Antioxidant and detoxifying fish enzymes as biomarkers of river pollution

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The activity of several antioxidant and detoxifying enzymes, superoxide dismutase (SOD), GSH peroxidase (GSHPx), GSSG reductase (GSR) and GSH-S-transferase (GST), the contents of thiobarbituric acid reactive substances (TBARS), and the SOD and GST isoenzyme patterns were studied in the livers of chubs (*Leuciscus cephalus*) from reference river areas and polluted urban sites. Livers of polluted fish contained higher concentrations of transition metals, especially copper and iron. Total GSHPx activity was 1.8-fold higher in the polluted fish than in reference animals, while the SOD and GSR activities and the TBARS content were not significantly changed. Three new SOD isoforms (pI 4.45, 5.1, 5.2) and a higher intensity of the band pI 4.2 were observed after isoelectrofocusing of polluted fish extracts. Total GST activity was higher in fish from polluted areas. The GST isoenzyme pattern was studied using subunit-specific substrates (DCNB, EPNP, EA, NPB, NBC) and by Western-blot using antibodies specific to rat GST subunits 1, 8 (Alpha class), 3 (Mu class) and 7 (Pi class). Reference and polluted fish lacked cross-reactivity towards Alpha-class GSTs. Reference fish displayed weaker cross-reactivity towards CST-7 and 2.3-fold lower activity with EA, while higher cross-reaction with GST-3 was observed in polluted fish.

Keywords: *superoxide dismutase, glutathione-S-transferase, isoenzyme pattern, biomarkers of river pollution, fish.*

Introduction

The rivers flowing through urban areas are becoming polluted by an alarming number of xenobiotics. Fish are exposed to pollutants of urban, industrial, mining and agricultural origin, many of which exert cytotoxic effects via production of reactive oxygen species (ROS; Di Giulio *et al.* 1989). These highly reactive compounds damage most biomolecules, namely lipids, proteins and DNA (Sies 1986), damage to the latter being responsible for genotoxic damage (Halliwell and Arouma 1991).

The extent to which ROS yield oxidative stress depends upon the effectiveness of antioxidant defences (Di Guiseppi and Fridovich 1984, Sies 1986), and significant damage occurs only if antioxidant defences are overwhelmed (Sies 1986, Halliwell and Arouma 1991). They include (i) various water and lipid-soluble free-radical scavengers of low MW, and (ii) several inducible enzymes, namely superoxide dismutase (SOD) (EC 1.15.1.1)—converting $O^{\cdot-}$ to H_2O_2, catalase (EC 1.11.1.16)—reducing the H_2O_2 to H_2O, and two glutathione peroxidases (EC 1.11.1.9), one seleno-enzyme, active with several hydroperoxides, including H_2O_2, and other Se-independent enzymes, active with different organic hydroperoxides (Lawrence and Burk 1978).

Several studies about the responses of antioxidant fish enzymes to pollutants enhancing ROS production have been reported, although often they have been inconclusive and shown wide individual differences (Gabryelak and Klekot 1985, Vig and Nemcsok 1989, Mather-Mihaich and Di Giulio 1991). Significant changes have been reported in marine fish by workers studying several molecular biomarkers, such as the glutathione redox status (Rodriguez-Ariza *et al.* 1993, 1994), the level of thiobarbituric acid reactive substances (TBARS; Pedrajas *et al.* 1995), the appearance of new SOD forms of higher electrophoretic mobility (Pedrajas *et al.* 1993, 1995), or the specific induction of new GST isoenzymes (Martínez-Lara *et al.* 1992, 1996).

Fish living in polluted aquatic environments are exposed to xenobiotics which are putative substrates for GSH-mediated detoxification (Kretzschmar and Klinger 1990). Conjugation of many electrophiles with GSH is catalysed by glutathione-S-transferases (EC 2.5.1.18), a multigene family of enzymes (Hayes and Pulford 1995). All eukaryotic species possess multiple cytosolic and membrane-bound GSH-S-transferase (GST) isoenzymes, each displaying distinct substrate specificities. Most mammalian cytosolic GSTs are classified into four classes, Alpha, Mu, Pi and Theta, encoded by different genes. Many xenobiotics transcriptionally activate GST genes through their antioxidant responsive element (Hayes and Pulford 1995). So the level of expression of certain classes of GST genes can represent an adaptive response mechanism to oxidative stress (Len'rtov' *et al.* 1996).

