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Single-step purification and h.p.l.c. analysis of glutathione transferase 8–8 in rat tissues

David J. MEYER,*§ Eoin LALOR,* Brian COLES,* Andreas KISPERT,* Per ÅLIN,† Bengt MANNERVIK‡ and Brian KETTERER*

*Cancer Research Campaign Molecular Toxicology Group, Department of Biochemistry, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1P 6DB, U.K., †Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden, and ‡Department of Biochemistry, Biomedical Center, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden

GSSG selectively elutes two GSH transferases from a mixture of rat GSH transferases bound to a GSH–agarose affinity matrix. One is a form of GSH transferase 1–1 and the other is shown to be GSH transferase 8–8. By using tissues that lack this form of GSH transferase 1–1 (e.g. lung), GSH transferase 8–8 may thus be purified from cytosol in a single step. Quantitative analysis of the tissue distribution of GSH transferase 8–8 was obtained by h.p.l.c.

INTRODUCTION

4-Hydroxyalkenals are prominent products of free-radical reactions occurring in biomembranes (Esterbauer, 1982). Such compounds react readily with thiols and show a variety of biological effects, including protein alkylation, mutagenesis associated with the formation of a DNA–guanosine adduct (Winter *et al.*, 1986) and lymphocyte chemotaxis (Curzio *et al.*, 1986). 4-Hydroxyalkenals react with GSH both non-enzymically and catalysed by GSH transferases (Ålin *et al.*, 1985), rat isoenzyme GSH transferase 8–8 having remarkably high activity (Jensson *et al.*, 1986; Danielson *et al.*, 1987). The action of GSH transferase 8–8 and other GSH transferase isoenzymes is thought to play an important role in the detoxication of products of free-radical damage to membranes (Tan *et al.*, 1984; Ålin *et al.*, 1985; Jensson *et al.*, 1986; Ketterer *et al.*, 1988).

In order to assess the GSH-dependent detoxication capacity of a given cell or tissue it is essential to know the quantitative GSH transferase subunit composition. To this end a combined affinity chromatography plus reverse-phase h.p.l.c. method was developed that was successful in quantification of subunits 1–7 (Ostlund Farrants *et al.*, 1987). In the present work it is shown that a fraction from h.p.l.c., previously thought to be a variant form of subunit 1, is due to subunit 8, thus allowing its rapid analysis. The present paper describes a novel single-step purification of GSH transferase 8–8 and an analysis of its tissue distribution.

EXPERIMENTAL

Preparation of GSH transferase fraction

GSH transferases were purified from various tissues of male Sprague–Dawley rats weighing 200–250 g. A combined GSH transferase fraction was prepared after the method of Vander Jagt *et al.* (1985). Tissues were homogenized in 2 vol. of buffer A (150 mM-KCl, 50 μM-phenylmethanesulphonyl fluoride, 1 mM-EDTA and 2 mM-dithiothreitol in 10 mM-potassium phosphate buffer, pH 7.0) and the supernatant cytosol fraction was prepared. After filtration through glass fibre, samples were applied directly to a 1.5 cm × 2.0 cm column of S-linked GSH–agarose equilibrated with buffer A. The column was washed with 5 vol. of buffer A, and the GSH transferases were then eluted with buffer B (5 mM-GSH and 1 mM-dithiothreitol in 50 mM-Tris adjusted to pH 9.1 with NaOH). With a small sample of cytosol, e.g. 6 ml, the recovery of GSH transferase activity is generally more than 95%.

For analysis of GSH transferases of erythrocytes, blood obtained by cardiac puncture was collected into heparinized tubes at 0–4 °C. The erythrocytes were collected by centrifugation, washed five times with 4 vol. of 0.15 M-NaCl and lysed with 4 vol. of a solution containing 1 mM-EDTA, 0.2 mM-dithiothreitol and 50 μM-phenylmethanesulphonyl fluoride. The lysate was centrifuged and the GSH transferases were isolated as described above.

