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Purification and Characterization of Multiple Forms of Rabbit Hepatic Glutathione S-Transferase

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Multiple forms and properties of glutathione S-transferase were investigated by using the liver from individual male or female rabbits. Most of the activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in the crude extract passed through a diethylaminoethyl (DEAE)-cellulose column. On carboxymethyl (CM)-cellulose chromatography, the flow-through fraction was resolved into at least four activity peaks which were designated as R1, R2, R3 and R4 in order of elution. The content of each component varied among the individual rabbit livers examined. The main components, R2 and R3, were further purified to homogeneity as judged by sodium dodecyl sulfate (SDS)/polyacrylamide-gel electrophoresis. R3 contained two forms, which were named R3a and R3b in order of elution from a hydroxylapatite column. R2, R3a and R3b each had a molecular weight of approximately 51000 as estimated by gel filtration. R2 and R3b appeared to be homodimers consisting of subunits of apparently different size (R2, Y2: 25000; R3b, Y3: 26500). On the other hand, R3a was a heterodimer composed of subunits with molecular weights of 24500 (Y1) and 26500 (Y3). The molecular weight of the subunit of R3b was identical with that of Y3. These enzymes showed the highest activity toward CDNB and much lower activities toward the other substrates tested, and the substrate specificities of all the enzymes were roughly similar to each other.

Our highly purified enzyme forms were compared with rat hepatic glutathione S-transferases with regard to substrate specificity, subunit species, kinetic parameters, isoelectric point and amino acid composition.

Keywords——glutathione; glutathione S-transferase; rabbit liver; multiple form; subunit; substrate specificity; isoelectric point; amino acid composition

Introduction

Glutathione S-transferases (EC 2.5.1.18) are a well-known family of enzymes existing in various tissues. These enzymes are multifunctional, being involved in catalyzing the conjugation of reduced glutathione (GSH) with a wide variety of exogenous compounds bearing an electrophilic center[1] as well as an endogenous compound, leukotriene[2]; they also show organic anion-binding activity[3-5] as well as non-selenium-dependent glutathione peroxidase activity.[6] Multiple forms of the enzyme have been purified to apparent homogeneity from the liver of rat,[1] human,[7] mouse,[8] monkey,[9] hamster,[10] chicken,[11] sheep,[12] guinea pig[13] and bovine,[14] and their properties have been clarified.

Rat hepatic glutathione S-transferases have been extensively investigated and seven forms (AA, A, B, C, D, E and ligandin) have been purified to homogeneity.[1,15,16] All of them possess basic isoelectric points and are apparently dimers of four different subunits, having molecular weights of 22000 (Ya), 23500 (Yb1 and Yb2) and 25000 (Yc).[17-20] Mannervik and Jensson suggested that these subunits possess distinct substrate specificities.[19]

Recently, Kubota *et al.*[21] reported the presence of a new type of subunit (Yn) in rat liver, having a slightly lower molecular weight than that of Yb. This enzyme seems to be the same as

the transferase S discovered by Hayes and Chalmers.[22] Kitahara *et al.*[23] reported that glutathione S-transferase P is not detectable in normal rat liver but is increased markedly in preneoplastic hepatic lesions and they suggested that this enzyme might be a useful marker for preneoplasia. Thus, many workers have studied the enzymes from rat liver in detail. However, the enzymes from rabbit have hardly been investigated except for the very recent report of Gawai and Pawar.[24] The rabbit is a very useful animal in *in vivo* experiments on the metabolites of xenobiotics and important studies using rabbit liver have been performed on the multiple forms and properties of the cytochrome P-450 monooxygenases[25-27] which are closely related with glutathione S-transferases.

The present work was carried out to investigate the multiple forms of rabbit hepatic glutathione S-transferase, and to ascertain the physico-chemical properties of the main forms.

Experimental

Materials——Male (body weight, about 2.4 kg) and female (body weight, 2.45—3.8 kg) Japanese white rabbits were purchased from Sankyo Labo. Service Corp. and maintained under a light-controlled environment (light, 8:00—20:00) for 3 weeks prior to use. Every animal was fed a standard diet (100 g/d, Clea Japan Inc.) and tap water (*ad libitum*). DEAE (DE-23, 1.0±0.1 meq/g) and CM (CM-52, 1.0±0.1 meq/g) cellulose were obtained from Whatman Chemical Separation Ltd. Sephadex G-75 (superfine), and Sepharose 6B and high pI calibration kit (pH 5—10.5) were from Pharmacia Fine Chemicals. Acetylated forms of cytochrome c (pI marker) were purchased from Oriental Yeast Co., Ltd. Ampholine (pH range of 3—10.5) from LKB-Produkter AB and GelBond PAG Film (0.2 × 124 × 199 mm) from Marine Colloids Division (Bioproducts Dept.) were used. Hydroxylapatite was prepared according to the method of Tiselius *et al.*[28] 1,2-Dichloro-4-nitrobenzene (DCNB), *p*-nitrobenzyl chloride (*p*-NBC) and benzalacetone (BA) were from Wako Pure Chemical Co. 1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-(*p*-nitrophenoxyl)propane (ENPP) were purchased from Tokyo Kasei Kogyo Co., and Eastman Kodak Co., respectively. Ethacrynic acid (EA) was obtained from Sigma Chemical Co. All other chemicals were of the highest purity available and were used without further purification.

