Leucoplast Isolation

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INTRODUCTION

While several aspects of primary metabolism are identical in plant and animal cells, others are distinctly different. The most prominent difference is the subcellular localization of biosynthetic reactions. Anabolism in animal and microbial cells takes place within the cytoplasm. Plant cells, however, have the anabolic reactions primarily localized within a double membrane-limited organelle, the plastid. In leaves these reactions include photosynthetic carbon fixation, nitrogen and sulfur assimilation, and biosynthesis of a wide range of lipids, amino acids, and nitrogenous bases (reviewed in 16).

For many years the study of plastids and their metabolism was limited to chloroplasts, with a few excursions into the proplastid -> etioplast -> chloroplast developmental sequence. Considering the global importance of carbon fixation/oxygen evolution, this early emphasis is not surprising. As our knowledge of plant metabolism improved, it became obvious that in many instances nongreen plastids provide a naturally amplified system, superior to chloroplasts, for the study of many plant processes (e.g. carotenoid biosynthesis by chromoplasts). As differences between green and nongreen plastids were noted, there was increasing study of the latter for comparative purposes. The results of these further studies have given rise to an increasing number of specialists who consider nongreen plastids as the plastids of interest.

In early studies of nongreen plastids, researchers adopted the isolation methods previously used with chloroplasts. It is necessary in most cases, due to the properties of nongreen plastids and the tissues in which they occur, to modify procedures designed for chloroplast isolation or to develop new methods. In this review I compare the major methods used for leucoplast isolation. In subsequent chapters other authors will delve into specific methods required for successful isolation of other nongreen plastids.
WHAT ARE LEUCOPLASTS?

Detailed ultrastructural and biochemical descriptions of the various plastid types have been previously published (e.g. 14,16), and will not be reviewed herein. A most succinct definition is that leucoplasts are mature, fully differentiated, nonpigmented plastids. Leucoplasts are generally found within specialized tissues. While leucoplasts are often specialized for particular metabolic roles, there is no functional component implicit in the definition. It has been suggested that as a class leucoplasts lack ribosomes, but this is not generally true. For example, the leucoplasts in *Ricinus communis* endosperm contain both proteins encoded by the plastome and typical 70S ribosomes. Additionally, it is now obvious that the number and functional state of plastid ribosomes change with changes in tissue age and developmental state (e.g. 3). In *situ* leucoplasts are typically ovoid, but are spherical when isolated under iso-osmotic conditions. Leucoplasts generally have a uniform granular matrix, few plastoglobuli, and no substantial stromal membrane elaboration.

Plastids from leaves of ribosome-deficient mutants such as *Albostrians* barley and *iojap* maize, or heat-bleached rye leaves, are mature, differentiated, and unpigmented, but are not strictly speaking leucoplasts. These plastids have, however, been used to study several aspects of plastid development and present many of the same problems in isolation and purification as do leucoplasts (11).

EXPERIMENTAL DESIGN

The first major consideration in experimental design is the choice of plant material. A distinction must be made between the study of leucoplasts *per se* and study of a particular plant species or process. If the aim is simply to study the organelle without other constraints, cauliflower florets (12) and legume primary roots (7) are simple, inexpensive, and readily available sources. If a particular metabolic process is to be considered, choosing a plant tissue and developmental stage where this process predominates will improve chances of success. Examples of the latter considerations include the use of seed storage tissues, at approximately the midpoint of development, to study glycolysis (6,19), and the choice of the exocarp of developing citrus fruits to study the synthesis of terpenoid flavor components (10). In some instances it is possible to manipulate a plant in order to activate or stimulate a particular aspect of plant metabolism. For example, infection of *Ricinus communis* endosperm with a fungal pathogen (or the appropriate pathogen-derived polysaccharide elicitor) will induce synthesis of the leucoplast synthesized diterpine phytoalexin casbene (18).

A second major consideration is that of experimental scale. Distinctly different strategies are employed in analytical versus preparative-scale experiments. Equilibrium density gradient centrifugation remains the method of choice for analytical experiments such as enzyme localization, but the capacity
of equilibrium gradients is small. In contrast, the capacity of simple rate-zonal sedimentation is virtually unlimited. It must be emphasized, however, that fractions prepared by rate-zonal sedimentation are not pure, but only enriched in plastids.

