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Isolation and Characterization of Organelles from Soybean Suspension Cultures[1]

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ABSTRACT

Whole homogenates from cells of *Glycine max* grown in suspension culture were centrifuged on linear sucrose gradients. Assays for marker enzymes showed that distinct peaks enriched in particular organelles were separated as follows: endoplasmic reticulum (density 1.10 g/cm³, NADH-cytochrome-c reductase), Golgi membranes (density 1.12 g/cm³, inosine diphosphatase), mitochondria (density 1.18–1.19 g/cm³, fumarase, cytochrome oxidase) and microbodies (density 1.21–1.23 g/cm³, catalase). In cells which had ceased to grow (stationary phase) only a single symmetrical catalase peak at density 1.23 g/cm³ was observed on the sucrose gradient. During the phase of cell division and expansion a minor particulate catalase component of lighter density was present; its possible significance is discussed.

Plant organs used for the isolation of organelles usually comprise a variety of differentiated tissues, and the cells are at different developmental stages. By contrast, cells growing in cell suspension culture under controlled conditions are considerably more uniform, and a suitable sampling sequence can provide a population of cells in which division, enlargement, or maintenance in a stationary phase is the predominant condition. It appeared to us that such cells might offer some advantages as starting material for the isolation of organelles and we hoped that they would yield to some gentle method of cell disruption which would allow the recovery of a more representative set of organelles than is possible when a differentiated organ is mechanically disintegrated. Out of a variety of disruption procedures which were tested, grinding in a mortar proved to be the only effective way of breaking the cells from suspension culture. This report describes the methods by which various organelles were separated and identified in such extracts and deals particularly with changes in the microbody fraction during the culture period.

MATERIALS AND METHODS

Culture Methods. *Glycine max* L. Merrill var. Acme was cultured as described previously (12), except that, for the experiments on organelle development, a larger inoculum (5.0 ml) was used for starting the cultures. This allows for the recovery of more cells at the early stages of growth and also reduces the lag period to approximately 2 days.

Fresh and dry weights were determined as described previously (12). Cell numbers were estimated on a haemocytometer following dissociation in 5% chromic acid (7). For isolation of organelles, the cells from several flasks were collected by filtration onto Miracloth and washed with glass-distilled water.

A variety of treatments was tried to achieve cell breakage. Initial attempts were made gently to break open the cells by passing them through a syringe needle after digestion of the cell wall by wall-degrading enzymes (Onozuka cellulase and Macerozyme, All Japan Biochemicals Co., Ltd.). This technique led to low yields of organelles although those obtained were largely intact. The use of a French pressure cell (American Instrument Co.) gave an increasing yield of protein with increasing pressure up to about 70% released at 2000 lb/in². At all pressures used, however, a high percentage of organelle disruption occurred. The method finally adopted was as follows. A weighed amount of cells was chilled on an icebath and then ground with a mortar and pestle for 3 min in an equal volume of grinding medium. The grinding medium contained 0.63 M sucrose, 0.15 M Tricine buffer (pH 7.5), 1 mM EDTA (pH 7.5), 10 mM KCl, and 1 mM $MgCl_2$. The grinding medium for the organelle development studies contained 0.5 M sucrose. Inclusion of dithiothreitol did not improve the yield of enzyme or alter the gradient profiles.

Density Gradient Separation. For the isolation of the total organelle population, the sample was filtered through Miracloth, centrifuged at 270*g*, and then layered directly onto a sucrose gradient. The gradient consisted of (*a*) a 5-ml cushion of 60% (w/w) sucrose, (*b*) a 20-ml linear gradient from 16 to 60% (w/w) sucrose, and (*c*) 10 ml of sample in a 37.5-ml centrifuge tube. All sucrose solutions contained 1 mM EDTA (pH 7.5). The gradients were centrifuged at 20,000 rpm for 4 hr in a Beckman L2-65B preparative ultracentrifuge with a Spinco SW 27 rotor, following which 0.6-ml fractions were collected.

