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Starch biosynthesis: sucrose as a substrate for the synthesis of a highly branched component found in 12 varieties of starches

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Abstract

D-[^{14}C]Glucose was incorporated into starch when 12 varieties of starch granules were incubated with [^{14}C]sucrose. Digestion of the ^{14}C-labeled starches with porcine pancreatic alpha amylase showed that a high percentage (16.1–84.1%) of the synthesized starch gave a relatively high molecular weight α-limit dextrin. Hydrolysis of the 12 varieties of starch granules by alpha amylase, without sucrose treatment, also gave an α-limit dextrin, ranging in amounts from 0.51% (w/w) for amylomaize-7 starch to 8.47% (w/w) for rice starch. These α-limit dextrins had relatively high molecular weights, 2.47 kDa for amylomaize-7 starch to 5.75 kDa for waxy maize starch, and a high degree of α-(1→6) branching, ranging from15.6% for rice starch to 41.1% for shoti starch. ADPGlc and UDPGlc did not synthesize a significant amount (1–2%) of the branched component, suggesting that sucrose is the probable substrate for the in vivo synthesis of the component and that sucrose is not first converted into a nucleotide-glucose diphosphate intermediate.

Keywords: Starch granules; Twelve varieties of starch; Sucrose as substrate for starch synthesis; Highly branched starch; α-Limit dextrin; Starch synthase; ADPGlc; UDPGlc

1. Introduction

Starch is found in plants in a granular form with a definite shape and size that is characteristic of the source.[1] The granules are water-insoluble and represent the storage form of D-glucose in plants that is formed in the photosynthetic process.[2] Two types of molecules are found in starch granules: a linear molecule, amylose, that has the D-glucose units joined together by α-(1→4) glycosidic linkages into chains and a branched molecule, amylopectin, that has α-(1→4) linked D-glucose chains that are joined to each other by α-(1→6) branch linkages.[3] The α-(1→6) branch linkages constitutes 5% of the linkages in the amylopectin molecule. The molecular size of the amylopectin molecule is considerably larger (10–100-times) that of the amylose molecule.[3]

In 1961, Leloir and co-workers[4] found that D-glucose from uridine 5′-(α-D-glucopyranosyl diphosphate) (UDPGlc) was incorporated into starch when incubated with starch granules. It was later found that adenosine 5′-(α-D-glucopyranosyl diphosphate) (ADPGlc) was a better substrate than UDPGlc for the incorporation of D-glucose into starch. It has been postulated that ADPGlc is the biological substrate for starch biosynthesis.[5] This was based on genetic studies in which a starchless *Arabidopsis* mutant was observed to be missing the gene for ADPGlc pyrophosphorylase, the enzyme that synthesizes ADPGlc from ATP and α-D-glucose 1-phosphate.[5]

Starch granules are known to contain active synthetic enzymes that synthesize starch from ADPGlc.[6–8] In the present study, we present evidence that the D-glucose unit of sucrose is incorporated into starch when starch granules from 12 different sources are incubated with sucrose, indicating that sucrose is a substrate for starch synthase(s) found in granules. The products that result are unusual and contain a highly branched species

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(15.6–41.1% branching) that makes up 16–84% of the synthesized material. It is also shown that this highly branched component is present in all 12 different varieties of starch.

2. Experimental

2.1. Materials

Adenosine 5′-(α-D-glucopyranosyl diphosphate) (ADPGlc), porcine pancreatic alpha amylase [EC 3.2.1.1] (Type VIIA), and [^{14}C]sucrose (8.2 mCi/mmol) were obtained from Sigma Chemical Co. (St. Louis, MO). ADP-[^{14}C]Glc (242 mCi/mmol) was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Isoamylase [EC 3.2.1.68] was obtained from Megazyme International, Wicklow, Ireland.

Starches were freshly prepared from the various sources, using standard procedures. Maize seeds (normal amylomaize [22% amylose and 78% amylopectin]) were obtained from Dr Martha James (Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University); amylomaize-7 (70% amylose and 30% amylopectin), and amylomaize-5 (53% amylose and 47% amylopectin) and waxy maize (100% amylopectin) seeds were obtained from National Starch and Chemical Co., Bridgewater, NJ. Rice, lima beans, and potatoes were purchased from local supermarkets. Wheat, barley, and rye berries were obtained from a local organic food store and the starches were isolated from them in the laboratory. Shoti starch was isolated from tubers obtained from M. Kitaoka, National Food Research Institute, Tsukuba, Japan. Rice and maize starches were prepared from seeds, using the established procedure[9] and potato starch was prepared, using an established procedure.[10] All of the starches were from mature materials.

Liquid scintillation cocktail contained 5.0 g PPO and 0.1 g POPOP in 1.0 L of toluene. All other chemicals were of the highest grade commercially available and were used without further treatment.

2.2. Reaction of starch granules with [^{14}C]sucrose

Starch granules (1.0 g) were suspended in 2.0 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.4), containing 1 μCi (0.12 μmol) of [^{14}C]sucrose. The starch was incubated in the sucrose solution at 30 °C for 24 h. The starch suspension was centrifuged, and the starch was washed with 5 mL of water five-times followed by 5 mL of anhydrous acetone five-times. Small amounts of acetone were removed by pulling a vacuum for 15 min. The acetone-dried granules were weighed and the radioactivity determined by adding 100 mg to 10 mL of toluene liquid scintillation cocktail and counting in a liquid scintillation spectrometer for 10 min or 10,000 counts, which ever came first.

2.3. Alpha amylase digestion of starch granules that were reacted with [^{14}C]sucrose

Porcine pancreatic alpha amylase was assayed by the addition of 0.1 mL of appropriately diluted enzyme to 1.9 mL of 1% (w/v) waxy maize starch in 40 mM imidazole–HCl buffer (pH 6.8). Aliquots (100 μL) were taken every 5 min for 30 min and added to 900 μL of 0.01 M NaOH. The reducing value, using maltose as a standard, was determined by the micro copper–bicinchoninate method.[11] One unit (U) of enzyme was defined as 1 μmol of α-(1→4) glycosidic bonds hydrolyzed per min.