Since the rivers are a major sink for numerous industrial spills, metals, pesticides and urban residues, the elucidation of the biochemical responses to pollutants by aquatic animals becomes very important. Such responses can provide reliable and sensitive biomarkers for exposure and toxicity in free-living organisms (Winston and Di Giulio 1991). The present study investigates some of the antioxidative enzymatic activities and the specific response of some additional molecular biomarkers in the livers of chub, a very common fish in Slovak rivers, in both polluted and non-polluted areas.

MATERIALS AND METHODS

Chemicals

All reagents, of the highest purity, were from Sigma, Merck and Boehringer. Rabbit antisera towards the rat GST subunits 1, 3, 7 and 8 were from Biotrin, Dublin (Ireland) and were purified on Protein A-Sepharose using a standar

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1354–750X/97 $12·00

Biological samples

Chub (*Leuciscus cephalus*) about 5 years old were captured with fishing nets from two rivers in the eastern region of the Slovak Republic in April (1995). A reference group of 12 fish came from the upstream non-polluted area of the river and another group of 12 fish was obtained from a metal-polluted urban area of the river. After killing the fish, the livers were excised and immediately frozen in liquid nitrogen until further analysis.

Preparation of soluble fraction

Pieces of liver were homogenized (20% w/v) in 10 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA, 2 mM GSH, 5 mM DTT and 10 mg PMSF per gram of liver using an Ultra-Turrax homogenizer. Homogenates were centrifuged at 105 000x *g* for 60 min and stored at –80 °C until used for further assays. Extracts for SOD assays were dialysed against 10 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM EDTA.

Enzyme assays and determination of proteins and TBARS

Superoxide dismutase (SOD) was assayed by measuring at 550 nm the inhibition of cytochrome C reduction using a xanthine/xanthine oxidase $O_2^{\cdot-}$ generating system (Flohé and Ötting 1984). One SOD unit was defined as the amount of enzyme causing 50% inhibition of cytochrome C reduction under such conditions. Glutathione peroxidase activity (GSHPx) was assayed at 37 °C in a coupled assay with glutathione reductase using cumene hydroperoxide (Se-independent) or H_2O_2 (Se-dependent) as substrates (Flohé and Gunzler 1984). Glutathione reductase (GSR) was assayed by following at 340 nm the oxidation of NADPH by GSSG (Pinto *et al.* 1984). Glutathione-S-transferase (GST) was assayed as described by Habig and Jakoby (1981) at 30 °C with the following substrates and final concentrations: CDNB and DCNB 1 mM, EPNP 0.5 mM, NPB and NBC 0.1 mM and EA 0.2 mM (37 °C for this assay); GSH was at 5 mM except in assays with CDNB (1 mM) and EA (0.25 mM). One unit of GSHPx, GSR or GST activity catalyses the formation of 1 μmol of product per minute under each assay conditions. Specific activity is defined as units of activity per mg of protein. Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as standard. Thiobarbituric acid reactive substances (TBARS) were determined as described by Gutteridge (1984), although incubations were made in 3 ml total volume and 2.8% (w/v) TCA was used instead of HCl. TBARS content is expressed as absorbance at 535 nm per g of wet tissues. The results are given as means ±SEM. Data were analysed using Student's test.

Quantification of metals

Metals were determined by atomic absorption spectroscopy (AAS) using a Varian spectrophotometer (Krupicer 1985).

Determination of SOD isoenzymes

Isoelectrofocusing was carried out with PhastSystem equipment; pI values of SODs were determined in gels with pH gradients of 4.0–6.5 and 5.0–8.0 using isoelectrofocusing calibration kits (Pedrajas *et al.* 1993). SOD activity was stained *in situ* using nitroblue tetrazolium (NBT), riboflavin and TEMED (Beauchamp and Fridovich 1971). Two types of SOD were distinguished by soaking the gels with 4 mM KCN: Cu,Zn-SODs were inhibited by CH^- while Mn-SODs were resistant to cyanide (Fridovich 1975).

Immunoblotting and Western blotting

Electrophoresis was carried out in 13% polyacrylamide gels (Laemnli 1970). Proteins were transferred onto nitrocellulose using a semi-dry system (Bio-Rad) (Towbin *et al.* 1979). Blots were probed with polyclonal antibodies raised in rabbits against rat GST subunits 1, 3, 7 and 8. Alkaline-phosphatase-conjugated goat anti-rabbit IgG served as the secondary antibody. The blots were developed with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP) and NBT (Blake *et al.* 1984).