Additional considerations in experimental design include the choice of methods to be used for analysis of plastid purity and integrity. The most commonly used methods for evaluation of organelle purity are electron microscopy and the measurement of marker enzymes. While electron microscopy is an important tool, the extended time necessary for sample preparation and extensive instrument requirements reduce its usefulness for routine sample evaluation. The marker enzyme concept was introduced by DeDuve (5). For effective use as a marker, an enzyme must be localized exclusively within a single subcellular compartment. Additionally, it is essential that marker activities and recoveries be related to the original total homogenate, and not cosmetically improved by summing the recoveries obtained from each of the derived fractions. Both total and specific activities should be reported.

Most of the commonly used chloroplast markers (e.g. chlorophyll, NADP-glyceraldehyde-3-phosphate dehydrogenase) are not present in nongreen tissues. RuBisCO is present in some, but apparently not all, nongreen plastids. If present, quantitation of either protein or catalytic activity is an excellent plastid marker. While triose-phosphate isomerase activity is often used to locate plastids on gradients, it is not suitable as a true plastid marker. The occurrence of both cytoplasmic and plastid isozymes of triose-phosphate isomerase within plant cells (6) makes any type of simple quantitation extremely difficult. Acetyl-CoA carboxylase is exclusively plastid localized in plant cells (3) and appears to be ubiquitous. These considerations plus the relative ease in measuring activity make this the marker enzyme of choice for nongreen plastids.

As with chloroplasts, the structural integrity of leucoplasts can be evaluated by phase-contrast light microscopy. The determination of functional integrity presents more of a problem. The method of ferricyanide-dependent oxygen evolution used for chloroplasts is obviously unsuitable for nongreen plastids. The best alternative would seem to be the measurement of enzyme latency. For latency measurements it is necessary to choose an enzyme for which the substrates cannot cross the intact plastid envelope. Activity measurements are made first under osmotic conditions which maintain organelle integrity, followed by osmotic or detergent lysis and a second measurement of total enzyme activity. The difference between the first and second measurements is then a measure of plastid intactness. Various enzymes could be used both as plastid marker enzymes and for determining intactness; acetyl-CoA carboxylase, RubisCO, the enzymes of starch metabolism, and possibly ferridoxin-linked glutamate synthase.
METHODS FOR LEUCOPLAST ISOLATION

There are strengths and weaknesses inherent in each method and the one used should be logically chosen. While this would seem obvious, the literature is replete with reports of research employing the use of inappropriate methods. At the very least, using the wrong method will impede the rate of scientific progress. Some methods place a premium on speed, others scale, etc. Enzyme localization studies require a high degree of purity in the isolated organelle fraction, but not necessarily high yields. Conversely, if one wishes to purify some biochemical beginning with an isolated plastid fraction, it is logical to sacrifice some initial purity for a larger amount of starting material. *In vitro* physiological studies generally require a high degree of membrane integrity and active transporters, while these factors are not major considerations in experiments emphasizing macromolecular analyses. Time spent in the initial planning stages is usually recovered many-fold through having utilized the appropriate method.

**Rate-zonal sedimentation.** Subcellular fractions have characteristic sedimentation coefficients, based upon size, shape, and chemical composition (16). Differences in these coefficients can be exploited in the preparation of plastid-enriched fractions by rate-zonal sedimentation. By manipulating centrifugation speeds and times it is possible to greatly reduce contamination by other organelles. This method should be primarily used for the large-scale preparation of a plastid-enriched fraction, or as the prelude to use of a secondary method of greater resolving power. The sedimentation behavior of nongreen plastids can vary with tissue age or developmental stage, and optimum centrifugation speeds and times must be determined empirically. A typical analysis of marker enzyme distribution in a leucoplast-enriched fraction prepared from developing *R. communis* endosperm by rate-zonal sedimentation is presented in Table I.

