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Enzyme modification of starch granules: formation and retention of cyclomaltodextrins inside starch granules by reaction of cyclomaltodextrin glucanosyltransferase with solid granules

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Abstract

Cyclomaltodextrin glucanosyltransferase (CGTase) was adsorbed into starch granules and allowed to react at 37 °C. The reaction was conducted with the granules removed from an aqueous environment, but containing 50% w/w water inside the granule. Reaction for 20 h gave a maximum of 1.4%, w/w of cyclodextrins (CDs) inside the granule. Waxy maize and maize starches gave the highest amounts of CDs (1.3 and 1.4%, respectively), with tapioca and amylomaize-7 starches giving about 50% less (0.9 and 0.6%, respectively). Reaction of a combination of CGTase and isoamylase with solid starch granules gave a 2.6-fold increase in the formation of CDs, with a maximum yield of 3.4 and 100% retention inside waxy maize starch granules.

Keywords: Starch granules; Cyclomaltodextrins; Cyclomaltodextrin glucanosyltransferase; Isoamylase; Reaction with raw starch

1. Introduction

Cyclomaltodextrin glucanosyltransferase (CGTase, EC 2.4.1.19) elaborated by *Bacillus macerans* produces cyclic maltodextrins (CDs) from starch that have six, seven and eight α-(1→4)-linked D-glucose residues. These CDs are commonly called α-, β- and γ-cyclodextrins. The CDs have interior cavities whose size and shape are determined by the number of D-glucose units [1]. The interior cavity has hydrophobic and electrophilic properties and will complex with many organic substances [1–3]. CDs, thus, can have a wide range of applications in complexing materials in foods, pharmaceuticals, plastics and agricultural products in which they can act as emulsifiers, anti-oxidants and stabilizing agents [3,4].

Glucoamylase [5,6], isoamylase [7] and CGTase [8] are enzymes that have a starch-binding domain, separate from their active sites. The starch-binding domain will bind with starch chains within the granules and produce products. The reaction takes place inside the granule and a certain percentage (8–32%) of the products remain inside the granule [9,10]. We have reported that glucoamylase acting inside solid waxy maize starch granules can produce 5–50% (w/w) of D-glucose that is

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100% retained inside the granule [11]. This reaction can be controlled to give different amounts of D-glucose inside the granule by inactivating the enzyme at different times of reaction [11]. The present study reports the formation of cyclomaltodextrins by the reaction of CGTase with raw starch granules in suspension and with solid starch granules and by the reaction of a combination of CGTase and isoamylase with solid starch granules in which the reaction with solid starch granules gave 100% retention of the CDs inside the granules.

2. Experimental

Materials

Enzymes. CGTase [EC 2.4.1.19] from *B. macerans* was obtained as an aq solution from Amano International Enzyme Co. (Troy, VA, USA), and it was dialyzed against 10 mM imidazolium–HCl (pH 6.0) containing 1 mM $CaCl_2$ to remove peptides, monosaccharides and other unwanted material. The dialysis membrane was obtained from Fisher Scientific and was Spectra/Por 3.1 regenerated cellulose, with a molecular weight cut-off of 3500 Da.

Enzyme activity of CGTase was determined by a modification of the method of Thoma et al. [12]; the reaction solution was composed of 0.3 mL of solution A (18 mM α-CD and 18 mM methyl α-D-glucopyranoside in 50 mM pyridine–acetate buffer (pH 5.0), 0.1 mL of solution B (10 IU/mL glucoamylase in 50 mM pyridine–acetate buffer, pH 5.0) and 0.1 mL of CGTase. After reaction at 37 °C for a period of time, 100 μL was taken and 20 μL of conc. HCl was added to obtain a pH of 1.5. This solution was kept at rt (20 − 22 °C) for 30 min and then neutralized. The amount of D-glucose produced was determined by the micro glucose oxidase method [13], and the CGTase activity in international units (IU) was determined as follows:

$$\text{CGTase activity (IU)} = \frac{\mu\text{mol of D-glucose/min}}{6}$$

Isoamylase [EC.3.2.1.68] from *Pseudomonas amyloderamosa* was obtained from Hayashibara Biochemical Laboratories, INC. (Okayama, Japan). The enzyme solution was prepared by dissolving the enzyme powder in distilled water and dialyzing it against 50 mM pyridine–acetate buffer (pH 5.0) for 12 h. Enzyme activity was determined by reaction with potato amylopectin (50 mg/mL) buffered with 50 mM pyridine–acetate buffer (pH 5.0) at 37 °C. Aliquots (100 μL) were taken with time and the increase in the reducing value was measured by the micro copper–bicinchoninate method [13]. One IU of isoamylase was that amount of enzyme that released 1 μmol of maltose equivalent/min from potato amylopectin as measured by reducing value.

