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In Vitro Synthesis of Ten Starches by Potato Starch-Synthase and Starch-Branching-Enzyme Giving Different Ratios of Amylopectin and Amylose

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Abstract

The objectives of this study was the use of two highly purified enzymes, potato Starch-Synthase (SS) and Starch-Branching-Enzyme (SBE) to obtain *in vitro* the syntheses of ten starches, with different ratios of amylopectin and amylose. The amylose was first synthesized by the reaction of SS with ADP [14C] Glc to give ten identical amyloses; the amylopectin was then synthesized by the action of SBE to give ten starches with different amounts of amylopectin. Two of the 14C-amyloses were reacted with 1.0 mIU of purified Starch-Branching-Enzyme (SBE) for 75 and 130 sec, respectively. In a second synthesis, 3 of the amyloses were autoclaved and reacted with 1.0 mIU for 15, 45, and 75 sec. In the third synthesis, 5 amyloses were reacted with different amounts of SBE from 0.50 to 0.01 mIU for 130 sec each. The 10 synthesized starches were separated into amylopectin and amylose fractions. The amylopectin and amylose ratios ranged from 99.9% amylopectin and 0.1% amylose to 10% amylopectin and 90% amylose. The results show that 10 different starches were synthesized, using only two enzymes, SS and SBE. No primers were involved. Glycogen and debranching enzymes were also not involved.

Keywords: Starch-Synthase; Starch-Branching-Enzyme; *In vitro* synthesis of starch; ADPGlc; Amylose; Amylopectin; Ten kinds of starches

Introduction

Starch usually is composed of a mixture of two polysaccharides: amylose and amylopectin. Amylose is a α-1, 4-linked D-glucopyranose polymer and amylopectin has a number of α-1,4-linked D-glucopyranose chains, joined to other α-1,4-linked D-glucopyranose chains by 5-6 % α-1,6 branch glycosidic linkages [1]. Most starches contain 75-80 % amylopectin with 25-20 % amylose, respectively. There are, however, some starches that contain 100% amylopectin (waxy varieties) and some that are high amylose starches, containing 50-80% amylose and 50-20% amylopectin, respectively. Amylopectins are branched 5-6 %, giving average branch chain lengths of 20-16 D-glucopyranose units, [1] respectively. Using pulse and chase reactions of highly purified Starch-Synthase (SS) with ADP-[^{14}C] Glc and nonlabeled ADPGlc, respectively, we recently have shown that starch chains are biosynthesized de novo from the reducing-end, without a primer requirement [2]. The synthesis starts by forming two D-glucopyranosyl covalent intermediates at the active-site of SS; the C-4-OH group of one of the glucopyranosyl intermediates then makes an attack on the C-1-carbon of the other glucopyranosyl intermediate to transfer the glucose and make a α-1,4-glycosidic linkage. The free catalytic group then attacks another ADPGlc unit and forms a D-glucopyranosyl-intermediate. The synthesis then proceeds processively, going back-and-forth between the covalent D-glucopyranosyl-C-4-OH groups and the growing D-glucopyranose-chains. The D-glucopyranosyl units are transferred to the reducing-ends of the growing chains. The synthesis is called a "two catalytic-site insertion mechanism" and results in the processive addition of D-glucopyranose from ADPGlc to the reducing-ends of growing starch chains [2] (Figure 1) During the pulse reaction, the reducing-ends become labeled with ^{14}C-D-glucose and that when the pulsed-polysaccharides are isolated, reduced with $NaBH_4$, and acid hydrolyzed, ^{14}C-D-glucitol is obtained; and in the chase reaction, the ^{14}C-D-glucose is chased from the reducing-ends into the chain, and in reduction and acid hydrolysis, the ^{14}C-D-glucitol is decreased, because nonlabeled D-glucose units are being added to the reducing-ends in the chase. If the synthesis had been to the nonreducing-ends of primers, no D-glucitol would ever have been formed in the pulsed reactions and therefore decreased in the chase reactions. In the present study, the *in vitro* biosynthesis of starch was performed by using only two highly purified potato starch enzymes: Starch-Synthase (SS) and Starch-Branching-Enzyme (SBE). The amounts of synthesized amylose was approximately the same for each reaction and ten different kinds of starches were obtained by the conversion of different amounts of amylose into amylopectin by the reaction of SBE with the amyloses for different lengths of time or by different amounts of SBE.

