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Isolation of Amyloplasts from Developing Maize Endosperm[1]

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ABSTRACT

Methods for the formation of protoplasts from developing maize endosperm and for the aqueous isolation of intact amyloplasts from such protoplasts are described. Protoplasts were obtained after incubating endosperm slices in a medium containing cellulase and pectolyase for 5 days at 4°C or 5 hours at 30°C. After purification in a Ficoll density gradient, the protoplasts were ruptured by forcing the suspension through a Nitex mesh (20 micrometer) positioned at the lower end of a modified disposable syringe. The resulting filtrate was layered on a discontinuous Ficoll density gradient of 30, 15, and 10%. Each Ficoll solution contained 0.7 molar sucrose, 10 millimolar arginine, 10 millimolar DL-dithiothreitol, 50 millimolar 2-(*N*-morpholino)ethanesulfonic acid (pH 5.6), and 2 millimolar $CaCl_2$. After 3 hours in the cold, an amyloplast fraction 50 to 93% intact and free from cytoplasmic, mitochondrial, and glyoxysomal contamination was recovered in the 15% Ficoll layer. Amyloplast intactness was estimated by fluorescent microscopy and activity of certain amyloplast marker enzymes before and after rupture of the amyloplast membrane. Starch branching enzyme, ADPG-pyrophosphorylase, and nitrite reductase were used as amyloplast marker enzymes.

In storage organs, as well as in other nonphotosynthetic tissues of higher plants, starch is synthesized and stored in organelles called amyloplasts (11). Synthesis of starch in these organelles differs from that of photosynthetic cells by its dependence on translocated carbon (17). In most cases, the carbon source for starch synthesis in reserve tissues is known to be sucrose; however, the enzymic pathways for the conversion of sucrose to starch precursors, and the form in which these precursors cross the amyloplast membrane, are not yet fully understood (16).

Isolation of intact amyloplasts should allow investigations into the distribution of the enzymes involved in the sucrose to starch interconversion at a subcellular level, as well as an insight as to which metabolites cross the amyloplast membrane. Initial attempts to aqueously isolate intact amyloplasts involved mechanical disruption of the tissue, resulting in a very low recovery of intact amyloplasts which were contaminated with cellular and subcellular constituents (18). More recent techniques by Duffus and Rosie (5), Williams and Duffus (22), and Fishwick and Wright (6) involved the use of density gradient centrifugation. Although these techniques are an improvement over the earlier methods, evidence to support their claims of amyloplast purity was not provided. Density gradient centrifugation was also used by MacDonald and apRees (12) who obtained a highly pure amyloplast fraction from suspension cultures of soybean. Although these authors (12) demonstrated amyloplast intactness prior to density gradient centrifugation, such a demonstration was not possible following centrifugation. In a more recent report, Gaynor and Galston (7) succeeded in separating intact amyloplasts from free starch grains by low gravity centrifugation in a Urografin density gradient. Such a separation was probably possible because these amyloplasts, obtained from etiolated pea internodes, contained multiple starch granules. Sack *et al.* (19) prepared a crude low gravity pellet from dark-grown maize coleoptiles which contained about 5% intact amyloplasts with multiple starch granules. Although purity of the fraction was not determined, amyloplast intactness was demonstrated by electron microscopy and by fluorescence microscopy following incubation with FITC-CF[2].

By using protoplasts instead of fresh tissue, Nishimura and Beevers (15) greatly improved plastid yield from castor bean endosperm. The use of protoplasts reduces the damage caused by mechanical rupture of the intact tissue.

From the information present in the literature, and our previous experience, it is clear that gentle homogenization and low gravity separation are essential for the preservation of the amyloplast envelope. In this communication, we report methods for the preparation of protoplasts from developing maize endosperm and for the aqueous isolation of intact amyloplasts.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L.) plants were grown in the Horticulture Department greenhouse facilities at The Pennsylvania State University, University Park, PA. All plants were hand pollinated and the ears were harvested 14 to 17 d after pollination.

Protoplast Formation. Developing maize kernels were excised from the ear, and the embryo and pericarp were removed. The endosperms were cut transversely into three sections with a sharp razor blade and collected in incubation medium (0.7 M sucrose; 0.2 mM $CaCl_2$; 0.5 mM DTT; 0.05% BSA; 25 mM Mes, pH 6.5; 10 mM arginine) until all sections were prepared (30 min). The endosperm sections were subsequently rinsed with the same solution to remove cellular contents from the cut cells.

