Carbohydrate Polymers 82 (2010) 14–20

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Extraction and dissolution of starch from rice and sorghum grains for accurate structural analysis

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ARTICLE INFO

Article history:
Received 28 January 2010
Received in revised form 3 April 2010
Accepted 7 April 2010
Available online 14 April 2010

Keywords:
Starch
Dissolution
Starch structure
Size exclusion chromatography
Molecular weight distribution

ABSTRACT

A complete dissolution without degradation and that removes non-starch components is important for accurate characterisation of starch molecular structure. Current milling and dissolution methods have limitations including incomplete dissolution and molecular degradation. An improved multi-step extraction/dissolution method was devised and tested, involving cryo-grinding, protease pre-treatment, dissolution in dimethyl sulfoxide solution containing 0.5% (w/w) LiBr (DMSO/LiBr) at 80 °C, centrifugation, ethanol precipitation, and, finally, re-dissolution in DMSO/LiBr at 80 °C. Cryo-grinding and dissolution in DMSO/LiBr were found to have negligible effects on the size distribution of rice starch molecules measured by size-exclusion chromatography (SEC). The peaks of non-starch components were removed or separated from the amylose and amylopectin peaks in the size distributions of rice and sorghum starches. The amylopectin component had a larger hydrodynamic radius than that obtained by conventional wet-milling and also that obtained without protease pre-treatment, suggesting that molecular degradation and aggregation were reduced with the new method. This new extraction/dissolution method allows a more accurate structural analysis of starch molecules from grains than conventional treatments.

1. Introduction

Starch is one of the most abundant polysaccharides in the nature. It is mainly comprised of two types of glucans: amylopectin and amylose. Amylopectin is the larger molecule with a high number of short branches and a molecular weight of ~10^8 Da, whereas amylose is the smaller molecule with a molecular weight of ~10^6 Da and a few long branches (Hizukuri & Takagi, 1984; Hoang et al., 2008; Yoo & Jane, 2002); the distributions of molecular weight and size are both broad. Starches from different botanical sources have different structures, including size and molecular weight distributions and ratio of amylose to amylopectin, which affect their thermal, pasting, and other properties (Castro, Dumas, Chiou, Fitzgerald, & Gilbert, 2005; Jane et al., 1999). These properties determine the suitable end uses of the starches for food, feed, or industrial applications, such as in the paper and textile industries. Thus, it is important to obtain the accurate structure of starch in order to understand structure–property relations and to discover new potential end uses of the starch materials.

Common techniques to analyse starch structure, including size exclusion chromatography (Hasjim & Jane, 2009; Yoo & Jane, 2002), anion exchange chromatography (Jane et al., 1999), field-flow fractionation (Hanselmann, Burchard, Ehrat, & Widmer, 1996; Roger, Baud, & Colonna, 2001), capillary electrophoresis (Hasjim, Srichuwong, Scott, & Jane, 2009; O'Shea, Samuel, Konik, & Morell, 1998), and hydrodynamic chromatography (Dias, Fernandes, Mota, Teixeira, & Yelshin, 2008; Klavons, Dintzis, & Millard, 1997), require a complete dissolution of starch molecules (Dona et al., 2007; Gidley et al., 2010) without degradation of the starch molecules in the parent sample and with removal of the non-starch components (proteins, lipids, and non-starch polysaccharides) that may interfere with the analysis. Starch aggregates, results of an incomplete dissolution, are usually filtered off and excluded from the analysis (Jackson, 1991; You & Lim, 2000), while entangled molecules may overestimate the molecular weight of starch; the dissolution process should be such as to avoid these problems.

Starch granules are relatively insoluble in water at room temperature. Heating (in water bath, autoclave, and microwave), high pressure treatment, physical shearing, and alkaline pH have all been used to improve starch solubility in water; however, they can also cause molecular degradation and, thus, underestimate the molecular weight of starch (Bello-Pérez, Roger, Baud, & Colonna, 1998; Han & Lim, 2004; Jackson, 1991; Kim, Huber, & Higley, 2006; You & Lim, 2000). In addition, the dissolved starch molecules in water, especially amylose, can reassociate with itself or with other starch molecules, an effect known as retrogradation (You & Lim, 2000).

