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RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM CITRUS LEAVES

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Key Word Index—*Citrus sinensis*; Rutaceae; orange tree; ribulose 1,5-bisphosphate carboxylase/oxygenase.

Abstract—Ribulose-1,5-bisphosphate carboxylase/oxygenase was purified to homogeneity from citrus leaves. Decreases of the carboxylase specific activity was observed throughout the purification process. Inactivation and loss of the enzyme occurred through shear involving manipulations, but these effects could be minimized using a high fresh material/extraction volume ratio and protective agents such as non-ionic detergents, urea and bovine serum albumin.

The enzyme had a $S_{20,w}=19.4$ S and a M_r of *ca* 520000, with subunits of 50000 and 15500. The holoenzyme dissociated spontaneously into its subunits at pH values between 3.5 and 5.5 with precipitation of the large subunit and leaving most of the small one in solution. The citrus carboxylase showed unusual kinetic features such as a high (0.34 mM) K_m(RuBP) and a low (7.6) pH optimum. $K_m(CO_2)$ was 21 μM. The carboxylase activity was strongly inhibited by ionic strengths higher than 0.1 M.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) (EC 4.1.1.39) catalyses both the carboxylation and the oxygenation of ribulose 1,5-bisphosphate (RuBP) thereby starting, respectively, the Calvin–Benson cycle and the photorespiratory pathway [1]. The enzyme from higher plants is an oligomer (M_r *ca* 530000) composed of eight large (LS) (M_r *ca* 53000) and eight small (SS) (M_r *ca* 14000) subunits [2]. The catalytic site for both reactions resides in the LS while the function of the SS remains unknown [3].

Rubisco has been characterized in a great variety of photosynthetic organisms [4]. In spite of structural similarity, notable differences in the catalytic activity of the enzyme have been found between enzymes from various species [5, 6]. Differences in the K_m (CO_2) seem to be related to the photosynthetic pathway (C_3, C_4 or crassulacean acid metabolism) while variations in the K_m (RuBP) are thought to follow taxonomic patterns [6]. The increase in the carboxylase/oxygenase activity ratio has been considered as an evolutionary improvement [7, 8] and expectations have been raised on the productivity enhancement of commercially important crops through the genetic engineering of the enzyme [9].

Rubisco is the most abundant component among soluble plant proteins [10] and it has been considered to play the role of a storage protein in many plants in view of its selective degradation and substantial contribution to the nitrogen mobilization during the leaf senescence [11]. In the evergreen citrus tree, rubisco has been reported to suffer preferential degradation during the reversible senescence of the leaves that takes place at the spring growth period [12]. The proteolytic activities involved in this degradative process have been partially characterized [13]. However, the characteristics of the citrus rubisco, which could be relevant for the correct interpretation of some proteolytic results, have remained unstudied up to the present. In this article we describe the general properties of the citrus rubisco and this is the first report on the characterization of this enzyme in a member of the Rutaceae.

RESULTS AND DISCUSSION

Purification and specific activity loss

The most significant characteristics of the three-step process used for purification of rubisco from citrus leaves are shown in Table 1. After a salt fractionation, the sucrose gradient centrifugation separated the rubisco from other proteins taking advantage of its high $S_{20,w}$. The DEAE-cellulose chromatography served mainly for eliminating nucleic acids. The isolated enzyme was finally 98% pure from other proteins (as checked by electrophoregram densitometry) and almost free from nucleic acids (as indicated by the A_{280}/A_{260} ratio).

The specific activity (referred to mass of rubisco) decreased throughout the different stages of the purification process (Table 1) indicating that inactivation of the enzyme took place. This fact, which has also been observed in rubiscos from other species [14–17], has been attributed by some authors to proteolytic degradation [18–20]. However, we have reported that proteolysis is not the main source of activity loss in citrus rubisco. The spontaneous inactivation of the enzyme, due to a partial unfolding that involves the oxidation of some cysteine residues, seems to be more important [21]. In addition,

Table 1. Purification of rubisco from citrus leaves

Step	Total protein* (mg)	% Rubisco	Recovery of rubisco (%)	A_{280}/A_{260}	Total activity* (nkat)	Specific activity (nkat/mg total protein)	Specific activity (nkat/mg rubisco)
Crude extract	332	51	100	—	1472	4.43	8.69
50% saturated $(NH_4)_2SO_4$ fraction	141	67	56	—	722	5.12	7.64
Sucrose gradient centrifugation	94	84	47	1.28	503	5.35	6.37
DEAE-cellulose chromatography	54	98	31	2.12	175	3.24	3.31

