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ABSENCE OF MULTIPLE FORMS OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE IN MUNG BEAN

STEVEN A. SCHLESINGER*, MICHAEL J. TOMANY and SAMUEL S. KENT†

Department of Microbiology and Biochemistry, University of Vermont, Burlington, Vermont 05405, U.S.A.

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Key Word Index—*Phaseolus aureus*; Leguminosae; mung bean; photosynthesis/photorespiration; ribulose 1,5-bisphosphate carboxylase/oxygenase; multiple forms.

Abstract—Ribulose 1,5-bisphosphate carboxylase/oxygenase has been reported to occur in multiple forms in mung bean (*Phaseolus aureus*) using Sephadex G-200 chromatography. We have isolated this enzyme by identical methodology. The profile from Sephadex G-200 chromatography shows only one peak in contrast to the previous report and we find no evidence to corroborate the conclusions. Where V_c, V_o and K_c, K_o represent V_{max} and Michaelis constants, respectively, the constant $V_c K_o / V_o K_c$ for the single form is 70 at 40 μM CO_2 and 1200 μM O_2.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) catalyses the reaction between ribulose 1,5-bisphosphate (RuBP) and CO_2 to form two molecules of 3-phosphoglycerate (3-PGA) [1]. 3-PGA can then enter the Calvin–Benson cycle. The enzyme also catalyses the reaction between RuBP and O_2, forming equimolar amounts of phosphoglycolate and 3-PGA [2]. RuBisCo regulates the balance point between photosynthesis and photorespiration and appears to be the limiting factor in photosynthesis [3]. Carbon dioxide and oxygen interact competitively for the respective carboxylase and oxygenase activities of the enzyme [4].

Rusinova *et al.* [5] have detected multiple molecular forms of RuBisCo in mung bean (*Phaseolus aureus*). This observation would have critical implications for agricultural productivity in that genetic selection of a 'superior form', i.e. that with more efficient carboxylation capacity, might be utilized to increase biomass. Such mutants would have higher growth rate potential. There is evidence of multiple forms of RuBisCo in the photosynthetic bacteria, *Rhodopseudomonas sphaeroides* [6], but no variation of molecular forms has been found in higher plants. The objectives of our work were to isolate RuBisCo according to the methodology of Rusinova *et al.* [5] and assay the carboxylase/oxygenase activity ratios by the dual label method [7].

RESULTS

Initially, enzymic activity was inhibited by 'browning'. This browning was due to the action of polyphenoloxidases [8]. By the addition of 10% (w/w) Dowex and 10% (w/w) PVPP [9] followed by extensive Sephadex G-25 chromatography, a clear enzyme supernatant appeared.

The methods of purification of RuBisCo are presented in Table 1. After Gel Chromatography I, there is only one apparent peak (Fig. 1) having a carboxylase activity of 30 nmol CO_2 fixed/min/mg protein. In Gel Chromatography II (Fig. 2), only one protein peak is evident, with a shoulder that resembles a second protein peak. The second peak, fractions 17 through 23, was pooled and rerun through the Sephadex G-200 column. The resulting peak (inset in Fig. 2) corresponds with the main peak in Fig. 2. A constant carboxylase specific activity of approximately 50 nmol CO_2 fixed/min/mg protein was observed across the peak.

Enzyme purity was monitored using disc gel electrophoresis. Disc gels were run on Ammonium Sulfate I, Gel Chromatography I and Gel Chromatography II samples. Only one major protein band is apparent at all three stages. Densitometer readings (Gel Scanner 1310, Instrumentation Specialties Co.) showed greater than 95% purity of the one major protein band in the gels. $A_{280/260}$ ratio equal to 1.25 indicated only 1.5% nucleic acid contamination calculated according to Cooper [10] after Ammonium Sulfate I.

Molecular weights of the large and small subunits of RuBisCo were determined by 10% SDS–PAGE, running standard proteins simultaneously for comparison. Molecular weights of 52 700 and 12 800 daltons were obtained for the large and small subunits, respectively (Fig. 3).

The v_c/v_o ratios were determined from $^3H/^{14}C$ specific activity ratios of [5-^{3}H] RuBP to $^{14}CO_2$ substrates and the doubly labeled 3-PGA product. 3-PGA is the only labeled product, i.e. phosphoglycolate is not labeled. The carboxylase/oxygenase (v_c/v_o) activity ratio was determined at 1200 μM O_2, 40 μM $^{14}CO_2$, 600 μM [5-^{3}H]RuBP, pH 7.9 and 25°. Reactions were terminated after 1 min. Where V_c, V_o and K_c, K_o represent V_{max} and Michaelis constants, respectively, for the enzyme, the expression $V_c K_o / V_o K_c$ was 70.

