Note

Binding Interaction of Porcine Pancreatic α-Amylase with waxy/amylose extender Double-mutant Rice Starch Granules Does Not Determine Their Susceptibility to Hydrolysis

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The starch from waxy/amylose extender (wx/ae) double-mutant rice is almost indigestible by porcine pancreatic α-amylase (PPA) and behaves like resistant starch, even though PPA digests waxy mutant (wx) and wild-type (WT) rice starches easily. To clarify the relationship between binding ability and susceptibility to PPA hydrolysis, we studied the PPA adsorption capacity of wx/ae starch. Results showed that the wx/ae starch adsorbed 80% more PPA than wx and WT starches, consistent with the ratio of their specific surface areas. This suggests that PPA adsorption capacity is determined by the specific surface area of starch granules. Thus, the resistance to digestion of the wx/ae starch is not due to adsorption capacity, but to the higher-order nanostructure of the starch granules. The morphological features of digested wx/ae starch granules observed by scanning electron microscopy (SEM) suggest that the higher-order nanostructure of wx/ae starch is similar to that of resistant starches, such as high-amylose rice and potato starches.

Keywords: waxy/amylose extender, porcine pancreatic α-amylase, adsorption, digestibility

Introduction

Starch is a digestible carbohydrate that also has components resistant to digestion called resistant starch (RS). RS is defined as the portion of the starch that is not broken down by digestive enzymes, mainly pancreatic α-amylase, in the small intestine. Some native starches, isolated from plants and without any structural modification, are resistant to digestion. Potato and high-amylose maize starches show greater resistance to hydrolysis by amylolytic enzymes than other native starches (Banks and Greenwood, 1975). We found that starch from the rice double-mutant waxy and amylose extender (wx/ae) is almost indigestible by α-amylase in vitro (Kubo et al., 2010). The wx/ae starch, along with potato and high-amylose maize starches, is classified as RS2, one of four types of RS. RS1 is physically inaccessible to digestion due to the presence of intact cell walls in the grains, seeds or tubers. RS2 is composed of native starch granules that are protected from digestion by the higher-order nanostructure of the starch granules. RS3 is composed of non-granular starch-derived materials that resist digestion and is usually formed during the retrogradation of starch granules. RS4 is generated by chemical modification to reduce digestibility (Sajilata et al., 2006).

Digestibility by amylases is affected by the composition and structure of starch granules, including amylose content, amylopectin double helices, crystalline domains, pores and other characteristics (Qi and Tester, 2016a). The wx/ae starch consists of pure amylopectin with relatively long unit chains (fewer with

Abbreviations: wx/ae: waxy/amylose extender; wx: waxy; WT: wild-type; PPA: porcine pancreatic α-amylase

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degree of polymerization (DP) 6-14 and more with DP > 15) and has a B-type polymorphic form (Nishi et al., 2001, Kubo et al., 2008, Kubo et al., 2010). The average mean diameter of wx/ae starch granules is 3.96 ± 1.22 μm (Kubo et al., 2010). The double helices of amylopectin cannot be digested unless they are untwisted (Gallant et al., 1992), and starch granules with the B-type polymorphic form are much more resistant to amylolysis than those with the A-type polymorphic form (Plancho et al., 1997). On the other hand, wx/ae starch has a larger specific surface area and more pores on its surface than waxy (wx) and wild-type (WT) rice starches (Takagi et al., 2017). The large surface area provides more access for enzymes to attach, resulting in increased hydrolysis (Tester et al., 2006, Qi and Tester, 2016b), and the pores on the granule surface might be sites of initial enzyme attack, allowing enzyme molecules access to the granule interior (Fannon et al., 1992). The resistance to digestion of wx/ae starch for α-amylase can be explained by its change in composition and crystal form (Kubo et al., 2010); however, its large specific surface area and many surface pores should increase its susceptibility to enzyme binding and hydrolysis.

