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FRUCTOSE-1,6-DIPHOSPHATASE FROM SPINACH LEAF CHLOROPLASTS: PURIFICATION AND HETEROGENEITY

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Abstract—The enzyme fructose-1,6-diphosphatase (FDPase), involved in the reductive cycle of the pentose phosphate pathway, has been purified from spinach leaves by heating (30 min at 60°), "salting out" with ammonium sulphate (between 30–70% of saturation), filtration through Sephadex G-100 and G-200, fractionation on DEAE-52 cellulose and preparative electrophoresis on polyacrylamide gel. Filtration through DEAE-cellulose led to the isolation of two active fractions (fractions I and II) with very close MWs and isoelectric points. By electrophoresis on acrylamide gel, both fractions gave two active fractions (fractions I_a–I_b and II_a–II_b). The fractions with low electrophoretic migration rate—I_b and II_b—are stable in acid and neutral pH, have a MW between 90000 and 110000 and constitute the native form of the photosynthetic enzyme. The fractions of faster migration rate—I_a and II_a—originate from the corresponding fractions I_b and II_b under alkaline conditions, show half the MW of the respective fractions, and behave as subunits of the original dimer form. Measured by electrofocusing, the four active fractions have isoelectric points in the range 4·10–4·30.

INTRODUCTION

FRUCTOSE-1,6-diphosphatase (E.C. 3.1.3.11; FDPase) is a key enzyme in the regulation of carbohydrate metabolism. Its occurrence has been demonstrated in many organisms. In yeast and animal tissues this enzyme has been mainly investigated because of its implication in the gluconeogenesis pathway. The enzyme from rabbit and rat liver has been extensively studied.[1–4] In plants FDPase activity has usually been related with photosynthesis, but the existence of an enzyme with the same action implicated in the reverse direction of glycolysis has been observed in germinating wheat germ,[5] in the endosperm of castor beans[6] and in the nonphotosynthetic tissues of spinach leaves.[7]

Data concerning the FDPase from photosynthetic tissues is scarce. The enzymes of *Opuntia ficus-indica* chloroplastic parenchyma,[8] and pea,[9] *Ricinus*[10] and navy-bean[11]

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leaves have been studied. The FDPase from spinach leaves has been studied in more detail; Racker and Schroeder[12] and Smillie[13] found its optimum pH to be about 8·0, the reaction being dependent on the presence of Mg^{2+} and showing a very high specificity for fructose-1,6-diphosphate as substrate. Similar results were obtained later with the enzyme from tapioca leaves.[14] Further work by Buchanan *et al.*[15,16] indicated the existence of a very sophisticated mechanism of enzyme regulation: reduced ferredoxin is needed for enzyme activity and, in cooperation with a "protein factor", converts inactive FDPase in the active form.

Working with the enzyme obtained from a crude extract of spinach leaves, we found several active fractions when the electrofocusing technique was employed. Further experiments demonstrated that all these fractions belong to the photosynthetic enzyme system, and the possible physiological role of this enzymic heterogeneity in photosynthesis prompted us to investigate them in more detail.

RESULTS

Figure 1 shows the elution pattern in DEAE-cellulose, when crude extract of whole spinach leaves was employed as starting material. Previously, with both Sephadex grades

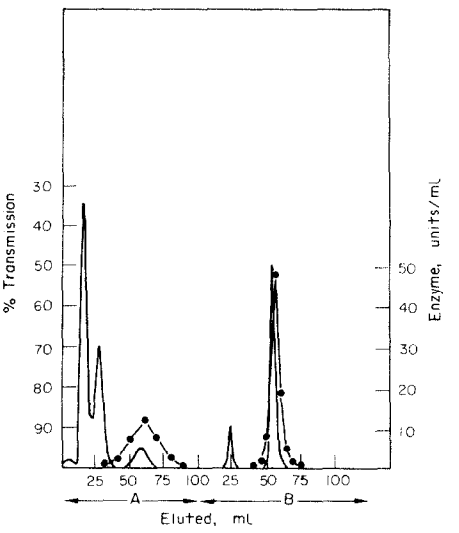

FIG. 1. CHROMATOGRAPHY ON DEAE-52 CELLULOSE (2 × 15 cm COLUMN).
Elution with 0·05 M acetic-acetate buffer pH 5·5, 0·25 M NaCl in the first 100 ml (A), and 1 M NaCl thereafter (B). Flow rate: 0·5 ml/min. Fractions of 5 ml. Values recorded at 280 nm in per cent transmittance, using a continuous flow cell with 3 mm path (——). Enzyme units/ml determined as referred in the text (●——●).

