Particulate structure of phytoglycogen studied using β -amylolysis

Hua Chen¹, Ganesan Narsimhan², Yuan Yao^{1*}

¹ Department of Food Science, Purdue University

² Department of Agricultural & Biological Engineering, Purdue University

* Corresponding author:

Address: 745 Agriculture Mall Drive, West Lafayette, IN 47907-2009

Email: yao1@purdue.edu

Phone: 765-494-6317

Abstract

Phytoglycogen (PG), a dendrimer-like glucan particulate, has a much higher dispersed molecular density than amylopectin (AP). In this study, β -amylase was used to investigate the effect of high molecular density of PG on its susceptibility to enzymatic hydrolysis. AP and PG reached the limit of β -amylolysis at 20 and 480 min, respectively, suggesting a much higher resistance of PG to β -amylase. The majority of PG β -amylolysis occurred in the initial 2 min, followed by a slow progression that implied low accessibility of internal particulate portion to enzyme. The chain length profile of PG β -limit dextrin showed only one population of long chains, indicating the absence of branch clusters with PG. At the limit of β -amylolysis, a substantial decrease in the molar mass was observed for both PG and AP, whereas only a slight reduction in the Z-average root mean square radius was observed for PG (24.5 to 23.1 nm) compared to that of AP (91.1 to 69.6 nm).

Keywords:

Phytoglycogen, particulate structure, beta-amylolysis, steric hindrance

1. Introduction

Phytoglycogen (PG) is a water-soluble, highly branched (1,4)(1,6)-linked α -D-glucan, which naturally occurs in various plants with a similar structure to the energy storage molecule glycogen in animals and microorganisms. It has been widely found in mutant species with compromised activity of isoamylase-type starch debranching enzymes (DBE), such as the *sugary-1* (*su1*) mutants of maize (Inouchi, Glover & Fuwa, 1987), sorghum (Boyer & Liu, 1983) and rice (Hamada, Suzuki, & Suzuki, 2014; Wong et al., 2003). Although the exact pathway for

PG generation remains unclear, compelling evidence has accumulated, highlighting the indispensable role of DBE in PG biosynthesis (Fujita et al., 2009; Kong, Corke, & Bertoft, 2009; Myers Morell, James, & Ball, 2000; Wattedled et al., 2005). The genetic analysis of maize has allowed the identification of several genes that code for isoamylase-type DBE. For example, the *sul-ref* gene codes for an isoamylase-type DBE, and the resulting mutation significantly modifies starch biosynthesis in maize endosperm (Gao, Wanat, Stinard, James, & Myers, 1998; James, Denyer, & Myers, 2003; James, Robertson, & Myers, 1995). For corn, mutations of *sul* lead to reduction in amylopectin content along with the generation of PG, suggesting a diversion from biosynthesis of normal insoluble branched glucan to the water-soluble form (Dinges, Colleoni, Myers, & James, 2001). For Arabidopsis, the gene *dbel* codes for an isoamylase-type DBE, and the resulting mutations prevent the leaf starch from accumulating to normal levels (Zeeman et al., 1998). Similarly, the rice *sul* gene codes for an isoamylase-type DBE, and the corresponding mutations lead to abundance of short side chains and increased accumulation of soluble α -glucans such as PG (Kubo et al., 2005; Nakamura et al., 1997; Nakamura et al., 1996). These studies showed that if DBE does not function properly, the glucosyl units normally incorporated into amylopectin are instead diverted into PG particulates.

As with amylopectin molecules, PG particulates are made up of individual linear glucan chains that are inter-connected through branch points. From the structural perspective, PG has been proposed to bear a resemblance to the glycogen isolated from animal sources (Ball et al., 1996; Powell et al., 2014; Sumner & Somers, 1944). According to the model proposed by Inouchi et al., PG is primarily composed of internal short B-chains attached by external short B- and/or A-chains, with no long B-chains (Inouchi et al., 1987). Up to now, evidences have shown that the overall chain length distribution of PG is unimodal, which makes it impossible for form

clusters as in the semi-crystalline structure of amylopectin (Lin et al., 2012). Additionally, it has been suggested that many B-chains of PG do not carry any A-chains, and the molar ratio of A- to B-chains for PG was < 1 (Gunja-Smith, Marshall, Mercier, Smith, & Whelan, 1970; Inouchi et al., 1987; Yun & Matheson, 1993). Specifically, it had been speculated that the average frequency of substitutions of B-chains over the whole molecule was > 2 for amylopectin and < 2 for PG (Yun & Matheson, 1993).

