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Influence of Cadmium on Rubisco Activation in *Canavalia ensiformis* L. Leaves

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Abstract We studied the effect of cadmium on chlorophylls and rubisco activation in *Canavalia ensiformis* L. leaves. Chlorophyll levels were reduced by 5.0 μM Cd. Rubisco activity at 5.0 μM Cd was significantly smaller than that at no treatment. Rubisco content showed patterns of change similar to rubisco activity. These data suggest that rubisco activity was associated with an amount of rubisco protein, and that the activation and induction of rubisco is inhibited by Cd. The degree of intensity of 50 and 14.5 kD polypeptides identified as the large and small subunit of rubisco by SDS-PAGE analysis at 5.0 μM Cd was significantly lower than that at control, indicating Cd had a effect on both subunits. Under the assumption that effects of Cd on rubisco may be related to rubisco activase, in addition to, its activity and content were determined. The rubisco activase activity at 5.0 μM Cd was more decreased than the control. A similar change pattern was also observed in content of rubisco activase. Remarkable differences in the intensitiy of both the 45 kD and 41 kD band were found between at control and Cd-treatment. These results suggest that the change in the levels of rubisco activase leads to a subsequent alteration of rubisco levels.

Keywords: cadmium, *Canavalia ensiformis* L., chlorophyll, rubisco, rubisco activase

INTRODUCTION

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase: EC 4.1.1.39) is an enzyme in the chloroplast-localised photosynthetic carbon assimilation and also in photorespiration [1]. Prior to catalysis, rubisco must be converted to an active carbamylated form by the binding of an activator CO_2 and Mg^{2+} to ε-amino group of Lys-201, [2]. In higher plants, this process is mediated by the rubisco activase [3]. ATP is required for activase activity [4], and the hydrolysis of this compound likely dissociates bound ribulose- 1,5-bisphosphate (RuBP) and other sugar phosphates from the active site [5]. Rubisco activase also catalyzes the removal of inhibitors such as CA1P (2-carboxyarabinitol 1-phosphate) and CABP (carboxyarabinitol 1,5-bisphosphate) from the active site of rubisco [6]. CA1P binds tightly to carbamylated rubisco [7], whereas CABP binds to both activated sites and inactive sites of rubisco [8].

Cadmium (Cd) is one of the major pollutants emanating in natural and agricultural environments from industrial and municipal wastes, sewage sludge. High concentration of Cd is recognized as one of the most phytotoxic heavy-metal contaminants [9]. Cd irreversibly replaces other metal ions in essential metalloenzymes [10].

Although not essential for plant growth, Cd is readily taken up by roots and translocated into aerial organs [11], then accumulates mainly in the cell vacuoles [12].

The application of Cd to plant induces water stress symptoms such as the decrease of stomatal conductance, transpiration rate and relative water content in plant leaves [13]. Cd also alters the synthesis of RNA, inhibits RNase activity [14], decreases catalase activity and increases ascorbate peroxidase activity [15]. Panković [16] have reported that Cd affected the RuBP regeneration capacity of the Calvin cycle.

The multiplicity of Cd effects on the photosynthesis can be explained by the variety of plant species and the concentration of Cd. Some reports have provided evidence that Cd is a potent inhibitor on the photochemical activity of photosynthesis in the chloroplast [17,18]. Conversely, different study has indicated that the photosynthesis is not sensitive to Cd [19].

In spite of the considerable literature on this subject, however, Cd toxicity on the photosynthesis at an enzyme level are not known with any certainty.

This study was performed to investigate the influence of Cd on photosynthetic pigments, and enzymes by measuring content and activity, and by analysis the peptides patterns of SDS-PAGE in *Canavalia ensiformis* L. leaves.

