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GLUTATHIONE S-TRANSFERASE FROM *HEVEA BRASILIENSIS*

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; GSH S-transferase; latex.

Abstract—Glutathione (GSH) S-transferase can be detected in a variety of tissues of *Hevea brasiliensis*. Lyophilized powders prepared from 20 000 *g* supernatants of latex adjusted to pH 5.0 contain substantial amounts of GSH S-transferase activity which is stable at −20° for up to 6 months. The enzyme has a broad pH optimum between 8.5 and 9.5. The K_m values for GSH and 1-chloro-2,4-dinitrobenzene are in the range of 33–45 and 150–200 μM, respectively. The enzyme has a MW in the range of 47 000–50 000 and an isoelectic point of 4.3. Although it appears homogeneous on analytical polyacylamide disc gel electrophoresis (PAGE) and isoelectric focusing, it resolves into five forms on DEAE-Sephadex chromatography.

INTRODUCTION

Glutathione S-transferase (EC 2.5.1.18) are a group of detoxicating enzymes that catalyse the conjugation of reduced glutathione (GSH) with a variety of electrophilic compounds [1–5]. These enzymes are known to be dimeric with MWs between 40 000 and 50 000 [5]. Several groups have suggested that the GSH S-transferases consist of various pairs of three subunits designated as Ya, Yb and Yc with MWs of 22 000, 23 500 and 25 000, respectively [6–9]. In addition, other workers have suggested that the minimum number of subunits needed to explain the multiple forms of rat liver isoenzymes is four [10, 11].

Although the properties and distribution of GSH S-transferases have been extensively studied in vertebrates [12–16] and to some extent in invertebrates [17–20], few such studies have been conducted in plants [21–23]. This may be due partly to difficulties encountered in extracting these enzymes from plant tissues.

In the rubber tree *Hevea brasiliensis*, the cell content of the latex vessels can be easily drained by paring a portion of the bark from the tree. This white milky liquid known as latex contains, amongst other substances [24], significant amounts of GSH and other thiols [25]. Since the role of GSH in detoxication in biological systems is well known, we were led to look for glutathione-conjugating activity in latex, as well as in a variety of other tissues of the rubber tree. This paper describes the detection and distribution of GSH S-transferase in a variety of tissues from *H. brasiliensis* in addition to latex. The enzyme, unlike other GSH S-transferases in animals and plants, behaves unusually in that it appears as a homogeneous protein in isoelectric focusing experiments and resolves into five chromatographically distinguishable forms on DEAE anion-exchange chromatography. It is only capable of conjugating GSH with aryl substrates such as 1-chloro-2,4-dinitrobenzene (DNCB) and 1,2-dichloro-4-nitrobenzene (DCNB).

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RESULTS

Preliminary studies conducted using several preparations of lyophilized powder revealed that the enzyme had a broad optimum pH between 8.5 and 9.5, with a gentle fall in activity on the acid and alkaline sides of the optimum, so that at pH values of 7.5 and 10.5 the activity was half that at the maximum. Routine enzyme assays of column eluants were conducted .in 0.1 M arginine–hydrochloric acid buffer of pH 8.3 at 28°. The enzyme was stable at 5° between pH values of 4.3–5.5 and 5.5–10.0 in 0.1 M sodium acetate–acetic acid and 0.1 M arginine–hydrochloric acid buffers, respectively, for up to 3 hr, which was the longest period tested. A dialysed preparation of an ammonium sulphate-fractionated enzyme lost only 20% of its activity when left to stand for 12 days at 5° at pH 6.5. Even when the enzyme was exposed to 37° for 4 hr at pH 8.3 in 0.1 M arginine–hydrochloric acid buffer it did not lose more than 10% of its activity. The specific activity of 37 different preparations of lyophilized powder prepared under similar conditions from latex collected from the same trees was 2.82 ± 1.50 nkat/mg protein at 28° in the same buffer. Using different preparations of the lyophilized powder the K_m for GSH was in the range 33–45 μM, while that for DNCB was in the range 150–200 μM at pH 8.3 under the same conditions. The ratio of activity for the same amount of enzyme towards DNCB and DCNB was approximately 4:1 at pH 8.3. Most of the enzyme activity could be precipitated from aqueous solutions of the lyophilized powder between 50 and 80% saturation by ammonium sulphate resulting in a 2- to 3-fold purification.

