Selective elution of rodent glutathione *S*-transferases and glyoxalase I from the *S*-hexylglutathione–Sepharose affinity matrix

John D. HAYES

University of Edinburgh Department of Clinical Chemistry, The Royal Infirmary, Edinburgh EH3 9YW, Scotland, U.K.

1. The major hepatic glutathione *S*-transferases (GSTs) from gerbil, guinea-pig, hamster, mouse and rat comprise Ya- (M_r 25 500–25 800), Yb- (M_r 26 100–26 400), Yc- (M_r 27 000–27 500) and Yf- (M_r 24 800) type subunits. 2. In all rodent species the GST subunits possess characteristic affinities for *S*-hexylglutathione–Sepharose and are eluted at distinct positions when a gradient of counter-ligand is employed to develop this affinity gel. The enzymes that bind to this matrix can be eluted, according to their subunit composition, in the order Ya-, Yc-, Yf- and Yb-containing GST; glyoxalase I, also retained by *S*-hexylglutathione–Sepharose, is eluted after the major GST YbYb peak. 3. Conditions are also described for the isocratic affinity elution of *S*-hexylglutathione–Sepharose that allow rat GST to be divided into four separate fractions (pools 1–4). A further fraction (pool 5) can be prepared from material that does not bind *S*-hexylglutathione–Sepharose and is obtained by chromatography on glutathione–Sepharose. 4. The sequential use of *S*-hexylglutathione–Sepharose and glutathione–Sepharose has facilitated the isolation of novel GSTs by enriching the various affinity-purified fractions with different subunits. This strategy allowed the Yk (M_r 25 000) and Yo (M_r 26 500) subunits from rat testis as well as Yl (M_r 25 700) from rat kidney to be rapidly purified. 5. The binding properties of GST subunits for *S*-hexylglutathione–Sepharose have been compared with their K_m values for GSH. The elution order from this matrix is inversely related to the K_m value. The GSTs that do not bind to *S*-hexylglutathione–Sepharose have considerably higher K_m values for GSH (i.e. > 2.0 mM) than do those enzymes that readily bind to the affinity gel (i.e. 0.13–0.77 mM). GST YkYk and YoYo, which have weak affinities for *S*-hexylglutathione–Sepharose, possess intermediate K_m values for GSH of 1.0 and 1.2 mM respectively.

INTRODUCTION

The glutathione *S*-transferases (GST; EC 2.5.1.18) have been widely studied because they play a central role in drug metabolism.

The GSTs are a complex multi-gene family of proteins (Mannervik *et al.*, 1985). The cytosolic enzymes each comprise two subunits and, in addition to the multiple homodimers, hybridization between a limited number of related subunits can also give rise to a series of GST heterodimers. To date, six GST subunit types have been described, namely Ya (M_r 25 500), Yb (M_r 26 300), Yc (M_r 27 500), Yf (M_r 24 800), Yk (M_r 25 000) and Yn (M_r 26 000) (Hayes & Mantle, 1986a). The Yf subunit has also been called Yp (Satoh *et al.*, 1985). Rat GSTs can be divided according to their structural and immunochemical properties into at least three different classes. The enzymes in Group I comprise YkYk subunits or binary combinations of Ya/Yc subunits. The Group II GSTs are composed of binary combinations of Yb/Yn polypeptides. Group III is composed of GSTs containing YfYf subunits.

Most work describing GSTs has used rat liver as the enzyme source. Human GST isoenzymes have also been studied by several groups (Stockman *et al.*, 1985; Suzuki *et al.*, 1987) and, more recently, mouse GSTs have

attracted attention (Warholm *et al.*, 1986; McLellan & Hayes, 1987). Little is known about the GSTs from other species.

Affinity chromatography using HexG–Ag is commonly employed as the central purification step for GST isolation. In most studies the GST enzymes have been eluted from this affinity matrix as a single pool of activity; individual isoenzymes are purified subsequently by chromatofocusing or by ion-exchange chromatography. However, gradient elution of human GSTs from HexG–Ag has been shown to result in the separate purification of the YaYa- and YbYb-containing enzymes (Hayes *et al.*, 1987a). This observation was potentially very important since, under certain circumstances, it makes possible a single-step purification of GST isoenzymes. As a result the optimization and performance of HexG–Ag has been studied further as a possible way of providing a comprehensive purification strategy applicable to many species.