Experimental

Materials

Adenosine 5'-diphospho-α-D-glucose (ADPGlc); dithiothreitol (DTT); polyvinyl alcohol 50K; PPO and PoPoP were obtained from Sigma-Aldrich Chem. Co. (St. Louis, MO. USA). ADP-[^{14}C]Glc (333 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63147, USA. Amylose was obtained by fractionation of potato starch as given in section, Separation of Amylose from Amylopectin, in the Methods Section [3], or obtained from Sigma-Aldrich Chem. Co. Starch-Synthase (SS) used was Fraction 23 [3] a highly purified enzyme, with a specific activity of 944 mIU/mg (1.0 IU is an International Unit that incorporates 1.0 µmole of D-glucose into starch/min) and was shown to be primer-free [4]. Starch-Branching-Enzyme (SBE) was Fraction 4 [3]. It had a specific activity of 198 mIU/mg, where 1.0 IU equal 1.0 µM of α-1,6 branch linkages formed/min and also was shown to be primer-free, along with

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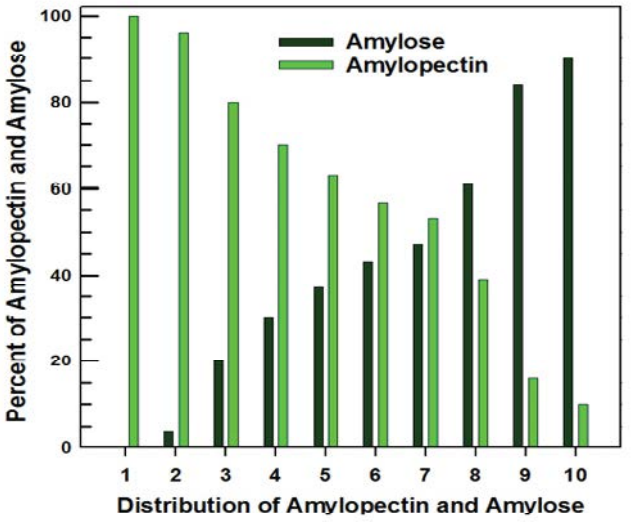

Figure 1: Distributions of amylopectin and amylose in the ten in vitro synthesized starches by highly purified potato Starch-Synthase and Starch-Branching-Enzyme.

the Standard Buffer [2]. The Standard Buffer was pH 8.4 and contained 10 mM glycine, 2 mM EDTA, 0.04% (w/v) polyvinyl alcohol 50K, and 1 mM DTT. Liquid Scintillation Cocktail was prepared, containing 5.0 g PPO and 0.1 g PoPoP in 1 L of toluene (Table 1).

Methods

Assay for starch-synthase (SS): The SS assay reagent contained (75 µL) Standard Buffer (pH 8.4) with 20 mM (0.05 µCi) ADP-[^{14}C] Glc; 25 µL of SS samples were added to the assay reagent and incubated at 37°C for 30 min; 25 µL aliquots were then taken in triplicate and each added to 1.5 cm^2 Whatman 3MM paper, which is immediately added to 100 mL of MeOH, with stirring for 10 min, to stop the reaction and precipitate the synthesized ^{14}C-amylose onto the paper, and wash away ADP-[^{14}C] Glc, and buffer material. The papers are washed 2-more times with 100 mL of MeOH and then dried and counted in 10 mL of toluene scintillation cocktail. A control blank is prepared by adding the Standard Buffer and the ADP-[^{14}C] Glc substrate in the same volume as the reaction digest, with Standard Buffer substituted for the SS enzyme sample. 1.0 mIU=1.0 nmole of D-glucose incorporated into amylose/min.