The enzyme when passed through a calibrated Sephadex G-200 column appeared as a single peak with an average MW of 47 000 ± 3000. This value was obtained not only for a solution of the lyophilized powder, but also for partially purified preparations of the enzyme obtained after ammonium sulphate fractionation or anion-exchange chromatography. The MW of the enzyme was not altered when it was chromatographed on a similar column equilibrated with buffer containing either 0.1 or 0.3 M sodium chloride.

The enzyme did not bind to a CM-cellulose column, and almost 98% of the activity loaded could be recovered in the washing buffer. When the column was subsequently washed with a solution of 1 M sodium chloride, no activity could be detected in the eluate. However, when the enzyme was applied to a DEAE-Sephadex column, it bound effectively and could be eluted in several peaks with a series of sodium chloride gradients (Fig. 1). The activity recovered in these experiments approached 100%. A similar result was obtained when either a solution of the lyophilized powder or the ammonium sulphate fractionated enzyme was loaded onto the DEAE-column and eluted with a linear sodium chloride gradient of 0–0.5 M (Table 2). In both cases five peaks were eluted: peaks 1, 4 and 5 being the major ones and peaks 2 and 3 the minor ones.

When partially purified preparations of the enzyme prepared by ammonium sulphate fractionation, gel filtration through Sephadex G-200, or ion-exchange chromatography on DEAE-Sephadex were dialysed and electrophoresed on polyacrylamide gels, a single peak migrating towards the anode with a relative mobility in the range 0.24–0.26 was obtained. Further, when solutions of the lyophilized powder (Fig. 2) or an ammonium

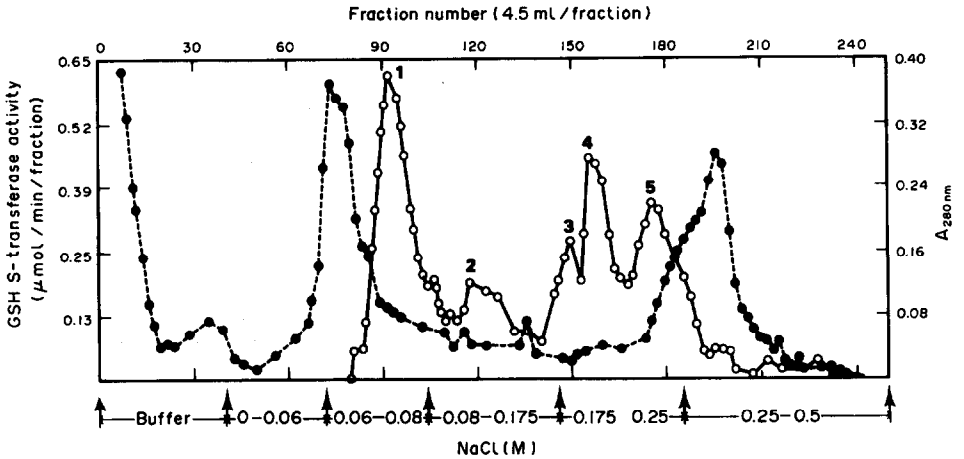

Fig. 1 A 50–80% ammonium sulphate protein fraction prepared from an aqueous solution of lyophilized powder was dialysed against 20 mM Tris–Hydrochloric acid buffer, pH 6.0, and then chromatographed (186 mg protein, 23 μmol/min of activity) on a DEAE-Sephadex column (33 cm × 1.5 cm). The series of sodium chloride gradients used (200 ml) is indicated. Fractions (4.5 ml) were collected and assayed for GSH S-transferase activity (O——O) and protein content (●——●) as described in the text.

