

Purification and characterization of eight glutathione *S*-transferase isoenzymes of hamster

Comparison of subunit composition of enzymes from liver, kidney, testis, pancreas and trachea

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Eight dimeric isoenzymes of glutathione *S*-transferase (GST) were purified from liver, kidney and testis of the Syrian golden hamster, using *S*-hexylglutathione affinity chromatography and chromatofocusing. The isoenzymes were characterized according to their substrate selectivity, physical properties and amino acid sequence analysis. Thus a classification into Alpha, Mu and Pi classes was made in analogy with GSTs of other species. Two Alpha-class GSTs were purified, termed A₁A₁ (pI 8.9) and A₁A₂ (pI 8.6). Four Mu-class subunits were detected (M₁–M₄), all forming homodimers, with M₂ and M₃ also forming a heterodimer. The isoelectric points ranged from 5.9 to 8.6. One Pi-class isoenzyme was purified and termed P₁P₁ (pI 6.8). Using h.p.l.c. analysis, the subunit composition was determined in a number of organs. The major subunits in liver were A₁ and M₁. Subunit A₁ was also the major subunit in the kidney. Subunit M₁ was not detected in kidney, while subunit P₁ was not found in the liver. Pancreas and trachea contained predominantly the Pi-class subunit, P₁. GST in the testis was mostly of the Mu class. The major subunit was M₄, and subunits M₂ and M₃ were exclusively detected in the testis.

INTRODUCTION

The glutathione *S*-transferases (GSTs) are a family of cytosolic and microsomal proteins which appear to exist in all aerobic organisms. Their enzymic function is the catalysis of the reaction of glutathione with both electrophilic xenobiotics and their metabolites [1], and endogenous compounds (prostaglandins, leukotrienes, steroids) [2–5]. In addition, they also serve as carrier proteins for organic substances [1].

Rat, mouse and human GST isoenzymes have been purified and characterized extensively [1,6–11]. However, relatively little is known about hamster GST isoenzymes, even though hamsters are frequently used as experimental animals. Smith and colleagues have described the purification of two enzyme forms from Chinese hamster liver with pI values of 9.0 [12]. Jensen *et al.* have described the purification of two Alpha-class enzymes and the existence of an unresolved set of Mu-class enzymes in the liver of Syrian golden hamsters [13]. Furthermore, Igarashi *et al.* have compared the S-Sepharose elution profiles of liver cytosolic GST activity from rats, mice, rabbits, hamsters and guinea pigs, and the corresponding bands obtained with SDS/PAGE in the different fractions from the investigated species [14]. Finally, Hayes described the selective elution of GSTs of several species from the *S*-hexylglutathione-Sepharose affinity matrix [15].

In this paper, the purification and characterization of eight different Syrian golden hamster GST isoenzymes are described. In addition, the subunit compositions of isoenzymes from liver, testis, kidney, pancreas and trachea are presented.

EXPERIMENTAL

Materials

S-Hexylglutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, ethacrynic acid and cumene hydroperoxide were obtained from Sigma (St. Louis, MO, U.S.A.). *Trans*-stilbene oxide was obtained from Aldrich (Milwaukee, WI, U.S.A.). Epoxy-activated Sepharose 6B, Pharmalyte 8–10.5 and Polybuffer 74 were purchased from

Pharmacia (Uppsala, Sweden). H.p.l.c.-grade acetonitrile was obtained from Promochem (Wesel, Germany) and h.p.l.c.-grade trifluoroacetic acid was from Baker (Deventer, The Netherlands). Glutathione peroxidase was purchased from Boehringer (Mannheim, Germany). Cyanogen bromide (CNBr) was obtained from Janssen Chimica (Geel, Belgium). All other chemicals were of the highest available quality.

Animals and organ selection

Syrian golden hamsters were obtained from the Central Institute for the Breeding of Laboratory Animals (Zeist, The Netherlands). Liver, testis and kidney were the organs selected for the purification of GST isoenzymes because of both their relatively high activity towards CDNB (90, 25 and 13 units/ml of cytosol respectively) and the presence of all the different detectable subunits in these organs.

