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# Identification, characterization, and ontogenic study of three novel zebrafish cytosolic sulfotransferases (SULTs)

Yasir Ihsan Mohammed  
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A Thesis

Entitled

Identification, Characterization, and Ontogenic Study of Three Novel Zebrafish  
Cytosolic Sulfotransferases (SULTs)

By

Yasir Ihsan Mohammed

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

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College of Graduate Studies

The University of Toledo  
May 2011

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An Abstract of  
Identification, Characterization, and Ontogenic Study of Three Novel Zebrafish  
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In mammals, sulfation as catalyzed by the cytosolic sulfotransferases (SULTs) is known to be involved in the metabolism and homeostasis of key endogenous compounds such as thyroid/steroid hormones, as well as in the detoxification of xenobiotics including drugs. The present study constitutes part of an overall effort in establishing the zebrafish as a model for studying drug sulfation. By searching GenBank database, we have identified sequences encoding three new zebrafish SULTs. These three novel zebrafish SULTs, designated SULT3 ST4, SULT3 ST5 and SULT1 ST9, were cloned, expressed, purified, and characterized. SULT3 ST4 showed strong activity toward endogenous compound such as dehydroepiandrosterone (DHEA), pregnenolone, and 17 $\beta$ -estradiol. SULT3 ST5 showed weaker, but significant, activities toward endogenous compounds such as DHEA and corticosterone, and xenobiotics including mestranol,  $\beta$ -naphthylamine,  $\beta$ -naphthol, and butylated hydroxyl anisole (BHA). SULT1 ST9, on the other hand, appeared to be mostly involved in the metabolism and detoxification of xenobiotics such as  $\beta$ -naphthol,  $\beta$ -naphthylamine, caffeic acid and gallic acid. pH-dependency and kinetic studies were

performed using these three enzymes with DHEA,  $\beta$ -naphthol,  $\beta$ -naphthylamine, and  $17\beta$ -estradiol as substrates. RT-PCR was carried out to investigate the expression of these three novel zebrafish SULTs during various developmental stages from embryogenesis to maturity.

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# **1. Introduction**

## **1.1 Overview of Drug Metabolism.**

Upon drug administration, the human body can metabolize and clear drugs through various biotransformation reactions. The metabolism of drugs involves the same enzymatic pathways and transport systems that are utilized in normal metabolism of dietary constituents. Many chemicals and xenobiotic (exogenous) compounds may come in contact with the human body through the exposure to the environmental contaminant and diet. The human body is equipped with various means to rapidly eliminate potentially harmful chemicals or xenobiotics. Drugs and other exogenous compounds, collectively called xenobiotics, are widely biotransformed in the body, which involves a large diversity of enzymes. Enzymes that metabolize xenobiotics have historically been called the drug-metabolizing enzymes, although they are involved in the biotransformation and excretion of many foreign chemicals to which humans are exposed (Gonzalez and Tukey, 2006).

## **1.2 Sites of Drug Metabolism.**

Enzymes that metabolize xenobiotics are present in most tissues in the body and the highest levels are found in the gastrointestinal tract and liver. Orally administered drugs are absorbed by the gut and taken to the liver, where the drugs can be extensively biotransformed and cleared. Most endogenous compounds (for example, cholesterol, steroid hormones, fatty acids, and proteins), and xenobiotics are metabolized by the liver,

which is considered as the major metabolic clearing house for these compounds (Gonzalez and Tukey, 2006).

### 1.3 The Two Phases of Drug Metabolism.

Historically, the drug-metabolizing enzymes have been categorized into two groups: Phase I and Phase II enzymes. Under normal circumstances, Phase I enzymes perform oxidation-reduction reactions or hydrolytic reactions, which may lead to the introduction of functional groups, such as -OH, COOH, -SH, -O- or NH<sub>2</sub> group, resulting in the modification of the drugs and other xenobiotics. The Phase II enzymes are involved in the conjugation reactions of the Phase I products. Through Phase II enzyme-catalyzed reactions, the water-solubility of the derivatized drugs or other xenobiotics will increase. The biological properties of the conjugated drugs may also be altered through Phase II reactions (Gonzalez and Tukey, 2006).

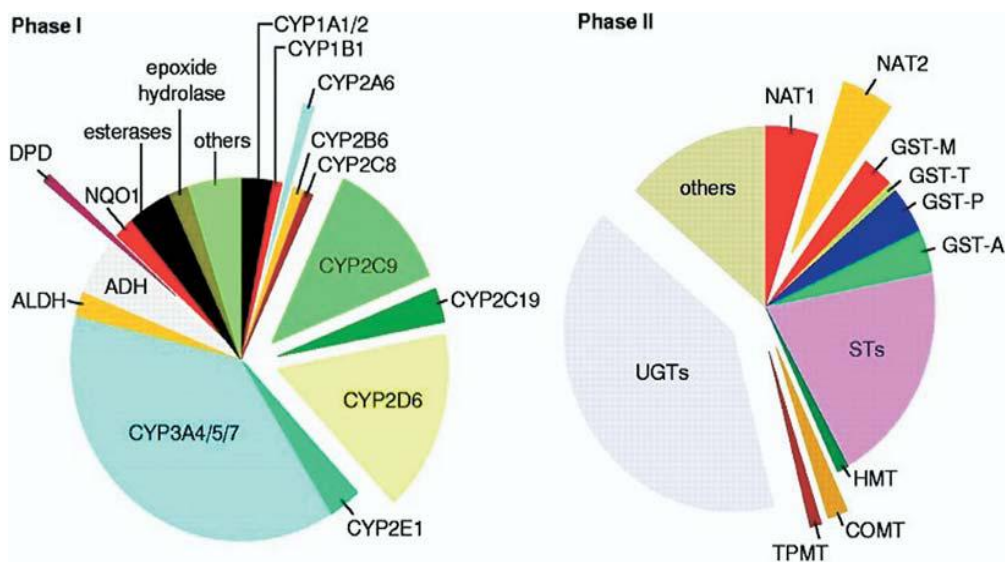


Figure 1-1: Drug-metabolizing enzymes involved in Phase I and Phase II metabolism (Juran *et al.*, 2006).

### 1.3.1 The Phase I Drug-metabolizing Enzymes.

The most common enzymes responsible for Phase I reactions are the cytochrome P-450 (CYPs) enzymes. Although these enzymes were first discovered in 1958, we still don't know completely how they work regarding the many fine details of their structure and function, despite a large number of studies that have been performed (Coleman, 2005). The CYPs constitute a gene family of heme-containing enzymes which are responsible for the biotransformation of a large variety of endogenous and xenobiotic compounds. More than 50 individual CYPs have been identified in humans and are classified into families depending on sequence homology (Blake *et al.*, 2005; Goodman and Gilman, 2006). Various CYPs families including CYP1, CYP2, CYP3, CYP4, CYP5, CYP7, CYP8, CYP11, and CYP17 are present in humans (Nishimura *et al.*, 2003). Most of the endogenous and xenobiotic compounds are metabolized by three families: CYP1, CYP2, and CYP3. Some distinct, but oftentimes overlapping, specificity of these enzymes towards substrates have been reported. The activities of CYPs can be affected by genetics, endogenous host, and environmental factors. These factors can lead to polymorphisms in metabolism between individuals (Pelkonen *et al.*, 2008). The substrates for CYP1 family tend to be planar molecules such as polycyclic hydrocarbons, and environmental pollutants including pesticide chlorpyrifos and coplanar polychlorinated biphenyls (PCBs). Some drugs such as acetaminophen and caffeine are metabolized by CYP1 family enzymes (Brown *et al.*, 2008). The CYP1 family can be classified into two subfamilies: CYP1A and CYP1B. Members of these subfamilies are CYP1A1, CYP1A2, and CYP1B1 (Omiescinski *et al.*, 1999). CYP1A1 is expressed in

many tissues but it is often detected upon induction with aryl hydrocarbon receptor as inducible gene or upon induction with 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD). CYP1A2 is mainly detected in the liver where it can be induced to a high level by aryl hydrocarbon receptor (Omiescinski *et al.*, 1999). CYP1B1 is a recently identified member and it is virtually expressed in nearly all human tissues such as colon and breast (Crofts *et al.*, 1997). CYP1A1 and CYP1B1 normally metabolize compounds like polyhalogenated aromatic hydrocarbons (Nebert and Dalton, 2006). The metabolism of these latter compounds may result in the formation of reactive metabolites which can irreversibly bind to cellular nucleophiles such as DNA (Shimada and Guengerich, 2006). The CYP2 family can be classified according to substrate classes into, for example, CYP2F1, CYP2E1, CYP2A6, and CYP2A13. Other enzymes including CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C18, and CYP2D6 have been shown to be involved in the metabolism of drugs (Brown *et al.*, 2008) Substrates of CYP2C8 and CYP2C9 in general have weakly acidic properties and multiple aromatic rings. Drugs such as ibuprofen and warfarin are metabolized by these enzymes, normally through hydroxylation. A particular substrate for CYP2C8, paclitaxel, undergoes a 6  $\alpha$ -hydroxylation and has been used as a substrate marker for this enzyme. A biologically active endogenous compound, arachidonic acid, is also metabolized to epoxyeicosatrienoic acid by CYP2C8. Tolbutamide is hydroxylated by CYP2C9 at the methyl group. CYP2C18 has low hepatic presence and its role in the metabolism of drugs is thus likely limited. CYP2D6 metabolizes drugs such as codeine and dextromethorphan, leading to their O-demethylation (Brown *et al.*, 2008). The genes encoding CYP2D6 and



CYP2C19 are highly polymorphic and different individuals exhibit differential drug metabolisms by these enzymes (Juran *et al.*, 2006). The CYP3 family contains probably the most important group of enzymes involved in the metabolism of the majority of xenobiotics (Brown *et al.*, 2008). The most clinically important of all CYP enzymes are the CYP3A subfamily enzymes, which can be found in many tissues particularly liver and small intestine, and account for about 30% of the total CYP content in the liver and intestine. About 50% of the most commonly used drugs are metabolized by the CYP3A4 subfamily of CYPs, which are considered the major hepatic cytochrome P-450 enzymes for Phase I metabolism (Blake *et al.*, 2005). Members of the CYP4 family are involved in metabolizing endogenous fatty acids and do not play a major role in the metabolism of xenobiotics (Brown *et al.*, 2008).

### **1.3.2 The Phase II Drug-Metabolizing Enzymes.**

Phase II reactions generally result in pharmacological inactivation and detoxification by conjugating xenobiotic compounds with chemical moieties like glucuronic acid, sulfate, acetyl group, etc. (Blake *et al.*, 2005). Some examples of the Phase II substrates are benzodiazepine, opiates, and paracetamol (Kaeferstein, 2009). In addition to their actions on xenobiotics, Phase II reactions may result in the conjugation of alcoholic or phenolic hydroxyl and amino groups of endogenous substrates (Kaeferstein, 2009). In Phase II reactions, the primary enzymes include UDP (uridine diphosphate)-glucuronosyl transferases (UGTs). UGTs are located in many tissues, with the highest concentrations being in the liver. UGT-mediated glucuronidation is one of the

main pathways of xenobiotic biotransformation. It occurs in the endoplasmic reticulum. Many endogenous compounds, including bilirubin, steroid hormones, and thyroxin, are metabolized by UGTs. Some xenobiotics, such as morphine, AZT, acetaminophen, ibuprofen and aspirin, are also metabolized by UGT. Glutathione S-transferases (GST) are also a major group of Phase II enzymes. Xenobiotics with electrophilic centers are directly subject to glutathione conjugation, due to their high susceptibility to nucleophilic attack by thiolate anion of glutathione. The result is a tripeptide conjugate which can be more easily eliminated. Glutathione can also conjugate reactive intermediates produced by CYPs and other enzymes. Glutathione therefore plays an important role in host defense mechanism. It is considered a scavenger of the electrophilic xenobiotics and their reactive metabolites (Kumar and Surapaneni, 2001).

