國立交通大學

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博士論文

亞硫酸基轉移酶之定位與功能分析

Localization and Functional Analysis of Sulfotransferase

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中華民國九十七年八月

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中文摘要

亞硫酸基轉移酶參與許多重要生理反應，如藥物代謝、類固醇與荷爾蒙調控、調節神經傳導物質功能和癌化過程。在亞硫酸基化反應中，磷酸腺苷酸磷酰硫酸鹽 (PAPS) 為亞硫酸基的來源，其受質可廣泛的包含醣類、蛋白質、多種內生性小分子及環境荷爾蒙等。 為了進一步探討亞硫酸基轉移酶在生物體中可能的生理與功能意義，本研究利用兩種重要的動物模型，果蠅及斑馬魚，作為亞硫酸基轉移酶之定位與功能分析之平台。首先，研究結果顯示在果蠅全腦中，類脫氫表雄甾酮亞硫酸基轉移酶之蛋白質 (DHEA ST-like protein) 選擇性地表現在果蠅腦中特殊的神經細胞及神經纖維，推測可能和記憶與學習迴路相關。在斑馬魚中，有兩種新的亞硫酸基轉移酶被確認。此兩種酶針對環境荷爾蒙，特別是羥化多氯化聯苯 (hydroxylated PCB) 有最強的亞硫酸基化能力，在斑馬魚的胚胎形成與發育過程中，此兩種酶大量表現在幼魚時期，推測在斑馬魚器官發育之初期，魚體已具有代謝環境荷爾蒙的能力。另外，本研究發展出一個針對酪胺酸亞硫酸基化之偵測方法，並發現三個恆常性血液蛋白有酪胺酸亞硫酸基化的現象。結果顯示，以果蠅及斑馬魚為平台，本研究詳細地探討亞硫酸基轉移酶之定位與功能分析，期能為日後之研究提供可用之相關資訊。

Localization and Functional Analysis of Sulfotransferase

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ABSTRACT

Sulfotransferases (SULTs) constitute a superfamily of related enzymes that play important roles in the regulation of detoxification, steroid hormone action, neurotransmitter function, drug metabolism, and carcinogenesis. Sulfation, catalyzed by SULTs, is the critical biotransformation process for transferring a sulfuryl group from the cofactor 3'-phosphoadenosine-5'-phophosulfate (PAPS) to a wide range of structurally diverse endogenous and xenobiotic compounds. It is believed that understanding the expression pattern and cellular distribution of SULTs, along with the functional and structural information, will assist in determining the physical and functional significance of SULTs. Here we utilized two ideal animal models, fruit fly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*), as the alternative testing systems for investigating the localization and functional significance of SULTs. Firstly, a systematical analysis demonstrated that dehydroepiandrosterone sulfotransferase-like protein was expressed in fruit fly, which so far has no evidence indicating the presence of cytosolic SULTs, and was abundant in the specific neural bodies as well as in several bundles of synapses in fruit fly neuronal circuits. Secondly, two novel cytosolic SULTs, SULT1 ST7 and SULT1 ST8, were identified and characterized in zebrafish. They both exhibited strong sulfating activities toward environmental estrogens, particularly hydroxylated polychlorinated biphenyls (PCBs), among various endogenous and xenobiotic compounds tested as substrates. Developmental expression experiments also revealed distinct expression patterns of SULT1 ST7 and ST8 during embryonic development and throughout the larval stage onto maturity. Lastly, to better understand the functional regulation of SULTs, a target-specific approach for the identification of tyrosine sulfation had been established. Three new tyrosine-sulfated hemostatic proteins were identified. Such a target-specific approach will allow for investigation of sulfated-tyrosine proteins of other biochemical/physiological pathways/processes and contribute to a better understating of post-translational modification by tyrosine sulfation.

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At last, I would like to dedicate this honor to my family. Certainly, without the love and support that they have shown me, I will not be able to reach this goal.

To my lovely family, especially my parents, and all the loved ones—I love you so much!

with loves,

An
August, 2008

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Structure of sulfated compounds

ABBREVIATIONS

3-OST-3A	glucosaminyl 3-*O*-sulfotransferase-3A
3-OST-3B	glucosaminyl 3-*O*-sulfotransferase-3B
3-OST-1	glucosaminyl 3-*O*-sulfotransferase-1
3-OST-2	glucosaminyl 3-*O*-sulfotransferase-2
ARS	aryl sulfatase
CNS	central nervous system
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHEA ST	dehydroepiandrosterone sulfotransferase
E2	17β-estradiol
EST	estrogen sulfotransferase
FGF	fibroblast growth factor
FTZ-F1	*Fushi Tarazu* factor-1 genes
GI	gastrointestinal tract
GlaNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
HMW	high molecular weight
MEM	minimum essential medium
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PAP	3'-phosphoadenosine 5'-phosphate
PCB	polychlorinated biphenyl
PNS	peripheral nervous system

PREG	pregnenolone
PROG	progesterone
PTM	post-translational modification
SULT/ ST	sulfotransferase
Sox9	SRY HMG box related gene 9
TLC	thin-layer chromatography
tPA	tissue-type plasminogen activator
TH ST	thyroid hormone sulfotransferase
TL PST	thermolabile phenol sulfotransferase
TS PST	thermostable phenol sulfotransferase
TPST	tyrosylprotein sulfotransferase
uPA	urokinase-type plasminogen activator

CHAPTER 1

INTRODUCTION

I. Background and significance

1. Sulfotransferases

1.1 General aspects of sulfotransferases

Sulfotransferases (SULTs) constitute a superfamily of related enzymes that catalyze the transfer of a sulfuryl group from the active sulfate donor, 3'-phophoadenosine 5'-phophosulfate (PAPS), to a substrate compound containing either a hydroxyl or an amino group (**Figure 1**) (Falany, 1997a; Coughtrie et al., 1998; Chapman et al., 2004). The reaction, usually referred to as "sulfation", occurs in many prokaryotic and vertebrate species and plays an important role in numerous biological processes including detoxification, homeostasis of neurotransmitters, activation and deactivation of hormones and carcinogens, as well as transport and metabolism of steroids in circulation. Sulfation reactions are usually classified by the acceptor group involved in sulfoconjugation, *e.g.*, *O*-sulfonation (ester) and *N*-sulfonation (amide) (Strott, 2002; Huxtable, 1986). *O*-Sulfonation, a major type of cellular sulfation reaction, involves an alcohol group and can occur with a numerous of relatively small endogenous and exogenous compounds such as hormones, steroids, catecholamines, drugs, and various xenobiotic agents. *N*-sulfonation,

although less predominant than *O*-sulfonation, is nevertheless a critical reaction in the posttranslational modification of macromolecules such as carbohydrates, peptides, and proteins (Strott, 2002).

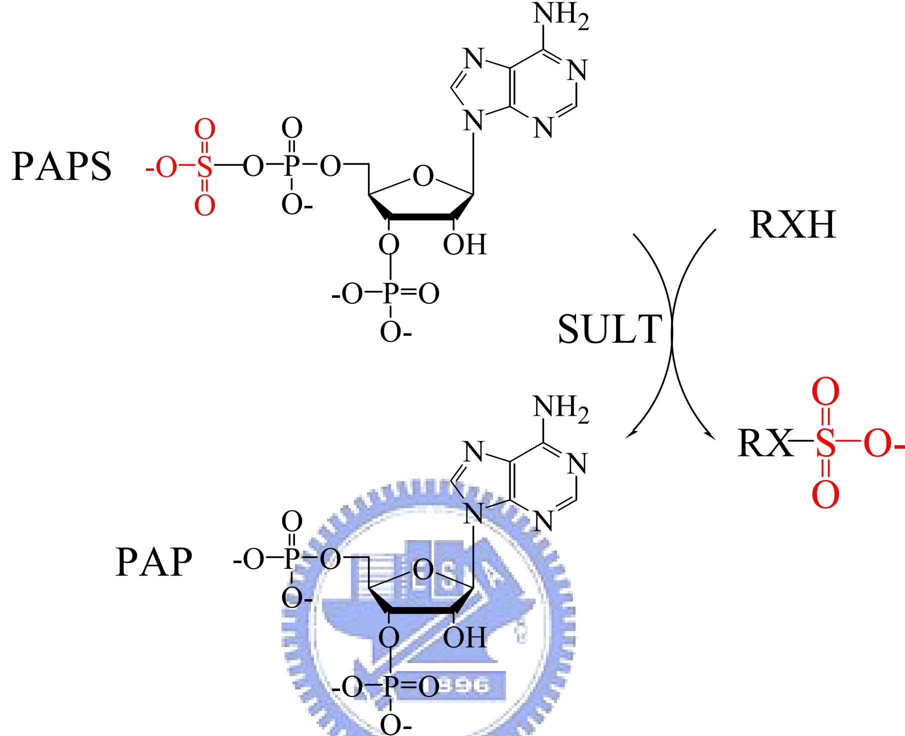

Figure 1. Physical sulfation catalyzed by SULT with PAPS as a cosubstrate.

1.2 Classification of sulfotransferases

In vertebrates, SULTs can be divided into two classes: (i) cytosolic SULTs that are responsible for the sulfation of small xenobiotics and endogenous substrates such as hormones, steroids, bile acids, neurotransmitters, as well as multitude of environmental chemicals (**Table 1**) (Falany, 1997b); (ii) membrane-bound SULTs that are resident transmembrane enzymes of the Golgi *trans*-network and catalyze the sulfation of

macromolecules such as peptides, lipids, proteins, and glycosaminoglycans, affecting both their structural and functional characteristics, as well as the molecular-recognition events and signaling transduction pathway (**Table 2**) (Chapman et al., 2004; Gamage et al., 2006; Negishi et al., 2001). There are enormous amount of sulfated molecules in biological system. However, we still understand very little about their biological functions in either metabolic pathway or physiological significance. In particular, how the interplay between various members of the SULTs and arylsulfatase (ARS) families, which are capable of the hydrolysis of sulfate esters, to regulate the availability and biological activity of xenobiotics and endogenous molecules *in vivo* remains poorly understood.

