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Proline Suppresses Rubisco Activity by Dissociating Small Subunits from Holoenzyme

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Proline caused irreversible inhibition (involving reduction in V_{max} without altering K_m for RuBP) in Rubisco activity. Proline-induced suppression in Rubisco activity did not exceed beyond ~65% of the original activity even upon exposure to higher levels of proline for prolonged duration. However, NaCl-induced reduction in Rubisco activity was reversible. Native PAGE analysis of Rubisco-incubated with proline showed the presence of two distinct bands corresponding to ~430 and ~28 kDa, but that incubated with NaCl showed a single band. SDS–PAGE analysis revealed that the ~430- and ~28-kDa bands represent octamers of large subunits and dimers of small subunits, respectively. These results demonstrated for the first time that proline suppresses Rubisco activity by bringing about dissociation of the small subunits from the octamer core of large subunits, probably by weakening hydrophobic interactions between them.

Proline is an imino acid that accumulates in a wide variety of organisms ranging from bacteria to higher plants under abiotic stresses. It is considered to be a compatible solute as (1) it is a small organic molecule with very high solubility and hence has potential to influence osmotic potential; (2) it has been shown to protect cellular enzymes and membranes against abiotic stress; (3) it acts as a good redox buffer capable of maintaining cellular pH and ratio of $NAD(P)^+$/NAD(P)H; (4) it scavenges and/or reduces the production of toxic oxygen species; and (5) it does not perturb regular metabolic events even when present at very high concentrations (1–10).

Proline has been well documented to protect a wide range of enzymes such as phospho*enol*pyruvate carboxylase, lactate dehydrogenase, malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, ribonucleases against various stresses (viz. salt, osmotic, temperature) (11–17). Further, in many of these reports it has been demonstrated that proline can bring about the protection of enzymes even when present at concentrations as high as 3 M. Earlier, we had demonstrated that the activity of Rubisco (the most vital enzyme for CO_2 fixation) from higher plants is susceptible to proline even when present at concentrations as low as 100 mM (18).

Chloroplasts are the major site for synthesis of proline that gets accumulated in plants under stress (19). Earlier, it had been demonstrated that proline protects photosystem II against photodamage or salt stress, by scavenging or lowering the production of toxic oxygen species (4, 5, 9). Contrary to this finding, we observed a negative role for proline on purified Rubisco from *Brassica juncea, Oryza sativa* and *Sesbania sesban* (18). This observation, which might have an adaptive significance, prompted us to investigate the mechanism(s) involved in the proline-induced suppression of Rubisco activity. In this communication we report that proline causes an irreversible inhibition in Rubisco activity under *in vitro* conditions by dissociating small subunits from the octamer core of large subunits.

MATERIALS AND METHODS

Rubisco was purified from the leaflets of two-year-old plants of *Sesbania sesban* (L.) Merrill (Fabaceae) growing in the campus of Jamia Millia Islamia, New Delhi.

Purification of Rubisco: The plant material was washed thoroughly with cold tris acetate (50 mM) buffer (pH 8) containing magnesium acetate (20 mM), EDTA (0.1 mM), β-mercaptoethanol (50 mM) and glycerol 10%. After removing the traces of the buffer by blotting, the plant material was homogenized in a buffer having same composition as above along with 2% polyvinylpolypyrrolidone. The homogenate was centrifuged at 16,000*g* for 20 min at 4°C and the supernatant used for the subsequent purification. Purification of Rubisco was done as described by Sivakumar *et al.* (1998).

Polyacrylamide gel electrophoresis and isolation of the protein components after proline treatment. The proline-treated Rubisco sample (~6 mg/ml) was subjected to molecular sieve chromatography on a Sepharose CL-6B column (20 × 2.5 cm).

