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Nucleoside Diphosphate Sugar-Starch Glucosyl Transferase Activity of *wx* Starch Granules[1]

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OLIVER E. NELSON, PREM S. CHOUREY,[2] AND MING TU CHANG[3]
Department of Genetics, University of Wisconsin, Madison, Wisconsin

ABSTRACT

Starch granule preparations from the endosperm tissue of all *waxy* maize (*Zea mays* L.) mutants tested have low and approximately equal capability to incorporate glucose from adenosine diphosphate glucose into starch. As the substrate concentration is reduced, however, the activity of *waxy* preparations relative to nonmutant increases until, at the lowest substrate concentration utilized (0.1 μM), the activity of the *waxy* preparations is nearly equal to that of the nonmutant preparation. The apparent K_m (adenosine diphosphate glucose) for starch granule preparations from *wx-C/wx-C/wx-C* endosperms was 7.1×10^{-5} M, which is compared to 3×10^{-3} M for preparations from nonwaxy endosperms. Starch granule preparations from three other *waxy* mutants of independent mutational origin have levels of enzymic activity approximately equal to *wx-C* at a given substrate concentration giving rise to similar apparent K_m estimates. We conclude that there is in maize endosperm starch granules a second starch granule-bound glycosyl transferase, whose presence is revealed when mutation eliminates activity of the more active glucosyl transferase catalyzing the same reaction.

It was reported in 1962 (9) that starch granules from the developing endosperms of homozygous *waxy* (*wx/wx/wx*) maize did not possess the starch granule-bound enzyme (nucleoside diphosphate sugar-starch glucosyl transferase, EC 2.4.1.21) transferring glucose from uridine diphosphate glucose (UDP-glc) to the nonreducing ends of starch molecules. This enzyme had been reported by Leloir *et al.* (6) to be present in starch granules isolated from bean cotyledons or maize endosperms. It was subsequently shown that adenosine diphosphate glucose (ADP-glc) is the preferred substrate for enzymes of this type (13).

Frydman (4) reported that with ADP-glc as a substrate, preparations of starch granules from developing *waxy* maize seeds had about one-fifth the activity of similar preparations from nonwaxy seeds. Nelson and Tsai (10) found about one-tenth as much activity in preparations from 17 different *waxy* mutants as in a preparation from a nonwaxy standard. The activity in the *waxy* mutants could be reduced somewhat by processing only the endosperms (minus the embryos and the seed coats which are not affected by the *waxy* mutation and which therefore contain starch granules as active as those from the same tissues in nonwaxy seeds). Nevertheless, the mutants have measurable enzymic activity even if only endosperm tissue is processed. This observation and the report (4) of higher enzymic activity in *waxy* starch granules relative to nonwaxy preparations than observed in this laboratory led to a reexamination of the ADP-glc-starch transferase activity in *waxy* and nonwaxy starch granules. This reexamination has shown that the relative enzymic activity of *waxy* starch granule preparations increases with decreasing substrate (ADP-glc) concentrations, until at the lowest substrate concentrations used (0.1-1.0 μM), the activities of *waxy* and nonwaxy starch granules are nearly equal in a 30-min incubation. This and subsequent observations have been interpreted as indicating the presence in maize endosperm starch granules of a second ADP-glc-starch glucosyl transferase with a low K_m (ADP-glc) which accounts for the transferase activity observed in the *waxy* starch granules (8).

MATERIALS AND METHODS

Biological Materials. Starch granule preparations were made from seeds resulting from the self-pollination of *wx/wx* or *Wx/Wx* maize (*Zea mays* L.) plants. The *Wx/Wx* plants were the single cross W64A × 182E in most experiments; the reference *waxy* allele *wx-C* was used for all experiments except that in which the enzymic activities of *waxy* alleles having independent origin were investigated. Except for the developmental studies, starch granule preparations were made from seeds harvested 22 days after pollination.

