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THE ACTIVITIES OF MAIZE α-1, 4 GLUCAN GLUCOSYLTRANSFERASES *IN VITRO*[1]

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Abstract

Three α-1, 4 glucan glucosyltransferase systems, phosphorylase, soluble adensosine diphosphate glucose-starch glucosyltransferase and the starch granule-bound nucleoside diphosphate glucose-starch glucosyltransferase, were assayed for their ability to transfer glucose from substrate into the amylose and amylopectin of natural starch granules, and to produce primer. Phosphorylase II is very active in the synthesis of an amylose-like substance in the absence of primer, but priming by natural starch granules is poor. Soluble adenosine diphosphate glucose-starch glucosyltransferase, when assayed under the unprimed condition, produces an amylopectin-like substance, but no amylose-like material. When assayed under primed conditions, and both amylose and amylopectin are offered simultaneously (natural starch granules) as primer for this soluble glucosyltransferase preparation, glucose is incorporated only into amylopectin. Starch granule-bound nucleoside diphosphate glucose-starch glucosyltransferase, on the other hand, transfers glucose from either adenosine diphosphate glucose or uridine diphosphate glucose into amylose more rapidly than into amylopectin.

Introduction

Three α-1,4 glucan glucosyltransferase systems, namely phosphorylase, soluble ADPglucose-starch glucosyltransferase and the starch granule-bound NDPglucose-starch glucosyltransferase, have been found to transfer glucose from various substrates into starch in α-1,4 glucosidic linkage. Phosphorylases use glucose-1-P. Soluble ADPglucose-starch glucosyltransferases use ADPglucose and the starch granule-bound NDPglucose-starch glucosyltransferase utilizes both ADPglucose and UDPglucose as substrates.

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(2) The following abbreviations are used in this paper: ADPglucose: adenosine diphosphate glucose; UDPglucose: uridine diphosphate glucose; NDPglucose: nucleoside diphosphate glucose.

The studies of starch-deficient maize mutants, *shrunken*-2 (Tsai *et al.*, 1966; Dickinson *et al.*, 1969) and *shrunken*-4 (Tsai *et al.*, 1969), have substantiated the importance of these three α-1, 4 glucosyltransferase systems for the synthesis of starch. However, the physiological role of these activities remains ambiguous. Phosphorylases II and III have been suggested as important enzymes for the synthesis of primer since they can produce an amylose-like substance in the absence of primer, and their activities are only present during the most rapid stage of starch biosynthesis (Tsai *et al.*, 1968; Tsai *et al.*, 1969). Recently, multiple forms of soluble ADPglucose-starch glucosyltransferase have been found in maize seed (Ozbun *et al.*, 1971). In the absence of primer, one of the isozymes is capable of producing a glucan in which the glucose units are largely α-1,4 linked, but with some α-1,6 linkages also. The starch granule-bound NDPglucose-starch glucosyltransferase has been shown to be a different enzyme from soluble ADPglucose-starch glucosyltransferase (Tsai, submitted). Since the bound NDPglucose-starch glucosyltransferase is deficient in a maize starch-forming mutant, *waxy* (Nelson *et al.*, 1962; Nelson *et al.*, 1964; Tsai, submitted), and this mutant synthesizes amylopectin exclusively (Sprague *et al.*, 1943), it has been suggested that the starch granule-bound NDPglucose-starch glucosyltransferase is responsible for the normal synthesis of amylose.

Starch can be readily separated into amylose and amylopectin by Sepharose column chromatography (Chuang, Tsai and Dalby, unpublished data). Therefore, it was anticipated that by incubating each of the α-1,4 glucosyltransferases with various substrates and natural starch granules (primer), and determining the extent of incorporation of glucose into the starch fractions, a better understanding regarding the function of these α-1,4 glucosyltransferases in starch biosynthesis might be obtained.

Materials and Methods

Preparation of enzymes

Samples of the normal (non-mutant maize, *Zea mays* L.) hybrid, B37×B14, were collected 22 days after controlled self-pollination, frozen on dry ice, and stored at −20°C until processing.

As the initial step in processing, kernels were cut from the cob. The embryo and the pericarp were removed from the kernel.

