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A Simplified Purification and Some Properties of Ribulose 1, 5-Diphosphate Carboxylase from Barley

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ABSTRACT

A rapid procedure was developed for purifying ribulose 1,5-diphosphate carboxylase from barley leaves. After $(NH_4)_2SO_4$ fractionation, the unique sedimentation properties of the enzyme were exploited to effect a single step purification to 90% homogeneity. High speed centrifugation pelleted the enzyme with complete recovery of activity. Residual impurities were then removed by diethylaminoethyl cellulose chromatography and density gradient centrifugation. The purified protein exhibited size heterogeneity due to polymerization. The polymerization products were enzymatically active aggregates of ribulose 1,5-diphosphate carboxylase and were precipitated by an antibody specific for the enzyme.

Present evidence indicates that ribulose-1,5-diP carboxylase is the main component of "fraction I protein," constituting up to 45% of the soluble protein of green bean leaves (14). Fraction I protein is readily pelleted by ultracentrifugation from the mixture of soluble leaf proteins because of its high molecular weight and high sedimentation coefficient. This unique property has not been used advantageously for purification, however, because the resulting pellet is difficult to solubilize and loses much of its enzymatic activity (6, 11). Reported here is a method of pelleting that retains the carboxylative activity of fraction I protein. This represents a rapid and easy means for obtaining ribulose-1,5-diP carboxylase at approximately 90% purity. It is separated from the other components of fraction I protein mixture: phosphoribulokinase, phosphoriboisomerase (17), and protochlorophyllide holochrome (3, 9).

Other investigators have occasionally observed multiple peaks of ribulose-1,5-diP carboxylase activity during filtration through Sephadex G-200 (17), or rapidly sedimenting peaks in an analytical ultracentrifuge (15, 16). It has been proposed that polymerization of the enzyme accounted for these components (17). Information is scant, however, on the nature and activity of these high molecular weight species. Evidence is given here that the purified enzyme forms size isomers and that each isomer retains enzymatic activity.

MATERIALS AND METHODS

Plant Materials. *Hordeum vulgare* L. var. Blanco Mariout was grown in vermiculite in 28- × 33-cm plastic pans. Cotton wicks connecting the vermiculite with a full strength nutrient solution (7) supplied the seedlings with moisture. The seedlings were grown for 7 days in a growth chamber at 24 C, 55% relative humidity, and a constant 21,000 lux.

Enzymatic and Protein Assays. Ribulose-1,5-diP carboxylase was assayed at 28 C by following the conversion of $KH^{14}CO_3$ into acid-stable products. The reaction mixture (0.1 ml) contained the following, in μmoles: ribulose-1,5-diP, 0.5; $KH^{14}CO_3$, 2.5, with a specific radioactivity of 0.455 μc/μmole; $MgCl_2$, 3.0; tris-sulfate buffer (pH 8), 6.0. The reaction was initiated by adding 0.1 ml of an appropriate dilution of the enzyme extract to 0.1 ml of reaction mixture. After 5 min the reaction was stopped by adding 0.05 ml of 1 N HCl. Aliquots of 0.1 ml were dried on strips of filter paper, placed in a toluene-base scintillator solution, and counted in a scintillation counter. Ribulose-1,5-diP was prepared as described previously (7). Phosphoribulokinase and phosphoriboisomerase were assayed as previously described (7). Protein was determined by precipitating with trichloroacetic acid (final concentration 5%) and assayed by the method of Lowry *et al.* (10) against bovine serum albumin as a standard.

Enzyme Purification. All purification procedures were carried out at 0–3 C. Two hundred fifty grams of leaves were ground in a Waring Blendor in 500 ml of 0.2 M tris-sulfate buffer, pH 8.0, and filtered through six layers of cheesecloth. Solid $(NH_4)_2SO_4$ was added to the supernatant solution, and the fraction precipitating between 35 and 60% was collected by centrifugation. The pellets were dissolved in 200 ml of 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer, pH 7.8, which contained 1 mM EDTA and 1 mM dithiothreitol.

