XENOBIOTICA , 1997, VOL. 27, NO. 6, 573–586

Purification and characterization of hepatic glutathione transferases from an insectivorous marsupial, the brown antechinus (*Antechinus stuartii*)

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Received 16 September 1996

1. Five unique glutathione transferase isoenzymes were purified from the hepatic cytosol of an insectivorous marsupial, the brown antechinus. The purified GSTs were characterized by structural and catalytic properties including apparent molecular weight and isoelectric point, specificity towards model substrates, kinetic parameters, sensitivity to inhibitors and cross-reactivity with antisera raised against human GSTs.

2. An alpha class GST, Antechinus GST 1-1, predominated in the hepatic cytosol, representing 71% of the total GST purified. The substrate specificity of Antechinus GST 1-1 was similar to that of other alpha class GSTs, particularly with respect to its high activity with cumene hydroperoxide. The mu class was represented by three GST isoenzymes, Antechinus GST 3-3, GST 3-4 and GST 4-4. These isoenzymes represented 8, 2 and 10% of the total GST purified respectively. A single GST, Antechinus GST 22, belonged to the pi class of GSTs and represented 12% of the total GST purified. The hepatic GST isoenzyme ratio (by class) observed in the brown antechinus was more similar to that observed in the human than in rat.

3. A previous study investigating a herbivorous marsupial, the brushtail possum (*Trichosurus vulpecula*) also identified a predominant hepatic GST belonging to the alpha class and displaying peroxidase activity. The evolutionary conservation of a similar predominant GST isoenzyme in these marsupials suggests that they play an important role in the detoxication metabolism of these unique mammals.

Introduction

The glutathione transferases (GSTs†, EC 2.5.1.18) are a multifunctional family of enzymes that by both catalytic activity and direct binding functions make a significant contribution towards cellular protection against the toxic challenges of endogenous compounds and a variety of xenobiotics (Daniel 1993). The main function of GSTs involves the catalysis of the conjugation of electrophilic, hydrophobic compounds with cellular glutathione, increasing aqueous solubility and thus facilitating excretion of the compound. This pathway results in the detoxication of many endogenous electrophiles as well as exogenously derived environmental chemicals (Motoyama and Dauterman 1980, Ketterer and Meyer 1989). GSTs may further function in a protective role by covalently binding electrophilic compounds, resulting in their inactivation in the cell (Mannervik and Danielson 1988). GSTs also non-covalently bind numerous non-substrate ligands

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† GST, glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; SBP, sulphobromophthalein; EPNPP, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane; EA, ethacrynic acid; Sbf-Cl, ammonium 4-chloro-7-sulphobenzofurazan; tPBO, *trans*-4-phenyl-3-buten-2-one; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

0049–8254/97 $12·00

and may thus serve as intracellular binding, storage and transport proteins (Litwack *et al.* 1971, Listowsky *et al.* 1988). Mammalian cytosolic GSTs are presently classified into four multigene classes (alpha, mu, pi and theta), each of which consist of two or more genes that encode different subunit types. The evolutionary events which have led to the present-day diversity of GSTs have still to be clearly defined, however the cytosolic GSTs are thought to have diverged from a common ancestral gene belonging to the theta class (Buetler and Eaton 1992, Taylor *et al.* 1993). Diversification into distinct classes is thought to have occurred prior to the divergence of mammals (Mannervik and Danielson 1988).

Investigation of glutathione transferases in the brushtail possum revealed significant differences in the expression of this detoxication enzyme in comparison to eutherian mammals (Bolton and Ahokas 1997). Of particular significance was the finding that the brushtail possum expressed a single predominant hepatic GST isoenzyme belonging to the alpha class of GSTs (Possum GST 1-1). The peroxidase activity shown by this isoenzyme suggested that it might play a significant role in the detoxication of allelochemicals present in the eucalypt diet of the brushtail possum. However, the lack of GST isoenzymes from other GST classes may result in susceptibility to particular types of environmental chemicals in this herbivorous marsupial.

If the major role of possum GST 1-1 is to contribute to the detoxication of eucalypt allelochemicals, then this may be considered an adaptation to the species' floral environment, and thus diet. The evolutionary significance of this finding, to all Australian marsupials, is unknown at present. In light of the possible disadvantages of the expression of a predominant GST isoenzyme, it is important to determine if the pattern observed in the brushtail possum represents an evolutionary divergence within the marsupial sub-class. Investigations of GST expression in a wider range of marsupial species will therefore contribute to determining the potential effects of environmental chemicals on Australian marsupials. Thus, purification of the hepatic glutathione transferases of an insectivorous marsupial, the brown antechinus, was undertaken. The brown antechinus is a small dasyurid marsupial which is common to the forests of south-eastern Australia and characterised by an unusual life history strategy in which all males of the species die at the end of the annual breeding season (Wood 1970).

