Mass spectrometric analysis of rat ovary and testis cytosolic glutathione S-transferases (GSTs): identification of a novel class-Alpha GST, rGSTA6*, in rat testis

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Cytosolic glutathione S-transferases (GSTs) from rat ovaries and testis were purified by a combination of GSH and *S*-hexylglutathione affinity chromatography. The isolated GSTs were subjected to reverse-phase HPLC, electrospray MS and N-terminal peptide sequencing analysis. The major GST isoenzymes expressed in ovaries are subunits A3, A4, M1, M2 and P1. Other isoenzymes detected are subunits A1, M3 and M6*. In rat testis, the major GST isoenzymes expressed are subunits A3, M1, M2, M3, M5* and M6*. Subunits A1, A4 and P1 are expressed in lesser amounts. We could not detect post-translational modifications of any GSTs with known cDNA sequence. The molecular masses of subunits M5* and M6*, two class-Mu GSTs that have not been cloned, were determined to be 25495 and 26538 Da respectively. An N-terminally modified protein from rat testis with molecular mass 25737 Da was isolated from the *S*-hexylglutathione column. Results from internal peptide sequencing analysis indicate that this is a novel class-Alpha GST that has not been previously reported. We designate this protein rGSTA6*.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are dimeric proteins that catalyse GSH-dependent conjugation and reduction [2,3] . The reactions are considered to be part of the cellular detoxification mechanism in the biotransformation of xenobiotics [4]. In addition, these isoenzymes possess non-catalytic binding functions [5,6].

Rat GSTs have been extensively studied. A total of 20 subunits have been detected and reported in the literature. These isoenzymes are grouped into six gene families, designated Alpha, Mu, Pi, Theta, Sigma and microsomal. For 13 of the subunits the cDNA (A1, A2, A3, A4, A5, M1, M2, M3, P1, T1, T2 and microsomal GST) or genomic sequence (M4) has been reported, and partial amino acid sequences have been published for three other isoenzymes (M5*, M6* and S1*) (for review, see [7]). A mitochondrial GST [8] that was previously identified as rGSTT3* was recently cloned by Pemble et al. [9]. Because of its unique DNA sequence, the gene product of this clone has been designated class-Kappa GST.

Post-translational modification has been suggested as a possible regulatory mechanism for GST activity. The N-termini of subunits A1, A2 and A4 have been shown to be acetylated [10,11]. Results from *in vitro* experiments have suggested that GSTs are substrates for phosphorylation [12] or methylation [13], and modified isoenzymes have altered activities. We have addressed the question of GST modification by carrying out careful MS analysis of GSTs isolated from rat livers [10] and kidneys [14]. The modifications that we have observed have been limited to N-terminal acetylation and C-terminal truncation.

Most studies on rat GSTs have been performed on hepatic tissues. The properties of testicular and ovarian GSTs have not been well defined. It has been suggested that sex hormones may be involved in the regulation of GST protein expression [15]. Awasthi et al. [16] speculated that testosterone/oestrogen may regulate the differential post-translational modification of murine testicular and ovarian GSTs. Recently, a glycosylated protein from rat testes was shown to have high sequence similarity to class-Mu GSTs and to utilize 1-chloro-2,4-dinitrobenzene as substrate [17]. In addition, rGSTM6*, the subunit that has been shown to undergo *in vitro* methylation [13], is found mainly in testis and brain tissues. Therefore we have extended our MS analysis of rat GSTs to testicular and ovarian tissues. The present studies were designed to detect tissue-specific expression and post-translational modification of GSTs.

MATERIALS AND METHODS

Materials

The tissues studied were from sexually mature Sprague-Dawley rats reared in the breeding colony of the Institute of Biomedical Sciences, Academia Sinica (Taiwan, Republic of China). GSH, *S*-hexylglutathione, epoxy-activated Sepharose 6B, bovine insulin, angiotensin II, adrenocorticotrophic hormone fragment (residue 18–39) and 4-hydroxy-α-cyanocinnamic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The GSH [18] and *S*-hexylglutathione [19] affinity columns were coupled as previously described. Vydac C_{18} reverse-phase columns were obtained from H-P Separations Group (Hesperia, CA, U.S.A.). Trifluoroacetic acid and heptafluorobutyric acid for HPLC analysis were obtained from Aldrich (Milwaukee, WI, U.S.A.). *Achromobacter* proteinase I was from Wako (Osaka, Japan). Problot membranes and chemicals for peptide sequencing

Abbreviations used: GST, glutathione S-transferase (the GST subunits are designated according to the class-based subunit nomenclature proposed by Mannervik et al. [1], which was originally devised for human GST); ES/MS, electrospray MS; LC/MS, liquid chromatography/MS; MALDI, matrix-assisted laser-desorption ionization; MIF, migration inhibitory factor.

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were obtained from PE-Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals used were reagent grade or better.