Results

Metal content

The concentrations of several transition metals were determined and used as a comprehensive index of total contamination. Table 1 shows the contents of Cu, Fe, Pb, Hg and Cd in the livers of reference and polluted fish. With the only exception of mercury, animals from upstream reference waters had low metal contents, in contrast to fish captured in polluted urban areas which showed much higher metal concentrations. Thus, the total metal load of animals from polluted areas was 12.8-fold higher than that of reference fish, with values ranging from an 8-fold higher lead to an 814-fold higher copper concentration.

Antioxidant enzyme activities and oxidative damage to biomolecules

The activities of several antioxidant enzymes were determined in the hepatic soluble fraction of reference and polluted fish (Table 2). Superoxide dismutase showed lower activity in polluted fish than in those from clean reference areas, although the difference was not statistically significant. Glutathione reductase activity was almost identical in both groups. In contrast, total glutathione peroxidase was 2.47-fold

Metal	Reference (R) *n* = 12	Polluted (P) *n* = 12	P/R *ratio*
Cu	0.010±0.002	8.140±1.465*	814.0
Fe	6.400±1.600	74.600±13.428*	11.7
Pb	0.010±0.003	0.080±0.024*	8.0
Hg	0.040±0.012	0.040±0.011	1.0
Cd	0.005±0.002	0.060±0.018*	12.8

Table 1. Metal content of fish liver.

Metal contents were determined in livers of fish as described in Materials and Methods. Data are expressed in mg of metal per kg of body weight ±SEM (*significant at $P < 0.05$).

Biomarker	Reference (R) $n = 12$	Polluted (P) $n = 12$	P/R *ratio*
Superoxide dismutase activity[b]	53.00±18.00	37.00±19.00*	0.70
Glutathione reductase activity[a]	32.00±4.00	28.00±4.00	0.88
Total[b]	13.00±3.00	32.00±9.00*	2.46
Se-dependent[b]	0.54±0.07	0.52±0.04	0.96
TBARS content[c]	0.09±0.06	0.09±0.07	1.00

Table 2. Levels of antioxidant enzymes and lipid peroxidation products in hepatic soluble fractions of fish from reference or polluted areas.

Hepatic soluble fractions were prepared from reference (R) or polluted (P) fish and their antioxidant enzyme activities and TBARS contents were assayed as described in Materials and Methods. The results are expressed as means ±SEM of three independent determinations in mU mg^{-1} [a], U mg^{-1} [b], or in A_{535} mg^{-1} [c] (*significant $P < 0.05$) of individual samples.

higher in polluted fish than in reference animals, a difference that was statistically significant, while the activity of Se-dependent GSHPx was nearly the same in both groups. The *changes in GSHPx and SOD* activities led us to study whether fish from polluted areas were subjected to higher oxidative stress. We first analysed malondialdehyde and other lipid peroxidation products by determining the levels of thiobarbituric acid reactive substances. Table 2 shows that TBARS contents were similar in both experimental groups. This result suggested that the higher GSHPx activity of polluted fish was able to prevent the increased production of TBARS.

Since no differences were seen in the lipid peroxidation of polluted fish, more sensitive biomarkers of oxidative damage were then investigated, namely the appearance of new isoforms of SOD, an enzyme highly sensitive to reactive oxygen species (ROS) (Pedrajas *et al.* 1993, 1995). A single Mn-SOD band (pI 4.8), unaffected by cyanide and of similar intensity, was seen in extracts from reference and polluted fish after separation by isoelectrofocusing and *in situ* staining (Figure 1). On the contrary, both groups showed marked differences in several Cu,Zn-SOD isoenzymes. While two intense Cu,Zn-SOD isoenzymes (pI 5.0 and 4.7) and one very faint form (4.2) were observed in reference fish, animals from polluted areas showed three new bands (5.2, 5.1 and 4.45) and a much more intense band of pI 4.2. Thus, at least three new Cu,Zn-SOD bands could be used as biomarkers of environmental pollution in the fish studied.

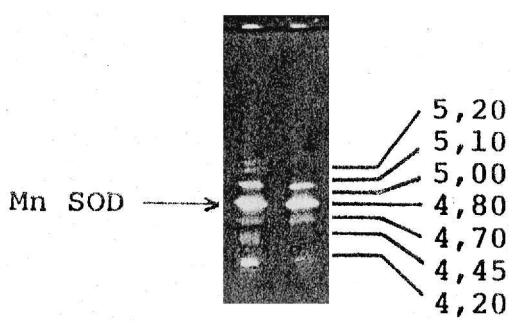

Figure [illegible] (R) and polluted (P) fish. The extracts were prepared, separated by isoelectrofocusing in gels with a 6.5 to 4.0 pH gradient, and stained *in situ* for SOD activity as described in Materials and Methods. pI values were determined by using IEF protein markers.