Materials and methods

Materials

CDNB, EA, SBP, EPNPP, Sbf-Cl, cumene hydroperoxide, glutathione, *S*-hexyl glutathione, dithiothreitol, EDTA, BSA, haematin, cibacron blue and Coomassie brilliant blue R were obtained from Sigma Chemical Co. (USA). DCNB, tPBO, tributyltin acetate, triethyltin bromide and triphenyltin chloride were obtained from Aldrich Chemical Co. (USA). Sepharose 6B, polybuffers, pharmalytes and the chromatofocusing resin PBE-118 were from Pharmacia (Sweden). Hydrogen peroxide was obtained from BDH Merck Chemicals (Australia), and trifluoroacetic acid from Applied Biosystems (USA).

Animals

Brown antechinus (*Antechinus stuartii*) were trapped in an area of sclerophyll forest near Powelltown, 70 km east of Melbourne, Victoria (Department of Conservation and Natural Resources permit RP-91-046). Elliot traps, baited with a mixture of oats and peanut butter, and containing shredded paper for nesting, were set at dusk and cleared at dawn. Ten adult male brown antechinus were trapped in mid–late June 1991, and nine adult female brown antechinus were trapped between late June and early August 1991 (winter). When in the field, animals were kept in the traps and food and bedding changed daily. Upon transportation to Melbourne, animals were housed in rat cages, with shredded paper bedding, and

provided with water and food daily. The food consisted of ground beef heart and liver, dry pet food and dried fruit as recommended by Woolley (1982). Animals were killed by cervical dislocation a maximum of 4 days after capture.

Preparation of cytosolic fraction

All procedures were carried out at 0–4 °C unless otherwise stated. Livers were perfused *in situ* with ice-cold 10 mM potassium phosphate buffer, pH 7·0, containing 1 mM EDTA, 2 mM dithiothreitol and 0·15 M KCl, then excised. Homogenization of the liver (10% w/v in the perfusion buffer) was performed using three passes on an Ultra-Turrax T25 homogeniser (Janke and Kunkel GMBH and Co., Germany). The homogenate was centrifuged at 10000 *g* for 20 min to remove cell debris, nuclei and mitochondria. The resulting supernatant was further centrifuged at 100000 *g* for 60 min. The cytosolic fraction used in this study was the resulting 100000 *g* supernatant. Cytosol was stored at −80 °C until used. This is a standard way of storing cytosol and no evidence of loss of activity was observed.

Enzyme assays

Glutathione transferase activity with the substrates CDNB, DCNB, EA, tPBO, EPNPP, and SBP was measured spectrophotometrically according to the method of Habig *et al*. (1974). Glutathione peroxidase activity towards cumene hydroperoxide and hydrogen peroxide was determined as described by Lawrence and Burk (1976). GST activity with the fluorescent substrate Sbf-Cl was determined as previously described (Bolton *et al*. 1994). All assays were carried out at 37 °C and specific activity expressed as μmol product formed/min/mg protein. Protein concentration of cytosols and pure isoenzyme samples were determined according to the methods of Lowry *et al*. (1951) and Bradford (1976) respectively. BSA was used as the standard.

Purification of glutathione transferases

All procedures were performed at 0–4 °C unless otherwise stated. Pooled samples of either male or female cytosols were diluted with 10 mM Tris-HCl, pH 7·8 and applied to an *S*-hexyl glutathione column in tandem with a glutathione-linked affinity column as described previously (Bolton and Ahokas 1997). Elution of glutathione transferases from the *S*-hexyl glutathione column was achieved using two elution buffers; 50 mM Tris-HCl containing 10 mM GSH, pH 7·0 followed by 50 mM Tris-HCl containing 10 mM GSH, pH 9·6 (Ooi *et al*. 1993). Two fractions of GSTs were therefore eluted, one containing acidic and near-neutral GSTs and the other containing basic GSTs. The glutathione column was washed with 22 mM phosphate buffer, pH 7·0 and the GSTs eluted using 50 mM Tris-HCl containing 5 mM GSH, pH 9·6. Purified GST samples were concentrated using an Amicon 8010 ultrafiltration unit with a YM 10 membrane (10000 molecular weight cut-off).

