XENOBIOTICA, 1993, VOL. 23, NO. 8, 851–861

Separation of multiple forms of glutathione *S*-transferase from the Blue Mussel, *Mytilus edulis*

P. J. FITZPATRICK† and D. SHEEHAN

Department of Biochemistry, University College Cork, Lee Maltings, Prospect Row, Mardyke, Cork, Ireland

Received 10 November 1992; accepted 25 March 1993

1. Glutathione *S*-transferase isoenzymes from *Mytilus edulis* and *M. galloprovincialis* have been partially purified by glutathione-sepharose affinity chromatography followed by Mono Q anion exchange fast protein liquid chromatography (f.p.l.c.).

2. The tissue distribution of glutathione *S*-transferase in *M. edulis* has been studied. Using 1-chloro-2,4-dinitrobenzene as substrate, highest specific activity is observed in the gill, the main feeding organ. Affinity-purified extracts of this organ give a characteristic f.p.l.c. profile. A similar profile is obtained with affinity-purified extracts of the digestive gland of *M. galloprovincialis*.

3. The subunit structure of the purified isoenzymes has been studied by SDS polyacrylamide gel electrophoresis and reversed-phase h.p.l.c. The subunits have similar molecular weights and h.p.l.c. retention times to rat glutathione *S*-transferases.

Introduction

A common mechanism for protection against electrophilic xenobiotics is by conjugation to glutathione (GSH) in phase II detoxication (Chasseaud 1979). This reaction, the first step in mercapturic acid synthesis (Boyland and Chasseaud 1969), is catalysed by the glutathione *S*-transferase (GSTs; for reviews see Mannervik and Danielson 1988, Pickett and Lu 1989). These enzymes are dimeric, mainly cytosolic proteins, structurally and kinetically related, displaying broad and overlapping substrate specificities and inhibition characteristics (Chasseaud 1979, Tahir and Mannervik 1986). In rat liver, GSTs are the most abundant cytosolic proteins (Jakoby 1978). As well as their catalytic role in detoxication, these enzymes also possess extensive binding properties (Bhargava *et al.* 1978) and are thought to play a role in maintaining bile salt homeostasis in liver (Takikawa *et al.* 1986).

One of the features of GSTs of interest to the toxicologist is that cells may respond to xenobiotic exposure by induction of particular isoenzymes (McLellan *et al.* 1991). This may result in tolerance of the xenobiotic and GSTs have indeed been implicated in a wide range of resistance phenomena such as resistance to antibiotics (Arca *et al.* 1988), insecticides (Lee 1991), and chemotherapeutic agents used in cancer treatment (Schisselbauer *et al.* 1990).

Although mammalian enzymes are the most studied, the GSTs are widely distributed in nature, being also found in bacteria and fungi (Sheehan and Casey 1993a), insects (Lee 1991) and in aquatic species such as fish (Lee *et al.* 1983). Because these enzymes are often inducible, it has been suggested that levels of detoxication enzymes in aquatic animals might be a useful index of exposure to chemical pollution. In particular, the use of cytochrome P450 as a measure of xenobiotic exposure has been extensively investigated (Livingstone 1991, Stegeman and Lech 1991).

†To whom correspondence should be addressed.

In our laboratory we have studied the GSTs of the Blue Mussel, *Mytilus edulis*, to assess the possible use of the level of this enzyme as an index of exposure to chemical pollution (Sheehan *et al.* 1991). *M. edulis* is frequently used as a bioindicator organism (Bayne 1978) and we have previously found that the GST specific activity of this mollusc varies with habitat pollution status. Our findings are in broad agreement with those obtained from studies on fish GSTs (Pascual *et al.* 1991). Interpretation of measurements of total specific activity is complicated, however, by the fact that several GST isoenzymes are present (Sheehan *et al.* 1991). A more detailed knowledge of these individual GSTs is therefore required to allow interpretation of the effects of xenobiotics on the levels of activity of these enzymes. We report here the tissue distribution of GST activity in *M. edulis*. We have partially purified and characterized a number of GST isoenzymes from *M. edulis*. Similar patterns of GST isoenzyme expression are found in the gill of *M. edulis* and the digestive gland of *M. galloprovincialis*, in which organs GST expression is likely to be particularly important in detoxication.

Materials and methods

Reagents

Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). The Mono Q column, sephadex G-25 and epoxy-activated sepharose 6B resins were purchsed from Pharmacia (Uppsala, Sweden). The GSH–sepharose affinity column was prepared using the method of Simons and Vander Jagt (1977). All other reagents were of the highest grade available. All solutions were prepared using milliQ water available in the laboratory.