Starch granules (500 mg) that had been reacted with [^{14}C]sucrose were dissolved in 2 mL of 88% (v/v) Me_2SO, followed by dilution to 50 mL with water. The solution was then dialyzed against three-changes of 2 L of water to remove the Me_2SO; 5 mL of 400 mM imidazole–HCl buffer (pH 6.2) and 30 IU of porcine pancreatic alpha amylase (PPA) were added, and the reaction was allowed to proceed for 15 h at 37 °C. The solution was filtered and concentrated to 1 mL by rotoevaporation. A 200 μL aliquot of the PPA digest was streaked along the center 15 cm section of Whatman 3MM chromatographic paper (22 × 56 cm), 7.5 cm from the top of the paper; 3 cm lanes on each side of the paper were marked off for maltodextrin standards. The chromatogram was irrigated with 65:35 volume proportions of 1-propanol–water in a descending mode for 30 h. Strips (3 cm from each side), containing the maltodextrin standards, were cut off and developed by the $AgNO_3$ dipping procedure.[12] The standards were lined-up along the sides of the paper and the radioactive compounds were located on the chromatogram and cut into strips. The strips of papers were rolled up and placed into 10 mL of toluene liquid scintillation cocktail for heterogeneous counting.

2.4. Large-scale preparation of the α-limit dextrin

Starch granules (5 g) were suspended in 10 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.5) and 170 mg of sucrose was added, making the concentration 50 mM in sucrose. The reaction was allowed to go 24 h at 30 °C. The progress of the reaction was followed by TLC, using Whatman K5 plates (2.5 × 5 cm), irrigated twice with 17:3 volume proportions of MeCN–water. The carbohydrates were visualized by dipping the dried plate into 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine/5% (v/v) H_2SO_4–methanol reagent, followed by heating at 120 °C for 10 min.[13] After reaction, the starch was

centrifuged and washed five-times with 10 mL of water and then dissolved by pouring a 10 mL slurry of the starch into 500 mL of boiling water. The solution was cooled and 20 mL of a 400 mM imidazole–HCl buffer (pH 6.2) and 150 U of PPA was added, and the reaction allowed to go at 37 °C for 24 h. The solution was concentrated to ≈50 mL and then dialyzed against 2 L of water, changed every 4 h for 24 h. The dialyzed solution was concentrated by rotoevaporation to ~10 mL and 2 volumes of EtOH were added to precipitate the α-limit dextrin. The α-limit dextrin was centrifuged and redissolved in 10 mL of water and reprecipitated with 2-volumes of EtOH. The precipitate was treated with 5 mL of anhydrous acetone five-times and once with 5 mL of anhydrous EtOH, followed by drying in a vacuum oven for 15 h at 40 °C.

2.5. Isolation of the α-limit dextrin component in non-sucrose treated starches

A slurry of starch granules (5 g) in 50 mL of water was poured into 500 mL of boiling water with stirring; boiling was continued for 20 min to gelatinize and solubilize the starch. The starch solution was cooled to 37 °C and 50 mL of 400 mM imidazole–HCl buffer (pH 6.8) was added. Porcine pancreatic alpha amylase (150 IU) was added to the starch solution, which was incubated at 37 °C for 15 h. The digest was centrifuged and the supernatant concentrated to 50 mL by rotoevaporation and then transferred to dialysis tubing (Spectra/Por, 29 mm diameter, 25 cm, MW cut off of 12,000–14,000 Da). The solution was dialyzed against six changes of 1 L of water and then concentrated to 20 mL. Two volumes of EtOH were added to precipitate the α-limit dextrin. The precipitate and solution were maintained at 4 °C for 15 h and then the precipitate was removed by centrifugation and treated six-times with 1 mL of acetone. The precipitated α-limit dextrin (100 mg) was dissolved in 10 mL of 40 mM imidazole–HCl buffer (pH 6.8) and 50 U of porcine pancreatic alpha amylase was added and incubated at 37 °C for 15 h. The digest was centrifuged and concentrated to 2 mL. The α-limit dextrin was precipitated with 2 volumes of EtOH, centrifuged, and treated 10-times with 1 mL of acetone, dried under vacuum, and weighed.

2.6. Structural analysis of the α-limit dextrins

The α-limit dextrin (10 mg) prepared as just described was dissolved in water and the average degree of polymerization (dp) obtained by determining the total carbohydrate by the micro phenol–H_2SO_4 method[11] and the reducing value by the micro copper–bicinchoninate method.[11] The average d.p. was calculated by the following:

$$\text{avg. d.p.} = \frac{\text{total carbohydrate}}{\text{reducing value, using maltose as a standard}} \times 1.9, \quad (1)$$

where the factor, 1.9, is the molecular weight of maltose divided by the molecular weight of D-glucose.

The α-limit dextrin was debranched by adding isoamylase (40 m IU/50 μg of α-limit dextrin) in 20 mM acetate buffer (pH 4.8) and 42 °C for 15 h. The average d.p. of the debranched α-limit dextrin solution was determined as just described. The percent branching was obtained by the following:

$$\%\ \text{branching} = \frac{\text{d.p. after debranching}}{\text{d.p. before debranching}} \times 100, \quad (2)$$

from which the average chain length of the branched chains was determined by the following:

$$\text{avg. chain length of the branched chains} = \frac{1}{\%\ \text{branching}} \times 100. \quad (3)$$

After debranching, the individual maltodextrins were identified by TLC, using Whatman K5 plates (20 × 20 cm) and 3-ascents (18.5 cm path length) of 85:20:50:50 volume proportions of MeCN–EtOAc–2-propanol–water. The carbohydrates were visualized as already described. The maltodextrins, resulting after debranching four of the α-limit dextrins, were analyzed by fluorescence-assisted capillary electrophoresis (FACE).

2.7. Fluorescence-assisted capillary electrophoresis of debranched maltodextrins

The maltodextrins, resulting from the debranching of the α-limit dextrin, were analyzed using FACE.[14] Maltodextrins (~1 mg) were dissolved in 1 mL of water; 40 μL containing ~40 μg of carbohydrate was taken to dryness, using a Speedvac evaporator; 2 μL of APTS (5 mg of 8-amino-1,3,5-pyrenetrisulfonic acid–48 μL of 15% acetic acid) and 2 μL of 1 M $NaCNHB_3$ in tetrahydrofuran were added and the mixture allowed to react for ~15 h at 42 °C; 46 μL of Milli Q-pure water was added and the solution centrifuged for 2 min. The supernatant (5 μL) was diluted with 195 μL of Milli Q-pure water and transferred to a 0.5 mL tube for capillary electrophoresis for 60 min, using a P/ACE MDQ Glycoprotein Electrophoresis System (Beckman Coulter, Fullerton, CA).