Endosperm sections (1.5 g) were placed in a 50-ml Erlenmeyer flask with 10 ml of incubation medium containing 1% Cellulysin (Calbiochem) and 0.1% Pectolyase Y-23. (The Pectolyase Y-23 was a gift from W. Lin.) For most experiments reported in this paper the tissue was vacuum infiltrated and the flasks placed in a cold room at 4°C for 5 d. Protoplasts were released from the endosperm slices by very carefully rotating the flask, and the digestion medium containing the protoplasts was filtered through a 140-μm nylon mesh to eliminate large debris and undigested tissue. The resulting filtrate was layered on a discontinuous Ficoll (mol wt 400,000; Pharmacia Co., Uppsala, Sweden) density

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[2] Abbreviations: FITC, fluorescein isothiocyanate; CF, cationized ferritin.

gradient of 30, 27, 21, 15, and 10%. Volumes for each layer were 1.0, 0.6, 1.0, 0.5, and 0.8 ml, respectively. The Ficoll solutions contained 0.7 M sucrose, 10 mM arginine, 10 mM DTT, 50 mM Mes (pH 6.5), 0.2 mM $CaCl_2$, and Ficoll at the appropriate concentrations. After a 3-h settling at 4°C, intact protoplasts had sedimented into the 27% Ficoll layer which was collected as the protoplast fraction.

Amyloplasts used to test sucrose synthase, ADPG pyrophosphyorylase, and the protection experiments reported in Table IV were from protoplasts produced and isolated as described above except that sorbitol was used instead of sucrose, $CaCl_2$ was increased to 100 mM, and the period and temperature of incubation was changed from 5 d at 4°C to 5 h at 30°C. The number of protoplasts and amyloplasts obtained was less than that obtained with the previously described method; however, the percentage of intact amyloplasts, using branching enzyme as a marker, was similar.

Amyloplast Isolation. The protoplast fraction (*i.e.* the 27% Ficoll layer) was diluted with 3 times its volume of incubation medium and passed through a nylon mesh (20 μm) which replaced the lower end of a 12-ml plastic syringe (Lancer, St. Louis, MO). The protoplasts were completely ruptured by a single passage. The resulting suspension was layered on a discontinuous Ficoll density gradient composed of 1.0 ml of 30%, 0.5 ml of 15%, and 1.0 ml of 10%. The Ficoll solutions contained the same components as those used above. The amyloplasts and starch granules were allowed to settle in the cold (4°C) for 3 h. The highest proportion of intact amyloplasts was collected in the 15% Ficoll layer.

Latency Experiments. The latency and protection experiments were patterned after those described by MacDonald and apRees (12). For these experiments, duplicate samples of the 15% Ficoll layer were used. In one, the intact amyloplasts were ruptured by the addition of Triton X-100 to a final concentration of 0.1% (v/v), while the other was kept intact. Triton X-100 at this concentration had no effect on the activity of soluble enzymes. Latency experiments were conducted with branching enzyme and nitrite reductase. The enzymic activity of the ruptured preparation minus that of the intact preparation is called latent activity. Per cent intactness (latency) is the latent activity divided by ruptured activity × 100 (12). All enzymic reactions were carried out in a reaction mixture containing 0.7 M sucrose.

Protection Experiments. One aliquot of the 15% Ficoll layer was preincubated with trypsin (4 mg/ml type III, Sigma Chemical Co.) and another aliquot (the control) was preincubated with trypsin (4 mg/ml) plus trypsin inhibitor (6 mg/ml type II-S, Sigma Chemical Co.) for 30 min at 30°C. Another control included an aliquot preincubated in trypsin plus Triton X-100. At the end of the preincubation period, trypsin inhibitor (6 mg/ml) was added to the samples that lacked it. An aliquot was also incubated on ice for 30 min and trypsin plus trypsin inhibitor added immediately prior to rupture and assay. Triton X-100 (0.1% final concentration) was added to all samples and branching enzyme activity was determined.

Fluorescent Microscopy. For fluorescent visualization of the amyloplast membrane, we used the method described by Sach *et al.* (19) with some modifications. Samples for fluorescent microscopy were collected from a 15% Ficoll layer that, in addition, contained 2.5% glutaraldehyde. These samples were incubated overnight in the glutaraldehyde containing 15% Ficoll layer. The following day, the samples were thoroughly washed with water and spread over several glass microscope slides. When dry, two drops of acetone were added to fix the material to the slide. After the acetone evaporated, 0.5 ml of FITC-CF was spread on top of the particles and the slides placed in a humid chamber for 30 min. FITC-CF was prepared by combining 0.5 ml of CF (25 mg/ml, Sigma Chemical Co.) with 1.5 ml of isolation medium containing 1.5 mg FITC. The mixture was stirred at room temperature for 30 min. The slides were removed from the humid chamber and the unbound FITC-CF was carefully rinsed off with distilled H_2O and the slide preparations dried on a hot plate at 40°C. The slides were examined with a Leitz fluorescent microscope (Ernest Leitz, New York, NY) and the micrographs recorded on Kodak Ektachrome 400 Daylight film.

