Characterization of pig liver glutathione S-transferases using HPLC–electrospray-ionization mass spectrometry

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We have characterized 11 porcine liver cytosolic glutathione S-transferase (GST) subunits from their precise molecular mass, immunoreactivity and partial amino acid sequence. Four Alpha-, six Mu- and one unexpected Pi-class GST subunits were found with average molecular masses of 24.984–25.228 kDa, 25.039–25.657 kDa and 23.510 kDa respectively. Molecular masses were established using electrospray-ionization mass spectrometry, with a precision of ± 3 –4 mass units. Glutathione (GSH) and S-hexylglutathione (ShGSH) were tested as affinity ligands in the

purification procedure. The binding selectivity of GSH was better than that of ShGSH, although non-GST proteins were retained on both matrices. As already described in other studies, a number of non-GST proteins bound to the affinity resins. Two of them were tentatively identified as mevalonate kinase and carbonyl reductase. The characterization of pig liver cytosolic GST subunits pattern achieved in this work should constitute a useful tool for rapid evaluation of these enzymes' expression in modulation studies.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of multifunctional inducible enzymes that catalyse the conjugation of intracellular GSH to a wide variety of electrophilic cytotoxic and genotoxic molecules from endogenous or exogenous origin. The increased hydrophilicity of the GSH-conjugates makes easier their further metabolism and elimination. GSTs are qualitatively and quantitatively expressed at various basic levels in most mammalian tissues, but are also inducible by various substances [1,2]. Other functions, such as binding and transport of non-substrate hydrophobic ligands, have been mentioned for these enzymes [3]. Moreover, their induction by anti-cancer drugs was shown to confer drug resistance to certain tumour cells [4,5].

Four species-independent classes of cytosolic GSTs (Alpha, Mu, Pi and Theta) have been described and characterized on the basis of their enzymic properties, immunological reactivity, subunit composition and molecular structure [1,6,7]. An additional microsomal trimeric form [8] and a Theta-class mitochondrial form [9] have also been described. So far, the presence of three classes of GSTs (Alpha, Mu and Pi) has been mentioned in pig liver, lung, lens, ovary and kidney [10–14]. Nevertheless, except for a lung Pi-class GST for which the three-dimensional structure has been characterized [15], very little structural information on porcine GSTs is available in the literature.

The present paper describes the purification of porcine liver cytosolic GST subunits using either a GSH or an S-hexylglutathione (ShGSH) affinity chromatography and the characterization of the complete pattern using a combination of HPLC, electrospray-ionization mass spectrometry (ESI-MS) and amino-acid-sequence analysis, a method previously validated on the rat model [16].

EXPERIMENTAL

Chemicals, enzymes and standard proteins

GSH, 1-chloro-2,4-dinitrobenzene (CDNB), trifluoroacetic acid

(TFA), dithiothreitol (DTT), as well as *Pseudomonas fragi* Asp-N endoprotease, equine myoglobin and horse heart cytochrome *c*, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ShGSH was synthesized as described [17]. Coupling of GSH and ShGSH to epoxy-activated Sepharose CL-6B (Pharmacia, Uppsala, Sweden) were achieved according to respective published methods [17,18]. Ultrafiltration and concentration of protein samples were performed on Microsep 30 from Filtron Technology Corporation (Northborough, MA, U.S.A.). Wistar rat GST subunit 1b was purified by using a previously described method [16].

Animals and subcellular-fractionation procedure

Male Large White castrated pigs (about 100 kg) receiving standard unmedicated feed were slaughtered and liver was chopped in pieces which were kept at -80°C for further use. Diced thawed samples were homogenized in ice-cold 0.1 M potassium phosphate buffer, pH 7.4 (4 ml/g of tissue) and subcellular fractionation performed by the method of Alin et al. [19]. The cytosolic fraction was deep frozen at -80°C .

