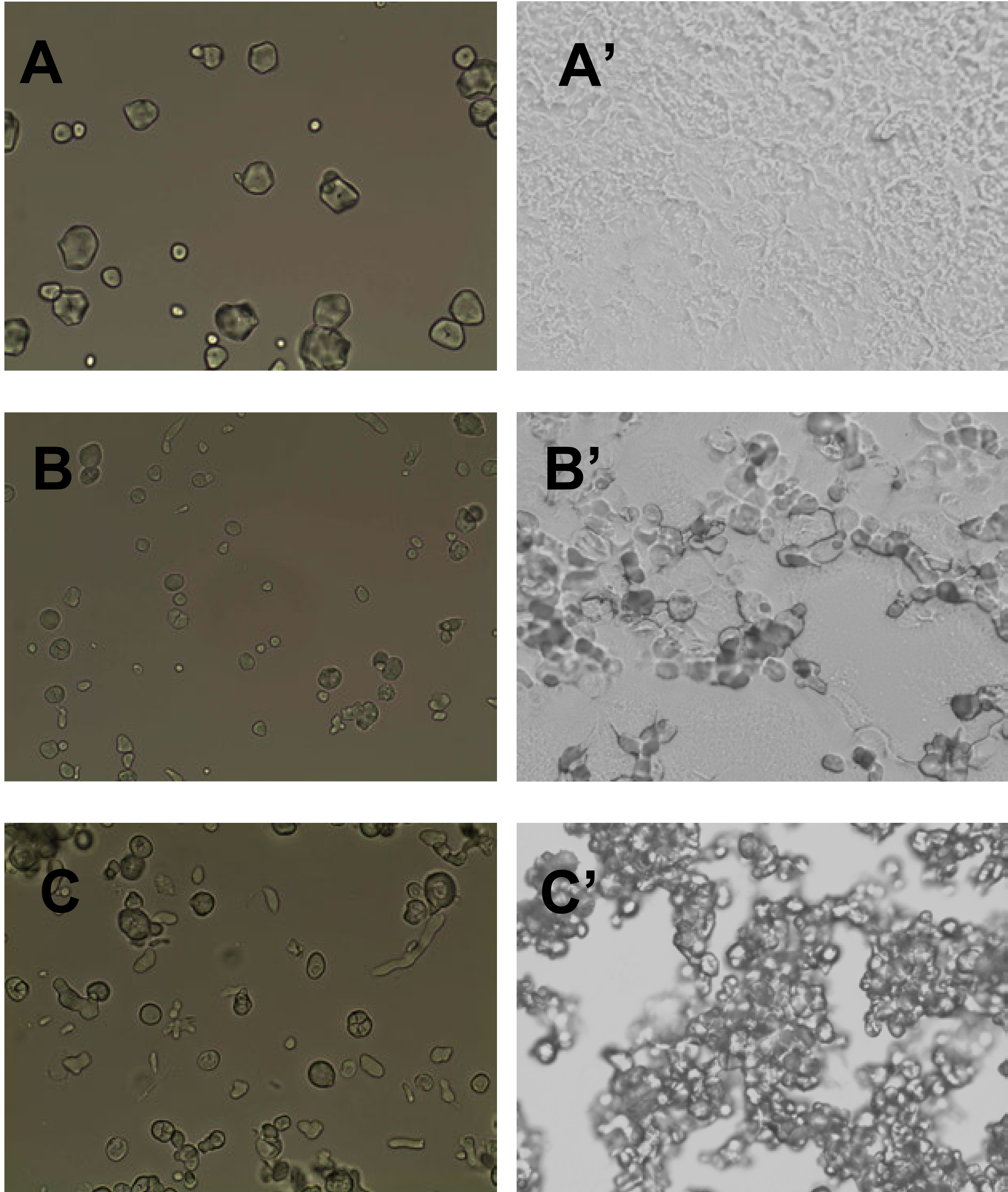

Figure 1.

