BBA 46391

EFFECT OF CHLOROPHYLLASE TREATMENT ON PHOTOOXIDATION OF P700 IN CHLOROPLAST FRAGMENTS

KEN-ICHIRO TAKAMIYA AND MITSUO NISHIMURA

Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812 (Japan)

(Received May 25th, 1972)

SUMMARY

The effects of chlorophyllase (chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14) treatment on the photooxidation of P700 in chloroplast fragments (180P particles) were investigated.

The magnitude of steady-state photooxidation of P700 was decreased by incubation with chlorophyllase in the presence of 25 % acetone. The amount of P700 in 180P particles was decreased by the treatment with chlorophyllase and at the same time the quantum yield of P700 photooxidation dropped. The decrease in the latter was greater than the loss of P700. Chemical analysis showed that chlorophyllase partially extracted chlorophyll from 180P particles in the presence of 25 % acetone.

Absorption and fluorescence emission spectra at 77 °K indicated that chlorophyllase altered the physical environment of chlorophyll molecules *in situ*. It was also suggested that alteration of the environment of chlorophyll and the decrease in molecular interaction lowered the efficiency of migration of excitation energy between chlorophyll molecules and thus caused a drop in the quantum yield of P700 photooxidation.

INTRODUCTION

Reversible photooxidation of P700 has been thought to be the primary oxidation–reduction reaction of photosynthesis in green plants and algae[1-3]. Since the process is photochemical in nature, the photooxidation of P700 is not affected by certain treatments such as sonication, ultraviolet light irradiation, heating, *etc.*, which generally affect enzymatic processes[2-4]. But it is probable that the alteration of the physical environment of bulk chlorophyll molecules or P700 affects the efficiency of photooxidation of P700.

Chlorophyllase (chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14) catalyzes hydrolysis of chlorophyll or phaeophytin to produce phytol and chlorophyllide or phaeophorbide *in vitro*[5-10]. Wakamatsu *et al.*[11] investigated the effect of chlorophyllase on the activity of the Hill reaction and $NADP^+$ photoreduction in isolated chloroplasts. They reported that $NADP^+$ photoreduction was more sensitive to the treatment

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

with chlorophyllase than the DCIP–Hill reaction. They also found that the ratio of fluorescence emission intensities at 690 to 730 nm at liquid-nitrogen temperature increased after chlorophyllase treatment. These observations led them to conclude that chlorophyll molecules belonging to Photosystem I are more susceptible to treatment with chlorophyllase than those belonging to Photosystem II.

In the present study, we investigated the effects of treatment with chlorophyllase on the photooxidation of P700 in particles obtained by sonication of chloroplasts. The effects of chlorophyllase on the absorption and fluorescence emission spectra at 77 °K were also investigated.

MATERIALS AND METHODS

Chloroplast fragments (180P particles) were prepared as previously described[4]. 180P particles were suspended in distilled water and stored at −20 °C in a deep-freeze.

Partially purified chlorophyllase, extracted from *Chlorella protothecoides*, was supplied by courtesy of Miss N. Hiromoto and Dr T. Sasa in our laboratory.

A typical reaction mixture was as follows; 5 mM Tris–HCl (pH 7.5), 6.5 ml; acetone, 2.5 ml; 180P particles, 0.5 ml; chlorophyllase, 0.5 ml; where the final concentration of chlorophyll was 50 to 100 μM and that of chlorophyllase was 0.03 to 0.05 mg protein per 10 ml of the reaction mixture. The original enzyme activity of chlorophyllase was about 30 mg chlorophyll *a* hydrolyzed per mg protein per h. The control was the same as described above except that 0.5 ml of chlorophyllase was replaced by 0.5 ml of distilled water. Incubation was carried out in a water bath at 30 °C.