Preparation of cytosol

Cytosol was prepared by homogenizing the various organs with 3 vol. of 0.01 M-Tris/HCl/0.14 M-KCl pH 7.4 with a Potter-Elvehjem tissue homogenizer and centrifuging for 75 min at 105000 *g*. The fat layer was removed from the supernatant and the cytosol was stored at –30 °C.

Enzyme purification

***S*-Hexylglutathione affinity chromatography.** The cytosol was applied to a *S*-hexylglutathione affinity column (liver: 160 ml, column volume 100 ml; testis: 135 ml, column volume 100 ml; kidney: 20 ml, column volume 30 ml). The column was equilibrated with 0.01 M-Tris/HCl/1 mM-EDTA/0.2 mM-dithiothreitol, pH 7.8. The flow rate was 1 ml/min. The column was washed with equilibration buffer containing 0.2 M-NaCl, and GSTs were eluted with equilibration buffer containing 0.2 M-NaCl and 2.5 mM-*S*-hexylglutathione. The fractions converting CDNB into its glutathione conjugate were pooled and concentrated using Amicon ultrafiltration cells with YM-10 membranes.

Chromatofocusing. The GST isoenzymes were separated by means of chromatofocusing using a Pharmacia h.p.l.c. system

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; CNBr, cyanogen bromide

equipped with a Mono-P HR 5/20 (20 cm \times 0.5 cm) column. The column was equilibrated with 0.025 M-triethylamine/HCl, pH 10.5. The isoenzymes were chromatofocused with Pharmalyte 8–10.5 (200-fold diluted, adjusted to pH 7.0 with HCl) and Polybuffer 74 (10-fold diluted, adjusted to pH 4.0 with HCl) (ampholyte/buffer change at pH 7.5). The flow rate was 1 ml/min and elution profiles were monitored at 280 nm. In total, 64 mg of liver GST (eight runs), 7 mg of kidney GST (two runs) and 40 mg of testis GST (four runs) were chromatofocused. Before application, the samples were diluted 5-fold with the diluted Pharmalyte 8–10.5 solution. The chromatofocusing eluate was analysed by the h.p.l.c. method described below, SDS/PAGE and isoelectric focusing. The purified GSTs A_1A_1 (liver), A_1A_2 (liver), M_1M_1 (liver/testis), M_2M_2 (testis), M_2M_3 (testis), M_3M_3 (testis), M_4M_4 (testis) and P_1P_1 (kidney/testis) chromatofocused at pH values of 9.2, 9.1, 8.8, 8.2, 6.7, 6.1, 5.8–5.4 and 8.5 respectively. The combined GST isoenzyme fractions of the different runs of each organ were pooled, concentrated using Amicon ultrafiltration cells (YM-10 membrane), diluted with 0.05 M-Tris/1 mM-EDTA/1 mM-dithiothreitol pH 7.3, concentrated and centrifuged for 10 min at 2500 g. Isoenzymes were stored at -80°C . They were named according to their classification into the GST classes Alpha (A), Mu (M) and Pi (P), and their order of elution from the Mono-P chromatofocusing column. Within the GST classes, each subunit received a different number according to the nomenclature recommended by Jakob *et al.* [16].

Gel electrophoresis

Isoelectric point, molecular mass and purity of the isoenzymes were estimated with SDS/PAGE and isoelectric focusing using the Pharmacia PhastSystem. The isoelectric points were determined using protein standards with known isoelectric points. Subunit molecular masses were determined by comparison with the marker GST subunits π (23 000 Da) and B_2 (25 000 Da).

Enzymic assays

The enzymic activities towards CDNB [17], ethacrynic acid [17], cumene hydroperoxide [18] and 1,2-epoxy-3-(*p*-nitrophenoxy)propane [19] were measured at 25°C . The enzymic activity towards *trans*-stilbene oxide was measured at 37°C [20].

Protein assay

The protein content of the purified GST isoenzymes was determined with the Lowry assay, using BSA as standard.