Animal dissection and preparation of cytosol

Mussels between 5 and 6 cm in length were collected from Fota Island in Cork harbour, Ireland. Whole mussel, gill and digestive gland tissue were dissected within 24 h of collection and immediately frozen in liquid nitrogen. The tissue was then stored at −70°C. *M. galloprovincialis* was collected from Venice, Italy. The digestive gland was dissected and stored in the same manner as the samples from *M. edulis*. Typically, 25 g of each tissue was used for preparation of cytosol for further purification. Tissue homogenization was carried out using 3 vol. of buffer (10 mM Tris–HCl (pH 7·2), 0·5 M sucrose, 0·15 M KCl, 1·0 mM EDTA, and 1·0 mM dithiothreitol (DTT)) with a polytron homogenizer. The homogenate was centrifuged for 1 h at 16000 *g* and the supernatant (cytosol) was further purified.

Affinity purification procedure

The cytosol was filtered through glass wool and the filtrate rapidly desalted using a Sephadex G-25 column (17 × 6·5 cm) pre-equilibrated in 10 mM sodium phosphate buffer (pH 7·2). The resulting eluate was passed through a GSH–sepharose affinity column pre-equilibrated with the same buffer. Non-specifically-bound material was removed by passing five column volumes of 10 mM sodium phosphate (pH 7·2) buffer containing 200 mM NaCl through the column. The specifically-bound material was eluted with 10 mM Tris–HCl (pH 9·0) buffer containing 200 mM NaCl and 30 mM GSH. All the specifically-bound material typically eluted in approx. 20 ml, and this was then passed through a second G-25 column (16 × 3·0 cm) pre-equilibrated with 10 mM Tris–HCl (pH 8·5) buffer, to remove the GSH and salt. The resulting active eluate was then stored at −70°C until subsequent fast protein liquid chromatography (f.p.l.c.) analysis.

Fast protein liquid chromatography analysis

F.p.l.c. is a high resolution intermediate pressure system designed specifically for the rapid separation of biopolymers (Richey 1982). It offers a number of advantages over h.p.l.c., e.g. high recovery of biological activity, compatibility with aqueous buffer and salt solutions, high protein loadings (up to 25 mg), and short separation times (15–45 min). The system is available in all the conventional chromatographic modes, e.g. ion exchange, gel filtration, chromatofocusing and reversed phase. F.p.l.c. has previously been widely used in purification of GSTs from mammalian sources (Alin *et al.* 1985, Meyer *et al.* 1991).

The desalted affinity-purified extract (10 ml, 3–5 mg) was applied to the f.p.l.c. system. Separation was achieved using Mono Q (HR 5/5) anion exchange chromatography. Protein was detected by measuring absorbance at 280 nm using a single path, dual beam flow-through UV-1 monitor (Pharmacia). The buffers used were as follows: buffer A, 10 mM Tris–HCl (pH 8·5); and buffer B, 10 mM Tris–HCl (pH 8·5) containing 1·0 M NaCl. Chromatography of *M. galloprovincialis* digestive gland extract was performed using the same procedure in the Biological Sciences Department of Plymouth University, UK.

Protein estimation

All protein estimations were carried out using a modification of the methodology of Lowry *et al.* (1951) with bovine serum albumin as standard.

Determination of glutathione-S-transferase activity.

GST activity was determined using the method of Habig *et al.* (1974) with GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates.

H.p.l.c. analysis

Reversed phase h.p.l.c. was performed on each of the peaks resulting from f.p.l.c. analysis of affinity-purified gill material. Analysis was carried out using a Superlco C8 reversed-phase column (150 × 4·6 mm internal diameter, particle size 5 μm (Bellefonte, PA, USA)) equilibrated with buffer A: 0·12% (v/v) aqueous trifluoroacetic acid, at a flow rate of 1 ml/min. Buffer B was composed of trifluoroacetic acid–water–acetonitrile (0·1 : 30 : 70 v/v). The concentration of acetonitrile was raised to 49% over 30 min using a linear gradient of buffer B.

Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) was carried out on whole mussel affinity-purified material and on each of the peaks from the f.p.l.c. analysis of the affinity-purified material from whole mussel, gill and digestive gland. Molecular weight standards; BSA(66 kDa), ovalbumin(45 kDa), glyceraldehyde-3-phosphate dyhydrogenase(36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor(20·1 kDa) and α-lactalbumin (14·2 kDa) were used for characterization of subunit molecular mass. In addition, rat liver affinity-purified GST extract (subunits Ya (25 kDa), Yb (26·5 kDa), Yc (28·0 kDa)) was also used as a standard. This extract is routinely prepared in our laboratory by affinity chromatography of liver cytosolic extract from male Wistar rats which have been starved overnight. The protein is prepared by trichloroacetic acid precipitation and stored at −20°C in 200 μg aliquots until required.

Results

Tissue distribution of GSTs in Mytilus edulis

Tissues were dissected and pooled from several mussels and the GST specific activity, with CDNB as substrate, of each extract was determined. CDNB was chosen as substrate because in preliminary experiments mussel extracts were found to display highest activity with this substrate compared with other reported GST substrates. Tissue distribution results are summarized in table 1. They indicate that the enzyme is expressed at the highest level in the gill, with significant amounts present in the digestive gland due to the relatively large size of the latter organ. Since it is known that in the rat there is a tissue-specific pattern of GST isoenzyme expression (Scully and Mantle 1981), it was of interest to separate and purify the GSTs both from whole mussel and from these particular tissues. The gill is the main feeding organ of mussels while the digestive gland has high amounts of cytochrome P450 activity (Livingstone and Farrar 1984, Livingstone 1985). These organs are therefore of some interest due to their likely roles in detoxication in *Mytilus* spp.