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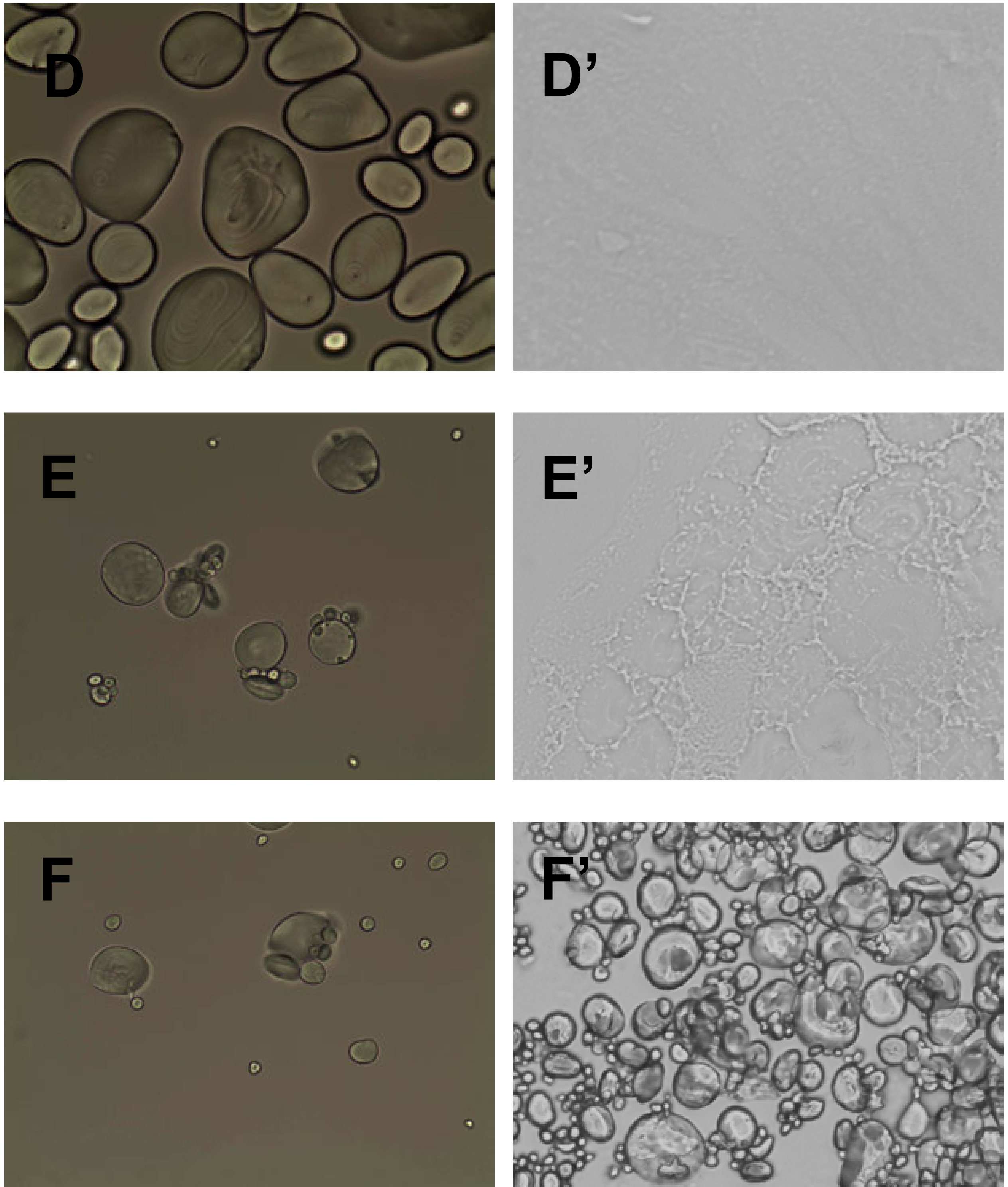

Figure 1.Selected light micrographs of raw and cooked starches showing increased granular resistance to cooking (40x magnification). A – A', NC, raw and cooked; B – B', HC, raw and cooked; C – C': RS5, raw and cooked; D – D', PS, raw and cooked; E – E' = WS, raw and cooked; F – F' = FS, raw and cooked

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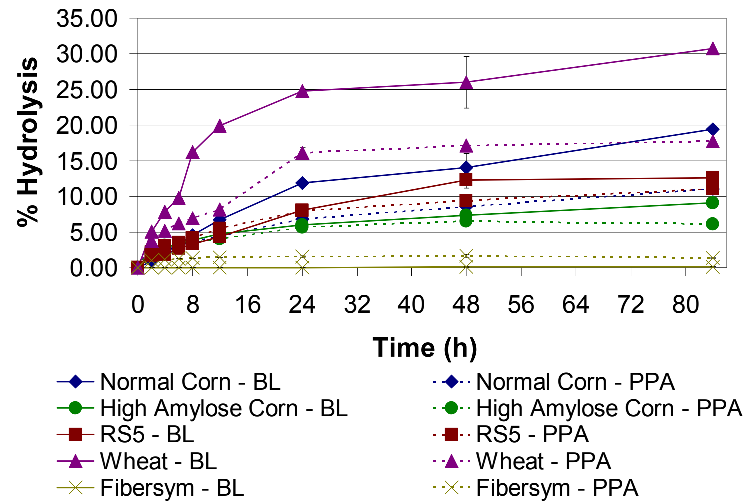

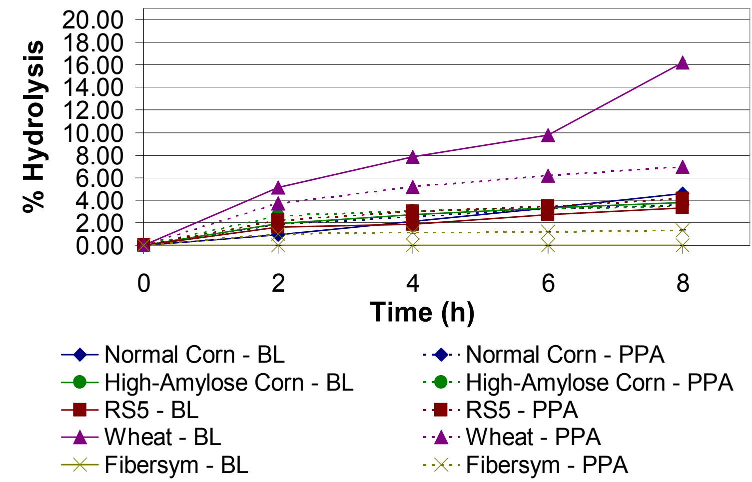

Figure 2.

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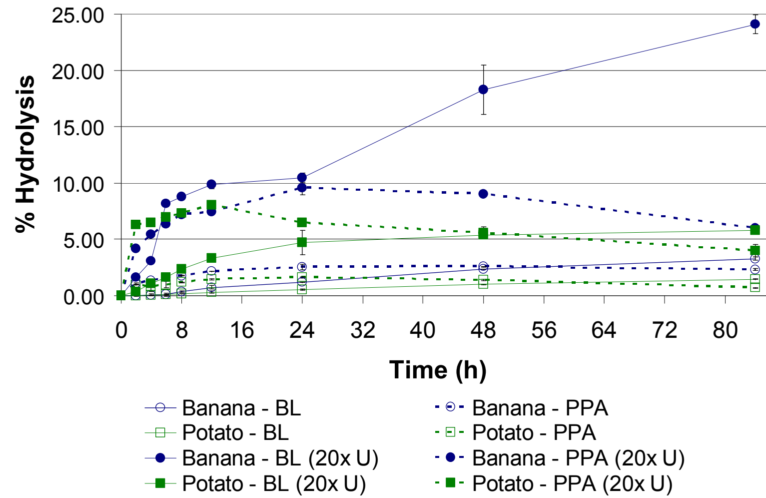
Figure 2 continued