Amino acid sequence analysis

Edman degradation of the first 20 amino acids was performed using a gas-phase protein sequencer (Applied Biosystems, model 475). The amino acid derivatives were identified by h.p.l.c. analysis. The *N*-terminal sequence of subunit M_2 was derived from the difference between the sequences of M_3M_3 and M_2M_3 , the latter showing two amino acid residues for a number of cycles. The *N*-termini of isoenzymes A_1A_1 , A_1A_2 and M_4M_4 were blocked. Therefore, peptide fragments of these isoenzymes were generated with CNBr. Isoenzyme A_1A_1 and M_4M_4 (25 nmol of each) were dialysed against 0.05 % formic acid and freeze-dried. Subunit A_2 (25 nmol) was purified from isoenzyme A_1A_2 by the h.p.l.c. method described below. Peptide fragments were generated by treatment with 1 ml of a solution containing 0.2 g of CNBr/ml of 70 % (v/v) formic acid for 24 h at room temperature. Subsequently, the samples were freeze-dried and dissolved in 30 % acetic acid. The peptide fragments were purified by the h.p.l.c. method described below, using a linear gradient from 10 to 60 % of acetonitrile in water in 60 min. The fragments showing the highest absorption were analysed by sequence analysis.

H.p.l.c. analysis

The GST-subunit profiles of the *S*-hexylglutathione eluates of liver, kidney, testis, pancreas and trachea were determined using the h.p.l.c. method described before [21,22]. A Vydac 218TP54 (250 mm \times 4.6 mm i.d.) chromatography column (Alltech, Deerfield, IL, U.S.A.) was used. The flow rate was 1 ml/min and absorbance peaks were detected at 214 nm. Subunits were separated using a linear gradient from 37 to 45 % acetonitrile in water in 18 min, followed by a linear gradient from 45 to 60 % acetonitrile in water in 12 min. Both acetonitrile and water contained 0.1 % trifluoroacetic acid. The fractions obtained with chromatofocusing were also analysed using this h.p.l.c. method. In addition, h.p.l.c. analysis was used for determination of isoenzyme purity.

RESULTS

Enzyme purification

The chromatofocusing elution profiles of the *S*-hexylglutathione eluates of liver, kidney and testis are presented in Fig. 1. The liver and kidney *S*-hexylglutathione eluates each contained three major isoenzymes. Both liver and kidney con-

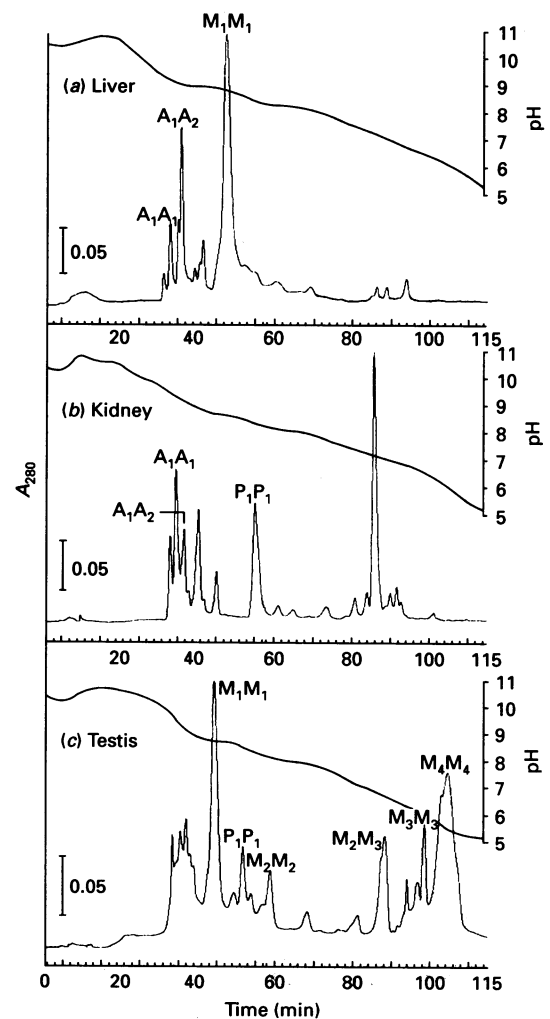


Fig. 1. Chromatofocusing elution profiles of the *S*-hexylglutathione eluates of hamster liver (a), kidney (b) and testis (c)

The chromatographic conditions are described in the Experimental section.

