Hepatic glutathione S-transferases from lamprey (Petromyzon marinus): purification and characterization

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Received 22 January 2003; accepted 23 April 2003

Abstract

Glutathione S-transferases constitute a very important family of biotransformation enzymes, which catalyse the conjugation of glutathione to a broad spectrum of xenobiotics. In this study, cytosolic glutathione S-transferases enzymes were purified from lamprey (Petromyzon marinus) liver, using an affinity chromatography method. Enzymatic activity was determined towards 1-chloro-2,4-dinitrobenzene, ethacrynic acid and 1,2-dichloro-4-nitrobenzene. The application of a HPLC system associated to electrospray ionization mass spectrometry allowed the identification of three different subunits, with Mrs between 22,300 and 25,300 Da. The one with the low Mrs was the main form, with a retention time of 29.5 min, a pi-related class isoenzyme.

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Keywords: Enzyme; Glutathione S-transferase; Lamprey; Liver; Purification; Characterization

1. Introduction

Pollution of aquatic environments by chemicals is widespread, possibly affecting the biological integrity of ecosystems as well as the physiological functions of individual organisms. Exposure of piscine organisms to pollutants has been typically...
determined by measuring external levels of contaminants in the surrounding water or sediments, but this determination is considered insufficient in order to establish the quality of these ecosystems, and it is often unable to predict toxicological consequences (Monod et al., 1988). To clearly assess the quality of the aquatic environment, the impact of contaminants in terms of biochemical responses reflecting their potential for impairment of physiological processes in different organisms has been examined (Everaarts et al., 1994). Research on effective biomarkers is a very relevant point of interest. A biomarker can be defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioural changes), which can be related to an exposure or a toxic effect of environmental chemicals, representing a specific answer to the degree and type of contamination (Petřivalský et al., 1997; van-Welie et al., 1992). There are many different parameters that can be used as markers of environmental contamination, the presence of an inductive or inhibitory process of phase I (monooxygenases) and II (conjugation) biotransformation enzymes could be an effective and sensitive biomarker of aquatic pollution (Roy et al., 1995; Vidal et al., 2002). Their study can help to understand the chemical toxicity at the individual, population or community levels in aquatic ecosystems (Malins and Ostrander, 1994).

Glutathione S-transferases (GST) constitute a multifunctional family of dimeric and mainly cytosolic biotransformation enzymes which play an important role in protecting tissues from oxidative stress (Yang et al., 2002). They catalyse the conjugation of intracellular glutathione (GSH: γ-Glu-Cys-Gly) with a great variety of chemicals possessing electrophilic centres (Dierickx, 1985), and the final GSH-conjugates have increased hydrophilicity, which facilitates their further metabolism and elimination (Rouimi et al., 1996). On the basis of protein sequence, substrate specificity and immunological properties, they have been classified into seven separate classes, identified as Alpha, Mu, Pi, Theta, Kappa and Zeta (Board et al., 1997). They have been extensively studied and characterized in different species (principally mammalian, like rat, mouse and human) as dimeric proteins (Alin et al., 1985), composed of subunits of about 24,000–28,000 Da, which dimerize by non-covalent interactions with other subunits of its class.

Hepatic and extrahepatic GST have been purified in different piscine species and the activity towards the model substrate CDNB has been quantified in all the elasmobranch and teleost fish species examined (Nimmo, 1987), although, in general, little information is available on the molecular characterization and role of piscine GST isoenzymes (Pérez-López et al., 2000). The GST expression levels in many species can be significantly increased by exposure to foreign compounds, suggesting that they could form part of an adaptive response to chemical stress, which could determine their availability to be used as effective biomarkers of aquatic contamination (Van der Oost et al., 1996; Nóvoa-Valiñas et al., 2002). It must be considered that development of diagnostic and predictive molecular biomarkers for their use in pollution monitoring depends on a fundamental characterization of these molecules (Goksøyr, 1995; Egaas et al., 1999). In this sense, molecular and biochemical approaches have been used here in order to purify and characterize the different GST
isoforms from lamprey liver cytosol, as an initial step to establish their use as a biomarker in ecotoxicology studies.

2. Material and methods

Lampreys weighing an average of 350 g were collected from the Miño river (Galicia, NW Spain), near its outlet. Sampled animals were transported to the laboratory in aerated polyethylene tanks of 100 l. Fish were anaesthetized using 3-aminobenzoic acid ethyl ester (MS-222, Sigma), the gall bladder was removed and the liver was then isolated. In order to avoid individual differences, the same organs of three different animals were pooled. Samples were stored at −80 °C until analyses were made. All subsequent purification steps were carried out at 4 °C.

