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Starch biosynthesis: experiments on how starch granules grow in vivo

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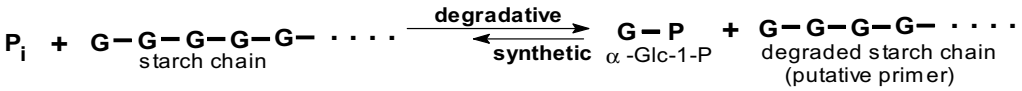
ABSTRACT

Four varieties of starch granules from potato, wheat, maize, and rice were fractionated into homogeneous 10-μm-sized ranges. The size with the largest amount of granules was reacted with ADP-[^{14}C]Glc, washed, and peeled into 7–9 layers, using a controlled peeling process, involving 90:10 volume proportions of Me_2SO–H_2O at 10 °C. All of the starches showed biosynthesis of starch throughout the granules. Starch synthase activities were determined for each of the layers. Three of the starches had a relatively large amount of synthase activity in the second layer, with only a small amount in the first layer. Potato starch had the largest amount of activity in the first layer. Starch synthase activity was found to alternate between higher and lower activities throughout all of the varieties of granules, showing that the synthesis was not uniform and also was not exclusively occurring at the surface of the starch granules, which had previously been hypothesized. From these results and our previous studies on the mechanism of starch chain elongation by the addition of D-glucose to the reducing end of a growing chain that is covalently attached to the active site of starch synthase, a hypothesis is proposed for how starch granules grow in vivo.

1. Introduction

Starch is usually composed of a mixture of two polysaccharides, a linear α-(1→4) glucan, amylose, and a larger α-(1→4) glucan with 5–6% α-(1→6) branch linkages, amylopectin, most frequently in the ratio of ~1:3. There are varieties with no amylose and 100% amylopectin (often called waxy starches) and there are varieties with much higher amylose to amylopectin ratios, such as ~1:1 and ~7:3, known as 'high-amylose starches'. Starch is found to be widely distributed in the leaves, stems, roots, tubers, and seeds of most plants, where it occurs as water-insoluble granules of different sizes and specific morphologies, depending on the source.[1] Starch is produced as an end-product of the photosynthetic process in which some of the energy of sunlight is conserved by its conversion into chemical energy contained in the D-glucose units of the starch molecules. Starch is found in all of the major food crops of the world. As such, it serves as the major source of energy for plants and non-photosynthesizing organisms, such as bacteria, fungi, and animals that are capable of breaking down the starch to D-glucose units and metabolizing it for energy and for the formation of other compounds.

In 1940, Hanes[2] reported that amylose or maltodextrin chains could be elongated by the reaction of potato phosphorylase (EC 2.4.1.1) with α-D-glucose-1-phosphate (α-Glc-1-P). Phosphorylase is an enzyme that catalyzes a reversible reaction, and it can be either synthetic or degradative, depending on whether the starting substrate is α-Glc-1-P or inorganic phosphate (P_i), respectively, as shown in the following reaction:



The synthetic reaction of the putative primer chain, however, will only occur when the ratio of P_i to α-Glc-1-P is less than the equilibrium value, which is 3.1 at pH 7.0.[3] In plant systems, this ratio is not obtained, as the concentration of P_i is 20- to 40-fold higher than the concentration of α-Glc-1-P.[4,5] The in vivo conditions, thus, greatly favor degradation rather than synthesis. Because a degraded starch chain is the product of the degradation reaction, the synthetic phosphorylase reaction requires a starch or a maltodextrin chain as a substrate, as shown in the synthetic reaction above. This is the origin for the primer requirement for starch biosynthesis from the nonreducing end of a primer that has been retained for almost 60 years, without much experimental evidence.[6–8]

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In 1960, some 20 years after Hanes' experiments, Recondo and Leloir[9] found that ADPGlc was the high-energy donor of glucose for the biosynthesis of starch chains, rather than α-Glc-1-P. Leloir and co-workers[9–11] also found that active starch synthesizing enzymes, starch synthase [EC 2.4.1.2] for the synthesis of linear α-(1→4) linked amylose chains and starch branching enzymes [EC 2.4.1.18] for the synthesis of the α-(1→6) branch linkages of amylopectin, were entrapped inside the starch granules. When they incubated ADP-[^{14}C]Glc with starch granules, starch was synthesized, and when this labeled starch was reacted with the exo-acting beta-amylase [EC 3.2.1.2], ^{14}C-labeled maltose was obtained. From this, Leloir and co-workers[9–11] concluded that the D-glucose from ADPGlc was being added to the nonreducing ends of starch primers. This experiment has been widely considered as a proof that starch is biosynthesized by the addition of D-glucose to the nonreducing ends of starch primer chains. This conclusion, however, is not necessarily correct in that if the starch chains had been synthesized de novo from the reducing end, without the need of a primer, instead of the addition of D-glucose from ADP-[^{14}C]Glc to the nonreducing end of a primer, the resulting synthesized starch chains would have had every D-glucose residue labeled with ^{14}C. Thus, when the starch granules were incubated with ADP-[^{14}C]Glc and then solubilized and reacted with beta-amylase, ^{14}C-labeled maltose would also have been obtained.