GST protein and subunit purification procedure

Cytosol samples were run through a Sephadex G-25 column equilibrated in 10 mM Tris/HCl (pH 7.8)/1 mM EDTA/0.2 mM DTT buffer (buffer A) and the unretained active fraction loaded on the affinity-chromatography column equilibrated in the same buffer. On the ShGSH-matrix, the gel was first rinsed with buffer A containing 0.2 M NaCl (buffer B) until no protein (estimated by A_{280}) could be detected in the effluent. Then, elution was performed using 5 mM ShGSH in buffer B. On the GSH-matrix, a single-step elution in 200 mM Tris/HCl/5 mM GSH/0.2 mM DTT, pH 9.2, was performed after extensive washing with buffer A. Active fractions were pooled and stored at -80°C . GST purity was checked by SDS/PAGE as described by Laemmli [20]. Protein concentration was estimated after trichloroacetic acid precipitation by the method of Lowry et al. [21]. GST

Abbreviations used: ESI-MS, electrospray-ionization mass spectrometry; GSH, reduced glutathione; ShGSH, S-hexylglutathione; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; TFA, trifluoroacetic acid; DTT, dithiothreitol; RP-, reversed-phase.

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Figure 1 Affinity-chromatographic purification of pig liver cytosol on ShGSH- and GSH-Sepharose 6B matrices

Samples of cytosol (10 and 10.8 ml) containing 31 mg of protein/ml were sequentially run through a Sephadex G25 (coarse grade) column (2.5 cm $\times$ 50 cm) and the unretained active fractions (about 25 ml) pooled and loaded on a ShGSH- (A) or GSH- (B) affinity column (1.6 cm $\times$ 18 cm) respectively. Fractions (1.6 ml) were monitored at 280 nm, and pools conserved at -80°C . Elution conditions are described in the Experimental section, and purification yields are given in Table 1(A).

subunit separation was performed at 22°C on a Vydac 201 TP 54 reversed-phase (RP-) HPLC column (4.6 mm $\times$ 250 mm) using a Waters 625 system (Waters S.A., St. Quentin-en-Yvelines, France) equipped with UV detection at 214 and 280 nm. Mobile phases were mixtures of acetonitrile in water (A = 3:7 and B = 7:3, both v/v) containing 0.1% TFA. Elution was performed at a flow rate of 1 ml/min using successive linear increases of B in A: 25% at 0 min, 38% at 5 min, 40% at 20 min, 80% at 35 min, 100% at 35.1 min and isocratic 100% for 10 min. Fractions corresponding to HPLC peaks were pooled and evaporated to dryness. Determination of peak protein contents was performed using the technique described by Schaffner and Weissmann [22].

Molecular-mass determination of GST subunits by ESI-MS

All mass spectra were obtained with a Nermag R10-10 H single quadrupole mass spectrometer (Nermag, Argenteuil, France) fitted with an ESI source from Analytica (Branford, CT, U.S.A.) and acquired using an HP ChemStation data system interfaced to the spectrometer (Quad Service, Poissy, France). Molecular masses were calculated from the multicharged peaks after smoothing with Gaussian filters [23] and using an average algorithm as described by Mann [24]. Analyses were performed in the positive-ion mode and in an acidic medium in order to enhance the protonation efficiency. HPLC-purified GST subunits were dried in a SpeedVac evaporator (Savant Instruments, Farmingdale, NY, U.S.A.) and solubilized in water/methanol/acetic acid (49:49:2, by vol.) to a final concentration of 20–40 μM . This solution was infused into the electrospray interface at a flow rate of 0.8 $\mu\text{l}/\text{min}$ using a Harvard apparatus (South Natic, MA, U.S.A.) model 22 syringe pump. The instrument was scanned over a mass range of m/z 700 to m/z 1700 in 15 s. Calibration was achieved using a 25 μM solution of myoglobin and checked before each measurement using a mixture of myoglobin and cytochrome c. Each spectrum was an average of 20 profiles acquired. The repeatability of the molecular-mass determination has been assessed from ten determinations of Wistar-rat GST subunit 1b (25.473 ± 0.003 kDa) [16]. Molecular-mass determination of each porcine subunit was deduced from at least three measurements.

