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Purification and characterization of multiple glutathione transferase isoenzymes from grey mullet liver

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Abstract. Fourteen isoforms of glutathione S-transferase (GST) have been separated and purified from mullet (*Mugil cephalus*) liver by scaling up an automatic analytical method based on anionic exchange chromatography. The activity of each isoenzyme with several substrates was determined. Dimeric combinations of six subunits make up this heterogeneous isoenzyme population. Five of these were resolved by reverse phase chromatography; four of them, named a, b, c and d, were present in more than one isoform, had the same apparent molecular mass (25.2 kDa) by SDS-PAGE, and were immunochemically related to plaice GST-A and possibly to rat GST-5 but not to plaice GST-B or any other rat GST subunit; they would belong to the theta class. Subunit e was only present in isoenzyme I which was basic, had an apparent molecular mass of 23.4 kDa and would belong to the alpha class, since it was recognized by antibodies towards plaice GST-B and rat GST-1 and GST-8 and less intensely by anti-(rat)GST-2. Another subunit, named f, with 25.2 kDa apparent molecular mass that could not be distinguished by reverse phase chromatography, was detected immunochemically by positive reaction with antibodies to rat GST-1 and GST-2 in addition to reaction with anti-(plaice)GST-A. As suggested by these results we discuss the existence of genetic polymorphism, the differential expression and the evolutionary relationships of mullet GSTs.

Key words. Biotransformation; glutathione conjugation; xenobiotic metabolism; reverse phase chromatography; immunochemical analysis; molecular biomarkers; *Mugil cephalus*.

Abbreviations. CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DCNB 1,2-dichloro-4-nitrobenzene; DTT, dithiothreitol; EA, ethacrynic acid; HNE, 4-hydroxynonenal; NBC, p-nitrobenzyl chloride; NPB, p-nitrophenethyl bromide.

Glutathione transferases (GSTs) are members of a multigene family of polyfunctional proteins found in all the organisms studied so far. They are involved in both the transport and biosynthesis of endogenous compounds and in the cellular defence against oxidative damage and peroxidation products of DNA and lipids (reviewed in refs 1–3). They also catalyse the conjugation of many electrophilic compounds with glutathione and constitute a major system for detoxification of drugs and pollutants via the excretion of their mercapturic acid derivatives [4].

The GSTs have been most extensively studied in terrestrial mammals, especially rat GST which is used as a reference. With the exception of a microsomal enzyme, the GSTs are soluble and dimeric comprising identical or different subunits of 25 to 28 kDa that are grouped into five classes according to structure: alpha (subunits 1, 1′, 2, 8 and 10), mu (subunits 3, 4, 6, 9 and 11), pi

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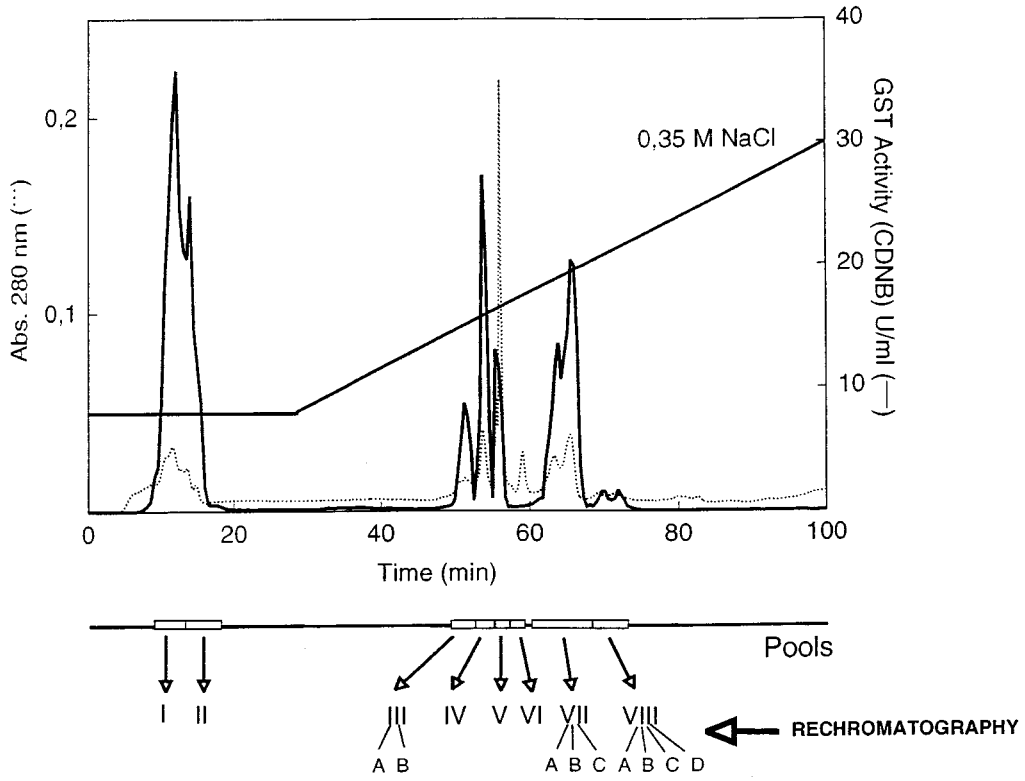

