A new class of glutathione S-transferase from the hepatopancreas of the red sea bream *Pagrus major*

Takafumi KONISHI*†, Keitaro KATO‡, Toshiyoshi ARAKI*, Kentaro SHIRAKI§, Masahiro TAKAGI† and Yutaka TAMARU*¹

*Department of Life Science, Faculty of Bioresources, Mie University, 1515 Kamihama, Tsu, Mie 514-8507, Japan, †School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292, Japan, ‡Fisheries Laboratory of Kinki University, 3153, Shirahama, Nishimuro, Wakayama 649-2211, Japan, and §Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

To elucidate drug deposition and metabolism in cultured marine fishes, in a previous study we isolated and purified the GSTs (glutathione S-transferases) from the hepatopancreas of the red sea bream *Pagrus major* that contained 25 and 28 kDa GST subunits. The 25 kDa GST subunits encoded by two genes (*GSTA1* and *GSTA2*) have been identified as Alpha-class GSTs. In the present study, we performed the molecular cloning and characterization of the *GSTR1* gene encoding the 28 kDa GST subunit from the *Pa. major* hepatopancreas. The nucleotide sequence of *GSTR1* was composed of an ORF (open reading frame) of 675 bp encoding a protein of 225 residues with a predicted molecular mass of 25.925 Da. A search of the BLAST protein database revealed that the deduced amino acid sequence of *GSTR1* was structurally similar to that of GSTs derived from other fishes such as largemouth bass (*Micropterus salmoides*) and plaice (*Pleuronectes platessa*). The genomic DNA containing

the *GSTR1* gene was found to consist of six exons and five introns quite distinct from mammalian Theta-class GSTs. We have purified and characterized the recombinant *GSTR1* enzyme (pmGST1-1) which showed activity only towards 1-chloro-2,4-dinitrobenzene, although it had no detectable activity towards cumene hydroperoxide, 1,2-dichloro-4-nitrobenzene, ethacrynic acid, 4-hydroxynonenal and *p*-nitrobenzyl chloride. Moreover, pmGST1-1 revealed remarkable heat instability (melting temperature $T_m = 30.3 \pm 0.11$ °C). Collectively, our results indicated that the characteristic GST genes including *GSTR1* have been conserved and functional in fishes. Therefore we designate them 'Rho-class', a new class of GSTs.

Key words: detoxification, glutathione conjugation, hepatopancreas, *Pagrus major*, red sea bream, xenobiotics.

INTRODUCTION

GSH is a tripeptide that acts during phase II metabolism to conjugate electrophiles and prevents damage to cell membranes and other macromolecules. Since GSH is ubiquitous in animals, plants and microorganisms, is water-soluble and is found mainly in the cell cytosol and other aqueous phases of the living system, it plays a key role in the regulation of the redox balance and can be used as an indicator of oxidative stress in the detoxification of xenobiotics [1]. GSTs (glutathione S-transferases) are a family of multifunctional enzymes involved in the cellular detoxification and excretion of a variety of xenobiotic substrates [2,3]. They catalyse the addition of GSH to substrates that have electrophilic functional groups. The GSH adducts produced have increased solubility in water and are subsequently degraded enzymatically to mercapturates and excreted [4,5]. Recent studies have suggested that certain GST isoenzymes may be involved in the regulation of stress-activated cell signalling pathways through direct physical association with c-Jun N-terminal kinase and apoptosis signal-regulating kinase-1 [6–8].

Most of the mammalian GSTs that have so far been purified and characterized exist in the cytosol, where they form homodimers or heterodimers with the subunits whose molecular masses range from 23 to 28 kDa [9]. Furthermore, mammalian GSTs have been particularly well characterized and are classified into at least eight classes such as Alpha, Kappa, Mu, Omega, Pi, Sigma, Theta and Zeta classes [10–15] on the basis of a combination

of criteria such as substrate, inhibitor specificity, primary and tertiary structure similarities and immunological identity [3]. Since many new GST sequences and their crystal structures from non-mammalian organisms including plants have been determined, their novel classes have also been identified and classified as Beta (bacteria); Delta, Epsilon, U (insects); Lambda, Phi and Tau classes (plants) [16]. So far, few complete cDNA sequences of piscine GSTs have been reported for plaice (*Pleuronectes platessa*) Theta-class-related *GSTA* (GenBank® Nucleotide Sequence Database accession number X63761) [17], largemouth bass GST (*Micropterus salmoides*, GenBank® accession number AY335905) [18] and zebrafish (*Danio rerio*) Pi-class GST (GenBank® accession number AF285098). Since some environmental compounds effect transcriptional activation of GST genes through either antioxidant responsive element or xenobiotic responsive element [19], particular GST isoenzymes have been utilized as valuable biomarkers for exposure to environmental pollutants [20,21]. Furthermore, the regulation and function of the GSTs have not been extensively studied, especially for marine fishes.

The red sea bream *Pagrus major* is one of the major marine fishes cultured in Japan and approx. 90 000 tons were raised in 2000. Previous studies in our laboratory indicated that two major GST isoforms, i.e. 25 and 28 kDa subunits, existed in the *Pa. major* hepatopancreas. We have recently cloned and sequenced two kinds of GST genes (*GSTA1* and *GSTA2*) coding for the 25 kDa subunit from the *Pa. major* hepatopancreas, which were classified as Alpha-class GSTs (T. Konishi, K. Kato, T. Araki,

Abbreviations used: 4HNE, 4-hydroxynonenal; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; DTT, dithiothreitol; EA, ethacrynic acid; GST, glutathione S-transferase; ORF, open reading frame; PNBC, *p*-nitrobenzyl chloride; poly(A)⁺, polyadenylated.