Assay for starch-branching-enzyme (SBE): Starch-Branching-Enzyme (SBE) (360 µL) in Standard Buffer (pH 8.4) is added to 200 µL (3 mg/mL) amylose and incubated at 37°C for 60 min; the reaction was stopped by placing the digest in a boiling water bath for 5 min and then cooled and centrifuged to remove denatured protein. The supernatant was removed and 2-10 µL of potassium triiodide reagent (2 mg iodine and 20 mg potassium iodide) was added to the digest supernatant to the point of just giving a slight blue triiodide color, and oxidize the DTT in the buffer. This was followed by a micro-titration (Hamilton Syringe) with 0.15 mM of silver nitrate, until the blue color just disappeared; the resulting silver iodide was removed by centrifugation. The pH was then made 5.2 by the addition of 10 µL of 40 mM pyridine-acetate buffer, containing 200 units of isoamylase (where 1 unit=1.0 nmole of α-(1→6) bonds hydrolyzed/min), and the isoamylase reaction was allowed to proceed for 30 min at 37°C. Three 100-µL aliquots were added to the wells of a microplate, containing 100 µL of copper bicinchoninate reagent, and the reducing value was determined, using maltose (20-200 µg/mL), as a standard [4]. The number of nmoles of α-(1→6) branched linkages formed/min by SBE was calculated from the reducing value obtained from the isoamylase reaction. A control blank was prepared by adding the Standard Buffer with the substrate, substituting water for the enzyme sample, and then carrying it through the isoamylase reaction, giving the exact reaction conditions used for the samples. One unit of SBE = 1.0 nmol of α-(1→6) branch linkages formed/min.

Separation of Amylose from Amylopectin in the synthesized starches [5]: Potato starch (1.0 g in 100 mL) was added to each of the enzyme digests as a carrier when the reaction was over. The pH was adjusted to 7.0 and stirred for 30 min at 21-22°C; 22 mL of 1-butanol was added and stirred for 24 hr at 22°C to precipitate the amylose/1-butanol complexes and were then centrifuged at 20K rpm for 30 min, washed with 20 mL of water; and the amylose was then suspended in 40 mL of water, and the 1-butanol was removed by heating to boiling with the addition of water to maintain the volume. The amylose was then precipitated by the addition of 3 volumes of ethanol and placed at 4°C for 24 hr. The amylopectin that remained in the supernatant from the precipitation of the amylose by butanol-1 was precipitated by the addition of 3 volumes of ethanol and placed at 4°C for 24 hr. The amylose and amylopectin were centrifuged and their pellets were washed with 40 mL of acetone/water, 80/20 (v/v), and then treated with 100 mL of acetone, and dried under vacuo for 24 hr.

In vitro synthesis of starch by SS reacting with ADP-[^{14}C] Glc and SBE reacting with the synthesized amylose to give amylopectin

Synthesis type I: Two reactions were performed. Both reactions had 1.05 mL containing 400 mIU of SS. The reactions were initiated by the addition of 30 µL of (25.4 mg, 0.1 µCi) ADP-[^{14}C]Glc and allowed to go for 135 min to give 1.2 Conversion Periods (CP), where 1.0 CP is the theoretical length of time for the complete reaction of ADP-[^{14}C] Glc. The two reactions were initiated by the addition of 30 µL of SBE (1.0 mIU) to each of the two amylose reactions. The first reaction was allowed to go for 75 sec and the second reaction for 130 sec. The reactions were stopped by the addition of 0.1 M trifluoroacetic acid (TFA) to give a pH of 2.0, and 1.0 g of carrier potato starch was added to each reaction, and the amyloses and amylopectins were separated as described under separation of Amylose from Amylopectin in the synthesized starches [5]. The amounts (20-100 mg) of synthesized amyloses and amylopectins were obtained by Liquid Scintillation Spectrometry. All of the pipetting for 100 µL and less for Synthesis Type I was done, using Hamilton Syringes to give the highest accuracy.

Synthesis type II: Three samples of amylose were synthesized identical to the amyloses in Type I. The reactions were allowed to go for 135 min, 1.2 Conversion Periods, where 1 CP is the theoretical length of time to give complete reaction of ADP-[^{14}C] Glc. The digests were then precipitated with 3 volumes of ethanol, which was washed 5-times with 80/20 acetone water, and dissolved in 0.6 mL of water by autoclaving at 121° C, followed by making the volume 1.05 mL by adding Standard pH 8.4 Buffer. The branching reactions were then started by the addition of 30 µL (1.0 mIU) of SBE to the three amylose samples and the reactions were allowed to go 15, 45, and 75 sec, respectively. The reactions were stopped by adding 0.1 M TFA to give pH 2, and 1.0 g of carrier potato