We focused on the relationship between enzyme adsorption and digestibility of wx/ae starch using porcine pancreatic α-amylase (PPA). We analyzed the amount of PPA adsorbed to determine whether or not the resistance to digestion of wx/ae starch is due to its PPA adsorption capacity, and also observed the starch granules after reaction with PPA to elucidate the patterns of hydrolysis involved.

Materials and Methods

Rice grain We used the amylase-free amyllose extender rice mutant line AMF18 (wx/ae), which is genetically defective in granule-bound starch synthase I (GBSSI) and starch branching enzyme IIb (BEIIb). It is derived from a cross between the waxy mutant line EM21 and the amyllose extender mutant line EM16 (Nishi et al., 2001, Kubo et al., 2008, Kubo et al., 2010). We also used the waxy mutant line EM21 (wx), which is genetically defective in GBSSI, and its wild-type japonica parental cv. Kinmaze rice (WT) (Nishi et al., 2001, Kubo et al., 2008, Kubo et al., 2010). The wx/ae mutant rice plants were grown in the summer of 2010 in a field in Shiga Prefecture, Japan. The wx and WT rice plants were both grown in the summer of 2007 in an experimental field at Osaka Prefecture University, Japan.

Starch granule isolation Starch granules were isolated according to a modified previous method (Kubo et al., 2010). Kernels (400 g) were polished to about 85% of their initial weight. Polished kernels were soaked in 0.05% (w/w) sodium hydroxide for 16 h and washed with distilled water several times until the supernatant had a neutral pH. The wet starch was homogenized using a mortar and pestle. The slurry was filtered with a 0.106 mm mesh sieve and starch granules were suspended in a 1.0% sodium dodecyl sulfate (SDS) solution and mixed by magnetic stirrer for 1 h, then they were collected by centrifugation at 620 G for 5 min and washed with distilled water several times to completely remove the SDS. Starch granules were then isolated by ethanol precipitation with 70, 90 and 99.5% ethanol. The isolated starch was dried in an oven at 50°C under vacuum and filtered through a 0.106 mm mesh sieve before use.

Enzyme binding property of starch The adsorption procedure is summarized in Fig. 1. Porcine pancreatic α-amylase (PPA, Product No. A4268), an endo-type amylase that hydrolyzes α-1,4-glucosidic linkages of starch randomly, was purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. The protein content was determined from the optical density at 280 nm using a molar adsorption coefficient of 13.8 x 10² M⁻¹ cm⁻¹ (Alkazaz et al., 1996). We also used bovine serum albumin (BSA, Sigma Aldrich) for the SDS poly-acrylamide gel electrophoresis (SDS-PAGE) analysis. Starch (10 mg) was placed in a 1.5 mL microtube and washed with 2.0% SDS solution to remove surface proteins and then washed with 20 mM phosphate buffer (pH 7.0) to remove the SDS. The starch granules were suspended in 200 μL of buffer, and 200 μL of enzyme solution was then added to the suspension. After mixing well, the sample was placed in an ice bath for 10 min. The suspension was separated by centrifugation at 15,000 G for 10 min at 4°C, and then the supernatant was collected as a soluble fraction (S fraction). The separated starch granules were resuspended in 200 μL of buffer. The supernatant was collected as a washed fraction (W fraction) by centrifugation under the same conditions as described above. The microtube was transferred to room temperature and 200 μL of 2.0% SDS solution was added to the tube. The starch was suspended by mixing, and the supernatant was collected as a bound fraction (B fraction) by centrifugation at 15,000 G for 10 min at 20°C.

Protein assay The S and B fractions collected in the adsorption step were analyzed by SDS-PAGE. The proteins obtained in each fraction were detected using Coomassie Brilliant Blue stain (CBB R250; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein contents of the S and W fractions were measured by the Bradford protein assay (Bio-Rad Laboratories, Inc.) with a micro-plate reader (iMark™, Bio-Rad Laboratories, Inc.). The protein content of the B fraction is the value of the added protein minus the S and W fractions.