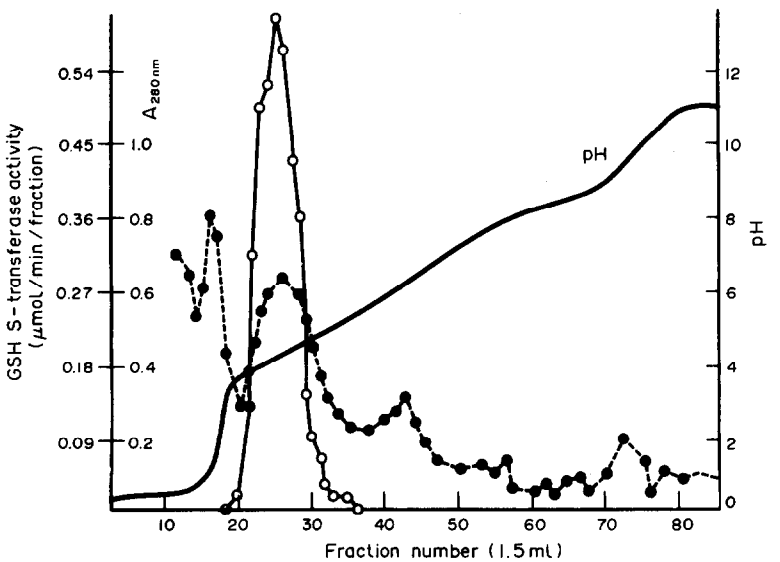

Fig. 2. A solution of lyophilized powder containing 30 mg of protein was electrofocused for 65 hr. Fractions were eluted manually and the pH of each fraction was determined at 5°. Protein contents were determined by the absorbance at 280 nm (●——●), while GSH S-transferase activity was measured with 100 μl aliquots as described in the text (O——O).

sulphate fractionated enzyme were electrofocused in ampholine of pH range 3.5–10.0, a single peak of activity was detected with an average isoelectric point of 4.32 ± 0.1. Again, when the five peaks resolved on the DEAE-column were pooled, dialysed and electrofocused in ampholine, a single peak with a similar pI was obtained. In all the electrofocusing experiments, between 60 and 80% of the activity loaded was recovered.

DISCUSSION

H. brasiliensis is perhaps the first lactiferous tree known to contain GSH S-transferase activity. The enzyme is present in both the young and mature tissues of the stem and leaves and also in mature seeds (Table 1). The highest specific activity was for the enzyme present in the supernatant from latex adjusted to pH 5.0. Both 20000 *g* as well as 100000 *g* supernatants from such acidified latex were found to contain the same amount of activity per unit volume of supernatant, and lyophilized powders prepared from them were found to be stable at −20° for up to 6 months, which was the longest period tested. No attempt was made to locate the presence of GSH S-transferase in organelles such as the mitochondria or microsomes as the presence of these in tapped latex has been reported to be rare [26, 27].

The addition of 1.0 mM potassium cyanide to freshly collected latex was useful in preventing it from developing a dark brown colour, which occurred on some occasions. The supernatant obtained from such samples was also found to be deeply coloured and this colour could not be removed by exhaustive dialysis. Such dark-coloured supernatants were always found to contain less GSH S-transferase activity than white latex. It is possible that the development of the dark brown colour could be due to the transformation of endogenous phenols to quinones since polyphenol oxidase, which can catalyse such transformations, has been shown to be present in substantial amounts in latex serum [28, 29]. It is interesting to note that vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone) and 1-naphthol were good inhibitors of latex GSH S-transferase (Table 3). Vitamin K and other quinones are also known to inhibit insect GSH S-transferase [19, 30] as well as GSH S-transferase from corn [22].

Latex GSH S-transferase appears to be highly specific in its substrate requirement. GSH conjugation only took place with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene, while methyl iodide, *p*-nitrobenzyl chloride, 1,2-epoxy-3-*p*-nitrophenoxypropane and bromosulphophthalein did not serve as substrates. A similar observation was also made for the corn enzyme, which was reported to have only 'aryl' transferase activity while 'alkyl' transferase activity with methyl iodide was absent [22]. The GSH S-transferase from pea seedlings catalysed conjugation with fluorodifen, but not conjugation with atrazine though this compound served as a substrate for the corn enzyme [31]. It may be possible that a high degree of substrate specificity is common to most plant transferases, unlike vertebrate liver transferases which show a broad degree of substrate specificity [4, 11]. In some respects, the latex enzyme has other properties that are in common with insect GSH S-transferases. The phthaleins, sulphophthaleins, fluoresceins and dicarboxylic acids which competitively inhibit insect GSH S-transferases [32, 33] also inhibit the latex enzyme. In this case their inhibition can be overcome by increasing the concentration of one of the substrates, namely glutathione (Table 3). These compounds are known to be poor inhibitors of vertebrate liver GSH S-transferases [32]. Bromosulphophthalein, on the other hand, which serves as a substrate for rat liver transferases, is a good inhibitor of GSH S-transferase from insects [32, 33], corn leaves [21], pea epicotyls [31] and the latex enzyme. Thus with regard to inhibition it seems that the latex enzyme resembles GSH S-transferases from other plants and insects. However, it behaves unusually when subjected to electrophoresis or ion-exchange chromatography.

