Nonaqueous Purification of Maize Mesophyll Chloroplasts

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ABSTRACT

A nonaqueous fractionation method to obtain highly purified mesophyll chloroplasts from lyophilized leaves of Zea mays L. is described. The levels of several metabolites including pyruvate were determined in the purified mesophyll chloroplast fractions which were prepared from leaves exposed to different light intensities. The role of pyruvate in the regulation of pyruvate, Pi dikinase in these chloroplasts under different light intensities is discussed.

Recently, changes in photosynthetic metabolite levels on whole maize leaves have been conducted during an induction period (14), during a course of a day (19), and under steady state photosynthesis with normal atmospheric conditions, varying CO₂ concentrations (15), and varying light intensities (16). Although these studies have increased our understanding of C₄ photosynthesis, a lack of information on the levels of photosynthetic metabolites in various compartments of the leaf is a stumbling block in further understanding of C₄ photosynthesis. Nonaqueous purification of mesophyll chloroplasts from C₄ plants has provided much information about metabolite levels in mesophyll chloroplasts under various conditions (e.g., 7). However, very few attempts have been made to purify chloroplasts from leaves of C₃ plants by nonaqueous methods. In an excellent pioneer work of Slack et al. (11), where they proposed a pathway of C₃ photosynthesis, mesophyll and bundle sheath chloroplasts from maize leaves were separated nonaqueously. However, there was substantial cross-contamination of the two fractions and the association with the mesophyll cytosol (represented by the activity of PEP carboxylase) varied from experiment to experiment. In certain experiments, the mesophyll chloroplast fraction (represented by the activity of pyruvate, Pi dikinase) was contaminated by the mesophyll cytosol. Slack et al. measured several metabolite levels after fractionating the nonaqueous gradient; however, it was almost impossible to estimate metabolite levels in specific compartments due to mutual contamination. Since their work, no attempt at nonaqueous separation of mesophyll and bundle sheath chloroplasts from maize leaves has been reported. The purpose of this report is to describe the details of a nonaqueous fractionation method to obtain highly purified maize mesophyll chloroplasts.

MATERIALS AND METHODS

Plant Material. Seeds of Zea mays L. (cv Chuseishu-B) were obtained from Nihonosogo, Tokyo, Japan. Plants were grown outside (April 27 to June 23, 1987) as previously described (14). The largest fully expanded leaves of plants 6 to 8 weeks old were used for the experiments.

Freeze-Drying of Leaf. The metabolism of the leaves was quenched by inserting them into liquid N₂ under sunlight as soon as the leaves were harvested. The midrib was removed from the frozen leaves. The frozen tissue was thoroughly homogenized using a mortar and a pestle prechilled with liquid N₂. Freeze-drying was carried out at −50°C (shelf temperature in a freeze-dryer) and 0.8 torr for 7 d. The dry material thus obtained (original lyophilized material) was stored until use in a closed plastic tube at −20°C in a desiccator containing P₂O₅.

Nonaqueous Density Fractionation. The following operations were carried out in a cold room at 2°C. About 300 mg of lyophilized leaf was homogenized in 25 ml of a mixture of hexane and carbon tetrachloride (density 1.25) with a Polytron (PTA 36/2 probe, setting 6) for 75 s in 5 s bursts followed by pauses of 5 s. The temperature of the homogenate was about −20°C. The combined homogenate from two extractions was filtered successively through 40 and 20 µm aperture nylon nets. The same volume of hexane was added to the filtrate, mixed (homogenate 1), and then centrifuged for 2 min at 3500g. The supernatant was discarded, and the sediment was resuspended in 5 ml of CCl₄/C₆H₁₄ (density 1.42). The suspension was homogenized with a Teflon/glass homogenizer, the homogenate was filtered through a 20 µm nylon net, and the residue was washed with 3 ml of CCl₄/C₆H₁₄ (density 1.42). Still, some materials were retained on the nylon net.

The following centrifugations were always carried out for 10 min at 15,000g using a swing rotor. The supernatant was collected very carefully because some material floated on the surface of the solution. Resuspension of the pellet was also done carefully to get a homogeneous suspension.