A distinct structural feature of PG is its remarkably high molecular density in aqueous dispersion. In rice, the dispersed molecular density (ρ) of PG (577 ± 12 to 2751 ± 57 g/mol·nm³) is about 10-fold that of amylopectin (71 ± 1 to 289 ± 4 g/mol·nm³) (Wong et al., 2003). Similarly, in maize, the ρ for PG was 871 g/mol·nm³, which is substantially higher than that of amylopectin (127 g/mol·nm³) (Scheffler, Huang, Bi, & Yao, 2010). In a later study, the particulate structure of PG was probed using amyloglucosidase, which demonstrated an obvious density increment towards the outer surface of the PG nanoparticle (from 950 to 1200 g/mol·nm³); whereas the ρ for amylopectin remained uniform (~ 60 g/mol·nm³) during the hydrolysis (Huang & Yao, 2011). The high molecular density of PG is consistent with its small particle size (30 - 100 nm) and high molecular weight ($\sim 10^7$ g/mol) (Huang & Yao, 2011).

One common structural property of starch that has been extensively investigated is the chain length profile. The chains are released by using debranching enzymes, and the chain length distribution is analyzed using chromatographic or electrophoretic technique (Bertoft, 2013). However, chain length profile of native starch does not directly provide information on branching pattern (Xia & Thompson, 2006). Therefore, the chain length profiles of β -limit dextrins (β -LD) are usually used to explore the distribution of branch points, and various

1 amylopectins have been characterized in this way (Bertoft, 2004; Kong et al., 2009; Tester & Qi,
2 2011; Yun & Matheson, 1993; Zhu, Corke, & Bertoft, 2011).

3 For amylopectin, exhaustive β -amylolysis would result in the production of β -limit
4 dextrans (β -LD), in which all A-chains are reduced to maltosyl (DP 2) and maltotriosyl (DP 3)
5 stubs attached to the branch points, depending on whether the A-chains originally contain an
6 even or odd number of glucosyl residuals (Klucinec & Thompson, 2002; Manners, 1989). The
7 external segments of the B-chains are shortened to 2, or 3 or 4 residues on the non-reducing side
8 from which branch point is projected (Bertoft, 2004; Kong et al., 2009; Tester & Qi, 2011; Yao,
9 Thompson, & Guiltinan, 2004). It has been suggested that in the completely debranched β -LD,
10 the total number of maltose and maltotriose equals to the number of A-chains, even though small
11 amounts of the shortest residual B-chains might account for maltotriose (Bertoft, 2013; Bertoft,
12 [Piyachomkwan, Chatakanonda, & Sriroth, 2008](#)). The chain length profile of β -LD, which is
13 usually obtained by size-exclusion chromatographic analysis of debranched β -LD, provides a
14 convenient way to analyze amylopectin branch structure, since the interferences from external
15 chains are very much eliminated.

16 The goal of this study was to understand the impact of high molecular density of PG
17 nanoparticles on the kinetics of β -amylolysis, and how β -amylolysis may affect the structure of
18 PG particulate. In this project, we hypothesized that the high molecular density of PG leads to
19 reduced rate of β -amylolysis, and the β -dextrans generated by hydrolysis allow us to better
20 understand the interactions between β -amylase molecules and PG particulates. In the study, we
21 monitored the maltose yield, the chain length distribution, and the particulate structural
22 parameters of β -dextrans of both PG and amylopectin during exhaustive β -amylolysis. Based on

the information obtained, we further hypothesize that β -amylase enzyme molecules, with a dispersed diameter around 6.9 nm, may not reach the central portion of PG nanoparticles.

2. Materials and Methods

2.1 Materials

Waxy corn starch (WCS, used as amylopectin) was obtained from the National Starch Food Innovation, now part of Ingredion (Bridgewater, NJ). Mature kernels of the sweet corn “Silver Queen” *sugary-1 (su1)* line were purchased from Burpee Co. (Warminster, PA). β -amylase (EC 3.2.1.2, from barley, E-BARBL, 28,400 U/mL), isoamylase (EC 3.2.1.68, from *Pseudomonas* sp., E-ISAMY, 280 U/mL) and pullulanase (EC 3.2.1.41, from *Klebsiella planticola*, E-PULKP, 1000 U/mL) were purchased from Megazyme International (Ireland).

2.2 Extraction of phytyglycogen

To extract phytyglycogen (PG), 320 g of dry kernels were ground and soaked in ten times their weight of deionized water. The suspension was homogenized using a high-speed blender (VARING Laboratory). The homogenates were collected and passed through a 270-mesh sieve, and the solids were further extracted twice using additional deionized water. All of the extracts were combined, the pH was adjusted to 4.8 - 4.9 using acetic acid and stored at 4°C for 2 h to induce protein precipitation. The extracts were centrifuged for 20 min at 4°C and $10,000 \times g$. The supernatants were re-centrifuged until no precipitate was present. The supernatants were combined, and the pH was adjusted to 7.0, followed by autoclaving at 121°C for 50 min. The denatured, insoluble proteinaceous material was removed again by centrifugation. To precipitate polysaccharides, three volumes of ethanol were added to the supernatant, followed by

centrifugation. The precipitate was re-suspended in ethanol and the supernatant was removed; the suspension-centrifuge operation was repeated for three cycles. The bulk ethanol was removed afterwards by vacuum filtration, and the solid pellets were placed in the hood to remove residual ethanol and used as PG.