Isolation of GSTs

Adult Sprague–Dawley rats (200–250 g) were used throughout the experiments. All purifications were performed at 4 °C. The GSTs were purified by coupled affinity chromatography as described by Yeh et al. [10] with minor modifications. Briefly, tissues were homogenized in 4 vol. of buffer A (10 mM Tris/HCl, pH 8.0, 0.2 mM dithiothreitol, 2 mM EDTA and 0.5 mM PMSF) with 20 % sucrose. Debris was removed by centrifugation at 200000 ***g*** for 1 h. The supernatant was then dialysed against buffer A overnight.

The isoenzymes in the diffusate were loaded on to a GSH–Sepharose affinity column that was connected in tandem with an *S*-hexylglutathione-Sepharose column. The columns were pre-equilibrated in buffer A and washed with buffer A containing 0.2 M NaCl after sample loading. The columns were then disconnected and eluted separately with buffer A containing 0.2 M NaCl, 5 mM GSH and 5 mM *S*-hexylglutathione. Isoenzymes from the GSH affinity column were analysed directly or stored immediately at −70 °C. Fractions from the *S*-hexylglutathione affinity column were concentrated with Centriplus 10 concentrators (Amicon) before analysis or storage.

Liquid chromatography/MS (LC/MS) analysis of GSTs

The GST isoenzymes were analysed on a Vydac C_{18} reverse-phase column (1 mm × 250 mm). Samples were introduced from a Prodigix-4P (Microtech Scientific) solvent-delivery system. The elution profile was monitored by UV absorbance at 214 nm with a model UVIS 204 detector (Linear Instruments) equipped with a 250 nl illuminated volume high-pressure flow cell. The column was equilibrated with 45 % solvent 1 (0.08 % trifluoroacetic acid in water) and 55 % solvent 2 (0.06 % trifluoroacetic acid in 80 % acetonitrile). After sample injection, the column was washed under equilibrating conditions for 10 min. A linear gradient of 45–75 % solvent 2 was developed over 60 and 90 min for the ovarian and testicular GSTs respectively at a flow rate of 28 μl/min.

Electrospray MS (ES/MS) analyses were performed on a VG Quattro-Bio-Q mass spectrometer (Micromass, Altrincham, Cheshire, UK). A splitter (1:3) was placed after the UV detector flow cell for sample collection, and approx. 6–8 μl of sample/min was introduced into the mass spectrometer. The instrument was set for positive ion detection and scanned from m/z 600 to 1600 in 8 s/scan. The multiply charged ion peaks from horse heart myoglobin (molecular mass 16951 Da) was used as an external reference for mass scale calibration. The collected data were summed according to the total ion current profile and process by the MaxEnt program [20].

HPLC purification of rGSTA6*

GSTs isolated from the *S*-hexylglutathione affinity column were separated on a Vydac C_{18} regular bore column (4.6 mm × 250 mm). The buffer system and the gradient employed were identical with those mentioned above. The column was developed at a flow rate of 600 μl/min and the UV-absorbing peak corresponding to rGSTA3 was collected.

The rGSTA3 fraction thus collected was further purified on a C_{18} narrow bore column (2.1 mm × 250 mm). The column was equilibrated with 55 % solvent 3 (0.08 % heptafluorobutyric acid in water) and 45 % solvent 4 (0.06 % heptafluorobutyric acid in 80 % acetonitrile). A linear gradient of 45–55 % solvent 4 in 10 min, then 55–80 % solvent 4 in 75 min was developed 10 min after sample loading at a flow rate of 128 μl/min. The elution profile was monitored at 214 nm as outlined above and the separated GST isoenzymes were collected manually.

Peptide mapping

The purified rGSTA6* was digested with *Achromobacter* protease I in 100 mM NH_4HCO_3 (pH 8.8)/2 M urea, at 45 °C for 18 h. The substrate/enzyme ratio was maintained at 20:1.

The protease digests were separated on the LC/MS system outlined above. The peptides were resolved on a microbore C_{18} reverse-phase column with a linear gradient of 0–70 % solvent 2 developed over 175 min at a flow rate of 28 μl/min. A quarter of the samples were subjected to on-line ES/MS analysis. The rest were used for Edman degradation and matrix-assisted laser-desorption ionization MS (MALDI-MS) analysis.

For peptide mapping, the mass spectrometer was calibrated in the positive ion mode with a NaI/CsI mixture (250–2050 Da) as external standard. Data were collected in centroid mode from m/z 250 to 1850 at a scanning rate of 6 s/scan [21].

Protein sequencing and MALDI-MS

Automated cycles of Edman degradation were performed with an Applied Biosystems Procise model 491 protein sequencer. The peptide samples were directly blotted on to precut (0.4 cm × 0.4 cm) Problot membranes under light suction from a water aspirator. The samples on the membranes were subjected to Edman degradation without further precycle treatment.