Because of these problems and the lack of definitive experiments supporting the nonreducing end primer elongation of starch chains, Mukerjea and Robyt[12–14] carried out a number of experiments to test the nonreducing end primer mechanism. The first set of experiments was a pulse with ADP-[^{14}C]Glc and a chase with nonlabeled ADPGlc of eight different kinds of starch granules.[12] The pulsed granules gave starch chains that on reduction with $NaBH_4$ and hydrolysis gave ^{14}C-D-glucitol and ^{14}C-D-glucose, and the chase experiments gave a significant decrease in the amount of label in D-glucitol. These experiments indicated that the starch chains were being elongated from the reducing end and not from the nonreducing ends of primers.

A second set of experiments by Mukerjea and Robyt[13] reported on the effects of adding the putative primers, maltose, maltotriose, and maltodextrin (DP 12) to the reaction of labeled ADP-[^{14}C]Glc with three varieties of starch granules. The reactions were examined in the absence and presence of increasing concentrations of the putative primers. Instead of stimulating starch biosynthesis, as would be expected for primers, all of the putative primers inhibited starch biosynthesis in increasing amounts, as the concentrations of the putative primers were increased.[13] The putative primers did undergo a reaction in which ^{14}C-labeled D-glucose was added in exponentially decreasing amounts to the nonreducing ends of the putative primers that inhibited the synthetic reaction. Only oligosaccharides were formed, and in no instance was a starch chain synthesized. It was concluded that the reaction of the putative primers was one of an 'acceptor' in which D-glucose was transferred to the acceptor, diverting it from being added to the growing starch chains, thus, giving inhibition of starch biosynthesis. These reactions were very similar to the acceptor reactions observed for dextransucrase by various oligosaccharides,[15] of which maltose also gave the inhibition of the synthesis of dextran.[16]

The third set of experiments performed by Mukerjea and Robyt[14] was the reaction of starch granules with labeled ADP-[^{14}C]Glc (pulsed starch). A second set of starch granules was reacted with labeled ADP-[^{14}C]Glc, followed by reaction with nonlabeled ADPGlc (chased starch). The labeled starches from the two reactions were then reacted with the exo-acting enzymes, glucoamylase [EC 3.2.1.3] and β-amylase [EC 3.2.1.2]. The two enzymes gave approximately equal amounts of ^{14}C-labeled products from the two kinds of labeled starch granules. If the addition of D-glucose had been to the nonreducing end of a primer chain, the second set of reactions with nonlabeled ADPGlc should have given no ^{14}C-labeled products when reacted with either glucoamylase or β-amylase. Because the two enzymes, respectively, formed ^{14}C-labeled D-glucose and ^{14}C-labeled maltose in approximately equal amounts from the two kinds of reactions by the action of the two kinds of enzymes, the addition of ^{14}C-labeled-D-glucose had to have been to the reducing end of a growing amylose chain and not to the nonreducing end of a primer chain. From these three studies, a de novo transglycosylation reaction by a two-catalytic-site insertion mechanism was proposed for the biosynthesis of starch chains by starch synthase.[12–14]

The potential growth of starch granules was reported many years ago by exposing $^{14}CO_2$ to a leaf of a plant for 2 h and then collecting the starch granules from the leaf 24 h later, followed by the preparation of radioautograms of the starch, which showed qualitatively that the radioactivity was higher in the outer layers than in the inner layers.[17,18] From this it was concluded that newly formed starch is deposited by apposition to the surfaces of the starch granules, rather than by intussusception, that is, growth from inside the granule outward. In both studies, however, this conclusion was drawn from very qualitative experiments that were obtained by radioautography of the starch granules 24 h after photosynthesis had been performed using $^{14}CO_2$.

In the present study, starch granules from potato, wheat, maize, and rice were reacted with ADP-[^{14}C]Glc, and the distribution of ^{14}C-D-glucose in the layers of starch granules was obtained by peeling the granules in Me_2SO–H_2O at 10 °C.[19] The distribution of starch synthase activity in the granules was also determined by peeling the surface of the granules and assaying the non-peeled parts of the granules, obtaining the synthase activities in the individual peeled layers. From this and the mechanism for the biosynthesis of starch,[12–14] a hypothesis for how starch granules grow in vivo is proposed.

