Glutathione S-transferases from rainbow trout liver and freshly isolated hepatocytes: purification and characterization

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Received 15 February 2000; received in revised form 15 October 2000; accepted 1 November 2000

Abstract

Glutathione S-transferases (GST) form an important family of biotransformation enzymes catalyzing the conjugation of glutathione to a great variety of xenobiotic compounds. The objective of this study was to compare the different characteristics of GST from freshly isolated rainbow trout hepatocytes with those corresponding to the total liver of the same fish, in order to establish the similarities. GST was purified by affinity chromatography and enzymatic activity was determined towards two substrates, 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (ETHA). The different isoenzymes were determined by HPLC associated with SDS-PAGE. Slight differences between the samples were obtained when the results corresponding to the enzyme activity were compared. HPLC results showed that all GST isoforms present in the total liver samples were represented in the isolated cells too, corresponding to isoforms with molecular masses of approximately 25.5 and 23.0 kDa.

Keywords: Glutathione S-transferases; Hepatocytes; Liver; Rainbow trout; Isoenzymes

1. Introduction

Freshwater appears to be contaminated with a great variety of inorganic and organic pollutants, possibly affecting the integrity of ecosystems, and the physiological functions of individual organisms. The impact of a number of contaminants on aquatic ecosystems can be assessed by the measurement of their external levels in the surrounding water or sediments, or by determining some biochemical parameters in fish and other organisms that respond specifically to the degree and type of contamination (Petčůvalský et al., 1997). One of these parameters may be the xenobiotic metabolizing enzymes, which biotransform different toxic agents to water-soluble products. Because of the inducibility of some of these biotransformation enzymes by various xenobiotics,
there has been a great effort to determine their usefulness in environmental monitoring. In vivo experiences have shown that these enzymes may be induced by various xenobiotics (Van der Oost et al., 1996), and freshly isolated liver cells, which account for up to 80% of the total liver volume (Hampton et al., 1989), are also expected to be used as a valuable model to study cellular regulation of metabolizing enzymes in fish (Masfaraud et al., 1992). Although in vitro systems are not substitutes for in vivo studies, they can be useful adjunct models to whole animal experiments in vivo and to ecotoxicological evaluation of the potential risk of aquatic pollutants (Pesonen and Andersson, 1997).

In vitro experiences with highly differentiated cells which possess a variety of mechanisms for xenobiotic metabolism offer a considerable advantage in terms of time, expense and reduction of animal experimentation, and because studies can be carried out under strictly controlled conditions (Carrera et al., 1998). Furthermore, a complete intracellular organization is maintained in isolated cells, and thus toxic metabolism reactions can be studied in relation to other cellular events (Pesonen and Andersson, 1991; Carrera et al., 1992). But to be truly representative when using this model, comparisons with the intact organ must be developed, because, for example, cytochrome P450 activities decline during the culture period when mammalian cells are studied (Maher, 1988; Donato et al., 1992).

Glutathione S-transferases form a family of multifunctional phase II biotransformation enzymes, present in the cytosol of most cells, catalyzing the conjugation of the tripeptide glutathione to a variety of compounds with an electrophilic group (George and Buchanan, 1990). These enzymes have been assigned to four separate classes, designated alpha, mu, pi and theta on the basis of primary structure, substrate specificity and immunological properties. The individual glutathione transferase isozymes exhibit different substrate specificity, rendering them suitable to different defence tasks (Mannervik and Danielsson, 1988). In fish and other marine animals, a great variety of compounds may be detoxified by this family of enzymes. Although different studies on cytochrome P450 in isolated hepatocytes of rainbow trout have been developed to establish the potential of this enzymatic system as a predictive biomarker for use in water pollution monitoring (Goksøyr, 1995), less information is available about other enzymatic pathways. In this sense, it has been observed that in primary cultures of hepatocytes, conjugating enzyme activities generally remain close to the levels in freshly isolated cells for 5 days (Pesonen and Andersson, 1991; Jensen et al., 1996).

The aim of the present work was to purify and characterize the different cytosolic glutathione S-transferases from freshly isolated hepatocytes of immature rainbow trout, and to compare these results with those present in the whole liver, where different cell populations are present, in order to determine if the enzymatic forms from isolated cells of rainbow trout may provide a useful system in toxicological research.

