A RAPID PROCEDURE FOR THE ISOLATION OF RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM SPINACH LEAVES

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1. Introduction

D-Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is usually isolated by $(NH_4)_2SO_4$ precipitation followed either by time-consuming chromatographic procedures or density gradient centrifugation [1–5]. PEG-4000 has also been used in the preparation of RuP_2 carboxylase from leaves of red kidney beans [6] but the procedure required more time and yield less enzyme than conventional methods.

Contemporary studies demand gram quantities of this large protein for kinetic studies and evaluation of the active site, as well as a rapid procedure for screening large numbers of samples in plant breeding research. This report describes a rapid two step isolation procedure for purifying RuP_2 carboxylase/oxygenase from spinach which involves precipitation with 18% (w/v) PEG-4000 and 20 mM $MgCl_2$, then chromatography on DEAE cellulose. Peak fractions had activities of $\geqslant 1.4$ μmol/min/mg protein and were ~ 95% homogeneous as analyzed by disc gel electrophoresis. Highly-purified enzyme, 1 g, can be prepared from 500 g spinach leaves in 14 h, and preparations with activity of at least 1 μmol/min/mg protein can be obtained in 2 h after a single step precipitation with PEG-4000 and $MgCl_2$.

2. Materials and methods

2.1. *Materials*

Spinach (*Spinacia oleracea* c.v. Viroflay 99 from Ferry-Morse Seeds) was grown under short day conditions in a greenhouse. RuP_2 was synthesized enzymatically from ribose 5-phosphate [7]. $NaH^{14}CO_3$ was from Amersham-Searle, PEG-4000 (mol. wt 3000–3700) was from J. T. Baker Chem. Co., ammonium sulfate (ultrapure grade) was from Schwartz-Mann and other reagents were from Sigma Chemical Co.

The grinding buffer contained 50 mM *N,N*-bis-(2-hydroxyethyl)glycine (Bicine), 1 mM $EDTANa_2$ and 10 mM 2-mercaptoethanol, adjusted to pH 8.0 at 4°C with KOH. The assay buffer contained 100 mM Bicine, 0.2 mM $EDTANa_2$, 20 mM $MgCl_2$, and 1 mM DTT, pH 8.0, at 30°C. A solution of 60% (w/v) PEG-4000 was prepared in distilled and deionized water. This material required warming and stirring to dissolve and gave a neutral solution. The saturated $(NH_4)_2SO_4$ solution was adjusted to pH 7.6 with concentrated ammonium hydroxide.

2.2. *Purification and assay of RuP_2 carboxylase*

All steps of the isolation were carried out at 4°C. Washed, de-veined spinach leaves, 500 g, were ground for 40 s at slow speed in a Waring blender with 1000 ml grinding buffer containing 2% (w/v) insoluble polyvinylpolypyrollidone. The homogenate was filtered through 5 layers of cheese cloth and then miracloth before centrifuging at 23 000 × *g* for 45 min. The supernatant was decanted through 2 layers of miracloth to remove some floating material. To the supernatant sufficient 60% (w/v) PEG-4000 was added with rapid stirring to make it 18% in PEG-4000. After stirring for 30 min the solution was centrifuged at 23 000 × *g* for 45 min and the precipitate discarded. A solution of 2 M $MgCl_2$ was then added to the clear supernatant to give final conc. of 20 mM

Abbreviations: RuP_2, D-ribulose-1,5-bisphosphate; PEG-4000, polyethylene glycol 4000, DTT, dithiothreitol

$MgCl_2$. A white protein precipitate containing RuP_2 carboxylase formed immediately upon addition of $MgCl_2$. After stirring for 30 min, the precipitated enzyme was removed by centrifugation at 16 000 × *g* for 30 min, and redissolved in ~100 ml grinding buffer. Insoluble matter was removed by centrifuging at 27 000 × *g* for 30 min. The enzyme was applied to a DEAE DE52 cellulose column (5.5 × 26 cm) equilibrated with grinding buffer. After washing the enzyme onto the column with 500 ml buffer, a 0–0.4 M $NaHCO_3$ linear gradient in 2 l grinding buffer was developed with a flow rate adjusted to < 300 ml/h. PEG, which was not absorbed by the ion exchanger, was washed through before the bicarbonate gradient was begun. Peak RuP_2 carboxylase fractions were determined from the A_{280} profile and mg protein/ml was calculated by multiplying the A_{280} (1 cm light-path) by 0.61 [8]. The enzyme was precipitated in 50% ammonium sulfate for storage and activity was retained for ⩾ 3 weeks.

Before assaying, solutions of RuP_2 carboxylase containing 2 mg/ml were activated by incubation for 10 min at 30°C in 10 mM $NaHCO_3$, 20 mM $MgCl_2$ and 2 mM DTT [2]. RuP_2 carboxylase assays were performed at 30°C in final vol. 0.25 ml [9]. To 0.22 ml assay buffer, 10 μl 0.25 M $NaH^{14}CO_3$ of known specific activity and 10 μl 12.5 mM RuP_2 were added. After the temperature had equilibrated, the reaction was initiated with 10 μl activated enzyme and after 1 min the reaction was stopped by the addition of 0.2 ml 2 N HCl. The contents of the vial were dried and counted in a liquid scintillation counter.