Figure 1. Separation of mullet liver GST isoforms by anionic exchange chromatography. 15 ml of GST affinity fraction were loaded onto a DEAE-Spherogel column. The flow rate was 6 ml/min and fractions of 6 and 0.24 ml were collected during flow-through and elution, respectively. Fractions with GST (CDNB) activity were pooled and numbered as indicated in the bar below.

(subunit 7), theta (subunits 5, 12 and 13) and sigma (reviewed in refs 3, 5–7).

Comparative studies of their occurrence and tissue distribution in a number of piscine species have been reported (reviewed in ref. 8). Multiple GST isoforms have been purified from two species of elasmobranch and four species of teleost fish [9–15]. They are dimeric proteins with similar molecular mass and catalytic activities to their mammalian counterparts, although there are important differences in properties and expression of GST classes not only between fish and mammals but also between different fish species [10, 14–19]. Immunological and aminoacid sequence data have shown that the predominant isoform in salmonids is a pi class homologue [15], whilst the predominant isoforms in a pleuronectid are theta class homologues [18].

Several mammalian GST isoezymes are induced by xenobiotics via transcription factors acting on specific DNA sequences present in their promoter regions and named AREs (antioxidant responsive elements) [20] or EpREs (electrophile responsive elements) [21]. Fish GST are also induced by a similar range of xenobiotics (reviewed in ref. 8) and the equivalent sequence elements have been found in the promoter regions upstream of the gene for GSTA from plaice [19].

Mullet (*Mugil* sp.) is a common fish on the South Atlantic Spanish coast. In the liver of mullet from polluted zones we found that certain isoforms were differentially induced, resulting in a greater total hepatic GST activity compared with fish from unpolluted areas [22]. This ability of environmental contaminants to elicit a differential induction of GST isoforms in fish liver was subsequently confirmed by controlled experimental exposure of giltheads (*Sparus aurata*) to model contaminants [23]. Thus, analysis of isoform profiles may be of use as a bioindicator of contamination in aquatic ecosystems. In the present study we present data on the characterization of the multiple GST isoenzymes present in mullet liver.

Materials and methods

Materials. All chemicals used were of analytical grade available commercially. S-hexylglutathione and S-hexylglutathione-agarose affinity matrix were obtained from

Table 1. Specific activity of GST isoforms from mullet liver with different substrates.

Isoforms	GST activity (U/mg)						
	Substrates						
	CDNB	DCNB	EA	NBC	NPB	CuOOH	HNE
I	60	0.2	1	9	0.1	0.1	nd
II	15	0.4	0.6	26	0.2	0	nd
IIIA	149	0.8	3.8	4	2.5	0	2.4
IIIB	153	1.2	1.6	3	0.5	1.3	2.1
IV	124	0.3	0.3	1	0	0.5	0.7
V	54	0	0.5	1	0	0.4	0.4
VI	77	0	2.9	3	0	1.7	1.3
VIIA	43	0	1.5	14	0	0	0.8
VIIB	71	0.3	0.7	1	0	0.7	0.5
VIIC	6	0.3	0.2	1	0	1	0.6
VIIIA	284	0	2.8	45	4.9	0	2.1
VIIIB	57	0	2.5	7	2.1	0	0.8
VIIIC	26	0.3	0.7	2	2.6	0	0.4
VIIID	1	0	1.1	8	1.2	0	0.3
NB	<1	0	0	<1	0	0	0.1

The isoforms are indicated using the nomenclature assigned after anionic exchange chromatography (fig. 1) and rechromatography (see text). nd = not determined, NB = not retained in the affinity matrix.

Sigma Chem. Co. HNE was a kind gift of Professor Esterbauer (University of Graz, Austria).