The organic solvent most commonly used to dissolve starch is dimethyl sulfoxide (DMSO). DMSO disrupts the intra- and

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doi:10.1016/j.carbpol.2010.04.014

inter-molecular non-covalent interactions in starch molecules by forming hydrogen bonding between its negatively charged oxygen atom and the hydroxyl groups on starch molecules as well as by forming hydrophobic interactions with starch through its methyl group (Erlander & Tobin, 1968). Starch dissolution in DMSO is usually carried out in the presence of a small amount of water or lithium salts (LiBr or LiCl). The presence of water in DMSO is to prevent granular swelling during dissolution (Jackson, 1991), but it also weakens the dispersion power of DMSO and slows down the dissolution kinetics (Dona et al., 2007; Erlander & Tobin, 1968). Furthermore, a DMSO-water system still requires heating in boiling water and hours of mechanical stirring for a complete dissolution, which can result in molecular degradation (Han & Lim, 2004; Jackson, 1991). DMSO-LiBr solution has been reported to be a better solvent for starch dissolution than DMSO-water solution (Zhong, Yokoyama, Wang, & Shoemaker, 2006). The lithium ions can interact with the hydroxyl groups of starch molecules and facilitate starch dissolution in DMSO at a milder temperature (Cave, Seabrook, Gidley, & Gilbert, 2009; Jane, 1993; Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009).

It is also important that the starch sample for structural study is not polluted by the presence of other biological components, such as proteins, lipids, and non-starch polysaccharides, at least in a way that significantly interferes with the target analysis method. Milling or grinding is required to disrupt protein and cell-wall matrices to release starch granules (Gidley et al., 2010). An extensive grinding process, however, can result in starch damage, which may affect the starch structure (Hasjim et al., 2009; Kumar, Malleshi, & Bhattacharya, 2008). Wet milling can isolate starch granules from cereal grains with minimal damage (Hasjim et al., 2009; Xie & Seib, 2002), but significant starch loss has been reported, which is found in the protein and bran fractions (Singh, Johnson, Pollak, Fox, & Bailey, 1997). These could alter the molecular weight and size distribution of starch. Hence, a grinding or milling process with minimal damage and loss is essential for starch structural analysis.

The objective of this study is to develop a starch extraction/dissolution method that does not produce an artificial result of starch structure. The improved extraction/dissolution method given here, which is derived in part from combining some features in various literature methods, involves the following (with details and sources, where appropriate, given in the later sections):

1. mild cryo-grinding to break up the protein and cell-wall matrices and release starch granules
2. protease pre-treatment to hydrolyse proteins
3. dissolution in dimethyl sulfoxide (DMSO) solution containing LiBr to dissolve starch granules
4. centrifugation to remove insoluble components (mostly non-starch polysaccharides)
5. starch precipitation using ethanol to separate starch from other soluble non-starch components (mostly proteins and lipids)
6. starch re-dissolution in the DMSO solution for injection into the desired structural analytical instrument (e.g. size-exclusion chromatography)

The effects of cryo-grinding and dissolution on starch structure are also analysed and discussed.

Two cereal grain samples, rice and sorghum, were used to provide variations in the chemical compositions (starch and protein contents) and structural arrangements in testing the new extraction/dissolution technique. In each case, the starting point was dehulled grains. The degree of starch dissolution, the amylose content of the dissolved starch, and the protein content of the treated samples were analysed to check the effectiveness of each step of the new extraction/dissolution method. Any structural changes that occurred during the extraction and dissolution were characterised using size exclusion chromatography (SEC, also known as gel-permeation chromatography, GPC).

2. Materials and methods

2.1. Materials

Rice and sorghum grains were used in this study to represent high- and low-starch grains (84% vs. 67%), respectively, that contain different types and arrangements of proteins. In rice, the protein content is small and does not play a major structural role, whereas in sorghum, the protein content is significantly higher, and the protein forms a protective layer around the starch. The medium-grain rice was obtained from a local grocery store; white sorghum was supplied by Professor Ian D. Godwin (The University of Queensland). Protease from *Streptomyces griseus* (type XIV) and LiBr (ReagentPlus) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMSO (GR for analysis ACS) was purchased from Merck & Co, Inc. (Whitehouse Station, NJ, USA). Total Starch (AA/AMG) assay kit was purchased from Megazyme International Ltd. (Co. Wicklow, Ireland). Other chemicals were reagent grade and used as received.

2.2. Cryo-grinding

Rice and sorghum grains (25 g) were ground using a cryogrinder (Freezer/Mill 6850 SPEX, Metuchen, NJ, USA) and liquid nitrogen (−210 to −196 °C) as the cryogenic freezing medium. Prior to grinding, the grain samples were allowed to freeze in liquid nitrogen for 5 min. Three grinding times were used for comparison in this study: 2 cycles of 5 min (CG5C2), 1 cycle of 10 min (CG10C1), and 2 cycles of 10 min (CG10C2). The grinding speed was set at 10 s^{-1}. In between cycles, the grain samples were allowed to re-freeze in the liquid nitrogen for 2 min to ensure the temperature of the samples did not rise significantly. The cryo-ground (CG) sample was sieved through a 250-μm screen.