Affinity purified GSTs were further fractionated by chromatofocusing using an LKB 2150 hplc system (Sweden) fitted with a Mono P HR 5/20 column (5 × 200 mm, Pharmacia, Sweden). The column was pre-equilibrated at room temperature with 25 mM diethanolamine, pH 9·6. The first elution buffer, 1 ml Pharmalyte 8–10.5 and 5·2 ml Polybuffer 96 diluted to 200 ml with water and adjusted to pH 8 with 1 M HCl, was run on the column (5 ml) prior to sample application. The sample was diluted in the elution buffer prior to injection. The second elution buffer, Polybuffer 74 diluted 20 times with water and adjusted to pH 4 with 1 M HCl, was run onto the column after the pH of the eluant was stable at pH 8. The flow rate during elution was 0·5 ml/min. Column effluent was continuously monitored at 280 nm using a Spectroflow 757 Absorbance Detector (Kratos Analytical Instruments, USA) and pH was continuously monitored using a glass combination electrode. Each fraction was measured for GST activity at 25 °C using substrate concentrations of 1 mM CDNB and 1 mM GSH. Ampholyte removal and concentration of pooled fractions was achieved using an Amicon 8010 ultrafiltration unit with a YM 30 membrane (30000 molecular weight cut-off) (Ooi *et al*. 1993). Each fraction was washed five times with 22 mM phosphate buffer containing 1 mM dithiothreitol, 1 mM EDTA and 20% glycerol, pH 7·0 (4 °C). Pure isoenzymes were stored at −80 °C until used.

Hplc analysis of GST subunits

Hplc analysis of brown antechinus GST subunits was performed according to the method of Östlund Farrants *et al*. (1987) using a Brownlee Aquapore 7 μm, C_{18} reverse-phase column (220 mm × 4·6 mm, Applied Biosystems, Australia). The mobile phase linear gradient (35–55% B over 60 min) of 0·05% (v/v) trifluoroacetic acid in water (solvent A) and 0·05% trifluoroacetic acid in acetonitrile (solvent B) was employed at a flow rate of 1 ml/min.

Inhibition studies

Investigation of the inhibitory effect of cibacron blue, SBP, *S*-hexyl glutathione, haematin, tributyltin acetate, triphenyltin chloride and triethyltin bromide on Antechinus GST 1-1 was performed essentially as described by Tahir *et al*. (1985). Assays were carried out at 37 °C using substrate concentrations of 6 mM glutathione (saturating concentration) and 1 mM CDNB.

Kinetic constants

GST assays were performed in quadruplicate and at 37 °C with varying concentrations of CDNB (0·1–3·0 mM) and glutathione (0·1–7·5 mM).

SDS-polyacrylamide gel electrophoresis

The apparent molecular weight of the purified Antechinus GST isoenzyme subunits was determined according to the procedure of Laemmli (1970) using SDS-PAGE in 12% acrylamide/2·6% bis-acrylamide. After electrophoresis, protein bands were visualized using Coomassie blue staining. The protein molecular weight standards used were 14·4 kDa (hen egg white lysozyme), 21·5 kDa (soyabean trypsin inhibitor), 31 kDa (bovine carbonic anhydrase), 45 kDa (hen egg white ovalbumin), 66·2 kDa (bovine serum albumin) and 97·4 kDa (rabbit muscle phosphorylase B) (Biorad Laboratories, USA).

Immunochemical methods

Antisera raised in rabbit to human alpha (GST A1-1), mu (GST M1-1) and pi (GST P1-1) GST were a kind gift from Professor Bengt Mannervik, Department of Biochemistry, Uppsala University. Brown antechinus GSTs were electrophoretically transferred onto nitrocellulose membrane according to the method of Towbin *et al.* (1979). Upon complete protein transfer (30 V for 16 h), the membrane was washed, blocked and the colour developed using a Biorad Immun-blot assay kit. All steps were performed according to the manufacturer's instructions. The nitrocellulose membrane was incubated for 1 h with human GST antisera (diluted 2000 times). The secondary antibody employed was goat anti-rabbit IgG alkaline phosphatase conjugate (Biorad).

Results

Purification of brown antechinus glutathione transferases

Affinity chromatography yielded two fractions containing partially purified glutathione transferases. Both fractions were obtained from the *S*-hexyl glutathione affinity column. Although only 81% of the starting CDNB activity was recovered from this column (table 1), the use of a glutathione affinity column loaded with the wash from the *S*-hexyl glutathione affinity column yielded no further glutathione transferases (measured via CDNB activity). The use of two elution buffers of differing pH allowed initial fractionation of GSTs into a basic and an acidic/near-neutral fraction. This method was particularly useful since the basic fraction was shown to contain a single isoenzyme, termed Antechinus GST 1-1. This fraction represented 70% of the total cytosolic GST purified (table 1) and hplc analysis showed a single subunit peak with a retention time of 37 min.

Hplc analysis of the acidic/near-neutral fraction showed the presence of four subunit peaks, with retention times of 25, 26, 32 and 37 min (figure 1). This fraction was further purified using chromatofocusing (figure 2) to yield five pure isoenzyme peaks. These isoenzymes are named in order of their purification. The first peak yielded a further small amount of Antechinus GST 1-1 and eluted in the void volume. Antechinus GST 2-2 yielded a single subunit peak at 25 min upon hplc analysis, and Antechinus GST 3-3 consisted of a single subunit peak at 26 min. Hplc analysis showed that the following protein peak consisted of two subunit peaks with retention times of 26 and 32 min. This isoenzyme is termed Antechinus GST 3-4, and was the only heterodimer purified. The final isoenzyme purified, Antechinus GST 4-4, consisted of a single subunit peak at 32 min when analysed by hplc.

