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The Use of Bromosulphonphthalein Glutathione as an Affinity Ligand for the Partial Purification of GSH S-transferase from the Latex of Hevea brasiliensis

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Glutathione S-transferase from the latex of Hevea brasiliensis *can be purified in large quantities using bromosulphonphthalein-glutathione as an affinity ligand. The fold of purification is affected by pH, flow rate and the volume of enzyme eluted through the column. High folds of purification can be obtained at a flow rate of approximately 14 ml per hour at pH 9.0 using large volumes of crude enzyme. The maximum specific activity so far obtained is in the region of 214 μmoles per minute per milligramme protein resulting in a 832-fold purification. This method can now be adapted commercially for the purification of latex glutathione S-transferase in large quantities.*

The conjugation of glutathione (GSH) with foreign compounds is one of the detoxification processes that has been well-studied[1]. The enzymic nature of this conjugation was first demonstrated *in vitro* in the supernatants of rat liver homogenates by Booth, Boyland and Sims[2]. The enzyme responsible was later named GSH S-transferase and subsequently designated EC2.5.1.18[3]. Hitherto its presence in a variety of vertebrates, invertebrates and a few plants has been well-reviewed[4].

Most of the studies on GSH S-transferases so far conducted were centred on purified preparations isolated from the cytosol of rat and human liver[3]. The first attempt to partially purify GSH S-transferase in a plant was done by Frear and Swanson[5] from corn leaves. They used differential centrifugation, $(NH_4)_2SO_4$ fractionation and gel filtration to partially purify the enzyme. Since then the enzyme has been purified from corn seedlings[6,7].

Recently significant amounts of GSH S-transferase activity was demonstrated in the latex of the rubber tree *Hevea brasiliensis*[8]. However purification of this enzyme was hampered by the appearance of multiple forms on DEAE-ion-exchange chromatography. This paper describes for the first time the use of affinity chromatography as an initial step in the purification of a plant enzyme from latex. This method can now be adapted commercially for the large-scale purification of GSH S-transferase from the latex of *Hevea brasiliensis*.

MATERIALS AND METHODS

Enzyme Assay

GSH S-transferase activity was assayed spectrophotometrically as described previously[8]. Enzyme assays were routinely carried out at 28°C in 0.05 *M* arginine-HCl buffer, pH 8.3, containing 2.0 mM GSH and 0.2 mM 2,4-dinitrochlorobenzene (DNCB). The rate of change in A_{344}nm of the conjugate of GSH and DNCB was followed in a Beckman Acta III recording spectrophotometer. In all cases non-enzymic blank reaction between the substrates was deducted from test values.

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Protein Estimation

Protein was estimated by the method of Bradford[9] or by A_{280}nm absorbance using bovine serum albumin as standard.

Affinity Chromatography

A sample of bromosulphonphthalein-glutathione (BSP-GSH) conjugate was prepared as described by Whelan *et al.*[10] The BSP-GSH conjugate was coupled to cyanogen-bromide (CNBr) activated Sepharose 4B (particle size 60-140μ from Pharmacia fine chemicals) by the method described by Brocklehurst *et al.*[11]

Crude enzyme solution (100 ml) was loaded onto a column (2.5 × 12.0 cm) of the gel (bed volume: 50 ml) at a flow rate of 14 ml per hour. The column was then washed with running buffer until the absorbance at 280 nm had fallen to zero.

The bound GSH S-transferase was then eluted with 200 ml of 5 mM glutathione in the running buffer adjusted to the required pH with 0.5 *M* NaOH. Fractions of 3.0 ml were collected and aliquots of 0.1 ml or 0.05 ml were used to assay for enzyme activity.

After each chromatographic run, the column was regenerated by washing with twenty bed volumes of 1*M* NaCl containing 0.1% EDTA. Finally the column was equilibrated with the running buffer.

Preparation of Crude Enzyme

Latex tapped from a number of trees was pooled and immediately treated with 1 mM KCN. All operations in the isolation of crude enzyme from latex were carried out at 5°C. The pH of the latex was adjusted to 5.0 with glacial acetic acid and the mixture left to stand for 15 min, after which it was centrifuged at 20 000 g for 1 hour. The coagulated rubber, which formed a layer at the top, was removed and the pale yellowish supernatant dialysed overnight against several changes of distilled water. Heavy precipitation occurred during dialysis. The dialysate was further centrifuged at 20 000 g for 30 min and the supernatant obtained used for chromatography. When required the supernatant was lyophilised and the lyophilised powder used as a source of GSH S-transferase.