Starches and cyclodextrins. The starches (waxy maize, maize, and amylomaize-7) were obtained from Cerestar USA, Inc. (Hammond, IN) and tapioca starch was obtained from National Starch and Chemical Co. (Bridgewater, NJ). Cyclodextrins were obtained from Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan).

Methods

Preparation of the enzyme reaction system giving CDs retained in the granule. Starch granules (50 mg) were suspended in 1 mL of 10 mM imidazolium–HCl buffer (pH 6.0), containing 1 mM $CaCl_2$ and 0.02% (w/v) NaN_3. CGTase (10 IU) was added and the suspension incubated at 4 °C for 30 min to allow the enzyme to be taken up by the granules. In case of the combination reaction of CGTase and isoamylase, 10 IU of CGTase and 2 IU of isoamylase were simultaneously added and the suspension incubated at 4 °C for 30 min to allow the enzymes to be taken up by the granules. The starch suspension was centrifuged and washed with 1 mL of buffer, and then filtered on a glass filter to obtain the enzymes inside the starch granules. The granules were sealed in a glass tube and incubated at 37 °C for various lengths of time. Starch granules (50 mg) contained 50 mg of water [11]. The reaction was stopped by opening the tube and adding 1 mL of 10 mM imidazolium–HCl buffer (pH 6.0) and 20 μL of 12 M HCl, and keeping it at rt (20 − 22 °C) for 30 min.

A similar reaction of CGTase with starch granules was conducted in an aq suspension.

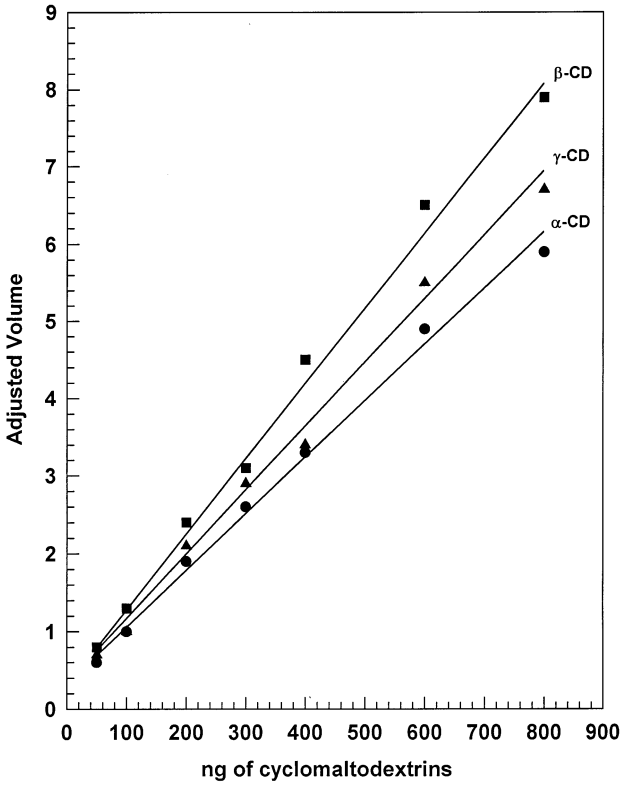

Fig. 1. TLC densitometric standard curves for α-, β- and γ-cyclomaltodextrins. The chromatography was performed on Whatman K5 TLC plates using two ascents of 18.5 cm of 85:25:50:45 acetonitrile–ethyl acetate–ethanol–water. The plates were dried and developed by dipping into 0.3% w/v *N*-(1-naphthyl)ethylenediamine and 5% v/v sulfuric acid in methanol, followed by heating for 10 min at 120 °C. The quantitative amounts of the cyclomaltodextrins were obtained by scanning densitometry.