Fish and tissue sampling. Grey mullet (*Mugil cephalus*) of 150 g body weight were obtained from CUPIMAR hatchery extensive cultures (San Fernando, Cádiz). Fish were killed, the livers were removed immediately, frozen and transported under liquid nitrogen. Once in the laboratory, the frozen livers were pooled and triturated with a mortar under liquid nitrogen; the resulting powder was kept at -80 °C and used for the preparation of crude extracts as required.

Enzyme assays and protein determination. Assays of the glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB), 1,4-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), p-nitrobenzyl chloride (NBC) and p-nitrophenethyl bromide (NPB) were carried out as described by Habig and Jakoby [24], and conjugation of 4-hydroxynonenal (HNE) was determined as described by Alin et al. [25]. Se-independent peroxidase activity with cumene hydroperoxide (CuOOH) as substrate was measured according to the method of [26].

Protein concentrations were determined by the Lowry method [27] with bovine serum albumin as standard or in column eluates by absorbance at 280 nm.

Enzyme purification. 40 g of frozen liver powder were homogenated in 4 volumes (w/v) of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM DTT and 5 mM GSH using an Ultra-Turrax rotating blade homogeniser. The resulting homogenate was centrifuged for 110 minutes at $105{,}000 \times g$ and the supernatant, which constituted the cytosolic fraction, was stored at -80 °C until use. Aliquots (25 ml) of the cytosolic fraction were purified by affinity chromatography by application to a 20 cm $\times$ 1 cm column containing S-hexylglutathione-agarose which was equilibrated with 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 2 mM DTT (buffer A) at a flow rate of 1 ml/min in a WATERS 650 E chromatography system. Unbound material was removed by washing with buffer A containing 50 mM KCl and the affinity-bound transferase was eluted with 5 mM S-hexylglutathione in 0.5 mM EDTA, 50 mM Tris-HCl, pH 9.6. The eluted GST-containing fractions were combined and dialysed against 0.2 mM EDTA, 0.5 mM DTT, 10 mM Tris-HCl, pH 8 (buffer B). This operation was repeated five times to process the whole cytosolic fraction. The affinity-purified GST-containing fractions were further resolved by preparative anion-exchange HPLC using a 15×2.15 cm DEAE-Spherogel TSK column (Beckman) equilibrated in buffer B at room temperature and a flow rate of 6 ml/min. 15 ml of sample were applied and the column was washed with 150 ml of buffer B while the unbound GST was collected in fractions of 6 ml; the column was eluted using a salt gradient from 0–0.35 M NaCl in buffer B, and 0.24 ml fractions were collected. GST activity with CDNB as substrate was measured in all the fractions and those constituting peaks of activity (eight) were pooled. Each of the eight pools was concentrated and dialysed against buffer B. The acidic ones were repurified by repetition of the anion exchange chromatography under the same conditions using a semi-analytical column (DEAE-Spherogel TSK; 7.5×0.75 cm). After elution, the active fractions were pooled, concentrated and dialysed against buffer B; they were stored at -20 °C or analysed immediately.

The subunit composition of the active GST-containing fractions was determined by reversed-phase HPLC using a 0.46×15 cm Vydac C4 214TP 5 μm column developed over 30 minutes with a linear 30–70% (v/v) acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid. Polypeptides were detected at 214 nm.

Electrophoretic analysis. Molecular mass analysis of mullet GSTs and GST subunits was performed by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS) using a PhastGel gradient (8–25% acrylamide, 2% cross-linking) in a PhastSystem apparatus (Pharmacia). Polypeptides were visualized by silver [28] or Coomasie Blue staining. Immunological analysis of GST subunits was performed by resolution of the polypeptides by SDS/PAGE on gels containing 12.5% (w/v) acrylamide and 0.3% (w/v) NN′-methylene-bisacrylamide, transfer to nitrocellulose and immunodetection as described previously for piscine GSTs [14, 17] using antisera raised against purified rat subunits 1, 3, 7 and 2 (Biotrin, Dublin), subunit 5 (gift from Dr. D. Meyer), subunit 8 (gift from Dr. J. Hayes) and against purified plaice GST-A and GST-B [14].