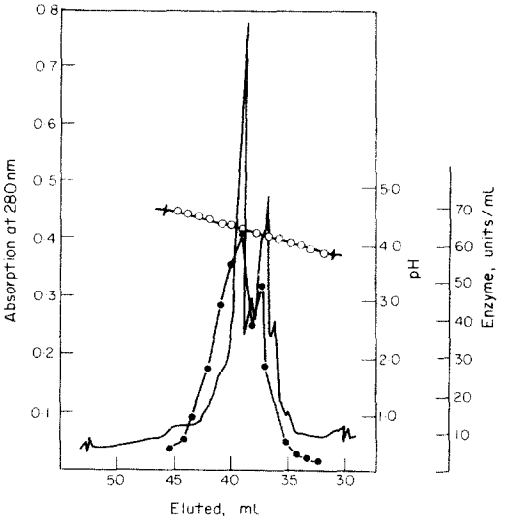

FIG. 2. ELECTROFOCUSING IN DENSITY GRADIENT AT pH 3–10 OF FRACTION I ISOLATED ON DEAE-CELLULOSE.
Absorption values recorded in a continuous flow cell with 5 m path at 280 nm (——). Enzyme units/ml determined as referred in the text (●——●). pH gradient after completion of the experiment (○——○).

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only one fraction with FDPase activity was obtained. However, on passage through DEAE-52 cellulose, two active fractions are clearly separated. The first one (fraction I) is collected at 0·25 M NaCl shortly after the elution of the bulk of non enzymic protein; the second one (fraction II) eluted when the NaCl concentration was increased to 1 M.

When chloroplast lysates were used, the protein and FDPase elution patterns in Sephadex G-100 and G-200 were very similar to those found with whole leaves, but in much smaller total amounts. However only the fraction II was obtained from DEAE-cellulose, probably due to the loss of enzymic material in the chloroplast isolation procedure.

When the active fractions I and II were developed in analytical acrylamide gel electrophoresis, each gave rise to two active fractions after detection *in situ* of FDPase activity. In addition, one inactive protein band appears between both as an impurity in the gels stained with amidoblack.

In accordance with this, preparative electrophoresis on polyacrylamide gel gave, from both fractions I and II, two highly purified active proteins. The greater, named subfractions I_a and II_a, eluted very fast and could be obtained fully active in acetate buffer (see Experimental). The minor subfractions, I_b and II_b, of low electrophoretic mobilities, eluted too late and were completely and irreversibly inactivated. Nevertheless its FDPase activity was clearly demonstrated *in situ* after its electrophoretic differentiation.

A similar, clear resolution of fractions I and II in the subfractions I_a–I_b and II_a–II_b was achieved by electrofocusing in a density gradient. The elution pattern of fraction I after electrofocusing in 3–10 pH gradient is shown in Fig. 2. Isoelectric focusing of fractions I_a and I_b gave pI values of 4·25 and 4·15, respectively. Similarly, the pI values of fractions II_a and II_b are 4·30 and 4·10.

The purification procedure starting from chloroplast lysates is summarized in Table 1. As mentioned above, only one fraction with FDPase activity is found in the DEAE-cellulose step, due to the reduced amounts of active starting material. Even so the specific activity of this fraction was calculated on the basis of the maximum protein content. The purification steps using crude extracts of spinach leaves as source of enzyme, are summarized in Table 2. As it was not possible to determine the specific activities of fractions I_b and II_b separated by preparative electrophoresis on polyacrylamide gel, all the specific activities have been calculated in the four active peaks isolated by electrofocusing in density gradient, taking for the calculation the fraction with highest absolute activity.

DISCUSSION

The characteristics and behaviour of the enzyme purified from chloroplast lysates unequivocally demonstrate that the enzyme isolated from whole leaf extracts corresponds to the photosynthetic FDPase, the gluconeogenic enzyme being completely inactivated and discarded. The low yield of the chloroplast isolation procedure and the high proportion of enzyme (often up to 90%) lost during the process because of the high solubility of the chloroplastidic FDPase,[17] makes it inadvisable to use isolated chloroplasts for preparative purposes. Consequently, whole leaf extracts were systematically employed as starting material.

