Ceylon J. Sci. (Bio. Sci.) 22, No. 1, 1992

NONAQUEOUS ISOLATION OF CHLOROPLASTS FROM SOYBEAN (*Glycine max* L. Merrill) LEAVES USING GLYCEROL AND POLYETHYLENE GLYCOL

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ABSTRACT

In a nonaqueous method of isolation of chloroplasts using a 5:5 v/v mixture of glycerol and polyethylene glycol (molecular weight 300) the final pellet contained about 50% of chloroplasts present in the original sample. Washing of pellet at high speed centrifugation removed most of the cytosol, but electron microscopic examination showed some cytoplasmic contamination. With respect to starch granule size, the isolation procedure yielded a population of chloroplasts not greatly different from those in the starting material. Chlorophyll extraction was also not so severe as with aqueous media.

INTRODUCTION

The chloroplast contains the basic photosynthetic machinery in the plant cell, and its inner envelope membrane functions as semi permeable barrier between the chloroplast and the cytosol. The carbon dioxide (CO_2) fixation occurring in the chloroplast involves an uptake of the substrates CO_2 and inorganic phosphate (P_1) from the cytosol and a release of the triose phosphate (TP) products from the chloroplast stroma. These TP are then converted to sucrose in the cytosol to be transferred to the distant sinks.

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Chloroplasts have been isolated in various aqueous media. Chloroplasts from pea shoots have been isolated in a medium which contained sorbitol, EDTA, $MgCl_2$, tris/methylglycine buffer and mercapto-ethanol (Lea *et al.*, 1979). Photosynthetically competent chloroplast preparations have also been isolated by means of density gradient centrifugation on the modified silica sol "Percoll" (Takabe *et al.*, 1979). Chloroplast obtained by these methods were 95% and 80% intact respectively.

Chloroplasts isolated aqueously had the advantage that they exhibited enzyme activity and O_2 evolution rates that would account for the whole cell photosynthetic rates. Conventional homogenization of tissue in aqueous media however introduces unavoidable analytical artifacts rendering assays for many important biological materials invalid. A major source of error in aqueous isolation is the leaching of some low molecular weight material from the organelles during isolation (Stocking, 1971). Also, when chloroplasts are isolated aqueously leaf tissue is generally kept in the dark for 24-48 hrs to destarch the plastids. In a study where the starch in the chloroplast becomes an important metabolite, aqueous chloroplast isolation becomes unsuitable. Therefore if metabolite distribution in the chloroplasts is to be studied they must be isolated by a procedure which does not require prior removal of the chloroplast starch and which does not allow loss of metabolites by leaching. Thus a nonaqueous isolation procedure is the method of choice.

It is reported that intact lyophilized nuclei are obtainable from a variety of tissue in a nonaqueous isolation procedure using a mixture of glycerol and 3-chloro-1,2-propanediol at 2°C (Kirsch *et al.*, 1970). A nonaqueous procedure using the same mixture was also developed to isolate starch granules with associated metabolites from maize endosperm (Liu *et al.*, 1989). A nonaqueous isolation procedure for chloroplasts using a hexane-carbon tetrachloride (CCl_4) medium and a pre-cooled petroleum ether and CCl_4 medium (Stocking, 1971) and nonaqueous gradient fractionation of spinach leaf tissue into chloroplast stroma, cytosol and vacuole using a mixture of CCl_4 and heptane (Gerhardt and Heldt, 1984) have also been reported. There was a severe loss of chlorophyll from the chloroplasts isolated in hexane-CCl_4 and petroleum ether-CCl_4 and considerable overlap of the cellular fractions in the procedure using CCl_4 and heptane. Thus, experiments were conducted to develop a nonaqueous procedure for isolation with better recovery of chloroplasts.

MATERIAL AND METHODS

Seeds of semi determinant soybean variety A2858 were grown under greenhouse conditions. At active pod filling time, leaves were removed, rinsed with distilled water and the large veins removed. The tissue was then frozen in Freon 12 (CCl_2F_2) cooled to its freezing point (-156°C) in liquid nitrogen. This tissue was freeze dried (5-10 μm Hg vacuum), and ground in a Cyclone Sample Mill. The ground leaves were then sifted through a 60 μm mesh sieve. The leaf powder thus obtained from sifting was stored at -25°C with silica gel until used.

For non aqueous isolation of chloroplasts from this tissue, several experiments were carried out with different homogenizing solutions, homogenizers, filters, centrifugation gradients, centrifugation speeds and times to establish the best possible procedure for isolation. Dry glycerol, mixture of glycerol and methanol, propylene glycol, mixtures of glycerol and potassium iodide, mixtures of glycerol and 3-chloro-

1,2-propanediol, mixtures of hexane and carbon tetrachloride, mixtures of glycerol and polyethylene glycol (PEG) (molecular weight 300) were used as homogenizing solutions. The mortar and pestle, glass grinder with glass pestle (Thomas Scientific Instruments Inc., Swedesboro, NJ, USA), a Polytron (Brinkman Instruments, Westbury, NY, USA), a size C Thomas Tissue Grinder with a Teflon Pestle (Thomas Scientific Instruments Inc.) were used as homogenizers.

Glass wool, Miracloth, 20 μm Nitex, 10 μm Nitex were used as filters. Combinations of the above homogenizing solutions, homogenizers and filters with different centrifugation speeds and times were tested in eighteen separate experiments and a "best" procedure for isolation of chloroplasts was determined.

