The Isolation of Photoactive Chloroplasts from Leaves of the Mangrove, Kondelio condel Druce

The Isolation of Photoactive Chloroplasts from Leaves of the Mangrove, *Kandelia candel* Druce

Norikatsu IKEHARA,* Fusako SASHIDA*
and Takako SOEISHI*

Summary

A method for isolating photoactive chloroplasts from mangrove leaves was investigated. Chloroplasts with the Hill reaction activity (photoreduction of ferricyanide) were isolated from the leaves of a mangrove plant, *Kandelia candel* Druce, by employing PEG** in the grinding medium. Some characteristics of these chloroplasts are presented in this paper.

Grinding medium for isolating photoactive chloroplasts is consisted of 1 M sucrose, 10% (W/V) PEG 4000, 0.025 M sodium iso-ascorbate, 0.05 M magnesium chloride and 0.05 M histidine buffered at pH 6.5 with HCl.

Chloroplasts isolated without PEG reduced ferricyanide considerably in the dark. However, ferricyanide reduction in the dark was decreased by addition of PEG in the grinding medium. A possibility of tannins as the reductant is examined.

1. Introduction

There are numerous reports on the studies on the Hill reaction in isolated chloroplasts from the leaves of herbaceous plants such as spinach.[1-3] In woody plants, however, only a few work has been done on the Hill reaction because of difficulties in isolating photoactive chloroplasts, probably due to the high content of resines, tannins and some other substances which affect the activity of the Hill reaction *in vitro*.

Oku and Tomita[4] has recently demonstrated that photoactive chloroplasts could be isolated from resine-rich pine leaves by employing PEG in the isolation medium. Chloroplasts with higher activities have been also isolated from cotton leaves by 10% (W/V) PEG instead of 0.5 M sucrose isolation medium.[5] On the other hand, Clendenning *et al.*[6] has improved a method for isolating photoactive chloroplasts from tannin-bearing leaves (*e.g. Rhus typhina*) with the aid of PEG. Thus, it is possible to obtain photoactive chloroplasts from the leaves of woody plants when some polymers such as PEG are added in the isolation medium.

Recieved : April 30, 1975

*Dept. of Biol., Sci. & Engr. Div., Univ. of the Ryukyus.

**Abbreviations: PEG, polyethylene glycol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea.

Hereupon, in the upper intertidal zone of tropical and subtropical shores, we can often find a special plant community collectively known as mangrove vegetation. Many studies on the ecology and physiology of mangrove vegetation have been made, for reasons of the adaptation of those woody plants in their high salinity habitats.[7-9] However, little has so far been done on the studies of subcellular particles such as chloroplasts in mangrove leaves, probably due to the abundance of tannins. These results prompted us to study the isolation of photoactive chloroplasts from the leaves of mangrove trees.

This paper deals with a method for isolating photoactive chloroplasts from the leaves of mangrove, *Kandelia candel* which dominates the mangrove vegetation in Okinawa Island. In this study, we succeeded in the isolation of chloroplasts with the Hill reaction activity from this plant by employing PEG in the 1 M sucrose grinding medium. Some characteristics of this chloroplast preparation and a possibility of tannins as some inhibitory factors are discussed.

2. Materials and Methods

The leaves of *Kandelia candel* Druce were used for isolating photoactive chloroplasts. They were collected at the mangrove swamps of Ishikawa river, Hijyagawa river and Fusozaki river and stored over night at 0-4° C to digest excess starch in the leaves. The leaves freed from midribs were cut into small pieces, washed with deionized water to remove excreted salts and then ground in a mixer with the medium (medium 1 in Fig. 1) which consists of 1 M sucrose, 0.05 M $MgCl_2$, 0.025 M Na-isoascorbate, 10% (W/V) PEG and 0.05 M histidine-HCl of pH 6.5. The homogenate was filtered through 4 layers of cheese cloth to remove leaf and cell debris, and the filtrate was then centrifuged at 4,000 x *g* for 10 min. The sediment which contains chloroplasts was suspended in the medium (medium 2 in Fig. 1) from which PEG and isoascorbate were omitted. The suspension was centrifuged at 2,000 x *g* for 3 min, and the sediment was discarded. This removed debris and some inhibitory factors. Chloroplasts with photoactivity were then sedimented from supernatant fluid by centrifugation at 10,000 x *g* for 15 min. The chloroplasts were suspended in small amounts of the same medium. All the procedures described above were carried out at 0-4° C.

