Isolation of Intact Chloroplasts and Other Cell Organelles from Spinach Leaf Protoplasts

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ABSTRACT
Freshly prepared spinach leaf protoplasts were gently ruptured by mechanical shearing followed by sucrose density gradient centrifugation to separate constituent cell organelles. The isolation of intact Class I chloroplasts (d = 1.21) in high yield, well separated from peroxisomes and mitochondria, was evidenced by the specific localization of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39), NADP triose-P dehydrogenase (EC 1.2.1.9), and carbonic anhydrase (EC 4.2.1.1) in the fractions. A clear separation of chloroplastic ribosomes from the soluble cytoplasmic ribosomes was also demonstrated by the band patterns of constituent RNA species in the polyacrylamide gel electrophoresis. Localization of several enzyme activities specific to leaf peroxisomes, e.g. catalase (EC 1.11.1.6), glycolate oxidase (EC 1.1.3.1), glyoxylate reductase (EC 1.1.1.26), glutamate glyoxylate aminotransferase (EC 2.6.1.4), serine glyoxylate aminotransferase, and alanine glyoxylate aminotransferase (EC 2.6.1.12) in the peroxisomal fractions (d = 1.25), was demonstrated. Overall results show the feasibility of the method for the isolation of pure organelle components in leaf tissues.

A number of recent investigations have been increasingly showing the potential usefulness of plant protoplasts for physiological as well as biochemical research (25). In previous papers (20, 21) we have reported the value of spinach leaf protoplasts for studying the mechanism of photosynthesis and photorespiration, the biggest advantage being the manipulation of homogeneous “naked leaf cell” preparations for such studies. We have also emphasized that the maintenance of interorganellar relationships in protoplasts is useful for the analysis of the cellular compartmentation mechanism in photorespiration which is believed to proceed in multiganelar fashion (28). As a next step, the separation of individual cell organelles having sufficiently high activities of enzyme reactions operating in photorespiration and the subsequent reassembling are crucial to understanding the overall processes. However, both structural complexity and fragility of organelles in leaf cells pose an obstacle for this task, and breakage of structural organization of organelles during isolation procedures is frequently encountered. Despite intensive trials, the isolation of pure chloroplast fractions retaining both structural and functional integrity has reached only a limited success (31). A clear separation of pure leaf peroxisomes free from other components has barely been accomplished (29). Since cell wall materials are enzymically degraded during the preparation of protoplasts, it is obvious that gentle breakage of the latter surrounded only by the plasmalemma is ideal for isolating the constituent organelles. In the work reported in this communication we have undertaken the isolation of intact chloroplasts and other organelles from spinach leaf protoplasts in an attempt to investigate the nature of the interorganellar relationship of photorespiration.

MATERIALS AND METHODS
Protoplasts. Protoplasts were prepared from freshly harvested spinach leaves (Spinacia oleracea L. var. Kyoho) essentially following the method reported previously (20). The only modification employed was that the concentration of mannitol was lowered to 0.7 M. The final preparations were shown to have photosynthetic activities of 35 to 70 μmol CO₂ fixation/mg Chl hr. The protoplasts (approximately 4 mg Chl) suspended in 2 ml of 0.05 M Tricine-NaOH buffer (pH 7.5) containing 0.5 M sucrose and 0.1% BSA were then ruptured through a syringe (0.5 × 4 cm, Terumo). A small piece of Miracloth (Calbiochem) was placed in the bottom of the needle (0.7 × 32 mm), and after each stroke samples released from the needle were examined by light microscope. Usually protoplasts were completely broken by three strokes (cf. Fig. 1).

Sucrose Density Gradient Centrifugation. The ruptured protoplast preparations (0.5 ml) were directly layered on top of 15 ml of the linear sucrose gradient (35–60%, w/w) dissolved in 0.02 M Tricine-NaOH buffer (pH 7.5) and run at 24,000 rpm for 3 hr at 4 C using a Beckman-Spinco SW 25-3 rotor. At the end of the run, 0.5-ml fractions were collected and aliquots were used for measuring the enzymic activities as well as the content of Chl, protein, RNA, and DNA (see below).

