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A Study of the Glutathione Transferase Proteome of *Drosophila melanogaster*: Use of S- Substituted Glutathiones as Affinity Ligands

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1. Introduction

Glutathione transferases (GSTs) are a widely distributed super-family of enzymes involved in detoxification, catalyzing the conjugation of a great range of electrophilic compounds with the tripeptide glutathione (Boyland and Chasseaud, 1969). Much of the interest in insect GSTs has, for many years, been focused on their role in the development of resistance to many insecticides (Enayati et al., 2005; Motoyama and Dauterman, 1980). The enzymes are involved in resistance to most of the major classes of insecticide. These include organophosphates (e.g. Lewis & Sawicki, 1971; Oppenoorth et al., 1977); organochlorines, especially DDT (Clark & Shamaan, 1984, Tang & Tu, 1994), chitin synthesis inhibitors (Sonoda & Tsumuki, 2005), and pyrethroids by both direct (Yamamoto et al., 2009b) and indirect (Vontas et al., 2001) mechanisms. There are many instances in which detoxication of insecticides has been shown to be catalyzed by GSTs of the Delta or Epsilon classes (Lumjuan et al., 2005; Tang & Tu, 1994; Wei et al., 2001) but GSTs from Omega and Zeta classes (Yamamoto et al., 2009a; Yamamoto et al., 2009b) have also been reported as being involved in insecticide resistance.

In addition to their well-established toxicological roles, it is becoming increasingly apparent that insect GSTs may be involved in a number of other important physiological processes. These include olfaction (Rogers et al., 1999), regulation of apoptosis (Adler et al., 1999; Udomsinprasert et al., 2004), eye pigment synthesis (Kim et al., 2006), haeme binding (Lumjuan et al., 2007) and wound healing (Li et al., 2002). In order to disentangle these multiple roles, it is desirable to develop methods to characterize as fully as possible the expression under differing conditions of the many insect glutathione transferases.

In a previous study (Alias & Clark, 2007) the glutathione conjugate of bromosulfophthalein (BSP), a strong inhibitor of several and a substrate of some GSTs (Prapanthadara et al., 2000) was employed as a ligand. The use of this matrix resulted in the purification of GSTs from Sigma, Delta and Epsilon families but many members from these families were not detected and no members of the Zeta and Omega families were detected at all. In the present work

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the use of different ligands is examined, to determine to what extent ligand choice influences the part of the GST proteome thus isolated.

Although the catalytic repertoires of GSTs tend to overlap, different families of GST are likely to have catalytic activity with substrate(s) characteristic principally of that family (Jakoby, 1978; Yu, 2002). Many, but not all, GSTs use 2,4-dinitrochlorobenzene (CDNB) as a substrate and the use of the product conjugate (S-2,4-(dinitrophenyl) GSH (DNP-SG)) as a ligand might be expected to isolate a wide range of isoforms. On the other hand, in *Drosophila*, the substrate 3,4-dichloronitrobenzene (DCNB) is used by a more restricted range of isoforms (Alias & Clark, 2007), so that a matrix employing its glutathione conjugate, S-(2-chloro-4-nitrophenyl) glutathione (CNP), might be expected to bind a narrower range of GSTs. Similarly, in using S-(4-nitrobenzyl) glutathione (NB-SG) as ligand, by analogy with mammalian results (Jakoby, 1978), it was anticipated that a different range of GSTs again would be selected. In the present work, we explore the potential of this approach to define the GST proteome.

2. Material and methods

2.1 Materials

Sepharose 6B, epichlorhydrin, L-glutathione (reduced), CDNB, DCNB, lactate dehydrogenase, "modified" sequencing-grade trypsin, Coomassie Brilliant Blue G-250, and Protease Inhibitor Cocktail (general use) were purchased from Sigma-Aldrich. Immobiline™ Drystrips, Destreak™ reagent, Vivaspin™ centrifugal concentrators and HiTrap™ desalting columns (5 ml) were obtained from GE Healthcare (NZ). Bradford Protein Assay reagent and SDS 2D PAGE protein standards were from BioRad Laboratories NZ. Aluminium-backed Silica Gel 60 plates were from Merck Ltd. (NZ). Benchmark™ protein ladder was obtained from Invitrogen (NZ) Ltd.

All other materials and chemicals used were of the highest purity available commercially. Chromatography was carried out using an Amersham Bioscience AKTA FPLC™.

Drosophila melanogaster, wild-type adult, 5-day post-emergence, flies were supplied by the School of Biological Science, Victoria University of Wellington. They were collected and stored at -20 °C until required.

2.2 Synthesis of glutathione conjugates

These conjugates were prepared by incubating CDNB and DCNB or p-nitrobenzyl chloride in ethanol with GSH in deionised water at pH 9.6, adapting the method of Vince *et al* (1971). The mixtures were kept at room temperature for 5-48 hr depending on the reactivity of the compound. After incubation, ethanol was removed from the mixture by rotary evaporation. The conjugates DNP-SG, CNP-SG or NB-SG were precipitated by decreasing the pH 9.6 from to 3.0. The precipitate was filtered, redissolved and reprecipitated twice and dried *in vacuo*. The final product was chromatographically homogeneous when examined on 0.25 mm layers of silica gel 60 in butanol: acetic acid: water (4:1:5 - upper phase) using ninhydrin: collidine in ethanol (0.3: 5 : 95 (w:v:v)) as a location reagent (Lato *et al.*, 1974). The conjugates were immobilised on epichlorhydrin-activated Sepharose 6B as described by for the BSP-SG conjugate (Clark *et al.*, 1990).

