J. nat. Rubb. Res., **8(2)**, 114 – 123

Purification and Properties of Glutathione S-transferase from Latex of Hevea brasiliensis

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Glutathione S-transferase was purified more than 500-fold with a recovery of 50% from the latex of Hevea brasiliensis. *The purification steps involved affinity chromatography, ion-exchange chromatography and gel filtration. The molecular weight of the native enzyme was 50 000 Daltons as determined by gel filtration while sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed the presence of one type of sub-unit with a molecular weight of 23 000 Daltons. Substrate specificity studies revealed the enzyme to have limited specificity. The N-terminal amino acid was determined to be glycine while the isoelectric point of this enzyme was estimated to be 4.3. The enzyme exhibited multiplicity on ion-exchange chromatography which could be abolished if the column was eluted with thiol reagents.*

Gluthathione (GSH) S-transferases are enzymes that facilitate nucleophilic attack of the sulphydryl group of glutathione on the electrophilic centres of a broad spectrum of compounds[1]. This conjugation has been shown to result in the synthesis of mercapturic acid derivatives in vertebrates and to some extent in invertebrates[2]. Although glutathione conjugation has been demonstrated in a wide variety of plants[3–9], the terminal conjugated products do not appear to be mercapturic acids. Shimabukuro[9] suggested that this may be due to the fact that plants lack an excretory system. GSH S-transferases have been studied in plants because of their role in herbicide tolerance mechanisms, and their general role in xenobiotic metabolism[1,4,10,11]. Recent studies in plants have shown GSH S-transferase activities to increase in response to herbicide and herbicide safener treatments[12–15]. To date GSH S-transferases have been purified to homogenity in seedlings of corn[12,13] and chickpea[10]. Since we had demonstrated the presence of high levels of GSH S-transferase in latex of *Hevea brasiliensis*[16], we were interested in determining if it had properties similar to other plant transferases and its possible role in the rubber tree.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB), ethacrynic acid, 2,3-epoxy-p-nitropenoxypropane, p-nitrobenzylchloride, trans-4-phenyl-3-buten-2-one, bromosulphonphthalein (BSP), standard proteins for gel filtration and electrophoresis were obtained from Sigma Chemical Company, St. Louis, Missouri.

Latex

Latex tapped from twenty mature trees was collected within 2 h, pooled and cooled in the laboratory to 4°C. Cysteine hydrochloride was immediately added to obtain a final concentration of 1 mM.

Isolation of the Crude Enzyme from Latex

The method for the preparation of the crude enzyme was similar to the method described previously[16] except that instead of lyophilising the clear latex supernatant it was allowed to stand for 48 h after which it was clarified by centrifugation at 20 000 × g for 30 min. The supernatant was used for all the studies.

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Enzyme Assays

GSH S transferase activity was assayed as described previously[16] All reaction rates were measured within the first minute after the addition of CDNB Stock solutions of GSH were prepared in distilled water and kept at 4°C throughout the experiment

Affinity Chromatography

The BSP GSH conjugate was prepared by the method described in the literature[17] The conjugate purified by paper chromatography, was coupled to cyanogen bromide activated Sepharose 4B as described by Brocklehurst *et al* [18] Enzyme samples were loaded onto the column at a flow rate of 10 ml/h The column was eluted with 0 05 *M* sodium bicarbonate buffer of pH 8 3 until the absorbance of the eluting fractions had fallen to zero The bound enzyme was eluted from the column with a 5 0 mM GSH solution in buffer The column was susbequently washed with 500 ml of 1 *M* sodium chloride solution containing 1 mM EDTA, followed by 500 ml of 0 1 m sodium bicarbonate buffer The column was ready to be re-used

DEAE-Sephadex Anion-exchange Chromatography

Samples of the enzyme partially purified through an affinity column were dialysed against 20 mM Tris-HCl buffer of pH 6 0 containing 1 mM GSH and loaded onto a DEAE column at a flow rate of 12 ml/h The column was subsequently washed with four bed volumes of the above buffer to ensure that the absorbance at 280 nm of the eluted fractions had dropped to zero The bound enzyme was then eluted with a 0–0 5 *M* sodium chloride gradient prepared in the same buffer This enzyme was subsequently used for characterisation studies