For the developmental studies, the homogenate was passed through Miracloth, centrifuged for 5 min at 270*g*, and the supernatant solution was recentrifuged for 15 min at 10,000*g*. The resulting crude particulate pellet was gently resuspended in a modified grinding medium containing 0.8 M sucrose and 2.5% Ficoll in addition to the other components. Two ml of this suspension were layered on top of a concave gradient of sucrose, and the tubes were centrifuged as above in a Spinco SW 25.2 rotor, following which 1.2-ml fractions were collected. The 40-ml gradients were prepared over a 10-ml cushion of 60% sucrose with an ISCO Dialagrad Model 380 gradient pump using a program in which the flow rates of 1 mM EDTA (pH 7.5), and of 60% (w/w) sucrose (in 1 mM EDTA, pH 7.5),

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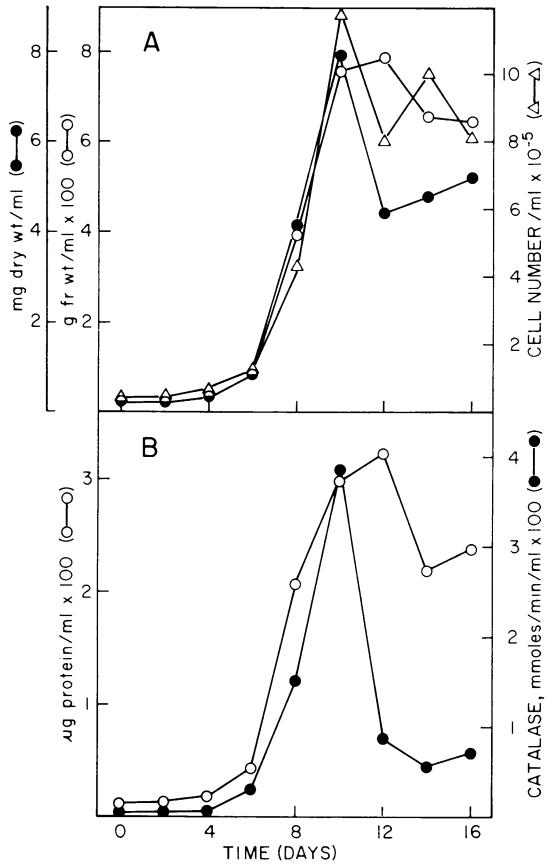

FIG. 1. Growth characteristics of soybean cells in suspension culture. Cultures were grown at 27 C in 250-ml flasks containing 100 ml of medium (12) and shaken at 150 rpm on a gyrorotary shaker. A: Dry weight, ●; fresh weight, ○; cell number, △; B: extractable protein, ○; catalase, ●.

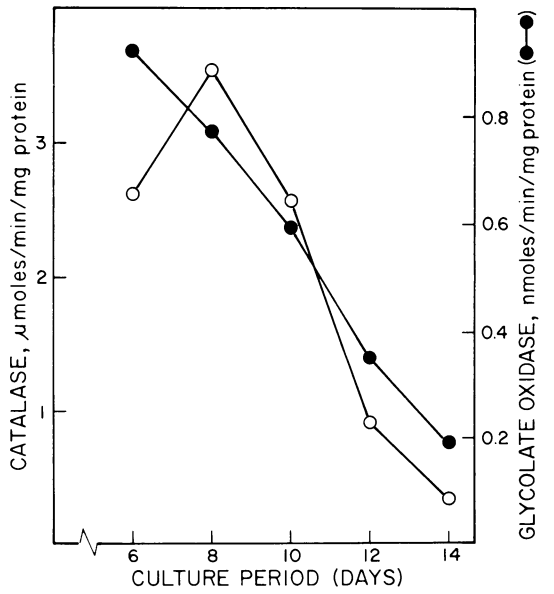

FIG. 2. Changes in the specific activities of two microbody enzymes over the culture period of the cells: catalase, ○; glycolate oxidase, ●.

set at the 11 dials were: 0, 8.5, 16.5, 23.5, 29.5, 34.5, 39, 43.5, 48, 52.5, 56.5 (4).