Table 1. Classification of human cytosolic SULTs

Family	Subfamily	Synonyms*	Substrate	Proposed biological function	References
SULT1A	SULT1A1	P-form PST; TS PST	Iodothyonines: T_2, T_3; Estrogens: E2	Inactivation of endogenous thyroids	Wilborn et al., 1994; Falany, 1997a
	SULT1A2	P-form PST2; TS PST2	Simple phenolic compounds: *p*-nitrophenol, N-hydroxy-2-AAF	Detoxification of xenobiotic compounds	Veronese et al., 1994
	SULT1A3	M-form PST; TL PST	Catecholamines: dopamine, norepinephrine	Homeostasis of dopamine; detoxification of dietary monoamines	Wood et al., 1994; Brix et al., 1999
SULT1B	SULT1B1	TH ST	Iodothyonines: T_2, T_3, r-T_3, T_4	Metabolism of thyroid hormones	Wang et al., 1999
SULT1C	SULT1C2	ST1C3	Simple phenols; carcinogens	Unknown	Her et al., 1997
	SULT1C4	ST	Simple phenols; carcinogens	Unknown	Sakakibara et al., 1998a
SULT1E	SULT1E1	EST	Estrogens: E1, E2	Inactivation and/or transport of endogenous estrogens	Aksoy et al., 1994; Falany et al., 1995
SULT2A	SULT2A1	DHEA ST	Hydroxysteroids: DHEA, androgens, pregnenolone, bile acids	Modulatory or bioactive effects of sulfated steroids on different receptors	Comer et al., 1993; Forbes et al., 1995; Otterness et al., 1992
SULT2B	SULT2B1a	-	DHEA, pregnenolone	AMPA receptor-mediated NO signaling	Her et al., 1998; Moloche and Falany, 2001; Kohjitani et al., 2008
	SULT2B1b	-	DHEA, pregnenolone, cholesterol, hydroxysteroids	cell proliferation and steroid hormone receptor expression	Her et al., 1998; He and Falany, 2007; Geese and Raftogianis, 2001
SULT4A	SULT4A1	Brain ST	Unknown	Unknown; may not have significant catalytic activity in vivo	Falany et al., 2000; Sakakibara et al., 2002; Allali-Hassani et al., 2007

*Abbreviations: P-form PST, simple phenol-form phenol sulfotransferase; M-form PST, monoamine-form phenol sulfotransferase; TS PST, thermostable phenol sulfotransferase; TL PST, thermolabile phenol sulfotransferase; TH ST, thyroid hormone sulfotransferase; EST, estrogen sulfotransferase; DHEA ST,

dehydroepiandrosterone sulfotransferase; T2, 3, 3'-diiodothyronine; T3, 3, 3', 5-triiodothyronine; r-T_3, 3, 3', 5'-reverse triiodothyronine; T_4, thyroxine; E1 , estrone; E2, β-estradiol; DHEA, dehydroepiandrosterone.

Table 2. Human membrane-bound SULTs

Name	Abbreviation	Substrate	Amino acid	Possible biological function
Glucosaminyl 3-*O*-sulfotransferase-3A	3-OST-3A	Heparan sulfate	406	Cell entry by HSV-1
Glucosaminyl 3-*O*-sulfotransferase-3B	3-OST-3B	Heparan sulfate	390	Cell entry by HSV-1
Glucosaminyl 3-O-sulfotransferase-1	3-OST-1	Heparan sulfate	307	Anticoagulation
Glucosaminyl 3-O-sulfotransferase-2	3-OST-2	Heparan sulfate	367	Anticoagulation
Uronosyl 2-O-sulfotransferase	UA2OST	Heparan sulfate	406	FGF binding, angiogenesis
Heparan sulfate 6-sulfotransferase-1	HS6ST-1	Heparan sulfate	401	FGF activation, angiogenesis
Tyrosylprotein sulfotransferase-1	TPST1	CCR-5; PSGL-1	370	Leukocyte adhesion, cell entry by HSV-1, P-selectin binding, enzyme dimerization
Tyrosylprotein sulfotransferase-2	TPST2	CCR-5; PSGL-1	377	Male infertility, hypothyroidism, Leukocyte adhesion, cell entry by HSV-1
GlcNAc 6-O-sulfotransferase	GlcNAc6ST	Sialyl Lewis X	484	L-selectin binding, leukocyte adhesion
Chondroitin 6-sulfotransferase	C6ST	Chondroitin sulfate	479	Cartilage development, neuronal functions, lymphocyte binding, T-cell response
HNK-1 glucuronic acid 3-OST	HNK-1 ST	HNK-1 sulfate	356	Expressed in nervous system
Keratan sulfate Gal-6-sulfotransferase	KSGal6ST	Keratan sulfate	411	Corneal transparency

1.3 Molecular mechanism of sulfotransferases

To date twenty-seven structures of human SULTs have been solved on twelve different isoforms. Crystal structures for the SULT1A1 (phenol sulfotransferase), SULT1A3 (catecholamine sulfotransferase), SULT1E1 (estrogen sulfotransferase), SULT1B1 (thyronine sulfotransferase), SULT2A1 (dehydroepiandrosterone sulfotransferase), two isozymes of SULT2B1 (pregnenolone sulfotransferase and cholesterol sulfotransferase), SULT4A1 (brain sulfotransferase), three subfamilies of SULT1C, as well as four isozymes of membrane-bound SULTs (Heparan Sulfate Glucosamine 3-O- Sulfotransferase isoform 1-3; Heparan Sulfate N-Deacetylase N-Sulfotransferase) have been characterized (Kakuta et al., 1997 and 1999; Bidwell et al. 1999; Pedersen et al., 2000; Gamage et al., 2003). These structures of SULTs, illustrated highly conserved regions of active sites and PAPS-binding sites, are similar to those of nucleotide kinases (Kakuta et al., 1997). The largest variation among these SULTs is found in the substrate-binding region. The substrate specificity and selectivity of these enzymes have a long and confusing biography due to a great deal of substrate overlap among the known SULTs. Although all SULTs carry out the same function on sulfation, their substrate specificity is significantly distinctive between the enzyme subfamilies, such as phenol and alcohol SULTs. Several recent studies have focused on this topic and implicated some characteristics of cytosolic SULTs toward their substrates, such as stereospecificity (Park et al., 1999; Pai et al., 2002) and enantioselectivity (Sheng and

Duffel, 2003). It is possible that the critical residues interacting with substrate may underscore different substrate specificity of SULTs. In addition to substrate specificity, several imperative amino acid residues in highly conserved region of PAPS-binding site were also found (Chapman et al., 2004). It was demonstrated that Ser134 is the key residue that enables SULT1A to discriminate PAP from AMP (Hsiao and Yang, 2002) and that SULT1A utilizes other nucleotides, besides PAPS, with much less catalytic efficiency (Lin and Yang, 2000). These studies barely focused on the type of nucleotides with adenosine, other nucleotides functioning with SULT1A are poorly understood. Another two structural features of cytosolic SULTs were found recently. One is a flexible loop that was proposed to control the in and out of PAP or PAPS during SULT1A catalysis (Su and Yang, 2003). Previous studies demonstrated that oxidation of SULT1A alters the enzyme's catalytic activity, pH optima and substrate activity (Marshall et al., 1997). The redox-responsive signal cascade is one of the post-translational modifications of protein, but the functions of this redox-sensing loop on SULT1A catalysis are still not clear. Cytosolic SULTs appeared to be capable of forming either homodimers or heterodimers in solution (Kiehlbauch et al., 1995). Recent articles indicated that there exists a common dimerization motif in cytosolic SULTs (Petrotchenko et al., 2001). However, the functional significance of the dimerization process is not known and the protein-protein interaction of SULTs has not been well studied.

2. Localization of human sulfotransferase

It is believed that knowing the expression pattern and cellular distribution of SULTs, along with the functional and structural information, will assist with determining the physical and functional significance of SULTs. To better understand the role of individual SULT isoforms in the regulation of various biological processes, a comprehensive study of cellular distribution of these enzymes *in vivo* is needed. Here an inclusive information has been studied in an attempt to illustrate the organ-specific distribution of individual SULT isoforms at mRNA and protein level (**Table 3**), and moreover, it may provide a useful information for further investigation of the physiological function of these enzymes.

2.1 SULT1A subfamily

SULT1A1 exhibits the highest expression level among all SULT1 isoforms in the liver. It has also been identified in brain, intestine, breast, endometrium, adrenal gland, placenta, jejunum, platelets, as well as kidney and lung. SULT1A3 is highly expressed in the jejunum and placenta with the exception of adult human liver. For the developmental perspective, a significant level of expression of both SULT1A1 and SULT1A3 appeared in the fetal liver, intriguingly, SULT1A3 nearly disappears in adult kidney and liver. The cellular localization and physiological significance of SULT1A2 is the least understood among the SULT1A members. Dooley et al. (2000) had shown the SULT1A2 encoding mRNA is present in the

liver, brain, kidney, ovary, lung, and gastrointestinal tract (GI) at the lower level. However, it appears that SULT1A2 mRNA expression does not translate into the formation of protein (Gamage et al., 2006).