2. Experimental

2.1. Materials

Potato, wheat, maize, and rice starches were freshly prepared from their mature sources, using standard procedures,[20] but without the addition of sodium bisulfite, ammonium oxalate, or mercuric chloride enzyme denaturants. Because the starches were not isolated with enzyme denaturants, the granules contained some hydrolytic enzymes adsorbed on their surfaces, as judged by the addition of the granules to a solution of 1 mg/mL maltohexaose and by the observation of the formation of D-glucose and maltodextrin hydrolytic products. The enzymes were inactivated and/or removed by treatment of the granules with 0.016% (w/v) sodium bisulfite for 10–30 min. The exception was potato starch for which the enzymes could be removed by washing the granules with EDTA/glycine buffer (pH 8.4) for 10 min, followed by washing with distilled water five times. Prior to the addition of the bisulfite or washing with buffer, it was observed that the granules were not degraded by the adsorbed enzymes as judged by incubation in buffer at 40 °C for 1 h, as no D-glucose or maltodextrins were observed by TLC. The washing and treatment with bisulfite only reduced the starch synthase in the granules by 5–10%.

Maize (dent corn) seeds were obtained from Dr. Thomas Binder of Archer, Daniel, Midland Co., Decatur, IL. Rice and potatoes were obtained from a local market. Hard, red spring wheat berries were purchased from a local organic food store. After isolation, the starch granules were assayed for starch synthase as follows: 100 mg of starch granules were suspended in 1.0 mL of 0.1 mM

EDTA–4 mM glycine buffer (pH 8.4). The reaction was initiated by adding 2 μmol (1 μCi) of ADP-[^{14}C]Glc and allowing the reaction to proceed at 40 °C for 30 min. The reaction was stopped by centrifuging the granules for 1 min, followed by washing five times with 1 mL of water, and then treatment with 1 mL of anhyd acetone five times and one time with anhyd ethanol. The granules were weighed, and the ^{14}C incorporated into the granules was determined by liquid scintillation counting. Starch synthase activity was defined as the nmoles of D-glucose from ADPGlc incorporated into 100 mg of starch granules per hour at pH 8.4 and 40 °C.[12]

Bacillus amyloliquefaciens α-amylase [EC 3.2.1.1] and adenosine α-D-glucopyranosyl 5′-diphosphate glucose (ADPGlc) were obtained from Sigma–Aldrich, St. Louis, MO. ADP-[^{14}C]Glc (333 mCi/mM) was obtained from GE Health Care (Amersham Biosciences), Piscataway, NJ. Liquid scintillation cocktail contained 5.0 g of PPO and 0.1 g of POPOP in 1.0 L of toluene.

2.2. Methods

2.2.1. Controlled peeling of starch granules

The starch granules were sized as previously described.[19] The granules were peeled from the granule surfaces by suspending each of the varieties (110 mg of starch, containing 10–15% w/w H_2O) in 1.0 mL of 90:10 volume proportions of Me_2SO–H_2O in 2-mL capped microcentrifuge tubes at 10 °C for various periods of time, as previously described.[19]

The samples in the capped microcentrifuge tubes were shaken in a horizontal shaker at 10 °C. The tubes were removed from the shaker at 1-h intervals for analysis. They were centrifuged 2 min at 4 °C, the supernatants were removed, and the starches were washed two times with 1.0 mL of water at 4 °C. Imidazole·HCl buffer (1.0 mL of 40 mM, pH 6.8) with 1 mM $CaCl_2$, containing 20–100 μL of *B. amyloliquefaciens* α-amylase (14–70 units, where 1.0 unit equals 1.0 μmol of α-(1→4) bonds hydrolyzed per min at pH 6.8 and 20 °C), was added to the starch suspension for reaction at 20 °C for varying lengths of time (see Table 2 in Ref. 19 for the various protocols for the amounts of enzyme and the reaction times). The starch suspension was centrifuged at 4 °C, and washed 5 times with 1.0 mL of water. At this point, the starches were treated differently, depending on whether the radioactivity was to be determined (Section 2.2.2) or whether the starch synthase activity was to be determined (Section 2.2.3).

2.2.2. Determination of the distribution of synthesized starch in the layers of the starch granules reacted with ADP-[^{14}C]Glc

For the determination of the distribution of synthesized starch in the granules, the sized starch granules (1.5 g) were suspended in 5 mL of 0.1 mM EDTA–4 mM glycine buffer (pH 8.4) containing 1.5 μCi (6.2 nmol) ADP-[^{14}C]Glc and were allowed to react for 30 min at 40 °C with shaking; the starch suspensions were centrifuged, the supernatants removed, and these were then washed five times with 5 mL of water and air dried at 20 °C. From these reacted starches, ten 110-mg samples of the starches were placed in the peeling solution and placed in a horizontal shaker at 10 °C. One of the ten samples was removed every hour for 1–10 h and treated as described above in Section 2.2.1 After washing each sample five times with water, the starches were treated five times with 1.0 mL of anhyd acetone and one time with anhyd ethanol. The samples were then dried at 37 °C for 18 h, followed by 15 h at 40 °C under vacuum. The dried starches were weighed and suspended in 10 mL of scintillation cocktail, and the radioactivity was determined by heterogeneous liquid scintillation counting for 10 min or 10,000 counts, whichever came first. The radioactivity in each layer of the granules was determined as described in Section 2.2.4. The experiments were repeated three times and gave differences that were between 1% and 2% for individual determinations.