In the present study conditions are described that permit the separate purification of the YaYa (or YaYc), the YbYb and the YfYf GSTs from the livers of five rodent species. GSTs in the rat have been resolved into several distinct fractions by using HexG–Ag and G–Ag. The G–Ag column has been used to isolate rat GSTs that fail to bind to HexG–Ag.

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; HexG–Ag, *S*-hexylglutathione–Sepharose; G–Ag, glutathione–Sepharose; PAGE, polyacrylamide-gel electrophoresis.

Fig. 1. Gradient elution of rodent GSTs from HexG-Ag

Hepatic cytosols (1.5–4.0 g of protein) from various species of rodent were applied to 1.6 cm $\times$ 40.0 cm columns of HexG-Ag. The affinity matrix was washed (32 ml/h) with about 800 ml of buffer A before the GST enzymes were eluted. Columns were developed in two stages, with two separate continuous gradients of *S*-hexylglutathione (i.e. 0–0.25 mM over 400 ml, followed by 0.25–5.0 mM over 150 ml). Fractions (4.8 ml) were collected and GST activity with CDNB ($\blacktriangle$) and absorbance at 280 nm ($\circ$) were measured. The *S*-hexylglutathione gradient is represented by a continuous line. In each panel the species examined is indicated and the fractions examined by SDS/PAGE are shown (I, II etc.).

The GSTs were purified from the HexG-Ag and G-Ag pools and their K_m values for GSH and CDNB were compared with their chromatographic behaviour.

MATERIALS AND METHODS

Chemicals

S-Hexylglutathione was synthesized by the method of Vince *et al.* (1971). The *S*-hexylglutathione affinity gel (HexG-Ag) and the glutathione affinity gel (G-Ag) were constructed by using respectively the methods of Mannervik & Guthenberg (1981) and Simons & Vander Jagt (1977).

Buffers

These were used at either 4 $^{\circ}\text{C}$ or 20 $^{\circ}\text{C}$; the pH quoted is that obtained at the working temperature.

Animals

The tissues studied were from sexually mature male rodents purchased from Bantim and Kingman, Hull, U.K. Animals were fed *ad libitum* and were killed by being placed in an atmosphere enriched with CO_2 .

Enzyme purification

Livers from gerbils, guinea-pigs, golden hamsters, mice (BALB/c) and Wistar rats were stored at -85°C for 1–26 weeks before study. In all cases GSTs were purified from the livers of between eight and 16 animals to help minimize the effects of inter-individual variation in GST expression from influencing the interspecies differences in GST. Rat extrahepatic organs were stored at -85°C for 1–3 weeks before being processed. Frozen tissues were allowed to thaw at room temperature before

being blended at 4 °C in 3 vol. of 50 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl (buffer A). All subsequent procedures were performed at 4 °C, unless otherwise stated.

Tissue extracts were centrifuged at 100 000 *g* and the supernatants were dialysed against four changes, each of 2 litres, of buffer A containing 0.6 mM-dithiothreitol. The dialysed samples were applied to 1.6 cm × 40.0 cm affinity columns of HexG-Ag that had been equilibrated with buffer A. This affinity matrix was washed (32 ml/h) with 800 ml of buffer A before the GSTs were eluted.

Solutions of *S*-hexylglutathione used to develop HexG-Ag were freshly prepared by dissolving 0.4 g portions of *S*-hexylglutathione in 10 ml of 2 M-NaOH. Once dissolved, this material was added immediately to buffer A (to yield a 5 mM solution of *S*-hexylglutathione) and the resulting solution was adjusted to pH 7.8 with 1 M-HCl. This stock solution was diluted with buffer A as required for experimental purposes (these are described below).