Table 1. Tissue distribution of GST activity of *Mytilus edulis*.

Tissue	Total protein (mg)	Total GST activity (U)	GST specific activity (U/mg)
Gill	1·4	5·5	3·93
Foot	0·5	0·9	1·80
Mantle	2·7	3·8	1·41
Digestive gland	16	14·9	0·93
Gonad	14·9	9·8	0·66

GST activity was determined with CDNB and GSH as substrates (Habig *et al.* 1974) while protein was measured by the method of Lowry *et al.* (1951).

Purification of GSTs from mussels

Affinity-purified extracts of fresh, whole *M. edulis* were prepared and fractionated by Mono Q f.p.l.c. An identical procedure was used to fractionate the GSTs of gill from *M. edulis* and of the digestive gland of *M. galloprovincialis*. These chromatographic patterns are shown in figure 1. Table 2 shows a purification table for each of the fractionations. This indicates that the affinity purification step alone results in a 10-fold purification for whole mussel, six-fold for gill, and 40-fold for

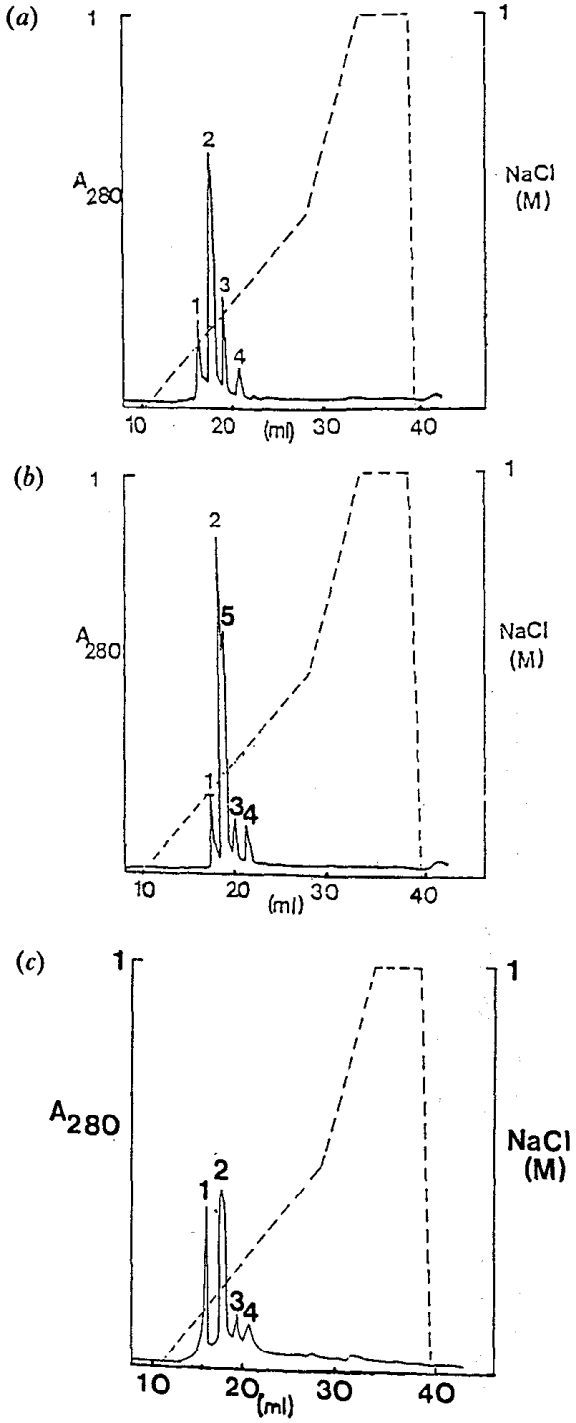


Figure 1. Mono Q f.p.l.c. chromatography of affinity-purified mussel extracts. Chromatography was performed in 10 mM Tris–HCl (pH 8·5), on extracts from (*a*) whole *M. edulis*, (*b*) *M. edulis* gill and (*c*) *M. galloprovincialis* digestive gland. Protein was monitored on-line as A_{280} (solid lines) while the NaCl gradient is shown as a dashed line. Fractions were collected automatically and subsequently used for SDS-PAGE analysis shown in figure 2.

Table 2. Purification of mussel GSTs.