Preparation of Epoxy-Activated Sepharose 6B——This adsorbent was prepared as described by Sundberg and Porath.[29] Sepharose 6B (30 ml) was washed on a glass filter-funnel with water (500 ml) and mixed with 1,4-butanediol diglycidyl ether (10 ml) and 0.6 N NaOH containing 2 mg/ml sodium borohydride (10 ml). The suspension was rotated at 25 °C for 8 h in a water bath, then the reaction was stopped by washing the gel with water (2 l). This epoxy-activated Sepharose 6B was stored at 4 °C in 1 M NaCl until used.

Synthesis of S-Hexylglutathione——S-Hexylglutathione was synthesized by the procedure of Vince *et al.*[30] GSH (6.14 g) was dissolved in water (20 ml) and mixed with 2 N NaOH (20 ml). Ethanol (about 60 ml) was added to the above solution to the cloud point. Iodohexane (4.3 g) was gradually added with vigorous stirring. After vigorous stirring for 3 h at room temperature, the pH of the reaction mixture was adjusted to 3.5 with 57% (w/v) hydroiodic acid and the mixture was chilled overnight in a refrigerator. The crystals thus obtained were collected on the filter (Toyo filter paper No. 101) and washed with cold water (500 ml). The product was recrystallized from water–ethanol (50:50, v/v) and dried at 4 °C in a desiccator under reduced pressure.

Preparation of S-Hexylglutathione-Linked Sepharose 6B——Epoxy-activated Sepharose 6B (30 ml) was washed with water on a glass filter-funnel. S-Hexylglutathione (1.2 g) was dissolved in 0.1 M sodium phosphate buffer, pH 10 (50 ml), and the resulting solution was readjusted to pH 10 with 1 N NaOH. The washed gel and S-hexylglutathione solution were mixed, and rotated gently for 24 h at 37 °C in a water bath. After the elimination of excess S-hexylglutathione from the gel by washing with water, the gel was further treated with 1 M ethanolamine (60 ml) for 4 h at 37 °C in order to block the remaining epoxy groups. Then, the gel was washed successively with 0.1 M bicarbonate buffer, pH 10, containing 0.5 M NaCl, water, 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl and water. This washing cycle was repeated three times. This affinity gel was stored in 20 mM Tris–HCl, pH 8.0 in the presence of 0.02% (w/v) sodium azide.

Enzyme Assay——Enzyme activity was spectrophotometrically determined by measuring the initial rate of conjugation of GSH with CDNB or other substrates. The assay conditions were adopted from the report of Habig *et al.*[1] except for the wavelength (320 nm) and the molar extinction coefficient (1.75 mM^{-1} cm^{-1}) of *p*-NBC. One unit of enzyme activity was defined as the amount of enzyme conjugating 1 μmol of substrate per min at 25 °C.

Protein Concentration——This was monitored during chromatographic elution by measurement of the absorbance at 280 nm in a quartz cell of 1 cm light path on a Shimadzu model UV-240 spectrophotometer. Protein concentration in the finally obtained pure enzyme solutions was determined by the method of Lowry *et al.*[31] with bovine serum albumin as a standard.

Estimation of Molecular Weight——Glutathione S-transferases (R2, R3a and R3b mixture, each about 1 mg)

and the molecular weight marker proteins (5—10 mg) were separately applied to a Sephadex G-75 column (2.7 × 80 cm) equilibrated previously with 0.1 M potassium phosphate buffer, pH 6.7, containing 0.2 M KCl, and fractions of 2.9 ml were collected. The marker proteins were cytochrome c (M_r, 12400), myoglobin (17200), α-chymotrypsinogen A (25000), ovalbumin (45000) and bovine serum albumin (66000). The void volume of the column was determined with Blue dextran 2000. The elution volumes were estimated by measuring the activity for glutathione S-transferases and by measuring the absorbance at 280 nm for the marker proteins.

The molecular weights of these enzymes were also examined by discontinuous SDS/polyacrylamide-gel electrophoresis.[32] Samples and the above marker proteins containing additional bovine carbonic anhydrase (M_r, 29000) were pretreated by heating for 5 min at 90 °C in 10 mM Tris–HCl buffer, pH 6.8, containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol. These proteins (5—10 μg) were applied to a vertical slab gel consisting of a 3% (w/v) stacking gel (0.2 × 2.5 × 13.5 cm) and a 12.5% (w/v) resolving gel (0.2 × 10 × 13.5 cm). The electrode solution was 0.025 M Tris–glycine buffer, pH 8.3, containing 1% (w/v) SDS, and bromophenol blue was added to the cathode buffer as a marker pigment. After electrophoresis, the gel slab was stained with 0.025% (w/v) Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (50 : 10 : 40, v/v) and destained in methanol–acetic acid–water (25 : 7 : 68, v/v).

Determination of Kinetic Parameters——A kinetic study was performed for glutathione S-transferases from rabbit and rat livers. The K_m value for GSH was determined in the presence of 1 mM CDNB because of the limited water solubility of CDNB; the CDNB concentration was almost equal to or less than the K_m value. Seven different concentrations of GSH were used in the range of 0.05 to 1.0 mM.

The K_m and V_{max} values for CDNB were determined in the presence of 1 mM GSH. Six different concentrations of CDNB were used in the range of 0.2 to 1.0 mM for rabbit glutathione S-transferases and rat glutathione S-transferase AA, and seven different concentrations were used in the range of 0.015 to 1.0 mM for the other transferases. A stock solution of CDNB (low solubility in water) was prepared in ethanol; the final ethanol concentration in the assay mixture was 3.3%. All assays were carried out in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C.

