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Effects of alpha-amylase reaction mechanisms on analysis of resistant-starch contents

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ABSTRACT

This study aimed to understand differences in the resistant starch (RS) contents of native and modified starches obtained using two standard methods of RS content analysis: AOAC Method 991.43 and 2002.02. The largest differences were observed in native potato starch, cross-linked wheat distarch phosphate, and high-amylose corn starch stearic-acid complex (RS5) between using AOAC Method 991.43 with *Bacillus licheniformis* α-amylase (BL) and AOAC Method 2002.02 with porcine pancreatic α-amylase (PPA). To determine possible reasons for these differences, we hydrolyzed raw-starch granules with BL and PPA with equal activity at pH 6.9 and 37 °C for up to 84 h and observed the starch granules displayed distinct morphological differences after the hydrolysis. Starches hydrolyzed by BL showed erosion on the surface of the granules; those hydrolyzed by PPA showed pitting on granule surfaces. These results suggested that enzyme reaction mechanisms, including the sizes of the binding sites and the reaction patterns of the two enzymes, contributed to the differences in the RS contents obtained using different methods of RS analysis.

1. Introduction

Starch is the major form of energy storage of higher plants. Starch consists 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 α-1,4 linked linear chains connected by α-1,6 branch linkages (Pérez, Baldwin, & Gallant, 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 et al., 1981) and serum insulin concentrations (Holt, Brand-Miller, & Petocz, 1997). Chronic repetition of these hyperglycemic and hyperinsulinemic responses contributes to health problems, such as diabetes, obesity, and the metabolic syndrome (Finley, Barlow, Halton, & Haskell, 2010). Diets containing starches that resist digestion may prevent the development of these health problems.

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 & Macfarlane, 1991). Additionally, RS acts as dietary fiber and contributes to fecal bulking (Phillips et al., 1995; Ai, Nelson, Birt, & Jane, 2013), shortens intestinal transit time of food bolus (Mathers, Smith, & Carter, 1997), and reduces development of cancerous lesions of the bowel in animal models (Zhao et al., 2011). RS is classified according to its structural characteristics. RS1 is physically inaccessible starch such as that in whole grains (Englyst, Kingman, & Cummings, 1992). RS2 is enzyme-resistant native starch-granules with the B or C-type polymorph, such as that of potato, green banana, and high-amylose corn (Englyst et al., 1992). RS3 is retrograded starch formed by recrystallization of starch chains during storage after cooking (Englyst et al., 1992). RS4 is chemically modified starch, such as starch cross-linked with phosphate diesters (Shin, Song, & Seib, 2004) or derivatized starch, e.g., octenyl succinic starch (Wang et al., 2011; Ai et al., 2013), acetylated starch (Annison, Illman, & Topping, 2003), and hydroxypropylated starch (Chung, Shin, & Lim, 2008). RS5 is the helical inclusion-complex formed between amylose and lipids (Hasjim et al., 2010).

Various *in vitro* methods for determination of RS contents of foods have been adopted in the food industry (Englyst et al., 2013; McCleary, 2007; McCleary, Sloane, Draga, & Lazewska, 2013; Seib & Woo, 1999). RS contents of foods analyzed using those methods, however, have shown discordant results depending on the method used (Englyst et al., 2013; Maningat, Seib, & Bassi, 2013; McCleary, 2007; McCleary et al., 2013; Seib & Woo, 1999). Because of the health benefits of RS in the human diet, accurate quantification of

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RS in foods is of critical importance. It is well documented that RS contents of native or modified starches obtained using different standard methods differ; reasons for causing the differences, however, are not well understood.

This study aimed to quantify RS contents of native and modified starches using two standard methods of analysis for dietary fiber/RS with two sources of enzymes: alpha-amylase from *Bacillus licheniformis* (BL) is used in AOAC Method 991.43, and porcine pancreatic α-amylase (PPA) in AOAC Method 2002.02. Methods of RS analysis used in this study were selected based on commonality of use and differences in sample treatments, including enzymes used, time of hydrolysis, and temperature of the hydrolysis medium. To the best of our knowledge, there are no papers reporting enzyme reaction mechanisms that cause differences in RS contents of starches analyzed using these standard methods in the literature.