Gel Filtration and Molecular Weight Estimation

The entire glutathione S-transferase peak from the DEAE step was pooled, dialysed, concentrated and loaded onto a column of Sephadex G-200 This column was then eluted with 50 mM Tris-HCl buffer of pH 7 0 at a flow rate of 12 ml/h The entire active peak was pooled, dialysed and lyophilised for determining its homogenity and sub-unit composition A Sephadex G-200 column was calibrated with a series of standard proteins and a sample of the purified enzyme eluted through this column The elution volume of the enzyme was fitted onto the calibration curve and its molecular weight read off

Electrophoresis

Analytical polyacrylamide gel electrophoresis was conducted according to Ornstein[19] and Davis[20] while sodium dodecylsulphate disc gel electrophoresis was done by the method of Laemli[21], using the purified preparation of GSH S-transferase In all cases, protein bands were stained with Coomassie Brilliant Blue while enzymic activity was located by slicing the gel and assaying each individual slice for activity using the assay method described above

Determination of the N-terminal Amino Acid

Identification of the N-terminal amino acid of GSH S-transferase was by modification of the method of Gray and Hartley[22] by two-dimensional thin layer chromatography on polyamide sheets using the solvent system of Woods and Wang[23]

Isoelectric Focussing

Isoelectric focussing on the purified sample of the enzyme was carried out on an analytical isoelectric focussing column of 110 ml capacity as described previously[16] in 1% (v/v) ampholine of pH range 4 to 6 Electrofocussing was carried out at a constant voltage of 300V for 48 h at 4°C

Substrate Cross Specificity Studies

A sample of the purified enzyme was assayed for conjugating activity using a variety of substrates These included CDNB, DCNB p-nitrobenzylchloride, ethacrynic acid, 2,3 epoxy p-nitrophenoxypropane and trans-4 phenyl-3 buten-2-one, which were all assayed according to the methods described by Habig *et al* [24]

RESULTS AND DISCUSSION

When a sample of the crude enzyme was eluted through a bromoslphonphthalein-glutathione column, all the GSH s-transferase activity was found to bind to it. A constant concentration of 5.0 mM GSH ensured complete elution of the enzyme from the column. This single step of purification resulted in enzyme preparations with specific activities of 190–230 μmoles/min/mg protein (*Figure 1*).

The enzyme purified by affinity chromatography was not homogenous. At least two major bands and five minor bands of proteins were noticed on analytical polyacrylamide get electrophoresis of the above preparation.

Further purification was effected by ion-exchange chromatography (*Figure 2*). Thus the enzyme purified by a combination of both affinity chromatography and ion-exchange chromatography resulted in a preparation with

