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Rat Brain Glutathione S-Transferases

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Rat brain glutathione S-transferases (GSTs) were studied and compared with rat hepatic GSTs in order to elucidate the mechanisms of detoxication of xenobiotics in the brain. In rat brain, the diethylaminoethyl (DEAE)-cellulose-bound fraction contained more glutathione S-transferase (GST) activity than the DEAE-cellulose-unbound fraction under conditions of 10 mM Tris–HCl, pH 8.0. The group of GSTs in the DEAE-cellulose-bound fraction was found to be resolved into at least three peaks. The GST in the main peak was partially purified by Sephadex G-75 and CM-52 column chromatography. The activity was eluted as a single peak on Sephadex G-75 gel filtration, and did not bind to a CM-52 column (10 mM potassium phosphate, pH 6.7). The molecular weight of the GST in the main peak was about 44000 daltons, and the enzyme consisted of YnYn (Yn subunit: M_r 24500) subunits as determined by SDS/polyacrylamide gel electrophoresis. The GST activities of this main peak were inhibited by antiserum raised against peak II (YbYb subunits; Yb subunit, M_r 25000) in the DEAE-cellulose-bound fraction of rat liver.

Keywords——glutathione S-transferase; rat brain; DEAE-cellulose; Sephadex G-75; CM-cellulose; YnYn subunit; substrate specificity; immunotitration

Introduction

Glutathione S-transferases (GSTs) exist mainly in cytosol. They are a group of enzymes which play an important role in the detoxication and excretion of xenobiotics.[1] In the rat, GST activity is much more abundant in the liver than in any other tissue, and many studies have been done on the hepatic GSTs.[2] However, relatively little work has been done on GSTs of brain. As regards the purification of GSTs from brain, there are two reports, one on pig brain,[3] and the other on the human brain.[4] Many antipsychotics and neurotoxins act on the brain,[5] and from the point of view of their metabolism and detoxication, it would be interesting to study brain GSTs.

In the case of rat liver, about 90% of the GST activity toward 1-chloro-2,4-dinitrobenzene flows through a diethylaminoethyl (DEAE)-cellulose column (10 mM Tris–HCl, pH 8.0),[6] while in the case of rat brain the DEAE-cellulose-bound fraction contains more GST activity than the DEAE-cellulose-unbound fraction.[7] We examined the GSTs in the DEAE-cellulose-bound fraction of rat brain and compared them with those in the same fraction of rat liver.

Experimental

Materials——Glutathione, 1-chloro-2,4-dinitrobenzene, *trans*-4-phenyl-3-buten-2-one and *p*-nitrobenzyl chloride were purchased from Wako Pure Chemical Ind. 1,2-Dichloro-4-nitrobenzene was from Tokyo Kasei Ind., Ltd. Ethacrynic acid was from Sigma Chemical Co.

Assay Method——GSTs were assayed according to the procedures of Habig *et al.*,[6] except that the wavelength and $\Delta\varepsilon$ value (mM^{-1}, cm^{-1}) used for *p*-nitrobenzyl chloride were 320 nm and 1.75, respectively. Enzyme activity was monitored through the purification steps by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with

glutathione (GSH). The assay mixture (3 ml) consisted of 1 mM CDNB, 1 mM GSH and 0.1 M potassium phosphate buffer, pH 6.5. The rate of increase in absorbance at 340 nm was monitored at 25 °C. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min at 25 °C. Protein content was estimated by measuring the absorbance at 260 and 280 nm (mg/ml = $1.45\ A_{280} - 0.74 A_{260}$).[8]

A) Preparation of GSTs from Rat Brain——Ten male Wistar strain rats (body weight 295–323 g) were killed by exsanguination. The following procedures were carried out at 4 °C. Brains (18.4 g) were stored at −80 °C and then homogenized in 5 vol. of 10 mM Tris–HCl pH 8.0 (at 20 °C) (buffer A) with a Teflon-glass homogenizer. The homogenate was centrifuged at 20000 × *g* for 60 min. The supernatant fraction was adjusted to a concentration of 10 mM Tris and a pH of 8.0. The brain extract (85 ml) was applied to a DEAE-cellulose column (DE-52, 2.6 × 38.5 cm). The column was washed with 650 ml of buffer A to remove the unbound GSTs (15 ml/fraction) and then the bound GSTs were eluted with a linear gradient formed from 1050 ml each of buffer A and buffer A containing 135 mM KCl (7 ml/fraction).

