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SEPARATION AND QUANTITATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE IN SPINACH LEAVES BY HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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SUMMARY

A rapid and highly efficient method is described for the separation and quantitation of ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBPCase; E.C. 4.1.1.39) in spinach leaves by hydrophobic-interaction chromatography. A TSK Phenyl 5 PW column was used for the separation of RuBPCase with magnesium sulphate solutions as the mobile phase. Different initial concentrations of the salt, pH values, buffer substances, and column temperatures were studied. After extraction of water-soluble proteins from powdered spinach leaves with 50 m*M* Bicine (pH 7.8) at a leaf-to-buffer ratio of 0.25 (g/ml), and after centrifugation of the homogenate, the supernatant was directly injected into the chromatographic column for the quantitative determination of RuBPCase. The chromatographic peak for RuBPCase was identified by its enzymatic activity and further characterized by stop-flow spectroscopy and by gradient polyacrylamide gel electrophoresis, with and without sodium dodecyl sulphate. The calibration curve for RuBPCase was linear for concentrations up to 300 μg of loaded enzyme. The recovery of the enzyme was greater than 90% in terms of activity.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBPCase) is the most abundant plant protein in nature[1]; it catalyzes two competing reactions of ribulose-1,5-bisphosphate (RuBP): carboxylation, which yields two phosphoglycerate molecules, and oxidation, which gives one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate. These reactions are at the heart of the photosynthesis and photorespiration processes, respectively[2,3]. The current interest in the enzyme is due to the key role of RuBPCase in carbon assimilation in relation to the general problem of increasing biomass productivity. Several reports[4–7] suggest that photo-

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synthesis under given conditions is regulated by the amount and activity of RuBPCase. Therefore, besides enzymologists and plant physiologists, researchers in different branches of biology and chemistry have shown an increasing interest in RuBPCase[1,8]. Studies of the enzyme require a rapid and convenient method for its quantitative determination. The methods previously described[9–11] are very time-consuming and complex.

Recently, weakly hydrophobic stationary phases were introduced for the separation of proteins by hydrophobic-interaction chromatography (HIC)[12–15]. HIC employs aqueous eluents under non-denaturating conditions. A gradient of decreasing salt concentration is used to elute the proteins in order of increasing hydrophobicity[16]. We have found that HIC on a high-performance stationary phase offers a rapid method for the quantitative determination of RuBPCase in spinach leaves, without apparent losses in activity.

MATERIALS AND METHODS

Plant material

Spinach seeds were sown in pots containing soil, sand and peat. The plants were grown in a controlled environment at 300 μEinstein/m^2 · s, 24°C, and 60% relative humidity, using a Hevitt nutrient solution[16] with a nitrogen content (as potassium nitrate) of 12 m*M*.

Chemicals

RuBPCase, as partially purified powder from spinach, D-ribulose-1,5-bisphosphate acid, and Bicine were obtained from Sigma (St. Louis, MO, U.S.A.). Labeled sodium bicarbonate was from Amersham (Bucks., U.K.). Water, HPLC-grade reagents, and all other chemicals were from Carlo Erba (Milan, Italy).

Equipment

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 liquid chromatograph was used with a Model LC-75 (autocontrol) variable-wavelength spectrophotometer, a Model 203 fluorescence detector, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector valve with a 35-μl sample loop, and a Perkin-Elmer Sigma 15 chromatography data station. A TSK Phenyl 5 PW (75 × 7.5 mm I.D.) column was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.). A circulating water bath (LKB, Bromma, Sweden; Model 2209) with variable temperature control was used to adjust the temperature of the column and of the eluent reservoirs, which were enclosed in 500-ml and 1-liter water jacket, respectively. The jackets were constructed from Plexiglas tubes and rubber stoppers. Ethylene glycol–water (30:70, v/v) was used as the circulating fluid.

Enzyme extraction

Fresh leaves were homogenized in a chilled mortar with quartz sand in 50 m*M* Bicine (pH 7.8) at a leaf-to-buffer ratio of 0.25 (g/ml). The homogenate was centrifuged at 1700 *g* and 4°C for 10 min. The supernatant was directly injected into the chromatographic column.

