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Exploring extraction/dissolution procedures for analysis of starch chain-length distributions

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ABSTRACT

The analysis of starch chain-length distributions (CLDs) is important for understanding starch biosythesis–structure–property relations. It is obtained by analyzing the number distribution of the linear glucan chains released by enzymatic debranching of starch α-(1→6) glycosidic bonds for subsequent characterization by techniques such as fluorophore-assisted carbohydrate electrophoresis (FACE) or size-exclusion chromatography (SEC). Current literature pretreatments for debranching prior to CLD determination involve varying protocols, which might yield artifactual results. This paper examines the two widely used starch dissolution treatments with dimethyl sulfoxide (DMSO) containing 0.5% (w/w) lithium bromide (DMSO-LiBr) at 80 °C and with aqueous alkaline (i.e. NaOH) solvents at 100 °C. Analyses by FACE with a very high range of degree of polymerization, and by SEC, of the CLD of barley starches with different structures show the following. (1) The NaOH treatment, even at a dilute concentration, causes significant degradation at higher degrees of polymerization, leading to quantitatively incorrect CLD results in longer amylopectin and in amylose chains. (2) Certain features in both amylopectin and amylose fractions of the CLD reduced to bumps or are missing with NaOH treatment. (3) Overestimation of amylose chains in starch CLD due to incomplete amylopectin dissolution with dilute NaOH concentration. These results indicate starch dissolution with DMSO-LiBr is the method of choice for minimizing artifacts. An improved pretreatment protocol is presented for starch CLD analysis by FACE and SEC.

1. Introduction

Starch is produced in plants by various types of biosynthetic enzymes (Ball & Morell, 2003). The result of this is a complex hierarchical structure, with up to six identifiable levels (Gilbert, 2011). Starch chain-length distribution (CLD), the first structural level, is the most analyzed structural feature. It is the number distribution of linear α-(1→4) linked glucan chains, released from digestion of starch with an isoamylase-type debranching enzyme, as a function of degree of polymerization (DP).

Quantitative analysis of starch CLD is desirable for a number of structure–property relations for starch-containing substances. For example, the gelatinization temperature, an important property for food preparation and digestion, correlates with differences in the proportion of glucan chains of DP 6–12 and DP 12–24 (Cuevas et al., 2010; Nakamura et al., 2002). Starch CLD is also used to estimate amylose content (e.g., Fitzgerald et al., 2009), a major determinant in many properties, such as those important for industrial applications such as viscosity modifiers, in digestibility and food manufacture. There is a correlation between starch digestion rate and features in the amylose CLD (Syahariza, Sar, Hasjim, Tizzotti, & Gilbert, 2013). CLDs can be used for elucidating the roles of starch biosynthetic and degradation enzymes (e.g., Delvalle et al., 2005; Regina et al., 2010). The conventional method for comparison between CLDs is the difference plot: subtracting one CLD from another. However, starch CLD is best presented as the logarithm of the number distribution as a function of DP, which brings out features at high DP and avoids artifacts from normalization (Castro, Dumas, Chiou, Fitzgerald, & Gilbert, 2005). The amylopectin fraction of CLDs, typically with DP of up to 100, can

Abbreviations: APTS, 8-aminopyrene-1,3,6,trisulfonate; CLD, chain-length distribution; DMSO, dimethyl sulfoxide; DP, degree of polymerization; FACE, fluorophore-assisted carbohydrate electrophoresis; M_p, peak molecular weight; SEC, size-exclusion chromatography.

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be parameterized by a mathematical model of starch biosynthesis which is useful for the understanding the underlying mechanisms for the rational design of starches desired for numerous applications (Morell & Myers, 2005; Wu & Gilbert, 2010; Wu, Morell, & Gilbert, 2013; Wu, Ral, Morell, & Gilbert, 2014). Such applications all require quantitative starch CLD analysis with minimal artifacts.

There are several techniques for starch CLD analysis (for a review, see for example Wu, Witt, & Gilbert, 2013): fluorophore-assisted carbohydrate electrophoresis (FACE), high-performance anionic-exchange chromatography (HPAEC), and size-exclusion chromatography (SEC). FACE is the method of choice for amylopectin chains. FACE data are superior to data from SEC for amylopectin chains, without the problems of band-broadening, calibration, and inaccuracies in the Mark-Houwink relation. However, it is limited to shorter chains. Longer chains (DP > 100), including extra long amylopectin and amylose chains, are currently best analyzed with SEC.

