MULTIPLE FORMS OF STARCH SYNTHETASE IN MAIZE VARIETIES AS REVEALED BY DISC-GEL ELECTROPHORESIS AND ACTIVITY STAINING

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1. Introduction

The *in vivo* formation of the 1,4-α-glucosidic bond of starch and phytoglycogen in plants is now generally considered to occur by the transfer of the glucose moiety of ADP(UDP)glucose to a 1,4-α-glucan chain [1–4], this transfer being catalysed by ADP(UDP) glucose: 1,4-α-glucan 4-α-glucosyltransferase (starch synthetase), although a participation of phosphorylase in the biosynthesis of starch cannot be ruled out at the present time [4–7]. Recent studies of starch biosynthesis have been complicated by the findings of multiple forms of starch synthetase which differ in their properties and which seem to vary in amount during the development of plant organs [4, 8–12]. One of the main impediments to more rapid progress in comparing the variety of synthetases in different plants, plant organs, subcellular organelles and at different stages of development, is a lack of a rapid screening technique applicable to minimum amounts of relatively crude material. In this communication we report the development of such a technique, the polyacrylamide disc electrophoresis of starch synthetases present in crude extracts, and the detection of these enzymes by an activity stain. The sensitivity of the method is such that less than 1×10^{-4} i of starch synthetase activity can readily be detected. The plant systems we have examined are the kernels of several mutants of maize which, because of the striking differences in their starch composition [13], are regarded as important objects of study in our efforts towards a better understanding of the biosynthesis of amylose, amylopectin and phytoglycogen. Comparative electrophoresis of extracts of maize varieties has now revealed differences in the pattern of synthetase activity which seem to parallel the starch composition.

2. Materials and methods

Isogenic lines of maize mutants with a W64A background were grown by Dr. P.A. Peterson of the University of Iowa. The material was frozen on harvesting at the 22-day stage after pollination and stored at −70°. Extracts of whole kernels, endosperm or embryo tissue were prepared by homogenisation in 50 mM Tris-HCl, 0.2 mM dithiothreitol, 2 mM EDTA, 10% sucrose, pH 7.6 (1 ml per 3 g tissue). The homogenates were passed through cheesecloth and centrifuged at 20,000 *g* for 20 min. Where only small amounts of material were available, the tissue was ground to a fine powder in liquid nitrogen prior to extraction; the cheesecloth step was omitted.

Disc gel electrophoresis was performed in a Buchler disc gel electrophoresis apparatus. The buffer system was based on that used by Davis et al. [14]. The gels (6% or 7% acrylamide) were cast in a buffer consisting of 7% sucrose in Tris-HCl, pH 7.9, such that the final chloride concentration was 30 mM. The bisacrylamide: acrylamide ratio was 1:37.5. After the gels were cast they were soaked 24 hr in the same buffer, with 1 mM dithiothreitol added, prior to use. This was to remove any residual ammonium persulfate, used as the polymerising agent, and which has been reported to give rise to artifacts [15]. The extracts (usually 50 μl) were diluted in a sample buffer and loaded directly onto the gels without the use of sample or spacer gels. The sample buffer consisted of 35% sucrose, imidazole-chloride pH 5.8, such that the final conc. of chloride was 0.24 M. The applied extracts usually contained about 0.02 IU/ml of starch synthetase activity [10] or about 2.5 mg/ml of protein. The running buffer was 1 mM dithiothreitol, 35 mM L-asparagine in 10% sucrose, adjusted to pH. 7.3 with 2 M Tris. Bromophenol blue