2.2 SULT1B subfamily

The predominant physical function of SULT1B1 appears to be in the regulation and biotransformation of thyroid hormones. Intriguingly, a significant expression of SULT1B1 mRNA and protein has been detected in the small intestine, colon, and is also found in liver and blood leukocytes at lower levels (Teubner et al., 1998; Wang et al., 1998).

2.3 SULT1C subfamily

The biological function of SULT1C is barely understood. Previous studies have indicated the presence of SULT1C2 in adult human kidney, stomach, thyroid, as well as fetal kidney and liver. It was also demonstrated that SULT1C2 protein was clearly detectable in stomach, ileum, jejunum, rectum, and cecum (Coughtrie, 2002). At the RNA level, the abundant expression of SULT1C4 was observed in fetal kidney and lung, as well as in the adult spinal cord, kidney, and ovary. However, the protein expression of SULT1C4 has not yet been demonstrated in any adult or fetal organisms or tissues (Sakakibara et al. 1998b).

2.4 SULT1E subfamily

SULT1E1 protein was detected in liver (Forbes-Bamforth and Coughtrie, 1994; Falany et al. 1995), endometrium (Falany et al. 1998), jejunum (Her et al. 1996), and mammary epithelial cells in primary culture (Falany and Falany, 1996). The significant level of mRNA encoding SULT1E1 on the immunoblots has been demonstrated in both human liver and jejunum cytosol, and mammary epithelial cells in primary culture (Forbes-Bamforth and Coughtrie, 1994; Falany et al., 1995). The enzymatic activity of SULT1E1 was also assessed by using β-estrogen as a substrate in the different human organs, such as fetal liver, kidney, lung (Adjei et al., 2008).

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2.5 SULT2A subfamily

Northern analysis has shown SULT2A1 is extensively present in many human tissues, such as liver, adrenal, small intestine, ovary, prostate, lover, stomach, small intestine, colon, as well as brain and bone marrow (Otterness et al., 1992; Tashiro et al., 2000). The immunohistochemical study has revealed that SULT2A1 is selectively expressed in the embryonic human hepatocytes and around the central vein (Baker et al., 1994). Paker et al. (1994) also reported that SULT2A1 expression was detected in the fetal and neocortical zones of the adrenal cortex, liver, testis, and intestine. Further, kidney SULT2A1 immunostaining was present in the proximal and distal tubules, loops of Henle, collecting

ducts, and their progenitors (Baker et al., 1994). Despite the localization of SULT2A1 were inclusively investigated, the comprehensively physiological implication of this isozyme remains ambiguous *in vivo*, suggesting a simple and convenient genetic model might be needed for the further investigation.

2.6 SULT2B subfamily

Both SULT2B isoforms mRNAs have been detected in many tissues including adrenal gland, placenta, ovary, prostate, lung, kidney, colon and skin (Her et al., 1998; Dooley et al., 2000; Meloche and Falany, 2001; Javitt et al., 2001; Geese and Raftogianis, 2001). Further, the results demonstrated that SULT1Bb was more extensively expressed than SULT1Ba in a variety of hormone-responsive tissues, such as stomach, small intestine, spleen, thymus, thyroid, and liver (Geese and Raftogianis, 2001). Additionally, the protein expression of SULT1Bb is present in prostate and lung, whereas SULT1Ba is present in prostate and placenta (Geese and Raftogianis, 2001).

2.7 SULT4A subfamily

SULT4A1 is also termed "brain sulfotransferase", which sharing an extremely high degree of sequence homology (97% amino acid identity) with the orthologous rat and mouse SULTs (Blanchard et al., 2004). Immunohistochemical staining of human brain

sections showed that SULT4A1 is located extensively, but exclusively, in a variety of brain regions including cerebral cortex (motor, cingulate, arontal), globus pallidus, medial temporal lobe (island of Calleja), amygdala pituitary, thalamus, cerebellum (dentate nucleus folia: vermis, granular layer), midbrain (oculomotor, red nucleus, magnocellular), and brainstem (Liyou et al., 2003). At present, the physiological substrate of SULT4A1 remains unknown.

2.8 TPST subfamily

TPSTs reside in the Golgi compartment and therefore have access to generate the posttranslational modification of secretory and membrane proteins transported through the *trans*-Golgi network (Kehoe and Bertozzi, 2000; Monigatti et al., 2006). Northern analysis has demonstrated that both human TPST1 and TPST2 are broadly expressed in many tissues including cerebellum, fetal brain, trachea, testis, spinal cord, thyroid gland, uterus, lung, kidney, salivary gland, prostate, skeletal muscle and uterus (Ouyang et al., 1998; Mishiro et al., 2006). However, due to the lack of the isoenzyme-specific antibodies or suitable analytic reagents and probes, the cellular distribution and related abundance of TPST isoforms at the protein level have not yet been studied.

Table 3. Tissue-specific distribution of human cytosolic SULTs and TPST isoforms

Name	Chromosome	Amino acids	Tissue-specific expression	
			mRNA level	Protein expression
SULT1A1	16p12.1	295	Epithelial cells within stomach, gastricpits, colon, crypts of small intestine	Very high in liver; brain, breast, intestine, endometrium, adrenal gland, platelets, placenta, kidney, lung, jejunum
SULT1A2	16p12.1	295	Liver, kidney, brain, lung, ovary, GI	-*
SULT1A3	16p11.2	295	Epithelial cells within stomach, gastricpits, colon, and crypts of small intestine	Very high in jejunum and colon; intestine, platelets, placenta, brain
SULT1B1	4q13.3	296	Liver, small intestine, colon, blood leukocytes	Highest expression observed in colon; also detected in liver and small intestine
SULT1C2	2q11.1-q11.2	296	Adult stomach, kidney, thyroid; fetal kidney > fetal heart, kidney, ovary, spinal cord	Stomach, ileum, jejunum, rectum, cecum
SULT1C4	2q11.1-q11.2	302	Adult kidney, ovary, spinal cord; fetal lung, kidney, heart	-
SULT1E1	4q13.1	294	Liver, kidney, lung, adrenal gland, intestine	Liver, endometrium, GI, trachea, pancreas
SULT2A1	19q13.3	285	Adrenal gland, ovary, prostate, lover, stomach, small intestine, colon, brain, bone marrow	Liver, adrenal, duodenum, central vein, brain *e.g.* thalamus, hypothalamus
SULT2B1a	19q13.3	350	Adrenal gland, placenta, ovary, prostate, lung, kidney, colon	Prostate, placenta
SULT2B1b	19q13.3	365	Adrenal gland, placenta, ovary, prostate, lung, kidney, colon, stomach, small intestine, spleen, thymus, thyroid	Prostate, lung
SULT4A1	22q13.2-q13.31	284	Cerebral cortex, frontal lobe, cerebellum, occipital lobe, temporal lobe, medulla, putamen, lowest in the spinal cord	Brain e.g. cerebral cortex, medial temporal lobe, amygdale pituitary, thalamus, hypothalamus, midbrain, cerebellum, lentiform nucleus, hippocampus, midbrain, brainstem

(Continued)

TPST1	7q11.21	370	Cerebellum, fetal brain, trachea, testis, spinal cord, thyroid gland, uterus, lung, kidney, salivary gland, prostate, skeletal muscle, uterus	-
TPST2	22q12.1	377	Cerebellum, fetal brain, trachea, testis, spinal cord, thyroid gland, uterus, lung, kidney, salivary gland, prostate, skeletal muscle, uterus	-

*Related study is currently not available.

3. Biological importance of sulfotransferases

3.1 Sulfation in biology

In vertebrates, two classes of SULTs can be distinguished. One of which, membrane associated SULTs, sulfonates endogenous macromolecular molecules and comprises mainly membrane-bound forms localized in Golgi apparatus. No xenobiotic-metabolizing properties have been reported insofar. The other class of enzyme is cytosolic SULTs which were responsible for sulfation of various small endo- and exogenous compounds, such as hormones, bioamines, drugs, and various xenobiotic agents (Falany, 1997a). Although the range of compounds which were sulfated is enormous, yet we still understand relatively little of the functions either of the metabolic pathway or physiological significance. Furthermore, large numbers of studies revealed the possible associations between the polymorphisms of SULTs and physiological disorders, for example, cancers, hypertension, neoplasias and neurodegenerative diseases, which were seriously epidemiological risks for human beings (Glatt and Meinl, 2004; Sachse et al., 2002; Wu et al., 2003; Peng et al., 2003; Steiner et al, 2000). Whereas sulfation is vital for various physiological regulations, hydrolysis of sulfate esters, catalyzed by ARS, also linked to many important cellular functions including bioactivation of endogenous compounds, cellular degradation and modulation of signaling pathways. Particularly, how the association between the members of SULT families and diseases regulates the availability and biological activity of

xenobiotics and endogenous chemicals remains to be clarified.

3.2 Hepatic detoxication and biotransformation

Sulfation is a major way *in vivo* for the biotransformation of a wide range of structurally diverse endo- and exogenous substrates including phenolic (17β-estradiol; E2), alicyclic hydroxysteroids (DHEA), and iodothyronines, phenols (2-naphthol), primary (ethanol) and secondary alcohols (2-butanol) (Gamage et al., 2006; Goldstein and Faletto, 1993; Chapman et al., 2004). The responsible enzymes, cytosolic SULTs, are traditionally known as a group of Phase II detoxifying enzymes widely found among various invertebrate and vertebrate species (Nowell and Falany, 2006). Conjugation with sulfate is generally considered a detoxication or inactivation mechanism (Goldstein and Faletto, 1993). The sulfated compounds may become more soluble in aqueous solution, less ability of the penetration of cell membrane, and therefore, can be more easily excreted from the body via the kidney or bile (Glatt et al., 2001). For example, acetaminophen, an analgesic, is chiefly excreted as sulfated conjugates in the urine (Glatt and Meinl, 2004). From this perspective, SULTs can be considered as a part of the chemical defense mechanism *in vivo* (Allali-Hassani et al., 2007; Gamage et al., 2006).