2. Materials and methods

2.1. Materials

2.1.1. Starches

Normal corn starch (NC, Cargill Gel™) and high-amylose corn starch (HC, AmyloGel™) were from Cargill, Inc (Minneapolis, MN). Novelose 330® (resistant corn maltodextrin, RS3) was from Ingredion (Westchester, IL). Green banana starch (BS) was from Professor A. R. Bonilla, University of Costa Rica. NC and HC were modified with 3% or 10% (wt%, dry starch basis, dsb) octenyl succinic anhydride (OSA) following the method of Ai et al. (2013) to prepare OS-NC (3%), OS-NC (10%), OS-HC (3%), and OS-HC (10%), respectively. Potato starch (PS) and normal wheat starch (WS) were from Sigma Chemical (St. Louis, MO). Fibersym™ (FS), a cross-linked wheat distarch phosphate (RS4), was from MGP Ingredients (Atchison, KS). High-amylose corn starch-stearic-acid complex (RS5) was prepared following the method of Hasjim et al. (2010).

2.1.2. Reagents and enzymes

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

2.2. Starch characterization

2.2.1. RS-content analyses

Starch samples were analyzed using AOAC Method 991.43 without determining soluble dietary fiber content (AOAC International, 2012). Samples were treated in a boiling water bath with 100 μL thermostable BL for 30 min. Samples were cooled to 60 °C in a water bath, 5 mg protease from *B. licheniformis* in 100 μL deionized water was added to each, and samples were treated for 30 min with shaking (100 rpm). Samples were removed from the water bath and cooled to room temperature. Sample pH was adjusted to 4.5 using 0.5 M HCl, 300 μL amyloglucosidase from *A. niger* was added, and samples were incubated in a 60 °C water bath with shaking (100 rpm) to hydrolyze soluble starch. The residue weight was taken as the RS content except in the case of RS5, which contained 10 wt% stearic acid (dsb). The RS content of RS5 was taken as the residue weight minus the original weight of stearic acid present in the sample.

Starch samples were analyzed for RS content using AOAC Method 2002.02 (Megazyme Resistant Starch kit) (AOAC International, 2012). Mixed enzyme solution (4 mL) containing PPA (10 mg/mL) and amyloglucosidase (3 U/mL) in sodium maleate buffer (0.1 M, pH 6.0) was added to starch samples (100 mg) in a 50-mL screw-cap centrifuge tube, and samples were incubated for 16 h in a 37 °C water bath with shaking (100 rpm). Absolute ethanol (4 mL) was added to the tubes to stop the enzyme reaction, samples were centrifuged at 4000 × *g*, and the supernatant was decanted and saved. RS residues were re-suspended in 50% ethanol using a vortex mixer, centrifuged as above, and supernatants were decanted and pooled. This washing step was repeated once. RS residue of each sample was dispersed in a potassium hydroxide solution (2 mL, 2.0 M) in an ice-water bath, with stirring. This dispersed residue was mixed with 8 mL acetate buffer solution (1.2 M, pH 3.8) and 100 μL amyloglucosidase in glycerol (3300 U/mL) using a vortex mixer and incubated in a 50 °C water bath with shaking (100 rpm). RS was quantified in AOAC Method 2002.02 using the GOPOD assay. An aliquot (100 μL) of RS residue hydrolyzate was added to 3 mL GOPOD solution, the sample was incubated in a 50 °C water bath for 20 min, and the absorbance was measured at 510 nm against a reagent blank using a UV–vis spectrophotometer (Beckman Coulter, Brea, CA). FS, however, could not be dispersed in the potassium hydroxide solution because of chemical cross-linkages, and the RS content of this starch was also determined gravimetrically. The resistant residue of RS5 was washed 3 times with ethanol following the AOAC Method 2002.02 before dispersing in the potassium hydroxide solution to minimize any influence of saponification. The RS content of RS5 was also measured without prior ethanol washing for comparison.

2.2.2. 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, db, respectively) with 3× distilled–deionized water (w/w). Pans were sealed and allowed to equilibrate for 2 h to fully hydrate starch prior to the analysis. Samples were heated at 10 °C/min from 10 °C to 110 °C (aluminum pans) or to 150 °C (stainless 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.

2.2.3. Amylose content analysis

Amylose contents of starches were analyzed using an autotitrator (Metrohm 702 SM Titrino, Herisau, Switzerland) following the method of Song and Jane (2000). Starches were dispersed and defatted using 90% dimethyl sulfoxide solution and precipitated using absolute ethanol (5×, v/v). The precipitate was collected by centrifugation (7000 × *g*) and dried overnight in a 40 °C convection oven. The defatted samples were ground using a mortar and pestle prior to the analysis. The amylose content of the defatted sample was calculated using the iodine affinity value divided by 0.2 (Takeda & Hizukuri, 1987).