The enzyme appears to be homogeneous on both PAGE and isoelectric focusing, but it resolves reproducibly into five peaks when chromatographed on a DEAE-anion exchange column. The five peaks were obtained both for a solution of the lyophilized powder and the ammonium sulphate fractionated enzyme (Table 2). The fact that the above two preparations had similar isoelectric points as well as relative mobilities on PAGE would imply that the enzyme molecules are identical with

Table 1. Distribution of GSH S-transferase in *H. brasiliensis**

Tissues	Activity (nkat/g fr. wt.)	Specific activity (nkat/mg protein)
Very young leaves from a 3-month-old seedling	6.92 ± 2.05	0.23 ± 0.06
Older leaves from a 3-month-old seedling	6.32 ± 2.30	0.11 ± 0.04
Very young stem from a 3-month-old seedling	1.78 ± 1.28	0.18 ± 0.13
Older stem from a 3-month-old seedling	0.22 ± 0.05	0.04 ± 0.01
Older leaves from 20-year-old trees	11.04 ± 3.83	0.30 ± 0.09
Mature seeds from 20-year-old trees	2.78 ± 2.08	0.18 ± 0.14
Latex from 20-year-old trees	3.52 ± 1.86	2.81 ± 1.50

*The results presented have been averaged from six determinations with the exception of latex where the data presented are an average of 37 different preparations: data are the mean ± s.d.

Table 2. Chromatography of GSH S-transferase from *Hevea* latex on DEAE-Sephadex A-50 columns*

Peak No.	Solution of a lyophilized powder: NaCl needed to elute fraction of maximum activity (M)	Solution of a lyophilized powder: Activity in each peak (%)	Dialysed solution of a 50–80% $(NH_4)_2SO_4$ fractionated enzyme: NaCl needed to elute fraction of maximum activity (M)	Dialysed solution of a 50–80% $(NH_4)_2SO_4$ fractionated enzyme: Activity in each peak (%)
1	0.07	29.6	0.07	28.2
2	0.12	6.0	0.10	8.5
3	0.17	15.3	0.16	11.5
4	0.20	28.7	0.20	25.5
5	0.28	22.1	0.24	27.3

*In both experiments the total amount of activity loaded was similar and the activity available under each peak was expressed as a percentage of the total activity recovered from the column. In both cases the columns (40 cm × 1.5 cm) were washed with three bed volumes of the equilibrating buffer followed by 1 l. of a 0–0.5 M linear sodium chloride gradient.

Table 3. Inhibition studies on GSH S-transferase from *Hevea* latex

Inhibitors (0.03 mM)	% Inhibition at 0.5 mM GSH and 0.1 mM DNCB	% Inhibition at 2 mM GSH and 0.1 mM DNCB
(a) Sulphophthaleins		
Bromocresol green	71	41
Bromophenol blue	53	19
Bromothymol blue	80	60
Chlorophenol red	72	61
Phenol red	29	19
(b) Phthaleins		
Phenolphthalein	26	15
3,3′-5,5′- Tetrabromo-phenolphthalein	33	25
Sulphobromo-phthalein	71	63
(c) Fluoresceins		
Fluorescein	12	10
Eosin	55	38
Rose Bengal	87	69
(d) Dicarboxylic acids		
Undecandioic acid	12	6
Brassylic acid	52	29
(e) Phenol and quinone		
Vitamin K_1	46	32
1-Naphthol	82	65

*Enzymic reaction velocities were appropriately deducted from test values and the fractional activity was measured after inhibition had been expressed as a percentage of the uninhibited value.

GSH, Glutathione; DNCB, 1-chloro-2,4-dinitrobenzene.

regard to net charge, and that ammonium sulphate treatment does not affect this property. Besides, only one peak was obtained when the five peaks were pooled, dialysed and electrofocused, thus suggesting that the electrophoretic homogeneity was not altered. In other species where a homogeneous GSH S-transferase has been detected by electrofocusing, such as with human erythrocyte [14], bovine lens [16] and *Galleria mellonela* larva [17], the enzyme has always appeared as a single peak on ion-exchange chromatography. Again in vertebrate liver, where multiple forms of GSH S-transferases were detected by ion-exchange chromatography, they could also be separated by isoelectric focusing [5]. It is this lack in conformity on ion-exchange chromatography and isoelectric focusing that makes the latex enzyme somewhat different from the transferases studied so far.