On the other hand, sulfation plays an important role in the bioactivation of a range of

compounds including as aminoazo dyes, benzidines, heterocyclic amines, hydroxymethyl polycyclic aromatic hydrocarbons, terpenes, β-aminoethyl alcohols, and 2-nitropropane (Michejda and Kroeger-Koepke, 1994; Gamage et al., 2006). For the N-hydroxy arylamines like 2-acetylaminofluorene (2-AAF), previous studies has demonstrated that the N-hydroxylated 2-AAF mediated by cytochrome 1A2 is a potent substrate of SULT1A2 and SULT1A1 (Glatt, 2000; Meinl et al., 2002). The sulfated metabolites were found to be more carcinogenic than the parent amide, suggesting the importance of SULTs as one of the metabolic pathways involved in the activation of 2-AAF (Meinl et al., 2002).

3.3 Regulation of sulfotransferase and its metabolites in nervous system

In 1913, Harvey Cushing, a giant of American medicine and brain surgery, presented his concept that "psychic conditions profoundly influence the discharges from the glands of internal secretion". He postulated that each glandular disorder would induce a typical psychopathology. The steroidal environment of the brain varies as a function of the life cycle or apart of intercurrent events such stress and illness means this can have profound consequences both directly on the integrity of the brain and on its resistance to toxic or noxious agents. The term "neurosteroids", apart from being synthesized by classical steriodogenic organs, such as progesterone (PROG), pregnenolone (PREG) and DHEA, were produced by *de novo* in central nervous system (CNS) and peripheral nervous system

(PNS) (Vallée et al, 2001). They were found in high levels and regulated the activity of genes and protein synthesis, cellular development, neuroendocrine system and behavioral pattern (De Nicola et al, 1993). SULT2A1 catalyzes the sulfonation of DHEA in its transition into dehydroepiandrosterone sulfate (DHEAS) (Adams et al., 1985; Longcope et al., 1982; Longcope, 1996). Both DHEA and DHEAS are critical precursors for several types of androgens and estrogens. They also involve in numerous neurophysical processes, such as increasing neuronal excitability, and enhancing neural plasticity and neuroprotective properties. Previous studies have reported that the concentrations of DHEA(S) in blood decrease markedly with ageing in humans (Orentreich et al, 1992; Vallée et al, 2000), and have been proposed to be the neuromodulators involved in age-related cognitive decline (Näsman et al, 1991; Sunderland et al, 1989). These findings have led to the hypothesis that elevated concentrations of steroid and its sulfate product may influence both physical and cognitive aging (Vallée et al, 2001).

3.4 Pathopharmacological properties of sulfotransferases

An increasing volume of studies have revealed the possible relationship between the expression pattern of SULTs and physiological disorders such as cancers, obesity, hypertension, neoplasias and certain neurodegenerative diseases. The altered levels of SULTs in individuals may greatly vary the cellular responsiveness to pathological

mechanisms. For example, it is believed that the administration of the progestin-derived anti-breast cancer drugs, medrogestone and tibolone, significantly induces the transcriptional regulation of SULT1E1 in estrogen-responsive breast cancer, which normally expresses lower levels of SULT1E1 than health adults (Chetrite and Pasqualini, 2001; Pasqualini and Chetrite, 2007). SULT1E1 catalyzes the estrogen into the biologically inactive estrogen sulfates that inhibit their action to responsive receptors, indicating the SULT1E1 plays a critical role in the estrogen homeostasis that may be disrupted in breast cancer (Gamage et al., 2006; Chetrite and Pasqualini, 2001; Pasqualini and Chetrite, 2007).

Statistically significant associations were also observed between the SULT1A1 genotype and age, obesity and certain neoplasias including mammary, pulmonary, esophageal and urothelial cancer (Glatt and Meinl, 2004; Sachse et al., 2002; Wu et al., 2003; Peng et al., 2003; Steiner et al, 2000). The elevated SULT activity phenotype was significantly more frequent in the cancer patient than in the controls (Glatt et al., 2001). Furthermore, there continues a broad interest in exploring the physiopathological and neuroregulatory role of SULT2A in the induction and maintenance of the endocrine-dependent cancers, including breast cancer and carcinoma of prostate gland (Qian et al., 1998; Purohit et al., 1999; Billich et al., 2000; Baulieu, 1998; Puia and Belelli, 2001). Theoretically, the sulfate metabolites of steroids, such as estrone, and testerone,

could be critical mechanism in modulating the availability of unconjugated parent molecules interact with corresponding receptors. Because only the unconjugated molecule has the growth-promoting abilities, abnormal regulation or metabolism of SULT2A, therefore, it may have the pathological implications (Strott, 2002). Nevertheless, the association with diseases (*e.g.* neoplasias, hypertension) and health-related parameters appears to be multifarious and varies between subgroups. It remains an important issue for us to investigate the physiological and pathopharmacological roles of SULTs in more detail.

4. Invertebrate versus vertebrate: potential application of non-mammalian models for sulfation analysis

Human SULT comprises of a multi-gene family of proteins containing at least thirteen distinct members that differ remarkably in their enzymatic properties, localization, regulatory and metabolic mechanism (Lindsay et al., 2008). Due to the multicellular complexity of higher species, the lack of suitable genetic models and increasingly moral concerns, however, studies in sulfation have difficulty circumstantiating the physical functions and regulatory mechanisms of those enzymes in mammals. A simple animal model for elucidating the genetic and molecular dissection of complex mechanism is therefore needed.

Over the past decades, non-mammalian models were thought to be far from the higher organisms for the study of biomedical science because of the phylogenic and functional diversities. To date, it has become abundantly clear that some of the non-mammals are not only convenient materials but are also shared with the physiological and pharmacological properties common to humans (Peterson et al., 2008). Here we utilized two premiere model organisms, *Drosophila melanogaster* and zebrafish, as the alternative testing systems for the study of the localization and functional analysis of SULTs.

4.1 *Drosophil. melanogaster*

4.1.1 *Drosophila*: systematical biomaterial for biomedical study

Cytogenetic studies in the complete mapping of the *Drosophila* polytene chromosomes and genomic sequence have altered our estimate of the evolutionary and physiological relationship between vertebrate and invertebrate (Reiter et al., 2001; Gilbert, 2008). Among the 929 distinct human disease genes, 714 are associated with 548 unique *Drosophila* genes, of which 153 are associated with known mutant alleles and 56 more are tagged by *P*-element insertions in or near the gene (Reiter et al., 2001; Gilbert, 2008). The rapid and high-throughput genetic screen of *P*-element insertion or deletion in *Drosophila* for the study of the mutagenesis and the genetic modifications has been widely utilized for many years (Igarashi, 2005; Gilbert, 2008; Castro and Carareto, 2004). This unique feature

revolutionizes the study of the transposition and regulation of a transposon in a eukaryote by using this tiny insect as a premiere model organism (Castro and Carareto, 2004). In addiction, *Drosophila* share most of the basic metabolic homeostasis found in vertebrates (Baker and Thummel, 2007). This tiny insect mimics the intricate carbohydrate, sterol, lipid metabolism, the timing and process of developmental events, as well as the pharmacological, neurobiological and pathological mechanisms in humans. Therefore, *Drosophila melanogaster*, with the functional similarities of genetic, developmental and metabolic characterizations, provides an important insight for the studies of the higher vertebrate systems (Baker and Thummel, 2007).

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4.1.2 SULTs in *Drosophila*

In *Drosophila*, several types of membrane-associated SULTs have been identified and characterized, and the physical functions and biological regulations modulated by the sulfate conjugates have been extensively investigated (**Table 4**) (Kushe-Gullberg and Kjellén, 2003; Xu et al., 2007; Kamimura et al., 2004 and 2006; Sergeev et al., 2001; Zhu et al., 2005; Sen et al., 1998). The sulfation of hexuronate and glucosamine units, usually *N*-acetylgalactosamine (GlaNAc) or *N*-acetylglucosamine (GlcNAc), has been shown to be essential for the fibroblast growth factor (FGF)-mediated tracheal formation, the stability or intracellular trafficking of Notch protein, the developmental processes and embryogenesis,

as well as differentiation and neuronal functions (Lander et al., 1996; Xu et al., 2007; Kamimura et al., 2006; Sen et al., 1998; Sergeev et al., 2001; Zhu et al., 2005). The findings have demonstrated that sulfation is a critical regulator for developmental and neuroregulatory functions in *Drosophila*, however, there has been no evidence clearly indicating that cytosolic ST(s) is(are) present or expressed in this important animal model. Recently, Hattori et al. have reported that four cytosolic SULT homologs, designed as *dmST1-4*, were identified by a research of the *Drosophila melanogaster* genome database (http://flybase.bio.indiana.edu/). Each of the four isozymes have classified into separate and novel gene family, as determined by the subsequent amino acid sequence alignment and molecular phylogeny analysis (Hattori et al., 2008). Despite the low amino acid sequence homology (less than 40%) between those isozymes, and also toward their vertebrate homologs, a degree of amino acid sequence similarity does exist between *dmSTs* and the human SULT family members suggests that, at least in part, the insect cytosolic SULTs share an common ancestral gene or conserved residues with other mammal SULTs (Hattori et al., 2008).

