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Amylolysis of large and small granules of native triticale, wheat and corn starches using a mixture of α-amylase and glucoamylase

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ABSTRACT

Starch granules from different sources differ in their morphology and structure, resulting in diverse amylolysis, which is closely related to bioconversion efficiency of starch to fermentable sugars during ethanol production. In this study, large and small granules were fractionated from native triticale, wheat and corn starches using a centrifugal sedimentation protocol and were hydrolyzed by a granular starch hydrolyzing enzyme at sub-gelatinization temperatures. Native triticale starches had bimodal granule size distribution with highest sizes of 14.9–15.9, 19.1–20.2, and 7.2 μm in diameter for overall, large and small granules, respectively, when compared to those of wheat and corn starches. The large granules in triticale starches contributed higher proportion of the total granules either by number (36–45%) or by weight (93–95%) than did those of wheat and corn starches. Surface pores and internal channels viewed by SEM and CLSM were more visible in large granules than in small granules of triticale, wheat, and corn starches, but corn starch generally displayed more pores and channels than triticale and wheat starches. Large granules contained significantly higher apparent amylose content and higher relative crystallinity than did small granules (23.0–28.5% vs. 12.4–21.0% and 24.9–26.8% vs. 20.8–24.4%, respectively) in all starches. Small granules were hydrolyzed significantly faster than did large granules initially (DH 9.3–18.0%, 24.7–40.3%, 17.7% higher in small granules than in large counterparts of triticale, wheat, and corn starches, respectively, at 55 °C for 1 h), however slowed down in later stage of hydrolysis. The final DH of large and small granules were similar in triticale (82.3–84.5%) and corn (80.8–83.4%) starches except wheat starches (90.8–95.1% in large and 79.6–86.2% in small granules) at 72 h hydrolysis. SEM and CLSM further revealed that the hydrolysis pattern differed between large and small starch granules and among starches, indicating diverse structure of large and small granules existed at macro, micro, and nano levels within and between species and cultivars. The study may be beneficial for precise control of liquefaction and saccharification in simultaneous hydrolysis and fermentation process of ethanol production.

1. Introduction

In the midst of fast growing global demand for energy and progressive depletion of fossil fuel warrants innovative research efforts toward alternative energy sources that are renewable and environmentally friendly. Biofuels are in shape to solve the aforementioned challenges. Of all biofuels, bioethanol is the most utilized liquid biofuel either as a fuel or as a gasoline extender/additive and its production from energy crops (e.g. sugar based and starchy materials) is referred to as the first-generation technique (Gomez, Steele-King, & McQueen-Mason, 2008). Starch is a cheap, clean, nontoxic and renewable carbon source, and available in abundance, thus is widely used as a feedstock in bioethanol production (Chen, Wu, & Fukuda, 2008; Sanchez & Cardona, 2008). Corn, wheat and cassava are used to a greater extent than barley, rye and triticale in bioethanol production (das Neves, Kimura, Shimizu, & Nakajima, 2007; Sanchez & Cardona, 2008). In Canada, most of the ethanol currently produced is distilled from corn and wheat (Natural Resources Canada, 2011). However due to the increasing cost of wheat and less availability of corn, triticale may be a best choice for bioethanol production in Canada (Alberta Agriculture and Rural Developemt, 2011; Wang, Thomas, Ingledew, Sosulski, & Sosulski, 1997). Triticale (× *Triticosecale* Wittmack) is a hybrid cereal species developed by crossing wheat (*Triticum turgidum* or *Triticum aestivum*) with rye (*Secale cereale*). Its agronomic advantages, which include high grain yield, high grain test weight, tolerance to climatic and soil-related abiotic stresses, resistance to disease and pest-related biotic stresses, and low input

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requirements compared to widely grown cereals (Pejin et al., 2009), have resulted in its adoption in more than 30 countries and a steady increase in world production. Triticale has been shown to be an economically favorable source of carbohydrate for industrial and energy end-uses perhaps due to the following reasons: (1) comparable biological value with the most suitable wheat varieties for bioethanol production, (2) a better reduction in net greenhouse gas emissions than wheat due principally to its lower nitrogen input, (3) savings in energy and technical enzymes due to its lower temperature requirement for liquefaction and saccharification, and (4) presence of high levels of autoamylolytic enzymes (Davis-Knight & Weightman, 2008; Pejin et al., 2009; Wang et al., 1997).

The conventional large-scale ethanol production is a batch process and it requires starch from agricultural crops to be enzymatically hydrolyzed (liquefaction at 90–110 °C followed by saccharification at 60–70 °C) completely to sugars, which are subsequently fermented to ethanol by yeast (Chen et al., 2008; Sharma, Rausch, Tumbleson, & Singh, 2007). On the other hand, the current trend in bioethanol production towards continuous process involves the saccharification of dextrin (liquefied starch) into sugars in parallel to yeast fermentation in a single reactor (Balat, 2009; das Neves et al., 2007). Regardless of technologies, the initial step in current bioethanol production that converts native starch into sugars (amylolysis) is expensive since excessive heat energy is used in gelatinizing starch. Recently a granular starch hydrolyzing enzyme (a mixture of α-amylase and glucoamylase) is developed to hydrolyze native starch granules into fermentable sugars at subgelatinization temperature. It could be used in simultaneous saccharification and fermentation (SSF) at low temperature, thus it reduces the cost and can effectively work on uncooked (raw) starches.

The structure and physicochemical properties as well as *in vitro* hydrolysis of large and small granules of cereal starches including triticale, wheat, barley and corn have been characterized (Ao & Jane, 2007; Bertolini, Souza, Nelson, & Huber, 2003; Dhital, Shrestha, & Gidley, 2010; Liu, Gu, Donner, Tetlow, & Emes, 2007; Peng, Gao, Abdel-Aal, Hucl, & Chibbar, 1999; Salman et al., 2009; Stevnebo, Sahlstrom, & Svihus, 2006; Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2001a; Utrilla-Coello, Agama-Acevedo, de la Rosa, Rodriguez-Ambriz, & Bello-Perez, 2010; Vermeylen, Goderis, Reynaers, & Delcour, 2005). Most recently, the characterization on density and dimension of surface pores and the distribution of internal channels, protein, and phospholipids of large and small granules of triticale and corn starches using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) has been studied (Naguleswaran, Li, Vasanthan, & Bressler, 2011). However, investigation on amylolysis of large and small granules of triticale starch using granular starch hydrolyzing enzyme has not been reported. SEM has been the main technique for investigating the morphological changes of hydrolyzed cereal starch granules. Recently, CLSM in conjunction with fluorescent staining using specific dyes has been used in studying enzyme hydrolyzed potato starch granules (Apinan et al., 2007; Varatharajan et al., 2011). However, no reports found on enzyme hydrolyzed cereal starches. The objective of this study was to understand the relationship between morphological, structural and physicochemical characteristics and degree of amylolysis of large and small starch granules from triticale, wheat and corn starches using a granular starch hydrolyzing enzyme at subgelatinization temperatures. The study may provide some new information for precise control of starch liquefaction and saccharification of bioconversions during simultaneous hydrolysis and fermentation process in bioethanol production and other industrial applications.

2. Materials and methods

2.1. Materials

Two cultivars of wheat (*T. aestivum* L.) grains, Canada Prairie Spring Red (CPSR) and AC Reed, were provided by Alberta Agriculture and Food, Barrhead, AB, Canada. Two cultivars of triticale (× *Triticosecale*) grains, Pronghorn and AC Ultima, were supplied by the Field Crop Development Centre, Alberta Agriculture and Rural Development, Lacombe, AB, Canada. Normal corn starch (Melojel) was donated by National Starch Food Innovation (Bridgewater, NJ, USA). Granular starch hydrolyzing enzyme, Stargen 002 (570 GAU/g), was donated by Genencor (Rochester, NY, USA). 8-Aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS) was purchased from Invitrogen (Molecular Probes, Eugene, OR, USA). All other chemicals and reagents used in this study were of ACS grade.