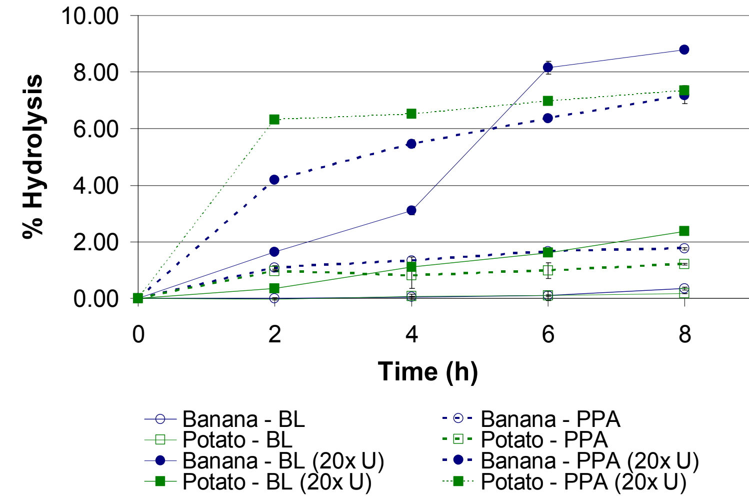


Figure 2. Extent of hydrolysis of raw starches up to 8 h (A', B') and 84 h (A, B); A/A', hydrolysis of NC, HC, RS5, WS, and FS by BL and PPA; B/B', hydrolysis of BS and PS by BL and PPA using low and high (20x) doses of enzymes.

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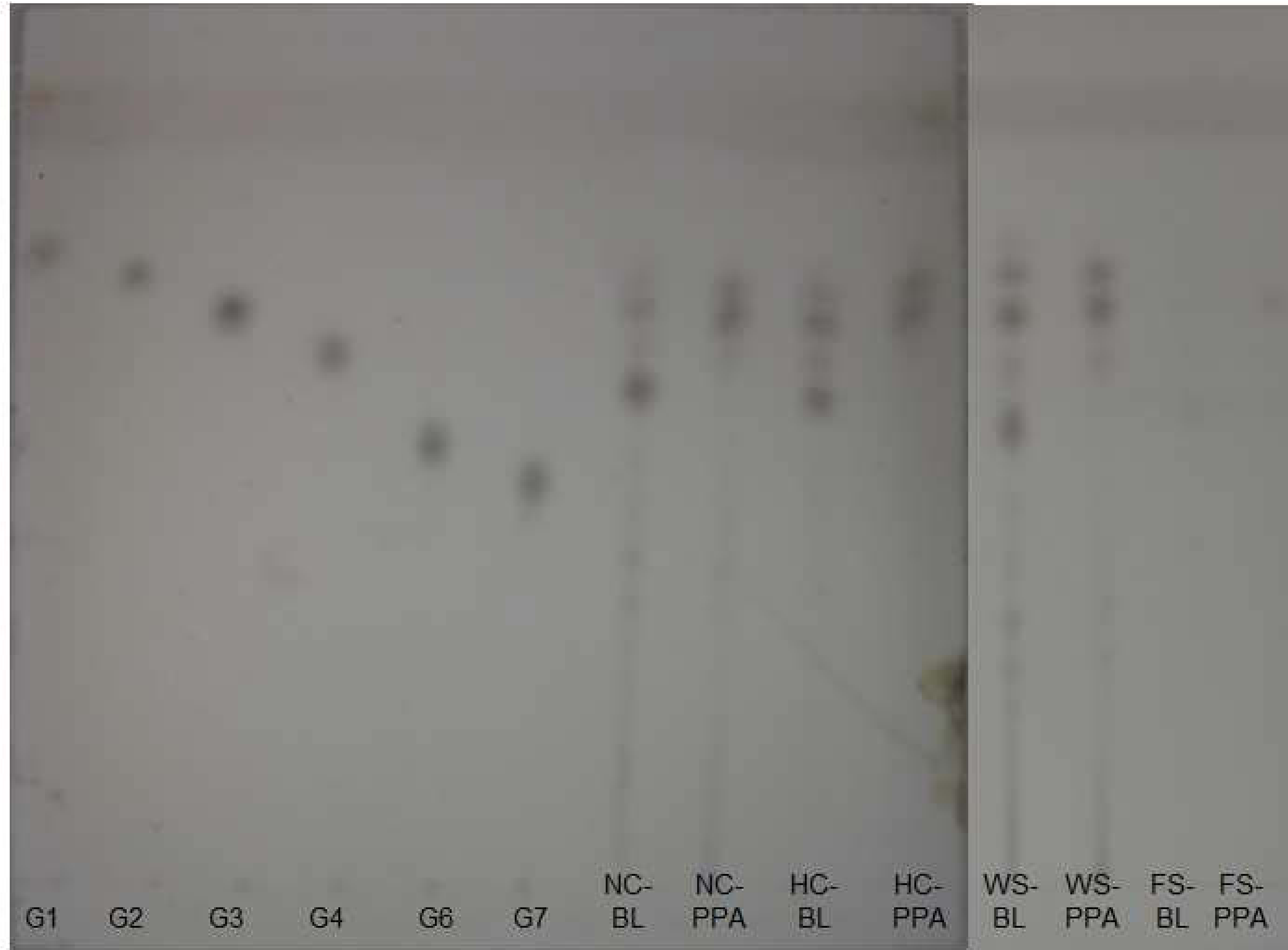

Figure 3. Thin-layer chromatography separation of BL- and PPA-hydrolysis end-products from raw starches using a single ascent. Left lanes show mono- and oligosaccharide standards G1 – G7 (excluding maltopentose, G5).Spots closest to the solvent line are glucose (G1).