Table 4. Membrane-associated SULTs and SULT-related proteins found in *Drosophila**

Drosophila	Functional similarity	Proposed functions	References
Hs2st	human Hs2st	FGF-mediated tracheal formation	Xu et al., 2007; Kamimura et al., 2006
Hs3st	human Hs3st	Notch signal	Kamimura et al., 2004
Hs6st	human Hs6st	Stability or intracellular trafficking of Notch protein	Kamimura et al., 2001 and 2006
PIPE-st1	human Hs2st	Probably nonfunctional protein	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st2	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st3	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st4	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st5	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st6	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st7	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st8	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
PIPE-st9	human Hs2st	Formation of embryonic dorsal-ventral polarity	Sen et al., 1998; Sergeev et al., 2001; Zhu et al., 2005
PIPE-st10	human Hs2st	Unknown	Sergeev et al., 2001; Zhu et al., 2005
Retinol dehydratase	SULT-related protein	Cytosolic SULT activity	Pakhomova et al., 2005

*Abbreviation: Hs2st, heparan sulfate 2-*O* sulfotransferase; Hs3st, heparan sulfate 3-*O* sulfotransferase; Hs6st, heparan sulfate 6-*O* sulfotransferase

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4.2 Danio rerio (zebrafish)

4.2.1 An idea vertebrate model for the genomic and embryonic analysis

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies (Beis and Stainier, 2006; Aleström et al., 2006; Strähle and Blader, 1994). The completion of the genome sequence and expression profiles of zebrafish has made it an ideal vertebrate model of choice for a wide spectrum of biological studies. Numerous scientists take advantages of this photogenic creature as an emerging model for functional genomics, pharmacology, developmental biology, and even human biomedical researches (Strähle and Blader, 1994). As a tropical teleost, zebrafish is remarkable for its many benefits: small size and ease of breeding, transparent embryo, rapidly developmental process, short generating intervals and sexually reproductive ability. The increasing popularity of zebrafish is also due to its amenability to well-characterized gene set of encoded proteins and to an eventual understanding of how they work to mimic the intricate genetic and physiological regulation in higher vertebrate animals. Exploiting the full potential of zebrafish is obligatory. In fact, zebrafish serves as a superior model than other invertebrate system, particularly in studies of large-scale mutagenesis and altered phenotype screening (Currie, 1996). By successful application of forward and reverse genetics, more than 2000 mutations are identified for perturbing the normal development of zebrafish.

4.2.2 Zebrafish cytosolic SULTs

The special characteristics make the zebrafish a premier model for a systematic investigation of the ontogeny, cell type/tissue/organ-specific expression, and physiological involvement of individual SULTs. A prerequisite for using zebrafish in these studies is the identification of the various cytosolic SULTs and their biochemical characterization. By searching the expressed sequence tag database, ten zebrafish cDNAs encoding putative cytosolic SULTs were systematically cloned, expressed, and characterized (**Table 5**) (Sugahara et al., 2003a, b, c, d; Liu et al., 2005; Yasuda et al., 2005a,b; Yasuda et al., 2006). Of the ten zebrafish SULTs, six fall within the SULT1 gene gamily (Sugahara et al., 2003a,b; Liu et al., 2005; Yasuda et al., 2005a,b); three belong to the SULT2 gene family (Sugahara et al., 2003c; Yasuda et al., 2006); and one (designated SULT X) appears to be independent from all known SULT gene families (Sugahara et al., 2003d). The analysis of amino acid sequence via BLAST search revealed that these zebrafish SULT isozymes display sequence homology to mammalian SULTs.

The sequence analysis also revealed that the recombinant zebrafish SULTs contains two sequences resembling the so-called "signature sequences" (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region) which are similar to mammalian SULTs. Of these two sequences, YPKSGTxW has been demonstrated by X-ray

Table 5. Features of the zebrafish SULT family

Group	Name	Gene ID	Locus	GenBank accession No.	Amino aicd	Reference
SULT1	SULT1 ST1	323424	chromosome 8	AY181064	299	Sugahara et al., 2003a
	SULT1 ST2	368269	chromosome 8	AY181065	301	Sugahara et al., 2003a
	SULT1 ST3	368270	chromosome 8	AY196985	301	Sugahara et al., 2003b
	SULT1 ST4	402915	chromosome 20	AY196986	304	Liu et al., 2005
	SULT1 ST5	619193	unknown	AY879099	293	Yasuda et al., 2005a
	SULT1 ST6	436872	chromosome 12	AY937249	308	Yasuda et al., 2005b
SULT2	SULT2 ST1	338214	unknown	AY181063	287	Sugahara et al., 2003c
	SULT2 ST2	777793	unknown	DQ640387	287	Yasuda et al., 2006
	SULT2 ST3	777792	unknown	DQ640388	288	Yasuda et al., 2006

crystallography to be responsible for binding to the 5'-phosphosulfate group of PAPS, a co-substrate for SULT-catalyzed sulfation reactions, and thus designated the "5'-phosphosulfate binding (5'-PSB) motif" (Chapman et al., 2004). The recombinant zebrafish SULTs also contain the "3'-phosphate binding (3'-PB) motif" responsible for the binding to the 3'-phosphate group of PAPS.

4.2.3 Enzymatic characteristics of the zebrafish cytosolic SULTs

Zebrafish cytosolic SULTs had been shown to involve regulation of endogenous hormones and detoxication of xenobiotic chemicals in past five decades (Nowell and Falany, 2006; Gamage et al., 2006; Armstrong, 1987). A variety of endogenous and xenobiotic compounds were tested as substrates for the zebrafish SULTs in many studies, and the activity data obtained are given in **Table 6**. In view of substrate specificity and selectivity (**Table7**), zebrafish SULT1 group was found to prefer catalyzing phenolic compounds, such as dopamine, thyroid hormones, and estrogen. Among zebrafish SULT1 group, all except SULT1 ST6 were able to catalyze thyroid hormones. Intriguingly, zebrafish SULT1 ST5 showed sulfating activities toward only thyroid hormones and their metabolites, including L-T_3, 3,3',5-triiodo-D-thyronine (D-T_3), 3,3',5'-triiodo-L-thyronine (L-rT_3), L-thyroxine (L-T_4), and L-thyronine.

Table 6. Substrate specificity of zebrafish cytosolic SULTs[a]

SULT family	Classification	Substrate		Homology
PST	SULT1 ST1	Hydroxychlorobiphenyls	*n*-Propyl gallate 3-Choloro-4-biphenylol 2-Naphthol	hSULT1A1 (49%)
	SULT1 ST2	Hydroxychlorobiphenyls	Estrone 17β-Estradiol $_L$-Dopa	hSULT1A1 (51%)
	SULT1 ST3	Phenolic compounds	Dopamine Thyroid hormone β-Naphthol	hSULT1A1 (50%)
	SULT1 ST4	Phenolic compounds	Thyroid hormone Estrone DHEA Flavonoids	hSULT1A1 (49%)
	SULT1 ST5	Thyroid hormone	$_D$-T_3 $_L$-T_3 $_L$-T_4	hSULT1B1 (50%)
EST	SULT1 ST6	Phenolic compounds	β-Naphthol Estrone 17β-Estradiol	mSULT1A2 (56%)[b]
AST	SULT2 ST1	Steroids	DHEA Pregnenolone Allopregnanolone	hSULT2B1b (43%)
	SULT2 ST2	Steroids	DHEA Pregnenolone	hSULT2B1a (43%)
	SULT2 ST3	Steroids	Corticosterone 17β-Estradiol	hSULT2B1a; hSULT2B1b (49%)

[a]References are listed in the *Chapter I, 4.2.2.*

[b]It cannot be classified into any of the existing subfamilies within the SULT1 family.

Previous studies had shown that dopamine was catalyzed by human SULT1A3 (Chapman et al., 2004). Among zebrafish SULT1 group, we found ST1, 2, and 3 can utilize dopamine as substrate, with the highest activity by SULT1 ST3 (Sugahara et al., 2003b). It is remarkable that only SULT1 ST2 was determined for the sulfating capacity of the precursor of dopamine, Dopa. On the other hand, estrone and 17β-estradiol, the key steroid hormones for sex determination, were also catalyzed by SULT1 group (except ST4 and 5). It should also be pointed out that, of the six zebrafish SULT1 isoforms (Sugahara et al., 2003a, b; Liu et al., 2005; Yasuda et al., 2005a, 2005b), the SULT1 ST2 also exhibited strong activities toward estrone and 17β-estradiol (Yanagisawa et al., 1998; Sugahara et al., 2003c). In addition, the SULT1 ST 6 appears to be the only zebrafish SULT isoform known to date that displays substrate specificity exclusively for endogenous estrogens. These findings implied that the SULT1 ST6 plays a critical role in the metabolism and homeostasis of endogenous estrogens *in vivo*. Except endogenous estrogens, xenobiotic plant-derived phytoestrogens and environmental estrogens were also catalyzed by SULT1 group.