Spectroscopic measurements were carried out with a Hitachi 356 dual-wavelength spectrophotometer. The photooxidation of P700 was measured in both the blue and red regions of the absorption spectrum. In the blue region, the "dual-wavelength" mode was used for measuring the light-induced absorbance change (reference wavelength, 450 nm). A guard filter, Corning 9782, was placed in front of the photomultiplier. Actinic light was provided by a tungsten iodine lamp (Ushio Co. Ltd, 650 W). The light was filtered through a red cut-off filter, Hoya R-68 or R-66 (which transmitted light of wavelengths longer than 660 nm and 640 nm, respectively), and a layer of water (6 cm). The sample in a four-sided cell (1 cm × 1 cm), which was placed in the first cell compartment (sample position 1) of the spectrophotometer was illuminated at right angles to the measuring beam. In the measurements of quantum yield of photooxidation of P700, the four-sided cell (measuring light path, 1 cm; width, 0.3 cm) was placed in the second cell compartment (sample position 2). The "split-beam" mode was used and the wavelength was fixed at 700 nm. A guard filter, Toshiba VR-69, which transmitted the light of wavelengths longer than 670 nm, was placed in front of the photomultiplier. Actinic light was provided by the tungsten iodine lamp (Ushio Co. Ltd, 650 W). The light was filtered through an interference filter (λ_{max} = 432 nm, half band width, 10 nm) and a layer of water (6 cm). The light intensity transmitted was $5 \cdot 10^{-10}$ Einstein/cm^2 per s. At sample position 1, the cell was placed close to the front surface of photomultiplier. However, the distance between sample position 2 and the detector was about 30 cm and thus the interference of fluorescence emission to absorbance measurement was minimized. The intensity of actinic light was attenuated by placing calibrated neutral density filters in front of

the interference filter. The energy of the incident light was measured with a calibrated Kipp and Zonen thermopile.

Chlorophyll concentrations were determined in 80% acetone extracts using the absorption coefficients reported by Mackinney[12]. The relative amount of P700 was spectrophotometrically determined from the absorbance difference of oxidized (ferricyanide)-*minus*-reduced(ascorbate) at 698 nm with a reference wavelength of 730 nm.

Absorption spectra at 77 °K were measured with the Hitachi 356 spectrophotometer equipped with a cryogenic cell holder. Fluorescence emission spectra were measured with a Hitachi MPF-2A fluorescence spectrophotometer. The exciting light from a monochromator was further filtered through a Corning 9782 filter and a cut-off filter (Toshiba UV-35) which removed light of wavelengths shorter than 330 nm. A red cut-off filter (Hoya R-61) which transmitted light of wavelengths longer than 590 nm, was placed in front of the shutter of the detecting monochromator to eliminate the actinic light. The detector used was Hamamatsu TV R136 (Ag-Bi-O-Cs type).

RESULTS AND DISCUSSION

The effect of chlorophyllase treatment on P700 photooxidation is presented in Fig. 1, in which the time courses of light-induced absorption changes at 430 nm are compared among samples incubated for 0, 2 and 4 h. In this measurement, 1 ml of the reaction mixture was taken into a cuvette at the indicated time and 2 ml of 50 mM Tris–HCl buffer (pH 7.5) were added to the cuvette. A few crystals of sodium ascorbate were added to reduce P700 completely. After 5 min of incubation in the dark, the actinic light was turned on. Unless otherwise stated, the light-induced absorption changes of P700 were measured in this manner in the present study.

