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Note

Reaction of enzymes with starch granules: reaction of isoamylase with native and gelatinized granules [1]

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Starch granules are generally considered to be resistant to amylase hydrolysis. Reports of starch granule hydrolysis by amylases have been variable [1–6]. Leach and Schoch [3] reported that starch granules from various botanical sources had widely different susceptibilities to hydrolysis by a bacterial alpha-amylase from *Bacillus subtilis*. They found that waxymaize starch was the most susceptible and potato and amylomaize-7 starches were the least susceptible. Valetudie et al. [5] found that B-type starches, such as potato starch, were only slightly eroded from the outside of the granule. Taniguchi et al. [7], however, found an unusual alpha-amylase from a *B. circulans* strain that was specifically induced by either solubilized potato starch or by potato starch granules. The enzyme was able to completely digest potato starch granules. It degraded the granule by acting on the exterior surface, producing ridges and furrows that ringed the granule. Hollinger and Marchessault [8] reacted potato starch granules with *B. amyloliquefaciens* alpha-amylase and found that this enzyme entered the granule and produced an ordered, lamellar structure with a central core inside the granule.

Kimura and Robyt [9] studied the reaction of *Rhizopus niveus* glucoamylase (GA1) on starches from waxymaize, maize, barley, tapioca, amylomaize-7, shoti, and potato. The starches fell into three groups based on their susceptibilities. Waxymaize starch was the most susceptible, being nearly 100% converted into D-glucose by 200 units/mL in

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32 h. Barley, maize, and tapioca starches were in an intermediate group, being converted into 82, 77, and 75% D-glucose by 200 units/mL in 32 h, respectively. The third and least susceptible were amylomaize-7, shoti, and potato starches, being converted into 21, 15, and 13% D-glucose by 200 units/mL in 32 h, respectively. The enzyme entered the granules through pores and reacted with the starch chains in the interior of the granules.

Kimura and Robyt [10] also have shown that gelatinization of the least susceptible group significantly enhanced their susceptibility to glucoamylase hydrolysis. The least susceptible, potato starch, was increased 6.7-fold to 87% conversion to D-glucose. Amylomaize-7 starch was increased to 45% conversion and shoti starch was increased to 21% conversion.

To date, the only studies of enzymes acting on native starch granules have been the α-(1 $\rightarrow$ 4) hydrolases, the alpha-amylases, and glucoamylase. In the present study, we report on the action of an α-(1 $\rightarrow$ 6) starch-debranching hydrolase, *Pseudomonas amyloderamosa* isoamylase (E.C. 3.2.1.68), with native starch granules and with gelatinized starch granules. Gelatinization, here, means the swelling and hydration of the starch granules by heating the granule in water for 1 h at the onset gelatinization temperature of the specific type of starch [11].

1. Experimental

Starches.—Waxymaize and maize starches were obtained from American Maize Co. (Hammond, IN). Amylomaize-7 (Hylon VII, high amylose maize starch with 70% amylose), tapioca, and potato starches were obtained from National Starch and Chemical Co. (Bridgewater, NJ). Large granule barley starch was a gift from Dr. J.-l. Jane of Iowa State University who obtained it from Professor K.S. Poutanen (VTT Food Research Laboratory, Espoo, Finland). Shoti starch is from the wild turmeric tuber of the curcuma species, *Curcuma zedoaria* [12] and was a gift to the Laboratory of Carbohydrate Chemistry and Enzymology at Iowa State University by the late Dr. T.J. Schoch. The granules contained 10–15% (w/v) H_2O.

Enzyme.—Pseudomonas amyloderamosa isoamylase was obtained from Hayashibara Biochemical Laboratories, Ltd. (Okayama, Japan); 0.1 g of enzyme powder was added to 1.0 mL of 0.5 M pyridinium acetate buffer (pH 5.0); this suspension was kept at 4 °C for ~ 15 h. The suspension was then centrifuged and the supernatant was dialyzed against 1 L of 0.2 M pyridinium acetate buffer (pH 5.0) at 4 °C for 2 days, with the buffer being changed every 12 h. Its activity was determined by reaction at 37 °C with potato amylopectin (50 mg/mL) buffered with 40 mM pyridinium acetate (pH 5). Aliquots were taken with time and the increase in the reducing value was measured by a micro copper–bicinchoninate assay [13]. One international unit (IU) of isoamylase was that amount of enzyme that released 1 μmol of maltose equiv/min from potato amylopectin. The activity of the dialyzed enzyme was 110–120 IU/mL.