2.2.3. Determination of the distribution of starch synthase activity in the layers of starch granules

The sized starch granules were peeled as described in Section 2.2.1. After peeling of each layer, the remaining parts of the non-peeled starch granules were washed five times with water and air dried 48 h at 20 °C. They were then suspended in 1.0 mL of 20 mM (0.2 μCi) ADP-[^{14}C]Glc, containing 0.1 mM EDTA/glycine buffer (pH 8.4) and incubated 4 h at 37 °C for the starch synthase assay. They were then centrifuged at 4 °C for 4 min, and the supernatants were removed, washed five times with 1 mL of water, three times with 1 mL of anhyd acetone, and once with 1 mL of anhyd ethanol. The samples were dried at 37 °C for 18 h and then at 40 °C for 15 h under vacuum. The dried starches were weighed to determine the weight percent of the granules in each layer. The samples were then suspended in 10 mL of scintillation cocktail, and the radioactivity was determined by heterogeneous liquid scintillation counting for 10 min or 10,000 counts, whichever came first. From the amount of ^{14}C incorporated into the starch granules, the number of units (1.0 U = 1.0 nmol of D-glucose incorporated into starch/h at pH 8.4 and 40 °C) was determined.[12] The starch synthase activity in each layer was determined as described in Section 2.2.4. The experiments were conducted in triplicate, and the average and standard deviations were determined.

2.2.4. Calculation of the amounts of ^{14}C incorporated from ADP-[^{14}C]Glc and the amounts of starch synthase activities in each of the gelatinized layers

The amount of ^{14}C incorporated into the granules (from Section 2.2.2) and the amount of starch synthase activity (from Section 2.2.3) in the 1st gelatinized layer were obtained by the subtraction of the activities of the granules after the 1st gelatinization (either the ^{14}C activity incorporated or the starch synthase activity) from the activities of the unpeeled (ungelatinized) starch granules to give the activities in the 1st layer, and the activities of the second sample of gelatinization (the 2nd layer) were obtained by subtraction of the activities of the 1st layer from the activities of the ungelatinized portion of the granule from the 2nd sample of peeling, and so forth for each of the subsequent samples of gelatinization to give the activities for each of the peeled layers.

3. Results and discussion

3.1. Description of the layers obtained by the Me_2SO–H_2O-peeling process at 10 °C for the four types of starch granules

The peeling process for potato starch granules gave 8–9 layers when the granules were peeled in 90:10 (v/v) Me_2SO–H_2O at 10 °C and sampled every hour. Figure 3 shows that there are nine crystalline layers in potato starch that had been treated with *B. amyloliquefaciens* α-amylase, and therefore nine crystalline/amorphous layers in untreated potato starch granules, as the α-amylase exclusively hydrolyzes the amorphous layers, possibly corresponding closely to the nine crystalline/amorphous layers observed in the potato starch granules. The other three kinds of starches gave a similar number of peeled layers when treated with 90:10 (v/v) Me_2SO–H_2O at 10 °C and sampled every hour: wheat starch gave nine layers, maize starch gave seven layers, and rice starch gave eight layers. It is, therefore, concluded that the peeling process used gives very close, if not exactly, the numbers of crystalline/amorphous layers found in the four kinds of starch granules used in this study.

3.2. Distribution of synthesized starch from the reaction of starch granules with ADP-[^{14}C]Glc

The distribution of the synthesized starch from the reaction of ADP-[^{14}C]Glc with four varieties of starches are shown in Figure 1A–D. For all of the starches, the majority of the synthesized starch was in the first one or two outer layers, with decreasing amounts in each of the following subsequent layers. This is what might be expected, as the volumes, and therefore, the amounts of starch and starch synthase in the layers decrease going from the outermost layers to the innermost layers of the granules. The results show that starch is synthesized in every layer throughout the entire granule, from the surface to the center, when ADPGlc is present. During the reaction, the granules were not exclusively synthesized at their surfaces, by apposition, as had previously been hypothesized by Badenhuizen and Dutton[17] and Yoshida et al.,[18] using $^{14}CO_2$, during photosynthesis, followed by radioautographic examination of the starch granules.