The photosynthetic FDPase has been obtained highly purified from very few sources: the photosynthetic bacteria *Rhodopseudomonas palustris*[18] and *Rhodospirillum rubrum*,[19]

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TABLE 1. PURIFICATION OF FRUCTOSE-1,6-DIPHOSPHATASE FROM SPINACH LEAVES CHLOROPLASTS

Purification step	Protein (mg)	Enzyme units	Specific activity	Purification (x)
Crude extract	485·0	52·4	0·11	1·0
Extract heated at 60°	310·8	41·1	0·13	1·2
Salting out with $(NH_4)_2SO_4$	56·1	39·0	0·69	6·4
Filtration through Sephadex G-100:				
fraction of highest activity	0·50	6·7	13·4	124
mixed active fractions	1·46	15·5	10·6	98
Filtration through Sephadex G-200:				
fraction of highest activity	0·14	4·3	30·7	284
mixed active fractions	0·37	8·7	23·5	217
Chromatography on DEAE-52 cellulose:				
active fraction II	<0·04	1·8	45*	417*

* Calculated on the basis of the maximum assumed of protein content.

TABLE 2. PURIFICATION OF PHOTOSYNTHETIC FRUCTOSE-1,6-DIPHOSPHATASE FROM SPINACH LEAVES

Purification step	Protein (mg)	Enzyme units	Specific activity	Purification (x)	Yield (%)
Crude extract	25402	4579	0·17	1·0	100
Extract heated at 60°	13171	3489	0·26	1·5	76
Salting out with $(NH_4)_2SO_4$	2143	1927	0·90	5·3	42
Filtration through Sephadex G-100:					
fraction of highest activity	19·4	126	6·5	38	—
mixed active fractions	364·0	1591	4·4	25	35
Filtration through Sephadex G-200:					
fraction of highest activity	9·3	97	10·4	61	—
mixed active fractions	124·6	1032	8·3	48	22
Chromatography on DEAE-52 cellulose:					
active fraction I	7·7	321	39·1	228	7
active fraction II	10·7	516	48·8	290	11
Electrofocusing in density gradient:					
fraction I_a	—	—	89·2	525	—
fraction I_b	—	—	104·3	610	—
fraction II_a	—	—	89·1	524	—
fraction II_b	—	—	111·2	654	—

the algae *Euglena gracilis*,[20] and castor-bean[10] and navy-bean[11] leaves. Racker and Schroeder[12] obtained for the first time a purified preparation from crude extracts of whole spinach leaves, and Preiss *et al.*[21] and Springer-Lederer *et al.*[22] from chloroplast lysates. More recently, El-Badry and Bassham[23] reported the crystallization of the enzyme, and Buchanan *et al.*[16] suggested the possible heterogeneity of the FDPase from spinach leaves.

The purification procedure we have developed takes advantage of two characteristics of this enzyme: high thermal stability and strong acid pI. Racker and Schroeder[12] found that the photosynthetic FDPase from spinach leaves was fully active after heating at 62° for 15 min at pH 5·8, and App and Jagendorf[20] working with the enzyme from *Euglena*

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gracilis retained the activity after treatment at 60° for 10 min at pH 5·1. We have found that heating the enzyme from spinach leaves at 60° for 30 min at pH 7·5 has no effect on the FDPase activity. Heating at more acid pH values progressively alters the enzyme, which precipitates nearly quantitatively at pH 4·5.[16] On the contrary, the FDPase from the photosynthetic bacteria *Rhodopseudomonas palustris* exhibits a considerable thermic sensitivity, highly purified preparations being inactivated more than 65% after 1 min at 50°.[18]

Also the acid characteristic of this enzyme have been widely used in its purification from different sources. Buchanan *et al.*[16] precipitated the spinach enzyme at pH 4·5, and other authors have used its strong affinity for the DEAE-cellulose.[20] Using the electrofocusing technique we have found that the pI values of the different active fractions of the spinach FDPase are between 4·10 and 4·30. This result explained the high ionic strength needed, even at pH 5·5, to elute the enzyme from the DEAE-cellulose column. In agreement with these pI values we have found that the supernatants obtained after isoelectric precipitation at pH 4·5, according to Buchanan *et al.*,[16] still show FDPase activity.[24]