Results

Purification of GST isoenzymes from mullet liver. GSTs from mullet liver were purified using two chromatographic steps; affinity chromatography on S-hexylglutathione-agarose and anion-exchange chromatography on DEAE-Spherogel. About 98% of the cytosolic GST activity (with CDNB as substrate) was retained by the affinity matrix and eluted as a single peak following application of the competing substrate, S-hexylglutathione, at alkaline pH. The lack of binding of 2% of the cytosolic GST activity was not due to column saturation. Subsequently, the affinity-purified GST-containing fraction was subjected to anion exchange chromatography using a preparative scale adaptation (column size × 6 and sample size × 200) of the previously described procedure [29] resulting in the partial resolution of eight groups of fractions which were combined and designated as pools I-VIII (fig. 1). Attempts were made to resolve further components of pools I and II, which were either unbound or eluted with the initial column washing, by cationic exchange HPLC using a 7.5×0.75 cm SP-TSK column, but protein and activity recoveries were very poor. Therefore the initial pools I and II were used directly for subunit characterization studies. Pools III-VIII were each rechromatographed on a smaller anion-exchange column and this resulted in further resolution of peaks III (to IIIA and IIIB), VII (to VIIA–C) and VIII (to VIII A–D). Thus, altogether 14 charge-variants (i.e. isoenzymes) of mullet GSTs which displayed CDNB-conjugating activity were purified.

Substrate specificity. In addition to CDNB-conjugating activity, each mullet GST isoenzyme was tested for activity towards six other compounds known to be relatively specific substrates of some mammalian GST subunits (table 1). There were some obvious differences between the isoforms: those of group VIII displayed prominent activity with NPB and with NBC but did not exhibit peroxidase activity with cumene hydroperoxide; isoforms of group III exhibited the highest activity with DCNB, a substrate relatively specific for class mu transferases. Isoform II displayed a higher conjugating activity towards NBC than any other substrate tested, and this was twice that with CDNB as substrate. Other isoforms also conjugated NBC. Isoforms III, VI and VIII conjugated HNE and EA at slightly higher rates than the rest of the isoforms. The fraction that was not bound to the S-hexylglutathione affinity matrix, designated NB, was not significantly active with any of the substrates assayed, indicating that all the major GSTs of mullet liver had been accounted for.

Subunit composition and molecular mass of GST isoenzymes. When analysed by SDS-PAGE (see fig. 5a) the affinity purified GST fraction contained three major polypeptide bands; the predominant component had an apparent Mr of 25.2 kDa and two other components had Mr's of 23.4 kDa and 22.3 kDa.

When reverse phase chromatography was employed to separate the GST subunits as described by Ostlund Farrants (1987) [30] it was found that the C18 column was too hydrophobic for the mullet GST fraction. The procedure was therefore adapted by use of a C4 column. Chromatography of the affinity-purified GST

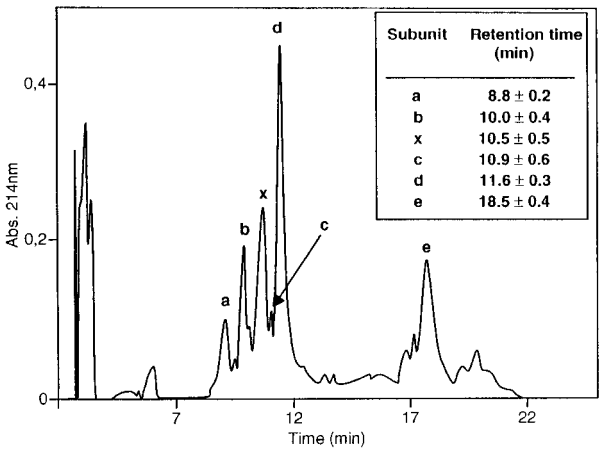

Figure 2. Separation of subunits from mullet liver GST. 150 μg of the GST affinity fraction was applied to a reverse-phase Vydac C_4 TP214 column and eluted as indicated in Materials and methods. The different subunits are named by a letter according to their order of elution (see text for details). The retention times are shown as $\bar{x} \pm SD$ of three chromatograms.