Robyt and co-workers[12–14] had previously shown that starch chains are biosynthesized by the addition of D-glucose from ADPGlc to the reducing ends of growing starch chains that are covalently attached to the active sites of starch synthase (see, Fig. 4 for the mechanism of the biosynthesis of starch chains). The starch chains are extruded from the active sites of the enzymes until they are released from the enzymes by hydrolysis. Starch chains, thus, do grow outward from each of the layers where starch synthase is located, but they do not grow exclusively, by apposition, from the outer surface of the starch granules. Starch chains

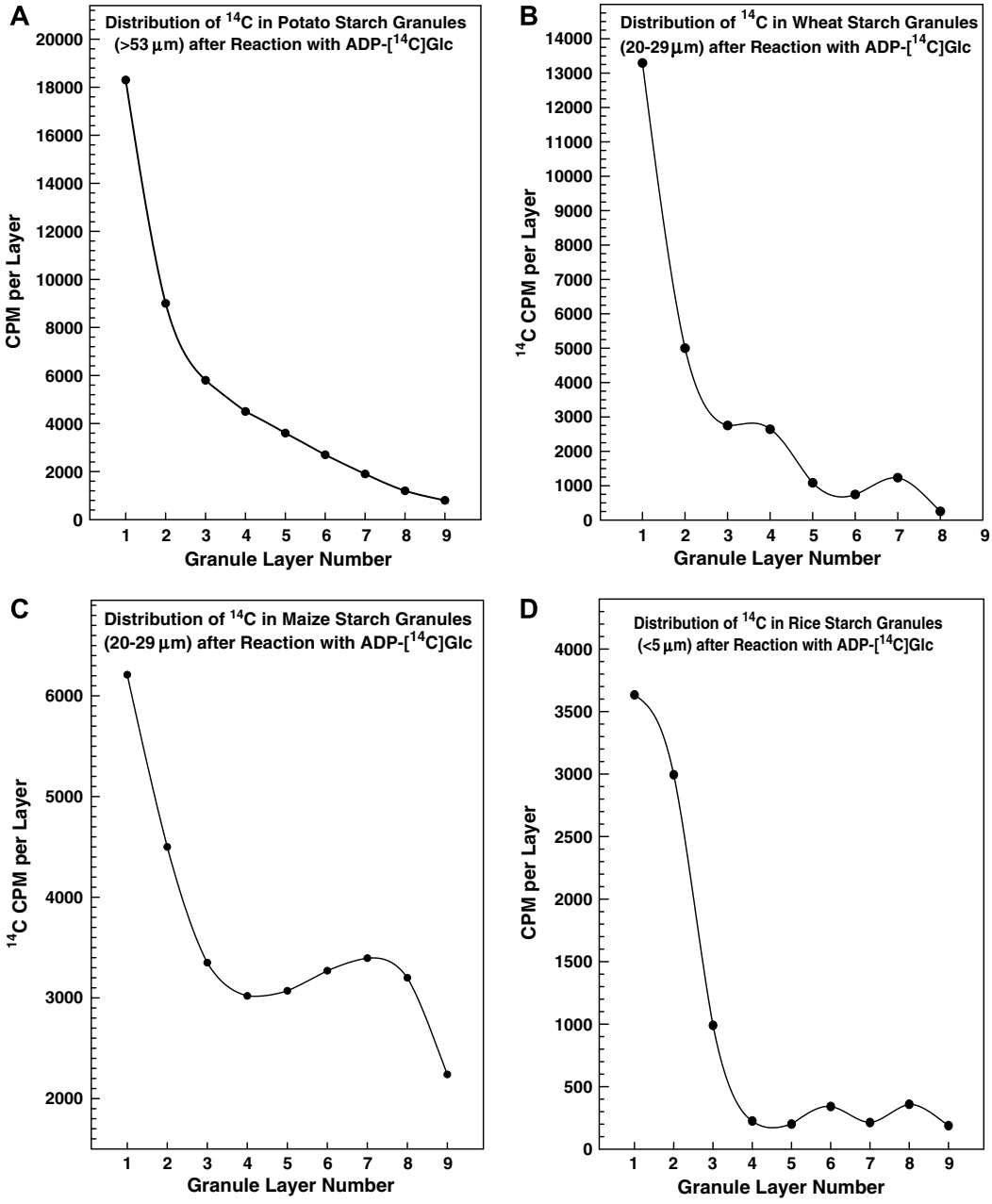

Figure 1. Distribution of ^{14}C in four kinds of starch granules after reaction with 1.5 μCi (6.2 nmol) of ADP-[^{14}C]Glc for 30 min at 40 °C: (A) potato starch; (B) maize starch; (C) wheat starch; and (D) rice starch.