For this "best" procedure, pulverized leaf tissue was ground in a 5:5 (v/v) glycerol/polyethylene glycol (PEG) (molecular weight 300) mixture using a glass grinder with a Teflon pestle. This was the centrifuged at 2000 x g for 20 min at 15°C. The supernatant from this was filtered through a 10 μm Nitex under vacuum. Grinding of the resulting pellet, centrifugation and filtering was repeated until most of the tissue passed through the 10 μm Nitex. The filtrate was centrifuged in a HB 4 rotor at 13,000 x g for 1 hr at 15°C. The pellet was washed twice by suspension in a 5:5 glycerol/PEG mixture and centrifugation as above for 30 min. The chloroplasts obtained in the final pellet were examined with light and transmission electron microscopy. A sample of the final chloroplast pellet was obtained for chloroplast counts on a 0.1 mm depth hemocytometer. The remaining pellet was washed twice with dry absolute methanol to remove glycerol and PEG. The washed pellets were extracted with 13:4:3 (v/v/v) methanol/chloroform/0.2 M formic acid (MCF) and 13:4:3 (v/v/v) methanol/chloroform/water (MCW) until the pellets were no longer green. These pellets were used for starch determination. The MCF and MCW extracts were combined and phase separated with chloroform and water. The chloroform phase was used for chlorophyll determination (Witham *et al.*, 1974). The aqueous phase was flash evaporated and used for metabolite analyses by enzyme assays.

Aliquots of the chloroplast preparations were examined with bright field, plane polarized and phase contrast using a Zeiss WL Research Microscope (Atlantic Instrument Co., PA, USA) and after preparation of chloroplasts for transmission electron microscopy they were examined with a Hitachi HU-11E-1 electron microscope (Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

All homogenizing solution except the 5:5 (v/v) mixture of glycerol and polyethylene glycol (PEG) (molecular weight 300) were unsatisfactory in many ways because they either did not pellet the chloroplasts satisfactorily or the recovery was extremely low. Also the extraction of chlorophyll from the plastids was very severe. Use of glass wool and Miracloth was also unsatisfactory because considerable amount of tissue and chloroplasts remained on them resulting in very low recovery of chloroplasts from the filtrate. Homogenization with mortar and pestle, and the glass grinder with the glass pestle were ineffective since the rupture of cells was minimal and release of chloroplasts was low.

The procedure determined as being the "best", homogenizing the freeze dried tissue with Teflon pestle and glass grinder in the 5:5 glycerol/PEG mixture released many chloroplasts. This was more effective

Table 1. Comparison of chloroplasts in original sample (whole cell homogenate) and non-aqueously isolated sample

	Original sample	Isolated sample
Number of chloroplasts x 10^9 per g dry weight tissue	5.70 ± 0.15	2.57 ± 0.06
Average number of starch granules/chloroplast	3.49 ± 0.41	3.81 ± 0.19
Length of starch granules in chloroplasts	1.03 ± 0.09	1.03 ± 0.06
Width of starch granules in chloroplasts	0.53 ± 0.03	0.63 ± 0.02

Size measurements were made on electron micrographs.
All starch granules in at least 200 chloroplasts were measured.

than any other grinding medium in that the higher viscosity of the medium produced better contact between the tissue and the grinder. Centrifuging at 2000 x g for 20 min at 15°C gave a pellet which contained the unbroken cells while the supernatant contained the free chloroplasts. Filtration through the 10 μm Nitex, passed the free chloroplasts while retaining much of the cell wall debris. With repeated homogenizations and centrifugations, it was possible to rupture most of the cells and recover the chloroplasts. Centrifugation at 13,000 x g for 60 min at 15°C produced a pellet which contained most of the free chloroplasts. The final pellet contained about 50% of the chloroplasts present in the original filtrate (Table. 1). Some chloroplasts were lost in the supernatants of these centrifugations. Washing the pellet from the high speed centrifugation presumably removed most of the cytoplasmic contamination. However, electron microscopic examination proved that some cytoplasm was still retained in pieces where the chloroplasts failed to separate from the wall fragments.

It is significant to note that neither the number of starch granules per chloroplasts nor their size differ between the original sample and the isolated chloroplast pellet (Table 1). The length and width of starch granules in the chloroplasts of samples from the whole cell homogenate and the final pellet were approximately the same (Table 1). Thus, with respect to starch granule size, the isolation procedure yielded a population of chloroplasts not greatly different from the original sample. Chlorophyll extraction with the glycerol/PEG procedure was not so severe as with other nonaqueous media (Gerhardt and Heldt, 1984; Stocking, 1971), e.g. hexane/CCl_4 used earlier, although chlorophyll was lost at every step in the isolation procedure - the highest amount being lost in the 60 min centrifugation of the original filtrate. The final chloroplast pellet contained approximately 25% as much chlorophyll per chloroplast as that in the original sample.

The advantage of the above method is that it is relatively easy. Effective homogenization could be

obtained with the glass grinder and 5:5 mixture. Since homogenization of freeze tissue in nonaqueous media is not the same as homogenization of fresh tissue in aqueous media, repeated grinding with slow centrifugation after each grinding also caused some cell wall contamination and introduced some accompanying cytoplasmic contamination as a disadvantage in the procedure.
The chloroplasts isolated by the above procedure were judged to be a representative sample of the chloroplasts in the original sample and were used to measure specific metabolites in a later study.

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