Table 1
Percent adsorption of CGTase into waxy maize starch granules at different conditions

Adsorption (°C)	Conditions [a] (pH)	Percent CGTase adsorbed [b]
4	5.0	62
4	6.0	60
4	8.0	34
37	5.0	50
37	6.0	49
37	8.0	46

[a] Starch granules (50 mg) were added to 1.0 mL of buffer, containing 10 IU of CGTase with various buffers: 10 mM pyridine–acetate (pH 5.0); 10 mM imidazolium–HCl (pH 6.0); and 10 mM Tris–HCl (pH 8.0). Adsorption was conducted for 30 min at 4 °C or 37 °C.

[b] The starch granules were removed and the activity in the supernatant determined from which the percent CGTase adsorbed was determined.

Starch granules (50 mg) were suspended in 1 mL of 10 mM imidazolium–HCl buffer (pH 6.0), containing 1 mM $CaCl_2$, 0.02% (w/v) NaN_3, 10 IU CGTase. The reaction was conducted at 37 °C for various lengths of time, and was stopped by adding 20 μL of 12 M HCl, followed by keeping it at rt (20 – 22 °C) for 30 min.

Analytical methods. The stopped enzyme reactions were neutralized and autoclaved to gelatinize the starch. CDs in the resulting solutions were separated by TLC (Whatman K5, 20 × 20 cm), using 2 ascents of 18.5 cm of 85:25:50:45, MeCN–EtOAc–EtOH–water. Carbohydrates were visualized on the TLC by immersing the dried plate into a solution of MeOH, containing 5% (v/v) H_2SO_4 and 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine, followed by heating for 10 min at 120 °C. The CDs were quantitated on the TLC plate by using an imaging densitometer (Bio Rad, model GS-670) [14].

3. Results

The α-, β-, and γ-CDs were readily separated by the TLC system and gave a linear densitometric response between 50 and 800 ng. Each type of CD gave a separate linear curve. Standard curves (Fig. 1) were prepared for each CD to quantitatively determine the amounts of CD produced in the enzyme reaction digests.

The amount of CGTase that was taken up by the starch granules was dependent on the pH of the enzyme solution and the temperature of incubation. When waxy maize starch was at pH 8.0 and 4 °C, the starch granules took up 34% of the CGTase, and when the pH was 6.0 at 4 °C, the starch granules took up 60%. There was very little effect by the different pH values on the amount taken up (46–50%), when the starch granules were at 37 °C (Table 1). The lower temperature (4 °C) for the absorption of enzyme was desirable to keep the amount of enzyme reaction at a minimum.

CGTase was reacted with starch granules that were suspended in an aqueous solution of the enzyme and with CGTase adsorbed into

Table 2
Percent CDs formed by the reaction of CGTase with starch granules in aqueous suspension and with solid starch granules at 37 °C

	Aqueous suspension % CD formed [a]	Solid granules % CD formed [b]		Solid granules w/isoamylase % CD formed [b]	
Starch	50 h	20 h	50 h	20 h	50 h
Waxy maize	4.6	1.3	1.1	3.4	3.4
Maize	4.7	1.4	1.1	2.9	3.1
Tapioca	3.4	0.9	0.9	1.7	1.8
Amylomaize-7	2.6	0.6	0.6	1.4	1.5

[a] Total CD formed in both the supernatant and in the granules.
[b] Total CD formed in the granules.

the starch granules, containing a relatively low amount of water. Starch granules from waxy maize, maize, tapioca and amylomaize-7 were used. Waxy maize starch granules gave the highest percent conversion to CDs in both reaction conditions, with maize starch granules a close second (see Table 2). Tapioca and amylomaize-7 starch granules gave approximately 50% the amount of CDs, as did waxy maize and maize starch granules. The percent of CDs formed increases up to about 20–22 h. Reactions for longer times in the solid granule reaction, for example 140 h, gave a significant decrease of approximately 50% in the amount of CD. This decrease is most likely due to cyclomaltodextrin acceptor reactions. The maximum amount of CD inside the granule is obtained after about 20 h of reaction (Table 2). To obtain the maximum amount of CD inside the granule, it is necessary to inactivate the enzyme(s) by heating for 20 min to 120 °C.