Rapid purification of GSH transferase 8–8 by using selective elution from GSH–agarose by GSSG

Cytosol preparations from different organs were applied to a column of GSH–agarose and washed with buffer A as described above. The column was then washed with 1 vol. of buffer A lacking dithiothreitol (buffer C). GSH transferases 8–8 (and a form of GSH transferase 1–1, when present) were selectively eluted with 5 mM-GSSG in buffer C. Buffer B could be used to elute the remaining GSH transferases.

Purification of GSH transferase 8–8 by anion-exchange chromatography

GSH transferase 8–8 was also purified from the combined GSH transferase fraction (obtained as described above) by desalting into 20 mM-Tris/HCl buffer, pH 8.0, containing 10% (v/v) glycerol and fractionation by anion-exchange f.p.l.c. on Mono Q (HR 5/5 column)

§ To whom correspondence should be addressed.

equilibrated in this buffer and eluting with a gradient of NaCl.

Analysis of GSH transferases

GSH transferase subunit composition was determined by reverse-phase h.p.l.c. essentially as described by Ostlund Farrants *et al.* (1987), except that for better resolution and greatly increased yield (approaching 100%) a 30 nm-pore-size Dynamax C_{18} column (30 cm × 4.6 mm) (Rainin Instrument Co., Woburn, MA, U.S.A.) was used. Elution was monitored at 214 nm and peaks were integrated by using a Hewlett–Packard Integrator 3390A. The protein content of peaks was then obtained by reference to those obtained with purified GSH transferase standards, for which the protein content was determined by the method of Bradford (1976), with ovalbumin as standard. Samples analysed contained 5–35 μg of protein. Apparent M_r values were determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Samples from h.p.l.c were neutralized with 0.5 M-NH_4HCO_3 containing a trace of Bromophenol Blue and dried under N_2 before SDS/polyacrylamide-gel electrophoresis.

GSH transferase activity was determined at 37 °C as described by Habig *et al.* (1974) and Ålin *et al.* (1985).

Direct amino acid sequence analysis of GSH transferase 8–8 in an Applied Biosystems gas-phase sequencer showed the *N*-terminus to be blocked. Peptides were generated by reaction with CNBr in 90% formic acid for 16 h and purified for sequencing by reverse-phase h.p.l.c. as described by Taylor *et al.* (1988).

RESULTS AND DISCUSSION

Rapid purification and characterization of GSH transferase 8–8

If a mixture of rat GSH transferases obtained from testis and kidney are bound to GSH–agarose under standard conditions and the matrix is washed with GSSG at neutral pH, two subunits are selectively eluted (Fig. 1). These correspond to subunits previously termed 1a and 1d (Ostlund Farrants *et al.*, 1987). The latter is shown below to be subunit 8 (Jensson *et al.*, 1986). Elution with GSSG therefore simplifies the purification of the homodimers 1–1 and 8–8, and with a tissue such as lung, which lacks subunit 1, GSH transferase 8–8 may be isolated in a single chromatographic step (Fig. 2). The enzyme so purified was identical with GSH transferase 8–8 (Jensson *et al.*, 1986) by the following criteria: (i) co-migration on SDS/polyacrylamide-gel electrophoresis (results not shown); (ii) similar specific activities towards 1-chloro-2,4-dinitrobenzene, ethacrynic acid and 4-hydroxynonenal (19, 18 and 170–240 units/mg respectively); (iii) the same retention time on h.p.l.c.; (iv) a blocked *N*-terminus and the presence of amino acid sequences characteristic of subunit 8, namely MEVKPLYYFQGRGR, MLLTQTRAIL and VEEVSAPVLSDFPLLQAF (cf. Ålin, 1988). Less than 1% of the GSH transferase activity towards 4-hydroxynonenal was present in the fraction that failed to bind to the affinity column.

The basal concentration of GSSG in cytosol of 10–450 μM (Kosower & Kosower, 1978), which in the laboratory gradually increases through oxidation of GSH, is likely to prevent efficient retention of GSH transferases 1–1 and 8–8 by GSH–agarose under the conditions described by Vander Jagt *et al.* (1985). This would explain why yields of GSH transferases 1–1 and 8–8 are improved by inclusion in the homogenization buffer of dithiothreitol to reduce GSSG.