Four milliliters of 35% sorbitol were brought to 60% of saturation with $(NH_4)_2SO_4$ and placed in the bottom of a 37-ml centrifuge tube. One milliliter of 35% sorbitol was layered over the 4 ml. Thirty-two milliliters of the protein solution were then layered on top, and the tubes were centrifuged 18 hr at 100,000*g* in an SW-27 rotor (Beckman) at 3 C. All sorbitol solutions were made up in the HEPES buffer described above. Sorbitol was used rather than sucrose to allow ready determination of phosphoriboisomerase activity.

The pellets were dissolved in 30 ml of HEPES buffer and dialyzed against 10 liters of 1 mM HEPES buffer for 6 hr to remove the $(NH_4)_2SO_4$ and eluted through a 2.5- × 30-cm column of DEAE-cellulose[1] equilibrated with 0.01 M HEPES buffer and 2.5 mM $(NH_4)_2SO_4$ with a linear gradient of $(NH_4)_2SO_4$ (2.5 mM–0.2 M). Ammonium sulfate was used as the eluting salt because of increased stability of the enzyme in the presence of the divalent sulfate ion (17). The fractions having carboxylase activity were combined and precipitated with $(NH_4)_2SO_4$ at 60% saturation.

The enzyme protein was dissolved in 0.05 M HEPES buffer, 2 ml of the solution were layered on top of 35 ml of a linear gradient (20–35%) of sorbitol, and the mixture was centrifuged

[1] Abbreviation: DEAE: diethylaminoethyl.

at 100,000g for 26 hr at 3 C. Table I gives results for a typical preparation. After fractionation the enzyme protein was concentrated by precipitation with 60% $(NH_4)_2SO_4$ and frozen in phosphate or HEPES buffer.

Preparation of Specific Antibody. The purified enzyme protein was diluted to 10 mg/ml in 0.02 M phosphate buffer (KH_2PO_4 and Na_2HPO_4), pH 7.2, and used as the antigen source for intravenous injection into four New Zealand white female rabbits weighing 4 to 5 pounds each. Two milliliters of the enzyme solution were injected into each of the four animals every other day for 3 weeks. Seven days after the last injection, rabbits were bled by cardiac puncture. The sera were collected by centrifugation, pooled, and stored in the frozen state. Three 2-ml injections of antigen were administered by the same route to each surviving animal on alternate days, and blood was collected 7 days later. The series of three injections, followed by a 7-day rest period, was repeated once more. Sera from all bleedings were combined and used as the source of antibody in this study. The pooled antiserum contained 450 μg of antibody nitrogen per ml. The specificity of the antibody was determined by the Ouchterlony diffusion technique (12). Double diffusion plates were prepared from 1% Oxoid-Ionagar No. 2 (Consolidated Laboratories Inc., Chicago Heights, Ill.) in 0.85% NaCl, and merthiolate was added to a final concentration of 0.01%. Six wells, 7 mm in diameter, at a distance of 11.7 mm from a center well, 10 mm in diameter, were cut in the agar plates using a Grafar Auto-Gel T/M Assembly (Grafar Corporation, Detroit, Mich.). Undiluted antibody and antigen (10 mg/ml) were added to the wells, and the agar plates were incubated for 5 days at 15 C.

Antigen-Antibody Standard Curve. A series of dilutions of purified ribulose-1,5-diP carboxylase were made containing 0 to 80 μg/0.2 ml. Antiserum (0.2 ml), diluted 3-fold with 0.85% NaCl solution, was added to each of the antigen dilutions, and the mixtures were incubated for 1 hr at 37 C. The precipitates were centrifuged and resuspended twice in 1-ml portions of saline solution, and the protein was assayed by the method of Lowry *et al.* (10). The resulting standard curve (enzyme protein *versus* antibody-antigen protein) was used to determine the amount of ribulose-1,5-diP carboxylase in the antibody-antigen precipitates during the purification.

RESULTS AND DISCUSSION

Table I shows the purification of ribulose-1,5-diP carboxylase during the four steps of isolation. Although fraction I protein from barley leaves can be pelleted by high speed centrifugation, the pellet is only slightly soluble and almost devoid of carboxylase activity. When fraction I protein was pelleted in the sorbitol-$(NH_4)_2SO_4$ layer, it was entirely soluble and activity of the enzyme was completely retained. Components of fraction II protein, such as phosphoribulokinase and phosphoriboisomerase, were further retarded by the upper layer of 35% sorbitol. The carboxylase readily penetrated this layer and was precipitated in the bottom layer by $(NH_4)_2SO_4$.