A summary of the purification of brown antechinus glutathione transferases is shown in table 1. Estimation of the proportion of total GST purified contributed by each isoenzyme shows that 71% of the GST purified was Antechinus GST 1-1. Antechinus GST 2-2 and Antechinus GST 4-4 represented 12 and 10% of the total GST protein, with Antechinus GST 3-3 and Antechinus GST 3-4 contributing lesser proportions of the total soluble hepatic GST content of this marsupial species.

Table 1. Purification of brown antechinus hepatic glutathione transferases.[a]

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)[b]	Specific activity (μmol/min/mg)[abc]	Fold purification	Yield (%)	Fraction of total GST protein purified (%)
Cytosol	49	210	3381	1·04	1		
Affinity chromatography							
Acidic fraction	19	0·4	380	60	58	11	28
Basic fraction							
(Antechinus GST 1-1)	26	1·0	2366	69	66	70	70
Chromatofocusing							
Antechinus GST 1-1	1	nd	20	69	66	0·6	1
Antechinus GST 2-2	3	nd	307	8·9	8·6	9·1	12
Antechinus GST 3-3	4	nd	4	0·7	0·6	0·1	8
Antechinus GST 3-4	4	nd	2	1·4	1·4	0·1	2
Antechinus GST 4-4	4	nd	25	3·5	3·5	0·1	10

[a] Starting material was pooled cytosol obtained from 10 male animals (5 ml cytosol from each individual animal).

[b] Activity was measured with the substrate CDNB at 25 °C using the assay conditions described in the Materials and methods section.

[c] For fractions in which protein content was not determined in individual experiments, specific activity is estimated from that of pooled samples (determined at 25 °C).

nd, not determined due to the small amount of material available.

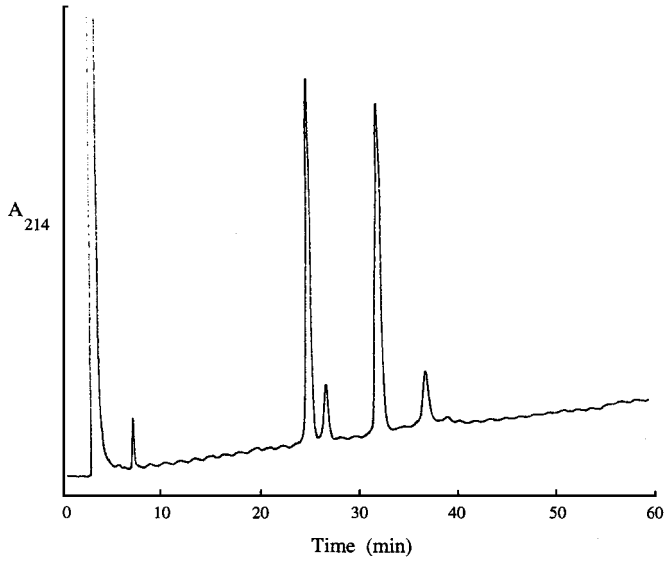

Figure 1. Analysis of brown antechinus glutathione transferase subunits by reverse-phase hplc. An aliquot (100 μl) of the acidic fraction obtained after affinity chromatography of pooled male cytosol was injected onto the hplc column (7 μm, C_{18} reverse-phase) under the conditions described in the Materials and methods section. When similar samples obtained from female animals were analysed, a clear sex-difference in the GST isoenzyme profile was not observed.

Molecular properties

The apparent isoelectric point of individual brown antechinus GSTs was determined as the pH of elution from the chromatofocusing column. Antechinus GST 1-1 eluted in the void volume, indicating that its *p*I was greater than the pH of the starting buffer (pH 9·6). A sample of this isoenzyme was chromatofocused between pH 11 and 8 using PBE-118 and eluted at pH 9·7. The remaining brown antechinus GSTs displayed a range of isoelectric points, from the basic Antechinus GST 2-2 (*p*I 8·7), to the near-neutral Antechinus 3-3 (*p*I 6·9) and acidic Antechinus 3-4 and 4-4 (*p*I 5·7 and 5·6 respectively).

The subunit molecular weights of brown antechinus GSTs were determined by SDS-PAGE (figure 3). Antechinus GSTs 1-1, 2-2, 3-3 and 4-4 were homodimers of subunits with M_r = 24800, 25700, 25700 and 27200 respectively. Although there was insufficient Antechinus GST 3-4 protein available to run on the gel, it is assumed based on hplc analysis that this isoenzyme consists of a heterodimer of two subunits with M_r = 25700 and 27200.