2.2.4. Hydrolysis of raw starches

Enzyme activities of BL and PPA were assayed using a NC dispersion (1%, w/w, db) cooked in a phosphate-buffer solution (100 mM, pH 6.9, containing 0.02% sodium azide). Enzyme activity was determined by measuring reducing sugars liberated from starch detected using 3,5-dinitrosalicylic acid reagent following the method of Miller (1959), and 1 U was defined as the amount of α-amylase that could release 1.0 mg of maltose equivalent from the solubilized NC in 1 min at pH 6.9 and 37 °C. BL and PPA (5.22 U) were used to hydrolyze raw starch suspensions (1%, w/w, dsb) in 10 mL phosphate buffer (100 mM, pH 6.9, containing 0.02% sodium azide)

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

Starch	AOAC method	
	991.43	2002.02
NC	0.65 ± 0.45[a]	1.45 ± 0.19[b]
OS-NC (3%)	1.44 ± 0.16[b]	1.35 ± 0.22[b]
OS-NC (10%)	1.85 ± 0.15[bc]	1.58 ± 0.13[b]
HC	32.92 ± 1.97[d]	39.84 ± 0.24[g]
OS-HC (3%)	16.07 ± 1.91[e]	36.29 ± 2.36[f]
OS-HC (10%)	10.22 ± 0.31[f]	31.46 ± 1.22[f]
WS	0.44 ± 0.09[a]	0.32 ± 0.51[a]
FS	90.57 ± 1.54[j]	0.32 ± 0.21[a]
	–	49.80[*] ± 0.10
BS	1.00 ± 0.47[ab]	27.17 ± 3.83[e]
PS	1.09 ± 0.78[b]	57.39 ± 3.45[c]
RS5	65.34 ± 0.76[g]	27.85[#] ± 0.21[d]
	–	29.75[^] ± 0.28
Novelose 330	46.58 ± 0.79[h]	45.23 ± 2.43[h]

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 starch-stearic-acid complex. Values with the same letter within a column were not significantly different ($\alpha = 0.05$).
[*] The RS content was measured gravimetrically.
[#] The RS content was determined without washing the residue with ethanol.
[^] The RS content was determined with washing the residue with ethanol.

at 37 °C for up to 84 h. Sample aliquots (0.25 mL) of the starch hydrolyzates were removed after 2, 4, 6, 8, 12, 24, 48, and 84 h and were analyzed for the reducing-sugar contents. After 84 h, absolute ethanol (2×, v/v) was added to stop the enzyme reaction, and samples were centrifuged at 1500 × *g* for 10 min. The residues were collected and dried overnight in a convection oven at 37 °C. The analysis was performed in duplicate.

2.2.5. Scanning electron microscopy (SEM)

Untreated and enzyme-hydrolyzed starch granules of selected samples were sprinkled on double-sided tape mounted on aluminum stubs and were sputter-coated under vacuum with palladium–gold alloy (60:40). Samples were imaged at an accelerator potential of 10 kV using a scanning electron microscope (Japan Electron Optics Laboratory, JEOL 5800LV, Peabody, MA).

2.3. 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 with the Analysis ToolPak add-in.

