An improved procedure for the isolation of intact chloroplasts of high photosynthetic capacity

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An improved procedure for the mechanical isolation of chloroplasts of high degree of intactness (90–95%) and photosynthetic capacity (25–50 mmol of O₂/s per mol of chlorophyll) is described. The combination of pea plants (*Pisum sativum* L.) as starting material and the high reproducibility of the procedure readily and cheaply yields reliable intact chloroplasts for photosynthetic studies.

Isolated chloroplasts exhibit different capacities for photosynthesis, depending on the preservation of their integrity during the isolation procedure (Lilley *et al.*, 1975). For most studies a homogeneous population of type A chloroplasts (Hall, 1972) capable of high rates of CO₂ fixation is preferred. This can be achieved by isolating them from protoplasts (Leegood & Walker, 1983), but, in general, mechanically isolated chloroplasts are preferred for several reasons: simplicity of isolation, stability, short time requirement, low cost. Apart from being restricted to a small number of species (mainly spinach and peas), the principal drawback of the mechanical method is the variability of degree of intactness and activity from preparation to preparation (Walker, 1980). Nakatani & Barber (1977) have largely overcome the problem of intactness by using ion-free isolation media, but the resulting preparations are very unstable and of variable activity (Jensen, 1979; Z. G. Cerović & M. Plesničar, unpublished work).

In the present communication we describe an isolation procedure yielding reproducibly and reliably uniform chloroplasts of high degree of intactness and photosynthetic capacity, with the variability between preparations restricted to that of the parental tissue.

Experimental

We have used pea shoots (*Pisum sativum* L. cv. Petit Provençal) because of the ease and low cost of growth in all seasons. Seeds were germinated for 3 days in the dark at 100% humidity and 25°C; then the plants were transferred to perforated trays, floating on tap water, and grown under controlled conditions: 11 h of light, average photosynthetic photon-flux density 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 16 or 23°C,

and 13 h of dark at 12°C. Before being harvested, plants were pre-illuminated for 20 min after a prolonged dark period of 36 h.

The isolation proper was a modification of Walker's (1964, 1980) method and proceeded as follows. The harvested pea shoots were immediately dipped into ice/water in a beaker covered with a piece of cheesecloth. When the last shoot had been harvested, they were withdrawn from the ice/water in the cheesecloth, which was then shaken to remove the excess of water. Plant material (50 g) was immersed in 200 ml of grinding medium, which had previously been brought to the consistency of melting snow (the medium was stirred in chilled antifreeze at about –15°C until it became a semi-frozen slush). A short grinding for 2–6 s followed. The grinding time depended on the apparatus employed: 2 s at about 12000 rev./min was appropriate for the Polytron (Kinematica), 3–4 s at maximal speed (20000 rev./min) for the Ultra-Turrax (Janke und Kunkel) and 5–6 s (three bursts of 2 s) in the kitchen blender (Girmi-Gorenje).

The brei prepared by one of these alternative grinding procedures was then squeezed through two layers of muslin and filtered through eight layers of muslin and a layer of cotton-wool. Chloroplasts were quickly separated from the supernatant by centrifugation in four tubes at 2000 g for 100 s (20 s at full speed).

A new low-cation medium was used both for grinding and washing. It contained 343 mM-sorbitol, 0.4 mM-KCl, 0.04 mM-EDTA and 2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.8, and was prepared daily by diluting the stock solution (containing 660 mM-sorbitol, 20 mM-KCl, 2 mM-EDTA and 100 mM-Hepes buffer, pH 7.9) 50-fold with 330 mM-sorbitol.

Table 1. *Characteristics of the isolated chloroplasts*

Reaction conditions were as described in the Experimental section. Data represent means $\pm$ S.D. for ten preparations.

Grinding apparatus	Day temperature (°C)	Intactness (%)	Rate of CO ₂ -dependent O ₂ evolution (intact chloroplasts) (mmol of O ₂ /s per mol of chlorophyll)	Rate of ferricyanide-dependent O ₂ evolution (envelope-free chloroplasts) (mmol of O ₂ /s per mol of chlorophyll)	Chloroplast efficacy
Kitchen blender	23	90 $\pm$ 2	36 $\pm$ 6	106 $\pm$ 12	0.34
Polytron	23	94 $\pm$ 2	39 $\pm$ 4	105 $\pm$ 11	0.37
Ultra-Turrax	16	92 $\pm$ 2	43 $\pm$ 5*	89 $\pm$ 10	0.48

* In old units: 172 $\pm$ 20 μ mol of O₂/h per mg of chlorophyll.

