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DOG LIVER GLUTATHIONE *S*-TRANSFERASE AND ITS STRONG IMMUNOREACTIVITY WITH RAT TRANSFERASE-P(7-7)

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Abstract—Dog liver cytosolic glutathione *S*-transferases (GSTs) were investigated to characterize their properties in comparison with rat liver transferases. Dog liver GSTs after the glutathione affinity column chromatography showed three subunit bands on SDS–polyacrylamide gel electrophoresis. These three subunits, designated as Yd1 (mol.wt 26,000), Yd2 (mol.wt 27,000) and Yd3 (mol.wt 28,000), were distinctly different from rat liver GST subunits, i.e. Ya(1) (mol.wt 26,500), Yb1(3)/Yb2(4) (mol.wt 27,500) and Yc(2) (mol.wt 28,500). Western blot analysis revealed that Yd1, Yd2 and Yd3 were immunoreacted with anti-rat GST 7-7, 1-1 and 3-3 antibodies, respectively. Four transferase activity fractions, I (pH > 7.63), II (pH 6.92), III (pH 5.80) and IV (pH 5.65), were obtained from affinity purified GSTs by chromatofocusing. Each fraction exhibited a characteristic substrate specificity. GST-II, III and IV were all strongly immunoreacted with anti-rat GST 7-7 antibody by immunoblotting, thus suggesting the occurrence of the heterogeneity of transferases immunologically related to rat GST subunit 7 in dog liver. Immunohistochemical examination showed that transferases immunoreacted with anti-GST 7-7 antibody have diffusely distributed throughout the lobule, while enzymes related to subunit 3 have been localized in a narrow range of cells around the central vein. These data suggest that GSTs immunologically associated with rat transferase subunit 7 may be major forms in dog liver.

The glutathione *S*-transferase (GST§; EC 2.5.1.18) is a group of enzymes which play an important role in drug biotransformation and metabolism of xenobiotics [1]. Multi-molecular forms of GST have been found in many species. Despite their multiplicity these enzymes from different tissue and species display a characteristic feature in that they are all dimeric proteins formed by binary combinations of various subunits. The rat cytosolic GSTs have been studied most, and eight subunits from 1 to 8 have been identified so far [2, 3]. It was recently demonstrated that GSTs from different mammalian species can be grouped by several criteria into three distinct species-independent classes named alpha, mu, and pi [4]. The alpha class involves the GST isozymes composed of subunits 1, 2 and 8, and mu class is associated with 3, 4 and 6 subunits. The pi class is a family of isozymes which contain only subunit 7. In addition to rats, each of mouse and man has been reported to contain at least one isozyme of each class [4]. Among these three classes, GST 7-7 (also named GST-P) has recently attracted particular attention as a new marker enzyme for (pre)-neoplastic lesions arising during chemical carcinogenesis in rat liver [5] and hamster pancreas [6] and human colonic carcinoma [7].

Since pharmacological and toxicological investigations are frequently performed in the dog, it was of much interest to characterize the GSTs in the dog. Dog liver GSTs have been recently separated into five isozymes and their heterogeneity has been demonstrated in several respects [8]. However, the relationship of these dog liver GSTs to well-characterized rat liver GSTs has not been elucidated. The present study was designed to conduct, for comparative purposes, dog and rat GSTs biochemically and immunochemically. In the present study, we report here the abundant presence of rat GST subunit 7 in normal dog liver.

MATERIALS AND METHODS

Animals. Adult male beagle dogs (10–16 kg) were used. They were freely given water and a commercial chow (CD-5®, Oriental East Co.). They were fasted for 18 hr and killed by exsanguination under anaesthesia with sodium pentobarbital. The livers were quickly removed and weighed, followed by perfusion with 1.15% KCl. Then 20% (w/v) homogenates were prepared after the addition of ice-cold 1.15% KCl in a Waring blender and, thereafter, a glass Potter–Elvehjem homogenizer with a Teflon pestle. Particles and lipid material were removed from the crude homogenate by filtration through one layer of gauze. The homogenate was centrifuged at 9000 *g* for 20 min. The resultant supernatant was further centrifuged at 105,000 *g* for 60 min. The resulting clear supernatant was used as the cytosol for all assays.

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§ Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GSH, reduced glutathione; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The male rat (Sprague–Dawley strain at seven–week-old) liver cytosol was prepared by the same procedure as for dog, described above.