2.2. Grain grinding and starch isolation

The triticale and wheat grains were ground into meals in a Retsch mill (Model ZM 200, Haan, Germany) using a ring sieve with an aperture size of 0.5 mm. Pure starch (purity >95%, w/w) was isolated from grain meal using the procedure described by Kandil, Li, Vasanthan, Bressler, and Tyler (2011).

2.3. Chemical composition of starches

Moisture and ash contents were determined by AACC Methods 44-15A and 08-01, respectively (AACC International, 2004). Starch content was estimated according to the total starch assay kit of Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland). The total nitrogen (TN) content of starch was determined by the Dumas combustion method (Rutherford, McCarthy, Arendt, & Figueiredo, 2008) using a Costech ECS 4010 Elemental Combustion System (Costech Analytical Technologies Inc., Valencia, CA, USA) and the protein contents were calculated by TN × 5.7. Starch lipid was determined by the procedures outlined in an earlier publication (Vasanthan & Hoover, 1992). Apparent amylose content of starch was determined according to the method described by Hoover and Ratnayake (2001). The amylose standard curve was prepared using pure amylose and amylopectin from potato starch (Sigma–Aldrich, Co., St. Louis, MO, USA) as described in the methodology.

2.4. Fractionation of large and small starch granules

Isolated triticale and wheat starches and commercial corn starch were fractionated into large and small granules using a centrifugal sedimentation protocol modified from Peng et al. (1999). In brief, starch was slurried in water (1:10, w/v) and centrifuged (Fisher Scientific accuSpin™ 400 bench top centrifuge, Germany) at 13 × *g* for 5 min. After centrifugation, the supernatant which contained small granules was collected and the residue was reslurried in water. The above centrifugal sedimentation step was repeated at least twenty times. Finally, the supernatant containing small granules and slurry of residue containing large granules were then passed independently through #41 Whatman™ filter paper (GE Healthcare UK Ltd., Buckinghamshire, UK) using a vacuum filtration system. The residue on the top of filter paper was washed few times with deionized water followed by washing with anhydrous ethanol. The fractionated small and large granules were then air-dried at 40 °C and stored in glass vials until further analyses.

2.5. Granule size analysis

Granule size and size distribution of unfractionated native starches was determined following the method of Li, Guiltinan, and Thompson (2007) using Beckman Coulter LS 13320 Particle Size Analyser (Beckman Coulter, Inc., Fullerton, CA, USA). The standard refractive indices applied for water and starch were 1.31 and 1.52, respectively. Number percentage of granules was recorded and weight percentage of starch granules was derived assuming all starch granules were spherical in shape.

2.6. X-ray powder diffraction analysis

X-ray diffractograms of starches (moisture content ≈10%) were obtained with cross beam optics (CBO) technology of Rigaku Ultima IV multipurpose X-ray diffraction meter (Rigaku Americas, The Woodlands, TX, USA) according to the method described by Gao, Vasanthan, and Hoover (2009).

2.7. Amylolysis of starches using granular starch hydrolyzing enzyme

Starch samples (0.3% db, w/v) were hydrolyzed with Stargen 002 enzyme (24 U/g starch) in 50 mM sodium acetate buffer (pH 4.0) at 55 °C for 1 h followed by at 30 °C for 72 h in a shaking water bath (Model BS-11, Jeio Tech Inc., Korea) according to the instructions given by enzyme manufacturer. The hydrolysates were withdrawn at 0, 1, 24, 48 and 72 h for the determination of degree of hydrolysis (DH). DH was expressed as a percentage of reducing value by the 3,5-dinitrosalicylic acid (1%, w/v) method (Bruner, 1964). The control starch samples were concurrently run without addition of enzyme.

2.8. Morphological and structural characterization of starch granules

The morphology and structure of unfractionated and fractionated (large and small) starch granules were characterized using SEM and CLSM according to the methods described by Naguleswaran et al. (2011). The enzymatically hydrolyzed starch residues were recovered with addition of anhydrous ethanol followed by centrifugation (Fisher Scientific accuSpin^{TM} 400 bench top centrifuge, Germany) at 5000 × *g* for 10 min. The starch granules without addition of enzyme (control) and the enzymatically hydrolyzed starch residues after 1 h and 24 h were examined microscopically. For SEM, starch samples were mounted on circular aluminum stubs with double-sided sticky tape, coated with gold to a thickness of 12 nm, and examined and photographed in a JEOL scanning electron microscope (Model JSM 6301 FXV, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV. In the CLSM procedure, the starch granules were stained with APTS dye and visualized under a confocal laser scanning microscope (Zeiss LSM 710, Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with a ×40 1.3 oil objective lens. Images of optical sections of starch granules were recorded and analyzed with ZEN 2009 Light Edition software (Carl Zeiss MicroImaging GmbH, Jena, Germany).

2.9. Statistical analyses

All treatments and analyses were carried out in triplicate. Analysis of variance using the General Linear Model (GLM) procedure and Pearson correlation statistics were performed using SAS Statistical Software, Version 9.1.2 (SAS Institute Inc., Cary, NC, 2004). Multiple comparisons of the means were carried out using Tukey's Studentized Range (HSD) Test at $\alpha = 0.05$.

3. Results and discussion

3.1. Composition of unfractionated and fractionated starches

As shown in Table 1, starches isolated from triticale and wheat meals had similar purity of starch (97%) and ash content (0.2%) but lower protein content compared to those of corn starch (0.40–0.52% vs. 0.86%). The lipid content in triticale starches (0.22%) was also lower than those of wheat (0.77–0.87%) and corn (0.69%) starches. For fractionated starches, large starch granules generally showed lower protein and lipid contents that those of small granules in triticale, wheat and corn starches. Similar results were also reported by other researchers (Liu et al., 2007; Raeker, Gaines, Finney, & Donelson, 1998; Soulaka & Morrison, 1985).

Table 1
Composition of triticale, wheat and corn starches (%, db).

Starch sources	Starch	Protein	Lipid	Ash
Triticale				
Pronghorn				
Unfractionated	$97.2^{a} \pm 0.5$	$0.50^{c} \pm 0.02$	$0.22^{gh} \pm 0.001$	$0.19^{bcd} \pm 0.008$
Large	$98.4^{a} \pm 0.3$	$0.43^{de} \pm 0.03$	$0.19^{hi} \pm 0.002$	$0.09^{f} \pm 0.002$
Small	$98.1^{a} \pm 0.4$	$0.47^{cd} \pm 0.01$	$0.23^{g} \pm 0.009$	$0.08^{f} \pm 0.003$
Ultima				
Unfractionated	$97.5^{a} \pm 0.9$	$0.40^{e} \pm 0.03$	$0.22^{gh} \pm 0.005$	$0.17^{de} \pm 0.008$
Large	$98.0^{a} \pm 1.0$	$0.32^{f} \pm 0.01$	$0.18^{i} \pm 0.006$	$0.08^{f} \pm 0.002$
Small	$98.4^{a} \pm 0.4$	$0.39^{e} \pm 0.01$	$0.22^{g} \pm 0.002$	$0.07^{f} \pm 0.013$
Wheat				
CPSR				
Unfractionated	$96.6^{a} \pm 0.1$	$0.52^{bc} \pm 0.01$	$0.87^{b} \pm 0.005$	$0.20^{bc} \pm 0.007$
Large	$97.9^{a} \pm 0.1$	$0.43^{de} \pm 0.01$	$0.76^{d} \pm 0.005$	$0.18^{cde} \pm 0.003$
Small	$98.3^{a} \pm 0.2$	$0.51^{c} \pm 0.01$	$0.90^{a} \pm 0.009$	$0.16^{e} \pm 0.002$
AC Reed				
Unfractionated	$96.8^{a} \pm 0.6$	$0.51^{c} \pm 0.03$	$0.77^{d} \pm 0.005$	$0.22^{a} \pm 0.001$
Large	$97.4^{a} \pm 0.7$	$0.42^{de} \pm 0.01$	$0.75^{d} \pm 0.006$	$0.19^{bc} \pm 0.002$
Small	$97.3^{a} \pm 0.3$	$0.50^{c} \pm 0.00$	$0.84^{c} \pm 0.009$	$0.18^{cde} \pm 0.002$
Normal corn				
Unfractionated	$97.1^{a} \pm 0.9$	$0.86^{a} \pm 0.01$	$0.69^{e} \pm 0.005$	$0.21^{ab} \pm 0.005$
Large	$97.7^{a} \pm 1.0$	$0.58^{b} \pm 0.02$	$0.64^{f} \pm 0.005$	$0.09^{f} \pm 0.005$
Small	$98.0^{a} \pm 1.0$	$0.83^{a} \pm 0.02$	$0.72^{e} \pm 0.007$	$0.08^{f} \pm 0.002$

Values are percentage of mean ± standard deviation in dry weight basis and values with the same superscript in the same column are not significantly different at $\alpha = 0.05$.