Enzyme Assays. (a) RuDP⁴ carboxylase (EC 4.1.1.39) activity was measured as described by Nishimura et al. (22). (b) NADP triose-P (EC 1.2.1.9) and NAD triose-P dehydrogenases (EC 1.2.1.12) activities were determined by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH or NADH by 3-PGA following the method of Heber et al. (10). (c) Carbonic anhydrase (EC 4.2.1.1) activity was assayed as previously described by Atkins et al. (4) using veronal-pH indicator buffer at 0 C and pH 8.2. (d) Catalase (EC 1.11.1.6) activity was assayed by measuring the disappearance of H₂O₂ spectrophotometrically at 240 nm following the method of Luck (18). (e) Glycolate oxidase (EC 1.3.1.31) assay was carried out

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2 Abbreviations: RuDP: ribulose 1,5-bisphosphate; DCPiP: 2,6-di-chlorophenolindophenol; 3-PGA: 3-P-glyceric acid.
anaerobically by measuring the reduction of DCPIP at 600 nm following the method of Tolbert et al. (30). \(f\) NADH-glyoxylate reductase (EC 1.1.1.26) activity was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH by glyoxylate following the method of Zelitch and Gotto (33). \(g\) Cyt c oxidase (EC 1.9.3.1) activity was determined by measuring the decrease of reduced Cyt c at 550 nm according to the method of Smith (26). \(h\) NAD-isocitrate dehydrogenase (EC 1.1.1.41) activity was assayed by measuring the increase of absorbance at 340 nm of NADH by isocitrate following the method of Cox (6). \(i\) NAD-malate dehydrogenase (EC 1.1.1.37) activity was measured as described by Asahi and Nishimura (3). \(j\) Glutamate glyoxylate aminotransferase (EC 2.6.1.4), serine glyoxylate aminotransferase, alanine glyoxylate aminotransferase (EC 2.6.1.12), and aspartate \(\alpha\)-ketoglutarate aminotransferase (EC 2.6.1.11) activities were determined following the procedures described by Reinfeld and Tolbert (23).

Except in the case of carbonic anhydrase, enzyme assays were carried out at 25 C for appropriate reaction periods, and activities are expressed as \(\mu\)mol substrates utilized or products formed/min·tube (0.5 ml) (cf. Figs. 3 and 4).

**Analytical Methods.** Chl content was determined according to the method of Arnon (2). Protein content was determined by the spectrophotometric method of Lowry et al. (17) using BSA as a standard.

The content of RNA and DNA in the fractionated samples was determined according to the method of Fleck and Munro (7). Each fraction (0.5 ml) was made to 5 ml with distilled H2O and 2.5 ml of cold 2.1 N HClO₄ were added. After 15 min the precipitate collected by centrifugation was washed twice with 0.1 ml of 0.7 N HClO₄. The precipitate was digested in an incubator at 37 C using 2 ml of 0.3 N KOH for 1 hr. At the end of incubation, the samples were neutralized with 10 N HClO₄ and acidified with 1 volume of 1 N HClO₄. The precipitate (DNA fraction) separated by centrifugation was washed twice with 1 ml of cold 0.5 N HClO₄. The combined supernatant fluid and washings (RNA fraction) were subjected to absorbance measurement at 260 nm and 275 nm. The RNA content was estimated by:

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\text{RNA (\mu g/ml) = 11.87 A}_{260} - A_{275} \times 10000
\]

The DNA fraction (precipitate) which was dissolved in 0.1 ml of 1 N KOH was assayed for DNA content by the diphenylamine method.

