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Identification and characterization of a zebrafish gluathione s-transferase pi

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A Thesis

Entitled

Identification and Characterization of A Zebrafish Glutathione S-Transferase Pi

By

Maryam S Abunnaja

Submitted to the Graduate Faculty as a Partial Fulfillment of the Requirement for the Master of Science Degree in Pharmacology and Toxicology

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An Abstract of

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Glutathione S-transferases (GSTs) are a major group of Phase II enzymes involved in the detoxification of certain endogenous compounds such as reactive oxygen species (ROS) generated during metabolism, as well as in the detoxification of xenobiotics including drugs. In recent years, the zebrafish has increasingly been used as a promising animal model for biomedical research. This study is part of an effort to establish the zebrafish as a model for studying GST-mediated glutathione conjugation of xenobiotics. By searching the GenBank database, we have identified sequences encoding fifteen zebrafish GSTs. Using a computer algorithm available at the Genebee website, a dendrogram of the fifteen zebrafish GSTs was generated. A zebrafish GST Pi was subsequently cloned, expressed, and purified. An enzymatic assay for purified GST Pi was established using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. A pH-dependency study was performed, and the results indicated that the zebrafish GST Pi was active over a pH range of 6 and 8 with optimal activity observed at pH 7.5. The expression of novel zebrafish

GST Pi during various developmental stages from embryogenesis to maturity was examined via reverse transcription-polymerase chain reaction (RT-PCR).

Acknowledgments

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Introduction:

1.1 Overview of Drug Metabolism.

Historically, the enzymes that metabolize xenobiotics have been called the drug-metabolizing or detoxifying enzymes, since they are involved in the metabolism and excretion of many foreign chemicals to which humans are exposed. These enzymes are found in nearly all tissues in the body and the highest levels are often found in the gastrointestinal tract and liver. The liver is considered the major metabolic clearing house for many endogenous and xenobiotic compounds (Gonzalez and Tukey, 2006). Two phases, designated Phase I and Phase II, are usually involved in the biotransformation of xenobiotics. In Phase I, cytochrome P-450 monooxygenase enzymes are responsible for mediating the oxidation, reduction or dealkylation of xenobiotic compounds. These reactions will introduce functional groups, such as hydroxyl or amino group to the compounds being metabolized. The resulting products will undergo further metabolism by Phase II enzymes (Knapen et al., 1999).

Cytochrome P-450s (CYPs), the major group of Phase I enzymes, are a superfamily of heme-containing enzymes proteins that are responsible for the metabolism and biosynthesis of many endogenous and exogenous compounds (Marechal et al., 2008). CYPs are the main enzymes involved in drug metabolism and about 75% of the overall

metabolism is carried out by CYPs. Based on amino acid sequence homology, CYPs are grouped into families and subfamilies. The most active CYPs involved in drug metabolism are those belonging to CYP2C, CYP2D, and CYP3A subfamilies. CYP3A4 is most abundantly expressed in the liver and is involved in the metabolism of over 50% of clinically used drugs (Gonzalez and Tukey, 2006).

Phase II reactions usually result in the formation of more water-soluble and less biologically active forms by conjugating the compounds with hydrophilic moieties (Knapen et al., 1999). Phase II conjugation reactions include methylation, sulfation, acetylation, glucuronidation, and glutathione conjugation (Mulder, 1989). It should be pointed out that although the drug-metabolizing enzymes are important for facilitating the elimination of xenobiotics from the body, in some cases they may convert certain chemicals to highly reactive and carcinogenic metabolites (Brunton et al., 2006).

1.2 Phase II Conjugating Enzymes.

As mentioned above, Phase II conjugation reactions usually lead to the formation of highly polar, readily excretable, and pharmacologically inert conjugates. In some exceptions, however, certain drugs may actually be activated through conjugation reactions. Benzodiazepine, opiates, and paracetamol are examples of the substrates for Phase II enzymes (Kaeferstein, 2009). Major classes of the Phase II drug metabolizing enzymes are: UDP-glucuronosyltransferases (UGTs), cytosolic sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs), and methyltransferases. The most important methyltransferases involved in drug metabolism

are thiopurine *S*-methyl transferase (TPMT) and catechol *O*-methyl transferase (COMT) (Jancova et al., 2010). Figure 1-1 shows the involvement of Phase II drug-metabolizing enzymes in the metabolism of clinically used drugs. Because drug interactions involving Phase II enzymes are relatively rare, these enzymes have attracted less attention in clinical pharmacology, compared with CYPs (Jancova et al., 2010).

Glucuronidation is one of the most important detoxification pathway of the drug metabolism in vertebrates that is mediated by (uridine diphosphate)-glucuronosyl transferases (UGTs). These enzymes catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid to the drug to form the glucoronides that can then easily be eliminated via bile or urine. Many endogenous compounds, including bilirubin, thyroid hormones, steroid hormones, fat-soluble vitamins and bile acids, and xenobiotics, such as drugs, chemical carcinogens, environmental pollutants and dietary substances, are metabolized by UGTs (Jancova et al., 2010). Sulfation is another major conjugation reactions mediated by the cytosolic sulfotransferase (SULT) enzymes. SULTs catalyze the transfer of sulfate from the sulfate donar PAPS (5-phosphoadenosine-3-phosphosulfate) to many drugs and other xenobiotics, as well as endogenous compounds including neurotransmitters and thyroid/steroid hormones (Hebbring et al., 2008). N-acetyltransferases (NATs) are involved in the metabolism of drugs and environmental agents that contain an aromatic amine or hydrazine group. NATs catalyze the transfer of the acetyl group from acetyl-CoA to a free amino group of the acceptor compound. Drugs such as isoniazid (an antitubercular drug), hydralazine (an antihypertensive drug) and sulphonamides (an antibacterial drug) are mainly metabolized by NATs. Thiopurine

S-methyltransferase (TPMT) is important in the metabolism of thiopurine compounds such as 6-mercaptopurine, 6-thioguanine and azathioprine that are used in treatment of acute leukemia and autoimmune disorders (Jancova et al., 2010). Catechol *O*-methyl transferase is responsible for methylation of catecholamines including epinephrine, norepinephrine and dopamine, as well as the metabolism of catecholic drugs such as L-dopa (used for treating Parkinson's disease) and methyldopa (used for treating hypertension) (Jancova et al., 2010).

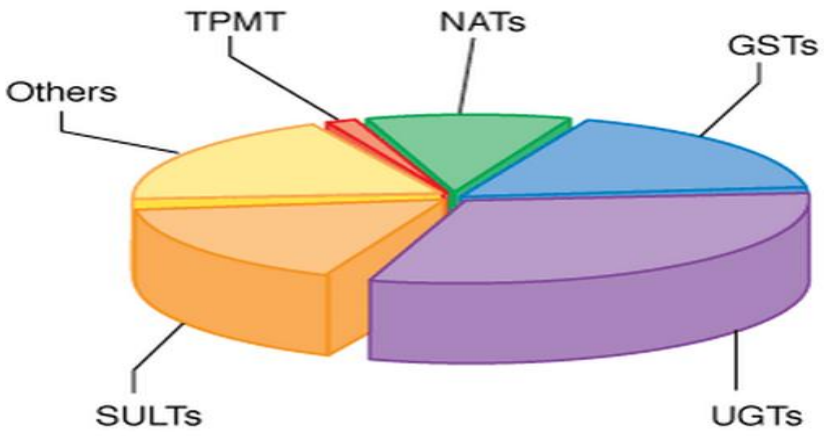

Figure 1-1: A schematic diagram showing the quantitative importance of major Phase II drug metabolizing enzymes in the metabolism of clinically used drugs (Gonzalez et al., 2011).