The low cation content of the medium was achieved by lowering the buffer concentration to a minimum instead of using two buffers of different pK_a (Nakatani & Barber, 1977). This allowed the use of Hepes, which is preferred to Tris (Good *et al.*, 1966), from the start.

The clean final pellet of chloroplasts was resuspended in 0.5–1.0 ml of resuspending medium (containing 330 mM-sorbitol, 10 mM-KCl, 1 mM-EDTA, 1 mM-MgCl₂, 1 mM-MnCl₂, 1% bovine serum albumin and 50 mM-Hepes buffer, pH 7.9) with the careful use of a very small paint brush, or by rolling the tubes on ice. The final concentration of chlorophyll was usually about 2 mM. The inclusion of Mg²⁺ and Mn²⁺ ions in the resuspending medium is essential for the stability of the preparation (Jensen, 1979; Z. G. Cerović, unpublished work). Orthophosphate (10 mM) can also be included in order to compensate for its efflux from chloroplasts during storage (Mourioux & Douce, 1981). Chloroplasts usually retained 80% of their initial activity after 5 h standing at 4°C.

CO₂-dependent O₂ evolution by intact chloroplasts was measured polarographically (Hansatech oxygen electrode) at 20°C in a reaction mixture containing 330 mM-sorbitol, 10 mM-KCl, 1 mM-EDTA, 50 mM-Hepes buffer, pH 7.9, 1 mM-ATP, 5 mM-pyrophosphate, 0.2 mM-orthophosphate and 33 μ M-chlorophyll (final volume 1 ml). For ferricyanide-dependent O₂ evolution, intact chloroplasts were osmotically shocked for 15 s in 10 mM-MgCl₂ and then O₂ evolution was measured in the presence of 330 mM-sorbitol, 10 mM-KCl, 1 mM-EDTA, 50 mM-Hepes buffer, pH 7.9, 5 mM-MgCl₂, 2.5 mM-ferricyanide, 5 mM-NH₄Cl and 33 μ M-chlorophyll. The photosynthetic photon-flux density was 1000 μ mol $\cdot$ m⁻² $\cdot$ s⁻¹.

Results and discussion

As shown in Table 1, the procedure described procured a reproducibly high degree of intactness, measured as ferricyanide-dependent O₂ evolution before and after an osmotic shock (Heber &

Santarius, 1970), independently of the apparatus used for grinding. The rates obtained for three types of preparation from plants that were grown under the same conditions were not significantly different. It is the low-cation medium and centrifugation that allow for selective separation of intact chloroplasts, independently of their content in the brei after grinding. However, more-efficient apparatus such as the Polytron is preferred, not only because it gives a higher yield and slightly higher degree of intactness, but also because the variability in rates of CO₂-dependent O₂ evolution is the same as the variability in electron-transport rates (10%). This indicates an absence of type-A₂ chloroplasts (Lilley *et al.*, 1975), i.e. re-sealed chloroplasts, from the preparation. The achieved high degree of chloroplast intactness and small degree of variability due to the preparation procedure itself allows the effect of plant growth conditions on chloroplast characteristics to stand out (Table 1). The growth of plants at lower temperature, in combination with high light-intensity, as stressed before (Walker, 1980), increases the rate of CO₂ fixation expressed on a chlorophyll basis, but, what is even more important, it increases considerably the chloroplast efficacy. The chloroplast efficacy, defined here as the ratio between the rate of CO₂ fixation and that of uncoupled electron transport, is independent of the basis for rate expression.

We have tested the described isolation procedure on spinach grown in water culture and three varieties of peas grown on vermiculite, and chloroplasts of the same high characteristics were obtained. The reproducibility and reliability, in combination now with the already mentioned simplicity, speed and low cost of mechanical isolation of chloroplasts, should allow an extended use of good-quality chloroplasts in studies, not only of carbon metabolism, but also of primary reactions of photosynthesis.

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