MATERIALS AND METHODS

Plant Culture

Seeds of jackbean (*Canavalia ensiformis* L.) were ger-

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minated and grown in growth chamber as described previously [20,21]. Plants were illuminated under mixed metal-halide and incandescent lamps at 800 to 1,200 $\mu M/m^2/s$ PFD, 350 ppm CO_2 for the entire 24-h period, and 26°C for the 16-h day and 18°C at night with a relative humidity of 60%. Plant leaves were sprayed manually with Cd (5.0 μM $CdCl_2 \cdot 2.5H_2O$) solution daily. Fully expanded leaves from mature plants were used for experiments.

Determination of Chlorophyll Content

Chlorophyll content was determined as described by Inskeep and Bloom [22]. For the chlorophyll estimation in leaves, the leaves were frozen in liquid nitrogen, and ground to a fine powder, extracted with DMF in the dark, and centrifuged for 5 min at 8,000 x *g*. Chlorophyll was measured spectrophotometrically using its specific absorption coefficients at 664.5 nm and 647 nm. The following equations were used to give the concentration of chlorophyll *a*, chlorophyll *b*, and total chlorophyll.

$$\text{Chlorophyll } a \text{ (mg/g fr. wt.)} = 12.70\, A_{664.5} - 2.79\, A_{647}$$
$$\text{Chlorophyll } b \text{ (mg/g fr. wt.)} = 20.70\, A_{647} - 4.62\, A_{664.5}$$
$$\text{Total chlorophyll (mg/g fr. wt.)} = 17.90\, A_{647} + 8.08\, A_{664.5}$$

Purification of Rubisco

The rubisco and rubisco activase were purified from soybean leaves following a modification of the method of Wang *et al.* [23]. Leaf tissue was ground to a fine powder with a pre-cooled mortar and pestle in liquid nitrogen and then extracted in the extraction buffer containing 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. The leaf slurry was filtered through four layers of cheesecloth and one layer of Miracloth. Filtered solution was centrifuged at 30,000 x *g* for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. The supernatant and pellet were collected by centrifugation at 8,000 x *g* for 10 min. The supernatant contains rubisco and the resuspended pellet contains rubisco activase.

The supernatant collected was brought to 55% saturation of $(NH_4)_2SO_4$ by addition of powder. The pellect collected by centrifugation at 8,000 x *g* for 10 min 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), and 50% PEG-10 K was added to a final concentration of 18%. The resulting precipitate was collected by centrifugation at 8,000 x *g* for 10 min and resuspended in buffer A.

Resuspended solution was loaded onto a Q-Sepharose column equilibrated with 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. The column was washed with the same buffer containing 0.1 M NaCl before starting elution with a linear gradient from 0.1 to 0.5 M NaCl at a flow rate of 1 mL/min. 3 mL fractions were pooled, and assayed for rubisco content and activity.

Purification of Rubisco Activase

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

The supernatants collected 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). Fractions (3 mL) were pooled, and assayed for rubisco activase content and activity.

All purification processings were done at 4°C except as indicated.

ELISA

The content of rubisco and rubisco activase were detected by ELISA. For coating of the antigen, 100 μL of different dilutions of two enzymes 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 with 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. To eliminate nonspecific binding, 250 μL of 0.1% BSA in 0.01 M PBS (pH 7.4) was added to each well and incubated for 1 hr at 37°C. After washing and adding of 50 μL of various dilutions of two enzymes in 0.01 M PBS (pH 7.4), 50 μL of different dilutions of a rabbit anti-rubisco and anti-rubisco activase antiserum as a primary antibody [21] was added to each well, respectively, 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 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).

Assay of Rubisco Activity

Rubisco activity was determined at 25°C by method of Racker [24]. The purified rubisco solution was added to assay medium of a final volume of 1 mL containing 1 M Tris buffer (pH 7.8), 0.006 M NADH, 0.1 M GSH, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025 M 3-phosphoglycerate kinase, 0.05% α-glycerophosphate

dehydrogenase-triose phosphate isomerase, 0.025 M RuBP, 0.2 M ATP, 0.5 M $MgCl_2$, and 0.5 M $KHCO_3$. Oxidation of NADH was monitored at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate. One unit was defined as the amount that catalyzed the cleavage of 1 μM RuBP per min.