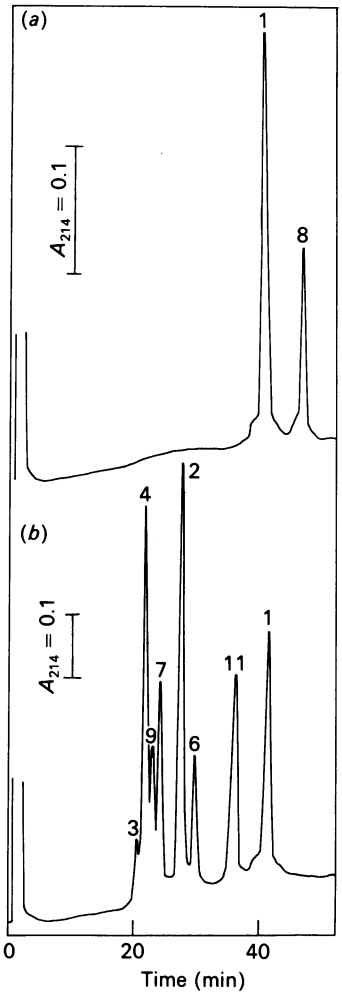

Fig. 1. Specific elution of GSH transferases from GSH–agarose by GSSG

A mixture of GSH transferases from cytosol preparations of kidney (1 ml) and testis (0.5 ml) was bound to a 1.1 cm × 1.5 cm column of GSH–agarose and washed with buffer A followed by buffer C as described in the Experimental section. GSH transferases were then eluted with 5 ml of 5 mM-GSSG in buffer C followed by 5 ml of 5 mM-GSH at high pH (buffer B). Samples (0.8 ml) of the GSSG eluate (*a*) and of the GSH eluate (*b*) were analysed by reverse-phase h.p.l.c. GSH transferase subunits are numbered according to the scheme of Jakoby *et al.* (1984).

The selective elution by GSSG may prove useful in identifying enzymes similar to rat GSH transferase 8–8 in other species.

We were unable to confirm the presence of two forms of subunit 1 in rat lung as reported by Singh *et al.* (1984): perhaps subunit 1 was confused with subunits 7 and 8, which had not been discovered at that time.

Two forms of subunit 8

If GSH transferase 8–8 is separated from the other isoenzymes by anion-exchange chromatography rather