RESULTS AND DISCUSSION

In a previous publication[8] it was reported that the GSH S-transferase from latex was inhibited by a variety of triphenyl methane compounds. Since these inhibitors have been regarded as isosters of GSH[12,13] and were found to be competitive inhibitors of the GSH binding site, an attempt was made to determine the inhibitor constant for BSP which was one of the most effective inhibitors among the phthaleins, for the latex enzyme[8]. The Ki experimentally obtained for BSP was in the region of 0.068 μM *(Figure 1)* suggesting that it might serve as a suitable ligand for affinity purification of this enzyme.

BSP was therefore linked onto cyanogen bromide-activated Sepharose 4B *via* glutathione to yield an affinity support capable of binding GSH S-transferases. When a sample of the crude enzyme was eluted through such a column the enzyme was found to bind to it tightly and could not be eluted with several bed volumes of buffer. When the column was subsequently eluted with a GSH gradient ranging from zero to 10 mM, the bound activity eluted at a GSH concentration of 2.5 mM *(Figure 2)*. Hence for all subsequent studies, bound enzyme was eluted from the column with a 5mM GSH solution in the appropriate buffer.

When volumes of crude enzyme solution between 50 ml to 850 ml were loaded onto the column and the bound enzyme eluted with 5 mM GSH *(Table 1)*, it was found that higher folds of purification were achieved when larger volumes of crude enzyme solution were loaded. The highest purification obtained was 832-fold when 850 ml of crude enzyme solution was loaded.

Varying the flow rate of the enzyme through the column also affected the specific activity of the enzyme obtained *(Table 2)*. When equal volumes of the enzyme solution (≃ 100 ml)

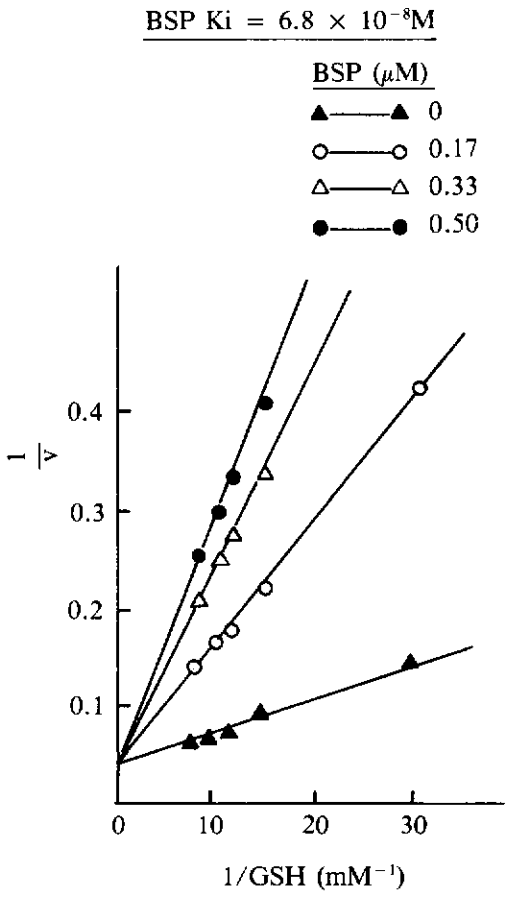


Figure 1. Inhibition of glutathione S-transferase by bromosulphonphthalein.

Double reciprocal plots show the mode of inhibition of the enzyme by BSP. Ki was determined by replotting the above values in a Dixon $[\frac{1}{v}$ *against (i)] plot*[14]*. Inhibition studies were carried out in 0.05* M *arginine-HCl buffer, pH 8.3 at 28°C in a total reaction volume of 3 ml containing 0.2 mM DNCB and varying GSH concentrations ranging from 33 μM to 125 μM.*

were loaded at different flow rates ranging from 14 ml to 42 ml per hour, it was found that higher purification folds were obtained at lower flow rates. At a flow rate of 14 ml per hour a purification of sixty-fold was obtained while at a flow rate of 42 ml per hour the purification was only four-fold. Although flow rates below 14 ml per hour could be used it was felt that such low flow rates would lengthen the duration of the experiment and thus effect the stability of the enzyme. Hence for subsequent experiments a flow rate of 14 ml per hour was maintained.

