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Enzyme modification of starch granules: in situ reaction of glucoamylase to give complete retention of D-glucose inside the granule

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Abstract

Starch granules have been modified in situ by using a reaction system in which glucoamylase reacts inside starch granules to give conversions of 10–50% D-glucose inside the granule. Waxy maize, maize, and amylomaize-7 starches were converted to the extent of 18.4, 15.0, and 9.4%, respectively, after reaction for 24 h and to 51.0, 33.8, and 24.4%, respectively, after reaction for 288 h. The reaction conditions, of solid granules with a relatively low amount of water and glucoamylase inside the granule, gave 100% retention of D-glucose inside the granule. The amount of D-glucose obtained inside the granule depends on the length of time of the reaction and the type of starch. The action of glucoamylase inside the granule can be terminated by heating at 110 – 120° C for 20 min. By stopping the reaction at selected periods of time, starch granules were obtained that contained 10–50% (w/w) D-glucose inside the granules.
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1. Introduction

Glucoamylase [1,4-α-D-glucan glucohydrolase, EC 3.2.1.3] is an exo-acting enzyme that produces D-glucose from the nonreducing ends of starch chains. It is capable of catalyzing the hydrolysis of both α-(1→4) and α-(1→6) glycosidic linkages of dissolved starch, thereby giving 100% conversion of starch to D-glucose [1]. Glucoamylase has a starch-binding domain that is distinct from the starch-hydrolyzing domain (active site) [2,3]. The starch-binding domain is essential for the enzyme to hydrolyze insoluble, whole starch granules [4,5]. Starch granules adsorb glucoamylase from solution in different proportions (33–62%), depending on the kind of starch [6].

In general, starch granules have been thought to be resistant to amylase hydrolysis. It has been found that different kinds of amylases produced different degrees of hydrolysis of starch granules [7,8]. Sandstedt and Gates [7] found that pancreatic alpha amylase was the most effective, followed in order by malt, bacterial, and fungal alpha amylases. Leach and Schoch [8] showed that the reaction of *Bacillus subtilis* (now known as *B. amyloliquefaciens*) alpha amylase with different starches from various botanical sources gave widely different degrees of hydrolysis, with waxy

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maize starch being the most susceptible and high-amylose maize starch being the least susceptible. It has also been shown that alpha amylase reacts with starch granules *inside* the granule to produce a series of concentric, resistant starch chains arranged around a core [9–11]. Glucoamylase also enters the starch granule to produce holes in the granules and granule shells or granule ghosts by reacting with the starch chains inside the granule [6].

Kimura and Robyt [6] found that starches can be divided into three groups, according to their susceptibilities toward glucoamylase hydrolysis: Group I, very susceptible (waxy maize starch), Group II, moderately susceptible (barley, maize, and tapioca starches), and Group III, relatively resistant (amylomaize-7, shoti, and potato starches). During the early stages of the glucoamylase reaction (for example, 2 h), 8–32% of the D-glucose remained inside the granule, but during the later stages (24 h), over 80% was in the reaction supernatant outside the granule.

The present study reports the reaction of glucoamylase inside the granules of waxy maize, maize, and amylomaize-7 starches to produce 100% of the D-glucose inside the granule. The amount of D-glucose obtained in the granules was dependent on the length of time of the reaction and the type of starch. Controlled conditions were developed to give 10–50% D-glucose inside waxy maize starch granules.

2. Experimental

Materials

Glucoamylase. *Rhizopus neivus* glucoamylase was obtained in a form free of alpha amylase from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan) and from Sigma Chemical Co. (St. Louis, MO). The latter was used after dialyzing against 33 mM pyridine–acetate buffer (pH 5.0). Glucoamylase activity was determined by reaction at 37 °C with soluble potato starch (50 mg/mL) dissolved in 33 mM pyridine–acetate buffer (pH 5.0). The amount of glucose released was measured every 5 min for 30 min by a micro glucose oxidase assay, using a microsample plate [12] and the slope of the resulting line determined. One international unit (IU) is defined as the amount of glucoamylase that released 1 μmol of D-glucose per min.

Starches. The three types of starches, waxy maize (containing 100% amylopectin), maize (containing 29% amylose and 71% amylopectin), and amylomaize-7 (containing 70% amylose and 30% amylopectin) were obtained from Cerestar International (Hammond, IN) and used without any further treatment. Soluble starch was prepared by heating 25% (w/v) potato starch in anhydrous ethanol with 0.36% (w/v) HCl at 60 °C for 60 min, neutralizing, filtering, and washing with water [13].