Zebrafish SULT2 was found to prefer catalyzing hydroxysteroids, such as DHEA, PREG, and corticosterone. To date, three zebrafish SULT2 isoforms had been cloned and characterized (as shown in **Table 8**). Similar to human SULT2A1, the zebrafish SULT2 ST1

Table 7. Specific activities of the zebrafish SULT1 subfamily toward endogenous and xenobiotic compounds*

Substrate	Specific activity ($nmol.min^{-1}.mg^{-1}$)					
	SULT1 ST1	**SULT1 ST2**	**SULT1 ST3**	**SULT1 ST4**	**SULT1 ST5**	**SULT1 ST6**
Dopamine	3.0 ± 1.2	0.3 ± 0.2	11.2 ± 1.0	ND**	ND	ND
3,3',5-Triiodo-$_L$-thyonine ($_L$-T_3)	7.9 ± 0.7	17.4 ± 1.4	22.9 ± 0.9	1.0 ± 0.1	17.4 ± 0.8	ND
Thyroxine (T_4)	0.3 ± 0.1	3.2 ± 0.5	3.5 ± 0.9	0.4 ± 0.1	4.3 ± 0.1	ND
17-beta-estradiol	0.7 ± 0.5	91.6 ± 5.9	2.4 ± 0.6	ND	ND	19.1 ± 0.6
Estrone	0.4 ± 0.1	83.9 ± 3.8	3.0 ± 0.5	1.5 ± 0.5	ND	30.2 ± 1.1
DHEA	0.2 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	ND	ND
L-Dopa	ND	1.5 ± 0.3	ND	ND	ND	ND
D-Dopa	ND	2.6 ± 0.7	ND	ND	ND	ND
p-Nitrophenol	10.1 ± 1.3	60.0 ± 4.7	15.0 ± 0.6	0.5 ± 0.3	4.7 ± 0.1	ND
2-Naphthol	122.0 ± 4.0	155.0± 4.0	110.0 ± 4.0	152.0 ± 2.0	12.3 ± 0.2	38.0 ± 0.5
2-Naphthylamine	16.9 ± 1.0	18.0 ± 0.4	17.4 ± 2.1	5.0 ± 2.0	0.4 ± 0.1	ND
Daidzein	13.1 ± 0.1	82.9 ± 3.5	249.0 ± 2.0	ND	9.8 ± 0.6	15.4 ± 1.0
Kaempferol	28.1 ± 3.2	91.2 ± 6.4	170.0 ± 2.0	150.2 ± 5.3	14.5 ± 0.7	ND
Caffeic acid	21.5 ± 1.4	12.1 ± 0.7	63.7 ± 0.2	1.6 ± 0.4	10.0 ± 0.2	ND
Chlorogenic acid	65.2 ± 4.2	4.7 ± 0.2	386.0 ± 1.0	ND	23.5 ± 0.5	ND

*References are listed in the *Chapter I, 4.2.2.*

** ND, not detectable.

Table 8. Specific activities of the zebrafish SULT2 subfamily with endogenous compounds as substrates*

Substrate	Specific activity ($pmol.min^{-1}.mg^{-1}$)		
	SULT2 ST1	**SULT2 ST2**	**SULT2 ST3**
DHEA	554.0 ± 48.0	579.0 ± 24.0	40.9 ± 2.5
Corticosterone	ND**	ND	510.0 ± 23.0
Pregnenolone	628.0 ± 59.0	47.4 ± 5.9	68.8 ± 4.0
17β-Estrodiol	63.2 ± 0.5	23.6 ± 2.7	51.7 ± 3.3
Allopregnanolone	245.0 ± 18.0	ND	37.8 ± 2.1
Estrone	ND	17.9 ± 0.4	ND
4-androstene-3,17-dione	122.0 ± 10.0	ND	ND
17α-Hydroxypregnenolone	44.8 ± 9.7	ND	ND
Alignment (%)	100	87.5	52.6

*References are listed in the *Chapter I, 4.2.2*.

** ND, not detectable

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can catalyze a wide range of neurosteroids, such as DHEA, PREG, allopregnanolone, 17α-hydroxypregnenolone, and 4-androstene-3,17-dione. It displayed strongest sulfating activity toward DHEA, but virtually no activities toward the rest of the endogenous and xenobiotic compounds tested, such as dopamine, T_4, Dopa, estrone, β-naphthol, daidzein, gallic acid, and *n*-propyl gallate (Sugahara et al., 2003c). It is notable that the SULT2 ST2 shares a high degree of sequence homology (87.5% amino acid identity) with the SULT2 ST1 and displayed strongest sulfating activity toward DHEA, however, the distinct substrate-catalyzing profile was found. Much lower sulfating activity toward PREG, and no activity toward allopregnanolone, 17α-hydroxypregnenolone, and 4-androstene-3,17-dione. The relatively low activity of SULT2 ST3 in catalyzing DHEA, PREG, allopregnanolone, 17α-hydroxypregnenolone, and 4-androstene-3,17-dione was found. Nevertheless, this isozyme appears to play an important role in the metabolism of corticosterone.

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CHAPTER 2

Immunohistochemical Analysis of a Novel Dehydroepiandrosterone Sulfotransferase-like Protein in *Drosophila* Neural Circuits

Sulfotransferases (SULTs) constitute a group of enzymes that catalyze the transfer of a sulfuryl group from the universal sulfate donor, 3'-phosphoadenosine-5'-phophosulfate (PAPS), to acceptor substrate compounds containing hydroxyl or amino groups. This reaction, usually referred to as "sulfation", occurs in many prokaryotic and vertebrate species and plays an important role in numerous biological processes including hormone regulation, maintaining the dynamic balance of neurotransmitters, as well as transport and metabolism of steroids in the circulation. The insect, *Drosophila melanogaster*, frequently is used as a model for the study of learning, memory and behavioral manifestations because it is able to mimic the intricate neuroregulation and recognition of the neuronal network system in human beings. However, there is no evidence indicating that cytosolic SULT is expressed in this important animal model. The aim of this study is to investigate whether or not cytosolic SULT is expressed in the *Drosophila* nervous system. Immunoblot analysis demonstrated that DHEA-like ST was expressed in *Drosophila* brain and a sensitive fluorometric assay detected its enzymatic activity. Moreover, immunohistochemical results illustrated that DHEA-like SULT was abundant in specific neural bodies as well as in several bundles of positive

synapses in *Drosophila*. The possible linkage between SULT and a neurotransmitter-mediated effect may help in alleviating amnesiac disorders in humans.

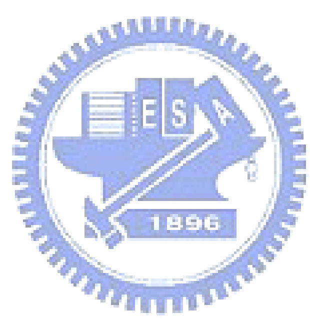
I. INTRODUCTION

Cytosolic sulfotransferases (STs) are enzymes that catalyze the transfer of a sulfuryl group from the universal sulfate donor 3'-phophoadenosine 5'-phophosulfate (PAPS) to a variety of endogenous and exogenous compounds, such as steroids, amines, and various xenobiotic chemicals (Chapman et al., 2004). The reaction, usually referred to as "sulfation", occurs in many prokaryotic and vertebrate species and plays an important role in numerous biological processes including hormone regulation, homeostasis of neurotransmitters, as well as transport and metabolism of steroids in circulation. The sulfation of steroids decreases their biological activity, rendering them incapable of binding and activating steroid receptors. These sulfated steroids, nevertheless, may serve as prohormones, which can be reactivated by desulfation (Falany, 1997). Although most steroids are synthesized in steroidogenic organs, a few, such as progesterone (PROG), pregnenolone (PREG) and DHEA, are produced *de novo* in the central nervous system (CNS) and peripheral nervous system (PNS) (De Nicola, 1993). These neurosteroids regulate specific gene expression and protein synthesis, cellular development, neuroendocrine functioning, and behavioral pattern (Valleé et al., 2001). Dehydroepiandrosterone sulfotransferase (DHEA ST) catalyzes the sulfation of DHEA, converting it to dehydroepiandrosterone sulfate (DHEAS) (Longcope, 1996). Both DHEA and DHEAS are critical precursors for the production of several types

of androgens and estrogens. DHEA can also be metabolized to form testosterone, estradiol and androstenediol (Young et al., 1997). DHEA and DHEAS are involved in numerous neurophysiological processes, such as increasing neuronal excitability, and enhancing neural plasticity and neuroprotective properties. Previous studies have demonstrated that the concentrations of DHEA and DHEAS in blood decrease markedly with age in humans, and have been proposed to be the neuromodulators involved in age-related cognitive decline (Legrain and Girard, 2003). These findings have led to the hypothesis that elevated concentrations of steroids may influence both physical and cognitive aging.

Drosophila melanogaster is a popular animal model for pathological and neuropharmacological research. At the molecular level, the nervous system of the *Drosophila* can mimic the intricate neuroregulation of the neuronal network in humans. In *Drosophila*, several types of carbohydrate STs have been cloned and characterized, and the physical functions and biological regulations modulated by the sulfate conjugates have been extensively investigated (Kushe-Gullberg and Kjellé, 2003). The sulfation of hexuronate and glucosamine units, usually *N*-acetylgalactosamine (GlaNAc) or *N*-acetylglucosamine (GlcNAc), has been shown to be essential for development and embryogenesis, as well as differentiation and neuronal functions (Lander, 1996). The recent studies have demonstrated that sulfation is a critical regulator for developmental and neuronal functions in *Drosophila*,

however, there has been no evidence indicating that cytosolic ST(s) is(are) present in this important animal model. In the present work, we used a specific DHEA ST antibody to determine the existence and distribution of DHEA ST-like protein in the nervous system of *Drosophila*. Moreover, we investigated and confirmed the presence of DHEA-sulfating activity of in *Drosophila* brain extracts by employing a continuous fluorometric assay.