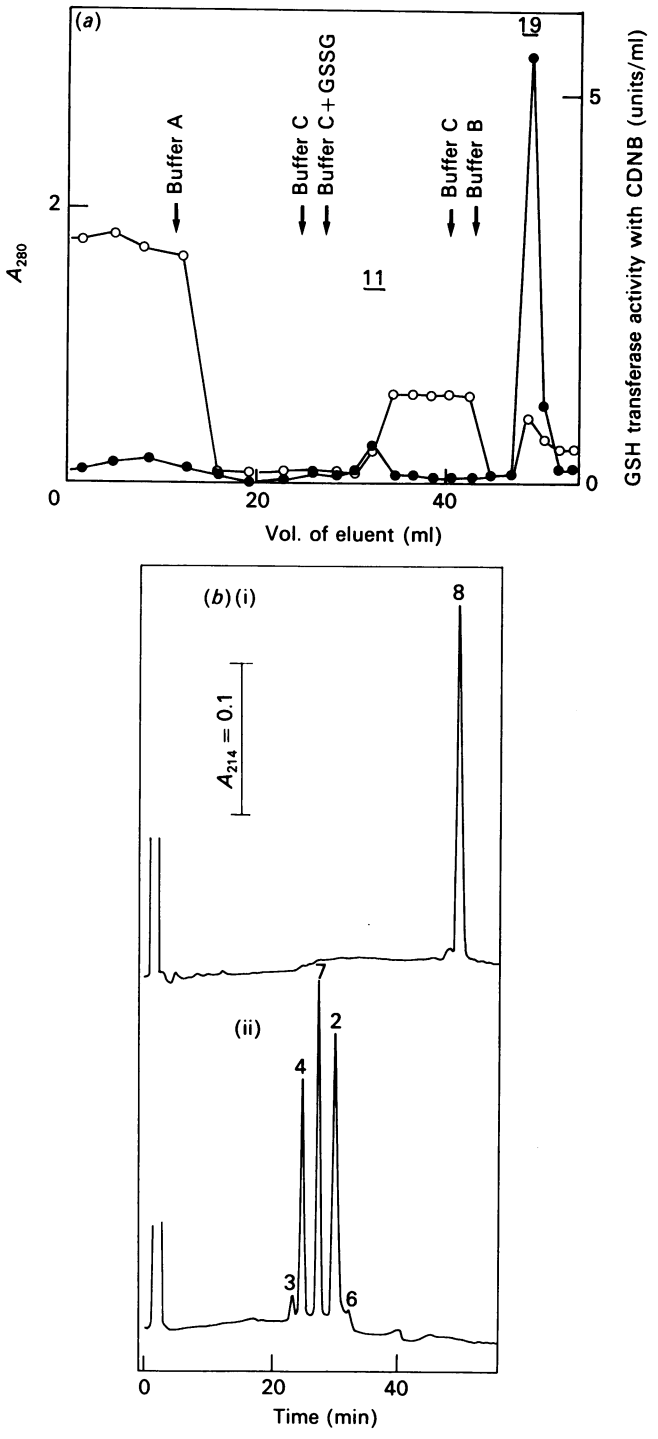

Fig. 2. Single-step purification of GSH transferase 8–8

The cytosol (15 ml) prepared from lungs of four rats (8 g) was fractionated on a 1.1 cm × 2.0 cm column of GSH–agarose (*a*). The column was washed with 15 ml of buffer A and 4 ml of buffer C, then GSH transferase 8–8 was eluted with 15 ml of buffer C containing 5 mM-GSSG. The column was washed again with 4 ml of buffer C, and the remaining GSH transferases were eluted with 10 ml of buffer B. Fractions were assayed for activity towards 1-chloro-2,4-dinitrobenzene (CDNB) (●) and for absorbance at 280 nm (○). The two peaks of activity were analysed by reverse-phase h.p.l.c. (*b*): (i) fraction 11 (300 μl), eluted by GSSG; (ii) fraction 19 (100 μl), eluted by GSH. GSH transferase subunits are numbered as indicated in Fig. 1.

