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Hepatic and branchial glutathione S-transferases of two fish species: Substrate specificity and biotransformation of microcystin-LR

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ABSTRACT

Liver and gills of roach (*Rutilus rutilus*) and silver carp (*Hypophthalmichthys molitrix*) were examined for glutathione S-transferases (GSTs) contents and their substrate specificity and capacity to biotransform microcystin-LR (MC-LR). GSTs and other glutathione (GSH) affine proteins were purified using a GSH-agarose matrix and separated by anionic chromatography (AEC). Substrate specificities were determined photometrical for 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (pNBC) and ethacrynic acid (ETHA). Biotransformation rate of MC-LR was determined by high performance liquid chromatography (HPLC). Roach exhibited different hepatic and branchial GST activities for used substrates (DNB, pNBC and DCNB) compared to silver carp but not for ethacrynic acid. It suggests that, both fish species have similar amount of pi and/or alpha class, which were the dominant GST classes in liver and gills. Gills of both fish species contained a higher number of GST isoenzymes, but with lower activities and ability of MC-LR biotransformation than livers. GST isoenzymes from roach had higher activity to biotransform MC-LR (conversion rate ranging up to 268 ng MC-LR min^{-1} mL^{-1} hepatic enzyme) than that isolated from silver carp. Without any prior contact to MC-LR or another GST inducer, roach seems to be better equipped for microcystin biotransformation than silver carp.

1. Introduction

Eutrophication of water bodies is often followed by excessive growth of phytoplankton. The phytoplankton community can change towards domination by cyanobacteria especially, if N:P ratio and CO_2 availability is low and pH high (Zurawell et al., 2005). Cyanobacterial mass developments (blooms) raise concern due to the potency of several strains e.g. of the genera *Microcystis*, *Planktothrix*, or *Anabaena* of producing bioactive or toxic secondary metabolites (Sivonen and Jones, 1999). One group of these toxins are the cyclic oligopeptide microcystins which are inhibitors of serine threonine protein phosphatases and hepatotoxic to vertebrates (MacKintosh et al., 1990). *Microcystis sp.* may be toxic to fish not only via gastrointestinal ingestion but also by absorption of the toxin microcystin from the water (Wiegand et al., 1999). Like in other vertebrates, microcystins rapidly accumulate in the liver of fish inhibiting the protein phosphatases causing damage by cytoskeletal disorganisation, cell blebbing, and cellular disruption followed by intrahepatic hemorrhage, that may lead to the death of the organism (Gupta et al., 2003).

Elimination of microcystins via biliary excretion has been reported in fish, whereupon metabolization to compounds that are more polar than the parent compound has been detected (Williams et al., 1997). Those compounds proved to be conjugates to tripeptid glutathione (GSH), catalysed by glutathione S-transferases (GST-isoenzymes), and represent a general detoxification pathway for microcystins in aquatic and terrestrial vertebrates, aquatic invertebrates and plants (Pflugmacher et al., 1998, 2001).

GST isoenzymes are major phase II detoxification enzymes found mainly in the cytosol. GST isoenzymes are expressed to varying extent from different species but also in different tissues within the same species. Hepatic GSTs have been purified and partially characterized in relatively few teleosts, including rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), plaice (*Pleuronectes platessa*) and Atlantic salmon (*Salmo salar*) (reviewed by George, 1994). Piscine GSTs have been found especially in liver, but GST activity has been also recorded in other fish tissues, e.g. gills, kidney, intestinal caeca and olfactory epithelium (Pérez-López et al., 2000). Piscine glutathione S- transferase are divided on the basis of their substrate specificities, immunological cross-reactivity and protein sequence data into six classes: alpha (Gallagher et al., 1996 in channel catfish (*Ictalurus punctatus*); Liao et al., 2006 in different carps and Nile tilapia), zeta (Board et al., 1997), theta (Landi, 2000), mu (Gallagher et al., 1996; Pérez-López et al., 2000 in rainbow trout (*O. mykiss*)), pi (Dominey et al., 1991 in salmonid species; Gallagher et al.,

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1996 in channel catfish; Pérez-López et al., 2000 in rainbow trout) and rho (e.g. Liang et al., 2007 in Nile tilapia, silver carp and grass carp). All of these GST classes except that of zeta are present also in common carp (Fu and Xie, 2006).