#### **1.4 Sulfation and Sulfotransferases.**

The primary physiological function for conjugation reactions is the formation of highly soluble and easily excreted metabolites. The desired properties for the urinary and biliary excretion of metabolites are water-solubility and lack of passive penetration of cell membrane to avoid reabsorption (Glatt, 2000). One of the major conjugation reactions is sulfation. Sulfation was first reported in 1876 (Baumann, 1876) when phenyl sulfate was isolated from the urine of a patient being treated with phenol as an antiseptic. This discovery had positioned the research on sulfation and the sulfotransferase enzymes within the area of pharmacology/toxicology for over a century. Sulfation may result in the inactivation of the substrate compounds and/or increase their water-solubility, thereby

facilitating their removal from the body (Liu *et al.*, 2010). Many xenobiotics and endogenous compounds such as thyroid/steroid hormones, bile acid, lipids, and neurotransmitters undergo sulfation, leading to biotransformation and detoxification of these compounds (Gamage *et al.*, 2006). It had been found that many endogenous and xenobiotic compounds are excreted as sulfate esters in the urine. Nowadays it is recognized that by the enzymatic transfer of a sulfonate group from the cofactor 5-phosphoadenosine-3-phosphosulfate (PAPS) to a nucleophilic moiety such as oxygen moiety of the substrate, the sulfate ester is produced. The oxygen moiety can be generated via hydroxylation reaction during Phase I metabolism or may already be present in the xenobiotics. In addition to oxygen moiety, the sulfonate group can also be transferred to other moieties like nitrogen and sulfur, resulting in the formation of sulfamates and thiosulfates, respectively. Since the ionization upon sulfation for these molecules occurs at normal physiological pH, this leads to an increase in their water-solubility and a decrease in their penetration of cell membrane, and an increase in their urinary and biliary excretion (Glatt, *et al.*, 2000; Klaassen and Boles, 1997; Cho *et al.*, 2004). As mentioned above, sulfation is mediated by the cofactor PAPS which donates its sulfonate group to a substrate. The synthesis of PAPS is mediated by two consecutive enzyme activities, the ATP sulfurylase and the adenosine phosphosulfate (APS) kinase. First ATP sulfurylase produces APS from inorganic sulfate and ATP, which is then phosphorylated by APS kinase to yield PAPS (Figure 1.3) (Cho *et al.*, 2004).

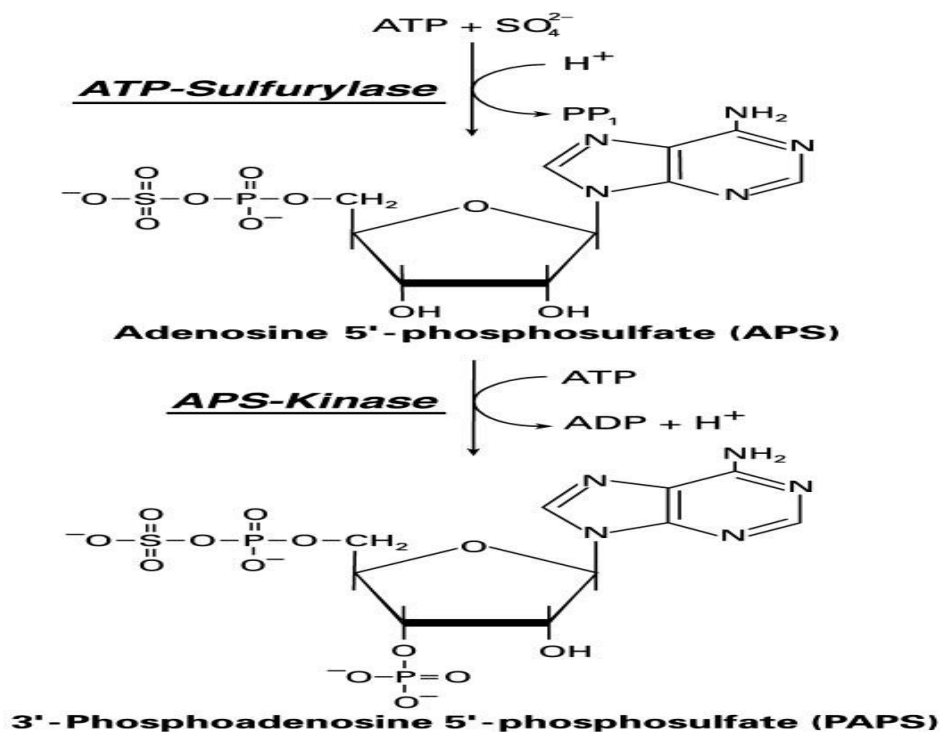


Figure 1-2: Two reactions involved in the synthesis of PAPS (Strott, 2002).

#### 1.4.1 Sulfation in Mammals.

Sulfation as mediated by the cytosolic sulfotransferases (SULTs) is considered as one of the most important Phase II detoxification pathways (Chen *et al.*, 2003). In mammalian species, the SULTs are distributed throughout the body, being present in the gut, liver, kidneys, adrenal glands, thyroid glands, lungs, reproductive organs, breast tissue, brain, and blood (Cole *et al.*, 2010). Besides their role in detoxification and biotransformation, SULTs have also been shown to be involved in the modulation and homeostasis of endogenous compounds such as thyroid and steroid hormones, catecholamine hormones/neurotransmitters, and cholesterol and its metabolites (Liu *et al.*, 2010). Sulfation of endogenous compounds and drugs and other xenobiotics may lead to the

decrease of their activities. In some unusual cases, however, sulfation of *N*-hydroxyl arylamines such as *N*-hydroxy-2-acetylaminofluorene may result in the formation of electrophiles, which may be mutagenic or cytotoxic (Falany and Kerl, 1990; Falany, 1991). The SULTs have been shown to be constitutive enzymes with the little known about the regulation of their enzymatic activities, except during early stage of development. Many gene families within the SULT gene superfamily have been categorized, based on the amino acid sequences of the identified SULTs (Sugahara *et al.*, 2003). In mammalian and avian species, more than 50 SULTs have been identified. These various SULTs have been cloned and sequenced. Between different SULT gene families, the SULT members have <40% sequence homology. Within each of subfamily, however, members display 60% or more homology in amino acid sequence (Nagata and Yamazoe, 2000; Nimmagadda *et al.*, 2006). Despite their similarity in amino acid sequence, different members appear to have different biological functions (Allali-Hassani, 2007). As shown in Figure 1.2, these enzymes can be subdivided into six families based on their amino acid sequences (Coughtrie, 2002).

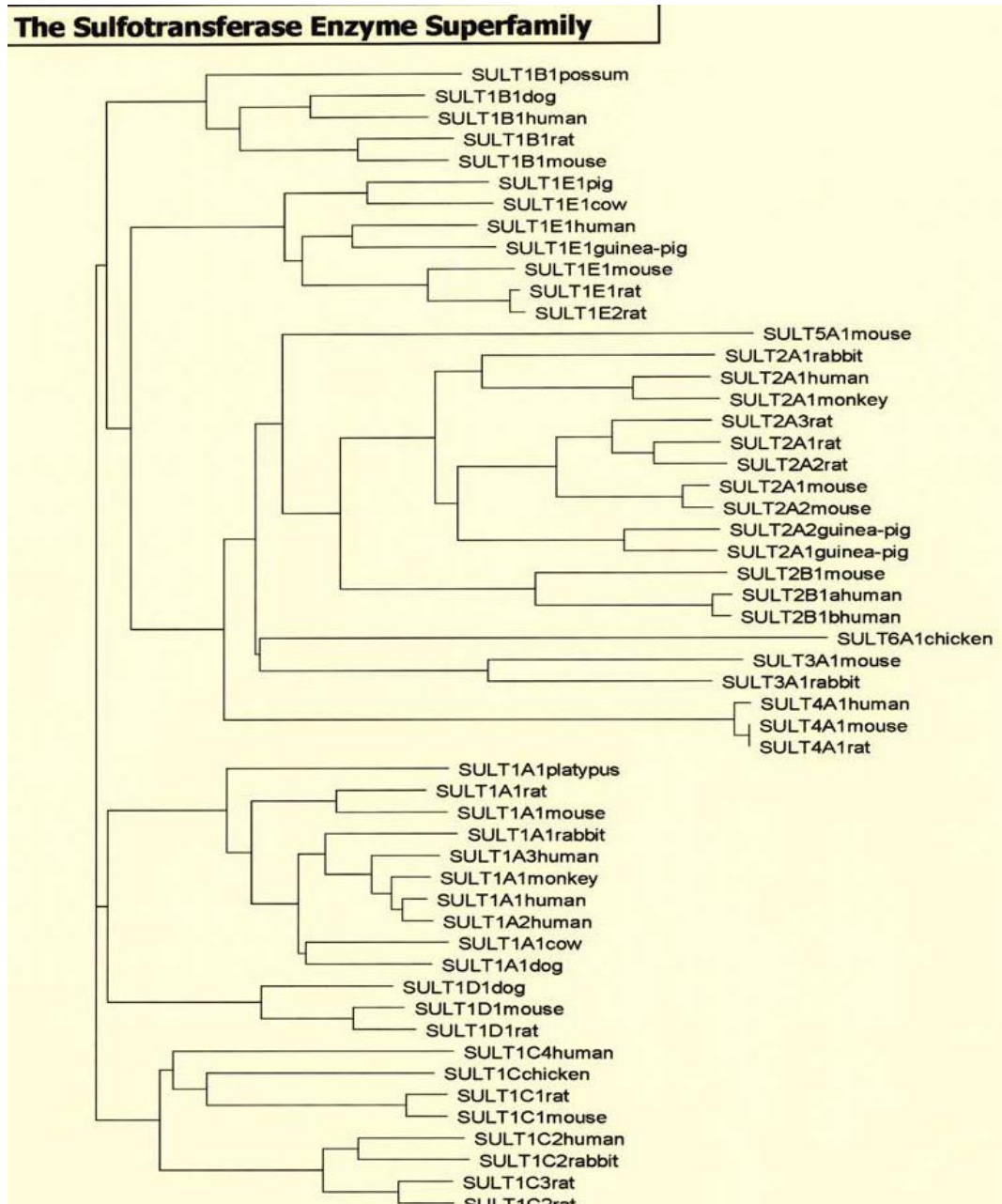


Figure 1-3: Classification of 53 members of mammalian and avian SULT gene (Coughtrie, 2002).

Five SULT families have been categorized in mammals: SULT1, SULT2, SULT3, SULT4, and SULT5 (Gamage *et al.*, 2005). Four major SULT families, SULT1, SULT2, SULT4, and SULT6 have been detected in human (Allali-Hassani, 2007). In humans, thirteen distinct SULT members have been identified and partitioned into these families. Two of the mammalian SULT families (SULT1 and SULT2) have been subjected to more extensive investigation. SULT1 family includes SULTs capable of sulfonating phenolic compounds including catecholamines (Strott, 2002). The SULT1 family can be divided into five sub-families. SULT1A represents phenolic SULT. SULT1B represents dopa/tyrosine (thyroid hormone) SULT. SULT1C can sulfonate hydroxyarylamine (acetylaminofluorene). SULT1D represents tyrosine ester SULT, and SULT1E can sulfonate estrogens (Yasuda, *et al.*, 2009). SULT1A isoforms are located in different tissues. High levels of SULT1A1 are found in the liver, while high levels of SULT1A3 are detected in the intestine, jejunum, and brain. Both SULT1A1 and SULT1A3 are present in lung, platelets, endometrium, and adrenal gland. Recently it has been demonstrated that both SULT1A1 and SULT1A3 are extensively distributed within developing human fetal brain and in neurotransmitter such as cholinergic, glutaminergic, GABA and  $\sigma$  opioid receptors (Gamage *et al.*, 2005). SULT1A2 has a low level of presence in liver (Glatt *et al.*, 2001). No endogenous substrates have been found for this enzyme, however. Furthermore, no equivalent form of this enzyme has been detected in species other than humans (Gamage *et al.*, 2005). Other enzymes such as SULT1B, SULT1C and SULT1E are found in certain tissues/organs including stomach, intestine, colon, liver kidney, and thyroids (Gamage *et al.*, 2005). Members of the SULT2 families

are found in tissues which are hormone-responsive and play roles in the metabolism of hydroxysteroids such as dehydroepiandrosterone (DHEA) and structurally-related compounds (Gamage *et al.*, 2005). SULT2 gene family consists of 3 subfamilies. SULT2A1 is highly expressed in liver, small intestine, and adrenal cortex. Many orally administered compounds are sulfated by SULT2A1 such as DHEA (Thomae *et al.*, 2002). SULT2B1a is localized in prostate and placenta, and it catalyzes the sulfation of cholesterol. SULT2B1b catalyzes the sulfation of pregnenolone (Liu *et al.*, 2010; Gamage *et al.*, 2005). It is to be noted that some members of the SULT2 family can also metabolize some drugs such as 4-hydroxyl-tamoxifen which is also a substrate for some SULT1 isoforms (Falany *et al.*, 2006). Of the two remaining human SULTs, one (a neuronal/brain SULTs) belongs to the SULT4 gene family and the other, yet to be fully characterized, belongs to the SULT6 family (Liu *et al.*, 2010; Falany, 2000).