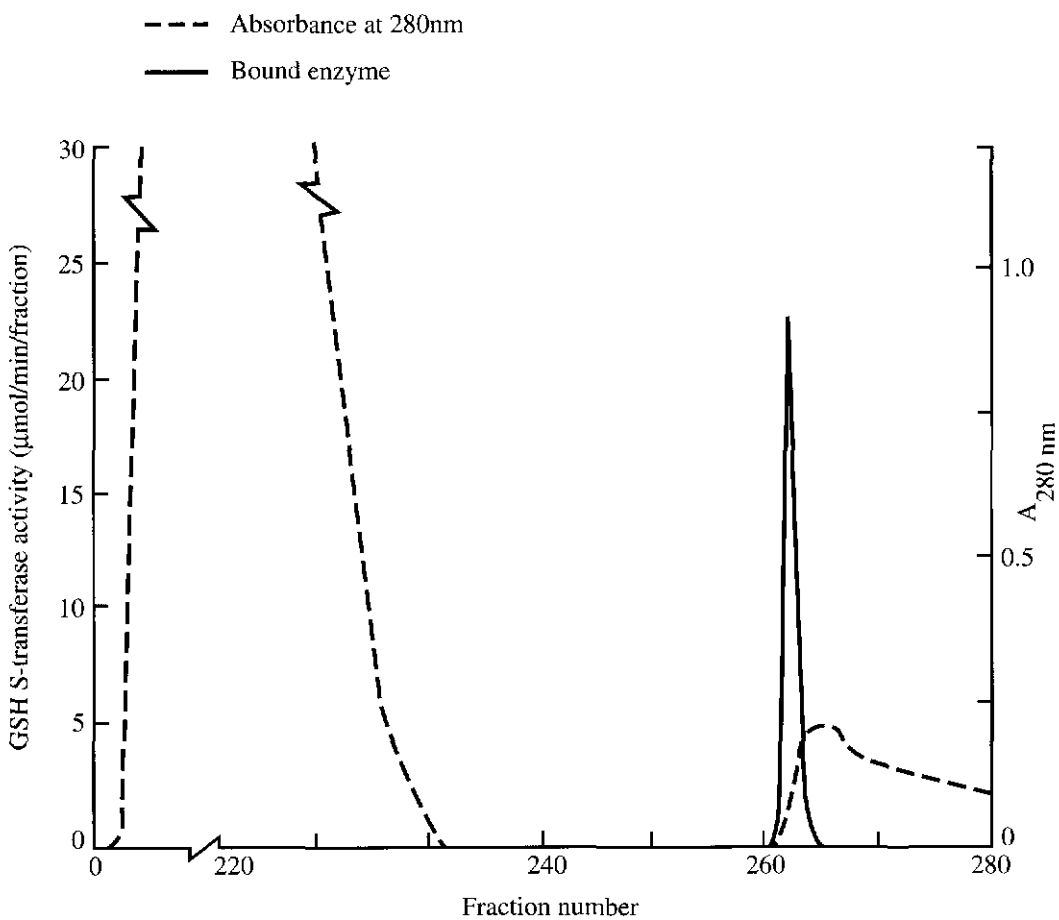

Figure 1. Affinity chromatography of the crude enzyme. A sample of the crude enzyme was charged onto a BSP-GSH linked affinity column (bed volume: 25 ml) that had been equilibrated with 0.1 M sodium bicarbonate buffer, pH 8.3. The column was eluted with buffer until the absorbance at 280 nm fell to zero. Bound enzyme was eluted with 100 ml of 5 mM GSH in buffer.