The mass of each peptide sample was confirmed by MALDI-MS on a Bruker Reflex time-of-flight mass spectrometer (Bremen, Germany) equipped with a 337 nm nitrogen laser and a Scout sample stage source. The analyser was used in the linear mode, at accelerating voltages of 10 kV. Data were acquired at a laser power just above that required for threshold irradiation of analyte ion generation. Low-mass matrix ions were deflected with a 5 μs voltage pulse. Angiotensin II (1047.2 Da), an adrenocorticotrophic hormone fragment (residue 18–39, 2466.7 Da) and bovine insulin (5734.6 Da) were used as external standards. The samples were embedded in 4-hydroxy-α-cyanocinnamic acid for analysis [22].

RESULTS AND DISCUSSION

Rat ovarian GSTs isolated by coupled affinity chromatography

Rat ovary cytosolic GSTs were purified on a combination of GSH and *S*-hexylglutathione affinity columns arranged in tandem [9]. The reducing agent in the purification buffers was purposely changed from 2-mercaptoethanol to dithiothreitol. The Cys-173 of rGSTM1 can be specifically modified by 2-mercaptoethanol (C.-H. Hsieh and M. F. Tam, unpublished work), and therefore this compound was replaced in all buffers to eliminate the possibility of modifying rGSTM1 or other isoenzymes during purification. With this purification procedure, we generally recover 75 μg of GST/g of tissue from both columns.

The GSH affinity column retained approx. 85 % of the total ovarian cytosolic GST isoenzymes. The chromatogram of these proteins is presented in Figure 1(a). The major GST subunits detected were A3, A4, M1, M2 and P1. Subunits A1, M3 and M6* were expressed in minor quantities. The molecular masses of these isoenzymes were determined by LC/MS and the results are summarized in Table 1. Except for rGSTM6*, the molecular masses obtained are identical with those for the isoenzymes from liver [10] and kidney [14], and similar to those predicted from

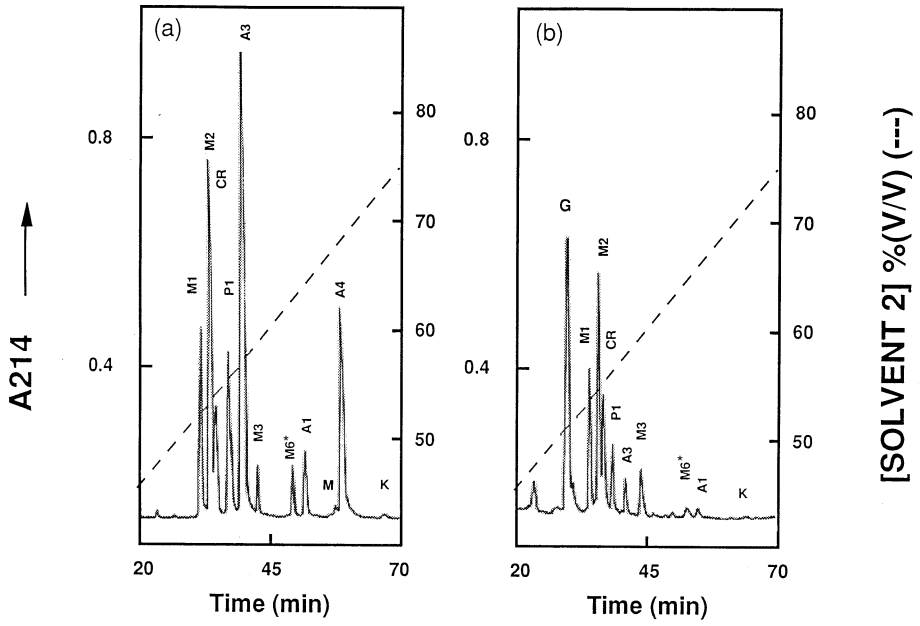

Figure 1 HPLC profile of rat ovary cytosolic GSTs

Rat ovary cytosolic GSTs were purified by coupled affinity chromatography. Fractions eluted from the GSH (**a**) and *S*-hexylglutathione (**b**) affinity columns were analysed on a C_{18} reverse-phase column. ——, A_{214}; – – – –, [solvent 2]. The GST subunits, carbonyl reductase (CR); glyoxalase I (G), keratin (K) and a possible class-Mu GST (M) are indicated.

Table 1 Molecular masses of rat testis and ovary cytosolic proteins isolated by GSH or *S*-hexylglutathione affinity column and determined by ES/MS

N.D., Not detected; MIF, migration inhibitory factor; hGSH, *S*-hexylglutathione.