1.3 Glutathione conjugation and Glutathione S-transferases.

Glutathione transferases (GSTs) were first discovered in the early 60s (Booth et al., 1961). In 1991, the first GST structure was determined (Oakley, 2011). GSTs catalyze the conjugation of glutathione (GSH) with a wide range of exogenous and endogenous compounds containing an electrophilic center (Oakley, 2011; Morel and Aninat, 2011) (Figure 1-2). Xenobiotic (exogenous) compounds that are substrates for GSTs include carcinogens, therapeutic drugs, and environmental pollutants (Hayes and Pulford, 1995). They include also some harmful compounds such as reactive oxygen species (ROS) which are products resulting from oxidative stress. ROS has been proposed as the main causative agent for many diseases that afflict humans (Knapen et al., 1999).

GSTs have been found in many species including: humans, rats, bacteria, yeast, molds, insects, plants, fish, and birds (Hayes and Pulford, 1995). There are three distinct types of GSTs: cytoplasmic GSTs, mitochondrial GST, and microsomal GSTs (Flanagan and Smythe, 2011). Different GSTs have been classified into families based on their amino acid sequences. GST isozymes within a family typically share more than 40% identity, and between families, GST isozymes share less than 25% identity (Flanagan and Smythe, 2011). The cytosolic GSTs have been grouped into alpha, beta, delta, epsilon, zeta, theta, mu, pi, nu, sigma, tau, phi, and omega families (Oakley, 2011). The mitochondrial GST constitutes a single kappa GST family. The microsomal GSTs are membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) (Oakley, 2011).

According to the dendrogram shown in Figure (1-3), mammalian cytosolic GSTs can be separated into two main evolutionary groups. The first group includes sigma, alpha, mu

and pi families and they all display high affinity for 1-chloro-2, 4-dinitrobenzene (CDNB) and with tyrosine residue in the active center of the enzyme which functions to stabilize the GSH thiol group. The second group includes omega, zeta and theta families which display low affinity toward CDNB and lack the tyrosine residue in the active center of the enzyme so that the enzyme displays low activity with GSH (Dourado et al., 2008). The mammalian mitochondrial kappa GST family has only one single kappa GST member (Hayes et al., 2005). This mitochondrial GST was first isolated from the rat liver mitochondrial matrix (Morel and Aninat, 2011). Six families involved in MAPEG in humans have been identified which include: microsomal glutathione transferase (MGST) 1, 2 and 3, leukotrience C4 synthase (LTC4), 5-Lipoxygenase activating protein (FLAP) and prostaglandin E synthase (Morel and Aninat, 2011). Human GST alpha family (GSTA) includes at least five members, GSTA1, GSTA2, GSTA3, GSTA4, and GSTA5 (Hayes et al., 2005). GSTA1-1 and GSTA2-2 are highly expressed in liver, intestine, kidney, adrenal gland, and testis; while GSTA3 is expressed in steroidogenic tissues and GSTA4 is expressed in many other tissues (Coles and Kadlubar, 2005). While the human Pi family contains only one member (GST P1), there are five different Mu-members, M1, M2, M3, M4, and M5, that have been identified (Sheehan et al., 2001). GST P1 is highly predominant in the lung, breast, placenta, heart, and spleen and is also expressed in the pancreas, testes, and kidney (Salinas and Wong, 1999). In humans, there are two different members of the GST theta-family (hGST1 and hGST2) with only 50% sequences identity (Sheehan et al., 2001). Furthermore, GST omega-family contains two distinct members, GSTO1 and GSTO2, and GST zeta-family has only one member (GSTZ1) (Hayes et al., 2005). Only one sigma family GST (GSTS1) has been identified in humans and it was

found in high levels in the adipose, placenta, lung, and fetal liver (Flanagan and Smythe, 2011).

Most of the cytosolic GSTs catalyze conjugation reactions of xenobiotics (Oakley, 2011). Some of them are, however, also involved in the metabolism of endogenous compounds. For example, GST Pi has other functions beside its role in xenobiotic detoxification. GST Pi has been reported to be involved in the regulation of kinase activity by interaction with mitogen-activated protein kinase (MAPK) c-jun NH2 terminal kinase (JNK). GST Pi can act as a negative regulator for this kinase since the resulting GST Pi–JNK complex has no kinase activity. Oxidative stress can result in the dissociation GST Pi–JNK complex and the activation of JNK enzyme. The c-terminus of JNK is very important in the interaction with GST Pi. Cell proliferation is associated with the JNK phosphorylation and subsequent activation of C-Jun transcription factors. This might explain the overexpression of GST Pi in the cancer cells that acquired resistance to the cancer drugs. The GST Pi also catalyzes the formation of the disulfide bond between the basic cysteine and GSH. This is called S-glutathionylation cycle and is considered a post-translational modification of proteins that is vital for the cell. Those proteins that have been shown to be S-glutathionylated include receptors, enzymes, transcription factors, and transports proteins (Tew and Townsend, 2011).

The mammalian mitochondrial kappa GST family is mainly present in mitochondria and peroxisomes and is involved in energy production and lipid metabolism (Morel and Aninat, 2011). MGST1 is one of several MAPEGs in human that has a role similar to cytoplasmic and mitochondrial GSTs. MGST1 can catalyze conjugation reactions of

GSH to many electrophilic compounds. Besides catalyzing conjugation reactions, other MAPEGs such as LTC4 and FLAP are also involved in biosynthesis of leukotriene and prostaglandin (Oakley, 2011).

In contrast to mammalian GSTs, there have been only a few studies on zebrafish GSTs. One of these studies showed that when zebrafish embryos were exposed to atrazine, a widely used herbicide, GST activity was highly induced. GST mediated the conjugation of atrazine to GSH and conjugated atrazine was rapidly eliminated from the embryos (Wiegand et al., 2001; Zhu et al., 2010). In contrast, the activity of GST became lower in response to treatment with cyanobacterial lipopolysaccaride which resulted in the decrease of the detoxification capacity in the zebrafish embryos (Best et al., 2002). Transcription of GST Pi in zebrafish embryos is induced upon exposure to electrophiles. This induction has been shown to be mediated by the Nrf2 transcription factor that directly binds to proximal antioxidant-responsive element (ARE)-like sequence which is located in the transcription initiation site of the zebrafish gstp1 gene (Suzuki et al., 2005).