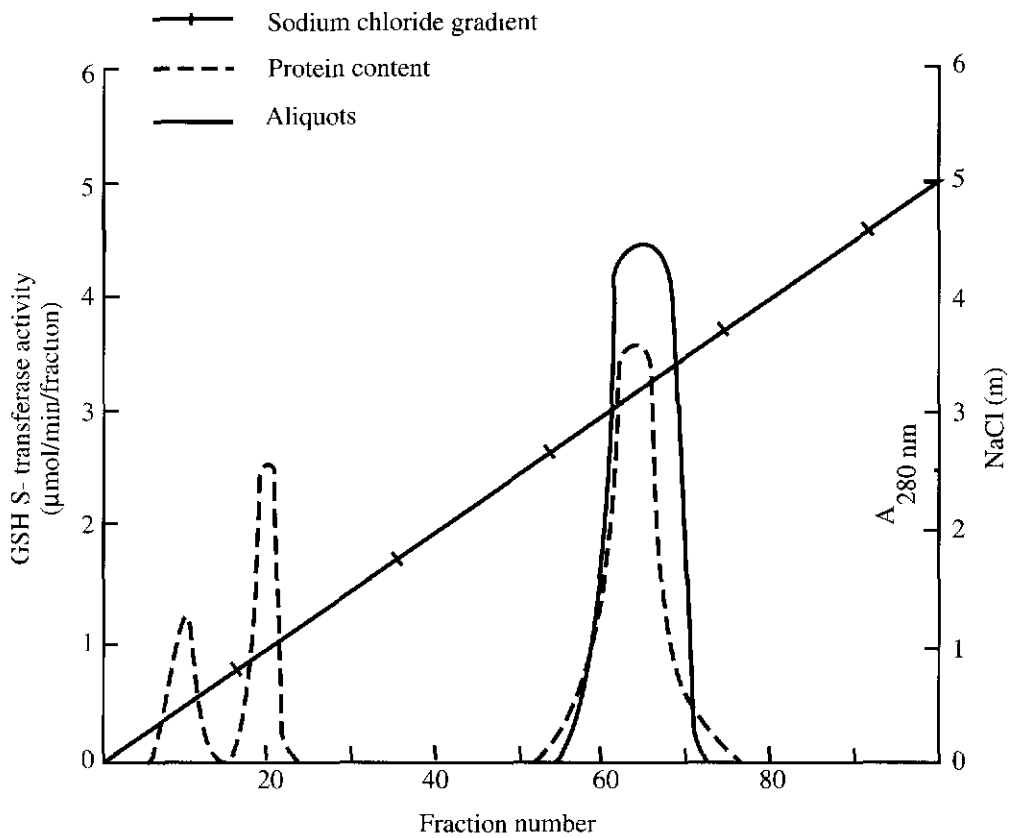

Figure 2. Ion-exchange chromatography of the affinity purified enzyme on DEAE-Sephadex. Pooled active fractions from the affinity chromatography step were equilibrated with 20 mM tris-HCL buffer, pH 6.0 containing 1 mM GSH and charged onto a column of DEAE-Sephadex (bed volume: 60 ml). After the passage of four bed volumes of this buffer, the column was eluted with a 500 ml sodium chloride gradient made in the same buffer. The protein content of each fraction was estimated by measuring the absorbance at 280 nm while 0.1 ml aliquots were removed for the assay of enzyme activity.

specific activities of 400–600 µmoles/min/mg protein. When the entire peak from this column was purified through a Sephadex G-200 column, the specific activity obtained was 600–800 µmoles/min/mg (*Table 1*).

When a desalted preparation of the purified enzyme obtained above was subjected to analytical polyacrylamide gel electrophoresis, a single protein band was observed. When a duplicate gel was sliced and assayed for enzyme, the activity peak coincided with the protein band (*Figure 3*).

When a sample of the partially purified enzyme was eluted through a calibrated Sephadex G-200 column, a molecular weight of 50 000 Daltons + 3000 Daltons was obtained (*Figure 4*). The molecular weight when estimated from ultracentrifugation studies was also in the region of 48 000 Daltons (data not shown). These values are not only in agreement with the values quoted for the same enzyme by Balabaskaran and Muniandy[16] but is also within the range quoted for the enzyme from other plant sources. It is

TABLE 1. PURIFICATION OF LATEX GSH S-TRANSFERASE

Purification step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Purification fold	Yield (%)
Crude enzyme	180	237	0.76	1	100
Affinity chromatography	133	0.65	205	270	74
Ion-exchange chromatography	102	0.24	425	559	56
Gel filtration chromatography	91	0.19	478	628.5	50.5

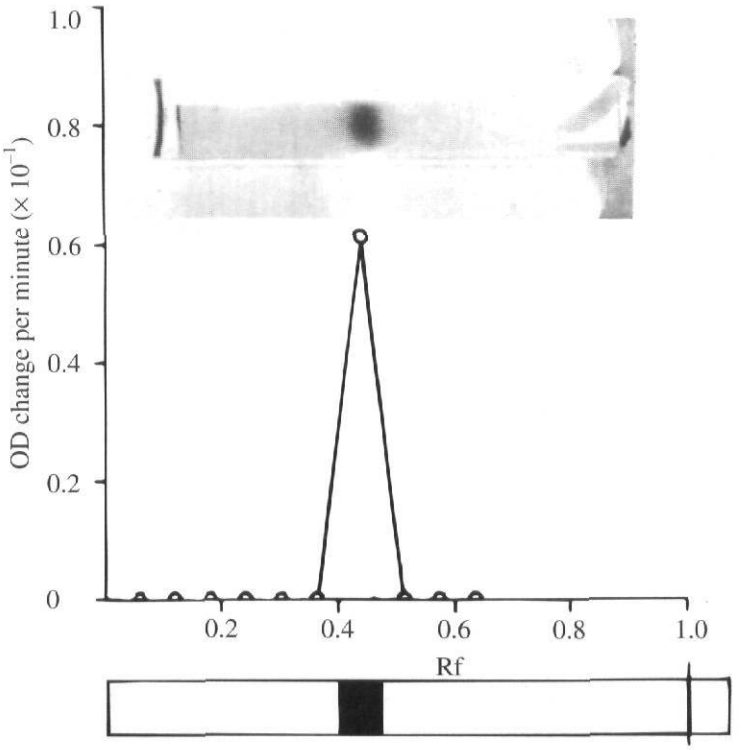

Figure 3. Analytical polyacrylamide gel electrophoresis. 100 μl of the purified enzyme containing 100 μg of protein was electrophoresed on duplicate tube gels. One gel was stained with Coomassie Brilliant Blue while the duplicate gel was sliced into 1 mm slices. Each slice was homogenised in 0.5 ml of assay buffer, centrifuged and the supernatant assayed for enzyme activity as described in the text. The inset shows the position of the protein band coinciding with GSH S-transferase activity assayed using the duplicate gel.