Reaction in the aqueous suspension of starch granules gave the highest yield of CDs (Table 2). CDs were found in both the aqueous solution and inside the granules, with the amount in the aqueous solution much higher than the amount inside the granule (Table 3). Reaction of CGTase in the solid granules gave a lower yield of CDs, amounting to 25–30% of the amount found in the aqueous suspension reaction, although 100% of the CDs were retained inside the granule. The yield of CDs formed inside the granules in the solid granule reaction was increased 2.6-fold by the addition of isoamylase (Table 2). The amount of CD formed, in the solid granule reaction of waxy maize starch with the combination of CGTase and isoamylase, was 74% the amount formed in the aqueous suspension reaction of waxy maize starch granules. Table 4 gives the percent composition of the three kinds of CDs (α, β and γ) that were formed. In all of the reactions, α-CD amounted to approximately 50% of the total, β-CD approximately 40%, and γ-CD approximately 10%.

4. Discussion

Enzymes can enter starch granules through pores in the granules and catalyze reactions inside the granules [9–11,15]. The pores are approximately 1000 Å in diameter on average and can easily allow large macromolecules, such as enzymes, to enter the granule and perform catalysis. Starch granules contain 10–15% w/w water of hydration. When starch granules are suspended in an aqueous solution

Table 3
Percent CDs released into the supernatant and percent retained in the granules for the aqueous starch granule suspension reaction with CGTase after 2 and 50 h

Starches		2 h [b]	50 h
Waxy maize	S [a]	88	91
	G	11	9
Tapioca	S	89	90
	G	11	11
Amylomaize-7	S	87	88
	G	14	12

[a] S = supernatant; G = granules.
[b] The percent of CDs formed were determined by quantitative TLC.

Table 4
Percent composition of the CDs produced by CGTase in 50 h of reaction at 37 °C in aqueous suspension of starch granules and in the solid starch granule reaction with and without isoamylase

	Aqueous suspension				Solid granules				Solid granules w/ isoamylase			
Starch	Total CD [a]	α-	β-	γ- [b]	Total CD [a]	α-	β-	γ- [b]	Total CD [a]	α-	β-	γ- [b]
Waxy maize	4.6	49	40	11	1.3	49	43	8	3.4	45	41	14
Maize	4.7	46	42	11	1.4	48	41	11	3.1	52	42	6
Tapioca	3.4	51	42	7	0.9	52	42	6	1.8	53	38	9
Amylomaize-7	2.6	47	43	10	0.6	55	38	7	1.5	43	44	13

[a] Total percent (w/w) CDs produced in the reaction with starch granules.
[b] Relative percent distribution of α-, β-, γ-CDs in the total amount of CDs produced.

of enzyme, water and enzyme molecules diffuse into the granule. When the granules are removed from the aqueous solution, the water and enzyme molecules that had entered the granules are retained in the granules. The enzyme then reacts with the starch chains inside the granule, utilizing the water if required. The products of the enzyme reaction are retained inside the granule until the granules are placed in water and then they rapidly diffuse out into the aqueous medium.

Although the enzyme preparation used in this study was a commercial preparation and was not highly purified, the preparation appeared to contain only a single enzyme, CGTase, that acted on starch to give CDs. Contaminating enzymes, such as alpha-amylase or glucoamylase did not appear to be present.

Glucoamylase [9] and isoamylase [10] have been reported to enter starch granules and carry out catalysis, leaving a percentage of their products inside the granules. We previously reported the controlled conversion of solid starch granules into 5–50% D-glucose, with 100% retention of the glucose inside the granule, by the reaction of glucoamylase [11].

In this study, we report the conversion of solid waxy maize starch granules into CDs, with 100% retention of the CDs inside the granules. The percent yield of CDs formed inside the granules appears relatively low compared with the yields of D-glucose that were formed inside waxy maize starch granules by glucoamylase [11]. Table 4 shows that the distribution of the three CDs formed by CGTase in waxy maize starch is 45% α-, 41% β- and 14% γ-CD.

