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# PURIFICATION OF GLUTATHIONE S-TRANSFERASES FROM RAT LIVER AND WALKER 256 MAMMARY CARCINOMA CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND A GLUTATHIONE AFFINITY COLUMN

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## SUMMARY

A novel method for the rapid purification of glutathione S-transferases (GST) from tissue and cell culture samples is reported. A high-performance glutathione affinity column was used and produced results comparable to those obtained with classical agarose affinity columns. Experiments with purified rat liver GST standards resulted in 87% recovery of total activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the affinity-purified GST was identical to the GST standard and revealed three major protein bands, corresponding to the Ya, Yb, and Yc subunits. A fourth protein band (relative molecular mass 25 000), migrating slightly faster than the Ya subunit, was present in both the standard and eluted GST samples. This polypeptide was tentatively identified as the Yk subunit. Successful purification from rat liver and Walker 256 rat carcinoma cell cytosols was also performed. Recovery of total GST enzymatic activity from Walker cell and rat liver cytosol was 49 and 58%, respectively. SDS-PAGE of these samples indicated a high degree of purity. This methodology requires less than 1 h and can be performed using small quantities of tissue. These features make this technique applicable to analysis of a broad range of biological applications including human biopsy material for GST content.

## INTRODUCTION

The glutathione S-transferases (GST; EC 2.5.1.18) are a family of dimeric isoenzymes whose importance in detoxification and cellular homeostasis is well documented [1–5]. The biological significance of these enzymes include the inactivation of hydrophobic and electrophilic xenobiotics by conjugation with glutathione (GSH) [1,2], prostaglandin isomerization [3], and binding non-substrate ligands such as heme and bilirubin [4–6]. Of particular interest is the observed over-expression of GST in certain malignancies with acquired or intrin-

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sic resistance to various anticancer drugs [7–9]. For example, in tumor cells resistant to chlorambucil the Ya subunit is specifically over-expressed [10]. Additionally, it was recently shown that GST could increase the rate and extent of detoxification of various antineoplastic agents [11,12].

The GST are ubiquitous enzymes exhibiting tissue-specific quantitative and isoenzyme expression [2]. The GST monomers, having relative molecular masses ($M_r$) of around 25 000, are the product of three gene families [13]. They exist as both homo- and hetero-dimers, but only subunits from an individual gene family can combine [13]. The GST monomers can be identified on the basis of their $M_r$ following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. They are Yc ($M_r$ 28 500), $Yb_1$ ($M_r$ 27 000), $Yb_2$ ($M_r$ 27 000), Yn ($M_r$ 26 500), Ya ($M_r$ 25 500), Yk ($M_r$ 25 000) and Yf ($M_r$ 24 500) [14].

In order to study the function of these enzymes, various isolation procedures have been employed. The most commonly used technique is affinity chromatography on agarose gel columns [15,16]. While this type of affinity chromatography results in high recovery of active enzyme, it is a tedious procedure, frequently taking 10–13 h to complete.

This paper reports a rapid high-performance liquid chromatographic (HPLC) affinity procedure, using a novel column packing, that yields results similar to the classical methodology in less than 1 h.

## EXPERIMENTAL

### *Materials*

Purified rat liver GST was prepared by the method of Simons and Vander Jagt [15]. This GST standard and all other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade water, used in the preparation of buffers, was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The GST affinity column (50 mm × 8 mm I.D.) was kindly donated by Showa Denko (Tokyo, Japan). It was custom synthesized and packed and contained GSH (45 μmol/g of packing) covalently bonded through the sulfur to a 1.5-nm spacer arm on a macro-porous (100 nm) polymethacrylate gel (particle size 15–20 μm) stationary phase.

### *Cell culture*

Walker 256 rat mammary carcinoma cells [17] were grown as cell suspensions in Dulbecco's modified eagle's medium supplemented with fetal bovine serum (10%), L-glutamine (4 m*M*), penicillin (50 U/ml) and streptomycin (50 μg/ml).