**Polyacrylamide Gel Electrophoresis.** Each of the chloroplast (No. 14 to 18 in Figure 5a) and the supernatant fractions (No. 27 to 31) were mixed with 1 volume of 90% phenol and stirred vigorously for 15 min at room temperature. Afterward the samples were thoroughly dialyzed against phenol-saturated water to remove sucrose. Then the samples were centrifuged and the water phase fraction was carefully collected and 2 volumes of ethanol were added. The precipitate formed was centrifuged and washed twice with 4 ml of 70% ethanol. The precipitate was dissolved in 100 \(\mu\)l of electrophoretic buffer and applied to the polyacrylamide gel electrophoresis following the method of Loening and Ingle (16). The whole leaf RNA fraction was extracted from spinach leaves by 50% phenol and treated in the same way. After electrophoresis (5 ramp/tube, 2 hr), the gel was stained with methylene blue. A Joyce Loebbe Chromo-Scan was used to obtain the densitometric tracings of the gels stained with dye.

**RESULTS**

**Isolation of Intact Chloroplasts.** The primary purpose of the present investigation was to isolate preparations of intact chloroplast after breakage of protoplasts. Several procedures for breakage were examined such as osmotic shock and mechanical breakage by Teflon homogenizer or passage through a syringe. The gentle mechanical shearing using a syringe was found to be the most satisfactory. Figure 1 shows the release of a homogeneous population of chloroplasts examined by light microscope after the syringe treatment. A single passage is compared with a triple passage preparation. By phase contrast microscopy the chloroplasts appeared brightly refractive, with a halo around the outer margin of the particles (27). A clear separation of a single band of chloroplasts (d = 1.21), practically free from striped chloroplasts, was achieved by sucrose density gradient centrifugation (Fig. 2), a nearly complete recovery of chloroplast fractions was achieved by this procedure (93% recovery from starting protoplasts on Chl basis).

**Cellular Localization of Enzyme Activities in Chloroplasts and Other Organelles.** A satisfactory resolution of chloroplasts and other constituent organelles can be supported from experimental results examining the enzyme localization in separated fractions. Figure 3 shows a sedimentation profile of the broken protoplasts preparations after sucrose density gradient centrifugation. The prominent peaks of chloroplastic marker enzyme exactly coincide with the peak of Chl (d = 1.21) (Fig. 3, a and b). Some activities of RuDP carboxylase and NADP triose-P dehydrogenase are detectable in the soluble supernatant fraction, but they comprise only a small proportion of the total. Sizable activities of carbonic anhydrase were found to be clearly associated with chloroplasts, but much activity remained in the top, soluble fractions. Presumably enzyme activities in each fraction represent the chloroplastic and cytoplasmic carbonic anhydrase (8), but that in the soluble fraction is so high that contamination by enzyme leached out, or from the surface, of chloroplasts must be considered.

Marker enzyme activities of peroxisomes (28, 30, 32), i.e. catalase, glycolate oxidase, and glyoxylate reductase, are clearly present in peroxisome fractions (d = 1.25), although much of these activities (approximately 70%) are also present in the supernatant fractions (Fig. 3c). This is probably due to the fragility of the single membranous organelles and leaching of the enzyme during the separation procedure. Tolbert reported (29) that leaf peroxisomes comprise 1 to 1.5% of the total protein, and the present results show that the content of peroxisomes recovered is approximately 3% on a protein basis (Fig. 3a). Employing the osmotic treatment of protoplasts followed by sucrose density gradient centrifugation, both glycolate oxidase and glyoxylate reductase activities were almost exclusively located in the top, soluble fractions of the centrifuged samples, although chloroplasts and mitochondria were fairly well preserved.