Step	*Mytilus edulis* whole mussel			*Mytilus edulis* gill			*Mytilus galloprovincialis* digestive gland		
	Total activity (U)	(% Yield)	Specific activity (U/mg)	Total activity (U)	(% Yield)	Specific activity (U/mg)	Total activity (U)	(% Yield)	Specific activity (U/mg)
Cytosol	29·6	100	$2{\cdot}24 \times 10^{-2}$	34·4	100	$6{\cdot}05 \times 10^{-2}$	41·6	100	$2{\cdot}05 \times 10^{-2}$
Sephadex G-25 #1	44·7	149	$3{\cdot}72 \times 10^{-2}$	37·4	109	$8{\cdot}09 \times 10^{-2}$	62·4	150	$4{\cdot}12 \times 10^{-2}$
Affinity Step	18·3	61	$2{\cdot}32 \times 10^{-1}$	16·2	47	$3{\cdot}42 \times 10^{-1}$	41·9	101	$8{\cdot}28 \times 10^{-1}$
Sephadex G-25 #2	16·57	55	1·00	12·9	37	1·13	40·7	98	2·52
f.p.l.c. peaks									
1	$7{\cdot}10 \times 10^{-1}$	2·4	1·53	$5{\cdot}83 \times 10^{-1}$	1·7	$9{\cdot}91 \times 10^{-1}$	14·2	34·1	3·78
2	$1{\cdot}67 \times 10^{-1}$	0·6	$5{\cdot}86 \times 10^{-2}$	$1{\cdot}47 \times 10^{-1}$	0·4	$3{\cdot}96 \times 10^{-2}$	7·7	18·5	$1{\cdot}99 \times 10^{-1}$
3	ND		ND	ND		ND	ND		ND
4	ND		ND	ND		ND	ND		ND
5				$5{\cdot}66 \times 10^{-2}$	0·2	$1{\cdot}46 \times 10^{-2}$			

ND, not detectable.

digestive gland. F.p.l.c. chromatography of these preparations separates a number of peaks of which the leading peak contains the bulk of the GST catalytic activity with CDNB as substrate. Peak 2 is the largest one in terms of total protein in each of the profiles. A peak (peak 5) is visible in the gill profile which is not apparent in either of the other chromatographies.

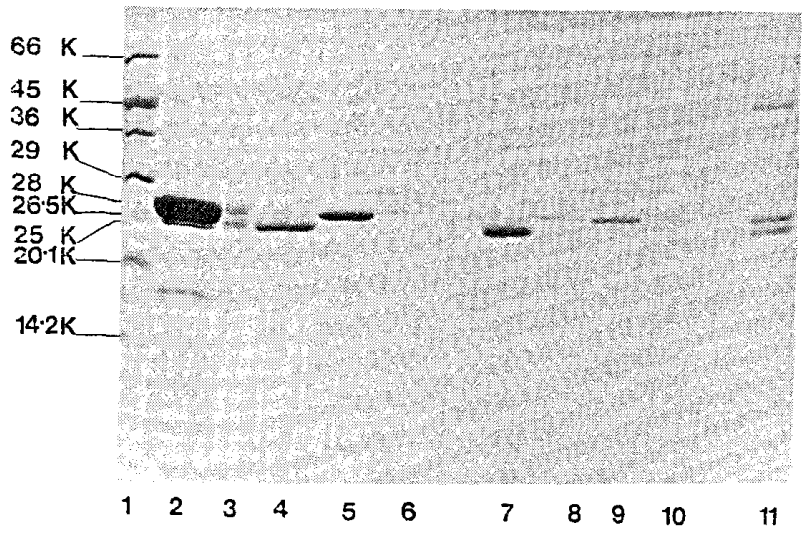
SDS-PAGE analysis of f.p.l.c. peaks

Figure 1 shows f.p.l.c. chromatographic patterns of affinity-purified extracts from A (*M. edulis*, whole mussel), B (*M. edulis*, gill) and C (*M. galloprovincialis*, digestive gland). Peaks 1–4 are the same in each case, i.e. they have the following retentions on f.p.l.c., (peak 1,120–130; peak 2,157–170; peak 3, 212–240; and peak 4, 270–290 mM NaCl). Peak 5 from f.p.l.c. of the *M. edulis* gill extract (retention 170–200 mM NaCl) is not present in the other chromatographies. Electrophoretic analysis of f.p.l.c. peaks is shown in figure 2. Whole *M. edulis* affinity-purified extract consists mainly of two polypeptides with Mrs of 26 500 and 24 500 by the criterion of SDS-PAGE. A minor band of approx. Mr 45 000 is also visible in this extract. This latter band immunoblots with antisera to rat GST-P and as such probably represents incompletely denatured GST dimers (not shown). Peak 1 of both whole *M. edulis* and gill affinity-purified extracts from this species consists mainly of a single polypeptide of Mr 24 500, while peaks 2 and 3 of these chromatographic patterns consist predominantly of polypeptides of Mr 26 500. Peak 5 of *M. edulis* gill affinity-purified extract also consists of a polypeptide of Mr 26 500.

