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Note

Isolation of plant phosphoenolpyruvate carboxylase by high-performance size-exclusion chromatography

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Phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) (PEPc), a widely distributed enzyme in higher plants[1,2], has been implicated to have numerous physiological rôles[3–5]. We have been concerned with the purification of PEPc isoforms from sorghum leaves: whereas etiolated leaves contain only one form of the enzyme, during greening a second form, exhibiting a 10–20-fold higher level of activity, develops[6]. These two isoforms display specific functional and regulatory properties[6].

For further studies on these enzymes and their subunits it was necessary to obtain highly pure protein in good yield. Immunoprecipitation together with gel electrophoresis led to the preparation of a pure protein (results not shown); however, these procedures are time-consuming and recovery of the protein from the gels is difficult. In a previous paper[7] we described a simple method for purification of PEPc from green sorghum leaves by using immunoadsorbent chromatography. However, the etiolated form of the enzyme and PEPc from other plants had less affinity for antibodies than the green sorghum form, thus significantly increasing contamination. Furthermore, repetitive manipulations were needed to obtain sufficient amounts of protein.

We describe here a new, simple and very fast method of purification of PEPc based on high-performance liquid Chromatography (HPLC) of immunoprecipitates. The method is of general use for plant PEPc and results in nearly quantitative recovery.

EXPERIMENTAL

Plant material

Conditions for the growth of *Sorghum vulgare* Pers. (cv INRA 450) have been described previously[6].

Enzyme extraction

Sorghum leaves were blended at 2°C with a Polytron homogenizer in 25 m*M* Tris–HCl buffer (1:10, v/v) pH 7.6 containing 5% glycerol, 100 m*M* β-mercaptoethanol (MET) and Polyclar AT (10% fresh weight of plant material). Extracts were filtered through gauze and centrifuged at 5000 *g* for 30 min. The supernatant was

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applied to a DEAE-cellulose column (12 × 2.5 cm) previously equilibrated with 25 m*M* Tris–HCl buffer, pH 7.6, containing 5% glycerol and 14 m*M* β-mercaptoethanol. After washing the column with the equilibrium buffer and then with the same buffer supplemented with 100 m*M* NaCl, the enzyme was eluted with a linear NaCl gradient (100–300 m*M*). Enzyme fractions were kept in 60% ammonium sulphate until use.

Immunoprecipitation procedure

A highly specific immune serum raised against the C_4 form of the enzyme from green sorghum leavces was used in this study[7]. Immunoprecipitations were performed as described[8]. Immunoprecipitates were rinsed with 1 ml of 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 and 50 m*M* NaCl, then twice with 1 ml of 10 m*M* sodium phosphate buffer. For protein determination, the pellets were dried, resuspended in a small volume of 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecylsulphate (SDS) and solubilized by heating at 90°C for 5 min in a water-bath. In order to obtain a complete dissociation for HPLC analysis, the samples were adjusted to 1% with respect to SDS and 5% β-mercaptoethanol. They were then heated at 90°C for various periods as shown below.

High-performance size-exclusion liquid chromatography

A Model 6000 A solvent-delivery system with a U6K injector (Waters Associates, Milford, MA, U.S.A.) was used. Chromatographic separations were performed using a μSpherogel TSK 4000 SW size-exclusion column (30 cm × 7.5 mm I.D., particle size 10 μm, Beckman).

The absorbance of the column eluate was monitored at 280 nm using a Model 450 variable-wavelength absorbance detector (Waters) and recorded on a linear chart recorder (Omniscribe B-5 000; Houston Instruments, Austin, TX, U.S.A.). All separations were carried out with buffer and samples passed through 0.22-μm Millipore filters prior to application to the column. Various volumes (5–100 μl) of samples were injected and chromatographed at room temperature. The mobile phase was 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.1% SDS, at a flow-rate of 0.5 ml/min. The eluate was collected in 200-μl fractions.

Standard proteins used as molecular weight markers were bovine serum albumin (M_r 66 kD), egg albumin (45 kD), pepsin (34.5 kD) and β-lactoglobulin (18.4 kD); all were from Sigma.

