PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASE FROM THE HUMAN HEPATOMA DERIVED PLC/PRF/5 CELL LINE

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ABSTRACT

PLC/PRF/5 cells, an established cell line derived from a human hepatoma, were cultured to large numbers in a cell factory for the isolation of glutathione S-transferase. This enzyme interacts in many toxicological important reactions. The enzyme was enriched by affinity chromatography, and further purified by cation exchange chromatography and anionic chromatofocusing. The enzyme activity was always recovered in one peak only. The sole transferase was identified as human glutathione S-transferase π by its pI (pH 4.4 on chromatofocusing), its kinetic properties, the effect of specific inhibitors, and by its subunit structure, as determined by electrophoresis (one band at M_r 26,000). The results are discussed in the view that glutathione S-transferase π is considered as a tumor marker. Since PLC/PRF/5 cells contain exclusively the glutathione S-transferase π isoenzyme, these cells cannot be used as an alternative model for intact human liver. However, they are a promising source for the study of specific glutathione S-transferase π -dependent metabolic processes, for example in relation to cancer.

Reduced glutathione (GSH) plays an important role in the detoxication of potentially toxic compounds. Its most widely known biological role is the conjugation with xenobiotics, including carcinogens, mutagens, toxic or pharmacologically active compounds, and their metabolites (3). This conjugation is catalyzed by glutathione S-transferase (GST, EC 2.5.1.18), of which multiple isoenzymes are described, as recently reviewed (14). GST is also capable of direct binding of toxic compounds, another important detoxication pathway (19). Moreover, GST has peroxidase activity for organic hydroperoxides and, therefore, the potential to detoxify lipid and DNA hydroperoxides arising from radical damage in the presence of oxygen (22). GST isoenzymes may also have diagnostic value as tumor markers (16).

The liver is the principal organ involved in the biotransformation and detoxification of xenobiotics. It is also the first organ after the gastrointestinal tract a drug passes after oral intake. Therefore, knowledge of hepatic metabolic pathways of xenobiotics is of major importance. Attempts to establish permanent cell lines of hepatocytic origin were unsuccessful so far, because typical biochemical functions are lost. GST activity, however, is found in any cell line investigated, albeit in highly different degrees (2, 17), and I previously reported that Hep G2 cells contain GST activity (4). Afterwards a four times higher GST activity was observed in PLC/PRF/5 cells, an established cell line, derived from a human hepatoma (13). An interesting aspect of these cells is that they have the biosynthetic capabilities of normal liver parenchymal cells (10).

They synthesize and secrete 11 of the major human plasma proteins (10) and produce the hepatitis B surface antigen (10, 13). Moreover they metabolize 3,4-benzpyrene and aldrin, proving that they contain at least two different forms of P₄₅₀ cytochromes (12). Since liver GST plays a central role in different detoxication pathways (3, 19, 22) and since cultured PLC/PRF/5 cells have many hepatocyte characteristics, I investigated the GST in this hepatoma cell line.

MATERIALS AND METHODS

The PLC/PRF/5 cells were provided by Dr Y.-J. Schneider, UCL, Louvain-la-Neuve, Belgium. Cell culture flasks were purchased from Nunc, and cell culture products from Gibco. GSH and bromosulfophthalein were obtained from Janssen Chimica (Beerse, Belgium), bovine serum albumin from Boehringer, chemicals for SDS-polyacrylamide gel electrophoresis from Bio-Rad, carboxymethyl cellulose (CM52) from Whatman, and epoxy-activated Sepharose 6B, PD-10 columns containing 10 ml Sephadex G-25 Medium, Polybuffer exchanger 94, Polybuffer 74, and the low molecular weight protein calibration kit from Pharmacia. Amicon furnished the YM-10 ultrafiltration membranes. Hematin came from Sigma, and all other chemicals from Merck, including triphenyltin chloride and the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB).

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ m/ml streptomycin (complete medium). They were incubated at 37°C in a 7% CO₂ atmosphere.

Specific GST activities were determined on cells cultured in tissue flasks with a growth surface of 175 cm². The same results were obtained when the cells were either loosened by trypsinisation or by scraping.