The yellow pigment in fraction I protein at this point was removed by elution through a DEAE-cellulose column. Phosphoriboisomerase activity was detected after chromatography with DEAE cellulose, but not phosphoribulokinase. The remaining phosphoriboisomerase activity was removed during the final step of density gradient centrifugation. Ribulose-1,5-diP carboxylase made up 46% of the soluble protein of the crude homogenate, and after pelleting in the sorbitol-$(NH_4)_2SO_4$ layer the enzyme made up 90% of the pelleted protein. Percentage

Table I. *Purification of Ribulose-1,5-diP Carboxylase*

Treatment	Protein	Activity	Ribulose 1,5-diP carboxylase[1]
	g	μmoles CO_2 fixed/min·mg protein	%
Homogenate	4.20	0.06	46
60% $(NH_4)_2SO_4$	1.68	0.07	53
18-hr centrifugation	0.82	0.13	90
DEAE-cellulose	0.61	0.14	98
Density gradient	0.44	0.15	100

[1] Percentage enzyme was determined by complete precipitation with specific antibody.

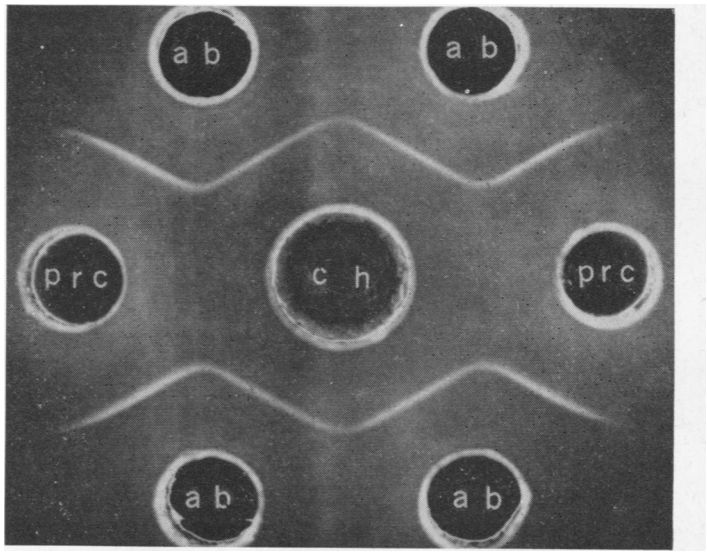

FIG. 1. Double immunodiffusion of antigen and antibody in agar. The large center well contained crude homogenate (ch), the two end wells purified ribulose-1,5-diP carboxylase (prc), and the remaining four wells antiserum (ab). See "Materials and Methods" for procedure.

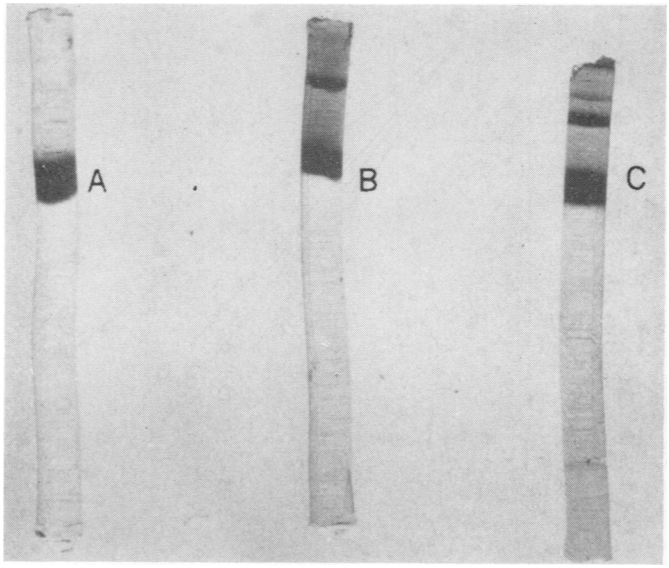

FIG. 2. Polyacrylamide electrophoresis of ribulose-1,5-diP carboxylase. Polyacrylamide (5% gel) electrophoresis was accomplished as described by Davis (4). Purified protein, 250 μg, was applied to the top of a 7.5 × 0.7-cm column. Electrophoresis was carried out at 4 ma per gel until the dye front reached the bottom. Gels were stained with Amido Schwarz 10B. A: Immediately after isolation; B: 1 month later; and C: 3 months later.

enzyme was determined by complete precipitation with the specific antibody. After the sorbitol density gradient centrifugation no other proteins were detected.