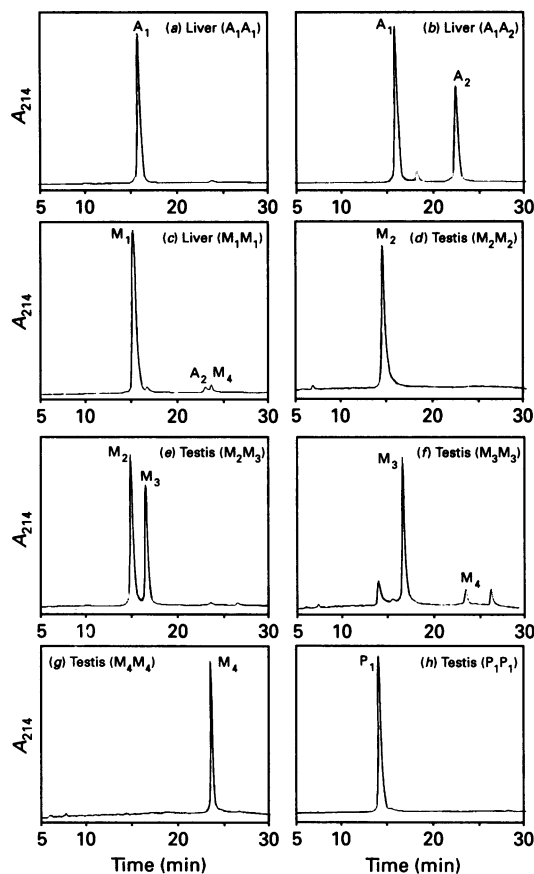
Table 1. Isoelectric points, chromatofocusing-pHs and subunit molecular masses of purified isoenzymes

Isoenzyme	Isoelectric point	Chromatofocusing pH	Subunit molecular mass (kDa)
A ₁ A ₁	8.9	9.2	27/27
A ₁ A ₂	8.6	9.1	27/25
M ₁ M ₁	8.6	8.8	23/23
P ₁ P ₁	6.8	8.5	23/23
M ₂ M ₂	6.8*	8.2	26/26
M ₂ M ₃	6.4	6.7	26/25
M ₃ M ₃	5.9	6.1	25/25
M ₄ M ₄	6.0–6.2	5.4–5.8	21/21

* Analysis of the chromatofocusing eluate.

tained isoenzyme A₁A₁, eluting at pH 9.2 (liver) and 9.4 (kidney), and isoenzyme A₁A₂, eluting at pH 9.1 (liver) and 9.2 (kidney). The third major isoenzyme in liver, M₁M₁, eluting at pH 8.8, was not detected in the kidney chromatofocusing eluate. The kidney eluate, however, contained isoenzyme P₁P₁, which was in turn not detected in the liver and which eluted around pH 8.4. Both in liver and kidney, the fraction eluting between pH 9.0 and 8.9 was found to be impure, containing components co-eluting with subunits A₁, A₂ and M₄ (h.p.l.c. analysis). Isoelectric focusing showed three distinct bands at pI 8.8, 8.7 and 6.3. It is likely that this fraction contained the homodimeric isoenzyme A₂A₂. However, this fraction was not further purified. The kidney eluate also contained a component having a rather large absorbance value at pH 7.2. This was owing to the presence of a haemoglobin in the *S*-hexylglutathione eluate.