The results from CLD analysis inevitably depend on the pretreatment procedure for obtaining the chains from starch or from starch-containing samples (flour, leaf, etc.). These pretreatments typically involve starch isolation (purification, including dissolution of purified starch, if starting with flour or leaf samples), and enzymatic digestion using isoamylase-type debranching enzyme.

There is a wide variation in the pretreatment procedures currently in use. Just to give a few examples, no purification for rice flour for CLD analysis (e.g., Lisle, Martin, & Fitzgerald, 2000) vs. using sodium dodecyl sulfate (SDS) to remove proteins from rice flour washed with water (Wong et al., 2003). Starch purification from barley flour has been carried out by removal of major groups of lipids and proteins in aqueous, saline, and alcoholic solvents (Schulman & Kammiovirta, 1991). A different pretreatment design has been to include an extra treatment with protease to digest proteins (Song & Jane, 2000). Wheat starch has been separated from flour in the form of dough by repeated washing with water (Regina et al., 2004). Maize starch has been purified from milled kernels by washing with excess ethanol and removed proteins by mixing the starch-containing ethanol solution with 0.1 M aqueous NaCl solution containing 10% toluene (Li, Blanco, & Jane, 2007).

Some procedures might cause artifacts from varying types of loss and/or degradation. For example, purification of starch from rice flour with protease and detergent causes a loss of higher molecular weight chains, whereas purification with protease and ethanol has been shown to be essentially artifact-free and to give enhanced instrument signal (Chiou, Martin, & Fitzgerald, 2002). There is a significant molecular degradation in corn starch when dissolved in aqueous NaOH, even just by vortexing the starch-NaOH mixture (Han & Lim, 2004a).

Complete dissolution of the purified starch is required for releasing α-(1→4) linked chains by debranching (enzymatically hydrolyzing the α-(1→6) branch points) starch. The crystalline region of the starch granule can inhibit the process. The purified starch is relatively insoluble in water at room temperature. There are two commonly used treatments for starch dissolution: the so-called alkali treatment (e.g. dissolving starch in aqueous NaOH solution) (Batey & Curtin, 1996; O'Shea & Morell, 1996; Wong et al., 2003) and DMSO treatment (i.e. dissolving starch in DMSO-based solutions) (Batey & Curtin, 1996).

There is a trade-off between starch dissolution and degradation. Maize starch can be effectively dissolved (up to 94.9%) in 1 M aqueous NaOH solution with 10 min vigorous vortexing at room temperature, however, this causes significant molecular degradation; vortexing for 2 min causes less molecular degradation; however, only 80.3% is dissolved (Han & Lim, 2004a). These authors also found that amylose is preferentially dissolved. It is well known that α-(1→4) and α-(1→6) glycosidic bonds can be hydrolyzed in the present of NaOH. The concentration of NaOH typically used in the alkali treatment is 50–250 mM, together with heating at 100 °C or higher for 5 min (Batey & Curtin, 1996; Lisle et al., 2000; O'Shea & Morell, 1996; Wong et al., 2003). It is probable that such a high temperature could speed up the degradation of the glycosidic bonds and leads to artifacts in the starch CLD. On the other hand, DMSO appears to dissolve starch without significant degradation (Han & Lim, 2004a,b). Syahariza, Li, and Hasjim (2010) devised a multi-step starch extraction procedure based on a DMSO-containing solvent for purification and dissolution of starch, or starch-containing samples, at a milder 80 °C. Detailed tests indicated removal of non-starch components, starch dissolution of up to 100%, and minimized degradation were achieved for both rice and sorghum. The purified and dissolved starch is then ready for debranching with isoamylase-type debranching enzyme (the type of enzyme most widely used for CLD characterization) for the release of linear chains (Batey & Curtin, 1996; Fontaine et al., 1993; Lisle et al., 2000; O'Shea & Morell, 1996; Streb, Eicke, & Zeeman, 2012; Syahariza et al., 2013; Wong et al., 2003).