Electron Microscopy. For ultrastructural visualization of negatively charged membranes with CF, the methods of Sack *et al.* (19) and King and Preston (10) were followed with some modification. Amyloplasts from the 15% Ficoll, 10% Ficoll, and upper layer fractions were separately collected in tubes and 25% glutaraldehyde was added to a final concentration of 2.45%. The tubes were kept for 30 min on ice and then gently centrifuged (500 rpm for 5 min). The supernatant fluids were replaced with a buffered fixative (1.8% glutaraldehyde, 1% formaldehyde, 0.3 M sucrose, and 0.1 M sodium cacodylate [pH 7.6]) and kept at 4°C overnight. The amyloplasts were pelleted by centrifugation as above, and washed first with 0.1 M cacodylate buffer containing 0.2 M sucrose and then washed with the same buffer containing 0.1 M sucrose. The washed amyloplasts were suspended in 2 ml of 50 mM Tris buffer (pH 7.6) and a sufficient amount of CF (10.4 mg/ml) was added to make a final concentration of 750 μg ferritin/ml suspension. The tubes were incubated for 15 min on ice and centrifuged as above. Three per cent agarose (kept at 46°C) was added to the pellets, mixed well, allowed to harden, and cut into tiny cubes. These were postfixed 3 h with 2% OsO_4 in 0.05 M K-phosphate (pH 6.8). The agarose cubes were dehydrated in a graded acetone series and embedded in Spurr's resin (20). Sections were cut on a Servall ultramicrotome, pick up on naked grids, and stained with lead citrate and uranyl acetate before being examined with a Hitachi HU-11 TEM at 75 kv.

Enzyme Assays. Nitrite reductase was measured by monitoring the disappearance of nitrite in the reaction mixture, as described by Hucklesby *et al.* (9). Branching enzyme activity was measured by following the amount of stimulation of α 1,4-glucan formation from [^{14}C]glucose-1-P catalyzed by phosphorylase *a* (8). Catalase and isocitrate dehydrogenase were measured as in Bergmeyer (1). Sucrose synthase was assayed in the breakdown direction as in Su and Preiss (21), except nonradioactive sucrose was used and the formation of UDPG was measured as in Bergmeyer (1). Formation of galactolipids by galactosyl transferase (UDP galactose:diacylglycerol transferase) was monitored as described by Douce (4); and ADPG pyrophosphorylase was assayed as in Dickinson and Preiss (3).

RESULTS

Preliminary attempts to produce protoplasts by more conventional methods using warmer temperatures, and shorter times of incubation in solutions containing mannitol or sorbitol were unsuccessful. Incubation at 25°C in media containing sorbitol or mannitol resulted in almost complete digestion of the tissue within 5 h; however, only a few small protoplasts remained intact. When sucrose replaced the sugar alcohols, digestion of the tissue occurred at much slower rates but a greater number of intact protoplasts was obtained. At cold temperatures (4°C), the time required for essentially complete digestion of the tissue was considerably extended (5 d); however, many more intact protoplasts were produced. Near the end of the studies reported here, we succeeded in producing acceptable quantities of protoplasts in 5 h when the incubation buffer was modified to include 100 mM $CaCl_2$ and sucrose was replaced with sorbitol.

Formation of Protoplasts. The procedures described here for the formation of protoplasts from developing maize endosperm yielded on the average 7.0 to 10.5 × 10^5 protoplasts/g fresh tissue. When observed under the microscope (Fig. 1A), the protoplasts appeared mostly spherical; however, irregularly