The extent of substitution was estimated by dissolving aliquots of the gels by heating to 100°C in 6M HCl for 30 sec. The hydrolysates were neutralized with 1M-NaOH. The extent of substitution was calculated using extinction coefficients for DNP-SG and CNP-SG of 9600, 8400 l.mol⁻¹cm⁻¹ at 340 and 344 nm respectively (Habig et al., 1974b) and for NB-SG of 4210 l.mol⁻¹cm⁻¹ at 280nm. The extent of substitution was 9.55 µmol/ ml of gel for DNP-SG, 9.65 µmol/ ml of gel for CNP-SG and 11.5 µmol/ ml of gel for NB-SG.

2.3 Enzyme assays

Enzyme activity was assayed by measuring the conjugation of CDNB, DCNB and ethacrynic acid (EA) with glutathione (Habig et al., 1974b). Assays with *p*-nitrophenol acetate (*p*-NPA) (Keen et al., 1976), *trans*-2-nonenal (TNE) (Brophy et al., 1989) and dehydroascorbic acid (DHA) (Kim et al., 2006) were also carried out to study the specificity of the partially purified GST preparations.

2.4 Protein determination

During the affinity chromatographic procedures, protein was monitored by measuring the extinction of the collected fractions at 280 nm. For specific activity determination, samples of known enzymatic activity were pooled and concentrated using VivaSpin centrifugal concentrators (10kD) and protein was assayed by using the Bio-Rad protein assay kit, with bovine serum albumin (BSA) as the standard protein (Bradford, 1976).

2.5 Enzyme preparation

Adult fruit flies (1 – 3 g) were homogenised in five volumes of cold 0.05 M phosphate buffer, pH 7.4 containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM phenylthiourea (PTU) by using a Polytron™ homogeniser. Protease inhibitor cocktail was added to the concentration specified by the manufacturer and cysteine (2mg/ml) was added to prevent the oxidative darkening of the homogenate. The homogenate was centrifuged at 100,000 × g for 1h at 4°C in a Beckman XL-80 ultracentrifuge. The supernatant was filtered through glass wool and passed over a 5 ml HiTrap Desalting column which had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.4, at a flow rate of 30 ml/h. This column bound potentially inhibitory pigments with sufficient affinity to separate them from the excluded proteins. In some experiments the supernatant was chromatographed on a column of Sephadex G-25 (5x 25 cm) to remove pigments. Fractions containing activity towards CDNB were pooled. Losses in activity were negligible during this procedure, but up to 20% of the activity was lost if the enzyme was allowed to stand for 24h at 4°C. For this reason, affinity chromatography followed immediately after this procedure.

2.6 Affinity chromatography

The initial stage of the analysis involved chromatography of the pooled fractions from the depigmentation stage on a GSH-Sepharose column (1.0 x 5 cm) at a flow rate of 1 ml/min. The enzyme sample was followed by 0.05 M sodium phosphate buffer (Buffer A) until the absorbance at 280 nm had fallen to a value close to zero. Five volumes of 1.0 M NaCl buffered as above at pH 7.4 were then applied and followed again by five volumes of buffer A. The bound GSTs were eluted with 20 mM glutathione in 0.05 M sodium phosphate

buffer, pH 9.6. Fractions of 3 ml were collected. See Fig. 1. This column employed as its ligand glutathione, attached via its sulfhydryl group to epichlorohydrin-activated Sepharose (Clark et al., 1990). This matrix removes specifically, and almost quantitatively, glutathione transferases S1, D1 and D3 which enables a subsequent clearer separation of GSTs from other classes (Alias and Clark, 2007). The extent of substitution of this column routinely used was 10 $\mu\text{mol/ml}$ gel but substitution levels of up to 30 $\mu\text{mol/ml}$ gel were tested.

The aryl-substituted GSH matrices were packed under gravity in disposable plastic syringes to form columns of bed volume 5 ml. Void fractions from the GSH affinity matrix having significant activity with CDNB were pooled and applied to the columns. The bound GSTs were eluted with 20 mM GSH at pH 9.6 as described above. Aliquots from each stage of the purification were reserved for testing for activity with the test substrates. Experiments were performed in triplicate. The ligands for these matrices, having aryl substituents on the sulfhydryl group of the glutathione and therefore acting as product inhibitors competing with glutathione, are linked to the epichlorohydrin-activated Sepharose via the glutamyl amino group, as has been described for the glutathione conjugate of bromosulphophthalein (Clark et al., 1990).