The traces of the "light-on" change consisted of two phases of absorption

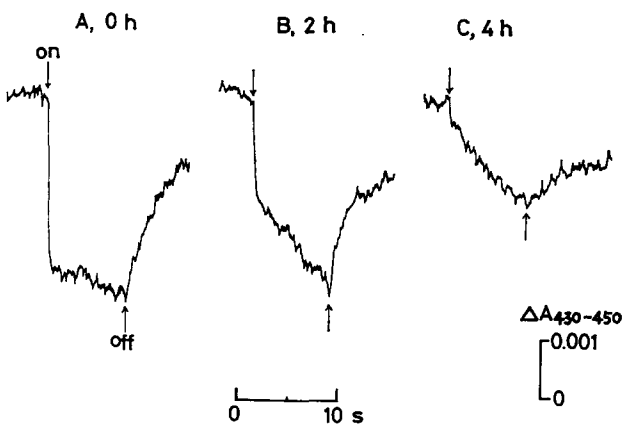

Fig. 1. Time courses of light-induced absorption changes at 430 nm after various incubation times with chlorophyllase. An aliquot of 1 ml was transferred to a cuvette from the reaction mixture at the indicated time, and 2 ml of 50 mM Tris–HCl buffer (pH 7.5) were added to the cuvette. A few crystals of sodium ascorbate were added to reduce P700 completely. After 5 min of incubation in the dark, the actinic light was turned on and off, respectively. Upward deflection of the traces corresponds to increase of absorbance. A, 0 h; B, 2 h; C, 4 h. Chlorophyll concentration, 22 μM.

decrease; one was fast and the other was slow phase. The magnitude of the fast absorption change markedly decreased and the slow phase became dominant after a 4-h incubation with chlorophyllase. The dark revovery of absorption decrease was not complete after the 4-h incubation.

In Fig. 2, the magnitudes of the fast absorption decrease were plotted against the incubation time. After 4 h of incubation, the magnitude of the fast absorption change decreased to about one-sixth of the original value. On the other hand, in the control experiment in which the enzyme solution was replaced by distilled water. the magnitude of the fast absorption decrease did not change after the 4-h incubation. In this experiment, the concentration of acetone in the reaction mixture was 25 % (v/v). Preliminary experiments (not shown here) showed that the rate of decrease in magnitude of P700 photooxidation was higher when the acetone concentration was increased in the 0–30 % range. A similar situation has been observed for the hydrolysis of pure chlorophyll by chlorophyllase[9]. However, at acetone concentrations above 30 % the decrease of magnitude of P700 photooxidation was observed without chlorophyllase.

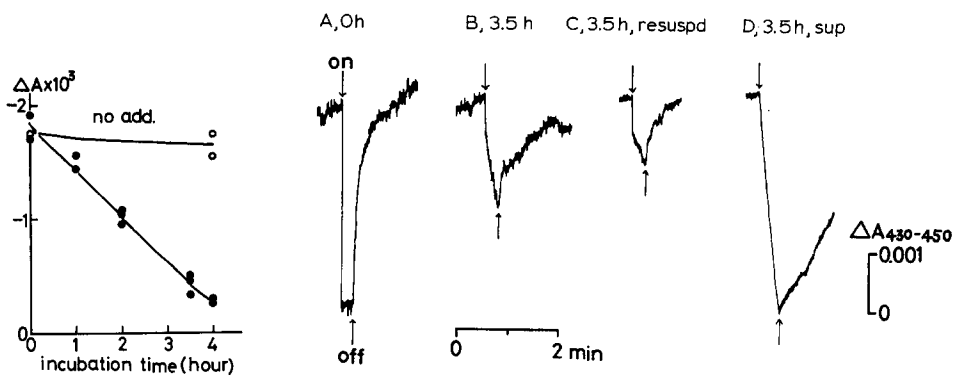

Fig. 2. Relationship between the magnitude of the fast light-induced absorption change at 430 nm and the incubation time. ●—●, in the presence of chlorophyllase; ○—○, in the absence of chlorophyllase. Experimental conditions were same as in Fig. 1.

Fig. 3. Time courses of light-induced absorption changes at 430 nm of the reaction mixture, resuspended particles and supernatant. A, reaction mixture, 0 h; B, reaction mixture, 3.5 h; C, resuspended particles, the reaction mixture, incubated for 3.5 h, was centrifuged at 180000 × *g* for 2 h and the pellet was resuspended in an equal volume of 50 mM Tris–HCl buffer; D, the supernatant solution. To a cuvette containing 2 ml of 50 mM Tris–HCl buffer (pH 7.5), a 1-ml aliquot from the reaction mixture, suspension of the resuspended particles or the supernatant solution after the indicated incubation time was added. A few crystals of sodium ascorbate were added to reduce P700 completely. After 5 min of incubation in the dark, the actinic light was turned on.

