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Impact of full range of amylose contents on the architecture of starch granules*☆

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ABSTRACT

The effects of amylose deposition on crystalline regions of barley starch granules were studied in granules containing zero to 99.1% amylose using "waxy" (WBS, 0% amylose), normal (NBS, 18% amylose) and amylose-only barley lines (AOS, 99.1% amylose). The effects were probed after hydrolysis of amorphous regions of starch granules in dilute HCl generating lintners, which typically represent the crystalline lamella of starch granules. Compared to NBS and WBS, AOS granules exhibited an irregular, multilobular morphology with a rough surface texture. AOS displayed lower rates of acid hydrolysis than WBS, and AOS reached a plateau at ~45 wt% acid hydrolysis. High-performance anion-exchange chromatography of lintners at equivalent levels of hydrolysis (45 wt%) revealed the average degree of polymerization (DP) of AOS lintners was 21, substantially smaller than that of NBS and WBS (DP 42). AOS lintners contained the lowest number of chains (NC) per molecule (1.1) compared to NBS (2.8) and WBS (3.3) and the average chain length of AOS, NBS and WBS lintners was 19, 15 and 13, respectively. Hence, both NC and the average chain length correlated with amylose content. The size distribution profile of AOS lintners revealed a repeat motif in the molecules corresponding to 5–6 glucose residues.

1. Introduction

Starch is the main storage polysaccharide in higher plants and is a globally important food and industrial material. Starch is cheap, renewable, biodegradable and composed of two main constituents, namely, amylose and amylopectin. Amylose is a mostly linear macromolecule containing α-(1,4)-linked D-glucosyl units, whereas amylopectin is a branched macromolecule composed of α-(1,4)-linked D-glucosyl chains connected by α-(1,6) branch points [1]. Despite this rather simple chemical composition, the structure and architecture of native starch granules varies significantly between plant species due to differences in the genes that encode starch biosynthetic enzymes, along with environmental factors [2]. Variability in starch structure drives numerous food and industrial uses of native starch, however, native starches do have natural limitations and modification can greatly enhance their desired functionality [3].

It was previously believed that the amylopectin fraction of starch was mandatory for the generation of starch granules [4], because double helices in amylopectin were shown to associate into crystallites of native starch granules [5]. However, it has recently been shown that semicrystalline starch granules can be synthesized with only the amylose component through the silencing of all known genes coding for starch branching enzymes (SBE I, SBE IIa, SBE IIb) in barley using a single RNAi hairpin [6]. Just like normal amylose [7], amylose-only starch (AOS) is slightly branched and has a clustered chain structure [8] permitting higher organized structures to be formed. However, AOS starch granules display an unorganized morphology [6]. Also, starch synthesis was slightly inhibited in the endosperm of grains with silencing of the SBE genes. When AOS was gelatinized, it contained very high contents of resistant starch (65%) compared to the control

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starch (29%) [6]. The high content of resistant starch is typically explained by decreased accessibility of amylose chains to digestive enzymes due to retrogradation, *i.e.*, the formation of stable double-helical segments during cooling following gelatinization [9]. AOS has great potential to be incorporated into food products designed to have a low glycemic impact, coinciding with a slow and sustained glucose release. Similarly, the grain endosperm starch showed increased resistance towards degradation by endogenous amylolytic enzymes during germination and early seedling establishment, which was retarded in amylose-only grains. This indicates that amylopectin is necessary for an apparent structural adaptation of starch for efficient remobilization of the energy and biomass stored in the grain starch [10].

Semicrystalline AOS granules exhibit an allomorphic composition corresponding to a mixture of V_h- and B-types [6] as opposed to the NBS and WBS granules that are of A-type [5,6]. However, the crystalline and molecular structures of AOS have not yet been fully studied. A common approach used to investigate the crystalline architecture of starch granules is based on the idea that the crystalline regions are more resistant towards mild acid hydrolysis than amorphous ones [11]. The insoluble residue after acid hydrolysis is referred to as lintners or Nägeli amylodextrins, depending on the type of acid that is used: 2.2 N HCl or 15% H_2SO_4, respectively [2]. The dextrins that compose the residue after acid hydrolysis are typically either linear (average degree of polymerization (DP) 13–17) or single-branched (average DP 24–30) [12]. The single-branched dextrins consist of chains of almost equal length [13]. In addition, a small amount of multiple-branched dextrins may also be present [14]. Investigating the kinetics of acid hydrolysis, as well as the structure of the resulting insoluble residues provides insightful information pertaining to the molecular structure, architecture, and dynamics of the crystalline components of starch in granules [15].