2.3. Protease pre-treatment

The sample was next pre-treated with protease to facilitate the protein removal, following the method of Chiou, Martin, and Fitzgerald (2002) with modification. The CG sample was incubated with protease (0.9 U/mL) in tricine buffer (20 mg/mL, pH 7.5, 250 mM) at 37 °C for 15 min. The digestate was centrifuged at 4000 × *g* for 10 min, and the protease-treated cryo-ground sample (CG-PT) was collected as precipitate.

2.4. Dissolution and removal of non-starch components

The CG and CG-PT samples were next dissolved in a DMSO solution containing LiBr (0.5%, w/w) (DMSO/LiBr) at a concentration of 2 mg/mL, following the method of Cave et al. (2009). The dissolution of sample (2 mg) for structural analysis using SEC was carried out in a shaking thermomixer (Thermomixer Comfort, Hamburg, Germany) running at 80 °C and 350 rpm for 24 h. The samples were shaken by hand every hour in the first 6 h to ensure the homogeneity of the mixture. The dissolution of a larger sample (20 mg) for analyses of starch dissolution, amylose, and protein contents was carried out in an oil or water bath at 80 °C with mechanical stirring at 350 rpm.

Further treatments were used to remove non-starch components (proteins, lipids, and non-starch polysaccharides) from the CG and CG-PT samples. The sample dissolved in the DMSO/LiBr solution was centrifuged at 4000 × *g* for 10 min. The supernatant was mixed with 3 volumes of absolute ethanol to precipitate starch, and the precipitate was collected through centrifugation at 4000 × *g* for 10 min. The isolated starches

were thus ethanol-precipitated cryo-ground (CG-EP) and ethanol-precipitated protease-treated cryo-ground (CG-PT-EP) samples; these were inverted on a paper towel to remove excess ethanol and immediately re-dissolved in the DMSO/LiBr solution for 2 h. For protein analysis, the CG-PT-EP and CG-EP samples were re-dissolved in 1.8 mL distilled water in a boiling water bath for 40 min instead of DMSO/LiBr solution, then frozen using liquid nitrogen, and dried using a freeze-drier.

2.5. Wet milling

Starch granules were isolated from rice and sorghum grains through wet milling following the method of Hasjim et al. (2009) with modifications for comparison with the new extraction/dissolution method. Rice grains (50 g) were steeped in a sodium metabisulfite solution (150 mL, 0.45%, w/v) overnight in a refrigerator. The rice grains in sodium metabisulfite solution were milled using a commercial food blender for 5 min. The blended slurry was mixed with 450 mL NaCl solution (0.1 M) and 50 mL of toluene. The mixture was stirred for 1 h and then allowed to stand until the starch granules settled at the bottom. The protein in the toluene and the NaCl solution layers was siphoned off and discarded, and this was repeated until all of the protein was removed, as indicated by a clear toluene layer after the starch granules settled at the bottom. The starch layer was then washed with water several times and subsequently with absolute ethanol. The wet-milled starch (WM) was recovered by filtration through Whatman #1 filter paper (Whatman International Ltd., Kent, UK), rinsed with ethanol several times, and air-dried. The same wet-milling procedure was also used for sorghum grains, with an additional step of filtration through a 75-μm screen after milling using the food blender for 3 min instead of 5 min; the fraction left on the screen, mostly germs and pericarps, was blended in the sodium metabisulfite solution and filtered again until no starch (white particle) was seen.

2.6. Size exclusion chromatography

The size distributions of starch from the CG, CG-PT, CG-EP, CG-PT-EP, and WM samples were analysed using SEC, following the method of Cave et al. (2009). The DMSO/LiBr solution was used as mobile phase after being filtered through a 0.45-μm hydrophilic Teflon membrane filter (Millipore, Billerica, MA, USA). The separation was carried out using an Agilent 1100 Series SEC (Agilent Technologies, Waldbronn, Germany) consisting of an isocratic pump, a series of separation columns (GRAM precolumn, GRAM 30, and 3000 analytical columns, Polymer Standard Services, Mainz, Germany), and a refractive index detector (PN3140, PostNova Analytics, Landsberg, Germany). The separation columns were held at 80 °C, the flow rate was set at 0.3 mL/min, and the detector was set at 45 °C. A series of pullulan standards (Polymer Standard Services, Mainz, Germany) with varying molecular weights ranging from 342 to 1,660,000 Da were used for calibration. The pullulan standards (1 mg) were dissolved in the DMSO/LiBr solution (1 mL) in the thermomixer at 80 °C and 350-rpm agitation for 3 h. The Mark–Houwink parameters for this eluent at 80 °C are $K = 2.424 \times 10^{-4}$ dL g^{-1} and $\alpha = 0.68$ (Cave et al., 2009).