The preparation and purification process of the subcellular cytosolic fraction was performed according to the method of Rouimi et al. (1996). Liver samples were weighed and homogenized into four times their volume of 25 mM Tris/HCl, 1.25 mM EDTA and 1 mM dithiothreitol (DTT) buffer (pH 7.6). The homogenate was centrifuged at 11,000 rpm for 20 min, and the resulting supernatant was further centrifuged at 34,000 rpm for 1 h. This last supernatant represented the cytosolic fraction of the isolated organ. These samples were applied to a column (4 × 25 cm) packed with a Sephadex G-25 gel, and eluted with 100 ml of 20 mM Tris/HCl, 1 mM EDTA, 0.2 mM dithioerythritol (DTE) buffer (pH 7.8). These partially purified fractions (identified as pool Sephadex G25) were applied to a GSH-agarose affinity column (1 × 10 cm), equilibrated and extensively washed with the same Tris–EDTA buffer. Finally, the bound cytosolic GST were eluted from this second column with 200 mM Tris/HCl, 10 mM GSH buffer (pH 9.2).

GST activity was assayed with CDNB (40 mM), ETHA (1 mM) and DCNB (40 mM) as substrates, by the method of Habig et al. (1974) in a thermostated (25 °C) Beckman DU 640 Spectrophotometer. The wavelength used to determine the different subcellular cytosolic fractions with GST activity were, respectively, 340, 270 and 345 nm for the three assayed substrates. The fractions resulting from the affinity column with GST activity were pooled and named as retained affinity pool. The fractions without GST activity, initially eluted from this affinity column, were collected and named as unretained affinity pool.

Protein concentrations were determined according to the general method described by Schaffner and Weissmann (1973) based on the staining of the fixed proteins by means of the Naphtol Blue Black (Sigma), and using bovine serum albumin (BSA) as a calibration standard.

An aliquot of the retained affinity purified fractions was subjected to reversed-phase HPLC (Waters, Beckman) using a Vydac 218TP54 C18 column (4.6 × 250 mm), and working at 214 nm. The mobile phases consisted of two mixtures of acetonitrile and water (A: 3/7; B: 7/3, v/v), containing 0.1% trifluoroacetic acid. Elution was performed at a constant flow rate of 1 ml/min, and the gradient consisted of a mixture of buffer B in A: 25–30% at 5 min, 30–34% at 7 min, 34–60% at 13 min, 60–100% at 5 min, and isocratic 100% for 10 min.
Molecular weight of GST subunits was determined by means of electrospray ionization mass spectrometry, as described by Rouimi et al. (1995) on a HP 1100 MSD mass spectrometer. Analyses were performed coupling the HPLC to the mass spectrometry using the electrospray in positive mode as ionization source. For the chromatographic separation, the same reversed-phase column indicated earlier has been used. The instrument was scanned over a mass range of $m/z$ 700–1800, and a solution of myoglobin was used for the calibration. Molecular mass determination of each enzymatic subunit was deduced from at least three different measurements.

3. Results

Table 1 shows the results corresponding to the protein content and the GST enzymatic activity determination in the different subcellular purified fractions from lam-prey liver. GST activity was determined against three substrates: CDNB, ETHA and DCNB. Except for the initial cytosolic fraction, GST activity was found to decrease dramatically after freezing and thawing or a few hours after keeping at 4 °C, thus requiring measurements on freshly isolated fractions.

The evolution of the protein concentration in each fraction indicates that the procedure was appropriate, with a recovery of 0.31% of total cytosolic conserved in the final retained affinity fraction.

GST enzymatic activities were expressed in terms of units of total activity (µmol of substrate conjugated per minute), and in terms of specific activity (units of total activity per mg of protein). A real value was considered as the difference between the obtained result in the determination of the activities from each sample and towards a blank determined in the same experimental conditions. The GSH-Sepharose matrix retained over 80% of the GST activity from liver cytosol, when measured with both CDNB and ETHA. The existence of a 14–16% of GST activity which did not fix to the affinity column matrix could be explained because certain GST (as for example some alpha or theta isoenzymes) are poorly linked to these columns (Hayes and Pulford, 1995; Rouimi et al., 1996). In general, higher activity values were observed for the CDNB than for ETHA.