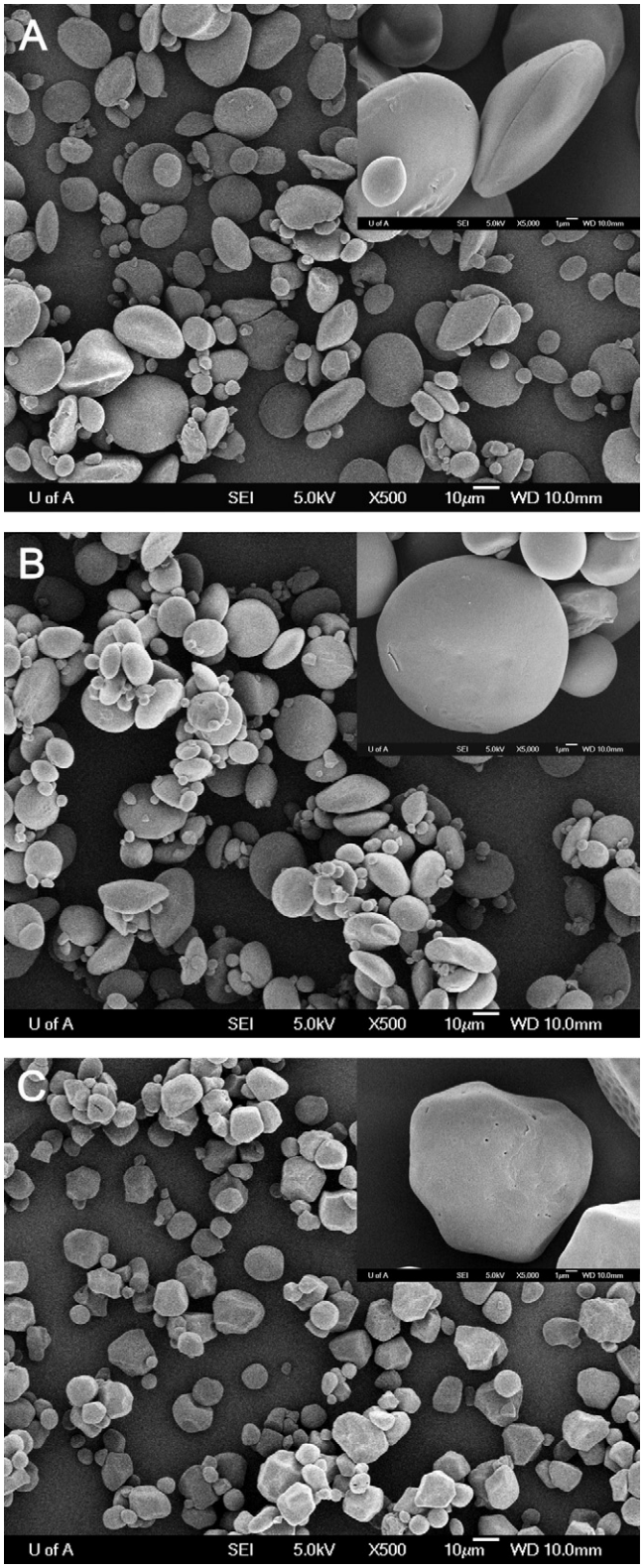

Fig. 1. Scanning electron micrographs of unfractionated triticale (Pronghorn) (A), wheat (CPSR) (B) and corn (C) starch granules. Inserts are individual granules at high magnification.

3.2. Granule size and size distribution of unfractionated starches

Triticale, wheat and corn starch granules showed a bimodal size distribution in the diameter range of 3–33 μm, 2–29 μm and 2–27 μm respectively (Fig. 1 and Table 2). The large granules (>10 μm) of triticale and wheat starches were lenticular and the large corn starch granules were polyhedral in shape. The small granules (<10 μm) in all starches were mostly spherical in shape. As shown in Table 2, the average granule sizes of triticale starches were larger than those of wheat and corn starches, whereas those of wheat and corn starches were comparable. The proportions of large and small granules by number and by weight varied with starch sources (Table 2). In triticale starches, large granules contained the highest proportion of total granules by number (36–45%) and by weight (93–95%) compared to those of wheat and corn starches (3–17% by number and 77–85% by weight). The lower number percentage of large granules in total starch represents major mass of the starch (Li, Vasanthan, Rossnagel, & Hoover, 2001). The granule size and size distribution of triticale, wheat and corn starch granules are in agreement with previous studies (Ao & Jane, 2007; Salman et al., 2009).

3.3. Morphology and microstructure of large and small starch granules

The morphological and microstructural features of fractionated large and small granules of triticale, wheat and corn starches were revealed by SEM (Figs. 2–4) and CLSM (Figs. 5–7). Under SEM, the surfaces of both large and small granules of triticale and wheat starches appeared to be relatively smooth with reduced furrows and shallow depressions (Figs. 2A, D and 3A, D). Surface pores were more frequently visualized on large granule surfaces of triticale and wheat starches. Oval-shaped pores along equatorial grooves and some aggregates of small pores with the appearance of slit-like cracks were present on some large granule surfaces of triticale and wheat starches (Figs. 2A and 3A). Compared to those of triticale and wheat starch granules, corn starch granules showed markedly numerous pores of varying size that were unevenly distributed on the granule surface. These pores were more pronounced on the surface of large granules (Fig. 4A).

Atomic force microscopy (AFM) has revealed that the starch granule surface is undulated as shown by protrusions and depressions even though there is presence of rather smooth, flat or low rough regions (Juszczak, Fortuna, & Krok, 2003; Tomoaia-Cotisel, Cioica, et al., 2010). The nitrogen absorption analyses of corn starch granules showed that the above protrusions are composed of large agglomerates (100–200 nm in size), which further consists of fine particles (20–30 nm in diameter) surrounded by much shallow depressions (Park, Xu, & Seetharaman, 2011), while dominant mesopores of 2–3 nm and macropores of 100–200 nm in diameter are also present on surfaces of corn starch granules (Sujka & Jamroz, 2009, 2010). AFM of triticale starch (Juszczak, 2003) has revealed that shallow depressions (<1 μm) with irregularly distributed pores in oval or circular or slit-like shape in a broad range of size (40 to >100 nm in diameter) as well as surface protrusion structure of 50–200 nm in size. In wheat starch granules, the surface protrusions consist of structures in the diameter range of 10–50 nm (Baldwin, Adler, Davies, & Melia, 1998; Baldwin, Davies, & Melia, 1997). The fine particle structure in the size of approximately 30 nm on starch granule surfaces commonly exists in cereal and tuber starches (Ohtani, Yoshino, Hagiwara, & Maekawa, 2000; Sujka & Jamroz, 2009). These surface structures are believed to be the ends of starch macromolecules within the crystalline amylopectin side chain clusters (Baldwin et al., 1998, 1997; Sujka & Jamroz, 2009), corresponding to the blocklet structure proposed by Gallant, Bouchet, and Baldwin (1997). Most recently, a hair-like structure in the length of 1–5 nm was found on the surface of fine particles of corn and potato starch granules by AFM when granules are exposed to iodine vapor under a humid environment

Table 2
Granule size distribution of unfractionated triticale, wheat and corn starches.