For the preparation of soluble ADPglucose-starch glucosyltransferase and phosphorylase II, a mixture of equal parts of endosperm and chilled 10 mM tris-maleate buffer (pH 7.0), was homogenized for 2 min in a blender (VirTis 45), strained through 2 layers of cheese cloth, and centrifuged for 20 min at 30,900 xg. From the supernatant fluid, the fraction precipitating below 45%

$(NH_4)_2SO_4$ saturation was collected by centrifugation for 20 min at 18,800 xg, suspended in chilled 10 mM tris-maleate buffer (pH 7.0), and dialyzed against the same buffer for 8 hr at 4°C.

About 40 mg of protein measured by the method of Lowry *et al.* (1951) was then added to a DEAE-cellulose column (1×20 cm) equilibriated with 10 mM tris-maleate buffer at pH 7.0. The proteins were eluted with a linear 0 to 0.5 M NaCl gradient buffered with the tris-maleate buffer, and 5 ml fractions were collected. The 5-ml fractions with the activity of soluble ADPglucose-starch glucosyltransferase or phosphorylase II were each pooled, and the protein fraction precipitating below 80% $(NH_4)_2SO_4$ saturation was saved. The pellet was resuspended in chilled 10 mM tris-maleate buffer, pH 7.0, and dialyzed against the same buffer for 8 hr at 4°C.

Starch granule-bound NDPglucose-starch glucosyltransferase was prepared as described previously (Tsai, submitted).

Preparation of starch granules free of bound NDPglucose-starch glucosyltransferase

About 500 mg of the normal starch granules was incubated with 20 ml of 0.2 M N,N-bis(2-hydroxyethyl)glycine (Bicine) buffer, pH 8.5, containing 0.1% pronase and 10 mM $CaCl_2$ for 12 hr at 37°C. Starch granules were collected by centrifugation and washed 3 times with 10 mM tris-maleate buffer, pH 7.0. To assure the complete removal of the bound NDPglucose-starch glucosyltransferase, the starch granules obtained were then subjected to the entire treatment again, starting with the pronase incubation. Starch granules free of bound NDPglucose-starch glucosyltransferase were also obtained by the combination of pronase and the dimethylsulfoxide treatment described previously (Tsai, submitted). These preparations were used as primers for soluble ADPglucose-starch glucosyltransferase and phosphorylase II.

Enzyme assays

For the assay of starch granule-bound NDPglucose-starch glucosyltransferase, 50 μl of the reaction mixture contained 9 μmoles of Bicine buffer, pH 8.5, 0.5 μmole of ADPglucose-^{14}C (200,000 cpm) or UDPglucose-^{14}C (600,000 cpm), and 3 mg of endosperm starch granules. Incubation was at 37°C for 2 hr.

Soluble ADPglucose-starch glucosyltransferase activity was assayed under the primed condition by the method similar to that described by Ozbun *et al.* (1971). The reaction mixture (0.2 ml) contained 50 μmoles of Bicine buffer, pH 8.5, 5 μmoles of potassium acetate, 2 μmoles of GSH, 1 μmole of EDTA, 0.25 μmole of ADPglucose-^{14}C (100,000 cpm) or 0.25 μmole of UDPglucose-^{14}C (300,000 cpm), 3 mg of pronase-treated starch granules, and 50 μl of enzyme preparation containing 55 μg of protein. Incubation was at 37°C for 30 min.

For both soluble and bound glucosyltransferase systems, the reaction was terminated by adding 0.5 ml of 0.1 N NaOH followed by 1.5 ml of methanol. The pellet collected by centrifugation was washed 3 times with 0.1 N NaOH followed by methanol precipitation and centrifugation.

Phosphorylase II activity was assayed either in the presence or absence of starch granules as primer. The reaction mixture (0.1 ml) contained 6 μmoles of 2-(N-morpholino)-ethanesulfonic acid·H_2O (MES) buffer, pH 5.9, 0.5 μmole of $MgSO_4$, 0.5 μmole of glucose-^{14}C-1-P (1,000,000 cpm), 3 mg of starch granules except in the case of unprimed reactions, and 50 μl of enzyme preparation containing 80 μg of protein. Incubation was at 37°C for 15 min. For both primed and unprimed reactions, the reaction was terminated by adding 0.5 ml of 0.1 N NaOH containing 1 mg of amylopectin followed by 1.5 ml of methanol. The pellet collected by centrifugation was washed 3 times with 0.5 ml of 0.1 N NaOH followed by methanol precipitation and centrifugation.