¹ To whom correspondence should be addressed (email ytamaru@bio.mie-u.ac.jp).

The nucleotide sequence data reported have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AB158412 and AB158415.

K. Shiraki, M. Takagi and Y. Tamaru, unpublished work). In the present study, we have isolated, cloned and sequenced the cDNA and genomic DNA encoding the 28 kDa GST subunit (GSTR1) that belongs to a new class of GSTs. Furthermore, properties of the recombinant GST (pmGSTR1-1) have been characterized and compared with recombinant Alpha-class GSTs from *Pa. major*.

EXPERIMENTAL

Materials

CDNB (1-chloro-2,4-dinitrobenzene), CHP (cumene hydroperoxide), NADPH and PNBC (*p*-nitrobenzyl chloride) were purchased from Nacalai Tesque (Kyoto, Japan). DCNB (1,2-dichloro-4-nitrobenzene), EA (ethacrynic acid) and GSH were purchased from Wako Pure Chemical Industries (Osaka, Japan), GSH reductase from Oriental Yeast (Tokyo, Japan) and restriction enzymes, DNA ligase and Ex *Taq*^{TM} DNA polymerase from Takara (Kyoto, Japan). KOD-Plus DNA polymerase was obtained from Toyobo (Osaka, Japan). DNA fragments were recovered after gel electrophoresis with GeneClean® II kit (Qbiogene, Carlsbad, CA, U.S.A.). *Escherichia coli* DH5 α and Epicurean coli^{TM} XL1-Blue MRF⁺ (Stratagene, La Jolla, CA, U.S.A.) were the host strains for cloning experiments. DYEnamic ET Terminators kit for DNA sequencing and Hybond-N+ nylon membranes were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). The V8 protease was purchased from Sigma (St. Louis, MO, U.S.A.).

Animals, RNA isolation and cDNA synthesis

Red sea breams *Pa. major* provided by the Fisheries Laboratory of Kinki University were bred in a net pen with marketed dry pellets; 3-year-old adult female fishes were used in the following study. Approximately 50 mg of the hepatopancreas stored in RNAlater® (Ambion, Austin, TX, U.S.A.) was used in the following manipulation. Total RNA was extracted by ISOGEN (Nippon Gene, Tokyo, Japan). Poly(A)⁺ (polyadenylated) RNA was purified with a QuickPrep® Micro mRNA Purification kit (Amersham Biosciences). The first-strand cDNA was synthesized using a PowerScript® reverse transcriptase, SMART® IV oligonucleotide and CDSIII/3'-primer. The second-strand cDNA was synthesized with a SMART® cDNA Library Construction kit (Clontech, Palo Alto, CA, U.S.A.). The synthesized cDNAs anneal to the 5'-PCR primer (5'-AAGCAGTGGTATCAACGCA-GAGT-3') and to the CDSIII/3'-primer (5'-ATTCTAGAGGCCG-AGGCGGCCGACATG-dT₃₀N₁N-3') in kit components.

Enzyme purification

All purification steps were performed at 4 °C. The hepatopancreas (8.0 g) from *Pa. major* was then homogenized in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.3). The homogenate was clarified by centrifugation at 12 000 g for 20 min. After the floating lipid was removed, the resulting supernatant was dialysed overnight against the PBS buffer. After filtration through a 0.45 μ m filter, the sample was applied to a GSH-affinity column, GSTrap® (Amersham Biosciences). Extensive washing was performed with the PBS buffer until no protein was detected in the effluent of absorbance *A* at 280 nm. The bound material was then eluted with 50 mM Tris/HCl (pH 8.0) containing 10 mM GSH. Active fractions were pooled and concentrated using ULTRAFREE®-15 Biomax-5 (Millipore, Bedford, MA, U.S.A.) by centrifugation at 2000 g for 1 h at 4 °C. The purity and determination of the subunit size of the enzyme preparation were confirmed by SDS/PAGE.

SDS/PAGE and Western-blot analysis

SDS/PAGE was performed by the method of Laemmli [22]. Migration of the proteins was determined in 12.5 % polyacrylamide gel. Kaleidoscope Polypeptide Standards (Bio-Rad, Hercules, CA, U.S.A.) and a low-molecular-mass SDS calibration kit (Amersham Biosciences) were used as the standard marker. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. For Western-blot (immunoblot) analysis, enzyme preparations resolved by SDS/PAGE were blotted on to a PVDF membrane (Atto, Tokyo, Japan) by semi-dried electrophoretic transfer. The membrane was probed with an anti-GST antibody raised against the *E. coli* GST (Amersham Biosciences) at a 1:1000 dilution for 1 h at 25 °C. The horseradish peroxidase-linked secondary anti-goat antibody (Biosource, Camarillo, CA, U.S.A.) was used at a 1:5000 dilution overnight at 4 °C and the membrane was developed with diaminobenzidine.