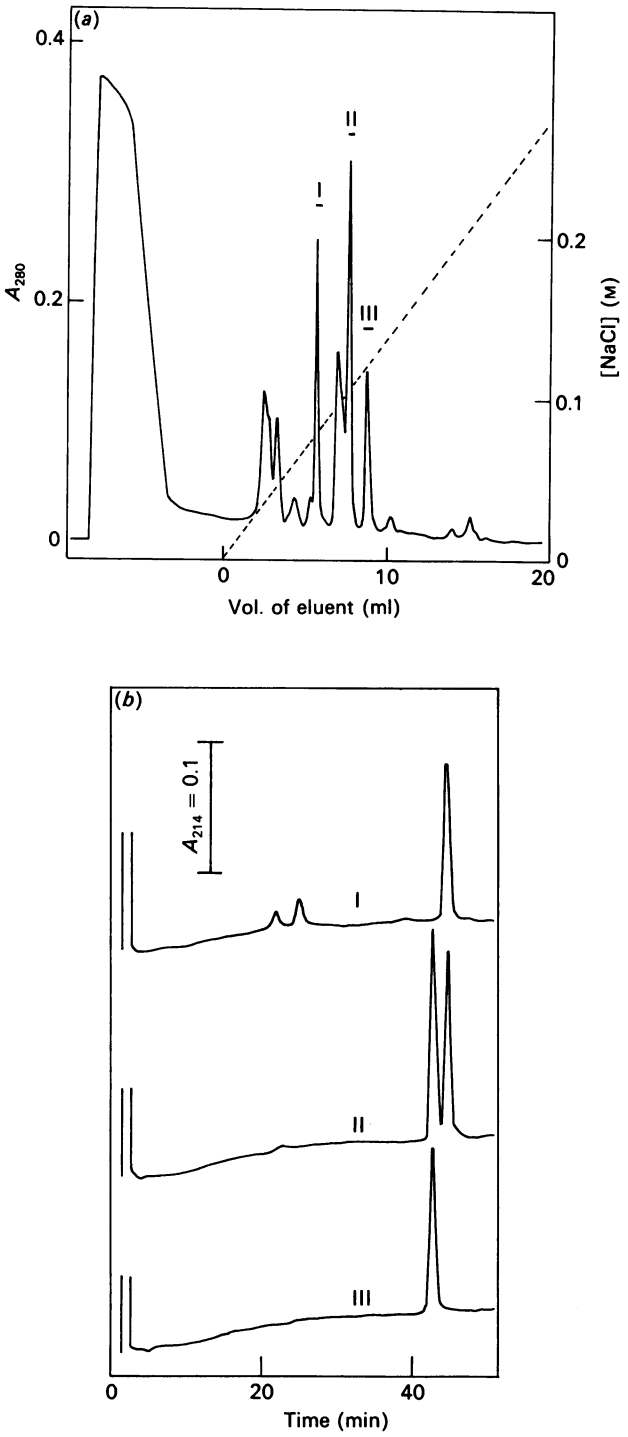

Fig. 3. Separation of GSH transferase 8–8 into three forms by anion-exchange f.p.l.c.

A combined GSH transferase fraction was prepared from 5.5 g of kidney by GSH elution from GSH–agarose as described in the Experimental section. GSH transferase 8–8 was then separated from the other isoenzymes by anion-exchange f.p.l.c. on a Mono Q column (HR 5/5) equilibrated in 20 mM-Tris/HCl buffer, pH 8.0, containing 10% (v/v) glycerol by using a gradient of NaCl (------) in the same buffer (*a*). Three fractions (I–III) containing GSH transferase 8–8 were identified by their high activity towards 4-hydroxynonenal and were further analysed by reverse-phase h.p.l.c. (*b*).

Table 1. Content in rat tissues of GSH transferase subunit 8

GSH transferase subunit 8 was quantified from reverse-phase h.p.l.c. analyses of the GSH transferase fraction obtained from various tissues by using GSH elution from GSH-agarose as described in the Experimental section.

Tissue or cell	Subunit 8 (μg/g of tissue or cells)
Liver	136
Testis	65
Kidney	41
Epididymis	40
Lung	17
Brain	14
Erythrocyte	10
Prostate	10

than by selective elution with GSSG, three fractions (I, II and III) are obtained (Fig. 3*a*), each having the characteristically high activities towards 4-hydroxynonenal and ethacrynic acid of GSH transferase 8–8. On h.p.l.c. analysis peak I gives a peak corresponding to that of the unfractionated preparation (Fig. 3*b*). Peak II gives the same peak together with an equivalent amount of another peak eluted 3 min earlier, whereas peak III gives only the earlier-eluted peak. This suggests that I is the native homodimer, III a modified homodimer and II the corresponding heterodimer. The formation of the earlier-eluted subunit appears to be an artifact of the purification procedure. Similar behaviour has been observed by Hayes (1986) for GSH transferase 8–8, termed YkYk, when analysed by hydroxyapatite chromatography.

Tissue distribution of GSH transferase 8–8

The GSH transferase 8–8 content of various rat tissues is given in Table 1. The results show that the subunit 8 content varies little more than one order of magnitude among the various tissues. The erythrocyte is noteworthy, since GSH transferase 8–8 is the predominant isoenzyme present (55% of the total by h.p.l.c. analysis) and corresponds to the form previously studied by Dirr & Schabort (1987). The relatively even distribution of GSH transferase 8–8 is consistent with its proposed role of detoxifying common products of lipid peroxidation in various tissues (Jensson *et al.*, 1986; Danielson *et al.*, 1987; Ketterer *et al.*, 1988).

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