### **1.5 Zebrafish as an Animal Model.**

Different animals have been used as models for research in different areas. Recently, the zebrafish has emerged as a popular animal model (Liu *et al.*, 2010). Compared with other animal models, the advantages of the zebrafish model include easy maintenance and breeding, requirement for only a small housing area, and short generation time (about 3 months). A female zebrafish can produce 100-200 eggs/day, which allows for performing assays needing a large number of experimental subjects. The embryogenesis of zebrafish is rapid, with the entire body plan established by 24 hrs post fertilization (hpf). The internal organs of zebrafish including heart, liver, intestine



and kidney are fully developed by 96 hpf (Ma *et al.*, 2003). This rapid development of the zebrafish is in contrast to the development of human embryo which takes ~3 months. Other advantages of the zebrafish model include the small in size, transparent embryo, and the simplicity of phenotypic analysis. With all these unique features, the zebrafish can be an excellent model for a systematic research of development stage-dependent and cell type/tissue/organ-specific expression as well as the physiological involvement of the SULTs (Yasuda *et al.*, 2008; Liu *et al.*, 2010). Zebrafish are tolerant to DMSO and readily can absorb compounds from water (Paul Goldsmith, 2004). Zebrafish can also be subjected to chemical mutagens. As a result, many mutants can be produced rapidly (Gerlai, 2003). It has been found that many genes in the zebrafish are evolutionarily conserved and include homologs found in mammals including human. This feature makes the zebrafish a good model for genetic studies. The various advantages of the zebrafish as an animal model are summarized in Table 1.

Table 1.1: Characteristics of the zebrafish as an animal model:

<b>Characteristics</b>	<b>Advantages</b>	<b>References</b>
High stocking density	Zebrafish are particularly genetically tractable.	Lieschke and Currie, 2007
Transparent embryo and Larva	The transparent embryo develops quickly outside the mother's body which helps the study of developmental stages of embryo	(Langheinrich, 2003)

	<i>in vivo.</i>	
Similar chromosome numbers with human	Zebrafish and humans have approximately the same number of chromosome (23 for human and 25 for zebrafish) which can reveal conserved gene functions and can be used as model for human disease and congenital malformation.	(Postlethwait <i>et al.</i> , 2000)  (Hill <i>et al.</i> , 2005)
Embryo and larval size	Embryo and larvae are small enough to use small amount of the drug (the small amount of the drug needed to be tested)	Langheinrich, 2003)
High fecundity	Allows the analysis of large numbers of animals	(Berghmans <i>et al.</i> , 2008)
Husbandry costs	Cost of zebrafish is relatively lower than other animals and the logistics and the cost of a screen that concentrate on one disease are not as high-priced as mouse.	Lieschke and Currie, 2007
Absorption of compounds from water across the skin at early life-stages	Small molecule compounds can be easily dissolved in fish water and diffuse into embryo.	(Ma <i>et al.</i> , 2003)

The above-mentioned characteristics therefore make the zebrafish a suitable model organism for genetic studies and research of the ontogeny, cell type/tissue-specific expression, and physiological involvement of the SULTs (Liu *et al.*, 2010)

### **1.5.1 Sulfation and the SULTs in Zebrafish.**

The goal of my thesis research is to identify and characterize novel zebrafish SULTs. In previous studies, fifteen zebrafish SULTs have been cloned, expressed, purified, and characterized. The sequence analysis by BLAST search revealed that these cloned zebrafish SULTs displayed considerable sequence homology to mammalian SULTs. Of the fifteen zebrafish SULTs that have been cloned, eight of them belong to SULT1 gene family, three belong to SULT2 gene family, two belong to SULT3 gene family and one appears independent from all identified SULTs (Yasuda *et al.*, 2006; Liu *et al.*, 2010). *In vitro* studies have been performed on members of the SULT1, SULT2 and SULT3, SULT4, and SULT6 families, with regard to their amino acid sequences and substrates specificity for various endogenous and xenobiotic compounds.

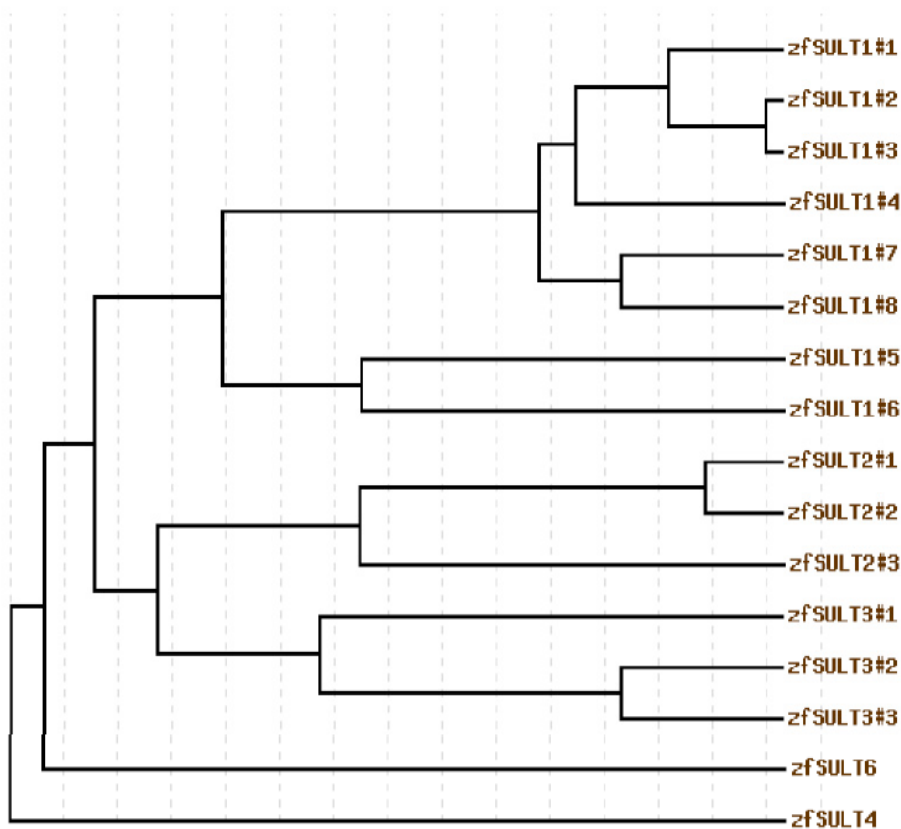


Figure 1-4: Classification of the Zebrafish SULTs based on their Amino Acid Sequences. (Liu *et al.*, 2010).

As mentioned above, different xenobiotic substrates including dietary, environmental and industrial compounds, are widely sulfated by human and other mammals SULT1 enzymes. The zebrafish SULT1 (ST1, ST2, ST3, ST4, ST5 and ST6) play a vital role in the metabolism and detoxification of xenobiotics such as hydroxylated PCBS (3-chloro-4 biphenylol and 3,3',5,5'-tetrachloro-4,4'-biphenyldiol. Drugs such as acetaminophen can also be sulfated by zebrafish SULT1 (Liu *et al.*, 2010). SULT1 isoform 2 displays strong sulfating activities towards estrone and 17 $\beta$ -estradiol. Similar to human SULT1A1, the zebrafish SULT1 isoform 2 showed sulfating activities to some endogenous compounds

such as L-DOPA, and thyroid hormone, in addition to xenobiotics (Yasuda *et al.*, 2005). Zebrafish SULT2 enzymes showed no activity towards xenobiotic and are active with endogenous compounds such as DHEA, corticosterone, and pregnenolone. In contrast to SULT2, SULT3 enzymes showed strong activities not only to endogenous compound, but also to xenobiotics including xenoestrogens (17  $\alpha$ -ethynylestradiol and diethylstilbestrol), as well as drugs such as dextrorphan and minoxidil (Liu *et al.*, 2010).

### **Objectives and Goals.**

As described above, recent studies indicated that the zebrafish SULTs are similar to many human SULTs in metabolism and detoxification of endogenous and xenobiotic compounds. Fifteen zebrafish SULTs have been identified, purified and characterized. In this research, three new zebrafish SULTs (designated SULT3 ST4, SULT3 ST5, and SULT1 ST9) have been identified, cloned, expressed, purified, and characterized. Ontogenic study was performed to reveal the expression of each of these novel SULT enzymes at different stages during the zebrafish developmental.

## 2. Material and Method

### 2.1. Materials.

Dehydroepiandrosterone (DHEA),  $17\beta$ -estradiol,  $17\alpha$ -ethynylestradiol, estrone, butylated hydroxyanisole (BHA), caffeic acid, gallic acid, mestranol, chlorogenic acid,  $\beta$ -naphthylamine, and  $\beta$ -naphthol, 3-(N-morpholino) propanesulfonic acid (MOPS), 2-morpholinoethanesulfonic acid (MES), N-2-hydroxylpiperazine-N2-ethanesulfonic (HEPES), 3-[N-tris-(hydroxymethyl) methylamino]-propanesulfonic acid (TAPS), 2-(cyclohexylamino) ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), were products of Sigma Chemical Company (St. Louis, MO). Ecolume scintillation cocktail, progesterone, pregnenolone, corticosterone, androstene-3,17-dione, carrier free sodium [ $^{35}\text{S}$ ]sulfate were products of MP Biomedical (Solon, OH). Cellulose thin-layer chromatography (TLC) plates were product from EMD chemicals (Gibbstown, NJ). Recombinant human bifunctional ATP sulfurylase/adenosine 5'-phosphosulfate kinase was prepared as previously described (Yanagisawa et al., 1998). TRI reagent was prepared from Molecular Research Center, Inc. Unfertilized zebrafish eggs, embryos, and larvae at different developmental stages from Scientific Hatcheries. Total RNA was prepared from different developmental stages of zebrafish eggs, embryos, larvae, and 3-month old fish. Tag DNA polymerase was a product of Promega Corporation. Oligonucleotide primers were generated by MWG Biotech.

## 2.2 Cloning, Bacterial Expression, and Purification of Recombinant Zebrafish SULTs.

By searching the GenBank database, three zebrafish sequences (GenBank Accession # XM\_001919250 (SULT1 ST9), XM\_6950520 (SULT3 ST4), and CR\_936460 (SULT3 ST5)) encoding putative SULTs were identified. To generate corresponding cDNAs for subcloning into the pGEX-2T or pMAL-c5x prokaryotic expression vector, sense and antisense oligonucleotide primers designed based on 5'- and 3'- regions of the respective coding sequences were synthesized with *Bam* HI restriction site incorporated at the end (Table 1). Using these primer sets, PCRs were carried out under the action of *EX Taq* DNA polymerase, with the first-strand cDNA reverse-transcribed from the total RNA of a 3-month-old zebrafish as template. Amplification conditions were 2 min at 94°C and 20 cycles of 94°C for 35 sec, 60°C for 40 s, and 72°C for 1 min. The final reaction mixtures were applied onto a 0.9% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product bands detected were excised from the gel, and the DNAs therein were isolated by spin filtration. Purified PCR products were subjected to *Bam* HI restriction and subcloned into *Bam* HI-restricted pGEX-2T (for SULT3 ST5) or pMAL-c5x (for SULT1 ST9 and SULT3 ST4) vector, and verified for authenticity by nucleotide sequencing [Sanger et al., 1977]. It should be pointed out that recombinant protein expression using pGEX-2T or pMAL-c5x allows for the production of glutathione S-transferase or maltose-binding protein fusion protein which can be conveniently purified by affinity chromatography using glutathione-Sepharose or amylose resin. To express the recombinant zebrafish SULT3 ST4,

competent *Escherichia coli* BL21 (DE3) cells transformed with pGEX-2T harboring the cDNA encoding SULT3 ST5 were grown in 1 L LB medium supplemented with 60 µg/ml ampicillin. After the cell density reached 0.6 OD<sub>600 nm</sub>, IPTG (0.1 mM final concentration) was added to induce the production of recombinant protein. After an overnight induction at room temperature, the cells were collected by centrifugation and homogenized in 25 ml ice-cold lysis buffer (10 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) were 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 GST-fusion protein was treated with 3 ml of a thrombin digestion buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2.5 mM CaCl<sub>2</sub>) containing 5 U/ml bovine thrombin at room temperature. Following a 10-15-min incubation with constant agitation, the preparation was subjected to centrifugation. The recombinant zebrafish SULT3 ST4 present in the supernatant was analyzed for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to enzymatic characterization. To express the recombinant zebrafish SULT1 ST9 and SULT3 ST5, competent *Escherichia coli* BL21 (DE3) cells transformed with pMAL-c5x harboring the cDNA encoding SULT1 ST9 or SULT3 ST5 were grown in 1 L LB medium supplemented with 60 µg/ml ampicillin. After the cell density reached 0.6 OD<sub>600 nm</sub>, IPTG (0.5 mM final concentration) was added to induce the production of recombinant maltose-binding protein (MBP)-SULT fusion protein. After a 5-hour induction at 37°C, the cells were collected by centrifugation and homogenized in 25 ml



ice-cold lysis buffer using an Aminco French Press. Twenty  $\mu$ l of 10 mg/ml aprotinin (a protease inhibitor) were 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 amylose resin, and the bound MBP-SULT fusion protein. Upon washing with lysis buffer to remove unbound proteins, the MBP-SULT1 ST9 or MBP-SULT3 ST5 fusion protein was eluted from amylose resin using a stepwise gradient of maltose (1 mM to 50 mM) in 50 mM Tris-HCl, pH 8.0. The MBP-SULT1 ST9 or MBP-SULT3 ST5 fusion protein present in eluted fractions was analyzed for purity by SDS-PAGE and subjected to enzymatic characterization.

