Title	Isolation of Physiologically Intact Chloroplasts from Euglena gracilis z
Author(s)	SHIGEOKA, Shigeru; YOKOTA, Akiho; NAKANO, Yoshihisa; KITAOKA, Shozaburo
Editor(s)	
Citation	Bulletin of the University of Osaka Prefecture. Ser. B, Agriculture and biology. 1980, 32, p.37-41
Issue Date	1980-03-31
URL	http://hdl.handle.net/10466/3138
Rights	

Bull. Univ. Osaka Pref., Ser. B, Vol. 32,
大阪府立大学紀要, 農学・生物学, 1980

Isolation of Physiologically Intact Chloroplasts from *Euglena gracilis* z

Shigeru SHIGEOKA, Akiho YOKOTA, Yoshihisa NAKANO, and shozaburo KITAOKA

Nutrition Laboratory, College of Agriculture

(Received October 31, 1979)

Abstract

Chloroplasts were isolated from *E. gracilis* z grown on a vitamin B_{12}-limiting medium. The procedure is consisted of partial trysin digestion of the *Euglena* cells, subsequent mechanical disruption of the cells and collection of chloroplasts by differential centrifugation. The isolated chloroplasts fixed CO_2 at a rate of 20 μmoles of CO_2 per mg chlorophyll per hr, corresponding to one-third of the photosynthetic activity of the original cells. Linear sucrose density gradient centrifugation of the crude cell homogenate obtained by the digestive method shows that chlorophyll and most of the activity of ribulose biphosphate carboxylase cosediment as only one peak to the fraction corresponding to an equilibrium density of 1.165 g/cm^3. Based on these results we disignate the chloroplasts isolated by the digestive method as the "intact" chloroplasts.

Introduction

Difficulties of isolating intact chloroplasts from photosynthetic organisms have hindered developments of biochemistry in such cells. While chloroplasts have been isolated intactly from higher plants by a few workers,[1,2] no one has been able to obtain physiologically intact chloroplasts from photosynthetic microorganisms, such as *Euglena* and algae.[3]

We have already established a method for the isolation of mitochondria with satisfactory respiratory control and nearly theoretical P/O ratio from *E. gracilis*.[4] The method of preparation of fuctionally intact chloroplasts from *E. gracilis*, described in the present paper, is an extention of the mitochondrial isolation method.

Materials and Methods

Organism and culture. E. gracilis z was cultured photoheterotrophically under vitamin B_{12}-limiting conditions according to Tokunaga et al.[4]

Preparation of chloroplasts. A partial trypsin digestion of pellicle, cell membrane of *E. gracilis*, and subsequent mild mechnical destruction of the cells having digested pellicle were employed according to the method of Tokunaga et al[4] with some modifications.

Euglena cells (6 g, wet basis) were suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.38 M mannitol and digested with 20 mg of trypsin (Sigma, type III) at 0°C for 1 hr with gentle stirring. The end point of the digestion was determined by watching the extent of cell disintegration microscopically. The digestion mixture was centrifuged at 250 × g for 3 min to collect unbroken cells. The cells were resuspended in 5 ml of 25 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.33 M mannitol and 10 mg trypsin inhibitor and the

suspension was stirred gently for 10 min with magnetic stirrer. The homogenate was centrifuged at 250 X g for 3 min to remove unbroken cells and paramylon and the supernatant was carefully decanted and centrifuged at 1,000 X g for 3 min to sediment chloroplasts. The pellet was gently suspended in the HEPES buffer containing 0.33 M mannitol with the use of a very flexible and fine brush and again centrifuged at 1,000 X g for 3 min. The washed chloroplasts were resuspended in the same buffer at a concentration of about 200 μg chlorophyll per ml.

Linear sucrose density gradient centrifugation. The cell homogenate obtained by the above method was subjected to linear sucrose density gradient centrifugation as described previously.[5]

Photosynthetic CO_2 fixation and ferricyanide reduction. Photosynthetic CO_2 fixation and ferricyanide reduction were measured following the procedures reported by Forsee and Kahn[6] and Neumann and Drechsler,[7] respectively.