Assay of Rubisco Activase Activity

Rubisco activase activity was assayed as the ability to produce ADP in an ATP-dependent reaction in absorption at 340 nm by procedure of Robinson and Portis [25]. The purified rubisco activase solution was added to a total volume of 0.4 mL of the activation reaction mixture containing 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. One unit was defined as 1 μM ATP hydrolyzed per min.

Electrophoresis and Molecular Weight

SDS-PAGE was performed in the presence of a 20% polyacrylamide gel at room temperature by the method of Laemmli [26]. The protein samples were boiled for 10 min before being loaded on the gel. Proteins were stained by Coomassie Brilliant Blue R-250, and then destained by 7.5% acetic acid.

The molecular weight was determined using the method of Weber and Osborn [27]. The molecular weight markers were rabbit muscle phosphorylase b (97 kD), bovine serum albumin (66 kD), chicken egg white ovalbumin (45 kD), bovine erythrocyte carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and bovine milk α-lactalbumin (14.4 kD).

RESULTS AND DISCUSSION

Chlorophyll Content

Negative effect of photosynthesis by Cd is due to the decreased amount of photosynthetic pigments [18]. In this study, chlorophyll content was determined from leaves of plants grown in both control and 5.0 μM Cd. Cadmium decreased the content of chlorophyll *a*. No significant difference in chlorophyll *b* was found between control and Cd-treatment. Chlorophyll *a*/*b* ratio showed a reduction at Cd-treatment. Total chlorophyll content was also reduced at Cd-treatment compared to control (Table 1). The decrease of chlorophyll in our study is due to inhibition of aminolevulinic acid synthesis [28] and of protochlorophyllide reduction [29]. These data suggest that Cd toxicity is associated with a marked reduction of chlorophyll level. These results were in agreement with those of Sandalio *et al.* [30] who showed that Cd produced a significant inhibition of chlorophyll content in leaves of pea. This situation could promote metabolic disturbances in photosystem I and II, leading to the generation of chlorophyll in chloroplast [30]. Dubé and Bornman [31] also observed a decrease in chlorophyll content in spruce when Cd was present.

Table 1. Effect of cadmium on chlorophyll content in jackbean leaves

Treatment	Chl. *a*	Chl. *b*	Chl. *a*/*b*	Total Chl.
	mg/g fr. wt.			
Control	13.9	21.7	0.64	35.6
Cd	12.2	21.6	0.56	33.8

Plants were grown for 15 weeks.

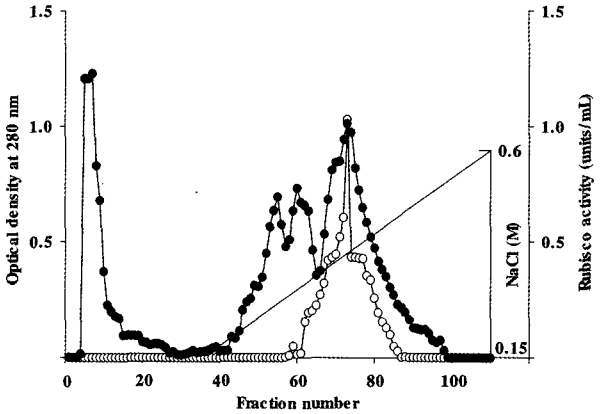

Fig. 1. Elution profile for protein (●) and activity (○) of rubisco from anion exchange chromatography on Q-Sepharose column. Rubisco was purified from jackbean leaves treated without Cd. It's activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0.15-0.6 M NaCl gradient in 20 mM BTP (pH 7.2).

Rubisco

Elution profiles for protein and activity of rubisco at control and Cd-treatment are seen in Fig. 1 and 2, respectively. The fraction with the highest peak of activity was used for measuring content and activity of rubisco.