Starch sources	Granule size range (μm)	Mean granule size (μm)			Number (%)		Weight (%)	
		Overall	Large	Small	Large	Small	Large	Small
Triticale								
Pronghorn	3–33	15.9	20.2	7.2	$44.6^{a} \pm 0.3$	$55.4^{d} \pm 0.3$	$95.2^{a} \pm 0.0$	$4.8^{e} \pm 0.0$
Ultima	3–31	14.9	19.1	7.2	$35.9^{b} \pm 0.1$	$64.1^{c} \pm 0.1$	$93.0^{b} \pm 0.1$	$7.0^{d} \pm 0.1$
Wheat								
CPSR	2–29	9.6	18.1	4.4	$3.0^{d} \pm 0.1$	$97.0^{a} \pm 0.1$	$76.7^{e} \pm 0.4$	$23.3^{a} \pm 0.4$
AC Reed	1.5–26	8.7	17.1	4.3	$4.1^{d} \pm 0.1$	$95.9^{a} \pm 0.1$	$80.7^{d} \pm 0.1$	$19.3^{b} \pm 0.1$
Normal corn	2–27	9.4	17.1	4.8	$16.8^{c} \pm 0.6$	$83.2^{b} \pm 0.6$	$84.9^{c} \pm 0.3$	$15.1^{c} \pm 0.3$

Values of number and weight percentages are mean ± standard deviation in number and weight basis, respectively. Values with the same superscript in the same column are not significantly different at $\alpha = 0.05$. Large starch granule was defined as granule diameter >10 μm.

(Park et al., 2011), which is believed to be extensions of either amylose or amylopectin molecules that are free to complex with iodine.

In agreement with our previous study (Naguleswaran et al., 2011), growth rings, internal channels, equatorial grooves, and a central amorphous region were easily distinguished in all starch granules by florescence intensity under CLSM (Figs. 5A, 6A and 7A). Recent studies on removal of channel proteins have shown the presence of tiny channels in both large and small granules of wheat, triticale and corn starches when viewed under CLSM (Kim & Huber, 2008; Naguleswaran et al., 2011). The CLSM has confirmed that granule surface and internal channels are rich in protein and phospholipids (Naguleswaran et al., 2011) which together with starch molecules may contribute to the complex nature of the granule surface (Debet & Gidley, 2007; Naguleswaran et al., 2011; Tomoaia-Cotisel, Cota, Mocanu, & Horovitz, 2010). The number, size and size distribution of pores, internal channels, and glucan polymer particles (topology) have been shown to vary with the botanical origin of starch (Juszczak et al., 2003).

3.4. Amylose content and relative crystallinity of large and small starch granules

Apparent amylose contents of unfractionated starches from triticale, wheat and corn were in a narrow range of 22.5–26.4% (Table 3). However, the apparent amylose content differed significantly between large and small granules of each starch (Table 4). The large granules of triticale, wheat and corn starches contained significantly higher apparent amylose content (23.0–28.5%, db) than the small granules (12.4–21.0%, db). The difference in amylose contents between large and small granules in triticale, wheat and corn starches were 2.4–10.0%, 8.0–13.4%, and 5% (percentage unit difference), respectively, indicating that the apparent amylose content of starch varied with starch granule size within and between starch sources. The present results are in agreement with previous studies, where the apparent amylose content of large granules was 5–9% higher than that of small granules in wheat (Ao & Jane, 2007; Bertolini et al., 2003; Liu et al., 2007), 10% in triticale (Ao & Jane, 2007), 1.3% in corn (Utrilla-Coello et al., 2010), and 3–7% in barley (Ao & Jane, 2007; Tang, Ando, Watanabe, Takeda, & Mitsunaga,

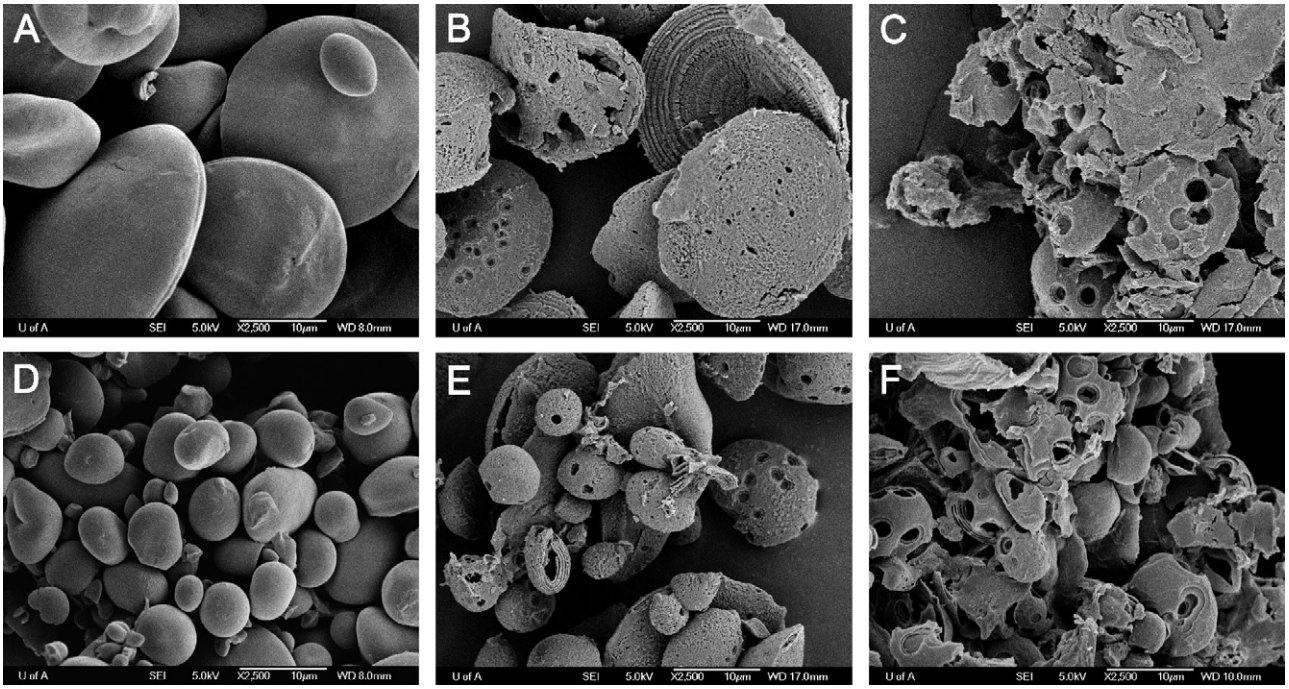

Fig. 2. Scanning electron micrographs of large (A–C) and small (D–F) triticale (Pronghorn) starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