	Molecular mass (Da)					
		Testis		Ovary		
Protein	Predicted	GSH column	hGSH column	GSH column	hGSH column	Reference
rGSTA1	25518†	25518±2	25518±1	25520±2	25519±1	pGTR261 [23]
rGSTA3	25188	25188±2	25188±3	25188±1	25191±2	pGTB42 [29]
rGSTA4	25550	25551±2	N.D.	25551±2	N.D.	λGSRA8 [27]
rGSTA6*		N.D.	25737±2	N.D.	N.D.	
rGSTM1	25782	25784±3	25783±2	25785±1	25785±3	pGTR200 [24]
rGSTM2	25571	25573±2	25572±2	25573±2	25573±2	λGTR4-1 [25]
rGSTM3	25549	25550±1	25552±3	25551±2	25551±2	Yb_3 [26]
rGSTM5*		25495±3	25496±2	N.D.	N.D.	
rGSTM6*		26538±3	26541±2	26542±1	26542±2	
rGSTP1	23307	23308±2	23308±1	23308±1	23311±2	pGP5 [28]
Glyoxalase I	20733	N.D.	20730±1	N.D.	20730±1	
MIF	12477	N.D.	12344±1	N.D.	12344±1	pR-MIF [31]
Carbonyl reductase		30469±5	30466±2	30466±1	30471±4	[30]
		30568±3	30566±2	30514±1	30516±6	
				30560±5	30565±8	
Keratin		45153±3	45153±2	45153±1	45151±3	
T		N.D.	28946±2	N.D.	N.D.	
X		25365±2	25367±3	N.D.	N.D.	
M		25425±3	N.D.	25424±3	N.D.	

† Molecular mass was predicted from pGTR261 with arginine and valine at positions 151 and 207 [13] respectively.

DNA sequences [23–29]. The results imply that, besides N-terminal acetylation of subunits A1 and A4, these isoenzymes were not post-translationally modified. We would also like to point out that subunit A1 exists as full-length not truncated forms in ovary and testes.

Subunit M6* has a molecular mass of 26541±2 Da. The

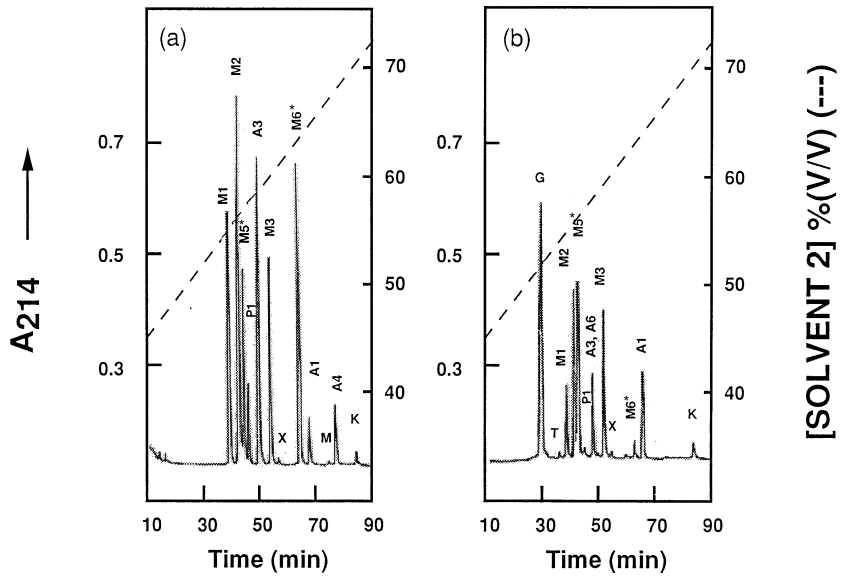

Figure 2 HPLC profile of rat testis cytosolic GSTs

Rat testis cytosolic GSTs were purified by coupled affinity chromatography. Fractions eluted from the GSH (**a**) and *S*-hexylglutathione (**b**) affinity columns were analysed on a C_{18} reverse-phase column. ——, A_{214}; – – – –, [solvent 2]. The GST subunits, an unidentified protein (X), keratin (K), a possible class-Mu (M) and a possible class-Theta GST (T) are indicated.

corresponding genomic or cDNA of rGSTM6* has not been isolated. Therefore this is the first accurate mass of this protein to be reported. Subunit M6* was purified from testis by Kispert et al. [32] as a labile class-Mu GST with a blocked N-terminus. We established the identity of this isoenzyme on the HPLC profile by internal peptide sequencing. We obtained sequences of SMVLGYWDIRGLAHAIRMLLEFTDTSYEEK and IAAFLQSDRK for two of the peptides isolated from an *Achromobacter* protease I digest. The sequences are identical with those of the CNBr-derived peptides obtained by Kispert et al. [32]. The sequence of the first peptide shares 83% identity with the N-terminus of rGSTM2. However, the N-terminal blockage on rGSTM6* could not be removed by treating the purified subunits with trifluoroacetic acid vapour at 60 °C for 30 min [33]. The result implies that rGSTM6* is not initiated with an acetylated serine. Further experiments are needed to establish the N-terminal residue(s) and the nature of the blockage of rGSTM6*.