2.3 Preparation of non-granular waxy corn starch

Non-granular waxy corn starch (WCS) consisting of amylopectin (AP) only was prepared. 60 g of WCS were dispersed in 20 times its weight of 90% dimethyl sulfoxide (DMSO). The suspension was continuously stirred in a boiling-water bath until it became clear. The suspension was then homogenized using a high-speed blender (VARING Laboratory). Three volumes of ethanol were added to the suspension to precipitate polysaccharides. The precipitate was re-suspended in ethanol and the supernatant was removed; the suspension-centrifuge operation was repeated for three cycles. The spent ethanol was removed by vacuum filtration, and the solid pellets were placed in the hood to remove residual ethanol. The collected solid was used later as the non-granular WCS.

2.4 β -Amylolysis of PG and WCS (AP)

PG and WCS (also called amylopectin, AP in the following texts), each of 780 mg, were fully dispersed in 7.8 mL of 90% DMSO by heating in a boiling water bath for 10 min. 57.2 mL NaAc buffer (20 mM, pH 6.0, 50°C) was mixed with the DMSO-dispersed samples, respectively. The mixture was then heated in a boiling-water bath for 20 min and cooled to 50°C in a shaking water bath. To each of the individual 65 mL-sample dispersions, diluted β -amylase solution was added at a dose of 2.84, 0.71, or 0.182 U/mg glucan substrate, respectively. Thereafter, the reactants were incubated at 50°C with constant agitation. To the blanks, only 65

mL NaAc buffer and β -amylase (2.84, 0.71, and 0.182 U/mg substrate) were added without including PG or AP, and the blanks were treated identically with the samples.

At various time intervals (0, 1, 2, 5, 10, 20, 30, and 60 min, and 2, 4, 8 and 24 h), a 5-mL aliquot of the hydrolysate was withdrawn, immediately added into 15 mL ethanol, and vortexed for 1 min. The mixtures were then centrifuged at $8000 \times g$ for 10 min; the supernatants were separated and saved for maltose measurement using high-performance size exclusion chromatography (HPSEC). The precipitated β -dextrins obtained as the precipitates at the above-mentioned time intervals were re-suspended using 80% (v/v) fresh ethanol, followed by centrifugation at $8000 \times g$ for 10 min. The suspension-centrifugation operation was repeated until no maltose could be detected by HPSEC in the ethanolic supernatant. This was to avoid contamination of the β -dextrins by the maltose released during β -amylolysis. Afterwards, the maltose-free β -dextrins were collected individually and evaporated to dryness in the hood.

2.5 Preparation of β -limit dextrins

To reach the limit of β -amylolysis, the hydrolysate after 24 h β -amylolysis was incubated in boiling-water bath for 10 min and cooled to 50°C. To each tube, an additional dose of β -amylase was added respectively as at the beginning of each β -amylolysis. The tubes were then returned to the 50°C shaking water bath for a continuation of β -amylolysis for another 24 h, and then heated in boiling water bath for 10 min to stop the reaction. The resulting hydrolysate presented the limit of β -amylolysis.

2.6 Maltose yield determination

Maltose resulted from β -amylolysis was quantified using HPSEC and converted to maltose yield based on the dry mass of PG and AP. A maltose standard curve was constructed

using maltose standard solutions prepared by a specific solvent consisting of 25% NaAc buffer and 75% ethanol (v/v) to mimic the condition when hydrolysates (5 mL) were withdrawn and added into ethanol (15 mL) at the end of each time interval of β -amylolysis. The concentration of maltose released during β -amylolysis was calculated using this standard curve. The mass of released maltose was divided by the PG or AP dry mass to calculate maltose yield (%).

2.7 Determination of weight-average molar mass, Z-average root mean square radius, and dispersed molecular density of β -dextrins and β -limit dextrins