During the study of rat GSTs, isoenzymes that failed to bind to HexG-Ag, and that were therefore recovered in the 'flow-through' fractions, were subjected to a second affinity-chromatography step on G-Ag. This was performed with columns (1.6 cm × 20.0 cm) of G-Ag equilibrated and eluted at 32 ml/h with buffer A; these G-Ag columns were washed with 800 ml of buffer A before being developed with 35 mM-GSH in 200 mM-Tris/HCl buffer, pH 9.5.

Individual rat GST isoenzymes isolated by affinity chromatography were resolved by the use of either anion-exchange or hydroxyapatite chromatography, both of which were carried out at room temperature. Anion-exchange f.p.l.c. was performed on Mono Q HR 5/5 with the integrated system marketed by Pharmacia, Milton Keynes, Bucks., U.K. The Mono Q columns were equilibrated with 20 mM-Tris/HCl buffer, pH 7.8, and developed with a salt gradient using 1 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.8, as the limit buffer. Hydroxyapatite h.p.l.c. was performed on HPHT columns (Bio-Rad Laboratories, Watford, Herts., U.K.) by using equipment and methods described elsewhere (Hayes *et al.*, 1987b).

Analytical

The standard GST enzyme assay was performed in a centrifugal analyser at 37 °C with 1 mM-CDNB and 2 mM-GSH. The K_m values for GSH were determined with the use of reaction mixtures containing 1 mM-CDNB and various concentrations (0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0 or 3.0 mM) of GSH. Likewise, the K_m values for CDNB were determined with the use of reaction mixtures containing 2 mM-GSH and various concentrations (0.1, 0.3, 0.5, 0.7, 1.0, 1.5 or 2.0 mM) of CDNB. Each experimental point was measured in quintuplicate, and the mean value as well as standard deviation was determined. The mean value obtained was used for further calculations if the coefficient of variation was less than 5% (the within-batch precision for this assay, with the use of a centrifugal analyser, is usually about 4.5%). Estimates of K_m were calculated by using a least-squares non-linear fitting procedure of the mean velocity against substrate concentration (Wilkinson, 1961). The calculations and graphical output were programmed on a Hewlett-Packard 9821 desk calculator with printer/plotter by Dr. A. F. Smith of this Department.

Table 1. GST content of rodent liver

GST activity was determined at 37 °C with CDNB.

Species	GST activity in hepatic cytosol (μ mol/min per mg)	Percentage of cytosolic protein retained by the HexG-Ag affinity column	Percentage of total protein bound represented by	
			Ya/Yc/Yl subunits	Yb/Yn subunits
Gerbil	3.36	5.2	46	53
Guinea pig	5.79	5.2	8	92
Hamster	5.79	2.9	38	62
Mouse	6.37	4.5	14	27
Rat	1.43	2.7	39	58

SDS/PAGE was calibrated by using rat lung GST subunits (Yf, Yb and Yc). Hamster GSTs were analysed by the use of SDS/PAGE utilizing a resolving gel of 16% polyacrylamide that incorporated 0.09% (w/v) *NN'*-methylenebisacrylamide instead of 0.32% (w/v) *NN'*-methylenebisacrylamide, since these conditions facilitate subunit identification in this species (Hayes & Mantle, 1986b). Isoelectric focusing was carried out in thin-layer 5% (w/v) polyacrylamide slab gels with the use of the Multiphor I system (LKB-Producter AB, Bromma, Sweden).

RESULTS AND DISCUSSION

HexG-Ag is a highly effective affinity matrix for the purification of GST isoenzymes and is widely employed for this purpose. Preliminary experiments during the present study showed that HexG-Ag retained at least 85% of the CDNB-GSH-conjugating activity in the hepatic cytosols from gerbil, guinea-pig, hamster, mouse, rat and man.

Affinity elution of HexG-Ag with gradients of counter-ligand

Gradient affinity elution of HexG-Ag was used to prepare the hepatic GSTs from rodent species, including gerbil, guinea-pig, hamster, mouse and rat; these profiles are compared in Fig. 1. In all the species examined GST activity was resolved into several peaks by gradient elution of HexG-Ag. Subunit analysis of these enzyme-containing peaks was achieved by using SDS/PAGE (Fig. 2) and dot blotting (results not shown).