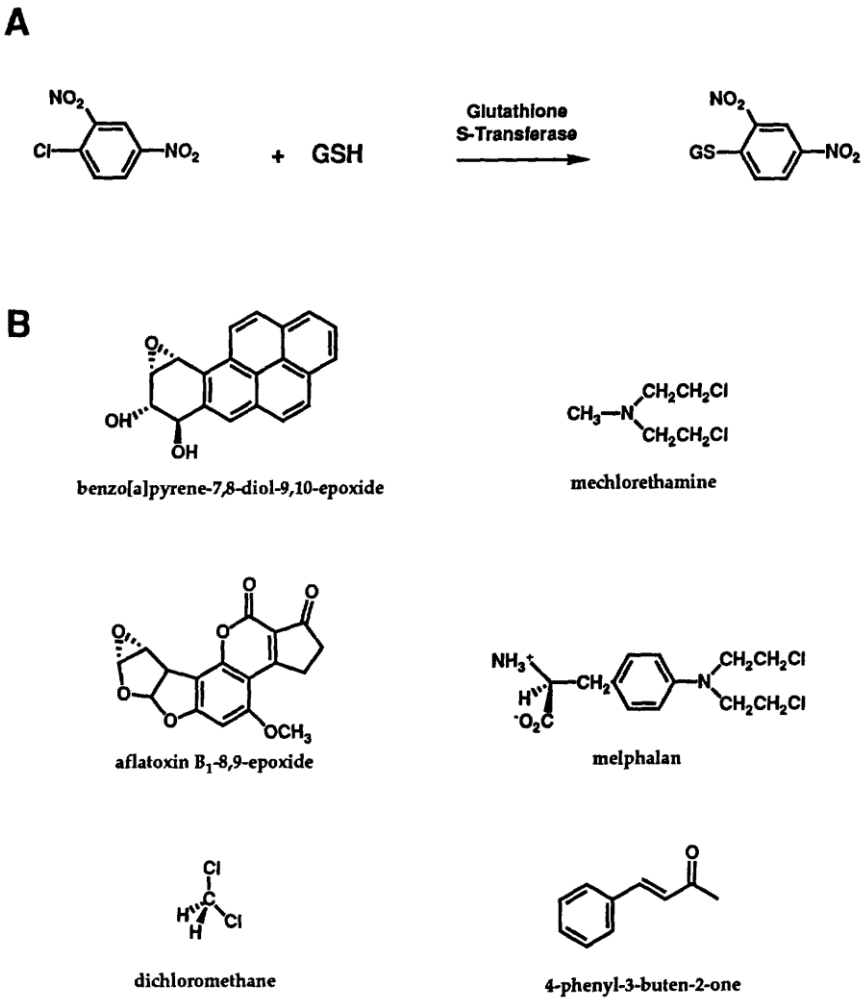

Figure 1-2: (A) A typical reaction catalyzed by glutathione S-transferase between a common substrate 1-chloro-2, 4-dinitrobenzene and glutathione (GSH), (B) Examples of GST substrates (Gulick and Fahl, 1995).

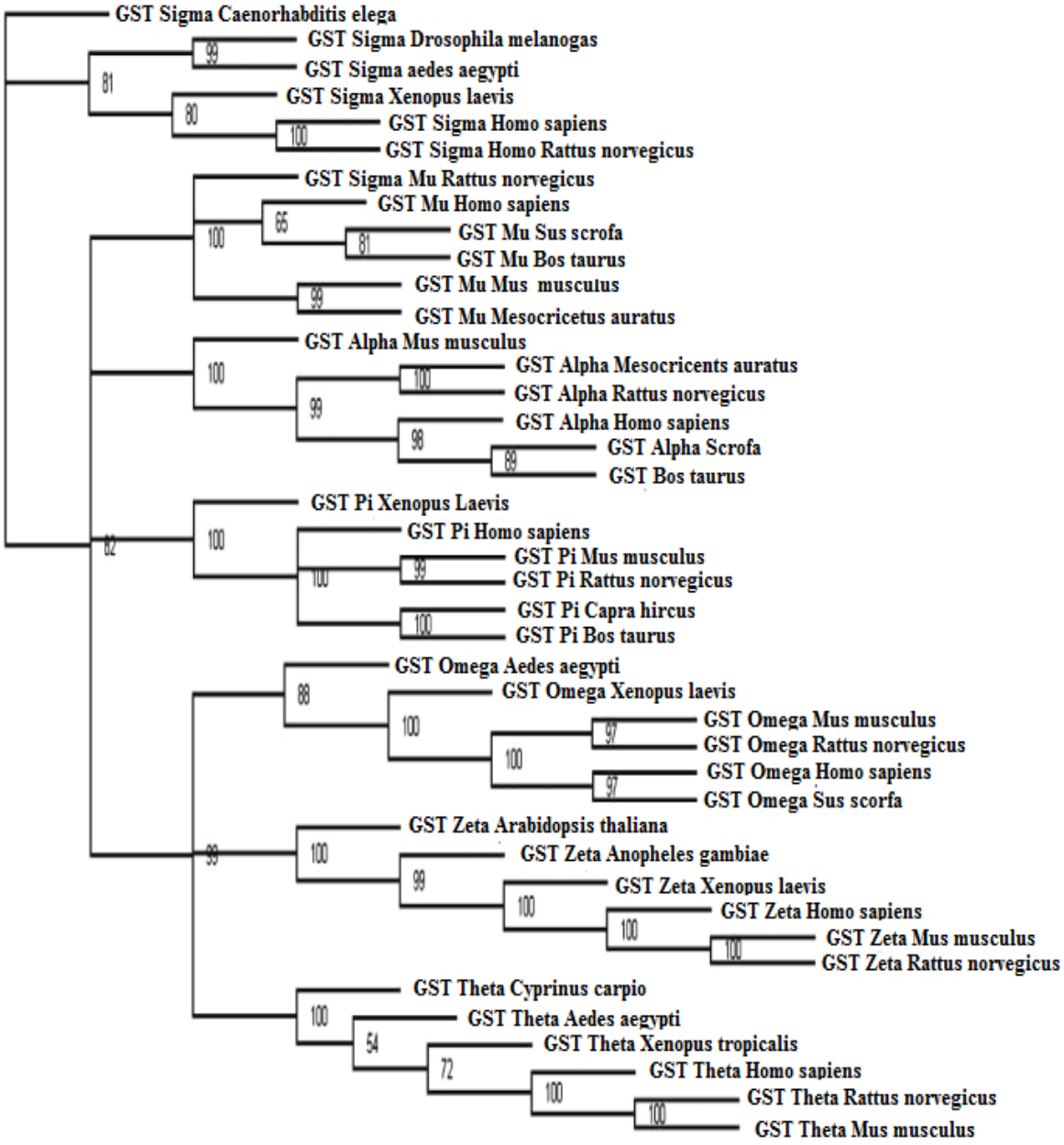

Figure 1-3: A phylogenetic tree showing different GST families in mammals (Dourado et al., 2008).

1.4 Physiological Roles of the Glutathione S-transferases.

1.4.1 The role of Glutathione S-transferases in Detoxification.

The critical role of GST is to conjugate the substrate compounds with GSH to produce less reactive metabolites that are more highly polar so as to be easily excreted. With a few GST substrates such as benzyl, phenyl and allyl isothiocyanate, the resulting GSH conjugates may instead be more reactive than the original compounds. This undesirable toxic conjugate had found its use in cancer chemotherapy (Hayes and Pulford, 1995). Tripeptide GSH (L-γ-glutamyl-L-Cysteinyl-glycine) has many important roles. As mentioned earlier, GSTs catalyze the conjugation of GSH with electrophilic chemicals to minimize the risks that may develop from the reactions of these chemicals with vital biomolecules like DNA. GSH has a sulfhydryl group which is responsible for its high nucleophilic potency. This nucloephilic potency allows the GSH to remove the hydrogen peroxide and other free radicals (Knapen et al., 1999). As an adaptive response to chemical stress, the expression of GST may be increased. It has been found that the GST can further induce the expression of other drug-metabolizing enzymes, such as UDP-glucuronosyl transferases and quinine reductase (Hayes and Pulford, 1995).