### *Purification of GST from Walker cell and rat liver cytosol*

All steps in the preparation of cytosol were performed at 4 °C . Walker cells ($3.4 \cdot 10^8$) were washed twice with three volumes of Hank's balanced salt solution. Both the Walker cell pellets and livers from male Fischer 344 rats were homogenized in three volumes of 10 m*M* Tris–HCl, pH 7.8 (buffer A). The resulting homogenate was centrifuged for 20 min at 10 000 *g* and the pellet discarded. The

supernatant was filtered through a plug of glass wool to remove floating lipid prior to centrifugation at 100 000 *g* for 1 h. The resulting microsome-free supernatant will be referred to as cytosol. Rat liver cytosol was diluted five times with buffer A before affinity chromatography (cytosol from Walker cells was used directly).

Chromatography was performed on a Hewlett-Packard 1090L liquid chromatograph (Palo Alto, CA, U.S.A.) equipped with a diode array detector. The column effluent was monitored at 280 nm. Samples were applied to the column via a Rheodyne 7010 sample injection valve (Cotati, CA, U.S.A.) equipped with a 1- or 5-ml sample loop. The column was equilibrated with buffer A (flow-rate 1 ml/min) until a stable baseline was obtained.

A sample of purified rat liver GST standard dissolved in buffer A (1 ml), Walker cell cytosol (1 ml) or rat liver cytosol (5 ml) was filtered through a 0.2-$\mu$m acrodisc membrane (Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to injection onto the column. After sample injection, the column was washed with buffer A until the UV absorbance at 280 nm returned to baseline. The mobile phase was switched to buffer A fortified with 0.2 *M* sodium chloride (buffer B) to elute any non-specifically bound material. GST was eluted with 5 m*M* S-hexyl glutathione dissolved in buffer B. This buffer system was identical to that described by Mannervik and Guthenberg [16] for purification of GST using an S-hexyl glutathione-Sepharose affinity column. Fractions were collected at 1-min intervals (volume 1 ml) and assayed for enzyme activity by measuring the rate of 1-chloro-2,4-dinitrobenzene conjugation to GSH [18]. Measurements of enzyme activity were performed using a Beckman DU-7 spectrophotometer (Fullerton, CA, U.S.A.) equipped with a kinetics module. The fractions which eluted with S-hexyl glutathione and contained activity were pooled, protein concentrations determined and dialyzed overnight against 2000 ml of buffer A. The Walker cell GST sample was also concentrated using a Micro-ProDiCon system (Bio-molecular Dynamics, Beaverton, OR, U.S.A.). Measurement of enzyme specific activity was subsequently performed. All protein determinations were performed using the Bradford [19] dye binding protein assay and bovine $\gamma$-globulin as a standard.

*Electrophoresis*

Identification of the specific GST subunits was based upon their $M_r$ determined following SDS-PAGE. Electrophoresis was performed as described by Laemmli [20] using a 12% polyacrylamide resolving gel. Gels were stained with silver [21] to identify polypeptide bands.

## RESULTS

*Purified rat liver GST*

In order to optimize the buffer system and evaluate the performance of the glutathione affinity column studies were initially performed using the purified rat liver GST standard. Fig. 1 shows a representative chromatogram and activity profile after injection of 1.75 mg of GST. A small amount of protein (2% of total injected) and some associated activity ($<1\%$ of total activity) was not retained by the column. The majority of the protein (and the corresponding enzymatic

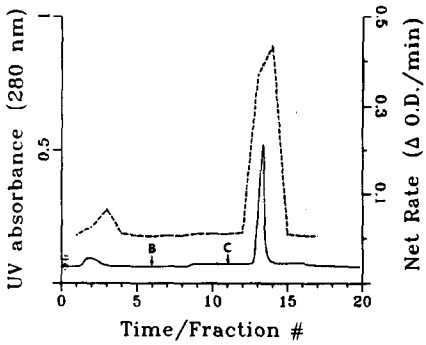


Fig. 1. Chromatogram and activity profile of fractions collected from HPLC affinity separation of rat liver GST (injection sample 1.75 mg). Solid line is UV absorbance at 280 nm of column effluent. Dashed line is rate of 1-chloro-2,4-dinitrobenzene conjugation determined for each fraction (100 $\mu$l). Starting mobile phase was 10 m*M* Tris–HCl, pH 7.8 (buffer A), at 6 min changed to buffer B (buffer A + 0.2 *M* sodium chloride) and at 11 min buffer C (buffer B + 5 m*M* S-hexyl glutathione).