2.7 Gel electrophoresis

Proteins in the material eluted from the affinity column were examined by SDS PAGE using standard conditions (Laemmli, 1970) in a 12% polyacrylamide gel and by 2D electrophoresis using the standard methods described below. In the first dimension isoelectric focusing was carried out in a Multiphor™ II Electrophoresis Unit (Pharmacia Biotech) apparatus. The precipitated proteins were mixed with rehydration buffer (8 M Urea, 2 % (w/v) CHAPS, 15 mM DTT, 30 mM thiourea) containing 2% pH 3-10 Ampholyte solution and Destreak™ reagent. Immobiline™ Drystrips (pI 4-7 and 3-10 for all samples) were rehydrated overnight and were focused in a three phase voltage programme. The first phase was at 200V for 1 min; second, 200-3500V ascending for 1.5h and the third phase was 3500V for 1.5h. Proteins in the gel were reduced with DTT and alkylated with iodoacetamide using standard methods. In the second dimension, the focused strips were run on SDS PAGE to separate proteins based on molecular mass. Proteins were located by staining with colloidal Coomassie Blue G-250 (Neuhoff et al., 1988). 2D gels were calibrated with respect to molecular mass using the BenchMark™ Protein Ladder and with respect to isoelectric point with BioRad SDS 2D PAGE protein standards.

2.8 Protein identification by MALDI-TOF

Protein spots were excised from gels and destained with 50 mM ammonium bicarbonate: acetonitrile (MeCN) (1:1). When destained, gel pieces were dehydrated and completely dried by vacuum centrifugation. The gel pieces were then rehydrated with 2 μl of 50 mM ammonium bicarbonate solution containing 0.125 μg of sequencing grade trypsin at room temperature. After 2-3 h, 30 μl of 50 mM ammonium bicarbonate solution was added to assist diffusion of peptides at room temperature overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by two washes of 30 μl for 1 h each with a solution containing 0.2% TFA and then with 0.2% TFA:MeCN (1:1). The extracts were then dried in a Speed-Vac for 2 h. The samples were stored at 4°C until required for analysis.

The MALDI-TOF analysis was performed using a PerSeptive Biosystem Voyager DE™ PRO Biospectrometry™ Workstation equipped with a delayed extraction unit. The mass spectra were obtained by using a Voyager Instrument Control Panel V 5.0 programme. The laser intensity was in the range of 1800-2100, and the accelerating voltage was 20 kV. Peptide spectra were obtained in positive reflectron mode in the range of 750 to 3500 *m/z*. The peptide solution was loaded onto the MALDI target plate by mixing with 2 µl of a matrix solution, prepared by dissolving 15 mg/ml alpha-cyano-4-hydroxycinnamic acid solution in 50% MeCN-0.1% trifluoroacetic acid (v/v), and allowed to dry. External calibration used Calibration Mixture 2 from Applied Biosystems. Each mass spectrum was generated by accumulating data from 150 to 200 laser shots. Database searches were carried out with the peptide masses against the non redundant NCBI database using the search programmes ProFound (<http://prowl.rockefeller.edu/>) (Zhang & Chait, 2000) and results were confirmed using MASCOT (www.matrixscience.com).

2.9 Assignment of GST family

GSTs identified by MALDI-TOF, which had not been explicitly assigned to particular families were matched against the *D. melanogaster* database in SwissProt Knowledgebase using the Basic Local Alignment Search Tool, BLAST (<http://web.expasy.org/blast/>). Family assignment was made on the basis of those known GSTs with which the test sequences had the greatest identity.

3. Results

Preliminary experiments showed that all three matrices bound GSTs from the Sigma, Delta, Epsilon and Omega classes. In order to reduce the complexity of the 2D gels obtained, an initial affinity purification of *D. melanogaster* extracts on GSH-agarose affinity was employed to remove the greater part of the Sigma and Delta sub-groups of GSTs. The GSTs removed had high specific activity for CDNB and 2D electrophoresis (not shown) confirmed earlier results that they consisted almost exclusively of GSTs S1, D1 and D3 (Alias and Clark, 2007). The use of matrix substituted with glutathione at up to 30 µmol/ml gel did not change the qualitative nature of the GSTs bound. As shown in Figure 1A and Table 1, a significant proportion of the material with CDNB activity (~40%) was not retained by the GSH affinity column.

The ability of the S-substituted matrices to adsorb the unbound activity was then tested. All of them did adsorb such activity but adsorption was not complete: between 25 and 50% of the applied activity (i.e. 10-25% of the original starting activity) was recovered in the void fractions. Material adsorbed and then eluted from these columns contained significant GST activity with CDNB. A typical elution profile is shown in Fig 1B in which the DNP-SG matrix was used. Material eluted from DNP-SG and CNP-SG columns contained significant activity with DCNB (Table 2) but the eluate from the NB-SG column did not show significant activity with this substrate.

Several model substrates were tested on the preparations and the results are shown in Table 2 below. All preparations showed activity with most of the substrates. The specific activities of the preparations from the DNP-SG and CNP-SG matrices with the different substrates were almost identical. That from the NB-SG matrix differed in that it showed no detectable activity with DCNB.

