

Purification and partial characterization of glutathione transferase from the teleost *Monopterus albus*

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Abstract

Glutathione transferases (GSTs) catalyze the transfer of glutathione to a variety of xenobiotic and toxic endogenous compounds. GSTs are phase II biotransformation enzymes and are proposed as biomarkers of environmental pollution. In this study, a cytosolic glutathione transferase (*ma*GST) was purified from liver of the freshwater fish *Monopterus albus* by affinity chromatography. The *ma*GST appeared to be a homodimer composed of two subunits each with a molecular weight of 26 kDa. This *ma*GST showed high activity towards the substrates 1-chloro-2,4-dinitrobenzene (CDNB) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Kinetic analysis with CDNB as substrate revealed a K_m of 0.28 mM and V_{max} of 15.68 $\mu\text{mol}/\text{min}$ per mg of protein. It had maximum activity in the pH range 7.0–7.5, a broad optimum T_m range of 30 °C–55 °C, and a high thermal stability with 77% of its initial activity at 45 °C. This high thermal stability of *ma*GST could be related to the physiological adaptation of *M. albus* to high temperatures in tropical and subtropical environments.

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1. Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) are a family of enzymes that catalyze the conjugation of the tripeptide glutathione (GSH) to a range of hydrophobic and electrophilic compounds (Armstrong, 1997). GSTs are major phase II detoxification enzymes found mainly in the cytosol (Sheehan et al., 2001). The primary function of the enzyme is generally considered to be the detoxification of both endogenous and exogenous alkylating agents such as α,β -unsaturated aldehydes and ketones, alkyl and aryl halides (Armstrong, 1991). GSTs are soluble proteins composed of two subunits with molecular masses of 22–27 kDa each. GSTs are mainly classified into several classes (alpha, mu, pi, theta, sigma, kappa, zeta and omega) based on their primary structure, immunological and kinetic properties (Myrnes and Nilsen, 2007). Different classes of GSTs vary in their functional role of protecting the cell

against exogenous and endogenous toxic compounds, including secondary metabolites of lipid peroxidation.

Contaminant input into the environment can affect the biochemical responses of the exposed organisms. Activity of conjugation enzymes, such as GSTs, has been proposed as a biomarker of susceptibility to the presence of potentially damaging xenobiotics (Perez Lopez et al., 2002; Luchmann et al., 2007). The GST expression levels in fish species have been reported to increase by exposure to foreign compounds, suggesting GSTs form part of an adaptative response to chemical stress, thus could be used as effective biomarkers of aquatic contamination (Hayes and Pulford, 1995; Perez Lopez et al., 2002; Hayes et al., 2005; Fu and Xie, 2006; Luchmann et al., 2007). The ricefield eel or Asian swamp eel (*Monopterus albus*) is a bony fish (family Synbranchidae; order Synbranchiformes) (Shi, 2005). It is not really an eel because it does not belong to the family Anguillidae of the order Anguilliformes (Tay et al., 2003). *M. albus* can be found in the tropics and subtropics from India to southern China, Malaysia and Indonesia (Ip et al., 2004). The fish live in rice fields, muddy

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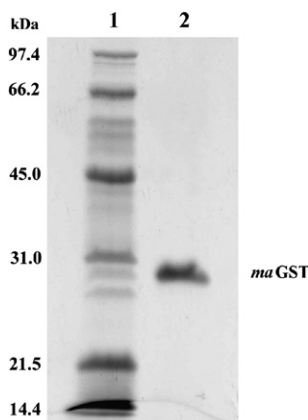


Fig. 1. SDS-PAGE analysis of the purified *maGST*. Lane 1, molecular mass markers (Bio-Rad) with the sizes shown on the left in kilodaltons; Lane 2, purified *maGST*.

ponds, swamps and canals (Tay et al., 2003). The species is also farmed for food consumption in SE Asia. In agriculture-intensive regions, *M. albus* is exposed to high concentrations of environmental contaminants (e.g. pesticides, herbicides and heavy metals) in rice fields. GST from *M. albus* could be a good candidate biomarker for contamination in freshwater ecosystems. The aims of the present work was to purify and characterize the enzymatic properties of the cytosolic GST from liver tissue of *M. albus*, and to compare its properties with other GSTs from different fish species to determine if the GST of *M. albus* provides a useful system in toxicological research and a good biomarker for contamination in freshwater ecosystems.