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Native PAGE was carried out using 6% polyacrylamide gels following the protocol described by Gabriel (20). Protein elution from the polyacrylamide gels was achieved by incubating the macerated gel strips containing the protein band of interest in 50 mM ammonium bicarbonate buffer (pH 7.8) containing 0.1% SDS for 1 h at 37°C. The preparation was centrifuged at 15,000*g* for 10 min at 37°C, and the supernatant was concentrated by the use of centrifugal ultrafiltration (Microsep, Pall Gelman, U.S.A.). SDS–polyacrylamide gel (10%) electrophoresis was carried out as described by Laemmli (21). The gels were stained with Coomassie brilliant blue R-250 for the visualization of the protein bands. All electrophoretic analyses of protein preparations were done on an equal protein basis, and protein content was estimated as per the method of Bradford *et al.* (22).

Measurement of Rubisco activity. Rubisco (20 μg) was preincubated for 5 min at 28°C in an assay mixture consisting of radioactive $NaH^{14}CO_3$ (0.376 mM, 0.325 μCi), Tris acetate (50 mM, pH 8.0), magnesium acetate (5 mM), DTT (0.1 mM) and sodium bicarbonate (6 mM) in a final volume of 250 μl. The reaction was started with the addition of 25 μl of 20 mM RuBP. The termination of the reaction was done after an incubation time of 10 min with the addition of 50 μl of glacial acetic acid (6 N). 50 μl of the resultant mixture was spotted on a Whatmann 3MM disc for determining radioactivity in the sample using a Wallac Liquid Scintillation Counter (Finland).

Treatment of Rubisco with proline. Equal amounts of Rubisco were incubated at 20 ± 2°C with different concentrations of proline for required time intervals.

The removal of proline from the medium was made possible by the use of centrifugal ultrafiltration (Microsep, Pall Gelman, U.S.A.). Alternatively, proline was also successfully removed by exhaustive dialysis. Apparent K_m for the substrate RuBP was measured by assaying Rubisco activity with varying concentrations of RuBP (0.1 to 2.0 mM) under saturating levels of bicarbonate (over 6 mM) in the presence and absence of 1 M proline.

All experiments were replicated and the data presented is the average from five independent experiments.

RESULTS AND DISCUSSION

Earlier, we demonstrated that incubation of purified Rubisco with proline for a short duration of 5 min results in a significant decline in its activity (18). This was ascertained with Rubisco purified from higher plant species viz. *Sesbania sesban*, *Oryza sativa*, and *Brassica juncea* belonging to three distinct families. Irrespective of the plant species to which it belongs, Rubisco showed decline in its activity even when exposed to proline at concentrations as low as 100 mM (18). However, living organisms have been shown to accumulate compatible solutes like proline, glycine betaine and certain polyols to the levels exceeding 1 M (23–25). More importantly, chloroplast is the major site for the synthesis proline in plants exposed to abiotic stresses (19).

To evaluate the extent to which Rubisco activity can be suppressed by proline, purified Rubisco from *Sesbania sesban* was exposed to higher concentrations of proline (ranging from 1 to 3.5 M) for 5 min and also for prolonged time intervals (up to 60 min). As indicated in Fig. 1A, there was a decrease in Rubisco activity with increase in the concentration of proline up to 2.5 M. Further, increase in the concentration of proline did not bring about any additional reduction in Rubisco activity. The extent of reduction in Rubisco activity also increased with duration of exposure to proline (Fig. 1B). However, regardless of the concentration of proline or the duration of exposure to proline, the extent of reduction in Rubisco activity did not exceed 65%. Thus, about 35% of Rubisco activity was retained even after exposure to high concentrations of proline for extended periods. In contrast, NaCl caused significantly higher loss in Rubisco activity (Figs. 2A and 2B). Upon exposure to 500 mM NaCl for 5 min ~80% reduction in Rubisco activity was observed (Fig. 2A). Further, almost total loss in Rubisco activity was noted upon prolonged exposure to NaCl (Fig. 2B).