The *S*-hexylglutathione eluate from testis contained six GST isoenzymes, eluting at pH 8.8 (M₁M₁), 8.5 (P₁P₁), 8.2 (M₂M₂), 6.7 (M₂M₃), 6.1 (M₃M₃) and 5.8–5.4 (M₄M₄). Of these, the GST isoenzymes containing subunits M₂ and M₃ were unique for testis. Although the isoenzyme containing subunit M₄ was not purified from the chromatofocusing eluates of liver and kidney, h.p.l.c. analysis showed the presence of this isoenzyme in both organs. Isoenzyme M₄M₄ was the major isoenzyme in the testis.

**Fig. 2. H.p.l.c. subunit elution profiles of purified GST isoenzymes**

The h.p.l.c. conditions are described in the Experimental section.

Enzyme purity

The h.p.l.c. elution profiles of the purified isoenzymes showed that P₁P₁ (kidney/testis), M₁M₁ (testis), M₂M₃ (testis) and A₁A₁

Table 2. N-terminal amino acid sequences and sequences of CNBr-derived peptide fragments compared with rat and human GST subunits [1,23–25]

Source	Residue ...	Amino acid sequence									
		N-terminal fragments				CNBr-derived fragments					
		1	10	20	30	51	60	70	80	90	100
Rat 1		SGKPVLYHYFIARGRM	CIRWLLAAAGVEFE			FDQVPMVEIDGMKLAQ	TRAILNYIATKYDLYGK	KDMKERALIDMYSEG	ILDLTE		
Rat 2		PGKPVLYHYFDGRGR	MEPIRWLLAAAGVDFE			FQQVPMVEIDGMKLVQ	TRAILNYIATKYNLYGK	DMKERALIDMYAEG	VADLDE		
Human B ₁		AEKPKLHYFNARGRM	ESTRWLLAAAGVEFE			FQQVPMVEIDGMKLVQ	TRAILNYIASKYNLYGK	DKERALIDMYIEGI	ADLGE		
Human B ₂		AEKPKLHYSNIRGR	MESIRWLLAAAGVEFE			FQQVP-VEIDG	KLVQTRAILNYIASK	YNLYGKDKERALID	MYIEGIADLGE		
Hamster A ₁				EFI--LLAAAGVEF-		VEIDGTTLVQTRAIL	NYIAT			YAEGIADLNE	
Hamster A ₂						YLIDGSRKITQSN	AIMRYLARKHHL	CGETE			
Rat 3		PMILGYWNVRGLTH	PIRLLLEYTDSYEEK			YLIDGSHKITQSN	AILRYLGRKHN	LCGETE			
Rat 4		PMTLGYWDIRGLA	HAIRLFLEYTDSYEDK			YLIDGSHKITQSN	AILRYLGRKHN	LCGETE			
Rat 11				VLGYWDIRGLA	HAIRLFLEYTDSYEEK						
Human μ		PMILGYWDIRGLA	HAIRLFLEYTDSYEEK			YLIDGSHKITQSN	AILRYLGRKHN	LCGETE			
Human testis/brain		MSCESMVLGYWD	IRGLAHAIRLFLEYTDSYEEK			YLIDGSHKITQSN	AILRYLGRKHN	LCGETE			
Hamster M ₁		PMTLLG				YLLDGKKNITGS	NAILCYIARKHN	MCGETE			
Hamster M ₂		PVIL-YWDV	RGLT-PIRMLL								
Hamster M ₃		PMTLGY-DI-	GLA-AI-LLL								
Hamster M ₄		VLGYW-	IRGLAHAI-			DGKNKITQSN	AILRYIARKHN-				
Rat 7		PPYTIVYFPV	RGRCEATRMLLADQ	QGSWKE							
Human π		PPYTIVYFPV	RGRCAALRMLLADQ	QGSWKE							
Hamster P ₁		PPYTIVYFPV	RGR-EAMRLL	LADQ							

- indicates no assignment.

Table 3. Specific activities of hamster GST isoenzymes towards a number of substrates and their classification

The specific activities of M_1M_1 (testis) and P_1P_1 (kidney) towards CDNB were 44.7 and 11.0 units/mg of protein respectively. No further substrates were investigated with these isoenzymes.