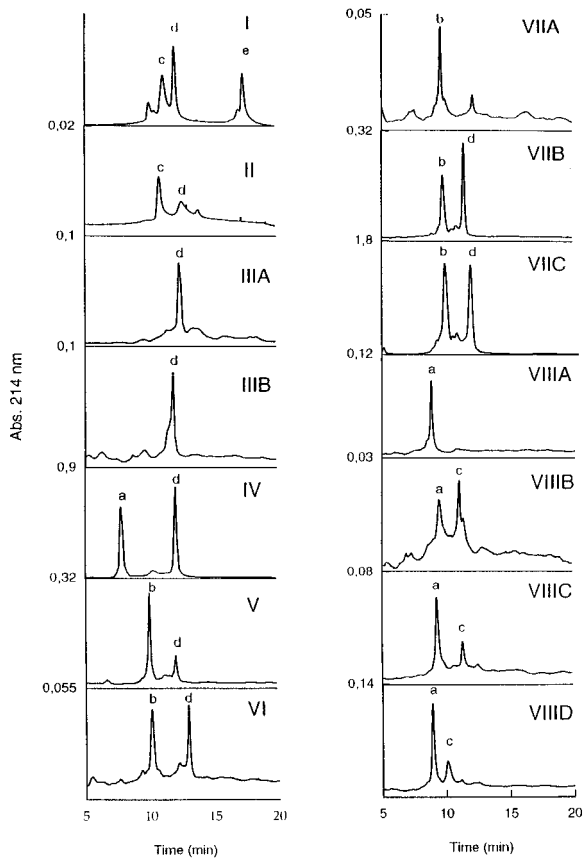

Figure 3. Subunit composition of mullet liver GST isoforms. Each of the isoforms separated by two successive anionic exchange rechromatographies (see text and fig. 1 for further explanation) was injected onto a Vydac C_4 column and eluted as indicated in Materials and methods. Each isoenzyme was chromatographed at least twice; retention times were averaged and names were assigned to the peaks according to those present in the 'GST Fraction' as given in figure 2. Representative chromatograms are shown.

fraction from mullet cytosol resolved several prominent peaks (fig. 2) and those that were subsequently identified as polypeptides of ca. 20–26 kDa after anion-exchange chromatography are designated a–e. Their retention times were determined precisely by averaging three chromatograms, and are also given in figure 2. One peak, named X, was not detected in any of the isoenzymes separated by anion-exchange rechromatography when they were analysed individually (see fig. 3). The minor peak that eluted around 3.5 mins (also present in isoenzymes IIIA and V) had a much smaller Mr; therefore, both peaks were considered as unidentified non-GST contaminants.

The subunit composition of each isoform, previously separated on the basis of the charge of the dimeric enzyme, was also analysed by reversed phase chromatography as shown in figure 3. Subunit a was present in isoform IV and those of group VIII; subunit b was evident in isoforms V, VI and those of group VII; subunit c was conspicuous in isoforms I, II and those of group VIII; subunit d was present in all isoforms except in those of group VIII; subunit e was only present in isoform number I.

SDS-PAGE analysis (see fig. 5a) showed that the component with a Mr of 22.3 kDa in the affinity purified fraction could not be detected in any isoform eluted from the anion exchange columns. Isoenzyme I uniquely contained a band of Mr 23.4 kDa which can be assigned as that of subunit e. All the isoenzymes except I displayed a single band with an apparent Mr of 25.2 kDa.

Immunological studies. Antisera raised against rat GST subunits 1, 2, 3, 5, 7 and 8 and against plaice GST-A and B were used to establish the immnunochemical relationships of the mullet GST subunits on Western blots.

Antibodies against the alpha class rat GST subunits 1, 2 and 8 crossreacted with the affinity-purified GST fraction from mullet liver whereas those against the rat GST-3 subunit (mu class) and subunit 7 (pi class) did not (fig. 4a–e). In the total cytosolic fraction of mullet liver, there was positive immunoreactivity with anti-(rat) GST-5 antiserum (theta class) of a 25.2 kDa polypeptide (fig. 4h) which also crossreacted with an antiserum towards plaice GST-A (fig. 4g), while a 23.4 kDa polypeptide crossreacted with a specific antiserum raised against plaice GST-B (fig. 4f). The immunoreactivities of the alpha class antisera were attributable to isoform I (fig. 5c) and none of the other isoforms made any significant contribution when analysed individually (not shown). More specifically, the 23.4 kDa subunit present in isoform I from mullet crossreacted with anti-(rat) GST-8 serum (fig. 5c); anti-(rat) GST-1 and 2 sera primarily crossreacted with the 25.2 kDa subunit and to a lesser extent with the 23.4 kDa subunit (fig. 5c). When analysed individually, the large 25.2 kDa subunit present in all the isoforms contributed to the reactivity towards anti(plaice)-GSTA (fig. 5b). The 25.2 kDa subunit from the basic isoform I was different from the large subunit in the rest of the acidic isoforms in that, in addition to crossreactivity with anti-(plaice)GST-A, it also crossreacted with anti-(rat)GST-1 and 2 (fig. 5c). We name this subunit *f*, although it could not be identified on the reverse phase chromatograms. Direct evidence of the apparent molecular mass (25.2 kDa) of subunits a, b, c and d and of their strong reactivity with anti-(plaice)GSTA is shown in figure 6. The smaller Mr subunit of 23.4 kDa, i.e. subunit e, which is only present in isoform I, was the only polypeptide which crossreacted with anti-(plaice) GSTB (fig. 5c), but did not crossreact with anti-(plaice) GSTA (fig. 6b).