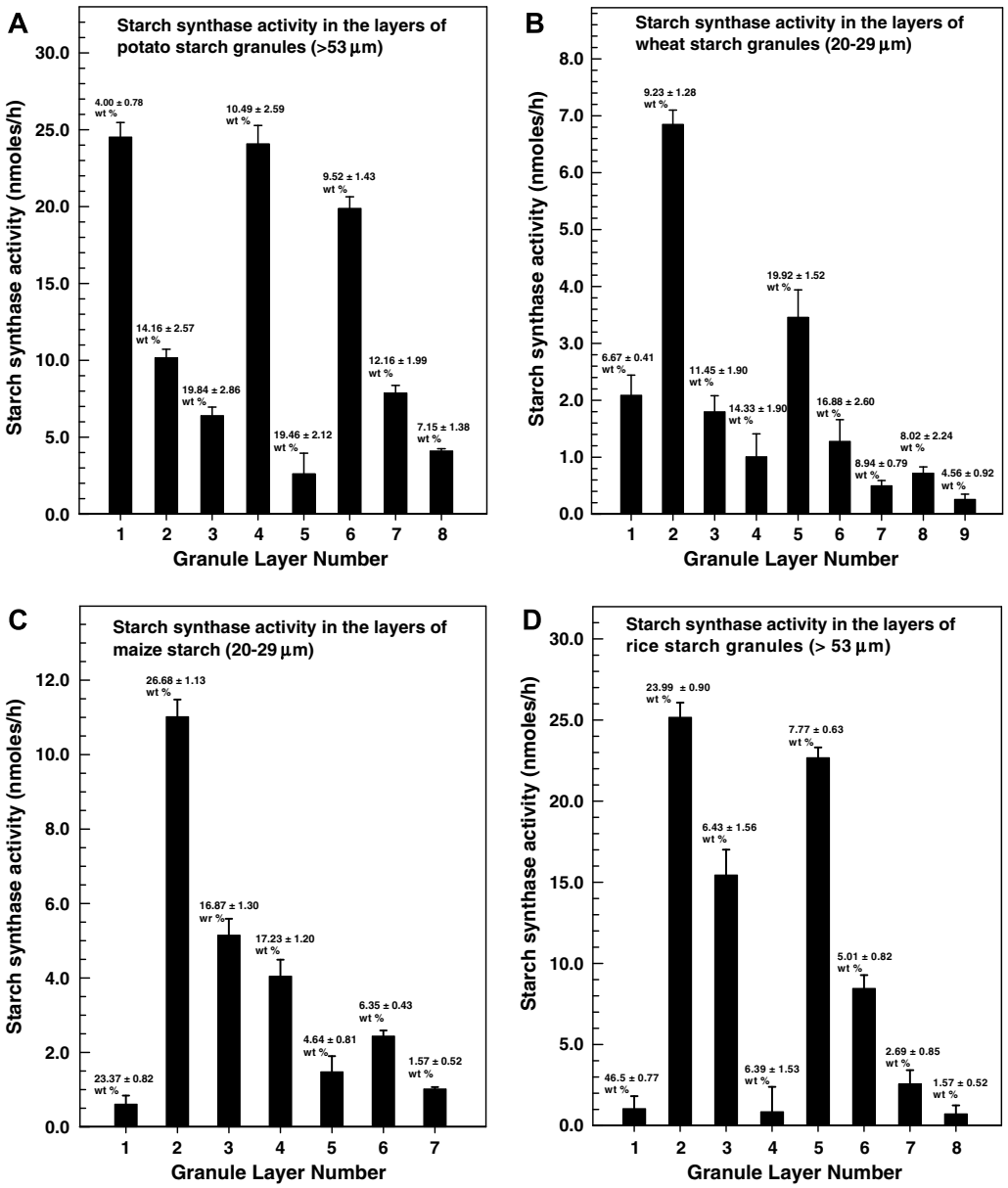

Figure 2. Distribution of starch synthase activity (nmoles Glc incorporated into starch granules/h) in the four kinds of starch granules: (A) potato starch; (B) maize starch; (C) wheat starch; and (D) rice starch. The percentages above the columns are the weight percents of starch in the individual layers that were obtained from the peeling process.

are synthesized in each of the layers, including the initiation site or hilum of the granules whenever ADPGlc, which can diffuse freely through the granules, is present.

