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Note

Reaction of enzymes with starch granules: enhanced reaction of glucoamylase with gelatinized starch granules [1]

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Glucoamylase [(1 → 4)-α-D-glucan glucohydrolase, E.C. 3.2.1.3] is an enzyme with three domains — an active-site domain, a glycosylated linker domain, and a starch-binding domain [1–4]. It is an exo-acting enzyme that produces β-D-glucose and can hydrolyze both the α-(1 → 4) and α-(1 → 6) glycosidic linkages, completely converting solubilized starch into D-glucose [5]. The presence of the starch-binding domain allows the enzyme (GA1) to hydrolyze whole starch granules. Removal of the starch-binding domain by proteolysis produces an enzyme (GA2) that can hydrolyze solubilized starch but can no longer hydrolyze whole starch granules [4].

The term "gelatinization" generally is used to describe the swelling and hydration of granular starches [6]. The process has also been described as the "melting" of the crystalline parts of the starch granule [7]. It is induced by heating the starch granule in water. The temperature at which the process is initiated is reported by the loss of birefringence and is called the *onset gelatinization temperature*. Gelatinization occurs over a narrow temperature range that is specific for each type of starch granule.

Previously, we showed [8] that starch granules from different botanical sources had widely different susceptibilities for hydrolysis by glucoamylase. The different starches from waxymaize, maize, barley, tapioca, potato, amylomaize-7, and shoti fell into three

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groups, based on their susceptibilities toward glucoamylase hydrolysis. Waxymaize starch was the most susceptible, being converted into 50, 95, and 98% D-glucose in 32 h by three concentrations of enzyme (2, 20, and 200 units/mL). Starches from barley, maize, and tapioca were in an intermediate group that was converted into 10–15, 60, and 75–80% D-glucose in 32 h of reaction by the three concentrations of enzyme, respectively. The third group from potato, amylomaize-7, and shoti was the least susceptible and was converted into 2–8, 9–16, and 13–21% D-glucose in 32 h by the three concentrations of enzyme, respectively.

In the present study, potato, amylomaize-7, and shoti starches were heated in water at their onset gelatinization temperatures for 0.5, 1, 2, and 4 h. This treatment increased the size of the granules and the loss of birefringence. The gelatinized granules remained particulate, but their susceptibility for hydrolysis by glucoamylase was significantly increased over that observed for the native starch granules.

1. Experimental

Enzyme.—*Rhizopus niveus* glucoamylase was obtained in pure-grade (free from alpha amylase) from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. This was the same enzyme preparation used in ref. [8]. Its activity was determined by reaction at 37 °C with soluble starch (50 mg/mL) that was prepared by heating potato starch at 65 °C in ethanol with 0.36% (w/v) HCl for 60 min [9]; the starch was buffered with 100 mM acetate (pH 4.6). Aliquots (150 μL) were taken with time (0–20 min) for determining the amount of D-glucose released, which was determined by a microsample plate glucose oxidase assay [10]. One international unit (IU) was the amount of glucoamylase that released 1 μmol of D-glucose per min.

Starches.—Potato starch and amylomaize-7 starch (Hylon VII, a maize starch with 70% amylose) were obtained from National Starch and Chemical Co., Bridgewater, NJ. Shoti starch was from the wild turmeric tuber *Curcuma zedoaria* [11] and was a gift to the Laboratory of Carbohydrate Chemistry and Enzymology at Iowa State University by the late Dr. T.J. Schoch. Shoti starch is an unusual starch that has flat, disc-like granules (24–60 nm) [12]. It has an onset gelatinization temperature of 70 °C and a very high ionic charge due to the presence of 0.2% covalently linked phosphate ester (which is three times that of potato starch). It also has a higher content of amylose (35–40%) than any other known root or tuber starch and a low lipid content (< 0.01%) [11].

Preparation of gelatinized starches.—Potato, amylomaize-7, and shoti starches were heated in water (10 g/100 mL) at their onset gelatinization temperatures, 60, 90, and 70 °C, respectively, for 0.5, 1, 2, and 4 h. After heating, the starches were removed by centrifugation and spread out on a plastic dish to dry at room temperature (20–21 °C) until a constant weight was obtained (about 7 days). The gelatinized granules remained particulate. The percent recovery of the gelatinized starches was obtained by dividing the weight of the gelatinized starch by the weight of the native starches after correction for their moisture content. The moisture content was determined by dissolving a known weight of the starches in water and determining the amount of dissolved starch by the micro phenol–sulfuric acid procedure [10]. The average percent of gelatinized granules