Fig. 3 shows the time courses of the light-induced absorption decrease at 430 nm of a reaction mixture at 0-h and 3.5-h incubation times, and those of particles and supernatant obtained after a 3.5 h incubation followed by centrifugation at 180000 × *g* for 2 h. The particles were resuspended in 50 mM Tris–HCl buffer (pH 7.5) in a volume equal to that before centrifugation. The magnitudes of initial fast absorption decrease of the reaction mixture after the 3.5-h incubation (B) and of resuspended particles (C) were similar to each other but the slow phase of absorption decrease was much larger in (B) than in (C). The green supernatant solution showed a gradual light-induced

absorption decrease and did not reach a plateau within 2 min of illumination. The slow phase of absorption decrease in (B) and (C) and the gradual light-induced absorption decrease in the supernatant are probably due to bleaching of free chlorophyll pigments.

The light-*minus*-dark difference spectrum (rapid phase) of the resuspended particles obtained after the 3.5-h chlorophyllase incubation had a maximal absorption change at 433 nm (Fig. 4). Although the location of maximal absorption change shifted 3 nm toward longer wavelengths compared to the original 180P particles[4], the shape of the difference spectrum of the chorophyllase-treated particles was similar to that of P700. Thus, the remaining light-induced absorption changes observed in the blue region seemed to be due to the photooxidation of P700.

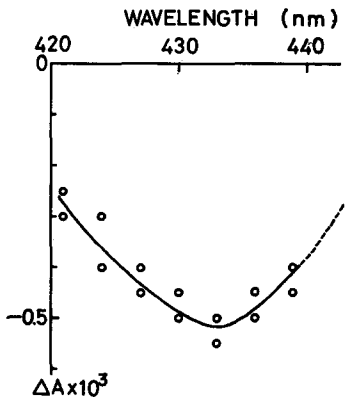

Fig. 4. Light-*minus*-dark difference spectrum of resuspended particles. 180P particles, incubated for 3.5 h with chlorophyllase, were centrifuged at 180000 × *g* for 2 h and resuspended in 50 mM Tris–HCl buffer. Reference wavelength, 450 nm. Chlorophyll concentration, 15.5 μM.

In general, the decrease in magnitude of the photooxidation of P700 may be caused by the following; (1) decrease of the amount of P700; (2) a lowering of the efficiency of photooxidation of P700; (3) a marked acceleration of the rate of dark recovery. As in Figs 1 and 3, the rate of dark recovery after a prolonged chlorophyllase treatment was similar to or somewhat smaller than that of the untreated sample. The overall half-recovery time became longer after the incubation. Thus, the third possibility was eliminated.

The total amount of P700 in the chlorophyllase-treated particles, resuspended in the 50 mM Tris–HCl buffer, was determined from the difference of absorbance at 698 nm between ascorbate-reduced and ferricyanide-oxidized particles. The shape of the reduced-*minus*-oxidized difference spectrum of the chlorophyllase-treated particles was similar to that of the original 180P particles in the region from 690 nm to 710 nm (not shown here).

Table I shows the magnitudes of the rapid light-induced absorption change at 430 nm and the chemically induced absorption change at 698 nm at the indicated incubation time. Table I also includes the magnitudes of the light-induced absorption change (430 nm, rapid phase) and of the chemically-induced absorption change (698 nm) of the resuspended particles and the supernatant after the 3.5-h chlorophyllase incubation. These were separated by a centrifugation at 180000 × *g* for 2 h and the particles were resuspended in 50 mM Tris–HCl buffer (pH 7.5) in a

TABLE I

EFFECT OF CHLOROPHYLLASE TREATMENT ON THE MAGNITUDE OF LIGHT-INDUCED AND CHEMICALLY INDUCED ABSORPTION CHANGE IN 180P PARTICLES MEASURED AT 430 nm AND 698 nm, RESPECTIVELY

Chlorophyll concentration of the reaction mixture at 0 h was 38.0 μM. For other details see text.

