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PHOSPHOENOLPYRUVATE CARBOXYLASE IN LUPIN NODULES AND ROOTS. IDENTIFICATION OF THE ENZYMATIC FORMS

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Phosphoenolpyruvate carboxylase (PEPC) EC 4.1.1.31 was extracted from nodules and roots of 2-day-old seedlings of lupin (*Lupinus luteus* L.). Chromatography on DEAE-cellulose of the nodule extract gave two forms of the enzyme: PEPC I and PEPC II eluted at 0.3-0.35 M and 0.41-0.53 M Tris buffer, respectively. A third form PEPC III from lupin roots was eluted from DEAE-cellulose column at the same buffer concentration as PEPC II from nodules. PEPC I and PEPC II eluted at 0.3-0.35 M and 0.41-0.53 M Tris buffer, more active in the 6-week-old nodules binding effectively nitrogen than in the 12-week-old ones.

Nitrogen fixation is an energy-intensive process which requires not only large inputs of reductant and ATP but also carbon skeletons for assimilation and transport of ammonia [4, 8, 13, 15, 16]. This process in annual legumes may consume up to 30% of the photosynthetic carbon and approximately 60% of the carbon transported to the nodules is lost as CO_2 through respiration [7, 12]. Non-photosynthetic CO_2 fixation *via* nodule PEP carboxylase results in partial reassimilation of respired CO_2 [10, 18]. Alfalfa root nodule PEPC provides as much as 25% of the carbon required for their assimilation of fixed N [10, 18]. The specific activity of alfalfa nodule PEPC was 6- to 12-fold that in roots [17]. Also in several other legumes PEPC activity was found to be higher in nodules than in roots [1, 2, 3, 5, 6, 11]. Vance *et al.* [19] claimed that alfalfa nodule PEPC is a nodule specific protein. However, it has recently [11] been reported that the enzyme from alfalfa roots and nodules was immunologically indistinguishable. In this work DEAE-cellulose chromatography was used for isolation of the enzymatic forms of PEPC from lupin nodules and roots.

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MATERIAL AND METHODS

Plant material. Lupin (*Lupinus luteus* L. cv. Ventus seeds were soaked for 6 h and germinated in the dark on Petri dishes for 48 h at 25°C. The apical, 1.5 - 2.5 cm-long root tips were used. The nodules were collected from the 6- and 12-week-old plants, grown in the field.

Purification of the enzyme from lupin roots and nodules. Nodules (15 g) or roots (30 g) were homogenized with two volume (w/v) of buffer A (50 mM Tris/HCl, pH 7.4, containing 400 mM sucrose, 5 mM DTT, 10% (w/v) glycerol, 2 mM EDTA, 0.1% bovine serum albumin and 10% (w/v) Polyclar AT). The extract was passed through two layers of miracloth and centrifuged for 30 min at 15000 *g*. The enzyme from clear supernatant was precipitated with solid ammonium sulfate at 0.6 saturation. The pellet was suspended in 5 ml of buffer B (100 mM Tris/HCl, pH 8.2, containing 10% (w/v) glycerol, 2 mM DTT and 0.2 mM EDTA), desalted on Sephadex G-10 and 5 - 7 ml (about 100 mg protein) was applied onto DEAE--cellulose column (2 × 32 cm) equilibrated with buffer B. Protein was eluted with 250 ml linear gradient of buffer B at concentration from 0.1 M (pH 8.2) to 0.6 M (pH 7.2). Fractions of 5.7 ml were collected at a flow rate of 24 ml/h.

Purification of the enzyme from the bacteroid-containing region of nodules. The bacteroid-containing region (1.5 g) of lupin nodules separated by manual dissection was homogenized with 10 ml of buffer A. The same isolation procedure was used as for roots and whole nodules. 2 ml of the desalted solution (30 mg of protein) was introduced to the DEAE-cellulose column (1.5 × 15 cm) equilibrated with buffer B. The enzyme was eluted using a linear gradient from 0.1 M to 0.6 M and from pH 8.2 to pH 7.2 respectively, in a total volume of 60 ml. Fractions of 2.4 ml were collected at a flow rate of 24 ml/h.

Enzyme assay. PEP carboxylase activity was measured spectrophotometrically by coupling with malate dehydrogenase and monitoring the absorbance at 340 nm at 28°C with the Specord (Carl Zeiss, Jena, G.D.R.). The standard reaction mixture (2 ml) contained: 100 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 3 mM PEP, 0.1 mM NADH, and 10 units of malate dehydrogenase. The reaction was initiated with the extract.