The relative incorporation of glucose-^{14}C into starch fractions was determined by dissolving the reaction products in 0.5 ml of 0.5 N NaOH, neutralizing with glacial acetic acid and fractionating on a Sepharose 4B column (1.7× 34 cm) by eluting with distilled water. Two-ml fractions were collected, transferred into scintillation vials containing Bruno and Christian's fluid (Bruno *et al.*, 1961) and counted with a Packard Tri-carb scintillation spectrometer.

For all the systems studied, there was no significant interference due to amylolytic, branching or debranching enzymes. The activity of the α-1,4 glucosyltransferases was linearly proportional to time of incubation and the amount of protein.

The profile of starch fractions obtained by Sepharose column chromatography was determined by the phenol-sulfuric acid method (Whistler *et al.*, 1962).

β-amylolysis of product formed

Peak fractions obtained from Sepharose columns were treated with β-amylase to characterize the product produced by various α-1,4 glucosyltransferases. To 1 ml of each of the peak fractions was added 1 ml of 0.2 M sodium acetate buffer, pH 4.8, containing 1 mg of β-amylase. After incubation at 37°C for 60 min, the reaction was terminated by adding 2 volumes of methanol and the soluble fraction was recovered by centrifugation and its volume reduced by evaporation. The soluble fraction and an authentic sample of maltose were spotted on a Whatman No. 1 paper and chromatographed with butanol-pyridine-water (6:4:3) (Jeanes *et al.*, 1951) as solvent for 40 hr.

The radioactivity was determined by counting 1 cm sections from the edge strip with a gas flow counter (Nuclear Chicago).

Results

Incorporation of glucose-^{14}C from ADPglucose-^{14}C or UDPglucose-^{14}C ginto starch fractions via starch granule-bound NDPglucose-starch glucosyltransferase

Chaung, Tsai and Dalby (unpublished data) have shown that starch may be fractionated according to molecular size into two major peaks using a Sepharose column. The composition of these two peaks has been identified by iodine absorption spectrum and β-amylolysis. The early peak corresponds to amylopectin, the later peak being amylose (Fig. 1).

As shown in Figure 1, either ADPglucose or UDPglucose can be used as a substrate for the starch granule-bound NDPglucose-starch glucosyltransferase. When ADPglucose is the substrate, the total incorporation of glucose into amylopectin and amylose is comparable. On the other hand, when UDPglucose is used as a substrate, the total incorporation of glucose into amylose is about two times more than into amylopectin. In determining the specific activity of these two starch fractions, glucose is incorporated more rapidly from either substrate, ADPglucose or UDPglucose, into amylose than to amylopectin via the starch granule-bound NDPglucose-starch glucosyltransferase (Table I). Similar results were obtained for rice using UDPglucose by Murata *et al.* (1964).

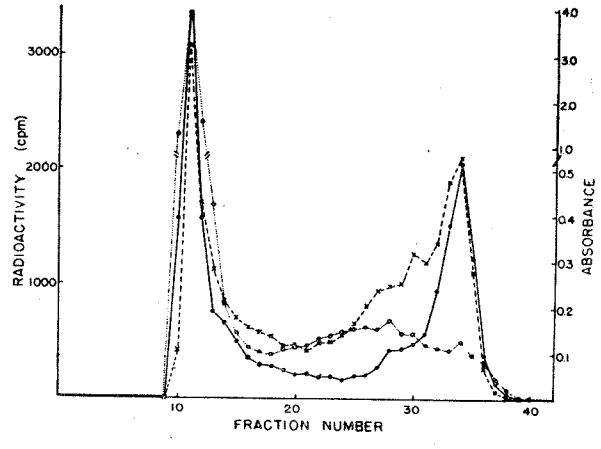

Fig. 1. Incorporation of glucose-^{14}C from either ADPglucose-^{14}C or UDPglucose-^{14}C into starch fractions by the starch granule-bound NDPglucose-starch glucosyltransferase.

o···o···o Carbohydrate profile.
•—•—• ADPglucose as a substrate.
×---×---× UDPglucose as a substrate.

Table I. *Incorporation of glucose-^{14}C into starch fractions by α-1, 4 glucosyltransferases*

Incubation time was 15 minutes for phosphorylase II, 30 minutes for soluble ADPglucose-starch glucosyltransferase and 2 hours for the starch granule-bound NDPglucose-starch glucosyltransferase.