Varying the pH of elution of the enzyme from the column also effected the specific activity of the enzyme obtained *(Table 3)*. At pH 7.0, the purification was eighty-four-fold with a maximum specific activity of 14.7 μmoles per minute per milligramme protein. At pH 8.0, the purification increased to ninety-seven-fold with a corresponding increase in specific activity to 17.3 μmole per minute per milligramme. Purification at pH 9.0 gave the highest fold of purification: 222 with a specific activity of 39 μmoles per minute per milligramme. At pH 10.0, there was totally no binding of the enzyme to the column. Hence a comparatively low fold of purification of fifty-six was obtained. Although the above observations point to pH 9.0 as the ideal operational pH two other factors had to be considered *(Figure 3)*.

The first concerns the amount of activity binding to the column at each pH. As shown in *Table 3,* the amount of activity bound to the column decreased with increasing pH. At pH 7.0, 8.0 and 9.0 approximately 93.2%, 76.4% and 55.8% of activity respectively bound to the column. At pH 10.0, however, there was no binding to the column at all.

The second factor that was considered is the stability of the enzyme at these pH values. The stability of the enzyme is reflected by the total activity recovered at each pH *(Table 3)*. At pH 7.0 almost all (98.5%) of the activity loaded could be recovered. At pH 8.0, 9.0 and 10.0, however, increasing quantities (22.2%, 34.3% and 48.5%) of the loaded activity were lost, in spite of the fact that protein recovery increased from 72% at pH 7.0 to 88% at pH 10. It is pertinent to mention that affinity gels prepared as described in the text do have variable binding capacities for the enzyme. In our experience the variations did not exceed by more than 40% from preparation to preparation.

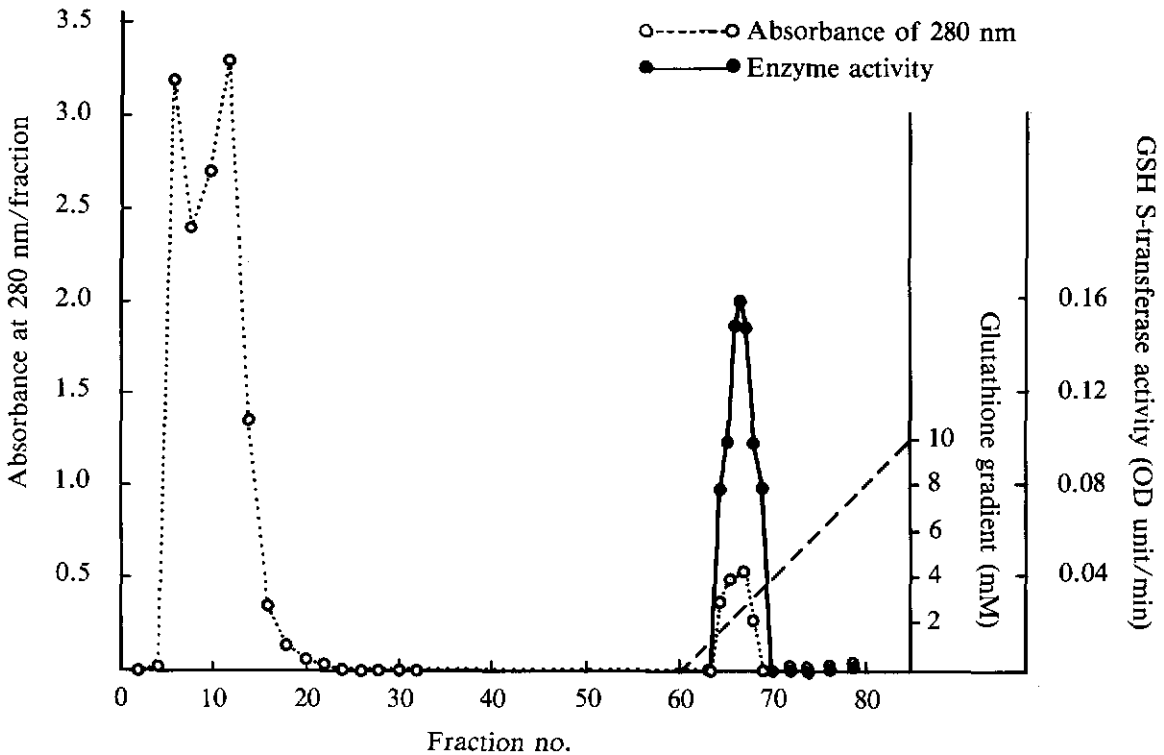


Figure 2. Elution of glutathione S-transferase from a BSP-GSH affinity column using a glutathione gradient.