For developmental studies, the inbred *M14 Wx/Wx* or *wx-C/wx-C* was used to provide a comparison between two stocks in which only the allelic state at the *waxy* locus differed. Ears were harvested at specified dates after pollination, the seeds cut from the ears and frozen immediately on dry ice. These seeds were then stored at 15 C until enzyme preparation.

In obtaining the starch granule preparations, the seed coat and embryo were removed from each seed, leaving only the endosperm except in the 12-day-old kernels from which the minute embryo was not removed. However, the nucellus and pericarp, which are maternal tissues and constitute an appreciable part of the developing seed at 12 days, were removed prior to homogenization. A number of endosperms were then processed to yield starch granules according to the method of Leloir *et al.* (6).

Reagents. The ADP-[^{14}C]glucose was purchased from New England Nuclear (228 mCi/mmol) or from Amersham (271 mCi/mmol). New England Nuclear was the source of UDP-[^{14}C]glucose (245 mCi/mmol). ADP-glc and UDP-glc were purchased from Sigma.

Assays. The reaction mixtures contained 3 to 5 mg of the starch granule preparation in 40 to 60 μl of a 0.1 M glycyl-glycine buffer solution (pH 8.5) that was 2 mM EDTA, 0.1 M KCl, and with the indicated concentration of ADP-glc or UDP-glc. Incubation was at 37 C for varying periods of time after which the reaction was terminated by the addition of chilled methanol (70%, v/v) containing 1% KCl. After centrifugation the starch pellet was resuspended in 0.2 M phosphate buffer, washed three times with buffer,

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[2] Present address: Department of Agronomy, University of Illinois.

[3] Present address: Department of Bacteriology, University of Wisconsin-Madison.

then trapped on a glass fiber filter, washed with 30 ml of H_2O, and counted in a Beckman scintillation counter. All assays were run in duplicate, and the mean values were corrected for the counts observed in reaction mixtures stopped immediately after addition of the substrate.

RESULTS

Effect of Varying Substrate Concentrations on Enzymic Activity. Substrate concentrations varied over five orders of magnitude in the assays reported in Table I. When ADP-glc was the glucose donor, nearly equal activities for the nonwaxy and *waxy* preparations and relatively high percentages of incorporation were observed at the lowest substrate concentrations. The data in Table I also show that there was a difference in the two preparations in their ability to utilize UDP-glc as a substrate compared to ADP-glc. The incorporation of 24.5% as much glucose from UDP-glc as from ADP-glc for the normal preparations at 1,000 μM substrate concentration agreed well with a previous estimate for a nonmutant (normal) starch preparation when K^+ was present in the reaction mixture (1). The *waxy* preparation had a much lower incorporation of glucose with UDP-glc relative to ADP-glc but with an ascending trend as substrate concentration increased. The activities of the mutant and nonmutant preparations with UDP-glc relative to ADP-glc were useful in attempting to determine whether the *waxy* preparation had a different enzyme from that present in the nonwaxy preparation.

With a low substrate concentration (1 μM ADP-glc), glucose incorporation by both preparations increased with increasing times of incubation although with decreased velocity with time (Fig. 1). The enzymic activity of the *waxy* preparation at this low substrate concentration approached that of the normal preparation in this instance as in the assays recorded in Table I.

K_m of *Waxy* Preparation. In Table I, the *waxy* preparation reached maximum velocity at a lower substrate concentration and hence had a lower apparent K_m. One should interpret with caution the estimates of K_m for an enzyme bound to or contained within a subcellular fraction. Nevertheless, distinctive and reproducible estimates of such an apparent K_m can be obtained from starch granules isolated from different tissues (embryo and endosperm) of plants of the same genotype (1). In assays intended to derive a more accurate estimate of the apparent K_m for the standard *waxy* mutant, *wx-C*, and using 3 mg of starch granules in a 10-min incubation (Fig. 2), the K_m was estimated as 7.1×10^{-5} M. The K_m of the nonwaxy preparation was estimated as 3×10^{-3} M. This figure agreed well with that previously found in our laboratory (1).