The weight-average molar mass (M_w) and Z-average root mean square radius (R_z) of PG and AP were determined using high-performance size exclusion chromatography equipped with a multi-angle laser-light scattering detector and a reflective index detector (HPSEC-MALLS-RI). For the measurement, 10 mg PG and AP and their corresponding dextrins were dispersed in 1 mL of deionized water and heated in a boiling-water bath for 10 min. The HPSEC consists of an isocratic pump (515 HPLC, Waters), an injector (Model 7725i with a 20- μ L sample loop, Rheodyne), a multi-angle laser-light scattering detector (MALLS, Dawn HELEOS-II, Wyatt Technology) with a He-Ne Laser source ($\lambda = 632.8$ nm), and a refractive-index detector (RI, OptilabrEX, Wyatt Technology). Two connected columns (PL Aquagel-OH 40 15 μ m and 60 15 μ m, Polymer Laboratories, Varian) with a guard column were used for separation. Deionized water (pH 6.8, containing 0.02% sodium azide) was used as the mobile phase at a flow rate of 1.0 mL/min. Samples, each of 50 μ L, were injected into the system. Astra software (Version 5.3.4.14, Wyatt Technology) was used to determine M_w and R_z using the first-order Berry model. The dn/dc value of 0.146 was used for calculations. Dispersed molecular density (ρ) was calculated as $\rho = M_w/R_z^3$. For each sample, triplicate injections were carried out.

2.8 Debranching of β -dextrins and β -limit dextrins

To debranch β -dextrins (β -Ds) and β -limit dextrins (β -LDs), 6 mg of each of dried materials (obtained at 1, 5, 20, 60 min, and 8, 24, and 48 h of β -amylolysis with the enzyme dose of 2.84 U/mg substrate) was mixed with 100 μ L of 90% DMSO in a 2-mL microcentrifuge tube. The tubes were then heated in a boiling-water bath for 10 min. While the DMSO-dispersed samples were still warm, 700 μ L NaAc buffer (20 mM, pH 4.75, 37°C) was added to each tube. The mixtures were heated in a boiling-water bath for another 10 min and then equilibrated at 37°C. To each tube, a 12- μ L aliquot of isoamylase (ISA) solution was added to achieve a final ISA dose of 0.02 U/mg glucan substrate. The tubes were then incubated at 37°C in a shaking water bath for 24 h. After that, the tubes were heated in a boiling-water bath for 10 min to deactivate ISA, and then transferred to 37°C shaking water bath. To each of the tubes, a 20- μ L aliquot of pullulanase (PUL) solution was added to achieve a final PUL dose of 0.02 U/mg substrate. The tubes were further incubated in the 37°C shaking water bath for 24 h. After that, samples were heated in a boiling-water bath for 10 min. Based on our preliminary HPSEC analysis, the combination of ISA and PUL treatment was sufficient to completely debranch β -Ds and β -LDs of PG and AP.

The tubes containing the debranched samples were then exposed to a flow of nitrogen gas to concentrate until around 300 μ L was left. The volume of the residue was then adjusted to 1 ml with DMSO. After vortex mixing, a 25- μ L aliquot for each sample was injected into the HPSEC system.

2.9 Analysis of debranched β -dextrins and β -limit dextrins by HPSEC

Completely debranched β -Ds and β -LDs of PG and AP were analyzed using HPSEC as described before (Yao et al., 2004). The HPSEC system contained two connected Zorbax gel PSM 60-S columns (6.2mm $\times$ 250mm, Agilent Tech.,). The flow rate was set to 0.5 mL/min with

DMSO as the mobile phase. The elution was detected with a Waters 2414 refractive index (RI) detector. To construct a standard curve, maltose (DP 2), maltotriose (DP 3), maltotetraose (DP 4), maltopentaose (DP 5), maltohexaose (DP 6), maltoheptaose (DP 7) (Sigma Chemical Co., St. Louis, MO), and pullulan standards with molecular weights of 5.9×10^3 , 1.22×10^4 , 2.28×10^4 , 4.73×10^4 , 1.12×10^5 , and 2.12×10^5 (Polymer Laboratories, Amherst, MA) were used. For each debranched sample, HPSEC data processing includes raw data exportation to an Excel spreadsheet and normalization of the chromatogram by the total area from the retention time of 10 - 20 min.

3. Results and Discussion

3.1 Maltose yield during β -amylolysis

The time course of β -amylolysis of phytoglycogen (PG) and amylopectin (AP) was studied by following the yield of maltose (**Fig. 1**). During the initial 20 min of β -amylolysis, PG exhibited a substantially lower rate of maltose release compared to AP at all of the three enzyme doses, showing an obvious resistance of PG to the action of β -amylase. At 20 min and beyond, maltose yield for AP reached a plateau at 59% at 2.84 U/mg, while a continually increasing yield trend was observed for PG at a much lower rate, leveling off at a maximum value of 38%. Taken together, these outcomes indicated that the limit of β -amylolysis for PG and AP was 38% and 59%, respectively, with PG being more resistant to the action of β -amylase. Our data were consistent with the work of Inouchi et al. (1987), who reported the limit of β -amylolysis of PG and AP to be approximately 40 and 67%, respectively (Inouchi et al., 1987). Other studies also confirmed that 55 - 58% of the AP was cleaved as maltose when the limit of β -amylolysis was

1 reached (Bertoft et al. 2008; Bello-Pérez, Paredes-Lopez, Roger, & Colonna, 1996; Pérez &
2 Bertoft, 2010; Tester & Qi, 2011).