1.4.2 Other roles of Glutathione S-transferases.

As mentioned above**,** GSH has the capacity to scavenge hydrogen peroxides and other free radicals. This process is catalyzed by a number of GST isozymes, involving the reduction of organic hydroperoxides to their corresponding alcohols. Fatty acid,

phospholipid, and DNA hydroperoxides generated upon lipid peroxidation or oxidative DNA damage, are examples of substrates that are subject reduction by GST isozymes. Many GSTs have isomerase activity. For instance, some can catalyze the isomerization of maleyl-acetone to fumaryl-acetone and maleylacetonacetic acid to fumarylacetoacetic acid. This latter reaction takes place in the tyrosine degradation pathway in mammalian liver (Hayes and Pulford, 1995). GSTs are also involved in the biosynthetic pathways of many endogenous compounds such as leukotrienes and prostaglandins (Knapen et al., 1999). It has also been found that GSTs may serve as transport proteins to help carry a wide range of nonsubstrate chemicals across different organs, e.g., across the liver to be excreted in the bile (Hayes and Pulford, 1995). These chemicals include steroid and thyroid hormones, bile acids, bilirubin, fatty acids, hemoglobin, and penicillin (Knapen et al., 1999). GSTs can also bind to glutathione S-conjugates. There might be two significant reasons for this binding. First, it might protect the cell from toxicity by preventing reactive conjugates from interacting with vital molecules present in the cell. The second one is to prevent the unstable conjugates to cleave their harmful substrates that might react again with cell components (Hayes and Pulford, 1995).

1.5 Zebrafish as an animal model.

Compared with other animals that have been used for research, the zebrafish recently emerged as one of the most popular vertebral models (Liu et al., 2010). The zebrafish has a small size and short generation time. A female zebrafish can produce hundreds of eggs at weekly intervals and the embryos can develop rapidly within 2-4 days. Transparency of the zebrafish embryos makes them easier to study under the microscope (Wixon,

2000). Zebrafish require only a small housing area and they are easy to maintain and breed (Ma et al., 2011). Husbandry cost of zebrafish is relatively lower compared to other experimental animals like mice and rats (Lieschke et al., 2007). The high productivity of zebrafish provides a large number of subjects to investigate and at early larva stages, there is no gender difference (Berghmans et al., 2008). Embryos can easily absorb a small amount of tested drugs through skin and gills (Langheinrich, 2003). Earlier, zebrafish had been used as a developmental and embryological model because of optical clarity of embryos and larva **(**Lieschke et al**.,** 2007).

Because both zebrafish and humans have about the same number of chromosomes and many of the zebrafish genes are orthologous to their human counterparts, zebrafish is now considered as a good model for study of human genetic diseases (Postlethwait et al**.,** 2000). In addition, there are several genomic resources that are available for zebrafish, including high density genetic maps for mapping induced mutations and the sequencing of zebrafish genome can significantly facilitate the studying of disease-cause **(**Lieschke et al**.,** 2007). Zebrafish has been used as a model to study infectious diseases, cancers, cardiovascular diseases, and many other human diseases (Liu et al., 2010). Zebrafish embryos can be used in high-throughput screenings for identification of new pharmacologically active compounds by screening those embryos for specific effects following exposure to a library of low molecular weight compounds (Langheinrich**,** 2003).

1.6 Objectives and goals.

As previously stated, GSTs are critical for detoxification and metabolism of the environmental pollutants and chemicals for both zebrafish and humans. Furthermore, many zebrafish genes are orthologous to corresponding human genes. It is therefore of interest and importance to gain further insights into zebrafish GSTs. In this study, amino acid sequences were identified for fifteen zebrafish GSTs by searching the GenBank database and a dendrogram of these sequences was performed using available computer softwares. A novel zebrafish GST (designated GST Pi) was cloned, expressed, purified, and characterized. Moreover, its expression during embryonic and larval development onto maturity was investigated.

2. Materials and Methods

2.1 Materials.

1-Chloro-2, 4-dinitrobenzene (CDNB) was purchased from Acros Organics (Belgium, USA). 3-(Cyclohexyl amino)-1-propan sulfonic acid (CAPS) was from Research Organic Company (Cleveland, OH). Dimethyl sulfoxide (DMSO) was a product from EMD Chemicals (Billerica, MA). Ethylenediamine tetraacetic acid (EDTA) was purchased from Alfa Aesar Company (Ward Hill, MA). Potassium phosphate was from Fisher Scientific Company (Pittsburgh, PA). 2-(N-cyclohexylamino) ethane-sulfonic acid (CHES), glutathione (GSH), N-2- hydroxylpiperazine –N2-ethansulfonic (HEPES), 2-morpholinoethanesulfonic acid(MES), 3-(N-morpholino) propansulfonic acid (MOPS), sodium acetate , 3-[N-tris-(hydroxymethyl) methylamino[-propanesulfonic acid (TAPS), were products of Sigma Chemical Company (Saint Louis, MO). TRI Reagent was from Molecular Research Center, Inc,. (Cincinnati, OH). Total RNA from a 3-month-old female zebrafish was prepared using the TRI Reagent based on the procedure described previously (Sugahara et al., 2003a). *Taq* DNA polymerase was a product of Promega Corporation (Madison, WI). Takara Ex *Taq* DNA polymerase was purchased from Fisher Scientific (Pittsburgh, PA). Oligonucleotide primers were synthesized by MWG

Biotech (High Point**,** NC). pETBlue-1 AccepTor Vector Kit and BL21 (DE3) competent cells were fromNovagen (Madison, WI). Glutathione-Sepharose 4B was a product of GE Healthcare (Swanton, OH).

2.2 Methods

2.2.1 Bioinformatics.

The Genbank Database at NCBI was searched to identify the amino acid sequences encoding different zebrafish GSTs. Sequence analysis by BLAST search using zebrafish GST Pi amino acid sequence was performed to identify the homology to various GSTs from different species. The dendrogram was constructed using Genebee computer algorithm.