Retention in ~35% Rubisco activity, in spite of its exposure to very high levels of proline for prolonged duration, could probably due to the dissociation of

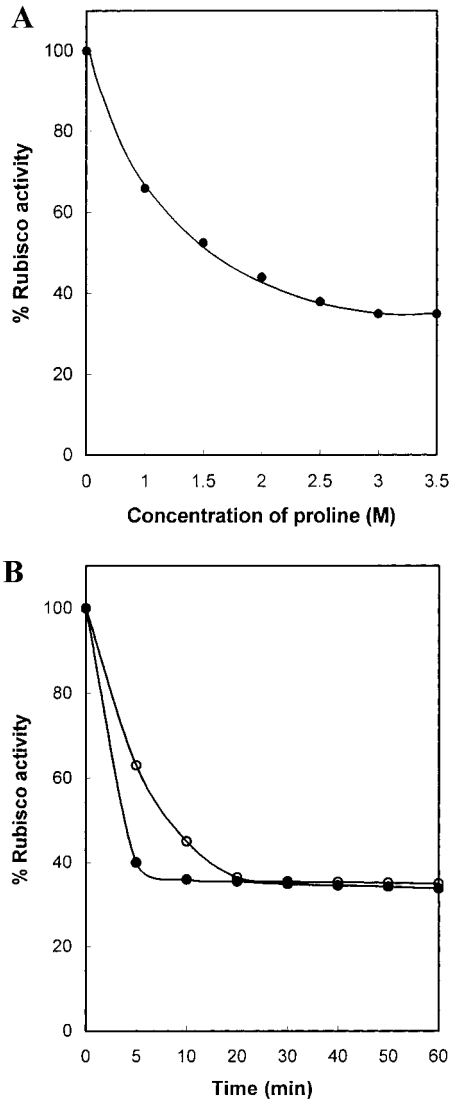

FIG. 1. (A) Effect of various concentrations of proline. (B) Effect of 1 M (○) and 3 M (●) proline on Rubisco activity with respect to time.

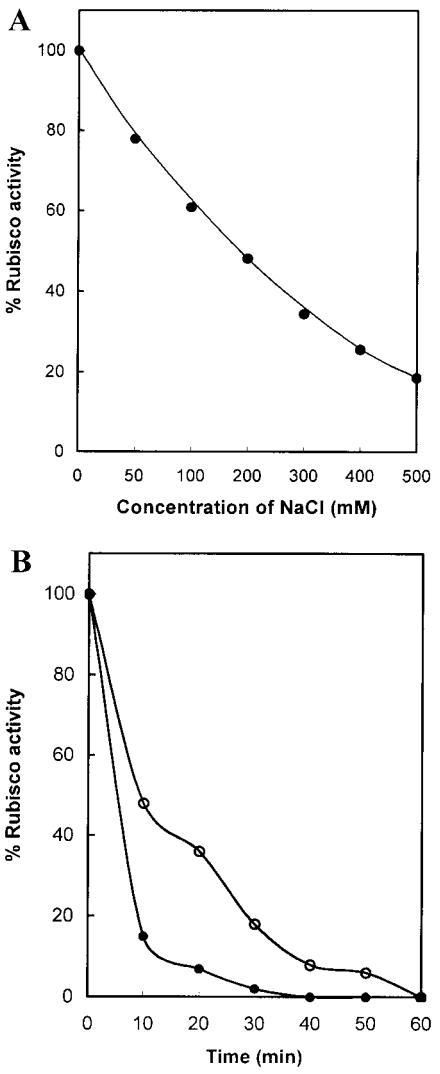

FIG. 2. (A) Effect of varying concentrations of NaCl on the activity of Rubisco purified from *Sesbania sesban*. (B) Change in the activity of Rubisco exposed to 200 (○) and 500 mM NaCl (●) for varying time intervals.

small subunits from the octamer of large subunits. Earlier, several groups demonstrated that the large subunit has actual catalytic sites and the small subunit basically is an allosteric/regulatory protein involved in enhancing the affinity of the large subunits to CO_2 (26–33). Further, a number of earlier investigators have clearly shown that the dissociation of small subunits from the higher plant Rubisco holoenzyme complex results in significant reduction in the catalytic functioning of large subunits (28, 34–38). Schneider *et al.* (33), Kaul *et al.* (35) and Aggarwal *et al.* (36) observed loss in the catalytic activity of the large subunits by 70% due to the dissociation of small subunits from Rubisco holoenzyme complex.