3. Results

3.1. RS contents

RS contents of starch samples analyzed using AOAC Method 991.43 and 2002.02 are shown in Table 1. Native normal starches (NC, WS, BS, and PS) were almost completely hydrolyzed using the AOAC Method 991.43 at the boiling-water temperature, but only the A-type cereal starches (NC and WS) were completely hydrolyzed using the AOAC Method 2002.02 at 37 °C. The B-type PS and C-type BS showed significantly larger RS contents analyzed using the AOAC Method 2002.02 at 37 °C (57.39% and 27.17% RS, respectively) than using the AOAC Method 991.43 at the boiling-water temperature (about 1% RS), which was expected because of the destruction of the native, crystalline granular structure at the boiling-water temperature. HC showed significantly higher RS content (32.92%) than the normal starches (0.44–1.09%) using the AOAC Method 991.43. RS contents of OS-NC and OS-HC (3 and 10 wt% OS) were increased and decreased, respectively, with increasing wt% OS-derivatives. Interestingly, RS contents of RS5 and FS were significantly greater when analyzed using the AOAC Method 991.43 (65.34 and 90.57% RS, respectively), in which BL was the main hydrolytic enzyme, than that using the AOAC Method 2002.02 and PPA was the hydrolytic enzyme. The large RS content of FS obtained using AOAC Method 991.43 was in agreement with previous reports (Maningat et al., 2013; McCleary et al., 2013). The RS content of FS obtained using the AOAC Method 2002.02 was very small (0.32%) because FS could not be dispersed in the 2 M KOH solution. Because of this problem, the RS content of FS was also determined gravimetrically (49.80%). The RS content of RS5 was 27.85% when determined using the AOAC Method 2002.02 without washing the remaining starch-residue with ethanol before dispersing in KOH, but the RS content was 29.75% after washing the starch residue with ethanol three times. The results indicated that the stearic acid remaining in the residue of the RS5 after hydrolysis by PPA interfered with the hydrolysis of the starch residue by amyloglucosidase and resulted in a smaller RS content measured using this glucogenic method. The RS contents of Novelose 330® were similar using both methods.

Optical micrographs of cooked starch granules showed that starches retaining granular character after 30-min cooking also had large RS contents when analyzed using the AOAC Method 991.43 (data not shown). These results, consistent with the gelatinization temperatures of the starches reported in Table 2 (except for FS), indicated that starch having T_c above the boiling temperature of water partially retained their granular and crystalline structures after boiling and were thus more resistant to enzyme hydrolysis by BL.

Table 2
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 330	109.16 ± 0.18	117.73 ± 0.62	–	124.97 ± 1.11	25.21 ± 3.54
RS5	96.43 ± 0.59	103.94 ± 0.47	–	109.74 ± 0.30	9.43 ± 3.47

T_o, T_p, T_c, Onset, peak, and conclusion gelatinization temperatures, respectively; 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™ cross-linked wheat starch; RS5, debranched high-amylose corn starch-stearic-acid complex.

Table 3
Amylose contents of native starches.

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 ($\alpha = 0.05$).

[#] The amylose content was analyzed using iodine potentiometric titration.

3.2. Amylose content and thermal properties of starch

Starch from corn with the amylose-extender (*ae*) gene mutation, HC, has a large amylose-content (Table 3), long amylopectin branch-chains, and is known to contain a larger RS content than the normal counterpart (Li, Jiang, Campbell, Blanco, & Jane, 2008). This is attributed to the presence of double-helical crystallites of amylose, which have T_c above the boiling temperature of water (Table 2) (Li et al., 2008; Jiang, Campbell, Blanco, & Jane, 2010). Addition of OS groups to the HC was observed to disrupt these thermally stable double-helices and decreased the gelatinization temperature of OS-HC (Table 2) with a net effect of decreasing RS contents (Table 1). Conversely, RS contents of OS-NC increased with increasing wt% substitution (Table 1), which was in agreement with results reported by Ai et al. (2013). These differences were attributed to that OS-derivatization of starch reduced the length of starch chains free of derivatives. Because enzymes could not bind starch chains carrying OS-derivatives at the binding-site and hydrolyze the starch, it resulted in increased RS contents. The RS5 displayed an additional peak (68.46–73.07 °C, data not shown in table) in the thermogram, which was due to the melting of stearic acid, and the main peak, 96.43–109.74 °C, was mainly attributed to dissociation of the amylose–stearic acid complex (Table 2) (Hasjim et al., 2010). The very high gelatinization temperature of Novelose 330® was due to the presence of thermally stable amylose double-helical crystallites (Table 2).

3.3. Alpha-amylolysis of raw starch granules

3.3.1. Starch hydrolysis rates

To understand whether the different RS contents determined using AOAC Method 991.43 and 2002.02 were related to the enzymes used for the analyses, we hydrolyzed raw native starches