Ultrathin-Layer Isoelectric Focusing on Polyacrylamide-Gel Plate——This was carried out according to the method of Radola.[33] The polyacrylamide-gel-forming solution was composed of 5% (w/v) acrylamide, 3% (w/w) *N,N'*-methylene-bis(acrylamide) with respect to total acrylamide, 13% (v/v) glycerol and 4% (w/v) Ampholine (pH 3.5—10). This solution (6 ml) was deaerated by stirring for 3 min under a vacuum generated by a water jet pump and polymerized on GelBond Film by addition of 3% (w/v) ammonium persulfate (0.15 ml) and *N,N,N',N'*-tetramethylenediamine (0.005 ml). The electrode strips were immersed in the following electrode solutions: 0.025 M aspartic acid and 0.025 M glutamic acid at the anode, and 2 M ethylenediamine containing 0.025 M arginine and 0.025 M lysine at the cathode. The gel plate (0.167 mm in thickness) covalently bound to the film was pre-focused for 30 min at a constant voltage of 250 V. The apparatus (Resolmax-Slab, Atto Corp.) was cooled with circulating cold water. Then samples (about 2 μg) and pI marker proteins (10—20 μg) were separately applied to a gel plate. Focusing was performed for 1 h at 500 V and further for 1.5 h at 1000 V. The pI markers used were acetylated forms of cytochrome c and a high pI calibration kit. After focusing, the gel plate was immediately fixed with a solution of 10% (w/v) trichloroacetic acid and 5% (w/v) sulfosalicylic acid, and rinsed with the universal solution (methanol : acetic acid : water = 25 : 10 : 65, v/v). Staining was performed with 0.1% (w/v) Coomassie Brilliant Blue R-250 in the universal solution and the gel was destained in the universal solution. A calibration curve was constructed by plotting the distance of marker proteins from the anode *versus* their pI values. The pI values of the samples were determined from the above calibration curve.

Amino Acid Analysis——Homogeneous enzymes (R2, R3a and R3b: each 0.2 mg) were hydrolyzed with 6 N HCl at 110 °C for 24 h in evacuated, sealed tubes. The amino acid composition of each was determined with a Hitachi model 835 amino acid analyzer according to the instruction manual.

Purification of Glutathione S-Transferases——The procedures were carried out at 4 °C according to the previous report,[34] in which separation of rat hepatic glutathione S-transferases was described.

1. Extraction: A male or female Japanese white rabbit was sacrificed by injecting 10 ml of air into the venous vessel of the ear. A portion (45 g) of liver from a single rabbit was cut into small pieces and homogenized with distilled water (5 ml per g wet liver) in a Teflon-glass homogenizer. The homogenate was centrifuged at 20000 × *g* for 1 h. The pellet was discarded and the supernatant was adjusted to pH 8.0 with 1 M Tris base.

2. Passage through a DEAE-Cellulose Column: The crude extract was applied to a DEAE-cellulose column (4 × 40 cm) equilibrated with 10 mM Tris–HCl, pH 8.0. The column was washed with 1.5 l of the same buffer and eluted with 1 l of the same buffer containing 0.5 M KCl. Fractions of 19 ml were collected. Most of the activity toward CDNB was present in the flow-through fraction. This fraction was packed in a cellophane tube (type 36/32 inch) and concentrated by dialysis against polyethylene glycol 6000. The concentrated sample (about 10 ml) was dialyzed against four changes, each of 2 l, of 10 mM potassium phosphate buffer, pH 6.7, for 2 h. Insoluble materials that precipitated during dialysis were removed by centrifugation at 20000 × *g* for 15 min.

3. CM-Cellulose Chromatography: The sample (35 ml) from the above step was applied to a CM-cellulose column (1.9 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.7. After being washed with 250 ml of the above buffer, the column was eluted with a linear gradient of 0 to 60 mM KCl in the same buffer (1 l). Fractions of

10 ml were collected. At least four activity peaks were observed and named R1, R2, R3 and R4 in order of elution. The major peaks, R2 and R3, were further purified as follows.

4. Affinity Chromatography: The two major fractions obtained from the above step were individually applied to S-hexylglutathione-linked Sepharose 6B columns (1.8 × 9 cm) previously equilibrated with 20 mM Tris–HCl, pH 8.0. The columns were washed with the same buffer containing 0.2 M KCl until the absorbance of the effluent at 280 nm was less than 0.01. The adsorbed proteins were eluted with the above buffer supplemented with 2.5 mM GSH and 5 mM S-hexylglutathione. Fractions of 5 ml were collected. Buffer exchange of each active eluate was performed on a Sephadex G-75 column (2.7 × 80 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.7.

5. Hydroxylapatite Chromatography: The above samples were separately chromatographed on hydroxylapatite columns (R2, 1.8 × 10 cm; R3, 2 × 15 cm) equilibrated with 50 mM potassium phosphate buffer, pH 6.7. Each column was eluted with a linear gradient of 50 to 200 mM potassium phosphate buffer, pH 6.7 (R2, 400 ml; R3, 800 ml). R3 showed two activity peaks which were designated as R3a and R3b in order of elution. Active fractions were individually collected and concentrated by ultrafiltration through a Diaflo PM-10 membrane. The buffer exchange of the concentrated samples was performed on a Sephadex G-25 column (2.5 × 45 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.7, and the resulting preparations were used in the following experiments as the finally purified enzymes.

