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Photosynthetic Activities of Spinach Leaf Protoplasts[1]

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ABSTRACT

Photosynthetic activities of protoplasts isolated from spinach leaf (*Spinacia oleracea* L.) were investigated. The protoplasts were stable up to 9 hr, without loss of the original activity of CO_2 fixation (33–75 μmoles CO_2/mg Chl·hr) and light-dependent O_2 evolution (33–40 μmoles O_2/mg Chl·hr), when stored in 0.8 M mannitol-0.05 M N-tris(hydroxymethyl)-methylglycine-NaOH buffer, pH 7, at 4 C in dark. The optimum pH of 8.5 for CO_2 fixation reaction carried out in the present experimental condition employed is about the same as that reported for intact spinach chloroplasts. The CO_2 concentration for half-maximal rate of CO_2 fixation by protoplasts. "K*m* (CO_2)," were determined to be 19.8 μM (pH 7) and 42 μM (pH 8.5) and are similar to those observed for intact spinach chloroplasts. Protoplasts showed postillumination CO_2 fixation. Over-all results indicate that spinach protoplasts are as active as the intact plant leaf tissues in their photosynthetic activities.

Since the initial report by Cocking (10) on the isolation of protoplasts from tomato root tip cells, many investigations have been conducted on various types of plant cells (2, 9, 11, 14, 18–20, 26–28, 32, 33). Protoplasts with normal metabolic activities are potentially useful for physiological research, and we were interested in their application pertaining to structure-function relationship of spinach leaf RuDP[2] carboxylase. We are attempting to elucidate the functional role of each individual subunit of the enzyme protein and the molecular interaction *in vivo* (1). Immunochemical techniques are being employed in this work (24, 25), and protoplasts appear to be an attractive system for such studies (2, 27, 33). As a basis for such research, spinach leaf protoplasts have been isolated, and their biochemical activities have been measured. Results reported below demonstrate that spinach protoplasts are potentially useful for research in photosynthesis.

MATERIALS AND METHODS

Photoplasts. Young leaves of freshly harvested spinach (*Spinacia oleracea* L. var Kyoho) were used for preparing protoplasts. Methods of the two-step enzymic maceration of spinach leaves using Macerozyme R-10 and Cellulase Onozuka R-10 (Kinki Yakult Co. Ltd, Osaka) were basically the same as those for preparing tobacco leaf (*Nicotiana tabacum*) protoplasts as reported by Takebe *et al.* (32). Two modifications employed were that the concentration of mannitol was raised to 0.8 M as recommended by Otsuki and Takebe (26) and that the washing medium did not contain 0.1 mM $CaCl_2$. Two g of spinach leaf segments (about 0.5 cm wide) devoid of lower epidermis were placed in a 50-ml Erlenmeyer flask containing 20 ml of maceration medium (0.5% Macerozyme R-10-1% K-dextran sulfate [18% S, Meito Sangyo Co. Ltd. Nagoya]-0.8 M mannitol [pH 5.8]). After vacuum infiltration of leaf samples for 2 min, the enzymic disintegration was carried out in a rotary shaker at 20 C. The spongy mesophyll cell suspension was collected by decantation of the greenish supernatant every 30 min, by substituting 10 ml of fresh maceration medium each time. At the same time, measurements of the absorbance increment at 680 nm as well as the phase contrast microscope inspection of the collected samples were carried out. This first step of the enzymic treatment usually goes to completion in 5 hr, and the whole supernatant fractions were filtered through a Nylon bolting cloth (35 mesh). The residues collected by centrifugation at 100*g* for 3 min were washed twice with 50 ml each of 0.8 M mannitol solution (pH 5.4). The spongy cell fractions obtained (Fig. 1a) were then subjected to the second enzymic treatment using a medium containing 2% Cellulase Onozuka R-10 and 0.8 M mannitol (pH 5.2). The enzymic reaction was carried out at 37 C with occasional stirring. The complete conversion to protoplasts occurs within 1 to 1.5 hr incubation, and the residues collected by centrifugation (100*g*, 3 min) were washed twice with 50 ml each of 0.8 M mannitol solution. Fig. 1b is a typical photomicrograph of the protoplast preparations. The final preparation was stored in 0.8 M mannitol at 4 C in dark until the time of analytical experiments.