Table 2.1: Oligonucleotide primers used in the cloning and the RT-PCR analysis of the new zebrafish SULTs

Target Sequence	Sense and Antisense Oligonucleotide Primers	
SULT1 ST9	Sense	5'CGCGGATCCATGGAAATCCAAGGCAAATCCTCTACTGATTTA-3'
	Antisense	5'CGCGGATCCTCACTCAGTAGGGAACTTGAGAGTGGAATTCT-3'
SULT3 ST4	Sense	5'CGCGGATCCATGGCTCAAGAGGAATGCAA AATGATTAGTGAC-3'
	Antisense	5'CGCGGATCCTCAACTATGCAGTTCTGTGATGTCCCAGACCAG-3'
SULT3 ST5	Sense	5'CGCGGATCCATGGCTCAGGAGGAATGCAA AATGATTAGTGAC-3'

	Antisense	5' <u>CGCGGATCCTCAGCCACGCAGTTCTGTGAT</u> GTCCCAGACCAG-3'
β-Actin	Sense	5'-ATGGATGAGGAAATCGCTGCCCTGGTC-3'
	Antisense	5'-TTAGAAGCACTTCCTGTGAACGATGGA-3'

\*Recognition sites of *Bam* HI restriction endonuclease in the oligonucleotides are underlined. Initiation and termination codons for translation are in bold type.

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

### 2.3 Sulfotransferase Assay.

All compounds tested as substrates were dissolved in DMSO, and diluted to 50 μM final concentration in the final reaction mixture. The protein concentration for SULT3 ST4 (in thrombin-cleaved form) was 0.725 mg/ml, and the protein concentrations of SULT3 ST5 and SULT1 ST9 (in MBP fusion form) were 0.73 and 1.68 mg/ml, respectively. The assays for zebrafish SULTs enzyme were performed using radioactive PAP[<sup>35</sup>S] as the sulfate donor. The reaction mixture for the standard enzymatic assay, prepared in a final volume of 25 μl, contained, 50 mM MOPS at pH 7.0, 14 μM of PAP[<sup>35</sup>S], 1 mM DTT, and 50 μM substrate. Controls with water or DMSO replacing substrate were also included. The reaction was started by the addition of 2.5 μl of the enzyme (1.8125 μg of SULT3 ST4, 1.825 μg of SULT3 ST5, and 4.2 μg of SULT1 ST9), allowed to continue at 28°C for 5 minutes, and terminated by placing the tube containing the assay reaction mixture on a heating block at 100°C for 1 minute. Heated reaction mixture was subjected to centrifugation to pellet down the precipitates formed. Afterwards, 2 μl of the reaction mixture was spotted on a cellulose TLC plate and the

spotted TLC plate was subjected to TLC analysis using a solvent system containing n-butanol, isopropanol, 88% formic acid and H<sub>2</sub>O in a ratio of 3:1:1:1 (by volume). Upon completion of TLC, the TLC plate was air-dried and autoradiographed by using a classic X-ray film. The radioactive spot corresponding to the sulfated product was located and cut out and eluted in 0.5 ml water in a glass vial using an orbital shaker. Four ml of scintillation liquid was added to the vial, mixed thoroughly, and then the radioactivity was counted by using a liquid scintillation counter. The cpm count obtained was used to calculate the specific activity in the unit of nmol of sulfated product/minute/mg enzyme for SULT3 ST4 and SULT1 ST9 and pmol of sulfated product/minute/mg enzyme for SULT3 ST5. The detection limit for the specific activity was about 0.1 nmol/min/mg enzyme.

#### **2.4 pH-dependency Study.**

For each of the three SULT enzymes, an endogenous compound and a xenobiotic were used as substrates to analyze their pH profiles. DHEA and  $\beta$ -naphthol were tested as substrates for SULT3 ST4. 17 $\beta$ -estradiol and  $\beta$ -naphthol were tested as substrates for SULT1 ST9. DHEA was tested as a substrate for SULT3 ST5. Different buffers used were: 50 mM sodium acetate at pH 4.5, MES at 5.5 and 6.5, HEPES at 7.5, TAPS at 8.5, CHES at 9.5, CAPS at 10.5 and 11.5. The experimental conditions for pH-dependency assays were the same as mentioned above, except for the buffer and pH used.

## **2.5 Kinetic Studies.**

In kinetics studies, different concentrations of DHEA or  $\beta$ -naphthol were tested for SULT3 ST4, and different concentrations of  $17\beta$ -estradiol and  $\beta$ -naphthol were tested for SULT1 ST9. 50 mM MOPS buffer at pH 7.0 was used. DHEA was diluted in DMSO to prepare stock solutions of 0.00666 mM, 0.008 mM, 0.001 mM, 0.0133 mM, 0.02 mM and 0.04 mM for use as substrates for SULT3 ST4. Stock solutions of  $\beta$ -naphthol at 0.00666 mM, 0.008 mM, 0.001 mM, 0.0133 mM, 0.02 mM, 0.04 mM were prepared and used as substrates for SULT1 ST9. 0.25 mM, 0.5 mM, 1 mM, 2.5 mM, and 5 mM of  $\beta$ -naphthol were prepared and used as substrates for SULT3 ST4. 0.1 mM, 0.2 mM, 0.25 mM, 0.333 mM, 0.5 mM, and 1 mM of  $17\beta$ -estradiol were prepared and used as substrates for SULT1 ST9. The reactions were allowed to proceed for 5 minute at 28°C and terminated by heating at 100°C, followed by the subsequent TLC analysis of the [ $^{35}$ S]sulfated products.

## **2.6 RT-PCR Analysis for the Developmental Stage-dependent Expression of the Zebrafish SULT3 ST4, SULT3 ST5, and SULT1ST9.**

The developmental stage-dependent expression of the zebrafish SULT3 ST4, SULT3 ST5, and SULT1 ST9 was investigated by employing reverse transcriptase-polymerase chain reaction (RT-PCR). Using the TRI Reagent, the total RNA from zebrafish embryos, larvae, and adult (male/female) at various developmental stages were isolated. The first-strand cDNAs were prepared using the total RNAs isolated and used as templates for the subsequent PCR amplification. The PCR reaction mixture, with a final

volume of 25  $\mu$ l, contained 2  $\mu$ l deoxyribonucleotide triphosphate (dNTP), 2.5  $\mu$ l of 10 x PCR buffer, 1.25  $\mu$ l each of the sense and antisense oligonucleotide primers, 0.125  $\mu$ l *Ex Taq* DNA polymerase, and 16.875  $\mu$ l water. The PCR amplification conditions were 2 min at 94°C for initial denaturation, followed by 35 cycles of 30 sec at 94°C for denaturation, 35 sec at 56°C for annealing, and 1 min at 72°C for extension. The final PCR reaction mixtures were subjected to 0.9% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR amplification of the sequence encoding zebrafish  $\beta$  actin was concomitantly performed as a control using the above described first-strand cDNAs as templates.

## **2.7 Miscellaneous Methods.**

The sulfate donor, PAP [ $^{35}$ S], was synthesized from ATP and carrier-free [ $^{35}$ S] using the bifunctional human ATP sulfurylase/APS kinase. The synthesized PAP [ $^{35}$ S] was then adjusted to the desired concentration and specific activity by the addition of nonradioactive (cold) PAPS. SDS-PAGE was performed on 12% polyacrylamide gels using the method of Laemmli [1970] and protein determination was based on the method of Bradford [1976] with bovine serum albumin as a standard.

### **3. Results.**

#### **3.1 Molecular Cloning, Expression, and Purification of the Zebrafish SULT3 ST4, SULT3 ST5, and SULT1 ST9.**

As described in the Materials and Methods section, the cDNAs encoding the zebrafish SULT3 ST4, SULT3 ST5, and SULT1 ST9 were amplified by RT-PCR and cloned into the pGEX-2TK or pMAL-c5x prokaryotic expression vector, and subjected to nucleotide sequencing for verifying their authenticity. Figures 3-1 through 3-3 shows the nucleotide and deduced amino acid sequences of the three newly cloned zebrafish SULTs. The open reading frames of SULT3 ST4, SULT3 ST5 and SULT1 ST9 encompass, respectively, 900, 900, and 903 nucleotides and code for 299-, 299-, and 300-amino acid polypeptides. Similar to other SULTs, these three new zebrafish SULTs contain sequences resembling the so-called “signature sequences” (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region) characteristic of known SULT enzymes [Weinshilboum *et al.*, 1997]. Of these two sequences, YPKSGTxW has been demonstrated by X-ray crystallography to be involved in the binding to the 5'-phosphosulfate group of the co-substrate for SULT-catalyzed sulfation reactions, PAPS [Lipmann, 1958], and has been named the “5'-phosphosulfate binding (5'-PSB) motif” [Negishi *et al.*, 2001]. Sequence analysis based on a BLAST pairwise search revealed that the deduced amino acid sequence of the zebrafish SULT3 ST4 and ST5 display, respectively, 46 and 46% amino acid sequence identity to mouse SULT3A1, and 49 and 48% identity to rabbit SULT3A1. Between the two zebrafish SULT3 STs identified, 89% amino acid sequence identity was observed. It is noted that the newly

cloned zebrafish SULT3 ST4 and ST5 display only 33-37% amino acid sequence identity to the previously identified zebrafish SULT1 STs, and 39-40% amino acid sequence identity to the previously reported zebrafish SULT2 STs. Sequence analysis based on a BLAST pairwise search revealed that the deduced amino acid sequence of the zebrafish SULT1 ST9 displays 52 and 49% amino acid sequence identity to mouse SULT1D and human SULT1A3, and up to 84% amino acid sequence identity to other zebrafish SULT1 STs. pGEX-2TK or pMAL-c5x harboring zebrafish SULT3 ST4, SULT3 ST5, or SULT1 ST9 cDNA was transformed into *E. coli* BL21 (DE3) cells for the expression of recombinant enzyme. Recombinant zebrafish SULT3 ST4, SULT3 ST5, and SULT1 ST9 were purified from the *E. coli* cell extract as thrombin-digested tag-free enzyme (for SULT3 ST4) or MBP-fusion protein (for SULT3 ST5 and SULT1 ST9). As shown in Figure 3-4, upon SDS-PAGE, purified SULT3 ST4, SULT3 ST5, and SULT1 ST9 migrated at approximately 35, 75, and 75 kDa positions. Taking into consideration of the 40 kDa molecular mass of the MBP portion in the MBP fusion proteins, these results are also in agreement with the predicted molecular weight (35,217, 34780 and 34,731) of SULT3 ST4, SULT3 ST5, and SULT1 ST9 based on their deduced amino acid sequences.