2. Materials and methods

2.1. Preparation of isolated hepatocytes

Isolated cells were obtained according to the general method described by Maitre et al. (1986), with slight modifications. Sexually immature rainbow trout (Oncorhynchus mykiss) weighing 200–250 g were maintained in 250-l tanks at 16°C under constant aeration. Animals were anaesthetized with 3-aminobenzonic acid ethyl ester (Sigma). After a few minutes, fish were removed from the solution and placed on an operating table. A ventral incision was made along the medial line. The portal vein was exposed and cannulated after placing a ligature around the vein 1–2 cm from the liver. The cannula, of 0.8 mm diameter, was introduced and ligated.

The liver was perfused using a peristaltic pump and a bubble trap at a flow rate of 15 ml min⁻¹ for 20 min. This first step cleared all the blood with a calcium-free solution A, containing Hepses 11.76 mM, NaCl 160.8 mM, KCl 3.15 mM and Na₂HPO₄ 0.33 mM, pH 7.65, maintained at a constant temperature of 20°C. The heart was cut, to avoid high pressure in the system. EGTA (0.5 mM) was added to solution A, and perfusion was continued at the same flow rate for 10 min, chelating the calcium in the medium. After that, solution A was perfused to the liver for a further 10 min. The perfusion buffer was then replaced with a collagenase solution (solution A supplemented with CaCl₂ 6.67 mM and collagenase 0.5 mg ml⁻¹), for 20 min, at a flow rate of 5 ml
min⁻¹. This solution was maintained at 20 ± 3°C. The liver was then carefully removed and floated in a covered beaker containing filter sterilized solution (A). The gall bladder was carefully removed, and hepatic cells were dispersed. The cell pellet was filtered through a 100-μm filter to allow viable cells to sediment while debris remained in suspension, after centrifuging at 50 × g for 3 min. The cell pellet was resuspended in 15 ml MEM medium added with Hanks’ salts, and submitted to a second centrifugation as before. A constant volume of 25 ml of MEM was added to the cell pellet, and cell viability was evaluated by the trypan blue exclusion test (Jauregui et al., 1981). Preparations with an initial viability of less than 85% were discarded.

2.2. Cytosolic preparation

Cytosolic fractions were obtained from sonicated isolated hepatocytes (for 2 min) or homogenized liver, by using a 25 mM Hepes, 1.25 mM EDTA buffer, pH 7.6. Samples were first centrifuged at 11000 × g for 20 min, and the supernatant was submitted to another centrifugation at 105000 × g for 1 h. This last supernatant represented the cytosolic fraction of the samples, and was purified according to the method described by Pérez-López et al. (1998). Cytosolic samples were run through a Sephadex G-25 column (4 × 25 cm) equilibrated with 20 mM Tris, 1 mM EDTA, 0.2 mM dithiothreitol, pH 7.8 solution. These partially purified and equilibrated fractions were loaded on a GSH-agarose affinity gel (Sigma), and equilibrated with the same buffer. After extensive washing, always with this 20 mM Tris–1 mM EDTA solution, the retained material was eluted with a 200-mM Tris, 10 mM GSH, pH 9.2. The presence of the different fractions containing cytosolic GST was determined according to the general method described by Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrinic acid (ETHA) as substrates. Protein determinations were carried out using bovine serum albumin as a standard according to Schaffner and Weissmann (1973), due to interferences when using the method of Lowry et al. (1951).

Separation of the different GST subunits was carried out on the retained affinity fractions by reversed-phase HPLC on a Vydac 218TP54 C18 column (4.6 × 250 mm), using mixtures of water and acetonitrile, containing 0.1% trifluoroacetic acid, as mobile phases (A, 3/7; B, 7/3, v/v). A protein amount of 0.017–0.019 mg (1000 μl of the affinity retained fraction) from both hepatocytes and whole liver affinity-retained subcellular fraction was loaded into the system. Elution was performed at a constant flow rate of 1 ml min⁻¹ using successive linear gradients of B in A: 25–30% in 5 min, 30–34% in 7 min, 34–60% in 13 min, 60–100% in 5 min, and isocratic 100% for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970) to determine the molecular mass of the enzymatic subunits present in the retained affinity fractions. A 50-μl portion of the affinity retained fraction (0.0008–0.0009 mg of protein) was subjected to electrophoresis and compared with molecular markers.