Enzyme and protein assays

Enzyme activity was measured by using CDNB as a substrate by the method of Habig et al. [23], which was assayed spectrophotometrically after the conjugation of CDNB with GSH at 340 nm ($\Delta\epsilon_{340}$ 9.6 mM⁻¹ · cm⁻¹). The reaction medium contained 0.1 M potassium phosphate buffer (pH 6.5), 1.0 mM GSH, 1.0 mM CDNB, 3 % (v/v) ethanol and an enzyme preparation in a total volume of 2.0 ml. The reaction incubated at 20 °C was initiated by the addition of CDNB, and the change in *A* at 340 nm was monitored for 60 s with a model V-550 spectrophotometer with EHC-477T temperature programmer (Japan Spectroscopic Company, Tokyo, Japan). Corrections for the non-enzymatic reactions were performed for all assays, and each reaction was performed at least three times. One unit of activity is defined as 1 μ mol of CDNB conjugated with GSH · min⁻¹. Protein concentrations were measured by the method of Bradford [24] using BSA as a standard. The assays with other model GST substrates (DCNB, EA and PNBC) were also performed at 20 °C under the standard condition. GST-4HNE (where 4HNE stands for 4-hydroxynonenal) activity was determined at 20 °C by the spectrophotometric method of Alin et al. [25]. Glutathione peroxidase activity was measured in a coupled assay system containing 0.1 mM NADPH, 1 unit of GSH reductase, 1 mM GSH and 1.2 mM CHP in 0.1 M potassium phosphate buffer (pH 7.0) [26].

N-terminal amino acid sequence analysis

After SDS/PAGE, the proteins stained in the gel were cut and transferred into a dialysis membrane tube (Wako Pure Chemical Industries) filled with SDS/PAGE buffer (25 mM Tris/HCl, 0.19 M glycine and 0.1 % SDS). The electroelution of the proteins was performed using a Mupid® electrophoresis system (Advance-Bio, Tokyo, Japan) at constant 50 V for 1 h. The eluted proteins were concentrated using a Microcon® YM-10 (Millipore) and dialysed against 50 mM ammonium bicarbonate (pH 7.8) at 4 °C for 5 h. The proteins were digested by V8 protease (1:50, w/w) at 37 °C for 12 h. The enzymatic cleavage products were separated by 15 % high-resolution Tricine-SDS/PAGE [27]. Semidry blotting on to a PVDF membrane (Atto, Tokyo, Japan) was performed at 2 mA · cm⁻² for 1 h. Peptides were stained with Ponceau S. The amino acid sequence of the peptides was determined by automated Edman degradation using an Applied Biosystems 476A instrument.

Cloning of the full-length cDNA

The primers for discovery PCR were designed on the internal sequence from two fragments digested by V8 protease, which corresponded to the 28 kDa GST subunit. A 16-degenerate sense

primer A designed as a 17-mer (5'-GTNATGAARATGAAYCC-3') was derived from the amino acid sequence VMKMNP which was located at the N-terminal region of the 28 kDa GST subunit, whereas a 64-degenerate antisense primer B designed as a 20-mer (5'-ACRTCNGCCATYTTYTGRTT-3') was designed from protein sequence NQKMADV, which was located at the C-terminal region of the protein. PCR was performed with two primers using total double-strand cDNA derived from the hepatopancreas. The PCR cycling condition with the degenerated primers and an Ex *Taq*^{TM} DNA polymerase was as follows: 95°C for 2 min and 40 cycles of 95°C for 30 s; 40°C for 60 s and 72°C for 105 s. A 206 bp PCR product was isolated and subcloned into pT7Blue T-vector (Novagen, Madison, WI, U.S.A.) and then sequenced in both directions by the chain termination method of Sanger et al. [28]. To obtain further the complete cDNA sequence of the *GSTR1* gene, gene-specific primers for 5'- and 3'-RACE (5' and 3'-rapid amplification of cDNA ends) were designed based on the 206 bp DNA sequence. The 3'-end of the *GSTR1* cDNA was amplified by PCR with a sense primer C (5'-TCCTGCCTTCAAACATGG-3') and the CDSIII/3'-primer, whereas the 5'-end of the cDNA was amplified by PCR with an antisense primer D (5'-TGAGTGAG-AGACCCTCAA-3') and 5'-PCR primer. By using the phosphorylated primers, PCR was performed with KOD-Plus DNA polymerase in the following condition: 94°C for 2 min and 30 cycles of 94°C for 15 s; 59°C for 30 s; and 68°C for 1 min. The PCR products were purified and cloned into pBluescript® II (SK-) vector and then sequenced in both directions.

The nucleotide sequence was subjected to the basic local alignment with a BLAST search provided by the NCBI (National Center for Biotechnology Information). Although a large number of GST sequences are available, to simplify computations and to promote legibility, representative GST amino acid sequences were selected from previously described GST classes. The sequences were obtained from GenBank® database and aligned using CLUSTAL W [29]. A phylogenetic tree was obtained by the neighbour-joining method. TREEVIEW software generated visual representations of clusters [30].

Cloning of genomic DNA

Genomic DNA was prepared with phenol extraction by lysing the whole blood of red sea bream, and the extracts were purified by ethanol precipitation. The PCR primers were designed by the sequence of cDNA clones in both directions. To clone the genomic DNA of *GSTR1*, a sense primer E (5'-CTTCTCCCACCACTC-ACA-3') and an antisense primer F (5'-CAATCTGCAGTTTC-AGTCTCA-3') were used. All the primers were phosphorylated before PCR was performed with the primers using KOD-Plus DNA polymerase and the genomic DNA as the template. The PCR cycle was performed as follows: 94°C for 2 min and 30 cycles of 95°C for 15 s; 59°C for 30 s; and 68°C for 150 s. The PCR products were purified and cloned into pBluescript® II (SK-) vector for sequence analysis.