While not much data are available regarding the separation of mitochondria from other organelles in leaf tissues, reported results from several laboratories are somewhat conflicting (11, 23, 24, 32). We have consistently observed that when the protoplast preparations suspended in sucrose solution without BSA were subjected to mechanical breakage followed by gradient centrifugation, the mitochondrial fractions (d = 1.22) overlapped with chloroplasts (d = 1.21), probably because of the absorption of solute (sucrose) through the outer membranes of mitochondria (T. Asahi, personal communication). Activity of Cyt c oxidase was found in the leading edge fractions of chloroplasts (data not shown). These results are judged to be basically similar to those of Rocha and Ting (24) and Huang and Beever (11); in such studies the density of mitochondria (d = 1.21) was shown to be larger than that of stripped chloroplasts (d = 1.17). On the other hand, we have found that inclusion of BSA in the suspending media exhibits a preservative effect on mitochondria, thereby the density of mitochondria becoming distinctly smaller than that of the chloroplasts. Figure 3d shows the specific localization of both Cyt c oxidase and NAD-isocitrate dehydrogenase in the fractions of d = 1.18. Figure 3e shows that activities of
NAD-malate dehydrogenase are detected in peroxisomes, mitochondria, and chloroplasts. In agreement with results of previous investigators (28, 32), the specific activity is highest in peroxisomes, but it must be pointed out that the enzyme activity is clearly detected in the chloroplastic fractions.

Activities of four aminotransferases which are reported to be operative in the glycolate pathway were assayed in the separated fractions and results are shown in Figure 4. In accordance with the results of Tolbert and his associates (23, 28, 32), a tight association of three aminotransferases, i.e. glutamate glyoxylate (Fig. 4a), serine glyoxylate (Fig. 4b), and alanine glyoxylate aminotransferases (Fig. 4c), with peroxisomes was clearly demonstrated. However, for each of these enzymes some activity was detected in the soluble supernatant fractions and small activities in other particulate fractions as well. Activity of aspartate a-ketoglutarate aminotransferase was detected in all three organelles: chloroplasts, peroxisomes, and mitochondria (Fig. 4d), presumably representing each individual isozyme (23, 32).

Isolation of Ribosomal RNA. An additional proof for the isolation of intact chloroplasts can be seen from the separation of chloroplastic and cytoplasmic ribosomal RNA. The profile of RNA analysis in the separated fractions is given in Figure 5a. In order to characterize the RNA components of the chloroplast and soluble cytoplasmic fractions, RNA species were resolved by polyacrylamide gel electrophoresis according to the method of Loening and Ingle (16). The stained electrophoresed gel samples

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**Fig. 1.** Photomicrographs of gently ruptured protoplasts. a and b show the spinach protoplasts ruptured by gentle shearing through syringe once and three times, respectively. In a arrows indicate unbroken protoplasts. Experimental procedures are described in the text.
trifugation of gently homogenized leaf cells using isotonic sorbitol solution (12, 31). It is inevitable that all such preparations are mixed populations of various organelles. It is unavoidable that these methods cause some structural damage of chloroplasts and other organelles during the step of breaking the leaf tissues. The use of leaf protoplasts as a starting material is different in principle from the previous commonly used methods, and the present investigation clearly shows that the gentle breakage of naked leaf cells (protoplasts) followed by sucrose density centrifugation is advantageous for separating three major organelles, namely chloroplasts, mitochondria, and peroxisomes, in pure form. The specific localization of some enzyme activities as well as the ribosomal RNA in the distinctly separated chloroplast

and their scan tracing are given in Figure 6 (b and c), in comparison with the picture of the total RNA fraction obtained from the whole spinach leaf (Fig. 6a). The pictures clearly show that the chloroplastic RNA (23s, 16s) is well separated from the cytoplasmic RNA (25s, 18s), indicating that the chloroplastic and cytoplasmic fractions contain chloroplastic rRNA and cytoplasmic rRNA, respectively. Percentage distribution of each RNA species in the individual fraction was calculated from the figure and the values are summarized in Table I. A small amount of cross-contamination between the two fractions can be seen, but we cannot exclude a possibility that cytoplasmic rRNA is associated with the chloroplastic outer membranes (1). The same type of association is clearly demonstrated in yeast mitochondria (13). Also, the presence of small amounts of unknown RNA species having smaller molecular size can be seen in Figure 6, b and c, and is probably due to the degradation of each respective rRNA species during the preparation of samples (16).