A protein eluted after rGSTM2 (Figure 1a; CR) was identified as carbonyl reductase. Internal peptide sequence analysis of the *S*-pyridylethylated protein generated the following results: GIGFAIVRDLCRK, QLQTEGLSP, VVDPTPFHIQA, TNFFGTQDVCK and KVEPW. The sequences of these peptides are identical with that reported for rat liver carbonyl reductase [30]. The results are not unexpected, since chick [34] and pig [35] liver carbonyl reductases have been reported to be co-purified with GSTs.

The molecular mass of rat carbonyl reductase as predicted from the cDNA sequence is 30447 Da, excluding the initiator methionine. MS analyses indicate that rat ovarian carbonyl reductase is a mixture of three polypeptides with molecular masses 30466 ± 1, 30514 ± 1 and 30560 ± 5 Da. Assuming that these proteins have equivalent ionization efficiency, they were present in the proportions 7:10:4 in our sample. The human carbonyl reductase is N-terminally acetylated and has a carboxyethyl-lysine at position 238 [36]. The molecular mass of the modified human enzyme is 114 Da higher than that predicted from the cDNA sequence. The rat carbonyl reductase is N-terminally blocked and 19–113 Da larger than predicted. The component with the highest molecular mass probably represents the N-acetylated and carboxyethyl-lysyl enzyme. However, the actual identities of the modification moieties on the rat enzyme remain to be investigated.

A protein of 25424 ± 3 Da was eluted from the reverse-phase column in front of rGSTA4 (Figure 1a; protein M). The N-terminal sequence of this protein is PMTLGYWFDRGL, showing a high sequence identity with other class-Mu GSTs. This protein is also expressed in rat spleen (C.-H. Hsieh and M. F. Tam, unpublished work). Rouimi et al. [37] have identified from rat liver a class-Mu GST (25443 Da) that was eluted from the reverse-phase column at a similar position. The N-terminal sequence (PMTLGYWDIRGLAHA) they obtained was identical with that of subunit M2 or M3. It is difficult to assess whether we are observing the same protein, since the molecular masses of the GSTs reported by Rouimi et al. [37] deviate by 2 (rGSTM2) to 29 (rGSTA4) Da from those predicted from the cDNA sequences.

A 45 kDa protein was eluted from the reverse-phase column near the end of the acetonitrile gradient (Figure 1a; protein K). Internal sequence analysis of this N-terminally blocked protein yielded sequences of IRELLQQME, SLAE/YFD and YEELEITAGRHGDSVR for three of the isolated peptides from an *Achromobacter* protease I digest. These peptides have high sequence similarity to residues 137–145, 293–300 and 303–319 respectively of mouse keratin as reported by Steinert et al. [38].

Rat ovarian GSTs retained on the *S*-hexylglutathione column were analysed by LC/MS, and the chromatogram generated by UV detection is presented in Figure 1(b). Except for subunit A4 and protein M, the *S*-hexylglutathione affinity column bound a similar set of GSTs to those retained on the GSH affinity column. The molecular masses of these GSTs are summarized in Table 1.

Other proteins identified from the *S*-hexylglutathione affinity column fraction are glyoxalase I (20730 ± 1 Da) and macrophase migration inhibitory factor (MIF, 12344 ± 1 Da). We have previously established the presence of glyoxalase I in a GST sample

prepared from rat liver tissues using the coupled affinity chromatography method [10].

The MIF co-migrated with glyoxalase I on the reverse-phase column but could be easily detected by MS. This protein has been shown to be related to GST [39]. The clone for rat MIF has been isolated by Sakai et al. [31]. The protein has a predicted molecular mass of 12477 Da, including the initiator methionine. Therefore, the MIF isolated by *S*-hexylglutathione affinity column chromatography was N-terminally processed but unmodified, even though two potentially N-linked glycosylation sites have been identified by Sakai et al. [31].

Rat testicular GSTs isolated by coupled affinity chromatography

Rat testis expressed GSTs at a higher level than ovary. Using the coupled affinity chromatography method, we routinely recovered 395 μg of GST/g of tissue. The yield is approx. 5-fold higher than that from the ovary. The HPLC elution profile of the fraction isolated from the GSH affinity column is presented in Figure 2(a). The molecular masses of the subunits as determined by LC/MS are summarized in Table 1.

Class-Mu isoenzymes are expressed predominantly in rat testis. The major subunits detected are M1, M2, M3, M5*, M6* and A3. Subunits A1, A4 and P1 are expressed in minor amounts but could be easily detected. The elution profile we obtained is similar to that reported by Johnson et al. [13]. The amount of subunit M1 recovered was significantly higher than that observed by Gandy et al. [40].

rGSTM5* appears to be specifically expressed in testis, as we could not detect it by LC/MS in ovary, kidney, liver, spleen, heart or brain tissues. This isoenzyme was isolated as rGSTM3-5* heterodimers by Ishikawa et al. [41], who also reported the partial sequence for the N-terminal 20 residues. We were able to obtain the sequence for the first 44 residues of the HPLC-purified rGSTM5*: PVTLGYWDIRGLGHAIRLLLEYTETSYEEKR YAMGDAPDYDRRQ. The molecular mass of rGSTM5* was determined to be 25495 $\pm$ 3 Da. The cDNA for this isoenzyme has not been isolated, and whether this protein is post-translationally modified remains to be investigated.

