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# Selective elution of soluble rat liver glutathione transferases from a glutathione-Sepharose affinity column

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## ABSTRACT

Glutathione transferases (GST) are dimeric enzymes that take part in many detoxification processes. A previous report described the use of a glutathione-Sepharose affinity matrix for the purification of human liver GST. The method involved the use of 5 m*M* glutathione in a high pH buffer, and the yields were nearly 100%. This method and adapted techniques have now been applied to rat liver GST. Selective GST elution can be obtained in several different ways: by stepwise change of the pH and/or glutathione concentration, and by linear gradient elution. Gel electrophoresis showed, however, that none of the fractions contained pure GST isoenzymes. Also, less than 50% of the total rat liver GST was eluted with 5 m*M* glutathione, in contrast to the results with human liver GST. A glutathione concentration of 30 m*M* is necessary for quantitative desorption of rat liver GST from a glutathione-Sepharose column.

## INTRODUCTION

Glutathione transferases (GST; E.C. 2.5.1.18) are widespread enzymes. They catalyse the conjugation of glutathione (GSH) with electrophilic compounds, including carcinogens, mutagens, toxic or pharmacologically active compounds, and their metabolites [1]. GST is also capable of directly binding to toxic compounds, another important detoxication pathway [2]. Moreover, GST has peroxidase activity for organic hydroperoxides and, therefore, the potential to detoxify lipid and DNA hydroperoxides arising from damage by free radicals in the presence of oxygen [3]. Multiple isoenzymes have been described, and recently reviewed [4]. GST proteins have a homo- or heterodimeric structure. GST 1 ($M_r$ 25 000), GST 2 ($M_r$ 28 000) and GST 3 and 4 ($M_r$ 26 500) are the most abundant subunits in rat liver, which is the best-investigated GST source [4]. GST 3 and 4 are indicated together as GST 3/4 in this paper, in the sense of GST 3 and/or GST 4.

GST has been purified from many sources. The purification procedure often includes an affinity step on GSH-Sepharose as described by Simons and Vander Jagt [5]. In this method human liver GST is first adsorbed on a GSH-Sepharose matrix. The column is washed with a neutral buffer, then GST is desorbed highly purified by 5 m*M* GSH in a high pH buffer with a nearly 100% yield. This

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technique was also used successfully for the purification of mammalian GST from many species, *e.g.* dog [6], rabbit [7] and cow [8], and also for the purification of GST from other animals, *e.g.* flies [9], freshwater worms [10], liver flukes [11], and even bacteria [12]. However, in these cases GST was not quantitatively desorbed. It was observed that only a part of the total rat liver GST was desorbed with 5 m*M* GSH, therefore the selective GST elution was investigated in detail. It was found that the GSH concentration allows a selective elution and that a much higher GSH concentration is necessary for quantitative desorption.

## EXPERIMENTAL

### *Materials*

Epoxy-activated Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden), GSH from Janssen Chimica (Beerse, Belgium), and chemicals for sodium dodecylsulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) from Bio-Rad (Richmond, CA, U.S.A.). Other chemicals, including 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Merck (Darmstadt, F.R.G.). They were of the purest form available.

### *Buffers*

Buffer A was a 22 m*M* solution of sodium phosphate buffer (pH 7.0) containing 1 m*M* EDTA.$NA_4$. Buffer B was a solution of 50 m*M* Tris–HCl (pH 9.6).

### *Methods*

Livers of male Wistar rats were homogenized in three times their volume of buffer A containing 0.25 *M* sucrose, with ten up-and-down strokes in a motor-driven Potter–Elvehjem homogenizer (1500 rpm), equipped with a PTFE pestle. The homogenate was ultracentrifuged (100 000 *g* for 1 h). The clear supernatant was stored in 5-ml fractions at −25°C. In these conditions no loss of activity was detectable after 3 months. A 5-ml aliquot of homogenate was used for most separations, although 10 ml were used in linear gradient elutions. All enzyme manipulations were performed at 0–4°C.