These experiments show that all the livers studied contained Ya- and Yb-type subunits. However, the relative amounts varied from species to species (Table 1). For example, guinea-pig liver was found to contain predominantly Yb subunits whereas gerbil, hamster and rat livers expressed similar amounts of both Ya and Yb subunits. Although mouse liver appears to possess less Ya and Yb polypeptides than other rodents, this is not the case; the low percentage values (Table 1) for mouse Ya and Yb subunits arise because of the contribution made to the total GST pool by the Yf subunit. The Yf polypeptide was only found to be expressed at a high concentration in mouse liver, but this subunit was also detected in gerbil liver. The Yc GST subunit was only found in significant amounts in hamster and rat liver, but trace amounts of Yc were also found in guinea-pig liver.

Fig. 2. SDS/PAGE analysis of rodent GST enzymes eluted from HexG-Ag

The GST enzymes that were resolved by gradient affinity elution of HexG-Ag were analysed by SDS/PAGE. The column fractions analysed (I, II etc.) correspond to those shown in Fig. 1. In all cases the total GST pool from rat lung (Yf, Yb and Yc subunits) was employed as internal standard and is designated Lu. In two cases rat liver GSTs (Ya, Yb and Yc subunits) were also used to calibrate the gel, the tracks being labelled Li. All gels, except (c), were composed of 12% (w/v) polyacrylamide that incorporated 0.32% (w/v) *NN'*-methylenebisacrylamide as cross-linker. The gel in (c) comprised 16% (w/v) polyacrylamide containing 0.09% (w/v) *NN'*-methylenebisacrylamide.

Table 1 shows that GSTs represent 2.7–5.2% of rodent liver cytosolic protein, gerbil and guinea-pig livers containing the highest concentrations. Glyoxalase I is present in high concentrations in mouse liver, but can

Table 2. Elution of GST enzymes from HexG–Ag

Abbreviation: N.D., not detected.

Species	Concentration of <i>S</i> -hexylglutathione at which protein was eluted (μM)			
	YaYa, YaYc or GSTs containing immunochemically related subunits	YfYf	YbYb (or YbYn)	Glyoxalase I
Gerbil	10–60	60	85–175	N.D.
Guinea pig	25–125	N.D.	750	N.D.
Hamster	25–60	N.D.	120	N.D.
Mouse	30	80	150	1000
Rat	5–60	N.D.	180	750
Man*	5–70	N.D.	200	1500

* Data from Hayes *et al.* (1987a).

also be detected in smaller amounts in rat and human livers (Hayes *et al.*, 1987a).

Table 2 shows that, although the concentration of counter-ligand at which a particular subunit was found to be eluted varied from species to species, the order of elution was the same in all cases. In the rat, hepatic Ya subunits were eluted slightly earlier than Yc subunits whereas the converse was the case with hamster liver (Fig. 2). When detected, glyoxalase I was found to be eluted from the HexG–Ag after the GST enzymes. Hence the order of elution was Ya/Yc, Yf, Yb/Yn and, lastly, glyoxalase I.

Isocratic elution of HexG–Ag and G–Ag to study rat GST isoenzymes

Figs. 1 and 2 show that affinity chromatography on HexG–Ag can be employed to separate hepatic GSTs. Extrahepatic GSTs also exist in the rat, notably Yf and Yn subunits, and their elution behaviour from HexG–Ag required investigation before general comments could be made about the resolving ability of this affinity matrix.

The GSTs usually represent a significantly smaller proportion of the cytosolic protein in extrahepatic tissues than in liver. To prevent unnecessary dilution of samples, the GSTs in cytosol from extrahepatic tissues were eluted

Fig. 3. Two-step isocratic elution of rat GSTs from HexG–Ag

Dialysed rat tissue cytosols were applied to 1.6 cm $\times$ 40.0 cm columns of HexG–Ag that had been equilibrated previously with buffer A. After the application of protein, columns were washed at (32 ml/h) with approx. 800 ml of buffer A until the A_{280} of the eluate was less than 0.025. The columns were then developed in two steps, with 550 ml of 0.25 mM-*S*-hexylglutathione followed by 150 ml of 5.0 mM-*S*-hexylglutathione (both eluents were made up in buffer A). Fractions (8.0 ml) were collected; the first elution step was initiated at fraction 1 and the second at fraction 70. The absorbance of the eluate at 280 nm ($\circ$) was monitored and GST activity ($\blacktriangle$) was measured with CDNB. The horizontal bars indicate the fractions that were combined for SDS/PAGE (Fig. 4).

