Ultrastructural study of chloroplasts isolated by various fractionation methods

J. LEGOCKA, A. WOŹNY and A. SZWEYKOWSKA

Laboratory of General Botany, Institute of Biology,
Adam Mickiewicz University, Poznań, Poland

(Received: April 12, 1975)

Abstract

The chloroplasts of kohlrabi and barley leaves isolated in conventional media and examined in electron microscope showed considerable impairments of their structure. The damages were not eliminated if the centrifugation number was limited and the tonicity of the medium was reduced gradually throughout the fixation and dehydration procedure. A good yield (about 90%) of structurally intact kohlrabi chloroplasts was obtained when they were isolated from leaves pre-fixed and homogenized in medium containing glutaraldehyde.

INTRODUCTION

During several past years plastids were objects of many investigations. For structural as well as physiological and biochemical studies they should be in a state as intact as possible (type A, according to nomenclature of Hall, 1972). The intactness of isolated chloroplasts is also an essential condition for their *in vitro* culture.

The electron microscopy studies carried out in our laboratory on isolated plastids prepared by several methods showed that these organelles were considerably impaired. Some investigators (e.g. Yoshida, 1969, Ridley and Leech, 1970) suggested that these damages of plastids were not due to isolation procedure but originated during the preparation for electron microscopy. The purpose of this study was to investigate the effect of various aqueous isolation media, of purification procedure and of the fixation moment for electron microscopy on structure preservation of chloroplasts.

MATERIAL AND METHODS

The material used in this study were mature kohlrabi leaves (*Brassica oleracea* L. var. *gongylodes* L.) and 10-day-old barley leaves (*Hordeum vulgare* L.). The leaves were destarched in dark for 24 hours before chloroplast isolation.

Following media were used for the isolation of chloroplasts:

STm : sucrose 0.4 M, Tris-HCl 0.05 M (pH 7.8), $MgCl_2$ 0.01 M, KCl 0.01 M and 2-mercaptoethanol 4 mM (Spencer and Whitfeld, 1967).

Hm : sucrose 0.25 M, Tris-HCl 0.025 M (pH 7.8), $MgCl_2$ 0.01 M, mercaptoethanol 4 mM, ficoll 2.5% and dextran 5% (Honda et al., 1966).

SPm : sucrose 0.4 M, phosphate buffer 0.15 M, pH 7.3 (Leech, 1964, modified).

SGm : sucrose-glutaraldehyde-buffer solution, containing 10 ml of 0.4 M sucrose, 10.5 ml of 0.4 M phosphate buffer (pH 6.5) and 6.5 ml of 25% commercial aqueous glutaraldehyde (Sabatini, 1963).

The leaf blades were washed with water, cut into small fragments (midribs removed) and carefully homogenized in cold mortar with STm, Hm, or SPm medium, resp. The crude suspension was filtered through 4 layers of gauze and centrifuged at $1000 \times g$ for 5 min. The pellets were designated according to the medium used as STm, Hm, and SPm pellet, resp.

The STm and Hm pellets were subjected to a purification procedure. They were washed by resuspending in 10 ml of the corresponding medium and centrifuged at $1000 \times g$ for 5 min. Each pellet was again suspended in medium, layered over a discontinuous sucrose gradient (20%, 30%, 45% and 60% in the same medium) and centrifuged at $1000 \times g$ for 10 min. The chloroplast fraction was carefully collected with Pasteur pipette (usually from 45% (60% sucrose layer) and sedimented at $3000 \times g$ for 10 min. The purified chloroplast fractions were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 45% sucrose, for 2 h at 4° C.

Another procedure consisted in using crude STm, Hm and SPm pellets. Each of them was divided in two parts. One of them was immediately fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.4 M sucrose, for 2 h at 4° C. The second part was first embedded in 1.5% agar according to Preston et al. (1972), cut into small fragments and then fixed in glutaraldehyde for 24 h at 4° C.

The fixed preparations were washed three times with 0.1 M phosphate buffer, pH 7.4, containing sucrose at 30%, 25% and 15%, resp., for STm and Hm purified fractions, or at 15%, 10% and 5%, resp., for STm, Hm and SPm crude fractions (Wellburn and Wellburn, 1971). They were centrifuged each time at $1000 \times g$ for 5 min., or — in the case of fractions embedded in agar — the solutions were each time decanted.

Post-fixation was carried out for 2 h in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 (containing 15% sucrose for STm and Hm purified fractions). The preparations were then washed three times with the phosphate buffer in the case of crude chloroplast fractions and with the same buffer containing sucrose at 15%, 10% and 5%, resp., in the case of purified fractions. All fractions were then embedded in agar (except of those which were embedded previously).