Chemicals. 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), *trans*-4-phenyl-3-buten-2-one and GSH were purchased from Wako Pure Chemicals Inc., Ltd., Tokyo. Cumene hydroperoxide were from Nakarai Chemicals, Ltd., Kyoto. Ethacrynic acid and 1,2-epoxy-3(*p*-nitrophenoxy)propane were from Sigma Chemical Co., St. Louis, MO. Other reagents used were of analytical grade.

Determination of GST activity. GST activities were assayed according to the method of Habig *et al.* [9], while the GSH peroxidase activity was measured by the method of Lawrence and Burk [10] using cumene hydroperoxide as a substrate.

Purification procedures for dog liver cytosolic GSTs. The cytosol was dialyzed with 20 mM Na,K-phosphate buffer (pH 7.0) containing 20% glycerol (buffer A) and applied onto a column, which previously equilibrated with the buffer A, of GSH-affinity resin, which was prepared from epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals) according to the method of Simons and Vander Jagt [11]. The bound GSTs were eluted with 50 mM Tris–HCl (pH 9.6) containing 15 mM GSH and 20% glycerol as reported by Koskelo *et al.* [12]. It is worth noting that on the GSH-affinity column approximately 35% of GST activity towards CDNB in dog liver cytosol was recovered in flow through fraction. The eluates were pooled and concentrated by ultrafiltration with a Diaflo PM-10 membrane. Then, the concentrated solution was desalted by using Sephadex G-25 column (PD-10) equilibrated with 25 mM imidazole–HCl (pH 7.4) and then applied to a column (1.0 × 29 cm) of chromatofocusing gel PBE 94 equilibrated with the same buffer. Elution was carried out with 400 ml of Polybuffer 74, which was diluted at a ratio of 1 to 8 with degassed water and adjusted to pH 4.0 with HCl. Fractions of 2.5 ml were collected. GST activity was measured with CDNB as a substrate.

SDS–polyacrylamide gel electrophoresis. Electrophoresis was performed as described by Laemmli [13] in 12.5% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue R-250.

Immunoblotting analysis. Antibodies against rat GST 1-1, 2-2, 3-3 and 4-4 were prepared as previously described [14]. Specific anti-rat GST-P(7-7) rabbit antibody was kindly provided by Professor Dr. K. Sato, Hirosaki University School of Medicine, Japan. Western blot analysis was carried out as described previously [15].

Immunohistochemical examination. Liver tissues of adult male beagle dog were fixed in 10% buffered formalin and then dehydrated in a graded ethanol series, and embedded in paraffin. The sections in thicknesses of 4 μm were cut, deparaffinized, and processed to ethanol as in routine processing. The sections were stained by the method of avidin–biotin–peroxidase complex [16] using anti-GST antibodies and were counterstained with hematoxylin.

Protein determination. Protein content was determined according to the method of Lowry *et al.* [17] or the Coomassie blue G-250 dye binding assay of Bradford [18] with bovine serum albumin as a standard.

RESULTS

Table 1 shows the substrate specificity of liver GST activity in rats and dog. When compared to rat liver GST activity, dog liver GST was found to have much higher activity towards DCNB and ethacrynic acid, while little activity was detected when *trans*-4-phenyl-3-buten-2-one and 1,2-epoxy-3-(*p*-nitrophenoxy)propane were used as substrates.

The analysis by SDS–PAGE and immunoblotting of dog liver GSTs is represented in Fig. 1. The GST fraction purified by affinity column chromatography displayed three subunits, but was clearly distinct from rat liver GST in their apparent subunit molecular weights. They were tentatively named Yd1, Yd2, Yd3 in the order of their increasing molecular weights. The subunit Yd1 was a little smaller than subunit 1, and Yd2 was between subunit 1 and subunits 3/4, and Yd3 was between subunits 3/4 and subunit 2. They gave an apparent molecular weight being smaller by about 500 daltons compared to rat subunits 1 (mol.wt 26,500), 3/4 (27,500) and 2 (28,500). The immunological examination with Western blot revealed that Yd1, Yd2 and Yd3 were immunochemically-associated with rat GST subunits 7, 1 and 3, respectively, and no cross-reactivity was observed between dog GSTs and antisera raised

Table 1. Comparison of glutathione *S*-transferase activities in liver cytosol of dog and rat

Substrate	Dog (N = 3)	Rat (N = 4)
1-Chloro-2,4-dinitrobenzene (μmol/min/mg)	0.963 ± 0.047	1.250 ± 0.058
1,2-Dichloro-4-nitrobenzene (nmole/min/mg)	115.0 ± 5.13	66.6 ± 2.3
Ethacrynic acid (nmole/min/mg)	32.5 ± 3.1	19.2 ± 0.7
trans-4-Phenyl-3-buten-2-one (nmole/min/mg)	<0.09	6.89 ± 0.34
1,2-Epoxy-3-(*p*-nitrophenoxy)propane (nmole/min/mg)	ND	55.2 ± 8.0

ND, not detected. Values represent mean ± SEM.