Expression and purification of the recombinant GST

The ORF (open reading frame) of the *GSTR1* gene was subcloned into pET22b(+) vector (Novagen) with *Nde*I and *Xho*I sites. *E. coli* BL21 (DE3)-competent cells (Novagen) were used as a bacterial expression host. The transformants were cultivated in 250 ml of LB (Luria-Bertani) medium at 37°C in the presence of 50 µg · ml⁻¹ ampicillin until the *A* at 600 nm reached approx. 0.6. At this point, the temperature was changed to 20°C, and expression of the His₆-tagged proteins was induced by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside, followed by another 4 h shaking of the culture. The cells were collected

by centrifugation at 6000 *g* for 15 min at 4°C. The cell pellet was resuspended in 20 ml of ice-cold PBS buffer and lysed by sonication. The cell lysate was clarified by centrifugation at 12000 *g* for 20 min. The recombinant enzymes were purified by a GStrap® column as described previously and dialysed with 0.1 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 5 mM DTT (dithiothreitol). Native size of the recombinant proteins was determined by gel filtration chromatography on a Superdex® 75 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM sodium phosphate and 150 mM NaCl. The molecular mass was estimated by using an LMW calibration kit (Amersham Biosciences) as a standard.

Effects of pH and temperature on enzyme activity

The optimum pH for the recombinant GST was evaluated by a spectrophotometric assay using a CDNB substrate as described earlier. Britton-Robinson buffers (40 mM each of acetic, boric and phosphoric acids, with pH adjusted using NaOH) were substituted with 0.1 M potassium phosphate buffer at different pH values (pH 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0). The optimum temperature for recombinant GST was evaluated by using the standard activity assay at different temperatures (5, 10, 15, 20, 25, 30, 35, 40 and 50°C).

Kinetic parameters and thermal stability

The *K_m* (app) values for GSH (0.1–1 mM) were determined with 1 mM CDNB, whereas those for CDNB (0.1–1 mM) were determined with 1 mM GSH. Lineweaver-Burk plots were used to determine the parameters.

A Jasco J-820 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan) was used to determine the *T_m* (melting temperature) curves for the recombinant enzymes. All experiments were performed using 0.1 mg · ml⁻¹ enzyme in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 5 mM DTT. The CD signal at 222 nm was monitored as the temperature increased in 0.1°C intervals from 10 to 80°C with the cuvette of 2 mm path-length in a thermostatically controlled cell holder.

RESULTS

Identification of the 28 kDa GST subunit from the *Pa. major* hepatopancreas

After purification of the GSTs from the *Pa. major* hepatopancreas by the GSH-affinity column, SDS/PAGE analysis revealed two protein bands corresponding to the 25 and 28 kDa GST subunits (Figure 1A, lane 1). Furthermore, as a result of Western-blot analysis, two bands were detected with the anti-GST antibody raised against the *E. coli* GST (Figure 1A, lane 2). In addition, the 28 kDa GST subunit strongly immunocross-reacted with the antibody compared with the 25 kDa GST subunit. After isolation of the 28 kDa GST subunit, the protein was digested by a V8 protease. At least five separated peptide bands were detected on the PVDF membrane (Figure 1B). The three amino acid sequences of the purified internal peptides from the 28 kDa GST subunit were determined as VMKMNPRGQLPAFKHGDK, GLSLNQKM-ADVIYYN and GERHDSAVKRNRE respectively. Eventually, the nucleotide sequences of the primers were designed as primers A and B from the underlined amino acid sequences as described in the Experimental section.

cDNA cloning of *GSTR1*

By using the two degenerate primers (primers A and B), a PCR fragment (206 bp) was isolated and coded for the partial cDNA

Figure 1 Analysis of native GSTs from the *Pa. major* hepatopancreas

(A) SDS/PAGE and Western-blot analysis. Lane M, a low-molecular-mass SDS calibration standard; lane 1, native GSTs from the *Pa. major* hepatopancreas; lane 2, immunocross-reacted bands with an anti-GST antibody raised against the *E. coli* GST. (B) Digested peptide pattern of the 28 kDa GST subunit by a V8 protease. Lane M, Kaleidoscope Polypeptide Standards; lane 1, the 28 kDa GST subunit digested by a V8 protease.