This study aims to develop a pretreatment protocol that is suitable for quantitative starch CLD characterization with FACE and SEC. We employ a modified version of the protocol devised by Syahariza et al. (2010) for starch purification. This was applied to a range of different barley samples, ranging from low to high amylose contents; barley was chosen because its CLD has been found (Chu, Hasjim, Hickey, Fox, & Gilbert, 2014) to have more distinct amylose fine-structure features than seen with some other grains, and which therefore should be very sensitive to changes in features caused by the extraction/preparation process. The purified starch is then subjected to the two widely used dissolution treatments: (1) DMSO containing 0.5% (w/w) lithium bromide (DMSO-LiBr) at 80 °C; or (2) aqueous NaOH at 100 °C dissolution treatments, and then debranched. The released chains are analyzed with both FACE and SEC. Our results confirm that the DMSO-LiBr dissolution treatment minimizes chain degradation and preserves features in the CLD which would otherwise be lost with aqueous NaOH dissolution treatment. Based on these results, we present a pretreatment protocol for obtaining glucan chains from starch or starch containing samples for quantitative characterization by FACE and SEC.

2. Methods and materials

2.1. Reagents

Wholemeal barley (*Hordeum vulgare* L.), flour of Waxiro (granule-bound starch synthase (GBSS) mutant), Golden Promise (normal barley), and ssIIa (starch synthase IIa mutant, *sex6*) (described in Morell et al., 2003) were kindly provided by CSIRO Plant Industry (Canberra, Australia). They contain starch with low, normal, high amylose contents, respectively. Protease from *Streptomyces griseus* (type XIV) and sodium cyanoborohydride were from Sigma–Aldrich (Castle Hill, NSW, Australia), and isoamylase from *Pseudomonas* sp. (E-ISAMY) were from Megazyme International Ltd. (County Wicklow, Ireland). 8-aminopyrene-1,3,6,trisulfonate (APTS), included in the Carbohydrate Labeling and Analysis Kit, was purchased from Beckman Coulter (Brea, CA, USA). Pullulan standards with peak molecular weights (M_p) ranging from 342 to 708,000 (PSS-pulkit), pullulan standards with M_p 1,300,000 (PSS-dpul1300k), and pullulan standards with M_p 2,560,000 (PSS-dpul2.5 m) were purchased from Polymer Standard Service (Mainz, Germany).

Dimethyl sulfoxide-lithium bromide (DMSO-LiBr) treatment. Barley starch was extracted from ~7 mg of the wholemeal barley flour and purified and solubilized according to a published method (Syahariza et al., 2010) with modifications. In case where the acquired samples were whole grains or leaf materials, grinding is needed. This can be achieved, with minimal structural degradation, with a cryogrinder and liquid nitrogen as the cryogenic freezing medium (Syahariza et al., 2010). The wholemeal flour was gently mixed by inverting with 0.5 mL protease (2.5 units/mL) in tricine buffer (pH 7.5, 250 mM) and incubated at 37 °C for 30 min in a 2-mL Eppendorf tube. The mixture was centrifuged at 4000 × *g* for 10 min, discarding the supernatant. The precipitate was then gently mixed with 0.5 mL sodium bisulfite solution (0.45%, w/w) and incubated at 37 °C for 30 min. The mixture was centrifuged at 4000 × *g* for 10 min, discarding the supernatant. To solubilize the starch in the precipitate, it was suspended in 1.5 mL DMSO containing 0.5% (w/w) LiBr (DMSO-LiBr) in a shaking thermomixer running at 80 °C and 350 rpm for 20 h (note: immediately vortex after adding DMSO-LiBr to avoid formation of lumps of starch gel). The suspension was occasionally inverted by hand to ensure no clumps of the precipitate adhere to the tube wall in the first 2 h. The solubilized starch was precipitated by washing with 10 mL absolute ethanol and centrifuged at 4000 × *g* for 10 min to remove non-starch components (proteins, lipids, and non-starch polysaccharides). This was repeated to further remove residual non-starch components and solvent.