Silver carp (*H. molitrix*), with its physiological features, the position of the mouth, the specialized branchial system with pharyngeal teeth and the length of the intestine, is considered as a phytoplanktonophagous fish species. Studies using isotopes techniques indicate that this fish digests green algae and cyanobacteria efficiently (Xie, 1999). In contrast, many other filter-feeding fish, e.g. roach (*R. rutilus*) ingests mainly zooplankton and non-animal food including cyanobacteria, macrophytes, and detritus is mostly only an alternative food source (Horppila and Peltonen, 1997). Due to feeding habits of silver carp, it can be supposed that its ability to conjugate microcystin could be higher than that in roach. Both fish species endure frequent exposure to cyanobacteria in their habitats (Ibelings and Havens, 2008). Hence, they should have various GST isoforms, which can influence the efficiency of the microcystin biotransformation, i.e. conjugation to glutathione.

The aims of this study were (1) to characterize the cytosolic GST activities in liver (the major organ of biotransformation) and gill (main feeding and simultaneously first contact organ with toxin of cyanobacteria) from roach and silver carp using four substrates and (2) to compare capability of roach and silver carp enzyme extracts to detoxify microcystin-LR (MC-LR) *in vitro*.

2. Material and methods

2.1. Chemicals

Most of the chemicals were obtained from Sigma (Germany). DCNB from Fluka (Switzerland) acetonitrile and TFA for HPLC from Roth (Germany) and pNBC were from Kodak (USA). All chemicals and solvents used were analytical grade (p.a.). Microcystin-LR was purchased from Alexis (Germany) with a purity of 98%.

2.2. Animals

In vitro experiments were performed with enzyme extracts of gills and liver from ten untreated roach (*R. rutilus* L., mean total length = 17 cm) originating from Lake Müggelsee (Berlin, Germany) early May before cyanobacterial development and 13 silver carp (*H. molitrix* Val., 10 cm mean total length), from aqua-global (Seefeld, Germany), respectively. To eliminate environmental stress prior to start of the experiment, fish were kept in tanks in reverse osmosis water containing 100 mg L^{-1} sea salt, 200 mg L^{-1} $CaCl_2$ $2H_2O$ and 103 mg L^{-1} $NaHCO_3$, with a light/dark regime of 14 h light to 10 h dark at 20 ± 2 °C for two (silver carp) or four (roach) weeks. Fish were fed once a day with live plankton (predominantly Cladocera and *Chaoborus sp.* larvae) originated from ponds. Fish were killed by a sharp blow on the head followed by cervical dislocation. Liver and gill filaments of both fish species were removed and immediately frozen in liquid nitrogen and stored until enzyme extraction at −80 ° C.

2.3. Enzyme extraction

Enzyme extracts were prepared at 4 °C according to the modified method of Wiegand et al. (2000). One g of fish tissue (gills or liver) (25% w/v) was homogenized on ice in 5 mL sodium phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol (v/v), 1.4 mM dithioerythritol (DTE), 1 mM ethylendiaminetetraacetic acid (EDTA) in a glass homogenizer. Cell debris was removed by centrifugation at 9000 × *g* for 10 min. The supernatant was centrifuged again at 105,000 × *g* for 1 h to obtain the microsomal fraction which was resuspended in sodium phosphate buffer (20 mM, pH 7.0 20% glycerol, 1.4 mM DTE) using a glass potter. Soluble proteins were concentrated by two steps of ammonium sulphate precipitation (35% and 80% (w/v) saturation at 0 °C) and centrifuged (83,000 × *g* for 30 min). The pellet was resuspended in 20 mM sodium phosphate buffer pH 7.0 and desalted by gel filtration on NAP-10 columns (Sephadex G-25) according to the manufacturers protocol (Amersham Pharmacia, Sweden). This soluble enzyme fraction of liver and gills of both species was further used for the soluble glutathione S-transferases (sGST) isolation. Several extractions per fish tissue were done and then pooled.