*Starting material was 125 g fresh weight of leaves.

shear-producing laboratory manipulations such as pipetting, stirring and vortex mixing, have been reported to disrupt the structure of the purified rubisco, thereby causing inactivation and loss of the enzyme through precipitation and adsorption to the container surface [21, 22]. This could account for loss of enzymatic activity during the purification process due to the shearing implicated in extraction and manipulation of the protein. Shearing treatments similar to those involved in extraction procedures caused enzyme loss and slight inactivation in crude and desalted extracts (Table 2). Both effects were more pronounced in diluted than in concentrated extracts (Table 2) suggesting some autoprotective effect. The carboxylase specific activity increased due probably to the preferential loss of inactive (or less active) forms of the rubisco as observed with the purified enzyme [21]. Non-ionic detergents, urea and bovine serum albumin proved useful in avoiding aggregation (as measured by turbidity) and preventing, to a greater or lesser extent, protein and activity loss, whereas thiols and glycerol were almost ineffective (Table 3). These results indicate that the inactivation and loss of rubisco during the extraction of fresh material and subsequent purification can be minimized using a high fresh weight to extraction volume ratio and adding some of the above mentioned protective agents.

Molecular properties and amino acid analysis

The M_r of the enzyme as determined by polyacrylamide gradient gel electrophoresis was 500 000. The M_r of the subunits by SDS polyacrylamide gel electrophoresis was 50 000 and 15 500 which gives a M_r of 524 000 for the holoenzyme assuming the well established 8:8 stoichiometry [2, 3]. The $S_{20,w}$ obtained for the citrus rubisco was 19.4 S and the partial specific volume calculated from the amino acid composition (Table 4) was 0.730 cm^3/g. These data are consistent with a spherical molecular shape having a diffusion coefficient of 3.3×10^{-7} cm^2/sec and a Stokes radius of 6.4 nm, which compares well with size and shape of the tobacco enzyme as determined from X-ray crystallographic data [26].

The observed extinction coefficient was $\varepsilon^{1\%}_{280} = 16.7 \pm 0.8$ which matches the one reported for the spinach

Table 2. Effect of shearing on inactivation and loss of rubisco in non-purified extracts

Extract	Concentration (mg/ml) Protein	Concentration (mg/ml) Rubisco	% remaining after shearing Rubisco	% remaining after shearing Total activity	% remaining after shearing Specific activity
Crude	3.13	1.54	86	96	112
	0.28	0.13	69	92	135
Desalted	2.10	1.08	84	94	112
	0.14	0.07	68	91	134

Shearing treatment consisted in 4 min vortex mixing of 2.5 ml of the crude or desalted extract at room temperature. The medium was 100 mM Tris–sulphate, 10 mM $MgSO_4$, 20 mM 2-mercaptoethanol pH 7.5 (crude extract) or 100 mM Tris–chloride, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 7.5 (pre-incubation buffer) (desalted extracts). To perform the carboxylase activity assay (pH 7.5) the crude extract was transferred to pre-incubation buffer after the shearing treatment by desalting in a Sephadex G-25 column. The low concentration extracts were achieved by dilution of the high-concentration ones with the appropriate buffer.

Table 3. Effect of different compounds on the aggregation, inactivation and loss of rubisco exposed to shearing

Added agent	Concentration	ΔA_{350}	% protein lost	% activity lost
None	—	0.38	27	43
Triton X-100	0.1 mM	0.00	1	2
Tween 20	25 mg/ml	0.02	N.D.*	0
Urea	2 M	0.03	12	13
1,4-Dithiothreitol	10 mM	0.28	13	34
2-Mercaptoethanol	20 mM	0.38	19	43
Glycerol	190 mg/ml	0.18	19	43
Bovine serum albumin	1 mg/ml	0.03	N.D.*	1

Purified rubisco (0.4 mg/ml in 100 mM Tris–Cl^-, 10 mM $MgCl_2$, 10 mM $NaHCO_3$ pH 7.5) was agitated for 2 min in a vortex mixer at room temperature. Carboxylase assay was performed at pH 7.5.

*N.D.; Not determined because of strong interference of the added agent on the protein measurement.