The results obtained by filtration through Sephadex G-100 and G-200 indicate the existence of a single enzymic protein in the experimental conditions used, with a MW around 90000–130000. This value is much lower than that of 195000 reported by Preiss and Kosuge,[25] and close to the values, 145000 and 130000, found by Buchanan *et al.*[16] using ultracentrifugation and filtration through Sephadex G-200, respectively. Scala *et al.*[10] reported a value of 120000–135000 for the photosynthetic enzyme of castor-bean leaves, and Springgate and Stachow[18] gave a value of 130000 for the FDPase from *Rhodopseudomonas palustris*. The MW of the gluconeogenic FDPase is very similar, with values of 100000, 127000 and 133000 for the enzymes from *Candida utilis*,[26] liver[27] and muscle[28] of rabbit, respectively.

The results obtained by chromatography through Sephadex and DEAE-cellulose suggest that both fractions I and II have very similar MWs and charges. Indeed, the pIs are in the very narrow range of 4·10–4·30 and, as we found in further experiments, the MW of fraction I is 92000 and that of the fraction II is 104000–110000. These are also the values we have found later for the two fractions of slow migration rate (I_b and II_b) in polyacrylamide gel electrophoresis. On the other hand, the MW of the fast-moving fractions (I_a and II_a) have been found to be 53000 and 59000, respectively.[24] The "Ferguson plots"[29] obtained by gel electrophoresis at different concentrations of acrylamide, confirm that these fractions have half the MW of the corresponding I_b and II_b.

The different behaviour found in the Sephadex filtration at pH 5·5 and in the electrophoresis conducted at pH 8·9, strongly suggest that the native enzyme gives rise to two subunits of half MW at alkaline pH. We have demonstrated in further experiments[24] that fraction II behaves as a completely homogeneous form, with MW of 110000, when filtered through a thin layer of Sephadex G-150 "superfine" at pH 5·5 and 7·1, but at pH 8·2 this molecular entity coexists with a subunit of MW 55000. Both the monomer and the dimer exhibit FDPase activity.

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Springgate and Stachow[30] reported that the FDPase of *Rhodopseudomonas palustris* behaves at pH 7·4 as an active dimer with MW of 130000, which is converted at pH 8·5 to a monomer of half the MW, with a specific activity three times higher. On the contrary, the enzyme from *Candida utilis* is fully active as a dimer of MW 100000 at neutral pH, whereas the subunits of 50000 formed at alkaline pH have only a residual activity.[26] The same occurs with the FDPase from rabbit liver, in which the active form is a dimer of MW 127000, fully active at neutral pH, although its dissociation in two inactive subunits is at acidic pH.[27]

Buchanan *et al.*[16] reported the detection of a low proportion of inactive subunit with half the original MW (130000–145000) when old purified spinach preparations were filtered through Sephadex G-200 at pH 7·3. Our results suggest that this subunit corresponds to fractions I_a and II_a which we have found to form at alkaline pH as dissociation products of the enzyme, and which already appears in small quantities at this pH.

On the other hand, Buchanan *et al.*[16] reported the existence of a strongly active dimeric fraction with high migration rate, followed by a monomeric and inactive diffuse tail, when a purified FDPase preparation is run at pH 8·3 in polyacrylamide gel electrophoresis. It is not clear why in these conditions the dimeric form exhibits a higher migration rate than the monomeric one: it suggests that the native dimeric enzyme is divided in a high proportion into the two monomeric subunits at this alkaline pH value, running ahead of the dimeric residual form. These authors were also unable to detect any activity in this slow fraction separated by electrophoresis on polyacrylamide gel. However, we have demonstrated the activity of this fraction by means of the *in situ* reaction, although a very rapid inactivation has been found in preparative electrophoresis.