The enzyme in the purified state was stable in the HEPES buffer solution for 3 to 6 months at 0 to 3 C and was stable in the frozen state for periods up to 1 year before loss of activity was noticeable.

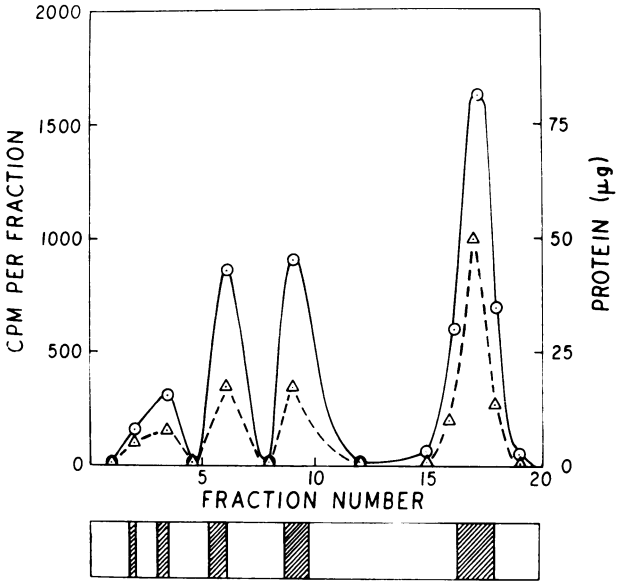

FIG. 3. Preparative acrylamide electrophoresis. Five milligrams of purified ribulose-1,5-diP carboxylase were applied to the top of a 5% acrylamide gel, 10 × 2.5 cm. Electrophoresis was carried out at 40 ma for 6 hr at 0 to 3 C. Banding patterns show five polymerization products. ⊙: Ribulose-1,5-diP carboxylase activity; △: antibody-enzyme protein. Experimental conditions are described in the text.

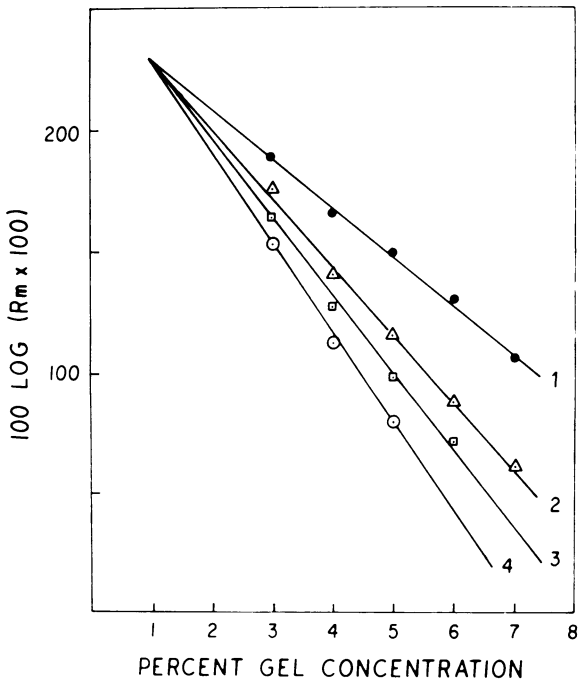

FIG. 4. The effect of different gel concentrations on the mobility of ribulose-1,5-diP carboxylase polymers. The experimental procedure was as used by Hedrick and Smith (5). Negative slopes of the polymers are: 1: 20.0; 2: 27.8; 3: 32.2; and 4: 37.0.