2.2.2 Cloning, Bacterial Expression, and Purification of Recombinant Zebrafish GST Pi.

By searching the GenBank database, a sequence encoding a zebrafish GST Pi (GenBank Accession # AB194127) was identified. To generate the cDNA for subcloning into the pETBlue-1 vector, sense and antisense oligonucleotide primers designed based on 5`- and 3`-regions of the coding sequence were synthesized (Table 2.1). Using these primers, a PCR reaction was carried out under the action of EX *Taq* DNA polymerase, with the

first-strand cDNA reverse-transcribed from the total RNA isolated from a 3-month-old adult female zebrafish as the template. Amplification conditions were 2 min at 94°C and 19 cycles of 94°C for 30 sec, 60°C for 35 sec, and 72°C for 45 sec, followed by a 5-min incubation at 72°C. The final reaction mixture was applied into a 1% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product band detected was excised from the gel, and the DNA therein was isolated by spin filtration. Purified PCR product was cloned into the pETBlue-1 vector and verified for authenticity by nucleotide sequencing. To express the recombinant zebrafish GST Pi, competent *Escherichia coli* BL21 (DE3) cells transformed with pETBlue-1 harboring the GST Pi cDNA were grown in 1 L LB medium supplemented with 60 μg/ml ampicillin. After the cell density reached 0.1 $OD_{600\ nm}$, IPTG (1 mM final concentration) was added to induce the production of recombinant zebrafish GST Pi. After an eight-hour induction at 37^{o}C, the cells were collected by centrifugation and homogenized in 25 ml ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using an Aminco French Press. Twenty μl of 10 mg/ml aprotinin (a protease inhibitor) was added to the crude homogenate. The crude homogenate was subjected to centrifugation at 10,000 x g for 15 min at 4°C. The supernatant collected was fractionated using 2.5 ml of glutathione-Sepharose, and the bound zebrafish GST Pi was eluted by a stepwise gradient (0, 0.1, 0.5, 1, 2.5, 5, 7.5, and 10 mM) of reduced glutathione in 50 mM Tris-HCl, pH 8.0. The recombinant zebrafish GST Pi was analyzed for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to enzymatic characterization.

2.2.3 Analysis of the development stage-dependent expression of the zebrafish GST Pi.

RT-PCR was employed to investigate the developmental stage-dependent expression of the zebrafish GST Pi. Total RNAs from zebrafish embryos, larvae, and adult (3-months-old male or female) fish at different developmental stages were isolated using TRI Reagent, based on manufacturer's instructions. Aliquots containing 5 μg each of the total RNA preparations were used for the synthesis of the first-strand cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). One microliter aliquots of the first-strand cDNA solutions prepared were used as the template for the subsequent PCR amplification. PCRs were carried out in 25 μl reaction mixtures using EX *Taq* DNA polymerase, in conjunction with gene-specific sense and antisense oligonucleotide primers (Table 2.1). Amplification conditions were 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 35 sec at 60°C, and 45 sec at 72°C. The final reaction mixtures were applied onto a 1% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. As a control, PCR amplification of the sequence encoding zebrafish β-actin was concomitantly performed using the above-mentioned first-strand cDNAs as templates, in conjunction with gene-specific sense and antisense oligonucleotide primers (Table 2.1) designed based on reported zebrafish β-actin nucleotide sequence (GenBank Accession No. AF057040).

Table 2.1: Oligonucleotide primers used for the cDNA cloning and RT-PCR analysis of the zebrafish GST Pi

Target sequence	Sense and antisense oligonucleotide primers used	
GST Pi	Sense:	5'-ATGGCTCCCTACACACTCACATACTTCGCAGTCAAAGGCA-3'
	Antisense:	5'-CGCGGATCCTTACTGTTTGCCGTTGCCGTTGATGGGCAGTTTCTTGA-3'
β-Actin	Sense:	5'-CGAGCTGTCTTCCCATCCA-3'
	Antisense:	5'-TCACCAACGTAGCTGTCTTTCTG-3'

*The sense and antisense oligonucleotide primer sets listed were verified by BLAST Search to be specific for the zebrafish GST Pi or β-actin nucleotide sequence.

2.2.4 Miscellaneous methods.

SDS-PAGE was performed on 12% polyacrylamide gels using the method of Laemmli (Laemmli, 1970). Protein determination was based on the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

2.2.5 GST Pi Enzymatic Assay.

The substrate, CDNB, was dissolved in DMSO, to form a 300 mM CDNB stock solution. Glutathione (GSH), used as a co-substrate, was dissolved in Tris-HCl, to form a 250 mM GSH stock solution. The protein concentration for GST Pi was 1µg/µl in the final reaction mixture. The reaction mixture, with a final volume of 1 ml, contained, 100 mM potassium phosphate buffer at PH 6.5, 1 mM EDTA, 2.5 mM GSH, and 3 mM CDNB. The reaction was started by adding 4.8 µl of the enzyme (GST Pi) to the reaction mixture. The formation of CDNB-GSH conjugate was measured using a Biospec-mini (Simadzu) spectrophotometer at 340 nm and the absorbance was recorded at one-minute interval for 10 minutes. The control sample was also prepared by using water instead of the enzyme. The specific activity was calculated, based on $\Delta\varepsilon$ =9.6 mM^{-1} cm^{-1} for CDNB-GSH, in the unit of µmol of the conjugated product/minute/mg enzyme for GST Pi.

2.2.6 pH-Dependency studies.

To analyze the pH profile for GST Pi, the GST Pi assay was carried out under above-mentioned assay conditions using CDNB as a substrate, but with different buffers replacing the potassium phosphate buffer. Different buffers used were: 250 mM sodium acetate at pH 5 and 5.5, MES at pH 6 and 6.5, HEPES at pH 7, 7.5, and 8, TAPS at pH 8.5, CHES at pH 9 and 10, and CAPS at pH 11.

3. Results.

3.1 Identification of zebrafish GST sequences.

By searching the GenBank Database at NCBI, 15 zebrafish sequences encoding putative GSTs were identified (Table 3-1). The dendrogram prepared using their deduced amino acid sequences (Figure 3-1) indicated that these fifteen zebrafish GSTs can be divided into three main groups. One group is consisted of omega, zeta and theta families, the second one included mu, pi and alpha families, and the third group contained only a single rho family.

Table 3.1: Putative zebrafish GSTs with their GenBank Accession #

GST type	Gene bank Accession #
GST Mu1	BC057526.1
GST Mu2	BC154218.1
GST Mu3	NM_001162851
GST Pi1	AB194127
GST Pi2	BC163150.1
GST Zeta1	NM_001030271
GST Omega1	NM_001002621.1
GST Omega2	NM_001007372
GST Alpha1	BC060914.1
GST Alpha2	BC150458.1
GST Alpha3	BC152202.1
GST Theta 1a	XM_687335.4