Glutathione-S-transferase activities

Total GST activity, assayed with CDNB as substrate, was significantly higher in fish from polluted areas in comparison to reference fish (844.0±250 versus 524.0±93 mU mg^{-1}). To determine the relative contributions of different GST isoenzymes to total GST activity, several substrates somewhat specific for the different rat GST subunits (Habing and Jakoby 1981, Hayes and Pulford 1995) were used (Figure 2). In contrast to the higher total GSTs, the activity assayed with ethacrinic acid, a substrate specific for rat GST isoenzymes 8–8 and 7–7 (George 1994), was 2.3-fold lower in fish living in polluted areas than in reference animals. No statistically significant differences were found between both experimental groups in the GST activities assayed with the substrates DCNB, EPNP, NPB, NBC.

To further confirm differential expression of fish GST isoenzymes, Western blottings were carried out using antisera specific for different rat GST subunits. Distinct cross-reactivities were displayed by reference and polluted fish (Figure 3). Animals from clean areas only showed cross-reactivity with antibodies specific for rat GST-7 subunit (Pi class), but no homology was observed with rat GST-3 subunit (Mu class). In contrast, fish from polluted areas cross-reacted with antibodies specific for rat GST-3, but cross-reactivity with rat GST-7 was much lower than in reference animals. No cross-reactivity was observed in control or reference fish with rat GST-1 or GST-8 subunits (Alpha class).

Discussion

In biological systems, reactive oxygen species (ROS) are formed by several processes involving environmental pollutants. Thus, biphenyls, quinones and nitroaromatics produce $O_2^{\cdot -}$ by redox cycling, while transition metals catalyse the reaction of $O_2^{\cdot -}$ with H_2O_2 to produce $HO^{\cdot}$ radicals by Fenton reactions (Aust *et al.* 1985, Sies 1988, Winston and Di Giulio 1991). Thus, fish inhabiting polluted aquatic environments are exposed to a variety of oxyradicals, leading to oxidative damage of different biomolecules such as lipids or proteins (Sies 1986). The marked increase total metal load, particularly copper and iron in fish from polluted areas in our experiment indicates that these metals may be involved in the activation of oxygen-related reactions.

Oxidative damage reflects an imbalance between the production of oxidants and removal or scavenging of those oxidants. Low molecular weight scavengers such as ascorbate, β-carotene, glutathione and vitamins A, E and C have been detected in several aquatic species and, in some cases, were shown to be elevated as a function of oxidative stress (Andersson *et al.* 1988, Partali *et al.* 1989, Ribera *et al.* 1989). Also induction of metallothionein synthesis by heavy metals has been demonstrated. Metallothion[illegible]