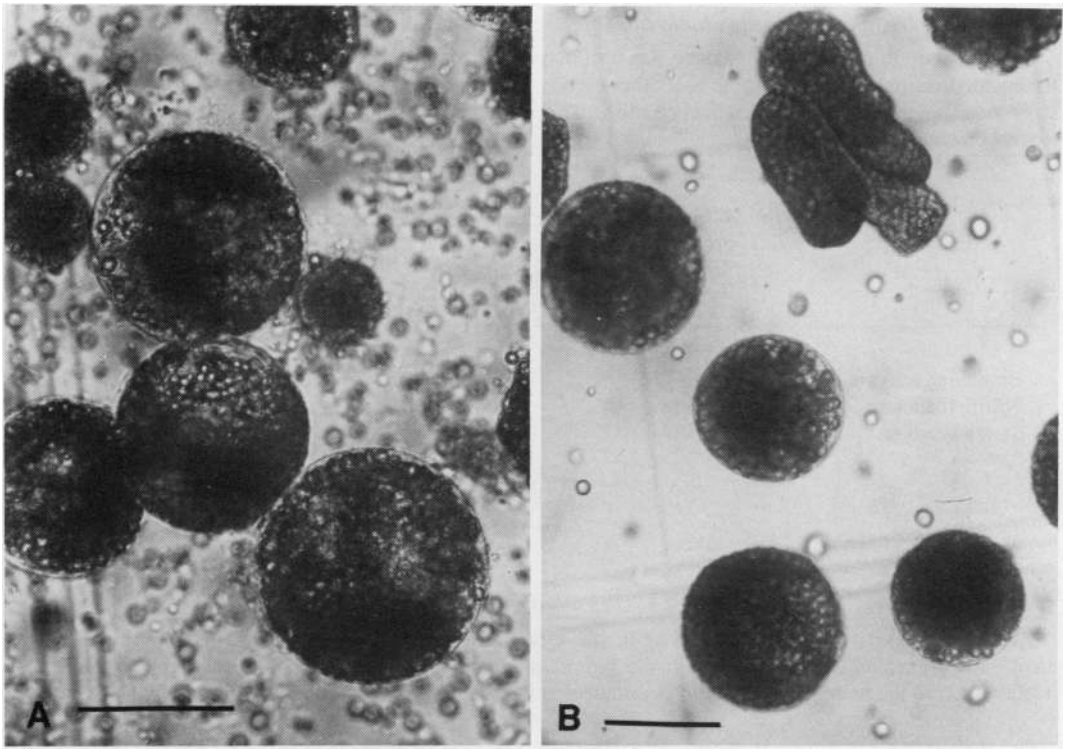

FIG. 1. A, Light micrographs of immature maize endosperm protoplasts prior to purification. B, Protoplasts after purification through Ficoll density gradient. Bars, 50 μm.

shaped cells were commonly seen (Fig. 1B). Large numbers of starch grains, amyloplasts, and cell debris were also present in the digestion medium (Fig. 1A).

The viability of the protoplasts was confirmed by three different methods. First, the protoplasts excluded Evans Blue dye; second, the highly dehydrated protoplasts showed rapid swelling when the sucrose concentration of the medium was reduced from 0.7 to 0.2 M; and third, cytoplasmic movement was observed.

The protoplasts were separated from the free starch grains, cellular debris, and the hydrolytic enzymes in the incubation medium by allowing them to settle through a Ficoll density gradient. The 27% Ficoll layer was enriched with intact protoplasts (Fig. 1B); however, some starch grains and amyloplasts were also present. The protoplasts are extremely fragile and the free starch and amyloplasts likely came from protoplasts which ruptured during manipulation of the sample after sedimentation.

Isolation of Amyloplasts. The protoplast sample, collected from the 27% Ficoll layer, was diluted with isolation medium so that it would remain on top of a 10% Ficoll solution. After the appropriate dilution, the protoplasts were gently ruptured and the resulting solution was layered on a second density gradient. After settling for 3 h, starch granules and the activities of amyloplast marker enzymes (branching enzyme, nitrite reductase, and starch synthase) were distributed throughout the gradient (Table I). Although the 15% Ficoll layer contained only about 10% of the total recovered activity, preliminary results indicated that it contained the highest proportion of intact amyloplast. Therefore, the 15% Ficoll layer was used as the amyloplast source for most studies.

Evidence of Purity. To test for the possible contamination of the amyloplast sample by other cellular components, a separate series of experiments were performed. In these experiments, the enzymes sucrose synthase, isocitrate dehydrogenase, and catalase, which are indicative of the presence of cytoplasmic, mitochondrial, and glyoxysomal contaminants, respectively, were tested. Samples of the protoplast fractions were ruptured accordingly, and each enzyme was assayed separately. We found all the above enzymes to be present in the protoplast samples. However, most of the activity was recovered in the upper layer with undetectable amounts in the 15% Ficoll (amyloplast) layer (Table I). Based on these results, the amyloplast fraction was judged to be free from cytoplasmic, mitochondrial, and glyoxysomal contamination.

Determination of Intactness. The percentage of amyloplast recovered intact from the 15% Ficoll layer was estimated by three different and independent methods. The first method is based on the assumption that amyloplast enzymes within the organelles are physically separated from the substrate and can not be measured. Thus, within a given sample, the percentage of the total activity of an enzyme marker within the membranes of the organelle (the latent activity) is equivalent to the percentage of intact amyloplasts in that sample. For these estimates, nitrite reductase, branching enzyme, and ADPG pyrophosphorylase were assayed. These three enzymes are known to be confined to plastids (12). Approximately 71% of the total branching enzyme activity and 66% of the ADPG pyrophosphorylase activity was latent and, thus, contained within intact amyloplasts (Table II). The results obtained with nitrite reductase suggested a lower percentage of amyloplast intactness. In these experiments, it was estimated that 47% of the total activity was latent. We believe, however, that this figure could be an underestimate of intactness due to the very difficult handling procedures required for this enzyme assay. The reaction mixture and the amyloplast sample had to be mixed extremely gently to prevent oxidation of the substrate and rupture of the amyloplast membrane. This great care probably resulted in an improperly mixed enzyme assay mixture.