Enzyme assays. Succinic semialdehyde dehydrogenase and glucose-6-phosphatase were assayed by the methods of Tokunaga et al[8] and de Duve et al[9], respectively. The activity of glutamate dehydrogenase was measured according to Corman and Inamoar.[10] Ribulose biphosphate ($Ru\text{-}P_2$) carboxylase was assayed by the method of Rabinowitz et al.[11]

Chlorophyll and protein were determined by the methods of Mackinney[12] and Lowry et al[13], respectively.

Results and Discussion

Table I shows photosynthetic activities of chloroplasts isolated from *E. gracilis* by the digestive method. The chloroplasts obtained were capable of fixing CO_2 at the rate of 20 μmoles of CO_2 per mg chlorophyll per hr. This photosynthetic activity corresponded to one-third of that of the whole cells used for separation of chloroplasts. In addition, ferricyanide was not reduced by the chloroplasts at all indicating that this compound did not penetrate into the organelles. When incubated in the reaction mixture free of mannitol, the chloroplasts reduced ferricyanide. The term, "intactness", is bestowed on chloroplasts which fix CO_2 actively and into which ferricyanide does not penetrate.[14,15] Accordingly, chloroplasts isolated from *Euglena* in the present experiments met these requirements.

Table I. Photosynthetic activities of isolated chloroplasts

CO_2 fixation[a)]	19.8 μmol/mg chlorophyll/hr
CO_2 fixation of original cells	60.0 μmol/mg chlorophyll/hr
Ferricyanide reduction	Not detected

a), $RU\text{-}P_2$ carboxylase activity; 127.4 μ mol/mg chlorophyll/hr.

Chloroplasts are divided into six types depending on the integrity of the organelle; from type A, complete chloroplasts, to type F, sub-chloroplast particles.[14] The chloroplasts isolated in the present works is designated as type A chloroplasts because of active CO_2 fixation and ferricyanide-resistance.

Integrity of isolated chloroplasts was also examined by linesr sucrose density gradient centrifugation. If the isolated chloroplasts are undamaged, a stromal enzyme, $Ru\text{-}P_2$ carboxylase, must be held in chloroplasts which migrate in a sucrose density gradient when the cell homogenate obtained by the digestive distruction of *Euglena* cells is applied to

sucrose density gradient centrifugation. In Fig.1, the cell homogenate free of unbroken cells and paramylon were subjected to 30-45% linear sucrose density gradient centrifugation. Succinic semialdehyde dehydrogenase, a marker enzyme for mitochondria[16], gave a sharp peak of the activity in Fraction 5, corresponding to an equlibrium density of 1.198 g/cm^3. A peak of the activity of a microsomal marker enzyme, glucose-6-phosphatase,[9] was seen at an equilibrium density of 1.13 g/cm^3. Glutamate dehydrogenase which is located solely in the cytosol in *E. gracilis*[16] showed the activity in Fractions 28 to 38. Chlorophyll showed only one peak at an equlibrium density of 1.165 g/cm^3. This means that none of chloroplasts are broken when isolated from *Euglena* cells. The major peak of the activity of Ru-P_2 carboxylase was present in the same fractions as those of chlorophyll. About 80% of the enzyme activity applied to the gradient centrifugation was recovered in the chloroplast fractions. The remaining activity was seen in the cytosol supernatant. These results indicate that chloroplasts released from *Euglena* cells maintain most of the stromal enzyme and that the digestive method employed in the present experiments is excellent for obtaining chloroplasts without damages. Salisburg et al[17] have reported the separation of chloroplasts of *Euglena* by isopycnic sedimentation in density gradient of Ludox AM, a silica sol. However, four chlorophyll-containing fractions were separated when crude chloroplasts were sedimented into the silica gradient.