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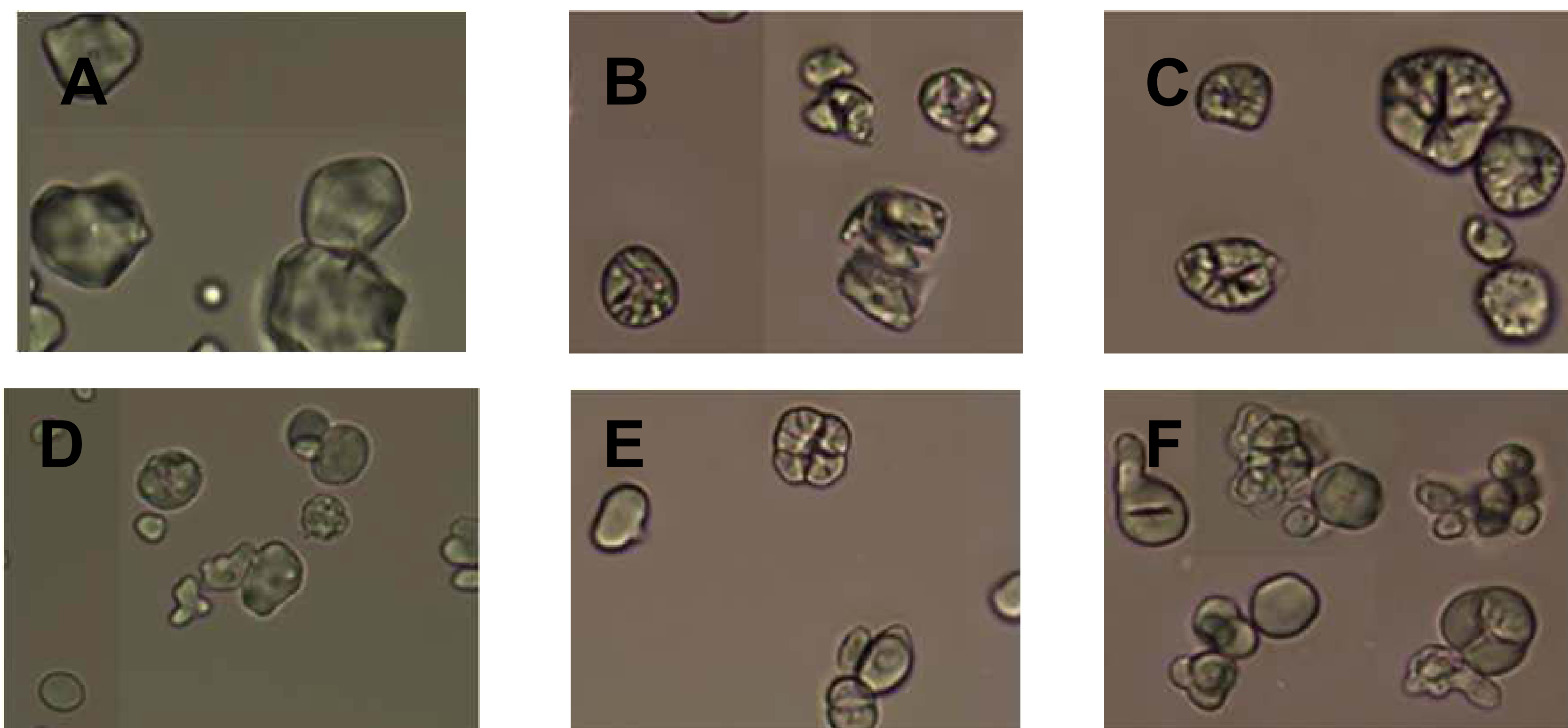

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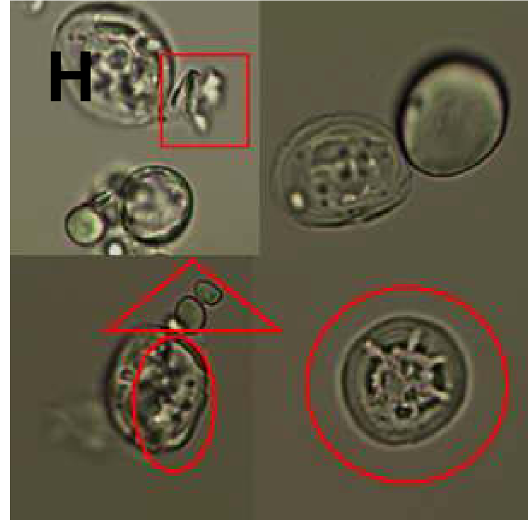