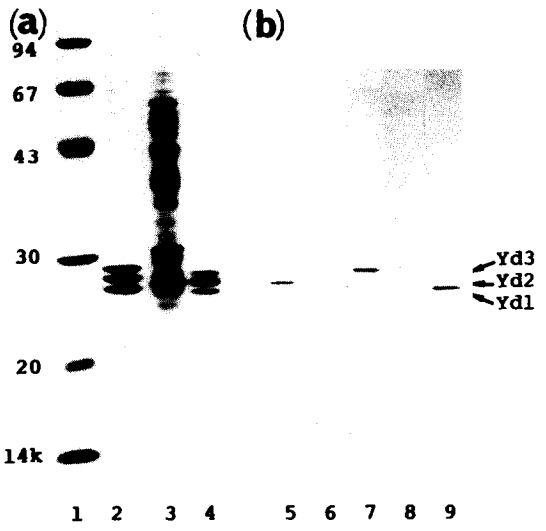

Fig. 1. SDS–polyacrylamide gel electrophoresis (a) and Western blot analysis (b) of dog liver glutathione *S*-transferases. Lane 1, marker proteins for molecular weight determination (products of Pharmacia Fine Chemicals: phosphorylase b, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20.1K; α-lactalbumin, 14.4K); 2, mixture of rat GST subunits Ya(1), Yb1(3)/Yb2(4) and Yc(2) bands; 3, dog cytosol (10 μg); 4, transferases (1 μg) obtained from the GSH-affinity column. Gels (lanes 5–9) to which similar amounts of GSTs (200 ng) obtained from affinity column had been applied were blotted to nitrocellulose membrane. Each nitrocellulose membrane of lanes 5–9 was incubated with antibodies raised against rat liver GSTs: Lane 5, anti-GST 1-1; 6, anti-GST 2-2; 7, anti-GST 3-3; 8, anti-GST 4-4; 9, anti-GST 7-7. Staining was achieved as described previously [15].

against the rat GST 2-2 and 4-4 (Fig. 1b).

The separation of dog liver GSTs obtained from affinity chromatography was carried out by chromatofocusing with PBE 94 (pH 7–4). The elution profile was shown in Fig. 2. Four peaks of GST activities towards CDNB were revealed and designated as GST-I, II, III and IV in the order of lowering pH. GST-I eluted in flowthrough fraction may be a basic isozyme, and GST-II to IV fractions were neutral and acidic isozymes, in which each peak was eluted at pH 6.92, 5.80 and 5.65, respectively. The total recovery of the GST activities of these four fractions was about 72% and each fraction from GST-I to IV was recovered 19%, 57%, 19% and 4% in that order, respectively. The peak of GST-I fraction showed only Yd2 subunit, while GST-II to IV possessed Yd1 subunit, but not Yd2 subunit. The Yd2 was a major subunit on SDS–PAGE as shown in Fig. 1, but this is not necessarily the case in the activity of every GST fraction after chromatofocusing.

Table 2 shows the substrate specificities of GST-I to IV in dog liver GSTs. GST activity towards five substrates was clearly distinct amoung four fractions. The CDNB activity was almost the same in GST-II to IV, but not in GST-I, having a low activity. The neutral and acidic isozymes, II, III and IV, were found to retain their much higher catalytic functional activity with ethacrynic acid than did GST-I. The glutathione peroxidase activity with cumene hydroperoxide was the highest in GST-I fraction and the lowest in IV fraction. The activity towards *trans*-4-phenyl-3-buten-2-one was, however, about 10-fold higher in GST-IV fraction than in I fraction.

Immunoblotting demonstrates that the Yd1 subunit of GST-II, III and IV was clearly immunoreactive with anti-GST-P(7-7) antibody and closely associated with subunit 7 (Fig. 3). These results indicate that GSTs, which have subunit 7, are a major species in dog liver.