The crude cytosol was applied to a GSH-affinity column (8.5 × 1.6 cm I.D.), packed with epoxy-activated Sepharose 6B that had been treated with GSH as described previously [5]. According to this procedure the remaining active groups were blocked with 1 *M* ethanolamine. The column was washed with buffer A and further treated under different conditions as described under Results, using a flow-rate of 0.4 ml/min. High flow-rates gave no reproducible separation patterns. All collected fractions were of 4.5 ml.

The GST samples were diluted with equal volumes of sample buffer (4% SDS, 0.1 *M* dithiothreitol, 80 m*M* Tris–HCl, pH 7.8, 20% glycerol and 0.01% bromophenol blue) for the analysis of the GST peaks by SDS-PAGE. Electrophoresis was carried out at 105 V, using 12% polyacrylamide slab gels modified from the

method of Laemmli [13]. The reference isoenzyme GST 1-2 was purified according to ref. 14. The gels were stained with Coomassie brilliant blue.

The GST activity was measured spectrophotometrically [15] using CDNB as substrate. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 $\mu$mol of product per minute under the assay conditions.

GSH concentrations were measured with 5,5′-dithiobis-(2-nitrobenzoic acid) according to Ellman [16] after precipitating the proteins by addition of an equal volume of 4% sulphosalicylic acid.

## RESULTS

The flow-through fractions contained 10–15% of the total GST activity in all experiments. In the original method the GSH-Sepharose column was washed with buffer A, after which GST was desorbed with 5 m*M* GSH is buffer B [5]. In order to estimate the importance of the pH-change, the elution patterns of GST with 5 m*M* GSH in buffer A and in buffer B were first compared (Fig. 1). Although the majority of GST was eluted with buffer B, an important fraction (14.3%) was also desorbed with buffer A. This fraction mainly consisted of GST 1 subunits (Fig. 2, lane 1), the four subunits being eluted with buffer B (Fig. 2, lane 2).

The influence of the GSH concentration in buffer B was also examined (Fig. 3). Only a small low-affinity fraction (3.7%) was obtained with pure buffer B, indicating the importance of the pH increase. The majority of GST was eluted with 5 m*M* GSH in buffer B. This eluate was composed of GST 1, GST 2 and GST 3/4 subunits (Fig. 4, lane 1). However, an important additional fraction (23–26%)

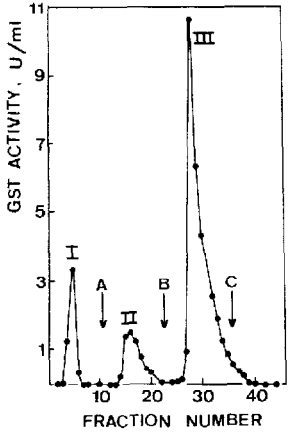



Fig. 1. Elution of rat liver GST activity from a GSH-Sepharose column. The column was first rinsed with buffer A, and then eluted with 5 m*M* GSH in buffer A (arrow A), 5 m*M* GSH in buffer B (arrow B), and 3 *M* NaCl (arrow C).