Preparation of Cell-free Extracts. One or 2 g of tissue were placed with a small portion of sand into a conical glass tissue grinder. Two ml of grinding medium without sucrose but containing 0.01% Triton X-100 were added. The tissue was ground until enzyme extraction was complete, incubated for 30 min at 4 C, and debris removed by centrifugation at 10,000*g*.

Analytical Methods. All enzymes were assayed spectrophotometrically, as described: catalase, cytochrome oxidase, and glycolate oxidase (3), hydroxypyruvate reductase (17), NADH-Cyt *c* reductase (15), and inosine diphosphatase (14).

Protein was determined by a modification of the Lowry procedure (11). Sucrose concentrations were determined refractometrically.

RESULTS

GROWTH OF CULTURES

Figure 1A shows the changes in fresh and dry weights and in numbers of cells during growth of the cultures used for all of the present experiments except for those in Figure 5, in which a heavier inoculum was used. These features have been described in detail in an earlier paper (12). The protein content of the cultures (Fig. 1B) rises sharply between days 6 and 10 and follows the changes in cell weight fairly closely. Catalase, a marker enzyme for microbodies, reaches its maximum activity as the cultures approach stationary phase (Fig. 1B), but subsequently a rapid fall occurs which is much more severe than that of total protein. Figure 2 shows that during the 11- to 14-day period the fall in specific activity of catalase is closely paralleled by that of glycolate oxidase, another microbody enzyme, suggesting that a specific destruction of these organelles occurs at this time.

CHARACTERIZATION OF ORGANELLES

The results shown in Figure 3 were obtained when the crude homogenate from 8-day-old cells was centrifuged on the linear sucrose gradient. The sucrose concentration of the successive fractions and the protein profile across the gradient are shown in Figure 3E. The protein (and enzyme activities) in fractions 1 to 18 represents truly soluble material and that solubilized from organelles during extraction; the major protein peak within the gradient is the mitochondrial band at a mean density of 1.18 g/cm^3.

NADH-Cyt-c Reductase (Fig. 3A). This enzyme is present in the soluble fraction but within the gradient two major peaks of activity can be distinguished. The smaller peak at density 1.18 g/cm^3 coincides with the mitochondrial band and its marker fumarase (Fig. 3C). The major peak, at density 1.10 g/cm^3 occurs slightly higher in the gradient than the enzyme from castor bean endosperm, which was shown recently to be strictly associated with the endoplasmic reticulum (10). We conclude by analogy that membranes derived from the endoplasmic reticulum of the soybean cells were present in this fraction.

Inosine Diphosphatase (Fig. 3B). The sharp single peak of activity within the gradient occurred at density 1.12 g/cm^3, clearly separated from the other enzyme activities. In previous work by others (2, 8, 14), inosine diphosphatase has been shown to be a marker enzyme for Golgi membranes. The equilibrium density of this fraction from the soybean cells is the same as that observed by Morré from onion root tips (13) but somewhat lower than that isolated from pea stems by Ray (14).

Fumarase (Fig. 3C). This was present only in the major protein band at density 1.18 g/cm^3. The fact that this mitochondrial marker was not present in the fractions at the top of the gradient indicates that the mitochondria were relatively undamaged.

Catalase (*Fig. 3D*). This enzyme, the classical microbody marker, also showed a single major peak, at density 1.22 g/cm³.

It therefore appears that on a single linear gradient, fractions greatly enriched in endoplasmic reticulum, Golgi membranes, mitochondria, and microbodies can be separated.