A sample of the crude enzyme solution was loaded onto the BSP-GSH affinity column. The column was washed with 10 mM tris-*HCl buffer, pH 8.3 till the absorbance at 280 nm had reached a zero value. The column was then eluted with a GSH gradient ranging from zero to 10 mM.*

TABLE 1. AFFINITY CHROMATOGRAPHY OF DIFFERENT VOLUMES OF 'CRUDE ENZYME SOLUTION'

Volume of enzyme solution loaded (ml)	Total activity loaded (μmoles/min)	Total protein loaded (mg)	Specific activity of enzyme loaded (μmoles/min/mg)	Specific activity of peak (μmoles/min/mg)	Fold of purification
50	48.6	300	0.162	5.2	32
100	97.1	600	0.162	12.2	75
200	194.3	1 200	0.162	20.75	128
300	273.6	1 800	0.152	33.06	217
850	801.0	3 114	0.257	213.9	832

The 'crude enzyme solution' was prepared as described in the section under 'Methods'. Different volumes of the enzyme solution were loaded onto the affinity chromatography column (2.5 × 12.0 cm). After loading was completed the column was washed with 20 mM *tris*-HCl buffer of pH 8.3 until the absorbance at 280 nm of the fractions returned to zero. The column was then eluted with 5 mM GSH which was prepared in the above buffer and the pH adjusted back to 8.3.

After each run, the column was washed with 2 *M* NaCl prepared in the above buffer before the next enzyme solution was loaded.

The flow rate of the column was maintained at 14 ml per hour for all the enzyme samples loaded.

Folds of purification are expressed in terms of the specific activity of the 'crude enzyme solution'.

TABLE 2. AFFINITY CHROMATOGRAPHY OF 'CRUDE ENZYME SOLUTION' AT DIFFERENT FLOW RATES

Flow rate (ml/h)	Activity loaded (μmoles/min)	Activity recovered (μmoles/min)	Activity unbound as % of activity recovered	Activity bound as % of activity recovered	Specific activity of bound enzyme eluted with GSH
14	91.42	61.26	0	100	10.52
21	95.43	68.60	32.7	67.3	8.89
31.5	88.73	71.87	37.5	62.5	4.88
42	92.46	78.6	40.6	59.4	0.67

The 'crude enzyme solution' was prepared as described in the section under 'Methods'. Exactly 100 ml was loaded in each run, and the flow rate was varied. Fractions of 3.0 ml were collected, and 0.1 ml aliquots were removed for enzyme activity measurements, using 1 mM GSH and 0.2 mM DNCB in 50 mM arginine-HCl buffer at pH 8.3. The 'crude enzyme solution' that was loaded had a specific activity of 0.18 μmoles per minute per milligramme.

