RECOVERY OF ACTIVE, HIGHLY PURIFIED PHOSPHOENOLPYRUVATE CARBOXYLASE FROM SPECIFIC IMMUNOADSORBENT COLUMN

Jean VIDAL, Guy GODBILLON+ and Pierre GADAL
Laboratoire de Biologie Végétale, ERA CNRS no. 799, +Laboratoire de Biologie Animale, Université de Nancy I, Case Officielle no. 140, 54 037-Nancy Cedex, France

Received 2 June 1980
Revised version received 7 July 1980

1. Introduction

Since its discovery in 1953 [1], PEP carboxylase (orthophosphate:oxaloacetate carboxylase, EC 4.1.1.31) has been the subject of numerous studies. Indeed, the carboxylase, which is ubiquitous among bacteria and plants, represents a means of incorporating inorganic carbon in a non-photosynthetic way. It has been implicated in numerous physiological roles such as, anaplerotic pathways [2], C_4 photosynthesis and crassulacean acid metabolism [3,4], stomatal aperture [5], ionic equilibrium in root cells [6], pH stat [7] and biological rhythms [8]. A number of isoenzymes of PEP carboxylase have been reported [9–13]. Many experiments have been and are being performed on the functional and regulatory properties of the enzyme and on its localization in cells and tissues [14].

We have been concerned with the purification of PEP carboxylase isoforms in bean and sorghum leaves and roots [15,16]. Immunochemical techniques provide an efficient method for enzyme purification and localization; unfortunately such techniques usually result in the complete loss of biological activity. Here we describe a new and simple method based on the elution of an active, highly purified enzyme from an immunoadsorbent column.

2. Materials and methods

2.1. *Plant material*

Sorghum plants were grown as in [16].

2.2. *Extraction and purification of the enzyme*

PEP carboxylase was extracted in 250 ml phosphate buffer from lyophilized sorghum leaves (5 g) [15,16]. The extract was deaerated by a stream of nitrogen and filtered through a column of polyclar AT. Purification was effected by using ammonium sulphate precipitation (40–55% saturation), elution from a hydroxylapatite (HTP) column (2.5 X 12 cm; equilibration buffer, 50 mM potassium phosphate (pH 7), 14 mM mercaptoethanol, 5% glycerol; elution buffer, the same except that potassium phosphate was 200 mM), followed by elution from a DEAE-cellulose column (2.5 X 12 cm: equilibration buffer, 10 mM Tris (Cl^-) (pH 7.6), 14 mM mercaptoethanol, 5% glycerol, 100 mM NaCl; elution buffer, the same with the addition of 200 mM NaCl). The active fractions were pooled and submitted to polyacrylamide gel electrophoresis [16]. Bands of the gels exhibiting carboxylase activity were cut out and homogenized in a Potter-Elvehjem homogenizer in 10 mM Tris (Cl^-) buffer (pH 7.6), containing 5% glycerol, 0.9% NaCl. This enzyme preparation was subsequently used for primary injections of the rabbits, as in section 2.3. For booster injections, the purification of the enzyme was slightly modified: polyacrylamide gel electrophoresis was replaced by chromatography on a DEAE-cellulose column. Elution was achieved with a linear NaCl gradient (0.1–0.3 M). Peak enzyme fractions were kept in 60% $(NH_4)_2SO_4$ until use. on a DEAE-cellulose column. Elution was achieved with a linear NaCl gradient (0.1–0.3 M). Peak enzyme fractions were kept in 60% $(NH_4)_2SO_4$ until use. Before injection, proteins were pelleted by centrifugation, dissolved in 10 mM phosphate buffer (pH 8) containing 0.9% NaCl and desalted on Sephadex G-25 gel equilibrated in the same buffer.