ORGANELLES IN THE CRUDE PARTICULATE FRACTION

Separation from Cells in Stationary Phase. The clearest separation of organelles present in the crude particulate fraction (10,000*g* pellet) was obtained from cultures in which cell division and enlargement had ceased (stationary phase). Cells from a 13-day-old culture gave the results shown in Figure 4. The distribution of protein and the slightly concave gradient are shown in Figure 4E. As shown by the distribution of fumarase and catalase, both the mitochondria and microbodies were recovered in symmetrical peaks, with respective densities of 1.18 g/cm³ and 1.23 g/cm³ and virtually no overlap. The distribution of glycolate oxidase (Fig. 4B) and hydroxypyruvate reductase (Fig. 4C), two other enzymes characteristic of microbodies (16) coincided precisely with that of catalase, and virtually no activity of any of these three enzymes was present elsewhere in the gradient. Thus a very satisfactory separation of intact mitochondria and microbodies was achieved. The distribution of triosephosphate isomerase, an enzyme present in proplastids, is shown in Figure 4D. The profile is rather irregular, and much of the activity was present at the top of the gradient, but a peak is recognizable at density 1.24 g/cm³. It seems that, in contrast to the other organelles, the proplastids are not recovered intact by this procedure. In younger cells, such as those used in the separations shown in Figure 3, no peak of triosephosphate isomerase was found within the gradient; all of the enzyme activity was present in the soluble fraction. It is possible that the presence of starch in the proplastids of younger cells makes

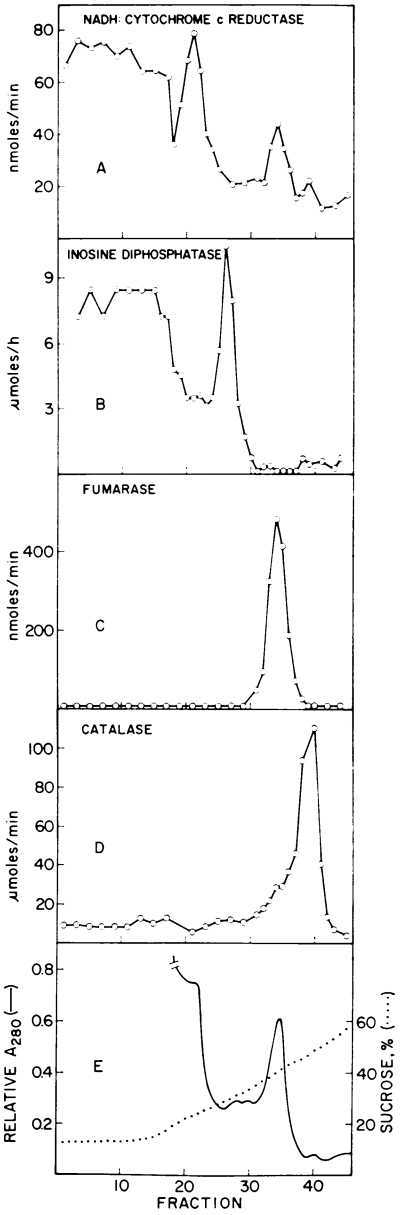

FIG. 3. Results of centrifuging a total cell extract on a sucrose gradient. The supernatant from a 270*g* centrifugation (10 ml) was layered over a linear 16 to 60% (w/w) gradient and centrifuged 4 hr at 20,000 rpm. The gradient was fractionated into 0.6-ml portions, and the indicated enzymes were assayed in each fraction.