SCHEME FOR ISOLATION OF CHLOROPLASTS

The third procedure consisted in homogenizing fresh leaves, or leaves preliminary fixed for 3 days in 5% glutaraldehyde, in a mortar with SGm buffer. The homogenate was filtered and centrifuged at $350 \times g$ for 10 min. to remove debris. The supernatants were spun at $1000 \times g$ for 10 min. to collect the chloroplast fractions. The pellets were washed with phosphate buffer + sucrose, similarly as in the case of STm, Hm

and SPM crude chloroplast fractions, post-fixed in osmium tetroxide, rinsed with phosphate buffer and embedded in agar.

All fixed preparations were dehydrated with alcohol and acetone series and embedded in Epon 812. Ultrathin sections were cut using Tesla BS 490 or LKB Ultratome III ultramicrotomes and contrasted with uranyl acetate and next with lead citrate (Reynolds, 1963). Photographs were taken in JEM 7A electron microscope.

The control material constituted electron micrographs prepared from intact leaf tissue fixed in the same way as experimental variants described above, with the omission of sucrose in the fixation mixture.

RESULTS AND DISCUSSION

The chloroplasts from kohlrabi and barley leaves, prepared by various methods, were submitted to electron microscope analysis. Comparison of electronograms of isolated chloroplasts with those of chloroplasts *in situ* (control material, figs 1 and 2) showed that the per cent of damaged kohlrabi chloroplasts was highest (about 90%) in fractions obtained with the use of the complete purification procedure. As a rule the plastids had no envelopes, their stromas were electron transparent, so that only a "framework" of thylakoids seemed to be present (Figs. 3—5). The results were similar whether STM or Hm (Honda medium) were used, although the Honda medium has been recommended by several authors as particularly protecting the structure of isolated chloroplasts. The only difference between the two media was a granular coating (probably of ficoll and dextran particles) around the chloroplasts from the Hm medium. To eliminate the possibility that the organelles were damaged during their purification, this step was omitted and the crude pellets of chloroplasts isolated with the use of buffers STM and Hm for kohlrabi or SPM for barley were directly fixed with glutaraldehyde. However, as can be seen from the electronograms on Figs. 6 and 8, most of the plastids were again damaged. Also a reduction of the centrifugation number and slowing down of the fixative penetration by embedding the samples in agar did not decrease the relative number of impaired chloroplasts. The occurrence of some intact chloroplasts, that occasionally could be seen, was probably a result of presence of fragments of cell walls in direct vicinity of plastids, which could have a protecting role during the isolation process (Figs. 7 and 9). However, also in these cases the structure of chloroplasts was different from that of chloroplasts observed *in situ* (presence of a great number of swollen thylakoids, Fig. 9).

The results indicated that the main damages of organelles were due to homogenization, i.e. they originated during the release of the chloroplasts from cells. It seemed thus that a strengthening of the chloroplast

Plate I

Fig. 1. Chloroplast in kohlrabi mesophyll cell (control material). Leaf tissue fixed in glutaraldehyde and OsO_4 . $\times 24\,000$.

Fig. 2. Chloroplast in barley mesophyll cell (control material). Leaf tissue fixed in glutaraldehyde and OsO_4 . $\times 28\,000$.

Plate II

Fig. 3. Chloroplast fraction from kohlrabi leaves, isolated in sucrose — Tris medium (STm) and purified in sucrose gradient. Fixation in glutaraldehyde and OsO_4 .
 $\times 14\,850$

Plate III

Figs. 4—5. Chloroplast fraction from kohlrabi leaves, isolated in Honda medium (Hm) and purified in sucrose gradient. Fixation in glutaraldehyde and OsO_4 . 4 — $\times 7750$. 5 — $\times 28\,800$

Plate IV

Figs 6—7. Crude chloroplast fraction from kohlrabi leaves, isolated in sucrose — Tris medium (STm). Fixation in glutaraldehyde and OsO_4 . 6 — $\times 10\,250$, 7 — $\times 45\,320$

Plate V

Figs. 8—9. Crude chloroplast fraction from barley leaves, isolated in sucrose — phosphate medium (SPm). Fixation in glutaraldehyde and OsO_4 . 8 — $\times 11\,670$,
9 — $\times 7230$

Plate VI

Figs. 10—11. Chloroplast fraction from kohlrabi leaves fixed in glutaraldehyde during the homogenization and isolation process in sucrose — glutaraldehyde — phosphate medium (SGm). Postfixation in OsO_4 . 10 — $\times 4200$, 11 — $\times 15\,000$

Fig. 12. Chloroplast fraction isolated in sucrose — glutaraldehyde — phosphate (medium (SGm) from leaves prefixed in glutaraldehyde. Postfixation in OsO_4 .
 $\times 11\,180$.

structure by fixing them with glutaraldehyde during or even before the isolation might be important for increasing the number of intact chloroplasts.