The Hill reaction activity was assayed spectrophotometrically by measuring the decrease in absorbance at 420 nm of potassium ferricyanide as Hill oxidant.[10] Basal reaction mixture contained the following per 2ml: histidine-HCl (pH 6.5), 100 μmoles; magnesium chloride, 50 μmoles; potassium ferricyanide, 2 μmoles; chloroplasts equivalent to 50-100 μg of chlorophyll. The mixture in test tube (10 x 90 mm) was illuminated at room temperature by light of 50,000 lux from a projector (ERMO-30), filtered through a 10-cm layer of 1% $CuSO_4$ solution. The reaction was stopped by adding 2 ml of 4% trichloroacetic acid after illumination. After precipitation of

denatured protein by centrifugation the amount of ferricyanide reduced in supernatant was determined. Chlorophyll concentrations were determined in 80% acetone extracts using the absorption coefficients reported by Mackinny.[11]

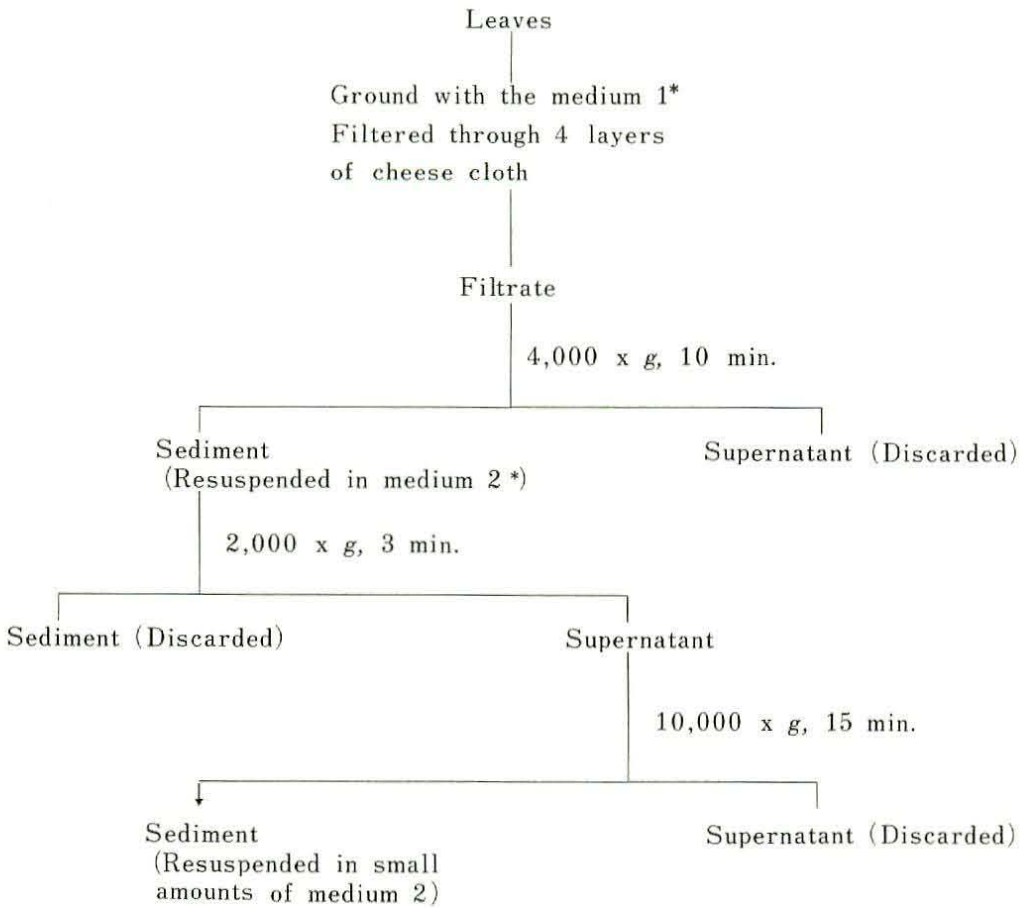
3. Results and Discussion

McClendon[12] has shown that polyethylene glycols, linear polymers of formula $HOCH_2(CH_2OCH_2)_nCH_2OH$, are beneficial in isolating chloroplasts having higher and more stable Hill reaction activity from marine algae and higher plants. Recently, Oku and Tomita[4] has also shown that the use of PEG 4000 (average molecular weight 2,400) was effective for the preparation of photoactive chloroplasts from the leaves of woody plants, conifers.

Then, we attempted to isolate photoactive chloroplasts from the leaves of mangroves which have a capacity to grow in their maritime habitat. However, there are some difficulties in isolating photoactive chloroplasts from mangrove leaves in buffered sucrose solution ordinarily used. They are (i) the coagulation of chloroplasts with other cytoplasm, (ii) the enzymatic browning in leaf macerates and (iii) the abundance of tannins in leaf. These phenomena, in general, inactivate the biochemical activities of chloroplasts, for instance, tannins are irreversible inhibitors of the Hill reaction.[6]

It has been shown that enzymatic browning in leaf macerates is resulted from the action of polyphenol oxidase upon tannins and related compounds, and this phenomenon is inhibited by PEG which have considerable affinity for tannins.[6] Clendenning *et al.*[6] has also shown that PEG removes tannins from the leaves.