Enzymatic assay

RuBPCase activity was measured in triplicate at 25°C by incorporation of $^{14}CO_2$ in the presence of RuBP. The final reaction mixture contained 0.1 *M* Bicine (pH 7.8), 200 m*M* magnesium chloride, 0.5 m*M* RuBP, and 20 m*M* $NaH^{14}CO_3$ in a total volume of 0.6 ml. The reaction was started by adding of 50 μl of a 6.0 m*M* RuBP solution after preincubation of the extract (50 μl) with the other constituents in the scintillation vials for 5 min. The reaction was stopped after 1 min by adding 200 μl of 2 *M* hydrochloric acid. After the samples were evaporated to dryness under an IR lamp, the vials were counted in a LKB scintillation counter. Control samples, assayed in the absence of RuBP, were also counted.

Chromatographic procedure

Chromatographic runs consisted of linear gradients from 1.2 *M* to 0 *M* magnesium sulphate in 50 m*M* Bicine (pH 6.8) in 20 min, proceeded and followed by isocratic elution for 2 min. The flow-rate was 1.0 ml/min. All solutions were filtered through a type HA 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed by sparging with helium. The column effluent was monitored with UV detection at 280 nm or with fluorescence detection at 340 nm (excitation at 295 nm).

Recovery of mass and activity

The mass recovery of the RuBPCase from the chromatographic column was established by the following method. Approximately 4 mg of RuBPCase were extracted from spinach leaves, purified by the present HIC method, ultrafiltered and lyophilized. The purity of the enzyme was measured by UV spectroscopy and gradient polyacrylamide gel electrophoresis with and without the addition of sodium dodecyl sulphate, following the methods described below. A RuBPCase stock solution was prepared by dissolving the purified enzyme in a solution of 0.26 *M* magnesium sulphate and 50 m*M* Bicine (pH 6.8), which was the mobile phase composition at which the enzyme was eluted. The concentration of this solution was 8.5 mg/ml, as determined by the method of Lowry[18]. Working standards were prepared by subsequent dilutions of the stock solution with the same solvent. All solutions were prepared on the day of use and stored in the refrigerator between manipulations. With the above standards, a calibration curve was obtained for the absorbance at 280 nm as a function of the RuBPCase concentration. For the determination of the mass recovery, 35 μl of the standard solution was chromatographed, and the appropriate fraction was collected in a 5.0-ml volumetric flask. The absorbance was measured at 280 nm in a 1.0-cm quartz cell and the protein concentration was determined from the calibration curve. In order to determine the recovery of the activity, a 35-μl aliquot of the standard solution was assayed for activity in the same way.

Electrophoresis

Electrophoresis was carried out in the presence of sodium dodecyl sulphate (0.1%) on 10–25% gradient polyacrylamide gel in 25 m*M* Tris, 192 m*M* glycine buffer (pH 8.3) at 6 mA for 24 h. Gels were fixed and stained overnight in a solution containing 0.09% (v/v) Coomassie Brilliant Blue and 4.3% (v/v) acetic acid in methanol–water (48:52, v/v). Gels were destained in 7% acetic acid.

Electrophoresis was also performed at pH 8.2 in the absence of sodium dodecyl sulphate on 30–15% gradient polyacrylamide gel.