3
4 **Fig. 1.** Maltose release profiles of phytoglycogen (PG) and amylopectin (AP) subjected to
5 different β -amylase doses. Left: time scale to 0 - 3000 min; Right: time scale to 0 - 60 min. Data
6 points are means of three replicates with error bars of standard deviation.

7 3.2 Chain length profiles of β -dextrins and β -limit dextrins of AP and PG

8 Fig. 2-(1) shows the chain length profiles of β -dextrins (β -Ds) of AP during β -amylolysis.
9 For the β -D generated after 1 min of β -amylolysis, substantial amounts of DP2 can be found in
10 the chain length profile, accompanied by a moderate presence of DP 3 and DP4 as well as a shift
11 of major populations to lower DP. This suggested that the external chains of AP were rapidly
12 hydrolyzed by β -amylase towards the branch points. Specifically, the DP2 and DP3 may
13 represent completely degraded residual A-chains, depending on whether the A-chains originally
14 contained an even or odd number of glucosyl residuals; while DP4 may originate from
15 incompletely degraded residual A-chain stubs.

16 As β -amylolysis proceeded, a complete disappearance of DP4 was noticed at 5 min,
17 accompanied by a concomitant increase in DP2 and DP3, indicating that the initial DP4 was

1 completely reduced to DP2. Particularly, the proportion of DP 3 increased rapidly, whereas DP2
 2 increased slowly. The increase of DP3 proportion gradually surpassed that of DP2 as β -
 3 amylolysis progressed. Notably, a small population persisted in the region of DP5 - 7 all
 4 throughout β -amylolysis, which agreed with previous observations that this population was
 5 commonly found for β -LD of starch and may be derived from the short residual B-chains
 6 (Bertoft, Koch, & Åman, 2012; Summer, 2005; Xia & Thompson, 2006; Yao et al., 2004; Yuan,
 7 Thompson, & Boyer 1993; Zhu et al., 2011).

9 **Fig. 2.** Time courses of chain length profiles of β -dextrins of amylopectin (1) and phytoglycogen
 10 (2) during exhaustive β -amylolysis, with β -amylase dose of 2.84 U/mg.

11 Fig. 2-(2) showed the chain length profiles of β -Ds of PG during β -amylolysis. At 1 min,
 12 β -D of PG barely showed any peak at DP2; instead, minor amounts of DP3 and DP4 were

detected. Conceivably, such an initial absence of DP2 was associated with a strong resistance of PG to β -amylase. It was also noted that in comparison with AP, PG exhibited a distinct peak at DP4 even after the 48-h digestion, indicating that there would be many incompletely degraded A-chains at the final stage of digestion. We believe that the incomplete conversion of DP4 to DP2 in PG was associated with the dense distribution of branches that exerted substantial steric hindrance against the binding of β -amylase. Consequently, certain portions of A-chains of DP4 were not accessible to enzyme even after exhaustive β -amylolysis.

Similar to those of AP, β -Ds of PG also showed a population at DP5 - 7 toward the limit of β -amylolysis, which may be associated with the common elements in the branching structures of PG and AP. Yao et al. (2004) reported the shortest chain length of B-chains from β -LD of AP was around 5.7 for several maize genotypes, with a possible internal chain length (ICL) of around DP3.2 (Yao et al., 2004). Other studies indicated that starch β -LDs from waxy maize and rice also exhibited a population at DP5 - 8 (ICL 2 - 3) which built up the inner parts of the smallest branched units of the clusters in AP (Bertoft, 2004; Kong et al., 2009; Pérez & Bertoft, 2010).

For PG, the nature of the DP20 shoulder deserves special discussion. One assumption is that this shoulder proportion originated mostly from external A- and B-chains. As noted in Fig. 2-(2), there was a prominent DP20 population in the chromatogram of (native) PG. 1 min of β -amylolysis slightly reduced this population while generating small amount of DP2 - 8. After 2 min, however, the DP20 shoulder was nearly removed, which coincided with the increasing presence of DP2 stubs. From 20 min to 48 h, the amount of DP3 stubs increased whereas the overall shape of the major population (around DP7 - 50) remained similar. Based on these observations, it was inferred that the chains corresponding to the DP20 shoulder might mostly

originate from the surface of the PG particulates. Upon extended β -amylolysis from 2 min to 24 h, the β -amylases “struggled” to attack the high-density branches, continuously but slowly releasing additional maltose (Fig. 1). Nevertheless, the overall chain length profiles of β -Ds didn’t change significantly over this period.