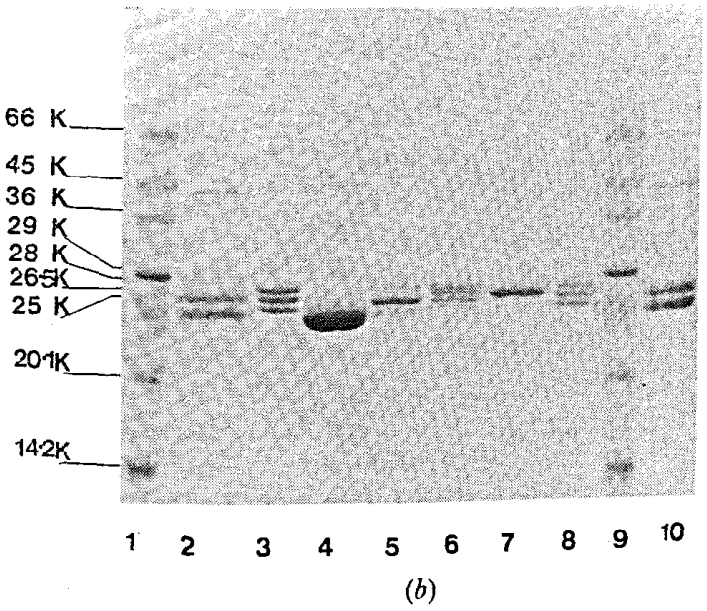
The main polypeptide of peak 1 from *M. galloprovincialis* has a mobility corresponding to a Mr of 24 500, although minor bands of Mrs 26 500 and 27 300 are also visible. Peak 2 consists mainly of a Mr 26 500 polypeptide while peak 3 is a heterogeneous mixture. Peak 4 from *M. galloprovincialis* consists of a slightly larger polypeptide with an apparent Mr of 27 300 which is not visible in SDS-PAGE of *M. edulis* extracts. These results indicate considerable similarity between *M. edulis* and *M. galloprovincialis*. The subunit Mrs observed in these mussels are very similar to those found in the rat (figure 2), fungi (Sheehan and Casey 1993b) and other sources (Mannervik and Danielson 1988). The values we quote are based on the behaviour of the rat affinity-extract run as a standard on SDS-PAGE gels. This rat affinity-extract contains the subunits Ya (25 kDa), Yb (26·5 kDa), and Yc (28 kDa). It should be noted that values are apparent Mrs since it is known that GSTs behave anomalously on SDS-PAGE (Hayes and Mantle 1986).

Reversed-phase h.p.l.c. analysis

GSTs from the f.p.l.c. separation of *M. edulis* gill affinity extract shown in figure 1 were further analysed by reversed-phase chromatography on a C-8 column. The samples were obtained from peaks 1, 2 and 5 and the chromatograms are shown in figure 3. Peaks 1, 2 and 5 gave single peaks with characteristic retention times 7·82, 6·36, and 6·42 min, respectively. GST subunits run as single polypeptides in reversed-phase h.p.l.c., and even very similar subunits are distinguishable by this technique (Ostlund Farrants *et al.* 1987). The major polypeptide of peaks 2 and 5 are similar to each other and distinct from that of peak 1. The polypeptides of peak 1 also migrate to a different Mr to that of peak 2 and 5 on SDS-PAGE analysis and these latter polypeptides are also indistinguishable by this criterion. These results indicate a pattern of multiple isoenzyme expression in *M. edulis* dominated by homo- rather than heterodimers.



(*a*)



(*b*)

Figure 2. SDS-PAGE analysis of f.p.l.c. peaks.

Samples were prepared by precipitation in 12·5% (w/v) trichloroacetic acid, analysed on 15% (w/v) polyacrylamide gels containing 0·1% (w/v) SDS and stained with Coomassie blue. The standard proteins are those described in Materials and methods and their molecular weights are shown. (*a*) Analysis of whole *M. edulis* and *M. edulis* gill fractions; 50 μg standard proteins (1); 40 μg rat liver affinity extract (2); 20 μg whole *M. edulis* affinity extract (3, 11); whole *M. edulis* f.p.l.c. peaks 1–3 (4–6); *M. edulis* gill f.p.l.c. peaks 1 (20 μg, 7), 2(20 μg, 8), and 5 (5 μg, 9). (*b*) Analysis of *M. galloprovincialis* digestive gland fractions: standard proteins (1, 9); 30 μg whole *M. edulis* affinity extract (2, 10); 40 μg rat liver affinity extract (3, 8); *M. galloprovincialis* f.p.l.c. peaks 1 (40 μg, 4), 2 (20 μg, 5), 3 (20 μg, 6), and 4 (20 μg, 7).