II. MATERIALS AND METHODS

Materials

MUS, MU, DHEA, PAP, PAPS, acrylamide/bis-acrylamide, tris[hydroxymethyl]aminomethane (Tris) and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Potassium phosphate (monobasic, dibasic), potassium chloride, sodium chloride, glycine, and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ 08865 U.S.A.). RC DC protein assay (RC reagent package) was acquired from Bio-Rad laboratories (CA94547, USA). *h*DHEA-ST antiserum was purchased from CALBIOCHEM® (San Diego, CA92121, USA). ECL™ plus western blotting detection system, ECL™ western blotting detection system reagents, ECL™ plus western blotting reagent pack, Hybond ECL™ nitrocellulose membrane and Hyperfilm™ ECL™ were obtained from Amersham Pharmacia Biotech (Buckinghamshire, England). All other chemicals were reagent grade.

Preparation of recombinant STs

Recombinant human phenol-preferring phenol sulfotransferase (*h*P-PST), human catecholamine-preferring phenol sulfotransferase (*h*M-PST), and *h*DHEA ST were cloned into an expression vector, pGEX-2TK and transformed into *Escherichia coli* BL21 (DE3). These

STs were expressed in the form of GST-fusion protein and purified by glutathione (GSH)-bound sepharose. The methods of expression and purification of these three STs were described previously (Sakakibara et al., 1998). Recombinant rat phenol sulfotransferase (*r*PST) was cloned into expression vector pET3c and transformed into *E. coli* BL21 (DE3). Briefly, DEAE, hydroxyapatite, and size-exclusion chromatography were used to purify the *r*PST (Su and Yang, 2003). Putative *Drosophila* cytosolic ST, *dm*CG5431, was found on the Flybase database (CG5431; http://flybase.bio.indiana.edu/). By using RT-PCR, a full length cDNA encoding *dm*CG5431 was cloned into pET-41b and transformed into *E. coli* BL21 (DE3). GST-fusion and His-tagged ST was expressed and purified by GSH-bound sepharose and Ni-chelating column. All purified STs were in homogeneous form and determined by SDS-PAGE.

Indirected enzyme-linked immunosorbent assay (IELISA)

Approximately 1 μg of the recombinant STs, *h*P-PST, *h*M-PST, *h*DHEA ST, *r*PST and *dm*CG5431 were coated on each well of an ELISA plate for screening using *h*DHEA ST antibody. Following washes with PBS, 50 μl of serially diluted solutions of *h*DHEA ST antibody were added to individual wells and incubated for 1 h. Afterwards, each well was washed three times with PBST (PBS containing 0.1% BSA and 0.05% Tween-20). Bound antibodies were then detected using a goat anti-rabbit IgG conjugated with horseradish

peroxidase (HRP) for 30 min in PBST. Finally, each well was washed and developed with 0.04% 2,2-azino-bis(3-ethylbenz- thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2 in PBS. For denatured conformation analysis, all steps were the same except that the recombinant STs were treated with 2% β-ME and heating prior to being coated on the wells.

Preparation of Drosophila brains homogenates

The *Drosophila* heads were freshly isolated by liquid nitrogen freezing and harvested through a sieve to separate the heads from the bodies. Approximately 0.2 g of frozen brain samples were homogenized using a mortar and pestle, dissolved in a lysis buffer (2 mM sucrose plus 3 mM β-ME, 0.2% Triton X-100 and 0.5% protease inhibitor cocktail in 10 mM HEPES buffer, pH 7.4) and then centrifuged to remove cell debris. The homogenate was centrifuged twice at 15,000 rpm for 20 min at 4°C. The supernatant was collected and the total protein concentration was estimated.

Protein estimation

Using BSA as a standard, protein quantitation of the homogeneous from *Drosophila* brains was estimated by a colorimetric assay (RC DC protein assay) on the basis of absorbence at 750 nm with a UV/Vis spectrophotometer. (Hitachi UV/Vis-3300, Japan).

Immunoblot analysis

Approximately 5μg of five recombinant STs, *h*P-PST, *h*M-PST, *h*DHEA ST, *r*PST and *dm*CG5431, were loaded onto individual wells of a 12% SDS-PAGE for electrophoresis according to the method of Laemmli (Laemmli, 1970). After electrophoresis, the separated proteins were electroblotted onto a nitrocellulose membrane and blocked with 5% skimmed milk for 1 h. The membrane was incubated with *h*DHEA ST antibody for 1 h and washed three times with PBST for 5 min. The membrane was then immersed in PBST containing antibody against rabbit IgG conjugated with HRP for 1 h. The bound antibodies were detected with an ECL western blotting reagents for chemiluminescent detection. The native immunoblot analysis procedure was similar to that described previously with minor modifications. All buffer solutions were free from the addition of SDS. Similarly, approximately 300 μg of *Drosophila* brain proteins and 20 μg of purified hDHEA ST were used for analyzing the protein expression of DHEA ST-like protein by native and SDS immunoblot. The steps were similar to those described previously.

Determination of sulfating activity of DHEA in Drosophila brain

DHEA-sulfating activity of *Drosophila* brain was determined by the continuous fluorometric assay developed by Chen et al. (Chen et al., 2005). By using DHEA as substrate, the activity of DHEA ST was determined by monitoring the fluorescence intensity of MU.

The standard assay mixture had a final volume of 1 ml, and contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM β-ME, 20 μM PAPS, 4 mM MUS, 5 μM DHEA and 3.2 mU K65ER68G, the recombinant β-form of PST. For use in the assay, a partially purified DHEA ST-like protein fraction, located by cross-reactivity with antibody against *h*DHEA ST, was prepared from *Drosophila* brain homogenates by using native gel electrophoresis. The intensity of MU was monitored using a spectrofluorometer (Hitachi F-4500, Japan).

Immunohistochemistry

The *Drosophila* brain was perfused with 4% paraformaldehyde for fixation and then penetrated with 30% sucrose. After washing with PBS, the brain was blocked with 1% BSA overnight at 4°C to prevent nonspecific staining. The sample was then incubated with *h*DHEA ST antibody at 4°C for 72 h, rinsed with washing buffer (containing 0.1% BSA and 0.2% Triton X-100 in PBS, pH 7.4) for 20 min three times, and then stained with biotinylated goat anti-rabbit secondary antibody. After an overnight incubation at 4°C, the sample was washed with washing buffer for 20 min. Tertiary antibodies (streptavidin-Cy5 and biotinylated HRP) were also incubated with the brain sample overnight at 4°C. Afterwards, the brain sample was mounted using FocusClear™ and examined by confocal laser scanning microscope photomicrographs. The *Drosophila* carries gene trap Gal4 (12423) and UAS-GFP to expresses GFP in the DPM neurons (green) was used to analyze the colocalization of DHEA-ST like

protein and DPM neuron. Steps were same as mentioned above (Wang et al., 2003).

III. RESULTS

Characterization of hDHEA ST antibody

The characterization of *h*DHEA ST antibody analyzed by IELISA and immunoblotting is shown in Figure 1 and 2. The *h*DHEA ST antibody showed stronger affinity toward recombinant *h*DHEA ST and *dm*CG5431 in native conformations than the others co-tested (**Figure 1A**). The dose-response curves showed that the *h*DHEA ST antibody was specific to *h*DHEA ST when the STs tested were first denatured by treating with β-ME and heating (**Figure 1B**). The binding of *h*DHEA ST antibody to PST isoforms was barely discernible. The specificity of antibody for *h*DHEA ST was determined by probing five different types of recombinant STs by immunoblotting (**Figure 2A**). The *h*DHEA ST antibody interacted with both *h*DHEA ST and *dm*CG5431 in their native forms, whereas neither *h*PST isofroms nor *r*PST was recognized. Furthermore, only *h*DHEA ST was identified by this specific antibody under the denatured conditions (**Figure 2B**).

Expression of DHEA ST-like Protein in Drosophila brains

As shown in Figure 3, the immunoblot of *Drosophila* brain extracts was analyzed by *h*DHEA ST antibody. The DHEA ST-like protein expressed in soluble extracts of *Drosophila* brains was recognized in its native form (**Figure 3A**). The SDS-PAGE

immunoblot showed that only *h*DHEA ST (positive control) was recognized when the native conformation was disrupted (**Figure 3B**).

Localization of DHEA ST-like Protein in Drosophila brains

The distribution and abundance of DHEA ST-like protein in *Drosophila* brain are shown in Figure 4. A total of six DHEA ST-like protein positive neurons were observed in the posterior section of *Drosophila* brain (**Figure 4A**). Two DHEA ST-like protein positive neurons were also detected in the dorsal part of the brain (**Figure 4B**). DHEA ST-like protein positive neuronal fibers, exhibiting the typical appearance of beaded nerve fibers, were seen throughout the entire *Drosophila* brain (**Figure 4C**). The continuous confocal images of *Drosophila* brain dissected by 2 μm interval were also exhibited in **Figure 5**. **Table 1** summarizes the relative localization and abundance of DHEA ST-like protein positive neurons and fibers. Additionally, the schematic frontal sections illustrating the selective distribution of DHEA ST-like protein immunoreactive cell bodies and fibers in *Drosophila* brain was shown in **Figure 6**. By using UAS-WGA as a trans-synaptic transmission marker and VAM-Gal4 as a driver, the colocalization of DHEA ST-like protein positive regions and DPM neuron were observed by transgenic fly that carries VAM-Gal4 and UAS-WGA and express WGA in the VAM neurons (**Figure 7**).