For the purification of GST the PLC/PRF/5 cells were cultured in a cell factory, consisting of 10 separate chambers, with a total culture surface of 6,000 cm². The cells were harvested when nearly reaching confluency. They were washed with 500 ml Hanks' balanced salt solution, trypsinised with 125 ml of 0.25% trypsin, suspended in 400 ml of com-

plete medium, and centrifuged at 1,000 g for 10 min. Further manipulations were carried out at 0–4°C. The cells were homogenized in 10 ml of 22 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose and 1 mM EDTA·Na₄, with 10 up-and-down strokes in a motor driven Potter-Elvehjem homogenizer (1,500 rpm), equipped with a Teflon pestle. A fat-free supernatant obtained after ultracentrifugation (1 h, 100,000 g) was applied to a GSH affinity column (1.6×9 cm), packed with epoxy-activated Sepharose 6B that had been reacted with GSH as described (18). The column was eluted with 22 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA·Na₄, until no further protein was detected by monitoring the absorbance at 290 nm. GST was then eluted with 15 mM GSH in 50 mM Tris-HCl buffer, pH 9.6 (23). The fractions with GST activity were pooled, concentrated to 2.5 ml by ultrafiltration on a YM-10 membrane, equilibrated with 5 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA·Na₄, on a PD-10 column, and applied to a carboxymethyl cellulose column (1.6×9.5 cm), equilibrated with the same buffer. The anionic proteins are not adsorbed when eluted with this buffer. The cationic proteins were later eluted with 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA·Na₄. No further GST was eluted with 1 M NaCl. The fractions with GST activity were pooled, concentrated again to 2.5 ml by ultrafiltration on a YM-10 membrane, equilibrated with 25 mM imidazole-HCl buffer, pH 7.4, on a PD-10 column, and applied to a Polybuffer exchanger 94 column (1.0×34 cm), equilibrated with 25 mM imidazole-HCl buffer, pH 7.4. Elution was carried out with Polybuffer 74, diluted 1/16, and adjusted to pH 4.0 with HCl. The pH was monitored with a Pharmacia pH monitor.

SDS-polyacrylamide slab gel electrophoresis was carried out on 12.5% polyacrylamide gels as described (11).

The GST activity was measured as described (6) using CDNB as substrate. Specific activity is expressed in units of enzyme activity/mg protein, as measured by the Bradford method (1) with bovine serum albumin as standard. Kinetic properties were examined measuring the initial velocities of GST at GSH and CDNB concentrations varying from

Fig. 1 Elution of PLC/PRF/5 GST activity from a GSH affinity column. The column was first rinsed with 22 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA·Na₄, and then eluted with 15 mM GSH in 50 mM Tris-HCl, pH 9.6 (arrow A). Thereafter the column was eluted with 3 M NaCl (arrow B). Fractions of 3 ml were collected.

Fig. 2 Chromatography of the by affinity chromatography enriched GST on a carboxymethyl cellulose column. The column was consecutively eluted with 5 mM and next with 100 mM (arrow A) sodium phosphate buffer, pH 7.0, containing 1 mM EDTA·Na₄, and 1 M NaCl (arrow B). Fractions of 4.5 ml were collected.

0.25 to 1 mM. They were analyzed by Lineweaver-Burk plots. The GST inhibitors were tested as described (21).

RESULTS

The specific GST activity in PLC/PRF/5 cells is 442 ± 41 nmol/min/mg protein. A sufficiently large quantity of cells was obtained after cultivation in a cell factory with a total growth surface of 6,000 cm². The 100,000 g supernatant of the cell extract was first enriched by GSH affinity chromatography (Fig. 1). GST was quantitatively bound to the matrix, since no trace of GST activity was observed in the flow-through fractions, and it was quantitatively desorbed with 15 mM GSH, since further elution with 3 M NaCl did

not further elute any GST activity.

The GST fractions which were eluted with 15 mM GSH from the affinity matrix were applied on a carboxymethyl cellulose column (Fig. 2). Anionic GST passed quantitatively through the column with the low ion-strength equilibration buffer of 5 mM sodium phosphate, pH 7.0, containing 1 mM EDTA·Na₄. None of both higher ion-strength elution solutions did further desorb any GST activity. This means that the PLC/PRF/5 GST is exclusively composed of anionic GST. Part (10%) of this purified GST was used for kinetic and inhibition experiments. The other part was further purified by anionic chromatofocusing (Fig. 3). Again, only one single GST peak was observed, eluting at pH 4.4. The SDS-polyacrylamide slab gel electrophoretogram

Fig. 3 Anionic chromatofocusing of PLC/PRF/5 GST on a Polybuffer exchanger 94 column. The column, equilibrated with 25 mM imidazole-HCl, pH 7.4, was developed with Polybuffer 74, diluted 1/16, and adjusted to pH 4.0 with HCl. Fractions of 3 ml were collected.

gram of PLC/PRF/5 GST is shown in Fig. 4. The Coomassie blue stained gel showed only one band with a M_r of 26,000. This indicates that PLC/PRF/5 GST is a homodimer, since GST enzymes have a dimeric structure (14).

