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Characterization of the time evolution of starch structure from rice callus

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ABSTRACT

Callus (formed when a plant tissue is wounded) is a promising system for studying starch biosynthesis and bioengineering; however the molecular structure of callus starch has been poorly characterized. Size-exclusion chromatography was used in this study to characterize the starch structure in rice calli from two cultivars and a mutant of one cultivar lacking starch branching enzyme IIb. There were major qualitative differences in the chain-length and whole-molecule size distributions between starch from grain and from callus. However callus starch was found to be able to simulate the starch metabolism from both leaves and endosperm and reveal the structural development of starch granules, and this was dependent on the culture system. During synthesis, trans-lamellar amylopectin chains in callus are synthesized earlier than single-lamella chains, while enzymatic degradation starts from outer to inner amylopectin chains. The outer layers of the callus–starch granules have larger molecules with lower amylose content and shorter amylopectin chains compared to further inside the callus–starch granules. Controlling starch granular number and size thus has potential for improving both the quantity and quality of plant starch.

1. Introduction

The use of plant calli has potential as a system for studying starch structure to both gain insight into starch biosynthesis and to bioengineer starch with more desirable characteristics (Carciofi, Blennow, Nielsen, Holm, & Hebelstrup, 2012; Kitahara, Antoku, Hori, Sedoshita, & Suganuma, 2002). Callus is developed on plant tissue after wounding, and will proliferate on artificial media with the addition of nutrients and phytohormones. Calli accumulate starch granules, particularly so when grown in a medium with a high concentration of sugar or under specific culture conditions, for example under a controlled temperature or with appropriate hormone treatment (Akihiro, Mizuno, & Fujimura, 2005; Hagen, Harrison, Muneta, & Letourneau, 1993; Miyazawa et al., 1999). The structure of starch extracted from calli is variable (Kitahara et al., 2002; Saravitz & Boyer, 1987), thus allowing a database of chain-length distributions (CLDs) to be constructed to study the relationships between different starch structures and starch biosynthetic enzymes. Moreover, constant culture conditions eliminate the influence of some environmental factors on starch development. The use of calli also has the potential to provide a relatively rapid and inexpensive preliminary platform to examine the effects of modified biosynthetic enzymes on starch structure in vivo, possibly allowing high-throughput screening before proceeding to tests in transgenic plants (Carciofi et al., 2012). Promising transgenic constructs could then be selected to be employed in a cycle of whole plant transformation and progeny testing.

However, there is little current knowledge of the structure of starches in calli: for example, there are no published data on the size distributions of amylose (AM) and amylopectin (AP) whole molecules. Published research has mostly focused on the granular morphologies of the starch, with attempts to improve the starch content in the calli (Akihiro et al., 2005; Hagen et al., 1993; Saravitz

Abbreviations: CLD, chain-length distribution; SEC, size-exclusion chromatography; DP, degree of polymerization; DMSO, dimethyl sulfoxide.

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& Boyer, 1987). While some recent investigations have focused on obtaining chain-length distributions (CLDs) from starch extracted from calli (Carciofi et al., 2012; Kitahara et al., 2002), the techniques used were not optimal, such as high performance anion-exchange chromatography with pulsed amperometric detection, which is only suitable for the characterization of short chains (up to degree of polymerization, DP, 80) and cannot be used quantitatively (e.g. for model fitting) without considerable effort (Vilaplana & Gilbert, 2010). However the CLD and size distribution of the whole molecule are major determinants of many functional properties and of some aspects of higher-level structures (Witt, Doutch, Gilbert, & Gilbert, 2012; Witt & Gilbert, 2014). The characterization of these structural levels is thus important to determine if callus is a useful model to study starch biosynthesis and bioengineering.

Size-exclusion chromatography (SEC, also known as GPC) was used here to characterize callus–starch structure, yielding the size distributions of whole AM and AP molecules and the CLDs of starch branches. Rice calli were used because of their high throughput regenerability from mature seeds (Nishimura, Aichi, & Matsuoka, 2006), allowing an efficient and flexible approach for research purposes. An optimal sucrose concentration was developed for Nipponbare rice calli, a general rice model *Japonica* genotype, to establish a medium promoting a high accumulation of starch with a stable chain-length distribution (CLD) over time, in an attempt to mimic the situation in the developing grain. The optimized medium was applied to accumulate starch from another rice cultivar and its mutant lacking starch branching enzyme IIb, with their starch structure also being tested.