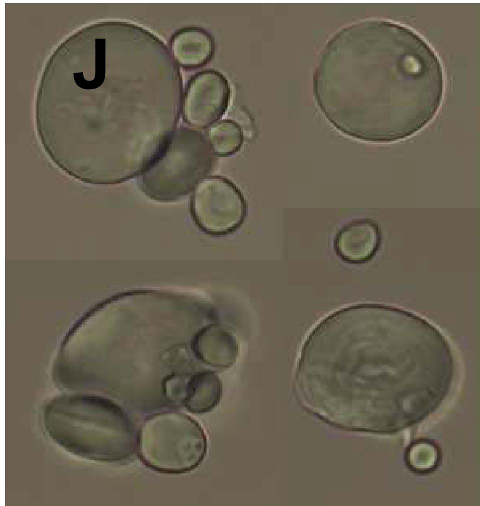

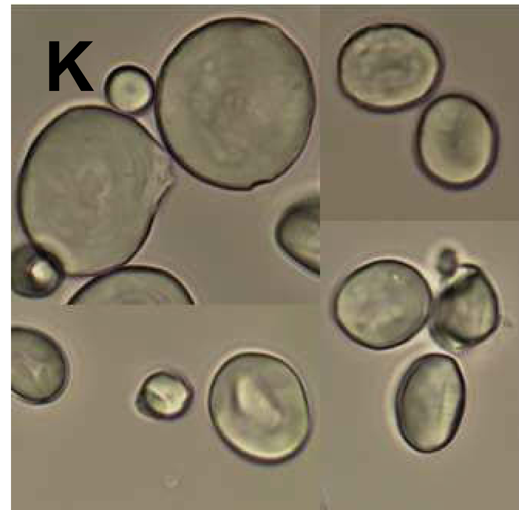

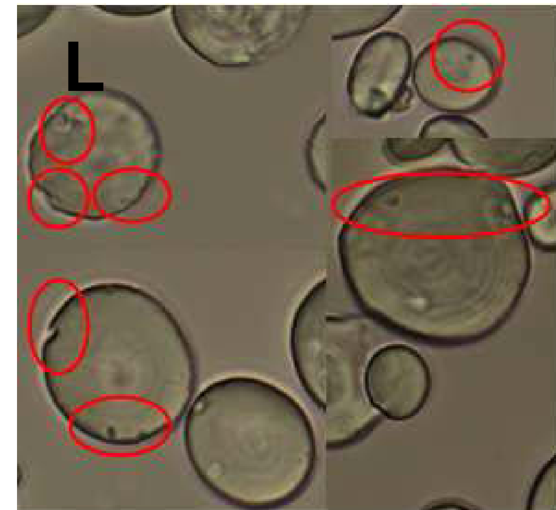

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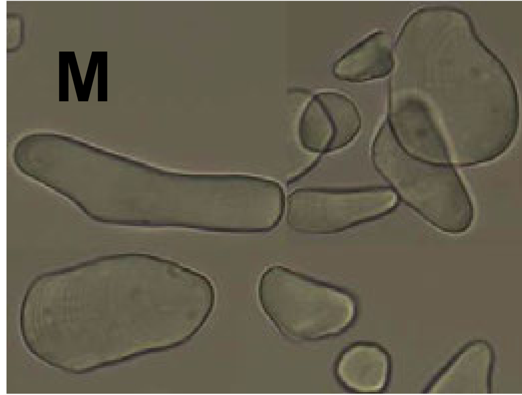

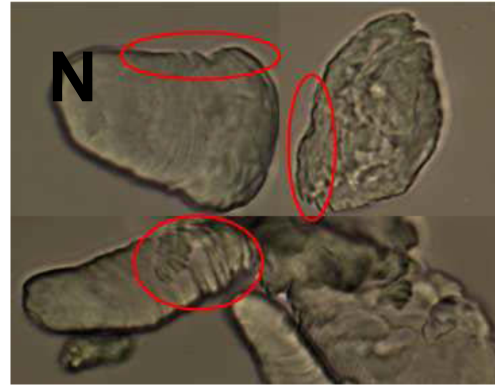

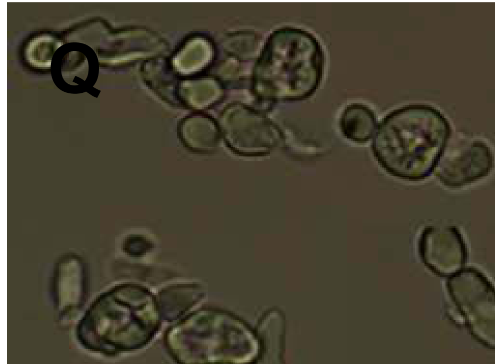

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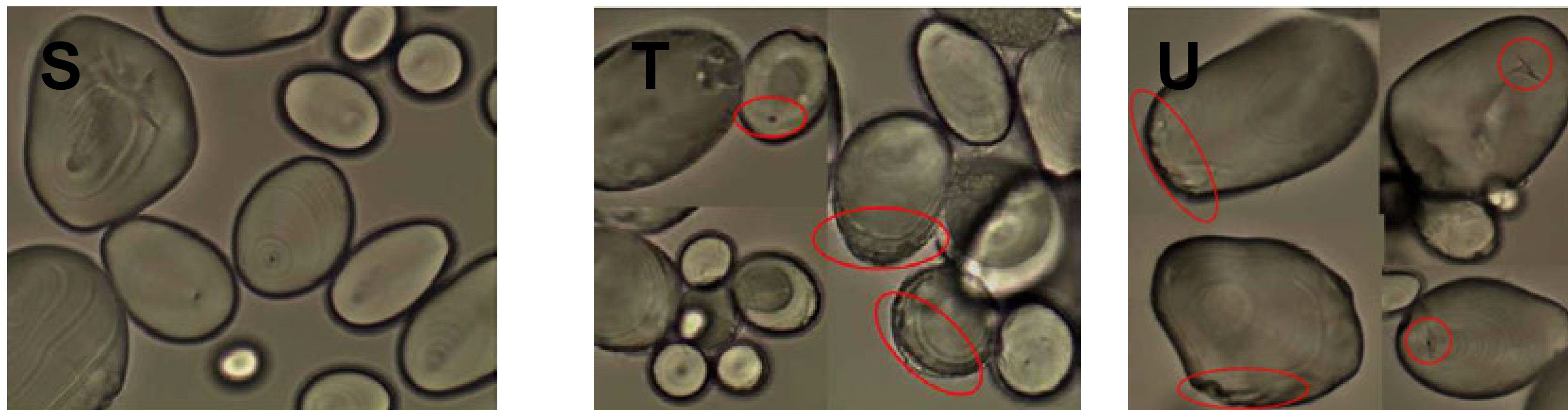