<table>
<thead>
<tr>
<th>Marker Enzyme</th>
<th>Total Activity</th>
<th>Distribution</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>S0.5</td>
<td>S10</td>
<td>P10</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>26</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Fumarase</td>
<td>6</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>Catalase</td>
<td>88</td>
<td>100</td>
<td>49</td>
</tr>
<tr>
<td>Cytochrome c reductase</td>
<td>3</td>
<td>98</td>
<td>92</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>100</td>
<td>100</td>
<td>32</td>
</tr>
</tbody>
</table>

Activities of alcohol dehydrogenase, succinate dehydrogenase and cytochrome c reductase are µmol min⁻¹ gfw⁻¹. Catalase activity is in Lück units. RuBisCO was determined immunochemically, with the amount in the total homogenate arbitrarily set at 100. Recoveries are percentages related to the total activities in the initial homogenate. S0.5 is the 500g supernatant, S10 is the 10,000g supernatant, P10 is the 10,000g pellet, WP10 is the 10,000g pellet washed by resuspension and repelleted.
Rate-zonal sedimentation in discontinuous sucrose density gradients. Isolation of leucoplasts by rate-zonal sedimentation in discontinuous sucrose gradients will result in preparations which are significantly more pure than those prepared by simple sedimentation (16). This method is relatively rapid and of variable capacity, being suitable for analytical through preparative scale isolations. The quality of the separations varies in direct correspondence to the ability to form sharp, well-defined discontinuities between solutions of different sucrose concentrations. I recommend using the "floating cork" method for forming the steps and use of a brief, low speed centrifugation prior to loading the sample. As with all of the gradient methods it is essential that the sample be uniformly resuspended prior to loading onto the gradient. While useful for separating plastids from cytosolic and mitochondrial contamination, this method will not resolve plastids and peroxisomes. In most instances sucrose is used as the gradient medium for isolation by density gradient centrifugation. In some instances, however, it can be advantageous to employ other nonpenetrating sugars such as Metrizamid or Nycodenz (8), sugar polymers such as Ficoll, or non-sugar media such as diatrizoic acid (9).

Rate-zonal sedimentation in discontinuous silica sol-gradients. The use of discontinuous density gradients of sucrose or other nonpenetrating sugars allows the preparation of plastids suitable for many studies. However, dehydration of the organelles caused by the high sugar concentrations results in osmotic damage to the membranes. This can result in plastids unsuitable for many physiological or transport experiments. The problem of dehydration can be overcome through the use of colloidal silica solutions such as Percoll rather than sucrose (16). The low osmolality of solutions of colloidal silica allows gradients that are nearly iso-osmotic throughout. There are two additional advantages to the use of colloidal silica. The low viscosity of the solutions allows equivalent separations in less time, and there appears to be a physical "scrubbing" of exposed membrane surfaces resulting in final preparations which are somewhat more pure. If necessary, the silica sol can be removed by dilution followed by low speed centrifugation. The position of single-membrane limited organelles is very sensitive to the inclusion of polyethylene glycol in the gradient solutions. Thus the position of peroxisomes in these gradients can be altered such that they are not a significant contaminant (Fig. 1). Leucoplasts isolated from developing *R. communis* endosperm and purified by rate-zonal sedimentation on discontinuous Percoll gradients have been used to study the uptake and processing of *in vitro* synthesized plastid protein precursors (2).

Rate-zonal sedimentation in linear sucrose-magnesium cogradients. It has been reported many times that incubation with divalent cations can alter the surface properties of membranes. This can result in clumping which is often a problem when attempting to isolate or purify organelles. Burden and Canvin (3), however, chose to exploit this alteration in surface properties during the purification of leucoplasts from developing *R. communis* endosperm. By preparing gradients which coincidentally increased in both sucrose and...
Fig. 1. The separation of leucoplasts and peroxisomes, in homogenates prepared from developing *R. communis* endosperm, by rate zonal sedimentation in discontinuous Percoll gradients. The gradients consist of 5 mL of 80%, 7.5 mL 35%, 7.5 mL 22%, and 7.5 mL 10% Percoll (all w/v), containing 20 mM TES-KOH, pH 7.5, and 400 mM sucrose. The gradient in panel A contains no PEG while that in panel B contains 3% (w/v) PEG. After sample application the gradients were centrifuged for 7 minutes at 9,000g using a Beckman JS-13.1 rotor in a J2-21 preparative centrifuge. After centrifugation, 1 mL fractions were collected by upward displacement and assayed for catalase activity (△) or RuBisCO protein (▲) using rocket immunoelectrophoresis.

magnesium concentration, it was possible to rapidly prepare plastids substantially free from other organellar contaminants. To the best of my knowledge this method has not yet been applied to the preparation of other nongreen plastids or leucoplasts from other sources. It is likely that ideal divalent cation concentrations would have to be determined empirically.

