Properties of Citrate-stimulated Starch Synthesis Catalyzed by Starch Synthase I of Developing Maize Kernels

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ABSTRACT

Chromatography of extracts of maize on diethylaminoethyl-cellulose resolves starch synthase activity into two fractions (Ozobum, Hawker, Preiss 1971 Plant Physiol. 48: 785-786). Only starch synthase I is capable of synthesis in the absence of added primer and the presence of 0.5 molar citrate. This enzyme fraction has been purified about 1,000-fold from maize kernels homozygous for the endosperm mutant amylose-extender (ae). Because ae endosperm lacks the starch-branching enzyme which normally purifies with starch synthase I, the final enzyme fraction was free of detectable branching enzyme activity. This allowed a detailed characterization of the citrate-stimulated reaction. The citrate-stimulated reaction was dependent upon citrate concentrations of greater than 0.1 molar. However, the reaction is not specific for citrate and malate also stimulated the reaction. Branching enzyme increased the velocity of the reaction about 4-fold but did not replace the requirement for citrate. Citrate reduced the \( K_m \) for the primers amylopectin and glycogen from 122 and 595 micrograms per milliliter, respectively, to 6 and 50 micrograms per milliliter, respectively. The enzyme was found to contain 1.7 milligrams of anhydroglucose units per enzyme unit. Thus reaction mixtures contained 1 to 5 micrograms (5 to 25 micrograms per milliliter) of endogenous primer. The citrate-stimulated reaction could be explained by an increased affinity for this endogenous primer. The starch synthase reaction in the absence of primer is dependent upon several factors including endogenous primer concentration, citrate concentration as well as branching enzyme concentration.

Previous reports have shown glycogen and starch synthase fractions from a number of sources catalyze \( \alpha \)-glucan synthesis in the absence of added primer, when in the presence of high concentrations of citrate (4, 6, 8). This reaction has been termed "unprimed" starch and glycogen synthesis. Citrate (0.5 m) was found to lower the \( K_m \) for the unprimed spinach leaf starch synthase 3,400-fold for glycogen and 270-fold for amylpectin (6). Likewise, citrate was found to lower the \( K_m \) of *Escherichia coli* glycogen synthase for glycogen 11- to 15-fold (8). Critical characterization of the *E. coli* enzyme showed that it contained large amounts of endogenous primer (4), thus possibly accounting for the unprimed synthesis. The unprimed reaction catalyzed by the *E. coli* glycogen synthase was found to be influenced by endogenous primer and citrate concentration as well as by glycogen-branching enzyme (8). Therefore, any observed reaction was the function of the interaction of several variables.

Two fractions of starch synthase have been isolated from maize endosperm by DEAE-cellulose chromatography (10). Starch synthase I is able to catalyze unprimed glucan synthesis in the presence of high citrate concentration whereas starch synthase II is inactive under these conditions. In addition, starch synthase I had greater activity with glycogen than with amylpectin as primer, while starch synthase II had greater activity with amylpectin. Schiefer et al. (13) showed that treatment of a starch synthase from sweet corn with \( \alpha \)-amylose abolished the unprimed starch synthase activity and suggested that primer had been removed. However, they were unable to characterize fully the high citrate reaction, because their preparations were contaminated with branching enzyme. The purpose of this report is to describe the properties of the citrate-stimulated starch synthase reaction of maize endosperm. The purification of starch synthase I virtually free of branching enzyme activity was made possible by using endosperm extracts of the branching enzyme mutant amylose-extender. A preliminary report of some of this work has already appeared (11).

MATERIALS AND METHODS

MATERIALS

The dent maize (Zea mays L.) inbred W64A and inbred W64A homozygous for the endosperm mutant amylose-extender (ae) were field grown in 1976. Plants were self- or sib-pollinated and ears were harvested at 22 days after pollination, quick frozen in dry ice, and stored at \(-15\) C until used. ADP-[\( ^{14} \)C]glucose was prepared enzymatically (12). Maize amylpectin (amylose-free) was purchased from Calbiochem; glycogen, \( \beta \)-amyrase, and rabbit muscle phosphorylase \( a \) were obtained from Sigma. Aminoalkyl-Sepharose resin was prepared by the procedures of Shaltiel and Er-El (14). All other reagents were of the highest purity available.

PURIFICATION OF ADP-GLUCOSE — STARCH SYNTHASE

Kernels (230 g) were ground with a porcelain mortar and pestle in the presence of 100 ml of cold 50 mM Tris-acetate buffer (pH 7.5) containing 2.5 mM DTT and 10 mM EDTA. Incompletely ground endosperm and pericarp material was collected by passing the homogenate through five layers of cheesecloth and the grinding repeated two additional times. This extracted solution constituted the crude extract. All additional procedures were carried out at 0 to 4 C.