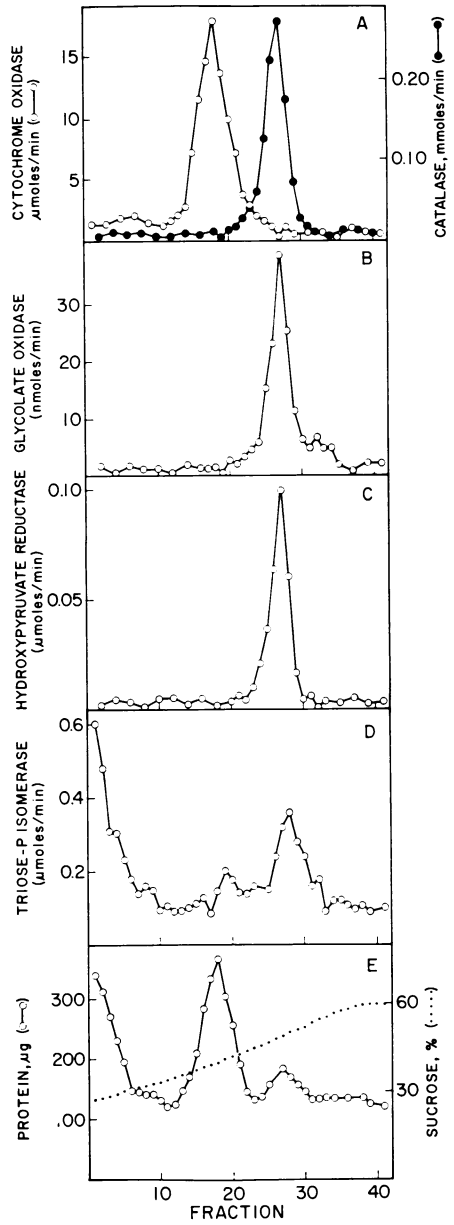

FIG. 4. Results of centrifuging a resuspended 10,000*g* pellet on a concave gradient. Details are described under "Materials and Methods."

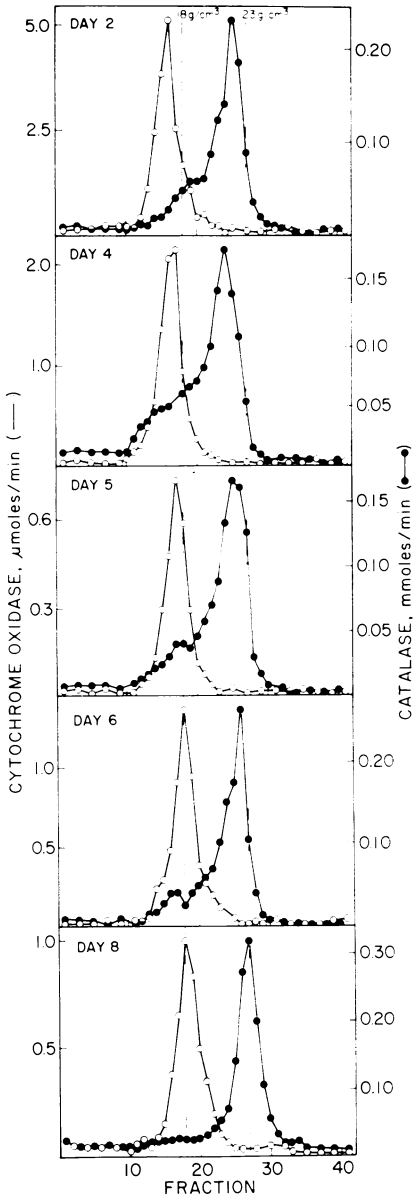

FIG. 5. Changes in the sedimentation behavior of the mitochondria (cytochrome oxidase) and microbodies (catalase) with increasing age of culture. The suspension cultures for the experiments in this figure were started with a heavier inoculum than those used for Figs. 1, 2, and 3, with the result that the lag phase was reduced by about 2 days (see "Materials and Methods").

their isolation more difficult (B. J. Miflin, personal communication), but it seems that in general these organelles are more fragile than the others which have been clearly separated.

Separation of Mitochondria and Microbodies from Cells of Different Ages. For these experiments, as indicated under "Materials and Methods," a heavier inoculum was used, and as a result the lag period was reduced from 4 to 2 days. Crude particulate fractions were prepared from cells harvested at 2, 4, 5, 6, and 8 days, and the resuspended pellets were centrifuged on the sucrose gradient as described. Assays for cytochrome oxidase and catalase in the successive fractions recovered from the gradient gave the results shown in Figure 5. By day 8 the cells under these cultural conditions were entering stationary phase, and the distribution of the enzymes marking the mitochondria and microbodies was identical to that observed earlier in the 13-day cells shown in Figure 4A; the mean densities were 1.18 g/cm^3 and 1.23 g/cm^3.