It is useful to recall that SEC separates molecules by size, not molecular weight. While there is a unique relation between molecular weight and molecular size for linear polymers, that is not the case for a complex branched polymer such as starch. For SEC, this size parameter is the hydrodynamic volume (V_h). For convenience, the data here are presented in terms of the corresponding hydrodynamic radius (R_h) with $V_h = 4/3\pi {R_h}^3$.

The resulting SEC chromatograms were analysed using PSS WinGPC Unity software (Polymer Standard Services, Mainz, Germany) and normalised to yield the same peak height of amylopectin. The size distribution [$w(\log V_h)$] was calculated from the detector signal and was plotted against $\log R_h$ following the method of Cave et al. (2009). Presenting the SEC distribution as a function of Rh (or Vh), which is a molecular quantity independent of machine set-up, enables these data to be reproduced, whereas presenting such data as elugrams in terms of elution time or volume cannot, because the elution varies with the particular machine set-up, and even from day-to-day with a given set-up. The SEC distributions used for comparison here were run on the same day to avoid day-to-day variations of the SEC instrument, which can affect the apparent distributions outside the calibration range (Cave et al., 2009); i.e. all the distributions in Fig. 1 were run on the same day, so were those in Figs. 2–4. Although it is unavoidable that the amylopectin component will suffer from some shear in SEC (Cave et al., 2009), as long as the comparison is made with samples run on the same day, this shear will be the same for every sample, and so the resulting distributions can be meaningfully compared; thus, for example, if one sample shows more larger molecules than another, then this is a true reflection of the actual size distributions of the polymers.

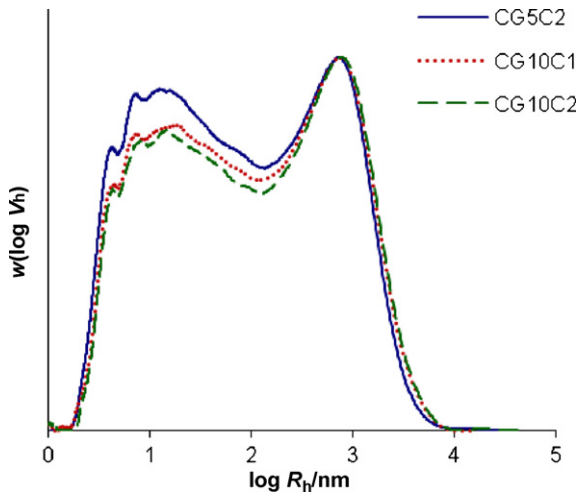

Fig. 1. SEC distributions of CG rice samples using different grinding times: 2 cycles of 5 min (CG5C2), 1 cycle of 10 min (CG10C1), and 2 cycles of 10 min (CG10C2). The CG samples were dissolved in DMSO/LiBr solution at 80 °C for 8 h and the distributions were normalised to the peak height of amylopectin.

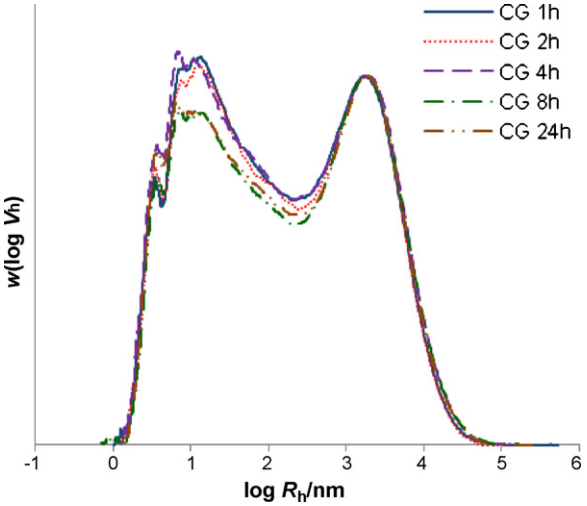

Fig. 2. SEC distributions of CG rice samples (CG5C2) after dissolution in DMSO/LiBr solution for different times. The distributions were normalised to the peak height of amylopectin.

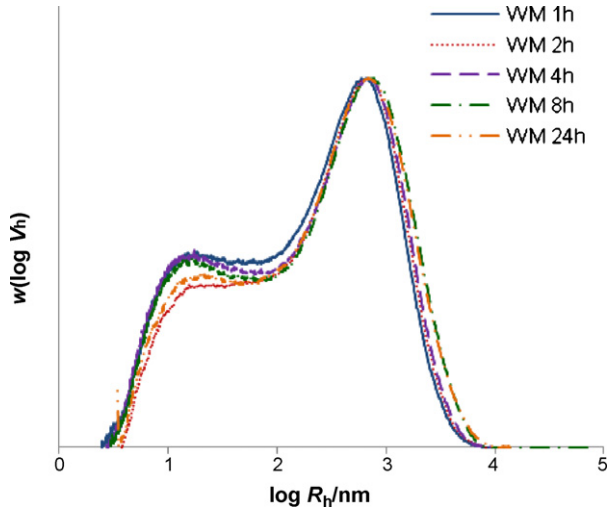

Fig. 3. SEC distributions of WM rice starches after dissolution in DMSO/LiBr solution for different times. The distributions were normalised to the peak height of amylopectin.