Results

Variation of Content of Glutathione S-Transferases in Individual Rabbit Livers

Table I shows the variation of content of glutathione S-transferases in individual rabbit livers. In female rabbits, activity toward CDNB surprisingly varied within the range of 44 to 362 μmol/min/g of wet liver. Similar experiments using male rabbits were done, although only twice, and very different values were obtained: 59 and 223 μmol/min/g of wet liver.

Purification of Glutathione S-Transferases from a Single Rabbit Liver

The result of a typical purification is summarized in Table II. This purification was performed by using 45 g liver of rabbit No. 2 in Table I. Approximately 90% of total protein in the crude extract was bound to a DEAE-cellulose column but almost all the activity was observed in the flow-through fraction. Only a few % of total activity was bound to the column. As shown in Fig. 1, the flow-through fraction on a DEAE-cellulose column was resolved into at least four activity peaks by CM-cellulose chromatography, and these were eluted at the concentrations of 0, 10, 21 and 34 mM KCl. These activity peaks were named R1, R2, R3 and R4 in order of elution. These four peaks were present in all the livers shown in Table I except for rabbit No. 3, but the content of each component, particularly R2 and R3, varied considerably from rabbit to rabbit. Different but typical CM-cellulose chromatographic patterns are shown in Figs. 2a and 2b. Thus, the main activity peaks were R2 and R3 in

Table I. Variation of Content of Glutathione S-Transferases in Rabbit Liver

Rabbit No.	Sex	Body weight (kg)	Liver weight (g)	Activity[a]	
				μmol/min/A_{280}	μmol/min/g liver
1	Female	3.60	96.58	0.62	114
2	Female	3.65	92.58	0.99	167
3	Female	3.80	99.18	2.21	362
4	Female	3.70	98.14	1.24	171
5	Female	3.80	110.89	0.40	44
6	Female	3.60	78.69	0.56	79
7	Female	2.45	77.35	0.65	98
8	Female	2.45	73.80	0.54	76
9	Male	2.45	63.30	1.34	223
10	Male	2.35	69.00	0.43	59

a) These values were calculated from the activities of the crude extract toward CDNB.

TABLE II. Summary of the Purification of Glutathione S-Transferases from Rabbit Liver

Purification step	Total		Specific activity (μmol/min/A_{280})	Yield (%)
	Protein (A_{280})	Activity (μmol/min)		
Crude extract	7639	7535	0.99	100
DEAE-cellulose	726	7714	10.6	102
CM-cellulose				
R1	132	753	5.7	10
R2	86	1678	19.5	22
R3	102	1885	18.5	25
R4	35	206	5.9	3
Affinity				
R2	24	1462	60.9	19
R3	60	1658	27.6	22
Hydroxylapatite				
R2	10 (13)[a)]	1184	118 (91)[b)]	16
R3a	21 (26)	461	22 (18)	6
R3b	16 (22)	392	25 (18)	5

The values in parentheses: *a*) and *b*) are mg and μmol/min/mg, respectively.

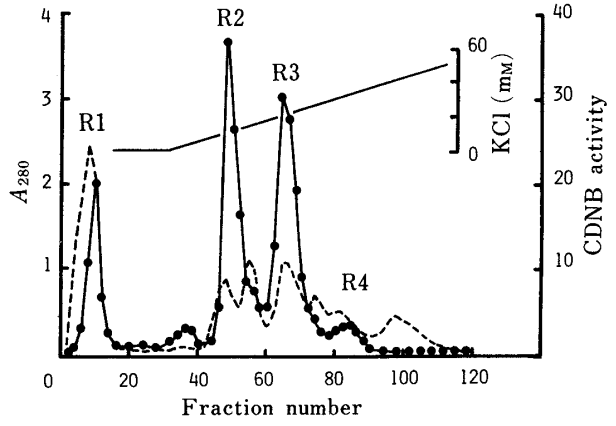

Fig. 1. Chromatography of Glutathione S-Transferases from Rabbit Liver on a CM-Cellulose Column

------, A_{280}; —●—, CDNB activity (μmol/min/ml); ——, KCl concentration. For details, see Experimental.

Fig. 1 (rabbit No. 2 in Table I), R2 in Fig. 2a (rabbit No. 3) and R3 in Fig. 2b (rabbit No. 6). We observed another activity peak eluted at lower KCl concentration (4 mM) than that of R2 (Fig. 1). However, this peak was only seen clearly twice (rabbits No. 2 and 7) among ten experiments. R2 and R3 were further purified on affinity and hydroxylapatite columns. All the activity of each sample was completely bound to an S-hexylglutathione linked-Sepharose 6B column and was not eluted with 20 mM Tris–HCl buffer, pH 8.0 containing 0.2 M KCl. Elution with the above buffer supplemented with 2.5 mM GSH and 5 mM S-hexylglutathione resulted in a single, sharp activity peak. When each eluate was separately applied to a hydroxylapatite column after buffer exchange by gel filtration, R2 showed a single activity peak which was eluted at the concentration of 65 mM potassium phosphate buffer, pH 6.7 (Fig. 3a, rabbit No. 2 in Table I). On the other hand, R3 was resolved into two activity peaks, eluted with 112 and 135 mM same buffer, which were designated as R3a and R3b, respectively (Fig. 3b, rabbit No. 2). The finally purified R2, R3a and R3b had specific activities of 91, 18 and 18 μmol/min/mg, respectively. The overall recoveries in CDNB assay were 16% for R2, 6% for R3a and 5% for R3b.