Carbonyl reductase appeared as a shoulder between subunits M2 and M5*. Unlike the corresponding enzymes from ovary, the testicular carbonyl reductase sample contained two polypeptides with molecular masses of 30469 $\pm$ 5 and 30568 $\pm$ 3 Da. The 30514 Da polypeptide, a major component of ovarian carbonyl reductase, was missing from the preparation.

Other proteins identified on the chromatogram were proteins X, M and K (Figure 2a). Proteins M and K can also be found in the ovarian tissues. Protein X was eluted after subunit M3 and has a molecular mass of 25365 $\pm$ 2 Da and a blocked N-terminus. We were unable to generate an internal peptide sequence for this protein because of the limited availability of the sample.

The HPLC profile of the GST isoenzymes bound to the *S*-hexylglutathione affinity column is presented in Figure 2(b). The molecular masses of the proteins as determined by LC/MS are summarized in Table 1. These isoenzymes represent approx. 3% of the total purified testicular GSTs. As for ovarian GSTs, subunit A4 was missing, whereas glyoxalase I and MIF were present in this fraction. We have identified two unique proteins in this fraction.

Protein T is eluted in front of subunit M1 from the reverse-phase column. This protein has a molecular mass of 28946 $\pm$ 2 Da and a blocked N-terminus. Proteinase digestion generated a peptide that yielded a sequence of YLDLLSQP/RSIYIFAR on Edman degradation. Except for serine and arginine at positions 11 and 17 respectively, the sequence of this peptide is identical

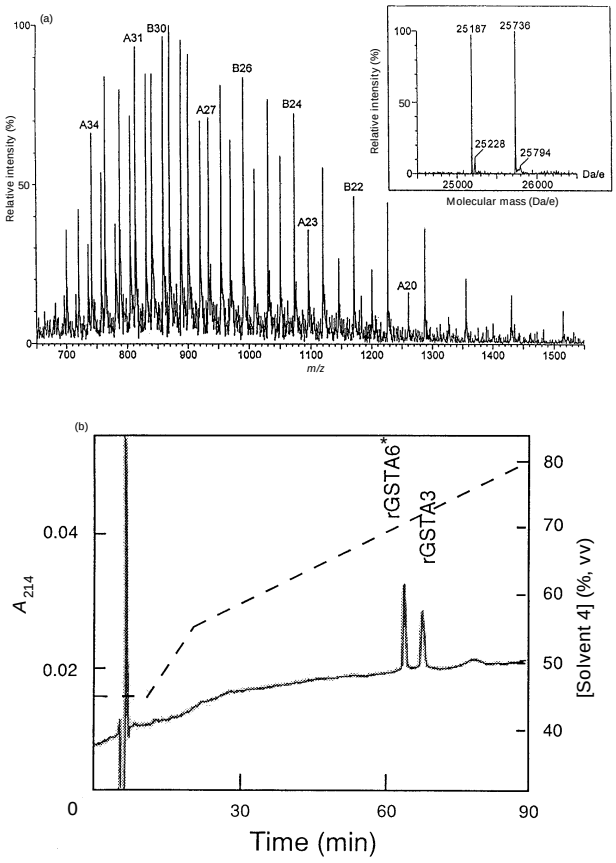

Figure 3 Analysis of rGSTA3 and rGSTA6*

(**a**) Combined scanning mass spectrum of the rGSTA3 and rGSTA6* mixture. The instrument was set for positive ion detection. Some of the ions and their charge states as contributed by rGSTA3 (A) or rGSTA6* (B) are designated. The deconvoluted mass spectrum with the molecular masses indicated is presented in the inset. (**b**) HPLC profile of the rGSTA3 and rGSTA6* fraction from Figure 2(b) rechromatographed on a C_{18} reverse-phase column. Heptafluorobutyric acid was used as ion-pairing reagent. ——, A_{214}; – – –, [solvent 4].

with that of rGSTT1 [42]. Subunit T1 has a cysteine for the corresponding unidentified residue on our peptide. In addition, this sequence differed from subunit T2 in only three positions. Residues 9, 12 and 17 were replaced with serine, valine and lysine in rGSTT2 [43]. Schroder et al. [44] have recently characterized an N-terminally modified class-Theta GST from human erythrocytes with unique substrate specificities. Therefore protein T probably represents a third and unidentified gene product of class-Theta GST.