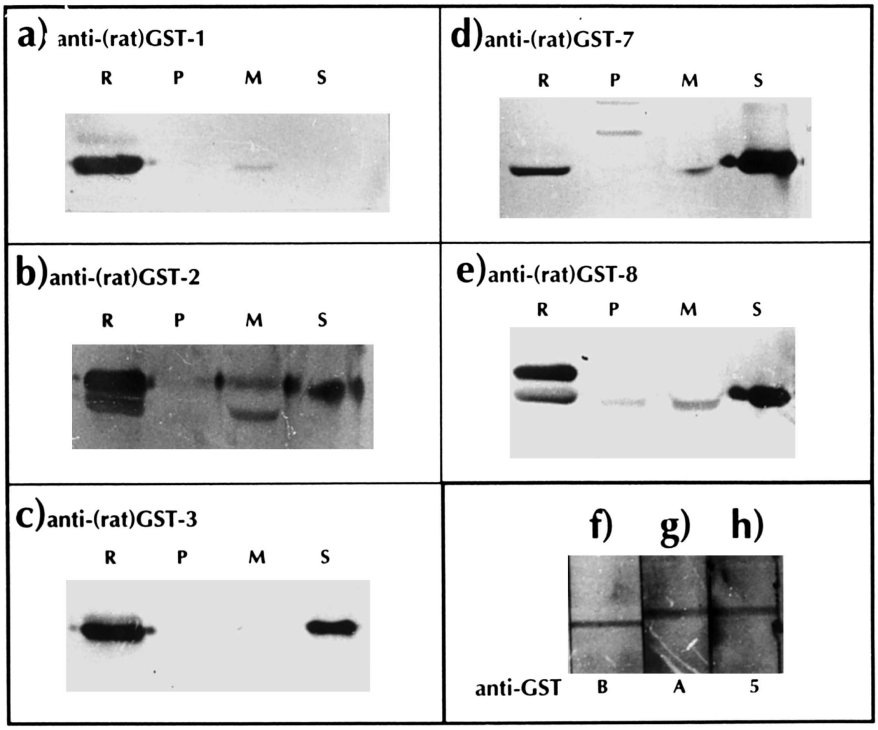

Figure 4. Immunochemical relationships between mullet, plaice and rat liver GST subunits. Aliquots of affinity GST fraction from mullet (M) and rat (R), whole cytosolic fraction from plaice (P) and mullet (*f*, *g*, *h*) and appropriate standards (S) were subjected to SDS-PAGE (12.5% acrylamide homogeneous gel) and transferred to nitrocellulose paper. The membranes were washed with antibodies specific for the following GST subunits (*a*) rat subunit 1; (*b*) rat subunit 2; (*c*) rat subunit 3; (*d*) rat subunit 7; (*e*) rat subunit 8; (*f*) plaice subunit B; (*g*) plaice subunit A; (*h*) rat subunit 5. In *b*, *c*, *d* and *e*, the standards were rat GST-2, rat GST-3, human placental GST and rat GST-8, respectively; no reference was run in *a*.

The immunochemical characterisation of subunits shown in figures 4, 5 and 6 is summarized in table 2.

Discussion

At least 14 isoforms of soluble GSTs could be separated by high resolution chromatography of mullet liver extracts. The elution profile of the preparative anionic exchange chromatography was strikingly similar to that obtained automatically on an analytical scale [29]. These isoforms were distinguished by charge, subunit molecular mass and immunoreactivity.

Analysis of enzymatically active GST dimers has been described for a great variety of tissues and organisms, and the number of isoforms obtained in each case varies depending on the source and between laboratories for the same source. Van der Jagt et al. [31] separated 13 isoforms from human liver; 12 were separated from rat liver by Tu and Reddy [32]; 11 from dog liver [33] and nine from chicken liver [34]. Other authors have separated between two and seven isoforms from different terrestrial organisms. In fish, crustaceans and mollusks, isolations of one up to five isoforms have been described [9–11, 15, 35–40].