Photosynthetic CO_2 Fixation. The reaction mixture for determining photosynthetic CO_2 fixation activity contained the following components (μmoles): Tricine-NaOH at various pH values, 20; $NaH^{14}CO_3$, 1.6 (2 μCi); mannitol, 160; and appropriate amounts of protoplasts (15–25 μg Chl) in a total volume of 0.2 ml. The reaction mixture except $NaHCO_3$ was incubated in the dark for 3 min at 25 C. After 5 min preillumination (3×10^4 lux of white light), $NaH^{14}CO_3$ was added and incubation continued at 25 C for 5 min. The reaction was then stopped by adding 0.1 ml of glacial acetic acid and radioactivity was measured by a Packard liquid scintillation spectrometer. The effect of various compounds on photosynthetic CO_2 fixation was examined by adding them to the standard assay mixture in the preincubation period.

Postillumination CO_2 Fixation. Postillumination CO_2 fixation activity was determined according to the method of Avron and Gibbs (5). After preillumination of the protoplast preparations for 10 min (3×10^4 lux of white light) at 25 C, test tubes con-

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[2] Abbreviation: RuDP: ribulose 1,5-diphosphate.

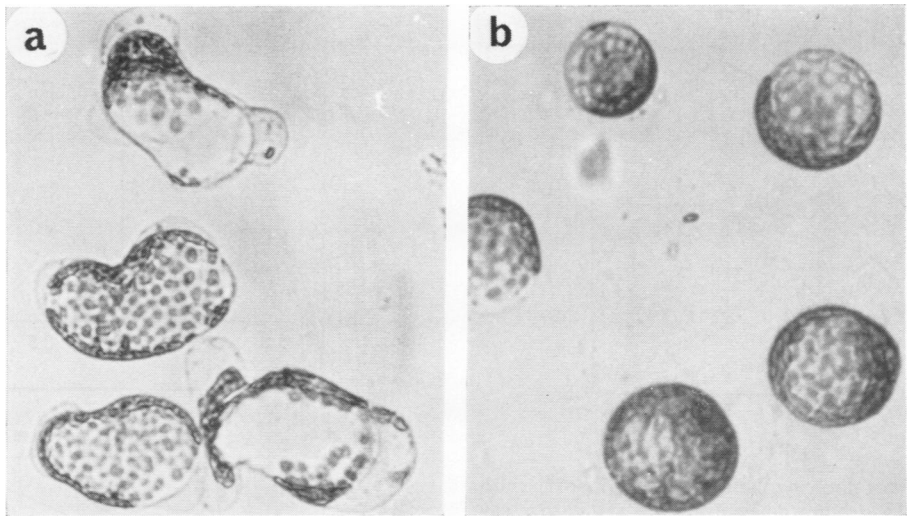

FIG. 1. Spinach protoplasts. Spinach leaf segments were first treated by pectinase (Macerozyme R-10) to give spongy cells (*a*), followed by treatment with cellulase (Cellulase Onozuka R-10) to give protoplasts (*b*). × 500.

taining reaction mixture were returned to the dark with the concomitant addition of $NaH^{14}CO_3$ (1.6 μmoles, 2 μCi), and subsequent time sequence of CO_2 fixation was determined at pH 7. A system without preillumination served as the control.

Photosynthetic O_2 Evolution. The same protoplast suspension used for photosynthetic CO_2 fixation was used for measuring O_2 evolution. The reaction mixture contained the following components (μmoles): Tricine-NaOH (pH 7), 100; $NaHCO_3$, 8; mannitol, 800; and protoplasts containing 18 μg of Chl in a total volume of 1 ml. The complete mixture was placed in the reaction chamber of a Rank oxygen electrode (Rank Bros., Bottisham, England), and the change of O_2 tension corresponding to the transient dark-light (3×10^4 lux of white light) condition was recorded at 25° C.

Chlorophyll. Chlorophyll content of protoplasts was analyzed chlorimetrically by the method of Arnon (4).

RESULTS

Photosynthetic CO_2 Fixation. The stability of the protoplasts was tested by withdrawing at 3-hr intervals aliquots of protoplasts in Tricine-NaOH buffer of five different pH values (7, 7.5, 8, 8.5, 9) at 4 C in the dark. Light-dependent CO_2 fixation by the preparations, which is linear up to 15 min, was measured at 25 C. As shown in Figure 2, the protoplasts kept in the buffer at pH 7 did not lose the original activity during 9 hr storage. At pH 8.5 and 9, however, there occurred a drastic decline of activity with only 13 to 15% remaining after 9 hr. Essentially the same results were obtained by using HEPES-NaOH buffer. On the other hand, when tris-HCl or phosphate buffer was used, activity of the light-dependent CO_2 fixation of protoplasts was markedly impaired at every pH. Therefore, preparations containing 0.8 M mannitol-0.05 M Tricine-NaOH buffer (pH 7) were used throughout the investigation.