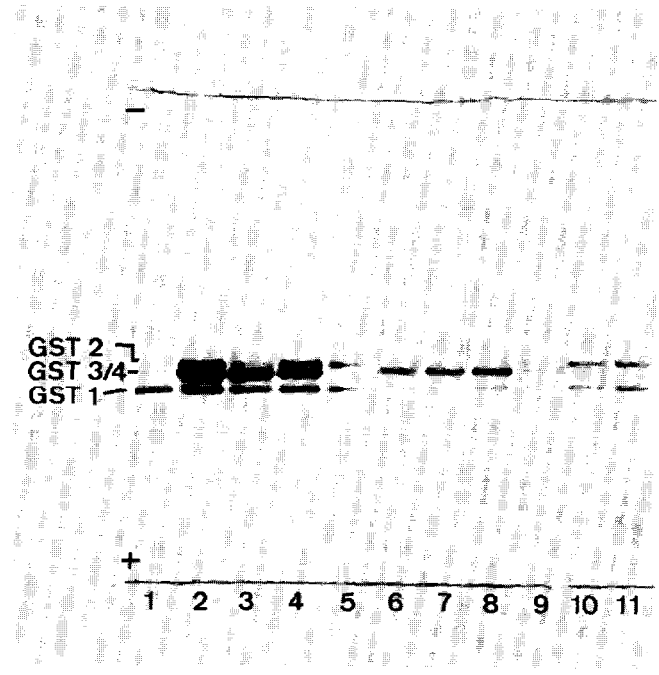



Fig. 2. SDS-PAGE showing the GST subunit patterns of GST peaks obtained in different ways from a GSH-Sepharose column. Lanes 1 and 2, peaks II and III from Fig. 1; lane 3 and 4 peak II and III from Fig. 7; lanes 5 and 11, purified GST 1-2; lanes 6–10, peak, II–VI from Fig. 5.

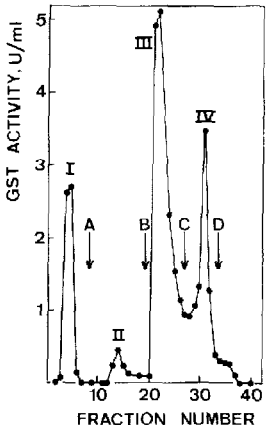



Fig. 3. Elution of rat liver GST acitivity from a GSH-Sepharose column. The column was first rinsed with buffer A, and then eluted with buffer B (arrow A), 5 m*M* GSH in buffer B (arrow B), 15 m*M* GSH in buffer B (arrow C), and 3 *M* NaCl (arrow D).

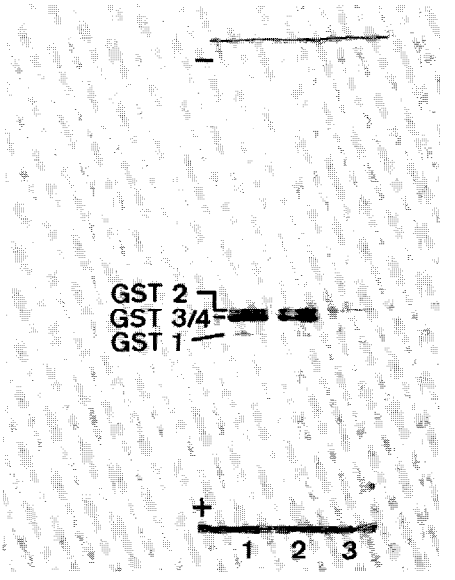



Fig. 4. SDS-PAGE showing the GST subunit pattern of GST desorbed with 30 m*M* GSH from a GSH-Sepharose column. Lane 1, peak II from Fig. 3, showing the three GST subunit sizes; lane 3, peak III from Fig. 6; lane 2, mixture of lanes 1 and 3.

was eluted with 15 m*M* GSH in buffer B (Table I). This experiment was repeated using a stepwise increase of the GSH concentration. Four GST peaks were observed when 0, 1, 3, and 5 m*M* GSH where added respectively to buffer B (Fig. 5). In the 0–5 m*M* GSH range the majority of GST was eluted with 1 and 3 m*M* GSH (Table I). Only a small fraction of GST was desorbed with 5 m*M* GSH. After SDS-PAGE of the different peaks, GST 3/4 subunits appeared to be eluted in the 0–3 m*M* GSH range (Fig. 2, lanes 6–8). GST 1 and GST 2 subunits were also eluted with 0 m*M* GSH (Fig. 2, lane 6), but more importantly with 3 and 5 m*M* GSH (Fig. 2, lanes 8 and 9) and not with 1 m*M* GSH (Fig. 2, lane 7). The GST peak that was desorbed with 15 m*M* GSH consisted mainly of GST 1 and GST 2 subunits, with only a minor amount of GST 3/4 (Fig. 2, lane 10).