With the aid of PEG 4000, we succeeded in the isolation of chloroplasts having Hill reaction activity (photoreduction of ferricyanide) from the tannin-bearing mangrove leaves. The method for isolating photoactive chloroplasts were shown schematically in Fig. 1. In order to maintain the osmotic environment, sucrose concentrations in the media increased to 1 M. This is prospected by the facts that the leaf sap of *K. candel* showed higher osmotic pressure (about 20-24 bar) than other land plants[13] and that bursting of chloroplasts in the leaves of mangrove, *Avicennia nitida*, could be avoided by 1 M sucrose.[14] The addition of 10% (W/V) PEG could greatly prevent the enzymatic browning in leaf macerates, but not completely. Further peroxidation could be avoided by adding ascorbate or isoascorbate. Coagulation of chloroplasts could also be avoided by PEG. These conditions gave active preparations for assay.



*See text for details.

Fig. 1. Scheme for the isolation of chloroplasts from mangrove leaves.

When the leaves were ground in PEG containing sucrose medium, cytoplasm is precipitated on the chloroplasts (*salting-out*) as reported by Clendenning.[6] However, suspending this chloroplast-cytoplasm sediment in PEG free sucrose medium reversed this phenomenon, and made it possible to isolate active chloroplasts by differential centrifugation of suspension as shown in Fig. 1.

Table 1 shows the activity of ferricyanide reduction by illumination of mangrove chloroplasts isolated in different grinding media with or without PEG. The chloroplasts of this plant exhibited considerable reduction of ferricyanide when these were isolated with the aid of PEG. However, the rates of photoreduction were small in comparison with that in spinach chloroplasts (*ca.* 400 μmoles/mg Chl·h). PEG 6000 was also effective in this respect. Fig. 2 shows the typical kinetics of ferricyanide

Table 1. Effects of different media on the Hill reaction in mangrove chloroplasts.

Medium	Ferricyanide reduced
	μmoles/mg Chl·h
Sucrose-succinate (pH 6.0)	—
+PEG	60.8
Sucrose-histidine (pH6.5)	5.5
+PEG	266.0
Sucrose-tris (pH7.5)	—
+PEG	103.4

Reaction conditions are described in Materials and Methods. Chlorophyll concentation, 100 μg per 2 ml volume.