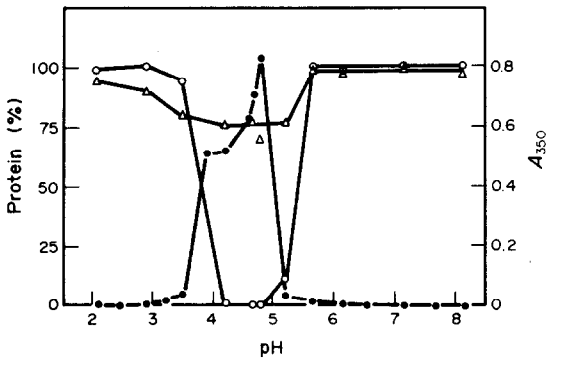

Fig. 1. Turbidity (as measured by A_{350}) (●) and percentage of LS (○) and SS (△) of the rubisco remaining in solution as a function of pH. Purified rubisco (0.26 g/l in 25 mM Tris–Cl pH 7.5) was mixed (equal volumes) with 0.2 M citrate–phosphate buffers of different pH's brought to constant ionic strength (I = 0.6 M) with NaCl. After 20 min the A_{350} was measured. Thereafter, the mixtures were centrifuged (1200 g × 20 min) and the subunit composition of the supernatant was determined by SDS-electrophoregram densitometry.

Table 4. Amino acid composition of citrus rubisco

Amino acid	% mass	Approximate number of residues (mol/mol protein)
Lys	5.63	227
His	4.05	153
Arg	8.80	292
Asx	8.93	402
Thr	5.76	295
Ser	5.26	313
Glx	10.59	425
Pro	4.48	239
Gly	4.68	424
Ala	5.44	396
Cys*	1.84	92
Val	4.15	217
Met	2.07	81
Ile	3.72	170
Leu	8.92	408
Tyr	5.39	171
Phe	5.05	178
Trp†	5.20	145
Total	100 00	4628

*Determined using the Ellman's reagent [23].

†Calculated from the Tyr/Trp ratio which was determined from the direct [24] and second-derivative [25] UV spectra.

enzyme [27]. However, the amino acid analysis (Table 4) shows that the citrus rubisco has a higher (Tyr + Trp)/Phe ratio than that of spinach [28]. This could account for the higher value (2.12) of the A_{280}/A_{260} ratio observed with the purified citrus enzyme (Table 1) when compared with that (1.9) reported for spinach [27]. The amino acid composition of the citrus rubisco (Table 4) was similar to that of other higher plant enzymes [28–30], showing some typical features such as the abundance of free cysteinyl residues and the relatively high Leu/Ile ratio. However, the citrus enzyme displayed several peculiar characteristics, in particular its Val content and Thr/Ser ratio which are rather low compared with those of the spinach, beet, tobacco [28], wheat [29] and maize [30] enzymes.

Subunit dissociation

Dissociation of the holoenzyme into its subunits occurred spontaneously by acidification of the enzyme solution (Fig. 1) as observed with other higher plant rubiscos [31]. The LS precipitated between pH 3.5 and 5.5 while most of the SS remained in solution. This pH range coincided with the one of maximum endoproteolytic activity observed by means of autodigestion experiments in citrus leaf extracts [13] as well as in those of other species [32–34]. This suggest that the pH dependence of proteolytic activity reflects more the susceptibility of the substrates (among which rubisco is the chief

component) than the optimum pH of the proteases. Moreover, the independent degradation of the citrus rubisco subunits observed in autodigestion experiments at acidic pH [13] now has a trivial explanation.

Kinetic parameters and effect of ionic strength

The effect of carbon dioxide concentration on the carboxylase activity of the rubisco is presented in Fig. 2, where a first phase (up to 0.2 mM CO_2) of activity increase according to Michaelis–Menten kinetics is followed by a second phase of substrate inhibition. Apparent $K_m(CO_2)$ calculated from values below 0.1 mM (Fig. 2 inset) was 21 μM which is a typical value for C_3 species [6]. In contrast, the apparent K_m(RuBP) for the carboxylation reaction was 0.34 mM (Fig. 3) which is 10- to 15-fold larger than those observed in other dicotyledons [6]. The enzyme showed a broad optimum pH between 7.4 and 8.0 (calculated at constant ionic strength and corrected for differences in carbon dioxide concentration [35]) which is half to one unit lower than the optimum reported for rubiscos from other species [35–37] and also than the pH measured in the stroma of illuminated chloroplasts [38].