The precipitate from the above was immediately dispersed in 0.9 mL hot water and heated in a boiling water bath until all precipitate was dispersed (roughly 10 min). Occasional mixing by inverting is required. This starch dispersion was cooled to room temperature before 0.1 mL acetate buffer (pH 3.5, 0.1 M) and 2.5 μL isoamylase were added. This mixture was briefly vortexed and incubated at 37 °C for 3 h. The 3 h is because at ~4.5 pH (our debranching condition), the optimal stable time is 4 h according to the specifications from Megazyme. The unit equivalent of isoamylase used is similar to that reported previously where a 2 h incubation was used (Batey & Curtin, 1996). The resulting debranched starch dispersion was adjusted to pH ≈7 with 0.1 mL NaOH (0.1 M). The dispersion was heated at 80 °C for 1 h. The dispersion was frozen in liquid nitrogen and freeze-dried overnight. Freeze-dried samples must be stored in desiccators if not used immediately.

Alkali treatment. As described above except that to solubilize the starch in the starch containing precipitate, 1.5 mL NaOH solution (0.05 M) was used to make the suspension. The suspension was heated at 100 °C for 5 min. Analogous tests with 0.25 and 0.75 M NaOH solution were also carried out.

2.2. APTS labeling of linear glucans

The freeze-dried linear glucans (0.2 mg) from both of the treatments above were derivatized with APTS in 2-mL Eppendorf tubes by the addition of 1.5 μL of a solution of APTS (0.2 M) in 15% glacial acetic acid (APTS powder was dissolved in 15% glacial acetic acid to obtain an APTS concentration of 0.2 M) and 1.5 μL of 1 M aqueous sodium cyanoborohydride. Our tests showed this gave consistent results compared to previously published conditions as described in (O'Shea, Samuel, Konik, & Morell, 1998) (data not shown). The mixture was vortexed and incubated at 40 °C for 20 h or 60 °C for 90 min in the dark for labeling of the linear glucans, as suggested by the Carbohydrate Labeling and Analysis Kit application guide. Our tests confirmed that both gave consistent results (data not shown). The solution of labeled glucans was diluted by adding 80 μL water and vortexing until all precipitate dissolved. The mixture was centrifuged at 4000 × *g* for 2 min and 50 μL of the supernatant was transferred to 200 μL microcentrifuge tubes for immediate fluorophore-assisted carbohydrate electrophoresis.

2.3. Fluorophore-assisted carbohydrate electrophoresis (FACE) of APTS-labeled linear glucans

The size distribution of the labeled linear glucans (prepared as described above) was analyzed with FACE to give the CLD, denoted $N_{de}(X)$ (the subscript "de" is used to indicate that the linear glucan were obtained by debranching starch; X = DP). Separation of the labeled linear glucans was performed on a PA-800 Plus System and monitored with a solid-state laser-induced fluorescence (LIF) detector with an argon-ion laser as the excitation source (Beckman-Coulter, Brea, CA, USA). The capillary used was a 50-μm diameter N–CHO coated capillary (included in the Carbohydrate Labeling and Analysis Kit). Carbohydrate separation buffer, also included in the kit, was used as the separating medium. The effective separation length of the capillary was 40 cm. The sample was introduced into the capillary by pressure injection for 3 s at 0.5 psi (3.4 kPa above atmospheric). Separation of the labeled linear glucans was achieved using an applied voltage of 30 kV (current ~14 mA) at 25 °C. 90 min of total separation time was used to separate the first ~160 peaks (Fig. S1). The areas of the peaks give the relative amount of glucans with different mass directly (the DPs of glucans in adjacent peaks differ by 1). Sample storage temperature in the PA-800 Plus System was at 18 °C. We noticed that if labeled samples were not analyzed immediately after derivatization, the relative amount of long chains decreased over time. This could be due to preferential retrogradation of long glucan chains. Retrograded chains probably migrate more slowly in the separation buffer. Diluted labeled glucans (as described in the previous section) may require further dilution if the raw electropherogram appears to be cut off as the detector response reaches a maximum, which can lead to an underestimate of the overloaded signals.