**Zebrafish SULT1 ST9**

```

      10      20      30      40      50      60
ATGGAATCCAAGGCAAATCCTCTACTGATTTACCCGATCGACCTGAGATATTTGAATTT
M E I Q G K S S T D L P D R P E I F E F
      70      80      90     100     110     120
GAGGGTATCTCAATGGTTCGAGCACTTCACTAAGAAGTGGGAAAATGTGAAAACTTTCAA
E G I S M V E H F T K N W E N V K N F Q
      130     140     150     160     170     180
GCAAGACCTGATGACATTCTAATCGCCACTTACCCCAAAGCAGGCACAACCTGGGTTTCT
A R P D D I L I A T Y P K A G T T W V S
      190     200     210     220     230     240
AATATTCTAGACCTTCTGTATTTTGGTAAGGAAGATCCAAAGCGCCAGACCACCAAGCCT
N I L D L L Y F G K E D P K R Q T T K P
      250     260     270     280     290     300
ATCTATAAAAGAGTGCCATTCCTGGAGTCTTGTTTTCCAGAGATGCAATCAGGCACAGAA
I Y K R V P F L E S C F P E M Q S G T E
      310     320     330     340     350     360
CTGGCTAATAACCTGCCAACCTCTCCTCGCCTAATCAAACCTCATTACCTGTTCAACTT
L A N N L P T S P R L I K T H L P V Q L
      370     380     390     400     410     420
GTTCCCCAGTCCCTTCTGGGAGAAGAACTCAAGGGTGGCATATGTAGCTCGTAATGCAAAA
V P Q S F W E K N S R V A Y V A R N A K
      430     440     450     460     470     480
GACAACGCGGTTTCCTATTTTTCATTTTAATCGAATGAACAAGGCACAACCTGAACCAGGA
D N A V S Y F H F N R M N K A Q P E P G
      490     500     510     520     530     540
GACTGGAACACCTTCCTGGAAGAATTTATGAAAGGAAAAATGGTGTGTTTGGTTCTTGGTTC
D W N T F L E E F M K G K M V F G S W F
      550     560     570     580     590     600
GACCATGTCTGTGGATGGTGGGAGAAAAAAAAAACATATCCTAACTTACACTACATGTTG
D H V C G W W E K K K T Y P N L H Y M L
      610     620     630     640     650     660
TACGAGGATATGGCGAAAGACATTAAGGGCGAAGTAGAAAGTTTGTGCACTTTCTTAAAA
Y E D M A K D I K G E V E S L C T F L K
      670     680     690     700     710     720
TTGTCACGTTTCAGACGAAGAGAAGGAGAAAAATAATAAATGGCATTTCAGTTTGATGCCATG
L S R S D E E K E K I I N G I Q F D A M
      730     740     750     760     770     780
AAACAGAACAAGATGACCAATTACTCCACTGTCCTAGTGATGGATTTCCACAATCTCGCCG
K Q N K M T N Y S T V L V M D F T I S P
      790     800     810     820     830     840
TTCATGCGGAAAGGTAAAGTTGGAGACTGGAAAAATCACTTTACTGTGGCACAGAATGAA
F M R K G K V G D W K N H F T V A Q N E
      850     860     870     880     890     900
CAGTTCAATGAGGACTACAAACAGAAGATGAAGAATTCCTCAAGTTCCCTACTGAG
Q F N E D Y K Q K M K N S T L K F P T E
      910
TGA
*
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Figure 3-1: The nucleotide and deduced amino acid sequences of the zebrafish SULT1 ST9.



**Zebrafish SULT3 ST4**

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      10      20      30      40      50      60
ATGGCTCAAGAGGAATGCAAAATGATTAGTGACAAACTGTTAAAGTACAAAGGAACTGTG
M A Q E E C K M I S D K L L K Y K G T V
      70      80      90     100     110     120
TTGACTGTGAATGACAACCAAGACATTACTCCAGAATATATTGACAGTATACAGGATTTT
L T V N D N Q D I T P E Y I D S I Q D F
      130     140     150     160     170     180
GAAACAAGGGATGACGATGTCTTTGTTGTGACATTTCCCAAATCTGGTACAGTATGGACC
E T R D D D V F V V T F P K S G T V W T
      190     200     210     220     230     240
CAGCGGATCATGACTTTAATATACGAGGAGGATTTCCCAGAGAAAGCCAAACAAATCACA
Q R I M T L I Y E E D F P E K A K Q I T
      250     260     270     280     290     300
TATGAGCAAATGCCCTGGATTGAGTATCGGGATAAAGGGAAAGATTACAGCACACGTCCA
Y E Q M P W I E Y R D K G K D Y S T R P
      310     320     330     340     350     360
TCTCCAAGACTCTTCTGTTTCGCATTTACTGGAGCCGCTGATGCCAGAGCTCTCCAGAGG
S P R L F C S H L L E P L M P R A L Q R
      370     380     390     400     410     420
AAAGGAAAAGTCATCTACGTCATGAGAAACCCCAAAGACGTCATGGTGTCTGATTTTTCAT
K G K V I Y V M R N P K D V M V S Y F H
      430     440     450     460     470     480
TTTTCCAACAAATTGGACAACCTGGATTCTTCTGAGAGCTACGATGAGATGTTAAAGAAA
F S N K L D N L D S S E S Y D E M L K K
      490     500     510     520     530     540
TTCATTACAGGATGCATGGTTGGTGGCTGCTGGTTTGACCATGTTAAAGGATGGGTGACA
F I T G C M V G G C W F D H V K G W V T
      550     560     570     580     590     600
AGTAAAGACAAATACAACATCCTGATCCTGACTTATGAAGAGATGATCAAAGACCTCAGA
S K D K Y N I L I L T Y E E M I K D L R
      610     620     630     640     650     660
TCTGTTCATTGTGAAAATCTGTAAGTTTGTGGCAAGAATCTGTCAGACGCAGCCATCGAT
S V I V K I C K F V G K N L S D A A I D
      670     680     690     700     710     720
AAAGTGGTGGAAAGAACAACATTC AAGCAAATGAAAGTAGACCCTGTGGCCAACTATGAG
K V V E R T T F K Q M K V D P V A N Y E
      730     740     750     760     770     780
TCCCTTTCTAAGGAGATCACAGATCAGCCGAAAGGAGCTTTTTTTCGCGCAAAGGAACAGTT
S L S K E I T D Q P K G A F L R K G T V
      790     800     810     820     830     840
GGAGACTGGAAGAACTCTTTAACCGTGGCTCAGAGTGAATGTGTTGATCGTGTCCCTTGAA
G D W K N S L T V A Q S E C V D R V L E
      850     860     870     880     890     900
GATAGAATGAAGGACGTACCTCTTAACCTGGTCTGGGACATCACAGA AACTGCATAGCTGA
D R M K D V P L N L V W D I T E L H S *

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Figure 3-2: The nucleotide and deduced amino acid sequences of the zebrafish SULT3 ST4.

### Zebrafish SULT3 ST5

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      10          20          30          40          50          60
ATGGCTCAGGAGGAATGCAAAATGATTAGTGACAAACTGTTGAAGTACAAAGAAACTGTG
M A Q E E C K M I S D K L L K Y K E T V
      70          80          90         100         110         120
TTGACTCTGGAACCCAGCTACGACATTACTCCAGAATATATTGACAGTATACAGGATTTT
L T L E P S Y D I T P E Y I D S I Q D F
      130         140         150         160         170         180
GAAACAAGGGATGACGATGTATTTGTTGTTACCTTCCCCAAATCTGGTACAGTATGGACC
E T R D D D V F V V T F P K S G T V W T
      190         200         210         220         230         240
CAGCGGATCATAACTTTAATTTATGAGGAGGATTTCCCGGAGAAAGCCAAACAAATCACA
Q R I I T L I Y E E D F P E K A K Q I T
      250         260         270         280         290         300
TTCGAGCAAATGCCCTGGATTGAGTATCGGAAAAAGGGGAAAGATTACAGCACACGTCCA
F E Q M P W I E Y R K K G K D Y S T R P
      310         320         330         340         350         360
TCTCCAAGACTCTTCTGTTTCGCATCTACTGGAGCCGTTGATGCCCAAACCTCTCAAAGG
S P R L F C S H L L E P L M P K T L K R
      370         380         390         400         410         420
AAAGGAAAAGTCATCTACGTCATGAGGAACCCCAAAGATGTCATGGTTTCATATTTTCAT
K G K V I Y V M R N P K D V M V S Y F H
      430         440         450         460         470         480
TTTTCTAAAAAATGAAAAATCTGGATTCTGCCAAGAGCTACGACGAGGTCTTAGAGAAT
F S K K M K N L D S A K S Y D E V L E N
      490         500         510         520         530         540
TTCTTAACAGGATGCATGGTTGGTGGCTCCTGGTTTGACCATGTTAAAGGATGGGGTGACA
F L T G C M V G G S W F D H V K G W V T
      550         560         570         580         590         600
AGTAAAGACAAATACAACATCCTGATCCTGACTTATGAAGAGATGATCAAGGACCTCAGA
S K D K Y N I L I L T Y E E M I K D L R
      610         620         630         640         650         660
TCCGTCATTGTGAAAATCTGTGAGTTTGTGGCAAGAATCTGTCAGACGCAGCCATCGAT
S V I V K I C E F V G K N L S D A A I D
      670         680         690         700         710         720
AAAGTGGTGGAAAGAGCAACATTCAAGCAAATGAAAGTAGACCCTGTGGCCAACTATGAG
K V V E R A T F K Q M K V D P V A N Y E
      730         740         750         760         770         780
TCCCTTCCTGTGGATATCACAGATCAGCCGAAAGGAGCTTTTATGCGCAAAGGAACGGTT
S L P V D I T D Q P K G A F M R K G T V
      790         800         810         820         830         840
GGGACTGGAGGAACTCTTTAACCATGGCTCAGAGTGAATGTGTTGATGGTGCCCTTGAA
G D W R N S L T M A Q S E C V D G A L E
      850         860         870         880         890         900
GAAAGAATGAAAGACGTGCCTCTTAACCTGGTCTGGGACATCACAGAACTGCGTGGCTGA
E R M K D V P L N L V W D I T E L R G *

```

Figure 3-3: The nucleotide and deduced amino acid sequences of the zebrafish SULT3 ST5.

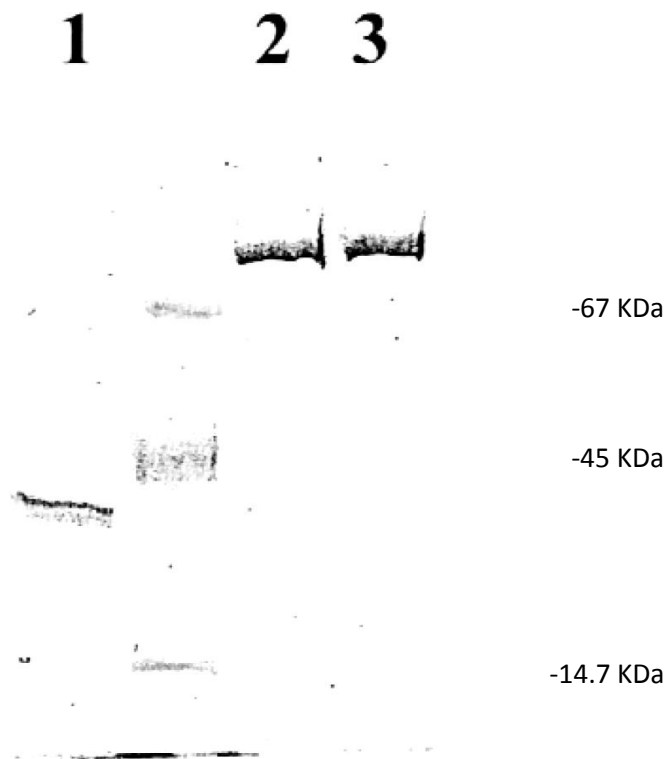


Figure 3-4: SDS-PAGE of Purified of zebrafish (1) SULT3 ST4, (2) SULT3 ST5 and (3) SULT1 ST9

### 3.2. Substrate Specificity.

A variety of endogenous and xenobiotic compounds were tested as substrates using the standard assay procedure described in the **Materials and Methods** section. SULT3 ST4, SULT3 ST5 and SULT1 ST9 were found to display differential sulfating activities toward the different substrates tested. SULT3 ST4 showed significant activities towards many endogenous compound including DHEA, pregnenolone,  $17\beta$ -estradiol,

corticosterone, androstene-3,17-dione, progesterone, and estrone. In addition, SULT3 ST4 also exhibited significant activity towards some xenobiotics including mestranol, butylated hydroxyanisole, 17 $\alpha$ -ethynylestradiol,  $\beta$ -naphthylamine, and  $\beta$ -naphthol.

SULT3 ST5 exhibited weaker, but significant, activities toward most of the endogenous and xenobiotic compounds (the specific activity for each substrate for this enzyme was calculated based on the unit of pmol product/minute/mg enzyme). SULT3 ST5 showed sulfating activities toward endogenous compounds including DHEA, corticosterone, androstene-3,17-dione, 17 $\beta$ -estradiol, pregnenolone, but no detectable activity towards estrone and progesterone. SULT3 ST5 displayed activities toward xenobiotics including butylated hydroxyanisole, mestranol, 17 $\alpha$ -ethynylestradiol,  $\beta$ -naphthylamine and  $\beta$ -naphthol, but no activity toward gallic acid, caffeic acid, and chlorogenic acid. SULT1 ST9 showed significant activities toward butylated hydroxyanisole, 17 $\beta$ -estradiol, 17 $\alpha$ -ethynylestradiol, estrone, but no activity toward DHEA, mestranol, corticosterone, pregnenolone, androstene-3,17-dione, and progesterone. Additionally, SULT1 ST9 exhibited activity toward both  $\beta$ -naphthylamine and  $\beta$ -naphthol. In contrast to SULT3 ST4 and SULT3 ST5, SULT1 ST9 exhibited stronger activities toward caffeic acid, gallic acid and chlorogenic acid.