Fig. 4. SDS/PAGE of affinity-purified protein

The subunit composition of protein isolated by HexG-Ag (pools 1–4, see Fig. 3) and G-Ag (pool 5) was investigated by SDS/PAGE. The 12% (w/v) polyacrylamide gel incorporated 0.32% (w/v) *NN'*-methylenebisacrylamide. The gels were all loaded with about 4 μ g of protein, the tracks from left to right being as follows: track a, pulmonary GST markers (Yf, Yb and Yc); track b, HexG-Ag affinity-purified pool 1; track c, HexG-Ag affinity-purified pool 2; track d, HexG-Ag affinity-purified pool 3; track e, HexG-Ag affinity-purified pool 4; track f, G-Ag affinity-purified pool 5; track g, pulmonary GST markers (Yf, Yb and Yc); track h, hepatic GST markers (Ya, Yb and Yc).

isocratically from HexG-Ag with a limiting concentration of counter-ligand. An initial step, involving 0.25 mM-*S*-hexylglutathione, was used to elute GSTs. A second step, involving 5.0 mM-*S*-hexylglutathione, was employed to elute glyoxalase I.

Certain rat GSTs have a poor affinity for HexG-Ag, the best-documented examples being GST E (Meyer *et al.*, 1984) and GST K (or YkYk; Hayes, 1986). However, some of these enzymes can be bound to G-Ag, and application of the 'break-through' fractions from HexG-Ag to G-Ag can lead to the isolation of additional

GSTs (see the Materials and methods section). It was decided that the enzymes that could be isolated in this manner from the 'break-through' fractions from HexG-Ag should be investigated, since study of these enzymes may allow a better understanding of why this matrix fails to bind certain GSTs.

Fig. 3 shows that a total of four peaks of GST can be resolved when HexG-Ag is developed isocratically by using the two-step elution procedure. However, four peaks were only observed when cytosol from rat testis was used as the enzyme source; these are referred to as T1–T4. In all other tissues the initial GST-containing peak (eluted between fractions 9 and 14 following the addition of 0.25 mM-*S*-hexylglutathione) was observed, but the relative amounts of the three other peaks varied considerably. It was decided, therefore, that the fractions that represented the ascending and descending portions of the initial GST peak in liver, kidney and spleen should be combined and analysed separately, to facilitate the identification of Yf and Yn subunits in these tissues. For example, in all organs other than rat testis the two portions of peak 1 are referred to as pool 1 and 2 (see Fig. 3 for definitions of other GST pools). Electrophoresis of these fractions showed that the order of elution of rat subunits from HexG-Ag was Ya, Yc, Yf, Yn and Yb. Rat glyoxalase I was found to be eluted after the GST subunits in these experiments, although its existence in Figs. 1 and 2 is difficult to demonstrate.

The material that failed to bind HexG-Ag was applied to G-Ag and the GST isolated by the second affinity matrix is referred to as pool 5.

SDS/PAGE (Fig. 4) showed that the affinity-purified pools (Fig. 3) contained large numbers of GST subunits and that the complexity varied considerably from tissue to tissue. The majority of the polypeptide bands were identifiable on the basis of their electrophoretic mobility, but certain bands did not appear to correspond to known GST subunits. Electrophoresis of the K1 pool revealed the existence of a polypeptide of M_r 25 700 (Yl) that has not previously been described in adult rat tissue. This subunit is immunochemically related to Ya and hybridizes to Yc. In the context of the present study it is interesting to note that both the physical properties of Yl and its elution position from HexG-Ag are consistent with it being a GST Group I (class Alpha) enzyme.

The testicular T5 pool obtained by G-Ag chromatography contains large amounts of an unique polypeptide of apparent M_r 26 500 (Yo) that migrates during electrophoresis between the Yb (M_r 26 300) and Yc (M_r 27 500) subunits; the Yo subunit was also detected in much smaller amount in the T1 and T2 pools. The reason why Yo possesses a poor affinity for HexG-Ag was investigated (see below).