Glucosyltransferase	Substrate (total ^{14}C added)	Starch fraction	Total activity recovered (cpm)	Glucose content (μmoles)	Specific activity (cpm/μmole)
Phosphorylase II	Glucose-1-P (0.50 μmole, 1,000,000 cpm)	Amylopectin	4,820	11.4	422.8
		Amylose	135,660	3.6	37,683.3
Soluble ADPG-starch glucosyltransferase	ADPglucose (0.25 μmole, 100,000 cpm)	Amylopectin	5,512	12.0	459.3
		Amylose	220	3.8	57.9
	UDPglucose (0 25 μmole, 300,000 cpm)	Amylopectin	231	12.0	19.2
		Amylose	0	3.8	0
Bound NDPG-starch glucosyltransferase	ADPglucose (0.50 μmole, 200,000 cpm)	Amylopectin	9,373	11.4	822.2
		Amylose	10,038	3.6	2,788.3
	UDPglucose (0.50 μmole, 600,000 cpm)	Amylopectin	9,606	11.4	842.6
		Amylose	16,548	3.6	4,596.7

When these two starch fractions were treated with β-amylase followed by paper chromatography, the radioactivity was found to migrate to a position characteristic of maltose. This observation is indicative of the formation of α-1,4 glucosidic linkage by the bound NDPglucose-starch glucosyltransferase.

Primed and unprimed activities of soluble ADPglucose-starch glucosyltransferase

Ozbun *et al.* (1971) have reported that two soluble ADPglucose-starch glucosyltransferases exist in the seed of maize *waxy* mutant. These two isozymes are separable by a DEAE-cellulose column. One of the isozymes is active only in the presence of a primer while the other is active either in the presence or absence of a primer depending on the conditions of assay. However, we have not been able to separate these two isozymes by column chromatography (Chuang, Tsai and Dalby, unpublished data). Only one peak of soluble ADPglucose-starch glucosyltransferase activity has been detected by DEAE-cellulose column fractionation, and this enzyme is active either in the presence or absence of primer, depending on the assay conditions employed.

When soluble ADPglucose-starch glucosyltransferase was assayed under primed conditions using natural starch granules as primer, glucose was incorporated from ADPglucose only into the amylopectin fraction, but not to the amylose fraction (Fig. 2). A similar result was obtained when the pronase-dimethylsulfoxide-treated starch granules were used as primer. On the other

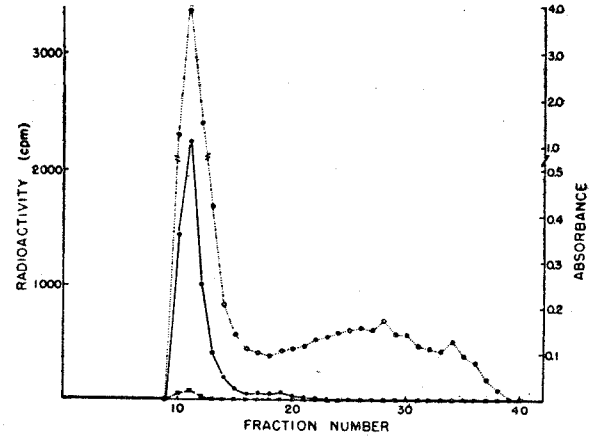

Fig. 2. Incorporation of glucose-^{14}C from ADPglucose-^{14}C into starch fractions by soluble ADPglucose-starch glucosyltransferase.

o···o···o Carbohydrate profile.
•—•—• ADPglucose as a substrate.
×---×---× UDPglucose as a substrate.

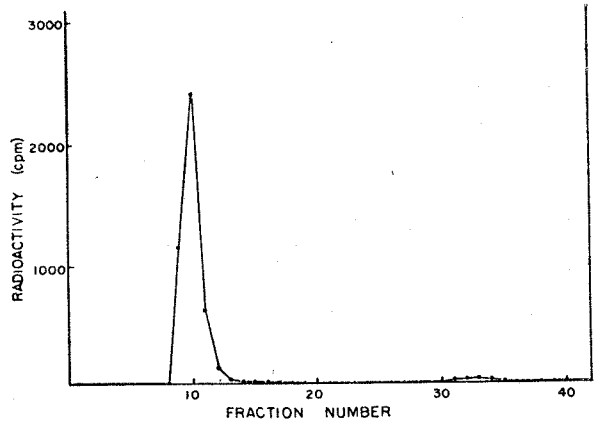

Fig. 3. Unprimed activity of soluble ADPglucose-starch glucosyltransferase.