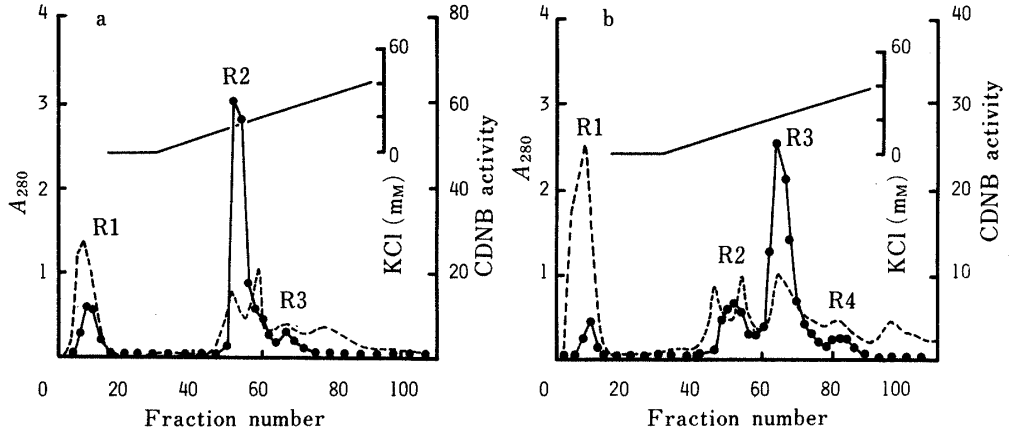

Fig. 2. Comparison of CM-Cellulose Chromatographic Patterns of Individual Rabbit Livers

a, Liver of rabbit No. 3 in Table I; b, liver of rabbit No. 6 in Table I. ----, A_{280}; —●—, CDNB activity (μmol/min/ml); ——, KCl concentration.

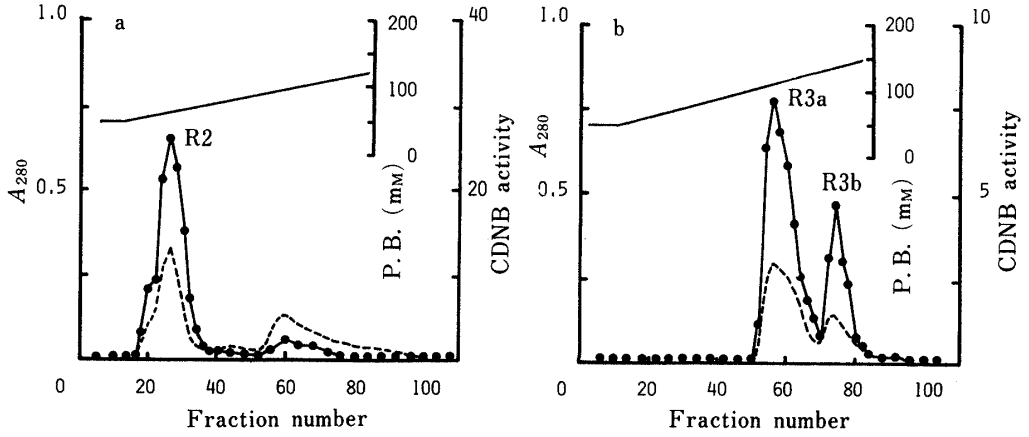

Fig. 3. Chromatography of R2 and R3 on a Hydroxylapatite Column

a, R2 (fraction size of 4 ml); b, R3 (fraction size of 8 ml). ----, A_{280}; —●—, CDNB activity (μmol/min/ml); ——, concentration of potassium phosphate buffer, pH 6.7 (P.B.). For details, see Experimental.

TABLE III. Substrate Specificities of Glutathione S-Transferases from Rabbit Liver

Enzyme	Substrate					
	CDNB	DCNB	*p*-NBC	EA	ENPP	BA
R2	91	0.186	2.05	0.036	0.008	—
R3a	18	0.044	0.08	0.036	—	—
R3b	18	0.039	0.05	0.053	—	—

These values are in μmol/min/mg. —, no activity was detected with about 0.1—0.4 mg protein.

Substrate Specificity

Table III presents the substrate specificities of the finally purified glutathione S-transferases. It is evident that CDNB was the most suitable substrate among those tested for each enzyme. R2 showed the highest activity toward CDNB, having approximately 5 times the specific activity of R3a or R3b. On the other hand, all the enzymes from rabbit had much

lower specific activities toward the other substrates, and their substrate specificities were similar to each other except that only R2 possessed a very low activity toward ENPP. The rabbit hepatic glutathione S-transferases were roughly similar to the enzymes from human liver[16] in that they possessed no or low activity toward DCNB, EA, BA and ENPP.

