PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASE FROM RAT BRAIN CYTOSOL: IDENTIFICATION OF FOUR ISOZYMES AND EVIDENCE FOR ABSENCE OF THE Ya SUBUNIT

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ABSTRACT
Glutathione S-transferases (GSTs) were purified from rat brain cytosol by a procedure including Sephadex G-75, glutathione-linked Sepharose 6B and CM-cellulose column chromatography. Three enzyme fractions were obtained by column chromatography on CM-cellulose; the fractions were apparently homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Its native form was a dimeric protein with a molecular weight of 45,000, and chromatofocusing of the enzymes gave four peaks at pI 6.7, 7.6, 8.9 and 10.1. Antibodies raised against GSTs of rat liver cytosol were used to identify the purified enzymes. It has been demonstrated that rat brain cytosol has no ligandin (GST-L2=YaYa), which is the major component of liver GST.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are important enzymes for detoxication because of their ability to conjugate a variety of electrophilic compounds with glutathione (9). The highest GST activity is present in the liver and detailed studies have been performed (9). Recent studies have revealed the presence of the enzyme in mammalian and avian brain (5, 17). Although it has been reported that brain GST binds with acrylamide, a neurotoxic agent (7), and the enzyme can be significantly induced by a prolonged treatment with phenobarbital or 3-methylcholanthrene (4), the physiological role of the enzyme has not been fully elucidated (7).

Our recent immunohistochemical study has shown that GSTs are exclusively localized in astrocytes and ependymal cells, but not in oligodendrocytes and neurons, of adult rat brain and they are especially abundant in the nucleus (14). The present study was undertaken to evaluate the structural properties of brain GST and it was found that rat brain cytosol contained no ligandin (GST-L2=YaYa), which is known to be the major component of GST in liver (8).

MATERIALS AND METHODS
Materials
The following chemicals were obtained from sources indicated: o-dinitrobenzene, ammonium sulfamate, and N-1-naphthylethylenediamine dihydrochloride from Nakarai; CM-cellulose (CM52) from Whatman; Sephadex G-75, chromatofocusing gel PBE 94 and 118, Pharmacia, and Polybuffer 74 and 96 from Pharmacia; GST purified from rat liver cytosol, glutathione-epoxy-agarose and ovalbumin from Sigma; reduced glutathione from Yamanouchi. All other chemicals were of analytical grade.

Preparation of Rat Brain Cytosol
Brains from 8-week-old Wistar-King rats weigh-
ing 100–200 g were excised and washed in cold 0.25 M sucrose. The brains were homogenized with a Potter-Elvehjem homogenizer fitted with a Teflon pestle in 10 vol of 0.32 M sucrose. The whole homogenate was centrifuged at 105,000 g for 90 min and the resultant supernatant was used as the cytosolic fraction for purification of GST.

**Analytical Methods**

Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was done by the procedure of Weber and Osborn (18). Double immunodiffusion tests were performed as described by Ouchterlony (13). Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as standard. GST activity was determined by measuring the rate of conjugation of o-dinitrobenzene with glutathione as described by Asaoka and Takahashi (2).

**Antibodies against GSTs**

The monospecific antibodies against various isozymes, GST-A₂ (YbYb), -B₁ (YcYc), -BL (YaYc), -AC (YbYb'), -C₂ (Yb′Yb') and -L₂ (YaYa), were kindly donated by Dr K. Sato (Hirosaki University, Hirosaki, Japan) (10, 15). These classifications of GST isozymes were followed by a nomenclature according to their subunits, which was proposed by Mannervik and Jansson (12).

**RESULTS**

**Purification of GSTs from Rat Brain Cytosol**

All purification steps were performed at 4°C, unless otherwise indicated. The brain cytosol (860 mg of protein) (fraction I) prepared as described in Materials and Methods was applied on a Sephadex C-75 column (4.5×85 cm) equilibrated with 0.1 M sodium bicarbonate (pH 8.0) and eluted with the same buffer (Fig. 1). The fraction containing GST activity, whose peak corresponded to the elution volume of ovalbumin (Mᵣ = 45,000), was pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) (fraction II).

Fraction II was applied on a glutathione-linked Sepharose 6B column (1×2 cm) (16) equilibrated with 10 mM sodium phosphate buffer (pH 6.0) (Fig. 2). After extensive washing until pro-
Fig. 2  Glutathione-affinity column chromatography. Fractions of 1 ml were collected at a flow rate of 25 ml/h. GST activity was determined in 5-μl aliquots. The bar above the active peak indicates the fraction which was combined and used for subsequent purification. See text for the detailed procedure.