TABLE I

PROTEIN AND ACTIVITY RECOVERY OF RAT LIVER GLUTATHIONE S-TRANSFERASE FROM GLUTATHIONE AFFINITY COLUMN

| Sample | Total protein[a] (mg) | Recovery of protein (%) | Specific activity[b] ($\mu$mol/min/mg) | Recovery of specific activity (%) | Total activity[c] ($\mu$mol/min/mg) | Recovery of total activity (%) |
|---|---|---|---|---|---|---|
| Injection sample[d] | 1.75 | (100) | 6.08 | (100) | 10.66 | (100) |
| Affinity-purified[e] | 1.60 ± 0.06[f] | 91 | 5.88 ± 0.24[f] | 96 | 9.37 ± 0.18[f] | 87 |

[a]Determination for recovery of protein performed on pooled fractions 13 and 14 before dialysis. Total volume of 2 ml.
[b]Reaction rate and protein determination performed on pooled fractions after dialysis for calculation of specific activity.
[c]Total activity = specific activity × total protein.
[d]Injection sample volume of 1 ml.
[e]Affinity-purified sample from combined fractions 13 and 14 from HPLC.
[f]Values are mean ± S.D. of three purifications.

activity) was eluted in fractions 13 and 14 with buffer C. Recovery of total protein, enzyme-specific activity and total GST activity were measured (Table I). The amount of protein recovered was 1.60 ± 0.06 mg (mean ± S.D., $n = 3$) and was equivalent to 91% of the injected protein. The eluted GST retained 96% of the original specific activity; 87% of the total enzymatic activity applied to the column was recovered in these fractions.

SDS-PAGE of samples from the purified GST separation (Fig. 2) revealed the affinity-purified GST contained three major protein bands of $M_r$ 28 500, 27 000 and 25 500 corresponding to the Yc, Yb and Ya subunits, respectively (lane e). A

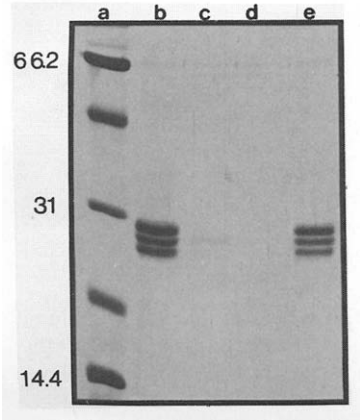


Fig. 2. SDS-PAGE of affinity-purified GST from standard solution: lane a, molecular mass markers; lane b, injection sample (1.75 mg/ml); lane c, column flow-through; lane d, salt wash; lane e, S-hexyl glutathione-eluted fraction. Proteins are silver-stained.

less prominent band of protein, migrating with an $M_r$ of 25 000, was also detected. Hayes [14] has recently described a GST subunit, isolated from rat liver by glutathione-Sepharose 6B affinity chromatography, that migrates slightly faster than the Ya subunit. He termed this monomer the Yk subunit. The 25 000 protein band was tentatively identified as the Yk subunit on this basis. The pattern of the affinity-purified GST was identical to that of the original injection sample (lane b). The column flow-through fractions (lane c) contained two faintly staining polypeptide bands with an $M_r$ of 27 000 and 25 000, corresponding to the Yb and Yk subunits, respectively, while the fractions from the salt wash contained no detectable protein (lane d).

*Rat liver cytosol purification*

The elution profile from a purification of rat liver cytosol is shown in Fig. 3. The majority of GST eluted at 34 min as a single peak of activity along with a band of protein. This peak was collected in two fractions (2 ml total volume) containing 58 and 3.9% of the total applied activity and protein, respectively (Table II). The specific activity of the affinity-purified rat liver GST was 4.24 μmol/min/mg (Table II).