3. Results

The two-step collagenase perfusion technique employed in this study yielded an average viability of the isolated hepatocytes greater than 90%. Table 1 shows the results obtained in the determination of the protein content and the GST cytosolic activity towards CDNB and ETHA in three different isolations, and compared with those assayed in trout liver. The interest in employing two different substrates to determine the GST enzymatic activity lies in the fact that GST isoenzymes have specific abilities to conjugate GSH with various electrophiles, and the use of various specific substrates to determine the GST isoenzymes activities might be an adequate tool for the recognition of xenobiotic-specific effects.

The total protein content was lower in isolated cells when compared with the whole organ preparation, not only in the cytosolic fraction, but also in the fraction retained on the affinity column. An average of 3.6% of protein was retained in the affinity fraction from isolated hepatocytes samples, whereas only 0.74% was quantified in the liver sample. This different % was probably due to the method when obtaining the isolated cells, because most of the subcellular components were eliminated when isolating the hepatocytes, not interfering with the final protein determination, and rendering the final protein percentage higher.

Total enzyme activity towards CDNB showed clear differences between biological systems: while only 9.80–11.56 units of total activity were recov-
Table 1
Purification procedures and quantitative data corresponding to three different cell isolations and whole liver from rainbow trout: protein content and GST enzymatic activities

<table>
<thead>
<tr>
<th>Sample</th>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Proteins mg</th>
<th>CDNB activity</th>
<th>ETHA activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>Total units a</td>
<td>Specific c,d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>Specific</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>units b</td>
<td></td>
</tr>
<tr>
<td>800 × 10⁶ cells</td>
<td>Cytosol</td>
<td>3.70</td>
<td>9.93 ± 0.08</td>
<td>100.00</td>
<td>11.41</td>
</tr>
<tr>
<td></td>
<td>Sephadex G25</td>
<td>13.50</td>
<td>8.78 ± 0.09</td>
<td>88.42</td>
<td>10.13</td>
</tr>
<tr>
<td></td>
<td>Unretained affinity</td>
<td>23.50</td>
<td>N.D.</td>
<td>100.00</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>Retained affinity</td>
<td>15.20</td>
<td>0.26 ± 0.04</td>
<td>2.62</td>
<td>8.16</td>
</tr>
<tr>
<td>660 × 10⁶ cells</td>
<td>Cytosol</td>
<td>4.00</td>
<td>6.43 ± 0.11</td>
<td>100.00</td>
<td>9.80</td>
</tr>
<tr>
<td></td>
<td>Sephadex G25</td>
<td>10.80</td>
<td>5.43 ± 0.09</td>
<td>84.45</td>
<td>8.55</td>
</tr>
<tr>
<td></td>
<td>Unretained affinity</td>
<td>22.00</td>
<td>N.D.</td>
<td>100.00</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Retained affinity</td>
<td>13.50</td>
<td>0.23 ± 0.05</td>
<td>3.58</td>
<td>7.26</td>
</tr>
<tr>
<td>500 × 10⁶ cells</td>
<td>Cytosol</td>
<td>3.70</td>
<td>5.84 ± 0.07</td>
<td>100.00</td>
<td>11.56</td>
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<td></td>
<td>Sephadex G25</td>
<td>10.80</td>
<td>5.11 ± 0.07</td>
<td>87.50</td>
<td>10.80</td>
</tr>
<tr>
<td></td>
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<td>N.D.</td>
<td>100.00</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>Retained affinity</td>
<td>15.20</td>
<td>0.26 ± 0.04</td>
<td>4.45</td>
<td>7.60</td>
</tr>
<tr>
<td>Liver</td>
<td>Cytosol</td>
<td>5.00</td>
<td>106.33 ± 7.23</td>
<td>100.00</td>
<td>67.20</td>
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<td></td>
<td>Sephadex G25</td>
<td>14.40</td>
<td>96.13 ± 7.17</td>
<td>87.59</td>
<td>64.00</td>
</tr>
<tr>
<td></td>
<td>Unretained affinity</td>
<td>26.00</td>
<td>N.D.</td>
<td>100.00</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td>Retained affinity</td>
<td>40.80</td>
<td>0.79 ± 0.05</td>
<td>0.74</td>
<td>60.97</td>
</tr>
</tbody>
</table>

a Values are mean ± S.D.
b One unit of enzyme activity: 1 μmol CBNB/ETHA conjugated min⁻¹.
c Units of total activity per mg of protein.
d N.D., not determined.