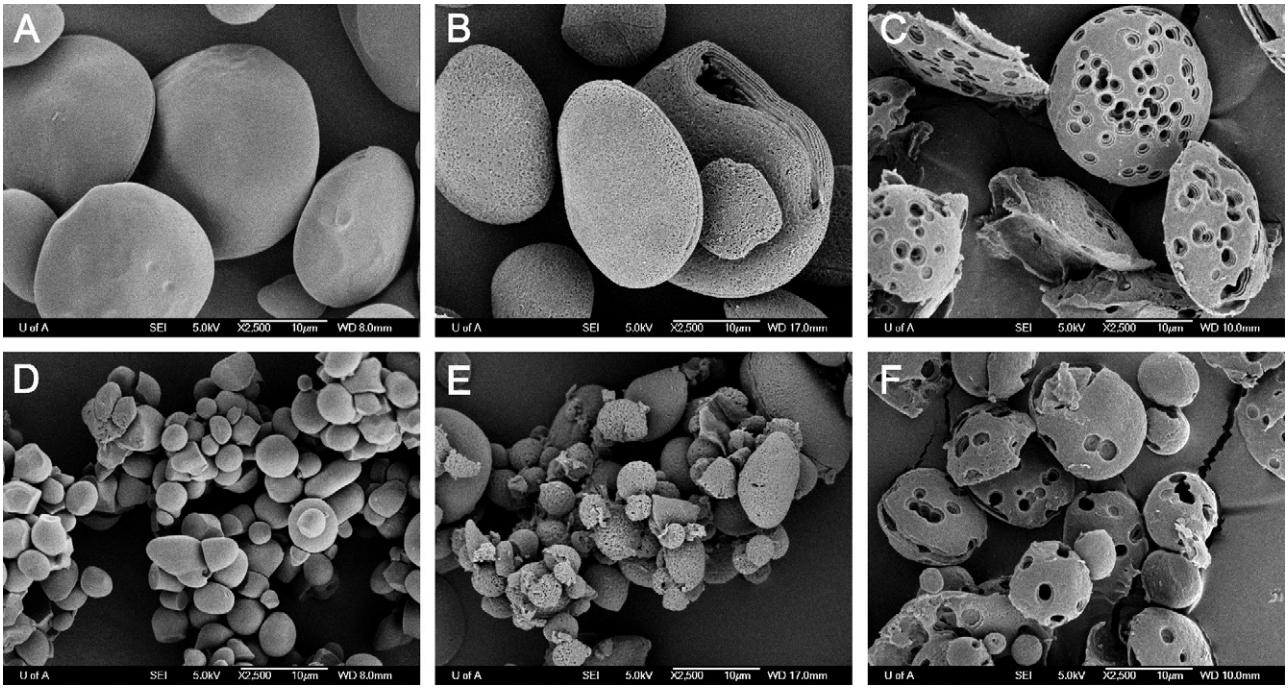

Fig. 3. Scanning electron micrographs of large (A–C) and small (D–F) wheat (CPSR) starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

2001b). Amylose content has been found to be positively correlated with the proportion of small granules and the overall granule size in barley starches (Li et al., 2001) and with the volume percentage of large granules (9.9–18.5 μm in diameter) in wheat starches (Raeker et al., 1998).

Amylopectin in large granules of barley starch showed higher molecular size, average chain length and number of chains than in small granules (Tang et al., 2001a, 2001b). Debranching of amylopectin molecules in wheat, triticale and corn starches indicated a longer average branch chain length, less short branch chains (A and B1 chains with DP 6–24) and more long branch chains (B2 and longer chains with DP ≥ 25) in large granules than in small granules (Ao & Jane, 2007; Liu et al., 2007). Salman et al. (2009) also reported a lower proportion of short chains (DP 6–12) and a higher proportion of long chains (DP 25–36) in large granules than in small granules of normal wheat starches. Thus, large starch granules differ from small granules in their composition and amylopectin structure (e.g. molecular size, branch

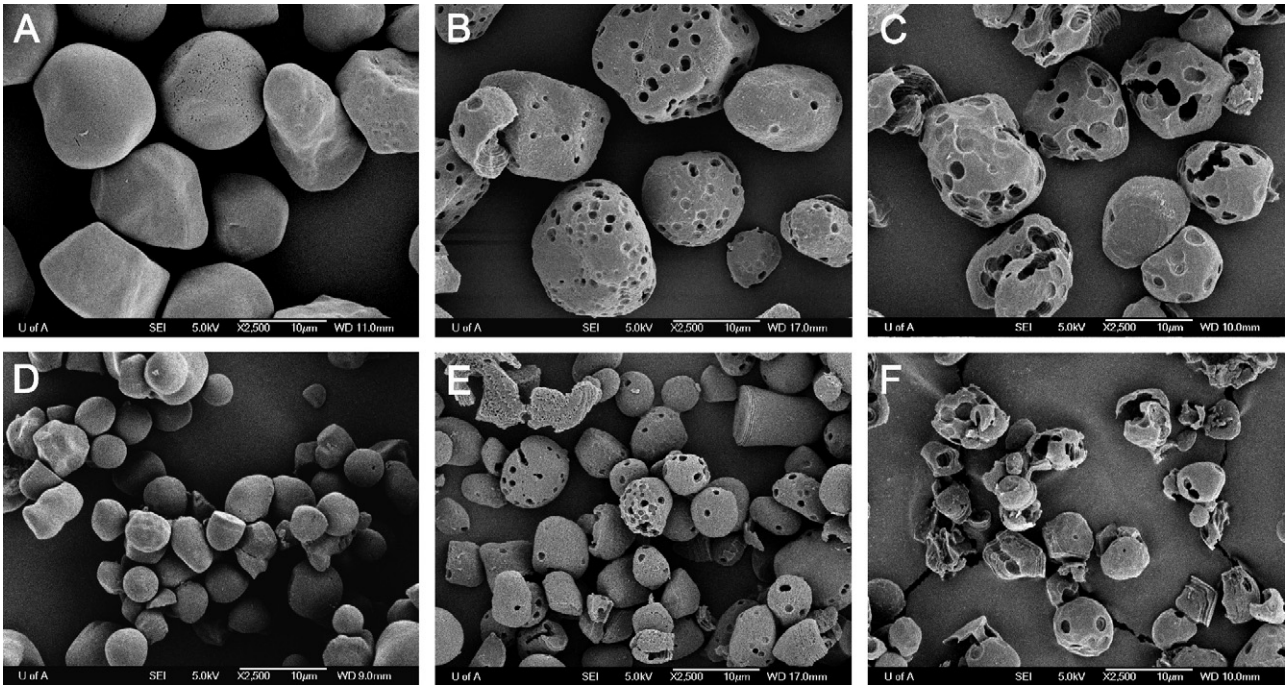

Fig. 4. Scanning electron micrographs of large (A–C) and small (D–F) corn starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

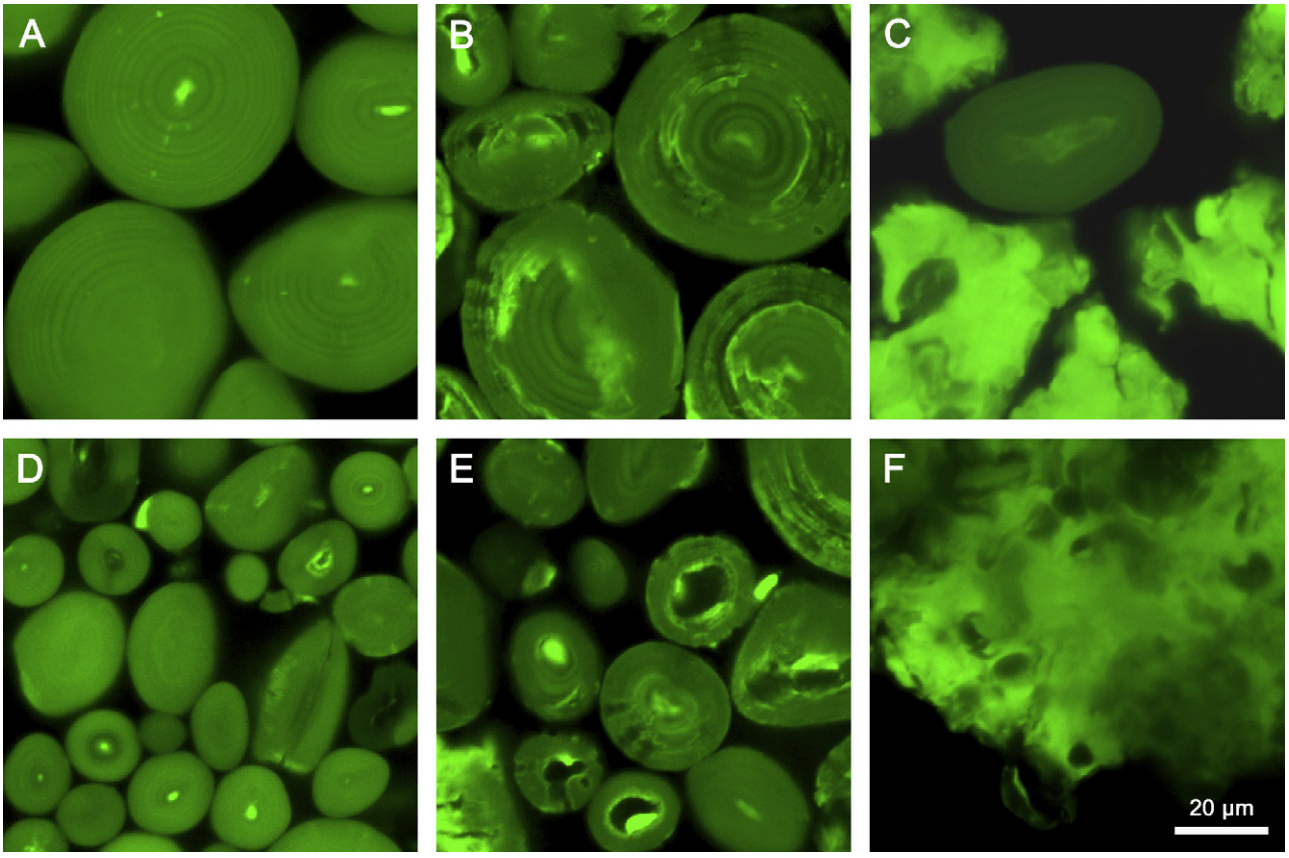

Fig. 5. Confocal laser scanning micrographs of large (A–C) and small (D–F) triticale (Pronghorn) starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

chain length and chain distribution of amylopectin, and crystallinity).

