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Decrease in Carbamylation of Rubisco by High CO_2 Concentration is Due to Decrease of Rubisco Activase in Kidney Bean

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Decrease in rubisco activation at high CO_2 concentration was caused by decrease in carbamylation of rubisco (Roh *et al.*, 1996). However, it is unclear whether decrease in carbamylation rate at high CO_2 concentration is due to decrease in activity itself or content of rubisco activase. To clarify this ambiguity, investigation was performed to determine effects of CO_2 concentration on rubisco activase with kidney bean (*Phaseolus vulgaris* L.) leaves grown at normal CO_2 (350 ppm) and high CO_2 (650 ppm) concentration. The analysis of Western blotting showed that the 50 and 14.5 kD polypeptides were identified immunochemically as the large and small subunits of rubisco in the preparation, respectively. For the 14.5 kD small subunit, the degree of intensity at high CO_2 concentration was similar to that at normal CO_2 concentration. For the 50 kD large sububit, however, the intensity of a band at high CO_2 concentration was significantly higher than that at normal CO_2 concentration, indicating that only the large subunit is affected by high CO_2 concentration. The analysis of Western immunoblotting showed two major polypeptides at 46 and 42 kD which were identified as rubisco activase subunits. The intensities of two bands were shown to be higher at normal CO_2 than high CO_2 concentration. These data indicate that decrease of carbamylation resulting from increase of CO_2 concentration was caused by rubisco activase. Finally, by employing ATP hydrolysis assay and ELISA, we also observed a significant decrease in both activity and content of rubisco activase as CO_2 concentration was raised from normal to high CO_2 concentration. These results suggest that decrease in rubisco carbamylation at high CO_2 concentration is caused by activity itself and/or content of rubisco activase.

Keywords: rubisco activase, high CO_2 concentration, carbamylation, Western blot, ELISA

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (rubisco) catalyzes not only the fixation of CO_2 in photosynthetic carbon reduction, but also the fixation of O_2 in photorespiration (Miziorko and Lorimer, 1983). The activation of rubisco involves formation of a carbamate which is binding of an activator CO_2 to the ε-amino group of lys-201 within the active site on the large subunit, followed by addition of Mg^{2+} (Lorimer and Miziorko, 1980; Andrews and Lorimer, 1987). The activation of rubisco *in vitro* is achieved spontaneously, while this activation *in vivo* is mediated by rubisco activase (Salvucci *et al.*, 1985) in the presence of ATP (Streusand and Portis, 1987) and RuBP (Portis, 1990).

The activation of rubisco by rubisco activase requires not only an appropriate pH, Mg^{2+}, and CO_2, but also the light-mediated association or interaction of rubisco activase with thylakoid membrane (Campbell and Ogren, 1992). Rubisco activation also requires electron transport through PSI and the presence of a transthylakoid pH difference (Campbell and Ogren, 1990).

Rubisco activase affects carbamylation by promot-

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ing ATP-dependent dissociation of tightly bound RuBP from the active site of decarbamylated rubisco (Wang and Portis, 1991). When bound to decarbamylated rubisco, RuBP prevents carbamylation and its removal by rubisco activase apparently converts rubisco into a form that has a high affinity for carbamylation (Wang and Portis, 1992). Rubisco activase also catalyzes a related reaction, the removal of 2-carboxyarabinitol 1-phophate (CA1P) from the active site of rubisco (Portis, 1992). Rubisco activase overcomes the inhibition by facilitating dissociation of CA1P from the rubisco active site (Portis, 1992).

Previous studies demonstrated that the decrease in rubisco activity at high CO_2 concentration was caused by decrease in carbamylation of rubisco (Roh *et al.*, 1996). However, it is unclear whether decrease in carbamylation rate in high CO_2 concentration is due to decrease in activity itself or content of rubisco activase. In this study, effects of high CO_2 concentration in the rubisco activase were investigated by studying the polypeptide profiles of SDS-PAGE and Western blotting, by measuring the activity and content of rubisco activase employing an ATP dependent hydrolysis assay and ELISA, respectively.

MATERIALS AND METHODS

Plant culture

Seeds of kidney bean (*Phaseolus vulgaris* L.) were germinated and grown in Conviron normal (350 ppm CO_2) and high (650 ppm CO_2) growth chambers as described previously (Roh *et al.*, 1996).