Protein measurement. Protein was determined by measuring absorbance at 280 nm and by the method of Lowry *et al.* [9], using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Nitrogen fixing legumes can be divided into two groups based on nitrogen transport products. First, the amide-transporting legumes (lupin,

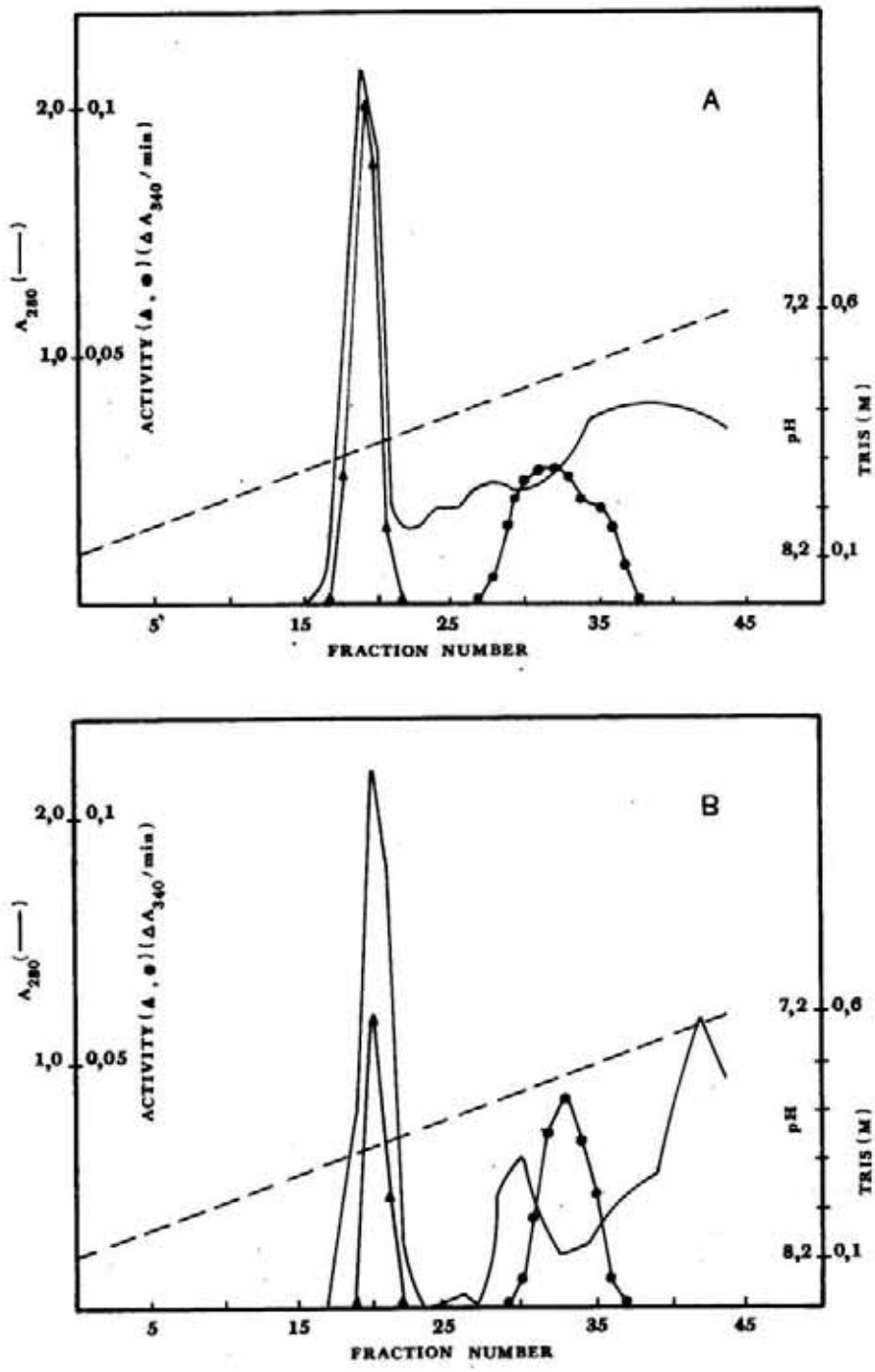

Fig. 1. DEAE-cellulose column chromatography of PEPC from lupin nodules from the A, 6-week-old plants.; B, 12-week-old plants. PEPC I, (▲). PEPC II, (●).

trefoil, alfalfa, peas) transport mainly asparagine, whereas the second ones the ureide-transporting legumes (French bean, cowpea, soybean) transport mainly ureides, allantoin and allantoic acid. These two groups differ in the major pathways of carbon metabolism. In the amide-transporting legumes CO_2 fixation in nodule provides oxalacetate, the product of PEP carboxylase for asparagine biosynthesis [10], whereas in the ureide transporting legumes allantoin and allantoic acid are products of purine metabolism.

Multiple forms of PEPC were reported in the nodules of various amide-transporting legumes: two in French bean [2] five in soybean [14] and two in alfalfa [17] although only one form from each species was purified. Christeller *et al.* [1] isolated one PEPC form from lupin and showed no substantial differences of enzymes from leaves, nodules and roots of lupin.