Since two soluble ADP-glc-starch glucosyl transferases are present in maize endosperms (12), it was necessary to consider the possibility that the activity observed in *waxy* starch granules may have been due to one or both of the soluble enzymes absorbed onto the starch granules. A soluble glucosyl transferase of rice endosperm is firmly bound by amylose but not by amylopectin (2). Starch formed by *waxy* endosperms is entirely amylopectin (15). Samples of starch granules were withdrawn at each stage of washing during the standard process of preparation. These samples were assayed for activity at a low substrate concentration, and the results are presented in Table II. Although there was some variation, it was apparent that activity was not attenuated by repeated washing. The failure to wash out any of the activity observed in *wx/wx/wx* starch granules indicated that the activity was not due to a soluble ADP-glc-starch glucosyl transferase loosely adsorbed to the starch granules. In addition, the soluble glucosyl transferases do not utilize UDP-glc as a substrate (12). The *wx/wx/wx* starch granules did, however, incorporate glucose from UDP-glc, albeit not efficiently (Table I).

The observations given are interpreted as indicating that there are two starch granule-bound glucosyl transferase enzymes. One

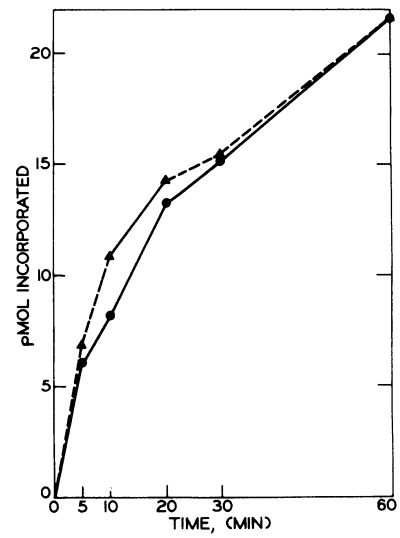

FIG. 1. Incorporation over time of glucose from ADP-[^{14}C]glucose into *wx/wx/wx* (●——●) and *Wx/Wx/Wx* (▲- - -▲) endosperm starch granule preparations. Five mg of starch granules were incubated for various periods of time in reaction mixtures containing 40 pmol (15,696 cpm) of ADP-glc in a total reaction volume of 40 μl. There were duplicate assays for each time period.

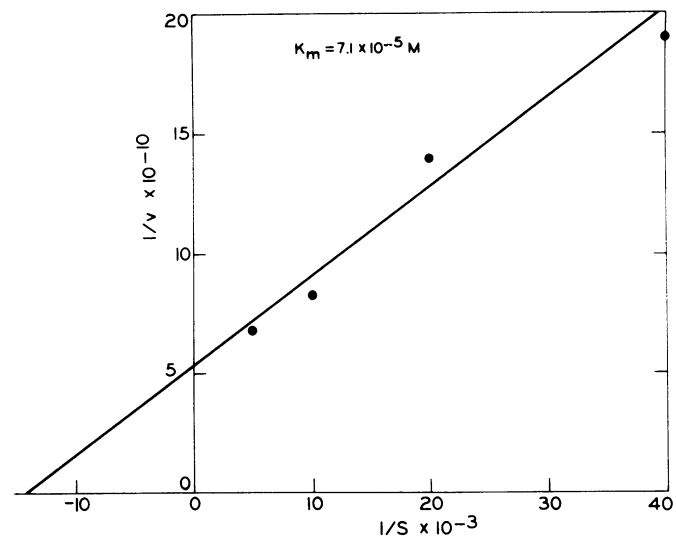

FIG. 2. Effect of ADP-glc concentration on the activity of starch granule preparations from *wx/wx/wx* endosperms (Lineweaver-Burk plot). Three mg of starch granules were incubated for 10 min in 50 μl containing different concentrations of ADP-glc. The substrate concentration (S) is micromolar while v is the pmol of glucose incorporated.