Isolation of crude extracts for rubisco and rubisco activase

The crude extracts containing rubisco and rubisco activase were isolated from kidney bean leaves following a modification of the method of Wang *et al.* (1992). Leaves (100 g) were deribbed and ground into the fine powder in liquid nitrogen. The frozen leaf powder was added into 200 mL of the extraction buffer [50 mM BTP (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP and 3 mM MBT] and stirred for 10 min after the ice was melted. Then the leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. Filtered solution was centrifuged for 40 min at 30,000 g. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. The supernatant and pellet were collected by centrifugation for 10 min at 8,000 g. The pellet was resuspended in 5 mL of 20 mM BTP (pH 7.0) containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT (buffer A). The supernatant contains rubisco and the resuspended pellet contains rubisco activase.

Purification of rubisco activase

50% (w/v) PEG-10,000 was added into the resuspended pellet obtained above to bring the final concentration to 18%, stirred 5 min, and centrifuged for 10 min at 8,000 g. The pellet was dissovled in 5 mL of buffer A. Solution was cleared by spinning for 10 min at 20,000 g. Pellet was resuspended again in 5 mL buffer A and the solution cleared again.

The supernatants collected above were loaded onto a 20 mL Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). The column was eluted with 40 mL of 20 mM BTP (pH 7.0) at a flow rate of 1 mL/min before continuing with 140 mL of a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). Only a single fraction (3 mL) having the highest rubisco activase activity was assayed by SDS-PAGE.

Assay of ATP hydrolysis by rubisco activase

ATP hydrolysis by rubisco activase was measured by procedure of Robinson and Portis (1989a). Production of ADP was measured in a coupled spectrophotometric assay. The reaction mixture contained 50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 40 units/mL pyruvate kinase, and 40 units/mL lactate dehydrogenase in a total volume of 0.4 mL.

The reaction was started with addition of rubisco activase and the rate of ADP appearance was determined from the rate of NADH oxidation as indicated by decrease in absorption at 340 nm. One unit was defined as 1 μM ATP hydrolyzed per min.

Preparation of kidney bean rubisco activase antibody

Antibody was produced against kidney bean rubisco activase as described by Harlow and Lane (1988). Purified rubisco activase obtained above was precipitated in 80% acetone at -20°C for 12 hrs and pelleted by centrifugation at 11,500 g for 20 min. The resulting pellet was resuspended in a phosphate-buffered saline (PBS) [137 mM NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4, 4 mM Na_2HPO_4 (pH 7.2)]. After boiling for 10 min, the samples were emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into the back of

a rabbit (three of 2.5 kg, male, New Zealand White rabbits were injected). A booster injection containing the protein emulsified in an incomplete Freund's adjuvant was given after 3 weeks. At two weeks after the booster injection, the rabbits were bled from the marginal ear vein. Rabbit antisera containing antibodies against rubisco activase were collected, aliquoted, and then either used immediately or stored at -80°C.

Electrophoresis

SDS-PAGE was carried out following the method of Laemmli (1970) using 13% gels.

Western blot analysis

Proteins were electroblotted from SDS-polyacrylamide gels onto 0.2 μm nitrocellulose membranes in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol) using a semi-dry transblot apparatus (Bio-Rad Lab. Hercules, CA, U.S.A.) for 1 hr at 0.25 A constant current. The efficiency of protein transfer was verified by Coomasie blue staining of the gels after transfer. After treatment with blocking buffer [5% (w/v) nonfat dry milk, 0.2% Tween 20, 0.02% sodium azide in PBS], the protein blots were incubated with a 1:1,000 dilution of a rabbit anti-rubisco activase antiserum as a primary antibody at room temperature for 1 hr, followed by four 5 min washes, each in PBS.

The membrane was incubated with a 1:5000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase as a secondary antibody at room temperature for 1 hr, and then washed for 10 min in Tris-buffered saline (TBS) [150 mM NaCl, 50 mM Tris (pH 7.5)]. The bound antibody was visualized by addition of 10 mL of alkaline phosphatase buffer [100 mM NaCl, 5 mM MgCl, 100 mM Tris (pH 9.5)] supplemented with 66 μL of 50 mg NBT/mL of 70% DMF and 33 μL of 50 mg BCIP/mL of 100% DMF. The reaction was terminated within 3 to 5 min by addition of PBS containing 20 mM EDTA which chelates the Mg^{2+}. The membrane was dried and photographed.

For analysis of rubisco, a rabbit anti-rubisco antiserum (gift from Dr. Daniel Klessig at Rutgers University, NJ, U.S.A.) was used as a primary antibody following the same procedure as described in rubisco activase analysis previously.