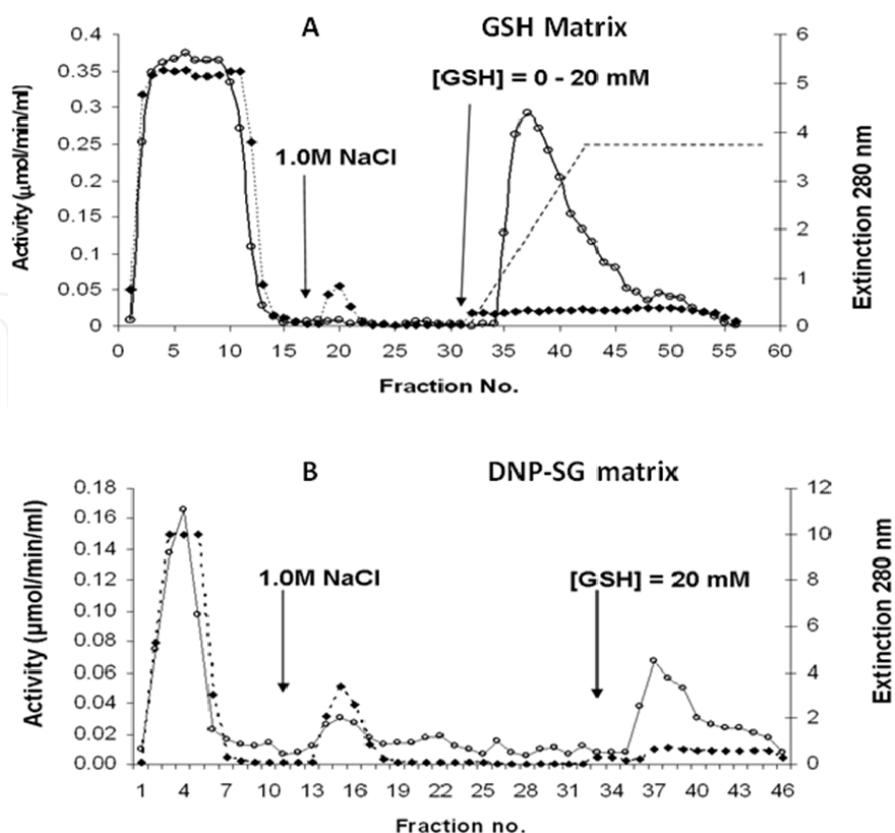


Fig. 1. Typical chromatography elution profiles from substituted glutathione affinity matrices. (A) shows chromatography on immobilised glutathione; (B) shows chromatography on the DNP-SG matrix. Open symbols, enzyme activity with CDNB. Closed symbols, extinction at 280 nm. Experiments were carried out in triplicate, as described in Section 2.6.

Procedure	Total activity ($\mu\text{mol}/\text{min}$) CDNB	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$) CDNB	Yield (%)	Purification (fold)
GSH matrix				
Applied	12.7 ± 1.4	0.132 ± 0.023	100	0.95
Unbound	6.00 ± 3.7	0.093 ± 0.04	43	0.67
Bound	7.00 ± 1.2	10.76 ± 4.67	51	77.9
DNP-GSH matrix				
Applied	3.00 ± 1.9	0.093 ± 0.04	100	1
Unbound	1.40 ± 0.5	0.051 ± 0.02	46	0.54
Bound	1.20 ± 0.6	3.529 ± 0.09	40	38
CNP-GSH matrix				
Applied	3.00 ± 1.9	0.093 ± 0.04	100	1
Unbound	1.70 ± 0.7	0.054 ± 0.02	56	0.58
Bound	1.30 ± 0.3	2.954 ± 0.77	43	32
NB-SG matrix				
Applied	0.86 ± 0.07	0.38 ± 0.03	100	1
Unbound	0.22 ± 0.03	0.24 ± 0.03	25	0.63
Bound	0.23 ± 0.01	7.9 ± 0.30	26	21

Table 1. Affinity chromatography of glutathione transferase activity from *D. melanogaster*. Chromatographic separations were carried out as described in Section 2.5 and 2.6. The results above are mean \pm S.D. from triplicate experiments.

Otherwise, the spectrum of activities was similar to that of the other two types of preparation. Of note were the high dehydroascorbate reductase activities that all preparations showed. The material eluted from the GSH column had the highest specific activity with CDNB.

The affinity-purified fractions obtained by using the DNP-SG and CNP-SG gels were concentrated, precipitated and subjected to 2D electrophoresis. The results from typical gels (pI = 4-7) after chromatography on these media are shown in Fig 2. Isoelectrofocusing over a 3-10 range of pI resulted in poorly resolved protein zones in the range 5-6 and these gels are not shown.