MS analyses revealed the presence of an unknown protein that was co-eluted with rGSTA3 on the reverse-phase column (Figure 3a). This protein has a modified N-terminus and a molecular mass of 25737 $\pm$ 2 Da. It can be separated from subunit A3 on a reverse-phase column by using heptafluorobutyric acid as ion-pairing reagent (Figure 3b).

MS and sequencing analysis of rGSTA6*

Subunit rGSTA6* was purified to apparent homogeneity and digested with *Achromobacter* protease I. The resulting peptides were subjected to LC/MS analysis (Figure 4), and fractions as indicated were collected for Edman degradation. The molecular masses of the peptides in these fractions were confirmed with MALDI-MS analysis in a time-of-flight mass spectrometer. The

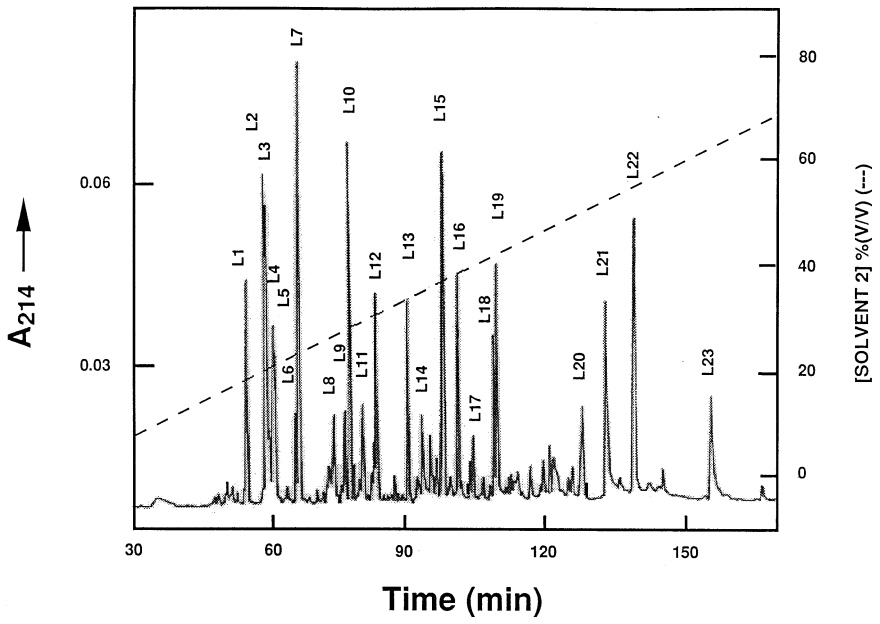

Figure 4 HPLC profile of protease digests of rGSTA6*

Purified rGSTA6* was digested with *Achromobacter* protease I and resulting peptides were subjected to LC/MS analysis. Fractions collected for amino acid sequencing analysis are indicated (L1–L23). ——, A_{214}; – – – –, [solvent 2].

Table 2 Amino acid sequencing and MS analyses of *Achromobacter* protease digests of rGSTA6*

Fraction	Sequence	Molecular mass (Da)	
		Observed	Calculated
L1	YDLYGK	757.5	757.8
L2	FLQPGSQRQPPVDEK	1725.7	1725.9
L3	FIHTNEDLEK	1246.7	1245.4
L4	KFLQPGSQRQPPVDEK	1853.6	1854.1
L5	TRMSDMPTIKK	1307.4	1307.6
L6	TRMSDMPTIK	1179.2	1179.4
L7	VFESHGQDYLVGNK	1592.6	1592.7
	EANIALMK	889.6	889.1
L8	No sequence	1768.4	
L9	No sequence	1344.6	
L10	ATNRYFPAFEK	1344.7	1343.5
L11	LVQTRAIMNYFSSK	1674.4	1658
L12	No sequence	—	
L13	LVQTRAIMNYFSSK	1657.7	1658
L14	No sequence	1625.7	
L15	No sequence	—	
L16	LRSDGVLMFQQVPMVEVDGMK	2379.4	2379.9
L17	PLFHYDEARGRMESVRWLLAAAGVEYEEK	3439.8	3423.9
L18	No sequence	3798.1	
L19	PLFHYDEARGRMESVRWLLAAAGVEYEEK	3423.5	3423.9
L20	No sequence	3429.8	
L21	ERALIDMYSEGLADLNEMFILYPFDPPGVK	3445.4	3445
L22	No sequence	3465.9	
L23	ADVHLVEMIYNMEELDQNILANFPLLSALK	3459.4	3445

results obtained from ES/MS and MALDI-MS deviate less than 1 Da from each other in all the samples tested. The sequencing and MS analysis results are summarized in Table 2.