Gel electrophoresis

Aliquots of the samples and of the eluted fractions were submitted to gel electrophoresis[9].

Protein determination

The protein content was measured spectrophotometrically at 205 nm[10].

RESULTS AND DISCUSSION

Gel electrophoresis of an immunoprecipitate of sorghum leaf PEPc yields three main protein bands after Coomassie Blue staining[8]. The subunits of the enzyme and those of the immunoglobulins (IgGs) can also be separated by HPLC using a TSK

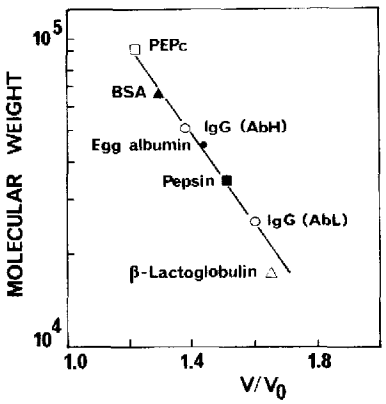

Fig. 1. Calibration graph of the molecular weight of proteins obtained by HPLC. BSA = Bovine serum albumin; AbH = heavy chain of antibodies; AbL = light chain of antibodies; PEPc = PEP carboxylase subunit. V = Retention volume; V_0 = void volume.

4000 SW size-exclusion column. By reference to a calibration performed on the same column (Fig. 1) with molecular weight markers, IgG subunits were found to have molecular weights of 25 and 52 kD, respectively; the molecular weight of the PEPc subunit was estimated to be around 90 kD. These values are in good agreement with our previous results[8].

The efficiency of the separation of the polypeptides present in the immunoprecipitates has been checked with respect to the flow-rate and the sample volume. It was found that a flow-rate of 0.5 ml/min and sample volumes up to 300 μl with a maximum protein content of 2 mg/ml gave the best results.

The influence of the heating time on the dissociation of the immunoprecipitates

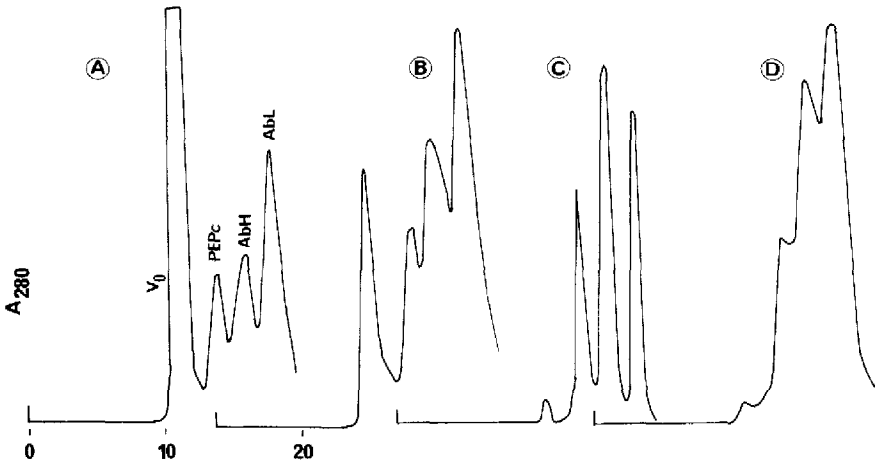

Fig. 2. Influence of the heating time at 90°C. Immunoprecipitates were dissociated by heating for 3 (A), 5 (B), 10 (C) and 20 min (D). V_0 represents the undissociated proteins.