2.4. Size-exclusion chromatography of linear glucans

The size distribution of the freeze-dried linear glucan from both of the treatments above were analyzed using an Agilent 1100 Series SEC system (Agilent Technologies, Waldbronn, Germany) equipped with GRAM precolumn, GRAM 30 Å, GRAM 100 Å and GRAM 1000 Å analytical columns (Polymer Standards Service (PSS), Mainz, Germany) set at 80 °C and a differential refractive index (DRI) detector (Optilab UT-rEX, WYATT, Santa Barbara, CA, USA). (Although not implemented here, a very recent paper has shown that even better columns can improve this technique (Ciric, Woortman, & Loos, 2014).) DMSO containing 0.5% (w/w) LiBr was used as the eluent, and the flow rate was set at 0.5 mL/min. The injected samples were prepared at concentration of 4 mg/mL in the same eluent. The pullulan standards were used for calibration, following the method of Cave, Seabrook, Gidley, and Gilbert (2009), to obtain the relation between SEC elution volume and the hydrodynamic volume (V_h) of the linear glucans. The calculation uses Mark-Houwink parameters $K = 0.0002427$ dL g–1 and $\alpha = 0.6804$ for the pullulan standards (Cave et al., 2009). The size distribution results were analyzed with ASTRA software (Wyatt) using a dn/dc value of 0.0576 mL/g, as measured by PSS for debranched amylopectin in the eluent used at 80 °C. Signals from the DRI detector gave the SEC weight distribution of the linear glucans as a function of log V_h, $w_{de}(\log V_h)$. It was converted to a function of log X (i.e. $w_{de}(\log X)$) as described in ref. (Li et al., 2013), using Mark-Houwink parameters $K = 0.015$ dL g–1 and $\alpha = 0.74$ for debranched starch. $N_{de}(X)$ and $w_{de}(\log X)$ are related by $w_{de}(\log X) = X^2 N_{de}(X)$, as explained in detail elsewhere, e.g. (Gaborieau, Gilbert, Gray-Weale, Hernandez, & Castignolles, 2007).