Experimental results of pH *versus* CO_2 fixation relationship shown in Figure 3 demonstrate that the photosynthetic activity was maximal at pH 8.5 under the experimental conditions employed. It will be recalled that Avron and Gibbs (5) reported that the pH optimum for CO_2 fixation by intact spinach chloroplast preparations is about 8.5 but dependent upon the reaction medium.

Several compounds were tested for their effect on photosynthetic CO_2 fixation, and results are summarized in Table I. They had no effect when added singly, but the combination

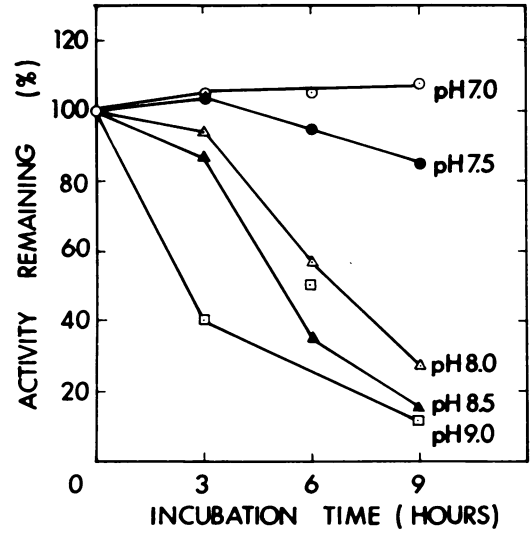

FIG. 2. Stability of spinach protoplasts. Protoplasts were suspended in 0.8 M mannitol-0.1 M Tricine-NaOH buffer of specified pH and stored in dark at 4 C. Aliquots withdrawn at 3 hr intervals were subjected to CO_2 fixation at 25 C for 5 min in the standard assay system (pH 8.5). Original activity of the protoplasts was 47 μmoles CO_2 fixed/mg Chl·hr (pH 8.5).

of 1 mM Mg^{2+} and 0.5 mM EDTA caused a significant inhibitory effect (23%).

"K*m*(CO_2)" of Protoplasts. The rate of CO_2 fixation by protoplasts as a function of bicarbonate concentration was studied to determine the CO_2 concentration for half-maximal rate of CO_2 fixation ("K*m*"), and "K*m*(HCO_3^-)" value of 0.9 mM (pH 7) and 2.5 mM (pH 8.5), respectively, was obtained (Fig. 4). Although there is no assurance that the "apparent K*m*" is comparable to real K*m*, the above "K*m*(HCO_3^-)" value is in the same range as that observed with the intact spinach chloroplasts having high affinity to CO_2 (16, 17, 35), which is evidently lower than that of the purified RuDP carboxylase reported by several workers (12, 23, 29, 31, 35). The "K*m*(CO_2)" calculated from the data according to Murai and Akazawa (23), 19.8 μM (pH 7.0) and 42 μM (pH 8.5), respectively, was nearly equivalent to that of the low K*m* form of RuDP carboxylase reported by Bahr and Jensen (7) and Badger *et al.* (6).

Postillumination CO_2 Fixation. Protoplast preparations pre-

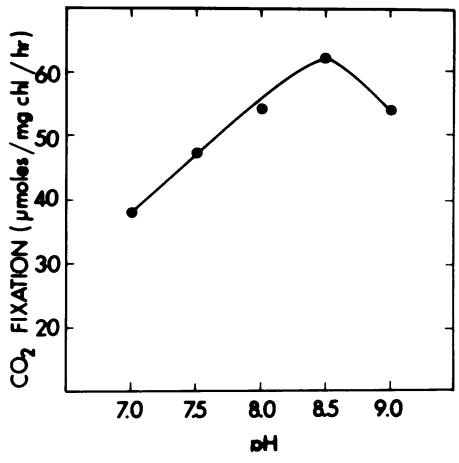

FIG. 3. pH dependence of photosynthetic CO_2 fixation. pH *versus* CO_2 fixation relationship was determined using the standard assay system described in text, including Tricine-NaOH buffer of varied pH. Original activity of the protoplasts was 64 μmoles CO_2 fixed/mg Chl·hr (pH 8.5).

Table I. *CO_2 Fixation under Various Reaction Conditions*

Various compounds were added to the assay mixture in the preincubation periods. Original activity (light-dependent CO_2 fixation) of the protoplasts was 55 μmoles CO_2 fixed/mg Chl·hr (pH 8.5).