Table 3.1: Zebrafish SULT3 ST4 specific activities towards different xenobiotic and endogenous compounds. The unit of specific activity in nmol/minute/mg of SULT3 ST4. Data represent mean  $\pm$  SD derived from 6 measurements. ND means on detected activities

Substrate (endogenous)	Specific Activity nanomol/min/mg	Substrate (Xenobiotic)	Specific Activity nomol/min/mg
DHEA	13.83 $\pm$ 0.20	17 $\alpha$ -ethynyl estadiol	0.58 $\pm$ 0.02
androstene-3, 17- dione	0.4 $\pm$ 0.02	caffeic acid	ND
17 $\beta$ -estradiol	3.45 $\pm$ 0.07	gallic acid	ND
progesterone	0.28 $\pm$ 0.01	mestranol	0.98 $\pm$ 0.07
estrone	0.21 $\pm$ 0.01	chlorogenic acid	ND
pregnenolone	9.04 $\pm$ 0.06	$\beta$ -naphthylamine	0.31 $\pm$ 0.01
corticosterone	0.8 $\pm$ 0.02	$\beta$ -naphthol	0.14 $\pm$ 0.01
		Butylated hydroxyl anisole (BHA)	0.13 $\pm$ 0.01

Table 3.2: Zebrafish SULT3 ST5 specific activities towards different xenobiotic and endogenous compounds. The unit of specific activity in pico mol/minute/mg of SULT3 ST5. Data represent mean  $\pm$  SD derived from 6 measurements. ND means no detected activities

Substrate (Endogenous)	Specific Activity picomol/min/mg	Substrate (Xenobiotics)	Specific Activity picomol/min/mg
DHEA	119.68 $\pm$ 4.76	17 $\alpha$ -ethynylestadiol	213.36 $\pm$ 3.97
androstene-3, 17-dione	21.06 $\pm$ 0.67	caffeic acid	ND

17 $\beta$ -estradiol	16.34 $\pm$ 0.37	gallic acid	ND
progesterone	ND	mestranol	33.12 $\pm$ 1.15
estrone	ND	chlorogenic acid	ND
pregnenolone	12.47 $\pm$ 0.23	$\beta$ -naphthylamine	6.11 $\pm$ 0.44
corticosterone	29.28 $\pm$ 1.09	$\beta$ -naphthol	11.44 $\pm$ 1.35
		butylated hydroxyl anisole (BHA)	23.49 $\pm$ 0.41

Table 3.3: Zebrafish SULT1 ST9, specific activities towards different xenobiotic and endogenous compounds. The unit of specific activity in nmol/minute/mg of SULT1 ST9. Data represent mean  $\pm$  SD derived from 6 measurements. ND means no detected activities.

Substrate (Endogenous)	Specific Activity nmol/min/mg	Substrate (Xenobiotics)	Specific Activity nmol/min/mg
DHEA	ND	17 $\alpha$ -ethynylestradiol	0.02 $\pm$ 0.01
androstene-3, 17- dione	ND	caffeic acid	1.57 $\pm$ 0.02
17 $\beta$ -estradiol	0.02 $\pm$ 0.01	gallic acid	0.16 $\pm$ 0.01
progesterone	ND	mestranol	ND
estrone	0.05 $\pm$ 0.01	chlorogenic acid	0.16 $\pm$ 0.01
pregnenolone	ND	$\beta$ -naphthylamine	0.08 $\pm$ 0.01
corticosterone	ND	$\beta$ -naphthol	4.43 $\pm$ 0.05
		butylated hydroxyl anisole (BHA)	0.02 $\pm$ 0.01

### 3.3 pH-Dependency Studies.

pH-dependency of SULT3 ST4 was examined using DHEA and  $\beta$ -naphthol as endogenous and xenobiotic substrates, respectively. Result from the pH dependence studies revealed that SULT3 ST4 with DHEA showed a broad pH optimum spanning pH 5.5 to 9.5, with a maximum sulfating activity at pH 6.5 (Figure 3-5 A). SULT3 ST4 with  $\beta$ -naphthol as a substrate also showed a broad pH optimum spanning pH 6.5 to 9.5, with a maximum sulfating activity at pH 7.5 (Figure 3-5B). In contrast, SULT3 ST5 with DHEA as a substrate exhibited a narrower pH optimum between pH 5.5 and 7.5, with a maximum sulfating activity at pH 6.5 (Figure 3-5 C). SULT1 ST9 showed a broad pH optimum spanning pH 6.5 to 9.5, with a maximum activity at pH 7.5 with  $\beta$ -naphthol as substrate (Figure 3-5D). With  $17\beta$ -estradiol as a substrate, SULT1 ST9 displayed strong sulfating activities between pH 7.5 and 9.5, with maximum activity at pH 8.5 (Figure 3-5 E).

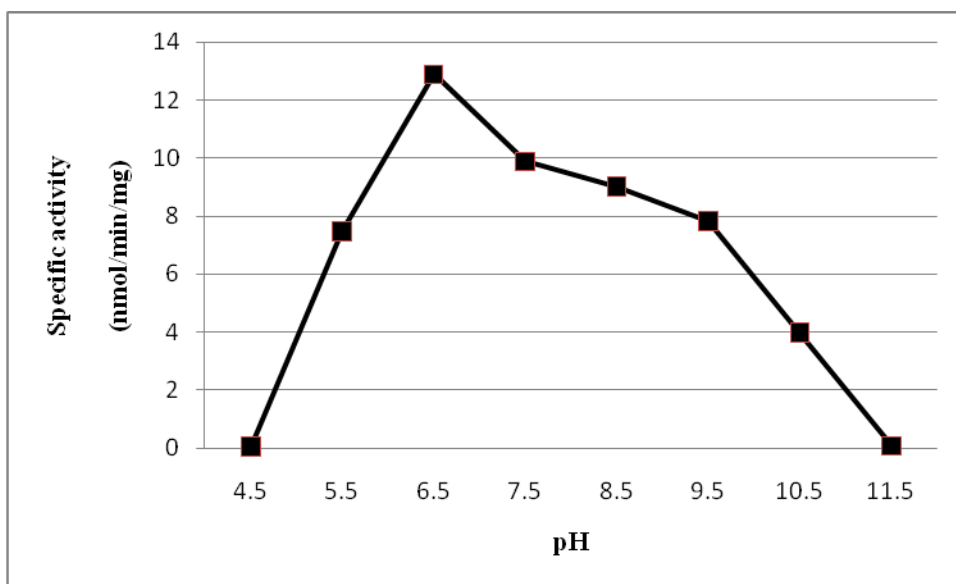


Figure 3-5(A): pH dependency profile of the sulfating activity of the zebrafish SULT3 ST4 with DHEA as a substrate.

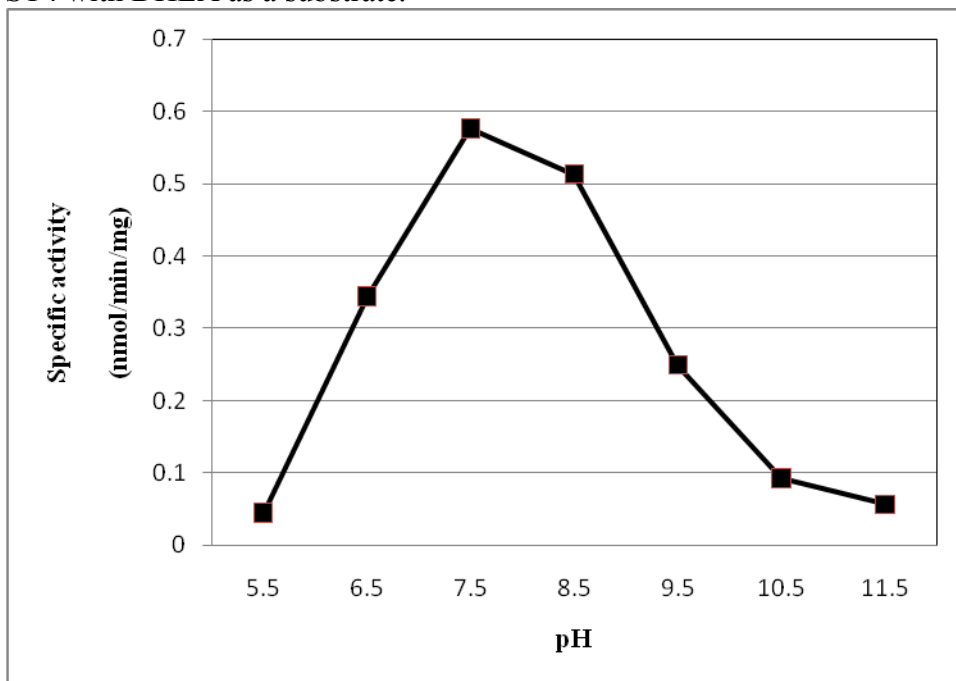


Figure 3-5(B): pH dependency profile of the sulfating activity of the zebrafish SULT3 ST4 with  $\beta$ -naphthol as a substrate.

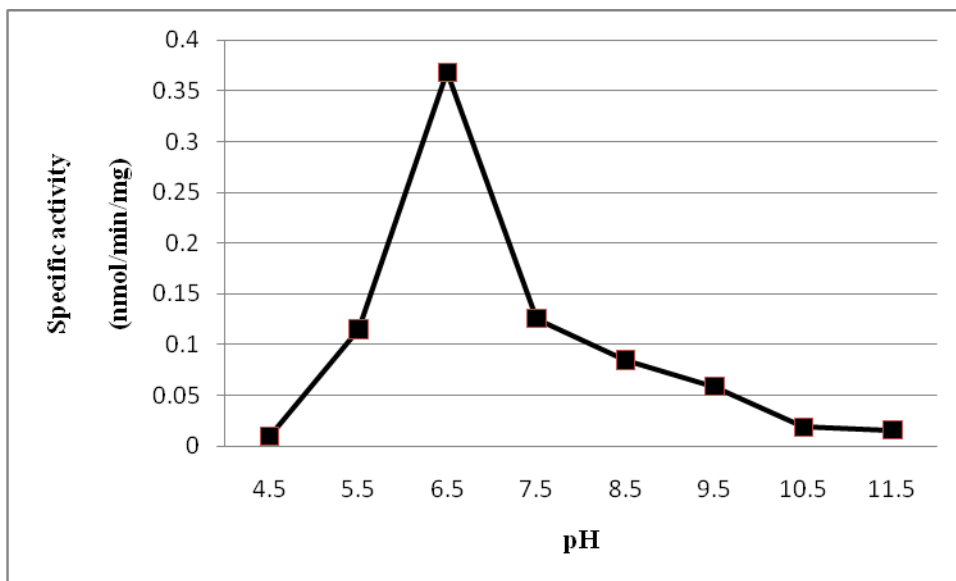


Figure 3-5(C): pH dependency profile of the sulfating activity of the zebrafish SULT3 ST5 with DHEA as a substrate.



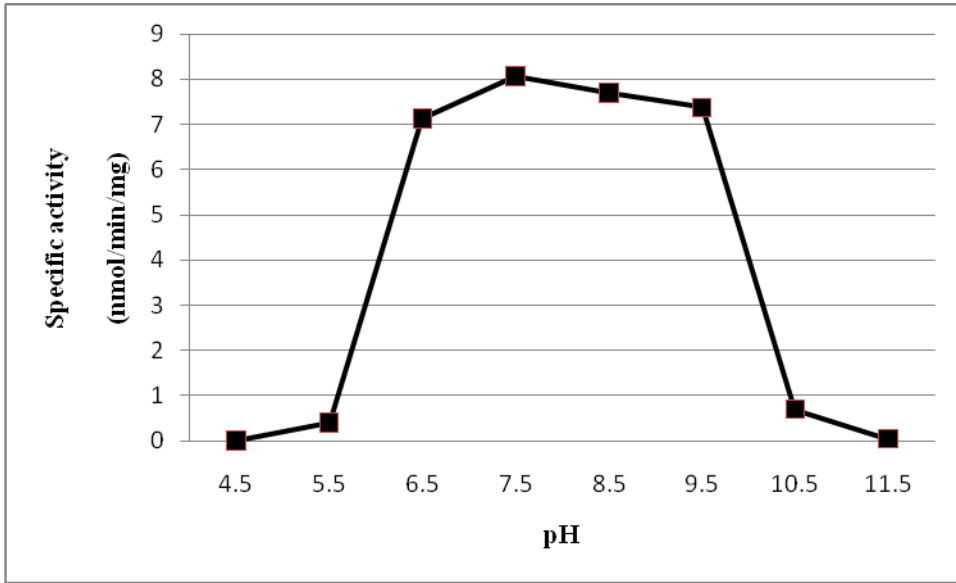


Figure 3-5(D): pH dependency profile of the sulfating activity of the zebrafish SULT1 ST9 with  $\beta$ -naphthol as a substrate.

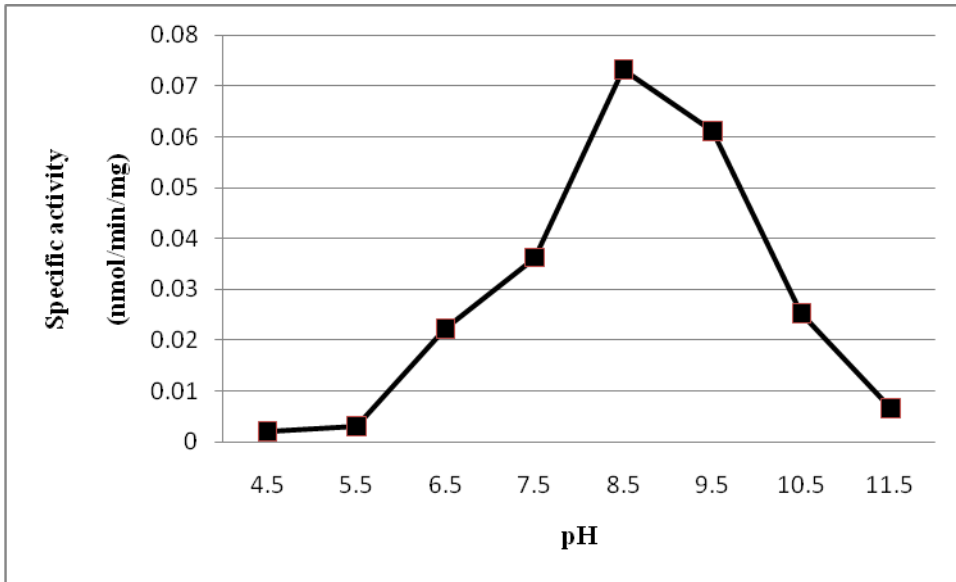


Figure 3-5(E): pH dependency profile of the sulfating activity of the zebrafish SULT1 ST9 with  $17\beta$ -estradiol as a substrate.