The *Achromobacter* protease digests were fractionated into 23 peaks on HPLC purification. Sequencing data were obtained from 15 fractions containing 16 peptides. The results covering 199 residues are listed in Figure 5 with other class-Alpha rat GSTs for comparison. The other eight fractions did not yield any sequence on Edman degradation, and two fractions within this group did not give any meaningful ionization products on MS analysis. The MS data for fractions L11, L17 and L23 are 14–16 Da higher than that predicted from peptide sequencing. Peptides L13 and L19 have the same sequences as L11 and L17 respectively, and their molecular masses match those predicted from their sequences. Therefore the additional mass on peptides L11, L17 and L23 is probably caused by oxidation of methionine residues.

Sequencing data show clearly that this protein is the product of a unique gene (Figure 5), and shares 58 (rGSTA4) to 69% (rGSTA5) sequence identity with other rat class-Alpha GSTs. We designate it rGSTA6* according to the class-based subunit nomenclature proposed by Mannervik et al. [1].

The structure of human GSTA1-1 has been elucidated by Sinning et al. [47]. Residues in the G- and H-site of the rat class-Alpha GSTs can thus be inferred from the human isoenzyme structure. It is noteworthy that the 10 G-site residues are mostly identical or conserved (Figure 5), whereas the H-site residues are rather diversified. In particular, rGSTA6* has an aspartic acid instead of phenylalanine at position 10. This should give it unique binding properties, if not enzymic activity, among the class-Alpha GSTs. Because of the low abundance of this enzyme in rat testis, its enzymic characterization must await cloning and expression experiments.

In the literature, there are three class-Alpha rat GSTs that have not been cloned and sequenced. These subunits were isolated on *S*-hexylglutathione columns. Hayes [48] identified a Y1 GST subunit from rat kidney. GST Y1 has a molecular mass of 25700 Da and is immunochemically related to class-Alpha GSTs. The other two subunits are GST A(6) from liver [11], and a

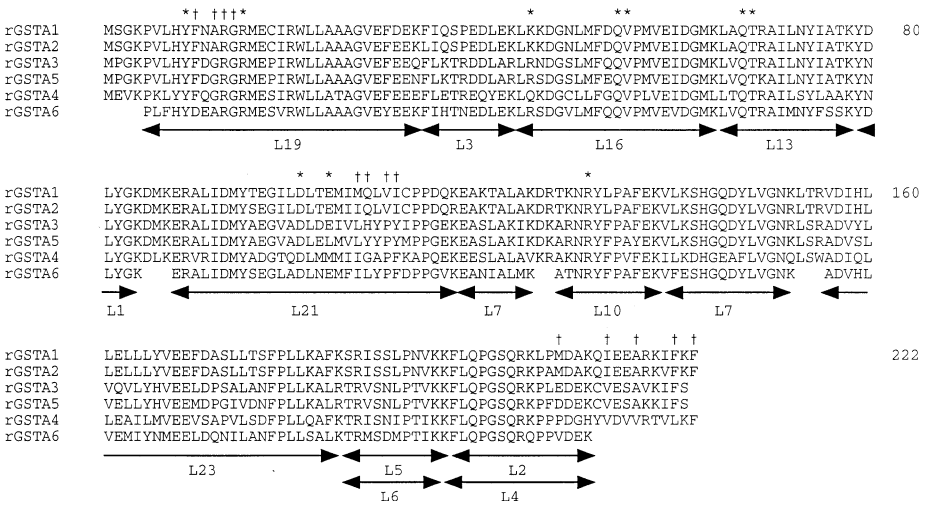

Figure 5 Comparison of the primary sequence of rGSTA6* with those of rGSTA1 [23], rGSTA2 [45], rGSTA3 [29], rGSTA5 [46] and rGSTA4 [27]

The amino acid sequences are given in single-letter code. The numbering of the amino acid residues includes the initiator methionine. Sequences of peptides from Figure 4 are indicated. The positions of G-site and H-site residues as identified by Sinning et al. [47] are indicated by * and † respectively.

protein with pI 5.8 from spleen [49]. We have reanalysed the male rat kidney and liver GSTs isolated by the coupled affinity chromatography method. We could not detect rGSTA6* in our kidney preparations. However, we did observe a protein with a molecular mass of 25736 ± 2 Da, which was eluted as a shoulder in front of rGSTA3 in the rat liver preparations (results not shown). The amount of protein expressed is at least 50-fold less than that detected in testis. Subunit A6* could also be detected in spleen. The amount of A6* expressed is similar to that in testis (results not shown). Therefore rGSTA6* and Y1 are probably different proteins. Whether subunit A6* is the equivalent of GST A(6) from liver [11] or subunit pI 5.8 from spleen [49] remains to be investigated.

In summary, we have analysed the GST isoenzymes from rat ovaries and testes by LC/MS and peptide sequencing. Rat ovaries have more class-Alpha than class-Mu GSTs. Testes express predominantly class-Mu GSTs. Subunit M5* is expressed specifically in testes. We provide the partial amino acid sequence for rGSTA6*, a subunit that has not been previously characterized.

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