Immunoblotting

Proteins (50–100 ng/track) were transferred from SDS/PAGE gels to nitrocellulose membranes using a PhastTransfer system (Pharmacia, Uppsala, Sweden). Rabbit anti-(porcine Alpha-class GST) primary antibody was generously given by Biotrin (Dublin, Ireland). The blot was probed using goat anti-rabbit secondary antibody coupled to peroxidase and FASTDAB (3,3'-diaminobenzidine) as substrate, both from Sigma Chemical Co.

Amino-acid-sequence determinations and comparisons, N-terminal deblocking and peptide mapping

Automated sequence analyses were carried out on a 477A sequenator connected to a 120A phenylthiohydantoin-derivative analyser, both from Perkin-Elmer (ABI, Foster City, CA, USA). RP-HPLC-purified GST subunit samples were dried and solubilized in acetonitrile/water/TFA (49:49:2, by vol.) before sequencing. N-Terminal sequences were compared with data from EMBL, Genbank, NBRF and Swiss-Protein databases. N-terminal chemical deblocking of the modified forms was performed on dried samples by incubation at 60°C for 30 min in TFA vapour according to a method recently described [25]. Undeblockable forms were digested with Asp-N endoproteinase for 16 h at 37°C in 0.1 M Tris/HCl, pH 8.2 (enzyme/substrate ratio 1:100, w/w). Digests were fractionated by RP-HPLC at 40°C on a Hypersil C18 3 μ narrow-bore column (2.1 mm $\times$ 150 mm) using a Hewlett-Packard (Böblingen, Germany) 1050TI HPLC apparatus with a 1040M diode-array detector. Elution was achieved at a flow rate of 0.3 ml/min with a linear gradient of 0–80% acetonitrile and 0.1–0.096% TFA in 80 min. Isolated peptides were submitted to amino-acid-sequence analysis as described above.

RESULTS

Purification of GSTs and HPLC separation of subunits

GST purification achieved on ShGSH- and GSH-affinity matrices (Figure 1) and subsequent HPLC separation of their constitutive

Figure 2 RP-HPLC separation of porcine liver GST subunits after purification on ShGSH- (A and B) or GSH-affinity (C) matrices

Protein samples (54, 79 and 52 μ g) from ShGSH-affinity bound pools I (A) and II (B) or GSH-affinity-retained fraction (C) were respectively loaded on a Vydac 201TP54 column (4.6 mm $\times$ 250 mm). Elution conditions are described in the Experimental section, and quantitative data are given in Table 1(B). Conditions: temperature, 22 $^{\circ}$ C; flow rate, 1 ml/min. Fractions were monitored at 214 nm.

subunits from contaminant proteins (Figure 2) were performed according to the procedures described in the Experimental section. CDNB-conjugation activity and/or protein content were evaluated in the eluates and revealed slight differences depending on the purification procedure used (Table 1).

Two groups of pig liver cytosolic proteins, differently bound to ShGSH-matrix, were named pool I and pool II according to their respective elution order from the column (Figure 1A). They were shown to contain distinct subunits when submitted to HPLC analysis. Pool I exhibited three peaks (Figure 2A), and pool II exhibited 11 peaks including three eluted at retention times similar to those in pool I (Figure 2B). When purification was achieved on GSH-Sepharose, there was no evidence for a shoulder in the elution pattern (compare Figures 1B and 1A), and ten of the 11 peaks (see Figure 2B) were identifiable on

HPLC (Figure 2C). The major peak 3 was absent using this procedure. Minor peaks 11 and 12 were less and more pronounced, respectively. The width of Peak 6 suggested it could correspond to more than one compound, so the first and second halves of the peak were collected separately and named 6A and 6B respectively. From SDS/PAGE, each peak appeared homogeneous, except the minor peak 1, which contained a protein with an apparent molecular mass of 30 kDa often contaminated by a 60 kDa molecule in ShGSH purifications (results not shown).