Substrate specificities and kinetic constants

Five male and five female cytosols were randomly chosen and cytosolic substrate activities measured (table 2). Significant sex differences were observed in both 1-chloro-2,4-dinitrobenzene (male > female, $p \leqslant 0{\cdot}007$) and ethacrynic acid (female > male, $p \leqslant 0{\cdot}004$) activity. The limited sample available for study necessitated the pooling of individual samples into sex-specific groups for the purification of

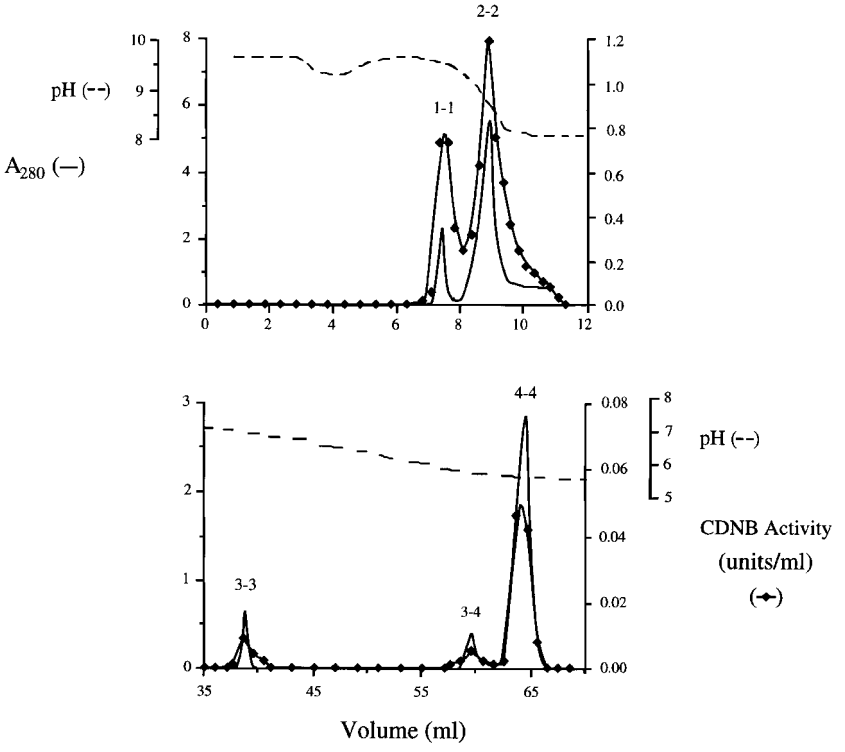

Figure 2. Separation of brown antechinus hepatic glutathione transferases using chromatofocusing. Preliminary runs showed that the final three peaks could be more easily visualized if the sensitivity of the absorbance detector was increased after the elution of the second peak. Thus, these two parts of the trace are shown on different axes. The acidic/near-neutral fraction of affinity purified GSTs was applied to a Mono P HR 5/20 column equilibrated with 25 mM diethanolamine, pH 9·6. Elution was performed using ampholine buffers at pH 8 and 4 (see experimental procedures). The absorbance of the eluant was monitored continuously at 280 nm, fractions collected and their CDNB activity measured. Individual GST isoenzymes were named according to their order of purification. Antechinus GST 3-4 was identified as a heterodimer using hplc analysis of the relevant fractions.

cytosolic GSTs. Hplc analysis of the pooled male and pooled female affinity purified GSTs did not indicate a clear sex difference in GST isoenzyme profile.

The substrate specificities of the pure antechinus GSTs are shown in table 3. CDNB displayed broad specificity for antechinus GSTs, although the greatest activity was observed with Antechinus GST 1-1. EA was also a substrate for all of the antechinus GSTs, with Antechinus GST 2-2 displaying the greatest specificity towards this substrate. Antechinus GST 1-1 was the only isoenzyme that displayed activity with the substrates DCNB and cumene hydroperoxide. Sbf-Cl activity was shown only by Antechinus GST 2-2.

Kinetic constants were determined for the three major brown antechinus GST isoenzymes using bisubstrate (CDNB and GSH) kinetic analysis (table 4.) Similarly to Possum GST 1-1 (Bolton and Ahokas 1997), the K_m for CDNB shown by Antechinus GSTs 1-1 and 4-4 was greater than this substrate's aqueous solubility, thus specific activities determined under standard conditions (1 mM CDNB and 1 mM GSH) are considerably below the V_{max}. The K_m for glutathione shown by Antechinus GST 4-4 was 10-fold lower than that of Antechinus GSTs 1-1 and 2-2

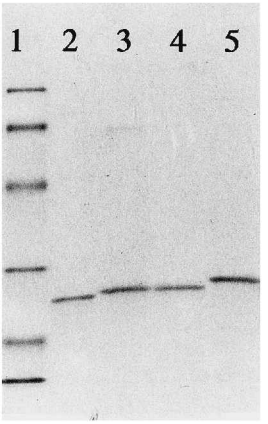

Figure 3. SDS/polyacrylamide-gel electrophoresis of brown antechinus glutathione transferases. Antechinus GST were analysed on a 12% SDS/polyacrylamide gel and proteins were visualized with Coomassie blue staining. Lane 1 contained protein molecular weight standards with M_r = 97400 (rabbit muscle phosphorylase B), 66200 (bovine serum albumin), 45000 (hen egg white ovalbumin), 31000 (bovine carbonic anhydrase), 21500 (soyabean trypsin inhibitor) and 14400 (hen egg white lysozyme). Lanes 2–5 contained approximately 0·1 μg Antechinus GSTs 1-1, 2-2, 3-3 and 4-4 respectively.