TABLE I. The incorporation of glucose from ADP-^{14}C-glucose into wx/wx/wx and Wx/Wx/Wx endosperm starch granule preparations.

Five mg of starch granules were incubated for 30 min in 40 μl reaction mixtures that contained different concentrations of ADP-glc plus ADP-^{14}C-glucose (228 mCi/mmol). At the three lowest substrate concentrations, labeled ADP-glc undiluted by unlabeled ADP-glc was used. There were duplicate assays for each substrate concentration.

Glucose Donor Concentration	Glucose Donor: ADP Glucose, Endosperm Source wx/wx/wx	ADP Glucose, Endosperm Source Wx/Wx/Wx	UDP Glucose, Endosperm Source wx/wx/wx	UDP Glucose, Endosperm Source Wx/Wx/Wx
μM	pmol glucose incorporated (% incorporation)			
0.1	1.7(43%)	1.6(40%)	0 (0.0%)	0.33(6.9%)
1.0	16.7(42%)	16.8(42%)	0.7 (0.2%)	2.65(7.1%)
10	107 (27%)	163 (41%)	0.78(0.2%)	21 (6.4%)
100	429 (11%)	1520 (38%)	7.8 (0.2%)	264 (6.6%)
1000	881 (2%)	10622 (27%)	50.3 (0.1%)	2641 (6.6%)
10000	818 (<1%)	51460 (13%)	210.0(<0.1%)	15230 (3.8%)

of these is the higher K_m enzyme observed in starch granule preparations from nonwaxy seeds 22 days postpollination. This enzyme is responsible for most of the activity observed in enzymic assays conducted at high substrate concentrations (0.1 mM or higher). The second enzyme is the enzyme with a relatively low K_m, which is largely responsible for glucose incorporation by nonwaxy preparations at low substrate concentrations (0.1–1 μM) and probably totally responsible for glucose incorporation by *waxy* preparations across the entire range of substrate concentrations employed.

Activity of Other *Waxy* Mutants. As a test of the hypothesis that the nonmutant starch granules have two glucosyl transferase enzymes, it was necessary to show that the enzyme present in the *waxy-C* starch granules was not the product of the mutant allele at the *waxy* locus. If it were the product of the mutant gene, one would expect different *waxy* mutants (those that occurred as independent mutational events) to have different amounts of activity ranging from zero to an activity somewhat higher than that observed with *waxy-C*. Furthermore, it would be most improbable that all mutant enzymes would have a lower K_m than normal since it is unusual for a mutant enzyme to have a greater affinity for the substrate than the nonmutant enzyme. Therefore, three other *waxy* mutants, *B7, C2*, and *R*, were selected for study. Two of these, *B7* and *R*, were spontaneous mutants, while *C2* was a putative x-ray-induced mutation. Each of these alleles had been shown by recombination tests to occupy a different position within the *waxy* locus (7). Each of the mutant preparations had approximately the same level of activity at a given substrate concentration (Table III); estimates of K_m from the data were 7.6×10^{-5} M for *wx-C*, 6.7×10^{-5} M for *wx-B7*, and 4.9×10^{-5} M for *wx-R*. These estimates were not only substantially below the K_m estimated for similar preparations from nonwaxy seeds, but they were also in general agreement with the value of *wx-C*. The similar enzymic activities for all of the mutant preparations indicated that the activity observed in the *waxy* preparations was not due to an altered enzyme produced by a mutant gene but, rather, was a second enzymic activity revealed when the *waxy* mutations resulted in the loss of the more active glucosyl transferase.