HPLC results of a constant injection volume (corresponding to a protein amount of approx. 0.017–0.019 mg) from both purified affinity retained fractions showed important similarities between the sample profiles. Five major isoforms were isolated, with highly reproducible retention times of 14, 19, 21, 25 and 26 min, respectively (Fig. 1). These similar results allow the determination that hepatocytes are the cells mostly responsible from the GST subunit composition in the total liver of rainbow trout, although non-parenchymal cells, which are eliminated during hepatocyte isolation, have been demonstrated to possess some post-oxidative enzyme activity (as, for example, glutathione S-transferases). This fact could explain the relative lower importance of peaks identified as 4 and 5, which are only slightly represented in the HPLC profile corresponding to the affinity retained fraction from the hepatocytes, whereas they are clearly observed in the whole liver, where different non-parenchymal cells are present. The main form, according to the general HPLC profile, corresponded in both cases.
Fig. 1. Reversed-phase HPLC profile of: (a) rainbow trout hepatic cytosolic GST subunits from affinity-retained pool; compared with (b) freshly isolated hepatocytes GST subunits. In both cases, 1000 µl of sample (0.017–0.019 mg of protein) was loaded into the system.

to the GST isoform identified as number 1, with a retention time of 14 min.

Fig. 2 shows the results for SDS-PAGE of affinity-purified glutathione S-transferases from freshly isolated rainbow trout hepatocytes, which always gave two distinct and highly reproducible bands, with apparent masses of 23000 and 25500 Da, respectively. Results are presented associated with a typical run from the cytosolic fraction of freshly isolated hepatocytes, where different bands corresponding to the cytosolic components are presented. These results indicated a high degree of purification of the GST enzymes. No results corresponding to the whole liver samples are presented, because no important differences were observed when comparing them with those obtained from the isolated hepatocytes.

4. Discussion

Our results showed that cytosolic GST isoforms from freshly isolated rainbow trout hepatocytes are comparable to those reported from the in vivo experience, where the whole organ is used, and in which case other cellular populations could pre-
sent different GST isoenzymes, interfering with the final determination. This fact represents an important finding, because in some species, a cell-related expression of different GST isoenzymes has been observed, showing the importance of an adequate isolation and characterization of these isoenzymes, because they may yield much higher variations than the global enzymatic activity, helping to find better biomarkers of contamination (Martínez-Lara et al. 1996). For example, it has been established that the major hepatic isoform in plaice, Pleuronectes platessa, has a well-defined cell-specific expression, and it is confined to the hepatocytes, not to other hepatic cells (George, 1994). In this sense, clear differences between individual GST isoenzymes with respect to their organ distribution (and also to the cellular population) have been demonstrated, directly related to a specific induction pattern towards xenobiotics (Sippel et al., 1996). This fact could explain why various GST subunits, with different expression levels and substrate affinity profiles, are present in specific cellular types, and have been developed to meet the requirements for broad coverage against various dangerous exotoxins. Similarly, a clear age- and maturation-dependent effect on antioxidant enzymes, such as GST, in various tissues of different animal species has been established, and these effects must be determined for the use of oxidative parameters as indicators of contaminant exposure in environmental studies (Otto and Moon, 1996). This fact explains the importance of working with sexually immature animals, in order to avoid this enzymatic variation, and because in salmonid species, several changes observed in GST activity indicate a weakening of these endogenous antioxidant defenses.

Steinberg et al. (1989), working on isolated rat liver parenchymal, Kupffer and endothelial cells, observed that all glutathione S-transferases isoenzymes present in the sinusoidal lining cells were also detected in the parenchymal cells, whereas Kupffer and endothelial cells lacked several GST isoenzymes present in hepatocytes. These authors showed that oxidative and post-oxidative drug-metabolizing enzymes such as GST in rat liver were not restricted to parenchymal cells, in spite the fact that GST enzymatic activity in hepatocytes was approximately 2.7-fold higher than in Kupffer and endothelial cells (using the CDNB as substrate). It must be noted that some GST isoenzymes isolated and identified in parenchymal cells were not observed in Kupffer and endothelial cells. As previously reported in Section 3, these results indicate the importance of an adequate characterization of the GST subunits from rainbow trout freshly isolated hepatocytes, in order to eliminate the interference of the non-parenchymal cells when isolating the cytosolic GST from the total liver.