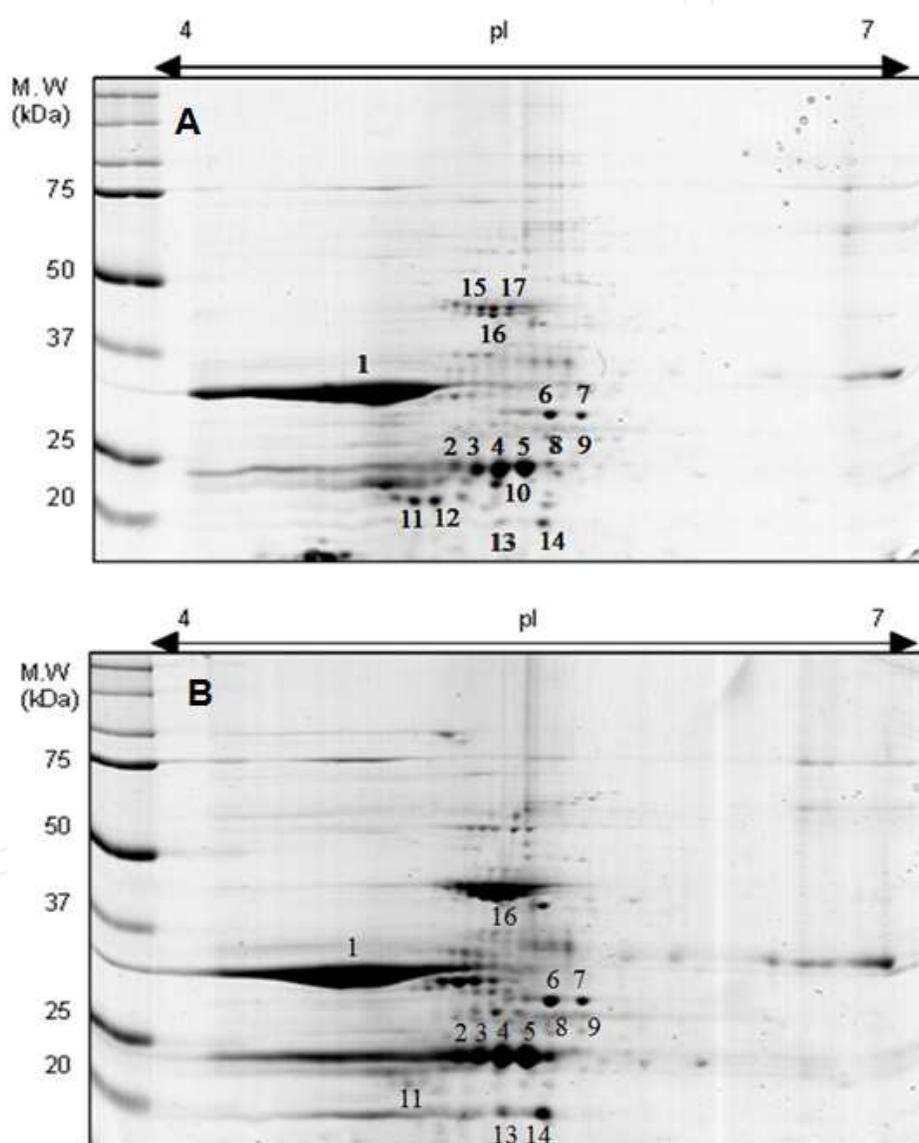


Fig. 2. Two dimensional gel electrophoresis on DNP-SG- (A) or CNP-SG- (B) affinity-purified *D. melanogaster* GSTs. Affinity-purified *D. melanogaster* GSTs were separated in the first dimension on a 7 cm pH 4-7 linear IPG strip and then in the second dimension on a 12% SDS-PAGE gel. The gel was stained with Coomassie Blue G250. The identification of the numbered spots can be found in Table 3 below.

Substrate	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)			
	Eluted from GSH	Eluted from DNP-SG	Eluted from CNP-SG	Eluted from NB-SG
CDNB	13.60 \pm 3.4	4.30 \pm 0.90	3.10 \pm 0.06	7.34 \pm 1.13
DCNB	n.s.	0.18 \pm 0.01	0.09 \pm 0.06	n.s.
NPA	1.00 \pm 0.40	1.30 \pm 0.46	1.50 \pm 0.52	1.99 \pm 0.75
TNE	0.84 \pm 0.48	0.35 \pm 0.16	0.16 \pm 0.04	0.41 \pm 0.12
DHA	3.30 \pm 0.70	11.1 \pm 6.4	12.65 \pm 0.43	27.4 \pm 6.3
EA	7.10 \pm 1.20	14.2 \pm 0.05	17.22 \pm 0.01	21.77 \pm 2.88

Table 2. Substrate specificity of glutathione transferases preparations from *D. melanogaster*. Activities were determined from triplicate experiments as described in Section 2.3

Proteins were identified by MALDI-TOF and are listed below in Table 3. Most marked are zones corresponding to DmGSTS1, not all of which had been trapped by the GSH column, and GST CG16936 (spots 2-5). The latter appears at four loci of the same apparent molecular mass and of differing pI. At higher isoelectric points (pI 6.7 and 8.0, not visible in gels covering the pH range 4-7) two Epsilon class GSTs, E3 and E9, were identified in DNP-SG preparations.

Spot No.	Flybase Annotation	Protein	Mr/pI (Theor.)	Mr/pI (Exp.)	P/C	Z score
1	CG8938	GST S1	27.6/4.6	33/4.6	8/47	2.43
2	CG16936	Epsilon GST	25.4/5.9	24/5.0	6/33	2.43
3	CG16936	Epsilon GST	25.4/5.9	24/5.1	9/49	2.35
4	CG16936	Epsilon GST	25.4/5.9	24/5.2	8/47	2.35
5	CG16936	Epsilon GST	25.4/5.9	24/5.3	7/38	2.35
6	CG6673	Omega GST	28.7/6.5	28/5.4	13/48	2.43
7	CG6673	Omega GST	28.7/6.5	28/5.5	15/46	2.43
8	CG6776	Omega GST	27.7/6.5	26/5.4	5/24	1.90
9	CG6776	Omega GST	27.7/6.5	26/5.5	6/22	2.09
10	CG10045	GST D1	23.9/6.9	23/5.2	4/26	2.43
11	CG3269	Drab2	23.7/5.8	20/4.8	7/37	2.43
12	CG32671	Rab9Fa	23.1/6.0	20/4.9	4/21	2.43
13	CG1707	Glyoxallase	20.14/6.1	19/5.2	11/60	2.37
14	C CG1707	Glyoxallase	20.14/6.1	19/5.3	13/72	2.39
15	CG8725	CSN4	46.7/5.9	46/5.1	11/33	1.40
16	CG8725	CSN4	46.7/5.9	46/5.2	9/29	2.43
17	CG9042	GPDH	44.7/6.4	46/5.2	13/38	2.00

Table 3. Identification of *Drosophila* GSTs after chromatography on DNP-SG or CNP-SG matrices. Protein zones are numbered as in Fig. 2. The proteins were identified using MALDI TOF MS peptide mass fingerprinting as described in section 2.7. The P/C column shows the number of peptides identified (P) and the percent coverage of the identified protein (C). Also shown are the theoretical and experimental values for Mr and pI of the identified proteins. A Z-score of greater than 2.34 indicates a probability of less than 1% that the result could have been attained by chance.