2.7. Starch content and degree of starch dissolution

The starch content and the degree of starch dissolution were analysed in duplicate using the Megazyme Total Starch (AA/AMG) assay kit. The CG, CG-PT, CG-EP, and CG-PT-EP samples dissolved in the DMSO/LiBr solution (2 mg/mL) were centrifuged at 4000 × *g* for 10 min. The supernatant (2 mL) of each sample was digested and the glucose released was determined following the procedure given by the manufacturer.

$$\%\,\text{starch dissolution} = \frac{\text{starch weight in DMSO/LiBr}}{\text{initial dry weight of starch}} \times 100$$

2.8. Amylose content

The amylose content of the dissolved starch in the DMSO/LiBr solution was analysed in duplicate using the method of Hoover and Ratnayake (2001) with some modification as follows. Iodine stock solution was prepared by mixing iodine (1 g) and potassium iodide (10 g) in acetate buffer (1 L, 0.1 M, pH 5.0). The iodine stock solution was diluted ten times before use. The dissolved CG, CG-PT, CG-EP, and CG-PT-EP samples in the DMSO/LiBr solution (2 mg/mL) were centrifuged at 4000 × *g* for 10 min. The supernatant (1 mL) was diluted with 111.5 mL distilled water and then mixed with 12.5 mL diluted iodine solution. The absorbance of resulting blue colour was measured at 640 nm.

$$\%\,\text{amylose} = \frac{\text{amylose weight in DMSO/LiBr}}{\text{starch weight in DMSO/LiBr}} \times 100$$

2.9. Protein content

The nitrogen contents of the CG, CG-PT, CG-EP, and CG-PT-EP samples were analysed in duplicate using a LECO CNS 2000 autoanalyser (LECO Corporation, St. Joseph, MI, USA) following the method of Jung et al. (2003). The protein content was calculated from the nitrogen content using a conversion factor of 6.25.

2.10. Statistical analysis

The mean values of the degree of starch dissolution, amylose content of the dissolved starch, and protein content in the CG sample after protease pre-treatment and/or starch dissolution in the DMSO/LiBr solution followed by precipitation using ethanol were analysed using analysis of variance (ANOVA) with the general linear model procedure (PROC GLM) in SAS (version 9.2, SAS Inst. Inc., Cary, NC, USA).