SEC and another size-separation technique, field-flow fractionation (FFF), have been widely used for starch structural characterization, e.g. Rubsam, Krottenthaler, Gastl, and Becker (2012). SEC can be used for solvent systems (dimethyl sulfoxide/LiBr) which have been proven to completely dissolve starch with molecular dispersion and with minimal degradation, but suffers from some shear scission of larger molecules. FFF has the advantage that it avoids shear scission but cannot be used quantitatively with DMSO/LiBr (Gilbert, Wu, Sullivan, Sumarriva, Ersch, & Hasjim, 2013). It was judged that the best methodology for the present purpose is SEC, with the procedure we have developed over the years for quantitative data parameterization and fitting (Gilbert et al., 2013).

2. Materials and methods

2.1. Plant material and callus induction

The calli were induced as described by (Nishimura et al., 2006) from mature seeds of *Japonica* rices Nipponbare, Zhonghua 11 (zh) and its derived *amylose-extender* (*ae*) mature rice seeds. Briefly, the seeds were dehulled and surface-sterilized by shaking in 70% (v/v) ethanol for 30 s, then in 1.5% sodium hypochlorite for 30 min, followed by five rinses with sterile water. Ten sterilized seeds were placed in each plate on the surface of N6D medium (Chu et al., 1975) containing 3% sucrose, and the plates sealed with surgical tape. The seeds were incubated on the medium at 27 °C for approximately 3–4 weeks after the yellow-white calli appeared, before subculturing to a fresh N6D medium in order to obtain a larger total callus mass.

2.2. Varying the sucrose content and growth rate calculation

A range of sucrose treatments was provided by subculturing 2-month-old calli on N6D with different concentrations of sucrose (3%, 6%, and 10%). Other components in the medium were kept constant. The calli were harvested after 3, 10 and 17 days and stored at–80 °C. Growth rates of callus are calculated based on the average fresh weights (FW) growth collected from five plates for each time point.

2.3. Measurement of starch content

Starch content was determined using the 'Total starch AOAC Method 996.11/AACC Method 76.13' kit from Megazyme International Ltd. (Wicklow, Ireland), using the protocol recommended by the manufacturer for samples containing resistant starch but no D-glucose and/or maltodextrins.

2.4. Starch extraction and purification

The calli were freeze-dried before starch extraction. Portions of the dried calli were added to plastic vials containing a grinding bead and pulverized for 5 min in a cryogrinder (Freezer/Mill 6850 SPEX, Metuchen, NJ, USA) containing liquid nitrogen. Prior to grinding, the callus samples were allowed to freeze in liquid nitrogen for 2 min in the plastic vials. Starch extraction and purification were performed as previously described (Syahariza, Li, & Hasjim, 2010), using a combination of protease, sodium bisulfite, dimethyl sulfoxide (DMSO) with 0.5% (w/w) LiBr (DMSO/LiBr), and an ethanol solution to completely dissolve starch molecules and remove non-starch components, i.e. proteins, lipids, and non-starch polysaccharides, with minimal degradation of starch molecules.

2.5. Whole molecular size distributions and chain-length distributions of starch

The SEC weight distributions and CLDs of the purified callus starch were obtained using SEC with a refractive index detector (RID, 235RID-10A, Shimadzu, Kyoto, Japan) following a previous method (Syahariza et al., 2010; Wu, Li, & Gilbert, 2014). A series of pullulan standards (Polymer Standard Services, Mainz, Germany) with molecular weights ranging from 342 to 2.35×10^6 was used for calibration, which fully covers the range of molar mass and size of the injected samples. Results are given in terms of the SEC separation parameter, the hydrodynamic radius R_h, or equivalently the DP inferred using the Mark–Houwink relation (Vilaplana & Gilbert, 2010). While the SEC data for the whole molecule suffer from unavoidable shear scission (Cave, Seabrook, Gidley, & Gilbert, 2009), the data interpretation for the present purpose only needs the semi-quantitative information on trends, which is reliable if (as at present) all data are obtained using the same set-up at similar times. Shear scission is only a problem for this system for R_h 50 nm, i.e. it is unimportant for all the CLD data and for the whole-molecule amylose data.

2.6. Amylose content calculation

The amylose content was calculated from the area under the curve of DP > 100 divided by the total area under the CLD (Vilaplana, Hasjim, & Gilbert, 2012).

3. Results

3.1. Starch accumulation optimization

Nipponbare calli grown on medium with different sucrose contents started with different growth rates but tended to reach a similar average growth rate after 17 days (Fig. 1A). The growth rate of calli on 6% sucrose medium was initially the lowest compared to the calli on the other two media, but increased significantly after 3 days and ended up as the highest growth rate, although it decreased slightly from day 10 to day 17. Conversely, the calli on 10% sucrose