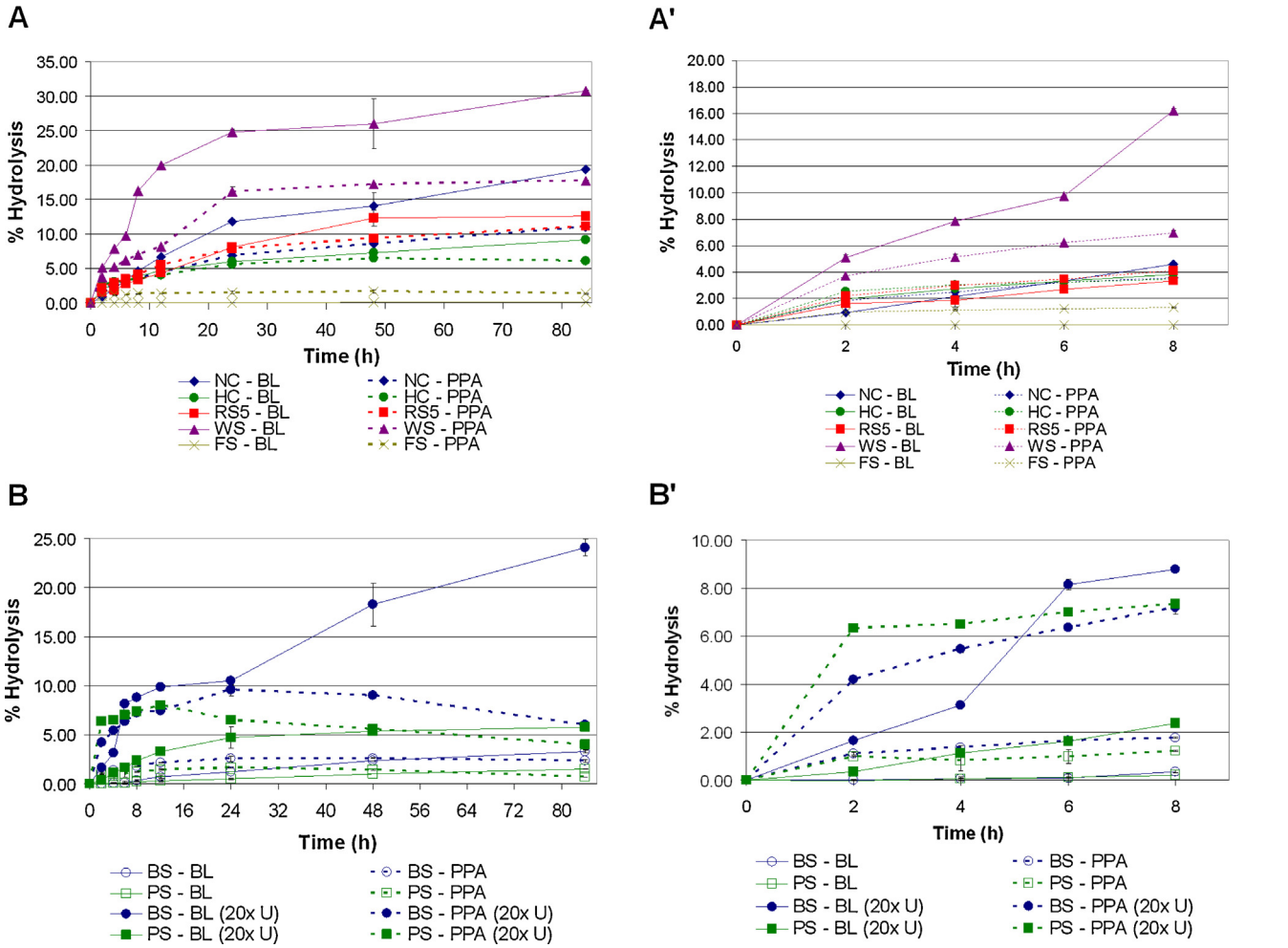

Fig. 1. Hydrolysis rates of raw starches up to 84 h ((A) and (B)); (A′) and (B′) are enlarged to show hydrolysis rates at the early stage. (A/A′), hydrolysis of 1% (w/w, dsb) normal corn (NC), high-amylose corn (HC), RS5, normal wheat (WS), and Fibersym™ (FS) starches by 5.22 U of *Bacillus licheniformis* (BL) and porcine pancreatic (PPA) α-amylases in phosphate buffer (100 mM, pH 6.9, containing 0.02% sodium azide) at 37 °C; (B/B′), hydrolysis of banana (BS) and potato (PS) starches by BL and PPA using low (5.22 U) and high (20×, 104.4 U) doses of enzymes in the same condition. One U of α-amylase was defined as the amount of enzyme that could release 1.0 mg of maltose equivalent from the solubilized NC in 1 min at pH 6.9 and 37 °C.