Substrate	Class ... Isoenzyme ... Source ...	Specific activity (units/mg)						
		Alpha		Mu				Pi
		A_1A_1 Liver	A_1A_2 Liver	M_1M_1 Liver	M_2M_3 Testis	M_3M_3 Testis	M_4M_4 Testis	P_1P_1 Testis
CDNB		8.01	8.10	47.0	36.8	36.5	16.5	15.2
Cumene hydroperoxide		3.93	2.65	0.23	< 0.013	< 0.035	< 0.005	< 0.011
<i>trans</i> -Stilbene oxide		0.0005	0.0005	0.0018	0.095	0.013	0.0093	0.0020
Ethacrynic acid		0.39	0.25	0.08	0.15	0.21	0.05	1.47
1,2-Epoxy-3-(<i>p</i> -nitro- phenoxy)propane		< 0.041	< 0.027	0.44	0.11	0.18	0.17	0.19

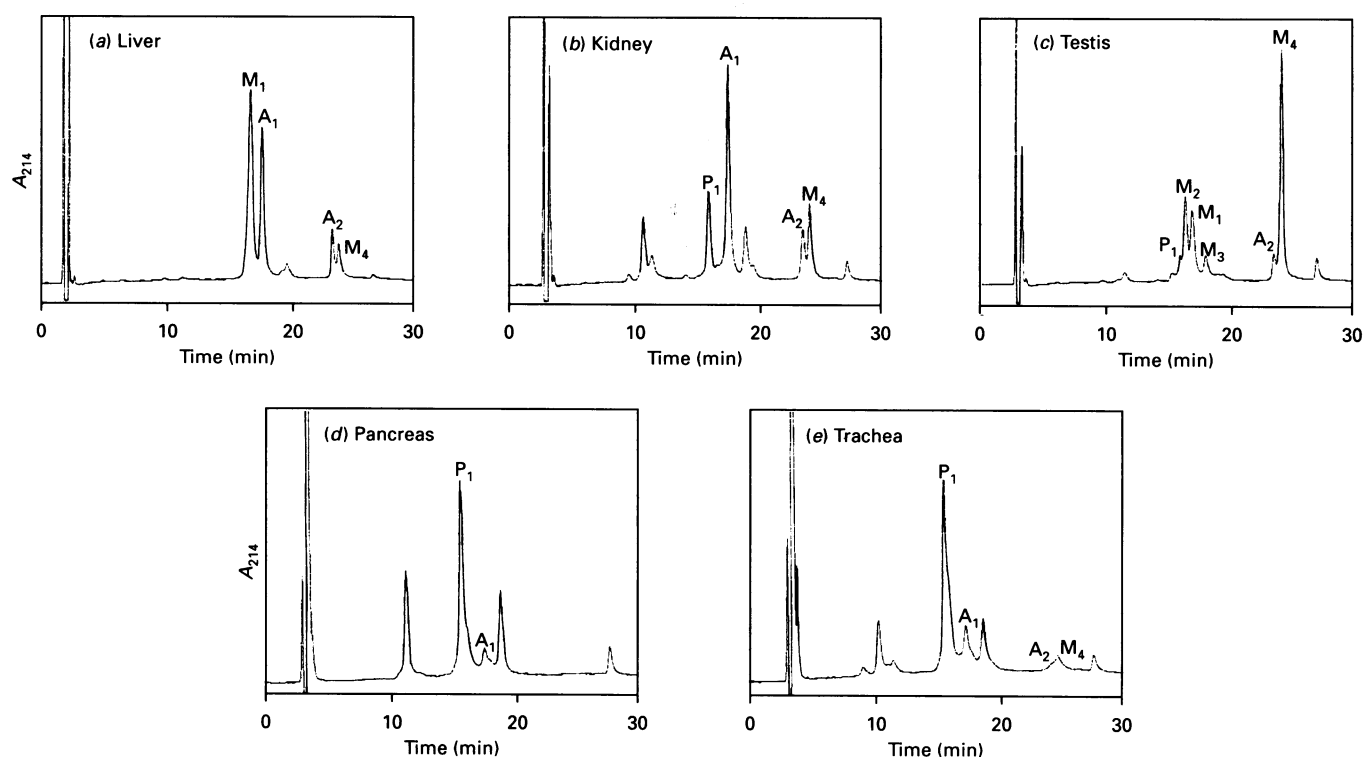


Fig. 3. H.p.l.c. elution profiles of the *S*-hexylglutathione eluates of liver (a), kidney (b), testis (c), pancreas (d) and trachea (e)

The h.p.l.c. conditions are described in the Experimental section.