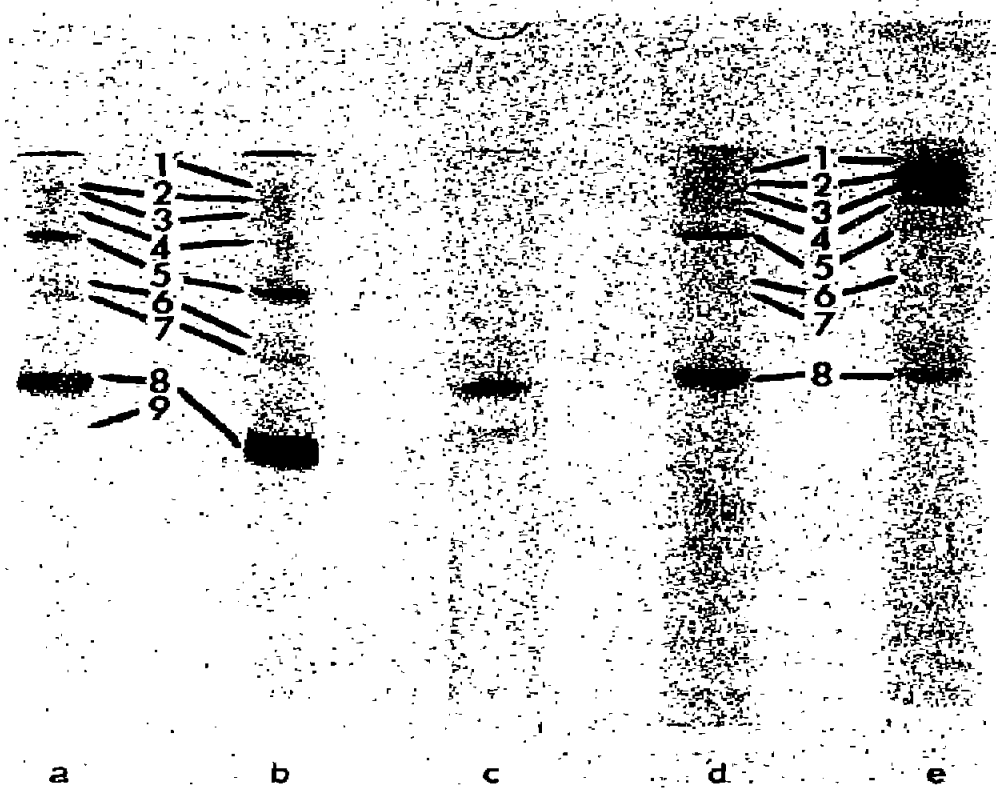

Fig. 1. Starch synthetase patterns of maize mutants on polyacrylamide gels. The bottom of the gels represents the position of the tracking dye. The bands were revealed by incubation with ADPglucose and subsequent staining with iodine (see text). a) Golden Bantam Iowa Belle 104, whole kernel, 7% gel; b) Golden Bantam Iowa Belle 104, whole kernel, 6% gel; c) *su*$_1$ maize, embryo, 7% gel; d) *su*$_1$ maize, endosperm, 7% gel; e) *ae* maize, endosperm, 7% gel.

was the tracking dye. Electrophoresis was carried out at 4° at a current of 2 mA per tube. After electrophoresis the gels were incubated for 15–20 hr at room temp. in 1 mM ADPglucose, 1 mM dithiothreitol, 2 mM EDTA, 25 mM Na_3-citrate, 25 mM Bicine [*N*, *N*-bis (2-hydroxyethyl) glycine], pH 8.0, and 0.01% amylose primer (Nutritional Biochemicals Corporation, degree of polymerisation ($\overline{DP}$) approx. = 300). The primer could be omitted by adding amylose (0.05 mg/ml) to the gels prior to polymerisation. After incubation the gels were placed in an iodine solution (0.2% iodine, 2% potassium iodide, 0.01 N HCl). The polysaccharides synthesized by starch synthetase were then revealed as dark brown bands. When lower iodine concentrations were used the bands were purplish or blue.

3. Results and discussion

3.1. *Procedure for the activity stains*

The procedure we have developed readily allows the electrophoretic examination of crude maize extracts for starch synthetase activity. An example of the results obtained is shown in fig. 1a for extracts of a commercial sweet corn. Amylose was used as the primer, since it gave the best results. It could be added to the incubation medium, or polymerised into the gels. No difference in the patterns was observed between these two procedures. In the absence of added primer, only very weak stains were obtained. Maltodextrin mixtures ($\overline{DP} = 6$) such as have been used as primers for the detection of phosphorylase activities [16] were unsuitable. In this case the synthesized 1,4-α-glucan appeared to be of a rather short chain length and diffused too readily out of the gels. This

was confirmed in an experiment where radioactively labelled ADPglucose was used; labelled 1,4-α-glucans were then detected in the incubation medium outside the gel. Amylopectin (from potato) and glycogen (from shellfish, Sigma Chemical Corporation) were also tested as primers at concentrations of 1 mg/ml. These were ineffective when added to the incubation mixture; presumably the molecules were too large to penetrate the gels. When the polysaccharides were polymerised directly in the gels, similar patterns were obtained as with the use of amylose, but the overall migration of the bands was markedly retarded.

It was necessary to confirm that the bands observed were due to starch synthetase activity, and not due to phosphorylase. Isozymes of the latter enzyme have been described in maize extracts [5, 6, 16], and it was possible that these might contribute to the patterns observed, either because of endogenous glucose 1-phosphate in the ADPglucose preparations used, or because of the breakdown of ADPglucose to glucose 1-phosphate during the incubation period. This possibility was eliminated by experiments in which 20 mM inorganic phosphate was added to the incubation mixtures. When this was done, the same patterns were observed on incubation with 1 mM ADPglucose as in the absence of inorganic phosphate. Phosphorylases were certainly present and could be detected by substituting 1 mM glucose 1-phosphate for the ADPglucose in the incubation mixture, but the phosphorylase activity bands were completely eliminated in the presence of 20 mM phosphate. In gels into which amylose had been polymerised, the light blue background color was bleached out at the position of the phosphorylase activities, when phosphate was present.