Following the initial extraction the enzyme was further purified by centrifugation (45,000g for 20 min) and ammonium sulfate precipitation (0-40%). The ammonium sulfate pellet was dissolved in a minimal volume of the above Tris-acetate buffer and dialyzed against 1,500 ml of the same buffer containing 5% sucrose for 12 h. This solution was then applied to a DEAE-cellulose column (2.5 x 24 cm) equilibrated with the above Tris-acetate, 5% sucrose buffer. The column was then washed with 200 ml of buffer and the enzyme was eluted in a 2,000-ml gradient of 0 to 0.4 M KCl. Twenty-ml fractions were collected, peak fractions combined.
overnight concentrated by ultrafiltration with an Amicon concentrator, and dialyzed overnight against the Tris-acetate buffer system plus 5% sucrose. This solution was absorbed onto a 4-aminobutyyl-Sepharose column (1.5 × 19.5 cm) which had been equilibrated with the same buffer. The column was washed with buffer and the enzyme eluted with an 800-ml gradient of 0 to 1.0 M KCl. Ten-ml fractions were collected and the pooled fractions were concentrated and dialyzed overnight against the Tris-acetate buffer-5% sucrose solution described above. This fraction was then applied to a Bio-Gel A-1.5m column (2.5 × 33 cm) and eluted with the Tris-acetate-5% sucrose buffer. Three-ml fractions were collected and fractions containing the enzyme were pooled and concentrated.

ASSAY OF ADP-GLUCOSE — STARCH SYNTHASE

Assay A. The transfer of glucose into primer from ADP-glucose was measured in reaction mixtures containing 140 nmol of ADP-[14C]glucose (500 cpm/nmol), 20 μmol of Bicine [N,N′-bis-(2 hydroxyethyl)glycine]-1 piperazineethanesulfonic acid buffer (pH 8.5), 5 μmol of K-acetate, 2 μmol of GSH, 1 μmol of EDTA, 1 mg of maize amyllopectin and enzyme in a final volume of 0.2 ml. Reactions were incubated at 30 C and the incorporation of [14C]glucose into methanol-insoluble polysaccharide was measured as described (5). Primer specificity was measured by subjecting different glucans for amyllopectin in the above mixture. This assay is referred to as the primed activity. For kinetic studies the specific activity of the ADP-[14C]glucose was doubled. One unit of starch synthase activity is defined as 1 μmol of glucose transferred per min.

Assay B. Incorporation of glucose into methanol-insoluble product in the absence of added primer was determined in reaction mixtures the same as described in assay A except that potassium acetate and amyllopectin were replaced with 100 μg of BSA and 100 μmol of sodium citrate. Reactions were stopped by heating at 100 C for 1 min. Carrier glycogen (1 mg) was added and radioactivity was measured as described (5).

ASSAY OF STARCH-BRANCHING ENZYME

Branching enzyme activity was determined by the stimulation of α-glucan formation from glucose-1-P by phosphorylase a as already described (6, 7). A unit of activity is defined as 1 μmol of glucose transferred per min.

PROTEIN DETERMINATION

Protein was assayed by the method of Lowry et al. (9) using BSA for a standard.

DETERMINATION OF POLYGLUCOSE IN THE STARCH SYNTHASE FRACTION

One ml of enzyme fraction was dialyzed against 4 liters of water for 12 h. The water was replaced three additional times with dialysis each time continuing for 12 h. The dialyzed fraction was divided into equal portions. To one was added 1.09 μmol of glucose as an internal standard and both fractions were made 2 N in HCl with reagent HCl and hydrolyzed for 1 h at 100 C. The samples were then frozen and lyophilized. The fractions were then dissolved in 1.0 ml of water and centrifuged. The concentration of glucose was determined by measuring NADPH formation when aliquots were incubated in reaction mixtures containing hexokinase and glucose-6-P dehydrogenase (1). The final amount was corrected for loss of the internal standard (usually 20%).

RESULTS

The purification of citrate-stimulated starch synthase from amyllose-extender (ae) is shown in Table I. This activity eluted as a single peak from DEAE-cellulose (2, 10), 4-aminobutyyl-Sepharose (Fig. 1A), and Bio-Gel A-1.5m (Fig. 1B) columns. The large loss in primed starch synthase activity (assay A) after DEAE-cellulose chromatography (90%) can be explained by the observation that a second starch synthase (starch synthase II [10]) eluted separately from the citrate-stimulated activity and showed only primed starch synthase activity. Therefore, the recovery in Table I does not reflect the total recovery of primed starch synthase activity. The loss of unprimed starch synthase activity after DEAE-cellulose was also more apparent than real (Table I). Kernels of mutant ae, although deficient in the starch-branching enzyme associated with the citrate-stimulated starch synthase activity, are not void of other branching activities (3). Separation of the citrate-stimulated activity from branching activity after DEAE-cellulose chromatography results in the observed decrease in unprimed activity.