These had previously been detected on the gels analysing the product of BSP-SG chromatography (Alias and Clark, 2007). Spots 6 and 7 were identified as Omega GST CG6673 (isoform B). Fainter protein zones (8 and 9) were identified, with lower confidence, as the Omega GST CG6776.

Non-GST proteins eluted from the DNP-SG matrix were also identified. Spots 13 and 14 were identified as CG1707, lactoyl glutathione lysase (Glyoxlase I). Also eluted from the matrix were Rab small GTP-binding proteins (spots 11, 12; CG3269 and CG32671) and higher molecular weight contaminants including glycerol 3-phosphate dehydrogenase (spot 17; CG9042) and the COP9 complex homolog subunit 4 (CSN4, spots 15,16).

The same experiments carried out using CNP-GSH as ligand produced very similar results: 2D gels were almost indistinguishable and specific activities of the enzyme preparations obtained from the two different procedures were almost identical (See Table 2).

Identities of corresponding protein zones in gels resulting from the two types of affinity chromatography were determined independently. These are shown in Table 3 below.

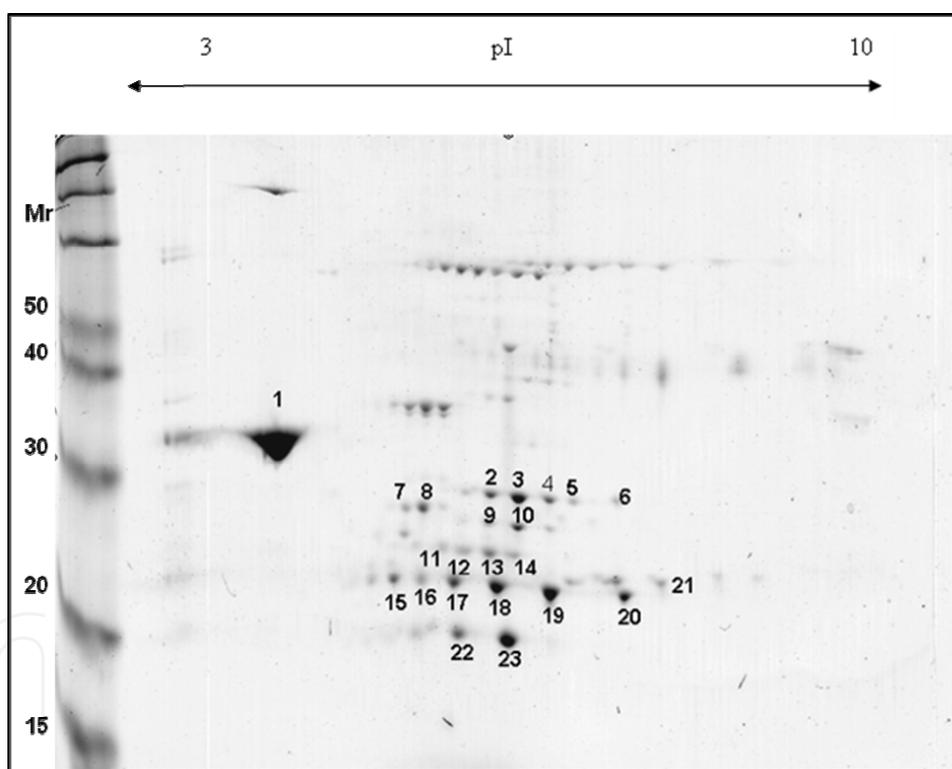


Fig. 3. Two dimensional gel electrophoresis of NB-SG affinity-purified *D. melanogaster* GSTs. Affinity-purified *D. melanogaster* GSTs were analysed as described in Section 2.7. The identification of the numbered spots can be found in Table 4 below.

When NB-SG was the affinity ligand, a rather different picture was seen (Fig. 3). As in the previous experiments Sigma GST had not been completely removed and there was detectable presence of GSTs D1 and D3. In addition to the Omega GSTs revealed by the other two matrices, the use of NB-SG showed the presence of CG6781, the Omega GST involved in synthesis of eye pigments (Kim et al. 2006).