In this study, the impact of amylose on starch granule morphology and crystalline and molecular structure was studied using lintners prepared from WBS, NBS and AOS. The results provide novel insights into how amylose, in the widest range of amylose contents, affects hydrolytic stability of the starch granule in terms of its crystalline sections. Plausible architectural explanations for the effects are given.

2. Materials and methods

2.1. Barley

Waxy barley (cv. Cinnamon, kindly provided by Lantmännen SW Seed, Sweden), normal barley (cv. Golden Promise) and amylose-only [6] barley were cultivated in a greenhouse with natural sunlight and artificial supplementing light using mercury lamps from 4 a.m.–8 p.m. at the University of Copenhagen, Denmark.

2.2. Starch extraction and composition

Starch was extracted from ground barley flour according to the method of Carciofi et al. [16] with modifications. Briefly, 5 g of milled barley was mixed with 25 mL of 5 mM dithiothreitol containing 1% sodium dodecyl sulphate for 30 min at room temperature, and subsequently centrifuged at 3300*g* for 15 min. The pellet was then washed twice with water and filtered through a 70 μm mesh cloth. The filtrate was then centrifuged and 50 mL of 20 mM NaH_2PO_4 buffer (pH 6.5) was added. The mixture was incubated in a 50 °C water bath for 5 min before the addition of 100 μL lichenase (to remove trace amounts of cell wall β-glucan), after which the sample was incubated for 1 h, with stirring every 15 min. After centrifugation (3300*g* for 15 min) the pellet was washed twice with distilled water, once with ethanol, followed by air-drying overnight. The content of amylose and amylopectin in normal and waxy barley starch were determined with chromatography following debranching with isoamylase and pullulanase. The debranched sample was applied on a column (1.6 × 90 cm) of Sepharose CL 6B (GE Healthcare, Uppsala, Sweden) and eluted with 0.5 M NaOH at 1 mL min^{-1}. Fractions (1 mL) were analysed for carbohydrate content according to Dubois et al. [17]. By dividing the chromatograms at the lowest points between the two peaks, the relative content of amylose and amylopectin were determined according to Sargeant [18].

2.3. Lintnerization

Linterization was conducted according to two methods. In method 1, 200 mg starch granules were suspended in 8 mL 2.2 N HCl and incubated at 35 °C or 40 °C according to Robin [19]. Starch suspensions were gently mixed daily. The rate of acid hydrolysis was determined by taking small aliquots (15 μL) at regular intervals, diluting to 1.5 mL with distilled water, centrifuging at 2000*g*, and analyzing the supernatant for carbohydrate content using the phenol-sulphuric acid reagent [17]. For structural analysis, large aliquots of sample prepared at 40 °C were taken at ~45% hydrolysis (*via* determination of solubilized carbohydrate content), neutralized with 0.1 M NaOAc, washed twice with distilled water, and the insoluble residue was recovered by lyophilization. In method 2, the native starch powders were also incubated at 35 °C in 2.2 N HCl but all resistant residues were collected after 35 days and extensively washed to neutrality by centrifugation in distilled water. These samples were kept in water at 4 °C.

2.4. Enzyme treatment

Freeze-dried lintners obtained by method 1 were enzymatically treated in three different manners. Firstly, lintners were treated with β-amylase (from barley) to obtain their β-limit dextrins [19]. Secondly, lintners were debranched with the addition of isoamylase (from *Pseudomonas amyloderamosa*) and pullulanase (from *Klebsiella pneumoniae*) [15]. Lastly, following debranching, lintners were treated with β-amylase to obtain the β-limit dextrin of debranched components [15]. All enzyme preparations were kind gifts from Megazyme International Ireland (Bray, Wicklow, Ireland).

2.5. Molecular size-distribution analysis

The molecular size distribution of lintners (obtained by method 1) and enzymatically treated lintners at 45% acid hydrolysis was determined by gel-permeation chromatography (GPC) using a column (1.6 × 90 cm) of Sepharose CL 6B as described by Bertoft [21]. The GPC column was calibrated with glucose, maltose, and maltoheptaose (Sigma-Aldrich, St. Louis, MO, USA), and larger α-glucans using debranched WBS, as quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). A standard curve for the GPC column was obtained by comparing the elution of dextrins from debranched WBS with HPAEC analysis on a weight basis, and the standard curve was extended linearly past the last clearly resolved DP-peak of 60 by HPAEC analysis to cover the remaining volume of the GPC column.

2.6. Anion-exchange chromatography

Lintners and enzymatically treated lintners (obtained by method 1) at 45% hydrolysis were analyzed by HPAEC-PAD (Dionex-ICS 5000^+, Sunnyvale, CA, USA) equipped with a CarboPac PA-100 column and analogous guard column [15]. Areas under