3.2. *Examination of starch synthetase patterns in maize varieties*

The three maize genotypes which are of particular interest in terms of their starch synthetase composition are sweet corn (su_1), waxy (*wx*), and amylomaize (*ae*). Sweet corn is a mutant of maize which is characterised by the accumulation of phytoglycogen [17], as well as a starch component with a normal amylose: amylopectin ratio (about 1 : 3). Waxy maize starch [18] consists almost entirely of amylopectin, while amylomaize starch [19] has an unusually high content of amylose (up to about 85%).

We have recently partly purified the starch synthetase of a commercial sweet corn (Golden Bantam Iowa Belle 104) [20] and have therefore chosen this variety of sweet corn for examination of the pattern of synthetase activities in crude extracts (fig. 1a and 1b). The starch synthetase composition of sweet corn is seen to be complex; as many as 17 bands of activity have been seen, of which the 9 most prominent are labelled in fig. 1. There is one major form of the enzyme (no. 8) which co-migrates with the partially purified enzyme [20], a second major form (no. 5), and a number of weaker bands (fig. 1a). The slow migrating bands (no. 1 to 4) were slightly better resolved on 6% gels (fig. 1b). Essentially the same patterns were observed when whole kernels of an isogenic line of sweet corn (su_1 maize) were examined. It was of interest to compare the patterns of activity in the isolated embryo and endosperm tissues of su_1 maize. These are shown in fig. 1c and 1d, respectively. The major embryo form of the enzyme (no. 8) is the same as the major form of the endosperm. The embryo pattern is characterised by the absence of the slower migrating bands, and also by the presence of band no. 9. This band, which is present in whole kernel extracts, is seen now to be present only in the embryo, and not in the endosperm.

The complexity of the pattern is not unique to su_1 maize. When *wx* and several other maize varieties of isogenic lines were examined (*du*, su_2, fl_1, fl_2 and sh_1), embryo and endosperm patterns very similar to that of sweet corn were observed, except that in the embryo band 5 was generally more pronounced than in su_1, and band 9, which is exclusive to the embryo, was much less pronounced than in su_1.

When *ae* maize was examined, the embryo pattern was similar to those of the other varieties, but the endosperm pattern was strikingly altered (fig. 1e). Bands 5 and 8, the major starch synthetase components in the other varieties, are the minor synthetase components in *ae*. The major synthetase forms are the slow moving bands, 1—4 (c.f. fig. 1d and 1c), that are the minor components in the other varieties. This is the first observation of such an enzyme difference in high amylose maize, and it suggests that the slow moving bands are specifically involved in the biosynthesis of amylose. This deduction was further supported by the iodine staining properties of the bands. In dilute iodine, the slow moving bands 1—4, and especially 1—2, had a bluish appearance, while bands 5 and 8

gave a purplish stain. Evidently the polysaccharide synthesized by the slow moving bands is linear, while that formed by the faster moving bands is branched. This suggests in turn that amylose, amylopectin, and phytoglycogen may be synthesised by different types of synthetase; amylose would be formed by the synthetases which migrate as the slow moving bands, present in all varieties; but especially prominent in *ae* maize. Amylopectin (and phytoglycogen) would be synthesized by the faster moving synthetases.

The purple stains of the fast-moving bands are indicative of the concomitant presence of a branching enzyme (Q-enzyme). One possibility is that this enzyme is widely distributed in the region of bands 5–8. Tests for the presence of branching enzyme, by incubation of gels containing amylose primer but no ADPglucose were negative, since this did not lead to the removal of the light blue background stain and the appearance of purplish bands. It may be, however, that Q-enzyme can act more rapidly on a growing chain than on a pre-existing amylose molecule. Another possibility is that synthetase and branching enzyme are present in the fast-moving bands as a two-enzyme complex. It may be noted that Preiss and coworkers [10] have obtained from waxy maize a starch synthetase which yields an amylopectin-like molecule after incubation with ADPglucose in the absence of added primer.

The evidence presented here for the existence of two types of synthetase is of considerable interest in relation to the genesis of amylose and amylopectin since there has not previously been any evidence to account for their side-by-side existence in the starch granule, or which would account for the variation in the amylose: amylopectin ratio between different starches. We suggest, with the pictorial support of the zymograms for su_1 (fig. 1d) and *ae* (fig. 1e), that amylose is synthesized by synthetase *per se*, while amylopectin is synthesized by a synthetase-branching enzyme complex. The ratio of amylose: amylopectin is a function of the ratio of the two forms of synthetase.

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