sequence of the 28 kDa GST subunit. Based on the 206 bp fragment, specific primers (primers C and D) were designed to obtain the complete cDNA sequence. Another PCR was performed with primers C and D towards the cDNA library derived from the *Pa. major* hepatopancreas. As a result of cDNA cloning, the PCR fragment (767 bp) was isolated with primer C and the CDSIII/3' primer, whereas the PCR fragment (433 bp) was isolated with primer D and the 5'-PCR primer. These PCR fragments included the DNA sequences in total agreement with the 206 bp PCR fragment with primers A and B. Since the 158 bp DNA sequence between the two fragments identically overlapped each other, the complete ORF of the 28 kDa GST subunit, named the *GSTR1* gene, was finally obtained. As shown in Figure 2, the nucleotide and deduced amino acid sequences of *GSTR1* are composed of 675 bp, encoding a protein of 225 residues with a predicted molecular mass of 25.925 kDa. The *GSTR1* gene also had 82 bp of 5'-non-coding region upstream of the ORF and 203 bp of 3'-non-coding region containing a consensus polyadenylation site (AATAAA) downstream of the ORF respectively. The deduced amino acid sequences were in complete agreement with the internal amino acid sequences of the 28 kDa GST subunit (Figure 2). Furthermore, a BLAST search indicated that the deduced amino acid of *GSTR1* showed 84% identity with largemouth bass GST (*Mi. salmoides*, GenBank® accession number AY335905) and 82% identity with plaice GST (*Pleu. platessa*, GenBank® accession number X63761). Furthermore, the *Pa. major* *GSTR1* shared high similarity with partial cDNA sequences of piscine GSTs such as fathead minnow (*Pimephales promelas*, GenBank® accession number AF274054) and gilthead sea bream (*Sparus aurata*, GenBank® accession number AY362762; Figure 3).

Comparison of the gene structure of *Pa. major* *GSTR1* with other GSTs

To clone the genomic DNA containing *GSTR1* from *Pa. major*, primers E and F were designed from the DNA sequences on both sides of the *GSTR1* cDNA. As a result of PCR cloning, the genomic DNA for *GSTR1* was found to be amplified and isolated as a single DNA fragment with primers E and F. The genomic DNA

consisted of 3486 bp encoding six exons coding for *GSTR1* and five introns. Table 1 shows a comparison of the number of exons for several GST genes from mammals and fishes and their amino acid residues of GSTs. The Theta-class-related GST genes from several fishes containing *Pa. major* were composed of six exons, whereas Theta-class GSTs from mammals had five exons. Furthermore, to investigate the relationship between piscine and other GSTs, we took a representative member of each of the 14 cytosolic GST classes (Alpha, Beta, Delta, Epsilon, Lambda, Mu, Omega, Phi, Pi, Sigma, Tau, Theta, U and Zeta) and used a CLUSTAL W program to align all of the amino acid sequences of GSTs. As shown in Figure 4, the results of alignment by the phylogenetic tree indicated that a group of piscine GSTs containing the *Pa. major* *GSTR1* was obviously distinct from other GST classes, although there were no Kappa-class GSTs because of very low sequence similarity among them.

Characterization of the recombinant *GSTR1*

As shown in Table 2, the recombinant *GSTR1* (pmGSTR1-1) purified by GSH-affinity chromatography yielded 4.6% of total cytosolic proteins from *E. coli*. SDS/PAGE analysis revealed that the purified pmGSTR1-1 without the His₆ tag gave a single protein band on SDS/PAGE after Coomassie Blue staining (Figure 5). The molecular mass of the pmGSTR1-1 was similar to that of the native 28 kDa GST subunit. On the other hand, size-exclusion chromatography revealed that the molecular mass of pmGSTR1-1 of the native forms was estimated to be 47.9 kDa by a Superdex® 75 HR 10/30 column (results not shown). Therefore the pmGSTR1-1 seemed to be composed of homodimers as the active form. Table 2 summarizes the kinetic parameters for the conjugation of CDNB with GSH as measured by three kinds of recombinant GSTs (pmGSTA1-1, pmGSTA2-2 and pmGSTR1-1) purified by the GSH-affinity column. The K_m and V_{max} values for pmGSTR1-1 with CDNB were 0.59 ± 0.06 mM and $7.9 \pm 0.65 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ respectively. For the co-substrate GSH, the K_m value for pmGSTR1-1 was 0.33 ± 0.024 mM, whereas the k_{cat}^{CDNB} value for pmGSTR1-1 was $3.56 \pm 0.29 \text{ s}^{-1}$. The utilization ratio (k_{cat}/K_m) for pmGSTR1-1 was approx. 0.8 and 4.2 times that for pmGSTA1-1 and pmGSTA2-2 respectively. As shown in Table 3, the GST activity of pmGSTR1-1 towards CDNB was detected at $4.99 \pm 0.08 \text{ unit} \cdot \text{mg}^{-1}$. On the other hand, enzyme activities of pmGSTR1-1 towards CHP, DCNB, EA, 4HNE and PNBC could not be detected. In addition, pmGSTR1-1 had no glutathione peroxidase activity.

To characterize further and compare the properties of pmGSTR1-1 with those of pmGSTA1-1 and pmGSTA2-2, the optimum pH and optimum temperature were assayed. The optimum pH and optimum temperature of pmGSTR1-1 towards CDNB as a substrate were 6.5 and 20°C respectively (Figure 6). Moreover, the temperature profile with pmGSTR1-1 indicated that relative activity at 30°C was only approx. 20% activity of that at 20°C. Unfolding studies on temperature were performed with three recombinant GSTs (Figure 7). Thermal denaturation curves were monitored by CD at 222 nm. The apparent T_m value for pmGSTR1-1 was 30.3 ± 0.11 °C, whereas the T_m values for pmGSTA1-1 and pmGSTA2-2 were 46.0 ± 0.20 and 50.4 ± 0.23 °C respectively. These results indicated that the temperature stability for pmGSTR1-1 was remarkably lower than those of pmGSTA1-1 and pmGSTA2-2.