After elution of rat liver GST from the GSH-Sepharose column with 15 m*M* GSH in buffer B, still another GST fraction, containing 29% of the activity (Table I) could be desorbed with 30 m*M* GSH in buffer B (Fig. 6). This fraction was composed of GST subunits with *M*r values similar to those of GST 2 and GST 3/4 (Fig. 4, lane 3). Increasing the GSH concentration to 50 m*M* accelerated the desorption initiated by 30 m*M* GSH, but did not desorb a further GST peak, nor did the addition of 3 *M* NaCl (Fig. 6).

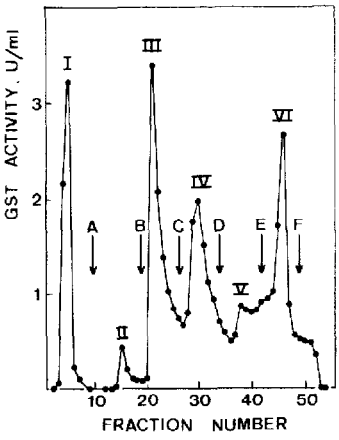
The experiments described so far show that the GST isoenzymes with different subunits are eluted differently as a function of the pH and of the GSH concentration. GST 1 is preferentially eluted at low pH when a fixed GSH con-

TABLE I

RELATIVE AMOUNTS OF GST ACTIVITY DESORBED FROM A GSH-SEPHAROSE COLUMN, AFTER DIFFERENT ELUTION PROCEDURES

| Experiment | Elution procedure | Percentage of total GST activity |
|---|---|---|
| A | 1) Buffer A | 11.1 |
| | 2) Buffer A + 5 m*M* GSH | 14.3 |
| | 3) Buffer B + 5 m*M* GSH | 74.6 |
| B | 1) Buffer A | 11.4 |
| | 2) Buffer B | 12.5 |
| | 3) Buffer B + 1 m*M* GSH | 30.7 |
| | 4) Buffer B + 3 m*M* GSH | 38.0 |
| | 5) Buffer B + 5 m*M* GSH | 7.4 |
| C | 1) Buffer A | 15.4 |
| | 2) Buffer B | 3.7 |
| | 3) Buffer B + 5 m*M* GSH | 54.9 |
| | 4) Buffer B + 15 m*M* GSH | 26.0 |
| D | 1) Buffer A | 14.1 |
| | 2) Buffer B | 2.2 |
| | 3) Buffer B + 1 m*M* GSH | 23.3 |
| | 4) Buffer B + 3 m*M* GSH | 25.5 |
| | 5) Buffer B + 5 m*M* GSH | 11.6 |
| | 6) Buffer B + 15 m*M* GSH | 23.3 |
| E | 1) Buffer A | 14.2 |
| | 2) Buffer B + 15 m*M* GSH | 56.6 |
| | 3) Buffer B + 30 m*M* GSH | 29.2 |
| | 4) Buffer B + 50 m*M* GSH | 0 |
| F | 1) Buffer A | 13.1 |
| | 2) Linear gradient: | 22.7 (peak II) |
| | 200 ml of buffer A, and | 64.2 (peak III) |
| | 200 ml of 5 m*M* GSH in buffer B | |
| G | 1) Buffer A | 10.6 |
| | 2) Linear gradient: | 1.7 (peak II) |
| | 200 ml of buffer A, and | 13.0 (peak III) |
| | 200 ml of 30 m*M* GSH in buffer B | 19.7 (peak IV) |
| | | 33.0 (peak V) |
| | | 22.0 (peak VI) |

centration is considered, GST 3/4 is desorbed at high pH and relatively low GSH concentrations, and GST 2 is desorbed at both high pH and high GSH concentrations. Therefore, we tried to obtain pure GST isoenzymes by gradient elution in one chromatographic step. Only two GST peaks were separated using a linear gradient formed by 200 ml of buffer A and 200 ml of 5 m*M* GSH in buffer B, albeit that peak II was highly asymmetric (Fig. 7). SDS-PAGE showed that the different GST subunits were nearly homogenously distributed in both GST peaks, although GST 2 had a lower concentration in peak II (Fig. 2, lanes 3 and