Methods

Reaction of the starches with glucoamylase. The reactions contained 50 mg of starch granules suspended in 1 mL of 33 mM pyridine–acetate buffer (pH 5.0), containing 0.02% (w/v) NaN_3; 2 IU of glucoamylase was added and the reactions incubated for 30 min at 37 °C to allow the enzyme to be adsorbed. The starch was then removed by centrifugation (1 min at 10,000*g*). The starch was washed by suspending in 2 mL of buffer for ~1 min and then filtered on a glass filter.

Three reaction conditions were studied: R-1 in which the starch granules were suspended in 1 mL of 33 mM pyridine–acetate buffer (pH 5.0); R-2 in which the starch granules were sealed in a glass tube; and R-3 in which the starch granules were in an open tube. The three types of reactions were placed in a 37 °C incubator for various lengths of time. The R-1 reaction was stopped by adding 160 μL of 0.2 M HCl (pH 2). The starch was removed by centrifugation and the supernatant decanted. The starch was washed and the washing added to the supernatant. The two, the supernatant and the starch suspended in 1 mL of water, were heated in a boiling-water bath for 10 min to inactivate the enzyme. They were then neutralized by the addition of 0.2 M NaOH in 0.5 M Tris–Cl to give a pH 6.9. The starch was dissolved by heating the suspension for 20 min at 121 °C in the autoclave. The amount of D-glucose in each of the samples, the supernatant and the dissolved starch, was determined by a micro glucose oxidase analysis [12].

The starch in the R-2 and R-3 reactions was treated in a similar manner. The starch samples were suspended in 1 mL of 33 mM pyridine–acetate buffer (pH 5.0) and 160 μL of 0.2 M HCl was added and they were heated in a boiling-water bath for 10 min. The samples were neutralized by the addition of of 0.2 M NaOH in 0.5 M Tris–Cl to give a pH of 6.9. The starch was dissolved by heating for 20 min to 121 °C in the autoclave and the amount of D-glucose in each of the samples was determined by micro glucose oxidase analysis [12]. The percent of hydrolysis of each starch was computed by the following:

$$\% \text{ Hydrolysis} = \text{mg D-glucose}/50 \text{ mg starch} \times 100$$

Large-scale solid granule enzyme reaction. For the large-scale enzyme reaction, using R-2 conditions, 10 g of waxy maize starch was suspended in 200 mL of 33 mM pyridine–acetate buffer (pH 5.0), containing 0.02% (w/v) NaN_3; 400 IU of glucoamylase was added and incubated for 30 min at 37 °C, and the suspension was then filtered through a glass filter. The starch granules were sealed in a glass vessel and incubated at 37 °C for a length of time necessary to obtain the desired percent conversion to D-glucose.

The reaction was stopped by first drying the starch granules in a stream of air for 30 min at 20 °C and then heat-treating the starch in a drying oven for 20 min at 120 °C to inactivate the glucoamylase. The length of heating time necessary to inactivate the glucoamylase was determined by sampling a reaction digest and heating for various lengths of time, and the amount of glucoamylase present determined. The heat-treated starch (10 mg) was suspended in 100 mL of 33 mM pyridine–acetate buffer (pH 5.0) to which 100 mL of 10% (w/v) soluble starch was added. After incubation for 2 h at 37 °C, the amount of D-glucose produced was determined by glucose oxidase analysis [12].

3. Results

The three reaction conditions (R-1, R-2, and R-3) contained the same amount of glucoamylase (1 IU/50 mg of starch) and the same amount of starch, but they produced different degrees of hydrolysis of waxy maize starch granules to D-glucose (Fig. 1). Reaction of waxy maize starch granules in the aqueous suspension (R-1) gave 83% conversion in 288 h of reaction and 98% conversion in 720 h of reaction. Reaction of waxy maize starch under the sealed, solid-granule reaction (R-2) gave a 51% conversion in 288 h and 53% conversion in 720 h of reaction. Reaction of waxy maize starch in the solid-granule, open-vessel reaction (R-3) gave a 24% conversion in 288 h of reaction that did not change on further incubation. The lower percentage conversion of the starch in the solid-granule reactions (R-2 and R-3) reflects the relatively low amount of water present inside the granule in contrast to the infinite amount of water present in the aqueous suspension. The further lower con-