2. Materials and methods

2.1. Chemicals

Glutathione, CDNB, ECA, 4-NPA, and NBD-Cl were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Sepharose 6B were purchased from Amersham Biosciences AB (Uppsala, Sweden).

2.2. Crude extract preparation from *M. albus*

Swamp eels (*Monopterus albus*, Synbranchidae) were caught from rice fields in southern China. Approximately 85 g of fresh livers were harvested from 30 individuals and washed once with 0.9% NaCl, then frozen in liquid nitrogen. The liver tissue was grinded into fine powder and suspended in 255 mL of buffer A (50 mM Tris-HCl, 1 mM EDTA, 3 mM DTT, pH 7.5). A crude extract was obtained by centrifuging the homogenate at 20,000 g for 30 min.

2.3. Purification of *M. albus* GST using affinity column

The affinity column was prepared according to the method described by Mannervik and Guthenberg (1981). The crude protein extract was applied to the GSH-Sepharose 6B affinity

column, equilibrated with buffer A. After sample application, the column was washed with buffer B (50 mM Tris-HCl, 1 mM EDTA, 3 mM DTT, 1.0 M NaCl, pH 7.8). GSTs were eluted from the column with buffer C (50 mM Tris-HCl, 1 mM EDTA, 3 mM DTT, 5 mM GSH, pH 7.8). The GST activity was monitored using GSH and CDNB as substrates. The purified GST protein was desalted using a PD-10 column (Amersham Pharmacia Biotech) in 10 mM Tris-HCl buffer, pH 8.0. The purified GST was stored at 4 °C. All the purification steps were performed at room temperature.

2.4. SDS-PAGE and mass spectrometry

The purity and subunit molecular weight of the purified protein was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in a 12% slab gel. About 8 µg of the purified protein was loaded on to the gel. The gel was stained with 0.1% Coomassie brilliant blue R-250. To accurately determine the molecular weight of the purified GST, Matrix-assisted laser desorption mass spectrometry (MALDI-TOF/MS) was obtained using an AXIMA-CFR™ plus mass spectrometer (Shimadzu, Kyoto, Japan).

2.5. Enzyme assays and protein determination

GST activities towards CDNB, ECA and 4-NPA were measured as described by Habig et al. (1974). The GST activity towards NBD-Cl was determined as described by Ricci et al. (1994). All assays were carried out at 25 °C. The dependence of GST activity on pH and temperature were measured as reported by Yuen and Ho (2001). Protein concentrations were determined by measuring absorbance at 280 nm.

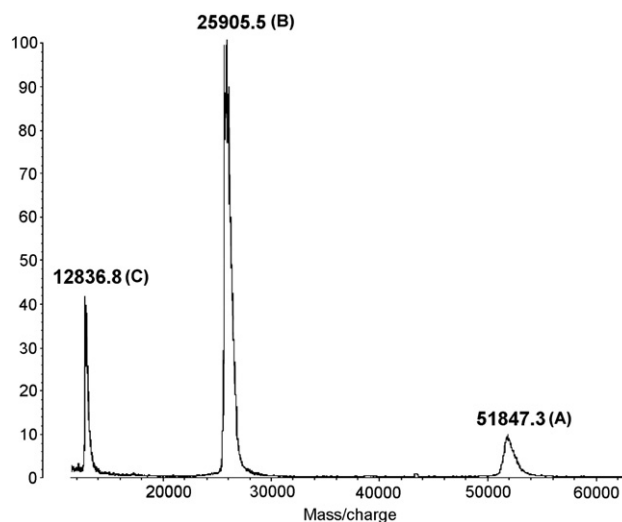


Fig. 2. MALDI-TOF/MS of *maGST*. Peak A correspond to the homodimeric *maGST*, Peak B correspond to the monomer *maGST*, Peak C is doubly charged $[M+2H]^{2+}$ which corresponds to the singly charged $[M+H]^+$ of peak B.