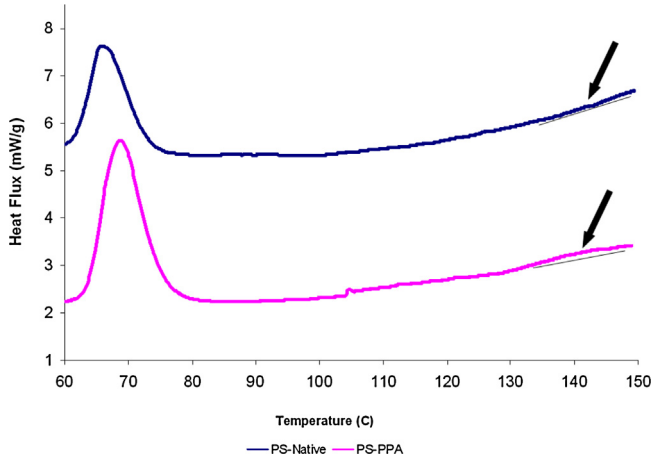

Fig. 2. DSC thermograms of native and PPA-hydrolyzed potato starch (PS). Arrows indicate retrograded amylose and drawn lines indicate the baseline. The magnitude of *Y*-axis units is arbitrary to show comparisons.

using 5.22 U of BL or PPA. Because native BS and PS were hydrolyzed very slowly under these conditions, we also used a higher enzyme-activity (20×, 104.4 U) to hydrolyze these two starches. Enzyme-hydrolysis rates of raw starches using PPA were greater than those using BL in the early stage of hydrolysis, up to about 8–12 h, except WS (Fig. 1A′). Native PS, a B-type polymorphic starch, showed a continuous increase in hydrolysis up to 84 h by BL, but PS was hydrolyzed more quickly by PPA in early stages at both the low and high enzyme doses (Fig. 1B′). Native BS, a C-type polymorphic starch, was hydrolyzed more slowly by BL than by PPA in early stages, which was similar to PS, but showed a drastic increase in hydrolysis rate after 4 h using the high dosage (Fig. 1B′). After 24–48 h, extents of starch hydrolysis by PPA, measured as soluble, reducing sugar content of the supernatant, either remained static, as shown in HC and WS, or decreased, as shown in BS and PS. The decrease in the hydrolysis rate by PPA was attributed to retrogradation of partially hydrolyzed amylose, which showed a characteristic peak at 130–150 °C in the DSC thermograms of PPA-hydrolyzed PS (Fig. 2). This was attributed to the multiple-attack hydrolysis pattern of PPA, which produced amylose with chain chains closer to the optimal length (DP ~100) for retrogradation (Robyt & French, 1967).

3.3.2. *Scanning electron micrographs of hydrolyzed starches*

BS, WS, and FS, were selected for study using SEM (Fig. 3) because the differences observed using optical microscopy following hydrolysis of these samples were the most readily visible (data not shown). Asymmetric removal of surface starch to expose growth rings was observed in BL-hydrolyzed BS granules (Fig. 3B), whereas pitting into the granule (Fig. 3C) followed by hydrolysis from the inside toward the surface (Fig. 3D), leaving an intact enzyme-resistant outer shell, was observed in PPA-hydrolyzed BS granules. Striations shown after hydrolysis using BL are consistent with studies of morphological changes in BS granules during ripening (Peroni-Okita et al., 2010) and in raw BS granules recovered from ileal effluents of rats (Sugimoto, Fujita, Takaya, & Fuwa, 1980) and humans (Langkilde, Champ, & Anddersson, 2002). This hydrolysis pattern could be due to looser packing of starch molecules at regions distal to the hilum as observed on polarized light micrographs (data not shown), which were biosynthesized later than the proximal region closer to the hilum. Surface hydrolysis exposing the contour of interior growth rings of BL-hydrolyzed WS granules was observed (Fig. 3F). Some BL-hydrolyzed WS A-granules were split following attack at the equatorial groove, the loosely-packed region of the granule (Fig. 3F). PPA-hydrolyzed WS, however, showed a preference for pitting and inside-out hydrolysis of the A-granules (Fig. 3G). PPA was the enzyme in this study observed to hydrolyze FS granules (Fig. 3J), which might occur at points on the granule surface with less dense cross-linking. In contrast, there was no sign of hydrolysis of FS using BL (Fig. 3I). This observation was consistent with the negligible hydrolysis of FS by BL (Fig. 1A and B).