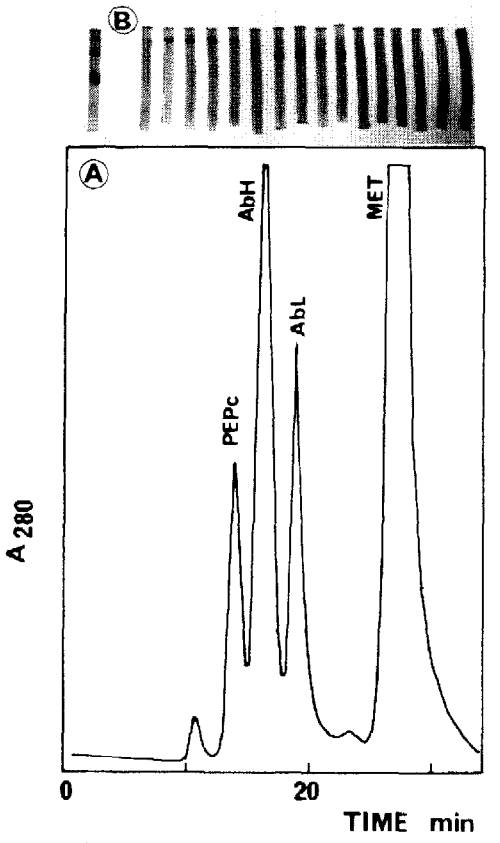

Fig. 3. Chromatography of an immunoprecipitate of PEPc from green sorghum leaves. A, 50 µl (0.5 mg/ml) were injected at a flow-rate of 0.5 ml/min; B, aliquots (100 µl) of the eluted fractions were analyzed on SDS-polyacrylamide gels.

has also been investigated (Fig. 2). The chromatogram in Fig. 2A shows that after 3 min of heating a significant amount of undissociated proteins was eluted at the void volume of the column. Increasing the heating time to 10 min (Fig. 2B, C) led to an almost complete dissociation of the immunoprecipitate and to a very good recovery of each protein peak. Fig. 2D shows that 20 min or more of heating partially destroyed the proteins; intermediate-molecular-weight peptides appeared which were not well separated by the column. When analyzed by SDS-gel electrophoresis, this preparation exhibited more than three protein bands. These data are consistent with those in a previous report[11], and indicate the importance of the heating time needed to dissociate the immunoprecipitates.

By using optimal conditions, very good separations of the proteins present in the immunoprecipitate were obtained for green sorghum leaf enzyme (Fig. 3A). Gel electrophoresis of aliquots of each fraction of the effluent showed the very high purity of the enzyme subunits (Fig. 3B). Similar results were obtained with the enzyme from etiolated sorghum leaves.

Additional information about the stability of the polypeptides separated by

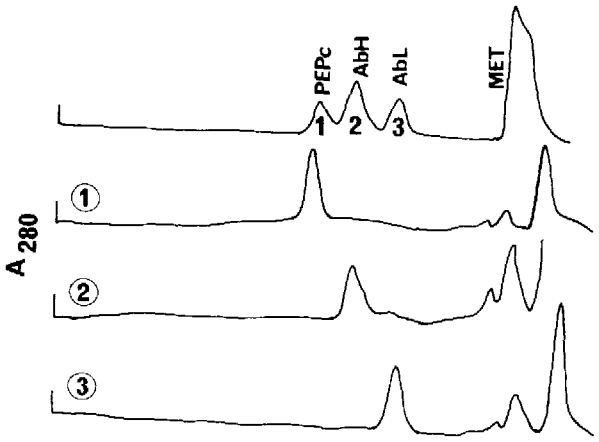

Fig. 4. Rechromatography analysis of PEPc immunoprecipitate fractions: 1, 2 and 3 correspond respectively to the peak fractions (200 μl) of each subunit.

this method was obtained by a rechromatography experiment (Fig. 4). Under these conditions, we did not detect any recombination of each protein into a supramolecular structure. In contrast, when the optimally dissociated immunoprecipitate (after 10 min of heating) was kept for 2 days without previous HPLC separation and further heating, a partial reassociation of the proteins was observed.

Repetitive injections of samples (2 mg/ml) of volume 300 μl gave similar results and permitted the purification of large amounts of each PEPc isoform subunits with near optimal yield. In a working day, more than 1 mg of each protein can be obtained. This method can be applied to plant PEPc and to other immunoprecipitated enzymes provided their subunits can easily be separated by HPLC from those of the IgG.

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