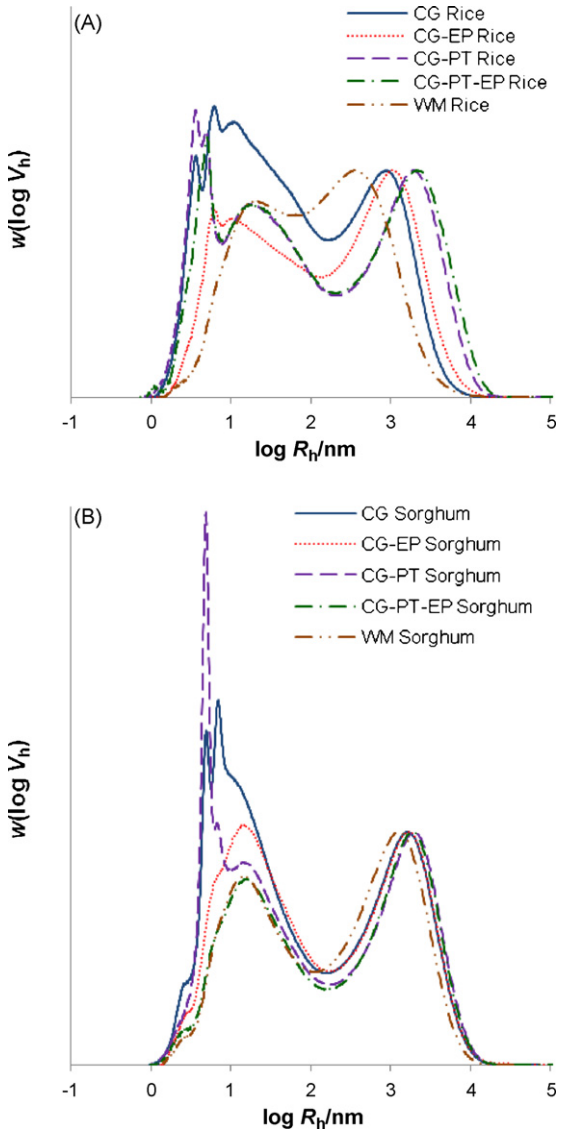

Fig. 4. SEC distributions of cryo-ground (A) rice and (B) sorghum samples after protease pre-treatment and starch dissolution in DMSO/LiBr solution followed by precipitation using ethanol. CG = direct dissolution of cryo-ground samples in DMSO/LiBr solution for 24 h, CG-PT = protease pre-treatment of cryo-ground samples followed by dissolution in DMSO/LiBr solution for 24 h, CG-EP = CG followed by starch precipitation using ethanol and re-dissolution in DMSO/LiBr solution for 2 h, CG-PT-EP = CG-PT followed by starch precipitation using ethanol and re-dissolution in DMSO/LiBr solution for 2 h, and WM = direct dissolution of wet-milled starch in DMSO/LiBr solution for 24 h. The distributions were normalised to the peak height of amylopectin.

3. Results and discussion

3.1. Effects of cryo-grinding process

Starch granules in grains or seeds are usually entrapped in protein and cell-wall matrices (Stenvert & Kingswood, 1977; Wursch, Del Vedovo, & Koellreutter, 1986). To achieve a complete dissolution, it is important to disrupt these matrices to release starch. Common processing methods to achieve this include wet milling and dry grinding (Chen, Lu, & Lii, 1999; Hasjim et al., 2009; Xie &

Seib, 2002). Both methods, however, are not appropriate for accurate structural analysis of starch molecules. Wet milling causes significant starch loss (Singh et al., 1997), which could change the average size distribution of starch molecules. Furthermore, lab-scale wet milling involves hours of soaking in water that could activate endogenous enzymes in the grains and cause molecular degradation. Dry grinding, on the other hand, commonly results in starch damage (Hasjim et al., 2009; Kumar et al., 2008), which could change the starch structure.

In this study, we opted to use cryo-grinding to break the grain structure. Dry grinding at cryogenic temperatures prevents the mechanical and heat damages on starch granules. The brittleness of cryogenically frozen sample also facilitates the dry-grinding process (Paul & Chattopadhyay, 1996), thereby minimizing tribological (frictional) degradation. Fig. 1 shows the SEC distributions of the CG rice samples, which were cryo-ground for 2 cycles of 5 min (CG5C2), 1 cycle of 10 min (CG10C1), and 2 cycles of 10 min (CG10C2), and then dissolved in the DMSO/LiBr solution for 8 h. The samples were re-frozen in between cycles to ensure the temperature and the brittleness of the samples during grinding. The results showed that the difference among the size distributions of these samples was minor (within the variation of repeated SEC distributions from a same sample as reported by Hoang et al. (2008)) even after additional 10-min cryo-grinding (CG10C2 vs. CG5C2) or without additional 2-min freezing during 10-min cryo-grinding (CG10C1 vs. CG5C2). This suggests that the effect of the cryo-grinding conditions used in this study on the starch structure is negligible. Based on these results, only the CG5C2 samples were used to represent the CG samples for further analyses.

3.2. Effects of dissolution time

Heating and agitation are important to ensure a complete dissolution of starch in DMSO solution (Han & Lim, 2004; Hoang et al., 2008). An incomplete dissolution, such as to leave aggregates or entangled molecules, can generate artificial results preventing an accurate characterisation of starch structure. Heating and agitation, however, can degrade starch molecules; this leads to an underestimation of the molecular weight of starch (Bello-Pérez et al., 1998; Han & Lim, 2004; Jackson, 1991; Kim et al., 2006; You & Lim, 2000).

The SEC distributions of the CG and WM rice samples directly dissolved in the DMSO/LiBr solution using a thermomixer at 80 °C with agitation for 1, 2, 4, 8, and 24 h are almost superimposable (Figs. 2 and 3, respectively), which indicates that there was no significant degradation during dissolution in the DMSO/LiBr solution. The same result was reported by Zhong et al. (2006) even though the starch dissolution was carried out in a DMSO solution with 50 mM LiBr and at a higher temperature (90–120 °C). The dissolution temperature at 80 °C was chosen because it is lower than those used in other studies, such as heating in boiling water, autoclaving, and microwave heating (Bello-Pérez et al., 1998; Han & Lim, 2004; Kim et al., 2006; You & Lim, 2000), and thus any thermal degradation of starch molecules during dissolution was reduced. Furthermore, shaking was used instead of mechanical stirring to minimise molecular degradation caused by shearing (Han & Lim, 2004). The degree of starch dissolution of the samples dissolved in the DMSO/LiBr solution did not change significantly after 2 months (data not shown), indicating that retrogradation of starch was minimised in the DMSO/LiBr solution.