Developmental Studies. With the identification of a second starch granule-bound glucosyl transferase having a low apparent K_m for ADP-glc, the question arose as to whether the two activities increased synchronously during development or whether one preceded the other. Starch granule preparations were therefore made from *Wx/Wx/Wx* and *wx/wx/wx* endosperms that had been frozen 12, 14, 16, or 24 days postpollination. These were assayed at a high substrate level (5 mM), and results are given in Table IV. No satisfactory starch granule preparation could be isolated from *wx/wx/wx* endosperms 12 days after pollination. The activity of the *Wx/Wx/Wx* preparation increased greatly from 12 to 14 days and somewhat more from 14 to 16 days. By contrast, the activity of the *wx/wx/wx* preparation decreased somewhat from 14 to 16 days. Since the activity of the low K_m system which contributed the enzymic activity of the *waxy* preparations dropped somewhat over the period assayed, it could be presumed to do so also in *Wx/Wx/Wx* preparations. The increase in activity in *Wx/Wx/Wx* starch granules therefore resulted from an increase in the high K_m enzyme in the period between 12 and 16 days. This was the period during which a large increase in the activity of enzymes participating in starch synthesis had been found (17). The results of the developmental study offered no basis for a decision as to whether one enzyme antedated the other during development. It was clear, however, that the major portion of the high K_m enzyme activity developed after 12 days postpollination while the activity of the low K_m enzyme was not increasing.

Identification and investigation of the activity present in *waxy* starch granules depend upon the absence from the starch granules of the more active enzyme utilizing the same substrates and effecting the same reaction. The absence (or near absence) of the more active enzyme is a consequence of homozygosity for the mutant alleles at the *waxy* locus. The probable role of the minor activity could be assessed most accurately if we succeeded in locating a mutant strain that had lost such activity. Such loss would be definitively established only in stocks which were also *wx/wx*. Therefore, it is necessary to carry out the search in double mutant stocks—homozygous for a *waxy* mutation and also for a second mutation whose effect one desires to investigate. Using such stocks in a genetically similar background, we have shown that neither the *dull* (*du*) mutation, nor the *sugary-1* (*su1*) mutation influenced the activity of the starch granules that were also *waxy* (Table V). A description of these mutants was given by Neuffer *et al.* (11). The *su1* mutation had seemed to be a possible candidate since the polysaccharide storage products in *su1/su1/su1* endosperms are approximately equal quantities (16) of starch and phytoglycogen. Such a pattern of carbohydrate storage products could conceivably be the consequence of difficulty in initiating starch synthesis early in development. Subsequent to the assays of these double mutants, we have also compared a *bt/bt/bt*; *wx/wx/wx* starch granule preparation to a *Bt/Bt/Bt*; *wx/wx/wx* preparation under somewhat different assay conditions (3 mg of starch, 10-min incubation in a total reaction mixture of 50 μl) and found

TABLE II. The glucosyl transferase activity in wx/wx/wx starch granules as affected by varying numbers of buffer and acetone washes during preparation.

Three mg of starch were incubated for 10 min in 50 μl of reaction mixture containing 50 μl of reaction mixture containing 50 pmol ADP-^{14}C-glc (27749 cpm).

Washes	Glucosyl Transferase Activity
	Net cpm ± SE
1 Buffer	6620 ± 290
2 Buffer	5610 ± 65
3 Buffer	6371 ± 194
3 Buffer + 1 Acetone	6418 ± 117
3 Buffer + 2 Acetone	5747 ± 334

TABLE III. The incorporation of glucose from ADP-^{14}C-glucose into starch granule preparations from the waxy mutants, wx-B7, wx-C2, and wx-R.

Three mg of starch granules were incubated for 10 min in a total reaction volume of 50 μl containing different concentrations of ADP-glc. The results are the mean of two different assays with duplicates within each assay.

ADP-glc Concentration	waxy mutant tested		
	wx-B7	wx-C2	wx-R
μM	pmol glucose incorporated/10 min/3 mg starch		
25	79	85	81
50	101	119	121
100	122	136	176
200	169	168	211
400	202	233	224

TABLE IV. The activities of Wx/Wx/Wx or wx/wx/wx starch granules from endosperms harvested at stated intervals post-pollination.