2.3. *Electrophoretic analyses*

Protein homogeneity was analyzed by disc gel electrophoresis with 4–50 μg protein/gel tube and with 5% or 7.5% polyacrylamide gel [10]. Electrophoresis was run at 3.5 mA/tube for 2–3 h. Gels were fixed and stained overnight in 0.1% Coomassie blue R stain, 10% (w/v) trichloracetic acid and 25% (v/v) isopropanol and destained in 10% (v/v) acetic acid containing Rexyn 1-300 mixed-bed ion-exchange resin (Fisher Sci. Co.).

3. Results and discussion

The complete procedure for the preparation of

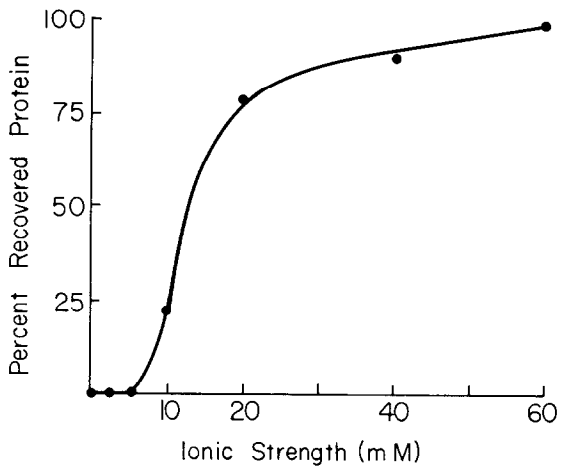

Fig. 1. Effect of ionic strength on the precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by PEG-4000. $MgCl_2$, 10 ml solutions of twice the final ionic strength were added to 10 ml purified RuP_2 carboxylase (10.33 mg) protein in 36% (w/v) PEG-4000. To simulate the components of the grinding medium in the enzyme isolation procedure, the salts were dissolved in 50 mM bicine, 0.4 mM $EDTANa_2$ and the pH adjusted to 8.0 at 22°C with KOH. After mixing and standing for 30 min, the precipitates were recovered by centrifugation for 30 min at 27 000 × *g*. Precipitates were dissolved in 2 ml grinding buffer and recovery determined by A_{280} or by carboxylase activity.

RuP_2 carboxylase/oxygenase consists of homogenization of the spinach leaves, precipitation by 18% PEG-4000 and 20 mM $MgCl_2$, and DEAE chromatography. Within 14 h 0.8–1.0 g highly purified protein can be obtained from 500 g spinach leaves. The specific activity of freshly-prepared enzyme was ⩾ 1.4 μmol CO_2 fixed/mg protein/min when assayed in 10 mM $NaHCO_3$, which is comparable with rates with the purified enzyme from spinach or tobacco leaves prepared by other longer procedures. The oxygenase activity was also similar to that of enzyme preparations by other procedures [2,3,5]. Values for A_{280}/A_{260} ratio approached 1.9, indicating the absence of nucleic acids. The enzyme preparations were nearly homogeneous by electrophoresis on 5% or 7.5% polyacrylamide gels. Beginning with 6 μg protein/gel up to 50 μg/gel, 2 faster moving minor protein bands became noticeable. The enzyme preparation was 95% pure, as estimated by a densitometer measurement of the protein bands. The DEAE chromatography gave a

single peak of enzyme activity for both the carboxylase and oxygenase which eluted in the same fractions as with other procedures [3].

After precipitation by the PEG-4000 and $MgCl_2$, the first step of the procedure, the enzyme had spec. act. $\geqslant 1$ μmol. This one step partial purification, which could be accomplished in 2 h, would be sufficient for assays concerning the carboxylase/oxygenase ratio or other possible changes associated with plant development.

The large precipitate which was discarded after making the homogenate 18% in PEG, appeared to have removed nearly all the nucleic acids, chlorophyll and other colored components. The precipitation of the carboxylase upon addition of 20 mM $MgCl_2$ was relatively specific, probably because of its high molecular weight relative to the other remaining proteins. There was no apparent salt specificity, as nearly 100% of the carboxylase was precipitated and recovered from the grinding solution upon addition of salts at 60 mM ionic strength (fig.1). The final ionic strength of the solution was >60 mM due to the bicine buffer in the grinding medium. Monovalent (K^+, Na^+) and divalent (Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}) cations, as either the chloride or acetate, were equally effective in precipitating the enzyme. $MgCl_2$ has been used routinely since $MgCl_2$ is required for subsequent activation and assay. Precipitation can also be achieved with an equal ionic strength from $NaHCO_3$. The function of the salt in the precipitation of this spinach enzyme can be explained by the steric exclusion principle as discussed for PEG [11]. In contrast, the crystallization of RuP_2 carboxylase from tobacco leaves involves dialysis to remove ions [12], but that procedure has never worked for crystallizing the enzyme from any other plant material. However, S. Johal and D. T. Bourque (personal communication) have used PEG-4000 to crystallize RuP_2 carboxylase from spinach leaves after first preparing it by longer conventional procedures.

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