Purification of GSTs for K_m determination

To allow an understanding of why HexG-Ag has a weak affinity for Yk and Yo subunits, but readily adsorbs the Ya, Yb, Yc, Yf, Yn and Yl subunits, YkYk and YoYo as well as other GSTs were purified and their K_m values for CDNB and GSH investigated. In testis pool 5 the Yk and Yo subunits were separately purified by anion-exchange f.p.l.c. on columns of Mono Q (Fig. 5); testicular YkYk (GST K) and YoYo (GST O) were eluted from Mono Q at different positions. The purity of these preparations was confirmed by SDS/PAGE (results not shown).

Fig. 5. Resolution of GSTs YnYn, YkYk and YoYo from rat testis by using anion-exchange f.p.l.c.

The testicular GST fraction T2, which bound to HexG-Ag and was eluted by 0.25 mM-S-hexylglutathione at 90–120 ml (see Fig. 3), was dialysed against 20 mM-Tris/HCl buffer, pH 7.8, containing 1 mM-2-mercaptoethanol. The GST fraction from rat testis that failed to bind to HexG-Ag but that was isolated by the subsequent use of G-Ag (i.e. pool T5) was also dialysed against the same buffer. Portions (5 ml) of the dialysed T2 (b) or T5 (a) pools were applied to the f.p.l.c. Mono Q HR 5/5 column that had been equilibrated with the dialysis buffer. The column was eluted at 0.5 ml/min and developed with a 0–1 M-NaCl gradient in the running buffer; this gradient was established in two steps, shown by the straight lines. Fractions (0.5 ml) were collected and the absorbance at 280 nm was monitored. The subunit compositions of the major peaks were determined by SDS/PAGE and are indicated.

Fig. 6. Hydroxyapatite h.p.l.c. preparation of YfYf and YcYc GST isoenzymes

The initial peak of kidney GST activity that was eluted from HexG-Ag (Fig. 3, fractions 9–13) was combined and dialysed against 10 mM-sodium phosphate buffer, pH 6.7. This material was subjected to h.p.l.c. on a column of Bio-Gel HPHT hydroxyapatite that had been equilibrated with 10 mM-sodium phosphate buffer, pH 6.7, containing 2 mM-2-mercaptoethanol, 0.4 mM-CaCl₂ and 0.1% (w/v) NaN₃. The column was developed (flow rate 0.5 ml/min)

GST YnYn was purified from the T2 pool by f.p.l.c. on Mono Q by using the method described for YkYk and YoYo (Fig. 5). GSTs YfYf and YcYc were purified from K1 (+K2) by hydroxyapatite h.p.l.c. (Fig. 6). These preparations appeared homogeneous when analysed by SDS/PAGE. Isoelectric focusing showed YfYf, YkYk, YoYo and YnYn have pI values of 7.4, 6.1, 6.0 and 5.9 respectively (Fig. 7). This gel also shows that GST YcYc has a pI value of about 9.0; the testicular and renal YcYc enzymes were found to co-migrate during isoelectric focusing (results not shown).

Comparison between K_m values and behaviour on affinity matrices

The rat subunits were eluted from HexG-Ag in the following order: Ya, Yc, Yf, Yn and Yb (Yb₁ and Yb₂ are co-eluted). Since this affinity gel is capable of interacting with both the GSH-binding site of GSTs and the lipophile (second substrate)-binding site, it is not clear which region in the active centre is responsible for the ordered elution of GST subunits from HexG-Ag. The K_m values of GST subunits for GSH and CDNB (Table 3) suggest that the GSH-binding site, rather than the lipophile-binding site, is primarily responsible for the elution order of GST subunits.

Data describing the inhibition of mouse GSTs by S-hexylglutathione (Warholm *et al.*, 1986) also argue against the lipophile-binding site of mouse liver GST being responsible for the different subunits being eluted at distinct positions; these workers reported that the I_{50} values of S-hexylglutathione (with CDNB as substrate)

with a 10–350 mM-sodium phosphate gradient (the continuous line indicates the shape of the gradient employed). The absorbance at 280 nm was monitored and the subunit compositions of the major peaks are indicated.