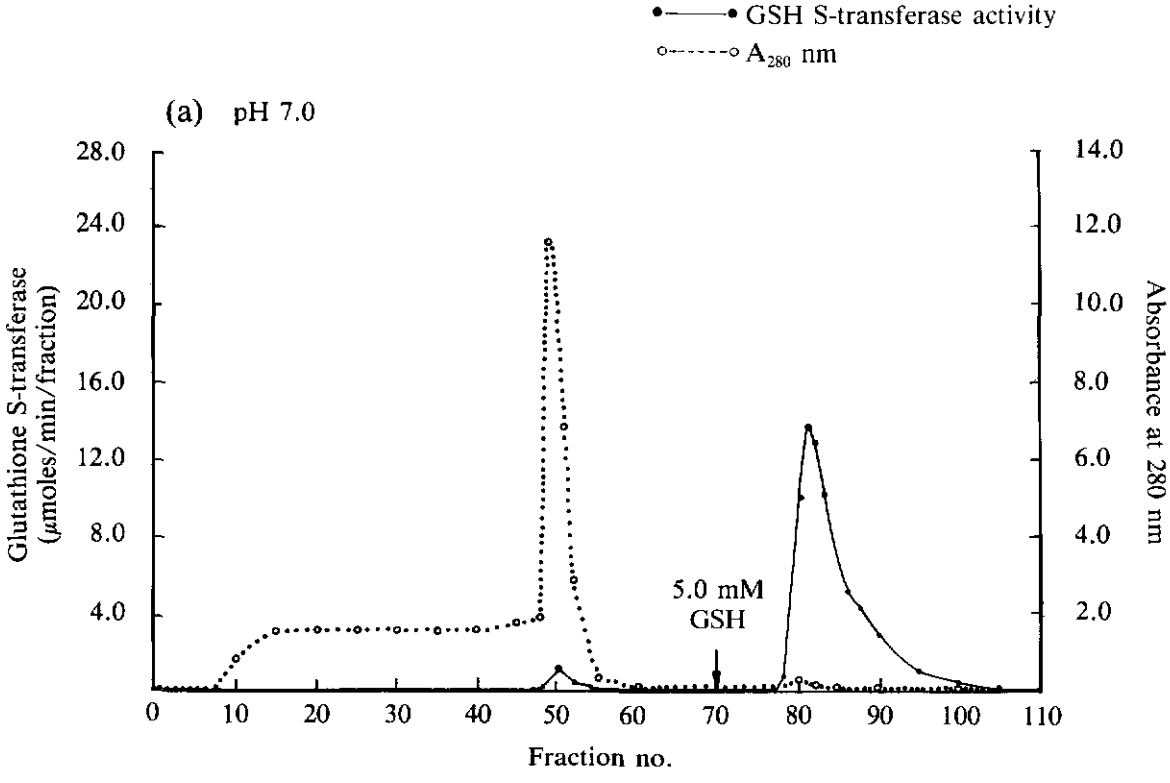
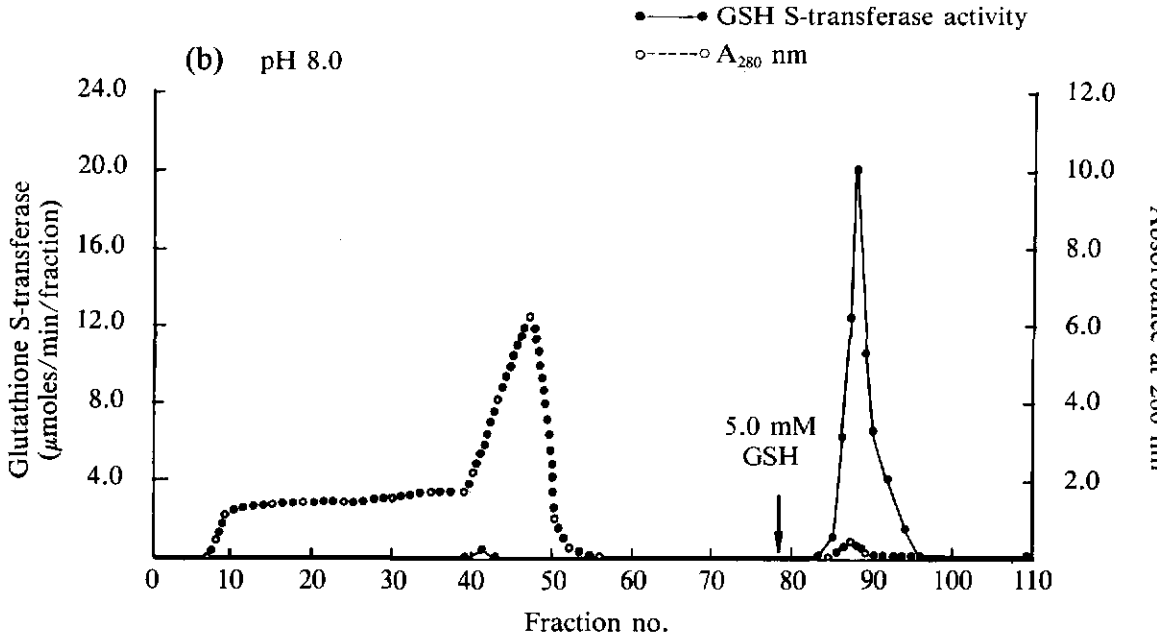


Figure 3. Elution profiles of glutathione S-transferase from a BSP-GSH affinity column at various pH values.