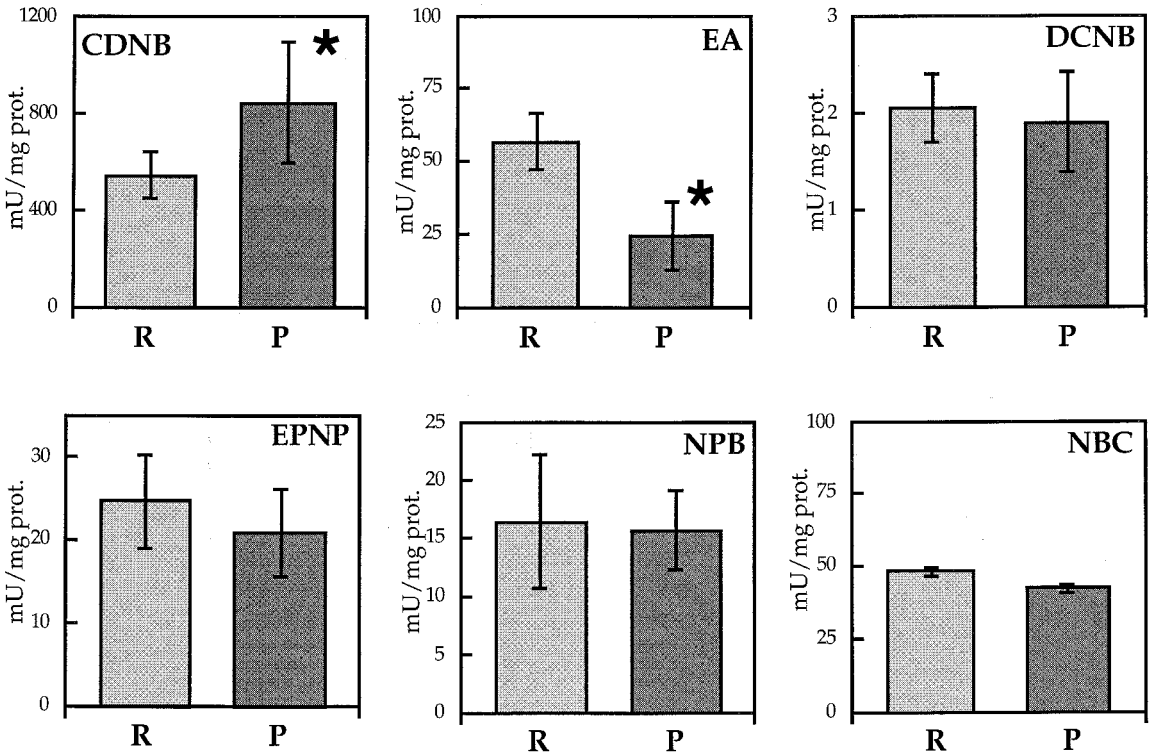

Figure 2. Activity of fish GST with various substrates. Cell-free extracts from reference (R) and polluted (P) fish were prepared and the GST activity was determined with different substrates as described in Materials and Methods. Significant statistical differences between two groups ($P < 0.05$) are indicated with asterisks.

zinc, copper and cadmium and is induced indirectly by heavy metals such as mercury, gold and platinum (Garvey 1990).

Several authors have previously investigated the effects of xenobiotics and metals on fish antioxidant enzyme activities (Vig and Nemcsok 1989, Rodríguez-Ariza *et al.* 1993, Thomas and Wofford 1993). Nevertheless, the lack of a clear correlation between lipid peroxidation and antioxidant activities indicate species or individual differences (Radi *et al.* 1985, Filho and Boveris 1993). The higher total GSHPx activity and lower SOD activity described in the present paper in fish heavily polluted with transition metals fully agree with previous paper. Thus, carps exposed to high Cu^{2+} doses showed higher GSHPx activities and MDA levels and lower SOD and catalase activities (Radi and Matkovics 1988).

The intensity of oxidative damages suffered by an organism depends on a fine balance among its individual antioxidant enzymes. Thus, IB cells overexpressing Cu,Zn-SOD were hypersensitive to ROS while consecutive transfection with catalase corrected their hypersensitivity (Amstad *et al.* 1991). Also increases in Cu,Zn-SOD were paralleled by higher Se-GSHPx activity in several systems. Amstad *et al.* (1994) have found that cells with a higher GSHPx/SOD ratio were highly protected from oxidant-induced damages. Only small deviations from this ratio had a dramatic effect on the resistance of cells to oxidant-induced damage.

In our studies the GSHPx/SOD ratio was 3.5-fold higher in polluted fish than in reference fish. This increased ratio and the unchanged TBARS levels could probably reflect an adaptation to the chronic oxidizing conditions to which polluted fish had been exposed.

SOD is an inducible enzyme key for dismutation of $O_2^{\cdot-}$ anions, although Cu,Zn-SOD is itself a primary target for ROS (Fridovich 1975). Fish living in contaminated rivers showed a more complex Cu,Zn-SOD isoenzyme pattern than reference animals, displaying a new intense band (pI 4.45), two new weaker bands (5.2, 5.1) and a higher intensity of a fourth band (4.2). H_2O_2 is converted by Cu,Zn-SOD into $HO^{\cdot}$ radicals which could attack a His residue, transforming it to its carbonyl derivative and yielding a more acidic protein (Salo *et al.* 1990, Sato *et al.* 1992). New more acidic SOD bands are also formed from human or bovine Cu,Zn-SOD after incubation with H_2O_2 (Sato *et al.* 1992, Sharonov and Churilova 1992). New SOD forms are also observed in fish (*Mugil* sp.) from metal-polluted areas of the Spanish littoral zone; such new SODs were reproduced by incubating the pure Cu,Zn-SOD isoforms with H_2O_2 (Pedrajas *et al.* 1993). In addition, fish (*Sparus aurata*) injected with model xenobiotics show new Cu,Zn- and Mn-SOD forms which are reproduced by incubating cell-free extracts with $O_2^{\cdot-}$ or H_2O_2-generating systems or with *t*-butylhydroxide (Pedrajas *et al.* 1995). Thus, the new SOD bands have been proposed as very sensitive biomarkers of pollutants producing oxidative stress (Pedrajas *et al.* 1993, 1995). Incubation of fish extracts with low MDA concentrations also reproduce the new Cu,Zn- and Mn-SOD forms (Pedrajas, unpublished results). Thus, the new Cu,Zn-SOD forms of pI 5.2, 5.1 and 4.45 detected in polluted fish could be due to reaction of original SOD forms with some reactive compound, perhaps of oxidative origin.

