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ESTIMATION OF *SORGHUM* LEAF PHOSPHOENOLPYRUVATE CARBOXYLASE PROTEIN USING AN IMMUNOADSORBENT COLUMN

JEAN VIDAL,† GUY GODBILLON* and PIERRE GADAL

Laboratoire de Physiologie Végétale Métabolique, ERA au CNRS no. 799, Université de Paris-Sud, Centre d'Orsay, Bât. 430, 91405 Orsay Cedex, France; *Laboratoire de Biologie Animale, Faculté des Sciences, Université de Nancy I, B. P. 239, 54506 Vandoeuvre les Nancy Cedex, France

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Key Word Index—*Sorghum vulgare*; Gramineae; immunoadsorption; estimation; phosphoenolpyruvate carboxylase.

Abstract—The present work describes a very simple technique for the isolation within 2–3 hr of inactive phosphoenolpyruvate carboxylase protein starting from a crude extract of *Sorghum* leaves by using an immunoadsorbent column prepared with a glutaraldehyde-activated gel. The conditions for the total elution of the enzyme protein are optimized. For quantitative determinations cyanogen bromide-activated gels should be avoided as the release of fixed immunoglobulin G (IgG) has been observed during washing with the acidic buffer needed to elute the enzyme. In that respect, glutaraldehyde-activated gel does not lose antibodies and consequently gives accurate results.

INTRODUCTION

Phosphoenolpyruvate carboxylase (EC 4.1.1.31), a widely distributed enzyme among plants and bacteria, has been extensively studied. Attention has been paid particularly to enzyme localization[1–10], functional and regulatory properties [11, 13], polymorphism [10, 15], participation in C_4 photosynthesis [4] and circadian rhythmicity in CAM plants[16]. In a number of cases the studies employed time consuming techniques for purification of the enzyme, and gave very low recovery[17, 18]. The present report describes the rapid purification of the carboxylase by the use of an immunochemical technique allowing determination of the amounts of enzyme protein in plant extracts.

RESULTS AND DISCUSSION

The high specificity of the immune serum raised against *Sorghum* leaf PEP carboxylase has been previously checked by the Ouchterlony double diffusion technique [19] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the results established that the immune serum reacted in a very specific manner with the carboxylase. Then, an immunoabsorbent column was constructed by linking the specific IgG to glutaraldehyde-activated Ultrogel. In the same preliminary work we were able to elute a catalytically active *Sorghum* leaf PEP carboxylase from the immunoadsorbent in a highly purified state[19]. However the cold water elution resulted only in a partial recovery of the enzyme; consequently, in order to obtain quantitative data we optimized a procedure for washing the immunoadsorbent with an acidic buffer which desorbed all the fixed protein enzyme in an inactive state.

In a standard experiment the carboxylase of an extract prepared as described in the Experimental was completely and strongly retained by the immunoadsorbent since it was not eluted with a phosphate buffer containing 2 M sodium chloride but only by a drastic procedure, using a very acidic buffer, citric acid–citrate buffer 0.1 M, pH 2.8; good results were also obtained by using the more classical acidic buffer glycocoll–hydrochloric acid, 0.2 M, pH 2.8. We checked the influence of buffer concentration on the desorption of the carboxylase. Enzymatic extract (1 ml) purified on hydroxylapatite containing 1.66 mg protein, 311 nkat, was passed through the immunoadsorbent. The column was rinsed as described in the Experimental and proteins were eluted with 24 ml of different concentrations of citrate buffer, pH 2.8 in 4 ml fractions at a flow rate of *ca.* 0.7 ml/min. In all experiments, no activity could be detected in the different washings. Protein amounts were determined according to the procedures described in the Experimental; it appeared that washing with 0.1 or 0.2 M solutions resulted in elution of about the same amount (680 μg) of inactive enzymatic protein. It was verified on sodium dodecyl sulfate-polyacrylamide gels that the effluent gave only one protein band exhibiting a monomeric MW of *ca.* 90 000, close to the value already reported[19, 22]. However at higher sample protein concentrations a second band occasionally appeared with a slightly higher elec-

†To whom all correspondence should be addressed.

trophoretic mobility. As the antibodies have been shown to be highly specific, we suggest a partial and variable proteolytic digestion of PEP carboxylase during the extraction process. A similar banding pattern has been shown for the maize leaf enzyme [23]. This problem needs further investigation.