Partial Purification of GST in the Main Peak (Peak 3): Sephadex G-75 Column Chromatography: Fractions 161 through 175 from the DE-52 column chromatography were pooled and concentrated by ultrafiltration through a PM-10 membrane. The solution was applied to a Sephadex G-75 column (superfine grade, 2.65 × 85 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.7 (buffer B) and eluted with buffer B (3 ml/fraction).

CM-52 Column Chromatography: The enzyme solution obtained from Sephadex G-75 column chromatography was applied to a CM-cellulose column (CM-52, 1.26 × 2.5 cm) equilibrated with buffer B. The column was washed with 45 ml of buffer B and then a linear gradient formed from 30 ml each of buffer B and buffer B containing 200 mM KCl was applied (3 ml/fraction).

B) Determination of Molecular Weight——The molecular weight of GST in the main peak (peak 3) was estimated by Sephadex G-75 (superfine grade, 2.65 × 85 cm) chromatography. Bovine serum albumin (M_r 66000 Sigma), ovalbumin (M_r 45000 Sigma) and myoglobin (M_r 17200 Sigma) were used as standards.

C) Sodium Dodecyl Sulfate (SDS)/Polyacrylamide-Gel Electrophoresis——This was done by the method of Laemmli.[9] Protein samples were subjected to electrophoresis in slab gel containing 12.5% polyacrylamide with SDS/Tris/glycine electrode buffer. Proteins were stained with 0.025% Coomassie Brilliant Blue R 250–50% (v/v) methanol–10% (v/v) acetic acid in water and destained with 25% (v/v) methanol–7% (v/v) acetic acid in water.

D) Immunotitration——Antiserum was raised against purified peak II (YbYb subunits) in the DEAE-cellulose-bound fraction of rat liver.[7] Immunotitration studies were done by incubating a fixed amount of enzyme with various amounts of antiserum. The reaction mixtures, total volume 0.6 ml, were incubated overnight at 4 °C and then for 2 h at room temperature. Subsequently, goat anti-(rabbit IgG) serum (20 μl) was added. The reaction mixtures were again incubated overnight at 4 °C and then centrifuged at 20000 × *g* for 30 min at 4 °C to precipitate the antigen–antibody complex. The GST activities of the supernatant solutions were assayed with CDNB. The incubation mixtures contained various volumes of a bovine serum albumin solution (57 mg/ml in 0.1 M potassium phosphate, pH 6.7) in order to obtain a constant protein concentration.[10]

Results

A) Preparation of GSTs from Rat Brain

The group of GSTs in the DEAE-cellulose-bound fraction was found to be resolved into four peaks (Fig. 1a). Peak 3 (main peak), peak 4 and peak 5 were eluted at approximately 50 mM, 67 mM, and 81 mM salt concentration, respectively. Experiments were done eight times with different brains. These three peaks were reproducible, except that their salt concentrations varied somewhat in all experiments. As to peak 2, in some cases it appeared as a shoulder peak and in some cases it could not be found. Another shoulder peak appeared in front of peak 5. In addition, a small peak was found at the position corresponding to peak II of rat liver in two of eight experiments, but the existence of this peak remains to be confirmed. Peak 3 was eluted as a single peak on Sephadex G-75 column chromatography, and did not bind to a CM-52 column (10 mM potassium phosphate buffer, pH 6.7).

B) Determination of the Molecular Weight

On Sephadex G-75 gel filtration of peak 3, a single activity peak was eluted just behind the ovalbumin peak, and its molecular weight was estimated to be about 44000.

C) SDS/Polyacrylamide-Gel Electrophoresis

As shown in Fig. 2, peak 3 gave a single band between Ya and Yb subunits. This position corresponded to that of Yn subunit.