It was initially felt that perhaps the enzyme had aggregated to different extents during the passage of increasing concentrations of sodium chloride through the DEAE-column, thereby creating molecular forms varying in net charge. Enzyme aggregation in the presence of high concentrations of sodium chloride has been observed with human liver α-galactosidase [34, 35] and plant β-galactosidase [36]. If the above were true with the latex GSH S-transferase, then that would explain why a homogeneous form of the enzyme was noticed in electrofocusing experiments when the five peaks were pooled, dialysed and electrofocused. However, high MW aggregates of the enzyme were not detected when solutions of the enzyme were passed through a Sephadex G-200 column that had been equilibrated with buffer containing 0.1 and 0.3 M sodium chloride, respectively. The MW of the eluted enzyme approximated to 50 000 in the above studies, suggesting that the multiple forms seen on DEAE-columns could not have been due to molecular aggregation.

Another possible explanation is that all the enzyme molecules had the same net charge, but had their charges

distributed about the protein in such a manner that their availability for binding varied. This difference in the availability of the binding sites on the protein(s) could either be an inherent property of the native enzyme(s) in the latex or an artefact created by conformational changes imposed on the protein by sodium chloride during the elution procedure. The latter is possible if the native enzyme had a number of disulphide bonds which dissociated to different extents at different sodium chloride concentrations to give the thiol forms. Very recently some support for this idea was obtained (Balabaskaran, S. and Muniandy S., unpublished work) by demonstrating that the multiple forms of GSH S-transferases resolved on the DEAE-column could be reduced to a single form by equilibrating the column with thiol reagents such as GSH or dithiothreitol. Further work is being carried out to understand the nature of this transformation in the presence of thiol reagents.

A role for GSH S-transferase in *H. brasiliensis* has still not been established. The fact that substantial quantities of GSH S-transferase are present not only in the latex but also throughout the tree suggests the possibility of an important role for this enzyme. Archer [37] reported the presence of a related GSH dependent enzyme, which he called a rubber transferase, that was involved in the incorporation of isopentenyl-pyrophosphate into the rubber polymer. This enzyme not only required GSH, but also had an identical MW, similar isoelectric point and binding property to DEAE-anion exchangers. It is not known if GSH S-transferase and rubber transferase are related enzymes or are one and the same protein having a number of roles to play in the rubber tree.

EXPERIMENTAL

Preparation of lyophilized powder from Hevea *latex.* Latex tapped from a number of trees was pooled and immediately treated with 0.1 mM KCN. All operations in the isolation of a partially purified preparation of the enzyme using the above latex were carried out at 5°. The pH of the latex was then adjusted to 5.0 with HOAc and the mixture left to stand for 15 min, after which it was centrifuged at 20 000 *g* for 1 hr. The coagulated rubber, which formed a layer at the top, was removed and the pale yellowish turbid supernatant dialysed overnight against several changes of distilled H_2O. Heavy precipitation occurred during dialysis. The dialysate was further centrifuged at 20 000 *g* for 30 min and the clear supernatant then lyophilized. The lyophilized powder, which was fluffy and white in colour, was used as a source of GSH S-transferase.

Enzyme extraction from tissues of H. brasiliensis. A variety of tissues were collected fresh from the field, weighed and thoroughly ground at 5° with a pre-cooled pestle and mortar in 100 mM arginine–HCl buffer, pH 8.3, in a ratio of 1 : 5 (w/v). The supernatants were filtered through three layers of cheesecloth and the filtrate was centrifuged at 20 000 *g* for 45 min at the same temp. Samples of 0.5 ml from each of the supernatants were used to assay for GSH S-transferase activity as described in the text.

Enzyme assay. GSH S-transferase activity was assayed spectrophotometrically by modifying the method described in ref. [38] using solns of the lyophilized powder in buffer. Enzyme assays were routinely carried out at 28° in 0.1 M arginine–HCl buffer, pH 8.3, containing 2.0 mM GSH and either 0.2 mM DNCB or 2.0 mM DCNB. The rate of change in $A_{344\,nm}$ of the conjugate of GSH and the aromatic substrate was followed in a Beckman Acta III recording spectrophotometer. In all cases non-enzymic blank reactions between the substrates were deducted from test values. The apparent K_m for GSH was estimated by varying its concn from 0.005 to 2.0 mM at a constant DNCB concn of 0.2 mM. Similarly, the apparent K_m for DNCB was estimated by varying its concn from 0.01 to 0.2 mM at a constant GSH concn of 2.0 mM.