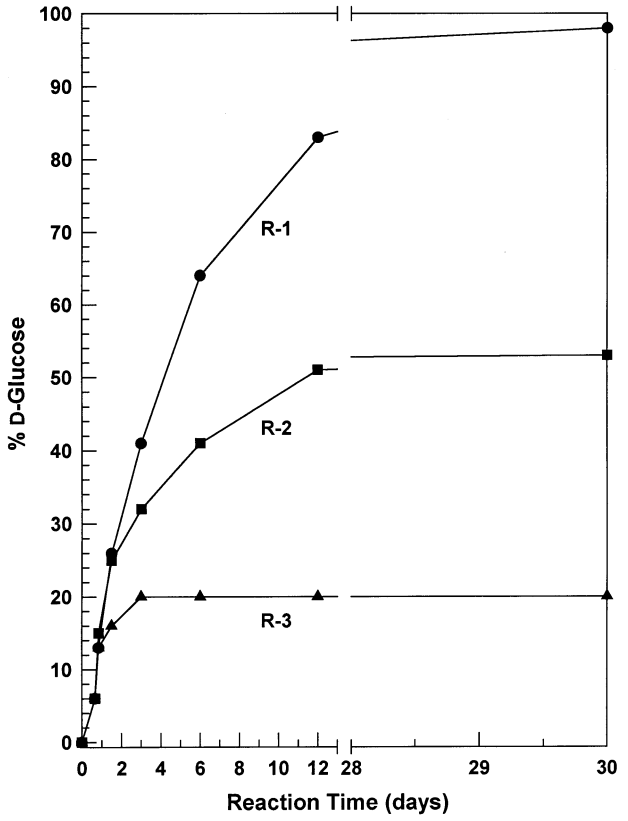

Fig. 1. Time course of the formation of D-glucose in the reaction of glucoamylase with waxy maize starch granules suspended in buffer and solid-granule reactions at 37 °C. R-1 is the reaction of 50 mg of waxy maize starch granules in 1 mL of 33 mM pyridine/acetate buffer (pH 5.0) with ~1 IU of glucoamylase; R-2 is the reaction of 50 mg of waxy maize starch granules with ~1 IU of glucoamylase in a sealed vessel; R-3 is the reaction of 50 mg of waxy maize starch granules with ~1 IU of glucoamylase in an open vessel.

Table 1
Percentage conversion to D-glucose and percentage remaining inside the waxy maize starch granules after reaction with glucoamylase in the three types of reaction conditions

Enzyme reaction [a]	Reaction time (days)			
	0.66	3	6	12
R-1	13.6 [b] (7.8) [c]	42.0 (4.6)	64.7 (2.6)	82.7 (0.4)
R-2	15.4 (100)	32.2 (100)	42.6 (100)	51.0 (100)
R-3	14.0 (100)	21.0 (100)	22.0 (100)	24.0 (100)

[a] R-1 reaction is a suspension of starch granules in an aqueous medium; R-2 reaction is starch granules (containing 50% water, w/w) in a sealed vessel; R-3 reaction is starch granules (containing 50% water, w/w) in an open vessel. All reactions had ≈ 1 IU of glucoamylase/50mg of starch and were incubated at 37 °C.

[b] The numbers refer to percentage conversion to D-glucose.

[c] The numbers in parentheses refer to percentage of D-glucose remaining inside the granule.

version of the starch granules in the open vessel reaction (R-3), as compared with the solid-granule reaction in the sealed vessel (R-2), was most probably due to loss of water by evaporation in the former.

In the solid-granule reactions (R-2 and R-3), 100% of the D-glucose was retained inside the granules, whereas in the suspension of the granules in an aqueous medium (R-1) only a very small amount of D-glucose remained inside the granule throughout the reaction (Table 1).

Fig. 2 and Table 2 give the percentage conversion to D-glucose of the three types of starches (waxy maize, maize, and amylomaize-7) reacting in the sealed-vessel, solid-granule reaction (R-2). The three starches gave different degrees of conversion from 51 to 24.4%. The amount of water in the granules was the same for the three types of starches. The differences in the percentage conversion to D-glucose of the three kinds of starches reacting with glucoamylase in the sealed-vessel, solid-granule reaction reflect differences in the composition of the three types of starches. Glucoamylase is an exo-acting enzyme that reacts with the nonreducing chain ends. The number of nonreducing chain ends decreases as the percentage of amylose increases. Waxy maize starch has 0% amylose and gave the