Electrophoresis of samples from the various steps of tissue purification reflected the increase in purity of the GST bands (Fig. 4). The affinity-purified GST (lane f) was free of contaminants and consisted of four bands of 28 500, 27 000, 25 500 and 25 000 corresponding to the Yc, Yb, Ya and Yk subunit classes. These were identical to the GST rat liver standard (lane b). Fractions from the salt wash which possessed GST activity were shown to contain small amounts of proteins with $M_r$ of 25 500 and 28 500, corresponding to the Ya and Yc subunits (lane e). The column flow-through fraction (lane d) had a protein pattern similar to the cytosol (lane c), but appeared to contain less protein in the $M_r$ range of the GST.

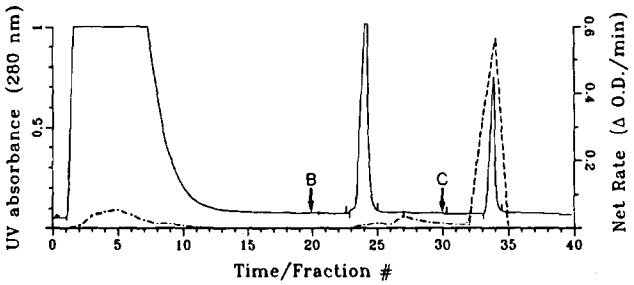


Fig. 3. HPLC and activity profiles of fractions collected from affinity purification of GST from rat liver cytosol. Solid line is UV absorbance at 280 nm of column effluent. Dashed line is rate of thioether formation determined for each fraction. At 20 min mobile phase changed to buffer B; at 30 min to buffer C.

TABLE II

AFFINITY PURIFICATION OF RAT LIVER AND WALKER CELL GST

| Sample | Volume (ml) | Total protein (mg) | Total activity (μmol/min) | Specific activity (μmol/min/mg) | Yield (%) | Purification[a] (fold) |
|---|---|---|---|---|---|---|
| Rat liver cytosol | 5.0 | 25.35 | 7.43 | 0.293 | | |
| Affinity-purified | 2.0 | 0.98 ± 0.04[b] | 4.43 ± 0.34[b] | 4.42 ± 0.31[b] | 58 | 15 |
| Walker cell cytosol | 1.0 | 15.8 | 0.262 | 0.017 | (100) | |
| Affinity-purified (concentrate) | 0.35 | 0.102 | 0.129 | 3.61 | 49 | 212 |

[a]Calculated as specific activity of purified GST divided by specific activity of cytosol.
[b]Values are mean ± S.D. of three purifications.

*Walker 256 rat mammary carcinoma cell purification*

The HPLC and activity profiles of the Walker cell extraction (not shown) was similar in appearance to the rat liver cytosol purification. Affinity-purified GST had a specific activity of 3.61 μmol/min/mg protein and was enriched 212-fold over cytosol (Table II). The recovery of total GST activity was 49% and electrophoresis revealed three bands ($M_r$ 28 500, 27 000 and 24 500) corresponding to the Yc, Yb and Yf subunits (lane f, Fig. 5). The polypeptide pattern of the flow-through (lane d) and the cell cytosol (lane c) fractions were nearly alike. These results were identical to those obtained in our laboratory using conventional affinity chromatographic methods [22].

## DISCUSSION

Affinity chromatography on GSH or S-hexyl glutathione-agarose columns is widely used to purify GST from tissue samples. This procedure provides recovery

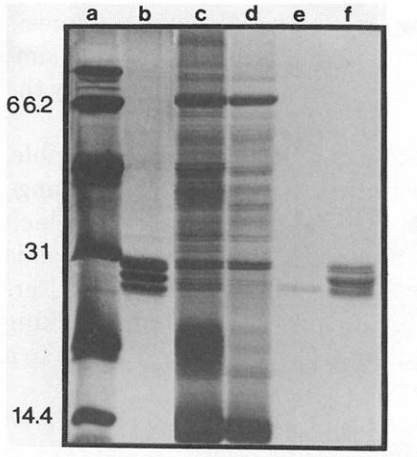


Fig. 4. SDS-PAGE of affinity-purified rat liver cytosol: lane a, molecular mass markers; lane b, rat liver GST standard; lane c, rat liver cytosol; lane d, column flow-through; lane e, salt wash; lane f, affinity-purified GST. Proteins are silver-stained.