Fig. 1 shows a typical HPLC profile corresponding to the injection of 500 µl of retained affinity purified samples. Three major peaks of GST subunits were separated, with retention times of 17.5, 28 and 29.5 min (identified from 1 to 3). The peak with a retention time of 29.5 min was the main one, but the two last subunits were eluted with a certain overlapping, that was not possible to resolve with other assay conditions.

Table 2 shows the molecular masses determined for the three isolated GST subunits. Peaks 1 and 2, respectively, showed one single mass value, whereas in peak 3, two distinct mass values were obtained, differing by 131 Da. As observed previously with similar studies with cytosolic GST from salmonid fish (Nóvoa-Valiñas et al., 2002), the presence of a methionine residue associated to the N-terminal sequence could be hypothesized, and explain the existence of these two masses. Specific advances on amino acid sequence determination should be realized to clearly establish this fact.
Table 1
Purification procedure and quantitative data corresponding to lamprey liver samples. Protein content and GST enzymatic activities towards three different substrates (CDNB, ETHA and DCNB)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytosol</th>
<th>Sephadex G-25</th>
<th>Unretained affinity</th>
<th>Retained affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>2.5</td>
<td>9.3</td>
<td>15.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>25.84 ± 5.01</td>
<td>6.58 ± 0.97</td>
<td>–</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>CDNB Totala</td>
<td>26.00 ± 4.98</td>
<td>17.79 ± 3.08</td>
<td>2.56 ± 0.67</td>
<td>14.54 ± 4.60</td>
</tr>
<tr>
<td>% Specificb</td>
<td>–</td>
<td>100</td>
<td>14.39</td>
<td>81.73</td>
</tr>
<tr>
<td>Yield</td>
<td>0.40 ± 0.09</td>
<td>0.29 ± 0.03</td>
<td>n.d.</td>
<td>17.31 ± 4.40</td>
</tr>
<tr>
<td>ETHA Totala</td>
<td>2.50 ± 0.13</td>
<td>1.24 ± 0.10</td>
<td>0.21 ± 0.09</td>
<td>1.05 ± 0.88</td>
</tr>
<tr>
<td>% Specificb</td>
<td>–</td>
<td>100</td>
<td>16.93</td>
<td>84.67</td>
</tr>
<tr>
<td>Yield</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>n.d.</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>DCNB Totala</td>
<td>0.35 ± 1.06</td>
<td>0.02 ± 0.01</td>
<td>0.51</td>
<td>–</td>
</tr>
<tr>
<td>% Specificb</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.42 ± 0.12</td>
</tr>
</tbody>
</table>

n.d.: not determined.

a One unit of enzymatic activity: µmol of substrate conjugated per minute.
b Specific activity: units of total activity per mg of protein.
Fig. 1. Reversed-phase HPLC profile of lamprey hepatic glutathione S-transferase subunits (identified as 1–3) from retained affinity pool. 500 ml of sample were loaded into the system.

Table 2
Molecular mass determination by electrospray ionization mass spectrometry of cytosolic GST subunits from lamprey liver

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Molecular mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25,334 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>25,003 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>22,384 ± 2</td>
</tr>
<tr>
<td></td>
<td>22,514 ± 3</td>
</tr>
</tbody>
</table>

4. Discussion

The present paper describes the purification of liver cytosolic GST subunits from the procordate lamprey using GSH-affinity chromatography, and their partial characterization by a combination of HPLC and mass spectrometry. The advances in the contamination studies of the aquatic ecosystems, submitted to great human stress, represent an important issue. The GST enzymes may have a great importance in order to determine their potential use as biomarkers in different protocols of ecotoxicology to assess chemical pollution of the environment (Vidal et al., 2002; Yang et al., 2002). These enzymes can help to indicate the exposure of piscine organisms in the wild to xenobiotic compounds and to evaluate the degree and risk of environmental contamination.

Protein concentrations were obtained by the Schaffner and Weissmann technique, minimizing the interference effect in all the subcellular fractions, including the
retained affinity fraction. Protein content (in %) in the last fraction corresponds to that found in comparable fractions from plaice, *Pleuronectes platessa* (George and Young, 1988). Similarly, George and Buchanan (1990) determined the amount of protein bound to the affinity gel, representing an average of 0.66% of total proteins from liver cytosol in place. Comparable results were obtained in rainbow trout, *Oncorhynchus mykiss* (Pérez-Lópe et al., 1998). It has been established that this final protein percentage can depend on a great number of factors, such as the biological species, the isolation and purification method, and the protein quantification method (Sugiyama et al., 1981; Rouimi et al., 1996).