The final enzyme fraction was purified 270-fold over the crude extract based on the unprimed assay (Table I). However, this enzyme fraction was not homogeneous. Disc gel electrophoresis revealed that the fraction contained many protein bands of near equal staining intensity. The fraction contained 2.1 mg of anhydroglucose (1.7 mg of anhydroglucose per enzyme unit). No branching enzyme activity was detected. The enzyme fraction was indistinguishable from citrate-stimulated starch synthase purified from nonmutant (normal) maize by the same procedures (Table II). Both enzyme fractions had the same Km for ADP-glucose and similar relative activities with different polysaccharide primers in the conditions of assay A. The high citrate reaction was characterized by only a slight lag with time (Fig. 2A) and was linear with respect to enzyme concentration (Fig. 2B). Although no

Table 1. Purification of Starch Synthase I from Amylose-Extender Maize

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Units*</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>Primed</td>
<td>Un-primed</td>
</tr>
<tr>
<td>Crude</td>
<td>365</td>
<td>4106</td>
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<td>2.71</td>
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<tr>
<td>45,000 g supernatant</td>
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<tr>
<td>(NH₄)₂SO₄ (0-40%)</td>
<td>52</td>
<td>681</td>
<td>10.5</td>
<td>2.52</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>36</td>
<td>93</td>
<td>1.24</td>
<td>1.30</td>
</tr>
<tr>
<td>4-aminobutyyl Sepharose</td>
<td>5.4</td>
<td>40</td>
<td>0.88</td>
<td>1.27</td>
</tr>
<tr>
<td>Bio-Gel A-1.5m</td>
<td>4.7</td>
<td>7.3</td>
<td>0.66</td>
<td>1.20</td>
</tr>
</tbody>
</table>

* One unit equals 1 μmol of glucose transferred per min at 30 C.

Fig. 1. Purification of amylose-extender maize starch synthase I by chromatography on 4-aminobutyyl-Sepharose (A) and gel filtration on Bio-Gel A-1.5mm (B).
branching enzyme had been detected, the product was only 80% converted to maltose by β-amylase.

The formation of glucan in the absence of added primer was dependent on a concentration of citrate of above 0.1 M in the reaction mixture (Fig. 3). Other salts at high concentrations such as NaF, (NH₄)₂SO₄, Na₂EDTA, Na- or K-acetate, were active while KSCN, NaClO₄, KNO₃, KI, KBr, and KCl were inactive (10). Thus, stimulation of the synthesis was not specific for citrate, and a similar curve was observed for DL-malate. The addition of branching enzyme did not alleviate the requirement for citrate or malate, but did increase the velocity of the reaction (Fig. 3). Increasing amounts of branching enzyme in the presence of 0.5 M citrate increased the rate of the unprimed reaction until a saturation point was reached (Fig. 4). The saturation point (0.0075 units of branching enzyme/0.2 ml) was at an approximate ratio of 10:1, branching enzyme units to starch synthase units. The maximum stimulation of the unprimed reaction varied from 300 to 400% and averaged 380%. The stimulation was not found to be specific for any branching enzyme fraction tested and any of the three branching enzymes previously observed in maize kernels (2) was equally effective. The specific activity of the purified citrate stimulated synthase was increased 1,000-fold over the crude extract when assay mixtures were optimal and included branching enzyme in addition to citrate.

The effect of citrate (0.5 M) was tested on the amylopectin and glycogen saturation of the enzyme. Because the levels of endogenous primer in the starch synthase fraction were so high, the enzyme levels had to be reduced to show primer saturation in the presence of citrate. Lineweaver-Burk plots of the amylopectin data are shown in Figure 5. The K<sub>v</sub> values of the enzyme for amylopectin and glycogen were reduced from 122 and 595 μg/ml, respectively, to 6 and 50 μg/ml, respectively, by the addition of citrate. The addition of branching enzyme to the reaction mixtures did not significantly further reduce the K<sub>v</sub> values for primer but did increase the reaction velocity at nonsaturating primer levels. No increase in reaction rate was observed at saturating primer levels (not shown).

**FIG. 4.** Effect of increasing branching enzyme concentration on the citrate stimulated reaction catalyzed by starch synthase I. Procedure is described in the text.

**FIG. 5.** Effect of amylopectin concentration on the formation of D-[14C]glucan by starch synthase I in the presence and absence of 0.5 M citrate: Lineweaver plots of the data A: minus citrate; B: plus citrate. Enzyme concentration is 2.5-fold less in the plus citrate experiment than the minus citrate experiment.

**FIG. 3.** Effect of citrate and malate concentration in the absence of presence of branching enzyme on formation of methanol-insoluble D-[14C]glucan by starch synthase I. (O) Citrate minus branching enzyme; (C) citrate plus branching enzyme; (A) malate minus branching enzyme; and (Δ) malate plus branching enzyme.