2.3. *Immunization procedure*

Three rabbits (Fauve de Bourgogne) each 2.5 kg, were immunized by 2 subcutaneous and 2 intramuscular injections. One injection (500 μl emulsion) was composed of 250 μl polyacrylamide gel suspension containing the enzymatically active fraction (~300 μg protein) and 250 μl complete Freund's adjuvant. Two intravenous booster injections of antigen purified by DEAE-cellulose chromatography (500 μl enzyme extract each, 500 μg protein) were given 1 month later at 2-day intervals. The rabbits were bled from the ear 6 days after the last injections. The blood was kept for 12 h at 4°C, then the immune serum was separated by centrifugation. The immunoglobulins precipitated by $(NH_4)_2SO_4$ (33% saturation) were collected by centrifugation, dissolved in 20 ml 50 mM borate buffer (pH 8.14), containing 0.9% NaCl, then dialyzed against the same buffer. This preparation (15 mg protein/ml), sterilized by filtration on a millipore membrane, was kept frozen at −20°C until use.

2.4. *Preparation of the immunoadsorbent column*

Immune serum 1.5 ml (22.5 mg protein) was mixed with 6 ml glutaraldehyde-activated Ultrogel (ACA 22, IBF) and the immunoglobulin G coupling was carried out by the method described in the practical guide published by Reactifs IBF. The column was equilibrated in potassium phosphate buffer, 50 mM (pH 7).

2.5. *Immunoadsorption chromatography*

The extract partially purified on hydroxylapatite (284 nKat/1.5 mg protein) was incubated for 30 min at 20°C [17], then fixed onto the immunoadsorbent. The column was successively rinsed by 10 ml 50 mM phosphate buffer (pH 7) containing 2 M NaCl and 10 ml 10 mM phosphate buffer (pH 7). At this stage, washing with cold distilled water resulted in desorption of the active enzyme. In order to keep the immunoadsorbent gel clean as long as possible, experiments were performed with the partially purified enzyme as cited above. However, identical results were observed when a crude enzyme preparation was used.

2.6. *Enzyme assay and determination of proteins*

PEP carboxylase activity was assayed in 1 ml 0.1 M Tris (Cl^-) buffer (pH 8) containing 5 μmol $MgCl_2$, 5 μmol $NaHCO_3$, 0.2 μmol NADH, 3 units malate dehydrogenase (from pig heart), 2 μmol PEP, using a double beam spectrophotometer (Beckman Acta C II) at 340 nm, 30°C. Enzyme units are expressed in nKat. Protein content was measured either by the method in [18] or [19].

2.7. *Electrophoresis of PEP carboxylase in SDS polyacrylamide gel*

To the peak fraction of the effluent (fig.2) was added Tris (Cl^-) buffer (final conc. 10 mM) (pH 7.6), SDS 0.1%, mercaptoethanol 1%, sucrose 10% and 10 μl bromophenol blue solution (0.01%). The mixture was placed for 5 min in a boiling-water bath and then submitted to electrophoresis on 10% polyacrylamide gels, containing 0.1% SDS, at 3 mA/tube. Marker proteins were treated similarly. After electrophoresis, the gels were stained for 2 h in Coomassie blue R-250 solution (0.25% in methanol–water–acetic acid, 5/5/0.2) and subsequently destained by electrophoresis.

3. Results and discussion

The specificity of the immune serum was checked by Ouchterlony double diffusion test. It can be seen in fig.1A that a crude extract from sorghum leaves gave only one sharp precipitation band in the presence of the antibodies; the serum of a non-immunized

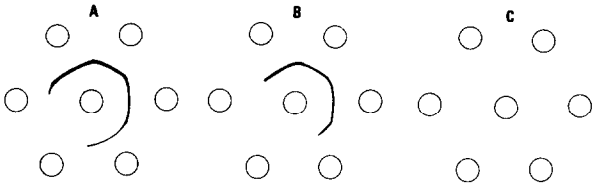

Fig.1. Ouchterlony double diffusion test: 3 immunodiffusion experiments were performed on agar plates (2%) according to the Ouchterlony technique. Homogenates of sorghum leaf concentrated by $(NH_4)_2SO_4$ precipitation at 55% saturation were prepared. Proteins were redissolved in a minimal amount of 50 mM borate buffer (pH 8.14), containing 5% glycerol. In each plate the central well contained 7 μl extract (1.5 nKat enzyme, 72 μg protein), the other wells contained different dilutions of the antiserum (1–1/16). The plates were placed for 48 h at 4°C in a humid atmosphere. (A) was rinsed for 24 h in saline and dried under filter paper at room temperature, it was stained with 0.4% azocarmin G in methanol–acetic acid (9/1) for 4 min and destained with 7% acetic acid in methanol. After diffusion, (B) and (C) were assayed for PEP carboxylase activity by adding some drops of reaction medium with (B) or without PEP (C). The agar was cut on either side of the precipitate line to facilitate the diffusion of the reagents.