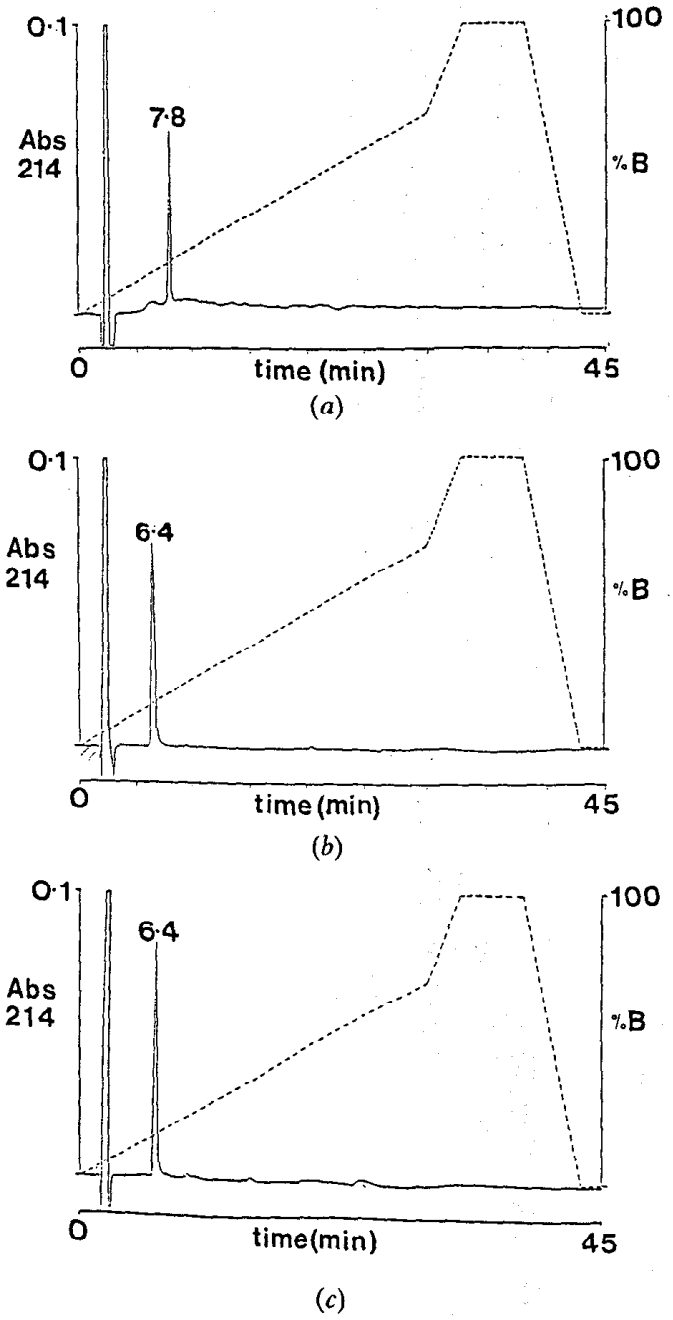


Figure 3. Reversed-phase h.p.l.c. of *M. edulis* gill fractions.

Column = Supelco C-8, flow rate = 1 ml/min, detection = 214 nm (solid line). Chromatographic patterns shown were obtained with peaks 1, 2 and 5 from f.p.l.c. of *M. edulis* gill as shown in figure 1. (*a*) Peak 1), (*b*) peak 2, (*c*) peak 5. Buffer A, 0·12% (v/v) aqueous trifluoroacetic acid; buffer B, trifluoroacetic acid–water–acetonitrile (0·1 : 30 : 70 v/v). %B (represented by dashed line).

Discussion

The GSTs are expressed in *M. edulis* as multiple isoenzymes (Sheehan *et al.* 1991). In the present paper we have separated these isoenzymes using a combination of affinity and ion exchange chromatography from *M. edulis* whole mussel tissue. Although peaks 1 (Mr 24 500) and 2 (Mr 26 500) display activity with CDNB, the other two peaks appear to be inactive with this substrate. It is possible that these latter peaks may represent non-GST enzymes which also happen to use GSH as a substrate (e.g. glyoxylases, GSH reductase, GSH peroxidase) and thus bind to the

GSH-sepharose affinity resin. However, in further assays we have found these peaks not to possess GSH reductase or GSH peroxidase activity (data not shown). It is also possible that peaks 3 and 4 may represent inactivated GST. A third possibility is that these enzymes are native GSTs which simply do not use CDNB as substrate. This has been reported as a diagnostic feature of the most recently identified GST class, the theta class (Meyer *et al.* 1991). In more recent experiments we have found that these polypeptides immunoblot with antisera to rat GST-P (not shown) which would seem to confirm that they are indeed GSTs. A definitive answer to this question will only come from detailed structural and kinetic studies on these proteins. Such studies are presently underway in our laboratory..

In assessing the contribution of an enzyme to detoxication in a simple organism such as *M. edulis*, it is important to know something of the tissue distribution of that enzyme. The distribution of the cytochrome P450 system of mussels has previously been investigated (Livingstone and Farrar 1984, Livingstone 1985). These studies showed that the activities associated with this system are most abundant in the digestive gland (hepatopancreas). In the present paper, by contrast, we find that the GST enzymes are preferentially expressed in the gill although they are also expressed in large amounts in the digestive gland. This is an interesting finding in view of the role of the gill as the main feeding organ in mussels and may indicate that detoxication by GSH conjugation is particularly important in this organ, perhaps facilitating rapid conjugate excretion and clearance from the animal.