The species dependence of the GST activity could be correlated to many physiological factors, such as the type of food ingested and the feeding behaviour. It must be remembered that lamprey is haematophagous, and the basal enzymatic activity could be affected in haematophagous species due to the presence of inductors in the blood of the other fish, which constitute their food, as previously related by Vindimian et al. (1991) for other fish species.

According to the purification process, Stenersen et al. (1987) established that a great variety of animals had GST activity towards CDNB, a minor and variable activity towards ETHA (some species seem to lack this activity) and that the activity towards DCNB was lower in piscine than in terrestrial animals. Moreover, it has been observed that both cartilaginous and bony fish had considerable GST activity with CDNB and not with DCNB (Stenersen and Øien, 1981; Fouremain and Bend, 1984). Undoubtedly, the CDNB has been invariably used as the adequate substrate in studies of modulations of GST activities by environmental pollutants due to their capacity to be used by almost all GST isoenzymes at various rates (Mannervik and Danielson, 1988).

Dominey et al. (1991) reported that the GST amounts in the last fraction of the purification process represented 72%, 82% and 79% of the total cytosolic enzymatic GST activity quantified, respectively, in *Salmo salar, Salmo trutta* and *O. mykiss* liver, and in accordance with those reported at the present work in lamprey liver.

Because of a high non-enzymatic reaction rate, low activity towards ETHA could easily pass unnoticed, whereas high activity was difficult to monitor with precision, because of curved progression lines. Measurements with this substance must, therefore, be regarded as lower-quantitative (Stenersen et al., 1987). In thorny-back shark (*Platyrhinoides triseriata*), in liver cytosol, GST activity with CDNB and ETHA was comparable with that from rat liver cytosol (Sugiyama et al., 1981). George and Buchanan (1990) found similar data, and reported that GST-specific activity towards ETHA was lower than that obtained with CDNB (approx. 1/1000).

Different results show the increased ETHA conjugation as a specific response to some specific contaminants, indicating that the ETHA–GST activity might be a selective biochemical marker of contamination by organic xenobiotics that did not induce CYP1A enzymes (Petřívalský et al., 1997). In this sense, Pérez-Lópe et al. (2002) observed an induction of cytosolic GST after intraperitoneal treatment of Atlantic eel with a commercial mixture of polychlorinated biphenyls. This effect was found with both CDNB and ETHA. According to this fact, research into compounds which are adequate substrates for piscine GST has two aims: to determine if these enzymes
can conjugate xenobiotics of ecological interest and to determine whether the enzymes have broad substrate specificities (Nimmo, 1987).

With respect to the HPLC and mass spectrometry results, Dominey et al. (1991) reported the isolation of two GST isoenzymes in *S. salar* (with retention times of 33 and 39 min) and *S. trutta* (eluting at 33 and 41 min), whereas three peaks were isolated in *O. mykiss*, with retention times of 33, 36 and 44 min. These peaks presented apparent Mr subunit values of 24,800 Da for the two species of the *Salmo* genus, and 25,700 Da for *Oncorhynchus*. On the other hand, Pérez-López et al. (2000) reported that in rainbow trout, cytosolic GST were composed of five different subunits with retention times ranging from 14 to 26 min and Mr values ranging between 23,200 and 26,000 Da, corresponding the main form to a pi-class, results which were confirmed by means of amino acid sequence determination. These results were similar to those in previous works, as subunits isolated in rainbow trout, with Mr values ranging between 21,700 and 24,500 Da (Ramage and Nimmo, 1984). Otherwise, George and Buchanan (1990) using SDS-PAGE showed that *P. platessa* possess GST subunits with Mr values of 25,000, 25,500 and 27,000 Da, appearing to be structurally related to an alpha-class GST.

At last, identification of the HPLC profile of GST subunits seems to constitute a reliable tool for ecotoxicological research, and it would be interesting to determine whether induced detoxification enzymes correlate with the potential capacity of piscine animals to be resistant to polluted water (Yang et al., 2002). As the CDNB conjugating activity is an integration of several GST isoenzymes, some of them can be affected by the quantitative and qualitative xenobiotic characteristics, showing significantly or not significantly induction ratio (Martínez-Lara et al., 1996). These data indicate that GST enzymatic activity towards CDNB is probably not directly usable as biomarker of aquatic pollution. Further research is needed to characterise such isoenzymes, associating HPLC and mass spectrometry characterization studies, as presented here.

Acknowledgements

This work was funded by a grant from the Xunta de Galicia (PGIDT99PX126101B). We are grateful to Ms Medina (Lugo, University of Santiago de Compostela) for her technical support.

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