In this study we have separated two forms of PEPC (PEPC I and PEPC II) from the extract of lupin nodules by DEAE-chromatography (Fig. 1A and 1B). PEPC I was eluted at 0.3 - 0.35 M Tris and PEPC II at 0.41 - 0.53 M Tris. The activities of PEPC forms depended on the vegetative stage of plants from which nodules were collected. The total PEPC activity decreased in flowering plants. In nodules from the 6-week-old plants PEPC I constituted about 60% of the total activity (Fig. 1A), while only about 35% in the older plants (Fig. 1 B). The extract from lupin roots revealed only one form of PEPC (PEPC III). It was eluted from the DEAE-cellulose column at the same concentration of Tris as that used for PEPC II from nodules (Fig. 2). The activity of PEPC I constituted almost 90% of the total activity in the bacteroid-containing regions of nodules (Fig. 3). Since no PEPC activity we detected in the bacteroid fraction [1, 3, 14] one can assume that PEPC I is located in the cell cytosol of this zone of a nodule. The trace activity of PEPC II detected in the bacteroid-containing zone probably originated from the contaminating parenchyma cells of nodules.

In the amide-transporting legumes the specific activity of PEPC in nodules is positively correlated with N_2 assimilation [1, 6, 18]. Also it has been reported [1] that the acetylene reduction activity was the highest in young nodules. The appearance of PEPC I in the bacteroid-containing zone and much higher PEPC activity in the nodules from younger plants suggest that this enzyme plays an important role in amino acid biosynthesis in lupin nodules. High PEPC activity detected in nodules of lupin [1] and soybean [14] was probably due to the activity of PEPC I.

A significant PEPC activity was detected in 10-day lupin nodules when the acetylene reduction activity was nearly zero [1]. Also low level of PEPC activity was detected in young alfalfa roots and nodules [11]. It may be assumed that PEPC activity in young nodules, ineffective yet in N_2 fixation represent PEPC II.

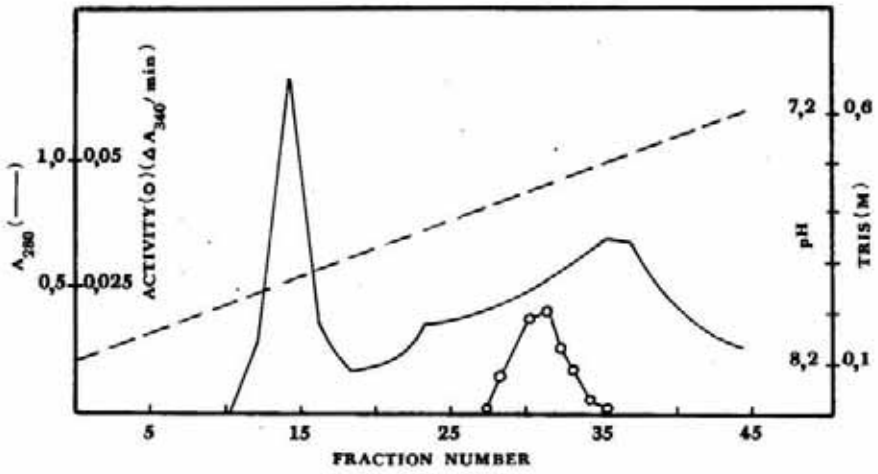

Fig. 2. DEAE-cellulose column chromatography of PEPC from the 2-day-old lupin roots. PEPC III (○).

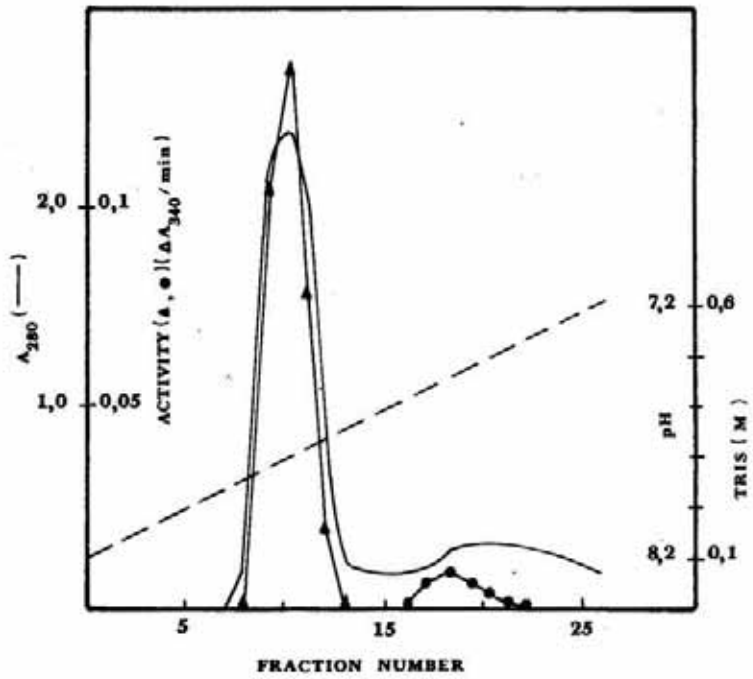

Fig. 3. DEAE-cellulose chromatography of PEPC from the bacteroid-containing region of the lupin nodule PEPC I (▲); PEPC II (●)

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