2.8. Competition reactions between [^{14}C]sucrose and ADPGlc with starch granules

Starch (1 g) was suspended in 2.0 mL of 0.1 mM EDTA–4 mM glycine buffer (pH 8.4), containing 7.6

mg of ADPGlc and 0.2 μCi of [^{14}C]sucrose, giving 6 mM ADPGlc and 6 μM sucrose. The reaction was conducted at 30 °C for 24 h and the suspension was then centrifuged, the supernatant removed, and the starch was washed five-times with 5 mL of water, followed by five washes with 5 mL of acetone. The starch was then placed at 37 °C for 2 h and a vacuum was pulled for a minimum of 20 min. The starch (200 mg) was added to 10 mL of toluene scintillation cocktail and the ^{14}C was counted in a liquid scintillation spectrometer. An identical experiment was performed with sucrose but without ADPGlc.

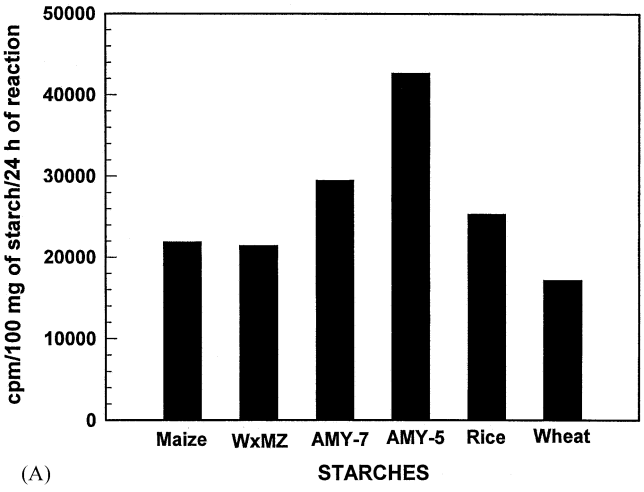
3. Results

3.1. Sucrose as a substrate for the biosynthesis of starch

When the twelve varieties of starch granules were incubated with [^{14}C]sucrose, D-[^{14}C]glucose was incorporated into all of the starches. Fig. 1(A and B) show the amounts of D-[^{14}C]glucose incorporated into 100 mg of the twelve varieties of starch granules reacting with [^{14}C]sucrose for 24 h. There was a wide variation in the reactivities of the different starches for incorporating D-glucose into starch from sucrose. The reactivities of the starches fell into two groups: those that incorporated more than 15,000 cpm (Fig. 1A) and those that incorporated less than 10,000 cpm (Fig. 1B). In the first group, amylomaize-5 incorporated 43,250 cpm; followed by amylomaize-7 with 29,520 cpm; rice with 25,100 cpm; maize with 22,130 cpm; waxy maize with 21,040 cpm; and wheat with 17,230 cpm. In the second group, rye had 8940 cpm; barley 3610 cpm; lima bean 1093 cpm; tapioca 1084 cpm; and shoti 198 cpm. Fig. 2 shows a comparison of the incorporation of D-glucose into maize starch granules from sucrose and from ADPGlc. ADPGlc was 6.2-times more reactive than sucrose in incorporating D-glucose into maize starch granules.

3.2. Reaction of the labeled starch with alpha amylase

To confirm that D-glucose was being incorporated into the starches from sucrose, the resulting ^{14}C-labeled starches were hydrolyzed by porcine pancreatic alpha amylase. The expected products were D-glucose, maltose, maltotriose, maltotetraose, and branched maltodextrins, B4, B5, B6, BB7, and BB8.[15–17] In addition to these expected alpha amylase products, we found that a significant amount of high molecular weight α-limit dextrin was also formed (Table 1).



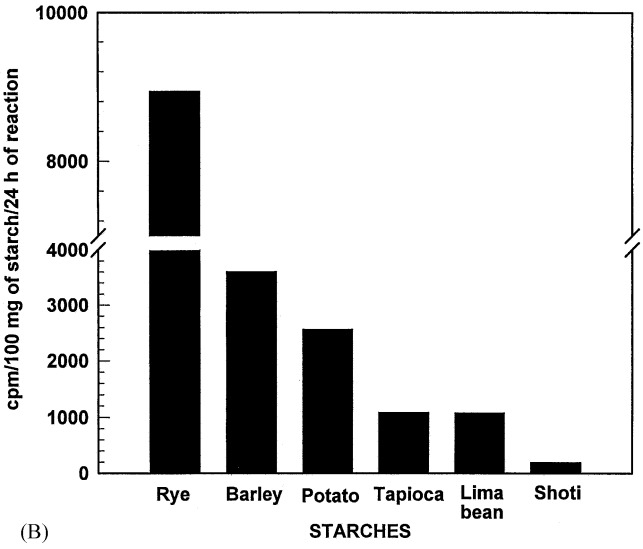


Fig. 1. Incorporation of D-[^{14}C]glucose into different starch granules after reacting with 1.0 μCi of [^{14}C]sucrose at 30 °C for 24 h. (A) Starches with relatively high reactivity, >15,000 cpm, (B) starches with relatively lower reactivity, <10,000 cpm.

3.3. Formation of α-limit dextrins from the reaction of alpha amylase with the labeled starch

The formation of the alpha amylase resistant product was not expected in the relatively high amounts that we observed. The percentages of the synthesized α-limit dextrins, formed by the reaction of each of the starch granules with [^{14}C]sucrose, are given in Table 1. The percentage synthesized varied with the type of starch granules. The α-limit dextrin represented 84.1% of the synthesized product formed by rice starch granules down to 16.1% for amylomaize-7 starch granules (Table 1). Reaction of maize starch granules with ADP-[^{14}C]Glc only gave 1.2% and reaction with UDP-[^{14}C]Glc only gave 1.8% of the of the α-limit dextrin.