The filtrate which passed through the 20 µm nylon net was centrifuged, and the sediment was suspended in CCl₄/C₆H₁₄ (density 1.42) (fraction 5). Then the supernatant was mixed with 0.7 volume of 1.20 g/cm³ CCl₄/C₆H₁₄ (giving a final density of 1.329) and was centrifuged. The sediment was suspended in CCl₄/C₆H₁₄ (density 1.37) (fraction 4). The supernatant was mixed with 0.5 volume of 1.20 g/cm³ CCl₄/C₆H₁₄ (giving a final density of 1.286) and was centrifuged. The sediment was suspended in CCl₄/C₆H₁₄ (density 1.31) (fraction 3). The supernatant was mixed with 0.56 volume of 1.20 g/cm³ CCl₄/C₆H₁₄ (giving a final density of 1.255) and was centrifuged. The sediment was suspended in CCl₄/C₆H₁₄ (density 1.28) (fraction 2). The supernatant was mixed with 0.5 volume of C₆H₁₄ (giving a final density of 1.237) and was centrifuged. The sediment was suspended in 1.20 g/cm³ CCl₄/C₆H₁₄ (fraction 1). The final supernatant was discarded. Each fraction was vigorously mixed, and one-tenth of it was saved for assay of marker enzyme activities. The rest of each fraction was used for

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2 Abbreviations: PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; MDH, malate dehydrogenase; ME, malic enzyme; PGA, 3-phosphoglycerate; UDPG, UDP-glucose.
determination of metabolite levels. After obtaining all fractions, they were dried at the same time; thus, the duration of exposure of the marker enzymes in each fraction to nonaqueous solution was exactly the same. The dried material was stored in a closed plastic vessel with P₂O₅ at −20°C for 1 to 2 d until assay.

**Assay of Marker Enzymes.** One ml of 50 mM Tris-HCl (pH 7.8), 1 mM MgCl₂, 0.1 mM EDTA, 5 mM DTE, 0.5% (w/v) BSA, and 1% (w/v) insoluble PVP were added to the dried samples, and the suspension was mixed thoroughly with a Teflon rod. The clear supernatant obtained after centrifugation was used for enzyme assay. To correct for partial inactivation of marker enzymes during nonaqueous fractionation, the original lyophilized material was also used for the enzyme assay. Twenty to 25 mg of the original lyophilized material was suspended in a test tube containing CCl₄/C₅H₁₂ (density 1.20) and kept on ice for the same amount of time as it took for nonaqueous fractionation after which the suspension was dried. The dried material was stored for 1 d at −20°C as described above. Fifteen mg of this dried material was homogenized in chilled mortar and pestle with 5 ml of the same extraction medium as mentioned above. The clear supernatant obtained after centrifugation was used for marker enzyme assay, unless otherwise mentioned. The supernatants from both the fractionated samples and the original lyophilized material were mixed with one volume of 0.1 M DTE and left on ice for 1 h and were used only for the assay of NADP-MDH. PEP carboxylase (marker for the mesophyll cytosol) was assayed in a mixture (1 ml) of 50 mM Tris-HCl (pH 8.0), 30 mM MgCl₂, 10 mM NaHCO₃, 0.2 mM NADH, and 10 units MDH. The reaction was initiated by adding PEP to a final concentration of 10 mM. NADP-MDH (marker for the mesophyll chloroplasts) and NADP-ME enzyme (marker for the bundle sheath chloroplasts) were assayed as previously described (13), and UDPG pyrophosphorylase was assayed according to Bergmeyer et al. (2). Preliminary experiments indicated that the percentages of partial inactivation of marker enzyme activities in the original lyophilized material after nonaqueous treatment for NADP-MDH, PEP carboxylase, UDPG-pyrophosphorylase, and NADP-ME were 35%, 23%, 15%, and 0%, respectively, compared to those found in the original lyophilized material without nonaqueous treatment.

**Measurements of Metabolites.** One and a half ml of 3% (v/v) HClO₄ was added to the dried samples, and the material was mixed thoroughly with a Teflon rod. The suspension was left for 1 h on ice, then centrifuged at 3,000g for 10 min at 4°C. The UV absorbances of the HClO₄ extracts of fractions 1 to 3 were quite low; however those of fractions 4 and 5 were extremely high. If necessary, charcoal treatments were carried out as before (14). The HClO₄ extract was neutralized to about pH 6.0 by the addition of 2.5 M K₂CO₃, and was centrifuged at 10,000g for 5 min at 4°C. The supernatant was immediately used for the enzymic determination of metabolites as previously described (14). Metabolite levels in the original lyophilized material were also determined and expressed on the basis of Chl units converted from phycoprotein (14).