Table 1
Percent of recovered gelatinized starch [a]

Gelatinization time (h)	Potato starch (%)	Amylomaize-7 starch (%)	Shoti starch (%)
0.5	88.0	90.5	92.1
1	85.6	94.5	93.2
2	85.2	99.0	89.8
4	89.5	94.8	93.5

[a] Percent recovered is based on the weight obtained after drying of the gelatinized starch at room temperature until a constant weight was obtained divided by the weight of the starting starch, each corrected for the amount of water in the granules.

recovered was 91%. The air-dried gelatinized granules contained 7–9% (w/w) water. The gelatinized granules were only weakly birefringent; scanning electron microscopy showed some distortion (≈ 10%) of the granules with a minimum of damage (≈ 5%).

Enzyme reaction with gelatinized starch.—Gelatinized starch (75 mg) was suspended in 150 μL of 0.2% (w/v) sodium azide. The reaction was started by adding 1.35 mL of glucoamylase (22.2 IU/mL in 44.4 mM acetate buffer, pH 4.6). The reaction was run at 37 °C, and 150-μL aliquots were removed at various times over a 32-h period. The pH of the aliquot was adjusted to 2 by the addition of 27.5 μL of 0.2 M HCl to stop the reaction; it was centrifuged for 1 min, and the supernatant was placed in a boiling-water bath for 5 min, and 35-μL 0.2 M NaOH in 0.1 M Tris–HCl was added. The centrifuged starch was suspended in 1.5 mL of water and placed in a boiling-water bath for 10 min and then neutralized by adding 5-μL 0.02 M NaOH in 0.1 M Tris–HCl. The starch was dissolved by autoclaving for 20 min at 121 °C. The amount of D-glucose formed was determined in each of the solutions, the supernatant and the dissolved starch, by using the micro glucose oxidase procedure [10].

2. Results

The percent of gelatinized starch recovered for the three types of starches heated at the four periods of time (0.5, 1, 2, and 4 h) is given in Table 1. The percent recovered was well over 88% in each case and averaged 91.3%. The length of time that the starches were heated did not affect the percent of starch recovered.

Table 2
Percent conversion to D-glucose by a 32-h reaction of 20 IU/mL of glucoamylase with starches gelatinized for different times

Gelatinization time (h)	Potato starch (%)	Amylomaize-7 starch (%)	Shoti starch (%)
0.5	64.2 [a]	38.3	13.9
1	69.4	39.7	14.8
2	71.5	41.9	15.3
4	71.8	44.9	17.4

[a] The data are means of three determinations and have an accuracy of ±0.1%.

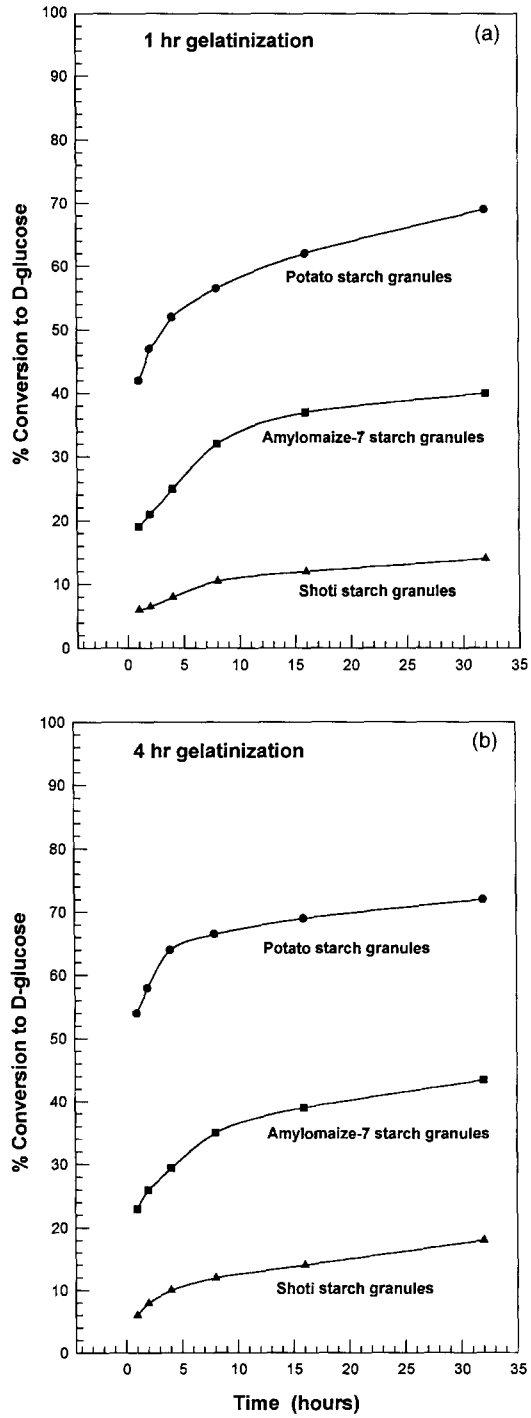
1 hr gelatinization
(a)
% Conversion to D-glucose
Potato starch granules
Amylomaize-7 starch granules
Shoti starch granules
4 hr gelatinization
(b)
% Conversion to D-glucose
Potato starch granules
Amylomaize-7 starch granules
Shoti starch granules
Time (hours)