Gelatinization of starch granules.—Potato, waxymaize, shoti, and amylomaize-7 starches were heated in H_2O (10 g/100 mL) for 1 h at their onset gelatinization temperatures of 60, 65, 70, and 90 °C, respectively. After heating, the starches were removed by centrifugation and spread out on a plastic dish to dry at room temperature

(20–21 °C) for 7 days. The gelatinized granules remained particulate and the percent recovered averaged 91%. The air-dried gelatinized granules contained 7–9% (w/v) H_2O. The gelatinized granules were only weakly birefringent; scanning electron microscopy showed some distortion ($\approx$ 10%) of the granules with a minimum of damage (5%).

Reaction of isoamylase with the starches.—Starch granules (either native or gelatinized, 30 mg) were suspended in 120 μL of isoamylase solution (50 IU/mL), containing 0.5 M pyridinium acetate buffer (pH 5.0) and 0.1% (w/v) sodium azide, and incubated at 4 °C for 1 h. Then, 480 μL H_2O was added and the digest incubated at 45 °C for 144 h. The final conditions of the digest were: 5% (w/v) starch, 10 IU/mL isoamylase, 0.1 M pyridinium acetate buffer (pH 5.0), and 0.02% (w/v) sodium azide. Two aliquots were taken from the digest: the first aliquot (150 μL) was centrifuged, the supernatant was removed, 300 μL H_2O was added, and it was then heated in a boiling-water bath for 7 min. The granules were centrifuged, resuspended in 1 mL H_2O and placed in a boiling-water bath for 10 min, and then solubilized by autocleaving for 20 min at 121 °C. The second aliquot (50 μL) was added to 950 μL H_2O, placed in a boiling-water bath for 10 min, and solubilized by autocleaving for 20 min at 121 °C. The reducing values were determined for the two aliquots by using the micro copper–bicinchoninate method [13].

The same procedure was followed for the reactions containing ethanol. In these digests, 480 μL of 25, 50, 75, 87.5, and 100% (v/v) EtOH was substituted for 480 μL H_2O to give 20, 40, 60, 70, and 80% (v/v) EtOH, respectively.

2. Results and discussion

Ps. amyloderamosa isoamylase hydrolyzes the α-(1 → 6) branch linkages of starch. It has the specificity to hydrolyze the α-(1 → 6) branch linkages of relatively long starch chains and can completely hydrolyze all of the branch linkages of amylopectin [14]. The enzyme is reported to be similar to glucoamylase and have a starch-binding domain [15]. The enzyme, thus, should be capable of hydrolyzing the α-(1 → 6) branch linkages found in starch granules.

Reaction of isoamylase with seven types of starches at 37 °C for 32 h showed that different types of starches had different susceptibilities (Table 1). The percent hydrolysis was based on the amount of reducing value obtained from the reaction with native starch granules divided by the reducing value obtained by reaction with solubilized starch. Two of the three starches that were quite resistant to glucoamylase hydrolysis, amylomaize-7 and shoti starches [9], gave the highest percent reaction with isoamylase, 11.9 and 11.6% reaction, respectively. Both of these starches have relatively low molecular weights and, thus, have shorter chains than most of the other starches.

Maize, waxymaize, and barley starches were intermediate in their susceptibility to isoamylase hydrolysis, giving 7.3, 7.8, and 6.2% reaction, respectively. Tapioca and potato starches were the least susceptible, giving 3.6 and 3.7% reaction, respectively. Potato starch was also the most resistant to glucoamylase hydrolysis, and tapioca starch was intermediate in its susceptibility to glucoamylase hydrolysis [9].