A high number of dimeric isoforms may arise from a small number of subunits given the proven capacity of heterodimer formation between members of the same family. The highest reported number of different subunits in the same tissue is that of Yeh et al. [41] who detected four major and four minor subunits in rat kidney by reverse phase chromatography. In addition, the existence of conformers of single subunits in mammalian tissues has been reported [32, 42–44] and microheterogeneity has also been observed in tissues from aquatic organisms [10, 15]. In most cases the differences have been detected as charge isomers by chromatofocusing or by ionic exchange chromatography. We have distinguished just five subunits, *a*, *b*, *c*, *d* and *e*, by the established reverse phase chromatography protocol, although the existence of an additional one, named *f*, was suggested from Western blotting data (see below). From the data in figure 3 it can be speculated that isoforms IIIA and B, VIIA and VIIIA are homodimers of subunits *d*, *b* and *a*, respectively, since they show one single

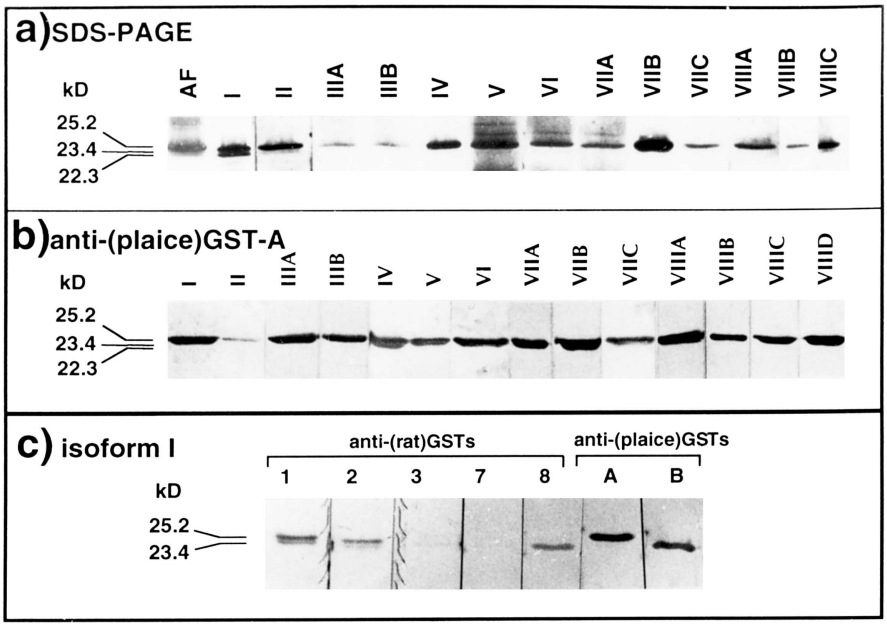

Figure 5. Immunochemical analysis of mullet liver GST isoenzymes. (*a*) SDS-PAGE analysis: the pools from the anionic exchange chromatographies shown in figure 1 were subjected to electrophoresis under denaturing conditions in 8–25% acrylamide gradient gels. The GST affinity fraction that was loaded onto the column (AF) is shown on the left for comparison. Proteins were visualized using silver staining. (*b*) The same pools were subjected to Western blotting and the nitrocellulose membranes were developed using anti-(plaice)GSTA. (*c*) Western blotting of pool I: approximately 0.7 μg/well was loaded and the membranes were developed using polyclonal antibodies raised against rat GST-1, 2, 3, 7, and 8 and against plaice GST-A and B, as indicated.

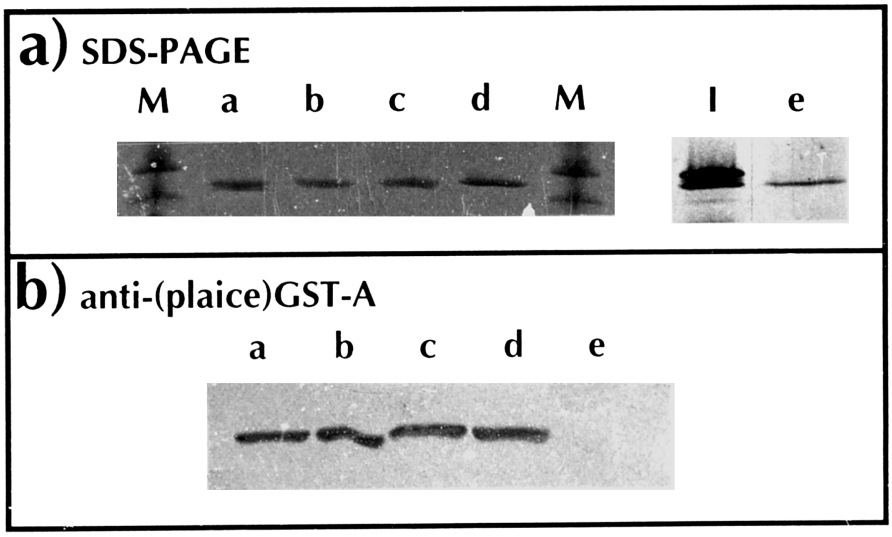

Figure 6. Immunochemical analysis of mullet liver GST subunits. (*a*) The eluate from the reverse-phase column was collected in fractions and those corresponding to the named peaks were concentrated, dialysed and analyzed by SDS-PAGE in 8–25% acrylamide gradient gels; protein was visualized by Coomassie Blue staining. M = molecular weight markers. Subunit e is shown together with whole isoform I for comparison; both blots were stained by the silver method. (*b*) The subunits were subjected to Western blotting and developed with anti-(plaice)GST-A antibody.