DISCUSSION

Gel chromatography of RuBisCo according to the

*Recipient of the undergraduate Biochemistry Award, College of Agriculture.

†To whom correspondence should be addressed.

Table 1. Stages of purification of RuBisCo

Stages of purification	Yield of protein (mg)	Carboxylase activity (nmol CO_2 fixed/min/mg)
Precipitation of proteins with ammonium sulfate, 35–55% of saturation	24	15
Gel chromatography on Sephadex G-200 (Gel I)	10.2	30
Gel chromatography on Sephadex G-200 (Gel II)	4.6	
Fraction 14		57
15		44
16		48

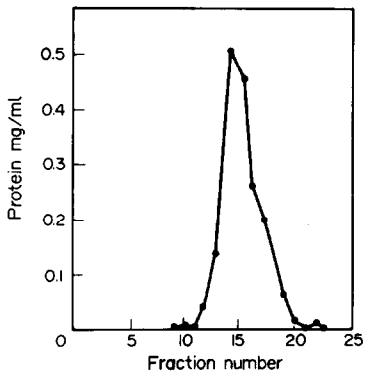

Fig. 1. Gel Chromatography I of RuBisCo from mung bean leaves. Protein content of fractions from Sephadex G-200 column.

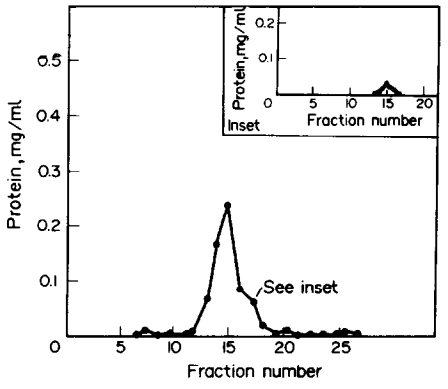

Fig. 2. Gel Chromatography II of RuBisCo from mung bean leaves. Protein profile of second Sephadex G-200 column. Inset: Gel Chromatography II of pooled fractions 17–23 from previous Gel Chromatography II of RuBisCo from mung bean leaves. Protein content off Sephadex G-200 column.

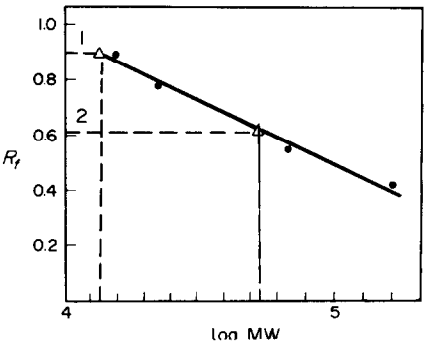

Fig. 3. Molecular weight determination by electrophoresis of RuBisCo from mung bean in 10% SDS–PAGE (1) small subunit and (2) large subunit. Standards used were: Aldolase, 154 000 daltons; BSA, 66 500 daltons; Soybean Trypsin Inhibitor, 21 000 daltons; and Lysozyme, 14 000 daltons.

methodology of Rusinova *et al.* [5], indicated a single form of the enzyme. The trailing effect off the Sephadex G-200 column in Gel Chromatography II was checked with a dye marker (Dextran Blue) and found to be a characteristic of the column. Carboxylase activity was associated with all protein peaks, verifying that these peaks are actually RuBisCo. Low activity in the samples appears to be due to inhibition by polyphenoloxidases that could not be totally eliminated. Alternatively, Seemann and Berry [11] find different K_{cat}'s for spinach and soybean suggesting that 'inhibition' may reflect inherently low activity. This inhibition appears not to affect enzyme kinetic parameters. Protein loss through purification is due to concentrating on the Amicon filters. Disc gel electrophoresis and SDS–PAGE indicate one molecular weight species with two subunit types.