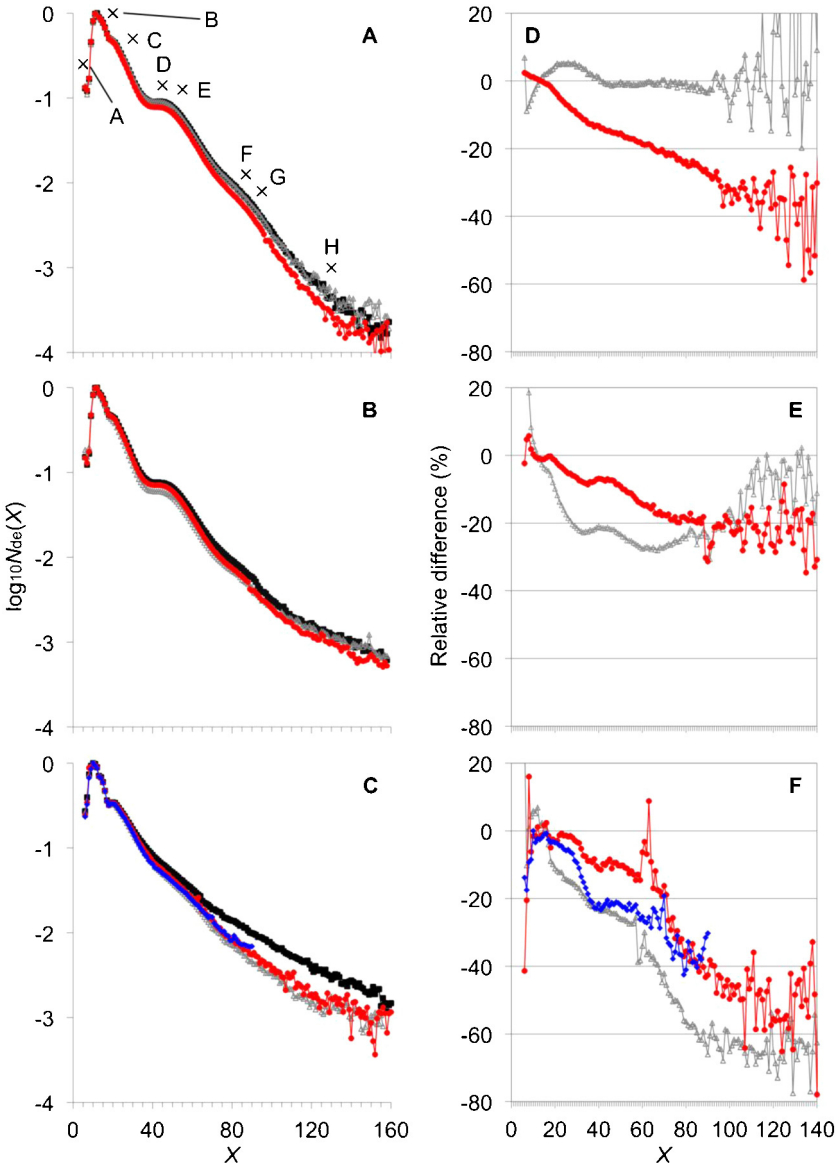

Fig. 1. Number CLD (arbitrary units) of low, normal and high amylose barley starch in the amylopectin range obtained with different dissolution solvents and analyzed with FACE. (A) Number CLD of waxy barley obtained by using DMSO/LiBr for starch dissolution ($N_{de.D}(X)$; black squares); the CLD obtained by using 0.05 M NaOH for starch dissolution ($N_{de.0.05N}(X)$; gray triangles); and that with 0.25 M NaOH ($N_{de.0.25N}(X)$; filled red circles). Crosses and letters mark the features in starch CLD. (D) Relative difference of $N_{de.0.05N}(X)$ and $N_{de.0.25N}(X)$ with respect to $N_{de.D}(X)$ of waxy starch. (B and E) are analogous to (A and D) for starch CLD of normal barley. (C and F) are analogous to (A and D) for starch CLD of HA barley. The filled blue diamonds show $N_{de.0.75N}(X)$. Replicate $N_{de}(X)$ gave very similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results and discussion

Linear glucans from the three types of barley starches (i.e. low, normal and high amylose contents) were analyzed by both FACE (with a typical electropherogram given in Supplementary information to show the baseline resolution), and SEC with a DRI detector. $N_{de}(X)$ are presented in Fig. 1 and $w_{de}(\log X)$ in Fig. 2. $N_{de}(X)$ obtained by the DMSO-LiBr dissolution treatment are designated $N_{de.D}(X)$; $N_{de.0.05N}(X)$ for the alkali dissolution treatment with 0.05 M NaOH; and $N_{de.0.25N}(X)$ for that with 0.25 M NaOH. Analogous notations are used for $w_{de}(\log X)$.

The FACE data obtained with the set-up used here goes to significantly higher DP than hitherto reported in the literature, in fact into what is usually thought of as the amylose region (DP > 100). Having such a high range is especially useful in the present context, as FACE data are free from band-broadening affects (Fig. S1 shows that individual peaks are well separated up to DP of ~160 for normal barley starch, which is also observed for waxy and high-amylose barley starches) which are unavoidable in SEC but which mask fine structural features which are distinguishing indicators of both biological processes and functional properties.

The data shown in both Figs. 1 and 2 reveals features in the starch CLD consistent with those previously published. (1) The features seen in $N_{de}(X)$ is consistent with that observed in other types of cereal starches (Wu & Gilbert, 2010; Wu, Morell, et al., 2013). (2) The features seen in $w_{de}(\log X)$ (i.e. AP1 and AP2 for the amylopectin fraction and AM1 and AM2 for the amylose fraction) is consistent with that observed in other types of starches with both DMSO and NaOH treatments (Butardo et al., 2011; Chu et al., 2014; Syahariza et al., 2013; Wang, Hasjim, Wu, Henry, & Gilbert, 2014; Ward, Gao, de Bruyn, Gilbert, & Fitzgerald, 2006). These suggest that the four peaks seen here are real.

The amylose fraction of the $N_{de}(X)$ and $w_{de}(\log X)$ agrees well with the previously measured amylose content (Regina et al.,