Table 2. Reactivity of mullet GST fractions with antibodies against rat and plaice GST subunits.

Mullet fraction	Anti-rat subunits						Anti-plaice subunits	
	1	2	3	5	7	8	A	B
Affinity fraction	(+)	+	−	[+]	−	+	+++	+++
25.2 kD subunits								
a, *b*, *c* and *d*	−	−	−	[+]	−	−	+++	−
f	+++	++	−	[+]	−	−	+++	−
23.4 kD subunit *e*	++	(+)	−	−	−	++	−	+++

Mr as determined by SDS-PAGE. Subunits a, b, c and d were present in isoforms II to VIII whereas subunits e and f were only present in isoform I. All the subunits were identified in reverse phase chromatograms except subunit f. −, negative; (+), faint positive; [+], the reactivity of the large subunit with anti(rat)-GST-5 was assayed only in the cytosolic fraction, not in purified isoforms or subunits.

or major peak. Isoforms IV, VI and VIIC are composed of equal amounts of two different subunits and could be heterodimers of subunit d with subunit *a* or *b*.

Four of the subunits, *a*, *b*, *c* and *d*, share several important properties: they had the same apparent molecular mass as determined by SDS-PAGE, reacted with antibodies against plaice GST A and were present in more than one isoform. Whether they are products of four separate genes or arose from post-translational modification is an interesting subject for further investigation.

N-terminal amino acid sequencing of each subunit was tried but unfortunately they were all blocked. Protease digestion and sequencing of internal peptides should be considered as a new project in the future.

Two forms of subunit 1 that are products of separate genes and differ by just eight amino acid residues have been identified in rat liver [45]. By LC/MS the presence of C-terminal one- and four-residue truncated forms of GST 1 in rat kidney that were undistinguishable by reverse phase chromatography has been demonstrated, and it was argued that they did not originate post-translationally [41]. Different but closely related genes are also present in fish: Leaver and George [19] have found three GST-A genes in a single fish but more must exist since there is also evidence of considerable genetic polymorphism [46]. This could apply to subunits a, b, c and d found in our study since the crude extracts were prepared from a pool of livers obtained from a number of individuals and it is likely that not all the isoforms are present in a single fish.

Subunit *e* was smaller and was just detected as part of isoform I, which is basic. It did not react with anti-(plaice)GST-A but was positive towards anti-(plaice) GST-B. It was also positive towards anti-(rat)GST 8 which is structurally similar to GST-B [14].

Our results point to the existence of another "large" subunit in mullet liver GST fraction with the same apparent molecular mass (25.2 kDa) but only present in isoform I: besides reactivity with anti-(plaice)GST-A it is distinguished from subunits a, b, c and d by its additional positive reactivity with anti-(rat)GST-1 and 2 and by its presence in the basic isoenzyme group. It may be a component of the GST population not definitively resolved by reverse phase chromatography. The basic isoenzyme I of mullet was markedly increased in the liver of fish from contaminated waters [47] and an equivalent isoenzyme was highly induced by experimental exposure of gilthead seabream (*Sparus aurata*) to Aroclor or copper [23]. Interstingly enough, an antioxidant response element (ARE) has been found in the promoter region of just one out of three genetic variants of plaice GST-A [19]. It would be worth investigating the identity and genetic regulatory elements of the subunit that make up this inducible isoenzyme in mullet.

Given the tight immunochemical relationships found to piscine GST-A, a theta class subunit [18], the large GST subunits from mullet liver can be classified into the theta class. This classification would explain the reactivity of mullet liver homogenate with anti-(rat)GST-5 (fig. 4h) and the widespread activity of mullet isoforms with NBC as substrate [6] found in this study (table 1).

From the immunochemical results reported here it would appear that mullet is evolutively close to the flat fish, according to the relatedness of its glutathione transferases to GST-B and GST-A from plaice and their lack of reactivity with anti-GST-7 from rat. Moreover, given its recognition by specific antibodies towards rat GST-8, the mullet appears to be more closely related to plaice than to turbot or flounder [17]. On the other hand, the mullet GST fraction reacted towards anti-GST-2 from rat and showed selenium-independent GSH peroxidase activity.

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