In vitro digestibility of starches with α-amylase Starch digestibility by PPA was analyzed by measuring the content of reducing sugars produced in enzyme reactions. The starch (40 mg) was suspended in 10 mL of 20 mM Tris-HCl buffer (pH 7.5) with 0.4 mg sodium azide. Then, 40 μg of PPA (50 U, according to the Certificate of Analysis from Sigma Aldrich) was added to the suspension, and the suspension was maintained at 37°C. The supernatant of each reaction mixture was collected by centrifugation at 10,000 G for 5 min at 4°C. The reducing sugar content was measured by the Somogyi-Nelson method. Digestibility is shown as the ratio (as a percentage) of reducing sugars produced in enzyme reactions. The starch (40 mg) was suspended in 10 mL of 20 mM Tris-HCl buffer (pH 7.5) with 0.4 mg sodium azide. Then, 40 μg of PPA (50 U, according to the Certificate of Analysis from Sigma Aldrich) was added to the suspension, and the suspension was maintained at 37°C. The supernatant of each reaction mixture was collected by centrifugation at 10,000 G for 5 min at 4°C. The reducing sugar content was measured by the Somogyi-Nelson method. Digestibility is shown as the ratio (as a percentage) of reducing sugars produced in enzyme reactions.
sugar content to the total reducing sugar content obtained from a reaction using gelatinized starch.

*Scanning electron microscopy (SEM)*  Starch was collected from the enzyme reaction mixture by centrifugation, washed with distilled water twice and then dried with ethanol. The starch was next placed on a sample stage using carbon tape and coated by Au-Pd using Ion Sputter (E1010; Hitachi High Technology, Tokyo, Japan). The starch surface was observed by SEM (SU1510; Hitachi High Technology) at accelerating voltage from 10 kV and 15 kV.

**Results and Discussion**

*Adsorption of amylases*  SDS-PAGE results and the ratio of S and B fractions in densitometric analysis after treatment by PPA are shown in Fig. 2. The same experiment was performed using BSA as a control for the adsorption analysis. BSA was detected in the S fractions of all starches, but not in the B fractions (data not shown). PPA was detected in both the S and B fractions of all starches. As shown in the densitometric result (Fig. 2 right), the B fraction band was denser and the S fraction band was lighter for the wx/ae starch than for the wx and WT starches. This indicates that the wx/ae starch has a stronger affinity to PPA, but is less digestible than the other two starches (Kubo *et al.*, 2010). In addition, another protein was detected with lower molecular weight than the main PPA protein (Fig. 2 left), possibly a pancreatic α-amylase-like isoform. In this study, we focused on the main PPA protein because its isoform protein was slightly detected in S and B fractions and the difference between starches was not clear.

The protein content adsorbed by wx/ae, wx and WT starches is shown in Fig. 3. This experiment was performed twice and similar results were obtained. Representative results are shown in Fig. 3.
The *wx/ae* starch adsorbed significantly more PPA at a PPA concentration of greater than 10 mg per g starch. At 55 mg/g starch, approximately 66% of PPA was adsorbed. The PPA was entirely adsorbed when its concentration was less than 17 mg/g starch; whereas, in the *wx* starch, the PPA was entirely adsorbed below 8.4 mg/g starch concentration. At 51 mg/g PPA concentration, the *wx* starch showed 39% adsorption. The WT starch showed the lowest adsorption; only 66% of PPA was adsorbed at low PPA concentrations and 37% at the highest concentration (61 mg/g starch).

Thus, the *wx/ae* starch adsorbed approximately 80% more PPA than the other two starches, and showed 80% greater specific surface area (Takagi *et al.*, 2017). We suggest that these two results are related. The amount of enzyme bound to the starch granule surface depends on granule size and surface area (Warren *et al.*, 2011). Warren *et al.* concluded that large granules have lower affinity for amylase than small granules. The greater capacity of *wx/ae* starch for PPA shown in our results is consistent with this previous report, with the exception of its digestibility. Our findings may provide a basis for further study of α-amylase binding to starch granules.