EXPERIMENTAL

Assay of the FDPase activity. Usually the reaction mixture was as follows: 0·1 M Tris–HCl buffer pH 8·8, 5 mM fructose-1,6-diphosphate, 5 mM $MgCl_2$, 1·6 mM EDTA, 5 mM cysteine and the enzyme preparation, in a final vol. of 2 ml. After 30 min incubation at 28°, 1 ml of a 5% trichloroacetic acid (TCA) soln was added and the Pi determined according to Fiske and Subbarow.[31] FDPase was also assayed by increase in absorbance at 340 nm, at 28°, in a reaction mixture containing 0·1 M Tris–HCl buffer pH 8·8, 1 mM fructose-1,6-diphosphate, 5 mM $MgCl_2$, 1·6 mM EDTA, 5 mM cysteine, 0·2 mM NADP, 0·50 units of glucose-6-phosphate dehydrogenase and 0·35 units of phosphohexose isomerase, in a final vol. of 1 ml. One enzyme unit is expressed as the quantity of enzyme which releases 1 μmol of Pi, or produces 1 μmol of NADPH, per min in the above experimental conditions. The sp. act. refers to the units of enzyme per mg of protein. Protein content was measured according to Lowry *et al.*[32]

Control of purity. In all the steps of the purification procedure the protein homogeneity was assayed by disc electrophoresis in polyacrylamide gel (7·5% of acrylamide and 0·375% of bis-acrylamide), using the discontinuous buffer system and method of Ornstein[33] and Davis.[34] Electrophoresis was performed at 4° with 5 mA per gel applied until the bromophenol blue used as a marker was about 1 cm from the anodic end. The gels were then stained with 1% amidoblack 10B in 7% HOAc, and afterwards decolorized by washing with HOAc.

The *in situ* detection of fractions with FDPase activity was accomplished by incubating the gels for 1 hr at 28° in the following soln: 0·1 M Tris–HCl buffer pH 8·8, 5 mM fructose-1,6-diphosphate, 5 mM $MgCl_2$, 1·6 mM EDTA and 5 mM cysteine. The Pi produced in the reaction was visualized by dipping the gels immediately in the following soln: 5% ammonium molybdate, 1% hydroquinone, 20% Na_2SO_3 and conc. H_2SO_4 (6:1:6:6).

In the last steps of the purification procedure, controls of homogeneity were also carried out by electrofocusing[35] in polyacrylamide gel (7·5% of acrylamide and 0·2% of bis-acrylamide). Gels of 6·5 cm of length and 5 mm diam. were used, with a pH gradient 3–10 and 350 V for 2 hr. Ampholines were eliminated by continuous washing

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of the gels with 5% TCA, and after that the protein fractions stained with amidoblack 10B. The *in situ* enzyme activity visualization is not possible when using the electrofocusing technique, because of the polyaminopolycarboxylic acids of the ampholines strongly inhibit this enzyme.

Materials and chemicals. Glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphate tetrasodium salt, and NADP were purchased from Boehringer; phosphohexose isomerase was from Calbiochem; Sephadex G-50, G-100 and G-200 were supplied by Pharmacia (Uppsala), and DEAE-52 cellulose by Whatman; acrylamide and bis-acrylamide were from BDH, and the ampholines of pH range 3–10 from LKB. The other products were of analytical grade. Batches (5 kg) of fresh spinach leaves, purchased in the local market, were washed with H_2O and kept overnight at 4° in plastic bags, in order to obtain the appropriate turgidity. The petioles and prominent veins were taken out and the leaves shredded (about 0·5 cm^2 pieces). Unless otherwise stated all the steps henceforth were carried out at 4°.

Preparation of chloroplasts lysates. The cut spinach leaves were homogenized (1:1 w/v) in 0·05 M Tris–HCl buffer pH 7·5, 5 mM $MgCl_2$, 5 mM cysteine, made isotonic with 0·35 M NaCl, for 1 min at maximum speed (Sorvall omni-mixer blendor). After filtering through 3 layers of nylon cloth, the extract was centrifuged at 200 *g* for 5 min. The supernatant was again centrifuged at 1000 *g* for 10 min, and the pellet of chloroplasts was collected into a hypotonic soln of 0·025 M Tris–HCl buffer pH 7·5, with 5 mM $MgCl_2$ and 5 mM cysteine. The lysis was completed by treating the suspension with glass beads in a vibratory apparatus (E. Bühler); it was then centrifuged at 20000 *g* for 30 min.

Preparation of crude extracts of the whole leaves. The cut spinach leaves were directly homogenized in 0·025 M Tris–HCl buffer, 5 mM in $MgCl_2$, pH 7·5 (1:1 w/v) in the same conditions as above. After filtering through nylon cloth the homogenate was centrifuged at 20000 *g* for 30 min.