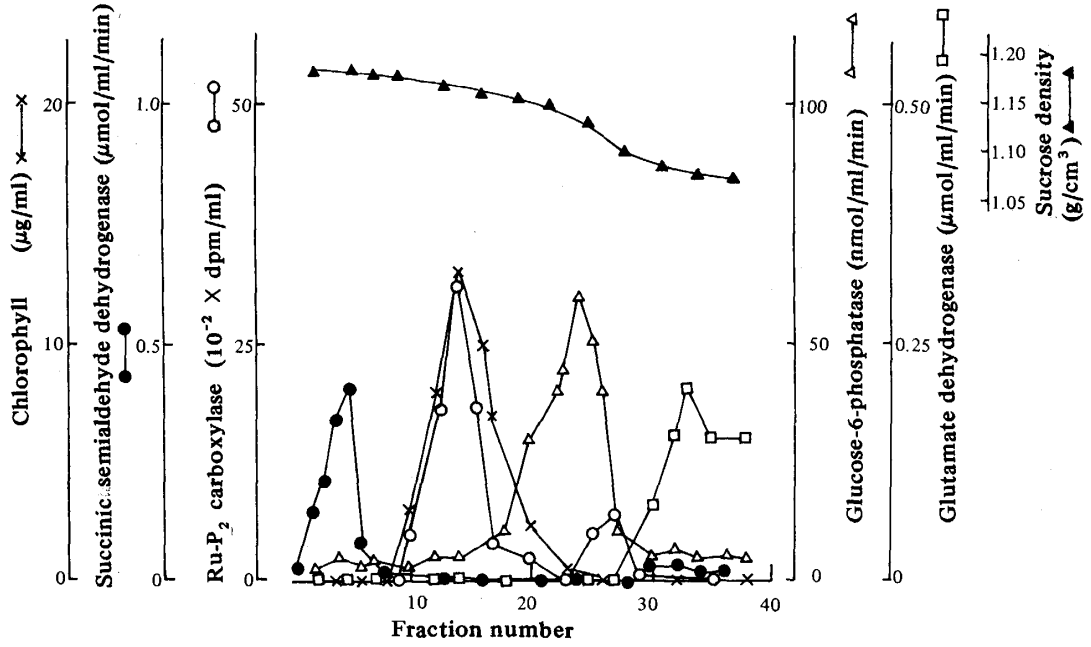

Fig. 1. Separation of chloroplasts isolated from *E. gracilis* by the digestive method on linear sucrose density gradient

It is known that individual cell organelles have their own osmotic pressures. The simultaneous isolation of various cell organelles has been thought to be difficult. In fact, subcellular localization of glyoxylate aminotransferase of rat liver changes depending on the sucrose concentration of the tissue-homogenizing medium.[18,19] In the present experiments, however, mitochondria was also isolated intactly, as shown by the distribution of the activity of succinic semialdehyde dehydrogenase on the sucrose gradient, in spite of the employment of a mannitol concentration suitable to chloroplasts (Fig.1). This may

indicate that preservation of intactness of cell organelles is not almost dependent on osmotic pressure of isolation medium. Mildness of cell disruption must be essential for the preservation, and the present digestive method satisfies this demand. The simultaneous isolation of chloroplasts and mitochondria has been used for the studies of subcellular distribution of enzymes in our laboratory.[20-22]

Acknowledgement: The authors thank Mr. Y. Isegawa for his technical assistance.