Incubation time (h)	*Light-induced absorbance change* $\Delta A \times 10^3$ *(430 nm)*	*Chemically induced absorbance change* $\Delta A \times 10^3$ *(698 nm)*
0	3.5	5.0
3.5	0.6	4.4
3.5 (resuspended)	0.6	2.6
3.5 (supernatant)	0.0	0.7

volume equal to that before centrifugation. The magnitude of the light-induced absorption change at 430 nm in Table I was that of the fast absorption decrease caused by the photooxidation of P700. After the 3.5-h incubation the magnitude of photooxidation of P700 decreased to 16 % of the original value at 0 h. The magnitude in the particles resuspended in the Tris–HCl buffer decreased similarly. The supernatant solution did not show any reversible, fast light-induced absorption change but a gradual irreversible decrease of absorption. On the other hand, the magnitude of the chemically-induced absorption change at 698 nm decreased only slightly after incubation for 3.5 h. The same sample (resuspended in the same volume of Tris–HCl buffer after centrifugation) showed about half the value of the chemically-induced absorption change of the original sample at zero time. The chemically-induced absorption change was also observed in the supernatant solution. In spite of P700 remaining in large amounts in the resuspended particles (about half of the initial amount of P700), the magnitude of the photooxidation of P700 decreased markedly. Although a part of the decrease of the magnitude of the light-induced absorption change at 430 nm could be attributed to partial solubilization of P700 into the supernatant solution, the decrease in efficiency of the photooxidation of P700 seems to be caused by another factor. This possibility was confirmed by measuring the quantum yield of photooxidation of P700. The rates of photobleaching of P700 at 700 nm were plotted against the number of quanta absorbed at 432 nm. The quantum yield was calculated from the tangent of initial slope of the curve. We did not measure the rates at 430 nm because it has been recently reported by Hiyama and Ke[13] that the light-induced absorption change at 430 nm does not solely consist of the photooxidation of P700 but is superimposed by a photoreduction of "P430". The interference of fluorescence of chlorophyll *a* was negligible in our measuring system as described in Materials and Methods. Lowering of the quantum yield of P700 photooxidation after chlorophyllase treatment is shown in Table II. The millimolar absorption coefficient at 700 nm of reduced P700 was assumed to be 64[14]. The quantum yield of photooxidation of P700 in the untreated sample at zero time was high, as reported by previous investigators[2]. After 2.5 h of incubation, the control (with no chlorophyllase) had the high quantum yield but the quantum yield of the chlorophyllase-treated sample was reduced to one-sixth of the control value. The quantum yield of the resuspended particles after 2.5 h of incubation with no chlorophyllase,

TABLE II

EFFECT OF CHLOROPHYLLASE TREATMENT ON THE QUANTUM YIELD OF P700 PHOTOOXIDATION

P700 photooxidation was measured at 700 nm. Excitation wavelength was 432 nm. For details see Materials and Methods.

Incubation time (h)		*Quantum yield*
0		0.61
2.5	Control	0.59
	Treated	0.09
2.5 (resuspended)	Control	0.33
	Treated	0.08

decreased to half the value of the reaction mixture incubated under the same conditions. On the other hand, the low quantum yield of the photooxidation of P700 in the reaction mixture incubated with chlorophyllase for 2.5 h remained unchanged after the particles were separated and resuspended in Tris–HCl buffer. Although an interpretation of the decrease in the quantum yield by resuspending the control 180P particles has not been made yet, the quantum yield of P700 photooxidation in chlorophyllase-treated resuspended particles was further reduced to one-fourth of that in the control resuspended particles. Thus, it is obvious that treatment with chlorophyllase reduces the efficiency of photooxidation of P700.

