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In Vitro Degradation of Ribulose Bisphosphate Carboxylase in Chloroplasts isolated from *Lemna minor* subjected to Sulfur Starvation

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Summary

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A study on the degradation of ribulose bisphosphate carboxylase (RuBP carboxylase - EC 4.1.1.39) was undertaken using density gradient isolated chloroplasts from *Lemna minor* grown in the presence or in the absence of sulfur for 12 days. The isolated chloroplast suspensions were treated with thermolysin in order to eliminate possible proteolytic activities exterior to these organelles. The treated chloroplasts were incubated for different periods of time at 25 °C and their proteins separated by SDS-PAGE and visualised by silver staining. Our results indicate that there is a proteolytic process responsible for RuBP carboxylase degradation in *L. minor* subjected to sulfur starvation specifically located in the chloroplasts.

Introduction

Besides its fundamental role in photosynthesis and photorespiration, ribulose bisphosphate carboxylase (RuBP carboxylase - EC 4.1.1.39) is often considered as a leaf storage protein (HUFFACKER & MILLER 1978). This enzyme often constitutes more than 50% of the protein present in photosynthetic tissues (ELLIS 1979) and is not subjected to degradation except under stress conditions (HUFFACKER & MILLER 1978). There is little information on the degradation of RuBP carboxylase under stress conditions (FERREIRA & TEIXEIRA 1992). Until now

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most studies have been performed using senescing tissues in which the degradation of the enzyme occurs as part of a process leading to cell death. Consequently, the mechanism responsible for RuBP carboxylase degradation remains unknown (MIYADAI & al. 1990).

Experiments performed recently in our laboratory have shown that under conditions of sulfur starvation there occurs a preferential proteolytic activity towards RuBP carboxylase in *Lemna minor*, which becomes particularly intense after about 12 days of stress. In this situation the enzyme is almost completely degraded during the first two weeks, with the plants remaining viable for at least four weeks (FERREIRA & TEIXEIRA 1992).

In the present work, a study on the degradation of RuBP carboxylase was undertaken using chloroplasts isolated from *L. minor* grown in the presence or in the absence of sulfur.

Materials and Methods

Chloroplasts were isolated from the higher plant *Lemna minor* L. (duckweed) grown autotrophically in complete growth medium (FERREIRA & DAVIES 1987) or submitted to sulfur starvation for 12 days. The isolation methodology involved an extraction, a differential centrifugation and an isopycnic Percoll density gradient centrifugation. Chloroplasts were obtained by grinding the plant tissue in ice-cold buffer (50 mM HEPES-KOH, pH 7.6, containing 0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$ and 1 mM NaH_2PO_4; 4 ml g^{-1} fresh weight). The suspension was filtered through gauze and cotton-wool and centrifuged twice at 300 *g* (plants grown in complete growth medium) or 250 *g* (plants subjected to sulfur starvation for 12 days) for 10 min at 2° C. The pellet was then resuspended in 0.15 ml grinding buffer g^{-1} fresh weight, carefully layered onto a 40 % (v/v, in grinding buffer) Percoll density gradient containing 0.02 g Ficoll and 0.1 g PEG ml^{-1}(previously formed by centrifugation at 14,500 *g* for 100 min at 2° C) and centrifuged at 5,000 *g* for 20 min at 2° C in a swinging-bucket rotor. The intact chloroplasts, forming a green band near the top of the gradient, were recovered, washed by centrifugation at 2,500 *g* for 5 min at 2° C, resuspended in 0.05 ml grinding buffer g^{-1} fresh weight and observed under a phase contrast microscope.

The phase contrast microscopy images (Fig. 1) show chloroplasts with a bright opaque appearance surrounded by a halo, which indicated a good integrity of the organelles (HALLIWELL 1981). The latter was also demonstrated by SDS-PAGE using RuBP carboxylase as a marker to assess the degree of intactness of the organelles (results not shown). Density gradient centrifugation of the chloroplast samples resulted in the elimination of various contaminants, e.g. the needle-shaped calcium oxalate crystals (Fig. 1).

The isolated chloroplast suspensions were then treated at 25° C with the protease thermolysin (EC 3.4.24.4; 50 µg ml^{-1}, in the presence of 2 mM $CaCl_2$) for 30 min in order to eliminate possible proteolytic activities adsorbed onto the outer surface of these organelles (MIYADAI & al. 1990). After addition of EGTA (10 mM final concentration) to inactivate thermolysin, the chloroplasts were pelleted and washed (2 centrifugations at 2,500 *g* for 1 min in a microcentrifuge). These organelles were finally resuspended in grinding buffer.

Electrophoresis was performed in the presence of SDS in 12.5% (w/v) acrylamide slab gels at 150 V, in a mini-gel apparatus, as previously described (FERREIRA & DAVIES 1987). After the separation, the proteins were visualised by silver staining (BLUM & al. 1987).

Abbreviations: RuBP carboxylase, ribulose bisphosphate carboxylase.

Results and Discussion

Chloroplasts isolated from *L. minor* grown in complete growth medium or subjected to sulfur starvation were treated with thermolysin, incubated *in vitro* for different periods of time at 25° C, lysed, and their proteins fractionated by SDS-PAGE.

The results obtained showed that the proteins in the chloroplasts isolated from *L. minor* grown in the presence of sulfur suffered no visible degradation during the 18 h period of incubation of the organelles (Fig. 2a). However, the large subunit of RuBP carboxylase in the chloroplast samples from sulfur starved *L. minor* was almost completely degraded after this period (Fig. 2b). Control experiments (data not shown) have demonstrated that the observed proteolytic process is not due to residual thermolysin activity.

