Biochimica et Biophysica Acta, 461 (1977) 510—512

BBA Report

BBA 41298

AN IMPROVED METHOD FOR ISOLATING CHLOROPLASTS RETAINING THEIR OUTER MEMBRANES

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(Received April 4th, 1977)

Summary

A new method of obtaining chloroplasts retaining their outer membranes is described. It is shown that the use of low cation media enhances the separation of intact and broken chloroplasts.

It is now quite clear that in order to gain a full description of both the light and dark reactions of photosynthesis it is necessary to understand the role of the chloroplast outer membranes, often called the envelope, in controlling the composition of the stroma [1,2]. The chloroplast envelope acts as a barrier to the free diffusion of enzymes, metabolites, nucleic acids and inorganic ions [1—5] as well as possessing specific translocators [4]. When isolated as an intact organelle, the chloroplast can reduce CO_2 at rates comparable to those observed with intact leaves and algae [5] and shows other photophysical phenomena seen with leaves but not normally seen with isolated chloroplasts which have lost their outer membranes. Clearly studies with isolated chloroplasts retaining their outer membranes are of considerable importance for relating both stromal and thylakoid associated phenomena with the normal photosynthetic activity of an intact system.

Over the years, a number of procedures have been developed for isolating intact chloroplasts from a variety of materials [6]. The criterion for the percentage intactness has been a comparison of the uncoupled rate of ferricyanide reduction before and after subjecting the preparation to an osmotic shock [7,8]. Since the chloroplast envelope is impermeable to ferricyanide, only the broken chloroplasts in a suspension will be able to reduce this electron acceptor. This test has revealed that most preparations always contain mixed populations of broken and intact chloroplasts [9] with a yield of intactness very rarely exceeding 80% and usually somewhat lower. The existence of mixed populations of chloroplasts in the experimental suspension has distinct disadvantages for many types of studies. In this communication

we report a new isolation procedure which regularly yields close to 100% intact chloroplasts as judged by the ferricyanide test.

We have used pea leaves (Feltham First) germinated and grown in vermiculite for about a week on a 12-h light-dark cycle and also used market spinach. The leaves were harvested and macerated in an ice-cold slurry of buffered sorbitol using a Polytron (type PT 35 0D) with the standard attachment (PT 35/2 0D) and speed setting of 5.5. A ratio of 35 g of leaves per 100 ml of buffer was routinely used and the buffer consisted of 0.33 M sorbitol, 0.2 mM $MgCl_2$, 20 mM MES (2(*N*-morpholino)ethane sulphonic acid) brought to pH 6.5 with tris(hydroxymethyl)aminomethane. The resulting slurry was filtered through 10 layers of muslin with the first two layers of muslin separated by a thin layer of cotton wool. The filtrate was then centrifuged in an MSE bench top centrifuge at 2200 × *g* for 30 s and the total time allowed for reaching the maximum speed and for hand braking being less than 90 s. The supernatant was discarded and most of the soft pellet was removed with the supernatant. The remaining pellet was routinely found to consist of 75% or more of chloroplasts retaining their outer membranes. However, if this pellet was resuspended in a cation free medium, then after centrifugation there was a regular and significant increase in the percentage intactness of the preparation. The procedure was to suspend the pellet in 0.33 M sorbitol brought to pH 7.5 with Tris base (approximately 0.5 mM) and centrifuged at 2200 × *g* for 20 s with the total time allowed for reaching maximum speed and for hand braking being about 60 s. The supernatant was aspirated, and the soft portion of the pellet was carefully removed and discarded before finally suspending the pellet in a small volume of the cation free medium. Fig. 1 shows typical traces of oxygen evolution measured in the presence of ferricyanide before and after osmotic shock. With this preparation the intactness corresponded essentially to 100%. Regularly preparations of 90% or greater intactness were obtained by this method. The supernatant which was normally discarded was often found to contain 50% intact chloroplasts and again if these mixed chloroplasts were resuspended in the cation free medium then after centrifugation a pellet could be obtained corresponding to approximately 85% intactness. We have studied CO_2 and phosphoglycerate-dependent O_2 evolution with chloroplasts isolated by the above procedure and obtained rates greater than 200 μmol O_2/mg chlorophyll per h. This would indicate that these preparations do not contain a significant amount of chloroplasts with resealed envelopes [10].