Chlorophyllase hydrolyzes chlorophyll or phaeophytin in aqueous acetone solution producing phytol and chlorophyllide or phaeophorbide. Preliminary experiments showed that chlorophyllase attacked 180P particles in 25 % acetone and released green pigments (chlorophyllides) from the particles into the supernatant solution. In the case of the experiment shown in Table II, only one-sixth of the total chlorophyll in 180P particles were removed from the particles after centrifugation into the supernatant solution. But an alteration of the physical environment of chlorophyll molecules may have occurred after treatment by chlorophyllase because the quantum yield of photooxidation of P700 in 180P particles was drastically reduced after the treatment. To investigate this possibility, the absorption and fluorescence emission spectra of chlorophyllase-treated and untreated particles were compared. Fig. 5 illustrates the absorption spectra at 77 °K of original 180P particles (solid line) and particles which were treated for 1.25 h with chlorophyllase and resuspended in Tris–HCl buffer after centrifugation at 180000 × *g* for 2 h (dashed line). Due to the chlorophyllase treatment, the red absorption peak shifted 7 nm towards the shorter wavelengths from 677 to 670 nm. The shoulder at 650 nm of the original 180P particles diminished after chlorophyllase treatment. Absorption around 485 nm, due to carotenoids, became prominent after the treatment since chlorophyll (especially chlorophyll *b*) was partially extracted from 180 P particles by chlorophyllase.

Fluorescence emission spectra at 77 °K of the original and chlorophyllase-treated (for 1 h) particles had a striking difference (Fig. 6). The shape of the fluorescence spectrum of the original 180P particles (solid line) was similar to those previously reported[15,16]. On the other hand, the emission spectrum of the chlorophyllase-treated

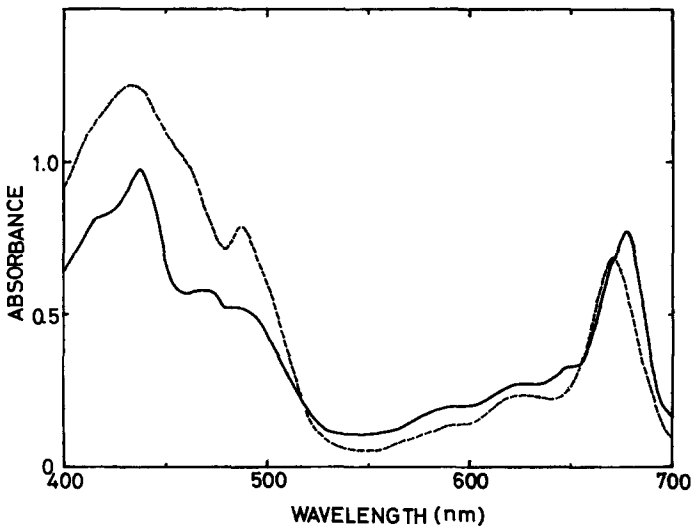

Fig. 5. Absorption spectra at 77 °K of 180P particles and chlorophyllase-treated particles. ———, 180P particles; — — —, chlorophyllase-treated (for 1.25 h) particles. Slit width, 1 nm; light path, 1 mm. Chlorophyll concentrations of 180P particles and of chlorophyllase-treated particles were about 20 μM.

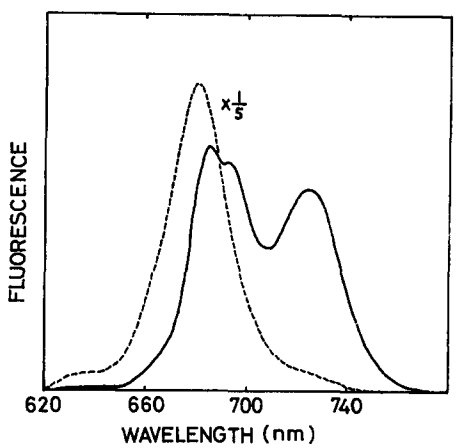

Fig. 6. Fluorescence emission spectra at 77 °K of 180P particles and chlorophyllase-treated particles. ———, 180P particles; – – – –, chlorophyllase-treated (for 1 h) particles. Wavelength of excitation, 430 nm (half band width, 20 nm). Half band width of detecting monochromator was 5 nm.