GST Theta 1b	BC056725
GST Theta 2	BC055612.1
GST Rho	BC139572.1

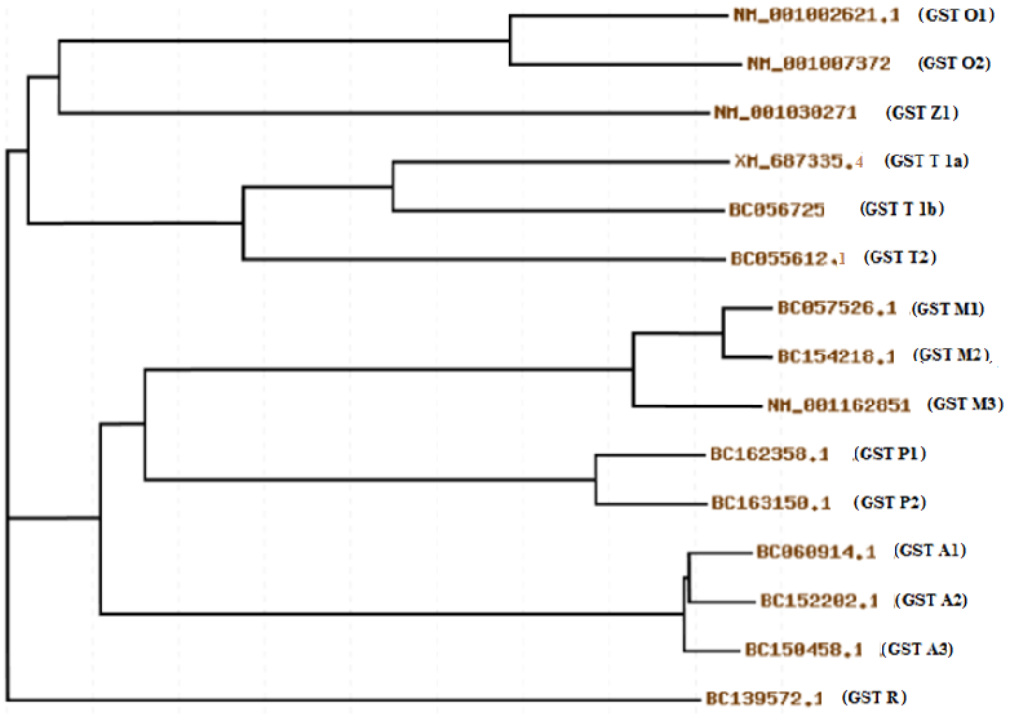

Figure 3-1: Classification of the Zebrafish GSTs based on their Amino Acid Sequences. The dendrogram was generated based on Greedy algorithm (Nikolaev et al., 1997).

3.2 Molecular Cloning, Expression, and Purification of the Zebrafish GST Pi.

By searching the GenBank database, a sequence encoding a zebrafish GST Pi (GenBank Accession # AB194127) was identified. Based on the sequence information, we designed and synthesized oligonucleotide primers corresponding to on 5`- and 3`-coding regions of the sequence. The first-strand cDNA was reverse-transcribed from the total RNA isolated from a 3-month-old adult female zebrafish. Using the first-strand cDNA as a template, cDNA encoding the zebrafish GST Pi was PCR-amplified. Purified PCR product was cloned into the pETBlue-1 vector and verified for authenticity by nucleotide sequencing. pETBlue-1 harboring the GST Pi cDNA was transformed into competent *Escherichia coli* BL21 (DE3) cells for expression of recombinant enzyme. The resulting PCR product was subject to nucleotide sequencing to confirm its authenticity. Figure 3-2 shows the nucleotide and deduced amino acid sequences of the newly cloned zebrafish GST Pi. Recombinant zebrafish GST pi was purified from *E. coli cell extract.* ST9). Figure 3-3 shows the SDS gel electrophoretic pattern of purified recombinant zebrafish GST Pi. Compared with the molecular weight markers co-electrophoresed, zebrafish GST Pi migrated at approximately 27 KDa position. This result is in agreement with the predicted molecular weight of 23529.61 of GST Pi based on its deduced amino acid sequences. Sequence analysis based on the BLAST pair wise search showed that the deduced amino acid sequence of the zebrafish GST Pi displays 60% amino acid sequence identity to mouse and human GST Pi, 61% to camel GST Pi, 71% to The Atlantic salmon GST Pi, 85% to common carp fish GST Pi, 89% to Gobio fish GST Pi, and 90% to silver carp fish GST Pi.

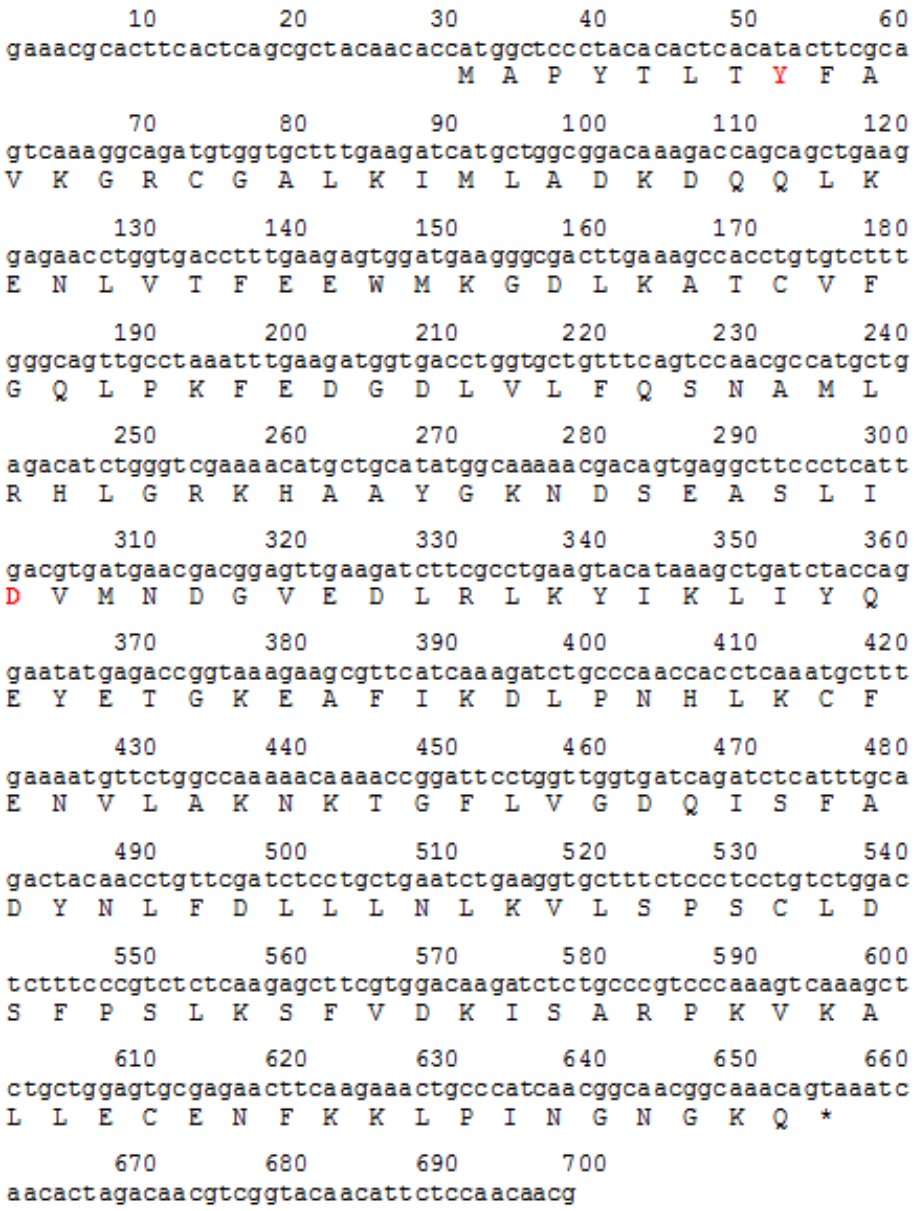

Figure 3-2: The nucleotide and deduced amino acid sequences of the zebrafish GST Pi.

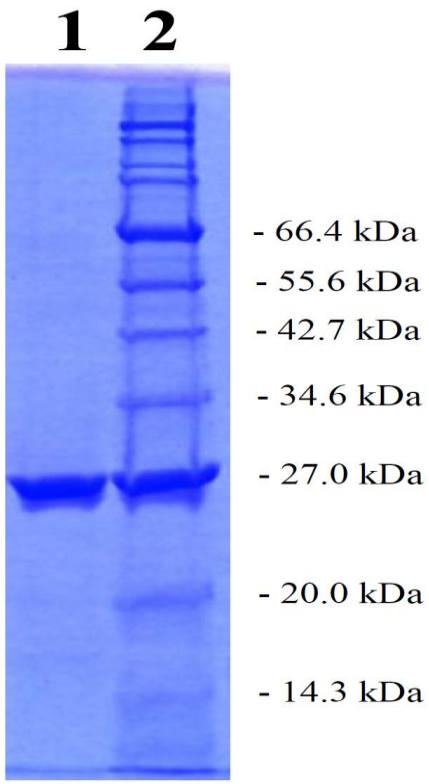

Figure 3-3: SDS-PAGE of purified zebrafish GST Pi.

