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Isolation of Intact Plastids from Protoplasts from Castor Bean Endosperm[1]

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MIKIO NISHIMURA[2] AND HARRY BEEVERS
Thimann Laboratories, University of California, Santa Cruz, California 95064

ABSTRACT

Protoplasts were prepared from castor bean (*Ricinus communis*) endosperm by treatment with a mixture of the commercial enzymes Macerozyme R-10 and Cellulose "Onozuka" R-10. The protoplasts were gently ruptured by forcing the suspension through a hypodermic needle and the homogenate centrifuged on a linear sucrose gradient. From such a homogenate the mitochondria are recovered at their typical isopycnic density of 1.18 g/ml, but the glyoxysomes are retained, with other membranes, at a density of 1.13. The plastids reach their typical density of 1.22 on the gradient and are thus clearly separated from other organelles. Moreover, since essentially all of the ribulose bisphosphate carboxylase activity on the gradient is present in this fraction it can be concluded that the plastids are intact and have been recovered in high yield.

Isolated protoplasts offer some advantages for experimental work in plant cell biology, among which is their use as starting material for the separation of organelles. When intact tissues are used, even with favorable material and propitious disintegration procedures, the forces necessary to bring about rupture of the cell wall result in damage to the more fragile organelles. Enzymic digestion of the wall removes this impediment and cell breakage can then be achieved by procedures that minimize this damage (2, 10, 12, 18).

The methods using castor bean endosperm that we have developed previously, in which chopping with razor blades is followed by centrifuging the total homogenate, give excellent preservation, separation, and yield of mitochondria, glyoxysomes and ER (3, 6). However, only a portion (<30%) of the plastids are recovered intact by this method (14, 17). We report here methods for the preparation of protoplasts from castor bean and the isolation of intact plastids in high yield uncontaminated with other organelles.

MATERIALS AND METHODS

Plant Material. Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in running tap water for 1 day and germinated in moist Vermiculite at 30 C.

Protoplasts. Endosperm halves, removed from 4-day-old seedlings, were used for preparing protoplasts. Sixteen endosperm halves (about 7 g) were cut into 1-mm slices with a razor blade and placed in a 50-ml Erlenmeyer flask containing 8 ml of enzyme solution (0.5% Macerozyme R-10 and 2% Cellulase "Onozuka" R-10 in 0.7 M mannitol [pH 5.5]). After vacuum infiltration (70 mm Hg) for 30 sec, enzymic digestion was carried out on a reciprocal shaker (72 stroke/min) at room temperature. As no protoplasts appeared during the first 1-hr treatment, the enzyme solution was removed and 7 ml of fresh enzyme solution was substituted. After a further 2-hr treatment, the enzyme solution containing protoplasts was collected by filtration through nylon bolting cloth (35 mesh). Seven ml of fresh enzyme solution was added to the slices and they were incubated for a further 2-hr period. The protoplast suspensions were combined and collected by centrifugation at about 200 g for 3 min and washed with 3 ml of 0.7 M mannitol (pH 5.5).

Disruption of Protoplasts. The protoplasts which appeared from 90 endosperm halves during the 4-hr enzyme treatment were collected and ruptured essentially following Nishimura *et al.* (10). The washed protoplasts were added to 1.5 ml of homogenizing buffer (150 mM Tricine-KOH [pH 7.5] −25% sucrose − 1 mM EDTA) and were passed through a syringe (0.5 × 4 cm Termo) with a small piece of Miracloth (Calbiochem) at the base of the needle (0.7 × 32 mm). The protoplasts were completely broken in one stroke (see Fig. 2).

Sucrose Density Centrifugation. The ruptured protoplast preparations (about 2 ml) were directly layered on gradients composed of: (*a*) a 1-ml cushion of 60% (w/w) sucrose; (*b*) 13 ml of sucrose solution increasing linearly in concentrations from 30 to 60% sucrose; and (*c*) 1 ml of 30% sucrose, contained in an 18-ml tube. All sucrose solutions were prepared in 0.1 mM EDTA (pH 7.5). The gradients were centrifuged at 21,000 rpm for 3 hr in a Beckman model L2-65B centrifuge with a Spinco model SW 27-1 rotor maintained at 0 C. Separation into serial 0.4-ml fractions and recording of the A_{280} were accomplished with an ISCO density gradient fractionator, model 640. Sucrose content of the fractions was determined refractometrically.