To ascertain the nature of inhibition brought about by proline, the apparent K_m for RuBP and V_{max} were determined. The activity of Rubisco (both control and proline treated) in the presence of varying concentrations of RuBP under saturating levels of bicarbonate is depicted in Fig. 3A. A significant reduction in V_{max} was observed due to the incubation of Rubisco with proline (Fig. 3A), but the apparent K_m for RuBP was unaffected. Substrate saturation curve as well as Lineweaver–Burk analysis clearly demonstrated that the mode of proline induced inhibition of Rubisco activity is basically either a non-competitive reversible type or an irreversible type of inhibition (39). Removal of proline by dialysis or centrifugal ultrafiltration did not bring about any recovery in Rubisco activity. In contrast, removal of NaCl from Rubisco brought about a ~95%

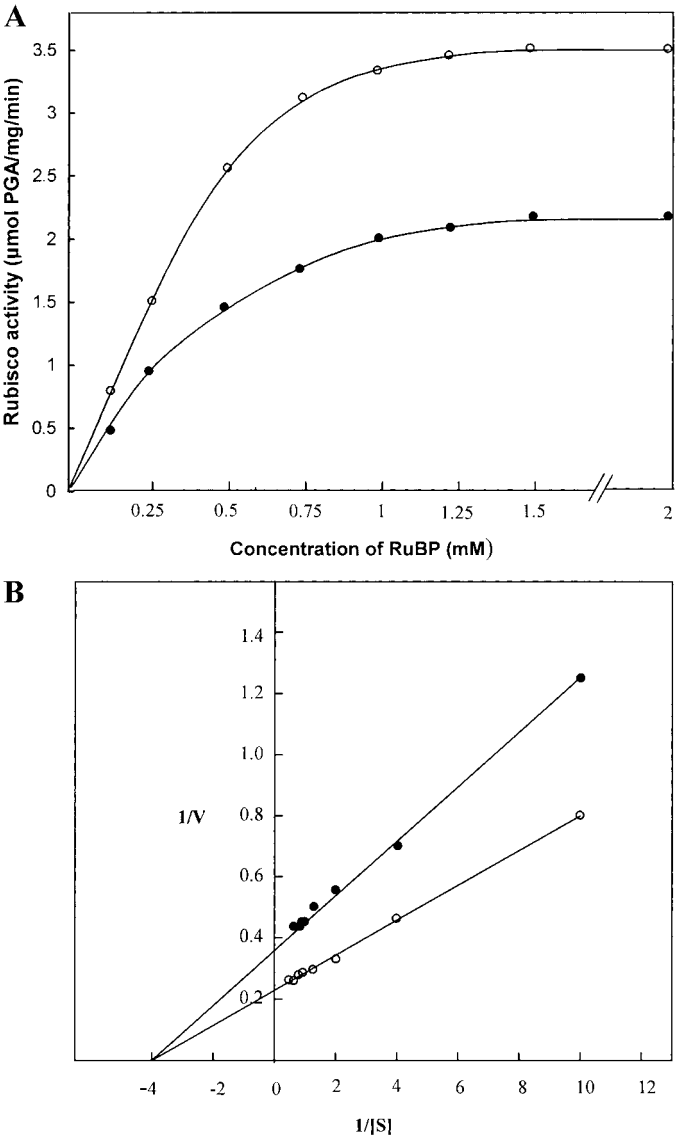

FIG. 3. (A) Activity of purified Rubisco exposed to 0 (○) and 1 M (●) proline, as influenced by varying concentrations of RuBP in the presence of saturating levels of bicarbonate. (B) Double reciprocal (Lineweaver–Burk plot) of the above data.