Even though all samples were obtained from the same initial fresh weight of *L. minor* fronds, that of plants grown without sulfur contained less proteins than that of plants grown with sulfur (Fig. 2). Two main reasons may have contributed to this observation: (1) the protein content of the plants submitted to sulfur starvation was lower than that of the plants grown in the presence of sulfur; (2) a lower yield of chloroplasts isolated from stressed plants due to an increased lability of the organelles when the plants are grown in the absence of sulfur.

We have detected *in vitro* a proteolytic activity inherent to the chloroplasts of *L. minor* subjected to sulfur starvation, which preferentially degrades RuBP carboxylase. Presently, experiments are being undertaken in our laboratory to characterise this proteolytic process.

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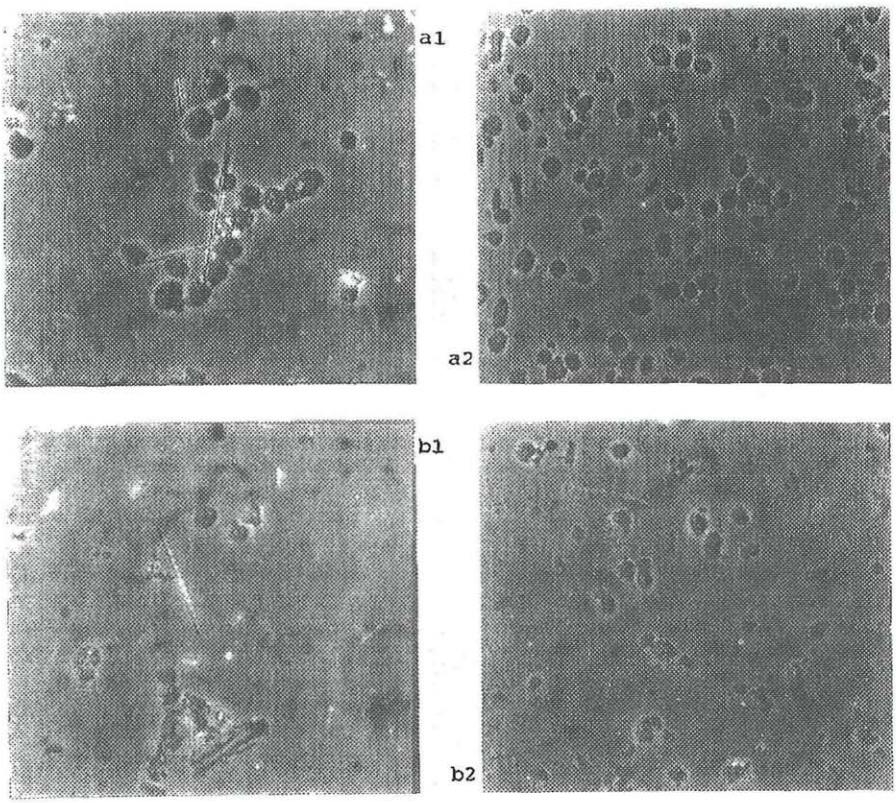

Fig. 1. Phase contrast microscopy of *L. minor* chloroplasts. Chloroplasts from plants grown in complete growth medium (a) or subjected to sulfur starvation for 12 days (b) were isolated as described in the text and observed under a phase contrast microscope (magnification: 400 X). 1 and 2: Chloroplast samples collected prior to and after the density gradient centrifugation, respectively.

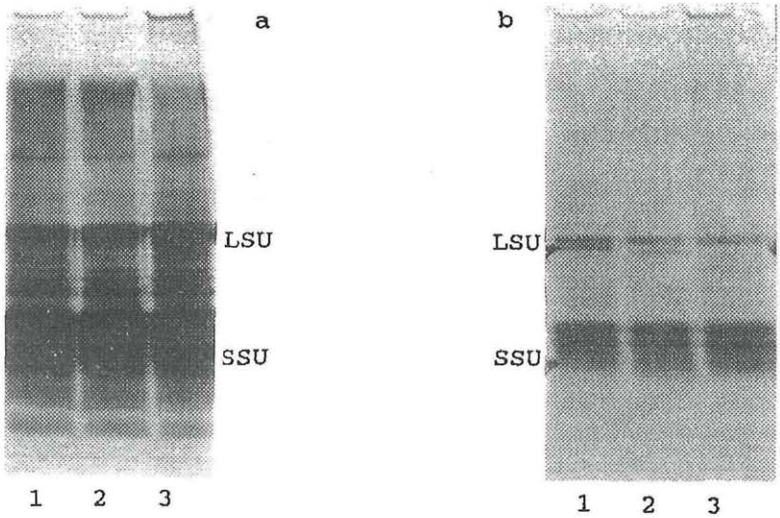

Fig. 2. *In vitro* proteolysis in chloroplasts isolated from *L. minor* subjected to sulfur starvation. Chloroplasts isolated from plants grown with (a) or without sulfur for 12 days (b) were treated with thermolysin and incubated *in vitro* at 25 °C. At intervals, samples (60 µl) were collected and subjected to SDS-PAGE. Lanes 1 to 3: chloroplasts incubated for 0, 2 and 18 h, respectively. LSU and SSU: large (52 kDa) and small (14.5 kDa) subunits of RuBP carboxylase, respectively.