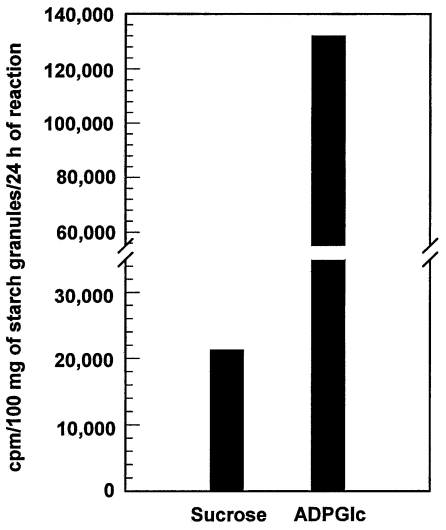


Fig. 2. Comparison of the incorporation of D-[^{14}C]glucose into maize starch granules after reacting with 1.0 μCi of [^{14}C]sucrose and 1.0 μCi of ADP-[^{14}C]Glc at 30 °C for 24 h.

Table 2 gives the percents of the α-limit dextrin formed by the reaction of maize starch granules with sucrose at various times. The percent of the α-limit dextrin increased from zero time to 24 h, where it peaked.

3.4. Structures of the α-limit dextrins

The starch granules were reacted with 50 mM sucrose for 24 h to obtain larger amounts of material for characterization. The reacted starch was digested with porcine pancreatic alpha amylase. Much to our surprise, we obtained more material than what was possible for the amount of sucrose that was added. A control experiment was then performed by reacting each of the 12 varieties of starch granules, without prior incubation with sucrose, with alpha amylase. It was found that each one of them contained a highly branched component in their structure that gave an α-limit dextrin. The percents of α-limit dextrins found in the 12 types of starch granules are given in Table 3. They ranged from 8.44% (w/w) for rice starch to 0.51% (w/w) for amylomaize-7 starch. The α-limit dextrins were highly branched, ranging from rice starch granules with 15.6% branching to shoti starch granules with 41.1% branching. Other starches with significantly high degrees of branching were lima bean starch with 25% branching, wheat starch with 27.2% branching, tapioca starch with 33.2% branching, and amylomaize-7 starch with 37.2% (Table 3). For the maize starches, the percent of the α-limit dextrin in the starch granule appeared to have a

Table 1
Porcine pancreatic alpha amylase hydrolysis of 200 mg of starch after reaction of starch granules with 1.0 μCi [^{14}C]sucrose for 24 h at pH 8.4 and 30 °C

Products →	Total ^{14}C [c]	G_1–G_4 &	B_4–B_8 [d]	HMW	α-LD [d]
Starches [a] ↓	cpm [e]	cpm [e]	%	cpm [e]	%
Rice	5545	580	(15.9)	4965	(84.1)
Waxy maize	16,770	6315	(37.7)	10,455	(62.3)
Maize	16,880	6660	(39.5)	10,220	(60.5)
Wheat	9224	5494	(59.6)	3730	(40.4)
Potato	922	632	(68.5)	290	(31.5)
Barley	4536	3121	(68.8)	1415	(31.2)
Amylomaize-5	34,160	23,700	(69.4)	10,460	(30.6)
Rye	5544	3919	(70.7)	1625	(29.3)
Lima bean	1576	1136	(72.1)	440	(27.9)
Shoti [b]	1740	1350	(77.6)	390	(22.4)
Tapioca	1600	1330	(83.1)	270	(16.9)
Amylomaize-7	22,080	18,515	(83.9)	3565	(16.1)
Maize/UDPGlc [b]	20,364	20,004	(98.2)	360	(1.8)
Maize/ADPGlc [b]	29,195	28,840	(98.8)	355	(1.2)

[a] Each of the starches (1 g) were reacted with 1.0 μCi [^{14}C]sucrose for 24 h and then 200 mg of each starch was hydrolyzed by porcine pancreatic alpha amylase and the products were separated by descending paper chromatography. The ^{14}C was counted on the paper by heterogeneous liquid scintillation spectrometry.

[b] For a comparison, 1 g maize starch granules were reacted with 1.0 μCi ADP-[^{14}C]Glc and 1.0 μCi UDP-[^{14}C]Glc and the starch was hydrolyzed by porcine pancreatic alpha amylase. The cpm's were figured on the basis of 200 mg of starch. The cpm's for shoti starch were figured on a 1 g sample rather than on 200 mg samples as was done for all of the other starches.

[c] Total amount of [^{14}C]glucose incorporated into the starch granules.

[d] G_1–G_4 = D-glucose and maltodextrins: maltose, maltotriose, and maltotetraose; B_4–B_8 = the branched tetra-, penta-, hexa-, hepta-, and octa-saccharide alpha amylase low molecular weight products; HMW α-LD = high molecular weight α-limit dextrin.

[e] Each of the samples were counted for 10 min or 10,000 counts, whichever came first.

Table 2
Porcine pancreatic alpha amylase hydrolysis of 1 g of maize starch granules after incorporation of D-[^{14}C]glucose from 1.0 μCi [^{14}C]sucrose at different times of reaction

Time of Rxn [a]	Total ^{14}C [c]	G_1–G_4 and B_4–B_8 [d]		HMW α-LD [d]	
	cpm [b]	cpm [b]	%	cpm [b]	%
Maize 12 h	11,634	9048	77.8	2586	22.2
Maize 18 h	14,240	10,340	72.6	3900	27.4
Maize 24 h	21,944	8658	39.5	13,286	60.5

[a] Maize starch granules (1 g) was incubated with 1.0 μCi [^{14}C]sucrose with samples taken at different times of reaction.
[b] Each of the samples were counted for 10 min or 10,000 counts, whichever came first; the cpm's are for 1 g of starch.
[c] Total amount of ^{14}C incorporated into maize starch granules after reaction for different times.
[d] G_1–G_4 = D-glucose and maltodextrins: maltose, maltotriose, and maltotetraose; B_4–B_8 = branched tetra-, penta-, hexa-, hepta-, and octa-saccharide alpha amylase low molecular weight products; HMW α-LD = high molecular weight α-limit dextrin.

correlation with the percent of amylopectin in the granule, with the high amylopectin maize starches, waxy maize and maize, having a high percent of the α-limit dextrin and with the low amylopectin maize starches, amylomaize-5 and -7, having a significantly lower percent of α-limit dextrin.