Estimation of Molecular Weight

By Sephadex G-75 gel filtration, the molecular weight of rabbit glutathione S-transferases (an R2, R3a and R3b mixture) was estimated to be approximately 51000 (Fig. 4). In addition, the molecular weights of the above enzymes were estimated by SDS/polyacrylamide-gel electrophoresis. For comparison with the enzymes from rabbit, rat hepatic glutathione S-transferases, prepared according to the method of the previous report,[34] were simultaneously electrophoresed. It is evident from Fig. 5a that R2 and R3b showed a single protein band, but R3a gave two equal protein bands. From this result, R3a was considered to be a heterodimer consisting of 24500-dalton and 26500-dalton subunits, while R2 and R3b seemed to be homodimers which consisted of 25000-dalton and 26500-dalton subunits, respectively (Fig. 5b). These subunits were named Y1, Y2 and Y3 in increasing order of molecular weight. Y1 and Y3 were clearly resolved by SDS/polyacrylamide-gel elec-

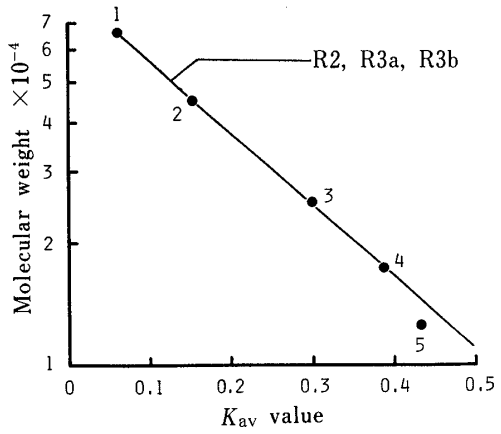

Fig. 4. Estimation of Molecular Weight of Glutathione S-Transferases by Gel Filtration

R2, R3a and R3b, purified enzymes from rabbit liver; ●, marker proteins: 1, bovine serum albumin; 2, ovalbumin; 3, α-chymotrypsinogen A; 4, myoglobin; 5, cytochrome c. For details, see Experimental.

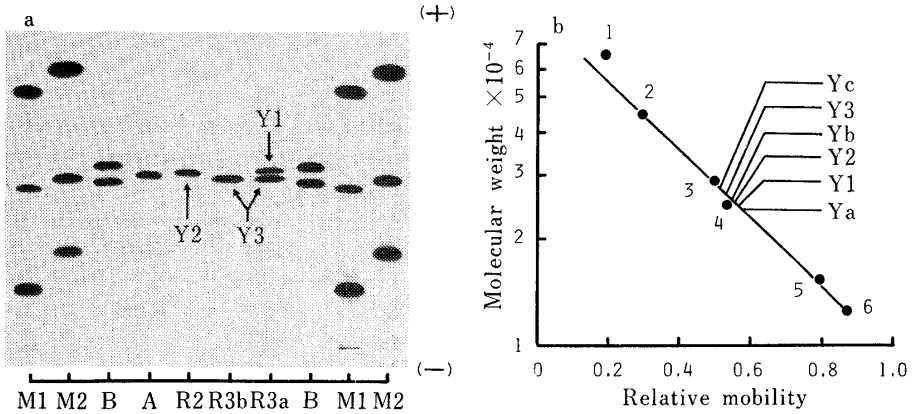

Fig. 5. SDS/Polyacrylamide-Gel Electrophoretic Pattern of Glutathione S-Transferases and Estimation of the Molecular Weight of Their Subunits

a, SDS/polyacrylamide-gel electrophoresis. R2, R3a and R3b, purified enzymes from rabbit liver; A and B, purified enzymes from rat liver; marker proteins, M1 (mixture of ovalbumin, bovine carbonic anhydrase and cytochrome c), M2 (mixture of bovine serum albumin, α-chymotrypsinogen A and myoglobin). b, Estimation of the molecular weight of subunits. Y1, Y2 and Y3, subunits of rabbit enzymes; Ya, Yb and Yc, subunits of rat enzymes; ●, marker proteins: 1, bovine serum albumin; 2, ovalbumin; 3, bovine carbonic anhydrase; 4, α-chymotrypsinogen A; 5, myoglobin; 6, cytochrome c. For details, see Experimental.

trophoresis. The electrophoresis of R2 and R3a mixture (Y1, Y2 and Y3 mixture) failed to resolve Y1 and Y2. However, Y1 always migrated a little earlier than Y2 even when R2 and R3b mixture and R3a were separately electrophoresed on the same gel plate (data not shown). From this result, Y1 and Y2 seem to be different from each other. Under these conditions, the three subunits of rat hepatic glutathione S-transferases, Ya, Yb and Yc were estimated to have molecular weights of 24000, 25500 and 27500 daltons, respectively. These values in the rat were similar to those of Tu *et al.*[35] and approximately 2000 daltons larger than those of Mannervik and Jensson,[19] and Bass *et al.*[36] Kitahara *et al.*[23] suggested that this difference was due to the marker proteins used. Therefore, it is desirable to compare directly the enzymes from different species.

Kinetic Parameters and Isoelectric Points of Glutathione S-Transferases from Rabbit and Rat Livers

The K_m and V_{max} values for CDNB were determined in the presence of 1 mM GSH. The apparent K_m value for GSH was determined in the presence of 1 mM CDNB. These results are

TABLE IV. Kinetic Parameters of Glutathione S-Transferases from Rabbit Liver

Enzyme		V_{max}[a] (μmol/min/mg)	K_m (mM) CDNB[a]	K_m (mM) GSH[b]
Rabbit	R2	155	0.66	0.14
	R3a	31	1.07	0.07
	R3b	35	1.47	0.13
Rat	AA	29	2.27	0.12
	A	56	0.04	0.13
	B	22	0.21	0.15
	C	38	0.07	0.11
	Lig	27	0.21	0.15

a) Determined with 1 mM GSH. *b*) Determined with 1 mM CDNB.