TABLE 1

Changes in the Specific Activity of Rubisco (Purified from Leaflets of *Sesbania sesban*) upon Exposure to Proline (1 M) and NaCl (500 mM) for 5 min at 28°C and after Their Complete Retrieval from the Vicinity of the Enzyme through Dialysis

	Specific activity (μmol PGA mg^{1} min^{-1})		
Inhibitor	Initial	In presence of inhibitor	After retrieval of inhibitor
Proline	4.09 ± 0.42	1.521 ± 0.21	1.532 ± 0.180
NaCl	4.09 ± 0.42	0.760 ± 0.066	3.890 ± 0.360

recovery in activity (Table 1). These results suggest that the proline-induced reduction in Rubisco activity under *in vitro* conditions is irreversible.

Based on these earlier reports, and our observation wherein about 35% of Rubisco activity was retained in spite of its exposure to high levels of proline for prolonged duration, we hypothesized that proline might be causing suppression in Rubisco activity by dissociating small subunits from the holoenzyme complex.

To confirm if proline induced suppression in the Rubisco activity is due to dissociation of small subunits from the octamer of large subunits as hypothesized above, we compared the electrophoretic mobility of Rubisco treated with proline and NaCl with untreated Rubisco on polyacrylamide non-denaturing gel. Interestingly, unlike untreated and NaCl-treated Rubisco, which formed a single band {corresponding to a molecular mass (MM) of ~550 kDa}, proline treated Rubisco resolved into two distinct bands corresponding to MM ~430 and ~28 kDa (Fig. 4). As no band corresponding to the holoenzyme complex (as in proline untreated

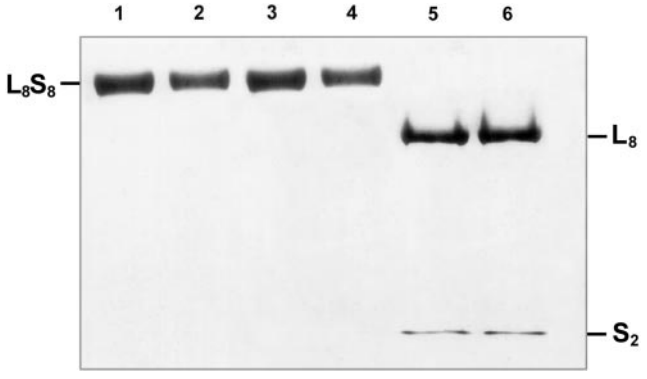

FIG. 4. Nondenaturing polyacrylamide gel electrophoretic analysis of the purified Rubisco from *Sesbania sesban*. Lanes 1 and 2 represent untreated Rubisco, lanes 3 and 4 represent Rubisco incubated with 500 mM NaCl and lanes 5 and 6 represent Rubisco incubated with 1 M proline. (Note a distinct change in the mobility of the proline-treated Rubisco with respect to untreated Rubisco and the presence of a distinct additional band).

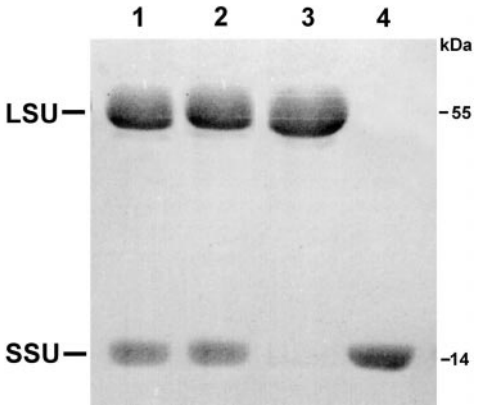

FIG. 5. SDS–PAGE analysis of purified Rubisco prior to and after various treatments. Lane 1 represents untreated Rubisco, lane 2 represents Rubisco treated with 500 mM NaCl, lanes 3 and 4 represent ~430 and ~28 kDa protein fractions of proline-treated Rubisco, respectively.