The α-limit dextrins are not small oligosaccharides. They ranged in size from 2367 Da for amylomaize-7 starch to 5753 Da for waxy maize starch (Table 3). The average chain lengths of the branched chains in the α-limit dextrins varied from 2.4 glucose residues for shoti starch to 6.4 glucose residues for rice starch (Table 3). The α-limit dextrins were debranched by reaction with isoamylase. The maltodextrins that resulted are shown in the TLC given in Fig. 3. Maltodextrins of d.p. 2–10 are formed after debranching, with the predominant dextrins being maltotetraose and maltopentaose. The maltodextrins obtained after debranching for four of the α-limit dextrins were also analyzed using FACE (Fig. 4). The maltodextrins that resulted from the debranching reaction by isoamylase were all linear, as they were converted to maltose and maltotriose on reaction with porcine pancreatic alpha amylase (data not shown). This shows that the isoamylase reactions with the α-limit dextrins were complete. The FACE analyses showed that G_5 was the predominant maltodextrin in the α-limit dextrins, followed by G_4 and G_3, with exponentially decreasing amounts of G_6–G_{20}. The four analyses were quite similar, with only small variations. For example, in the maize sample, the amounts of G_7 and G_8 were equal, whereas the other three samples, G_8 was less than G_7, following the exponential decrease as the size of the maltodextrins increased. Even though there were variations in the percents of α-limit dextrins in the different varieties of starch granules, differences in their molecular weights, and differences in the degrees of branching, all twelve of the α-limit dextrins appear to have a similar structure as judged by TLC and FACE analyses of the debranched α-limit dextrins, with similar amounts of branched maltodextrin chains from G_2–G_{20}.

3.5. Competition reactions between sucrose and ADPGlc in reacting with starch granules

The amounts of ^{14}C-labeled D-glucose incorporated into the 12 starches from [^{14}C]sucrose in the presence of 1000-fold molar excess of ADPGlc and in the absence of ADPGlc were determined and the results are presented in Table 4. ADPGlc inhibited the incorporation of D-glucose into 11 of the 12 starches. The amount of inhibition varied widely for the 12 starches. The greatest amount (84%) of inhibition occurred for lima bean starch. From there on, the amount of inhibition decreased from 54.1% for rice starch down to 7.6% for waxy maize and wheat starches. ADPGlc did not inhibit the incorporation of D-glucose into rye starch.

4. Discussion

In this study, it is shown that when starch granules from 12 varieties were incubated with [^{14}C]sucrose, D-[^{14}C]glucose was incorporated into the starches (Fig. 1A and B, Table 1). When all 12 of these labeled starches were treated with alpha amylase, a relatively high percent of the synthesized product, for example, 84.1% for rice starch, 62.3% for waxy maize starch, and 60.5% for maize starch, was resistant to hydrolysis, giving an α-limit dextrin (Table 1). These α-limit dextrins were also produced when the 12 varieties of the starches were reacted with alpha amylase without any prior treatment with sucrose. The α-limit dextrins had relatively high molecular weights of 2367–5753 Da, depending on the particular variety of starch (Table 3). They also had very high degrees of branching, ranging from 15.6% for rice starch to 41.1% for shoti starch (Table 3). The amount of α-limit dextrin obtained from the untreated starches varied from 0.51% for amylomaize-7 starch to 8.47% for rice starch.

Some types of starches, for example the *sugary* varieties and sweet corn produce a non-granular,

Table 3
Properties of the α-limit dextrins obtained by porcine pancreatic alpha amylase hydrolysis of 12 kinds of starches

Starches	(g) α-Limit dextrin in 4.5 g starch [a]	(%) α-Limit dextrin in starch	Avg. d.p. [b]	Avg. MW (Da) [c]	Avg. d.p. after debranching	Degree of branching [d] (%)	Avg. chain length of the branched chains [d]
Rice	0.318	8.47	33.9	5510	5.3	15.6	6.4
Waxy maize	0.308	6.84	35.4	5753	5.8	16.4	6.1
Potato	0.104	2.31	29.8	4846	5.2	17.4	5.7
Maize	0.167	3.71	30.9	5023	5.5	17.8	5.6
Amylomaize-5	0.064	1.42	24.6	4003	5.3	21.5	4.7
Barley	0.112	2.49	26.5	4311	5.9	22.3	4.5
Rye	0.113	2.51	23.4	3809	5.9	25.2	4.0
Lima Bean	0.317	8.24	23.6	3841	5.9	25.0	4.0
Wheat	0.114	2.53	21.3	3469	5.8	27.2	3.7
Tapioca	0.332	7.38	20.2	3290	6.7	33.2	3.0
Amylomaize-7	0.023	0.51	14.5	2367	5.4	37.2	2.7
Shoti	0.224	4.98	15.8	2578	6.5	41.1	2.4

[a] Starch (4.5 g) was solubilized and reacted extensively with 150 U of porcine pancreatic alpha amylase (PPA) at pH 6.8 and 37 °C for 15 h; the digest was concentrated, dialyzed, and precipitated with 2 volumes of ethanol. The dried precipitate (100 mg) was dissolved in buffer and treated with 50 U of PPA at pH 6.8 and 37 °C for 15 h and then the α-limit dextrin was precipitated with 2 volumes of ethanol, dried, and weighed.

[b] The average d.p. (degree of polymerization) of the α-limit dextrins and the d.p. after debranching were determined by measuring the reducing value by the micro copper–bicinchoninate method, using maltose as the standard, and by measuring total carbohydrate by the micro phenol/sulfuric acid method, using maltose as the standard. The average d.p. of the α-limit dextrin was calculated from (total carbohydrate ÷ reducing value) × 1.9.

[c] Average MW of the α-limit dextrin = (avg. d.p. × 162) + 18.

[d] The degrees of branching (%) of the α-limit dextrins were calculated from: (d.p. after debranching ÷ d.p. before debranching) × 100. The average chain lengths of the branch chains in the α-limit dextrins were calculated from: (1 ÷ % branching) × 100.