At earlier periods, however, the distributions were notably different. First, the mean density of mitochondria from the younger cells was lower; it increased progressively from 1.17 g/cm^3 at day 2 to the value typical of mature cells (1.18 g/cm^3) by day 6. A similar shift, from 1.21 g/cm^3 to 1.23 g/cm^3 occurred during growth in the density of the main microbody peak. In addition, however, it was clear that not all of the catalase was recovered in the main band from younger cells. At day 2, the end of the lag phase, there was a minor broad secondary peak at density 1.18 g/cm^3 to 1.19 g/cm^3. By day 4, when the number of cells was increasing most rapidly, this secondary peak was more noticeable, spreading broadly from density 1.16 g/cm^3 to 1.19 g/cm^3. Subsequently as the phase of cell division was completed, and the density of the main microbody peak approached its final value of 1.23 g/cm^3, the secondary peak declined and had disappeared entirely by day 8 (Fig. 5). When organelles from tissue of the same age as that in Figure 5, day 4, are separated by the whole homogenate method (see "Materials and Methods"), the catalase profile is similar to that shown in Figure 5. Further, the distribution of glycolate oxidase is close to that of catalase (not shown).

DISCUSSION

The preparative procedure adopted after preliminary experiments was a fairly standard one in which a crude total homogenate of cells was obtained by grinding in a buffered sucrose solution in a mortar and removing the debris by centrifuging at 270*g*. When such a total homogenate from cells approaching stationary phase was centrifuged to equilibrium on a linear sucrose gradient, clear peaks of enzyme activities corresponding to endoplasmic reticulum, Golgi, mitochondria, and microbodies were obtained. The enzyme activities in the nonparticulate fractions at the top of the gradient showed that essentially all of the mitochondria and microbodies had been recovered intact. Some leakage of marker enzymes from the endoplasmic reticulum and the Golgi fractions may have occurred, but the soluble activity might also be due to other nonspecific enzymes. Proplastids were not recovered successfully in such gradients.

The isolated microbodies have a limited enzyme composition and the relatively low glycolate oxidase activity is typical of microbodies of undefined function (9). The sedimentation behavior of microbodies changed significantly during the growth period. In cells which had reached stationary phase the equilibrium density was 1.23 g/cm^3 and the distribution of catalase, the marker enzyme, was quite symmetrical. In younger cells, the major peak of catalase was at a distinctly lower density, 1.21 g/cm^3. The other striking feature of the catalase distribution from these cells (Fig. 5) is the leading shoulder or peak higher in the gradient. As shown, this portion of the catalase activity is most noticeable during the phase of rapid cell growth and diminishes in a regular fashion during aging.

The behavior of the lighter catalase component may be accounted for in several ways. Possibly, it is a trapping artifact, or derives from microbodies broken during extraction or separation on the gradient. Although these possibilities cannot be ruled out, they are difficult to reconcile with the observations that this component is not seen in organelle separations from older cells and that no soluble catalase is observed, even on gradients from whole homogenates, when the secondary catalase peak is most evident. An alternative possibility, which requires further investigation, is that the lighter catalase component seen in growing cells represents unfinished or incomplete microbodies which have not yet acquired their final protein content. Such an interpretation was suggested for the

increasing density of microbodies during the development of wheat leaves (4); a similar increase in density of microbodies has been described in developing Arum spadix (1) and may be related to the changes seen in microbody structure in electron-micrographs of developing bean leaves (6).

The development of catalase and glycolate oxidase is not coordinately regulated, but upon entering stationary phase both decline in parallel (Fig. 2). Since this destruction is not accompanied by the appearance of soluble catalase, or of particulate catalase at a lighter density, it appears that, as in the castor bean endosperm (5), the microbodies are selectively destroyed as wholes at this time.

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