The continual synthesis of starch in the inner layers contributes to a higher density of starch and crystallinity in these layers than the outer layers, as they continue to incorporate glucose from ADPGlc every time it is available to the granules, especially those with the low volumes at the core or hilum and adjacent layers of the granule, in contrast to the larger volumes of the outermost layers. This was observed in our previous study on the peeling of starch granules in aqueous Me_2SO, in which the first or second layers were usually more rapidly peeled than the subsequent inner layers, indicating less structural order and integrity in the outer layers, compared with the inner layers that were peeled more slowly.[19]

Potato starch was the largest sized granules, having a size of >53 μm; maize and wheat starch granules were intermediate, having a size of 20−29 μm; and rice starch was the smallest sized granules, having a size of ⩽5 μm. The differences in the distributions of the amounts of starch synthesized for the four starches are reflected by the differences in the sizes and the volumes of the layers. Potato starch granules had a relatively high amount of starch synthesized in the first layer, followed by a nearly exponential decrease in the subsequent eight layers (Fig. 1A). Rice starch (Fig. 1D), the smallest granules, on the other hand, had the highest amount of starch synthesized in the first two layers, with very low and close to the same amounts in the subsequent seven layers, which had smaller volumes. Wheat and maize starches (Fig. 1B and C) were approximately in between, with the highest amounts in the first two layers, followed by much lower and closer amounts of starch formed in the subsequent seven layers. The data show that all four of the starches had formed ^{14}C-labled starch through out the entire granule and not just at the surface of the granules when ADPGlc was present.

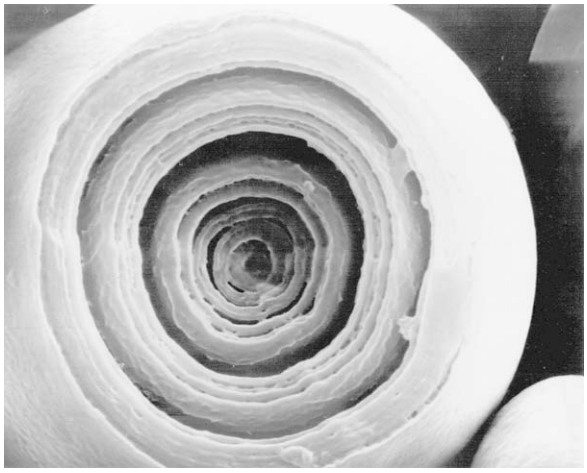

Figure 3. Scanning electron micrograph of a potato starch granule that has had the amorphous areas of the granule removed by treatment with *Bacillus amyloliquefaciens* α-amylase, showing the non-hydrolyzed, highly ordered crystalline ring structures of the internal parts of the granule.

3.3. Location of starch synthase activity in the layers of the starch granules

The starch synthase activity located in the various layers of the four varieties of starch granules is shown in Figure 2A–D. Because the outer one or two layers are of much greater volume than the inner layers, there is a higher amount of starch synthase present in one of the two outer layers than in the inner layers. This is observed for potato starch (Fig. 2A); for wheat, maize, and rice starches, the second layer had the highest starch synthase activity (Fig. 2B–D). This probably resulted from a lower amount of starch synthase being produced just before the plant materials had stopped synthesizing starch.

It is interesting to note that the amount of starch synthase activity in the granules was not necessarily present in the layer that had the highest weight percentage of the granule. The highest amount of starch synthase activity present in potato starch granules occurred in the first layer, which only represented 4.00 ± 0.78 wt % of the granules (Fig. 2A). The highest starch synthase activity of wheat starch occurred in the second layer that only had 9.23 ± 1.28 wt % of the granules (Fig. 2B). Maize starch also had the highest starch synthase activity in the second layer that had 26.68 ± 1.13 wt % of the granules and had the lowest amount of starch synthase in the first layer that had 23.37 ± 0.82 wt % of the granules (Fig. 2C). Rice starch also had the highest starch synthase activity in the second layer that had 23.99 ± 0.90 wt % of the granules and had the lowest starch synthase activity in the first layer that had the highest 46.15 ± 0.77 wt % of the granules. Thus, the amount of starch synthase activity in the layers is not correlated with the amount of starch in the layers.

For all four of the starch varieties, the starch synthase activities in the granules had an interesting pattern of alternating higher and lower enzyme activities. For potato starch (Fig. 2A), the starch synthase activities were quite high in the 1st, 4th, and 6th layers and much lower activities in the 2nd, 3rd, 5th, 7th and 8th layers. For wheat starch (Fig. 2B), starch synthase activities were relatively high in the 2nd, 5th, and 8th layers and relatively low in the 1st, 3rd, 4th, 6th, 7th, and 9th layers. For maize starch (Fig. 2C), starch synthase activities were relatively high in 2nd and 6th layers and relatively low in the 1st, 3rd, 5th, and 7th layers, and for rice starch (Fig. 2D), starch synthase activities were relatively high in the 2nd, 3rd, 5th, and 6th layers and relatively low in the 1st, 4th, 7th, and 8th layers.