Spot	Flybase Annotation	Protein	pI/Mr (Theor.)	pI/Mr (Exp.)	P/C	Z score
1	CG8938	GST S1	4.6/27.65	4.5/32.4	27/67	2.43
2	CG6673-PB	Omega GST	6.5/28.76	6.75/29.5	19/65	2.43
3	CG6673-PB	Omega GST	6.5/28.76	7.1/29.5	17/55	2.43
4	CG6673-PB	Omega GST	6.5/28.76	7.1/29.5	13/51	2.43
6	CG6673-PA	Omega GST	7.1/29.17	8.1/29.5	11/62	2.27
7	CG6781	Omega GST	5.5/24.87	5.9/28.6	5/22	2.43
8	CG6781	Omega GST	5.5/24.88	6.1/28.6	15/21	2.43
9	CG6776	Omega GST	6.5/27.76	6.75/27.4	7/18	1.35
10	CG6776	Omega GST	6.5/27.77	7.1/27.4	10/47	2.43
11	CG5224	Epsilon	5.6/25.36	6.25/26.0	9/30	1.71
12	CG11784	Epsilon	6.1/25.98	6.5/26.0	6/35	2.43
13	CG11784	Epsilon	6.1/25.99	6.75/26.0	9/48	2.43
14	CG11784	Epsilon	6.1/25.10	7.0/26.0	6/38	2.22
15	CG4381	GST D3	5.3/22.89	5.8/24.2	9/38	2.43
16	CG10045	GST D1	6.9/23.89	6.1/24.2	3/24	1.03
17	CG10045	GST D1	6.8/24.02	6.45/24.2	8/33	2.43
18	CG10045	GST D1	6.8/24.03	6.85/23.7	11/45	2.43
19	CG10045	GST D1	6.8/24.04	7.5/23.4	12/45	2.43
20	CG10045	GST D1	6.9/23.89	8.25/23.4	10/40	2.43
21	CG17524	GST E3	7.0/24.93	8.6/24.2	9/55	2.3
22	CG1707	Glyoxallase	6.1/20.14	6.5/21.7	8/44	2.43
23	CG1707	Glyoxallase	6.1/20.15	7.0/21.5	9/59	2.43

Table 4. Identification of *Drosophila melanogaster* GSTs after chromatography on the NB-SG matrix. Proteins identified from the NP-SG affinity-purified fractions of *D. melanogaster* separated over a pH range 3-10. Protein zones are numbered as in Fig. 3. The proteins were identified using MALDI TOF MS peptide mass fingerprinting as described in Methods. The P/C column shows the number of peptides identified (P) and the percent coverage of the identified protein (C). Also shown are the theoretical and experimental values for Mr and pI of the identified proteins. A Z-score of greater than 2.34 indicates a probability of less than 1% that the result could have been attained by chance.

Particularly noticeable was the absence of the putative GST CG16936 a protein which was prominent in gels of preparations isolated with the other two matrices. Multiple forms of the closely related protein, CG11784 were seen in these gels. These two proteins as well as CG5224 are closely related to the Epsilon class. Sequence analysis using the programme BLAST shows CG16936 to be closely aligned with GSTs E4 and E9, having 45 % identity with both: CG5224 was most similar to E9 and E10 (scores of 42% with both) and GCG11784 was most similar to E7 and E5 (scores of 37 and 36% respectively). As with the other matrices, the NB-SG matrix also bound higher molecular weight contaminants including glycerol 3-phosphate dehydrogenase (GPDH).

4. Discussion

The multiplicity and overlapping substrate specificities of the GSTs (Habig et al., 1974a; Jakoby 1978) renders them an extremely effective defence against many toxic compounds but this, along with overlapping cellular roles, may render it difficult to attribute particular functions to any individual or group of GSTs and in many contexts, it may in fact be more appropriate to regard them as acting as a complex, rather than as individual enzymes. The current work investigates the potential of the use of S-substituted glutathiones as affinity ligands to isolate and characterise the expression of the members of this super-family, selectively.

Several affinity chromatography systems have been used to isolate GSTs from many sources. Ligands tested have included glutathione immobilised through its sulphur group (Clark et al., 1990; Fournier et al., 1992) and glutathione (Yu, 1989) and S-substituted glutathione immobilised through the γ -glutamyl amino group. Ligands in the latter category have included the glutathione conjugate of the dye bromosulphophthalein (BSP-SG) (Alias & Clark, 2007; Clark et al., 1977), S-hexyl glutathione (Grant & Matsumura, 1989; Guthenberg et al., 1979). S-(2,4-dinitrophenyl) glutathione appears to have been employed as an affinity ligand only in the isolation of the ATP-dependent glutathione transporter (Awasthi et al., 1998).

In few studies on the affinity chromatography of insect GSTs has a detailed examination of the GST proteome thus isolated been made: the focus has usually been on the isolation of particular types of GST. Studies on *Musca domestica* (Clark et al., 1990; Fournier et al., 1992) and *D. melanogaster* (Alias & Clark, 2007) GSTs showed that the use of GSH immobilised through its thiol group led to the isolation and identification of a limited number of GSTs from only the Sigma and Delta families. These were, in the case of *D. melanogaster* S1, D1 and D3. The use of BSP-SG as ligand isolated more GSTs from the Delta and Epsilon families (Alias & Clark, 2007). These included D2 (elevated after treatment of the insects with phenobarbital), D1, D3, E3, E6, E7, E9 and an additional, putative Epsilon class GST, CG16936. A small fraction of the Sigma GST was also bound by this matrix. Several non-GST proteins were also bound. Of these, CG1707 (Glyoxallase I) was unsurprising since its substrate is lactoyl glutathione and it binds, presumably, via its affinity for the glutathione moiety. However, this matrix also bound a number of dehydrogenases including CG12262 (acyl CoA dehydrogenase), CG32954 (alcohol dehydrogenase), CG4665 (dihydropteridine reductase). The reason for binding of these proteins is not clear: they resist elution by high concentrations of salt and whether they bind through specific interactions with the matrix or by non-specific hydrophobic interaction or whether it may be through specific protein-protein interaction with bound GST proteins is not currently known.