We also carried out GST purification from the gill of *M. edulis* and the digestive gland of *M. galloprovincialis*, a closely related species. Our f.p.l.c. profiles indicate that these tissues express a pattern of GST isoenzymes essentially similar to that of the whole mussel. Reversed-phase h.p.l.c. of samples from peaks 1, 2 and 5 of *M. edulis* gill extracts indicated that these isoenzymes are composed of homodimers and that peaks 2 and 5 are very similar by this criterion. A tissue-specific peak (peak 5) was observed in this extract. These results are consistent with a tissue-specific pattern of isoenzyme expression of some complexity and, unusually, dominated by the expression of homodimers. Such a pattern probably arises from tissue-specific control of GST gene expression similar to that known to occur in more advanced species (Scully and Mantle 1981). We are now extending these studies to other tissues from *M. edulis* and *M. galloprovincialis*.

The cytochrome P450 system is frequently used as a bioindicator of xenobiotic exposure in marine species but the levels found in mussels and other molluscs are usually lower than those of fish and the system does not seem to be as inducible as that of fish (Livingstone *et al.* 1989, Stegeman and Lech 1991). Moreover, activities associated with this system in molluscs display considerable seasonal variation (Livingstone 1985, Stegeman 1985). Consequently, the use of this activity as a bioindicator of xenobiotic exposure is unsuitable.

In the present work we have studied the distribution and separation of phase II GST activities in mussels from different sampling locations and species. Our results indicate that these enzymes may be routinely separated and rapidly quantified by the techniques described. We have found that affinity-purified extracts may be stored at $-70°C$ for periods of months without deterioration or loss of activity. F.p.l.c. analysis of samples may be carried out rapidly (<1 h) and if an autosampler is used many samples may be analysed per day. We are presently carrying out a detailed biochemical characterization of purified mussel GSTs, and investigating the possibility of using specific GST isoenzymes as bioindicators for xenobiotic

exposure. Our preliminary results indicate that f.p.l.c. patterns of gill GSTs from *M. edulis* and of digestive gland from *M. galloprovincialis* and *M. edulis* do seem to vary with the pollution status of the sampling site. In similar studies on fish Pascual *et al.* (1991) have found that the h.p.l.c. patterns of affinity-purified GSTs do seem to vary with extent of habitat pollution. Cytosolic enzymes such as the GSTs may present fewer problems in such measurements than mussel cytochrome P450. For example, as membrane proteins, particular isoenzymes of cytochrome P450, are much more difficult to purify and therefore to quantify. Our data further underlines the importance of GSH and its associated enzymes in detoxication.

Acknowledgements

We acknowledge the funding of an f.p.l.c. system by EOLAS, The Irish Science and Technology agency. P.J.F. received an EOLAS basic science award studentship and also spent a period of time at the University of Plymouth on the ERASMUS programme. We thank Dr D. R. Livingstone, National Marine Laboratory, Plymouth, for providing us with samples of *M. galloprovincialis* digestive gland.

References

ALIN, P., JENSSON, H., GUTHENBERG, C., DANIELSON, U. H., TAHIR, M. K., and MANNERVIK, B., 1985, Purification of major basic glutathione transferase isoenzymes from rat liver by use of affinity chromatography and fast protein liquid chromatography. *Analytical Biochemistry*, **146**, 313–320.

ARCA, P., RICO, M. BRANA, A. F., VILLAR, C. J., HARDISSON, C., and SUAREZ, J. E., 1988, Formation of an adduct between fosfomycin and glutathione: A new mechanism of antibiotic resistance in bacteria. *Antimicrobial Agents and Chemotherapy*, **32**, 1552–1556.

BAYNE, B. L., 1978, Mussel watching. *Nature*, **275**, 87–88.

BHARGAVA, M. M., LISTOWSKY, I., and ARIAS, I. M., 1978, Bilirubin binding and glutathione S-transferase activity are independent processes. *Journal of Biological Chemistry*, **253**, 4112–4115.

BOYLAND, E., and CHASSEAUD, L. F., 1969, Role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. *Advances in Enzymology*, **32**, 173–219.

CHASSEAUD, L. F., 1979, Role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Advances in Cancer Research*, **29**, 175–274.

HABIG, W. H., PABST, M. J., and JAKOBY, W. B., 1974, Glutathione S-transferases; the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, **248**, 7130–7139.

HAYES, J. D., and MANTLE, T. J., 1986, Anomalous electrophoretic behaviour of the glutathione S-transferase Ya and Yk subunits isolated from man and rodents. *Biochemical Journal*, **237**, 731–740.

JAKOBY, W. B., 1978, The glutathione S-transferases: a group of multifunctional detoxification proteins. *Advances in Enzymology*, **46**, 383–414.

LAEMMLI, U. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

LEE, K., 1991, Glutathione S-transferase activities in phytophagous insects: induction and inhibition by plant phototoxins and phenols. *Insect Biochemistry*, **21**, 353–361.