The localization of dog liver GST was investigated immunohistochemically using the polyclonal antibodies raised against GST 1-1, 2-2, 3-3, 4-4 and 7-7. The results are summarised in Table 3. The liver lobule was stained against anti-GST 7-7, 1-1, 2-2, and 3-3 antibodies, but negative with GST 4-4 antiserum. The staining intensity of the cytoplasm was most marked in GST 7-7, followed by GST 3-3 and GST 1-1 or 2-2. In addition, GST 7-7 diffusely distributed throughout the cytoplasm, but GST 3-3 localized in the central zone of the hepatic lobule.

DISCUSSION

Dog and rat liver GST were significantly different from each other in their substrate specificities, SDS–PAGE pattern and immunological properties. Dog liver GSTs have a high activity in the conjugation of DCNB and ethacrynic acid. The DCNB and ethacrynic acid are known to be good substrates for subunits 3 and 7, respectively [19]. The abundance of these subunits 3 and 7 in dog liver was ascertained by their immunohistochemical distribution. Immu-

Table 2. Substrate specificities of dog liver glutathione *S*-transferases

Substrate	Specific activity (μmol min^{-1} mg^{-1})			
	I	II	III	IV
1-Chloro-2,4-dinitrobenzene	4.02	15.05	17.97	12.50
1,2-Dichloro-4-nitrobenzene	0.009	ND	ND	ND
Ethacrynic acid	0.06	1.52	2.32	1.36
trans-4-Phenyl-3-buten-2-one	0.002	0.008	0.019	0.028
Cumene hydroperoxide	2.10	0.44	0.19	0.03

ND, not detectable.

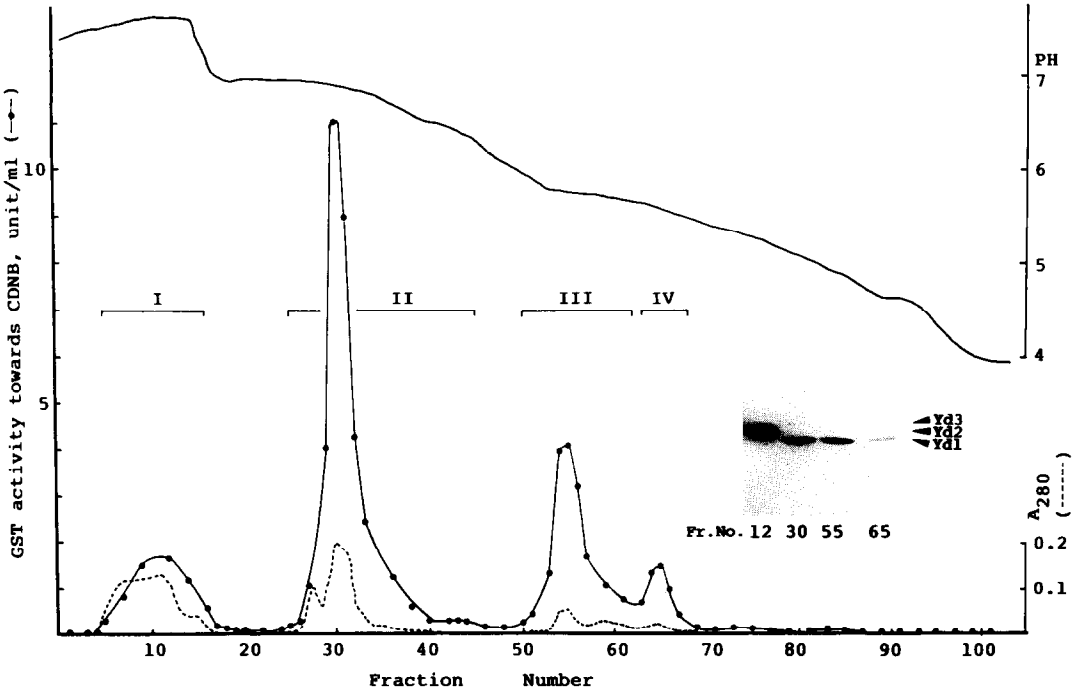

Fig. 2. Chromatofocusing profile of dog liver glutathione *S*-transferases from the glutathione affinity column. The inset shows the SDS–PAGE of each fraction as indicated.

nohistochemical localization of GSTs present in normal dog liver showed GST 7-7 and GST 3-3 related isozymes to be localized diffusely and pericentrally, respectively. Redick *et al.* [20] have demonstrated that in normal rat liver GST 1-2, 3-4 and 5-5 are not distributed uniformly throughout the liver lobule and that each GST is localized in the greatest concentration within the centrilobular region of the lobule. Later, Wolf *et al.* [21] also reported that GST 3-4 was concentrated in a narrow area around the central vein, while GST 1-2 were distributed in a large centrilobular area.