Enzyme Assays. All enzyme assays were carried out spectrophotometrically at room temperature except for RuP_2 carboxylase, which was measured radiochemically using $NaH^{14}CO_3$ at 30 C. The methods employed were those described in the literature as follows: catalase (7), fumarase (15), isocitrate lyase (1), RuP_2 carboxylase (11).

Oxygen Consumption. The reaction mixture contained the following components: Tricine-KOH (pH 7), 50 μmol; mannitol, 700 μmol; and protoplasts ($1.0–1.5 \times 10^4$) in 1 ml. The O_2 consumption was measured with a Rank oxygen electrode at 25 C.

Cell counting was done using a hemocytometer.

RESULTS AND DISCUSSION

Preparation of Protoplasts. The preparation, as described, from 4-day-old castor bean enzyme is shown in Figure 1. Protoplasts from 3- to 7-day-old castor bean endosperm can be prepared by the same method, but protoplasts from dry seed and 1- to 2-day-old endosperm are much more labile and cannot be prepared in this way. Table I shows the effect of vacuum infiltration and time of incubation on the preparation of protoplasts from 4-day-old endosperm. It is clear that the yield of protoplasts was maximal at 30-sec vacuum infiltration and that the bulk of protoplasts were

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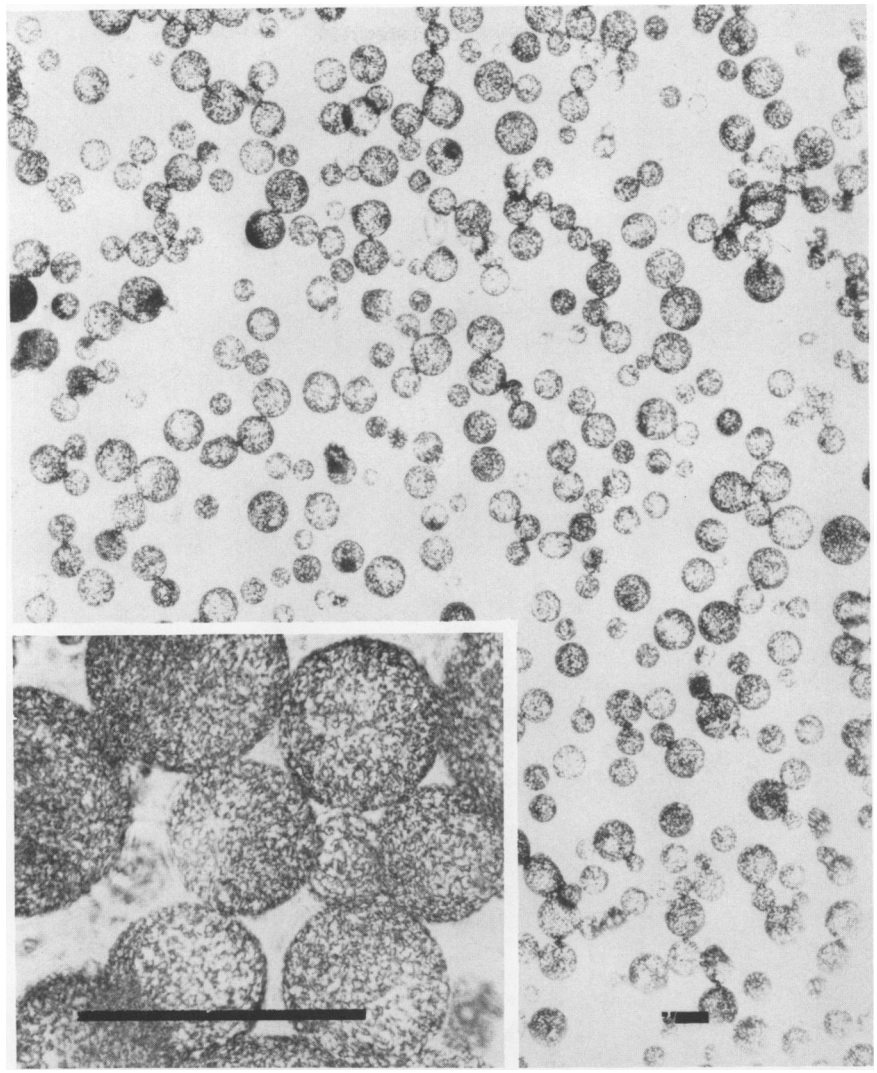

FIG. 1. Photomicrographs of protoplasts from 4-day-old castor bean endosperm. Bars are 100 μm.