(liver) were more than 99% pure. Isoenzyme M_1M_1 (liver) contained a 3% impurity of subunits A_2 and M_4 , while A_1A_2 (liver) contained an unknown impurity of about 4%. Isoenzyme M_4M_4 (testis) contained a 4–5% impurity of subunit M_3 , and M_3M_3 (testis) contained a 6% impurity of subunit M_4 . The h.p.l.c. elution profiles of A_1A_1 , A_1A_2 and M_1M_1 (liver) and M_2M_2 , M_2M_3 , M_3M_3 , M_4M_4 and P_1P_1 (testis) are presented in Fig. 2. The impurities, with retention times of 14.2 and 26.9 min, in M_3M_3 could not be identified. The component eluting at 14.2 min was most probably a low-molecular-mass (< 10000 Da) component, because its concentration decreased during ultrafiltration.

Isoelectric focusing of isoenzyme M_4M_4 showed a rather broad band, which was also observed when chromatofocusing this isoenzyme. However, SDS/PAGE and h.p.l.c. analysis showed only distinct single bands. Immediately after chromato-

focusing, A_1A_2 showed a single band. After prolonged storage, two more bands were observed, indicating that rehybridization of the subunits A_1 and A_2 to homodimers had taken place.

Physical and chemical properties

The isoelectric points and the subunit molecular masses of the purified hamster GSTs, together with their chromatofocusing pHs, are presented in Table 1.

The *N*-terminal amino acid sequences of the subunits M_1 , M_2 , M_3 and P_1 are presented in Table 2. The (partial) sequences of the CNBr-derived peptide fragments of subunits A_1 , A_2 and M_4 are also presented. Corresponding sequences of rat and human GST subunits are also included [1,23–25]. The matching peptide fragments of subunits A_1 , A_2 and M_4 were found in the *N*-terminal region and the region between positions 51 and 103. The sequences showed that the isoenzymes containing subunits A_1

Table 4. Concentration of hamster GST subunits in liver, kidney, testis, pancreas and trachea

– indicates not detectable. Subunit compositions were determined by the h.p.l.c. method as described in the Experimental section.

Source	Subunit ...	Subunit concentration ($\mu\text{g/g}$ of tissue)						
		A ₁	A ₂	M ₁	M ₂	M ₃	M ₄	P ₁
Liver		2327	592	3596	–	–	536	–
Kidney		1228	234	–	–	–	350	487
Testis		–	67	274	246	115	588	70
Pancreas		38	–	–	–	–	–	235
Trachea		137	8	–	–	–	35	443

and A₂ were Alpha-class enzymes, the enzymes containing subunits M₁, M₂, M₃ and M₄ were Mu-class enzymes, while P₁P₁ could be classified as a Pi-class enzyme.

Substrate specificity

The specific activities of the purified enzymes towards different substrates are presented in Table 3. Isoenzyme M₂M₂ was not purified in sufficiently high quantities to allow determination of substrate specificities. The substrate specificities confirmed the results obtained by amino acid sequence analysis. The Alpha-class enzymes showed a relatively high activity towards cumene hydroperoxide, the Mu-class enzymes towards *trans*-stilbene oxide, with the exception of M₁M₁, and the Pi-class enzyme P₁P₁ towards ethacrynic acid.

GST subunit composition of various organs

The GST-subunit patterns of liver, kidney, testis, pancreas and trachea are presented in Fig. 3. The subunits present in the different *S*-hexylglutathione eluates were identified by co-elution with purified isoenzymes. The tissue subunit concentrations of the various organs are summarized in Table 4.