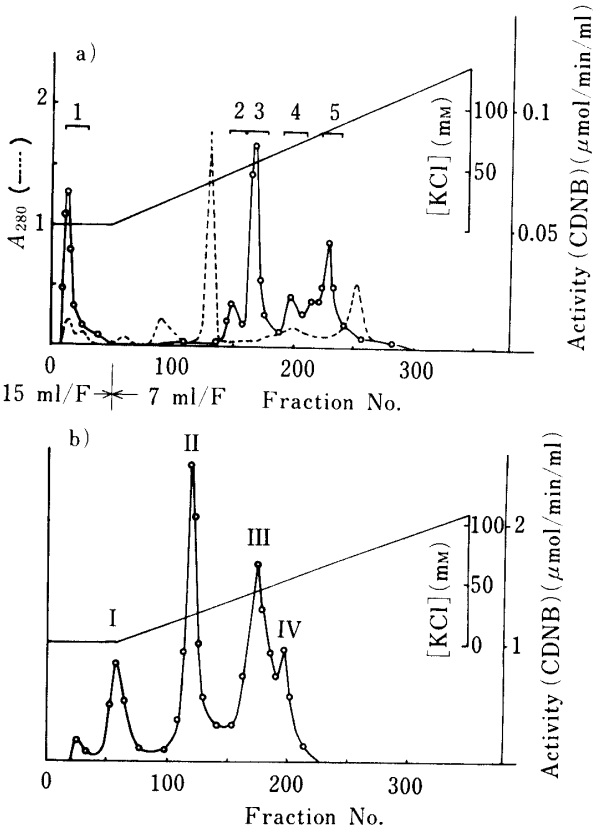

Fig. 1. Elution Pattern of Rat Glutathione S-Transferases from a DEAE-Cellulose Column

a) Brain. b) Liver (Fig. from ref. 7). Liver homogenate was first applied to a DEAE-cellulose column (DE-23), and, after the unbound activity had been removed, the bound activity was reapplied to a DEAE-cellulose column (DE-52).
—○—, activity toward 1-chloro-2,4-dinitrobenzene (CDNB); ------, absorbance at 280 nm.

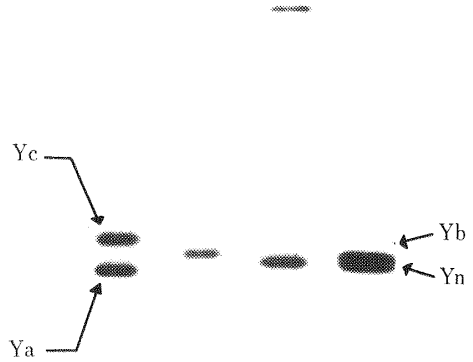

Fig. 2. SDS/Polyacrylamide-Gel Electrophoresis

The slots, from left to right contained GST B, peak II (liver), peak 3 (brain), peak IIIb (liver).

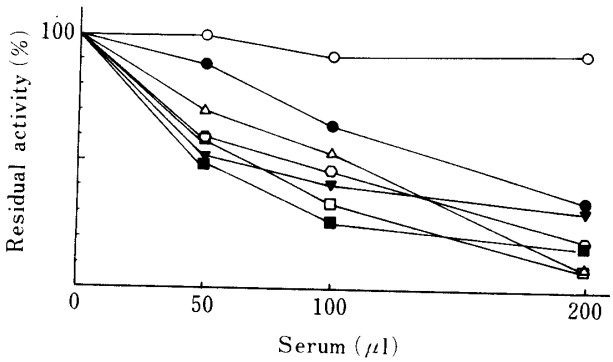

Fig. 3. Immunotitration of Rat Brain Glutathione S-Transferases by Antiserum Raised against Peak II (Liver)

○, homogenate supernatant in the presence of normal serum. The following components were tested in the presence of antiserum, ●, homogenate supernatant; ■, peak 1; □, peak 2; △, peak 3; ▼, peak 4; ○, peak 5.
Peaks 1—5 were obtained from the DEAE-cellulose chromatography step.

TABLE I. Substrate Specificities of Rat Brain Glutathione S-Transferases[a]

	Specific activity (units/mg protein)[b] Substrate[c] CDNB	Relative activity (%)				
		CDNB	DCNB	*t*-PBO	*p*-NBC	EA
Peak 1	0.288	100	11.8	0.2	18.8	2.4
Peak 3	0.475	100	15.2	0[d]	3.7	1.1
Peak 4	0.080	100	8.6	0[d]	16.5	9.3
Peak 5	0.122	100	9.2	0[d]	0[d]	0[d]

a) The samples used were the enzyme fractions obtained from the DE-52 column chromatography. *b*) Protein content was estimated by measuring the absorbance at 260 and 280 nm. *c*) CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; *t*-PBO, *trans*-4-phenyl-3-buten-2-one; *p*-NBC, *p*-nitrobenzyl chloride; EA, ethacrynic acid. *d*) Zero activity indicates no significant enzymatic reaction under the conditions used.