particles had only one peak. Its position (680 nm) shifted 5 nm towards the shorter wavelengths from the short wavelength emission band of untreated particles (685 nm). On the chlorophyll base, the intensity of fluorescence of treated particles was about 5 times stronger than that of the original. These results of our fluorescence measurements were generally in agreement with the results of Wakamatsu *et al.*[11]. But our results were more drastic in that the fluorescence emission spectrum of the treated particles lacked the peak around 725 nm. This may be partially due to the difference of

incubation conditions, *e.g.* incubation temperature (30 °C compared with 23 °C) and composition of reaction mixture (25 % acetone compared with 25 % methanol). The emission spectrum of control 180P particles (incubated in 25 % acetone for 1.5 h without chlorophyllase) was generally similar to that of the original 180P particles except that the peak at 695 nm was not clear.

The shape of the fluorescence emission spectrum of the chlorophyllase-treated particles was rather similar to that of detergent-solubilized chlorophyll *a*[17,18]. The highly fluorescent property of the treated particles also supports the possibility that chlorophyll *a* in the treated particles has a much decreased degree of molecular interaction, as in the detergent-solubilized state or in solution in organic solvents. This implies that besides partial removal of chlorophyll molecules from 180P particles, the enzyme modifies the environmental conditions of remaining chlorophyll molecules. It is probable that this alteration of the physical environment of chlorophyll molecules and the lower degree of molecular interaction cause the drop in efficiency of excitation energy migration between chlorophyll molecules, thereby inducing the drop in the quantum yield of P700 photooxidation.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Miss N. Hiromoto and Dr T. Sasa in our laboratory for providing chlorophyllase. This work was supported in part by a grant from the Ministry of Education and from the Matsunaga Science Foundation.

REFERENCES

1 B. Kok, *Biochim. Biophys. Acta*, 22 (1956) 399.
2 B. Kok and G. Hoch, in W. D. McElroy and B. Glass, *Light and Life*, The Johns Hopkins Press, Baltimore, Md., 1961, p. 397.
3 H. T. Witt, A. Müller and B. Rumberg, in Centre National de la Recherche Scientifique, *La Photosynthèse*, 1963, p. 43.
4 K. Takamiya and M. Nishimura, *Plant Cell Physiol.*, 13 (1972) 35.
5 R. Willstätter und A. Stoll, *Untersuchungen über Chlorophyll*, Springer-Verlag, Berlin, 1913, p. 172.
6 M. Holden, *Biochem. J.*, 78 (1961) 359.
7 A. O. Klein and W. Vishniac, *J. Biol. Chem.*, 236 (1961) 2544.
8 S. Shimizu and E. Tamaki, *Bot. Mag. Tokyo*, 75 (1962) 462.
9 Y. Chiba, I. Aiga, M. Idemori, Y. Satoh, K. Matsushita and T. Sasa, *Plant Cell Physiol.*, 8 (1967) 623.
10 I. Aiga and T. Sasa, *Plant Cell Physiol.*, 11 (1970) 161.
11 K. Wakamatsu, Y. Kobayashi and T. Sasa, *Bot. Mag. Tokyo*, 84 (1971) 101.
12 G. Mackinney, *J. Biol. Chem.*, 140 (1941) 315.
13 T. Hiyama and B. Ke, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 1010.
14 T. Hiyama and B. Ke, *Biochim. Biophys. Acta*, 267 (1972) 160.
15 J. C. Goedheer, *Biochim. Biophys. Acta*, 88 (1964) 304.
16 N. Murata, M. Nishimura and A. Takamiya, *Biochim. Biophys. Acta*, 126 (1966) 234.
17 N. K. Boardman, S. W. Thorne and J. M. Anderson, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 586.
18 T. Ogawa, L. P. Vernon and H. H. Mollenhauer, *Biochim. Biophys. Acta*, 172 (1969) 216.