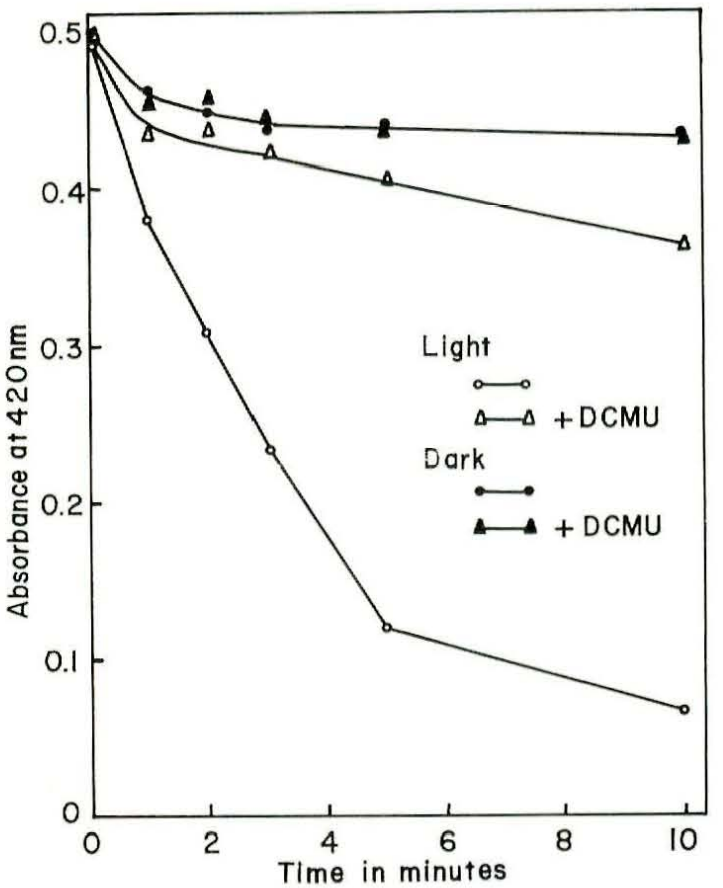


Fig. 2. Time course of ferricyanide reduction in the light and dark. Reaction conditions are the same as in Table 1. Chlorophyll concentration, 100 μg per 2 ml volume. 10μμM of DCMU were added when indicated.

reduction in mangrove chloroplasts. The rates increased linearly up to 5 min illumination. The activity of photoreduction was inhibited by DCMU (10 μM) which is known as potent inhibitors of the Hill reaction. Ferricyanide reduction also occurred in the dark which ceased within 3 min, suggesting the presence of endogenous reductant as discussed below.

Chloroplasts isolated without PEG exhibited coagulation and did not show any photochemical activities, but greatly reduced ferricyanide in the dark (Table 2). The rates were not influenced by light, and by DCMU (Fig. 2). Even the boiled preparations exhibited the dark reduction. The photoactive chloroplasts isolated with the aid of PEG as described above also showed a weak reduction of ferricyanide in the dark (Fig. 2), and the rates were closely dependent on the concentrations of chlorophyll. These results suggest the presence of an endogenous reductant such as gossypol in the cotton chloroplasts suggested by Fry.[5] This endogenous reductant could be partially removed by employing PEG in the grinding medium as shown in Table 2.

Table 2. Effect of polyethylene glycol on the dark reduction of ferricyanide in mangrove.

Polyethylene glycol (%)	Dark reduction
	μmoles/mg Chl·h
0	226
5	63
10	23
20	25

Reaction mixture contained the following components in μmoles; histidine-HCl, pH 6.5, 100; $MgCl_2$, 50; potassium ferricyanide, 2 and chloroplasts equivalent to 100 μg of chlorophyll in a volume of 2 ml. Histidine-HCl buffer (pH 6.5) was used for isolation of chloroplasts.

At concentrations 10 to 20% (W/V), PEG in the medium adequately removed dark reductant indicating the optimum concentration of PEG for isolating photoactive chloroplasts being at 10%.