Changes in Reaction Mixture	Light-dependent CO_2 Fixation	Postillumination CO_2 Fixation
	%	
Complete	100	100
With 0.1 mM Mg^{2+}	95	93
With 1 mM Mg^{2+}	92	94
With Mg^{2+} and 0.5 mM EDTA	77	82
With 0.5 mM EDTA	88	96
With 0.1 mM Pi	93	94

viously illuminated for 10 min exhibited the activity of CO_2 fixation in dark (Fig. 5). The specific activity was approximately 13% of the light-dependent CO_2 fixation. The behavior is essentially analogous to the intact cells of higher plants (21) as well as green algae (34). The results can be interpreted on the basis that a steady state level of RuDP in the preilluminated protoplasts sustains the CO_2 fixation reaction in the subsequent dark period. The effect of various compounds on the postillumination CO_2 fixation was basically the same as that on light-dependent CO_2 fixation (Table I).

Photosynthetic O_2 Evolution. A complete structural assembly of photosynthetic activities in protoplasts can be envisaged from the light-dependent O_2 evolution as shown in Figure 6 (3). The specific activity of 33 to 40 μmoles of O_2 evolved/mg Chl·hr (pH 7) is nearly in the range observed with intact chloroplasts (13, 22), and the activity was not lost during 9 hr storage in 0.8 M mannitol buffer (pH 7). The photosynthetic O_2 evolution by the protoplast preparations was strongly inhibited by 10 μM DCMU. However, it will be noted that there is a DCMU-insensitive O_2 uptake in the dark (12.7 μmoles of O_2 uptake/mg Chl·hr), which is approximately the same rate as that observed by Aono *et al.* (3) using spinach mesophyll cells.

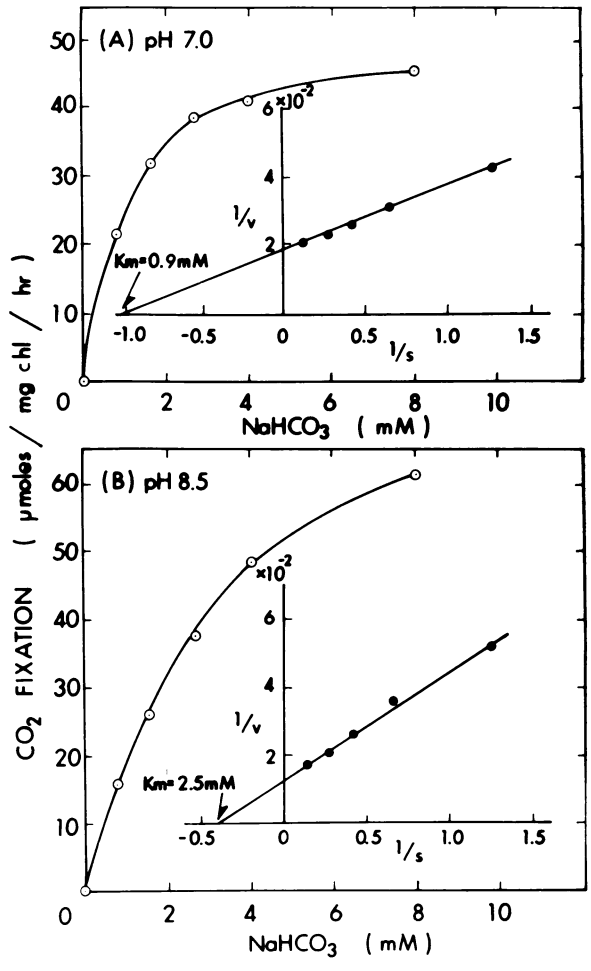

FIG. 4. Effect of bicarbonate concentration on CO_2 fixation by protoplasts. Experimental systems were the same as those described in Fig. 3, except for varied levels of bicarbonate. Activities were measured at both pH 7 and 8.5. In order to calculate the "K*m*(CO_2)" values, the concentration of CO_2 at each pH was computed according to the method of Murai and Akazawa (23). Original activity of the protoplasts was 60 μmoles CO_2 fixed/mg Chl·hr (pH 8.5).