**Chl Determination and Expression of Enzyme Activities.** A sample of original lyophilized material was weighed and extracted with 80% acetone, and Chl was measured by the method of Arnon (1). Thus, Chl content on the basis of dry weight of the original lyophilized material was determined. The marker enzyme activities in the original lyophilized material described above were expressed on the basis of Chl using the relationship between Chl content and dry weight.

**RESULTS AND DISCUSSION**

**Nonaqueous Fractionation of Mesophyll Chloroplasts.** The recovery of each marker enzyme (PEP carboxylase, NADP-MDH, and NADP-ME) and metabolite (pyruvate, PEP, and DHAP) in fractions 1 to 5 was more than 80% of that found in the crude extract (homogenate 1). Some materials were retained on the 20 μm nylon net before sequential centrifugations (see ‘Materials and Methods’). Thus, the recoveries of enzyme activities and metabolites after nonaqueous fractionation were sufficiently high. Distribution of marker enzyme activities in fractions 1 to 5 is shown in Figure 1. The activity of NADP-MDH was greatest in the lighter fraction (fraction 3, with density between 1.329 and 1.286 g/cm³), while the highest activities of PEP carboxylase and NADP-ME were found in the heaviest fraction (density greater than 1.42). About half of the activity of NADP-MDH was found in fraction 3 (Fig. 1a) with slight contamination by PEP carboxylase and NADP-ME. In fraction 2 the amount of NADP-MDH recovered was about half of that found in fraction 3, while contamination by the other two enzymes was very small. These
results suggested that mesophyll chloroplastic material was highly purified in fraction 2 without significant contamination by mesophyll cytosolic and bundle sheath chloroplast material. However, bundle sheath chloroplastic material was isolated with the mesophyll cytosolic and chloroplastic material. Distribution of bundle sheath cytosolic material in fractions 1 to 5 was not evaluated, because there was no appropriate marker enzyme for bundle sheath cytosol.

The activity of UDPG-pyrophosphorylase was very low in fraction 2, and it was mostly found in fractions 4 and 5 similar to the distribution of the activity of PEP carboxylase (data not shown). UDPG-pyrophosphorylase activity was found both in mesophyll and bundle sheath cells of maize leaves (17). It is located in the cytosol in mesophyll cells of maize (17) and in bundle sheath cells of Panicum maximum (20). These results suggested that this enzyme in maize bundle sheath cells is also in the cytosol. All the results suggest that the mesophyll chloroplastic material recovered in fraction 2 was free from mesophyll and bundle sheath cytosolic and bundle sheath chloroplastic material. Similar results were also obtained with leaves exposed to a lower light intensity (50–60 μE · m⁻² · s⁻¹) for 30 min (data not shown).

The percentage distribution of the total marker enzyme activities in Figure 1a only shows the relative distribution of each enzyme and does not indicate the absolute amount in each compartment. I estimated the actual amount of each compartment recovered in fractions 1 to 5 by comparing marker enzyme activities found in these fractions to those found in the original lyophilized material after nonaqueous treatment (enzyme activities were expressed on the basis of Chl content, see “Materials and Methods”). The amount of mesophyll chloroplastic and cytoplasmic material and bundle sheath chloroplastic material was expressed by Chl content in each cell type, assuming that in maize leaves 60% of the Chl is in mesophyll chloroplasts and the rest in the bundle sheath chloroplasts (8). Figure 1b shows the distribution of mesophyll chloroplastastic and cytosolic material and bundle sheath chloroplastic material in fractions 1 to 5. Fraction 2 contained practically only mesophyll chloroplastic material and no mesophyll cytosolic material or bundle sheath chloroplastic material. All these results indicated that mesophyll mesophyll chloroplasts were highly purified by this nonaqueous fractionation. The principle of this nonaqueous fractionation method is essentially the same as that of Slack et al. (11). However, careful sequential centrifugations are very important in this process in order to obtain a pure preparation of mesophyll chloroplasts. In C₃ plants, the mesophyll chloroplasts were often separated with some cytosolic material (7) in a fraction with heavier densities compared to the present results with maize mesophyll chloroplasts. This could be due to the fact that C₃ mesophyll chloroplasts contain starch granules which are lacking in maize mesophyll chloroplasts.