important to mention that no high molecular weight forms of the enzyme were detected in this work, noticed with the GSH S-transferase from pea seedlings[8]. In this species, in addition to the 40 000 Dalton form, other forms with molecular weight of 75 000 Daltons and 120 000 Daltons were also detected. The latter two forms were believed to be the dimers and trimers of the 40 000 Dalton form of the enzyme. This unusual behaviour of molecular aggregation has so far not been observed with GSH S-transferases from other plant sources including that from latex.

On SDS polyacrylamide gel electrophoresis, the purified enzyme migrated as a single band of molecular weight of 23 000 Daltons (*Figure 5*). This appeared to be the sub-unit molecular weight of the enzyme. Since the native enzyme had a molecular weight of 50 000 Daltons, the enzyme appears to be a homodimer. It is interesting to mention that in other plants, GSH S-transferase appears in multiple forms with some isoenzymes appearing as homodimers and heterodimers. For instance in chickpea, five isoenzymes can be separated on DEAE-Sephacel and if each of the purified forms is subjected to SDS-PAGE, the presence of at least two types of sub-units can be detected for GST III, GST IV and GST V while GST I and GST II appear to be homodimers[10]. In corn, GST I and GST II appear to be homodimers of 29 kDaltons and 26

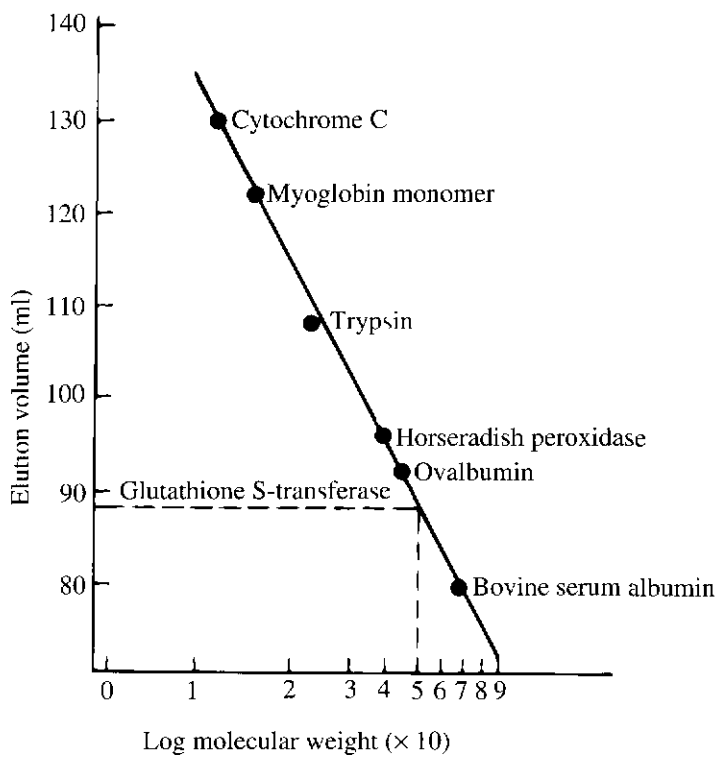

Figure 4. Estimation of the molecular weight of GSH S-transferase by chromatography on Sephadex G-200. 5 mg of each standard protein was separately charged onto the column and the elution volume of each standard protein was determined by monitoring the absorbance at 280 nm. The elution volume of the enzyme was fitted onto the calibration curve.