Elisa

The optimum dilution of antigen to be coated on the microplate was determined by using multiple rubisco activase dilutions. For coating of the antigen, 100 μL of different dilutions of rubisco activase in 0.1 M sodium carbonate-bicarbonate coating buffer (pH 9.5) was added to each well of microplate. After overnight incubation at room temperature, the plate was washed six times with washing solution [0.05% Tween 20 in 0.01 M PBS (pH 7.4)]. To eliminate nonspecific binding, 250 μL of 0.01 M PBS (pH 7.4) containing 0.1% BSA was added to each well and incubated for 1 hr at 37°C. Then the plate was washed with the washing solution six times. After adding of 50 μL of various dilutions of rubisco activase in 0.01 M PBS (pH 7.4), 50 μL of different dilutions of a rabbit anti-rubisco activase antiserum as a primary antibody was added to each well, and incubated for 30 min at 37°C. The plate was again washed as described above, then 100 μL of peroxidase-conjugated goat anti-rabbit IgG diluted to 1:20,000 in 0.01 M PBS (pH 7.4) containing 0.1% BSA was added and incubated for 30 min at 37°C. The plate was washed eight times as previously described and 100 μL of peroxidase substrate [OPD tablets in 10 mL of 0.05 M citrate/0.1 M sodium phosphate buffer (pH 5.0) containing 30% of H_2O_2] was added. After incubation at room temperature in the dark for 10 to 20 min, the reaction was terminated by addition of 0.1 mL of 1 N HCl. The absorbance at 490 nm was determined by a ELISA microplate reader (Bio-Rad Model 3550-UV).

All procedures were carried out at 4°C except as indicated.

RESULTS AND DISCUSSION

The concentration of CO_2 in the atmosphere is one of the major limiting factors in photosynthesis. To understand photosynthesis in the plant grown at high CO_2 concentration, it is necessary to define the rubisco activity related to carbon fixation in photosynthesis. CO_2 fixation is controlled by rubisco that is active in calvin cycle (Perchorowicz *et al.*, 1981).

Decrease in rubisco activation at high CO_2 concentration in kidney bean (Roh *et al.*, 1996) is consistent with reports showing that rubisco activity declines in transgenic tomato (Micallef *et al.*, 1995) and in pea (Majeau and Coleman, 1996) grown at elevated CO_2 concentration. Decrease of rubisco activity occur during extended exposure to high CO_2 concentration in plants with a relatively high source-sink ratio such as clover or sunflower (Stitt, 1991; Morin *et al.*, 1992; Woodrow, 1994). Similar studies were also reported by Vu *et al.* (1983) in tobacco and by Bowes

(1991) in tomato and wheat.

The reduction in rubisco activity was associated with a reduced amount of rubisco protein at the higher CO_2 level (Downton *et al.*, 1980). To verify that increased CO_2 concentration causes decrease in rubisco amount, we have preliminarily tested SDS-PAGE rubisco profile in kidney bean leaves grown at normal CO_2 and high CO_2 concentration. Two major polypeptides of 50 and 14.5 kD detected by SDS-PAGE analysis were identified as the large and small subunits of rubisco, respectively. Previously, Roh *et al.*, (1996) reported that a few differences exist in the intensity comparing high CO_2 concentration to normal CO_2 concentration in the both 50 kD and 14.5 kD bands. We assume that this preliminary experiment was not accurate enough to distinguish slight difference in the intensity of bands. In this experiment, therefore, we have performed the immunoblotting assay using the antibodies for the large and small subunits to clarify this ambiguity.

As shown in Fig. 1, the profiles of the preparation by analysis of Western immunoblotting showed two major polypeptides at 50 and 14.5 kD which were identified as the large and the small subunits of rubisco. For the 14.5 kD small subunit, the degree of intensity at high CO_2 concentration was similar to that at normal CO_2 concentration. For the 50 kD large sububit, however, the intensity of band at high CO_2 concentration was significantly higher than that at normal CO_2 concentration, indicating only large subunit is affected by high CO_2 concentration.

Fig. 1. Comparison of rubisco in normal CO_2 (lane 1) and high CO_2 concentration (lane 2) by immunological detection. Proteins (10 μg) were separated by 13% SDS-PAGE and then transferred onto nitrocellulose membranes. LS, large subunit; SS, small subunit.