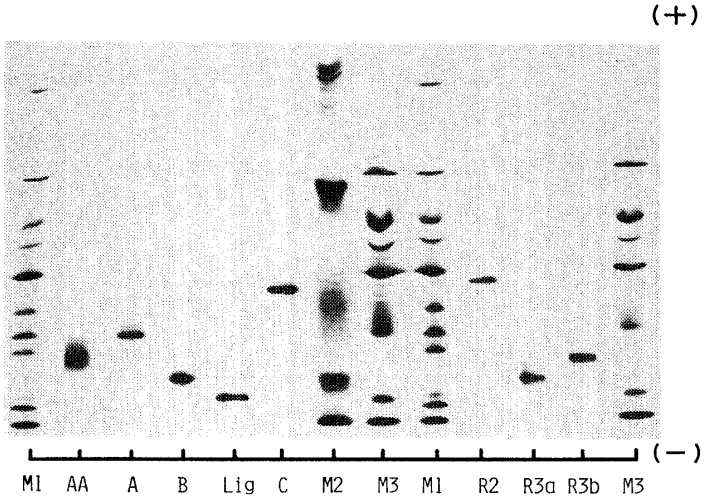

Fig. 6. Ultrathin-Layer Isoelectric Focusing Pattern in Polyacrylamide-Gel Plate

AA, A, B, Lig and C, purified enzymes from rat liver; R2, R3a and R3b, purified enzymes from rabbit liver; marker proteins, M1 [high pI calibration kit containing cytochrome c (pI 10.25), trypsinogen (pI 9.30), lentil lectin (pI 8.65, 8.45 and 8.15), myoglobin (pI 7.35 and 6.85), human carbonic anhydrase (pI 6.55), bovine carbonic anhydrase (pI 5.85) and β-lactoglobulin A (pI 5.20)], M2 [acetylated forms of cytochrome c (pI 10.6, 9.7, 8.3, 6.4, 4.9 and 4.1)], M3 [mixture of cytochrome c, ribonuclease, myoglobin (horse and whale) and carbonic anhydrase (human and bovine)]. For details, see Experimental.

listed in Table IV. All of the enzymes, prepared according to the method of the previous report,[34] from rabbit and rat livers showed similar K_m values for GSH. However, the enzymes from rabbit liver showed higher K_m values (0.66—1.47 mM) for CDNB than the enzymes (0.04—0.21 mM) from rat liver except for AA (2.27 mM). The V_{max} values for CDNB of R3a and R3b (31 and 35 μmol/min/mg) were similar to those (22—56 μmol/min/mg) of the rat liver enzymes, while R2 showed the highest V_{max} value, 155 μmol/min/mg, among the enzymes examined.

Figure 6 shows the result of ultrathin-layer isoelectric focusing of glutathione S-transferases from rabbit and rat livers on a polyacrylamide-gel plate. AA from rat liver showed a broad protein band, but all the other enzymes from rabbit and rat livers showed sharp protein bands. The pI values of these enzymes were determined by using two sorts of pI

TABLE V. Isoelectric Points of Glutathione S-Transferases from Rabbit Liver

Enzyme		pI value			
		Pharmacia	Oriental	a	b
Rabbit	R2	7.5	8.1		
	R3a	9.1	9.7		
	R3b	8.8	9.3		
Rat	AA	8.6	9.2	9.6	10
	A	8.4	8.9	8.4	8.9
	B	9.0	9.6	9.1	9.8
	C	7.7	8.2	7.7	8.0
	Lig	9.2	9.9		

The pI values of enzymes were determined with a high pI calibration kit from Pharmacia and with acetylated forms of cytochrome c from Oriental. a, cited from ref. 39. b, cited from ref. 38.

TABLE VI. Amino Acid Analysis of Glutathione S-Transferases from Rabbit Liver

Amino acid	Rabbit enzyme			Rat enzyme[a]		
	R2	R3a	R3b	A	B	C
	Residues per 51000			Residues per 45000		
Lysine	36	49	48	34	36	35
Histidine	4	7	8	6	6	6
Arginine	23	25	26	21	22	21
Aspartic acid	49	45	46	45	37	44
Threonine	23	14	16	13	11	12
Serine	20	14	12	20	18	17
Glutamic acid	43	57	62	42	46	41
Proline	27	23	22	22	20	22
Glycine	29	24	24	19	21	21
Alanine	19	31	32	20	31	20
Half-cystine	4	4	4	6	4	6
Valine	19	24	22	11	25	12
Methionine	13	15	13	10	8	10
Isoleucine	20	22	23	22	18	19
Leucine	60	57	54	45	50	45
Tyrosine	23	13	12	23	13	22
Phenylalanine	29	19	20	20	17	22
Tryptophan[b]	—	—	—	6	9	6

These values are averages of results from triplicate samples. *a*) Cited from ref. 1. *b*) Tryptophan was not measured.

markers, namely acetylated forms of cytochrome c from Oriental and a high pI calibration kit from Pharmacia. As shown in Table V, when acetylated forms of cytochrome c were used, the pI values of R2, R3a and R3b were 8.1, 9.7 and 9.3, respectively. On the other hand, R2, R3a and R3b showed pI values of 7.5, 9.1 and 8.8 against the high pI calibration kit, respectively. Similarly, the pI values of rat enzymes calculated from the calibration curve of cytochrome c were approximately 0.6 unit higher than those based on the high pI calibration kit. The higher pI values (in both cases) for the rat enzymes were similar to those of ref. 38, while the lower pI values determined from the Pharmacia kit were similar to those of Hales *et al.*[39] In our experiment on the rat liver, ligandin, but not AA showed the highest pI value. The increasing order of pI values of the rat enzymes was identical with the result of Mannervik and Jensson.[19]

Amino Acid Analysis

As shown in Table VI, the amino acid compositions of rabbit and rat hepatic glutathione S-transferases were generally similar; both groups had high contents of aspartic acid, glutamic acid, leucine and lysine. R3a and R3b showed very similar amino acid compositions.