The content of rubisco was detected by immunological method using an antibody. Rubisco content at 5.0 μM Cd was significantly smaller than that at no treatment (Fig. 3). Rubisco activity showed patterns of change similar to rubisco content (Fig. 4). These data suggest that rubisco content was connected with an activity of rubisco protein, and that the activation and induction of rubisco is inhibited by Cd. It was suggested that Cd could irreversibly bind to SH-groups on the active site of rubisco, thus lowering its activity [32]. This result is consistent with that of Stiborova *et al.* [33] who reported that rubisco activity was significantly decreased. The inhibition of rubisco activity has been considered to be the primary plant response to Cd stress [34].

To verify the inhibitory degree of rubisco, the accumulation of rubisco at control and Cd-treatment was moni-

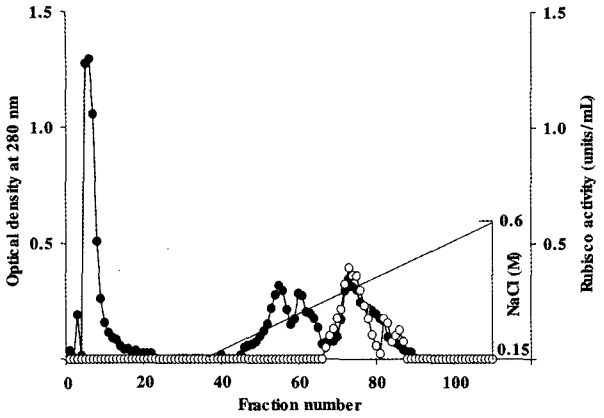

Fig. 2. Elution profile for protein (●) and activity (○) of rubisco from anion exchange chromatography on Q-Sepharose column. Rubisco was purified from jackbean leaves treated with 5.0 μM Cd. It's activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0.15-0.6 M NaCl gradient in 20 mM BTP (pH 7.2).

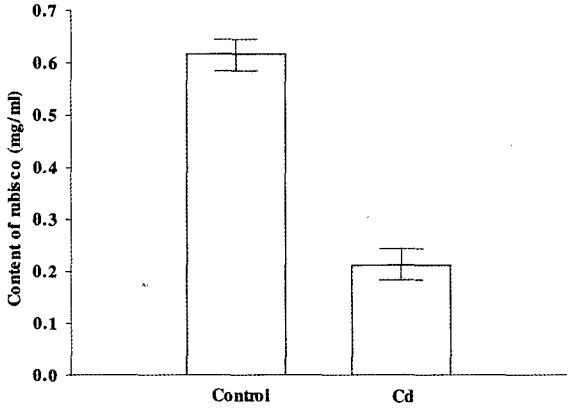

Fig. 3. Effect of cadmium on the content of rubisco in jackbean leaves. Plants were grown on Hoagland's nutrient solution for 15 weeks.

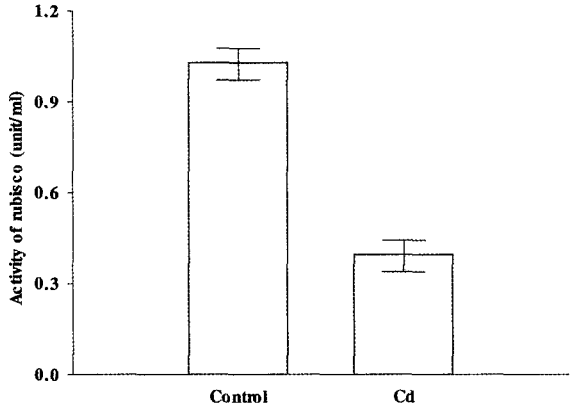

Fig. 4. Effect of cadmium on the activity of rubisco in jackbean leaves. Plants were grown on Hoagland's nutrient solution for 15 weeks.