### 3.4 Kinetic Studies.

Kinetic studies for zebrafish SULT3 ST4 were performed using DHEA and  $\beta$ -naphthol as substrates. The enzymatic assays were performed using the same procedure as in the Materials and Method section, except different substrate concentrations were used. The final concentrations tested were 0.00066 to 0.004 mM for DHEA and 0.025 to 0.5 mM for  $\beta$ -naphthol (Table 3.4). Kinetic study for zebrafish SULT1 ST9 was performed using  $17\beta$ -estradiol and  $\beta$ -naphthol as substrates. The final concentrations tested were 0.00066 to 0.004 mM for  $\beta$ -naphthol and 0.01 to 0.1 mM for  $17\beta$ -estradiol (Table 3.5). Data obtained were used to generate Lineweaver-Burk double-reciprocal plots in order to calculate the values of  $K_m$ ,  $V_{max}$ , and  $V_{max}/K_m$  for each of these two enzymes in catalyzing the sulfation of indicated substrates. Figures 3-6 and 3-7 show the Lineweaver-Burk double-reciprocal plots of zebrafish SULT3 ST4 with DHEA and  $\beta$ -naphthol, respectively, as substrates. Figures 3-8 and 3-9 show Lineweaver-Burk double-reciprocal plots of zebrafish SULT1 ST9 with  $\beta$ -naphthol and  $17\beta$ -estradiol, respectively, as substrates. The calculated values of  $K_m$ ,  $V_{max}$ , and  $V_{max}/K_m$  for the SULT3 ST4 and SULT1 ST9 are compiled in Table 3.6.

Table 3.4: List of the substrate concentrations used in the study of kinetic properties of the zebrafish SULT3 T4

Zebrafish SULTs	Substrate	Final concentration (mM)
SULT3ST4	DHEA	0.00066, 0.0008, 0.001, 0.00133, 0.002, 0.004

	$\beta$ -naphthol	0.025, 0.05, 0.1, 0.25, 0.5
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Table 3.5: List of the substrates and the concentrations used to study the kinetic properties of the zebrafish SULT1 ST9.

Zebrafish SULTs	Substrate	Final concentration (mM)
SULT1 ST9	$\beta$ -naphthol	0.00066, 0.0008, 0.001, 0.00133, 0.002, 0.004
	17 $\beta$ -estradiol	0.01, 0.02, 0.025, 0.0333, 0.05, 0.1

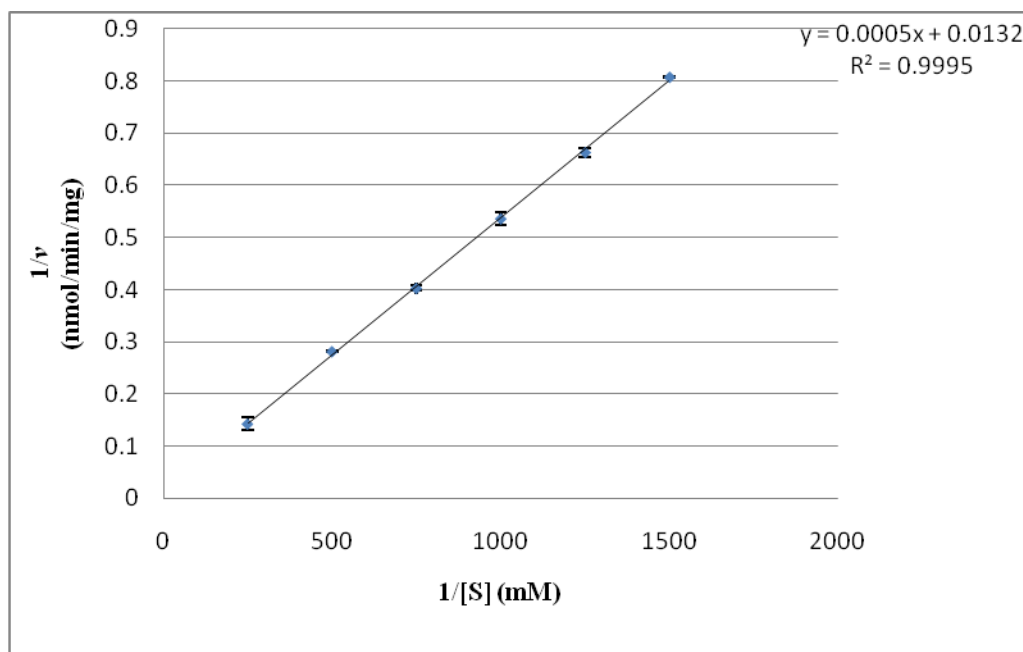


Figure 3-6: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT3 ST4 with DHEA as a substrate. The final concentrations of the DHEA were 0.000666, 0.0008, 0.001, 0.00133, 0.002, and 0.004 mM. The velocities of the reactions are expressed in nmol/min/mg of the enzyme.

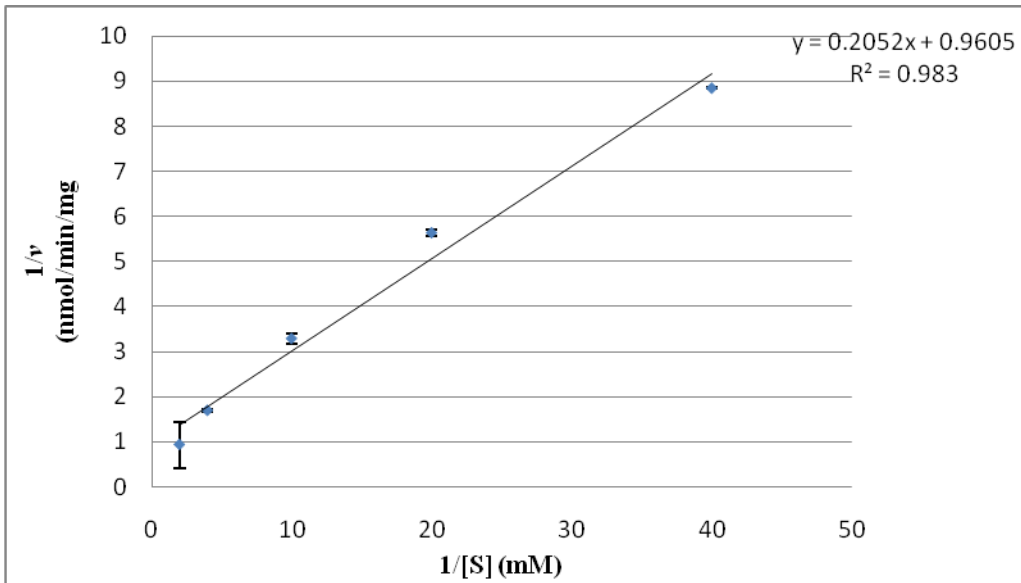


Figure 3-7 Lineweaver-Burk double-reciprocal plot of the zebrafish SULT3 ST4 with  $\beta$ -naphthol as a substrate. The final concentrations of the  $\beta$ -naphthol were 0.025, 0.05, 0.1, 0.25, 0.5 mM. The velocities of the reactions are expressed in nmol/min/mg of the enzyme.

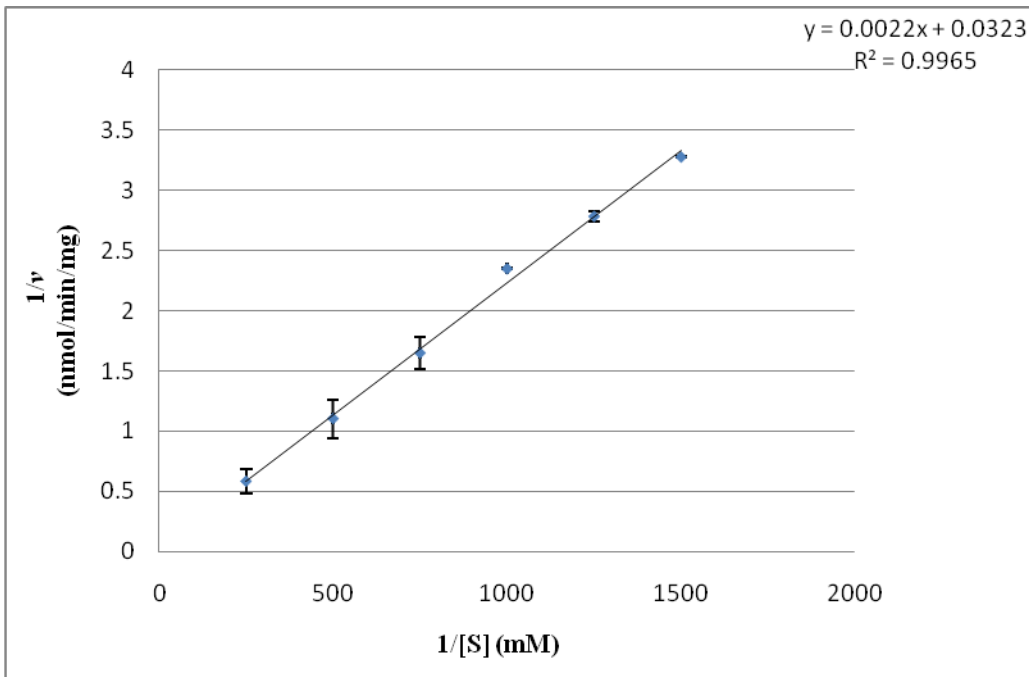


Figure 3-8: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 with  $\beta$ -naphthol as a substrate. The concentrations of the  $\beta$ -naphthol were 0.000666, 0.0008, 0.001, 0.00133, 0.002, and 0.004 mM. The velocities of the reactions are expressed in nmol/min/mg of the enzyme.

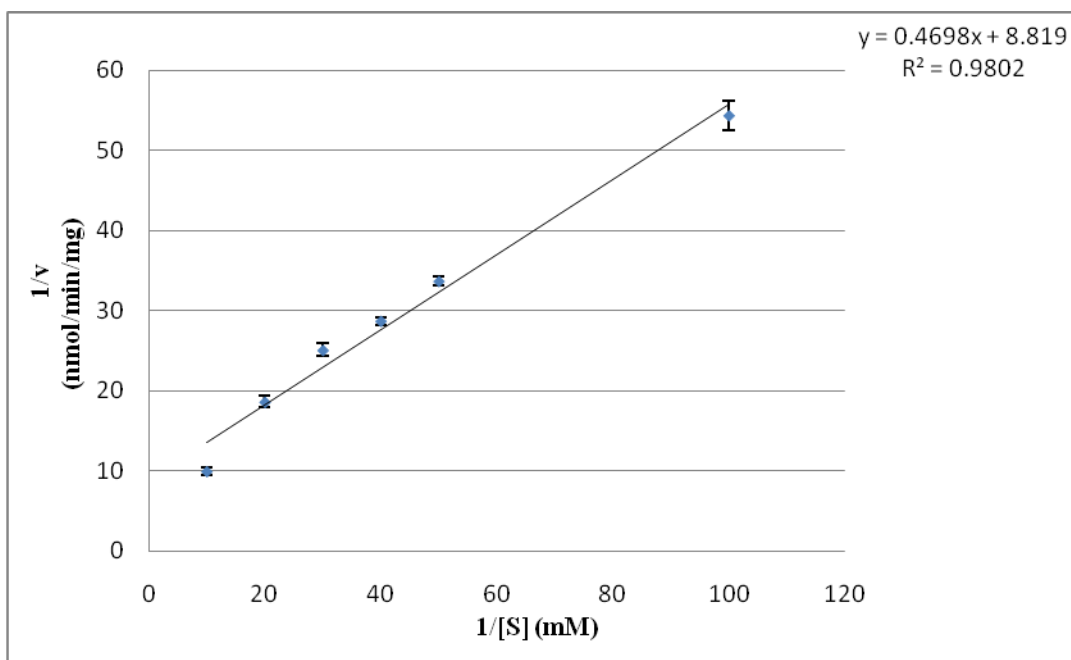


Figure 3-9: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 with  $17\beta$ -estradiol as a substrate. The final concentrations of the  $17\beta$ -estradiol 0.01, 0.02, 0.025, 0.0333, 0.05, and 0.1 mM. The velocities of the reactions are expressed in nmol/min/mg of the enzyme.