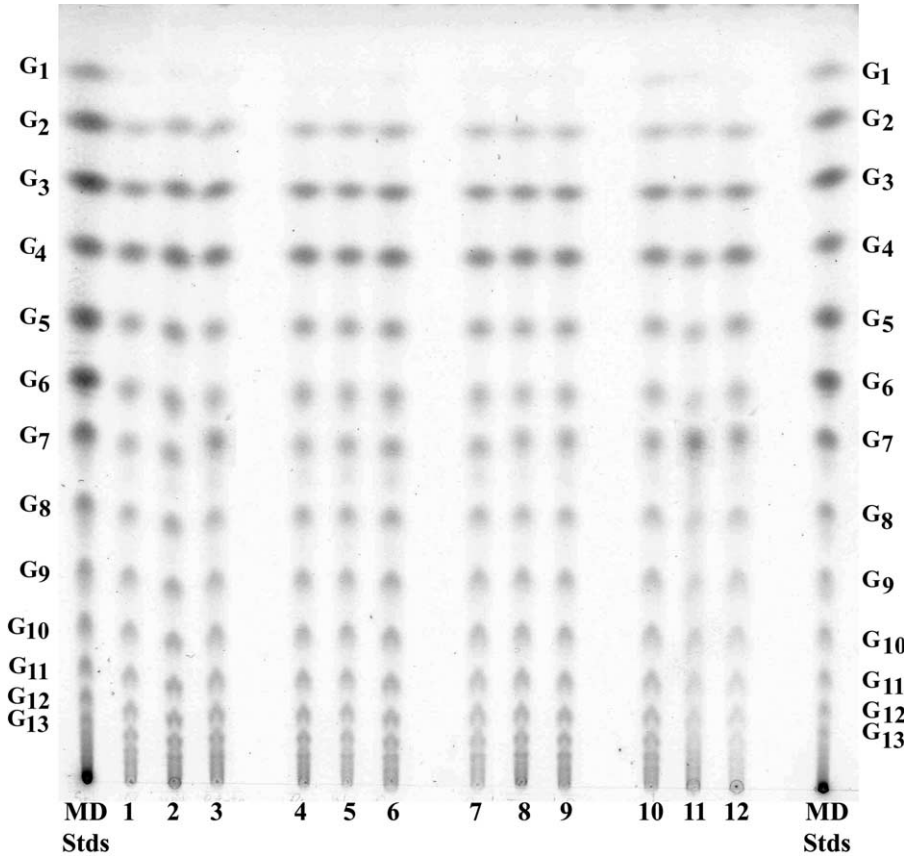


Fig. 3. TLC of the maltodextrins released from the α-limit dextrins of 12 different starches after reaction (debranching) with isoamylase. MD/Stds = maltodextrin standards, G_1–G_{13}; starches in lanes 1–12: (1) maize; (2) waxy maize; (3) potato; (4) rice; (5) wheat; (6) rye; (7) barley; (8) tapioca; (9) lima bean; (10) amylomaize-5; (11) shoti; (12) amylomaize-7.

soluble, glycogen-like polysaccharide, called *phytoglycogen*.[18–21] Glycogens usually have a degree of branching of 7–10%[22] and also have some resistance to alpha amylase hydrolysis.[23] The branched material that we have found, synthesized by reaction of sucrose with the 12 varieties of starch and present in all 12 varieties of starch prior to reaction with sucrose, appear to be quite different from phytoglycogen. They represent a smaller amount of material, have a lower molecular weight, have much higher degrees of branching and make up a part of the starch in the granule.

Reaction of maize starch granules with ADPGlc and UDPGlc, the usual substrates considered for starch biosynthesis, did not form a significant amount of the α-limit dextrin (Table 1). This and the high percentage of the highly branched material that was formed by reaction with sucrose, suggests that sucrose is the most probable substrate for the in vivo synthesis of this highly branched starch component that was found in all 12 varieties of starch granules. Further, the absence of significant synthesis of the highly branched component by ADPGlc and UDPGlc (Table 3) indicates that sucrose is the primary substrate for the synthesis of the highly branched material and that sucrose is not first being converted into a nucleotide-glucose diphosphate intermediate.

Sucrose is known to be a high-energy donor of D-glucose for the synthesis of other polysaccharides by enzymes, such as dextransucrase that synthesizes dextran,[24] amylosucrase that synthesizes glycogen[25] and amylose,[26] alternansucrase that synthesizes alternan,[27] and mutansucrase that synthesizes mutan.[28] Sucrose is also known as a high-energy donor of D-fructose for levansucrase and the synthesis of levan.[29] Sucrose is very pervasive and present in all plants. So, it is feasible from both the energy of the glycosidic linkage and availability that sucrose could be a substrate for the in vivo synthesis of starch, as is suggested by our experiments.

Plastids, chloroplasts, and amyloplasts are the sites for the biosynthesis of starch in plants. The presence of sucrose in these organelles has been debated. Gerrits and co-workers[30] recently reported the introduction of enzymes into these organelles that utilize sucrose as a substrate. They introduced *Bacillus subtilis* levansucrase into plastids, chloroplasts, and amyloplasts and found a high level of accumulation of levan in these organelles, indicating that sucrose is indeed present in these organelles. The introduction of levansucrase into amy-