3.3. Effects of protease pre-treatment

The CG and WM rice samples, when dissolved in the DMSO/LiBr solution, showed different SEC size distribution patterns (Figs. 2 and 3, respectively). Size distributions of starch generally show two peaks, which are associated with amylose (smaller molecule, $\log R_h$/nm between 0.2 and 2.5, $R_h \sim 1.5$–300 nm) and amylopectin (larger molecule, $\log R_h$/nm between 2.5 and 4.5, $R_h \sim 300$–30000 nm) (Cave et al., 2009). The CG rice sample showed a larger peak area with additional peaks in the R_h range of amylose than did the WM rice starch. This discrepancy was assigned to the presence of non-starch components in the CG samples, such as proteins, lipids, and non-starch polysaccharides, which had molecular size in the range of amylose or smaller. The same was also observed with the CG sorghum sample (Fig. 4B), which agreed with the supposition about the presence of non-starch components.

Additional steps were then devised for the dissolution of the CG samples to remove the non-starch components from starch and to give a more accurate size distribution of starch molecules. The protease pre-treatment hydrolyses proteins and increases the protein solubility in the DMSO/LiBr solution, thus facilitating the protein removal. Dissolution in the DMSO/LiBr solution dissolves starch molecules and should open up the compact structure of some grain components such as protein and cell-wall matrices. The centrifugation step then removes insoluble components, especially non-starch polysaccharides, and thereafter the starch precipitation using ethanol should separate starch molecules from other DMSO-soluble components, such as protein fragments and lipids.

Table 1 shows that the degree of starch dissolution in the CG rice sample increased after protease pre-treatment because the protein matrix was weakened and, thus, made starch granules easier to dissolve. The sorghum sample, however, had high degree of starch dissolution (~100%) even without protease pre-treatment, which could be due to the different types of proteins present in the sorghum and rice grains, for example, rice protein was less soluble in polar solvent (Paraman, Hettiarachchy, Schaefer, & Beck, 2007; Shih & Daigle, 1997), and/or their interactions with starch. In general, the degrees of starch dissolution of the CG-EP and CG-PT-EP samples were lower than those of the CG and CG-PT samples, suggesting that some starch might be lost during precipitation using ethanol and/or dissolution in the DMSO/LiBr solution. The amylose contents of the dissolved starches from the CG, CG-PT, CG-EP, and CG-PT-EP samples, however, were not significantly different. This indicates that the starch loss during extraction and dissolution was not selective, and did not change the ratio of amylose to amylopectin.

The protein content did not change significantly after protease pre-treatment (CG vs. CG-PT) of sorghum, and apparently slightly increased in the rice sample (Table 1). The protease pre-treatment introduced some additional protein (in the form of protease) to the CG samples. However, protein content was decreased significantly, in general, after dissolution in the DMSO/LiBr solution and starch precipitation using ethanol with or without protease pre-treatment. The results suggest that the protein hydrolysate in the CG-PT samples might still be trapped in the compact grain structure, and was difficult to remove. The dissolution in DMSO/LiBr solution destroyed the compact structure and thereby facilitated protein removal.

Similar to Figs. 1 and 2, the SEC distributions of the CG samples showed a larger peak area of amylose than those of their respective WM samples (Fig. 4). The peak area of amylose of the CG samples was also larger than those of their respective CG-EP, CG-PT, and CG-PT-EP samples even though the amylose content of these samples were not significantly different for each grain sample (Table 1). Beside amylose and amylopectin, the CG samples also showed additional peaks at $\log R_h$/nm ~ 0 to 1. The additional peaks became smaller or reduced in R_h after protease pre-treatment, which is ascribed to the hydrolysis of proteins in the CG samples. The disappearance of some or all the additional peaks in the CG rice and sorghum samples after starch precipitation using ethanol, with

Table 1
Degree of starch dissolution, amylose content of dissolved starch, and protein content of cryo-ground samples after protease pre-treatment and starch dissolution in DMSO/LiBr solution followed by precipitation using ethanol.[a].

Treatments[b]	Degree of starch dissolution (%)[c]	Amylose content of dissolved starch (%)[d]	Protein content (%)[e]
Rice			
CG	92.8 ± 1.0 b	12.1 ± 0.4 a	6.1 ± 1.6 ab
CG-EP	84.6 ± 0.7 c	13.6 ± 1.3 a	3.0 ± 0.2 b
CG-PT	101.0 ± 2.2 a	10.3 ± 0.1 a	9.8 ± 0.5 a
CG-PT-EP	85.9 ± 0.6 c	13.0 ± 0.9 a	4.4 ± 2.3 ab
Sorghum			
CG	108.8 ± 0.2 a	15.5 ± 0.9 a	11.2 ± 0.0 a
CG-EP	100.2 ± 2.2 a	14.7 ± 1.2 a	4.1 ± 0.7 b
CG-PT	102.8 ± 4.8 a	16.6 ± 0.8 a	12.3 ± 0.0 a
CG-PT-EP	94.6 ± 1.6 b	16.0 ± 0.9 a	2.7 ± 0.0 c