Table 3.6: Kinetic parameters of Zebrafish SULT3 ST4 and SULT1 ST9 using DHEA,  $\beta$ -naphthol, and  $17\beta$ -estradiol as substrates

Zebrafish SUTLs	Substrates	$K_m$ (mM)	$V_{max}$ nmol/min/g	$V_{max}/K_m$
SULT3 ST4	DHEA	0.038	75.76	1993.7
	$\beta$ -naphthol	0.21	1.04	4.95

SULT1 ST9	$\beta$ -naphthol	0.068	30.96	455.3
	17 $\beta$ -estradiol	0.053	0.11	2.08

### **3.5 Developmental Stage-Dependent Expression of the Zebrafish SULT3 ST4, SULT3 ST5 and SULT1 ST9.**

The expression of mRNAs encoding SULT3 ST4, SULT3 ST5, and SULT1 ST9 from embryogenesis to maturity (cf. Table 3.7) was examined using RT-PCR in order to gain insight into their physiological involvement. For SULT3 ST4, there was no detectable level of its coding mRNA in unfertilized egg. Upon fertilization, a low level of the SULT3 ST4 mRNA was detected in the zygote period, which then disappeared in the cleavage period. Afterwards, a significant level the SULT3 ST4 mRNA was detected during blastula, gastrula, segmentation, pharyngula and hatching periods. It then started to increase in the larval stages and on to maturity in both male and female adult fish (Figure 3-10 A). In contrast, the expression of the mRNA encoding SULT3 ST5 was detected at all stages from embryogenesis on to maturity (Figure3-10 B). The expression mRNA encoding SULT1 ST9 was not detected in unfertilized eggs and embryos up to phyaryngula period. It appeared at a low level in hatching stage, then increased in larval period and decreased in the first week of maturity. Afterwards, significant expression was detected from the second week of larval development on to maturity to adult fish. Interestingly, the expression of the mRNA encoding SULT1 ST9 was detected only in female, but not in male adult fish (Figure 3-10 C). As a control,  $\beta$ -actin, a housekeeping

protein, was found to be constantly expressed throughout the all developmental stages (Figure 3-10 D).

Table 3.7: List of zebrafish embryonic developmental stages.

Developmental stage	Time of development
Zygote period	0-h pf
Cleavage period	1-h pf
Blastula period	3-h pf
Gastrula period	6-h pf
Segmentation period	12-h pf
Pharyngula period	24-h pf
Hatching period	48-h pf
Larval period	72-h pf

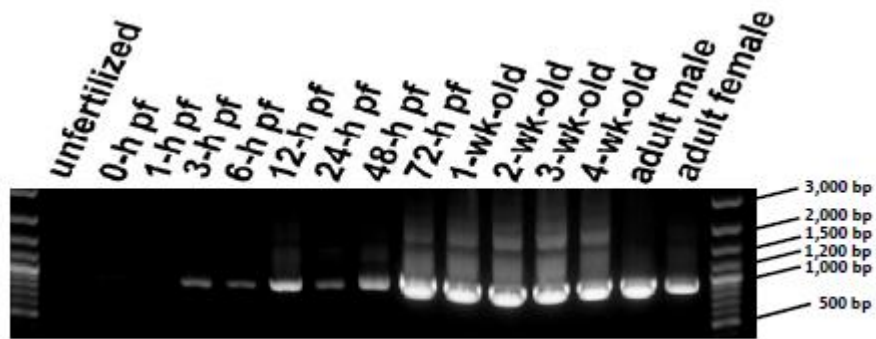


Figure 3-10 A: RT-PCR analysis of the expression of mRNA encoding SULT3 ST4 during the zebrafish development

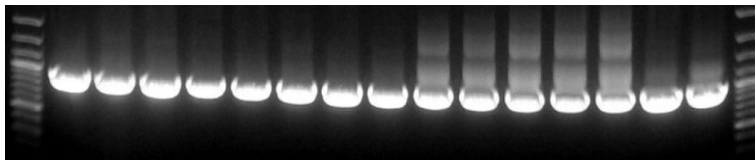


Figure 3-10 B: RT-PCR analysis of the expression of mRNA encoding SULT3 ST5 during the zebrafish development

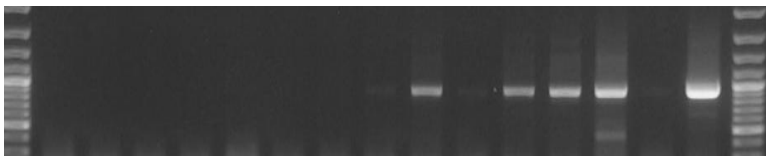


Figure 3-10 C: RT-PCR analysis of the expression of mRNA encoding SULT1 ST9 during the zebrafish development

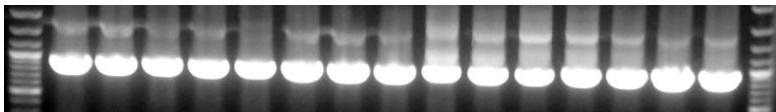


Figure 3-10 D: RT-PCR analysis of the expression of the zebrafish  $\beta$ -actin during the zebrafish development



#### 4. Discussion.

Among the Phase II conjugation reactions, sulfation as mediated by the SULTs is known to be involved in the regulation and biotransformation of many endogenous compounds such as thyroid hormones and steroid hormones, as well as in the detoxification of xenobiotics including drugs. In this research, three novel zebrafish SULTs, SULT3 ST4, SULT3 ST5, and SULT1 ST9, were cloned, purified, and characterized. The results from substrates specificity experiments showed that the zebrafish SULT3 ST4 displayed strong sulfating activities toward DHEA and pregnenolone, among a panel of endogenous compounds that were tested as substrates. In view of the structural similarity between DHEA, pregnenolone, and  $17\beta$ -estradiol, it can be proposed that SULT3 ST4 preferred to sulfate hydroxysteroids, although it also displayed significant sulfating activities toward other endogenous compounds and xenobiotics such as  $17\alpha$ -ethynyl estradiol, mestranol, butylated hydroxyl anisole,  $\beta$ -naphthol, and  $\beta$ -naphthylamine. SULT3 ST5 also displayed strong activities toward DHEA and  $17\alpha$ -ethynylestradiol. In contrast to SULT3 ST4, however, SULT3 ST5 showed no activity toward estrone and progesterone. Unlike SULT3 ST4 and SULT3 ST5, SULT1 ST9 displayed the strongest activities towards  $\beta$ -naphthol, while showing significant activity toward other xenobiotics and no activity towards many of the endogenous compounds tested as substrates, except estrone and  $17\beta$ -estradiol. As mentioned previously, the zebrafish SULT1 STs (ST1 through ST8) had been shown to display sulfating activities toward xenobiotics (Liu *et al.*, 2010). In this research, it was demonstrated that SULT1 ST9 exhibited sulfating activity towards all xenobiotics, except mestranol, that were tested as substrates. It therefore can be

concluded that SULT1 ST9 appeared to dedicate primarily to the sulfation and detoxification of xenobiotics. pH-dependency experiments revealed that, with DHEA as a substrate, SULT3 ST4 showed a broad pH optimum spanning pH 5.5 to 9.5. With  $\beta$ -naphthol as substrate, SULT3 ST4 showed also a broad pH optimum spanning 6.5 to 8.5. These results appeared to be consistent considering the similarity between DHEA and  $\beta$ -naphthol with regard to the presence of planar, aromatic ring in their structures. DHEA was tested as a substrate for SULT3 ST5 in pH-dependency experiments. Interestingly, SULT3 ST5 exhibited a narrower pH optimum from pH 5.5 to 7.5. As mentioned above, SULT 1 ST9 was found to sulfate primarily xenobiotics. With  $\beta$ -naphthol as substrate, SULT1 ST9 showed a broad pH optimum spanning pH 6.5 to 9.5, whereas with 17 $\beta$ -estradiol as substrate, a pH optimum spanning 7.5 to 9.5 was observed. The different pH ranges where each of the three zebrafish SULTs remained catalytically active may imply that the three enzymes may function in different zebrafish cell types/tissues/organs where the local pH environments may vary within those pH ranges. Kinetic parameters for the sulfation of DHEA and  $\beta$ -naphthol by SULT3 ST4 were determined based on Lineweaver-Burk double-reciprocal plots. The  $K_m$  for DHEA (0.038 mM) was lower than the  $K_m$  for  $\beta$ -naphthol (0.21 mM), indicating the higher affinity of SULT3 ST4 for DHEA than that for  $\beta$ -naphthol. Kinetic parameters for the sulfation of  $\beta$ -naphthol and 17 $\beta$ -estradiol by SULT1 ST9 revealed that the  $K_m$  for 17 $\beta$ -estradiol was almost the same as that for  $\beta$ -naphthol, indicating that SULT1 ST9 has comparable affinity toward these two substrates. With regard to  $V_{max}$ , DHEA as substrate for SULT3 ST4 and  $\beta$ -naphthol as

substrate for SULT1S T9 have higher  $V_{max}$  than 17 $\beta$ -estradiol and  $\beta$ -naphthol as substrates for SULT1 ST9 and SULT3 ST4, respectively. These results indicated that SULT3ST4 and SULT1 ST9 have higher intrinsic catalytic activity in sulfating DHEA and  $\beta$ -naphthol, respectively. The ratio of  $V_{max}/K_m$  is known to reflect the catalytic efficiency of an enzyme in mediating a reaction at sub-maximal substrate concentrations. The calculated  $V_{max}/K_m$  for DHEA by SULT3 ST4 was found to be the highest (1993.7), therefore implying that SULT3 ST4 has the highest efficient in catalyzing the sulfation of DHEA under physiological conditions. As mentioned above, while SULT1 ST9 displayed comparable  $K_m$  values for 17 $\beta$ -estradiol and  $\beta$ -naphthol, the  $V_{max}/K_m$  with  $\beta$ -naphthol (455.3) is considerably higher than that (2.08) for 17 $\beta$ -estradiol. It therefore can be concluded that SULT1 ST9 is more catalytically efficiency with  $\beta$ -naphthol than with 17 $\beta$ -estradiol as substrate. The expression of the mRNA encoding SULT3 ST4, SULT3 ST5, and SULT1 ST9 during the zebrafish development from embryogenesis to the maturity was investigated using RT-PCR in order to find out whether the expression of these enzymes may correlate with the development of the endocrine system or with the detoxification mechanism of the zebrafish. As mentioned above, hydroxysteroids (DHEA, and pregnenolone) are preferred substrates for SULT3 ST4. Steroid hormones, including sex steroids and mineralocorticoids play an important role in the regulation of homeostasis, differentiation in sex, and mineral balance. In addition to being (precursors of) steroid hormones, DHEA and pregnenolone (and perhaps other steroids as well) are present in the nervous system where these “neurosteroids” play important roles in cellular development, behavior pattern, regulation of gene expression and protein synthesis, and

in the functioning of neuroendocrine system (Yasuda *et al.*, 2009). Interestingly, a significant level of the mRNA coding for SULT3 ST4 was detected in segmentation period during which the endocrine system of the zebrafish starts being developed. Thereafter, high levels of SULT3 ST4 mRNA were expressed throughout hatching, larval, and adult stages. Therefore, it is possible that SULT3 ST4 may play a role in the regulation of hydroxysteroids at those developmental stages. For SULT3 ST5, the expression of the coding mRNA was detected throughout all developmental stages of the zebrafish from embryogenesis to maturity. It is possible that this enzyme is critical to the metabolism of hydroxysteroids at all times during the zebrafish development. SULT1 ST9 was shown to be a detoxifying enzyme based on enzymatic characterization. No expression of SULT1 ST9 mRNA was detected in unfertilized egg and during the early phase of embryonic development. Significant levels were detected only at later developmental stages as mentioned in section 3.4. Interestingly, similar to SULT1 ST7 (Liu *et al.*, 2008), mRNA encoding SULT1 ST9 was detected only in adult female, but not male fish. The functional implication of this finding remains to be clarified.

In conclusion, three new zebrafish SULT enzymes, designated SULT3 ST4, SULT3 ST5 and SULT1 ST9, were identified, expressed, and purified. The two SULT3 STs (ST4 and ST5) were found to display sulfating activities toward hydroxysteroids, whereas the SULT1 ST9 exhibited sulfating activities toward mostly xenobiotic compounds. The developmental stages expression of these three novel zebrafish SULTs was examined. The identification and characterization of these enzymes and their developmental expression is essential for establishing the zebrafish as an animal model for the

investigation of the metabolism of key endogenous compounds such as steroid hormones, as well as xenobiotics including drugs. With further understanding about the correlation between zebrafish and human SULTs, the information obtained using the zebrafish enzymes can eventually be extrapolated to the human system.

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