Results from the present experiments show similarities to those obtained with the BSP matrix (Alias & Clark, 2007) but there are differences. There is, following use of the DNP-SG or CNP-SG materials a substantial zone of Sigma GST, larger than seen with use of the BSP matrix. This suggests that these materials have a stronger affinity for the Sigma GST than did the BSP matrix. The matrices currently under consideration appeared to have a differing selectivity for Epsilon class GSTs compared with the BSP-GSH matrix. Of this family, only E3, E9 and CG16936 are seen in common in preparations made with these matrices, the last-named protein being the most abundant.

A major difference from studies with the BSP-SG matrix is the appearance of four zones containing proteins from the Omega GST family, two identified as CG6673, isoform B (spots

6 and 7) and two (spots 8, 9) as CG6776. The Omega isoform CG6673B has a high dehydroascorbate reductase activity (Kim et al., 2006) and accounts for the high reductase activity in the material eluted from these columns. These isoforms also have minimal catalytic activity with CDNB (Kim et al., 2006) suggesting that GSTs may bind to GSH conjugates of a substrate with which they have little or no catalytic activity. This is supported by the observation that the matrices carrying conjugates of CDNB and DCNB, substrates that discriminate strongly between differing GSTs, appear to bind almost identical groups of proteins, both GSTs and non-GSTs. From this it may be concluded that differences in catalytic performance may be generated by relatively subtle changes in the active site that have little impact on the binding of product analogues. With regard to the Omega GSTs, this view is supported by the work of Whitbread et al. (2005) who demonstrated that the change of Cysteine 32 to Alanine resulted in an increase of activity with CDNB of over an order of magnitude.

A striking feature of the studies of the affinity chromatographic analysis of *D. melanogaster* GSTs is in the small number so far identified. In the works cited here, the number of GSTs positively identified is fourteen, only one third of the number possible (Tu & Agkul, 2005). This may reflect the modest expression of some isoforms. Heavy loading of gels indicates the presence of as yet unidentified proteins in the appropriate Mr range on 2D gels. It may also be that some isoforms have low affinity for the media so far studied. That might explain the absence of any members of Zeta or Theta families from our list of identified proteins.

5 Conclusions

5.1 The present work

The primary aim of this work was to determine whether affinity chromatography systems could be designed to characterise the proteome of the superfamily of glutathione S-transferases, with their multiple toxicological and metabolic roles, in a target organism. This aim has been partially successful. These experiments, in combination with the results from Alias & Clark, 2007 show that the use of different S-substituted glutathiones as affinity ligands results in the isolation of differing, but overlapping groups of GSTs from the Delta, Epsilon, Sigma and Omega families. It has not been possible to predict which will bind to a given matrix. The use of these media demonstrates the expression *in vivo* of a number of GSTs, particularly those related to the Epsilon class, the production of which has not previously been shown. Their use also establishes the presence of multiple forms of several GSTs. In the case of the Sigma and D1 isoforms it seems probable that these result from post-translational modifications (Alias & Clark, 2007; Pal & Clark, unpublished).

The proteins identified account for only about 50% of the Sigma, Delta, Epsilon and Omega families. This may be due to low levels of expression or to low affinity for the matrices of some isoforms. A further disappointing aspect of the work has been the failure to identify members of the Zeta or Theta families.

5.2 Future work

The overlap in specificities of the media discussed here suggests that their use in combination should lead to a greater coverage of the GST proteome than the use of any

single medium. Alternatively, their use in sequence should lead to the preferential isolation of particular types of GST (CG16936 would be a possible target for such a study) and sequential use might also be employed to enrich preparations in isoforms expressed at too low a level to be identified in the current experiments.

It is possible that Theta and Zeta families have not been detected because of a low affinity for the S-aryl glutathiones. Different types of ligand need to be investigated for the isolation of members of these families.

This work has involved the study of *D. melanogaster* as a model insect but the techniques described have been developed with a view to applying them to insect pests that are of economic or health significance. This proteomic approach generates information that does not depend on knowledge of the genome of the organism in question. Arthropod genomic databases are sufficiently extensive so that cross-database matching of proteomic data may be sufficient to obtain a provisional characterisation of GSTs isolated in this fashion from a pest species. The approach may thus serve as a tool for preliminary studies in organisms for which there is no genomic database. It enables preliminary characterisation of GSTs that are produced to a significant extent and are thus, perhaps, most immediately worthy of individual study.

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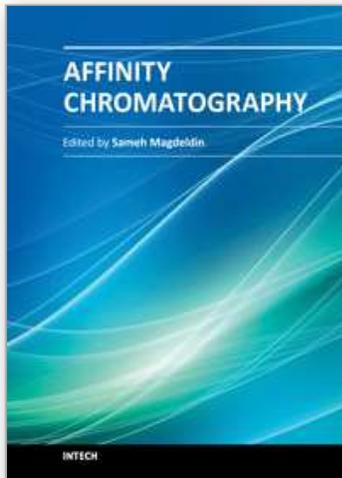
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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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