Although it was not positively proved, tannin can be considered as the reductant. The reasons for this are that PEG removes leaf tannin which is known as one of the inhibitors of the Hill reaction, and that it also inhibits tannin absorption by chloroplasts through its affinity for tannin.[6] On the other hand, PEG is known to cause precipitation of some proteins in solution.[15] Hence, it is possible that a part of tannins in the leaf may be bound to the proteins during isolation of chloroplasts,

and then precipitated on the chloroplasts by the action of PEG.[6] This tannin-protein complex subsequently resulted in a weak dark reduction of ferricyanide. The use of polyethylene glycols make it possible to isolate photoactive chloroplasts from the leaves of the mangrove plant by relieving some inhibitory factors such as tannins and related compounds during the isolation of chloroplasts.

It has been suggested that PEG also resulted in the uncoupling of photophosphorylation in chloroplasts.[16] Satoh *et al.*[17] reported that in the *Euglena* chloroplasts in which phosphorylation is uncoupled from electron flow, divalent cations such as Ca^{2+} caused pH-dependent acceleration of Hill activity, being optimum at pH 6.5. These results prompted us to study the effects of divalent cations on the ferricyanide reduction in the mangrove chloroplasts isolated in the medium containing PEG. Effects of the salts, $CaCl_2$ and $MgCl_2$ on the rates of ferricyanide reduction in the mangrove chloroplasts were shown in Fig. 3. Both $CaCl_2$ and $MgCl_2$ caused Prominent

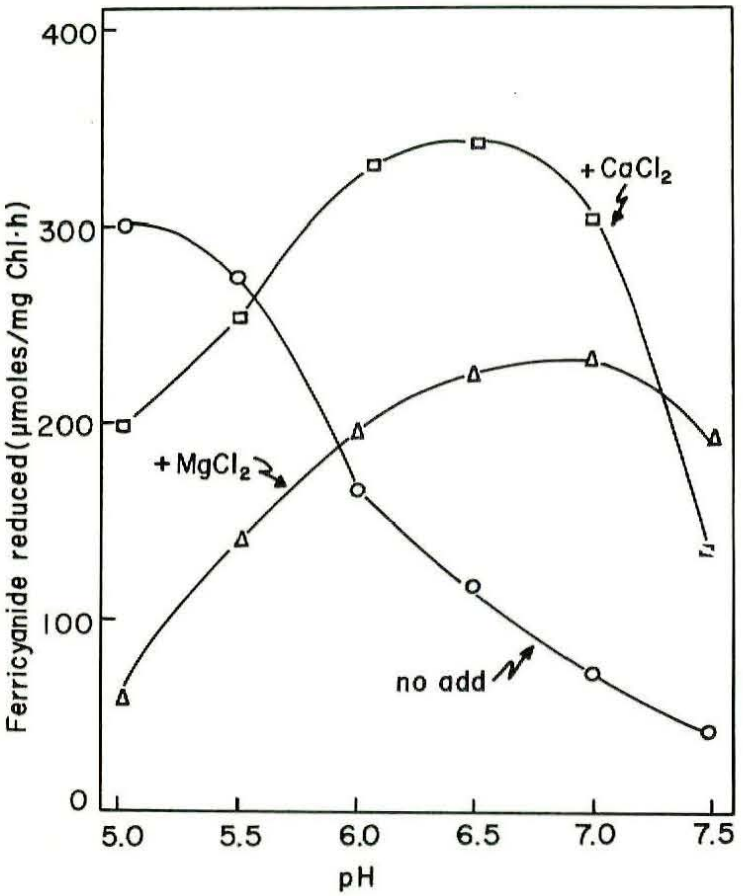


Fig. 3. Effects of $CaCl_2$ and $MgCl_2$ on the Hill activity at various pH. The reaction mixture contained following : potassium ferricyanide, 2 μmoles and chloroplasts containing 100 μg chlorophll in volume of 2 ml. 100 mM of $CaCl_2$ or $MgCl_2$ was added.