Table 1
Comparison of liver GSTs activities towards CDNB among different fish species

GST	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	References
<i>Monopterus albus</i> maGST	13.07	This study
<i>Paralichthys californicus</i> GST	0.23	Donham et al. (2007)
<i>Micropterus salmoides</i> recGST	7.0	Doi et al. (2004)
<i>Rivulus marmoratus</i> rm-GST-T	9.94	Lee et al. (2006)
<i>Rivulus marmoratus</i> rm-GST-A	4.84	Lee et al. (2006)
<i>Platichthys stellatus</i> GST	2.6	Gallagher et al. (1999)
<i>Ictalurus punctatus</i> GST	10.4	Gallagher et al. (1996)
<i>Pagrus major</i> pmGSTA1-1	9.64	Konishi et al. (2005a)
<i>Pagrus major</i> pmGSTA2-2	2.88	Konishi et al. (2005a)
<i>Dicentrarchus labrax</i> DL-GST-8.2	10.2	Angelucci et al. (2000)
<i>Dicentrarchus labrax</i> DL-GST-6.7	14.5	Angelucci et al. (2000)
<i>Scolopsis bimaculatus</i> GST	16.56	AlGhais (1997)
<i>Salmo trutta</i> liver total GST	27.69	Novoa-Valinas et al. (2002)

2.6. Kinetic studies

The apparent K_m and V_{max} values for GSH were determined using a GSH range from 0.2 to 1.0 mM and a fixed CDNB concentration of 1.0 mM. The apparent K_m and V_{max} values for CDNB were determined using a CDNB range from 0.2 to 1.0 mM and a fixed GSH concentration of 1.0 mM. The kinetic parameters K_m and V_{max} were calculated using non-linear regression method described by Zeng et al. (2005).

2.7. Thermal stability

Thermal stability of the purified GST (0.093 mg/mL in 10 mM Tris-HCl, pH 8.0) was measured by incubating the protein for 15 min at various temperatures from 30 °C to 60 °C with 5 °C intervals. GST activity towards CDNB was determined at the end of each incubation.

3. Results and discussion

3.1. Purification of *M. albus* GST

M. albus GST was purified about 300-fold by GSH-affinity chromatography, with about 14% recovery of total activity using CDNB as substrate. The purified GST (maGST) showed an enzymatic activity of 13.07 $\mu\text{mol}/\text{min}$ per mg towards substrate CDNB. When the purified protein was analyzed by SDS-PAGE, a single band corresponding to a molecular mass of

Table 2
Steady-state kinetic constants of the purified maGST using CDNB and GSH as substrates

Substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min}$ per mg)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \text{min}^{-1}$)
CDNB	0.28 \pm 0.03	15.68 \pm 0.37	146.54	523.36
GSH	0.20 \pm 0.02	7.57 \pm 2.84	229.39	1146.95

26 kDa was detected after Coomassie staining (Fig. 1). Further analysis on MALDI-TOF/MS revealed a molecular mass of 52 kDa (Fig. 2, Peak A) for MaGST, and a 25.9 kDa (Fig. 2, Peak B) for its subunits (monomer). The peak C in Fig. 2 is the double charged $[M+2H]^{2+}$ signal of m/z 12.8 kDa corresponding to the single charged $[M+H]^+$ of 25.9 kDa (Fig. 2, Peak B). These results indicate that the maGST is a