In contrast, Vandenberghe et al. (1988) re-
ported increased levels of GST subunit 7 from rat hepatocytes after culture for 4 days, after absence in the whole liver, and changes in the GST isoenzyme pattern have been observed when comparing total liver, freshly isolated and cultured rat hepatocytes (Rogiers et al., 1990). Nevertheless, Hatayama et al. (1991) demonstrated that the placental form GST-P (GST 7-7), the only GST of the pi family present in rats, was undetectable in normal rat hepatocytes, but was detected at high levels in preneoplastic hepatocytes, associated with the presence of epidermal growth factor or insulin in the added serum. Although all these results were obtained for mammalian liver, results are interesting, because in our work, hepatocytes and whole liver samples showed great similarities in their GST subunit composition.

GST specific activity towards CDNB in freshly isolated hepatocytes was significantly higher than results obtained in primary cultures, where a specific activity of 0.10 μmol min⁻¹ mg⁻¹ of protein was determined in the cytosolic fraction (Pesonen et al., 1989). The enzymatic activity decreased gradually from the initial value found in the freshly isolated cells and after 5 days in culture, GST specific activity was 0.079 μmol min⁻¹ mg⁻¹ protein. Laurén et al. (1989) determined in whole rainbow trout liver that GST cytosolic specific activity towards CDNB was 0.348 μmol min⁻¹ mg⁻¹ protein, clearly lower than our results in isolated cells, and in the whole organ (Table 1).

Jensen et al. (1996) measured different enzyme activities in primary monolayer cultures of rainbow trout hepatocytes. They established that GST specific activity was approximately 1.0–1.5 μmol min⁻¹ mg⁻¹ protein, quite constant during the experiment (0–144 h in primary culture), at two different acclimatization temperatures, with only slight differences between individual fish. This investigation concluded that cultures of rainbow trout hepatocytes could be obtained with stable activities of different biotransformation enzymes for 6–7 days. Jensen et al. (1996) also established a ratio of 3 for in vivo/in vitro specific activity, while we found a corresponding ratio of approximately 2. Similarly, GST activity in a primary culture of rainbow trout hepatocytes was stable for 120 h after cell isolation, with an average value of 87.5 nmol min⁻¹ mg⁻¹ protein, allowing us to conclude that trout liver cell culture provides a reliable model for studying the metabolic fate of xenobiotics and the balance between biotransformation enzymes (Pesonen and Andersson, 1991).

The results corresponding to the electrophoresis profile are consistent with those previously presented by Pérez-López et al. (1998), where electrospray-ionization mass spectrometry was developed to determine the molecular mass of each enzymatic subunit from rainbow trout liver cytosol, demonstrating that the major GST isoform was related to a pi-class (23385 ± 2 Da). In this sense, they are in accordance with the results obtained by Ramage and Nimmo (1984), who determined that the major isoforms of cytosolic GST from rainbow trout liver possessed a molecular mass of approximately 22,400 and 24,500 Da. Sugiyama et al. (1981) established that the major isoform present in elasmobranch liver corresponded to a subunit with a molecular mass of 24,000 Da, showing the importance of this GST family in many groups of fish.

Finally, the induction of GST in organisms by exposure to certain xenobiotics is a process used for adapting to changes in the environment, and in this sense freshly isolated hepatocytes systems obtained from rainbow trout seem to be an adequate model for analyzing the biotransformation of xenobiotics in ecotoxicology studies. In this sense, the perfect knowledge of these cytosolic enzymes is absolutely necessary for the development of different in vitro studies, because not all GST subunits are affected to the same extent by drugs or environmental pollutants. Comparative studies of the expression and function of piscine biotransformation systems are likely to be fruitful for advancing understanding of toxicological mechanisms.

Acknowledgements

The authors wish to thank Xunta de Galicia, who financially supported this study by project PGIDT99PX126101B.

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