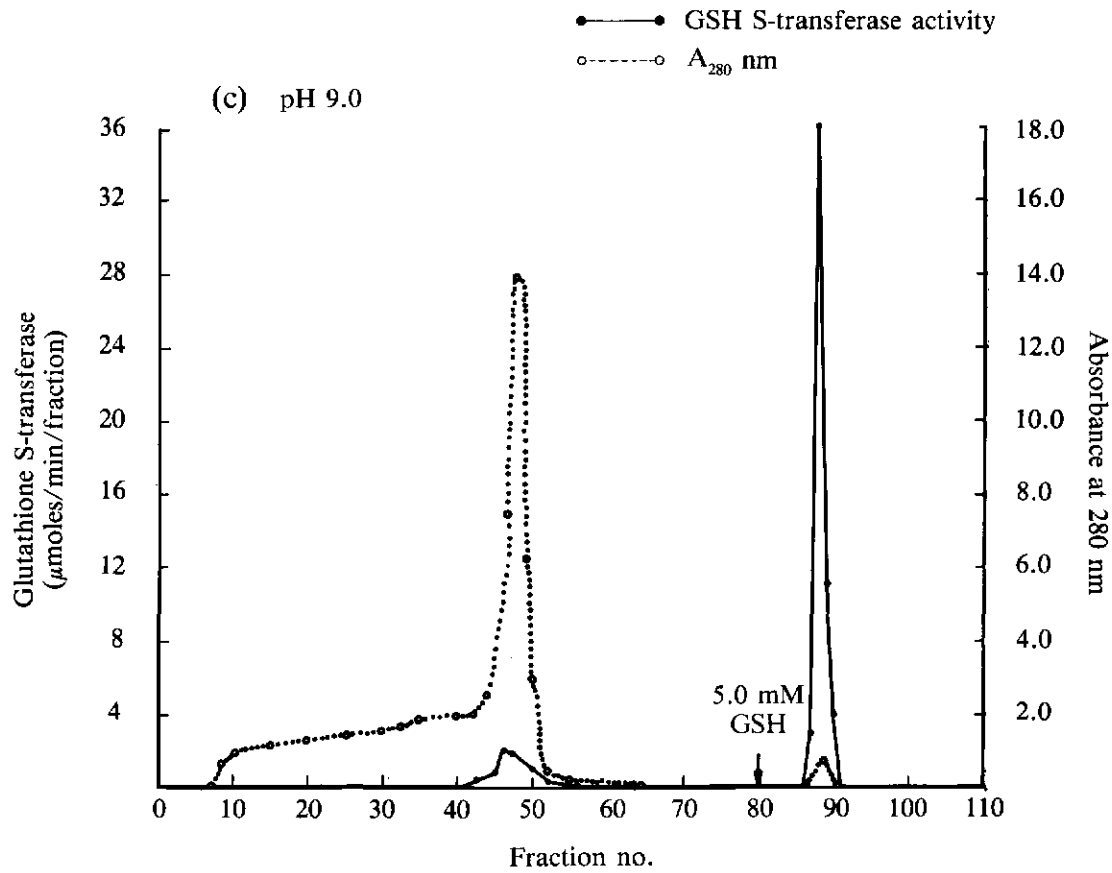


Figure 3. Elution profiles of glutathione S-transferase from a BSP-GSH affinity column at various pH values (contd).