Although the success of the above method for consistently producing chloroplast preparations of high percentage intactness does depend on the maceration, filtration and centrifugation procedures, as also found by others, the use of a cation free medium is of prime importance. When the salt content of the medium is raised, a greater proportion of the pellet contains broken chloroplasts. It seems that with low cation media there is a better centrifugal separation of intact and broken chloroplast which almost certainly reflects differences in the surface charges on the envelope and exposed

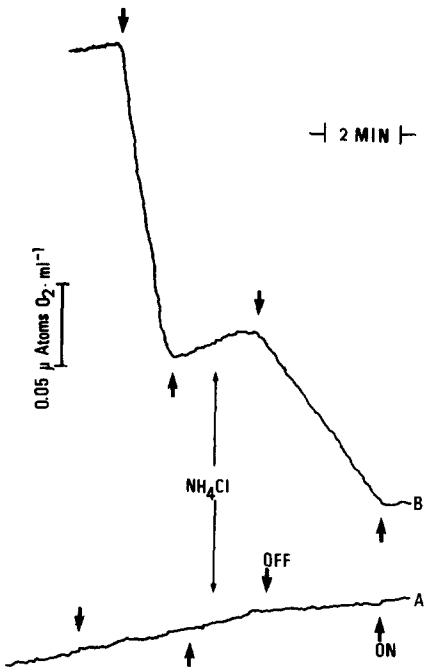

Fig. 1. Ferricyanide-dependent O_2 evolution by intact (A) and envelope-free (B) chloroplasts. 2.5 mM NH_4Cl was added during the dark period. Assay medium contained 0.33 M sorbitol, 1 mM $MgCl_2$, 50 mM HEPES/KOH, pH 7.6, 2 mM EDTA, 20 μg chlorophyll/ml and 1.0 mM potassium ferricyanide.

thylakoid membranes of the broken chloroplasts. It is these charges which control changes in membrane adhesion or stacking processes under different ionic conditions [11].

Acknowledgements

We wish to thank the Science Research Council and the EEC Solar Energy Research and Development Programme for financial support. We also are grateful for fruitful discussions with Dr A. Telfer and Professor P. Thornber and in particular to Professor D.A. Walker, for his assistance with the CO_2-dependent O_2 evolution studies.

References

1 Barber, J. (1976) in The Intact Chloroplast, Topics in Photosynthesis. (Barber, J., ed.) Vol. 1, pp. 89—134, Elsevier, Amsterdam
2 Heber, U. (1974) Ann. Rev. Plant Physiol. 25, 393—421
3 Ellis, R.J. (1976) in The Intact Chloroplast, Topics in Photosynthesis. (Barber, J., ed.) Vol. 1, pp. 335—364, Elsevier, Amsterdam
4 Heldt, H.W., Fliege, R., Lehner, K., Milovancev, M. and Werdan, K. (1974) in Proc. 3rd Int. Cong. Photosynthesis (Avron, M. ed.) pp. 1369—1379, Elsevier, Amsterdam
5 Walker, D.A. (1974) in Med. Tech. Publ. Int. Rev. Sci. Biochem. (Northcote, D., ed.) Ser. 1, Vol. II, pp. 1—49, Butterworth, London
6 Walker, D.A. (1971) in Methods in Enzymology (San Pietro, A. ed.) Vol. 23, pp. 211—220, Academic Press
7 Cockburn, W., Baldry, C.W. and Walker, D.A. (1967) Biochim. Biophys. Acta. 143, 614—624
8 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch 25b, 718—728
9 Hall, D.O. (1972) Nature 235, 125—126
10 Lilley McC. R., Fitzgerald, M.P., Rienits, K.G. and Walker, D.A. (1975) New Phytol. 75, 1—10
11 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174—181