2.4. GST activity assay and protein determination

Activity of sGST was detected according to the methods of Habig et al. (1974). Substrate specificities of sGST isoenzymes were quantified colorimetrically at 25 °C using 4 substrates: 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM, λ = 340 nm); 1,2-dichloro-4-nitrobenzene (DCNB, 1 mM, λ = 345 nm), ethacrynic acid (ETHA, 0.5 mM, λ = 270 nm) and 4-nitrobenzyl chloride (pNBC, 0.9 mM, λ = 310 nm). Concentration of glutathione (GSH) was 0.4 mM when measured with ETHA and 1.9 mM for all other substrates. In the AEC fractions with the highest protein content, glutathione reductase (GR) and glutathione peroxidase (GPx) were also measured. GR activity was assayed according to Tanaka et al. (1994) and activity of GPx according to Drotar et al. (1985), using H_2O_2 as substrate. Enzyme activity was calculated in katal (kat), which is the conversion rate of 1 mol of substrate per s and is given in nanokatals per mg (nkat mg^{-1}) protein. Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard. All determinations were conducted in triplicate on a Konton/Uvikon 922 spectrophotometer in quartz cuvettes with a total measurement time of 5 min with single points every 30 s. Protein determination was conducted as endpoint measurement after 20 min in plastic cuvettes.

2.5. Isolation of GST — affinity chromatography

Cytosolic enzyme extracts were first separated on the basis of their affinity to GSH on GSH-agarose affinity column (GSTrap, 1.6 × 2.5 cm, Pharmacia, Germany). The column was equilibrated at 20 °C with 5 column volumes of 20 mM Tris/HCl (pH 7.8) before applying of cytosolic enzyme extract (1 mL). The column was then washed with the same buffer to remove all non GSH-affine proteins (5 column volumes). Thereafter the bound GSTs and other GSH-affine enzymes (e.g. glutathione reductase, glutathione peroxidase) were eluted with 20 mL of 25 mM GSH in 20 mM Tris/HCl (pH 7.8) buffer at a flow rate 1 mL min^{-1}. The eluent was fractionated in 1 mL steps (yielding 51 fractions). The proteins remaining in the column were removed with 10 mL of 1 M NaCl. GST activity in all fractions was assayed using CDNB as described above. The fractions showing highest GST activities (e.g. fractions: 25–29, see Fig. 1) were pooled and concentrated using 10 kDa ultrafiltration membrane (Centrisart I, Sartorius, Germany) at 2500 g for 10 min (Haraeus Multifuge 1sr, Germany).

2.6. Separation of GST isoenzymes — anionic chromatography (AEC)

Affinity-concentrated GSTs and other GSH-affinity-separated proteins were separated by anionic chromatography (BioRad, Germany) using two columns (UNO-Q-1, Bio-Rad, Germany and MONO-Q HR 5/5, Pharmacia, Sweden) connected in series and online UV detection at 280 nm. Buffer system consisted of buffers A: 20 mM Tris/HCl (pH 7.8), and B: 1 M NaCl in 20 mM Tris/HCl (pH 7.8). Affinity-concentrated GSTs (250 µl, i.e. 12 mg protein on average) was applied to the columns in buffer A. Separation was achieved by using a linear gradient of 0 to 25% buffer B within 16 mL, followed by 25–100% buffer B within 8 mL at a flow rate of 0.5 mL min^{-1}. Fractions of 0.5 mL were collected. GST activity in all fractions was assayed again using CDNB and ETHA as substrates.