DISCUSSION

To elucidate drug deposition and metabolism in cultured marine fishes, we have studied the structure, function and regulation of

aagcagtggatcaacgcagagtggccattacggccgggactcct
cacttctcccaccactcacaatttcagccgcgtcaggttaacacttcactctgaaacc

ATGGCCAAGGACATGACTCTGCTGTGGGGCTCCGGCTCTCCTCCCTGCTGGAGGGTGATG 60
M A K D M T L L W G S G S P P C W R V M 20

ATCACTCTGGAGGAGAAGAACCTGAAGGGCTACAACCAAACTGCTTTCTTTTGAAGAA 120
I T L E E K N L K G Y N Q K L L S F E K 40

ATGGAGCACAAGTCCGAGGAAGTCATGAAGATGAACCCAGGGGACAGCTTCCTGCCTTC 180
M E H K S E E V M K M N P R G Q L P A F 60

AAACATGGAGACAAGGTCTGAATGAGTCTACGCTGCCTGCCTGTACCTGGAGAACCAG 240
K H G D K V L N E S Y A A C L Y L E N Q 80

TTCAAGTCCCAGGAAACAAGCTGGTCCCTGACTGCCCCGCTGAAGTGGCAATGATGTAC 300
F K S Q G N K L V P D C P A E L A M M Y 100

CAGCGCATGTTTGGGGTCTCTCACTCAACAGAAAATGGCGGACGTCATCTACTACAAC 360
Q R M F E G L S L N Q K M A D V I Y Y N 120

TGGAAGGTCCCAGAGGGAGAGACACGACTCTGCTGTGAAGAGAAACAGAGAGAGTCTG 420
W K V P E G E R H D S A V K R N R E S L 140

ACTGCTGAGCTCAAGCTGTGGGAGGGTTACCTGCAGAAGACTTCAGGCGGTTTCTGGCA 480
T A E L K L W E G Y L Q K T S G G F L A 160

GGAAAGACCTTTTCACTGGCTGATGTGTGTGTTTATCCAAGCATCTGTTATGTCTTCCGT 540
G K T F S L A D V C V Y P S I C Y V F R 180

TTTGGGTTATGCGAGGAGCGTTACCCTAAACTGGCATCTTACTATAACAGTCTGAAGGAC 600
F G L C E E R Y P K L A S Y Y N S L K D 200

AGACCCAGCATCAAAGCCAGCTATCCTCCAACCTGGCTGGAGAACCACAGGGACAAGAT 660
R P S I K A S Y P P T W L E N P Q G Q D 220

GTGCTGAAAGACATCTGAgatgctgacacactggaacagttcctgttgtaaaatagttaa 675
V L K D I * 225

ccactccttgcatgtgactactgtagctggcagcattttataagaaatttgcatgtgctt
ttactgtggttttttgatttcttaacacaatttggttgagactgaaactgcagattgtgc
tgtataacggaagcaataaaaaacaacttgagctaaaaagcaaaaaaaaaaaaaaaaaaaaa
aaaaaaaaaaaacatgtcgccgcctcgccctctagaat

Figure 2 Nucleotide and deduced amino acid sequences of *Pa. major* GSTR1

The deduced amino acid sequence is shown in single-letter amino-acid notation below each codon. The amino acid sequences in the shaded boxes indicate identity to the deduced amino sequences with the native protein by a V8 protease. The translation-termination codon is marked by an asterisk. Poly(A)⁺ addition signal is underlined. The sequences of both terminal primers are shown with a broken line.

Fathead minnow (partial)	1:-----MIALEBKNRSGYNRHHFSEDRNEHQCAVKAIFRLVVFPHGDLIVNESFAACLYLESAPKSCQTRPIESBPACQLWV	81
Gilthead sea bream (partial)	1:-----PCMRVHIALEBKNRSGYNRHHFSEDRNEHQCAVKAIFRLVVFPHGDLIVNESFAACLYLESAPKSCQTRPIESBPACQLWV	86
Largemouth bass	1:MAKDMTLWGSGSPFCMRVHIALEBKNRSGYNRHHFSEDRNEHQCAVKAIFRLVVFPHGDLIVNESFAACLYLESAPKSCQTRPIESBPACQLWV	100
Plaice	1:MAKDMTLWGSGSPFCMRVHIALEBKNRSGYNRHHFSEDRNEHQCAVKAIFRLVVFPHGDLIVNESFAACLYLESAPKSCQTRPIESBPACQLWV	100
Red sea bream	1:MAKDMTLWGSGSPFCMRVHIALEBKNRSGYNRHHFSEDRNEHQCAVKAIFRLVVFPHGDLIVNESFAACLYLESAPKSCQTRPIESBPACQLWV	100
Fathead minnow (partial)	82:QRMFETNNLQONKADVIYYIMKVPEGBRHDSAVKRNRESITAEIKLMWEGYLQKTSG-CFLAGHTFSLADVCTYEBHCQVFPGLCEERYPKLAAYYNSL	180
Gilthead sea bream (partial)	87:QRMFEGTLQONKADVIYYIMKVPEGBRHDSAVKRNRESITAEIKLMWEGYLQKTSG-CFLAGHTFSLADVCTYEBHCQVFPGLCEERYPKLAAYYNSL	184
Largemouth bass	101:QRMFEGTLQONKADVIYYIMKVPEGBRHDSAVKRNRESITAEIKLMWEGYLQKTSG-CFLAGHTFSLADVCTYEBHCQVFPGLCEERYPKLAAYYNSL	198
Plaice	101:QRMFEGTLQONKADVIYYIMKVPEGBRHDSAVKRNRESITAEIKLMWEGYLQKTSG-CFLAGHTFSLADVCTYEBHCQVFPGLCEERYPKLAAYYNSL	198
Red sea bream	101:QRMFEGTLQONKADVIYYIMKVPEGBRHDSAVKRNRESITAEIKLMWEGYLQKTSG-CFLAGHTFSLADVCTYEBHCQVFPGLCEERYPKLAAYYNSL	198
Fathead minnow (partial)	181:KDRPSIKASGPPFTMLENPQGQDLKDI	186
Gilthead sea bream (partial)	185:KDRPSIKASGPPFTMLENPQGQDLKDI	211
Largemouth bass	199:KDRPSIKASGPPFTMLENPQGQDLKDI	225
Plaice	199:KDRPSIKASGPPFTMLENPQGQDLKDI	225
Red sea bream	199:KDRPSIKASGPPFTMLENPQGQDLKDI	225