GSH conjugation with other substrates such as 2-epoxy-3-(*p*-nitrophenoxy)propane [39], *p*-nitrobenzyl chloride [2], bromosulphophthalein [40] and methyl iodide [41] were carried out with solns of the lyophilized powder according to the methods described in the lit.

Inhibition studies. A variety of compounds known to inhibit insect GSH S-transferases were tested as potential inhibitors of the latex enzyme. Both inhibited and uninhibited velocities were measured using solns of the lyophilized powder at two GSH concns at a constant concn of DNCB and inhibitor, in a total reaction vol. of 3.0 ml in 0.1 M arginine–HCl buffer, pH 8.3, at 28°. Each assay contained 0.25 mg of lyophilized powder.

MW estimation. A Sephadex G-200 column (68 cm × 2.6 cm) was equilibrated with 20 mM Tris–HCl buffer, pH 8.3, and calibrated with human transferin (90 000); bovine serum albumin (67 000), ovalbumin (45 000), bovine pancreatic trypsin (24 000) and cytochrome *c* (12 400). The MW of GSH S-transferase was estimated by passing a soln of the following preparations of enzyme which had been previously dialysed in the eluting buffer: (a) a soln of lyophilized powder; (b) $(NH_4)_2SO_4$ fractionated enzyme; and (c) conc. soln of the five peaks pooled from the DEAE-column through which was passed an $(NH_4)_2SO_4$ fractionated enzyme. In a similar expt, a Sephadex G-200 column (81 cm × 2.5 cm) was equilibrated with the above buffer but containing either 0.1 or 0.3 M NaCl, respectively. Serum bovine albumin and ovalbumin were eluted through this column before and after the passage of a soln of the lyophilized powder and an $(NH_4)_2SO_4$ fractionated enzyme.

Ion-exchange chromatography. Both DEAE-Sephadex A50 (equilibrated with 10 mM Tris–HCl buffer, pH 6.0) and CM-cellulose (equilibrated with 10 mM phosphate buffer, pH 6.7) columns (33 cm × 1.5 cm) were used to partially purify GSH S-transferase from a soln of the lyophilized powder prepared in the appropriate equilibrating buffer. Proteins were eluted from the columns first with the above buffers, then with the same buffers plus NaCl. The NaCl concn required to elute the fraction of maximum enzyme activity was graphically estimated. Enzyme activity in the fractions was assayed as described above.

Analytical PAGE. This was conducted essentially according to the method described in ref. [42]. Electrophoresis was conducted at 5° in 10% gels at pH 8.3 in Tris–glycine buffer at a constant current of 2 mA/tube until the tracking dye was *ca* 0.5 cm from the bottom of the tube. At the end of electrophoresis, the gels were dislodged and the dye front was marked before they were sliced into 1.0 mm slices. Each slice was homogenized in 0.5 ml 50 mM arginine–HCl buffer, pH 8.3, and allowed to stand for 2 hr at 5° after which a suitable aliquot was assayed for GSH S-transferase activity, as described in the text, using microcuvettes. The maximum amount of protein loaded on the gels was not more than 200 μg. Protein samples that were electrophoresed included: (a) a soln of the lyophilized powder; (b) $(NH_4)_2SO_4$ fractionated enzyme; (c) Sephadex G-200 purified enzyme; and (d) a conc. soln of the five peaks pooled from a DEAE-column through which was passed an $(NH_4)_2SO_4$ fractionated enzyme.

Isoelectric focusing. Isoelectric focusing was carried out in an LKB 8101 column of *ca* 110 ml capacity according to the method of Vesterberg and Svenson [43] in a sucrose density gradient (0–50% w/v) superimposed with an ampholine (1% v/v) pH gradient ranging from 3.5 to 10.0, at a constant voltage of 300 V from between 20 and 70 hr at 5°. The quantity of protein loaded in these expts varied from 10 to 100 mg. The samples of enzyme

focused on the column were: (a) a soln of the lyophilized powder; (b) $(NH_4)_2SO_4$ fractionated enzyme; and (c) a dialysed soln of the five peaks pooled from a DEAE-column through which was passed an $(NH_4)_2SO_4$ fractionated enzyme.

Protein estimation. Protein was estimated by the method of Lowry *et al.* [44] or by $A_{280\,nm}$ using bovine serum albumin as standard.

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