**DISCUSSION**

The above results (Table II) show that the kinetic properties of the starch synthase I isolated from maize kernels homozygous for ae did not differ from starch synthase I from normal kernels. By using ae kernels it has been possible to purify maize starch synthase I free from detectable branching enzyme activity. Although no branching enzyme was detected in the starch synthase fraction, the product formed in the unprimed reaction by this enzyme was only 80% converted to maltose by β-amylase. Therefore, some branching enzyme activity may still have been present. However, complete β-amylolysis is probably due to retrogradation of the linear amyllose-type chains produced. Nevertheless, this fraction allowed the complete characterization of the variables of the citrate-stimulated reaction characteristic of maize starch
synthase I. The enzyme from *ae* kernels was purified over 1,000-fold on the basis of the citrate-stimulated reaction in the presence of branching enzyme. During purification only a single peak of unprimed starch synthase activity was observed at each step (Fig. 1) and thus the enzyme appeared to be a single entity.

The citrate-stimulated reaction was found to be dependent upon several variables. First, the concentration of citrate (or malate) had to be greater than 0.1 mM (Fig. 3). The reaction velocity of the unprimed reaction was further increased by the addition of branching enzyme (Figs. 3 and 4). Finally, the level of the endogenous primer found associated with the starch synthase enzyme was important. The observations that citrate lowered the $K_m$ for primer to 6 and 50 $\mu$M/ml for amylopectin and glycogen, respectively, and that glucan associated with the starch synthase was present at a level to give glucan concentrations of 5 to 25 $\mu$M/ml in reaction mixtures indicated that endogenous primer could account for the apparent primer independence of this reaction. Similar conclusions were reached for an unprimed starch synthase isolated from sweet corn which lost unprimed synthesis activity when treated with $\alpha$-amylase (13). Therefore, the citrate-stimulated reaction of maize endosperm does not appear to be truly *de novo* and is similar to the citrate-stimulated reactions reported for enzymes from *E. coli* and spinach leaves (6, 8).

The citrate-stimulated starch synthase reaction did have some very interesting properties, however. Although the $K_m$ for amylopectin was lower than the $K_m$ for glycogen (3- to 4-fold), the $V_{\text{max}}$ for amylopectin was also observed to be lower (Table II). These two parameters of the reaction appeared to be independent and must be intrinsic to differences in the structures of the two glucans. One possible explanation might be that the number of nonreducing ends on the surface of the primer molecule is important in determining $K_m$, but the chain length of these terminal chains is important in determining $V_{\text{max}}$. Thus, the structure of the glucan primer is important in determining the reaction characteristics also. Starch synthase I isolated from normal and waxy kernels was observed to have unprimed starch synthase activity severalfold higher than the primed activity using amylopectin as a primer (2, 6). Therefore, the endogenous primer may be more similar to glycogen (*i.e.*, more highly branched) than to amylopectin.

Another interesting observation was that even at reduced enzyme levels the velocity of the citrate-stimulated reaction at low amylopectin levels exceeded the velocity in reactions without citrate (Fig. 5). Similar enhancement of the reaction velocity at low primer levels has been observed using *E. coli* glycogen synthase (8). Therefore, low amounts of starch synthase I are capable of transferring glucose to small amounts of endogenous primer. However, this reaction is dependent on high salt and branching enzyme. It is possible that the starch synthase is present *in vivo* in a high ionic strength environment and therefore is effective at very low primer concentrations. Although the *in vitro* reaction does not appear to be *de novo*, the role of maize starch synthase I in the initiation of or early steps in starch synthesis cannot be ruled out at this time.

**LITERATURE CITED**

8. Kawaguchi D, J Fox, E Holmes, C Boyer, J Preiss 1978 "De novo" synthesis of Escherichia coli glycogen is due to primer associated with glycogen synthase and activation by branching enzyme. Arch Biochem Biophys 190: 385-397

Table II. Comparison of Starch Synthase I Fractions from Normal (Nonmutant) and Amylose-Extender Maize

<table>
<thead>
<tr>
<th>Property</th>
<th>Source of Starch Synthase I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>$K_m$ (ADP-glucose)</td>
<td>0.10 mM (±0.01)</td>
</tr>
<tr>
<td>Per cent activity with:</td>
<td></td>
</tr>
<tr>
<td>Amylopectin</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit liver glycogen</td>
<td>431</td>
</tr>
<tr>
<td>Oyster glycogen</td>
<td>150</td>
</tr>
<tr>
<td><em>E. coli</em> glycogen</td>
<td>242</td>
</tr>
</tbody>
</table>

1042

BOYER AND PREISS

Plant Physiol. Vol. 64, 1979

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