Figure 3 Alignment of the *Pa. major* GSTR1 with other GSTs

The sequences correspond to similar sequences of GSTs of fathead minnow (*Pi. promelas*, GenBank® accession number AF274054), gilthead sea bream (*S. aurata*, GenBank® accession number AY362762), largemouth bass (*Mi. salmoides*, GenBank® accession number AY335905), plaice (*Pleu. platessa*, GenBank® accession number X63761) and red sea bream (*Pa. major*, GenBank® accession number AB158412; the present study). Identical amino acid sequences are indicated by highlighting.

Table 2 Kinetic parameters of recombinant GSTs from the *Pa. major* hepatopancreas

All reactions were assayed using CDNB as a substrate at 20 °C in 0.1 M potassium phosphate buffer (pH 6.5). The results represent means $\pm$ S.D. for triplicate determinations.

Enzyme	Specific activity* (units $\cdot$ mg ⁻¹)	Protein yield† (%)	K_m^{GSH} ‡ (mM)	K_m^{CDNB} § (mM)	V_{max}^{CDNB} (μ mol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹)	k_{cat}^{CDNB} (s ⁻¹)	k_{cat}/K_m^{CDNB} § (s ⁻¹ $\cdot$ mM ⁻¹)
pmGSTA1-1	9.64 $\pm$ 0.22	23.8	0.15 $\pm$ 0.016	1.36 $\pm$ 0.14	22.4 $\pm$ 1.90	9.92 $\pm$ 0.84	7.28 $\pm$ 0.13
pmGSTA2-2	2.88 $\pm$ 0.02	5.9	0.69 $\pm$ 0.077	7.69 $\pm$ 2.35	24.9 $\pm$ 7.40	10.9 $\pm$ 3.3	1.43 $\pm$ 0.01
pmGSTR1-1	4.99 $\pm$ 0.08	4.6	0.33 $\pm$ 0.024	0.59 $\pm$ 0.06	7.9 $\pm$ 0.65	3.56 $\pm$ 0.29	6.01 $\pm$ 0.14

* Unit $\cdot$ mg⁻¹ is the specific activity of the purified enzymes expressed at 20 °C.

† Protein yield, given as a percentage of the total cytosolic proteins, represents the fraction of GSTs purified by affinity chromatography.

‡ K_m for GSH was determined with a concentration of 0.1–1.0 mM GSH and 1 mM CDNB.

§ K_m for CDNB was determined with a concentration of 0.1–1.0 mM CDNB and 1 mM GSH.

Figure 5 SDS/PAGE of purified pmGSTR1-1

Samples were separated on 12.5% polyacrylamide gels. Lane M, molecular-mass standards (molecular masses are indicated at the left); lane 1, affinity-purified native GST from the *Pa. major* hepatopancreas; lane 2, affinity-purified pmGSTR1-1.

Table 3 Substrate specificity for pmGSTR1-1

All the reactions were performed at 20 °C.

Substrate	GST class	Substrate (mM)	GSH (mM)	pH	Specific activity (units $\cdot$ mg ⁻¹)*
CDNB	Overall GST	1.0	1.0	6.5	4.99 $\pm$ 0.08
CHP	Alpha, Theta	1.2	1.0	7.0	n.d.†
DCNB	Mu	1.0	1.0	7.5	n.d.†
EA	Alpha, Pi	0.2	0.25	6.5	n.d.†
4HNE	Alpha	0.1	0.5	6.5	n.d.†
PNBC	Mu, Theta	1.0	1.0	6.5	n.d.†

* 1 unit = 1 μ mol of product produced $\cdot$ min⁻¹.

† n.d., no detectable activity.