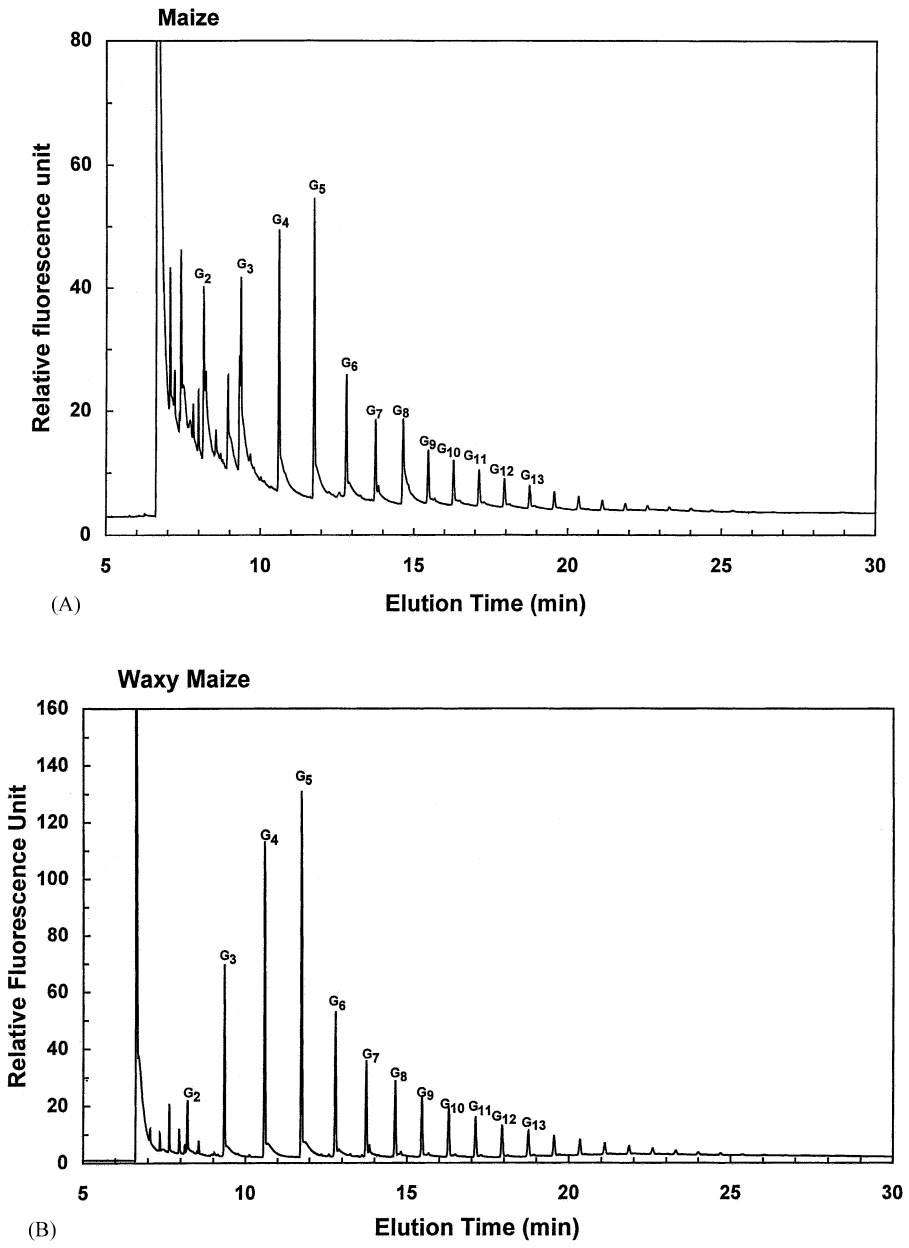
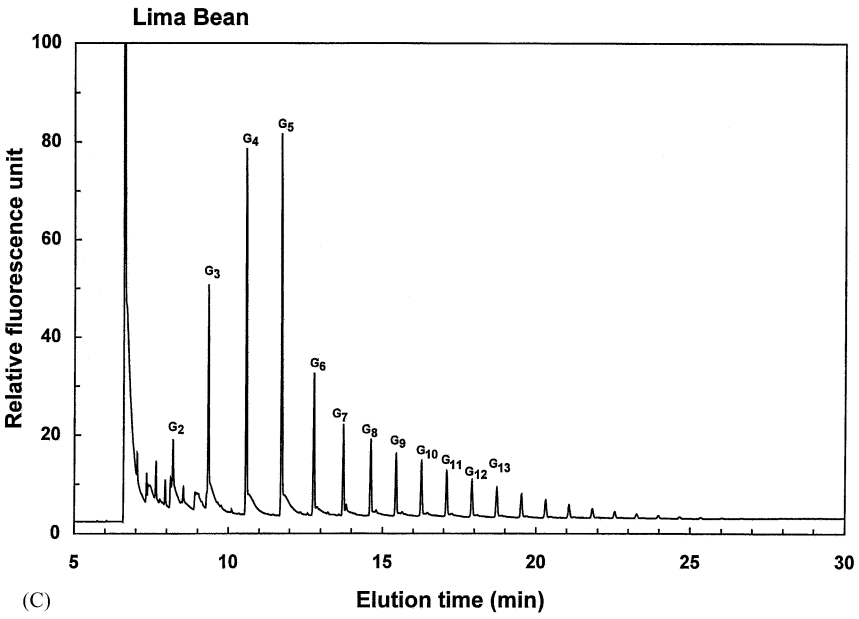


Fig. 4. FACE of the maltodextrins released from the α-limit dextrins of four different starches after reaction (debranching) with isoamylase: (A) maize starch; (B) waxy maize starch; (C) lima bean starch; (D) tapioca starch.

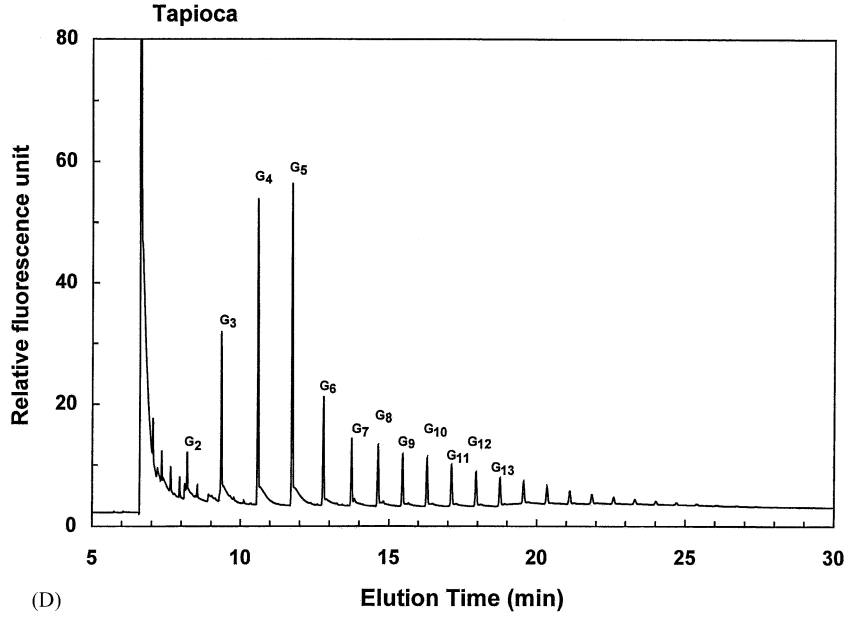
loplasts resulted in an altered starch granule. They also expressed yeast invertase into potato tuber amyloplasts and found an 80% reduction of total tuber sucrose. The utilization of sucrose by both levansucrase and invertase indicates that the sucrose was not being converted into hexose phosphates or other metabolic compounds on its transport into the organelles and that it remains there as sucrose.