3.3 Changes of M_w , R_z and ρ of PG and AP during β -amylolysis

Evidence on chain length profile may complement the analysis of the particulate structure pertaining to the β -Ds of PG. In this study, the weight-average molar mass (M_w), Z-average root mean square radius (R_z), and dispersed molecular density ($\rho = M_w/R_z^3$) of PG and AP during β -amylolysis at β -amylase dose of 2.84 U/mg were determined using HPSEC-MALLS-RI system.

Fig. 3. Weight-average molar mass (M_w) of phytoglycogen (PG) and amylopectin (AP) during β -amylolysis. The changes in M_w during the overall 48 h (left) and the initial 60 min (right) are displayed separately. Data points are means of three replicates with error bars of standard deviation.

The change of M_w of PG and AP over the course of β -amylolysis is shown in Fig. 3. AP displayed significantly greater M_w than PG over the 48-h β -amylolysis. For both PG and AP, the majority of the change of M_w occurred in the initial 2 min. The M_w of AP decreased by 50% in 2

1 min, having an overall reduction of 62% in 48 h; whereas for PG, M_W was reduced by 28% in 2
 2 min, in comparison with an overall reduction of 42% in 48 h. At 48h, β -limit dextrin of PG and
 3 AP showed a M_W reduction of 42% and 62%, respectively, correlating with their maltose yields
 4 of 38% and 59%.

6 **Fig. 4.** Z-average root mean square radius (R_z) of phytylglycogen (PG) and amylopectin (AP)
 7 during β -amyolysis. The changes during the overall 48 h (left) and initial 60 min (right) of β -
 8 amyolysis are displayed separately. Data points are means of three replicates with error bars of
 9 standard deviation.

Fig. 5. Dispersed molecular density (ρ) of phytoglycogen (PG) and amylopectin (AP) during β -amylolysis. The changes during the overall 48 h (left) and the initial 60 min (right) of β -amylolysis are displayed separately. Data points are means of three replicates with error bars of standard deviation.

Fig. 4 and Fig. 5 illustrated the impact of β -amylolysis on R_Z and ρ of PG and AP, respectively. PG exhibited significantly lower R_Z (24.5 nm) yet appreciably larger ρ (1408.56 g/mol·nm³) than AP (R_Z = 91.1 nm, ρ = 49.95 g/mol·nm³). Similar results have been reported (Wong et al., 2003; Huang and Yao, 2011). Notably, the R_Z of AP decreased dramatically from 91.1 to 71.4 nm within 60 min of β -amylolysis; it then stabilized with a minor further reduction (to 69.6 nm) towards 48 h. In contrast, only a slight decrease in R_Z of PG was noticed (from 24.5 to 23.1 nm) over 48 h.

The value of ρ was derived from M_W and R_Z ($\rho = M_W/R_Z^3$). For PG, the change of R_Z was almost negligible, thus inferring that the change of ρ was closely correlated with that of M_W . Therefore, the decline of ρ also occurred mostly in the first 2 min from 1408.56 to 1088.4 g/mol·nm³, paralleling the reduction in M_W , followed by a gradual reduction to 973.09 g/mol·nm³ at 48h.

3.4 Can β -amylase reach the internal region of PG particulate?

During the initial 2 min of β -amylolysis of PG, 68% of the total M_W reduction was achieved. The DP20 shoulder population of PG, representing the external chains of the particulate, was almost totally eliminated over this period, giving rise to DP2 stubs. From 2 min to 24 h, β -amylase appeared to slowly but steadily release minor amount of maltose from PG. The molecular hydrodynamic diameter of the barley β -amylase (molar mass 58,300 Da) in the

hydrolysis buffer was measured to be 6.9 nm. With this size, the enzyme molecules may react readily with the external chains packed at the surface of PG particles and subsequently achieve a shallow penetration inward (Fig. 6) to seek additional, accessible glucan chains to react on. We speculate that the enzyme molecules would be unlikely to fully reach the central region of PG particulate, given the steric barrier exerted by the high density of the glucan branches.

In contrast, an AP molecule is essentially a collection of clusters connected with inter-cluster chains. While AP molecules are branched, their molecular flexibility associated with multiple inter-cluster chains provides sufficient accessibility to β -amylases. Therefore, the enzyme molecules are able to quickly reach all branches to achieve the limit of β -amylolysis, even at relatively low enzyme doses (i.e. 0.71 and 0.182 U/mg).

Fig. 6. Schematic of β -amylolysis of phytoglycogen at the stages of initial and exhaustive hydrolysis, showing limited penetration of enzyme molecules in a phytoglycogen particle.

