Density Gradient and Differential Centrifugation Methods for Chloroplast Purification and Enzyme Localization in Leaf Tissue

The Case of Citrate Synthase in Pisum sativum L.*

B.A. Elias** and Curtis V. Givan
Department of Plant Biology, The University, Newcastle upon Tyne NE1 7RU, U.K.

Abstract. Intact chloroplasts, isolated by differential-centrifugation and sucrose density-gradient methods, have been used to study the degree of apparent artifactual adsorption of citrate synthase (EC 4.1.3.7) to the organelles. Unfractionated homogenates layered directly on to sucrose density gradients gave elution profiles showing definite citrate synthase activity in the intact and broken plastid regions, along with the major mitochondrial peak. Nonreversible triose-phosphate dehydrogenase (EC 1.2.1.9), a cytosolic marker, showed no activity in any particulate region of the gradient. Crude chloroplast pellets and twice washed (resedimented and resuspended) chloroplasts layered on to the gradient gave progressively reduced citrate synthase activity in the plastid regions. In addition, the peak in the mitochondrial region of the gradient was virtually eliminated when washed chloroplasts were fractionated on the gradient. Differences in protein binding behavior on the chloroplasts may necessitate the inclusion of a washing step in chloroplast purification procedures. Moreover, repeated sedimentation and resuspension can also be a useful procedure to reduce mitochondrial contamination of chloroplast preparations.

Key words: Centrifugation methods - Chloroplasts - Citrate synthase - Enzyme adsorption - Pisum.

Introduction

Density-gradient techniques based on aqueous buffers now constitute the most popular method for purifying plant cell organelles, e.g. when determining the subcellular location of enzymes in plant tissues. Organelles purified by density-gradient procedures are usually purer than those obtained by the older differential-centrifugation methods, where the organelle of interest is not further purified once it has been sedimented (cf. Leech, 1977). The principal advantage of the density-gradient method is that it can resolve populations of different organelles from one another, with little or no particulate cross-contamination (cf. Givan & Harwood, 1976; Leech, 1977; Yamazaki and Tolbert, 1970).

While density-gradient methods definitely should continue to be employed in careful enzyme-localization studies, the gradient method alone may not invariably provide a completely accurate picture. Marker enzymes are obviously essential to confirm the purity of gradient fractions. But even using markers it is often difficult to rule out the possibility that an enzyme may associate with an organelle band owing to artifactual adsorption of a soluble enzyme originating from elsewhere. Nearly all tissue-homogenizing procedures break organelles, thereby causing solubilization of organelellar enzymes. Also, unfractionated homogenates contain soluble cytosolic enzymes. Adsorption of soluble or solubilized enzymes to organelles may produce a potentially misleading picture in the gradient elution profile. In the case of nitrate reductase, Dalbling et al. (1972) found that bovine serum albumen (BSA) prevented the adsorption of the soluble enzyme to chloroplast membranes. BSA has, therefore, tended to become a routine ingredient in media used in enzyme-localization work (e.g. Miflin and Beevers, 1974; Elias and Givan, 1977). Leech (1977) has expressed concern lest BSA cause different types of organelles to stick together irreversibly; however, Miflin and Beevers' work indicates that there need be no serious drawback of this kind.

In this paper we examine the behavior of the enzymes citrate synthase and nonreversible, NADP-linked triose-P dehydrogenase on sucrose density gradients. We suggest that density-gradient procedures can and should be supplemented by differential centrifugation methods. In particular, repeated resuspen-
Chloroplast Isolation and Purification

Materials and Methods

*Pisum sativum* cv. Laxton Superb seedlings were grown as described earlier (Givan, 1975), and leafy shoots homogenized for a few seconds in a mechanical blender. The homogenizing buffer comprised D (-) sorbitol, 330 mM; BSA, 0.1% (w/v); HEPES, 50 mM, pH 6.5. After filtration through Miracloth, the crude brei was either layered directly onto the density gradient or was centrifuged at 3000 g for 15 s (excluding acceleration time) with manual braking. The pellet ("crude chloroplasts") was then either layered directly onto the density gradient, or alternatively, resuspended in grinding medium (20 ml) and resedimented at 3000 g for 30 s (excluding acceleration time), resuspended and again resedimented as before. The resuspension and resedimentation procedure was termed "washing".

The density gradient procedure was based on Miflin & Beevers (1974) and has been used previously by us (Elias and Givan, 1977). In this procedure, the centrifugation time is short (15 min), so that intact and broken (=envelope-free) chloroplasts approach equilibrium positions, whereas peroxisomes and mitochondria do not; after a 15 min spin, the latter two organelles thus band higher up the density gradient than either intact or broken chloroplasts.

**Enzyme and Chlorophyll Assays**

Non-reversible glyceraldehyde-3-P dehydrogenase (NADP dependent) (EC 1.2.1.9) was assayed by the method of Kelly and Gibbs (1973). The assay mixture contained 2.2 ml 120 mM glycylglycine-NaOH buffer, pH 7.7; NADP, 18 μmoles; triose-P isomerase (Sigma), 20 units, plus 0.5 ml eluted gradient fraction in a total volume of 3.0 ml. The assay was run at 30°C, and the reaction was initiated by addition of 1 μmole dihydroxyacetone-P.

Citrate synthase (EC 4.1.3.7) was assayed by the spectrophotometric method of Ochoa (1955). In this assay, citrate synthase was coupled to malate dehydrogenase, and the increase in absorbance at 340 nm followed.

Chlorophyll was determined according to Arnon (1949).

**Results and Discussion**

Marker–enzyme elution profiles for density gradients of the type used here were originally determined by Miflin and Beevers (1974), with particular reference to organelle-localized enzymes. The marker data of Miflin and Beevers have been essentially confirmed by us (Elias and Givan, 1977) and by Bryan et al. (1977). Intact chloroplasts, possessing envelopes and retaining stromal protein, move quickly to a near-equilibrium position well down in the gradient and are well separated from broken chloroplasts, peroxisomes and mitochondria. Separation between mitochondria and broken chloroplasts is less good, but some resolution is possible if the spin is halted in time to prevent mitochondria from passing into the region of broken (envelope-free) chloroplasts.

In order to check on the extent of nonspecific (i.e. generalized and indiscriminate) adsorption of soluble or solubilized enzymes to chloroplasts, we examined the elution profile of the cytosolic marker NADP-linked glyceraldehyde 3-P dehydrogenase (nonreversible) (Kelly and Gibbs, 1973). Figure 1 shows elution profiles for chlorophyll and for the nonreversible dehydrogenase, obtained when a Miracloth-filtered leaf homogenate was layered directly onto the sucrose gradient and centrifuged through it. The three chlorophyll peaks represent intact chloroplasts (A), broken (envelope-free) chloroplasts (B), and small chloroplast fragments (C) (Elias, 1977). Previous work has shown that particulate triose-P isomerase, a marker for intact chloroplasts, is found only in association with chlorophyll band A (Miflin and Beevers, 1974; Bryan et al., 1977; Elias and Givan, 1977).

The cytosolic marker nonreversible glyceraldehyde-3-P dehydrogenase was entirely at the top of the gradient and was not associated with any chlorophyll peak. One can, therefore, reasonably conclude that Miflin and Beevers (1974) were correct in assuming that intact chloroplasts purified in this way did not suffer from gross, indiscriminate contamination by soluble or solubilized enzymes.

Figure 2 shows elution profiles for chlorophyll and citrate synthase, when the Miracloth-filtered leaf homogenate was layered directly onto the sucrose gradient (without prior preparation of a plastid-enriched