The specific activity of the enzyme varied from one extraction to another depending on the physiological state of the plant as well as on the age and history of the extract as pointed out above. The maximum specific activity measured by us in purified enzyme solutions was about 35 nkat/mg rubisco.

Increasing the ionic strength of the assay medium by addition of sodium salts resulted in inhibition of the carboxylase activity (Fig. 4). Observed differences between the effect of the various anions may be attributed to the different ability for sequestering the activator Mg^{2+} present in the assay medium. Curiously, a slight but consistent activation in the presence of sodium citrate was observed at low ionic strength (Fig. 4). Magnesium chloride had also an inhibitory effect similar to that of the sodium salts (Fig. 4). This suggests that the inhibition by both an excess of the activator Mg^{2+} and the substrate carbon dioxide (see Fig. 2) (which implies the presence of the bicarbonate ion) reported in the literature [29, 36, 37] are not specific but rather due to the increase of ionic strength.

When compared with other species, the most outstanding features of the citrus carboxylase are its low pH optimum and high K_m (RuBP). Since both the pH and the RuBP level are thought to be important effectors on the carboxylase activity *in vivo* [39], the unusual characteristics of the citrus rubisco could reflect a peculiarity of its regulation pattern. While the low optimum pH may relieve the carboxylase from inhibition through stromal pH decrease occurring in darkness, the high K_m(RuBP) may sensitize the rubisco activity to depletion of the RuBP pool which happens also in the dark. Hence, the effect of RuBP concentration seems to be more relevant than the pH changes in the dark/light regulation of the citrus rubisco. It has been suggested that variations in K_m(RuBP) are related to taxonomic patterns [6]; therefore it appears that the high value found in citrus could be a characteristic of the Rutaceae. Since no other rubisco from a member of this family has been characterized hitherto, further studies are needed to corroborate this conjecture.

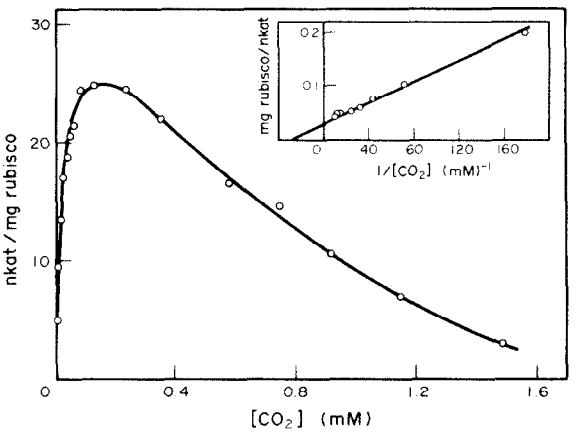

Fig. 2. RuBP carboxylase specific activity versus CO_2 concentration at pH 8.2. Experimental points corresponding to concentrations of carbon dioxide below 0.1 mM were used for double reciprocal plot (inset) and adjusted by weighted least squares for K_m (CO_2) determination.

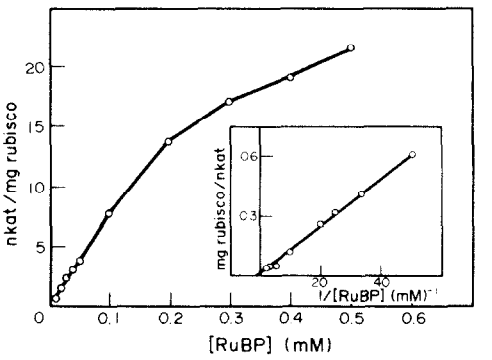

Fig. 3. RuBP carboxylase specific activity versus RuBP concentration at pH 8.2. Experimental points were adjusted by weighted least squares to a straight line on the double reciprocal plot (inset) for K_m(RuBP) determination.

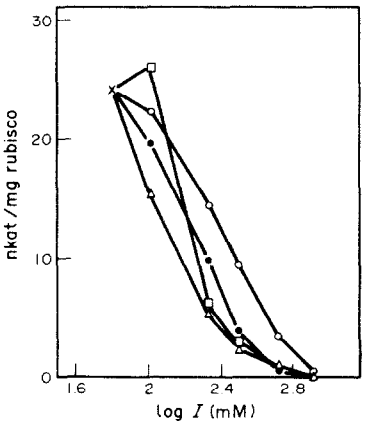

Fig. 4. RuBP carboxylase specific activity as a function of the ionic strength (I) of the assay medium at pH 8.2. Ionic strength was increased by adding the following salts: none (×), NaCl (○), Na_2SO_4 (△), Na_3citrate (□) and $MgCl_2$ (●).