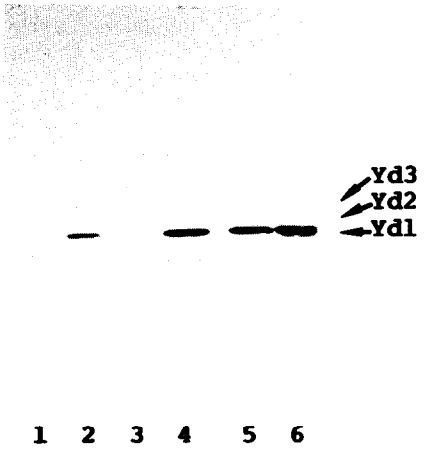

Fig. 3. Western blot analysis of dog liver GST-I, II, III and IV using anti-GST 7-7 antibody. Lane 1, mixture of subunits Ya(1), Yb1(3)/Yb2(4) and Yc(2); 2, GSTs (200 ng) after affinity column; 3, 4, 5 and 6, GST-I, II, III and IV, respectively, each 200 ng of protein.

Dog livers have apparently three GST subunits, which all migrated to different positions from rat GSTs on SDS–PAGE. The GSTs in dog liver cytosol have been separated into four fractions by chromatofocusing. Differences between the four forms are manifested in substrate specificities and isoelectric points. Dog neutral and acidic GST forms, II, III and IV, were all closely immunologically related to rat GST subunit 7, thus strongly suggesting the heterogeneity of GST 7-7-related isozymes in dog liver. The present study also demonstrates that neutral and acidic isozymes represent the major component of total conjugating activity in dog liver. To our knowledge, this is the original paper to demonstrate the presence of isozymes immunoreacted with GST 7-7 in normal dog liver, while Roomi *et al.* [22] have demonstrated that dog liver cytosol gave a weak band on Ouchterlony double immunodiffusion analysis using anti-GST-P antibody. The GST 7-7 is almost undetectable in normal rat liver, and as far as liver GSTs are concerned, only mouse liver has, up to now, been demonstrated to have GST 7-7 [23, 24]. Transferase 7-7 was also isolated from rat lung [25] and kidney [26], and hyperplastic nodules in rat liver [27]. The mouse GST MII (pH 8.9), rat GST 7-7 (pI 7.0) and the human GST π (pI 4.8) all have been proposed by Mannervik *et al.* [4] to be same pi groups.

The present study also suggests that GST 4-4 is not a major form available in dog liver. The species lacking a particular form of GST may be more susceptibile to toxicant injuries. The GST 4-4 has been reported to be the most active isozyme ident-

Table 3. Immunohistochemical staining of the beagle dog liver against anti-GST antibodies

	Hepatocytes		Erythrocytes	Epithelium cells of the bile ducts	Epithelium of the gall bladder	
	Cytoplasm	Nucleus			Cytoplasm	Nucleus
GST 7-7	++	±	+	+	+	+
1-1	±	+	+	−	+	+
2-2	±	+	+	−	+	+
3-3	+a (±b)	+	+	+	+	+
4-4	−	−	−	−	−	−

Remarks: −, negative; ±, slight; +, moderate; ++, marked. a, central zone; b, periportal zone.

ified in the GSH conjugation of (±)-7β,8α-dihydroxy-9α-10α-oxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene((±)-anti-BPDE) [29]. However, GST 7-7 has recently been demonstrated to have 7-fold greater activity with (±)-anti-BPDE than GST 4-4 [28]. The presence of GST 7-7 in dog liver might therefore partially compensate for the lower overall GST 4-4 activity of dog liver. Further investigations on the dog liver GST isozymes are now in progress in this laboratory.

Since GST is an important enzyme for the detoxification of xenobiotics, the drastic species differences in substrate specificity of this enzyme system may become an important concern in choosing a laboratory animal for the evaluation of biochemical transformation and toxicity (or carcinogenesis) of xenobiotics.

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