Molecular-mass determination of GST subunits by ESI-MS

Each protein obtained from HPLC peaks was directly analysed by ESI-MS as described in the Experimental section. The precision of the measures was ± 3 –4 Da, in accordance with typical ESI-MS conditions [16] (Table 2). Except for peaks 1, 6a, 6b, 8 and 10, which contained two distinguishable proteins, a single value was obtained from the others. The molecular mass of the protein in peaks 7 and 12 could not be determined, owing probably to very low amounts combined with bad solubility. As already expected from HPLC retention times, proteins from the ShGSH-affinity pool I were shown to be identical with the corresponding subunits 4, 5, 6a and 6b in pool II. This indicates that proteins in peaks 1, 2, 3, 7, 8, 9, 10, and 11 are specific to pool II. GSH-affinity provided polypeptides with HPLC retention times and molecular masses corresponding to subunits 2, 4, 5, 6a, 6b, 8a, 8b, 9, 10a, 10b and proteins in peaks 1 and 11 retained on the ShGSH matrix (Table 2). All the masses measured, but those in peaks 1, 3 and 11 from ShGSH affinity (20.644, 27.328 and 42.360 kDa respectively) and peak 7 (30.507 kDa) from GSH-affinity eluates, corresponded to typical GST molecular masses described in other organisms. It should be mentioned that Na adducts were sometimes observed on ShGSH- but not GSH-purified proteins, due probably to the additional NaCl elution step in the former method.

Immunoblotting

SDS/PAGE analysis was performed on each HPLC-purified subunit and non-GST proteins. The blot screening using anti-(porcine Alpha-class GST) antibody clearly confirmed that subunits in peaks 1, 5, 6a and 6b belongs to this class. Weak cross-reactivity was observed with the Mu-class subunits in peaks 4, 8 and 9, but also with mevalonate kinase in peak 11 and carbonic anhydrase from the Pharmacia standard protein calibration kit. In contrast, the Mu-class subunits in peak 10 were not recognized. Unknown proteins in HPLC peaks 1, 3 or 12 and carbonyl reductase in peak 7 did not cross-react with this antibody (Table 3).

Amino-acid-sequence determinations, N-terminal unblocking and peptide mapping

The proteins retained on the ShGSH-affinity matrix were first directly submitted to amino-acid-sequence analysis. From the 11 peaks present on the HPLC profile, clear sequences could be obtained only from peaks 2, 4, 8 and 11, which indicated possible N-terminal modifications on the other proteins (Figure 3). As N-terminal acetylation is a common modification described on GSTs, the remaining proteins were incubated in TFA vapour as described in the Experimental section. Thus the peak-9-subunit N-terminal end was successfully deblocked, suggesting the presence of an acetyl group. As expected from the described method [25] a serine residue was found at position 1. Peptide mapping

Table 1 Purification procedures and quantitative data

(A) Purification of pig liver cytosolic GSTs using GSH- and ShGSH-affinity matrices

Purification step	Fraction volume (ml)	Total protein		CDNB activity		Yield (%)	Purification (fold)
		(mg)	(%)	Total (units*)	Specific (units*/mg)		
ShGSH-matrix							
Cytosol	10.0	310.0	100.0	848	2.7	100	1.0
Sephadex G-25	30.0	278.9	90.0	749	2.7	88	
Affinity pool I	7.3	3.9	1.3	302	77.0	36	
Affinity pool II	13.9	8.3	2.7	207	24.9	24	
Total affinity (I + II)	21.2	12.2	4.0	509	41.7	60	15.5
GSH-matrix							
Cytosol	10.8	334.0	100.0	916	2.7	100	1.0
Sephadex G-25	21.0	303.1	90.7	855	2.8	93	
Affinity	11.1	11.4	3.4	513	45.0	56	16.6

* A unit of activity is 1 μ mol of CDNB conjugated/min.