Figure 4. Light micrographs of untreated and hydrolyzed starches (40x magnification). A, Native NC; B, BL-hydrolyzed NC; C, PPA-hydrolyzed NC; D, native HC; E, BL-hydrolyzed HC; F, PPA-hydrolyzed HC; G, untreated RS5; H, BL-hydrolyzed RS5; I, PPA-hydrolyzed RS5; G, Native WS; H, BL-hydrolyzed WS showing generalized surface damage (ovals), granule fragments (square), and relatively undamaged small B-granules (triangle); I, PPA-hydrolyzed WS showing pitting (ovals), exposure of surface striations (rectangles); J, untreated FS; K, BL-hydrolyzed FS, showing minimal changes after 84 h hydrolysis; L, PPA-hydrolyzed FS showing a limited extent of surface pitting (ovals); M, Native BS; N, BL-hydrolyzed BS showing asymmetric hydrolysis and granule damage in distal regions of the granule (ovals); O, PPA-hydrolyzed BS showing pitting and damage on the granule surface (ovals); P, untreated RS5; Q, BL-hydrolyzed RS5; R, PPA-hydrolyzed RS5; S, Native PS; T, BL-hydrolyzed PS showing surface damage on granules and wearing away of surface starch (ovals); U, PPA-hydrolyzed PS showing minor pitting and granule damage localized at hilum regions of large granules (ovals)

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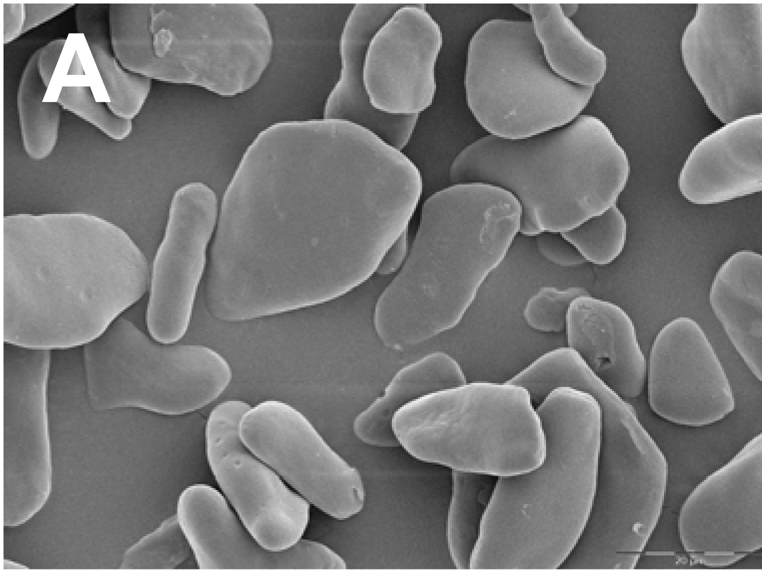