Starch Sample Number	Synthesis Type[a]	Percent Amylopectin Synthesized[b,c]	Percent Amylose Synthesized[b]	Amount of SBE used mIU[c,d]	Time of SBE Reaction Sec[d]
1	I	99.9	0.1	1.0	130
2	III	96.0	4.0	0.5	130
3	III	80.0	20.0	0.3	130
4	II	70.0	30.0	1.0	75
5	II	63.0	37.0	1.0	45
6	III	57.0	43.0	0.2	130
7	I	53.0	47.0	1.0	75
8	III	39.0	61.0	0.1	130
9	II	16.0	84.0	1.0	15
10	III	10.0	90.0	0.01	130

[a]For each of the types of reactions, 400 mIU (1.0 mIU = 1.0 nmole of α-1,4 linkages synthesized/min) of Starch-Synthase was first allowed to go for 135 min or 1.2 CP, where 1.0 CP is the theoretical length of time necessary to convert 100% of the ADPGlc into an identical amount of amylose for each of the ten syntheses.

[b]Presented in decreasing amounts of amylopectin and increasing amounts of amylose, respectively.

[c]The resulting amounts of amylopectin and amylose synthesized is dependent on the amount of Starch-Branching-Enzyme (1.0 to 0.01 mIU) and the length of time (130 sec to 15 sec) that it was allowed to react.

[d]1.0 mIU = 1.0 nmole of α-1,6 branch-linkages synthesized per min.

Table 1: Percent distribution of amylopectin and amylose in ten in vitro synthesized starches, obtained by the reaction of potato Starch-Synthase and Starch-Branching-Enzyme in different amounts or for different lengths of time.

starch was added to each reaction, and the amyloses and amylopectins were separated as described under Methods. The amounts (20-100 mg) of synthesized amyloses and amylopectins were obtained by Liquid Scintillation Spectrometry. All of the pipetting for 100 µL and less for Synthesis Type II was done, using Hamilton Syringes to give the highest accuracy.

Synthesis type III: Five amyloses were synthesized as in Type I. Varying amounts (0.5 mIU, 0.3 mIU, 0.2 mIU, 0.1 mIU, and 0.01 mIU) of SBE were added and the reactions were allowed to go for 130 sec. They were stopped by the addition of 0.1 M of TFA to give pH 2, followed by the addition of 1.0 g of carrier potato starch to each reaction, and the amyloses and amylopectins were separated as described under Separation of Amylose from Amylopectin in the synthesized starches [5]. The amounts (20-100 mg) of synthesized amyloses and amylopectins were determined by Liquid Scintillation Spectrometry. All of the pipetting for 100 µL or less for Synthesis Type III was done, using Hamilton Syringes to give the highest accuracy.