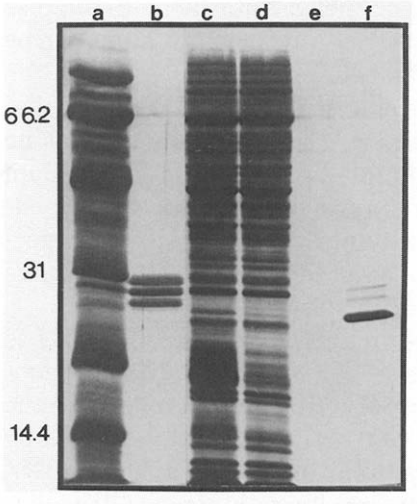


Fig. 5. SDS-PAGE of affinity-purified Walker 256 rat mammary carcinoma cells: lane a, molecular mass markers; lane b, GST standard from rat liver; lane c, Walker cell cytosol; lane d, column flow-through; lane e, salt wash; lane f, Walker cell affinity-purified GST. Proteins are silver-stained.

of active enzyme in the range 31–95% [15,16,22]. However, the time involved prohibits analysis of a large number of samples. One of the advantages of HPLC is the higher flow-rates obtainable resulting in shorter analysis times.

In initial experiments, performed using purified rat liver GST, a small amount of protein and related activity did not bind to the column, however, this represented only 2% of the total GST protein and less than 1% of the total enzymatic activity injected onto the column. In addition, these values were probably overestimates because the injection sample did contain an unknown amount of GSH

which can interfere with the protein and activity assays used. Gel electrophoresis of these fractions showed minute amounts of protein, migrating with an $M_r$ similar to those reported for the Yb and Yk subunits, which were not retained by the column (Fig. 2).

The contribution of this loss to the total recovery of activity was negligible. Very good recovery of total GST activity was obtained (87%), with little change in specific activity (97%). There was a 9% loss of GST protein, the fate of which is unknown. It is possible the protein could have adhered to the walls of the plastic collecting tubes or the stainless-steel components of the HPLC apparatus. Alternatively, the GST may have become irreversibly bound to the column packing material. These findings indicate that the loss of total GST activity was due to a small loss of protein rather than a change is enzyme activity.

S-Hexyl glutathione was used to elute the bound GST because GSH interfered with UV detection at 280 nm, resulting in a large baseline offset. In addition, it has been shown GSH can alter the electrophoretic mobility of GST, perhaps by the formation of mixed disulphides [23]. However, there is no a priori biochemical reason why GSH could not be substituted for S-hexyl glutathione in the elution buffer if a different detection system were utilized. Hayes [14] has recently shown that the Yk subunit has low affinity for the S-hexyl glutathione-Sepharose 6B affinity column. However, this study suggests that S-hexyl glutathione can be used to elute the Yk subunit from a GSH affinity column.

The addition of 0.2 *M* sodium chloride to the elution buffer was necessary because the recovery of purified GST standard with 5 m*M* S-hexyl glutathione alone was low (33%) and the peak volume was large (4 ml). A wash with 0.2 *M* sodium chloride was added before the elution step to remove any non-specifically adsorbed protein that would contaminate the affinity-purity GST. This was important when studies with cell and tissue extracts were performed because a large peak of non-GST protein was eluted during the salt wash.

The purification of GST from rat liver cytosol produced results similar to those obtained using agarose-based affinity columns in our laboratory (data unpublished) and elsewhere [15]. About 58% of the total activity was recovered in the affinity-purified sample. Less than 5% of the total activity was not retained by the column and a small amount ($<1\%$) was present in the salt wash (Fig. 3). Dialysis of the cytosol sample prior to chromatography, to remove endogenous GSH which may interfere with binding to the affinity ligand, had no effect on the recovery or elution pattern. A substantial amount of protein lacking GST activity was also eluted during the wash with buffer B indicating the advantage of a salt wash before GST elution. The resulting affinity-purified sample contained the same subunit composition, as shown by SDS-PAGE, as the purified GST standard (cf. Fig. 4). The increased proportion of activity present in the column flow-through, compared to that obtained during separation of the purified rat liver GST standard, may be due to association of GST with other proteins present in the cytosol mixture. Other experiments suggested that lower flow-rates did not result in better recovery of GST from tissue samples (data not shown). The specific activity of the affinity-purified rat liver GST (4.42 $\mu$mol/min/mg of protein, Table II) was similar to the value obtained from purified GST rat liver standard