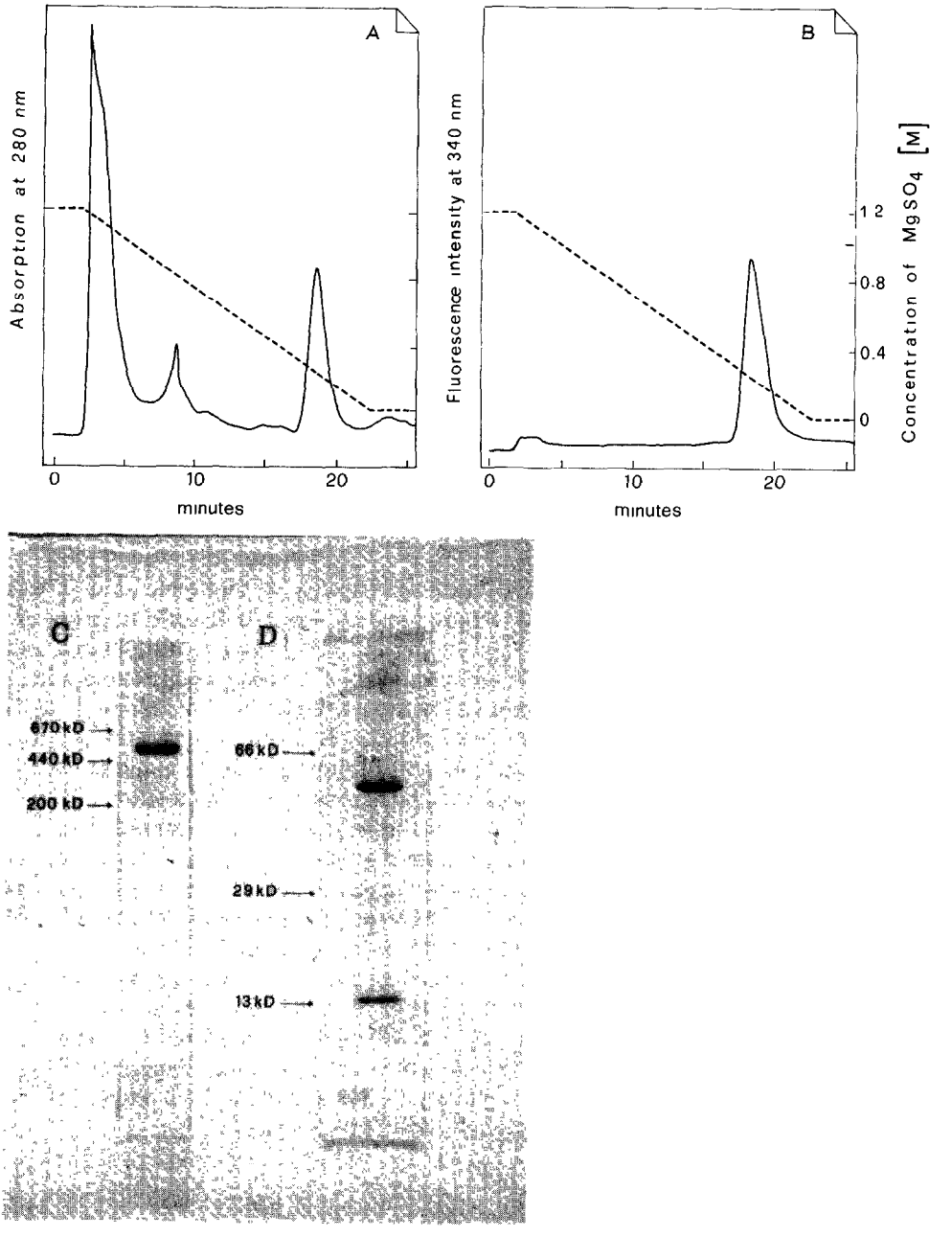

Fig. 1. Chromatogram of a spinach leaf extract containing 8.0 mg of RuBPCase per g of spinach leaves (fresh weight); Column TSK Phenyl 5 PW, 75 × 7.5 mm, flow-rate 1.0 ml/min, temperature 25°C; linear gradient from 1.2 *M* to 0 *M* magnesium sulphate in 50 m*M* Bicine (pH 6.8) in 20 min, preceded and followed by isocratic elution for 2 min. (A) UV detection at 280 nm, 0.5 a.u.f.s., (B) fluorescent detection at 340 nm (excitation wavelength 295 nm); (C) and (D) analysis of the isolated enzyme by non-denaturating gradient polyacrylamide gel electrophoresis (3–15%) (C) and by gradient polyacrylamide gel electrophoresis (10–25%) in the presence of sodium dodecyl sulphate (0.1%) in 25 m*M* Tris 192 m*M* glycine buffer (pH 8.2) at 6 mA for 24 h; approximately 20 μg of the enzyme was run in each lane. kD = kilodaltons.

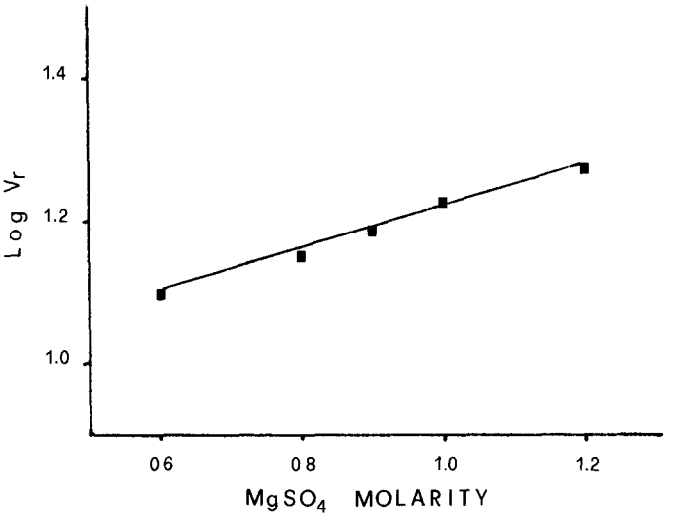

Fig. 2. Plot of the logarithmic retention volume (V_r) of RuBPCase against the concentration of magnesium sulphate in 50 m*M* Bicine (pH 6.8), used as the starting eluent; linear gradient from 0–100% final eluent in 20 min, preceeded by 2 min of isocratic elution; final eluent 50 m*M* Bicine (pH 6.8); flow-rate 1.0 ml/min; temperature 25°C; samples 35 µl of a standard solution of the purified enzyme (2.8 mg/ml).