3.3 Analysis of the development stage-dependent expression of the zebrafish GST Pi

The expression of the mRNA encoding GST Pi throughout different developmental stages was examined by employing the RT-PCR technique (Figure 3-4). Results from RT-PCR showed that there was no expression of the GST Pi mRNA in the unfertilized egg (lane1). Following fertilization, the expression of the GST Pi mRNA started and the levels of expression remained constant throughout the early embryonic development until the blastula period (3-h pf; lane 4). The level of GST Pi mRNA started to decrease in

gastrula period (6- h pf; lane 5), segmentation period (12-h pf; lane 6), and pharyngula period (24-h pf; lane 7). At hatching stage, the level of the GST Pi mRNA increased again (48- h pf; lane 8) and this increase lasted until the late (4-week) larval stage (lane13). Interestingly, in the adult zebrafish, the GST Pi appeared to be expressed at a very low level in male fish (lane 14), in contrast to the significantly higher level detected in female fish (lane15). β-actin, a housekeeping protein, was used as a control and it was found to be constantly expressed throughout the all developmental stages (Figure 3-5).

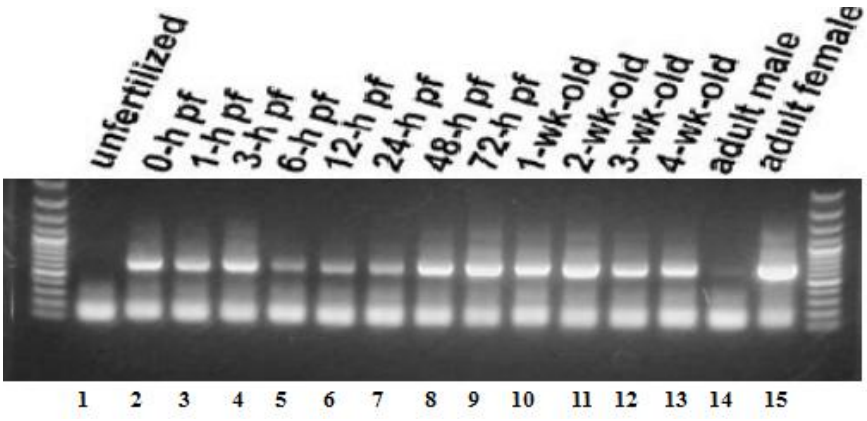

Figure 3-4: RT-PCR analysis of the expression of mRNA encoding GST Pi during the zebrafish development.

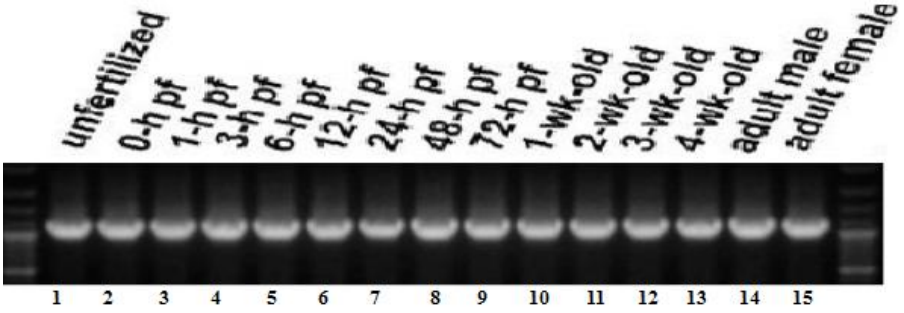

Figure3-5: RT-PCR analysis of the expression of the zebrafish β-actin during the zebrafish development.

3.4 The pH-Dependency studies.

The pH-dependency of GST Pi was examined using CDNB as a substrate. Results from the pH-dependency studies revealed that GST Pi had no activity at pH 5 and 5.5. The activity was detected at pH 6 and continued to increase and reached the maximum at pH 7.5. Then the GST Pi activity decreased at pH 8 and disappeared at pH 8.5 and higher (Figure 3-6).

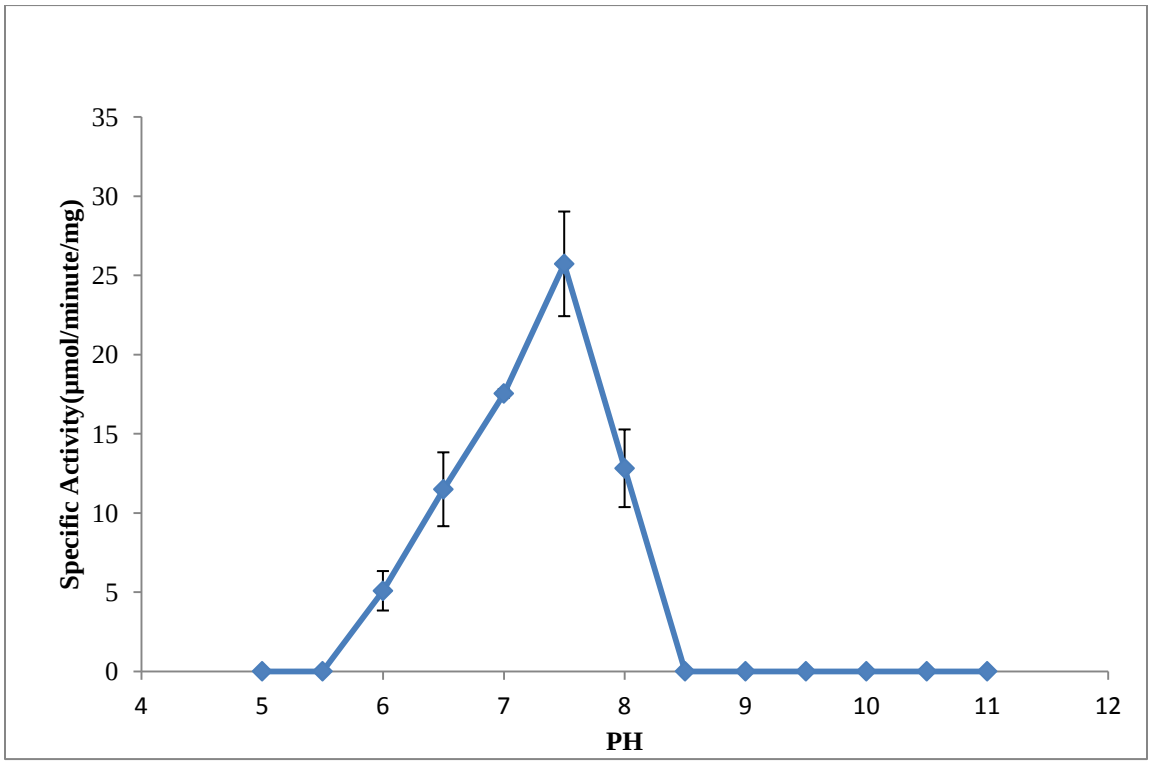

Figure 3-6: pH dependency profile of the glutathione S-transferase activity of the zebrafish GST Pi with CDNB as a substrate.