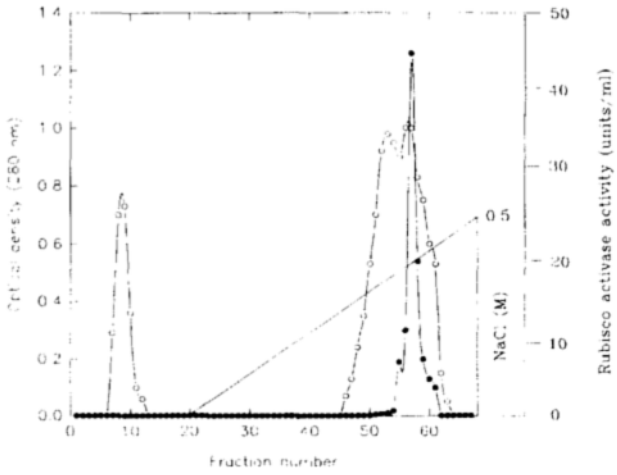

Fig. 2. Elution profile of rubisco activase from anion exchange chromatography on Q-Sepharose column. The straight line indicates the 0~0.5 M NaCl gradient in 20 mM BTP (pH 7.0). The protein content (○) of each fraction was measured at 280 nm. The activity of rubisco activase (●) was detected by ATP hydrolysis at 340 nm.

For preparation of rubisco activase antibody, the rubisco activase was purified on a Q-Sepharose column after PEG-10,000 fractionation. The elution profiles for protein and activity of rubisco activase are seen in Fig. 2. A single peak of activity was detected eluting at 0~0.5 M NaCl. For the rubisco activase, the maximal elution is at 0.38 M.

The various fractions in the purification steps were analyzed by SDS-PAGE as shown in Fig. 3. As shown in Fig. 3, the fraction with the highest rubisco activase activity from the ion exchange Q-Sepharose (Fig. 2) was confirmed to have only 46 and 44 kD bands which were found in most of higher plant species except maize (Somerville *et al.*, 1982; Salvucci *et al.*, 1987; Robinson *et al.*, 1988).

The specificity of the antibody produced from the purified activase was confirmed by probing the nitrocellulose replicates of crude extracts of kidney bean separated by SDS-PAGE. Bands correspondig to the 46 kD and 42 kD polypeptides in the protein gel were visible on two immunoblots of crude leaf extracts from kidney bean grown at normal CO_2 (Fig. 4) and high CO_2 concentration (Fig. 5).

Rubisco activase promoted carbamylation of rubisco in the presence of RuBP (Salvucci, 1989; Portis, 1990) and controlled release of RuBP from the ac-

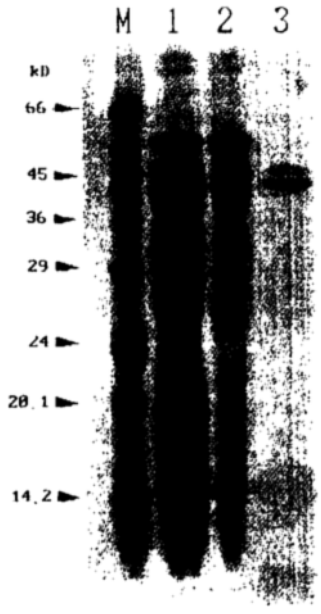

Fig. 3. SDS-PAGE analysis of fractions in purification of rubisco activase as described in "Materials and Method". Lanes contained 10 μg protein. M, molecular weight standards; lane 1, crude extracts; lane 2, 50% PEG-10,000; lane 3, high peak fraction from Q-Sepharose anion exchange chromatography. Protein was stained with Coomassie blue.

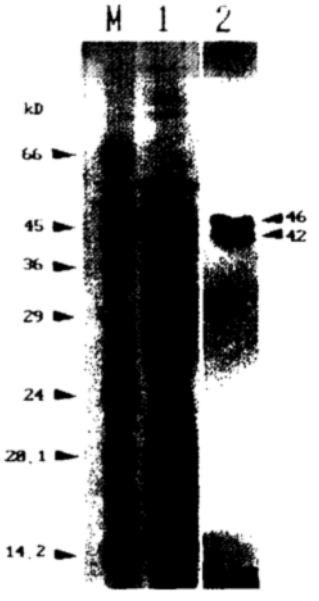

Fig. 4. Immunological detection and SDS-PAGE analysis of rubisco activase in normal CO_2 concentration. Lanes contained 10 μg protein. M, molecular weight standards; lane 1, SDS-PAGE pattern of crude extracts; lane 2, Western blotting assay of lane 1. Protein was visualized by Coomassie blue. Nitrocellulose membrane was stained with BCIP and NBT as substrate. Rubisco activase indicated by an arrow.