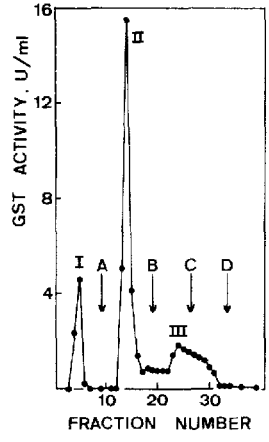




Fig. 5. Elution of rat liver GST activity from a GSH-Sepharose column. The column was first rinsed with buffer A, and then eluted with buffer B (arrow A), 1 m*M* GSH in buffer B (arrow B), 3 m*M* GSH in buffer B (arrow C), 5 m*M* GSH in buffer B (arrow D), 15 m*M* GSH in buffer B (arrow E), and 3 *M* NaCl (arrow F).

Fig. 6. Elution of rat liver GST activity from a GSH-Sepharose column. The column was first rinsed with buffer A, and then eluted with 15 m*M* GSH in buffer B (arrow A), 30 m*M* GSH in buffer B (arrow B), 50 m*M* GSH in buffer B (arrow C), and 3 *M* NaCl (arrow D).

4). Finally, a linear gradient formed by 200 ml of buffer A and 200 ml of 30 m*M* GSH in buffer B was applied (Fig. 8). It is noteworthy that the final pH remains below 8 under these experimental conditions, showing that high GSH concentrations (From 12.5 m*M*) affect the buffering capacity of buffer B. Five GST peaks were separated by this gradient following the first peak of GST activity in the flow-through fractions. However, SDS-PAGE (results not shown) revealed that peak II corresponded to the GST peak desorbed with pure buffer B in Fig. 5, peaks III and IV with peaks II and III in Fig. 7, peaks V and VI respectively with

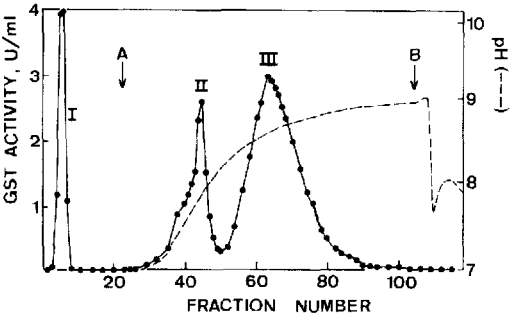



Fig. 7. Elution of rat liver GST activity from a GSH-Sepharose column. The column was first rinsed with buffer A, and further developed with a linear gradient formed by 200 ml of buffer A and 200 ml of 5 m*M* GSH in buffer B. Arrow A, start of the gradient; arrow B, elution with 3 *M* NaCl.

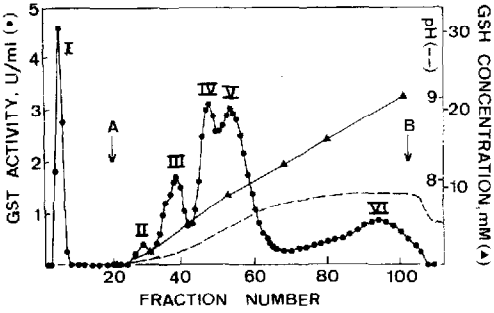



Fig. 8. Elution of rat liver GST activity from a GSH-Sepharose column. The column was first rinsed with buffer A, and further developed with a linear gradient formed by 200 ml of buffer A and 200 ml of 30 m*M* GSH in buffer B. Arrow A, start of the gradient, arrow B, elution with 3 *M* NaCl.

the peaks desorbed with 15 m*M* GSH in buffer B (Fig. 5) and the peak desorbed with 30 m*M* GSH in buffer B (Fig. 6). Attempts to obtain pure GST isoenzymes in one chromatographic step have thus failed.