As expected, the liver contained the subunits A₁, A₂ and M₁. In addition, the *S*-hexylglutathione eluate also contained subunit M₄. Besides subunits A₁, A₂ and P₁, the kidney eluate also contained subunit M₄. The two peaks in the kidney eluate, eluting at 10.8 and 19.0 min, were caused by the haemoglobin impurity. The testis *S*-hexylglutathione eluate contained the GST subunits P₁, M₁, M₂, M₃ and M₄. In addition, the testis eluate contained subunit A₂. Whether hamster testis also contained subunit A₁ could not be established because of the almost equal retention times of subunit A₁ and M₃. However, as a result of chromatofocusing, the testis eluate may contain minor amounts of both subunits A₁ and A₂. To confirm this, further investigation will be needed.

In hamster pancreas and trachea the major subunit was subunit P₁. In addition, both organs contained subunit A₁. Subunits A₂ and M₄ seemed to be completely absent in the pancreas, while the trachea eluate contained minor amounts of these subunits. Furthermore, both in pancreas and trachea the peak shoulders after the subunits P₁ and A₁ co-eluted together with the subunits M₂ and M₃ respectively. Therefore, pancreas and trachea may contain minor amounts of subunits M₂ and M₃. As observed with kidney, the two peaks eluting around 10.5 and 18.5 were due to the presence of haemoglobin.

DISCUSSION.

So far, only a few hamster GST isoenzymes have been purified. Smith *et al.* [12] have purified two isoenzymes from Chinese hamster liver with pI values of 9.0, named forms II and III in

order of their elution from carboxymethylcellulose, with physical similarities to basic GSTs. These are probably the same as the Alpha-class isoenzymes SG-1 and SG-2, purified from Syrian golden hamster liver by Jensen & MacKay [13]. Furthermore, Jensen & MacKay have described an unresolved set of Mu-class isoenzymes [13]. In this paper, the existence of six homodimeric and two heterodimeric GST isoenzymes was demonstrated.

With our purification procedures, two Alpha-class isoenzymes, A₁A₁ and A₁A₂, and one Mu-class isoenzyme, M₁M₁, could be purified from Syrian golden hamster liver. A₁A₁ is probably identical to the SG-1 isoenzyme. The homodimeric SG-2 isoenzyme, presumably equal to GST A₂A₂, was not purified in our experiments, whereas the heterodimeric GST A₁A₂ was clearly identified in the chromatofocusing eluate. Contrary to the results of Jensen & MacKay [13] we only found one Mu-class isoenzyme eluting at pH 8.8, which was confirmed by isoelectric focusing and h.p.l.c. analysis.

On comparing the purified and characterized hamster GST isoenzymes with rat and human isoenzymes, the hamster Alpha-class isoenzymes A₁A₁, A₁A₂ and A₂A₂, although only partially sequenced, show a high degree of similarity to the rat isoenzymes 1-1, 1-2 and 2-2, and the human isoenzymes B₁B₁, B₁B₂ and B₂B₂. Furthermore, on the sequence data available, the hamster Pi-class enzyme P₁P₁ could be a homologue of the Pi-class enzymes rat 7-7 and human π . Sequence similarity between the hamster Mu-class enzymes and the rat and human Mu-class enzymes is not very obvious. Possibly isoenzyme M₄M₄ shows resemblance with the rat Mu-class enzyme 11-11 [24]. The *N*-termini of both hamster M₄M₄ and rat 11-11 were blocked. Both enzymes showed a rather similar tissue distribution, although the relative amounts of hamster M₄ in liver (7%) and kidney (13%) were higher than the relative amounts of rat 11-11 in these organs (0.8 and 1% respectively). However, GST 11-11 is enzymically unstable [24], which was not the case for GST M₄M₄. The human Mu-class enzyme purified from testis and brain [25], may be the human homologue to rat 11-11 and hamster M₄M₄. Thus, sequence similarity of hamster Mu-class GSTs with rat and human GSTs is not as obvious as the similarity of the Alpha- and Pi-class enzymes with rat and human GSTs.

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