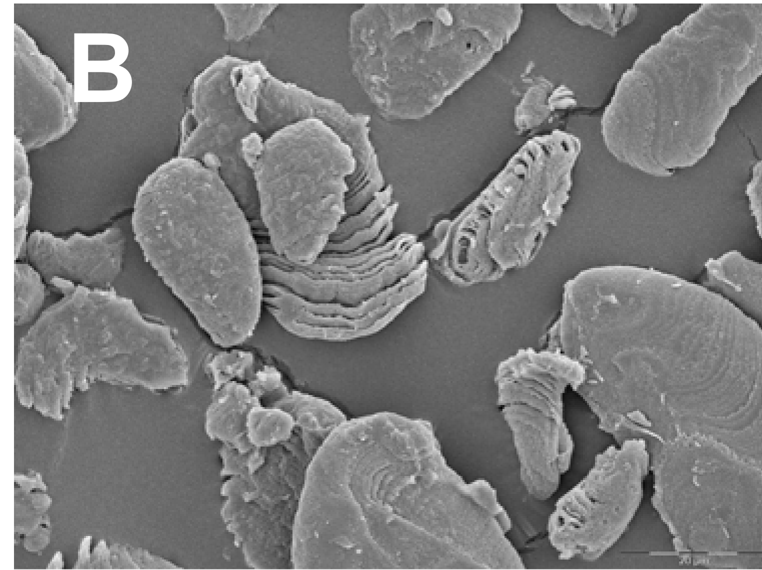

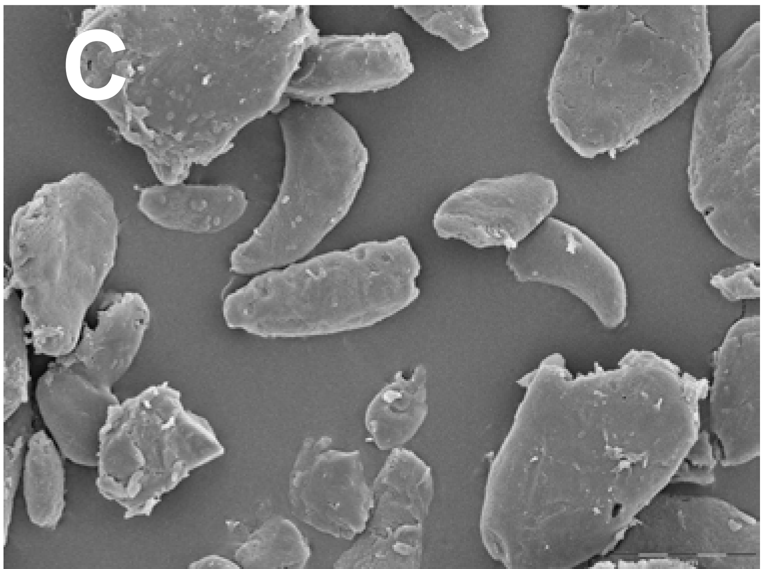

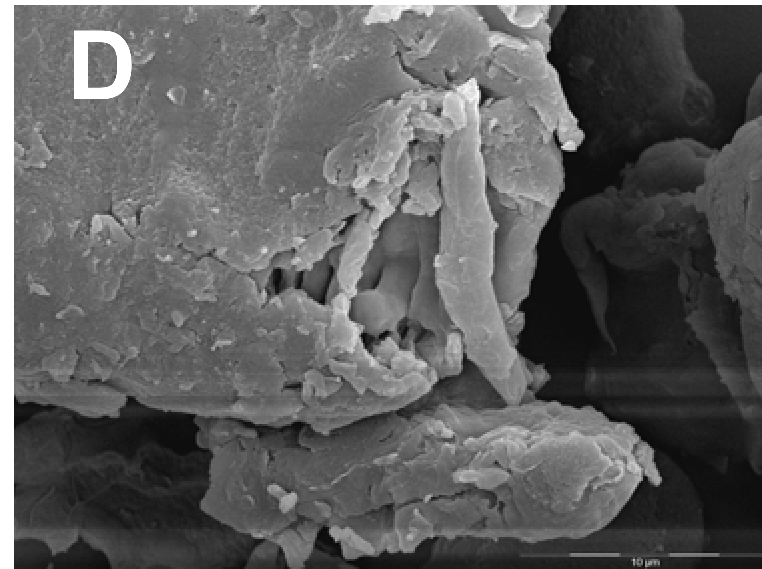

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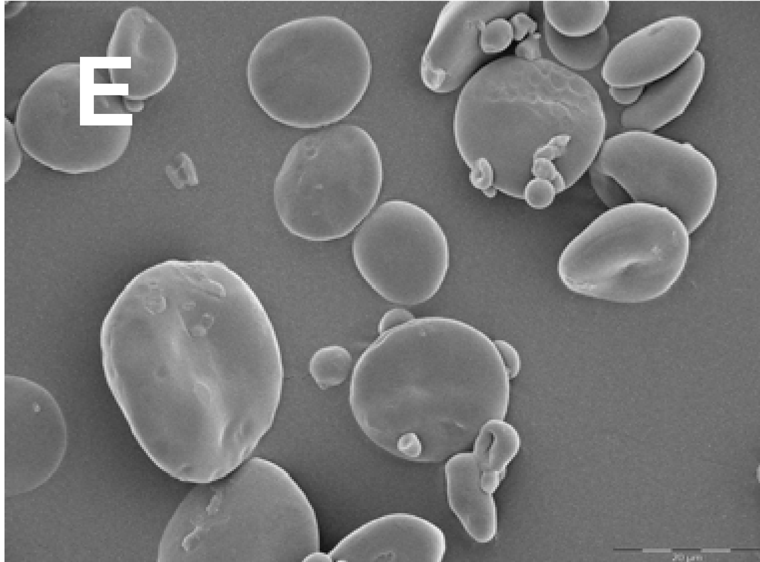