4. Conclusions

In the present study, β -amylase was used to study the structure of PG using AP from WCS as a reference. The pattern of maltose release and change of chain length profile during β -amylolysis suggested a rapid hydrolysis of the external chains at the surface of PG particulate,

followed by a slow and steady hydrolysis to release additional whereas minor amount of maltose. This result was specifically supported by the change of M_w , R_z , and ρ in the progression of β -amylolysis. Our results indicated that the action of β -amylase was likely to occur at the surface of PG particles as well as toward a shallow depth inward, which can be attributed to the high density of PG glucan chains that constitutes a spatial barrier against the penetration of enzyme molecules to the central region. The use of β -amylase treatment allows for glucan trimming at surface without causing substantial structural changes inside the PG particle. This study offered important information on the structure of PG, a naturally occurring glucan particle, as well as provided a potential enzymatic approach to modify this type of biomaterial.

Acknowledgement

The National Science Foundation grant No. 1006301 to Yuan Yao and Ganesan Narsimhan provided the financial support for this study.

References

- Ball, S., Guan, H. P., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P. & Preiss, J. (1996). From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. *Cell*, 86, 349-352.
- Bello-Pérez, L., Paredes-Lopez, O., Roger, P. & Colonna, P. (1996). Molecular characterization of some amylopectins. *Cereal chemistry*, 73, 12-17.
- Bertoft, E. (2004). On the nature of categories of chains in amylopectin and their connection to the super helix model. *Carbohydrate Polymers*, 57, 211-224.

- 1 Bertoft, E. (2013). On the Building Block and Backbone Concepts of Amylopectin Structure.
2 *Cereal Chemistry*.
- 3 Bertoft, E., Koch, K. & Åman, P. (2012). Building block organisation of clusters in amylopectin
4 from different structural types. *International journal of biological macromolecules*.
- 5 Bertoft, E., Piyachomkwan, K., Chatakanonda, P. & Sriroth, K. (2008). Internal unit chain
6 composition in amylopectins. *Carbohydrate Polymers*, 74, 527-543.
- 7 Boyer, C. D. & Liu, K. C. (1983). Starch and water-soluble polysaccharides from sugary
8 endosperm of sorghum. *Phytochemistry*, 22, 2513-2515.
- 9 Dinges, J. R., Colleoni, C., Myers, A. M. & James, M. G. (2001). Molecular Structure of Three
10 Mutations at the Maizesugary1 Locus and Their Allele-Specific Phenotypic Effects.
11 *Plant Physiology*, 125, 1406-1418.
- 12 Fujita, N., Toyosawa, Y., Utsumi, Y., Higuchi, T., Hanashiro, I., Ikegami, A., Akuzawa, S.,
13 Yoshida, M., Mori, A., Inomata, K., Itoh, R., Miyao, A., Hirochika, H., Satoh, H. &
14 Nakamura, Y. (2009). Characterization of pullulanase (PUL)-deficient mutants of rice
15 (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice
16 endosperm. *Journal of Experimental Botany*, 60, 1009-1023.
- 17 Gao, M., Wanat, J., Stinard, P. S., James, M. G. & Myers, A. M. (1998). Characterization of
18 dull1, a maize gene coding for a novel starch synthase. *The Plant Cell Online*, 10, 399-
19 412.
- 20 Gunja-Smith, Z., Marshall, J., Mercier, C., Smith, E. & Whelan, W. (1970). A revision of the
21 Meyer-Bernfeld model of glycogen and amylopectin. *FEBS letters*, 12, 101.
- 22 Hamada, S., Suzuki, K. & Suzuki, Y. (2014). Development of a new selection method and
23 quality improvement of sugary-1 rice mutants. *Breeding science*, 63, 461.

- 1 Huang, L. & Yao, Y. (2011). Particulate structure of phytoglycogen nanoparticles probed using
2 amyloglucosidase. *Carbohydrate Polymers*, 83, 1665-1671.
- 3 Inouchi, N., Glover, D. & Fuwa, H. (1987). Chain Length Distribution of Amylopectins of
4 Several Single Mutants and the Normal Counterpart, and Sugary-1 Phytoglycogen in
5 Maize (*Zea mays* L.). *Starch-Stärke*, 39, 259-266.
- 6 James, M. G., Denyer, K. & Myers, A. M. (2003). Starch synthesis in the cereal endosperm.
7 *Current opinion in plant biology*, 6, 215-222.
- 8 James, M. G., Robertson, D. S. & Myers, A. M. (1995). Characterization of the maize gene
9 sugary1, a determinant of starch composition in kernels. *The Plant Cell Online*, 7, 417-
10 429.
- 11 Klucinec, J. D. & Thompson, D. B. (2002). Structure of amylopectins from ae-containing maize
12 starches. *Cereal chemistry*, 79, 19-23.
- 13 Kong, X., Corke, H. & Bertoft, E. (2009). Fine structure characterization of amylopectins from
14 grain amaranth starch. *Carbohydrate Research*, 344, 1701-1708.
- 15 Kubo, A., Rahman, S., Utsumi, Y., Li, Z., Mukai, Y., Yamamoto, M., Ugaki, M., Harada, K.,
16 Satoh, H. & Konik-Rose, C. (2005). Complementation of sugary-1 phenotype in rice
17 endosperm with the wheat isoamylase1 gene supports a direct role for isoamylase1 in
18 amylopectin biosynthesis. *Plant Physiology*, 137, 43-56.
- 19 Lin, Q., Huang, B., Zhang, M., Zhang, X., Rivenbark, J., Lappe, R. L., James, M. G., Myers, A.
20 M. & Hennen-Bierwagen, T. A. (2012). Functional interactions between starch synthase
21 III and isoamylase-type starch-debranching enzyme in maize endosperm. *Plant*
22 *physiology*, 158, 679-692.

