OptiPrep™ Application Sheet S59

Membrane trafficking and proteomic analysis of plants and plant cells

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- The purification of plant protoplasts that is often a prelude to subcellular membrane fractionation is covered in **Application Sheet C18** (see Mammalian and Non-mammalian Cell Index)
- **This Application Sheet describes methods for analysis of membrane trafficking, protein processing and proteomic analysis; it covers, plasma membrane, ER and Golgi in particular.**
- **Application Sheet S58 covers the purification of the following organelles: chloroplasts, peroxisomes and glyoxysomes, mitochondria, amyloplasts, leucoplasts, tonoplasts and vacuoles. For nuclei see Application Sheet S10.**
- **For information on lipid rafts see Mini-Review MS11 "Lipid-rich detergent-resistant membranes from non-mammalian sources – a bibliography"** lists all the published papers. The methodology has been adapted to various **plant sources.**
- To access MS11 return to the initial list of Folders and select "**Mini-Reviews".**

1. Flotation gradients

1a. Two-layer discontinuous format

Background

There are many situations where it is necessary to provide an efficient separation of either a total membrane fraction and the cytosol or a specific membrane type and the cytosol. In both cases the aim is to be able to allocate one or more proteins to one or other compartment or both compartments. A simple discontinuous gradient flotation strategy, first used with Nycodenz®, has been adapted to the use of OptiPrep™. Such gradients are widely used in studies with cultured animal cell studies. An advantage of iodixanol gradients is that because of the lower osmolality of iodixanol solutions, compared to those of Nycodenz®, the difference in density between cytoplasmic proteins (approx. 1.26 g/ml) and all membrane vesicles and most membrane organelles (<1.18 g/ml) is enhanced. With animal cells a post nuclear supernatant is usually adjusted to approx. 30% (w/v) iodixanol and overlayered with 25% iodixanol (approx 1.14 g/ml); membrane-bound particles float through the lower density layer and the proteins will tend to sediment from the load zone. While this has not been extrapolated exactly to plant cells and tissues, some similar procedures have been adopted.

Methodology

In order to carry out a 2D electrophoresis analysis of *Arabidopsis* membranes Mahon and Dupree [1] overlaid a crude fraction with a discontinuous gradient of 25% and 9% (w/v) iodixanol and centrifuged was at 90,000 *g* for 1.5 h; to band the membranes at the upper interface. This flotation strategy would work less well for the densest organelles (nuclei), thus Liu et al [2] layered the crude *Arabidopsis* fraction (after clarification through a 100 µm filter) over 15% and 45% (w/v) iodixanol and centrifuged at 1,500 *g* for 15 min; the nuclei banded at the interface 15%-45% interface. Routinely the OptiPrep™ is diluted with extraction buffer to provide the gradient solutions; in this case 400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, 0.1% Triton X100, 0.1 mM DTT, 10 mM MES-NaOH, pH 5.3 (plus protease inhibitors).

1b. Flotation through continuous iodixanol gradients

Analysis of tonoplast, ER, Golgi and protein bodies

BY-2 cells and tobacco (*Nicotiana benthamiana*) leaf protoplasts have been homogenized in a standard medium (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4 supplemented with 10 mM DTT). Unbroken cells and nuclei were removed at 400 *g* for 10 min and the supernatant adjusted to 35% (w/v) iodixanol. This was layered beneath a 0-30% (w/v) iodixanol gradient and centrifuged at 114,000 *g* for 16 h [3] in 13 ml tubes for the Beckman SW41 rotor. The tonoplast banded at the top of the gradient and ER/Golgi in the middle. In this gradient tobacco mosaic virus 130K and 180K banded

in the tonoplast and ER/Golgi regions but also in the bottom fractions, which probably contained predominantly soluble proteins. By reducing the volume of the gradient to 2.2 ml the centrifugation time can be reduced to 2h at 106,000 *g* [4].

Tonoplasts from *Pteris vittata* [5] and apple tissue [6] may be purified by flotation through discontinuous iodixanol gradients (**see Application Sheet S58**)

2. Sedimentation gradients

Protein bodies

Endoplasmic reticulum-sourced Protein bodies have been analyzed in a discontinuous gradient of 1.11, 1.17, 1.19, 1.21, 1.23 and 1.25 g/ml; this is equivalent to the following % (w/v) solutions prepared by diluting OptiPrep™ with the homogenization medium (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0): 17%, 29%, 33%, 37%, 41.4% and 45.5%. The gradients (approx. 13 ml), which were centrifuged at 80,000 *g*, for 2 h, were used to analyze the protein bodies from tobacco (*Nicotiana benthamiana)* leaves. This method was first reported by Llop-Tous et al [7] in studies on the N-terminal proline-rich domain of the maize storage protein γ-zein (Zera) fused to other proteins. A dense fraction at the 1.19/1.21 g/ml interface contained Zera linked to enhanced cyan fluorescent protein, while other leaf proteins remained in the supernatant and lower density fractions (microsomes). **Iodixanol solutions were prepared from OptiPrep™ as described in Application Sheet S01**. More recently protein body-targeting of Zera linked xylanase [8] and linked to DsRed [9] were studied using the same gradient system.

3. Self-generated gradients

- For more information on the creation of self-generated gradients of iodixanol see **Application Sheet S04**.

3a. Organelles and proteomic analysis of *Arabidopsis thaliana*

The first example of the use of a self-generated iodixanol for the analysis of *Arabidopsis* membranes was by Dunkley et al [10]. After homogenization of the plant tissue in 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 mM HEPES-NaOH, pH 7.4, a post-nuclear supernatant was obtained by centrifugation at 2200 *g* for 5 min (carried out twice). A working solution of 50% (w/v) iodixanol containing 1 mM EDTA, 1 mM DTT, 10 mM HEPES-NaOH, pH 7.4 was diluted with the homogenization medium to produce solutions of 16% and 18% (w/v) iodxanol. The 2200 *g* supernatant was layered over 18% (w/v) iodixanol and the total membranes concentrated at the interface by centrifugation at 100,000 *g* for 2 h [10,11]. Most of the supernatant was removed and the residual material mixed to provide a crude membrane suspension in 16% iodixanol. This was transferred to a 13 ml tube for a Beckman VTi65.1 and a gradient created by self-generation at 350,000 *g* for 3 h [10].

The gradient successfully resolves the Golgi and smooth ER membranes from the denser mitochondria membranes and this method has been used by and been reported by several groups [12-17]. The method has also been the subject of many review articles [18-23] and described in detail in refs 21-23.

Oil bodies from *Arabidopsis thaliana* have also been successfully purified on discontinuous iodixanol gradients [27]

3b. Wheat-grain endosperm

The source material was homogenized in 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 10 mM HEPES-NaOH, pH 7.4 and the suspension centrifuged twice at 2200 *g* for 5 min to remove rapidly sedimenting material and debris. A microsome fraction was obtained by sedimentation on to an 18% (w/v) iodixanol barrier (OptiPrep™ diluted with the homogenization medium) at 100,000 *g* for 2 h in a swinging-bucket rotor. Golgi fractions were then isolated in a self generated iodixanol gradient (starting concentration 14% (w/v)). It was formed by centrifugation ay 200,000 *g* for 12 h in a Beckman 70Ti rotor. Although self-generated gradients are normally formed at higher *g*-forces for much shorter times, it is known that some fixed angle rotors, particularly those with low angles such as the 70Ti (23°) can be a suitable substitute. Golgi and ER markers banded distinctively under these conditions [28].

- Iodixanol gradient analysis of plant tissue has played an important part in identifying the subcellular localization of many plant proteins [29].

4. References

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