Table 2. Hepatic cytosolic glutathione transferase activity in the brown antechinus.

	Specific activity[a] (μmol/min/mg protein)	
Substrate	Male (n = 5)	Female (n = 5)
1-Chloro-2,4-dinitrobenzene	1·32±0·10[b]	0·84±0·09
1,2-Dichloro-4-nitrobenzene	0·02±0·00	0·02±0·00
Ethacrynic acid	0·03±0·00	0·06±0·01[c]
trans-4-Phenyl-3-buten-2-one	nd	nd
Sulphobromophthalein	nd	nd
Ammonium 4-chloro-7-sulfobenzofurazan	nd	nd
Cumene hydroperoxide	0·15±0·01	0·13±0·01
Hydrogen peroxide	0·07±0·00	0·07±0·00

[a] Values are shown as mean±SE.
[b] Males significantly greater than females, $p \leqslant 0{\cdot}007$.
[c] Females significantly greater than males, $p \leqslant 0{\cdot}004$.
nd, Activity not detected.

indicating differences in the catalytic efficiency and GSH-binding site between these brown antechinus GST isoenzymes. The ratio of k_{cat} to K_m was in the order of 10^4 for all of the antechinus GSTs investigated, indicating that these isoenzymes are efficient in their catalysis of this substrate.

Inhibitor sensitivity

The sensitivity of Antechinus GST 1-1 to a range of inhibitors is shown in table 5. Activity of Antechinus GST 1-1 toward the substrate CDNB was extensively inhibited by tributyltin acetate, haematin and triphenyltin chloride. Intermediate inhibition was observed with triethyltin-bromide and cibacron blue whereas the binding affinity of this GST for SBP and *S*-hexyl glutathione was low.

Table 3. Substrate specificities of brown antechinus glutathione transferases.

Substrate	Specific activity (μmol/min/mg protein)[a]				
	Antechinus GST 1-1	Antechinus GST 2-2	Antechinus GST 3-3	Antechinus GST 3-4	Antechinus GST 4-4
1-Chloro-2,4-dinitrobenzene	71·38 ± 1·10	24·87 ± 1·39	3·11 ± 0·27	11·03 ± 0·17	11·69 ± 0·07
1,2-Dichloro-4-nitrobenzene	0·37 ± 0·04	nd	nd	nd	nd
Ethacrynic acid	0·76 ± 0·05	2·83 ± 0·01	0·95 ± 0·04	2·07 ± 0·40	0·5 ± 0·02
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	nd	nd	nd	nd	nd
trans-4-Phenyl-3-buten-2-one	nd	nd	nd	nd	nd
Sulphobromophthalein	nd	nd	nd	nd	nd
Ammonium 4-chloro-7-sulphobenzofurazan	nd	0·05 ± 0·00	nd	nd	nd
Cumene hydroperoxide	5·45 ± 0·01	nd	nd	nd	nd
Hydrogen peroxide	nd	nd	nd	nd	nd

[a] Values shown as mean ± SE, where $n \geqslant 5$.
nd, Not detected.

Table 4. Kinetic parameters of brown antechinus glutathione transferases.[a]

Isoenzyme	Glutathione[b]		1-Chloro-2,4-dinitrobenzene[c]		
	K_m^{GSH} (mM)[d]	K_m^{CDNB} (mM)[d]	V_{max} (μmol/min/mg)[d]	k_{cat} (s^{-1})[d]	k_{cat}/K_m ($M^{-1}s^{-1}$)
Antechinus GST 1-1	0·73 ± 0·03	3·96 ± 0·45	351 ± 27	290 ± 22	$7·3 \times 10^4$
Antechinus GST 2-2	0·29 ± 0·04	1·21 ± 0·30	90 ± 3	77 ± 3	$6·4 \times 10^4$
Antechinus GST 4-4	0·03 ± 0·00	3·20 ± 0·48	44 ± 2	40 ± 2	$1·2 \times 10^4$

[a] Apparent kinetic parameters are reported since saturating concentrations of 1-chloro-2,4-dinitrobenzene were greater than its aqueous solubility.
[b] Measured with 3 mM 1-chloro-2,4-dinitrobenzene.
[c] Measured with saturating glutathione concentration.
[d] Mean ± SE, where $n = 4$.