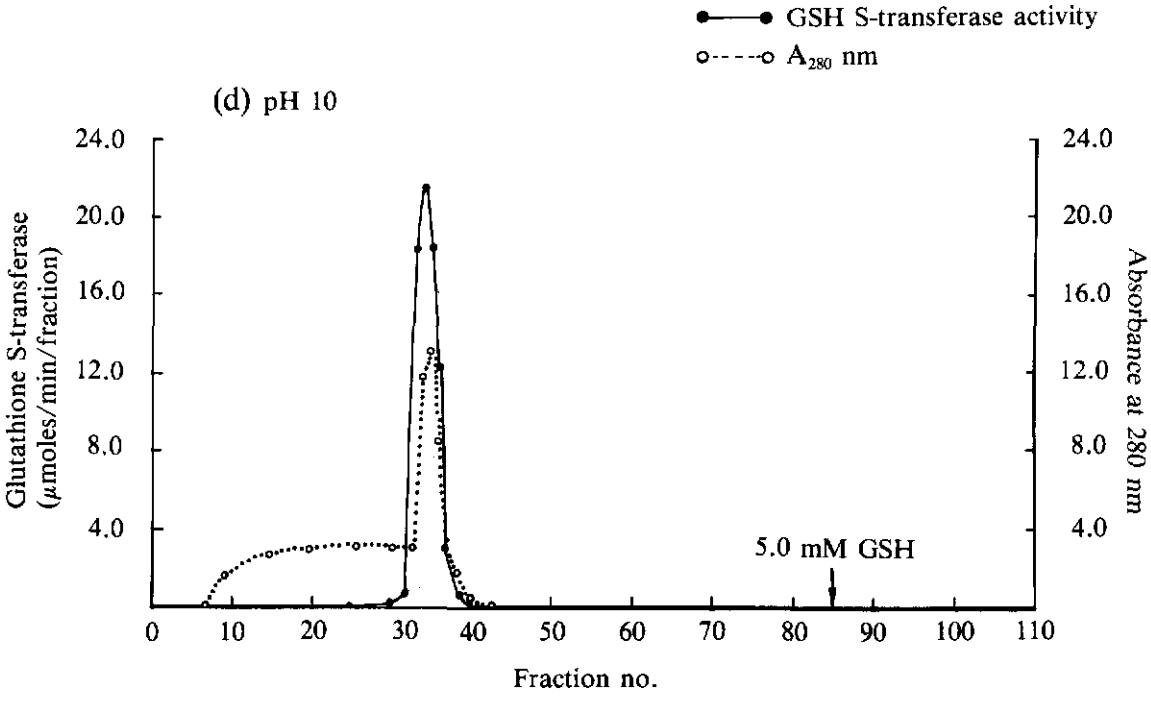


Figure 3. Elution profiles of glutathione S-transferase from a BSP-GSH affinity column at various pH values (contd).

100 ml of the crude enzyme solution, prepared as described in the text, was loaded onto the affinity column which had been previously equilibrated with the appropriate buffer (pyrophosphate buffer was used at pH 7.0 while in all other studies bicarbonate buffer was utilised. Buffer concentration in all cases was 50 mM at a constant flow rate of 14 ml per hour. As soon as the sample had percolated below the gel surface, the column was eluted with buffer. Elution was continued until the A_{280} nm of the fractions had dropped to zero. The column was then eluted with 200 ml of 5 mM solution of GSH in the same buffer. Fractions of 3 ml were collected and assayed for GSH S-transferase activity as described in the text. The A_{280} nm of each fraction was also measured.

TABLE 3. AFFINITY CHROMATOGRAPHY AT VARIOUS pH VALUES

Item	pH 7.0	pH 8.0	pH 9.0	pH 10.0
Total enzyme activity load (μmoles/min)	97.5	100	98.2	112.7
Total enzyme activity recovered (bound and unbound) (μmoles/min)	96.0	77.8	64.5	58.0
Total protein loaded (mg)	560	560	560	560
Total protein recovered (bound and unbound) (mg)	402	458	466	493
Specific activity of crude enzyme loaded on column (μmoles/min)	0.17	0.18	0.18	0.20
Specific activity of enzyme eluted from column with GSH (μmoles/min)	14.7	17.3	39	11.2
Purification achieved by enzyme eluted with GSH (fold)	86	96	216	56
Enzyme activity bound to gel column (%)	93.2	76.4	55.8	0

The values shown are in reference to *Figure 3.*

Thus from the above results, a set of optimised conditions for affinity chromatography can be inferred. The volume of crude enzyme loaded should be maximised while flow rates close to 14 ml per hour should be maintained for as long as the operational pH of the column is maintained at pH 9.0. These conditions are ideally suited for a large-scale partial purification of this enzyme that would otherwise be difficult using conventional methods. However, it must be maintained that should any one of the above parameters be altered, due consideration must also be given to possible changes in other parameters discussed above.

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