X-ray diffraction revealed that both large and small granules of triticale, wheat and corn starches exhibited the typical A-type polymorph pattern with characteristic peaks at 2θ angles of 15°, 17°, 18°, and 23° (crystallograms are not shown). As shown in Table 4, the relative crystallinities (RC) of large and small granules of triticale, wheat and corn starches were in the range of 24.9–26.8% and 20.8–24.4%, respectively. In the present study, the RC of large starch granules was significantly higher than those of small starch granules in all starches. Vermeylen et al. (2005) also reported higher degree of crystallinity in large starch granules of different wheat varieties. Ao and Jane (2007) proposed that the more long-branch chains (B2 chains) and lesser short-branch chains (A and B1 chains) of amylopectin in large granules form cylindrical shaped amylopectin molecules. The cylindrical shaped amylopectin molecules better align in a parallel arrangement into disk-shaped granules, which are expected to expose greater percentage of RC when compared to cone-shaped amylopectin molecules arranged radially in small spherical granules. Small-angle X-ray scattering (SAXS) and DSC study suggested that small granules of wheat starch consist of denser crystalline lamellae and longer double-helices connected to shorter single-stranded chains of the amylopectin backbone than larger granules (Vermeylen et al., 2005), which may hinder or delay enzyme absorption and diffusion in the later stage of hydrolysis. Compared to small granules, large granules of wheat starches had thicker lamellae and larger repeat distance, which may be related to the higher proportion of medium (DP 13–24) and long amylopectin chains (DP 25–36) (Salman et al., 2009).

Table 3
Apparent amylose content and degree of hydrolysis of unfractionated triticale, wheat and corn starches.

Starch sources	Apparent amylose (%, db)	Degree of hydrolysis (%)			
		1 h[1]	24 h[2]	48 h[2]	72 h[2]
Triticale					
Pronghorn	$23.0^{b} \pm 0.8$	$76.5^{a} \pm 1.5$	$89.3^{ab} \pm 0.8$	$92.2^{a} \pm 0.7$	$92.4^{b} \pm 1.0$
Ultima	$23.2^{b} \pm 0.7$	$69.0^{b} \pm 1.1$	$87.5^{b} \pm 1.6$	$92.2^{a} \pm 1.6$	$92.5^{b} \pm 0.9$
Wheat					
CPSR	$22.5^{b} \pm 1.1$	$53.8^{c} \pm 1.1$	$91.3^{a} \pm 0.5$	$92.8^{a} \pm 0.5$	$96.9^{a} \pm 0.9$
AC Reed	$26.4^{a} \pm 0.7$	$22.1^{d} \pm 1.3$	$88.6^{b} \pm 0.3$	$89.7^{b} \pm 0.9$	$96.2^{a} \pm 0.5$
Normal corn	$24.4^{ab} \pm 0.9$	$3.5^{e} \pm 0.4$	$59.8^{c} \pm 1.0$	$84.9^{c} \pm 0.4$	$90.6^{b} \pm 1.4$

Values are mean ± standard deviation and values with the same superscript in the same column are not significantly different at $\alpha = 0.05$.
[1] Degree of hydrolysis at 55 °C for 1 h.
[2] Degree of hydrolysis at 30 °C for 24, 48, and 72 h, respectively.

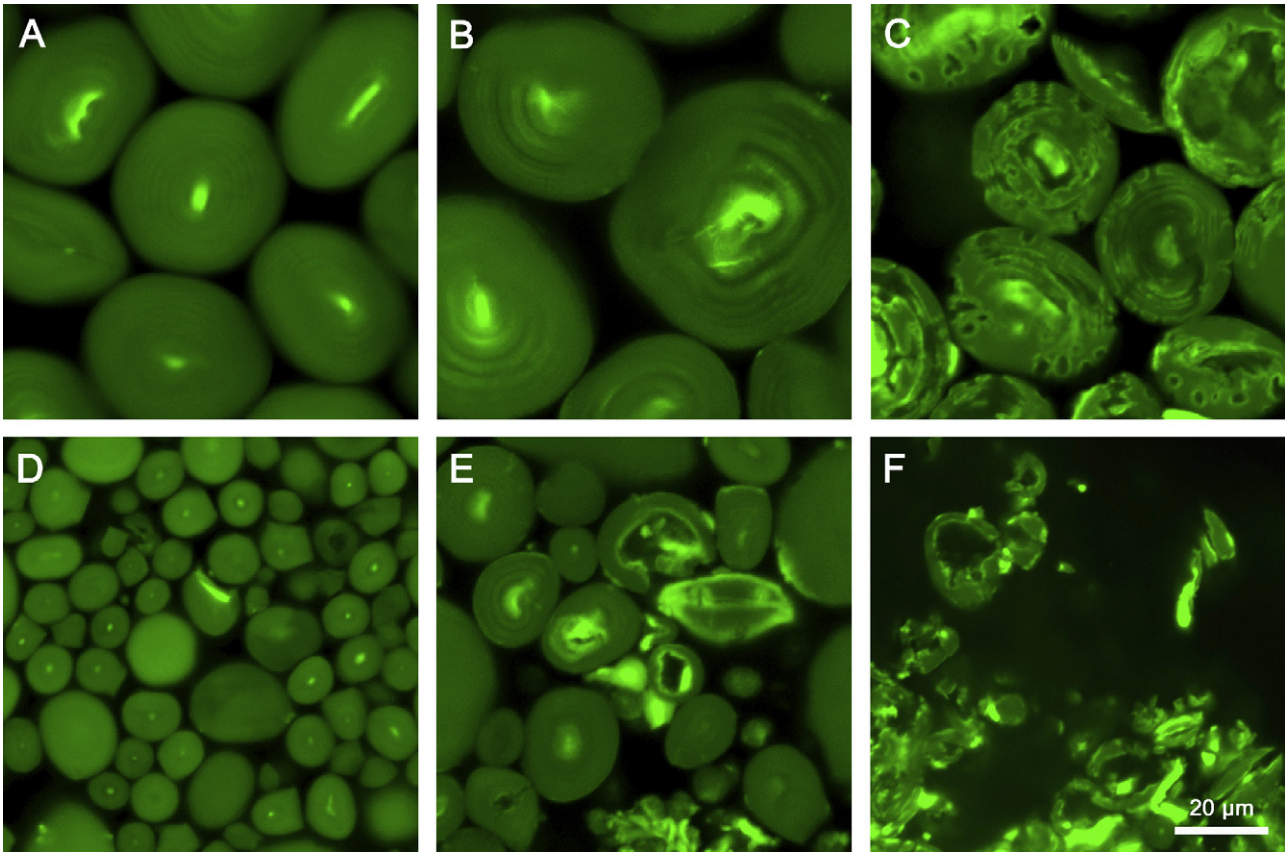

Fig. 6. Confocal laser scanning micrographs of large (A–C) and small (D–F) wheat (CPSR) starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

3.5. Amylolysis of large and small starch granules

The unfractionated and fractionated large and small granules of triticale, wheat and corn starches were hydrolyzed to evaluate their susceptibilities toward a granular starch hydrolysing enzyme at sub-gelatinization temperatures (55 °C for 1 h and then at 30 °C for up to 72 h). The degree of hydrolysis (DH) as a percentage of reducing value is summarized in Table 3 for unfractionated starches and in Table 4 for fractionated large and small starch granules. Unfractionated triticale starches showed rapid hydrolysis (DH 76.5% for Pronghorn and DH 69.0% for Ultima) followed by wheat (DH 53.8% for CPSR and DH 22.1% for AC Reed) and corn (DH 3.5%) starches when hydrolyzed at 55 °C for 1 h (Table 3). However, wheat starches were hydrolyzed to a greater extent (DH 96.2–96.9%) than triticale and corn starches (DH 90.6–92.5%) at 30 °C for 72 h. For each starch, small granules were hydrolyzed significantly faster than

Table 4
Apparent amylose content, relative crystallinity and degree of hydrolysis of large and small granules of triticale, wheat and corn starches.