Some kinetic parameters of the PLC/PRF/5 GST are summarized in Table 1. The apparently pure GST has a very high specific activity. Linear Lineweaver-Burk plots ($r > 0.99$) were always observed in the kinetic experiments. The PLC/PRF/5 GST was further characterized by measuring the influence of a set of discriminating GST inhibitors (Table 2). Gossypol acetic acid had the least inhibiting effect, while hematin was the strongest inhibitor.

DISCUSSION

Our results show that PLC/PRF/5 cells have only one GST isoenzyme, since the GST activity was always found in a single peak, without any trace of activity in other fractions. This situation sharply contrasts with different biological tissues and cells, where several isoenzymes are found (14). At least 15 different GST isoenzymes were demonstrated in human liver, although not all forms were detectable in all liver sample (24). The multiplicity of GST isoenzymes is assumed to result from the need to conjugate numerous types of substances differing in the nature of their electrophilic centre and their molecular struc-

Fig. 4 SDS-polyacrylamide electrophoretic gel showing one subunit for PLC/PRF/5 GST. Lane A: PLC/PRF/5 GST, lane B: standard proteins

ture (9). Therefore, the investigated cell line can by no means be considered as a valuable alternative model for the study of GST-dependent interactions in the human liver, although the cells have many other hepatocyte characteristics (10, 12, 13).

Another important difference is that the majority of the human liver GSTs are cationic forms (24), whereas the PLC/PRF/5 GST is only found in one single anionic peak, with pI 4.4 (Fig. 3). A human anionic GST was first isolated from erythrocytes (15), and later on from many different tissues as placenta, lung, kidney, and lens. These proteins correspond to the same enzyme and are now designated as GST π (14), which shows very close homology with GST 7-7 from the rat (8).

The GST isolated from PLC/PRF/5 cells can be identified as GST π on the basis of the following criteria. The pI (Fig. 3) and the K_m values (Table 1) correspond with those of GST π (5). The homodimeric structure with two identical subunits of M_r 26,000 (Fig. 4) agrees with that of GST π (20), although other authors reported a slightly lower M_r (5, 14). Finally, the IC_{50} values from Table 2 corre-

Table 1 *Some Kinetic Parameters of PLC/PRF/5 Glutathione S-Transferase*

Specific activity ^a	114 $\mu\text{mol}/\text{min}/\text{mg}$ protein
Apparent K_m towards CDNB ^b	2.19 mM
Apparent V_{max} towards CDNB ^b	118.5 $\mu\text{mol}/\text{min}/\text{mg}$ protein
Apparent K_m towards GSH ^c	0.17 mM
Apparent V_{max} towards GSH ^c	66.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein

^aMeasured with both CDNB and GSH at 1 mM. ^bMeasured at a fixed concentration of 1 mM GSH. ^cMeasured at a fixed concentration of 1 mM CDNB

Table 2 *Inhibition Parameters for PLC/PRF/5 Glutathione S-Transferase*

Inhibitors	IC_{50} (μM)
Gossypol acetic acid	460
Triphenyltin chloride	13
Bromosulphophthalein	49
Hematin	5
S-Hexylglutathione	19

The IC_{50} is the concentration of inhibitor giving 50% inhibition of enzyme activity, measured with GSH and CDNB at 1 mM.

spond very well to those of GST π (21). They exclude the possibility that another previously described GST isoenzyme, other than GST π , was isolated from PLC/PRF/5 cells. No trace of other GST activity containing peaks was detected during the different purification steps (Figs. 1-3), although usually at least a small part of GST activity freely passes the affinity matrix (24). Therefore it is concluded that the GST in the investigated hepatoma derived PLC/PRF/5 cell line is entirely composed of GST π . Although GST π is reported as the major GST isoenzyme in different cell lines (17), no established cell line has been described, to the best of my knowledge, in which GST π is the sole GST isoenzyme.

GST π (and its rat homologue GST 7-7) are now generally considered as a tumor marker in a variety of tissues. GST π content is higher in human hepatic tumors than in surrounding non-tumor tissue, in normal liver, or in liver tissue of patients with liver diseases other than hepatic tumors (20). It is also the major isoenzyme in a variety of human tumors from different tissues (17). GST 7-7 has become established as one of the best markers for early detection of putative preneoplastic and neo-

plastic cells in rat chemical hepatocarcinogenesis (16). It was introduced as a marker for analysis of carcinogenic processes and for screening of carcinogens and carcinogen modifiers (7, 16).

In conclusion, the human hepatoma derived PLC/PRF/5 cells cannot be considered as an alternative model for intact human liver. However, being derived from an hepatoma, they contain exclusively GST π , specific for tumors. Therefore they are a promising source for the study of specific GST π -dependent metabolic processes, for example in relation to cancer.

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