RESULTS AND DISCUSSION

Optimization of the chromatographic parameters

The aim of this work was to develop a specific, fast and reproducible method for the determination of RuBPCase in spinach leaf extracts. Several reports[19–21] have indicated that magnesium sulphate stabilizes the carboxylase activity of this enzyme. Therefore, we used this salt in HIC for the isolation of RuBPCase. All experiments were performed under linear gradient elution conditions, with brief isocratic elution periods before and after. This facilitated the separation of the enzyme from the other components of the extract in a single chromatographic run. The best separation of

TABLE I

RETENTION VOLUME OF RuBPCase ON A TSK PHENYL 5 PW COLUMN (75 × 7.5 mm I.D.) WITH INCREASING COLUMN TEMPERATURE

Linear gradient from 1.2 *M* to 0 *M* magnesium sulphate in 50 m*M* Bicine (pH 6.8) in 20 min, preceded and followed by isocratic elution for 2 min; flow-rate 1.0 ml/min; samples 35 µl of standard solutions of the purified enzyme (2.8 mg/ml).

Temperature (°C)	*Retention volume (ml)*
3	16.0
3.5	16.4
15.0	18.6
25.0	18.4
35.0	19.0

RuBPCase was obtained by using magnesium sulphate at a starting concentration of 1.2 *M* in 50 m*M* Bicine (pH 6.8). Chromatograms obtained with UV and fluorescent detection are shown in Fig. 1A and B. The enzyme was eluted with a retention time of 18.4 min and gave the only fluorescent peak detectable at 340 nm (excitation wavelength 295 nm). As shown in Fig. 2 and Table I, the elution was found to follow the expected trends[22,23] for HIC with variations in the salt concentration and the column temperature, respectively. Variations in pH between 5 and 7 and changing the buffer to 50 m*M* phosphate or 50 m*M* Tris–HCl, had no effect on the chromatographic behavior of RuBPCase. Bicine was selected as the buffer in the mobile phase because of its compatibility with the reaction mixture used in the enzymatic assay of RuBPCase. By using the chromatographic procedure described here more than 90% of the activity was recovered.

Characterization of the enzyme

The RuBPCase peak was identified by its enzymatic activity and its identity was further confirmed by other means. The absorbance ratio at 280/260 nm was 1.80, which is consistent with that of the spinach enzyme purified by the classical techniques[23] and of the three times crystallized tobacco RuBPCase[25]. The ratio was measured by a stop-flow procedure[26]. Non-denaturing gradient polyacrylamide gel electrophoresis of the fraction containing the chromatographic peak gave a single, slow-moving, narrow band in the 3–15% gradient gel. The same experiment, performed in the presence of sodium dodecyl sulphate in the 10–25% gradient gel, gave a major band with a molecular mass of 56 000 daltons and a minor band with a molecular mass of 14 000 daltons. The results are in agreement with the finding that RuBPCase is composed of eight large subunits, each 56 000 daltons, and eight small subunits, each 14 000 daltons, so that its molecular mass is 560 000 daltons[8].

Quantitative determination

The mass recovery of the enzyme from the chromatographic column was greater than 96%. The peak area of RuBPCase increased linearly ($r = 0.9998$) with the concentration of the protein in spinach leaf extracts up to a sample load of 300 μg enzyme. The reproducibility of the method was investigated by quantitating the RuBPCase content on seven leaf extracts from the same spinach plant. The results of these analyses were 8.3, 8.4, 8.2, 8.1, 8.0, 8.5 and 7.9 mg of RubPCase per g of spinach leaves (fresh weight) (mean: 8.2 mg/g; relative standard deviation: 2%).

CONCLUSIONS

The method developed for the determination of RuBPCase in spinach leaves permits very simple and fast analysis of the enzyme without loss of activity. The method was also used for the preparation of milligram amounts of purified RuBPCase with high enzymatic activity. Preliminary attempts to use this method on other plant extracts have indicated that it can be used for the determination of RuBPCase in pea, maize, and with some modification, in alfalfa, tomatoe and bean.

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