## DISCUSSION

Less than 50% of the total rat liver GST activity is eluted with 5 m*M* GSH in buffer B (Table I, experiments C–E). This is in sharp contrast to human liver GST, from which nearly 100% is desorbed in a highly purified state from the GSH-Sepharose column [5]. A GSH concentration of 30 m*M* is necessary for quantitative elution of rat liver GST (Fig. 6). This observation has important consequences because this affinity chromatographic procedure is often used in purification protocols [6–12]. Therefore, the desorption of GST from other sources should also be examined at higher concentrations than 5 m*M*.

A low-affinity (25%) and a high-affinity (75%) set of human liver GST isoenzymes was described after elution of the GSH-Sepharose column with respectively buffer B and 5 m*M* GSH in buffer B [17]. This was also observed for rat liver GST, but the low-affinity set was much smaller (Table I, experiment C), proving again that rat liver GST is more strongly bound to the affinity matrix than human liver GST. A low- and an high-affinity set of GST isoenzymes were obtained in the same way from nematode homogenates [18]. The importance of the pH increase for the desorption of GST is shown in Fig. 1.

Any of the different chromatographic procedures described in this paper shows that GST can be selectively eluted from a GSH-Sepharose column. Separations can be obtained both by stepwise changes in the pH and/or the GSH concentration (Figs. 1, 3, 5, and 6), and by linear gradient elution (Figs. 7 and 8). Different GST fractions were obtained previously from extracts of earthworms, cabbage fly larvae and mealworms by using different GSH concentrations for the GST desorption of a GSH-Sepharose column [19]. However, as found by Stener-

sen *et al.* [19], we never obtained pure GST isoenzymes (Figs. 2 and 4), indicating that although the selectivity of the GST desorption from a GSH-Sepharose matrix can be obtained in different ways, it is not specific enough for pure GST isoenzymes to be isolated.

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REFERENCES

1 L. F. Chasseaud, *Adv. Cancer Res.*, 29 (1979) 175.
2 G. J. Smith and G. Litwack, *Rev. Biochem. Toxicol.*, 2 (1980) 1.
3 K. H. Tan, D. J. Meyer, J. Belin and B. Ketterer, *Biochem. J.*, 220 (1984) 243.
4 B. Mannervik and U. H. Danielson, *CRC Crit. Rev. Biochem.*, 23 (1988) 283.
5 P. C. Simons and D. L. Vander Jagt, *Anal. Biochem.*, 82 (1977) 334.
6 T. Igarashi, E. Nanba, F. Sagami, K. Tsudikate, T. Fukuda, T. Horie, T. Satoh and H. Kitagawa, *Biochem. Pharmacol.*, 24 (1988) 4713.
7 C. J. Serabjit-Singh and J. R. Bend, *Arch. Biochem. Biophys.*, 267 (1988) 184.
8 P. R. Young and A. V. Briedis, *Biochem. J.*, 257 (1989) 541.
9 B. J. Cochrane, J. J. Morrissey and G. A. LeBlanc, *Insect Biochem.*, 17 (1987) 731.
10 P. J. Dierickx, *Arch. Int. Physiol. Biochim.*, 93 (1985) 193.
11 M. J. Howell, P. G. Board and J. C. Boray, *J. Parasitol*, 74 (1988) 715.
12 C. Di Ilio, A. Aceto, R. Piccolomini, N. Allocati, A. Faraone, L. Cellini, G. Ravagnan and G. Federici, *Biochem. J.*, 255 (1988) 971.
13 U. K. Laemmli, *Nature*, 227 (1970) 680.
14 B. Mannervik and H. Jensson, *J. Biol. Chem.*, 257 (1982) 9909.
15 W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. Biol. Chem.*, 249 (1974) 7130.
16 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
17 D. L. Vander Jagt, L. A. Hunsaker, K. B. Garcia and R. E. Royer, *J. Biol. Chem.*, 260 (1985) 11603.
18 A. Papadopoulos, P. M.Brophy, P. Crowley, M. Ferguson and J. Barrett, *FEBS Lett.*, 253 (1989)76.
19 J. Stenersen, S. Kobro, M. Bjerke and U. Arend, *Comp. Biochem. Physiol.*, 86C (1987) 73.