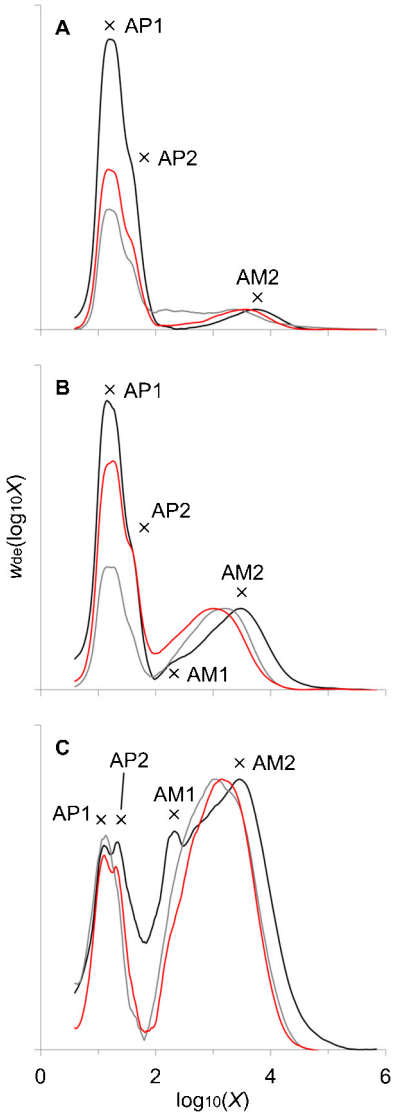

Fig. 2. SEC weight CLD of waxy, normal and high amylose barley starch obtained with different dissolution solvents and analyzed with SEC coupled with DRI detector. (A) SEC weight CLD of waxy barley obtained by using DMSO/LiBr for starch dissolution ($w_{de.D}(\log X)$; black line); that obtained by using 0.05 M NaOH ($w_{de.0.05N}(\log X)$; gray line); and that obtained by using 0.25 M NaOH ($w_{de.0.25N}(\log X)$; red line). Crosses mark the features in $w_{de}(\log X)$. (B) is analogous to (A) for SEC weight CLD of normal barley. (C) is analogous to (A) for SEC weight CLD of HA barley. AM1, AM2 and lines mark the peaks of the features in the amylose fraction in $w_{de.D}(\log X)$ (i.e. at $\log_{10}(X) > 2$). All $w_{de}(\log X)$ are normalized to AM2. Replicate $w_{de}(\log X)$ gave very similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2012). Samples with higher amylose contents showed elevated long chains. It is noted that the amount at the higher end of the amylopectin fraction in $N_{de}(X)$ (at $X \sim 100$) increases with amylose content (Fig. 1A–C), which is consistent with there being no clear separation between amylopectin and amylose chains (Vilaplana, Hasjim, & Gilbert, 2012).

3.1. Alkali dissolution treatment degrades long glucan chains

For each of the three types of starches, $N_{de.0.05N}(X)$ shows a reduction in amounts of chains with certain DPs compared to $N_{de.D}(X)$ (Fig. 1A–C). This increases up to 60% at DP of around 100 for ssIIa barley starch (Fig. 1F); reduction of up to 20% was seen for GP barley starch (Fig. 1E). However, it was interesting to find that $N_{de.0.05N}(X)$ of waxy barley was not significantly different from the corresponding $N_{de.D}(X)$ (Fig. 1D). The difference in CLD were unlikely to be caused by incomplete debranching, as the debranching processes of DMSO-purified starch and NaOH-purified starch were exactly the same, and NMR measurements have shown that there are no more than 0.5% residual branches with this technique (Ward et al., 2006) (although early work had suggested that debranching might be incomplete with a somewhat different method (Hizukuri, Takeda, Yasuda, & Suzuki, 1981)). NaOH degrades starch by facilitating the hydrolysis of α-(1→4) and α-(1→6) glycosidic bonds. This readily occurs at a pH ~ 10 (Lee, Han & Lim, 2009). Our results indicate that long chains are preferentially degraded during starch dissolution with aqueous NaOH, most probably simply because the longer the chain, the greater the number of α-(1→4) links. Compared to $N_{de.0.05N}(X)$, $N_{de.0.25N}(X)$ appears to have a higher relative amount of long chains (Fig. 1E–F). Interestingly, it appears that the waxy starch is less susceptible to degradation when dissolved in 0.05 M aqueous NaOH. In this present work, we did not determine if this is true for all low-amylose starches. However, it will be seen later from examining $w_{de}(\log X)$ that dissolution in 0.05 M aqueous NaOH causes degradation to the amylose fraction of the starch CLD.