Geiger and co-workers[31] observed that sucrose stimulated the accumulation of starch in potato tubers and Weber and co-workers[32] also observed the same thing for bean seeds. This sucrose stimulation of starch accumulation may actually be the synthesis of the highly branched component, which may act as a matrix or nucleation site for the formation of the starch granule.

The competition experiments between sucrose and 1000-fold molar excess ADPGlc showed that ADPGlc inhibited the incorporation of D-glucose into starch from sucrose for 11 of the 12 starches. Table 4 shows that the inhibition decreases from a high of 84% for lima bean starch down to 7.6% for waxy maize and wheat starches and there was no inhibition at all for rye starch. Because ADPGlc inhibited the incorporation of D-glucose into starch granules from sucrose to widely differing amounts for the various kinds of starches, sucrose must be reacting with two different kinds of



(C)



(D)

Fig. 4 (*Continued*)

enzymes. For one or more of the enzymes, sucrose and ADPGlc are both substrates and a 1000-fold molar excess of ADPGlc inhibits the incorporation of D-glucose into starch from sucrose. The decrease in the ADPGlc inhibition for the various starches indicates that sucrose is also reacting with an enzyme for which it is a specific substrate. It is possible that it is this enzyme that is responsible for catalyzing the synthesis of the highly branched starch component.

All 12 of the starches had a component that had a high degree of branching, but three of the starches had a component with an extremely high degree of branching, namely tapioca starch with 33.2%, amylomaize-7 starch with 37.2%, and shoti starch with 41.1%. The highly branched component in tapioca starch had one branch linkage for every 3 glucose residues, amylomaize-7 starch had one branch linkage for every 2.7 glucose residues, and shoti starch had one branch linkage for every 2.4 glucose residues. The amylopectin component for most starches has one branch linkage for every 20 glucose residues and most glycogens have one branch linkage for every 10 glucose residues.

The fact that the different starches had widely differing percents of branching, ranging from 15.6%

Table 4
Incorporation of D-[^{14}C]glucose into 12 starches from [^{14}C]sucrose in the presence of 1000-fold molar excess of ADPGlc and in its absence

Starches [a]	With ADPGlc [b]	Without ADPGlc [b]	% inhibition [c]
Lima bean	679 ± 18	4239 ± 179	84.0
Rice	4517 ± 133	9837 ± 232	54.1
Barley	1220 ± 34	1799 ± 80	32.2
Tapioca	693 ± 22	875 ± 10	20.8
Potato	798 ± 19	1006 ± 56	20.7
Amy-7	17,515 ± 377	21,190 ± 544	17.3
Shoti	234 ± 8	278 ± 8	16.8
Maize	2554 ± 163	2899 ± 116	11.9
Amy-5	6087 ± 162	6613 ± 637	8.0
Waxy maize	10,039 ± 282	10,862 ± 541	7.6
Wheat	3473 ± 164	4477 ± 326	7.6
Rye	3697 ± 226	3620 ± 171	0

[a] Each of the starches (1 g) were incubated with 6 μM (0.2 μCi) [^{14}C]sucrose and 6 mM ADPGlc at pH 8.4 and 30 °C for 24 h, washed, dried, and counted. [^{14}C]sucrose (0.2 μCi) was also incubated with the starches (1 g) without ADPGlc. Amy-5 = amylomaize-5 and amy-7 = amylomaize-7 starches.

[b] [^{14}C]Glucose (cpm) incorporated into 1 g of starch in the presence of ADPGlc and in the absence of ADPGlc. The samples were counted for 10 min or 10,000 counts, whichever came first.

[c] % Inhib. = percent inhibition by ADPGlc for the incorporation of D-glucose into starch granules from sucrose, which was computed by 100 − [(cpm w ADPGlc ÷ cpm w/o ADPGlc) × 100].

for rice starch to 41.1% for shoti starch, also suggests that the individual varieties of starch have a specific enzyme system (synthase + branching enzyme) that utilizes sucrose as a substrate for the synthesis of the highly branched component. It, however, could be that because the reaction of sucrose with starch synthase is slower than the reaction with ADPGlc, the branching enzyme has a greater frequency of branching the linear chain, resulting in high degrees of branching.

It previously has been reported that a starchless *Arabidopsis thaliana* mutant also lacked the gene for ADPGlc pyrophosphorylase. From this, it was concluded that ADPGlc was the exclusive substrate for starch biosynthesis.[5,33] It should be pointed out, however, that the test for starch was the I_2–KI reagent to give blue or maroon color for amylose and amylopectin. A starch component, having 15.6–41.1% branching would not give any color with the I_2–KI reagent and, thus, even this so-called starchless mutant could have been synthesizing the highly branched starch from sucrose and not have been detected. We have recently shown by pulse and chase studies of eight varieties of starch granules that the starch chains are elongated by the addition of glucose from ADPGlc to the reducing-end of a growing chain and saccharide primers that were previously postulated are not involved.[8] This highly branched component, thus, cannot be acting as a primer for starch biosynthesis.

In conclusion, we have found that sucrose is a substrate for starch synthesis in 12 different varieties of starch granules. The synthesized product contains a high percentage (16.1–84.1%) of a high molecular weight (2367–5753 Da) α-limit dextrin that has a high degree of branching (15.6–41.1%), depending on the variety of starch. These highly branched components are not synthesized from ADPGlc or UDPGlc to any significant amount (~1–2%). The highly branched component was found in all 12 of the varieties of starch granules that were not treated with sucrose in amounts from 0.51 to 8.47% (w/w). It is suggested that this highly branched component that is resistant to alpha amylase hydrolysis might act as a matrix or nucleation site for the formation of starch granules in chloroplasts, amyloplasts, and plastids, where sucrose has recently been found to occur.

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