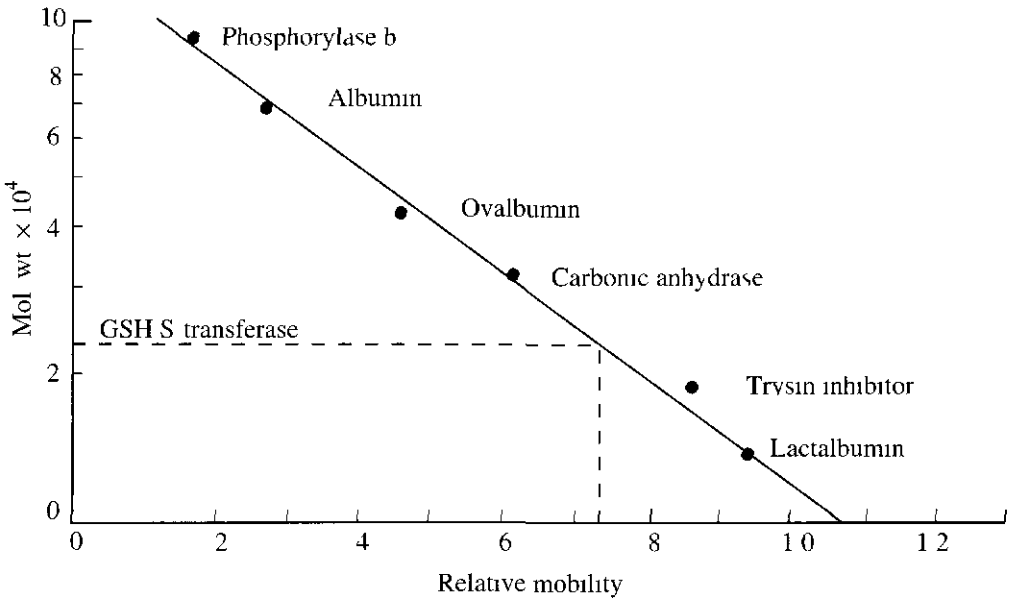

Figure 5 Estimation of the sub unit molecular weight of GSH S of GSH S transferase by S D S polyacrylamide gel electrophoresis The relative mobility of each of the protein standards was plot ted against the log of its molecular weight The relative mobility of the protein band obtained when the purified preparation of latex GSH S-transferase was electrophoresed was determined and the sub unit molecular weight read off from the calibration plot

kDaltons respectively while GST III appears to be a heterodimer of 29 kDaltons and 27 kDaltons[13]

The N-terminal amino acid of the latex GSH S-transferase was determined to be glycine The detection of the single amino acid was not only confirms the purity of the enzyme preparation but also indicated that both sub units of the enzyme had identical N-terminal residues

When a sample of the enzyme was electro-focussed, a single symmetrical peak with an isoelectric point of 4 3 was obtained as was reported previously[16] A similar value was noted by Edwards and Owens[15] for the corn enzyme

Although the isoelectric points of many plant transferases are not known, most of them do bind to DEAE anion exchange columns[7-25] This would suggest that they too probably have acidic pI values

In spite of the fact that GSH S-transferase from *H brasiliensis* had been focussed as a single form in electrofocussing experiments, it exhibited multiplicity on ion-exchange chro matography on DEAE-Sephadex When the DEAE column was eluted with a 0–0 5 *M* sodium chloride gradient, five peaks of activi ty were detected with *Peak 1* being the least negatively charged and *Peak 5* being the most negatively charged If a similar column was loaded with the same amount of enzyme and eluted with a similar sodium chloride gradient containing 1 0 mM of either glutathione, mercaptoethanol thioglycollic acid or dithio-threitol only a single peak of activity was detected (*Figure 6*) Since the total amount of activity recovered in both experiments was similar it must mean that the less negatively