4. Discussion.

In the present study, fifteen zebrafish GST sequences were identified by searching the Genbank Database at NCBI website. The dendrogram of zebrafish GSTs (Figure 3-1) shows three groups, in contrast to two groups shown in the dendrogram of human GSTs (Figure 4-1). The first group of zebrafish GSTs includes omega, zeta and theta families; the second group includes mu, pi and alpha families; and the third group includes only a single rho family. For human GSTs (Figure 4-1), the first group includes six families: omega, zeta, theta, alpha, mu, and pi families; whereas the second group contains kappa, microsomal and prostaglandin E-synthase families. For both zebrafish and humans, the omega family has two members: omega 1 and omega 2, and the theta family also has two members: theta 1 and theta 2. The zebrafish theta 1 member contains theta 1a and theta 1b. While the zebrafish mu family has only three members: mu1, mu2, and mu3, the human mu family consists of five members: mu1, mu2, mu3, mu4 and mu5. The zebrafish pi family has two members: pi 1 and pi 2, but the human pi family only has one, designated simply as pi. The zebrafish alpha family consists of three members: alpha 1, alpha 2 and alpha 3. In contrast, the human alpha family consists of five members: alpha1, alpha 2, alpha 3, alpha 4 and alpha 5.

In regard to the structure, glutathione S-transferases in general contain two binding sites; a glutathione binding site (G-site) and a substrate binding site (H-site). There are a number of amino acid residues present at the G-site that are highly conserved among different GSTs and have been shown to be important for GSH binding. On the contrary, the residues that formed at the H-site are not highly conserved between different families (Gulick and Fahl, 1995). A global analysis of sequences and structural similarity of the cytosolic GSTs classified the GSTs into two major subgroups: Y-GSTs and S/C-GSTs. While the Y-GSTs use tyrosine residue in their interaction with GSH, the S/C-GSTs utilize serine or cysteine in their interaction with GSH (Oakley, 2011). Alpha, mu and pi GST families are Y-GSTs where the Tyr residue is conserved in the active site of the human and mouse GSTs. Tyr6 for human mu GSTs, Tyr7 for human pi GSTs and Try9 for human alpha GSTs (Salinas and Wong, 1999). Based on the amino acid sequence analysis, the conserved regions are found among the fifteen zebrafish GSTs enzymes. The conserved tyrosine residue, as mentioned above, is present in members of different families: Tyr7 for mu family, Tyr8 for pi family and Tyr9 for alpha family. Zebrafih has a rho family that is not found in humans, and this family also has a conserved Tyr at position 8. This conserved tyrosine is believed to be important for stabilizing GSH binding by forming a hydrogen bond with the sulfur atom of GSH (Gulick and Fahl, 1995). Omega family GSTs are grouped under C-GSTs since the active site of the human omega family has a Cysteine32 which can form a mixed disulfide bond with the sulfur group of GSH (Sheehan et al., 2001). This function is performed by Cysteince 31 of members of the zebrafish omega family. Theta and zeta families are categorized under S-GSTs where a serine residue is located in their active site to form hydrogen bonding with

GSH. The crystal structure of human zeta family GST has a serine14 in the active site of the enzyme (Sheehan et al., 2001). The serine residue is conserved at position 15 for zebrafish zeta family and zebrafish theta2 family, and position 16 for zebrafish theta1 family. In addition to the conserved tyrosine residue in the G-site of most of the GSTs, the G-site also contains a highly conserved aspartic acid at position 96 that forms a hydrogen bond with the GSH to further increase the GSH binding (Gulick and Fahl, 1999). For zebrafish pi and rho families, the conserved aspartic acid is located at 91 position. For zebrafish alpha, omega and zeta families the aspartic acid is located at 93; and for zebrafish mu family the aspartic acid is located at 98.

In this thesis research, a zebrafish GST, GST Pi, was cloned, purified, and characterized. The developmental expression of the zebrafish GST Pi mRNA was examined from embryogenesis to maturity by RT-PCR (Figure 3-4). Except the unfertilized eggs, the GST Pi mRNA was expressed throughout all developmental stages. Interestingly, the adult female showed a much higher expression of the mRNA than in the male. Previous studies have shown that GST Pi exhibited gender-related differences in various species. For example, female mouse ovary has been shown to have higher expression of GST Pi than male mouse testis (Mitchell et al., 1997). Conversely, female mouse liver expressed less GST Pi compared to male mouse liver (Singhal et al., 1992). Furthermore, the specific activity of female colon GST Pi towards CDNB was higher than male colon GST Pi which suggested the significant differences in GST Pi expression between male and female colon tissue (Singhal et al., 1992). In the pH-dependency study, the results indicated that the GST Pi was active over a pH range from 6 to 8, with maximum activity detected at 7.5 pH.

To summarize, by searching the Genebank Database at NCBI, amino acid sequences of fifteen zebrafish GSTs were identified in this study. For comparison purposes, a dendrogram was performed using computer software. One zebrafish GST, designated GST Pi, was identified, cloned, expressed, purified, and characterized. These results are expected to contribute to the establishment of the zebrafish as an animal model for investigating the Phase II metabolism of drugs and xenobiotics through glutathione conjugation.

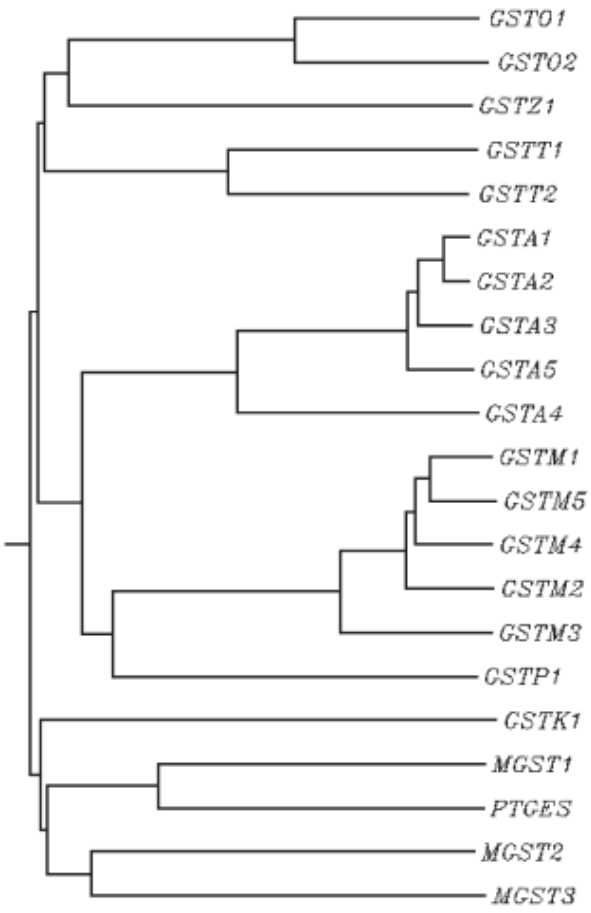

Figure 4-1: Dendrogram of the 21 human GSTs (Nebert and Vasiliou, 2004)

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