(B) Protein distribution between the peaks obtained from HPLC separation of ShGSH- and GSH-affinity preparations of GSTs

Affinity ligand	Peak...	Protein in HPLC peaks (μ g/100 μ g of purified GSTs)											
		1	2	3	4	5	6a + 6b	7	8	9	10	11	12
ShGSH*	1.01	0.55	7.64	15.52	14.92	46.52	1.26	5.01	3.90	2.68	0.96	0.01	
GSH	0.41	0.29		22.13	17.50	46.92	1.99	4.63	3.64	2.36	0.11	0.03	

* Values from pools I and II were added (see Figures 2A and 2B).

Table 2 Molecular-mass determination of pig liver cytosolic proteins with affinity for ShGSH- and GSH-affinity matricesDetailed conditions are given in the Experimental section. The precision of the measures was ± 3 –4 Da. Abbreviation: n.d., not determined.

HPLC peak name	Molecular mass (kDa) determined from:		
	ShGSH affinity		
	Pool I	Pool II	GSH-affinity pool
1	—	20.644	n.d.
2	—	23.510	23.511
3	—	27.328	—
4	25.043	25.039	25.042
5	25.028	25.027	25.026
6a	25.228	25.226	25.225
6b	24.987	24.984	24.984
7	—	n.d.	30.507
8a	—	25.614	25.613
8b	—	25.599	25.598
9	—	25.657	25.656
10a	—	25.411	25.412
10b	—	25.514	25.515
11	—	42.360	n.d.
12	—	n.d.	n.d.

Table 3 Immunoblotting of pig liver cytosolic GST and non-GST proteins eluted from ShGSH-affinity matrix using anti-(porcine Alpha-class GST) antibody

Conditions for SDS/PAGE, blotting and screening are given in the Experimental section. The primary rabbit anti-(porcine Alpha-class GST) and the horseradish peroxidase-conjugated secondary goat anti-rabbit antibodies were diluted 1:400 and 1:1000 respectively.

HPLC fraction name	Protein identified from amino acid sequence comparison	Cross-reactivity with anti-(porcine Alpha-class GST) antibody*
1	Alpha-class GST (+ unknown)	++
2	Pi-class GST	—
3	unknown	—
4	Mu-class GST	—
5	Alpha-class GST	+++
6a	Alpha-class GST	++++
6b	Alpha-class GST	++++
7	Carbonyl reductase	—
8a + 8b	Mu-class GST	++
9	Mu-class GST	++
10a + 10b	Mu-class GSTs	—
11	Mevalonate kinase	+
12	Unknown	—

* Evaluated from relative staining using 3,3'-diaminobenzidine as substrate

was performed on peaks 1, 3, 5, 6a, 6b, 7 and 10 proteins. At least two well-defined peptides from each form were collected and submitted to amino-acid-sequence analysis. Each partial sequence obtained was compared with those of the GSTs of other species, which allowed identification of subunit classes (Figure 3).

Thus GST subunits were found under nine HPLC peaks and characterized as belonging to the Alpha- (peaks 1, 5, 6a and 6b), Mu- (peaks 4, 8, 9 and 10) and Pi- (peak 2) classes. All the peptides analysed from the heterogeneous peaks 8 and 10 (see above) revealed sequence homologies only with Mu-class subunits. The protein in peak 3, absent from the GSH-affinity eluate, exhibited an unknown partial structure. Unknown and Alpha-class GST sequences were found in peak 1 protein peptides. The