Table 1
Reaction of native starch granules with 10 IU/mL isoamylase at 37 °C for 32 h

Starch	% In supern. [a,b]	% In granule [a,b]	% Total reaction [b,c]
Tapioca	61.1	38.9	3.6
Potato	68.2	31.8	3.7
Barley	75.8	24.2	6.2
Maize	43.8	56.2	7.3
Waxymaize	64.1	35.9	7.8
Shoti	47.4	52.6	11.6
Amylomaize-7	56.3	43.7	11.9

[a] Percent in the supernatant and in the granule represents the percent of the product obtained from the total reaction.
[b] The data are reported as the means of triplicate measurements and have an accuracy of ±0.1%.
[c] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.

It is interesting to note that different percents of the products were released into the reaction solvent and there was a certain amount of the amylodextrin product that remained in the granule (Table 1). Maize, amylomaize-7, and shoti starches had 56, 44, and 53%, respectively, of the amylodextrin chains remaining in the granule.

It was found that the temperature of the reaction could be increased to 45 °C when the reaction involved either native starch granules or gelatinized starch granules. In a study of the action of glucoamylase [10] with gelatinized starch granules, it was found that gelatinization times beyond 1 h did not significantly change the glucoamylase susceptibility of the granules. Hence, in this study, we only gelatinized the granules for 1 h. The gelatinized starch granules were reacted with isoamylase at 45 °C for 144 h. The percent reaction increased significantly with the gelatinized granules (Table 2). Gelatinized potato starch had a dramatic 15.2-fold increase to 56% reaction. Amylomaize-7, waxymaize, and shoti starches also increased, although not as dramatically, to 36, 36, and 22% reaction, respectively. The two native starch granules from amylomaize-7 and shoti, which had the highest percent reaction, gave only a moderate 3-fold and 2-fold

Table 2
Reaction of gelatinized starch granules with 10 IU/mL isoamylase at 45 °C for 144 h

Starch	% Reaction of gelatinized granules [a,b]	% Reaction of native granules [a,b,c]
Potato	56.2	3.7
Amylomaize-7	36.4	11.9
Shoti	21.8	11.6
Waxymaize	36.4	7.8

[a] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.
[b] The data are reported as the means of triplicate measurements and have an accuracy of ±0.1%.
[c] The native starch granules are the results of reaction with isoamylase at 37 °C for 32 h.

Table 3
Effect of various concentrations of ethanol on the reaction of gelatinized waxymaize starch granules with 10 IU/mL isoamylase at 45 °C for 144 h

% EtOH	% In supern. [a,b]	% In granule [a,b]	% Total reaction [b,c]
0	84	16	36
20	82	18	35
40	56	44	33
60	10	90	31
70	2	98	31
80	0	100	31

[a] Percent in the supernatant and in the granule represents the percent of the total reaction obtained.
[b] The data are reported as the means of triplicate measurements and have an accuracy of $\pm 0.1\%$.
[c] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.

increase, respectively, when gelatinized; and potato starch, whose native starch granules had a low susceptibility (3.7%) to isoamylase hydrolysis, had its susceptibility dramatically increased to 56% when gelatinized. This is similar to the results that were obtained for the reaction of glucoamylase with native and gelatinized starches [10].

Gelatinized waxymaize starch, the most highly branched starch on a weight basis, was reacted with isoamylase at 45 °C for 144 h in the presence of different percents of ethanol (Table 3). The reason for doing this was to see if a high percentage of the hydrolyzed amylodextrin chains could be kept in the granule after the α-(1 → 6) linkages were cleaved. Without any ethanol, only 16% of the hydrolyzed chains remained in the granule. With 20% EtOH, there was a slight increase to 18% of the hydrolyzed chains in the granule. As the concentration of ethanol was increased further, there was a rapid increase of the percent of chains that remained in the granule, although there also was a slight decrease in the overall percent of the reaction. At 60% EtOH, 90% of the chains remained in the granule; at 70% EtOH, 98% of the amylodextrin chains remained in the granule; and at 80% EtOH, 100% of the chains were in the granule.