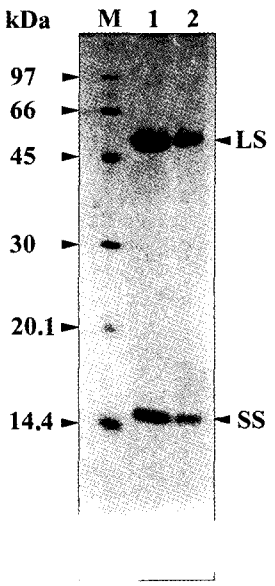

Fig. 5. SDS-PAGE analysis of rubisco purified from jackbean leaves. Proteins were (40 μg) separated on 20% SDS-PAGE gels. M, molecular weight standards; lane 1, no treatment; lane 2, Cd treatment. Protein was stained with Coomassie blue. LS, large subunit; SS, small subunit.

tored by SDS-PAGE analysis. 50 and 14.5 kD polypeptide were identified as the large and small subunit of rubisco at both control and Cd-treatment (Fig. 5). The degree of intensity of both polypeptides identified at Cd was significantly lower than that detected at control, indicating Cd had a effect on both subunits.

Rubisco Activase

The activation of rubisco *in vivo* is catalyzed by rubisco activase [35] in two sequential steps of the presence of ATP [4] and RuBP [3]. Rubisco activase is involved in the light-regulated activation of rubisco [36]. Rubisco activation by rubisco activase also requires electron transport through photosystem I and the presence of a transthylakoid pH difference [37].

Under the assumption that the effects of Cd on rubisco may be related to rubisco activase, this enzyme was purified on a Q-Sepharose column after PEG-10K fractionation, and then its content and activity were determined. The elution profiles and an ATP-dependent activity of rubisco activase at control and Cd-treatment are seen in Fig. 6 and 7, respectively. The rubisco activase activity was detected in a single peak at 0-0.5 M NaCl.

The rubisco activase content at 5.0 μM Cd was more decreased than the control (Fig. 8). A similar change pattern was also observed in activity of rubisco activase (Fig. 9). The content and activity of rubisco activase showed patterns of change which were similar to those of rubisco. These results suggest that the change in the levels of rubisco activase leads to a subsequent alteration of rubisco levels.

SDS-PAGE analysis of purified rubisco activase identi-

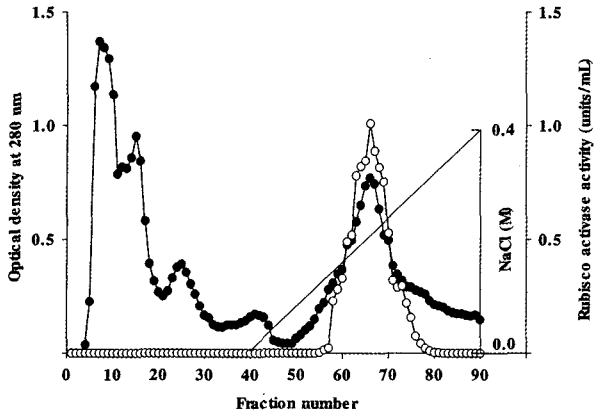

Fig. 6. Elution profile for protein (●) and activity (○) of rubisco activase from anion exchange chromatography on Q-Sepharose column. Rubisco activase was purified from jackbean leaves treated without Cd. It's activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0-0.4 M NaCl gradient in 20 mM BTP (pH 7.0).