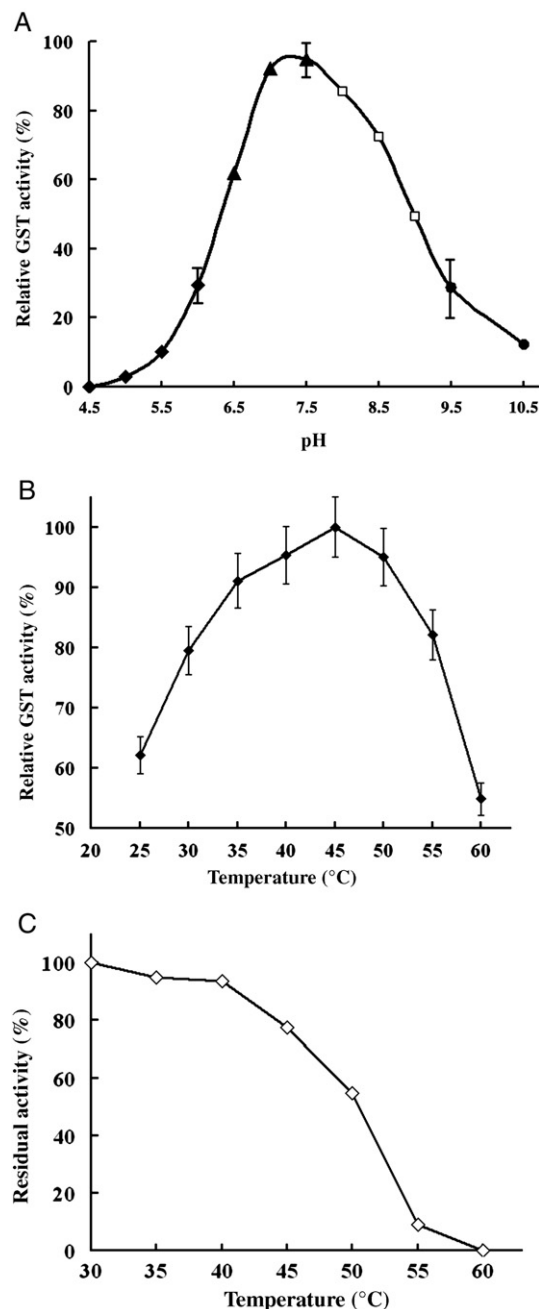


Fig. 3. A) Effect of pH on CDNB-conjugating activities of maGST. Different symbols represents different buffers used. (\blacklozenge) 0.1 M citrate–0.2 M sodium phosphate, pH, 4.5–6.0; (\blacktriangle) 0.1 M potassium phosphate, pH, 6.0–7.5; (\diamond) 0.1 M Tris–HCl, pH, 7.5–9.5; (\bullet) 0.1 M carbonate–bicarbonate buffers, pH, 9.5–10.5. (B) Effect of temperature on CDNB-conjugating activities of maGST. The enzyme activity of the control was expressed as 100%. (C) Thermal stability of maGST, based on the retention of enzymatic activity towards the substrate CDNB following heat treatment.

homodimer of 25.9 kDa subunits. This GST configuration is similar to those from other fish and bivalve where homodimeric GSTs are isolated (Yang et al., 2002; Yang et al., 2003; Konishi et al., 2005b; Lee et al., 2006; Myrnes and Nilsen, 2007). However, heterodimeric GSTs have been observed in freshwater carp (Dierickx, 1985) and marine flatfish (George and Buchanan, 1990). To date, most GSTs isolated from fish, mammals and bivalves are homodimers and only minor cases are heterodimers (Sheehan et al., 2001). However, in plants many GSTs are naturally expressed as heterodimers (McGonigle et al., 2000; Sheehan et al., 2001). The transcriptional regulation of individual subunits is considered to influence the formation of GST homodimers and heterodimers (Dixon et al., 2002).

After affinity purification step, a single protein was obtained suggesting this protein was the predominant GST expressed in *M. albus* liver. In salmonid fish livers, a similar pattern is observed where the predominant GST expressed comprised subunits of M_w 24.8 kDa (Dominey et al., 1991). In another study on digestive gland of Icelandic scallop, also a single GST protein is detected by SDS-PAGE after GSTrap chromatography (Myrnes and Nilsen, 2007). However, two and four GST isoenzymes are observed in Gilthead Seabream livers (Martinez-Lara et al., 1996) and rainbow trout livers (Melgar Riol et al., 2001), respectively. These studies indicate that the livers of different fish species have different GST isoenzyme expression patterns and/or the expression of GSTs are induced by different environmental factors.