Fig. 3  CM-cellulose column chromatography. Fractions of 1 ml were collected at a flow rate of 25 ml/h. All fractions were lyophilized and dissolved in 0.5 ml of distilled water, and 10-μl aliquots were used for the GST assay. The bars above the active peaks A, B and C indicate the fractions which were combined and used as final enzyme preparations. See text for the detailed procedure.
Table 1  Purification of Glutathione S-Transferases from Rat Brain Cytosol

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (nmol)</th>
<th>Yield (%)</th>
<th>Specific activity (nmol/mg)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Cytosol</td>
<td>50</td>
<td>860</td>
<td>33,226.0</td>
<td>100</td>
<td>27.9</td>
<td>1</td>
</tr>
<tr>
<td>II Sephadex G-75</td>
<td>86</td>
<td>363.0</td>
<td>26,315.0</td>
<td>79.2</td>
<td>72.5</td>
<td>2.6</td>
</tr>
<tr>
<td>III GSH-affinity</td>
<td>32</td>
<td>11.9</td>
<td>33,824.1</td>
<td>101.8</td>
<td>2,848.6</td>
<td>102.1</td>
</tr>
<tr>
<td>IV CM-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak A</td>
<td>3</td>
<td>0.9</td>
<td>4,086.8</td>
<td>12.3</td>
<td>4,598.0</td>
<td>164.8</td>
</tr>
<tr>
<td>Peak B</td>
<td>3</td>
<td>1.0</td>
<td>5,382.6</td>
<td>16.2</td>
<td>5,630.2</td>
<td>201.8</td>
</tr>
<tr>
<td>Peak C</td>
<td>3</td>
<td>1.3</td>
<td>4,917.4</td>
<td>14.8</td>
<td>3,682.8</td>
<td>132.0</td>
</tr>
</tbody>
</table>

Protein was reduced to nearly zero, GSTs were eluted by 5 mM glutathione in an equilibration buffer. The fraction containing GST activity was pooled and dialyzed against the equilibration buffer (fraction III).

Fraction III was applied on a CM-cellulose column (2×8 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.0) (Fig. 3). After extensive washing, GSTs were eluted by a linear concentration gradient (0-0.2 M) of NaCl in an equilibration buffer. The active enzyme peaks A, B and C were collected separately and concentrated by ultrafiltration using an Amicon macrolute concentrator (B15) (fraction IV). Rat brain GSTs were purified approximately to 130-200-fold with 12-16% yield. Results of the typical purification procedures are summarized in Table 1.

Homogeneity and Molecular Weight

The final enzyme preparation showed an apparent homogeneity upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 4a). The molecular weight under denaturating conditions was: peak A, 23,500 and 24,500, peak B, 23,500, peak C, 23,500 and 25,000 (Fig. 4b). Therefore, its native form whose molecular weight was determined to be approximately 45,000 (Fig. 5), seems to be a dimeric protein.

Chromatofocusing of Brain GSTs

Peaks A, B and C in Fig. 3 were subjected to chromatofocusing on a column (1.2×18 cm) of PBE 94 (A) and PBE 118 (B, C) equilibrated with 0.025 M imidazole/HCl, pH 7.4 and 0.025 M triethylamine/HCl, pH 11, respectively (Fig. 5). GSTs were eluted with 12.5% Poly buffer 74/ HCl, pH 7.4 (A) and 2.2% Pharmalite/HCl, pH 7.0 (B, C), respectively. The pH of GST eluted in this chromatofocusing was 6.7 for peak A, 7.6 for peak B, and 8.9 and 10.1 for peak C.

Immunological Characterization of the Subunit Pattern

Immunological investigations were carried out to identify the subunit pattern of the purified enzymes using monospecific antibodies raised against the various liver GST isozymes. By the double immunodiffusion technique, peaks B and C showed a single precipitin line with anti-GST-C2, and anti-GST-A2 and -B2, respectively (Fig. 6). However, peak A did not show any precipitin line. Furthermore, it has been demonstrated that no peaks showed any precipitin line with anti-GST-L2, whose antigen is known to be the major component of liver GST (8).

Based on these electrophoretic mobility and immunological characterization (Table 3), it has been concluded that rat brain cytosol contains a new type of GST not found in liver cytosol.