Table 3
Comparison of the percent conversion to D-glucose of native and gelatinized starches using 20 and 200 IU/mL amounts of glucoamylase

Type of starch	Native starch [a]		Gelatinized starch [b]	
	20 IU/mL (%)	200 IU/mL (%)	20 IU/mL (%)	200 IU/mL (%)
Potato	8.9 [c]	12.6	69.4	86.6
Amylomaize-7	15.6	20.9	39.7	45.0
Shoti	11.2	14.7	14.8	21.1

[a] Data from ref. [8]
[b] Starches were gelatinized for 1 h; potato starch at 60 °C; amylomaize-7 starch at 90 °C; and shoti starch at 70 °C.
[c] The data are means of three determinations and have an accuracy of ±0.1%.

Each of the gelatinized starches was reacted with glucoamylase for 32 h and the percent D-glucose produced was measured to determine the effect of the gelatinizing time on the susceptibility of the starches to glucoamylase hydrolysis. The results are shown in Table 2.

The time course of the reactions of glucoamylase on the three starches that were gelatinized for 1 and 4 h are given in Fig. 1a and b, respectively. A comparison of the reaction of the native starch granules and gelatinized starch granules for 32 h of reaction with two concentrations of enzyme (20 and 200 IU/mL) is given in Table 3. Of the three starches, gelatinized potato starch had the highest percent conversion to D-glucose, being converted 7.8- and 6.9-fold over the conversion of the native potato starch for the two concentrations of enzyme, respectively. In comparison, gelatinized amylomaize-7 starch was converted 2.5- and 2.2-fold for the two concentrations of enzyme over native amylomaize-7 starch, and gelatinized shoti starch was only converted 1.3- and 1.4-fold for the two concentrations of enzyme over native shoti starch.

The results of Fig. 1 and Tables 2 and 3 show that gelatinized potato starch was the most susceptible to glucoamylase hydrolysis, being converted in 32 h of reaction by 20 IU/mL of glucoamylase to 69.4, 71.5, and 71.8% D-glucose after gelatinization times of 1, 2, and 4 h, respectively. This is a significant 8-fold increase over the 8.9% conversion of the native starch granules in 32 h of reaction with 20 IU/mL of enzyme. The increase in the susceptibility of the gelatinized potato starch was not very great after heating for an additional hour, increasing only 2.1% for 2 h of gelatinizing the starch and only an additional 0.3% after 4 h of gelatinizing the starch.

After 32 h of reaction with 20 IU/mL of glucoamylase, gelatinized amylomaize-7 starch was converted to 39.7, 41.9, and 44.9% D-glucose for granules gelatinized for 1, 2, and 4 h, respectively. This represents a 2.5-fold increase over the 15.6% conversion of the native amylomaize-7 starch granules in 32 h of reaction with 20 IU/mL of enzyme. The increase in the susceptibility of amylomaize-7 starch after heating for 4 h

Fig. 1. Time course of the reaction of 20 IU/mL of glucoamylase at pH 4.6 and 37 °C with gelatinized potato, amylomaize-7, and shoti starches: (a) reaction with starch gelatinized for 1 h; and (b) reaction with starch gelatinized for 4 h. The data are means of three determinations and have an accuracy of ±0.1%.

was greater than the potato starch, increasing 5.2%, although the overall conversion of 45.0% using 200 IU/mL of glucoamylase was significantly lower than that of potato starch, but significantly higher than native amylomaize-7 starch or gelatinized shoti starch.

In 32 h of reaction, gelatinized shoti starch was converted to 14.8, 15.3, and 17.4% D-glucose for granules gelatinized for 1, 2, and 4 h, respectively. This represents only a very modest increase of 1.4-fold over the 11.2% conversion of the native shoti starch granules to D-glucose in 32 h of reaction with 20 IU/mL of enzyme.