The relatively low yields of CDs formed by the reaction of CGTase with solid starch granules compared with the yields of D-glucose formed by the reaction of glucoamylase may be due to several factors. While both enzymes are *exo*-acting and have about the same number of glucose-binding subsites of seven to eight, glucoamylase is a hydrolase and CGTase is a glucanosyltransferase. Glucoamylase cleaves (hydrolyzes) the first α-(1→4) glycosidic linkage from the nonreducing-end of the starch chain. CGTase cleaves the 6th, 7th or 8th α-(1→4) glycosidic linkage from the nonreducing-end glucose unit and then transfers the cleaved reducing-end unit intramolecularly to the C-4-OH group of the nonreducing-end glucose residue to give CDs. Because of these differences in action, the steric requirements for the two enzymes are very different. Glucoamylase requires only the first two or three glucose residues at the nonreducing-end of the starch chain to perform catalysis. CGTase, on the other hand, requires a relatively long chain of 9–11 D-glucose residues to give CDs. Reacting with amylopectin, CGTase, therefore, requires the outermost part of a starch B-chain and nearly a complete starch A-chain of about 10–12 D-glucose residues. Further, glucoamylase can hydrolyze α-(1→6) branch linkages and thereby hydrolyze a complete starch chain. CGTase cannot cleave the α-(1→6) branch linkages and therefore cannot by-pass the branch linkage. CGTase's action, thus, stops when a branch linkage is reached. The formation of products by CGTase, therefore, is limited, whereas the formation of D-glucose by glucoamylase is substantial.

The addition of isoamylase with CGTase increased the amount of CDs that were formed from all of the starches in the solid starch granule reaction by 2.6-fold, giving maximum yields of 3.4% CDs from waxy maize starch granules, with 100% retention. The reaction with waxy maize starch gave an amount of CDs that was 74% of the amount obtained in the aqueous suspension of waxy maize starch granules. The reaction of other solid starch granules from maize, tapioca and amylomaize-7 with CGTase and isoamylase gave amounts of CDs that were 64–50% of the amounts formed in the aqueous suspension reaction with these kinds of starch granules.

Rendleman used debranching enzymes (pullulanase or isoamylase) to enhance the yield of CDs by the reaction of CGTase with gelatinized starches and maltodextrin syrups in the presence of CD complexing agents [16]. He stated that the enhancement by debranching enzymes may involve two potential factors: first, debranching eliminates steric barriers that retard the effective contact of CGTase molecules with the large, bulky, highly branched amylopectin molecules, and second, debranching produces linear amylodextrin molecules that can be more easily utilized by CGTase in the cyclization reaction. This is probably even more the case for the reaction of CGTase in solid starch granules where the molecules are held together by α-(1$\rightarrow$6) branch linkages, hydrogen bonding, and hydrophobic interactions between the chains in the granules. The action of isoamylase, which itself is also relatively low [10], most probably improves the steric ability of CGTase to interact with the starch chains, giving an increase in the amount of CDs formed.

Kimura and Robyt previously reported [9] that starch granules from different botanical sources had different susceptibilities to hydrolysis by glucoamylase. Three general categories were found: high susceptibility (waxy maize starch granules), intermediate susceptibility (maize and tapioca starch granules), and low susceptibility (amylomaize-7 starch granules). In the reaction of CGTase (with and without isoamylase), waxy maize and maize starches were similar in their susceptibility. Amylomaize-7 starch was the least susceptible. Tapioca starch granules, however, were only about half as susceptible as maize starch granules. The order of the susceptibility of these starch granules with CGTase was different than the order of susceptibility with glucoamylase.

The formation of cyclomaltodextrins in situ, with retention of the CDs in the granule, provides a new material that has the properties of both starch granules and cyclomaltodextrins. The cyclomaltodextrins have the capability of forming complexes with a very large number of organic molecules. The formation of complexes with these organic molecules could provide stabilization of light, heat and oxygen-sensitive materials in the starch granules as well as a mechanism for their slow release. They could also provide special tastes, odors and flavors to the starch granules. The material, thus, has the potential for applications in foods, drugs, and agricultural products [4].

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