Purification of the enzyme. Unless otherwise stated, the chloroplast lysates and the whole crude extracts were treated in the same way through all the purification procedure. In the steps where chromatography or electrophoretic techniques were used, the elution was followed by A 280 nm using a continuous flow cell. The purification procedure consisted in the following steps: (a) Thermic treatment. The supernatant (about 60 ml in the case of the chloroplast lysates, and 2·7 l. when the whole extracts were used) is heated at 60° for 30 min. The precipitate is centrifuged off at 2800 *g* for 10 min. The enzyme activity remains unaltered in the supernatant. (b) $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to 30% saturation, and the ppt. was removed at 2800 *g* for 20 min. $(NH_4)_2SO_4$ was again added to the supernatant to 70% saturation. After centrifugation at 2800 *g* for 20 min the ppt. was dissolved in 40 ml of 0·05 M acetate buffer pH 5·5. (c) Sephadex G-100 chromatography. The soln was filtered through a column of Sephadex G-100 (3 × 50 cm) equilibrated and afterwards eluted with 0·05 M acetate buffer pH 5·5. 5 ml fractions were collected with a flow rate of 0·5 ml/min. The enzyme eluted soon after the void vol., and the active fractions (*ca* 240 ml) were combined. (d) Sephadex G-200 chromatography. The active soln was now filtered through a column of Sephadex G-200 (3 × 50 cm) in the same conditions as stated above. The enzyme was recovered at the end of the eluted protein peak, and all the active fractions (*ca* 470 ml) were combined. (e) Chromatography on DEAE-52 cellulose. The soln was applied to a DEAE-52 cellulose column (2 × 15 cm), equilibrated beforehand with 0·05 M acetate buffer pH 5·5. The elution of fixed material was carried out with 100 ml of the same buffer made 0·25 M in NaCl, increasing the NaCl concentration 1 M. Fractions of 5 ml were collected at a flow rate of 0·5 ml/min. The active chromatographic peaks were separately dialyzed for 24 hr against 0·015 M acetic-acetate buffer pH 5·5, and then lyophilized. (f) Preparative acrylamide electrophoresis. Due to insufficient enzymic material, this step was only carried out with the active peaks obtained from whole spinach leaves as starting material. The lyophilized fractions were dissolved in a 10× smaller vol. of 0·015 M HOAc, resulting in a protein concentration of 1–3 mg/ml in acetate buffer pH 5·5. A Canalco "Prep-Disc" apparatus was used, with a 7·5% acrylamide gel and a ratio acrylamide/bis-acrylamide of 20:1, in a column of 3·2 cm^2 of cross section and 2 cm height. The gel was prepared in 0·375 M Tris–HCl buffer pH 8·9. The buffer of the anode and cathode compartments was 0·025 M Tris–0·2 M glycine pH 8·3. Samples (2 ml), previously made 10% in sucrose, were used in each fractionation procedure. 6 mA for 30 min was applied, then increased to 15 mA. Elution was with 0·025 M Tris–HCl buffer pH 7·5, at a flow rate of 2 ml/3 min. 2 ml Fractions were collected.

As it was observed that the enzyme activity was inhibited by the buffer system Tris–glycine within a short time, the active fractions were filtered immediately after collection through small columns (2 × 25 cm) of Sephadex G-50 "fine", equilibrated and eluted with 0·05 M acetate buffer pH 5·5. (g) Electrofocusing in density gradient. This procedure was also only employed with active fractions obtained from whole leaves. As its resolution was found to be inferior to the acrylamide electrophoresis method, the electrofocusing technique was not used for preparative purposes. A 110 ml LKB column was used, with ampholines of 3–10 pH gradient and 310 V for 70 hr. The column was eluted at 1 ml/min. Fractions of 1 ml were collected and their pH measured in a pH-meter at the same temperature at which the electrophoretic focusing took place. The isoelectric point of each peak was that of the fraction with highest sp. act. As the ampholines were found to act as strong inhibitors of the enzyme activity, they were eliminated by filtering the active fractions through a Sephadex G-50 "fine" column (2 × 25 cm), stabilized then eluted with 0·05 M acetate buffer pH 5·5.

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