Class Alpha GSTs

hGSTA1 F E E K F I K S A E D L D K L R N D G Y L M F Q Q V P M V E I D G M K L V
 hGSTA2 F E E K F I K S A E D L D K L R N D G Y L M F Q Q V P M V E I D G M K L V
 rGSTA1a F D E K F I Q S P E D L E K L K K D G N L M F D Q V P M V E I D G M K L A
 rGSTA1b F E E K L I Q S P E D L E K S K K D G N L M F D Q V P M V E I D G M K L A
 rGSTA2 F E E Q F L K T R D D L A R L R N D G Y L M F Q Q V P M V E I D G M K L V
 rGSTA3 F E E E F L E T R E Q Y E K L Q K D C L L F G Q V P L V E I D G M L L T
 mGSTA1 F E E K F I Q S P E D L E K L K K D G N L M F D Q V P M V E I D G M K L V
 rbGSTA1 F D E K F M E T A E D L D K L R N D G S L M Y Q Q V P M V E I D G M K L V

Frac. 6a

Frac. 6b

Frac. 1

D A K F I K T P E N L F R D G S L L F Q Q V
 D G S L L F Q Q V
 D G S L L F Q Q V
 hGSTA1 R A I L N Y I A S K Y N L Y G K D I K E R A L I D M Y I E G I A D L G E M
 hGSTA2 R A I L N Y I A S K Y N L Y G K D I K E R A L I D M Y I E G I A D L G E M
 rGSTA1a R A I L N Y I A T K Y D L Y G K D M K E R A L I D M Y T E G I L D L T E M
 rGSTA1b R A I L N Y I A T K Y D L Y G K D M K E R A L I D M Y S E G I L D L T E M
 rGSTA2 R A I L N Y I A S K Y N L Y G K D I K E R A L I D M Y A E G V A D L D E I
 rGSTA3 R A I L S Y L A A K Y N L Y G K D L K E R A R I D M Y A D G T Q D L M M M
 mGSTA1 R A I L N Y I A T K Y D L Y G K D M K E R A L I D M Y S E G I L D L T E M
 rbGSTA1 R A I L N Y V A N K H N L Y G K D M K E R A L I D M Y T E G V A D L Y E L

Frac. 6a

Frac. 6b

Frac. 5

A I L N Y I A D M Y T E G V A
 D M Y T E G V A

Class Mu GSTs

rGSTM1 P M I L G Y W N V R G L T H P I R L L L Y D R S Q W L N E K F K L G L D
 rGSTM2 P M T L G Y W D I R G L A H A I R L F L Y D R S Q W L S E K F K L G L D
 rGSTM3 P M T L G Y W D I R G L A H A I R L L L F D R S Q W L N E K F K L G L D
 hGSTM1b P M I L G Y W D I R G L A H A I R L L L Y D R S Q W L N E K F K L G L D
 mGSTM1 P M I L G Y W N V R G L T H P I R M L L F D R S Q W L N E K F K L G L D
 mGSTM2 P M T L G Y W D I R G L A H A I R L L L Y D R S Q W L S E K F K L G L D
 cGSTM2 V V T L G Y W D I R G L A H A I R L L L F D P S D W T N E K E K L G L D
 gpGSTM P M T L G Y W N I R G L T H P I R L T L Y D R S Q W T N E K F K L G L D

Frac. 4

Frac. 8

Frac. 9

Frac. 10

M I L G Y W D I R G L D M I L G Y W D I R G L A H A I R L L L
 S M I L G Y A D N S Q W L S E K F A L G
 D I L G Y

Class Pi GSTs

pGSTP1 P P Y T I T Y F P V R G R C E A M R M L
 hGSTP1 P P Y T V V Y F P V R G R C A A L R M L
 rGSTP1 P P Y T I V Y F P V R G R C E A T R M L
 bGSTP1 P P Y T I V Y F P V Q G R C E A M R M L
 mGSTP1 P P Y T I V Y F P V R G R C E A M R M L

Frac. 2

P P Y T I T Y F P

Other identified proteins

Mevalonate kinase

rMK M L S E V L L V S A P G K V I L H G E H A V V
 hMK M L S E V L L V S A P G K V I L H G E H A V V
 Frac. 11 M L S Q V L L V S A P G K V I L H G E H S V V
 R

Carbonyl reductase

hCR R D V T R G Q A A V Q Q L Q A E G L S P W V R T D M A G P K A
 Frac. 7 D V A X G Q A A V Q Q L Q A E G L S P D M A G P K A