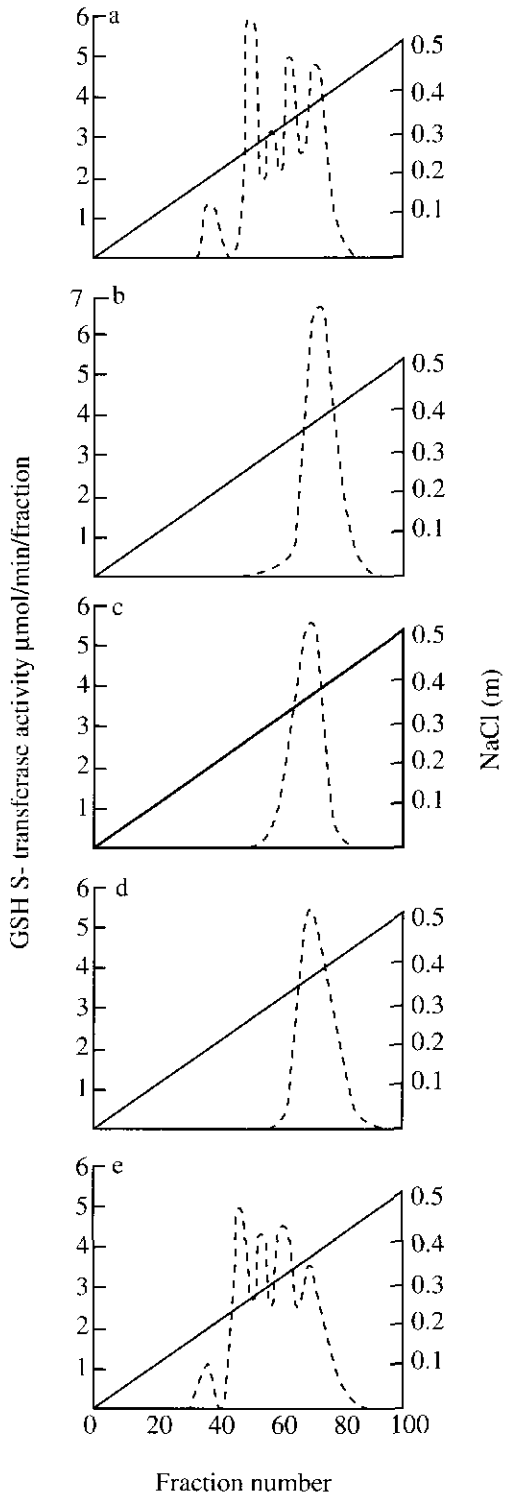

Figure 6. Ion-exchange chromatography of GSH S-transferase on DEAE-Sephadex. a) A sample of the enzyme, prepared as described in the text was equilibrated with 20 mM tris-HC1 buffer, pH 6.0 and eluted through the column. The column was washed with the same buffer until the absorbance at 280 nm returned to zero. Bound enzyme was eluted with 500 ml of a sodium chloride gradient made in the same buffer. b), c), d) and e) refer to the elution profiles obtained when both the sample equilibration and elution buffers as well as the sodium chloride gradient contained 1 mM of reduced glutathione, mercaptoethanol, thioglycollic acid or oxidised glutathione respectively.

charged forms (*Peaks 1–4*) noticed with sodium chloride alone must have been transformed to the highly negatively charged form (*Peak 5*) by the thiol reagents. These thiol reagents are known to cleave labile disulphide bonds in proteins and hence the transformation of the less negatively charged forms to the highly negatively charged form (*Peak 5*) is probably due to the cleavage of disulphide bonds in them. However, when a homogenous preparation of the enzyme, purified as described above, was titrated for free thiol groups with N-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoate) and p-chloromercuribenzoate essentially according to the methods described by Glazier[26], no free thiol groups could be detected, suggesting that if cysteine residues are present, they are probably engaged in disulphide bonds or are totally incaccessible to these reagents. The fact that oxidised GSSG could not abolish the multiple forms does suggest that a free thiol group in GSH is necessary for catalysing the transformation of the multiple forms of GSH S-transferase to a highly negatively charged form (*Peak 5*). Although the thiol reagents did help to abolish multiplicity it is possible that *Peak 5* obtained in the presence of 1.0 mM of the thiol reagents is not absolutely homogenous and could contain small amounts of the less negatively charged forms of the enzyme contaminating the ascending parts of *Peak 5*.

Substrate specificity studies done on the purified enzyme revealed the limited specificity of the latex GSH S-transferase. The enzyme only showed activity against CDNB and DCNB, while it was inactive against p-nitrobenzylchloride, ethacrynic acid, 2,3-epoxy-p-nitrophenoxypropane and trans-4-phenyl-3-buten-2-one. Similar observations had been reported for the crude preparation earlier[16]. The ratio of activites against CDNB and DCNB was 12 to 1 in both the crude and the purified enzyme. In this regard, latex GSH S-transferase resembles other plant tranferases in lacking the broad substrate specificity enjoyed by most vertebrate enzymes with respect to the above preparation.

Date of receipt: March 1993
Date of acceptance: May 1993

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