Table I. The effect of time of incubation and vacuum infiltration on recovery of protoplasts

Following vacuum infiltration for the periods shown, 7 g samples of endosperm slices were incubated with enzyme solution. Fresh enzyme solution was used for each successive period of treatment.

	Vacuum infiltration		
Enzyme treatment	60 sec	30 sec	0
	Number of protoplasts recovered x 10^{-4}		
1 (1 hr)	0	0	0
2 (1-3 hr)	40	45	12
3 (3-5 hr)	27	34	21
4 (5-7 hr)	12	16	18
5 (7-9 hr)	4	9	14

obtained in the period 1 to 5 hr. From the relationship between catalase activity in the isolated protoplasts and that in the original endosperm tissue, it was calculated that 3% of the cells were recovered as protoplasts. The protoplast suspended in 0.7 M mannitol showed a stable O_2 uptake (18 μmol O_2/hr · 10^7 protoplasts) for at least 6 hr. This value is similar to that observed with *Acer pseudoplatanus* cells from tissue culture (13 μmol O_2/hr · 10^7 cells) (4) and higher than that with pea root cells *in vivo* (4.8 μmol O_2/hr · 10^7 cells) (16).

Disruption of Protoplasts. The protoplasts from 4-day-old castor bean endosperm were broken by a gentle procedure of slow extrusion through a hypodermic needle following Nishimura *et al.* (10). Castor bean protoplasts were more fragile than spinach protoplasts since only a single passage through the syringe caused complete destruction (Fig. 2), whereas three passes were necessary for spinach protoplasts (10). The homogenate was directly applied to the sucrose density gradient.

Enzyme Activities in the Sucrose Gradient. Figure 3 shows the enzyme profile of the broken protoplast preparations after sucrose density gradient centrifugation. The prominent protein peak (d = 1.18) coincides exactly with fumarase activity, the marker enzyme of mitochondria. Only 3% of the total fumarase activity was detected in the supernatant fraction. RuP_2 carboxylase is recognized as a marker enzyme of plastids in castor bean endosperm (14, 17). This enzyme activity was clearly present in a fraction with peak density 1.22 and was barely detectable in the supernatant fraction. This indicates that the proplastids are recovered intact by this method. Surprisingly, the marker enzyme activities of glyoxysomes, catalase, and isocitrate lyase are localized in a fraction at peak density of 1.13, with 30 to 40% of these enzyme activities present in the supernatant fraction. In the standard procedure (3, 6, 9, 14, 17), the glyoxysomes are recovered in sucrose gradients at a mean density of 1.25 g/ml, partially overlapping the unbroken plastids. Thus, for the preparation of plastids by the method described above, it is a fortunate chance that the glyoxysomes show this anomalous behavior. The explanation appears to lie in the fact that when protoplasts in homogenizing medium are disrupted by passing them through a needle, the glyoxysomes remain or become associated with other membranes