3.4. Concluding discussion and the development of a hypothesis for how starch granules grow in vivo

When starch granules are incubated in an aqueous environment with ADP-[^{14}C]Glc, the starch granules become labeled throughout the granule. ADPGlc freely diffuses through the granule, binding to the active sites of starch synthase that was entrapped in the granules during their original biosynthesis. There is a greater amount of starch synthesized in the outer layers than in the inner layers, primarily due to the much larger volumes in the outer layers, and decreasing volumes in the inner layers toward the core of the granule. Nevertheless, the synthesis of starch occurs in all parts of the granule when ADPGlc is present, giving a higher density of starch, with a higher level of structural order and integrity in the inner layers than in the outer layers.

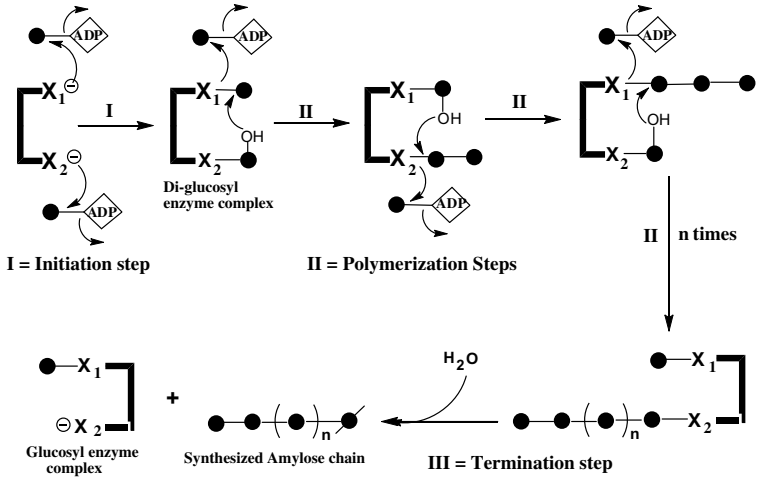

Figure 4. The biosynthesis of the amylose chain from the reducing end by a two-catalytic-site insertion mechanism. The black circles represent D-glucose residues, and the circle with a slash through it represents a free hemiacetal D-glucose reducing end. $\mathbf{X}_1$ and $\mathbf{X}_2$ are the two carboxyl catalytic groups at the active site of starch synthase. There are three steps in the synthesis: **I,** the initiation step in which the D-glucose units from ADPGlc are added to $\mathbf{X}_1$ and $\mathbf{X}_2$; **II**, the polymerization steps that occur many times to give the amylose chain by the nucleophilic attack of the C4–OH group of D-glucose onto C1 of the covalently linked D-glucopyranosyl residue of the amylose chain, giving transglycosylation of the amylose chain and the increase in the size of the chain by one D-glucose unit each time that it occurs; and **III**, the termination step in which the amylose chain is released from the active site by hydrolysis.

The data from the measurement of the amounts of starch synthase activity in the various layers of the starch granules showed an interesting alternating pattern of higher and lower starch activities for the four starches. We hypothesize that the results from these studies indicate how starch granules are formed and grow in vivo. It is proposed that the granules start out as very small aggregates of starch synthase that synthesize starch by forming covalent intermediates, postulated from previous studies[12–14] and add D-glucose from ADPGlc to the reducing ends of the growing starch chains by the two-catalytic-site, insertion mechanism, which was previously demonstrated by three distinct experimental procedures.[12–14] The starch chains, thus, are extruded outward from the active sites of the starch synthase that are in the granules and are eventually branched by starch branching enzymes, and then released from the active sites of the starch synthases by hydrolysis. The synthesis eventually slows down and ceases when the supply of ADPGlc is diminished. At some later point, these small synthesized starch particles then absorb new starch synthesizing enzymes (starch synthase and branching enzymes), and new starch chains are synthesized from newly supplied ADPGlc. The new starch chains are extruded outward in three dimensions, and then starch synthesis again slows down, and stops; and then again with new adsorption of enzymes and the formation of ADPGlc, synthesis occurs again, slows down, and stops. These successive processes occur to build the starch granules and give successive layers of starch chains that grow outward, giving the typical alternating internal starch granule crystalline structures and amorphous structures that result from the initial rapid synthesis of starch chains and the eventual slowing down of the synthesis that gives fewer chains, further apart and less associated, respectively, as described by French,[21] which results in the so-called starch granule growth rings shown in Figure 3 for the potato starch granule. These putative growth rings have a high degree of similarity to the growth rings observed for trees and their wooden branches. Further, during the successive syntheses of starch granules, the inner layers of the starch granules continue to synthesize starch to give a higher density and a greater structural order, while the outer layers have a larger volume and a consequent lower concentration of ADPGlc and starch synthase, and a lesser amount of time for chain synthesis, due to the fact that the inner layers of the starch granules have chain synthesis every time ADPGlc is present. This gives rise to lesser amounts of intermolecular chain interaction in the outer layers and the outer parts of the inner layers, producing less dense and less structured order, and the formation of amorphous starch chains in each of the layers.

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