The second method utilized for determining amyloplast integrity consisted of performing protection experiments. In these

Table I. *Activities of Enzyme Markers in Different Fractions Collected during Amyloplast Isolation*

The distribution of branching enzyme was routinely included as a check of the gradient separation when testing the fractions for the distribution of other marker enzymes. Therefore, we have presented the average ± SD of the mean for seven individual fractionations. The results of two fractionations are presented for the other marker enzymes.

Enzyme	Activity in Protoplast Fraction	Per Cent Total Activity Recovered from Gradient			Sum of All Gradient Fractions (% of Protoplast Activity)
		Upper layer[a]	10% Ficoll	15% Ficoll	
	nmol/min·fraction				
Branching enzyme	2468 ± 285[b]	71 ± 5	27 ± 5	10 ± 2	100[b]
Nitrite reductase	28–18	63–57	28–31	9–12	82–77
Starch synthase	17–16	86–78	8–9	5–13	64–71
		% of Protoplast Activity			
Catalase	93–91	98–87	—	traces	98–87
Isocitrate DH	55–67	85–86	NA[c]	NA	85–86
Sucrose synthase	562–728	113–98	NA	NA	113–98

[a] The upper layer represents the activity in the ruptured protoplasts which did not settle into the gradient. [b] Branching enzyme activity in the protoplast fraction was calculated from the sum of gradient fractions due to the presence of inhibiting substances in the protoplast preparations. [c] No activity.

Table II. *Activity of Branching Enzyme, Nitrite Reductase and ADPG Pyrophosphorylase in the Presence or Absence of Triton X-100*

Enzyme	Activity per 15% Ficoll Fraction			Per Cent Intactness
	Ruptured (+ Triton)	Intact (− Triton)	Latent	
	nmol/min			%
Branching enzyme				
Exp. 1	198	68	130	66
Exp. 2	244	59	185	76
Average	221	63	157	71
Nitrite reductase				
Exp. 3	2.49	1.22	1.27	51
Exp. 4	2.20	1.25	0.95	43
Average	2.34	1.23	1.11	47
ADPG pyrophosphorylase				
Exp. 5	0.334	0.117	0.217	65
Exp. 6	0.267	0.090	0.177	66
Average	0.301	0.104	0.197	66

Table III. *Activity of Branching Enzyme following Pretreatment with or without Trypsin and/or Trypsin Inhibitor*

Amyloplast Pretreatment[a]	Branching Enzyme Activity per 15% Ficoll Fraction		
	Exp. 1	Exp. 2	Average
	nmol/min		
None	195	192	193
Cold	185	175	180
Lysed + trypsin	NA[b]	NA	0
+ Trypsin + Trypsin inhibitor	135	232	184
+ Trypsin − Trypsin inhibitor	36	68	52
% Intactness	27	29	28

[a] Trypsin and/or trypsin inhibitor were added to all pretreated samples, as needed, prior to rupture of the amylopast membranes with Triton X-100 and addition of [^{14}C]G-1-P to start the assay reaction. [b] No activity.

experiments, it is assumed that the membrane of an intact organelle would protect the proteins within against degradation by externally added trypsin. This protection would not be expected for those proteins found outside an intact organelle. Simply preincubating the amyloplast on ice for 30 min prior to the addition of trypsin, trypsin inhibitor and Triton X-100 had very little effect on branching enzyme activity of the ruptured amyloplasts (Table III). Likewise, the trypsin inhibitor effectively protected the branching enzyme from inactivation although there was more variation between the two experiments. Preincubation of Triton X-100 lysed samples and nonlysed samples with trypsin destroyed 100% and 70% of the total branching enzyme activity. Thus, trypsin effectively destroyed all unprotected enzyme. The remaining activity (28%) in the nonlysed samples represents that protected by the plastid membranes. The lower percentage of intactness determined by this method is probably the result of membrane rupture during the preincubation period due simply to aging at 30°C or due to the effect of trypsin on structural membrane proteins.