3. Discussion

Recently, Kimura and Robyt [8] reported that potato, amylomaize-7, and shoti starches were quite resistant to hydrolysis by *R. niveus* glucoamylase. In contrast, waxymaize starch was nearly quantitatively converted into D-glucose by reaction with 20 IU/mL of glucoamylase for 32 h, and maize, barley, and tapioca starches were significantly converted (75–80%) into D-glucose by reaction with 200 IU/mL of glucoamylase for 32 h.

Jane and Robyt [13] showed that alpha amylases hydrolyze the amorphous regions of amylose-V, single-helical complexes and the amorphous regions of the double-helical, retrograded amylose. The crystalline regions of these forms of amylose remained resistant to amylase hydrolysis.

The resistance of potato, amylomaize-7, and shoti starch granules toward hydrolysis by glucoamylase suggests that they have a high degree of crystallinity in their granules in comparison with the other starches that were more susceptible to glucoamylase hydrolysis. When the three relatively resistant starches were gelatinized, potato starch became significantly more susceptible, being hydrolyzed 7- to 8-fold over native potato starch. Although gelatinized amylomaize-7 starch and gelatinized shoti starches became more susceptible to hydrolysis by glucoamylase, the increase over the native starch granules were modest with a 2.4- and a 1.4-fold increase, respectively.

Potato starch is relatively easy to gelatinize, having a relatively low gelatinization temperature of 60 °C, in contrast to 90 °C for amylomaize-7 starch and 70 °C for shoti starch. This, along with the differences in their susceptibilities to glucoamylase hydrolysis of their gelatinized starch granules, suggests that the crystalline character of the three resistant starches also differs.

Although potato starch has been reported to have a relatively low degree (22%) of crystallinity [14], its high resistance to glucoamylase and to amylase hydrolysis in general would suggest a higher degree of crystallinity. The relative ease of gelatinizing potato starch granules, however, supports the low degree of crystallinity. The resistance of native potato starch granules to glucoamylase hydrolysis could be due to a high degree of double-helical chains in potato starch granules that involve both the amylose and the amylopectin components, but a high percentage of these double-helical chains are "isolated" from each other so that they are not highly associated with each other in large crystalline net works. When potato starch granules are gelatinized, the nonassociated, double-helical chains unravel and become susceptible to glucoamylase hydrolysis.

The amylomaize-7 starch has a high (70%, w/w) amylose content [15] and a relatively low degree of polymerization (dp) of 400–450 as determined by reducing value measurements, using the copper bicinchoninate procedure [10]. These molecules could have a double-helical structure that has a high degree of inter-double-helical chain association, giving them resistance to gelatinization and to glucoamylase hydrolysis. Shoti starch also has a higher than normal percentage of amylose (39–43%) [11] and a relatively low dp of 350–400. Further, it has the highest known content (0.2%) of covalent phosphate ester [11]. The relatively high amylose content of low dp also suggests that shoti starch too could have highly inter-associated, double-helical chains that are difficult to gelatinize. This, combined with the high covalent phosphate content, is the likely cause for the low susceptibility of gelatinized shoti starch to glucoamylase hydrolysis.

For potato and shoti starches the lipid contents are very low (essentially non-detectable), and for amylomaize-7 starch it is $< 0.5\%$. Thus, the lipid contents for the three starches are quite low and are not significantly affecting the susceptibility to glucoamylase hydrolysis.

In summary, potato, amylomaize-7, and shoti starch granules were gelatinized by heating them in water at their onset gelatinization temperatures for 0.5, 1, 2, and 4 h. The recovery of the gelatinized starches averaged 91.3%. The gelatinized granules had increased the susceptibility for hydrolysis by *R. niveus* glucoamylase. Hydrolysis of gelatinized potato starch was increased 7-fold from 13 to 87%; amylomaize-7 starch was increased 2-fold from 21 to 45%; and shoti starch was increased 1.4-fold from 15 to 21%. The increases in the susceptibility of gelatinized starch granules to glucoamylase hydrolysis were interpreted as being due to a decrease in the crystallinity of the granules as intermolecular hydrogen and hydrophobic bonds between double helices of the starch chains are broken during the melting process of gelatinization. The differences observed between gelatinized potato starch and the amylomaize-7 and shoti gelatinized starches are interpreted as being due to the higher amylose content of lower molecular size of the latter two starches. Further, the relatively greater resistance of shoti starch to gelatinization and susceptibility to glucoamylase hydrolysis is further interpreted as being due to the relatively high covalent phosphate content of shoti starch.

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