EXPERIMENTAL

Enzyme purification. Fresh leaves from field orange trees (*Citrus sinensis* L. Osbeck cv Washington Navel) were extracted in a blade homogenizer with 4 ml/g fr. wt of 100 mM Tris–SO_4^{2-}, 10 mM $MgSO_4$ and 20 mM 2-mercaptoethanol pH 8.0 (extraction buffer). The homogenate was filtered through cheesecloth and stirred for 5 min with insoluble PVP (20 mg/ml). After centrifugation (8000 $g \times 20$ min) the supernatant (crude extract) was brought to 50% $(NH_4)_2SO_4$ saturation. After 30 min the ppt. was collected (10 000 $g \times 20$ min), dissolved in the minimum volume of extraction buffer and desalted in a Sephadex G-25 (PD-10) column equilibrated with the same buffer. Aliquots (2 ml) were applied on the top of 16 ml linear gradients (0.2–0.8 M) of sucrose in a 10 mM Tris–SO_4^{2-}, 10 mM $MgSO_4$, 10 mM $NaHCO_3$ and 1 mM 2-mercaptoethanol pH 8.0 soln, and centrifuged in a fixed angle rotor at 132 000 g for 4 hr. Fractions containing the leading peak were pooled, loaded onto a DEAE-cellulose column (0.9 × 21 cm, charge capacity 30 meq) equilibrated with 10 mM Tris–SO_4^{2-} (pH 7.8) and eluted with a 0–0.15 M $(NH_4)_2SO_4$ linear gradient in the same buffer. All steps were carried out at 4–6°.

Electrophoresis. Gradient (5–30%) polyacrylamide gel electrophoresis of native proteins was carried out in 375 mM Tris–Cl^- buffer pH 8.9 for 2000 V hr. SDS-polyacrylamide gel electrophoresis was performed according to Conejero and Semancik [40]. Staining and corrected quantitation of the gels were done as previously reported [13].

Protein. This was determined by the Coomassie Blue method [41] or by A_{280} in highly purified rubisco soln using the extinction coefficient reported in Results.

Amino acid analysis. Hydrolysis of the rubisco was carried out in 6 N HCl, 0.1% (w/v) phenol at 105° for 20, 24 and 28 hr. Amino acids were separated and quantified in an automatic analyser. Cysteine and tryptophan were determined as indicated in Table 4.

Partial specific volume. This was calculated from the amino acid composition according to Cohn and Edsall [42].

Extinction coefficient. This was calculated from the absorbance of protein solutions whose concentrations were determined gravimetrically [43].

Standard sedimentation coefficient. This was calculated by the method of Martin and Ames [44] using an isokinetic sucrose gradient in a swinging rotor. Marker proteins were egg white lysozyme, bovine hemoglobin, rabbit muscle aldolase, beef liver catalase and bovine thyroglogulin with $S_{20,w}$ 1.9, 4.3, 7.6, 11.3, and 21.7 S, respectively [45].

Enzymatic assay. RuBP carboxylase activity was determined according to Lorimer *et al.* [46] with some modifications. 200 μl of enzyme soln (*ca* 20 μg rubisco in 100 mM Tris–Cl^-, 10 mM $MgCl_2$, 10 mM $NaHCO_3$ containing 20 units of bovine carbonic anhydrase at pH 8.2 or without carbonic anhydrase at pH 7.5) were preincubated in plastic vials for 10 min at 30° in a thermostated waterbath. The assay was started by adding 50 μl of 100 mM Tris–Cl^-, 2.5 mM RuBP, 10 mM $MgCl_2$ and 55 mM (pH 7.5) or 10 mM (pH 8.2) [^{14}C]-$NaHCO_3$ (*ca* 48 MBq/mol). The reaction was stopped one minute later with 50 μl of 2 M HCl. The excess of [^{14}C]–CO_2 was removed in a steam bath and the stable radioactivity was measured in a scintillation counter. [CO_2] in the assay medium was calculated from the bicarbonate content using 6.35 as pKa [47].

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