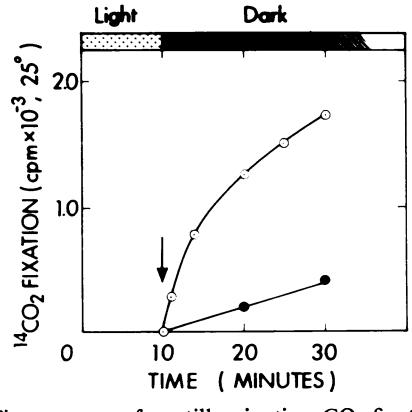

FIG. 5. Time course of postillumination CO_2 fixation by protoplasts. Reaction conditions are described in text. Reaction components containing protoplasts (18 μg Chl) were same as those in Fig. 1. Original activity of the protoplasts was 60 μmoles CO_2 fixed/mg Chl·hr (pH 8.5). Preillumination for 10 min (⊙); without preillumination (●).

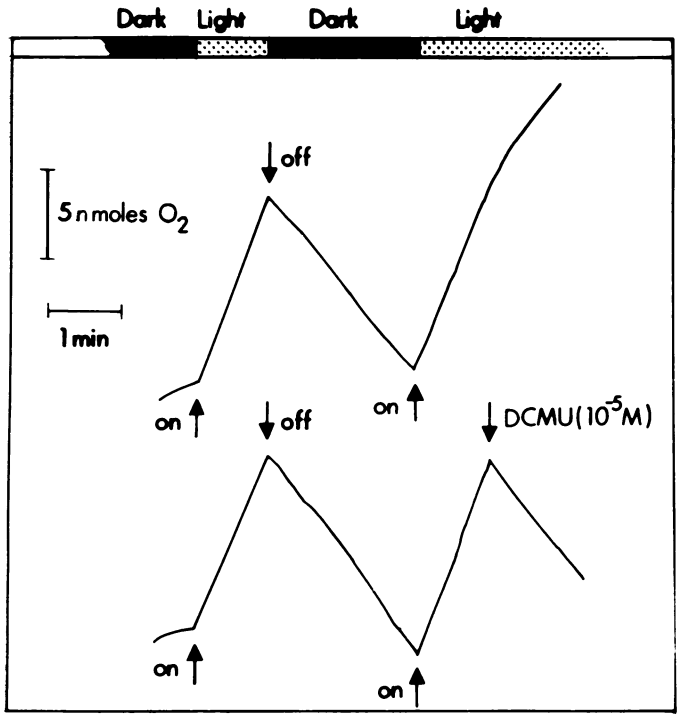

FIG. 6. Photosynthetic O_2 evolution by protoplasts. Reaction conditions are described in text. To examine the effect of DCMU, the reagent dissolved in ethanol was added to the reaction mixture at the final concentration of 10 μM at arrow. Original activity of the protoplasts was 75 μmoles CO_2 fixed/mg Chl·hr (pH 8.5) and 40 μmoles O_2 evolved/mg Chl·hr (pH 7), respectively.

DISCUSSION

With the advent of knowledge permitting the interpretation of the regulatory mechanism of photosynthetic reactions in molecular terms, much current interest in photosynthesis research is directed at correlating molecular mechanisms based on enzymic studies (*in vitro*) with those operating in living plant cells (*in vivo*) (1, 12, 35). Many experiments using intact chloroplast preparations having high photosynthetic activities have shown that they are profitable for such research purposes (12, 16, 17, 22, 35). Chloroplasts, however, are vulnerable to environmental conditions during isolation steps. Isolated chloroplasts are classified into several types depending on the degree of intactness or breakage, and changes in structural characteristics are reflected in impairment of their biochemical activities (15, 22, 30). It will be noted that there is a discrepancy between chloroplasts and intact plant cells with respect to their photosynthetic activities, *e.g.* postillumination CO_2 fixation (5). One major reason for this phenomenon is that metabolic channeling is cut off between the chloroplast and cytoplasm. Our results on protoplasts show that they have reasonably high photosynthetic activities and high affinities to CO_2. Penetration of macromolecular metabolites into protoplasts appears to be another advantage in their usage for biological research. In a series of investigations, Takebe and his associates (2, 27, 28, 33) clearly established the multiplication of tobacco mosaic virus in tobacco leaf protoplasts by means of penetration of virus particles and anti-tobacco mosaic virus-γ-globulin into protoplast preparations. In order to disclose the functional role *in vivo* of constituent subunits of the spinach leaf RuDP carboxylase molecule, we are now examining the effect of antibodies raised against each subunit molecule on photosynthetic CO_2 fixation reactions in protoplasts. Because several investigations have demonstrated that the site of synthesis of two subunits of RuDP carboxylase is different (8, 13), we feel that protoplasts may also provide a chance of studying the biosynthetic mechanism of this important protein in plant leaf cells.

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