About 50% of intact and apparently normal plastids were obtained with the use of fixation during the homogenization process of kohlrabi leaves. The remaining 50% were plastids in different stages of damage (up to the presence of only a "framework" of thylakoids, Fig. 10). The best results were obtained when chloroplasts were isolated from prefixed material (Fig. 12). In this case about 90% of plastids showed an apparently normal structure with all typical features of kohlrabi chloroplasts *in situ*, as described previously by Młodzianowski and Kwintkiewicz (1973) and shown in Fig. 1. They were spindle shaped and their internal structure showed differentiation into thylakoids of grana and stroma. The number of compartments per granum amounted in some cases to 20 or even more, but in most cases grana were composed of 4—10 compartments. A large number of ribosomes and occasionally small but numerous plastoglobules occurred in the stroma. Plastids with 3—6 nucleoid-like regions were frequently observed. A similar result was obtained by Yoshida (1969) with chloroplasts isolated from wheat and by Sprey (1973) with etioplasts from barley.

On the basis of the experiments described above one can conclude that isolation of kohlrabi and barley chloroplasts in conventional media with the use of gentle homogenization method and a proper tonicity of the solution throughout the fixation procedure does not prevent them from being damaged in about 90%. The traditionally used media did not show to be satisfactory for the preservation of chloroplast structure. On the other hand, the fixation with glutaraldehyde prior to or during the isolation process preserved the structure of chloroplasts quite efficiently and made the chloroplasts more resistant to the isolation procedure.

Acknowledgement

The authors express their thanks to Dr. Fortunat Młodzianowski for preparing photographs in Figs. 1 and 2.

REFERENCES

- Hall D. O., 1972. *Nature New Biology* 235: 125—126.
Honda S. I., Honglandarom T., Laties G. G., 1966. *J. Exp. Bot.* 17: 460—473.
Leech R. M., 1964. *Biochim. Biophys. Acta* 79: 637—639.
Młodzianowski F., Kwintkiewicz M., 1973. *Protoplasma* 76: 211—226.
Preston J. F., Parenti F., Eisenstadt J., 1972. *Planta* 107: 351—367.
Reynolds E. S., 1963. *J. Cell Biol.* 17: 208—212.
Ridley S. M., Leech R. M., 1970. *Nature* 227: 463—465.

- Sabatini D. D., Bensch K., Barnett R. J., 1963. *J. Cell Biol.* 17: 19—58.
Spencer D., Whitfield P. R., 1967. *Arch. Bioch. Biophys.* 121: 336—345.
Sprey B., Gietz N., 1973. *Z. Pflanzenphysiol.* 68: 397—414.
Wellburn A. R., Wellburn F. A., 1971. *J. Exp. Bot.* 23: 972—979.
Yoshida Y., 1969. *Plant Cell Physiol.* 10: 555—561.

Author's address:

Prof. dr Alicja Szwejkowska,

mgr Jolanta Legocka,

mgr Adam Woźny

Institute of Biology

Adam Mickiewicz University

Al. Stalingradzka 14; 61-713 Poznań; Poland

*Badanie ultrastruktury chloroplastów izolowanych przy pomocy
różnych metod frakcjonowania*

Streszczenie

Analizowano ultrastrukturę chloroplastów izolowanych z liści kalarepy i jęczmienia przy użyciu konwencjonalnych środowisk wodnych. Pomimo zastosowania łagodnych metod homogenizacji liści (mieszanie w moździerzu), właściwej toniczności roztworów w czasie procedury utrwalania oraz ograniczenia liczby wirowań i zwolnienia tempa penetracji utrwalacza przez zatopienie frakcji w agarze, uzyskane chloroplasty były silnie uszkodzone. Zastosowanie aldehydu glutarowego przed lub w czasie procesu izolacji wzmacniało strukturę chloroplastów i czyniło je bardziej odpornymi podczas uwalniania z komórki, co pozwalało uzyskać około 90% chloroplastów w stanie nie uszkodzonym.