References

1) TAKABE, T., NISHIMURA, M. and AKAZAWA, T. (1979). Isolation of intact chloroplasts from spinach leaf by centrifugation in gradient of the modified silica "Percoll". *Agric. Biol. Chem.*, **43**, 2137-2142.
2) ROBINSON, S. P. and WALKER, D. A. (1979). Rapid separation of the chloroplast and cytoplasmic fractions from intact leaf protoplasts. *Arch. Biochem. Biophys.*, **196**, 319-323.
3) MIYACHI, S. (1976). Chloroplasts. In: "Mehtods in plant enzymes and proteins", (ed. by MORITA, U., SHIN, M., ASADA, K. and IDA, S.). Kyoritsushuppan, Tokyo, 64-69.
4) TOKUNAGA, M., NAKANO, Y. and KITAOKA, S. (1976). Preparation of physiologically intact mitochondria from *Euglena gracilis* z. *Agric. Biol. Chem.*, **40**, 1439-1440.
5) YOKOTA, A. and KITAOKA, S. (1979). Occurrence of glycolate dehydrogenase in mitochondria and microsomes in streptomycin-bleached mutant of *Euglena gracilis* z. *Agric. Biol. Chem.*, **43**, 855-857.
6) FORSEE, W. T. and KAHN, J. P. (1972). Carbon dioxide fixation by isolated chloroplasts of *Euglena gracilis*. 1. Isolation of functionally intact chloroplasts and their characterization. *Arch. Biochem. Biophys.*, **150**, 296-301.
7) NEUMANN, J. and DRECHSLER, S. (1967). Inhibition of photoinduced electron transport and related reactions in isolated chloroplasts by phenol. *Plant Physiol.*, **42**, 573-577.
8) TOKUNAGA, M., NAKANO, Y. and KITAOKA, S. (1976). Separation and properties of the NAD–linked and NADP-linked isozymes of succinic semialdehyde dehydrogenase in *Euglena gracilis* z. *Biochim. Biophys. Acta*, **429**, 55-62.
9) de DUVE, C., PRESSMAN, B. C., WATTAUX, R. G. and APPLEMAN, F. (1955). Tissue fractionation studies. 6. Intracellular distribution pattern of enzymes in rat-liver tissue. *Biochem. J.*, **60**, 604-617.
10) CORMAN, L. and INAMOAR, A. (1970). Glutamate dehydrogenase. *Method. Enzymol.*, **17a**, 844-850.
11) RABINOWITZ, H., REISFELD, A., SAGHER, D. and EDELMAN, M. (1975). Ribulose diphosphate carboxylase from autotrophic *Euglena gracilis*. *Plant Physiol.*, **56**, 345-350.
12) MACKINNEY, G. (1941). Absorption of light by chlorophyll solution. *J. Biol. Chem.*, **140**, 315-322.
13) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, T. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
14) HALL, D. O. (1972). Nomenclature for isolated chloroplasts. *Nature New Biol.*, **235**,

125-126.
15) HALLIWELL, B. (1978). The chloroplast at work. A review of modern developments in our understanding of chloroplasts metabolism. *Prog. Biophy. Mol. Biol.*, **33**, 1-54.
16) TOKUNAGA, M., NAKANO, Y. and KITAOKA, S. (1979). Subcellular localization of the GABA-shunt enzymes in *Euglena gracilis* strain z. *J. Protozool.*, **26**, 471-473.
17) SALISBURG, J. L., VASCONCELOS, A. C. and FLOYD, G. L. (1975). Isolation of intact chloroplasts of *Euglena gracilis* by isopycnic sedimentation in gradients of silica. *Plant Physiol.*, **56**, 399-403.
18) HSIEH, B. and TOLBERT, N. E. (1976). Glyoxylate aminotransferase in peroxisomes from rat liver and kidney. *J. Biol. Chem.*, **251**, 4408-4415.
19) NOGUCHI, T., MINAMIKAWA, Y., TAKADA, Y., OKUNO, E. and KIDO, R. (1978). Subcellular distribution of pyruvate (glyoxylate) aminotransferases in rat liver. *Biochem. J.*, **170**, 173-175.
20) YOKOTA, A. and KITAOKA, S. (1979). Occurrence and operation of the glycollate-glyoxylate shuttle in mitochondria of *Euglena gracilis* z. *Biochem. J.*, **184**, 189-192.
21) SHIGEOKA, S., NAKANO, Y. and KITAOKA, S. (1979). Some properties and subcellular localization of L-gulono-γ-lactone dehydrogenase in *Euglena gracilis z. Agric. Biol. Chem.*, **43**, 2187-2188.
22) SHIGEOKA, S., NAKANO, Y. and KITAOKA, S. (1980). Metabolism of hydrogen peroxide in *Euglena gracilis* z by L-ascorbic acid peroxidase. *Biochem. J.*, **186**, 377-380.