Results and Discussion

Three types of syntheses were performed. All three start with the same amounts of amylose, synthesized by highly purified potato SS to give ^{14}C-labeled amylose. The results of the different percentages of amylopectin and amylose for the ten syntheses are given in (Table 1). It should be noted that the 75 sec reaction of SBE in the Type I synthesis gave different amounts of amylopectin than did the 75 sec reaction of SBE in Type II synthesis. Type I synthesis gave 53% amylopectin, whereas Type II synthesis only gave 16% amylopectin. In the Type II synthesis, the SS had been completely denatured by precipitating and autoclaving before its addition. The SS in the Type I synthesis was not autoclaved and SS activity remained after SBE had been added and amylose continued to be synthesized during the branching reaction by SBE to give much larger amounts of amylopectin by some synergistic mechanism. In (Table 1), the percent distribution for each synthesized amylopectin and amylose are given. The synthesized starches had (a) "very high amylopectin" (99.9% amylopectin and 0.1% amylose); (b) "normal" amounts of amylopectin (80% amylopectin and 20% amylose) and (70% amylopectin and 30% amylose); (c) intermediate amounts of amylopectin and amylose (57% amylopectin and 43% amylose) and (53% amylopectin and 47% amylose); (d) "high amylose starch" (39% amylopectin and 61% amylose); and (e) "very high amylose starch" (16% amylopectin and 84% amylose) and (10% amylopectin and 90% amylose). These results show that various kinds of starches, containing different amounts of amylopectin and amylose, have been synthesized, *in vitro*, by only two highly purified enzymes, potato SS and SBE, in which the SBE was allowed to react with the same amounts of amylose for different lengths of time or with different amounts of activity for a constant length of time. Previous studies of starch biosynthesis [6-10] involved various amounts of primers, phosphorylase, and glycogen. During the course of these studies, Hanes found that if he started with α-Glc-1-P and starch, without any phosphate, some glucose units were added to the nonreducing-ends of the starch chains, giving synthesis. This, in essence, was the origin of the requirement for a primer in polysaccharide biosynthesis. It was found shortly thereafter [7], however, that only a few (2-3) glucose units were added to the nonreducing-ends of the starch chains. In plants and animals, it also was found that the concentration ratio of Pi to α-Glc-1-P was 20-40 folds higher *in vivo*, and phosphorylase was exclusively a degradative enzyme rather than a synthetic enzyme [7-10]. Hanes' observation [6], however, set the stage for the primer concept for polysaccharide biosynthesis and many investigators made futile searches for the putative primers for polysaccharide biosynthesis. One such study was by Koepsell et al. [11], who added isomaltose and maltose to digests of sucrose and Leuconostoc mesenteroides B-512F dextransucrase, and found that these reactions formed isomaltodextrins and maltosyl-isomaltodextrins, with maltose at the reducing-ends, respectively. Both also inhibited the biosynthesis of dextran and were therefore not primers. Likewise, Mukerjea and Robyt [12] found that maltose, maltotriose, and maltododecaose (G12) also inhibited starch biosynthesis by maize, wheat, and rice starch granules, very similar to the findings of Koepsell et al. [11] for the biosynthesis of dextran by dextransucrase.In the early 1960s, Leloir et al. [13-15] also found that active Starch-Synthase, was entrapped inside starch granules, and when reacted with adenosine diphospho-^{14}C-glucose (ADP-[^{14}C]Glc), ^{14}C-starch chains were synthesized in the granule. The ^{14}C-labeled starch was dissolved in buffer and reacted with the exo-acting ß-amylase that hydrolyzes starch from the nonreducing-ends, to give ^{14}C-labeled maltose. From this result, it has been widely assumed that the starch was being biosynthesized by the addition of ^{14}C-labeled glucose to the nonreducing-ends of primers. Later, Mukerjea and Robyt, [12] suggested that the Leloir et al. [15] conclusion that the formation of ^{14}C-maltose by ß-amylase action on their synthesized ^{14}C-starch, which suggested that the addition of glucose was to the nonreducing-ends of primers, was not necessarily correct: if the starch chains had been synthesized de novo by the addition of D-glucose to the reducing-ends, without the involvement of a primer, the entire starch chains would have been labeled with ^{14}C-D-glucose and ß-amylase would also have produced 14C-maltose.In 1948, Wolf et al. [16] assumed that sweet corn starch apparently originated from globules of polysaccharide on the surface of sweet corn. These globules were assumed to be glycogen and, therefore, the starch arose from glycogen. The globules, however, were never shown to be glycogen and could very well have been pre-amylopectin. Nevertheless, from their observation, it was proposed that glycogen was an intermediate in the biosynthesis of amylopectin and amylose. In 1951 Hobson et al. logically indicated, however, that