To test for such a possibility, the per cent intactness, measured as latency, was determined following preincubation of the amyloplasts at 30°C for 15 to 45 min prior to assay (Table IV). Concurrently, another set of amyloplast samples were incubated with trypsin (4 mg/ml) for similar time intervals. At the appropriate time, trypsin inhibitor was added to those samples preincubated with trypsin and branching enzyme assayed. The results of these experiments (Table IV) show about a 25% decline in total activity during the first 30 min of preincubation without trypsin but only a moderate decrease in the per cent intactness. In the presence of trypsin, however, the loss of total activity was much greater than in its absence with the greatest loss occurring in the first 15 min.

These results are interpreted as indicating that there is a natural but gradual breakdown of the amyloplast membranes for 30 min and the released enzymes undergo inactivation. However, trypsin dramatically increased the loss of amyloplast intactness and enzyme inactivation. Thus, we conclude that the low per cent intactness calculated based on the protection experiment (Table

Table IV. *Activity of Branching Enzyme and Per Cent Intactness following Pretreatment of the Amylopast with or without Trypsin*

Where presented, the two values represent results of two experiments. The single values are the result of one experiment. The 100% activity of the two experiments were 211 and 174 nmol/min·15% Ficoll fraction

Time of Preincubation	Per Cent Intactness[a]	Per Cent Total Activity	
		− Trypsin	+ Trypsin
min		%	
0	93.3–93.0	100	—
15	90.0–88.9	74.8–61.6	38.8
30	87.7–85.3	70.2–83.6	25.6
45	80.9	46.0	21.7

[a] Per cent intactness was based on the difference in activity of amyloplasts incubated in the presence and absence of Triton X-100 as in Table II.

III) is low because of the detrimental effect of trypsin on the amyloplast membrane.

The use of fluorescent microscopy enabled us to estimate the degree of intactness by direct observation. A recent report by Sack *et al.* (19) demonstrated the existence of negative changes at the amyloplast membranes. CF, which binds to negative changes on membrane surfaces, was tagged with a fluorescent label (FITC). After incubation of the amyloplast samples with FITC-CF, fluorescence was observed to be bound to 45 to 60% of the particles. Figure 2 shows examples of intact and ruptured amyloplasts as seen with the fluorescent microscope. The intact amyloplasts appear much brighter than the free starch granules.

A final method for confirming amyloplast intactness was by EM after staining with CF. The CFs were inevitably present on the peripheries of almost all amyloplasts from the 15% Ficoll fraction, although the circle of CF around some amyloplasts was incomplete. The thickness of the CF particles around starch granules varied from a single layer to multiple layers (Fig. 3, B and C). The layers of CF were present at variable distances from the borders of the starch granules (Fig. 3, B and C). Many starch granules from the 10% Ficoll fraction were not surrounded by CF, indicating that the membrane was lost prior to fixation (Fig. 3A). Although we could not observe membranes in electron micrographs of CF-treated amyloplasts, the presence of membranes was detected in preparations from fresh endosperm tissue. Therefore, membranes were probably modified during the isolation and processing of the amyloplasts. How and when the membranes were modified merit further investigation.

To test for the presence of outer membrane in the amyloplast preparation, the enzyme galactosyl transferase (an outer membrane marker for chloroplasts [4]) was tested. The activity of the enzyme (30 pmol/min·fraction) suggests the existence of outer membrane components within the amyloplast preparation; unfortunately, it does not give indications of the structural integrity of the membrane.

DISCUSSION

Earlier attempts to isolate intact amyloplasts from fresh tissue resulted in a very low percentage recovery (6, 7, 12). The failure to obtain a significant number of intact amyloplasts is probably due to a combination of factors, such as the fragile nature of the organelle (6), and to the forces necessary to mechanically disrupt the cell walls of the intact tissue (15). Following suggestions of Nishimura and Beevers (15) that the use of protoplasts reduces the exposure of plastids to such disruptive forces, we proceeded to devise a method for protoplast formation from maize endosperm.

Motoyoshi (13) described a method for maize endosperm protoplast production from endosperm callus tissue cultured for over 7 years. However, maize endosperm callus produces little starch, and when starch is induced to accumulate, the amylose percentage is much different from that produced by normally developing kernels (2). To our knowledge, this is the first description of a procedure for the production of protoplasts from normally developing cereal endosperm.