We compared the percent of the chains that remained in the granule when the reaction was conducted in H_2O and in 80% EtOH for four gelatinized starches from potato, amylomaize-7, waxymaize, and shoti (Table 4). For potato, waxymaize, and shoti starches, 100% of the chains remained in the granule when the reaction was conducted in 80% EtOH. For amylomaize-7 starch, 98% of the amylodextrin chains remained in the granule and 2% of the chains was released into the reaction solvent. These released amylodextrin chains must have a relatively low degree of polymerization. The percent of reaction for amylomaize-7 and shoti starches dropped significantly when the reaction was conducted in 80% EtOH. The percent of reaction from potato and waxymaize starches also dropped but not as extensively. The addition of ethanol to concentrations of 60–80% (v/v) most probably prevents the release of the amylodextrin chains from the granule by precipitation of the chains as they are released from the starch by the isoamylase-catalyzed hydrolysis of the α-(1 → 6) branch linkage.

Table 4
Reaction of gelatinized starch granules from various botanical sources with 10 IU/mL isoamylase at 45 °C for 144 h in H2O and in 80% EtOH

Starchesa	Solvent	% In supern. [b,c]	% in granule [b,c]	% Total reaction [d]
Potato (60 °C/1 h)	H_2O	57	43	56
	80% EtOH	0	100	41
Amylomaize-7 (90 °C/1 h)	H_2O	76	24	36
	80% EtOH	2	98	13
Shoti (70 °C/1 h)	H_2O	57	43	22
	80% EtOH	0	100	10
Waxymaize (65 °C/1 h)	H_2O	73	27	36
	80% EtOH	0	100	31

[a] Starches were gelatinized by heating at the temperature in parentheses for 1 h.
[b] Percent in the supernatant and in the granule represents the percent of the total reaction obtained.
[c] The data are reported as the means of triplicate measurements and have an accuracy of $\pm 0.1\%$.
[d] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.

The results show that *Ps. amyloderamosa* isoamylase will hydrolyze the α-(1 → 6) branch linkages in native starch granules to a limited extent (3.6–11.9%), depending on the type of starch. Similar to glucoamylase, there is a variation in the degree of reaction with the different types of starch granules. There was not a correlation, however, between the types of starches that were susceptible to glucoamylase hydrolysis and the types of starches susceptible to isoamylase hydrolysis. There was an increase in the percent of hydrolysis when the starches were gelatinized. In this case, there was a correlation with the hydrolysis of gelatinized starch granules by glucoamylase. Potato starch became dramatically susceptible to both glucoamylase [10] and to isoamylase. The former had an 8-fold increase and the latter had a 16-fold increase.

Even though both glucoamylase and isoamylase have starch-binding domains that allow them to attack native starch granules, they have obvious differences in their action mechanisms. Glucoamylase is an exo-acting enzyme that *requires* a non-reducing-end glucose residue for effecting hydrolysis of either the α-(1 → 4) or α-(1 → 6) glycosidic linkages. Isoamylase is an endo-acting enzyme that *requires* the binding of two starch chains to effect hydrolysis of the α-(1 → 6) branch linkage, the starch chain to which the branch chain is attached and the branch chain itself. This requirement for binding the two chains may be the impediment for a more extensive action of isoamylase in the granule (in comparison with the amount of hydrolysis that is observed for solubilized starch), due to the structure and conformation of the chains in the granule. The percent of hydrolysis of both native and gelatinized starch granules by isoamylase is undoubtedly dependent on the steric nature of the starch chains around the α-(1 → 6) branch linkage. The resistance of at least some of the α-(1 → 6) branch linkages to isoamylase hydrolysis is most likely due to the starch chains being involved in double helices and in intermolecular associations that are part of the crystalline regions in the granule. These crystalline regions do not form productive complexes with the active-site of isoamylase. Nevertheless, it remains remarkable that relatively large molecules the size of glucoamy-

lase (M_r = 74,000 Da) and isoamylase (M_r = 86,000 Da) do penetrate into the granule and perform catalysis on linkages inside the granule to the extent observed.

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