acceleration of the rates at pH values higher than 6.0; the optimum pH of the acceleration being at 6.5 and 7.0 for $CaCl_2$ and $MgCl_2$ respectively. It is well known that chloride ion stimulates the Hill reaction in isolated chloroplasts. However, it is unlikely that acceleration of Hill activity by $CaCl_2$ or $MgCl_2$ in the mangrove chloroplasts is caused by the stimulating effect of chlorine, since addition of NaCl or KCl to this reaction mixture did not change the rates of ferricyanide reduction. These results agree well with those of the *Euglena* chloroplasts isolated by Satoh *et al.*[17] This indicates a possibility that the mangrove chloroplasts isolated with the medium containing PEG are probably uncoupled as suggested by Miflin and Hageman for the maize chloroplasts.[16] However, for the reasons that the uncoupling action of PEG was not complete in the cotton chloroplasts[5], another possibility that prominant acceleration of Hill activity by Ca^{2+} or Mg^{2+} in chloroplasts is related to the activation of ATPase activities in chloroplasts[18] is also considered. The finding that Na^+-K^+-ATPase activities in the leaves of *Avicennia nitida* were largely stimulated by Mg^{2+}at pH 5.5 and 6.75 is of interesting in this respect. More detailed studies on the photoactivities in the mangrove chloroplasts isolated with PEG, however, are necessary in order to confirm these possibilities, and such studies are in progress.

References

(1) Avron, M.: Photophosphorylation by Swiss-chard chloroplasts. *Biochim. Biophys. Acta* 40: 257-272 (1960).

(2) Walker, D. A.: Photosynthetic activity of isolated pea chloroplasts. In *Biochemistry of Chloroplasts* 2. Edited by Goodwin, T. W. Academic Press, London and New York. p. 53-69 (1967).

(3) Gockburn, W., D. A. Walker and C. W. Baldry: The isolation of spinach chloroplasts in pyrophosphate media. *Plant Physiol.* 43 : 1415-1418 (1968).

(4) Oku, T. and G. Tomita: The preparation of photoactive chloroplasts from pine leaves. *Photosynthetica* 5: 28-31 (1971).

(5) Fry, K. E.: Some factors affecting the Hill reaction activity in cotton chloroplasts. *Plant Physiol.* 45: 465-469 (1970).

(6) Clendenning, K. A., T. E. Brown and E. E. Walldov: Causes of increased and stabilized Hill reaction rates in polyethylene glycol solutions. *Physiol. Plant.* 9: 519-532 (1956).

(7) Chapman, V. J.: Respiration studies of mangrove seedlings. I. Material and some preliminary experiments. *Bulletin of Marine Science of the Gulf and Caribbean.* 12: 137-166 (1962).

(8) Joshi, G. V., M. Pimplaskar and L. J. Bhosale: Physiological studies in germination of mangroves. *Botanica Marina* 15: 91-95 (1972).

(9) Stoddart, D. R., G. W. Bryan and P. E. Gibbs: Inland mangroves and water chemistry, Barbuda, West Indies. *J. nat. Hist.* 7: 33-46 (1973).

(10) Ikehara, N. and E. G. Uribe: A pH dependent alteration of photosystem II activity in tris washed chloroplasts. *Fed. Eur. Biol. Soc. Lett.* 9: 321-323 (1970).

(11) Mackinney, G.: Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140: 315-322 (1941).

(12) McClendon, J. H. : The physical environment of chloroplasts as related to their morphology and activity in vitro. Plant Physiol. 29: 448-458 (1954)

(13) Ikehara, N.: Unpublished data.

(14) Kylin, A. and R. Gee: Adenosine triphosphatase activities in leaves of the mangrove *Avicennia nitida* Jacp. Influence of sodium to potassium ratios and salt concentrations. *Plant Physiol.* 45: 169-172 (1970).

(15) Stocking, C. R.: Precipitation of enzymes during isolation of chloroplasts in Carbowax. *Science* 123: 1032-1033 (1956).

(16) Miflin, B. J. and R. H. Hageman: Demonstration of photophosphorylation by maize chloroplasts. *Plant Physiol.* 38: 66-70 (1963).

(17) Satoh, K., S. Katoh and A. Takamiya : Effects of chloride ion on Hill reaction in *Euglena* chloroplasts. *Plant & Cell Physiol.* 11: 453-466 (1970).

(18) Ikehara, N. and M. Nishimura: Anion-dependent changes of energy transfer in chloroplasts I. Effects of Tris salts on electron flow and phosphorylation. *Plant & Cell Physiol.* 14: 61-75 (1973).