DISCUSSION

The highest GST activity is present in the liver, but all other tissues examined contain the enzyme activity (3). In addition to their multiple catalytic functions, GST binds a number of nonsubstrate ligands (9). Subsequent studies have also shown a biological role of brain GST in conjugation and binding of a potent neurotoxic agent, acrylamide (5, 7, 17). Rat brain GST activity is age-dependent (19), and varies moderately in different brain regions (6). On the other hand, mouse brain GST activity varies considerably among the strains examined (19). In porcine brain, Asaoka and Takahashi (1) reported that GST activity was distributed fairly evenly in various regions of the brain, and was especially abundant in the cytosol fraction. However, considerable enzyme activity was also detected in the
Fig. 4 Polyacrylamide disc gel (10%) electrophoresis in the presence of 0.1% sodium dodecyl sulfate. a: A, peak A in Fig. 3; B, peak B; C, peak C; D, commercial rat liver GST. Five micrograms (as protein) of each sample were applied to the gel. b: Molecular weight determination. The standard proteins were 1, ovalbumin (45,000); 2, carbonic anhydrase (31,000); 3, soybean trypsin inhibitor (21,500) and 4, lysozyme (14,400). Each standard was electrophoresed in triplicate. Arrows indicate the relative mobility of the purified enzymes.

microsomal and mitochondrial fraction, but not in the synaptosomal fraction (1). Theodore et al. reported three, one cationic (pI 8.3) and two anionic (pI 5.5 and 4.6), forms of GST present in human brain (17). This communication has revealed that rat brain contains four forms of GST, one anionic (pI 6.7) and three cationic (pI 7.7, 8.9 and 10.1). The subunit composition of the anionic form was found to be different from any other GST previously obtained from rat liver.
By immunohistochemical staining, we have recently reported that GST is exclusively localized in astrocytes and ependymal cells of adult rat brain and is especially abundant in the nucleus (14). However, oligodendrocytes and neurons were not stained to a detectable extent. Considering the detoxification ability of GST (9), the enzyme in astrocytes and ependymal cells presumably performs important physiological functions in the blood brain barrier. The enzyme may protect the brain from toxic effects of a wide variety of xenobiotics.

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REFERENCES
Fig. 6 Double immunodiffusion test of purified enzymes with antibodies against various GST isozymes consisting of a known subunit. The center well contained purified peak A, B or C (20 μg) as indicated; the contents (10 μg) of the outer well were as follows: 1, anti-rabbit glutathione GST-L₂ (YaYa); 2, -BL (YaYc); 3, -C₂ (YbYb); 4, -AC (YbYb); 5, -A₂ (YbYb); and 6, -B₂ (YcYc).

Table 2 Immunological Analysis on Isozyme Pattern of GSTs Purified from Rat Brain Cytosol

<table>
<thead>
<tr>
<th>Anti-GSTs</th>
<th>Precipitin line formed</th>
<th>Theoretical immunoprecipitin reaction of each GST isozyme with antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak A</td>
<td>Peak B</td>
</tr>
<tr>
<td>Anti-L₂ (YaYa)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-BL (YaYc)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-A₁ (YbYb)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-AC (YbYb)</td>
<td>-</td>
<td>++</td>
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<tr>
<td>Anti-C₁ (YbYb)</td>
<td>-</td>
<td>-</td>
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*Results from double immunodiffusion experiments (Fig. 6). Analytical data from reference 10.

Table 3 Characterization of GSTs Purified from Rat Brain Cytosol

<table>
<thead>
<tr>
<th>GSTs</th>
<th>Molecular weight</th>
<th>pH eluted from chromatofocusing column</th>
<th>Subunit composition</th>
<th>GST isozyme identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Subunit</td>
<td>SDS-PAGE</td>
<td>Ouchterlony</td>
</tr>
<tr>
<td>Peak A</td>
<td>45,000</td>
<td>23,500</td>
<td>24,500</td>
<td>6.7</td>
</tr>
<tr>
<td>Peak B</td>
<td>45,000</td>
<td>23,500</td>
<td>24,500</td>
<td>7.6</td>
</tr>
<tr>
<td>Peak C</td>
<td>45,000</td>
<td>23,500</td>
<td>25,000</td>
<td>8.9, 10.1</td>
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</table>

N.I., not identified

4. CHAND P. and CLAUSEN J. (1982) Effects of phenobarbital and sodium salicylate on cytochrome P₄₅₀ mixed function oxygenase and glutathione S-