hand, when this soluble enzyme preparation was assayed under unprimed conditions, where the enzyme requires a very high salt concentration to be active, a glucan was obtained. This glucan eluted from a Sepharose column at the position characteristic of amylopectin (Fig. 3, data adapted from Chuang, Tsai and Dalby, unpublished). This observation agrees with the work reported by Ozbun and his co-workers (1971). No appreciable radioactivity was detected in those fractions where amylose would elute.

As has been reported previously (Tsai, submitted), soluble ADPglucose-starch glucosyltransferase utilizes ADPglucose as a sole substrate. No signifi-

cant incorporation of glucose from UDPglucose into either starch fraction can be detected.

For all the glucose-^{14}C incorporated, either under primed or unprimed condition, the radioactivity was found to be associated with both β-limit dextrin and maltose when fractions were treated with β-amylase.

Primed and unprimed activities of phosphorylase II

As shown in Figure 4, when phosphorylase II is assayed in the absence of a primer, this enzyme can produce an amylose-like substance as was reported previously (Tsai *et al.*, 1968). No radioactivity was detected in the fractions where amylopectin would elute. On the other hand, when phosphorylase II is assayed in the presence of natural starch granules as primer, some radioactivity is detected in association with the amylopectin fraction. However, most of the radioactivity is eluted in the fractions characteristic of amylose. The specific activity of the amylose is about 100 times higher than that of the amylopectin (Table I). One explanation for this observation might be that the amylose fraction in the starch granules is a better primer than the amylopectin. Alternatively, natural starch granules may stimulate phosphorylase II to produce primer. As shown in Table II, the latter seems to be the case. When phosphorylase II was assayed either in the presence or absence of natural starch granules as primer, and the reaction terminated by methanolic amylopectin precipitation as described in the "Methods" section, the total radioactivity detected in the pellet of the primed reaction was about 4 times more than that of the unprimed reaction. This result is similar to that shown in Figure 4. A primed control, in which the starch granules were sedimented at the end of reaction without methanolic amylopectin precipitation, was run

Table II. *Primed and unprimed activities of phosphorylase II*

Assay conditions are as described under "Methods". For A and B systems, the reaction was terminated by adding 0.5 ml of 0.1 N NaOH containing 1 mg of amylopectin followed by methanol precipitation and centrifugation in order that the soluble material produced would be co-precipitated. In the C system, the reaction was terminated by adding 0.5 ml of 0.1 N NaOH followed by centrifugation to recover the starch granules only.

Primer	Substrate	Methanolic amylopectin precipitation	^{14}C in the pellet (cpm)
A. No primer	Glucose-1-P (0.5 μmole, 100,000 cpm)	yes	3,448
B. Starch granules	Glucose-1-P (0.5 μmole, 100,000 cpm)	yes	13,182
C. Starch granules	Glucose-1-P (0.5 μmole, 100,000 cpm)	no	500

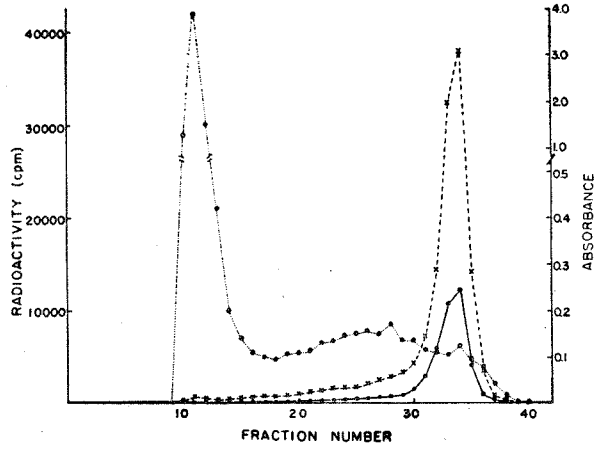

Fig. 4. Unprimed and primed activities of phosphorylase II.
o···o···o Carbohydrate profile.
•—•—• Reaction contained no primer.
×---×---× Reaction contained starch granules as primer.

simultaneously. Very low radioactivity was found associated with the starch granules in the control, presumably most of the radioactivity was associated with the soluble material which remained in the supernatant fraction upon centrifugation. This observation suggests that the higher radioactivity eluted in the position characteristic of amylose in the primed, co-precipitated reaction is probably due to a higher rate of primer synthesis, instead of direct incorporation into the amylose fraction of starch granules.