4. Discussion

Hydrolysis rates of raw starches using BL and PPA may provide insight into the mechanisms underlying the observed differences in RS contents in this study. The B-type polymorphic structure of PS granules is known to have long-chain double helices and a homogeneous internal structure without voids (Jane, 2009), which are the features responsible for the enzyme resistance of this raw starch (Gallant, Bouchet, Buléon, & Pérez, 1992). NC and WS are of the A-polymorph and have shown great internal porosity (Jane, 2009), which facilitates enzyme penetration and increased rates of enzyme hydrolysis (Kim & Huber, 2010). PPA exhibits a multiple-attack reaction pattern of starch hydrolysis (Robyt & French, 1967; Thoma, 1976), indicating a high probability of consecutive scissile events per enzyme-substrate binding (Butterworth, Warren, & Ellis, 2011). In contrast, BL exhibits a random-attack reaction pattern (Kandra, Gyémánt, Remeyik, Hoyánszki, & Liptak, 2002). The multiple-attack reaction pattern of PPA likely facilitates the binding and progressive hydrolysis of starch chains and results in the formation of pits on the surface of starch granules and the more rapid hydrolysis in the early stage observed in most starches treated with PPA.

The large A-granules of WS have a loosely-packed equatorial groove that is highly susceptible to enzyme attack (Fannon, Hauber, & BeMiller, 1992). The equatorial groove may thus facilitate hydrolysis and account for the faster initial hydrolysis rate and splitting of WS granules observed using BL. The continuous increase in extent of hydrolysis of the B-type polymorphic PS by BL likely corresponded to a surface-hydrolysis process. A similar continuous increase was observed during the hydrolysis of C-type polymorphic BS by BL up to 12 h in the low dosage and 4 h in the high dosage, after which the rate of hydrolysis rapidly increased. It is therefore plausible that BS granules may have an enzyme-resistant outer shell similar to that of PS, whereas the granule interior is more digestible.

Phosphate cross-linking reactions of starch occur mainly on the surfaces of starch granules (Huber & BeMiller, 2001). Cross-linking may therefore represent a reinforcement of the starch granule surface and reduction in length of starch chains free of cross-linkages for enzyme binding and hydrolysis (Butterworth et al., 2011). BL and PPA differ in the size of glucose-binding sub-sites for enzyme binding and subsequent hydrolysis: BL has a binding-site of nine glucose-units (Kandra et al., 2002), whereas PPA has that of five glucose-units (Robyt & French, 1967). Reduced starch chain-length free of phosphate cross-linkages on the surface of granules, which could be bound by PPA but were not long enough for the binding by BL, may explain the lack of hydrolysis of FS by BL compared with that by PPA. The moderate increase in RS content of OS-NC with increasing wt% OS was also attributed to the reduction in the length of starch chains free of OS-derivatives, which were less available to bind with enzymes. OS-derivatization, however, facilitated swelling of the granules (Ai et al., 2013). Consequently, OS-HC had decreased RS content compared with the native HC. Phosphate cross-linking inhibited α-amylolysis of the starch to a greater extent than the OS-derivatization, resulting from restricted swelling of the starch granules by the cross-linking. The helical complex of high-amylose corn starch with stearic acid (RS5) also showed restricted swelling and lipid coating on the surface of