Fig. 7. Isoelectric focusing of GSTs YoYo, YkYk, YnYn and YcYc

Isoelectric focusing was performed with a broad-range gel (pH 3.5–9.5) in thin-layer 5% (w/v) polyacrylamide. The top of the photograph represents the cathodal (i.e. basic) end of the gel. All the GST enzymes, with the exception of renal YfYf, were from rat testis. Each of the GST enzymes was reduced with 1 mM-dithiothreitol (at 4 °C for 16 h) before analysis. The samples were loaded (about 15 µg) from left to right as follows: track 1, GST YoYo; track 2, pI calibration standards; track 3, GST YkYk; track 4, GST YoYo; track 5, GST YfYf; track 6, pI calibration standards; track 7, GST YnYn; track 8, GST YcYc; track 9, GST Yb₂Yb₂; track 10, pI calibration standards. The positions of the calibration proteins soya-bean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin basic band (pI 7.35), lentil lectin acidic band (pI 8.15), lentil lectin basic band (pI 8.65) and trypsinogen (pI 9.30) are shown.

Table 3. K_m values of rat GST enzymes and their behaviour on HexG–Ag

The GST enzymes are listed according to their order of elution from HexG–Ag. The K_m values (given as means ± S.E.M.) were all determined at pH 6.5. Abbreviation: N.D., not determined.

Enzyme	Subunit M_r	Order of elution from HexG–Ag	K_m (mM)	
			GSH	CDNB
E (5–5)*	26 300	Fails to bind	8.1	N.D.
YoYo	26 500	Weak affinity	1.19 ± 0.08	3.4 ± 0.8†
YkYk	25 000	Weak affinity	1.02 ± 0.02	6.0 ± 0.5†
YaYa	25 500	1	0.77 ± 0.07	0.95 ± 0.12
YcYc	27 500	2	0.58 ± 0.05	0.97 ± 0.01
YfYf	24 800	3	0.42 ± 0.01	5.2 ± 0.9†
YnYn	26 000	4	0.53 ± 0.05	0.91 ± 0.08
Yb ₁ Yb ₁	26 300	5 =	0.22 ± 0.01	0.10 ± 0.01
Yb ₂ Yb ₂	26 300	5 =	0.13 ± 0.01	2.5 ± 0.6†

* Data for GST E are from Fjellstedt *et al.* (1973) and Meyer *et al.* (1984). All other data are from previous publications from this laboratory or were determined during the present study.

† K_m values for CDBN above 2.0 mM cannot be accurately determined owing to the limited solubility of this substrate.

for the Ya, Yf and Yb subunits were very similar, being respectively 7 µM, 10 µM and 7 µM.

CONCLUSION

The use of HexG–Ag has been very important in studies of GST isoenzymes. Most workers have used this

affinity matrix to isolate GST as a single pool of activity.

The present study has shown that when HexG–Ag is affinity-developed, either with a gradient of counter-ligand or isocratically with limiting concentrations of counter-ligand, the GST subunits are eluted in a specific order. The ordered elution of GST subunits is observed in rat, gerbil, guinea-pig, hamster, mouse and man, showing that this behaviour may be conserved between species. The order of GST elution may reflect the gene family encoding the subunits, and this phenomenon is likely to be mediated by the GSH-binding site.

The selective use of affinity matrices greatly facilitates the preparation of purified GST isoenzymes, and this study has described the optimized use of HexG–Ag. This work has also served to emphasize the value of G–Ag in isolating subunits (e.g. Yk and Yo) that, in the rat, have a poor affinity for HexG–Ag. Most importantly, the data presented suggest that GSTs with K_m values for GSH greater than about 1.0 mM may not adsorb on HexG–Ag. The simple purification scheme devised for testicular GST YkYk and GST YoYo, by anion-exchange f.p.l.c. on Mono Q columns, demonstrates the value of using HexG–Ag followed by G–Ag as affinity matrices from which to isolate GST subunits.

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