[a] Mean ± standard deviation. Different letters in the same column represent significant difference at $p > 0.05$ for each grain sample (rice or sorghum).
[b] CG = direct dissolution of cryo-ground samples in DMSO/LiBr for 24 h, CG-PT = protease pre-treatment of cryo-ground samples followed by dissolution in DMSO/LiBr for 24 h, CG-EP = CG followed by starch precipitation using ethanol and re-dissolution in DMSO/LiBr for 2 h, and CG-PT-EP = CG-PT followed by starch precipitation using ethanol and re-dissolution in DMSO/LiBr for 2 h.
[c] The percentage was on the basis of dry starch weight in the CG before treatment(s).
[d] The percentage was on the basis of dry starch in the DMSO/LiBr solution.
[e] The percentage was on the basis of dry sample weight of after treatment(s).

or without protease pre-treatment, verifies the removal of non-starch components. The amylose peak of the CG-PT-EP sorghum sample was superimposable with that of the WM sorghum starch regardless of starch loss during extraction and dissolution (Table 1). Although the proteins were not completely removed in the CG-PT and CG-PT-EP rice samples, the amylose peaks had the same R_h as that of the WM rice starch. This implies that the accurate size distribution of the amylose component can be achieved using this new extraction/dissolution method. The different effects of the new extraction/dissolution methods on protein removal of the rice and sorghum CG samples could be attributed to the different solubilities of rice and sorghum proteins and/or their structural interaction with starch.

In general, the amylopectins of the WM starches had smaller R_h values than those of the corresponding CG samples (Fig. 4), which suggests that molecular degradation and/or starch loss might take place during the wet-milling process (Cave et al., 2009; Singh et al., 1997). On the other hand, the amylopectins had larger R_h values when the CG samples were subjected to the protease pre-treatment. This difference could not be the effect of molecular entanglement, because the protease pre-treatment was carried out on undissolved CG samples, where starch was still in granular form and molecular mobility was restricted. Furthermore, the dissolution in DMSO/LiBr solution did not seem to cause molecular entanglement, since the same results were not observed with the CG and CG-EP samples, which were subjected to the same dissolution method. The possible explanations are that (1) the large, unhydrolysed proteins might associate with starch molecules and thus lead to greater shear scission in the SEC separation column, or (2) the large, unhydrolysed proteins might exclude the large amylopectin molecules from the pores in the SEC separation column, thus excluding them from the analysis.

The new extraction/dissolution method proves to provide the solution to the problems of starch loss, incomplete dissolution, and/or molecular degradation that are common with the previous methods to extract and dissolve starch for structural analysis. It also allows the study of starch structure in grains without the need of isolating starch granules prior to the analysis, which is usually the prerequisite in the previous starch structural studies. The starch structural changes during extraction and dissolution are substantially minimised, and more accurate structural information of starch molecules from grains can be obtained without artifactual result or interference from the non-starch components. The optimal procedure may vary with different grains, and the methodology given here can be used to optimise a similar procedure for any starch source.

4. Conclusion

A new multi-step extraction/dissolution method has been developed for starch structural analysis. This method involves mild cryo-grinding, protease pre-treatment, dissolution in DMSO/LiBr solution, centrifugation, starch precipitation using ethanol, and, finally, re-dissolution in DMSO/LiBr solution. Cryo-grinding is used to disrupt protein and cell-wall matrices to facilitate starch dissolution in the DMSO/LiBr solution. The effect of cryo-grinding on the starch structure was found to be negligible when the grain samples were ground for a range of times and conditions. Furthermore, the dissolution of starch in DMSO/LiBr solution using a thermomixer at 80 °C and 350-rpm agitation did not change the size distribution of starch up to 24 h dissolution. The new extraction/dissolution method removed or separated the peaks of non-starch components from the amylose and amylopectin peaks in the SEC distributions, which allowed the analysis of starch structure without pollution from the non-starch components in the grains. The new method also gives a more accurate amylopectin size than the wet-milling method. This new extraction/dissolution method, although significantly more complex than other techniques, allows a more accurate analysis of starch structure from grain samples, and minimises artifacts from loss and degradation of starch during the extraction and dissolution steps.

Acknowledgements

The authors thank the Australian Research Council (DP0985694 and DP0986043) for funding, Professor Robert G. Gilbert (The University of Queensland) for constructive comments, and Professor Ian Godwin (The University of Queensland) for supplying the sorghum samples.

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