Fish from polluted urban areas showed significant increase in total GSTs activity assayed with th

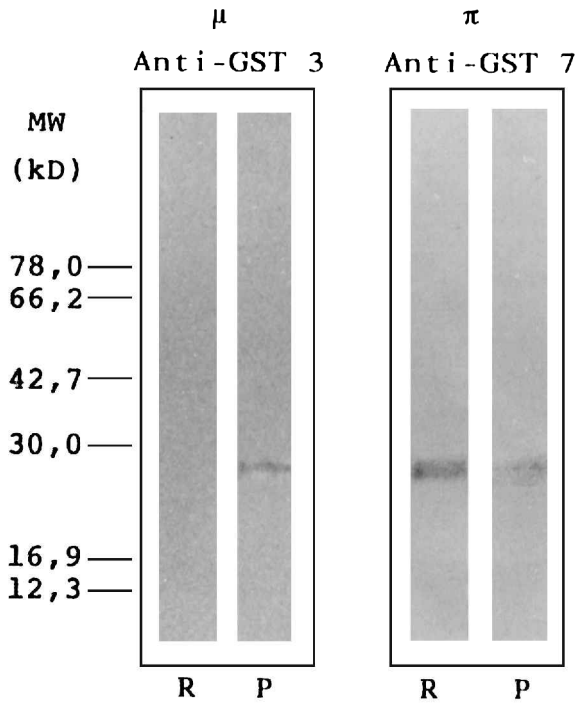

Figure 3. Western blot analysis of liver tissue extracts after SDS/PAGE. R: reference group, P: polluted group. Blots were immunostained with rabbits polyclonal antisera against rat GST subunits 3 (Mu class) and 7 (Pi class). The molecular mass of the marker proteins in kD is indicated on the left.

CDNB. The differential expression of GSTs genes determines cell sensitivity towards a variety of toxic chemicals. Particular GSTs contribute to resistance to carcinogens, antitumour drugs, environmental pollutants and products of oxidative stress. In addition, H_2O_2 induces certain GSTs genes in plant and mammalian cells (Hayes and Pulford 1995). The present paper shows differences in the activity of certain GST isoenzymes between references and polluted fish. The lower cross-reactivity with rat GST-7 found in animals from polluted areas agreed well with the 2.3-fold lower GST activity assayed with ethacrynic acid, a substrate reported specific for rat GST-7 (Pi class) and 8 (Alpha class) (George 1994). Among all GSTs, subunit 7, active with lipohydroperoxides (Meyer *et al.* 1985), contains a Cys-SH group sensitive to ROS ($O_2^{\cdot-}$, H_2O_2 or $HO^{\cdot}$) and to the GSH/GSSH levels (Sato *et al.* 1990). Such a residue is well conserved within Pi class GSTs (e.g. pig and bovine) and its modification leads to inactivation (Dirr *et al.* 1991, Nishihara *et al.* 1991). Apart from multiple regulatory elements in the gene, including those controlled by oncogene expression, GST-7 activity can also be chemically modulated in a reversible manner (Sato *et al.* 1990).

A band cross-reacting with rat GST-3 was found in polluted fish, but was missing in reference animals. Mu class GSTs, particularly GST-3 and GST-4, are very active with several mutagenes and DNA hydroperoxides. Subunit 3 is a highly active aryltransferase which reacts with DCNB, although in polluted fish we did not detect increased activity with DCNB or NBC as substrates. GST subunit 4 shares 80% of its amino acid identity with subunit 3, but does not react with DCNB; thus it is plausible that GST-4 were preferentially expressed in polluted fish instead of GST-3, a possibility that will be checked as soon as GST-4 rat specific antibodies are available.

The differences in antioxidant activities between polluted and reference fish lead us to propose that induction of some isoenzymes, especially of SOD and GSTs, could be useful biomarkers for monitoring environmental pollution in rivers.

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Received 18 November 1996, revised form accepted 10 February 1997