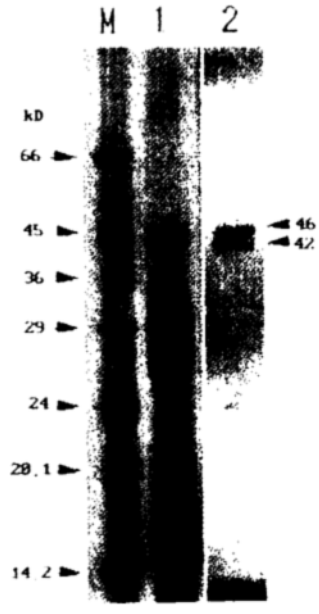

Fig. 5. Immunological detection and SDS-PAGE analysis of rubisco activase in high CO_2 concentration. Lanes contained 10 μg protein. M, molecular weight standards; lane 1, SDS-PAGE pattern of crude extracts; lane 2, Western blotting assay of lane 1. Protein was visualized by Coomassie blue. Nitrocellulose membrane was stained with BCIP and NBT as substrate. Rubisco activase indicated by an arrow.

tive site of rubisco in a process requiring the hydrolysis of ATP (Lilley and Portis, 1990; Portis, 1992). This action serves to "activate" rubisco by freeing the rubisco active site of compounds that either hinder carbamylation of the activator lysine or block RuBP from binding to the carbamylated rubisco (Hartman and Harpel, 1994). In the absence of rubisco activase, only 20 to 40% of rubisco catalytic sites are carbamylated, leading to a significant inhibition of photosynthesis (Salvucci, 1989; Portis, 1990). However, full carbamylation of rubisco can occur if CO_2 are increased to high levels in the absence of RuBP (Andrews and Lorimer, 1987). The carbamylation of rubisco was inhibited under the condition in which RuBP was present but rubisco activase was absent, even at high concentrations of CO_2 (Portis *et al.*, 1986; Robinson *et al.*, 1988). For these reasons, we postulated that decrease in carbamylation rate in high CO_2 concentration is due to decrease of rubisco activase. Therefore, to answer this question, Western immunoassay of the rubisco activase prepared in normal CO_2 concentration and high CO_2 concentration were performed with antiserum in the present study. As seen in Fig. 6, the intensity of both bands in nor-

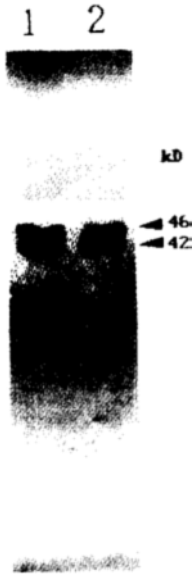

Fig. 6. Comparison of rubisco activase in normal CO_2 (lane 1) and high CO_2 concentration (lane 2) by immunological detection. Proteins (10 μg) were separated by 13% SDS-PAGE and then transferred onto nitrocellulose membrane for Western blotting assay. Rubisco activase indicated by an arrow.

mal CO_2 concentration was significantly higher than that of corresponding bands in high CO_2 concentration. This data indicates that high CO_2 concentration affects rubisco activase. Moreover, these results suggest that decrease of carbamylation due to increase of CO_2 concentration is caused by rubisco activase.

Further experiments were done to measure activity and content of rubisco activase in order to clarify whether the rubisco activase decreases carbamylation or not. The assay for rubisco activase activity is based on its ability to catalyze the hydrolysis of ATP (Robinson and Portis, 1989b; Salvucci, 1992), while content of rubisco activase was measured by ELISA. The activity and content of rubisco activase in normal CO_2 and high CO_2 concentration is shown in Table 1. When CO_2 concentration was raised from normal to high, activity and content of rubisco activase decreased: from 41 to 23 units for activity; from 3.337 to 2.042 ng for content. These results suggest that decrease in carbamylation of rubisco at high CO_2 concentration is caused by activity itself and/or contents of rubisco activase.

Table 1. The activity and content of rubisco activase in normal CO_2 and high CO_2 concentration

	Activity (units)	Content[a] (ng)
Normal CO_2	41	3.337
High CO_2	23	2.042

[a]Content of rubisco activase was detected by ELISA.

All of the above results strongly suggest that increase in CO_2 concentration results in decreases in activity and content levels of rubisco activase, which leads to a subsequent reduction of rubisco activation and carbamylation.

Further studies should be carried out to determine the sequence of DNA and/or protein in rubisco acivase purified from normal CO_2 and high CO_2 concentration.

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