D) Immunotitration

The activities of peaks 1, 2, 3, 4, 5, and homogenate supernatant were 0.075, 0.037, 0.080, 0.054, 0.047, and 0.042 (units), respectively. The activity of every peak was inhibited by anti-peak II serum (Fig. 3), though the residual activities were various. The titration curve of peak 3 showed a roughly straight line, but curves of peaks 1 and 4 reached a plateau at 100 μl of serum.

E) Substrate Specificities

These are listed in Table I for peaks 1, 3, 4, and 5 which were obtained by DE-52 column chromatography. The amount of peak 2 was insufficient to ascertain the substrate specificities. Among the compounds tested as substrates, CDNB was most effectively conjugated with GSH.

Discussion

Dierickx has found several GSTs in rat brain by using CM-cellulose column chromatography after passing the homogenate through a DEAE-cellulose column in 100 mM Tris–HCl, pH 8.0.[11] We, however, resolved rat brain GSTs by DEAE-cellulose column chromatography because, as we previously reported,[17] the DEAE-cellulose-bound fraction of rat brain contains more GST activity than the DEAE-cellulose-unbound fraction under conditions of 10 mM Tris–HCl, pH 8.0. We obtained at least three peaks in the DEAE-cellulose-bound fraction. Peak 3 of brain was eluted at a salt concentration similar to that of peak III or IV of liver. We could not find peaks in the liver corresponding to peaks 4 and 5 in brain. When peak 1 was applied to a CM-52 column equilibrated with buffer B and eluted with a linear gradient of 0—0.1 M KCl in buffer B, one peak appeared in the CM-52-unbound fraction and four peaks appeared in the CM-52-bound fraction (data not shown). Though an accurate comparison between our result and Dierickx's result is not possible, we infer that the GST D+E and the anionic GST he reported correspond to the GSTs in the DEAE-cellulose-bound fraction and in the CM-52-unbound fraction of peak 1 that we resolved.

The molecular weight of the GST in the main peak (peak 3) was about 44000. On SDS/polyacrylamide-gel electrophoresis, peak 3 was found to have YnYn subunits. We reported that the DEAE-cellulose-bound fraction of rat liver contained a GST having YbYn subunits (peak IIIb),[7] which was probably identical with GST S reported by Hayes and Chalmers.[12] However, we could not find the GST having YnYn subunits in rat liver. It is interesting that the GST in the main peak of rat brain has YnYn subunits.

It was reported that rat GSTs could be classified into two groups, which did not cross-react with each other immunologically.[13] The GSTs in group I consist of combinations of Ya and Yc subunits, while the GSTs in group II consist of Yb subunits.[13]

Recently, Yb subunit was reported to consist of Yb_1 and Yb_2 subunits.[13c-e,14] These two subunits could not be separated on SDS/polyacrylamide-gel electrophoresis by the method of Laemmli. It is inferred that GST A, GST C and the GST in the DEAE-cellulose-bound fraction may consist of combinations of Yb_1 and Yb_2: Yb_1Yb_1, Yb_1Yb_2 and Yb_2Yb_2, respectively. We and Hayes found several GSTs having Yb subunits and a GST having YbYn subunits (peak IIIb or GST S) in the DEAE-cellulose-bound fraction of rat liver.[7,12] In addition, we found a GST (peak 3) having YnYn subunits in the DEAE-cellulose-bound fraction of rat brain. The result that the GST activity of peak 3 was inhibited by the anti-peak II (YbYb) serum seems to indicate that it belongs to group II. However, this is uncertain, because it is not clear which group peak 3 (YnYn) belongs to. We are planning to examine it in detail.

GSTs of rat brain were reported to catalyze the conjugation of acrylamide with GSH.[15]

Several antibiotics, as well as antipsychotic, antidepressant and antianxiety drugs, were reported to produce a considerable *in vitro* inhibition of the GST activity of guinea-pig brain.[5] The physiological significance of brain GSTs requires further investigation.

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