Starch sources	Apparent amylose (%, db)	Crystallinity (%, db)	Degree of hydrolysis (%, db)			
			1 h[1]	24 h[2]	48 h[2]	72 h[2]
Triticale						
Pronghorn						
Large	$23.0^{c} \pm 1.2$	$26.2^{b} \pm 0.0$	$44.5^{e} \pm 1.1$	$76.6^{d} \pm 0.9$	$80.7^{de} \pm 0.8$	$82.3^{def} \pm 0.8$
Small	$20.6^{de} \pm 0.2$	$24.4^{d} \pm 0.2$	$62.5^{c} \pm 1.2$	$81.7^{bc} \pm 1.2$	$83.2^{bcd} \pm 0.4$	$83.9^{cd} \pm 0.9$
Ultima						
Large	$28.5^{a} \pm 0.7$	$24.9^{c} \pm 0.2$	$64.5^{c} \pm 0.7$	$79.5^{c} \pm 1.0$	$81.4^{cde} \pm 1.0$	$84.5^{cd} \pm 0.4$
Small	$18.5^{e} \pm 0.7$	$20.8^{f} \pm 0.2$	$73.8^{a} \pm 1.1$	$82.4^{ab} \pm 0.7$	$83.4^{bc} \pm 0.8$	$84.3^{cd} \pm 0.6$
Wheat						
CPSR						
Large	$25.8^{b} \pm 0.4$	$25.2^{c} \pm 0.1$	$44.0^{e} \pm 1.1$	$84.4^{a} \pm 0.9$	$85.3^{b} \pm 0.8$	$90.8^{b} \pm 1.4$
Small	$12.4^{f} \pm 1.4$	$23.7^{e} \pm 0.2$	$68.7^{b} \pm 0.8$	$84.9^{a} \pm 0.7$	$85.6^{b} \pm 1.1$	$86.2^{c} \pm 1.2$
AC Reed						
Large	$27.6^{ab} \pm 0.4$	$25.9^{b} \pm 0.2$	$14.3^{g} \pm 1.0$	$83.8^{ab} \pm 0.4$	$91.3^{a} \pm 0.7$	$95.1^{a} \pm 1.4$
Small	$19.6^{de} \pm 0.9$	$24.4^{d} \pm 0.0$	$54.6^{d} \pm 1.5$	$74.0^{d} \pm 1.1$	$77.2^{fg} \pm 1.1$	$79.6^{f} \pm 1.0$
Normal corn						
Large	$26.0^{b} \pm 0.9$	$26.8^{a} \pm 0.1$	$3.2^{h} \pm 0.4$	$51.8^{f} \pm 0.9$	$75.3^{g} \pm 1.1$	$83.4^{cde} \pm 1.3$
Small	$21.0^{cd} \pm 0.3$	$23.5^{e} \pm 0.3$	$20.9^{f} \pm 0.6$	$66.5^{e} \pm 1.1$	$79.2^{ef} \pm 0.8$	$80.8^{ef} \pm 0.1$

Values are mean ± standard deviation and values with the same superscript in the same column are not significantly different at $\alpha = 0.05$.
[1] Degree of hydrolysis at 55 °C for 1 h.
[2] Degree of hydrolysis at 30 °C for 24, 48, and 72 h, respectively.

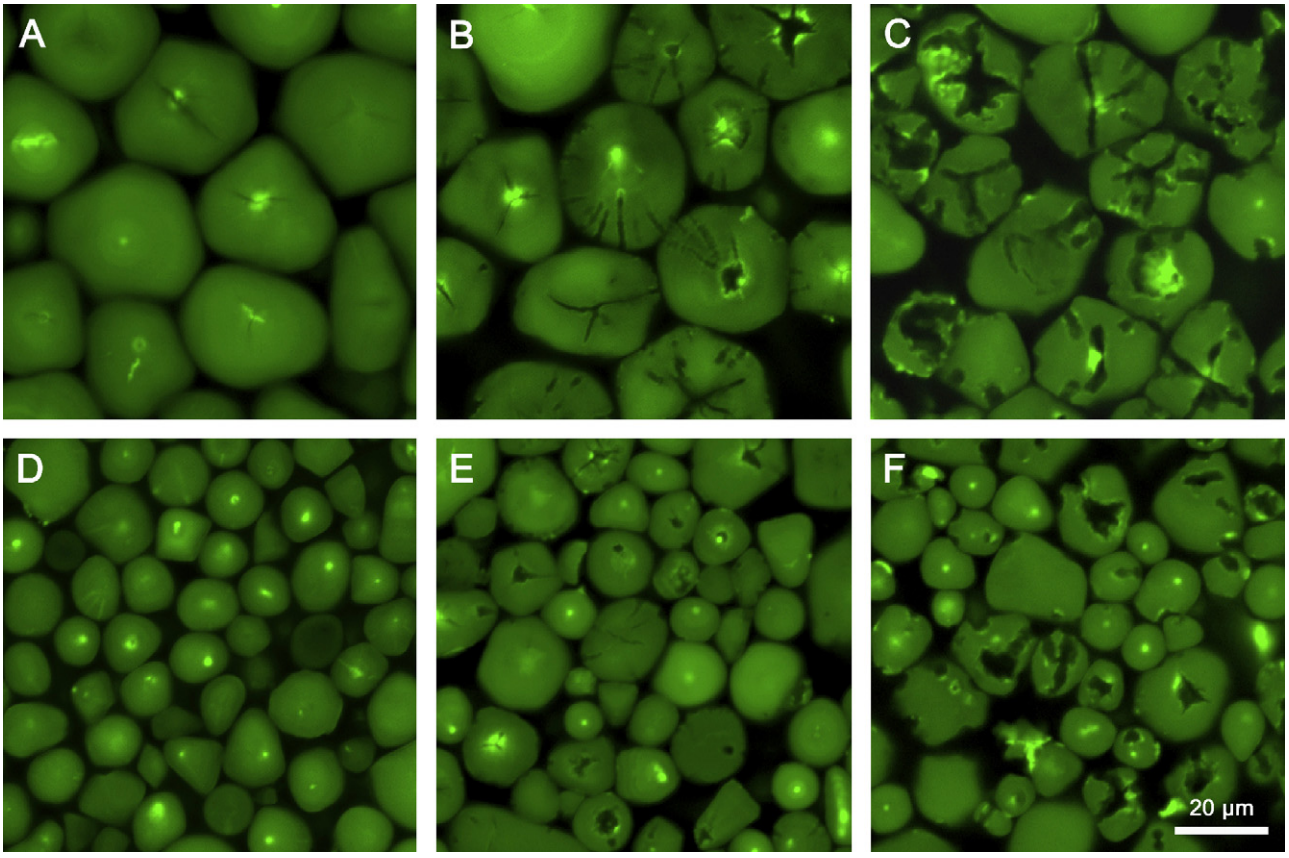

Fig. 7. Confocal laser scanning micrographs of large (A–C) and small (D–F) corn starch granules hydrolyzed by granular starch hydrolyzing enzyme at 55 °C for 0 h (A and D), 1 h (B and E) and at 30 °C for 24 h (C and F).