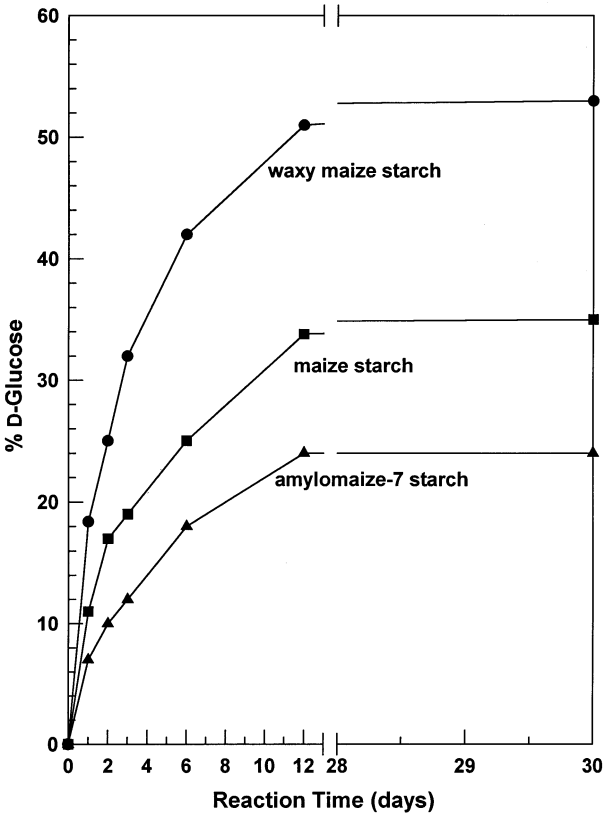

Fig. 2. Time course of the formation of D-glucose in the reaction of glucoamylase with three kinds of starch granules (waxy maize, maize, and amylomaize-7) in a sealed vessel at 37 °C.

highest percentage conversion (51%); maize starch has 29% amylose and gave an intermediate percentage conversion (33.8%); and amylomaize-7 has 70% amylose and gave the lowest percentage conversion (24.4%).

Fig. 3 shows the percentage conversion of D-glucose produced in a large-scale reaction under the sealed, solid-granule reaction conditions (R-2). The reactions were stopped at

Table 2
Percentage conversion to D-glucose for the reaction of three kinds of starch granules with glucoamylase in a sealed vessel at 37 °C

Type of starch [a]	Reaction time (days)			
	1	3	6	12
Waxy maize	18.4	32.2	42.6	51.0
Maize	15.0	24.0	32.0	33.8
Amylomaize-7	9.4	15.0	19.6	24.0

[a] Reactions were conducted with 50 mg of starch granules and ≈ 1 IU of glucoamylase in a sealed vessel at 37 °C.

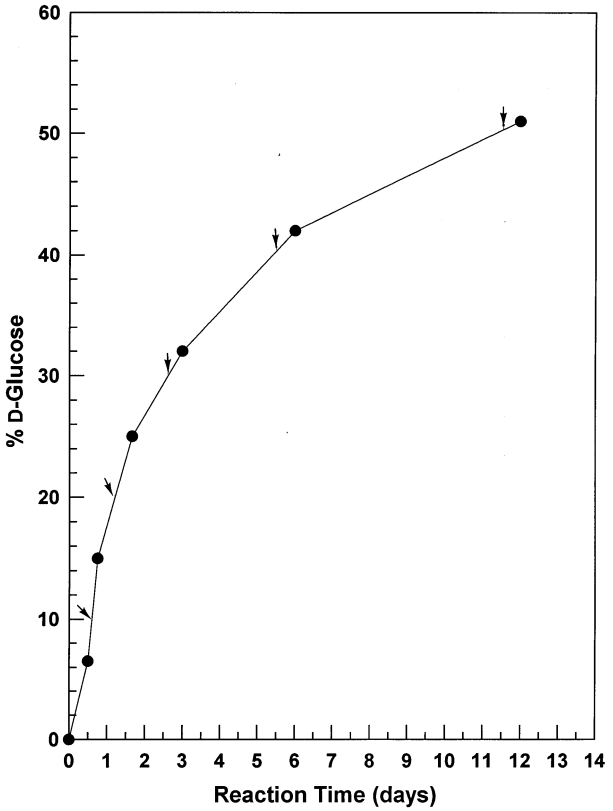

Fig. 3. Formation of varying amounts of D-glucose on a larger scale by reaction of glucoamylase with waxy maize starch granules in a sealed vessel. The arrows indicate times when the reaction was stopped to give various percentage conversions to D-glucose.

various times, as indicated in Fig. 3 by the arrows, by drying in a stream of air for 30 min at 20 °C and then heat-treatment for 20 min at 120 °C. Heating at this temperature for 10 min gave a small amount of residual glucoamylase activity, but heating for 20 min gave no residual glucoamylase activity (Fig. 4). By stopping the reaction at various times, we were able to obtain starch granules containing varying amounts of D-glucose from 10 to 50% (w/w) inside the granules.