**Isopycnic banding in linear density gradients.** Upon centrifugation, organelles will migrate through a density gradient until they reach their...
isopycnic point, that of equilibrium density as determined by their chemical composition and permeability properties. The primary advantage of isopycnic banding is the ability to separate all of the major classes of organelles in a single step and with relatively high recoveries (16). Isopycnic banding is generally the method of choice for studies of enzyme localization. The isopycnic point of \( R. \textit{communis} \) leucoplasts is 1.21 g mL\(^{-1}\), the same as that of green plastids. The major disadvantages of isopycnic banding are that relatively long centrifugation times are required and that the capacity of the gradients is low.

**Other Methods.** In some instances, such as studies of metabolite levels, it is desirable to isolate plastids using nonaqueous conditions. Typically, the tissue is frozen in liquid nitrogen and the resulting powder homogenized in glycerol or an organic solvent (e.g. hexane). After filtration to remove unbroken cells and wall fragments, the homogenates can be layered onto gradients of \( \text{CCl}_4 \) in hexane and the plastids purified by either rate-zonal or isopycnic centrifugation. Other nonaqueous separation methods have employed 3-Cl-1,2-propanediol (15), and gradients of KI in anhydrous glycerol (1). Additionally, it is possible to isolate plastid enriched fractions on a small scale by using centrifugal filtration through silicon oil.

Noncentrifugal methods for plastid isolation include gel permeation chromatography, phase partition, and unit gravity sedimentation. Phase partition has not yet been used for the isolation of nongreen plastids, and unit gravity sedimentation will be addressed in a subsequent chapter by J.C. Shannon (17). Gel permeation chromatography using Sephadex G-50 was initially introduced as a method for the isolation of highly intact chloroplasts. It was subsequently demonstrated that this method was time consuming, yields were low, and the only contaminating fraction removed from the plastids was the cytosol. With improvements in gel permeation media, this method was reexamined for the purification of \( R. \textit{communis} \) leucoplasts (16). By using Sephacryl S-1000 as the chromatography matrix, it was possible to separate the leucoplasts from mitochondria and peroxisomes, as well as the cytosol. While highly intact, yields were significantly lower than any of the centrifugal methods.

Thus, gel permeation is not really a suitable method for plastid isolation. There is, however, a significant improvement in the purification of leucoplasts by isopycnic banding, through the use of a relatively short (i.e. 2.6 x 20 cm) column of Sepharose 4B as a replacement for the initial pelleting step (Miernyk, unpublished observation). It seems likely that this method rapidly removes cytoplasmic contamination and also “scrubs” the plastid exterior free of organellar contamination. Samples prepared by Sepharose column chromatography are substantially diluted in comparison with resuspended pellets, so adjustments must be made in the amount of sample subsequently loaded onto density gradients.
EMERGING TECHNIQUES

This section will be very brief. There are very few new methods being used in cell fractionation, and none have been employed for the isolation and purification of nongreen plastids. This is more indicative of the paucity of research on these organelles than a reflection upon the creativity of the scientists working in the area. In many instances application of variations of methods developed for chloroplast isolation has been adequate for research on nongreen plastids. There remains, however, substantial room for improvement.

For example, I do not believe that there have been any published reports of the use of immunochemical aids to plastid isolation. Antibodies have been prepared against plastid outer envelope proteins (e.g. 13) and such antibodies might be effectively used in plastid isolation and purification. Immobilized antibodies could be used to select plastids from a mixture of organelles or antibodies to other membrane proteins could be used to remove contaminants from a plastid fraction. Another potential method would employ antibodies to alter the sedimentation properties or equilibrium density of plastids. By altering the behavior of the plastids, persistent contamination by peroxisomes and/or protein body fragments could be avoided.

CONCLUSION

While similar in many ways to the more thoroughly studied green plastids, the peculiarities of leucoplasts require some modification to standard isolation procedures. The methods employed for leucoplast isolation and purification must be compatible with subsequent experimental plans. As our knowledge of nongreen plastids increases, it becomes increasingly clear that as an object of scientific curiosity, these organelles are at least the equal of chloroplasts.

LITERATURE CITED