Pleu. platessa (GenBank® accession number X63761) was the first to be reported among the GSTs, the deduced amino acid sequences of *GSTA* showed high similarity to the GSTs from insects and plants and Theta-class GST isoforms from mammals [17]. Therefore the *Pleu. platessa* *GSTA* has been classified as a Theta-class-related GST. However, since the genes homologous

Figure 6 Effect of pH and temperature on pmGSTR1-1 activity

Effect of pH (A) and temperature (B). Rates were determined by spectral assay of CDNB conjugation with GSH; $\blacktriangle$, pmGSTA1-1; $\blacksquare$, pmGSTA2-2; $\bullet$, pmGSTR1-1.

with the plaice *GSTA* have been isolated from certain marine flatfish and freshwater species such as English sole (*Pleu. vetulus*), starry flounder (*Platichthys stellatus*) and largemouth bass (*Mi. salmoides*), these genes seem to be conserved among several fishes [31,32]. On the other hand, our results suggested that these piscine GST genes including *GSTR1* should be reclassified as a new class of GSTs for aquatic fishes. In addition, native forms of a new class of GSTs from both plaice and red sea bream could bind to

Figure 7 Thermal denaturation profiles of the recombinant GSTs

All experiments were performed using 0.1 mg·ml⁻¹ purified enzymes in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 5 mM DTT. The elliptical curve was monitored at 222 nm. The temperature was increased from 10 to 80 °C; Δ , pmGSTA1-1; $\square$, pmGSTA2-2; $\circ$, pmGSTR1-1.

a GSH-agarose matrix and were active with CDNB unlike most Theta-class GSTs [33]. Furthermore, although several fishes have so far been found to possess both Alpha- and Theta-class-related GSTs, we believe that the *Pa. major* GSTs should be classified as Alpha-class and as a new 'Rho-class' of GSTs. In this context, we mention that this new 'Rho-class' has no relationship to human red cell GST Rho, which is now called GSTP1-1 [34].

There are no clearly established criteria concerning the extent of sequence similarity required for placing a GST in a particular class. Therefore it is generally accepted that GSTs share greater than 40% identity within a class, and those with less than 25% identity are assigned to separate classes [16]. In fact, since Theta-class GSTs show generally lower amino acid sequence identities compared with other classes, many GSTs would be classified under this class. According to the recent genome database of zebrafish (*D. rerio*), the genes for mammalian-like Theta-class GSTs have been found: *gstt1* (GenBank® gene ID 378843), *zgc:65964* (GenBank® gene ID 393556), the related expression sequence tag clone (GenBank® accession number CF416980) and the complete cDNA clone (GenBank® accession number BC056725). In fact, since the deduced amino acid sequences of these GST genes shared approx. 50% identity with mammalian Theta-class GSTs, it seems that the zebrafish GSTs should be classified as Theta-class GSTs. On the other hand, a BLAST search analysis of the whole genome shotgun assembly sequences of zebrafish with the Ensembl software system (<http://www.ensembl.org>) [35] indicated that the expression sequence tag clone (GenBank® accession number CK239092) and the predicted gene (Ensembl accession number ENSDARG 00000017538) showed high similarity to Rho-class GSTs. The deduced amino acid sequence of the predicted gene shared approx. 55% identity with that of Rho-class GSTs, whereas it shared 20% identity with that of mammalian Theta-class GSTs. On the other hand, there are differences in the number of exons between mammalian Theta-class and the new class of GSTs (Table 1), even though Alpha-class GSTs from *Pa. major* consisted of six exons of length in

total agreement with mammalian Alpha-class GSTs (T. Konishi, K. Kato, T. Araki, K. Shiraki, M. Takagi and Y. Tamaru, unpublished work). Moreover, phylogenetic relationship between the GSTs indicated that Rho-class GSTs including the *Pa. major* GSTR1 are located far from mammalian Theta-class GSTs (Figure 4). Therefore these results indicate that aquatic organisms have unique GST genes that may play a different role in drug deposition and metabolism. Interestingly, marine organisms containing a green alga (*Coccomyxa* sp. PA, GenBank® accession number U42463) and an amphioxus (*Branchiostoma belcheri tsingtaunense*, GenBank® accession number AY279519) were also located close to Rho-class GSTs.

By expression and purification of the recombinant GSTR1 (pmGSTR1-1) from *E. coli*, the properties of pmGSTR1-1 have been characterized. Although the pmGSTR1-1 exhibited high activities towards CDNB as the model substrate, it exhibited no detectable activity towards other common prototypical xenobiotic substrates (CHP, DCNB, EA, 4HNE and PNBC; Table 3). The catalytic profile of pmGSTR1-1 was similar to that of the recombinant GST proteins derived from plaice [36] and bass [32], except for the fact that pmGSTR1-1 showed no activity towards EA, CHP and 4HNE. On the other hand, the utilization ratio (k_{cat}/K_m) of pmGSTR1-1 was efficient at its optimal temperature (20 °C) as well as that of the recombinant Alpha-class GSTs (pmGSTA1-1) from the *Pa. major* hepatopancreas (Table 2). However, pmGSTR1-1 revealed remarkable heat instability ($T_m = 30.3 \pm 0.11$ °C; Figure 7), indicating that residual activity of pmGSTR1-1 resulted in 43.4% irreversible inactivation after incubation at 30 °C for 1 min (results not shown). These results suggested that the *Pa. major* GSTR1 might have been adapted to cold temperatures because of their environmental habitat. Since we pointed out that zebrafish (*D. rerio*) as a tropical fish might have a Rho-class GST in the genome, comparison of the properties of GSTs between *Pa. major* and *D. rerio* is interesting in terms of the relationship between temperature adaptation and evolution. On the other hand, the protein properties should be compared between native GST and recombinant GST in our future work. Further molecular properties and the physiological functions of *Pa. major* GSTs should be studied to elucidate their drug-metabolizing system.

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