large granules at 1 h hydrolysis. The DH of small granules were 9.3–18.0%, 24.7–40.3%, 17.7% (percentage unit difference) higher than those of large granules of triticale, wheat, and corn starches, respectively, at 55 °C for 1 h (Table 4). The higher DH of small granules can be attributed to their relatively larger surface area per unit mass. Surface pores and internal channels of granules are assumed to increase effective surface area for fast enzyme diffusion. However, the presence of minor components, such as proteins and lipids on granule surface and in channels largely block the binding sites of enzyme (Naguleswaran et al., 2011), thereby reducing the rate of hydrolysis, especially in larger granules which have numerous pores and channels. With the progress of hydrolysis, the hydrolysis was more rapid in large granules possibly due to the gradual release of protein and lipid from associated glucan molecules, resulting in a narrowed difference of DH between large and small granules after 24 h (Table 4). In later stage, the densely packed crystalline lamellae (Vermeylen et al., 2005) and higher concentration of protein and lipid (Table 1) in small granules may greatly reduce hydrolysis rate. The difference in DH between large and small granules became minimal in triticale after 48 h and in corn after 72 h (Table 4). In wheat, the difference in DH minimized at or less than 24 h (Table 4). However, there was a significant difference in DH between small and large granules of CPSR and AC Reed wheat starches observed at or after 48 h (Table 4). The result for wheat starches is in agreement with Salman et al. (2009). Significant negative correlations at $p < 0.05$ were found between the amylose content of small granules and the DH at 24 h ($r = -0.62$), 48 h ($r = -0.6$), and 72 h ($r = -0.65$) and between the relative crystallinity of large granules and the DH at 1 h and 24 h ($r = -0.83$ and -0.75, respectively, $p < 0.01$). Salman et al. (2009) reported that the DH of wheat starches was positively correlated with the proportion of short amylopectin branch chains (DP 6–12) and negatively correlated with the proportions of medium (DP 13–24) and long chains (DP 25–36) at initial hydrolysis but positively correlated with the proportion of medium chains at longer time of hydrolysis. This study and previous studies (Ao & Jane, 2007; Dhital et al., 2010; Naguleswaran et al., 2011; Salman et al., 2009) suggested that amylolysis of large and small starch granules is closely related to granule morphology, composition and structure at granular micro, and nano levels, such as shape, size, pores, channels, amylose content, associated protein and lipid, degree of crystallinity, lamellae size, and ratio of long and short amylopectin chains. It was noticed that the variation of DH between unfractionated starches (Table 3) and those of fractionated large and small granules (Table 4) differed among starches. This could be attributed to the presence of diverse proportion of large and small granules in each unfractionated starch and some cross contamination of large and small granules in fractionated starches.

3.6. Morphological and microstructural changes of hydrolyzed large and small starch granules

The morphological and microstructural changes of hydrolyzed starch granules (0, 1 and 24 h) revealed by SEM and CLSM are shown in Figs. 2–4 and Figs. 5–7, respectively. One hour hydrolysis resulted in roughly pitted honeycomb-like structures on the surfaces of both large and small triticale (Fig. 2B and E) and wheat starch granules (Fig. 3B and E), even though individual granules were unevenly hydrolyzed. A few enlarged surface pores were observed on the surfaces of large and small triticale starch granules (Fig. 2B and E) and even fewer on the surfaces of large and small wheat starch granules (Fig. 3B and E). Hydrolysis occurred extensively along the equatorial groove of large triticale and wheat

starch granules, resulting in frequent appearance of half pieces of granules at 1 h hydrolysis (Figs. 2B and 3B). Corn starch was also roughened after 1 h hydrolysis showing more perforated erosion pits and enlarged pores on the surfaces of large and small granules and much less granule fragments (Fig. 4B and E). Generally, the visible layered structures were more pronounced on large granule surfaces (Figs. 2B, 3B and 4B) than on small granules (Figs. 2E, 3E and 4E). SEM suggested that enzymatic hydrolysis initiated from granule surfaces by generating pits, size enlargement of existing pores, and penetration into granule interior. The roughened surfaces in hydrolyzed corn starch granules may have been due to uneven shortening of amylopectin molecules by the action of α-amylase (Sujka & Jamroz, 2009). Hydrolysis for 24 h at 30 °C resulted in degradation of most large granules into fragments in triticale (Fig. 2C) and layered residual granules with many enlarged hollows in wheat and corn (Figs. 3C and 4C). However, many smaller granules were degraded into thin layered structures with less number of hollows (Figs. 2F, 3F and 4F).

CLSM showed that the enzyme degradation pattern of triticale and wheat starches were different to that of corn starch (Figs. 5–7). In triticale and wheat starches, enzyme erosion occurred along both channels (radially) and growth rings (perpendicularly), forming a large central cavity in the center of small granules (Figs. 5E and 6E and F), while uneven hydrolysis took place in the outer layers of large granules, resulting in fragments with internal ring structure (Figs. 5B and 6B and C). Hydrolysis was more extensive in triticale starches (Fig. 5C and F) than in wheat starches (Fig. 6C and F). Nearly complete loss of structure of both large and small granules was observed in triticale starch after 24 h hydrolysis (Fig. 5C and F). Compared to triticale and wheat starches, erosion of the granule interior in corn starch occurred mainly along internal channels resulting in hollowed intact granule residues (Fig. 7B, C, E and F). With respect to corn starch, enlarged channels irregularly crossed in different size and depth in large granules, while a relatively large central cavity with less number of channels was formed in small granules after 24 h hydrolysis (Fig. 7C and F). The disappearance of internal structure of large and small granules in corn starch revealed by CLSM was consistent with the DH as shown in Table 4 (3.2% vs. 20.9% for 1 h and 51.8% vs. 66.5% for 24 h hydrolysis).

Hydrolysis patterns revealed by SEM and CLSM were caused by synergistic degradation of α-amylase and glucoamylase. Each enzyme erodes starch granules in a manner of exocorrosion and endocorrosion but at different hydrolysis rate and extent between starches (Kimura & Robyt, 1995; Li, Vasanthan, Hoover, & Rossnagel, 2004; Li, Gao, Wang, Jiang, & Huang, 2011; Quigley, Kelly, Doyle, & Fogarty, 1998). As reported previously (Li et al., 2004, 2011), α-amylase preferentially hydrolyzes the amorphous regions of the granule leaving granule residues with a sharp saw-toothed layer structure and a large cavity in the granule center, whereas glucoamylase hydrolyze amorphous and crystalline regions of the granule simultaneously by formation of shallow and circular pits with much smooth edges of internal layered structure due to confined hydrolysis along tangential direction of the granule. Separated and fragmented layer structure revealed by CLSM in hydrolyzed starch granules indicated that α-amylase played a predominant role in hydrolyzing amorphous regions during amylolysis. This is also supported by X-ray diffraction and amylose content analysis (Blazek & Gilbert, 2010; Chen, Huang, Tang, Chen, & Zhang, 2011; Shariffa, Karim, Fazilah, & Zaidul, 2009; Uthumporn, Zaidul, & Karim, 2010), in which an increases of crystallinity intensity and a decrease of amylose content in hydrolyzed starches by both enzymes were found. Thus the accessibility of α-amylase and amyloglucosidase toward large and small starch granules differ with starch origin, contributing to different hydrolysis kinetics (rate and extent) and hydrolysis patterns.

4. Conclusions

Large and small granules of triticale, wheat and corn starches differed with granule morphology (granule size, size distribution, number and size of surface pores and internal channels), amylose content, presence of protein and lipid, relative crystallinity, amylolysis pattern, and hydrolysis rate and extent, indicating that the nature of starch granules (composition and macro-, micro-, nano-structures) controls starch amylolysis. Triticale starch was comparable to wheat and corn starches in terms of granular starch hydrolysis for use in ethanol production. The current study suggests that the genetic manipulation of grains with different ratio of large and small starch granules could be used for precise control of liquefaction and saccharification in simultaneous hydrolysis and fermentation process. Further research is ongoing to investigate the molecular characteristics (e.g. amylose molecular size and amylopectin branch chain length distribution) of small and large starch granules and their relationship with starch hydrolysis in order to fine-tune the use of triticale in bioethanol production.

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