LEE, M. J., GHIM, G. J., LEE, J. K., and LEE, T. H., 1983, Comparative studies on the glutathione S-transferases of some animal tissues. *Korean Journal of Biochemistry*, **15**, 31–36.

LIVINGSTONE, D. R., 1985, Responses of the detoxication/toxication enzyme systems of molluscs to organic pollutants and xenobiotics. *Marine Pollution Bulletin*, **16**, 158–164.

LIVINGSTONE, D. R., 1991, Towards a specific index of impact by organic pollutants for marine invertebrates. *Comparative Biochemistry and Physiology*, **100C**, 151–155.

LIVINGSTONE, D. R., and FARRAR, S. V., 1984, Tissue and subcellular distribution of mixed function oxygenase and benzo[*a*]pyrene metabolism in the common mussel *Mytilus edulis* L. *Science of the Total Environment*, **39**, 209–235.

LIVINGSTONE, D. R., KIRCHIN, M. A., and WISEMAN, A., 1989, Cytochrome P-450 and oxidative metabolism in molluscs. *Xenobiotica*, **19**, 1041–1062.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., 1951, Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry*, **193**, 267–275.

MANNERVIK, B., and DANIELSON, U. H., 1988, Glutathione transferases—structure and catalytic activity. *CRC Critical Reviews in Biochemistry*, **23**, 283–337.

MCLELLAN, L. I., KERR, L. A., CRONSHAW, A. D., and HAYES, J. D., 1991, Regulation of mouse glutathione S-transferases by chemoprotectors. *Biochemical Journal*, **276**, 461–469.

MEYER, D. J., COLES, B., PEMBLE, S. E., GILMORE, K. S., FRASER, G. M., and KETTERER, B., 1991, Theta, a new class of glutathione transferases purified from rat and man. *Biochemical Journal*, **274**, 409–414.

OSTLUND FARRANTS, A., MEYER, D. J., COLES, B., SOUTHAN, C., AITKEN, A., JOHNSON, P. J., and KETTERER, B., 1987, The separation of glutathione transferase subunits by using reverse-phase high-pressure liquid chromatography. *Biochemical Journal*, **245**, 423–428.

PASCUAL, P. MARTINEZ-LARA, E., TORIBIO, F., LOPEZ-BARREA, J., and BARCENA, J. A., 1991, HPLC isoenzyme patterns of glutathione S-transferase from marine fish with different pollution levels. *Biochemical Society Transactions*, **19**, 302S.

PICKETT, C. B., and LU, A. Y. H., 1989, Glutathione S-transferases: gene structure, regulation and biological function. *Annual Review of Biochemistry*, **58**, 743–764.

RICHEY, J., 1982, FPLC: a comprehensive separation technique for biopolymers. *American Laboratory*, **14**, 104–129.

SCULLY, N. C., and MANTLE, T. J., 1981, Tissue distribution and subunit structure of multiple forms of glutathione S-transferase in the rat. *Biochemical Journal*, **193**, 367–370.

SCHISSELBAUER, J. C., SILBER, R., PAPADOPOULOS, E., ABRAMS, K., LACRETA, F. P., and TEW, K. D., 1990, Characterisation of glutathione S-transferase expression in lymphocytes from chronic lymphocyte leukemia patients. *Cancer Research*, **50**, 3562–3568.

SHEEHAN, D., and CASEY, J. P., 1993 a, Microbial glutathione S-transferases. *Comparative Biochemistry and Physiology*, **104B**, 1–6.

SHEEHAN, D., and CASEY, J. P., 1993 b, Evidence for alpha and mu class glutathione S-transferases in a number of fungal species. *Comparative Biochemistry and Physiology*, **104B**, 7–13.

SHEEHAN, D., CRIMMINS, K. M., and BURNELL, G. M., 1991, Evidence for glutathione S-transferase activity in *Mytilus edulis* as an index of chemical pollution in marine estuaries. In *Bioindicators and Environmental Management*, edited by D. W. Jeffrey and B. Madden (London: Academic Press), pp. 419–425.

SIMONS, P. C., and VANDER JAGT, D. L., 1977, Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. *Analytical Biochemistry*, **82**, 334–341.

STEGEMAN, J. J., 1985, Benzo[*a*]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, **89**, 21–30.

STEGEMAN, J. J., and LECH, J. J., 1991, Cytochrome P-450 monooxygenase systems in aquatic species; carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environmental Health Perspectives*, **90**, 101–109.

TAHIR, M. K., and MANNERVIK, B., 1986, Simple inhibition studies for distinction between homodimeric and heterodimeric isoenzymes of glutathione transferase. *Journal of Biological Chemistry*, **26**, 1048–1051.

TAKIKAWA, H., STOLZ, A., SUGIMOTO, M., SUGIYAMA, Y., and KAPLOWITZ, N., 1986, Comparison of the affinities of newly identified human bile acid binder and cationic glutathione S-transferase for bile acids. *Journal of Lipid Research*, **27**, 652–657.