Unknown proteins

Frac. 1*

D A K V A S T E

Frac. 3

D P A V S A L H I E P P
 D L Y L Q N S P G A
 E

Figure 3 Comparison of porcine liver GST partial amino acid sequences with other animal GST structures

The subunits are assigned to separate classes based on amino acid sequence similarities to published structures of mevalonate kinase [26,27], carbonyl reductase [28,29] and GSTs [30–32] indicated by enclosed boxes. *Italic* letters indicate uncertain determinations. Abbreviations used: h, human; r, rat; m, mouse; p, pig; b, bovine; c, chicken; rb, rabbit. *, Contaminant protein in HPLC peak 1.

60 kDa contaminant sometimes observed on SDS/PAGE analysis of this peak (see above) should correspond to the latter unknown peptide. Unexpected partial amino acid sequences of

carbonyl reductase and mevalonate kinase were characterized in peak 7 and 11 respectively. Porcine liver mevalonate kinase exhibited a very high sequence similarity to the corresponding

human [26] and rat [27] enzymes. On the other hand, carbonyl reductase peptides, which were very similar to the monomeric form described in human placenta and breast-cancer cell lines [28,29] were different from other aldoketoreductases. In addition, molecular masses measured by ESI-MS for these two proteins were close to those described for other species (Table 2) [26–29].

DISCUSSION

We have determined for the first time the precise molecular mass and partial amino acid sequence of 11 porcine liver cytosolic GST subunits purified from two different affinity-chromatography matrices. From their structural similarity to various mammalian published structures [30–32], four Alpha-, six Mu-class major and one minor Pi-class subunits were identified in pig liver. The latter form has not been reported in a previous study by Williamson [10], probably owing to low expression level and/or to the purification procedure used, which included additional ion-exchange steps. The molecular mass and partial sequence of this Pi-class GST are identical with those of the pig lung form previously characterized by Dirr et al. [11]. Most of the liver subunits were blocked on their N-terminal end. As suggested by the result obtained from subunit 9, the modified subunits should be acetylated. This is in accordance with the N-terminal modification commonly described for the GSTs of other species [1,16,33]. Unresolved subunits with identical partial amino acid sequences have been characterized in heterogeneous HPLC peaks, e.g. peaks 6, 8 and 10. These variants are probably expressed from different genes, as the occurrence of *in vivo* post-translational processing of GSTs, other than N-terminal acetylation, has never been established [1,33].

The selectivity of porcine GST binding to the affinity matrix is found to be improved when GSH instead of ShGSH is used as ligand. Contamination of GST preparations with other proteins was precisely evaluated and found less important with the GSH–than with ShGSH–matrix. Thus the latter ligand revealed to bind specifically a major unknown 27.328 kDa polypeptide. The presence of mevalonate kinase and carbonyl reductase identified as minor compounds in both GSH- and ShGSH-affinity preparations remains unexplained, as these enzymes have not been described as GSH-dependent proteins. These results seem to indicate a real recognition of GSH by these enzymes, and especially for carbonyl reductase, not due to the *S*-hexyl substitution of the ligand or to its binding position on the matrix. A similar case has been mentioned for Δ^3, Δ^2 -enoyl-CoA isomerase, which copurifies with human GSTs under similar conditions [34]. Like mevalonate kinase with the anti-(porcine Alpha-class GST) antibody in our study, Δ^3, Δ^2 -enoyl-CoA isomerase was shown to slightly cross-react with an anti-(Alpha-class GST) antibody raised in human and other mammalian organs and species [34–36]. As the apparent unspecific binding and immunoreactivity of these non-GST proteins cannot be attributed to primary-structure identity, putative tertiary-structure similarities may be considered and remain to be investigated.

We thank Biotrin International for generously providing a new anti-(porcine Alpha-GST) antibody, A. Paris for providing pig liver samples and G. Bories for reviewing the manuscript before its submission.

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