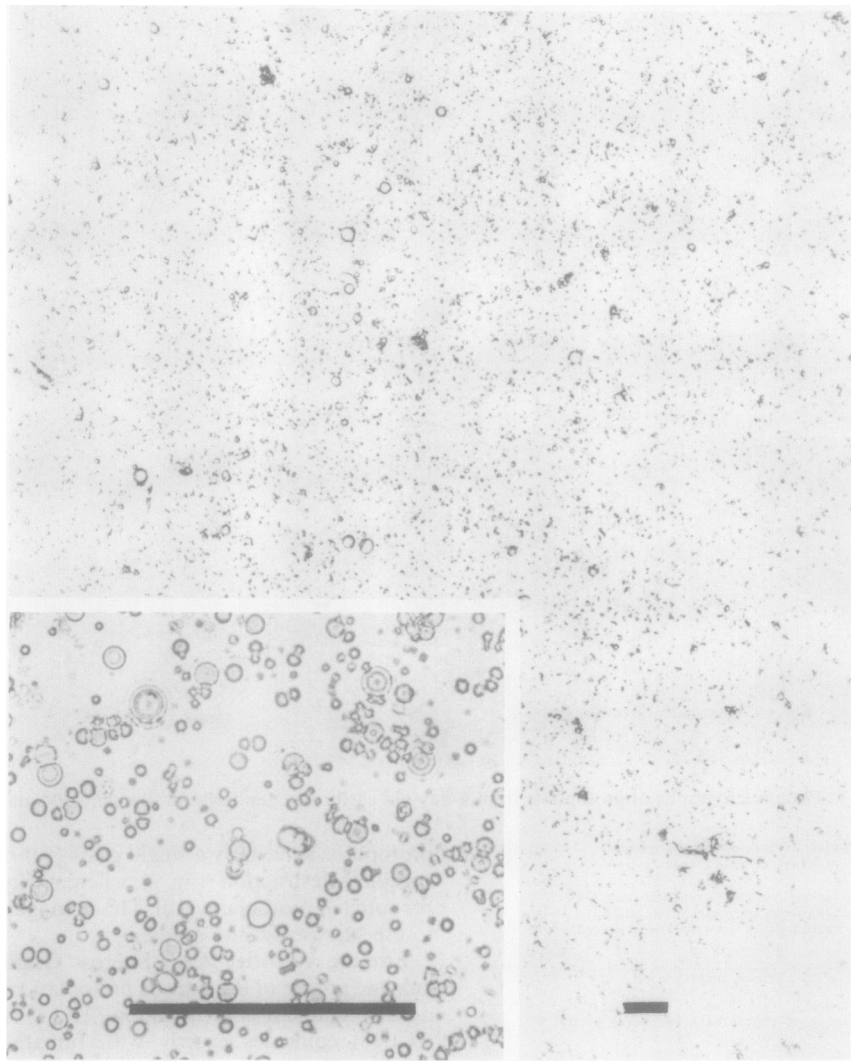

FIG. 2. Photomicrographs of ruptured protoplasts. Bars are 100 μm.

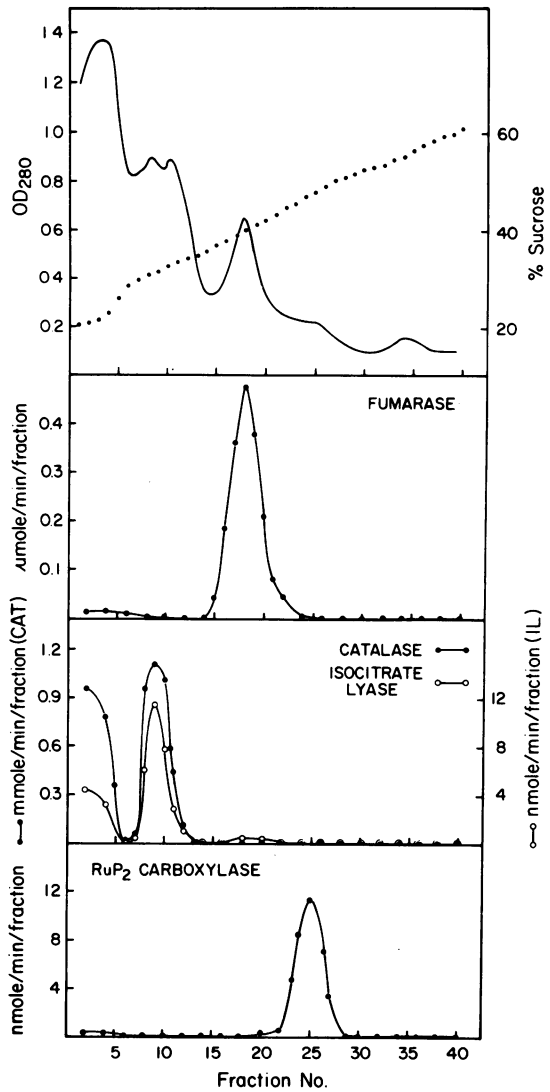

FIG. 3. Localization of enzyme activities in the separated fractions (0.4 ml) after sucrose density gradient centrifugation of mechanically ruptured protoplasts.

or organelles and are recovered high in the gradient even after 3 hr. When the gradient fractions were analyzed for acid lipase, which is a constituent of the spherosome membrane (9, 13), it was found that the fractions containing intact glyoxysomes also contained this enzyme. In the standard gradients obtained after razor blade chopping virtually none of this lipase appears in the gradient. In support of this explanation it was found that when the protoplasts were disrupted more vigorously by repeated passage through the needle, glyoxysomes were then recovered at their typical density of 1.25, although 70% glyoxysomes were broken by this treatment.

For the present, the important outcome is that we now have a method for the preparation of intact plastids uncontaminated with other organelles and these can be used for more precise work on enzyme localization than has been possible previously (5, 8).

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