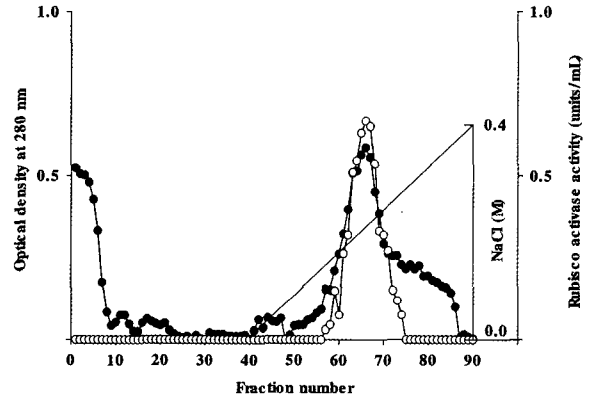

Fig. 7. Elution profile for protein (●) and activity (○) of rubisco activase from anion exchange chromatography on Q-Sepharose column. Rubisco activase was purified from jackbean leaves treated with 5.0 μM Cd. It's activity was detected by oxidation of NADH at 340 nm. The straight line indicates the 0-0.4 M NaCl gradient in 20 mM BTP (pH 7.0).

fied two polypeptides at 45 kD and 41 kD (Fig. 10). Similar results were observed in a study of rubisco activase using anti-RCA antibody in spinach, tobacco and rice [38]. In contrast to our result, however, two polypeptides, at 46 and 42 kD, have been reported during the immunological and electrophoretical detection of rubisco activase from leaves of soybean [39-41] and kidney bean [21], and from barley rubisco activase expressed in transformant *E. coli* [42] separated by SDS-PAGE. Bands corresponding to 45 kD and 41 kD polypeptide at control were visible at Cd-treatment. Remarkable differences in the intensitiy of both 45 kD and 41 kD band were found between at control and Cd-treatment, indicating Cd had a effect on rubisco activase.

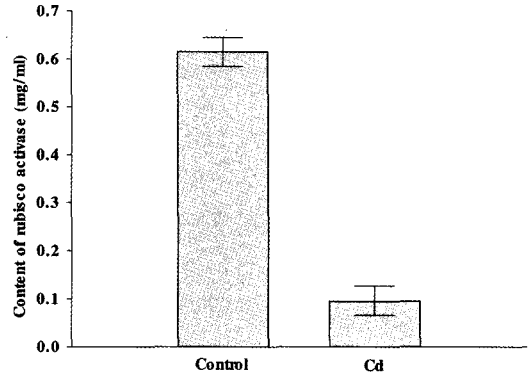

Fig. 8. Effect of cadmium on the content of rubisco activase in jackbean leaves. Plants were grown on Hoagland's nutrient solution for 15 weeks.

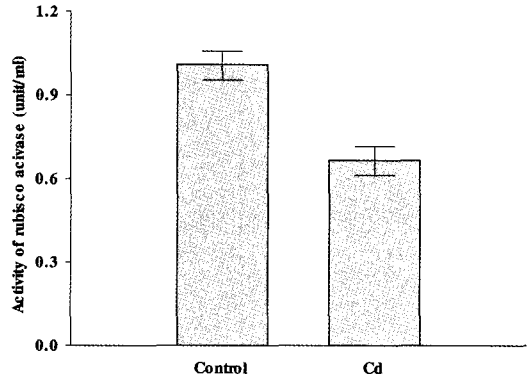

Fig. 9. Effect of cadmium on the activity of rubisco activase in jackbean leaves. Plants were grown on Hoagland's nutrient solution for 15 weeks.

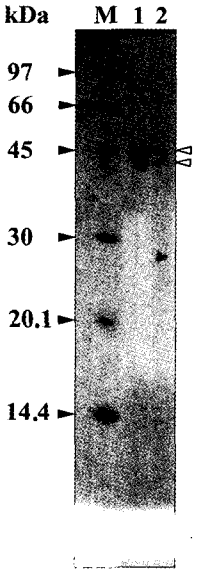

Fig. 10. SDS-PAGE analysis of rubisco activase purified from jackbean leaves. Proteins were (40 μg) separated on 20% SDS-PAGE gels. M, molecular weight standards; lane 1, no treatment; lane 2, Cd treatment. Protein was stained with Coomassie blue. Rubisco activase are indicated by an arrow.

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