Using these experimental conditions, different volumes of the leaf extract purified on hydroxyl apatite, containing 3.32 mg protein, 622 nkat–0.83 mg protein, 155 nkat, have been successively fixed onto the immunoadsorbent. It was established that the amount of protein recovered by acid washing was proportional to the total amount of protein in the extracts. Moreover, it could be noted that the sum of the two experimental values 'fixed protein + unfixed protein' was found to be equal to the total amount of protein loaded onto the column. These results established that the elution method was valid, as it resulted in total and proportional desorption of fixed PEP carboxylase. In accordance with the data reported in[24] we have found in previous experiments that cyanogen bromide-activated gel released significant quantities of IgG during the acid wash; this resulted in the recovery of a larger amount of protein than that fixed on the column, and contamination of the enzyme by antibodies. We then switched to glutaraldehyde-activated gel and following this procedure antibody desorption was no longer observed.

By using the immunoadsorbent column we have estimated some quantitative parameters of *Sorghum* leaf PEP carboxylase. It was found that 1 mg of enzymatic protein exhibits 455 nkat of activity; it was then calculated that 1 g of 15-day-old *Sorghum* leaf tissue (250 nkat of activity) contains *ca.* 0.55 mg PEP carboxylase, i.e. 5.5% of the total soluble leaf protein; these values are close to those reported for the maize leaf enzyme [17] and in perfect agreement with the data recently obtained in our laboratory using an immunoprecipitation method [22].

EXPERIMENTAL

Plant material. The method for growth of *Sorghum* plants has been described [14].

Preparation of the immunoadsorbent column. Methods for extraction and purification of the enzyme and preparation of the antibodies are described elsewhere[19]. Linkage of the IgG to glutaraldehyde-activated Ultrogel (ACA 22, IBF) was performed as indicated by the manufacturer. In all expts described in this paper the column was 1.4 cm in diameter, 2.8 cm in height.

Immunoadsorption chromatography. The leaves (15-days old) were homogenized using a Polytron homogenizer in Tris (Cl^-) buffer 0.1 M, pH 7.6 (1 g/26 ml) containing 0.1 M mercaptoethanol and Polyclar (10% fr. wt). The brei was squeezed through cheesecloth and centrifuged at 50000 *g* for 30 min. The supernatant fluid was fixed onto a hydroxylapatite column equilibrated with 0.01 M Pi buffer, pH 7; the adsorbent was rinsed many times with the buffer, then PEP carboxylase was recovered quantitatively by washing the column with 0.2 M Pi buffer, pH 7. The total enzyme activity was recovered through this purification step; the exact was clarified in order to keep the immunoadsorbent clean as long as possible. 311 nkat (1.66 mg protein) of enzyme were fixed on top of the immunoadsorbent. To remove non-specifically adsorbed proteins the column was successively rinsed with 10 ml 50 mM Pi buffer pH 7, 10 ml of the same buffer made 2 M in NaCl and 10 ml 10 mM Pi buffer pH 7. Adsorbed PEP carboxylase was eluted at this stage with 24 ml 0.1 M citric acid-citrate buffer, pH 2.8. This vol. of buffer was shown to be sufficient for total recovery of the protein enzyme.

Determination of protein amounts and enzyme activity. The amount of protein was determined using Lowry's method[20] by reference to a standard curve established with bovine serum albumin dissolved in the citric acid–citrate buffer 0.1 M pH 2.8, or by Scopes' method [21].

The procedure for determination of enzyme activity and the definition of the enzyme unit have been reported elsewhere[19].

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