amylose had to be synthesized first and then part of the amylose converted into amylopectin [17]. However, in 1958 Erlander [18], stated that cumulative evidence indicated that glycogen was the precursor for the formation of the linear amylose chains by the action of isoamylase or debranching-enzyme with the glycogen to give amylose. The released chains, however, that would be released would be quite short, ~10-12 D-glucose units, and did not come close to the number average degree of polymerization (dpn) values obtained for several amyloses: the smallest known was from amylomaize-VII, a high amylose starch, in which the amylose has a dpn of ~400 glucose units; then amylomaize starch, with 20% amylose, has a dp_n of ~800 glucose units; potato starch, with 25% amylose, has dp_n of ~1,000 glucose units; and wheat starch, with 25% amylose, has a dp_n of ~4,000 D-glucose units. In 1990, Baba et al. [19] found that glycogen was the best primer for starch biosynthesis. This observation furthered the hypothesis that starch arose from glycogen. In 1996, Ball et al. [20] proposed that amylopectin was synthesized from glycogen. Glycogen is branched 10-12%, having an average of 8-10 glucose units per chain. They proposed that glycogen was debranched to give 5-6% branching and the chains were then elongated at the nonreducing-ends by Starch-Synthase to give an average of 20-25 glucose units in amylopectin. In 1998, Ball et al. [21] further hypothesized that the amylose, that was initially released from the glycogen was also elongated by Starch-Synthase addition of several hundred glucose units to the nonreducing-ends to give high-molecular weight amyloses. Ball and Morell [22] proposed that glycogen was a precursor for the formation of starch. There are a number of problems with these hypotheses. One problem is that experimental attempts to elongate linear maltodextrin chains and branched maltodextrin chains from the nonreducing-ends by Starch-Synthase only gives the addition of a few (1-3) glucose units to the nonreducing-ends of linear and branched putative primers [23,24] Mukerjea and Robyt [12] further found, a compounding result for Starch-Synthesis, in which different concentrations of the putative maltodextrin primers (maltose, maltotriose, and maltododecaose) inhibited starch biosynthesis, instead of stimulating it, as would have been expected for primers. Using pulse and chase techniques with ADP-[^{14}C] Glc, Mukerjea and Robyt [2] recently showed that Starch-Synthase synthesizes linear amylose chains, de novo, by the addition of D-glucose units to the reducing-ends of growing amylose chains and not to the nonreducing-ends of a primer. Further, Robyt et al. [25] showed that the need for a primer in starch biosynthesis had been perpetuated for over 70-years by a primer myth that arose from the use of Tris-type buffers (Tris, Bicine, and Tricine), which they have recently shown to be potent inhibitors for Starch-Synthase [25]. It was then shown that the inhibition can be partially reversed, giving ~10% Starch-Synthase activity for Tris and Bicine, but 0% for Tricine buffers by the addition of the putative primers that actually are activators that release a small percentage of the ADPGlc substrate that is complexed with the Tris-buffers, producing inhibition [25]. Along with the carbohydrate putative primers, a high concentration of a non-carbohydrate, 500 mM Na-citrate, had also been found by Pollock and Preiss to give about the same degree (10%) activation of SS in the presence Tris-type buffers as did 10 mg/mL glycogen [26]. Na-Citrate is not a carbohydrate and its structure could, therefore, not be acting as a primer. Thus, it was shown that these so-called primers (glycogen, starch, maltodextrins, and Na-Citrate) were not primers, but activators that partially reversed the Tris-buffer inhibition [25]. In the present study, the *in vitro* biosynthesis of ten different kinds of starches, having different percent ratios of amylopectin and amylose, were obtained using only two highly purified potato enzymes, Starch-Synthase and Starch-Branching-Enzyme. The amounts of amylopectin and amylose synthesized are given in Table 