We found it necessary to test for the viability of the protoplasts

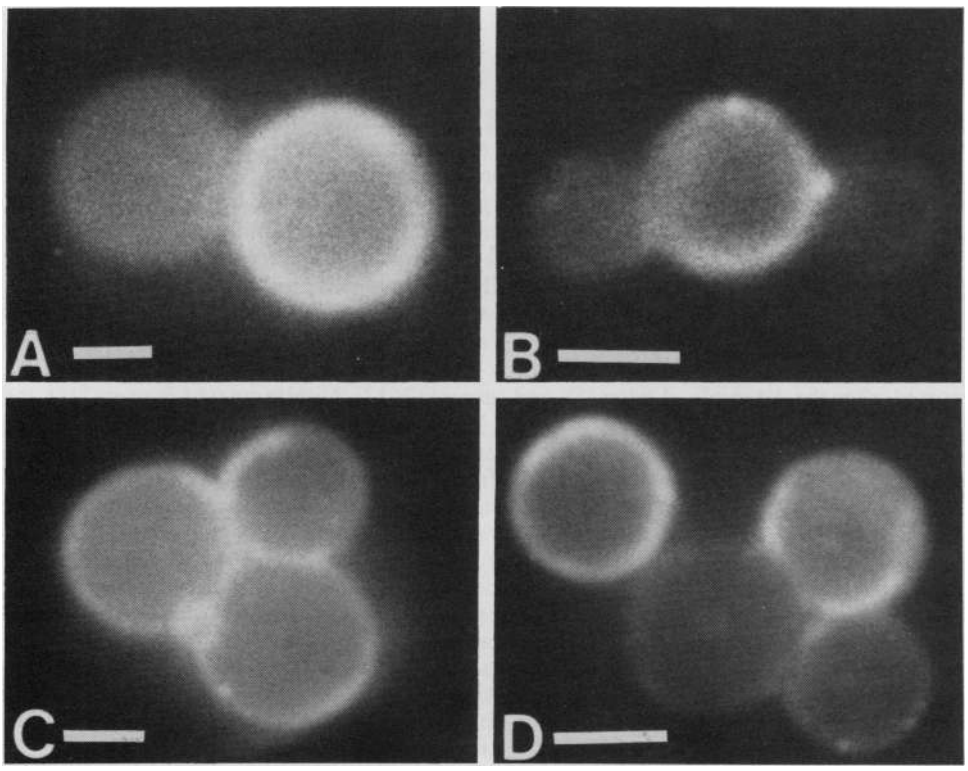

FIG. 2. Fluorescent micrographs of amyloplasts and starch grains isolated from developing maize endosperm protoplasts. Intact amyloplasts are completely encircled by FITC-CF fluorescence. Bars, 5 μm.