Figure 5b shows the results of DNA analysis. There are two major DNA-containing fractions, I and II, but none of them is fully characterized. Although we found that fraction I is positive to the Feulgen staining, the sedimented pellets in centrifuge tubes are also positive to this stain. It will be noted that since the absorption spectra of peak II fractions analyzed by the diphenylamine method showed the presence of two prominent peaks at 650 nm and 600 nm, there is a possibility that non-DNA substances are present in these fractions.

DISCUSSION

The hitherto reported structurally intact and functionally active chloroplasts were preparations obtained by low speed cen-
ORGANELLE SEPARATION FROM PROTOPLASTS

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FIG. 4. Localization of various aminotransferase activities in the separated fractions after sucrose density gradient centrifugation of mechanically ruptured protoplast preparations. The preparation used for mechanical breakage and sucrose density gradient centrifugation was the same as that shown in Fig. 3. Aliquots of separated fractions were subjected to the following enzyme assays: a: glutamate glyoxylate aminotransferase; b: serine glyoxylate aminotransferase; c: alanine glyoxylate aminotransferase; and d: aspartate α-ketoglutarate aminotransferase. Experimental details for the enzyme activity measurements are described in the text, and all enzyme activities are expressed as μmol products formed/min-tube (0.5 ml).

Fig. 5. Localization of RNA and DNA in the separated fractions after sucrose density gradient centrifugation of mechanically ruptured protoplast preparations. The preparation used for mechanical breakage and sucrose density gradient centrifugation was the same as that of Fig. 2. Aliquots of separated fractions were analyzed for (a) RNA and (b) DNA.

fractions (d = 1.21) is evidence that the isolated fractions are pure. However, the present method has its own limitations. As is generally recognized, gradient separation of chloroplasts on a dense sucrose solution for a prolonged period (3 hr) causes deterioration of their biochemical activities. The light-dependent CO₂ fixation activity is usually low. The final chloroplast preparations (d = 1.21) obtained in the present study exhibited fixation of about 5 μmol CO₂/mg Chl·hr. Therefore, in spite of the fact that chloroplasts appear to retain their structural integrity microscopically, the type of preparations isolated can be classified as Class I according to Spencer and Unt (27). An additional factor causing the decline of photosynthetic activity is likely to be the time lapse during preparation of protoplasts (4 hr), probably resulting in the consumption of photosynthetic intermediates during this step. It will be recalled that previously...
several investigators attempted to enhance the CO₂ fixation capacity of chloroplast preparations by supplementing some P esters, e.g., fructose-diP, ribose-5-P, and 3-PGA (5). Furthermore, one can assume that the contact of cytoplasmic and other cellular inclusions with chloroplasts during the preparative step of chloroplasts may result in both enzymic and nonenzymic degeneration of the membranous structure of the latter. Recently reported procedures for the isolation of active chloroplast preparations are all based on very rapid separation using a medium such as silica gel (19) or two phase dextran-polyethylene glycol (14), but the homogeneity of separated organelle fractions has not been well documented in these studies. Although the present procedure appears to be far superior to others to the extent of isolating enzymically and structurally pure chloroplasts, further technical improvements are needed in order to obtain fully functional Class A chloroplasts (9).

It is known that single membranous organelles such as peroxi- some are particularly fragile and are difficult to isolate in the intact form. Lips (15) reported that enzyme content of plant microbodies is liable to be affected by experimental procedures such as homogenization or sedimentation. It must be pointed out that there are no satisfactory criteria for the intactness of peroxi- some. The present method is considered to be useful for separa- ting them free from other cellular constituents. From the specific localization of seven major component enzymes with the fractions of $d = 1.25$ the separation of intact peroxisomes free from other organelles can be achieved by the present technique. However, some enzymic activities are leached out into the soluble fractions located at the top of the gradient, whereas they may represent specific location in the cytosol in vivo.

**Note.** After completion of this manuscript our attention was drawn to the paper by Rathnam and Edwards which described the isolation of chloroplasts from various grasses (Rathnam, C. K. M. and G. E. Edwards. 1976. Protoplasts as a tool for isolating functional chloroplasts from leaves. Plant Cell Physiol., 17: 177-186).

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**LITERATURE CITED**