3.2. Characterization of *maGST*

The specific activities for *maGST* towards CDNB and NBD-Cl were 13.07 and 5.54 $\mu\text{mol}/\text{min}$ per mg, respectively. A comparison of enzymatic activities of *maGST* and other fish liver GSTs towards the substrates CDNB is summarized in Table 1. The *maGST*, DL-GST-6.7 and *Scolopsis bimaculatus* GST share a similar high activity. The high specific activities of *maGST* toward CDNB and NBD-Cl suggest this GST plays an important role in the detoxification of xenobiotics in *M. albus*. However, the *maGST* did not show any enzymatic activity towards ECA and 4-NPA. Similar results are observed in liver GSTs from *Dicentrarchus labrax*, *Branchiostoma belcheri* and *Platichthys stellatus* (Gallagher et al., 1999; Angelucci et al., 2000; Fan et al., 2007). In general, few studies on fish GSTs have tested the enzymatic activities towards ECA and 4-NPA, thus difficult to make any generalization on fish GST properties toward these groups of substrates.

The enzyme's steady state kinetics were studied in assays with various concentrations of GSH and CDNB. At fixed GSH concentrations, the K_m and V_{\max} values were 0.28 mM and 15.68 $\mu\text{mol}/\text{min}$ per mg of protein, respectively, for CDNB. At fixed CDNB concentrations, the K_m and V_{\max} values were 0.20 mM and 7.57 $\mu\text{mol}/\text{min}$ per mg of protein, respectively, for GSH (Table 2). Compared to other fish GSTs, *maGST* shared similar K_m^{GSH} with catfish intestine GST (0.19 mM) (Gadagbui and James, 2000), indicating these GSTs have similar affinity for the substrate GSH.

The effect of pH on the enzyme activity was evaluated using CDNB as a substrate. The activity of *maGST* was pH-dependent (Fig. 3A). The highest activity was observed in the pH range 7.0–7.5. At pH 6.5 and 8.5, *maGST* still had 62% and 72% of its maximum activity, respectively, suggesting a broad pH optimum. The temperature profile of *maGST* indicated a optimum temperature for enzymatic activity at 45 °C (Fig. 3B). It still had 80% of its maximum activity at 30 °C and 55 °C, suggesting a broad temperature optimum of the enzyme. Similar results are observed in ns-GST-O and ns-GST-T from the polychaete *Neanthes succinea* (Rhee et al., 2007). Thermal stability of the *maGST* is shown in Fig. 3C. It retained 90% of its initial activity at 40 °C, indicating *maGST* is stable below 40 °C. At 45 °C and 50 °C *maGST* retained 77% and 55% of its initial activity, respectively. Similar results were observed in the rm-GST-T and rm-GST-A from *Rivulus marmoratus* livers (Lee et al., 2006). However, two GSTs from hepatopancreas of *Pagrus major* showed much different optimum temperature (35 °C and 40 °C) (Konishi et al., 2005a). These results indicate different GST members in fish are induced by different temperature environments. The fact that different GSTs in fish have different thermal stability supports the hypothesis that GST isoenzymes response to temperature changes for physiological adaptation in various environments.

As GSTs are widely distributed in all organisms in nature, their expression/induction could be utilized for early detection of stress responses in organisms exposed to organic compounds. The procedure for GST purification from liver tissue and the methods of GST assays are usually simple and convenient, and can be standardized for cross laboratory comparisons. Thus, GST is considered as a good marker system for contamination evaluation in aquatic ecosystems. The purified *maGST* from *M. albus* has the potential for this application in SE Asia region where agriculture practice is intensive and water pollution has become a increasingly important ecological concern. The expression patterns after exposure to model contaminants will be further studied in order to validate its use as biomarkers of contamination in freshwater ecosystems.

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