1. The ratios range from 99.9% amylopectin and 0.1% amylose to 10% amylopectin and 90% amylose. All ten starches were biosynthesized in this study by first synthesizing identical amounts of amylose by Starch-Synthase and ADP-[^{14}C] Glc, and then having different amounts or different lengths of time of reaction for Starch-Branching-Enzyme to synthesize the α-1,6-branch linkages with the amylose chains, to give 10 different kinds of ^{14}C-labeled starches, with different percentages of amylopectin and amylose see Table 1. The amounts of amylose and amylopectin are also given in Figure 1 so that a comparison can be readily made between the ten synthesized starches. It should be noted that the relative activity of Starch-Branching-Enzyme must be considerably less than the activity of Starch-Synthase, as amylopectin is only branched 5-6%, although the amount of amylopectin is usually considerably larger than the amount of amylose; however, the amount of branching enzyme activity must then be considerably less than the amount of starch synthesizing activity to obtain a starch with significant amounts of amylose e.g., 20-25%.Starch-Debranching-Enzyme(s) that supposedly would react with glycogen and amylopectin were not present in the reactions, and the putative primers (glycogen, maltodextrins, or pre-formed starch) were also not present. The earlier hypotheses put forward by Ball et al. [20,21], in which it was proposed that glycogen was a precursor for the biosynthesis of both amylose and amylopectin, by the action of a Debranching-Enzyme, followed by the elongation of the chains of the partially debranched glycogen and the debranched amylose chains by Starch-Synthase to give amylopectin and an extensively elongated debranched amylose chains also by Starch-Synthase shows that the Ball et al. [20,21] hypotheses are impossible and do not occur because (i) Debranching-Enzyme(s) are not involved in the synthesis and have not been shown to be involved in any study and certainly was not involved in the present study, and (ii) it has been shown that Starch-Synthase also does not elongate starch chains from the nonreducing-ends [2], as is required by the Ball et al. hypotheses [21,22]. Starch-Synthase synthesizes linear, α-1,4 linked chains, de novo, processively from the reducing-end, as shown by Mukerjea and Robyt [2]. The present study, thus, shows that glycogen is not involved in starch biosynthesis as was proposed by Ball and Morell [22], and linear α-1,4-glucose chains are not synthesized from the nonreducing-end of a primer, as has been hypothesized by Ball et al. [21] and many others.While the *in vitro* biosynthesis is not identical to the *in vivo* biosynthesis that occurs in chloroplasts and amyloplasts, this study closely mimics the *in vivo* biosynthesis, capable of giving different kinds of starches. An important point here is that if this synthesis, to give ten different kinds of starches, with different ratios of amylopectin and amylose, can occur *in vitro*, using only two enzymes (highly purified Starch-Synthase and Starch-Branching-Enzyme), it certainly should be able to also occur *in vivo* in chloroplasts and amyloplasts. The study, further, shows that glycogen, Debranching-Enzyme(s), and amylose elongation from the nonreducing-ends, are not involved in starch biosynthesis, and the synthesis only requires two kinds of enzymes, Starch-Synthase(s) that synthesize amylose chains, de novo, from the reducing-end, and Starch-Branching-Enzyme(s) that synthesize α-1,6-linked branches to give amylopectin from the amylose in different proportions to produce different kinds of starches. What the study does not show is how the Starch-Branching-Enzyme is regulated and controlled *in vivo* to give the different ratios of amylopectin and amylose. In this study, it was controlled by first synthesizing amylose and then adding small amounts of Branching-Enzyme for varying lengths of time to convert a portion of the amylose into amylopectin. The major synthesis was performed by Starch-Synthase synthesis (400 mIU of SS reacting with 400 nmoles of ADP-[^{14}C] Glc reaction for 135 min) to synthesize ^{14}C-amylose; and the