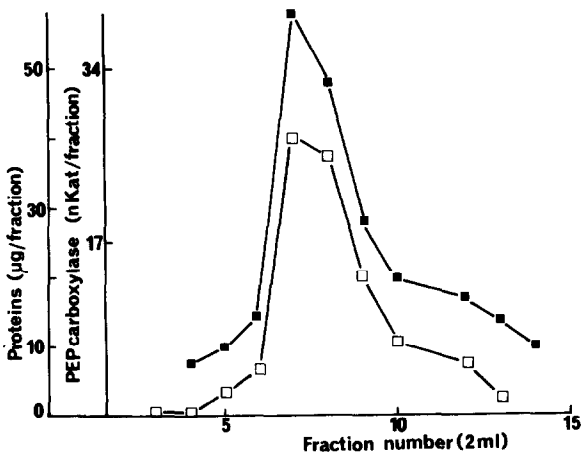

Fig.2. Elution of active PEP carboxylase from the immunoadsorbent column. Partially purified extract, 1 ml (1.5 mg proteins, 284 nKat) was fixed onto the immunoadsorbent column; the gel was rinsed as in section 2 and the enzyme eluted by distilled water. Fractions (2 ml) were collected: protein (■—■); enzyme activity (□—□).

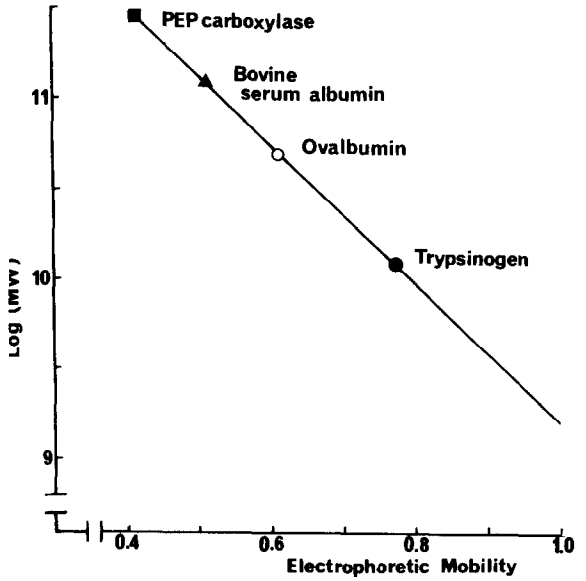

Fig.3. Subunit molecular weight determination of purified PEP carboxylase by SDS–polyacrylamide gel electrophoresis.

rabbit did not react with the enzyme. In addition, the protein bound to antibodies in the precipitate was characterized by its catalytic activity (fig.1B,C) in the presence of fast violet B [16]. The results strongly suggest that the antibody preparation against PEP carboxylase is monospecific.

When an enzyme extract purified on hydroxylapatite (1.5 mg protein exhibiting PEP carboxylase activity of 284 nKat) was filtered through the immunoadsorbent column subsequently washed as in section 2, no enzyme activity could be detected in the effluent; of the many different attempts we made to elute the enzyme, the one described here proved to be the best. It consists of washing the column with cold distilled water.

It can be seen in fig.2 that the enzyme activity is eluted as a sharp peak on which a protein peak is superimposed. Because the enzyme is very unstable in water, preliminary experiments gave a poor recovery of activity, as little as 8.3 nKat/232 µg proteins. However, we were able to improve the recovery by stabilizing the enzyme; to this end, the effluent was poured in an equal volume of 100 mM potassium phosphate buffer (pH 7), containing 5% glycerol, 14 mM mercaptoethanol, and ammonium sulfate at 40% saturation. In this case, about 60 nKat enzyme activity could be recovered and the purification factor was 9.28 (table 1, expt. 1). This relatively low value can be explained by the fact that phosphoenol-