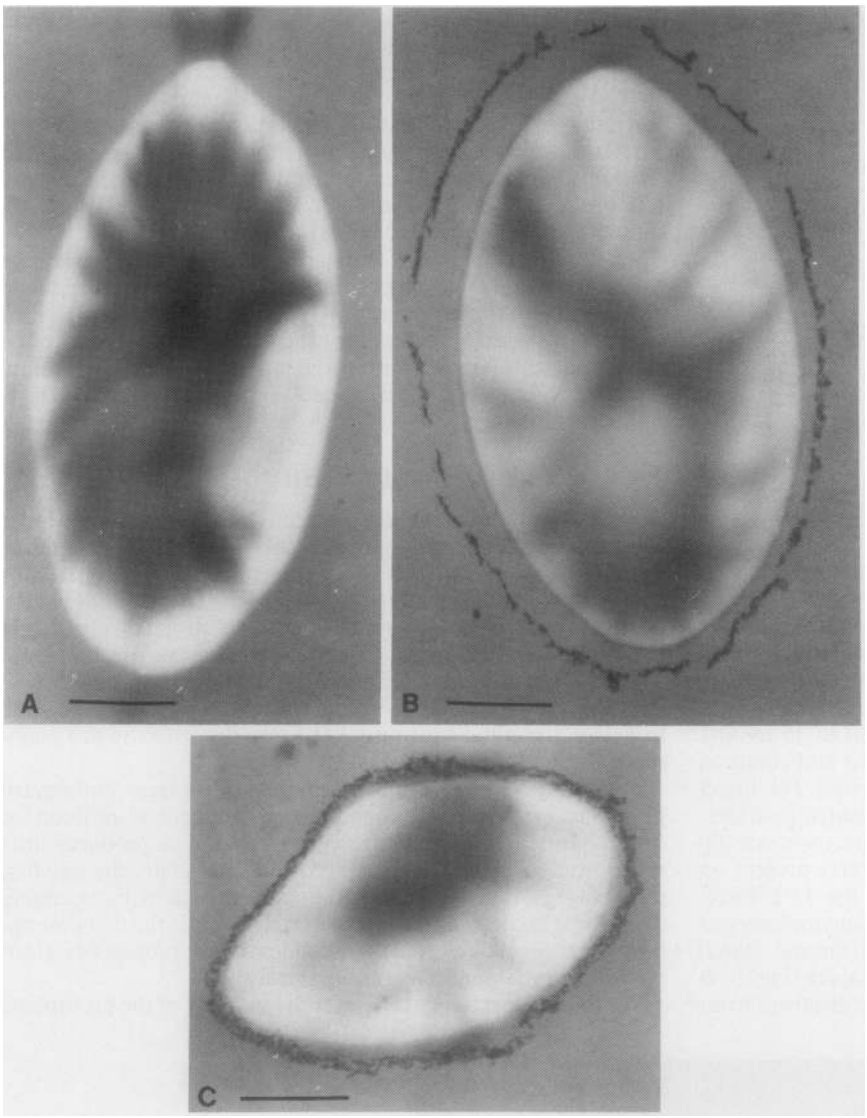

FIG. 3. Electron micrographs of CF-stained amyloplasts and starch grains from developing maize endosperm protoplasts. A, Starch granule with no CF staining membrane. B, Amyloplast with partially CF-stained membrane. C, Amyloplast with complete CF-stained membrane. Bars, 1 μm.

due to the unusually long period of incubation required for the formation of intact protoplasts. Since the protoplasts excluded Evans Blue dye, showed rapid swelling in response to dilution of the osmoticum, and had cytoplasmic movement, we concluded that the protoplasts remained alive and functional. We have to note, however, that in a separate set of experiments (data not shown) we observed a rapid decline in glucose incorporation into starch when endosperm slices were preincubated in the cold in the protoplast isolation medium (minus hydrolytic enzymes) for various periods of time prior to incubation in [^{14}C]glucose. The greatest decline in starch formation occurred after the 1st d of preincubation and thereafter remained relatively constant until the 5th d of preincubation. At this time we do not know the site(s) in the pathway from glucose to starch affected by the preincubation treatment.

Although a significant number of protoplasts were obtained by the method described here, we observed that such protoplasts containing starch granules are extremely delicate and had to be handled with particular care. A protocol including centrifugation such as the one employed by Nishimura and Beevers (15) ruptured all protoplasts and could not be used. However, the protoplasts were successfully separated from the cellular debris and from the hydrolytic enzymes present in the incubation medium by 1 *g* settling through a Ficoll density gradient. Since the heavy starch containing protoplasts settled more rapidly than the remaining cellular organelles and debris, we were able to collect an almost pure protoplast fraction.

The preparation of intact amyloplast free from other subcellular organelles and cytoplasmic contaminants was achieved by again eliminating all centrifugation steps from the purification procedure. Initially, we attempted procedures that had been used by other researchers, but we were unable to obtain a satisfactory amyloplast yield or purity. These methods involved the use of gradients of high density compounds such as Urografin (5, 7), Ludox (5), and sucrose (7, 12). Nishimura and Beevers (15) and MacDonald and apRees (12) used protoplasts as the starting material for isolation of plastids from castor bean endosperm and amyloplasts from soybean cell cultures, respectively. Plastids from the castor bean protoplasts do not contain starch and were purified in high yield on a sucrose density gradient (15). Similarily, starch containing amyloplasts from the soybean cell culture were purified by centrifugation through a sucrose density gradient (12). The resulting amyloplast preparation contained 20 to 30% of the cellular plastid marker enzyme and was free from other cytoplasmic contaminants. One-half to two-thirds of the amyloplasts in the unfractionated lysates were determined to be intact; however, after purification on the sucrose gradient, the resuspended amyloplasts were no longer intact (12).

Our method for amyloplast separation took advantage of the relatively high density of the amyloplast which allowed them to settle through a Ficoll gradient. The differential sedimentation of cellular components resulted in an amyloplast fraction containing undetectable amounts of mitochondrial, cytoplasmic, and glyoxysomal marker enzymes. However, this procedure does not completely separate free starch grains from intact amyloplasts. Fishwick and Wright (6) also used gravity settling to purify amyloplasts from potato tubers. They report that of the starch granules within their 'amyloplast' preparation, approximately 16% were within intact amyloplasts. In our study, the 15% Ficoll layer contained only about 10 to 15% of the total amyloplast marker enzyme, but within that fraction up to 93% of the amyloplasts were intact. The low estimate of per cent intactness obtained with the protection experiments (Table III) are not considered to be an accurate measurement of amyloplast integrity. It is clear from later studies (Table IV) that trypsin was disrupting the membrane structure and causing rapid amyloplast breakdown. Mourioux and Douce (14) reported a similar effect of proteases on chloroplast membranes. In the protection experiments performed by MacDonald and apRees (12), protection of the amyloplastic enzymes by the amyloplast membrane was probably the result of using an unfractionated cell extract. These extracts would be expected to contain much larger amounts of protein than the purified amyloplasts used in our study. This additional protein could contribute some protection of the amyloplast membranes from trypsin.

Although our total recovery of intact amyloplasts is relatively low, the ability to isolate a fraction with a high proportion of intact amyloplasts should allow us to complete studies on enzyme compartmentation and membrane transporters.

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