sample) was observed in proline treated sample it can be concluded that retention of 35% Rubisco activity is neither due to the presence of some intact Rubisco molecules nor due to partial dissociation of small subunits from the octamer of large subunits. The two bands obtained from the proline treated Rubisco might be corresponding to octamer of large subunits and dimer of small subunits. The Rubisco holoenzyme complex in all higher plants is composed of eight large subunits (which form an octamer) and eight small subunits (34, 38). Small subunits have been reported to form dimers, which then associate with an octamer of large subunits (34, 37, 38).

To confirm the composition of each of the two bands obtained from proline treated Rubisco, these bands were eluted from the gel and after SDS treatment, each of these protein samples subjected to SDS–PAGE. The results obtained are shown in Fig. 5. As expected, Rubisco which had not been treated with NaCl and proline formed two distinct bands corresponding to large subunits with a MM of ~55 kDa and small subunits with a MM of ~14 kDa (Lanes 1 and 2 of Fig. 5). However, the ~430 kDa protein fraction formed only a single band corresponding to that of large subunits with MM of ~55 kDa (Lane 3 of Fig. 5) suggesting that this ~430 kDa protein fraction is an octamer of large subunits. Additionally, ~28 kDa protein fraction of proline treated Rubisco also formed a single band on SDS gel. This band corresponded to that of small subunit with a MM of ~14 kDa (Lane 4 of Fig. 5) suggesting that the ~28-kDa fraction basically was a dimer of small subunits. These results demonstrate for the first time that the decrease in Rubisco activity associated with proline involves dissociation of dimers of small subunits from the octamer of large subunits in Rubisco holoenzyme complex.

It has been demonstrated that one of the major interactive forces between the large and small subunits of Rubisco is hydrophobic in nature. Additionally, it is also known that hydrophobic interactions between large subunits are stronger than that between large and small subunits (33, 40). Therefore, there is a possibility that proline causes dissociation of small subunits from the octamer of large subunits by directly interfering with weaker hydrophobic interactions between them. Rajendrakumar *et al.* (16) had proposed that proline with its hydrophobic backbone can interact with hydrophobic regions of enzymes such as lactate dehydrogenase. However, the presence of only a single band of large subunit corresponding to a MM of ~430 kDa in native gel suggests that proline does not have the potential to disrupt the stronger hydrophobic interactions between the large subunits of Rubisco.

Reconstitution of higher plant Rubisco after dissociation of the small subunits from the large subunits under *in vitro* conditions has not been successful so far (34–37). This has been attributed to the lack of chaperones, which are considered to be vital for the assembly of large and small subunits to form the Rubisco holoenzyme (41–46). Therefore, irreversible inhibition in Rubisco activity caused by proline could be due to the lack of factors that are involved in assembly of small subunits with the octamer of large subunits, as we used Rubisco, which had been purified to electrophoretic homogeneity. However, we cannot rule out the possibility of proline-induced inhibition to be noncompetitive reversible type under *in vivo* conditions as chaperones will be readily available and the level (turnover) of proline in cells/chloroplasts is regulated depending on the conditions to which plants are exposed (47, 48). As no organism prefers to synthesize any molecule in excess unless and until it is beneficial, we believe that proline induced suppression in Rubisco activity also must be having some significance for better survival of the plant under abiotic stresses that promote proline accumulation (2, 5, 8, 9, 18). We are presently making efforts to understand the probable significance of proline induced suppression in Rubisco activity in plants.

In summary, in this communication we provide strong experimental evidence that demonstrates that proline suppresses the activity of Rubisco by influencing its structural integrity through the dissociation of dimers of small subunits from octamer of large subunits of the holoenzyme complex.

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