(5.88 μmol/min/mg of protein, Table I), another indication that the HPLC affinity purification procedure yields results similar to those obtained using agarose gel columns.

To evaluate further the performance of this procedure, the GST from Walker 256 rat mammary carcinoma cells were purified. The Walker Cell system was selected because our laboratory had previously investigated the GST content of this cell line [22]. Purification of GST in that study was by affinity chromatography on an S-hexyl glutathione-Sepharose column [22]. The findings from both methods were comparable. The HPLC method results were as follows: specific activity, 3.61 μmol/min/mg; yield, 49%; 212-fold purification. The S-hexyl glutathione-Sepharose 6B affinity procedure gave similar findings: specific activity, 2.06 μmol/min/mg; yield, 33%; 171-fold purification [22]. The protein patterns obtained after SDS-PAGE of the samples were identical, further evidence that the two methods produce analogous results.

The most obvious advantage of this HPLC procedure is the time required to perform a purification. Table III illustrates the various techniques employed to isolate the GST and the time required to run a separation. The HPLC affinity chromatography method produces results similar to affinity chromatography on agarose gel columns in one tenth the time. Four purifications (including tissue homogenization and centrifugation steps) have been performed in 8 h which would take at least four days using classical techniques.

Another significant advantage is the cost effectiveness of the HPLC system. While the initial investment for an HPLC column (2.5 ml bed volume, 3.5 mg capacity) is approximately three times that of a glutathione-Sepharose column with the same capacity, the durability and labor savings more than compensate

TABLE III

COMPARISON OF RECOVERY AND TIME REQUIRED FOR VARIOUS GST PURIFICATION METHODS

| Method | Recovery (range) (%) | Time (h) | References |
|---|---|---|---|
| DEAE-cellulose chromatography | 10–53 | 38 | 24–28 |
| Gel permeation | 34–95 | 13–18 | 24–27, 29–31 |
| CM-cellulose | 59 | 13–18 | 22–27, 29–31 |
| Chromatofocusing | 40 | 13–18 | 22–27, 29–31 |
| Isoelectric focusing | 10 | 96 | 26, 28, 32 |
| Affinity chromatography on agarose gels | 30–95 | 10–13 | 15, 16, 22 |
| High-performance affinity chromatography | 49–87 | <1 | |

for the difference in cost. For example, preparation of a Sepharose gel column involves swelling the gel in buffer overnight and packing the column by gravity. The column is then washed sequentially with three different buffers before use, a process taking at least 8 h. Our experience with these columns is that the performance begins to deteriorate after ten uses making it necessary to discard the resin and begin again. To use the HPLC column the packing buffer is washed out, the column equilibrated with starting buffer (approximately 30 min) and the purification executed. To date, this column has been used for twenty separations with purified GST (to optimize conditions) and forty purifications from cytosol of different tissues. Between each purification and after each day of use the column was washed with 1.0 *M* sodium chloride in 10 m*M* Tris, pH 7.8, to remove any strongly retained contaminants. The protein that was eluted from the column during this wash did not posses GST enzymatic activity suggesting that it did not represent a form of GST with higher affinity for the GSH ligand. The column was stored for prolonged periods in 10 m*M* sodium phosphate, pH 7.0, at 4°C. The column has been used in this way for almost nine months without a decrease in column efficiency.

The present study has reported results on the purification of GST from rat tissue. This methodology is also currently being used for the rapid analysis of GST in various human tissue samples. Work in progress includes GST determinations in human chronic lymphocytic leukemia and ovarian carcinomas. We believe this methodology may have clinical utility if a relationship between GST content and resistance to anticancer drugs is established.

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