The reaction mixtures contained 0.25 μmol ADP-glc in a total of 50 μl plus 5 mg of starch granules. Incubation was for 30 min at 37 C.

Time after Pollination	Source Endosperm Genotype	
	wx/wx/wx	Wx/Wx/Wx
Days	pmol glucose incorporated	
12	-	4,200
14	1,725	16,800
16	765	20,550
24	945	19,650

TABLE V. The incorporation of glucose from low substrate concentrations of ADP-glc into Wx/Wx/Wx; wx/wx/wx; wx/wx/wx, du/du/du; wx/wx/wx, sul/sul/sul; and wx/wx/wx, su2/su2/su2 starch granules.

Five mg of starch granules were incubated for 30 min in a total reaction volume of 40 μl at 2 substrate concentrations.

Source	ADP-Glucose Concentration	
	4 pmol(2,348 cpm)	40 pmol(20,717 cpm)
	net cpm incorporated	
Wx	1,300	11,915
wx	752	9,166
wx, du	1,213	10,082
wx, sul	1,201	10,458
wx, su2	405	2,984

no apparent difference between preparations of the two genotypes.

One mutation, *sugary-2* (*su2*), was found to affect the minor activity. Starch granule preparations from the double mutant endosperms (*su2/su2/su2*; *wx/wx/wx*) had less than half of the activity of similar preparations from *Su2/Su2/Su2*; *wx/wx/wx* endosperms (Table V). The observed reduction in enzymic activity in *su2/su2/su2*; *wx/wx/wx* endosperms might have resulted from the presence of more than one low K_m enzyme with the normal allele at the *su2* locus specifying one of the enzymes. Hence its loss, through substituting the mutant allele, would have still left observable enzymic activity. A second possibility is that the normal allele at the *su2* locus did specify a single low K_m enzyme but the mutation to the particular *su2* allele that we were investigating did not abolish enzymic activity but reduced it substantially. A third possibility is that the effect of the *su2* mutation on enzymic activity may have been indirect, *i.e.* through an alteration in the structure of the starch granule which in turn influenced enzymic activity.

DISCUSSION

The exact steps by which starch synthesis is initiated in the endosperms of cereals have not been elucidated since most enzymes capable of forming α-1,4 linkages between glucose molecules require an oligosaccharide or polysaccharide primer. It has been suggested that the apparent primer-independent phosphorylase (18) or the soluble starch synthetase with no primer requirement (12) might initiate starch synthesis with subsequent synthesis being carried out by other enzymes. However, it has been demonstrated both for the phosphorylase (3) and for the soluble starch synthetase (14) that the apparent ability to initiate starch synthesis depends on an endogenous primer.

Lavintman *et al.* (5) have reported the isolation from potato tubers of a particulate fraction (presumably largely proplastids) that contains three enzymic activities not previously identified. These activities are the transfer of glucose from micromolar concentrations of UDP-glc to a protein acceptor in α-1,4 linkages, the transfer of glucose from millimolar concentrations of UDP-glc to a protein acceptor in α-1,4 linkages, and finally a Mg^{2+}- or Mn^{2+}-dependent, glucose-1-P-stimulated transfer of glucose from UDP-glc to a protein acceptor in α-1,4 linkages. ADP-glc, which is the preferred or obligatory substrate for the other starch synthetases, is not a substrate for these enzymes. ADP-glc can, however, stimulate incorporation of glucose from UDP-glc by the third enzyme but to a lesser extent than can Glc-1-P. One or several of these enzymes may have a role in the initiation of starch synthesis. In preliminary investigations we have not been able to find such activities in comparable fractions from developing maize endosperms, however.

The existence in maize starch granules of an enzyme that is capable of transferring glucose from micromolar concentrations of ADP-glc into a α-1,4 glucan linkages and that is present in starch granules at early stages of endosperm development (while the major portion of the higher K_m enzyme activity develops later) is also suggestive of a role in starch synthesis early in development of the endosperm.

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