**Digestibility of starches by PPA** The digestibility of starches by PPA is shown in Fig. 4. The *wx* and WT starches were entirely digested by PPA in 8 h, whereas the *wx/ae* starch was digested by approximately 32% after 24 h. As shown in a previous report (Kubo *et al.*, 2010), *wx/ae* starch is less digestible than the other two starches. The interaction between the physical properties of starch and its hydrolysis by amylases has been studied (Qi and
Starch amylosis depends on the composition of starch granules (amylose/amylopectin ratio and lipids), the structural features of starch granules (amylopectin double helices, crystalline domains, blocklets, pores and growth rings), molecular structure (phosphorylation) and structural modification by chemical or physical processes. The wx/ae starch consists of pure amylopectin with relatively long unit chains, and thus has more amylopectin double helices (Kubo et al., 2010). Further, it has a B-type polymorph pattern like potato starch, which is an indigestible native starch. These changes at the molecular and crystal levels might explain its resistance to PPA hydrolysis. The wx/ae starch granules have many pores on their surfaces (Takagi et al., 2017). Surface pores enable hydrolysis by amylases, and may act as sites of initial enzyme hydrolysis and the pathway to the granule interior for enzymes (Fannon et al., 1992). Maize starch, which contains surface pores, is hydrolyzed by PPA faster than rice starch, which is characterized by relatively few pores (Kong et al., 2003). As shown in previous reports, pores on the starch surface affect starch digestibility; however, wx/ae starch is resistant to PPA hydrolysis. This implies that the surface pores of wx/ae starch differ from those of other starches, such as maize.

**Morphology of partially digested starches**

SEM images of approximately 20% and 35% digested starches are shown in Fig. 5. Starch samples of the former correspond to 8, 0.5 and 0.5 h reactions of wx/ae, wx and WT, respectively, and those of the latter correspond to 24, 2 and 2 h reactions. Many pores were observed on the surfaces of digested wx and WT starches, but not digested wx/ae starch, which was instead characterized by numerous surface cracks. The pores on the granule surface of wx and WT starches shared the typical pattern of α-amylase hydrolysis, which is in good agreement with previous studies (Gallant et al., 1992, Oates, 1997, Zhang et al., 2006, Zhu et al., 2011, Man et al., 2013). We propose that following hydrolysis, the surface pores have been enlarged by PPA or that weak areas (e.g., the amorphous lamellae) were
degraded by PPA. The cracking and erosion observed on the digested \(wx/ae\) starch surface are similar to the amylase hydrolysis patterns of high-amylose rice and potato starches (Man et al., 2013, Dhital et al., 2014), which, like the \(wx/ae\) starch, are B-type polymorphs. Man et al. discuss differences in digestion from the viewpoint of the higher-order structure of starch granules. They concluded that high amylose rice starch is resistant to PPA hydrolysis because of the B-type crystallinity and the higher-order structure in the peripheral region of subgranules and the surrounding band.

Perhaps differences in the hydrolysis pattern of \(wx/ae\) starch might be attributable to its higher-order nanostructure, which is related to the primary and crystal structures resulting from the double mutation. It is necessary to explain why the \(wx/ae\) starch shows greater resistance to digestion than the other two starches despite having many surface pores. Some of the genes coding for \(\alpha\)-amylases are upregulated by more than 100% 15 days after pollination (Takagi et al., 2017). Perhaps the digestible part of starch granules is eroded during maturation and pore development. Further research is needed to clarify the process of pore development and the behavior of pores in hydrolysis by amylolytic enzymes.

**Conclusions**

We revealed that \(wx/ae\) starch has 80% more capacity to bind PPA than \(wx\) and WT starches. We attribute this large binding capacity to the 80% larger surface area of the \(wx/ae\) starch. The \(wx/ae\) starch showed only 32% digestion by PPA in 24 h, while the other two starches were almost completely digested after 8 h. The hydrolysis pattern of \(wx/ae\) starch also differed from that of the other two starches, which showed the typical pattern of hydrolysis by \(\alpha\)-amylases, and was similar to that of other B-type starches, such as high-amylose rice and potato.

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**References**


