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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).