It has been argued that a higher concentration of NaOH provides a better amylopectin dissolution (Nagamine & Komae, 1996). To test whether dissolution was the limiting factor, we tested an even higher concentration of NaOH for starch dissolution treatment (i.e. 0.75 M NaOH). However, the result shows a further reduction in the relative amount of high DP chains in HA barley starch CLD (Fig. 1C and F). This indicates that the fewer amounts of long chains are not due to incomplete dissolution and that it due to degradation. A smaller range of the HA barley starch CLD is presented in Fig. 1C. This is because dissolution treatment with 0.75 M NaOH resulted in a poorer signal-to-noise ratio. Thus it was felt that applying this to the other two types of starches would not serve any purpose. Hydrolysis of α-(1→6) branch points, in theory, should not affect the overall CLD. However, one possibility is that the branch point of long chains is hydrolyzed and the chains washed away by the ethanol precipitation step (described in Section 2).

It is noted that in $w_{de}(\log X)$ for all three types of starches, the relative amount of amylopectin chains increasing with an increase in the concentration of NaOH used during the dissolution treatment. This is consistent with the fact that amylopectin is less soluble relative to amylose in dilute aqueous NaOH (Han & Lim, 2004a). If using the starch CLD for amylose content estimation, NaOH treatment could lead to an overestimation. It could be argued that the incomplete dissolution of amylopectin gives a higher relative amount of long chains and that, as the concentration of NaOH is increased, short chains are more fully released, which would cause the relative amount of long chains to decrease (Fig. 1). This possibility can be discounted, as the DMSO-LiBr treatment has been shown using NMR (Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009) to give effectively 100% starch dissolution, and we see here that the relative amount of long chains is higher than with NaOH treatment (Fig. 1). This is consistent with the notion that NaOH treatment degrades starch by facilitating the hydrolysis of α-(1→4) bonds, and that long chains are preferentially degraded.

The amylose fraction of starch CLD also suffers from significant degradation when treated with NaOH for starch dissolution. Two peaks are seen in the amylose fraction of $w_{de}(\log X)$, and are designated AM1 and AM2. For each of the three types of starches used here, AM2 in $w_{de.0.05N}(\log X)$ is degraded to a lower DP when compared to $w_{de.D}(\log X)$ (Fig. 2A–C).

It is also apparent that certain features in the amylose fraction in $w_{de}(\log X)$ of starch are less apparent with the NaOH dissolution treatment (Fig. 2B and C). AM1, the lower-DP amylose peak, presenting as a distinct peak in $w_{de.D}(\log X)$ of the normal barley starch, is missing in $w_{de.0.05}(\log X)$; moreover, the peak is reduced

to a bump in the high-amylose barley starch. AM1 is completely missing in $w_{de.0.25}(\log X)$ in both normal and high-amylose starches. These results indicate that the NaOH dissolution treatment yielded artifactual results. On a separate note, waxy barley starch appears to lack the AM1 feature. As expected, AM2 appears at an even lower DP in $w_{de.0.25N}(\log X)$ (Fig. 2A–C), which is again consistent with NaOH degradation.

Our results show that the commonly used alkali dissolution treatment is not suitable for quantitative characterization of starch CLD for the following reasons. (1) Degradation of glucan chains is significant even with dilute NaOH concentration and is selective towards higher DP. (2) features such as the peaks indicating amylose fine structure can be lost. (2) A low concentration of NaOH does not offer adequate dissolution of amylopectin and leads to an overestimation of the amylose chains.

4. Conclusions

It is important to avoid artifactual results from sample pretreatment procedures for quantitative analysis of starch CLD. We have demonstrated that the DMSO-LiBr dissolution treatment is superior in several ways compared to commonly used alkali (i.e. aqueous NaOH) dissolution treatment. Data obtained from the alkali treatment are useful for purposes of semi-quantitative comparison but are not quantitatively accurate, especially for longer chains, which are important both for nutrition and other functional properties. The common use of difference plots between ranges of CLDs will be especially sensitive to inaccuracies in the relative amounts of longer and shorter chains. The conclusions reached here make use of data from FACE which go to a much higher range (into the amylose region) than seen previously in the literature; FACE data in this high range are free from the unavoidable band-broadening affects in SEC which can mask fine structural features which are distinguishing indicators of both biological processes and functional properties. This study presents a detailed pretreatment protocol for quantitative starch CLD characterization with both FACE and SEC coupled with a DRI detector. Although not performed here, the freeze-dried linear glucans could also be analyzed with high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.001.

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