Again, when all the fractions were treated with β-amylase, radioactivity was found to be associated with maltose. This result suggests that the product formed by phosphorylase II was a glucan with α-1,4 glucosidic linkages.

Discussion

The studies of maize mutants, *shrunken*-4 (Tsai *et al.*, 1969), having a much reduced phosphorylase activity and only about 33% of the normal quantity of starch, have substantiated the importance of the phosphoryla e system for the synthesis of starch. Phosphorylase II is present, along with other enzymes presumably implicated in starch synthesis, only at the most rapid stage of starch biosynthesis (Tsai *et al.*, 1970), and this enzyme can produce an amylose-like substance in the absence of a primer (Tsai *et al.*, 1968). Although the properties of phosphorylase II seem to change along with the steps of purification, Burr and Nelson (personal communication) have shown that a 2,000-fold purified phosphorylase II still retains its unprimed

activity when assayed in the presence of high salt concentrations. Experimental data presented above show that phosphorylase II is very active in the synthesis of primer, which is an amylose-like substance, and this enzyme will not incorporate glucose into natural starch granules efficiently. These observations suggest that phosphorylase II might be an important enzyme for the synthesis of primer.

It is unlikely that phosphorylase II is responsible for the physiological synthesis of amylose or amylopectin for the following reasons: a) natural starch granules seem to be a poor primer, b) phosphorylase II has been shown to be inhibited very strongly by ADPglucose or UDPglucose (Tsai and Nelson, in preparation). These are substrates for the starch granule-bound NDPglucose-starch glucosyltransferase, and ADPglucose is the substrate for soluble ADPglucose-starch glucosyltransferase, c) The *waxy* mutant, that has normal phosphorylase II activity (Tsai, unpublished data), synthesizes no amylose (Sprague *et al.*, 1943).

The normal synthesis of amylose may proceed via the starch granule-bound NDPglucose-starch glucosyltransferase (Tsai, submitted) since the *waxy* mutant has been shown to be deficient in this enzyme activity (Nelson *et al.*, 1962; Nelson *et al.*, 1964; Tsai, submitted), and synthesizes amylopectin exclusively (Sprague *et al.*, 1943). Furthermore, the starch granule-bound NDPglucose-starch glucosyltransferase transfers glucose from either ADPglucose or UDPglucose more rapidly into amylose than into amylopectin (Table I).

Soluble ADPglucose-starch glucosyltransferase system, on the other hand, may be responsible for the synthesis of amylopectin. When assayed under unprimed conditions, this enzyme preparation can only produce an amylopectin-like substance that could be a primer for the synthesis of amylopectin. When this enzyme preparation is assayed under primed conditions, glucose is only incorporated from ADPglucose into the amylopectin fraction but not the amylose fraction of the natural starch granule.

Although purified amylose or amylopectin can both be used as primers for these α-1,4 glucosyltransferases, the incorporation of glucose into a particular fraction of the natural starch granule appears to be highly enzyme specific.

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玉米三種 α-1, 4 glucan glucosyltransferase 合成澱粉的研究

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在試管中探測三種 α-1, 4 glucosyltransferase 對於利用基質的特性，以及他們合成澱粉和形成 primer 能力的結果。發現 phosphorylase II 的主要功用可能與 primer 的合成有關。Soluble ADPglucose-starch glucosyltransterase 只能用 ADPglucose 做基質，這個酵素在沒有 primer 的狀態下可以合成一種類似 amylopectin 的多醣物，但是以澱粉粒做爲 primer 時該酵素的活性只能表現在 amylopectin 的合成。反之 starch granule-bound nucleoside diphosphate glucose-starch glucosyltransferase 除了可以利用 ADPglucose 做爲基質之外也可以利用 UDPglucose. Nucleoside diphosphate glucose-starch glucosyltransferase 雖然也具有促進 amylopectin 合成的活性，但是促進 amylose 合成的活性比較高。