Double immunodiffusion in agar showed one line of precipitation between wells containing purified ribulose-1,5-diP carboxylase (prc) and wells containing antibody (ab) (Fig. 1). This line showed serological identity with the line that developed between the crude enzyme homogenate (ch) and the antiserum (ab). No other lines appeared between the antiserum and the crude homogenate. This shows that the antiserum contained no heterologous antibodies to other proteins which might have been present in the antigen preparations. When dilutions of purified enzyme were placed in wells surrounding undiluted antiserum, no heterologous lines developed (not illustrated).

Used as another criterion of purity was polyacrylamide electrophoresis (Fig. 2). The appearance of a single band on disc gel electrophoresis is not unequivocal evidence of protein homogeneity. However, a protein may be considered size-homogeneous if a single band is observed at several different gel concentrations (5). The purified enzyme showed one major and sometimes a minor band on disc gel immediately after isolation (Fig. 2) but polymerized with time (Fig. 2B, 1 month later; 2C, 3 months later). To show that polymerization could account for the heterogeneity, a preparative acrylamide gel was cut lengthwise, one-half was stained with Amido Schwarz, and the other half was cut into 3- to 5-mm sections (Fig. 3). The small sections were macerated in 2 ml of HEPES buffer and allowed to diffuse for 2 hr at 0 C, and the enzymatic activity and antibody reaction were measured on the filtrate. Extracts of all bands showed enzymatic activity, and the protein was precipitated with the antibody.

A log plot of relative mobility *versus* gel concentration for the protein bands yields four lines intersecting near zero gel concentration (Fig. 4). This plot shows that the protein bands were size isomers with similar ratios of charge to mass. If the protein of the bands differed in both size and charge, the lines of Figure 4 would intersect at other than 0 gel concentration (5). Molecular weight of the polymers was not determined from the gel data, because suitable high molecular weight standards were lacking.

Further evidence of polymerization was obtained by analytical ultracentrifugation (Fig. 5). The two protein peaks showed sedimentation coefficients of 17.2 and 25.2, which give estimated molecular weights of 500,000 and 900,000 for spherical protein molecules.

K*m* values were determined for the purified enzyme by varying the concentration of a substrate over a range of 50- to 500-fold (1–50 mM for $KHCO_3$ and 0.001–0.5 mM for ribulose-1,5-diP) while maintaining the other substrate at saturating concentrations, 0.5 mM for ribulose-1,5-diP and 60 mM for $KHCO_3$. The respective K*m* values of 0.145 mM and 22 mM for ribulose-1,5-diP

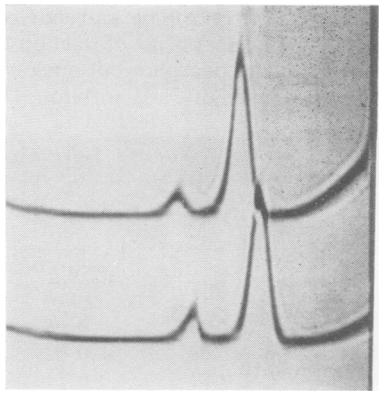

FIG. 5. Analytical centrifugation pattern of purified ribulose-1,5-diP carboxylase from barley leaves. Synthetic boundary cell, 16 min at 59,780 rpm, phase angle 70°, 0.2 M tris-sulfate buffer, pH 8.0, 25 C. Calculated S values for the monomer and dimer are, respectively, 17.2 and 25.2. Sedimentation is from right to left.

and $KHCO_3$ are in good agreement with values reported for this enzyme from other plant sources (13, 18).

The specific activity of the purified enzyme from barley was 0.15 μmole of CO_2 fixed per min per mg of protein, which compared closely to that from spinach reported by Trown (0.13–0.15) (17). Specific activities on the same basis from other sources ranging from 3 to 0.03 have been reported (1, 2, 8, 11, 13, 18). Because of differing assay temperatures and bicarbonate concentrations in the assay mixtures, the reported specific activities are not directly comparable.

In this method of purification a single step high speed centrifugation yielded ribulose-1,5-diP carboxylase at 90% homogeneity. DEAE-cellulose chromatography and density gradient centrifugation removed the remaining impurities. The purified enzyme was very stable when frozen in HEPES buffer but polymerized with time. The polymerization products were enzymatically active aggregates of ribulose-1,5-diP carboxylase.

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