**Metabolite Levels in the Mesophyll Chloroplast Fraction.** Various fractions were isolated from leaves exposed to high or low light intensity, and the metabolite levels in mesophyll chloroplasts were determined by analysis of fraction 2 (Table I). The actual concentrations of metabolite in the mesophyll chloroplasts were calculated based on the estimated amount of mesophyll Chl recovered in fraction 2 (Fig. 1b) and assuming a stromal volume of 25 μl · mg⁻¹ Chl (Table I). Pyruvate concentration was estimated to be 10 and 2 mM under 600 to 700 and 50 to 60 μE · m⁻² · s⁻¹, respectively. Concentrations of pyruvate in maize mesophyll and bundle sheath cells under high light were previously reported to be 6 and 5 mM, respectively (12). Recently, light-dependent active uptake of pyruvate into mesophyll chloroplasts was demonstrated (6, 10). Taken together, these results suggest that pyruvate is actively accumulated in mesophyll chloroplasts and that there may be a sufficient concentration gradient between bundle sheath cytosol and mesophyll cytosol to facilitate pyruvate transport between the two cell types.

The state of activation of the C₄ mesophyll chloroplast enzyme, pyruvate, Pi dikinase, is known to be regulated by light (5), and it decreases with decreasing light intensities to a point just sufficient to accommodate photosynthesis (18). Thus, under low light intensities, this enzyme is partially inactivated. Adenylate energy charge (9) and pyruvate (3, 4) have been suggested to have an important role in regulation of activation-inactivation or dephosphorylation-phosphorylation of the enzyme. In vitro, pyruvate concentrations greater than 1 mM have been found to inhibit the inactivation or phosphorylation completely (3). In this study, under low light intensity, 2 mM pyruvate was present in

| Table I. Metabolite Levels under Two Different Light Intensities in Whole Leaves and in Nonaqueously Purified Mesophyll Chloroplasts of Maize Leaves |
|-----------------|-----------------|-----------------|-----------------|
| Metabolites     | Light Intensity | Metabolites     | Light Intensity |
|                 | (μE · m⁻² · s⁻¹)|                 | (μE · m⁻² · s⁻¹)|
|                 | Whole Leaves    | Fraction 2⁶      | Mesophyll      | Whole leaves    |
|                 |                 |                   | chloroplasts³  |                 |
|                 |                 |                   |                 |                 |
|                 | nmol · mg⁻¹ Chl | nmol             | mm             | nmol · mg⁻¹ Chl | nmol             | mm             |
| Pyruvate        | 340             | 118              | 10.2           | 48              | 33              | 2.1            |
| PEP             | 110             | 29               | 2.5            | 33              | 13              | 0.8            |
| PGA             | 828             | 158              | 13.6           | 275             | 55              | 3.5            |
| DHAP            | 243             | 74               | 6.4            | 39              | 26              | 1.7            |
| FBP             | 20              | 1.0              | 0.09           | 14              | 0.6             | 0.04           |
| Glucose-6-P     | 89              | 7.7              | 0.7            | 97              | 21              | 1.4            |
| Fructose-6-P    | 40              | 2.5              | 0.2            | 24              | 5.1             | 0.3            |

* Mesophyll chloroplastic material (equivalent to 0.46 mg mesophyll Chl), mesophyll cytoplasmic material (equivalent to 0.017 mg mesophyll Chl), and bundle sheath chloroplastic material (equivalent to 0.004 mg bundle sheath Chl) were found in this fraction 2.

** Mesophyll chloroplastic material (equivalent to 0.63 mg mesophyll Chl), mesophyll cytoplasmic material (equivalent to 0.048 mg mesophyll Chl), and bundle sheath chloroplastic material (equivalent to 0.008 mg bundle sheath Chl) were found in this fraction 2.

Metabolite concentrations in mesophyll chloroplasts were estimated assuming that 60% of the Chl of maize leaves is in mesophyll chloroplasts (8) with a stromal volume of 25 μl · mg⁻¹ Chl.

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mesophyll chloroplasts (Table I). These results indicated that even with a concentration of pyruvate sufficient to prevent inactivation of pyruvate, Pi dikinase, the enzyme was still partially inactivated under low light intensity. Thus, some factor(s) other than pyruvate may have a regulatory role in the inactivation of the enzyme under low light. A study of changes in the adenylate energy charge in mesophyll chloroplasts under different light intensities may help elucidate the regulatory mechanism of this enzyme.

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