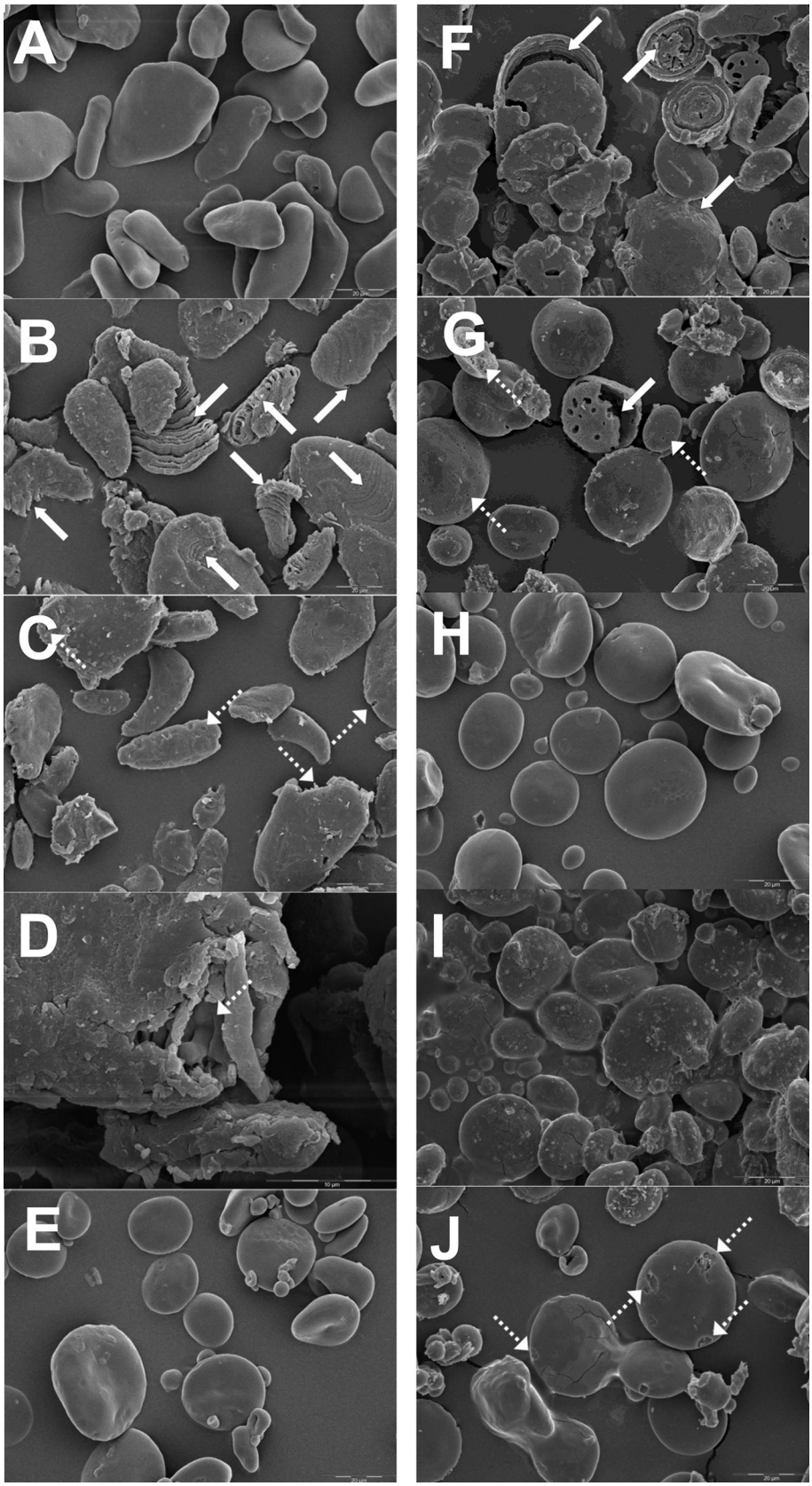

Fig. 3. Scanning electron micrographs of untreated and enzyme-hydrolyzed starches using BL and PPA after 84 h. The same magnification (1500×) is used in all micrographs except for (D) (5000×). (A) Native banana starch (BS); (B) BL-hydrolyzed BS; (C) and (D) PPA-hydrolyzed BS; (E) native wheat starch (WS); (F) BL-hydrolyzed WS; (G) PPA-hydrolyzed WS; (H) untreated Fibersym^{TM} cross-linked wheat starch (FS); (I) BL-hydrolyzed FS; (J) PPA-hydrolyzed FS. Solid arrows show hydrolysis of starch granule surfaces and exposure of interior growth rings, and dashed arrows show pitting and widening of surface pinholes on starch granule surfaces. BL and PPA were used to hydrolyze 1% (w/w, dsb) starch suspended in phosphate buffer (100 mM, pH 6.9, containing 0.02% sodium azide) at 37 °C.

granules. Therefore, RS5 was substantially less hydrolyzed by BL using the AOAC 991.43 than by PPA in the other method.

5. Conclusion

RS contents of starches varied depending on starch botanical origin, the type and extents of chemical modification, and the method of analysis used. Hydrolysis rates of raw starch granules by BL and PPA used in the standard methods of RS content analysis explained the wide differences in RS contents determined using AOAC Method 991.43 and 2002.02. Hydrolysis at 37 °C by BL was mostly localized on the surface, whereas hydrolysis by PPA produced visible pitting on the surface of starch granules, followed by hydrolysis from the inside-out. These differences were ascribed to differences in the number of glucose-binding subsites of the enzymes, the reaction patterns of enzymes (i.e. random or multiple attack), and availability of starch chains free of derivatives for enzyme binding and hydrolysis.

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