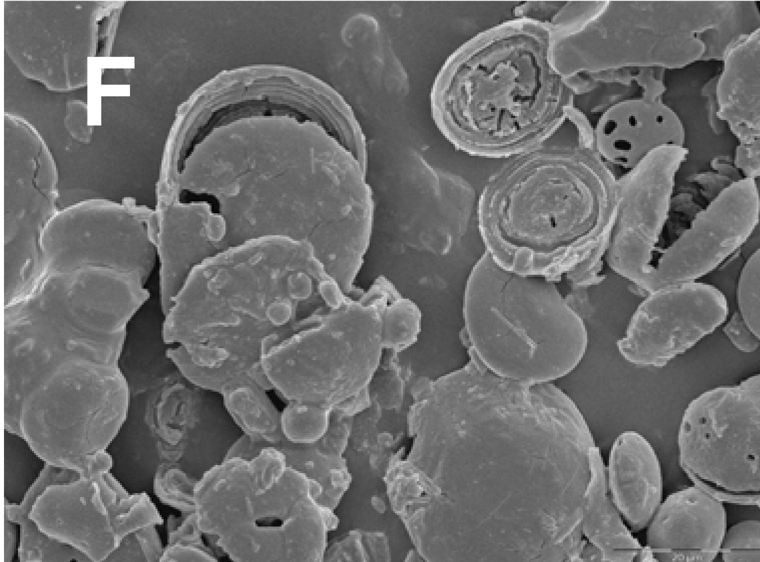

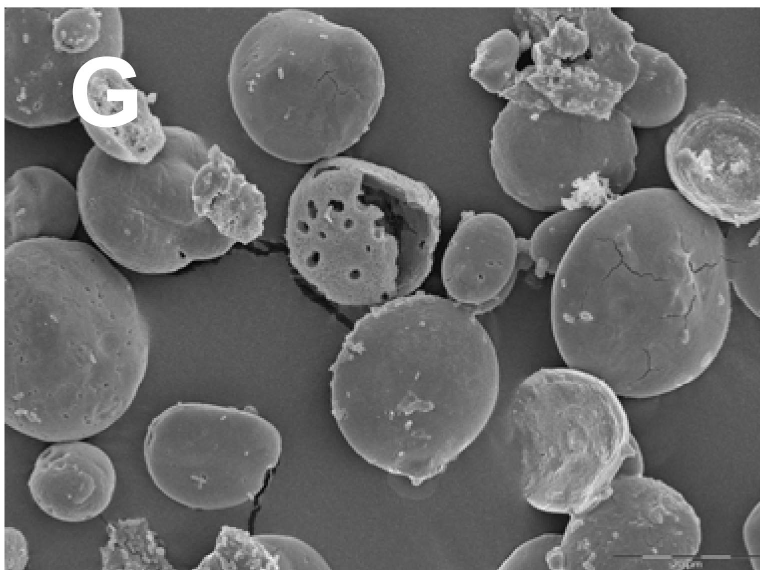

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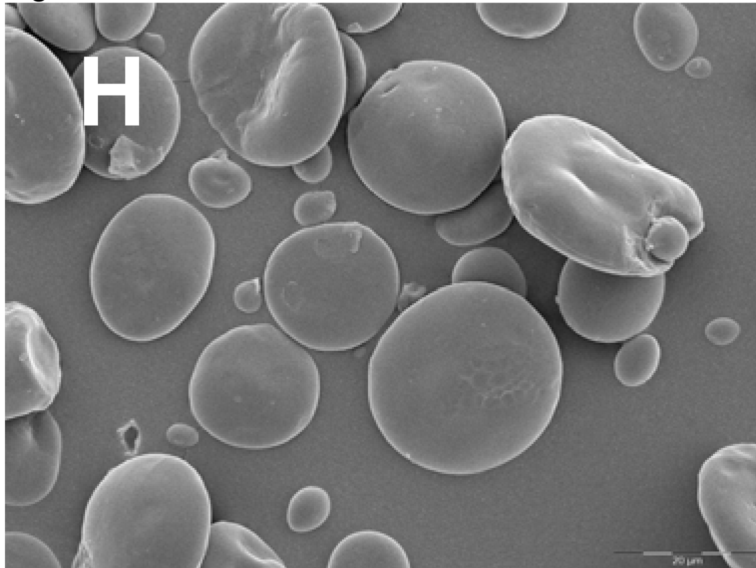

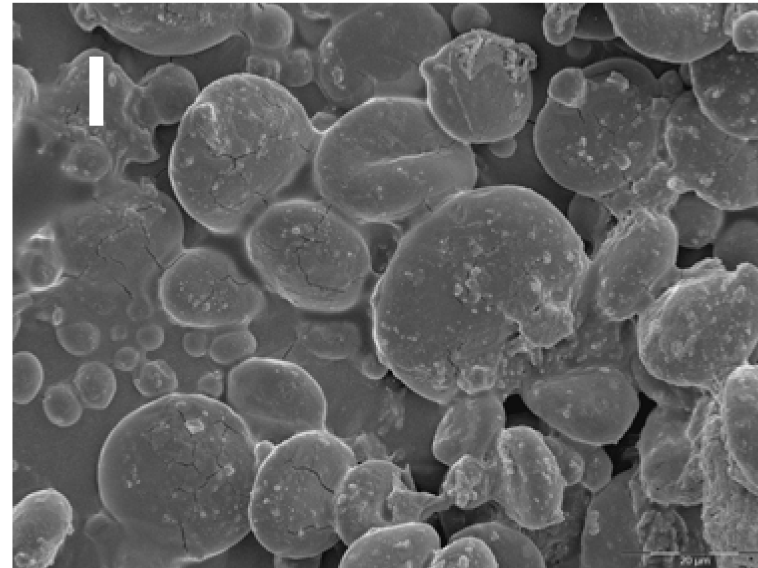

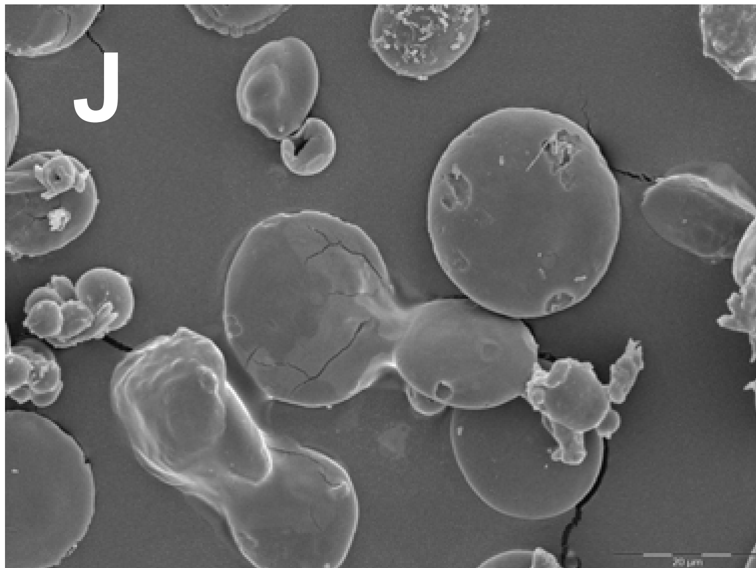

Figure 5. Scanning-electron micrographs of untreated and hydrolyzed starches treated with BL and PPA after 84 h. The same magnification with scale bar of 20 µm are shown in all micrographs except for D (10 µm). A, Native BS; B, BL-hydrolyzed BS; C,D, PPA-hydrolyzed BS; E, Native WS; F, BL-hydrolyzed WS; G, PPA-hydrolyzed WS; H, Raw FS; I, BL-hydrolyzed FS; J, PPA-hydrolyzed FS

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CONCLUSIONS

RS contents of starches varied depending on the method used, starch botanical origin, and type and extent of chemical modification. Extent and rate of hydrolysis of raw starch granules by BL and PPA enzymes, commonly used in standard methods of RS content analysis, provided an explanation for wide differences in RS contents determined using AOAC Method 991.43 with BL, or AOAC Method 2002.02 and the Englyst method with PPA. Hydrolysis by PPA produced visible pitting on the surface of starch granules, whereas hydrolysis by BL was mostly generalized on the surface. These differences were ascribed to differences in number of glucose-binding subsites in the enzyme actives sites, the propensity of enzymes to random or multiple attack, and availability of starch chains free of derivatives to enzyme hydrolysis.

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