4. Discussion

There have been several reports that starch granules have pores on their surface that go into the granule [14–16]. Fannon et al. [14] reported that the pores facilitate amylase hydrolysis and some chemical reactions of the granules. Pancreatic alpha amylase [14,17], glucoamylase [6,17,18], and isoamylase [19] have been reported to penetrate into the granule through these pores and perform catalysis inside the granule. Kimura and Robyt [6] reported that the pores appeared to be enlarged by the action of glucoamylase. They also reported that during the early stages of the reaction with glucoamylase (2 h with 2 IU/50 mg of starch), a substantial amount ($\sim 20\%$) of the D-glucose remained inside the granule, but with longer reaction times, the majority of the D-glucose diffused into the aqueous reaction medium.

In the present study, we have developed a method to retain 100% of the D-glucose inside the granule by decreasing the water to an amount equal to the weight of starch, that is 50 mg of water/50 mg of starch. This gives what we have termed a solid-granule reaction system. The relatively low amount of water inside the granule is used for hydrolysis, but the solid granules do not permit diffusion of the D-glucose out of the granule. Further, only very low amounts ($< 0.01\%$) of reversion products (maltose, isomaltose, and nigerose) were formed as estimated by TLC analysis (data not presented). By using a reactor with the starch sealed inside a vessel, we were able to convert waxy maize starch granules into

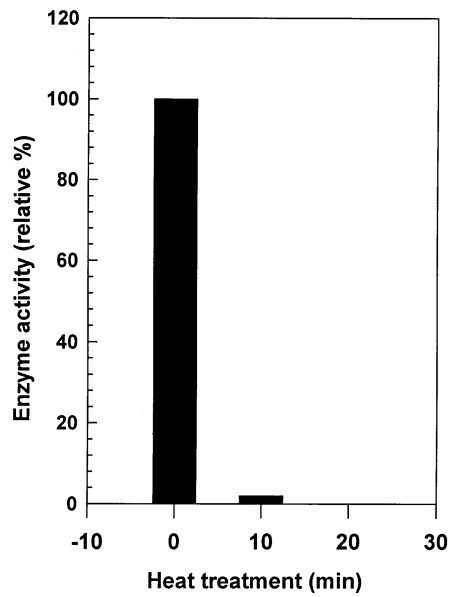

Fig. 4. Inactivation of glucoamylase adsorbed onto waxy maize starch by heating at 120 °C for various times. Starch granules were removed from the reaction at 37 °C, dried in a stream of air at 20 °C for 30 min and then heated, and the amount of glucoamylase activity remaining in the granules determined.

51% D-glucose by reaction of 1 IU of glucoamylase/50 mg of starch at 37 °C for 288 h. If the reaction is conducted in an open vessel, the reaction stops after 72 h, giving only 24% conversion to D-glucose, apparently due to the evaporation of the water from the granules.

Using the sealed, solid-granule reaction system, we obtained different maximum degrees of hydrolysis for waxy maize, maize, and amylomaize-7 starches in which the amount of conversion to D-glucose decreased in the order listed for the three types of starches. Glucoamylase does not give complete conversion of the starches due to the ultrastructure and crystallinity of the starches. The differences observed in the degrees of conversion to D-glucose of the three starches could be attributed to differences in their ultrastructure and degrees of crystallinity. Interestingly, however, the decreasing conversion to D-glucose was directly proportional to the decreasing number of nonreducing chain ends as the amount of amylose increased in the three starches. Glucoamylase, being an exo-enzyme, would have less reaction sites in the starches with increasing amounts of amylose and would thus produce less D-glucose in these starches.

We have been able to control the reaction of the solid granules in the closed vessel by heating the starch for 20 min at 110–120 °C to inactivate the glucoamylase. By using this technique and stopping the glucoamylase reaction at various times, we have obtained waxy maize starch granules that have 10, 20, 30, 40, and 50% D-glucose inside the granules. The glucoamylase-modified waxy maize starch granules, with 20% and higher amounts of D-glucose, have a sweet taste.

When the starch granules containing D-glucose are suspended in water, the D-glucose rapidly diffuses out of the granule. We are currently investigating methods for controlling the release of D-glucose from the granules at different rates.

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