Discussion

This work describes the purification of major forms of glutathione S-transferases from rabbit liver and their characterization in comparison with the enzymes from rat liver. The content of CDNB activity in the rabbit liver was higher than those in the other species reported hitherto except for the mouse liver,[8] but varied markedly among individuals within the range of 44—362 μmol/min/g of wet liver (Table I). Davies *et al.*[40] reported that the enzyme activity in the mouse liver varied in a circadian manner (with DCNB as a substrate). The rabbits, therefore, were sacrificed at 11:00 a.m. in all our experiments and the crude extracts were prepared in the same manner. Thus, we consider that the variation of activity content may be due to individual differences.

To minimize the loss of enzyme activity during the separation procedures, the procedures from the sacrifice to the separation of the multiple forms of the enzyme on the CM-cellulose column were performed within 4 d under the same conditions as regards column size, dialysis time, *etc.* However, as shown in Figs. 1 and 2, the relative intensity of each activity peak toward CDNB was different in each chromatogram, although three activity peaks were observed in all experiments. This difference was not correlated with the content of activity in the crude extract (data not shown). Consequently, the different patterns of CM-cellulose chromatograms also appear to be due to individual differences as concluded in the case of the content of enzyme activity. In the previous report,[34] we found a sex difference of glutathione S-transferases in the rat liver as follows. Normal male rat liver contained two more enzymes than normal female liver. The activity toward CDNB in the male rat liver could be increased much more significantly by phenobarbital treatment than that in females, and the forms of enzymes induced were different between the sexes. However, no sex difference in the rabbit liver could be observed with respect to the number of enzymes. The possibility of sex difference in induction has not yet been examined.

Recently, Gawai and Pawar[24] indicated the existence of four forms of hepatic glutathione S-transferase in rabbits treated with phenobarbital and characterized the major form which was eluted at the highest concentration of KCl from the CM-cellulose column (concentration not given). As their separation procedure was essentially similar to ours, their major form might correspond to our R4. However, R4 (bound most tightly to the CM-cellulose column in our experiment) was not a major form; R2 and/or R3 were the major form(s). R4 may be induced by phenobarbital treatment because this treatment is well-known

to induce some forms of enzyme in rat liver.[34,37,41,42]

As regards the substrate specificity (Table III), every purified enzyme showed the highest activity toward CDNB among the substrates examined. In particular, the specific activity of R2 was higher than that of any enzyme from rat,[34] human,[7] chicken,[11] monkey,[9] hamster[10] or sheep[12] liver, but not higher than that of any enzyme from mouse liver.[8] However, the rabbit enzymes all exhibited little or no activity toward DCNB, *p*-NBC, EA, ENPP or BA. In addition, the crude extract showed similar substrate specificity (data not shown). This suggests that the substrate specificity of each enzyme was unaffected through the purification steps. Interestingly, the different forms of enzyme from rat liver showed significant differences in substrate specificity.[1,19,34] For example, AA and B, containing Yc subunit, possess higher specific activity toward EA, whereas A and C, containing Yb2 subunit, are more active toward BA. Thus, the different forms of these enzymes can be distinguished in terms of substrate specificity. In this respect, rabbit enzymes resemble those from human liver which all exhibit poor activity toward these substrates except for CDNB.

An R2, R3a and R3b mixture was eluted as a single activity peak from a Sephadex G-75 column. Its molecular weight was approximately 51000. However, this method gave molecular weights of 50000 for R2, 48000 for R3a and 53000 for R3b when these enzymes were separately applied to the column (data not shown). The sum of molecular weights of the subunits (determined by SDS/polyacrylamide gel electrophoresis) was 50000 for R2, 51000 for R3a and 53000 for R3b (Fig. 5). Both methods gave the same molecular weights of 50000 for R2 and 53000 for R3b, but different values for R3a: 48000 by the former method and 51000 by the latter. The reason for this difference is not yet clear.

All known basic glutathione S-transferases[7-14] appear to be homodimers except for the rat hepatic enzymes.[17-20] In the case of the rat hepatic enzymes, the multiple forms are homodimers and heterodimers of subunits with three distinct molecular weights. Similarly, R2 and R3b were homodimers of Y2 and Y3 subunit, respectively, whereas R3a was a heterodimer of Y1 and Y3 subunits. Since the subunit of higher molecular weight in R3a coincided with that in R3b, a homodimer of Y1 subunit might exist in the R1, R4 peak fractions of DEAE-cellulose-bond fraction. We are planning to investigate the number of enzymes present and the subunit compositions of R1 and R4.

Determination of pI values by using two sorts of pI marker proteins gave two different values for each enzyme: every pI value determined by using the pI marker proteins from Oriental was 0.6 pH higher than that based on the Pharmacia kit (Table V). According to the manufacturers' instructions, the pI values of pI marker proteins from Oriental had been determined at 4 °C, while the temperature of 24 ± 1.5 °C had been adopted for determining the pI values of the pI marker proteins from Pharmacia. Fredriksson[43] reported that when the pI value of a basic protein was determined, the measurement at 4 °C gave a 0.6 unit higher pI value than that at 25 °C. Therefore, this can account for the different values in our experiment. It is important to note the temperature at the time of determining the pI value of a protein, because the pI value is considerably temperature-dependent.

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