The ratio of enzymic activities (v_c/v_o) may be calculated from the change in the isotope ratio ($^3H/^{14}C$) of the 3-PGA product relative to the original substrates [7]. v_c/v_o is equivalent to $A/(B-A)$, where A and B represent the $^3H/^{14}C$ isotope ratios. A = [5-^{3}H]RuBP/$^{14}CO_2$, and B = $^3H/^{14}C$ PGA. The enzymic activity ratio, v_c/v_o, is proportional to the ratio of the molar concentrations of CO_2 and O_2 in the reaction, $[CO_2]/[O_2]$, the proportionality constant (K) being V_cK_o/V_oK_c [12]. K constant is determined from the following equation:

$$\frac{A}{B-A} = \frac{v_c}{v_o} = K\frac{[CO_2]}{[O_2]}$$

The K constant from mung bean is 70. This corresponds well with other values for C_3 plants [13, 14].

The results of our investigations indicate that RuBisCo from Sephadex G-200 gel chromatography exists in only one molecular form characteristic of higher plants. The

findings of Rusinova *et al.* [5] might have been artifactual or due to an unusual genetic variant of mung bean; we find no evidence to corroborate their results.

EXPERIMENTAL

Purification of RuBisCo. Mung bean leaves were homogenized with 1–3 vols of 0.01 M Tris–HCl buffer (pH 8.5) containing 0.2 M NaCl, 0.0005 M EDTA, 0.01 M $MgCl_2$ and 0.01 M DTT (Buffer A). 10% PVPP (w/w) and 10% AG 1-X10 Anion Exchange Resin (Dowex) (Bio-Rad) (w/w) were added to help eliminate the problem of 'browning' caused by polyphenoloxidases; preparations were gassed under N_2. The homogenate was centrifuged at 10000 *g* for 10 min. The supernatant was passed over a Sephadex G-25 column to remove phenols; enzyme was then eluted with Buffer A. Proteins of the eluate were fractionated with ammonium sulfate at 35–55% saturation, followed by centrifugation for 15 min at 10000 *g* (Ammonium Sulfate I).

The final ppt. was taken up in Buffer B (0.05 M Tris–HCl buffer, pH 8.5, containing 0.2 M NaCl, 0.0005 M EDTA, 0.01 M $MgCl_2$ and 0.01 M DTT). The enzyme was desalted on a Sephadex G-25 column (40 × 0.5 cm), eluting with Buffer B. The eluate was concd on an Amicon P100: 25 mm filter and applied to a Sephadex G-200 column (90 × 1 cm) with reverse flow. Elution was conducted with Buffer C (0.025 M Tris–HCl, pH 8.5, containing 0.01 M NaCl, 0.0005 M EDTA, 0.01 M $MgCl_2$ and 0.01 M DTT) at a rate of 0.1 ml/min, collecting 3 ml fractions. This is Gel Chromatography I.

Eluate from Gel Chromatography I was concd on an Amicon PM10: 43 mm filter, precipitated with 55% ammonium sulfate and centrifuged at 10000 *g* for 15 min. The pellet was dissolved in Buffer C and desalted on Sephadex G-25, eluting with Buffer C. This eluate was concd on an Amicon P100: 25 mm filter and applied to the Sephadex G-200 column, eluting with Buffer C as in Gel Chromatography I. This is Gel Chromatography II.

Carboxylase activity. Carboxylase activity was assayed by classical acid-stable CO_2 fixation. The reaction chamber contained 0.6 mM RuBP, 10mM [^{14}C] $NaHCO_3$ at 2 μCi/μM and 10–50 μg activated enzyme. Reactions proceeded for 1 min and were quenched with glacial HOAc. Radioactivity was monitored using a Beckman LS 7000 Scintillation Counter.

Protein determination. Protein was determined by the Bio-Rad Protein assay system, measuring the absorbance at 595 nm. A standard curve was prepared with purified RuBP carboxylase for comparison.

Electrophoresis. Disc gel electrophoresis was perfomed at different steps of purification. 25 μg of protein was applied to 5% gels and run at a constant current of 3 mA per tube. Gels were stained with Coomassie Brillant Blue and destained with distilled water. SDS–PAGE gels (10%) were used for determination of MW of the subunits of RuBisCo. Standard MW compounds (aldolase, bovine serum albumin, soybean trypsin inhibitor and lysozyme) were run simultaneously for comparison. A constant voltage of 400 V was applied for *ca* 3 hr. The gels were stained as above.

v_c/v_o *ratios.* v_c/v_o ratios of the enzyme were conducted according to the dual label methodology of Kent and Young [7] using the $^{14}CO_2$/[5-^{3}H] RuBP system. The reaction was run at 1200 μM O_2 and 40 μM CO_2 for 90 sec at 25° and pH 7.9.

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