Table 1
Purification of sorghum leaf PEP carboxylase

Expt.	Purification step	Spec. act. (nKat/mg protein)	Purification (-fold)	Activation by glucose-6-phosphate (-fold)
1.	Crude extract	28		
	HTP	189	6.66	3.4
	Immunoadsorbent	260	9.28	3.4
2.	Crude extract + Ammonium sulfate precipitation (30–60%)	34		
	Immunoadsorbent	260	7.6	

The enzyme was assayed as in section 2. Glucose-6-phosphate was added at 6 mM

pyruvate carboxylase is present at high levels, ~10% total proteins, in leaves of C_4 plants [20]; it corresponds well with 9.25% reported [20] for the pure corn leaf enzyme. Comparable data were obtained with extracts purified only by ammonium sulfate precipitation (table 1, expt. 2). On SDS–polyacrylamide gel electrophoresis, only one protein band appears, corresponding to M_r ~90 000 (fig.2), a figure very similar to the subunit M_r we found (unpublished) or reported, using an enzyme preparation purified by conventional techniques [20,21]. This establishes that the application of immunoadsorbent allows one to obtain rapidly a highly purified PEP carboxylase. We checked as to whether the purification process modifies the enzyme. As with the enzyme obtained by conventional methods, a 3.4-fold activation was found in the presence of glucose-6-phosphate (table 1, expt. 1); hence, the enzyme appears to be unaltered at least in its regulatory properties.

Water elution of PEP carboxylase from the immunoadsorbent may be due to the very low ionic strength, and to pH ~5.5, factors known to reduce both the hydrogen and hydrophobic bonds implicated in the stability of the antigen–antibody complex.

Acknowledgements

The authors express their appreciation to Ms. Joëlle Bouville for her excellent technical assistance; they thank Ms Ann Oaks and Mr Jean Daussant for fruitful discussions and evaluation of the manuscript. Thanks are also due to Professor Frentz for cooperative assistance. This work was supported by a DGRST grant no. 78 70362.

References

[1] Bandurski, R. S. and Greiner, C. M. (1953) J. Biol. Chem. 204, 781–786.
[2] Ozaki, H. and Shiio, I. (1969) J. Biol. Chem. 66, 297–311.
[3] Hatch, M. D. and Slack, C. R. (1970) Ann. Rev. Plant Physiol. 21, 141–162.
[4] Sutton, B. G. and Osmond, C. B. (1972) Plant Physiol. 50, 360–365.
[5] Willmer, C., Kanai, R., Pallas, J. E. jr and Black, C. C. jr (1973) Life Sci. 12, 151–155.
[6] Jacobson, L. (1955) Plant Physiol. 30, 264–269.
[7] Bonugli, K. J. and Davies, D. D. (1977) Plant 133, 281–287.
[8] Queiroz, O., Celati, C. and Morel, C. (1972) Physiol. Veg. 10, 765–781.
[9] Mukerji, S. K. and Ting, I. P. (1971) Arch. Biochem. Biophys. 143, 297–317.
[10] Mukerji, S. K. (1977) Arch. Biochem. Biophys. 182, 343–351.
[11] Ting, I. P. and Osmond, C. B. (1973) Plant Physiol. 51, 448–453.
[12] Chen, J. H. and Jones, R. F. (1970) Biochim. Biophys. Acta 214, 318–325.
[13] Brulfert, J., Arrabaca, M. C., Guerrier, O. and Queiroz, O. (1979) Planta 146, 129–133.
[14] Davies, D. D. (1979) Ann. Rev. Plant Physiol. 30, 131–158.
[15] Vidal, J. and Cavalié, G. (1974) Physiol. Veg. 12, 175–188.
[16] Vidal, J., Cavalié, G. and Gadal, P. (1976) Plant Sci. Lett. 7, 265–270.
[17] Hatch, M. D. and Oliver, I. R. (1978) Aust. J. Plant Physiol. 5, 571–580.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[19] Scopes, R. K. (1974) Anal. Biochem. 59, 277–282.
[20] Uedan, K. (1976) Plant Physiol. 57, 906–910.
[21] Jones, R., Wilkins, M. B., Coggins, J. R., Fewson, C. A. and Malcolm, A. D. B. (1978) Biochem. J. 175, 391–397.