Table 5. Inhibition characteristics of brown antechinus glutathione transferase 1-1.

Inhibitor	I_{50} (μM)[a]
Cibacron blue	2·10
Tributyltin acetate	0·22
Triethyltin bromide	1·23
Triphenyltin chloride	0·80
Sulphobromophthalein	7·93
Haematin	0·77
S-hexylglutathione	19·30

[a] The I_{50} is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6·5 and 37 °C, with 1 mM CDNB and 6 mM glutathione as substrates.

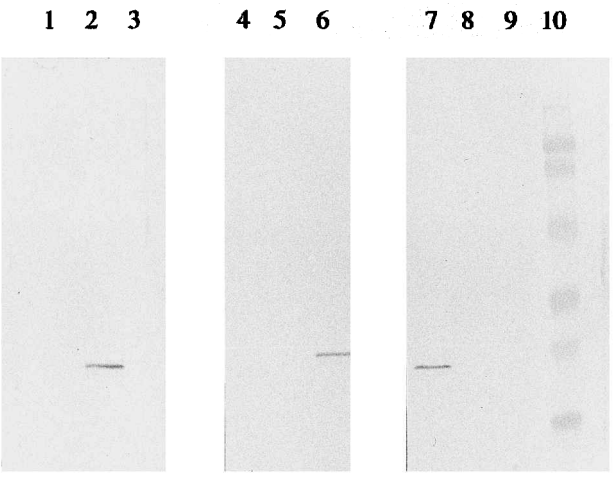

Figure 4. Immunological relationships of brown antechinus glutathione transferases. Antechinus GSTs 1-1, 2-2 and 4-4 were investigated for immuno-cross reactivity with antisera raised against human alpha, mu and pi GSTs using Western blotting. Lanes 1–3, 4–6 and 7–9 were treated with antisera raised against human pi, mu and alpha GST respectively. Lanes 1, 4 and 7 contained 0·1 μg Antechinus GST 1-1. Lanes 2, 5 and 8 contained 0·1 μg Antechinus GST 2-2. Lanes 3, 6 and 9 contained 0·1 μg Antechinus GST 4-4. Lane 10 contained prestained molecular weight standards (Novex).

Immunochemical analysis

Immuno-cross reactivity of Antechinus GSTs 1-1, 2-2 and 4-4 with antisera raised against human alpha, mu and pi GST was investigated using Western blotting techniques (figure 4). Antechinus GST 1-1 showed immuno-cross reactivity with antisera raised against human alpha GST, whereas Antechinus GST 2-2 and 4-4 displayed immuno-cross reactivity with antisera raised against human pi and mu GSTs, respectively.

Discussion

Five GST isoenzymes were detected in hepatic cytosol from adult male and female brown antechinus. All forms were purified to apparent homogeneity using a combination of affinity chromatography and chromatofocusing.

Antechinus GST 1-1 represented 71% of the total GST protein purified (table 1) and was characterized by a combination of catalytic and structural properties.

Comparison of these characteristics with those of GSTs of known class purified from other mammalian species indicates that Antechinus GST 1-1 belongs to the alpha GST family. Similarly to Possum GST 1-1 (Bolton and Ahokas 1997) and other members of the alpha class of GSTs, Antechinus GST 1-1 displayed a highly basic apparent isoelectric point, an intermediate molecular weight, substrate specificity towards an organic hydroperoxide (cumene hydroperoxide) (table 3), high affinity towards haematin and low affinity toward cibacron blue (table 5) (Mannervik *et al*. 1985, Mannervik and Danielson 1988).

The classification of Antechinus GST 1-1 as an alpha class GST was confirmed by investigation of structural characteristics of the enzyme. Antechinus GST 1-1 cross-reacted with antisera raised against human alpha GSTs and showed no reaction with antisera raised against human mu and pi GSTs (figure 4).

Four minor GST isoenzymes were purified from brown antechinus hepatic cytosol (table 1). These isoenzymes were characterized by catalytic and structural properties and their classification was achieved using immunological cross-reactivity with antisera raised against human alpha, mu and pi GST's. Antechinus GST 2-2 displayed cross-reactivity with antisera raised against human pi GST, whereas Antechinus GST 4-4 showed an immunological cross-reaction with antisera raised against human mu GST (figure 4). Insufficient samples were available to investigate the immunological properties of Antechinus GST 3-3 and 3-4. Dimeric structures with combinations of subunits from different GST families have not been observed in other mammalian species (Mannervik and Danielson 1988). Since Antechinus GST 4-4 is a mu class GST, and Antechinus subunit 4 forms a heterodimer with Antechinus subunit 3, it is assumed that both Antechinus GST 3-3 and 3-4 also belong to the mu GST family. Thus, brown antechinus alpha class GSTs were represented by a single isoenzyme that contributed 71% of the total hepatic soluble GST content. Mu class GSTs were represented by three isoenzymes and contributed a total of 20% of the GSTs purified. Pi class GSTs were represented by one isoenzyme that constituted 12% of the total hepatic soluble GST content in the brown antechinus. Clearly, the liver of the brown antechinus contains three distinct GST classes, formed by at least four unique subunits. Assuming similarity to findings in other mammals (Mannervik *et al*. 1985, Board *et al*. 1990), the distinct proteins belonging to each GST class are most likely the products of at least three separate genes, a speculation that remains to be confirmed.