amylopectin fraction was then synthesized by the addition of 1.0-0.01 mIU of Starch-Branching-Enzyme for 130 sec to 15 sec with the ^{14}C-amyloses to give the different percentages of the amylopectin fractions. The action of the Branching-Enzyme is, thus, only a fraction of the Starch-Synthase reaction, as amylopectin is only branched to the extent of 5-6% and, therefore, only a relatively small amount of the branching enzyme activity is required to obtain the varying amounts of the amylopectin fractions, to give the different ratios of amylopectin and amylose (Table 1 for the individual number of SBE units and number of seconds that they reacted to obtain the 10 starches). Schwall et al. [27] published a paper in 2000, in which a very high-amylose, potato starch was obtained by simultaneously inhibiting two SBE, A and B isoforms, to give about 1% of the wild-type activities. This project and result further indicates that it is SBE that controls the amount of amylopectin and not a Debranching-Enzyme action on glycogen, followed by extension of the nonreducing-ends of the resulting debranched glycogen and short amylose chains by Starch-Synthase.

References

1. Whistler RL (1965) Starch: Chemistry and Technology Chap. 1, pp. 1-8 in, Vol. I Whistler RL, Paschall EF, BeMiller JN, Roberts HJ, eds., Academic Press, New York; and/or Robyt JF (2008) Properties and Occurrence of Starch in General Properties, Occurrence, and Preparation of Carbohydrates, Chap. 1.2, Glycoscience II, 2nd Ed : 72-73, (Fraser-Reid B, Tatsuta K, Thiem J, eds.) Springer-Verlag, Berlin, Heidelberg.
2. Mukerjea R, Robyt JF (2012) De novo biosynthesis of starch chains without a primer and the mechanism for its biosynthesis by potato starch-synthase. Carbohydr Res 352: 137-142.
3. Mukerjea Ru, Falconer DJ, Yoon SH, Robyt JF (2010) Large-scale isolation, fractionation, and purification of soluble starch synthesizing enzymes: Starch-synthase and branching enzyme from potato tubers. Carbohydr. Res 345: 1555-1563.
4. Fox JD, Robyt JF (1991) Miniaturization of three carbohydrate analyses using a microsample plate reader. Anal Biochem 195: 93-96.
5. Gilbert L, Gilbert GA, Spragg SP (1964) Separation of amylose and amylopectin fractions of starch, in Methods Carbohydr. Chem Starch, Whistler RL, Smith RJ, Be Miller JN, eds: 25-27, Academic Press, New York, Vol. IV
6. Hanes CS (1940) The reversible formation of starch from glucose-1-phosphate catalyzed by potato phosphorylase. Proc Roy Soc Bull 129: 174-208.
7. Green DE, Stumpf PK (1942) Starch Phosphorylase from Potato. J Biol Chem 142: 355-366.
8. Trevelyan WE, Mann PF, Harrison JS (1952) The phosphorylase reaction. I. Equilibrium constant: principles and preliminary survey. Arch Biochem Biophys 39: 419-439.
9. Ewart MH, Siminovitch D, Briggs DR (1954) Studies on the Chemistry of the Living Bark of the Black Locust in Relation to its Frost Hardiness. VIII. Possible Enzymatic Processes Involved in Starch-Sucrose Interconversions. Plant Physiol 29: 407-413.
10. Liu TT, Shannon JC (1981) Measurement of Metabolites Associated with Nonaqueously Isolated Starch Granules from Immature Zea mays L. Endosperm. Plant Physiol 67: 525-529.
11. koepsell HJ, Tsuchiya HM, Hellman NN, Kazenko A, Hoffman CA, et al. (1953) Enzymatic synthesis of dextran; acceptor specificity and chain initiation. J Biol Chem 200: 793-801.
12. Mukerjea Ru, Robyt JF (2005) Starch biosynthesis: the primer nonreducing-end mechanism versus the nonprimer reducing-end two-site insertion mechanism. Carbohydr Res 340: 245-255.
13. Rongine De Fekete MA, Leloir LF, Cardini CE (1960) Mechanism of starch biosynthesis. Nature 187: 918-919.
14. Recondo E, Leloir LF (1961) Adenosine diphosphate glucose and starch synthesis. Biochem Biophys Res Commun 6: 85-88.
15. Leloir LF, De Fekete MA, Cardini CE (1961) Starch and oligosaccharide synthesis from uridine diphosphate glucose. J Biol Chem 236: 636-641.
16. Wolf MJ, MacMasters MM, Hubbard JE, Rist CE (1948) Comparison of corn starches at various stages of kernel maturity. Cereal Chem. 25: 312-325.
17. Hobson PN, Whelan WJ, Peat S (1950) A 'de-branching' enzyme in bean and potato. Biochem J 47: xxxix.
18. Erlander SR (1958) A proposed mechanism for the synthesis of starch from glycogen. Enzymologia 19: 273-283.
19. Baba T, Noro M, Hiroto M, Arai Y (1990) Properties of primer dependent starch synthesis catalyzed by starch synthase from potato tubers. Phytochemistry 29: 719-723.
20. Ball S, Guan HP, James M, Myers A, Keeling P, et al. (1996) From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. Cell 86: 349-352.
21. Van de Wal M, D'Hulst C, Vincken JP, Buléon A, Visser R, et al. (1998) Amylose is synthesized in vitro by extension of and cleavage from amylopectin. J Biol Chem 273: 22232-22240.
22. Ball SG, Morell MK (2003) From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. Annu Rev Plant Biol 54: 207-233.
23. Damager I, Denyer K, Motawia MS, Møller BL, Blennow A (2001) The action of starch synthase II on 6"'-alpha-maltotriosyl-maltohexaose comprising the branch point of amylopectin. Eur J Biochem 268: 4878-4884.
24. Damager I, Olsen CE, Blennow A, Denyer K, Møller BL, et al. (2003) Chemical synthesis of methyl 6'-alpha-maltosyl-alpha-maltotrioside and its use for investigation of the action of starch synthase II. Carbohydr Res 338: 189-197.
25. Mukerjea R, McIntyre AP, Robyt JF (2012) Potent inhibition of starch-synthase by Tris-type buffers is responsible for the perpetuation of the primer myth for starch biosynthesis. Carbohydr Res 355: 28-34.
26. Pollock C, Preiss J (1980) The citrate-stimulated starch synthase of starchy maize kernels: purification and properties. Arch Biochem Biophys 204: 578-588.
27. Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, et al. (2000) Production of very-high-amylose potato starch by inhibition of SBE A and B. Nat Biotechnol 18: 551-554.