- Manners, D. J. (1989). Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers*, 11, 87-112.
- Myers, A. M., Morell, M. K., James, M. G. & Ball, S. G. (2000). Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiology*, 122, 989-998.
- Nakamura, Y., Kubo, A., Shimamune, T., Matsuda, T., Harada, K. & Satoh, H. (1997). Correlation between activities of starch debranching enzyme and α -polyglucan structure in endosperms of sugary-1 mutants of rice. *The Plant Journal*, 12, 143-153.
- Nakamura, Y., Umemoto, T., Takahata, Y., Komae, K., Amano, E. & Satoh, H. (1996). Changes in structure of starch and enzyme activities affected by sugary mutations in developing rice endosperm. Possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. *Physiologia Plantarum*, 97, 491-498.
- Pérez, S. & Bertoft, E. (2010). The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch-Stärke*, 62, 389-420.
- Powell, P. O., Sullivan, M. A., Sweedman, M. C., Stapleton, D. I., Hasjim, J. & Gilbert, R. G. (2014). Extraction, isolation and characterisation of phytoglycogen from su-1 maize leaves and grain. *Carbohydrate Polymers*, 101, 423-431.
- Scheffler, S. L., Huang, L., Bi, L. & Yao, Y. (2010). In Vitro Digestibility and Emulsification Properties of Phytoglycogen Octenyl Succinate. *Journal of agricultural and food chemistry*, 58, 5140-5146.
- Summer, R. E. 2005. *Action of β -amylase on branched oligosaccharides*. IOWA STATE UNIVERSITY.

- 1 Sumner, J. B. & Somers, G. F. (1944). The water soluble polysaccharides of sweet corn. *Arch.*
2 *Biochem*, 4, 7-9.
- 3 Tester, R. F. & Qi, X. (2011). β -limit dextrin-Properties and applications. *Food Hydrocolloids*.
4 Wattebled, F., Dong, Y., Dumez, S., Delvallé, D., Planchot, V., Berbezy, P., Vyas, D., Colonna,
5 P., Chatterjee, M. & Ball, S. (2005). Mutants of Arabidopsis lacking a chloroplastic
6 isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. *Plant*
7 *physiology*, 138, 184-195.
- 8 Wong, K. S., Kubo, A., Jane, J., Harada, K., Satoh, H. & Nakamura, Y. (2003). Structures and
9 properties of amylopectin and phytoglycogen in the endosperm of sugary-1 mutants of
10 rice. *Journal of Cereal Science*, 37, 139-149.
- 11 Xia, H. & Thompson, D. B. (2006). Debranching of β -dextrins to explore branching patterns of
12 amylopectins from three maize genotypes. *Cereal chemistry*, 83, 668-676.
- 13 Yao, Y., Thompson, D. B. & Guiltinan, M. J. (2004). Maize starch-branching enzyme isoforms
14 and amylopectin structure. In the absence of starch-branching enzyme IIb, the further
15 absence of starch-branching enzyme Ia leads to increased branching. *Plant physiology*,
16 136, 3515-3523.
- 17 Yuan, R., Thompson, D. & Boyer, C. (1993). Fine structure of amylopectin in relation to
18 gelatinization and retrogradation behavior of maize starches from three wx-containing
19 genotypes in two inbred lines. *Cereal Chemistry*, 70, 81-81.
- 20 Yun, S. H. & Matheson, N. K. (1993). Structures of the amylopectins of waxy, normal, amylose-
21 extender, and wx: ae genotypes and of the phytoglycogen of maize. *Carbohydrate*
22 *Research*, 243, 307-321.

- 1 Zeeman, S. C., Umemoto, T., Lue, W.-L., Au-Yeung, P., Martin, C., Smith, A. M. & Chen, J.
2 (1998). A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both
3 starch and phytylglycogen. *The Plant Cell Online*, 10, 1699-1711.
- 4 Zhu, F., Corke, H. & Bertoft, E. (2011). Amylopectin internal molecular structure in relation to
5 physical properties of sweetpotato starch. *Carbohydrate Polymers*, 84, 907-918.
- 6