Like the brushtail possum (Bolton and Ahokas 1997), the brown antechinus expresses an alpha class GST as the predominant hepatic isoenzyme. In contrast to the possum, the brown antechinus also expresses isoenzymes belonging to both the mu and pi GST class, although these isoenzymes are relatively minor in their contribution to the overall soluble hepatic GST content. In comparison with the eutherian mammals, the marsupials studied clearly express a different GST isoenzyme profile, in which a single (alpha) GST isoenzyme predominates. However, when the isoenzyme pattern is compared in terms of GST classes, a similar profile is observed in man and the brown antechinus, in which alpha GSTs predominate (71 and 75% for antechinus and man respectively), mu GSTs are intermediate (20 and 30% respectively) and pi GSTs are a minor contributor (12 and 4% respectively) (van Ommen *et al*. 1990). In contrast, the rat profile shows that alpha and mu GSTs are both major representatives (37 and 54% of total hepatic GST respectively) (Jernström *et al*. 1988).

The evolutionary conservation of an isoenzyme pattern in which a dominant

hepatic alpha GST isoenzyme is expressed suggests that alpha GSTs have a functional role important to the successful survival of these marsupials. Both Antechinus GST 1-1 and Possum GST 1-1 display specificity towards organic hydroperoxide and it has been suggested that this activity is important for the detoxication of plant allelochemicals and their oxidised metabolites (Bolton and Ahokas 1997). Antechinus GST 1-1 lacks the inorganic hydroperoxide activity displayed by Possum GST 1-1, a finding that may result from the different diets of these marsupials. The brown antechinus does not feed directly on *Eucalyptus* foliage, but on insects which may have ingested eucalypt leaves (Fox and Archer 1984). Therefore in the brown antechinus, the need for detoxication of plant allelochemicals is concomitant to the need to detoxify xenobiotics found in the insect diet, and may thus result in expression of members of the mu and pi GST families. Alternatively, the expression of GST isoenzymes in these species may be influenced by presently unknown endogenous functions of these enzymes. Wildlife permit restrictions resulted in an unavoidable period (up to 4 days) when the animals were fed an unnatural diet and this may also have affected the GST expression profile in this species.

Previously, it had been postulated that the expression of a single predominant hepatic GST by the brushtail possum may result in a limited ability to detoxify a wide range of xenobiotics. The brown antechinus relies on its predominant hepatic GST to a lesser extent and expresses isoenzymes from three different GST classes. Thus, these findings indicate that the brown antechinus may have a greater adaptive ability for metabolism of environmental chemicals in comparison to the brushtail possum. Further investigation of detoxication enzymes in marsupials will thus contribute to environmental management in Australia.

Sex differences in GST isoenzyme profile were indicated by significant differences in cytosolic substrate activities for CDNB and ethacrynic acid (table 2). Since all brown antechinus GST isoenzymes displayed significant but varying specificity towards CDNB, the greater cytosolic activity with this substrate observed in male brown antechinus may represent an increased expression of one or more GST isoenzymes. These animals were trapped at around the time of the start of the annual breeding season. In males of the species, this period coincides with an increase in free corticosteroid concentration in the plasma (Lee *et al*. 1977). Glucocorticoids have been shown to transcriptionally up-regulate GST expression in the mouse (Rushmore *et al*. 1993, Rushmore and Pickett 1993). Sex-dependent regulation of GST isoenzyme expression has been observed in several mammalian species (Igarashi and Satoh 1989) and is thought to be influenced by a range of hormones (Srivastava and Waxman 1993). The mechanisms that regulate GST isoenzyme expression in marsupials may include both endogenous and exogenous influences, and remain to be elucidated. Importantly, sex-specific expression of xenobiotic metabolising enzymes may be of particular significance in a species, such as the brown antechinus, in which all males die at the end of the annual breeding season and GST expression during this period in the life history of the brown antechinus is worthy of further investigation.

In summary, five GST isoenzymes representing the alpha, mu and pi classes were purified from the hepatic cytosol of an insectivorous marsupial, the brown antechinus. The predominance of an alpha GST, similarly to that observed in an herbivorous marsupial, the brushtail possum, suggests an important functional role for these GSTs which is yet to be elucidated.

Acknowledgements

R.M.B. is the recipient of a John Storey Junior Memorial Scholarship (1991) and an Australian Postgraduate Research Award (1992–4).

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