Determination of the DHEA-sulfating activity in Drosophila brains

The sulfating activity of DHEA in partially purified *Drosophila* brain extract was assessed using a continuous fluormetric assay (**Table 2**). The complete system (I) could detect not only sulfating but also desulfating activities due to the presence of MUS. The DHEA-sulfating activity could not be observed in the absence of PAPS, and therefore reaction condition II gave a background activity exhibited mainly by arylsulfatase. As a result, the specific activity and total activity of DHEA-sulfating activities in *Drosophila* brain were determined to be 57.7 ± 12.1 pmole/min/mg and 0.7 ± 0.2 nmole/min/g, respectively. It is to be noted that a high level of arylsulfatase activity (specific activity and total activity, 319.1 ± 7.5 pmole/min/mg and 6.8 ± 0.1 nmole/min/g, respectively) in the fraction was detected as well.

IV. DISCUSSION

To date twenty-three structures of cytosolic STs have been solved on eleven different isoforms. Crystal structures for the ST1A1 (phenol ST), ST1A3 (catecholamine ST), ST1E1 (estrogen ST), ST1B1 (thyronine ST), ST2A1 (DHEA ST), two isoenzymes of ST2B1 (pregnenolone ST and cholesterol ST), ST4A1 (neuronal ST) and three subfamilies of ST1C have been characterized. Structure-based sequence alignments indicate that the PAPS binding site, and structural fold, is highly conversed, albeit the homology of the amino acid sequences between different ST isoforms is not high (Rath et al., 2004). On the basis of the characteristic pattern of the STs, we demonstrated the possible presence of a cytosolic ST-like protein in *Drosophila* neural circuits by the specific recognition of the *h*DHEA ST antibody. The results obtained from immunoblot analysis and IELISA are close agreement with conserved nature of STs and indicate that STs may exert similar biological functions in various animals.

Drosophila is an excellent experimental model to systematically study the neuroregulative mechanisms in human CNS. Many scientists have placed much effort into the molecular characterization and physical relevance of STs in this tiny creature, however, the biological significance of STs in *Drosophila* remains obscure. In general, sulfate conjugation is apparently involved in the metabolism of juvenile hormones and ecdysteroids in insects (Sannasi and Karlson, 1974). In *Prodenia eridania*, sulfate conjugation of ecdysteroids seems

to play a critical role in embryonic development and puparium formation (Slade and Wilkinson, 1974). In the present study, the protein partially purified from *Drosophila* brain extracts was demonstrated to be capable of transferring a sulfuryl group from a sulfate donor, PAPS, to an analog of ecdysteroid, DHEA, and the result was consistent with that reported for *Mosquito*, *Aedes togoi* (Shampengtong and Wong, 1989) suggested that sulfation in various insect species may exhibit similar biological functions in the metabolism of free hormones and post-stage embryogenesis. Earlier studies had also revealed the existence of cytosolic STs in flies of *Diptera*, *Prodenia eridania* (Yang and Wilkinson, 1972). In addition to its activity on *p*-nitrophenol, the ST prepared from *Prodenia eridania* gut was significantly active in the sulfation of the steroids, such as DHEA, oestrone, and insect moulting hormones α-ecdysone and 22,25-bisdeoxyecdysone. It appears logical to suggest that STs comprehensively regulate the biochemical transformations for the purpose of detoxication, others may have important physiological implications in insects. Besides, it is noted that DHEA-sulfating activity of in *Drosophila* was significantly lower than that in rat and human (Aldred and Waring, 1998; Sugahara et al., 2003). This is to be expected because DHEA is a more common substrate for DHEA ST in mammalians than in insects. Furthermore, the validity of such comparison is always open to some question in consideration of differences in enzyme preparation and enzymatic assay. Nevertheless, It is noteworthy that the significant level of arylsulfatase activity was determined (**Table 2**). In agreement with these previous findings, the low or

undetectable DHEA ST activities may be due to high levels of steroid sulfatase in soluble extracts, thereby interfering with the determination of DHEA ST *in vitro* (Janer et al., 2005).

Several neural regions of *Drosophila* brain were found to have DHEA ST-like immunoreactivity in this study. The immunoreactivity was selectively localized in the neurons of posterior and dorsal part of *Drosophila* brain, and nerve fibers indicating the relevant molecular and neuronal mechanism between this enzyme and its metabolites. In *Drosophila*, there are several types of projection neuron that forward information out of the antennal lobes. Medial and outer antennocerebral tracts (mACT and oACT, respectively) protrude into the ill-defined region and the lateral horn, while the others carry information along the inner antennocerebral tracts (iACT) to the mushroom body (MB). In our study, we observed an abundance of *Drosophila* DHEA ST-like protein expressed in the lateral horn and iACT (**Table 1**). The results implied that the DHEA ST-like protein may act as a neuromodulator of the ecdysteroids, which are involved in memory formation in *Drosophila*. The finding is also consistent with the result reported by Johnson et al (Johnson et al., 2000). The sulfate-conjugated steroid is essential to the process of memory retention and significantly enhanced the cognition and learning in rats. Basically, they act through γ-aminobutyric $acid_A$ ($GABA_A$) receptors, *N*-methyl-D-aspartate (NMDA)-type glutamatergic receptors and sigma receptors to induce excitatory cellular actions or inhibit cellular properties.

Increasing evidences have suggested that DPM neuron may co-release *amnesiac* neuropeptide and acetylcholine in the *amnesiac* mutant flies (Keene et al., 2004). Transgenic expression of the *amnesiac* gene in the DPM neurons rescues the *amnesiac* memory phenotype, establishing a possible route between DPM neuron function and *amnesiac*-dependent memory. Additionally, the paired conditioning of unconditioned stimulus (US) (electric shock) and conditioned stimulus (CS) (odor stimulus) increases odor-evoked calcium signals and synaptic release from DPM neurons (Yu et al., 2005). These observations indicated that DPM neurons not only respond to the US pathway, but that they are also "odor generalists", responding to all odors that were tested. The colocalization of DHEA ST-like protein and DPM neuron implied that DHEA ST-like protein may not merely play the role as a neuroregulator in the process of odor-specific memory trace in *Drosophila* but also involve in the modulation of specific memorial and behavioral formations.

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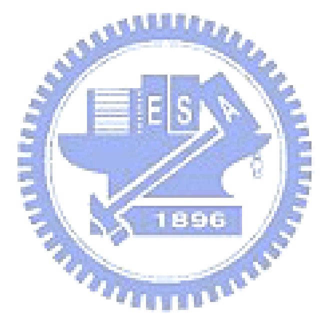


Table 1. Distribution and cellular colocalization of DHEA ST-like protein in *Drosophila* brain[a]

Brain region	Specific areas[b]	Neural cytoplasm[c]	Fibers[c]
Anterior brain			
αα'	MB's vertical lobe	−	−
s m pr	superior medial PR	−	−
m bdl	median bundle	−	−
mb sat neu	MB satellite neuropil	−	−
a ot tu	anterior optic tubercle	−	+
v l pr	ventrolateral PR	−	+
b lb	MB's medial lobe	−	−
ant lob	antennal lobe	−	−
ant n	antennal nerve	−	−
v bo	ventral body	−	−
Middle brain			
ped	pedunculus	−	+
f b	fan-shaped body	−	+
s l pr	superior lateral PR	−	++
v bo	ventral body	−	++
s m pr	superior medial PR	−	+
trito	tritocerebrum	−	++
s a	superior arch	−	+
ant glt	antennoglomerular tract	−	++
e b	ellipsoid body	−	+
no	nodulus	−	++
inf l deu	inferior lateral deutocerebrum	−	+
lo	Lo	−	++
me	Medulla	−	+
Posterior brain			
lo	Lo	−	−
lo p	LoP	−	++
ca	MB calyx	−	−
pr br	PR bridge	+++	+
ocl n	ocellar nerve bundle	−	−
i act	inner antennocerebral tract	−	+
l ho	lateral horn	+++	−

(Continued)

p l fasc	posterior lateral fascicle	−	++
sog	SOG	−	+
vs	axons of vertical cells of the LoP	−	++
hs	axons of horizontal cells of the LoP	−	+
sog n	SOG nerves	−	−

[a]The distribution of DHEA ST-like protein in *Drosophila* brain was investigated by the continuous sections of the confocal laser scanning microscope photomicrographs. Detailed procedures were mentioned under *Materials and Methods* (14).

[b]Abbreviations: MB, mushroom body; PR, protocerebrum; Lo, lobula; LoP, lobula plate; SOG, subesophaeal ganglis; SOG nerves, roots of nerves from the fused subesophageal ganliea.

[c]The relative intensity of labeling was ranked by two independent observers. Ratings reflect mainly the density of DHEA ST-like protein labeled cell and fibers. Negative, −; weak, +; moderate, ++; strong +++.

Table 2 Sulfate activity in *Drosophila* brain[a]

Fraction					
Reaction conditions	Enzyme activity involved	Specific activity (pmole/min/mg)		Total activity (nmole/min/g)	
I. Complete[b]	DHEA-sulfating protein + arylsulfatase	376.8	± 9.5	7.5	± 0.2
II. – PAPS[c]	arylsulfatase	319.1	± 7.5	6.8	± 0.1
I – II	DHEA-sulfating protein	57.7	± 12.1	0.7	± 0.2
III. –Lysates	K65ER68G			16.6 ± 1.7	

[a]For use in the assay, a partially purified fraction which cross-react with antibody against *h*DHEA ST, was isolated from *Drosophila* brain homogenates by using native gel electrophoresis.

[b]Detailed procedures were described under *Coupled-enzyme assay for alcohol sulfotransferase (AST)* in *Materials and Method* (Chen et al., 2005). Specific activity referred to MU produced following the addition of extract whose protein concentration was determined by absorption at A_{280}. Total activity referred to MU produced with one gram of *Drosophila* brain extracts.

[c]AST activity was eliminated in the absence of PAPS (Chen et al., 2005).

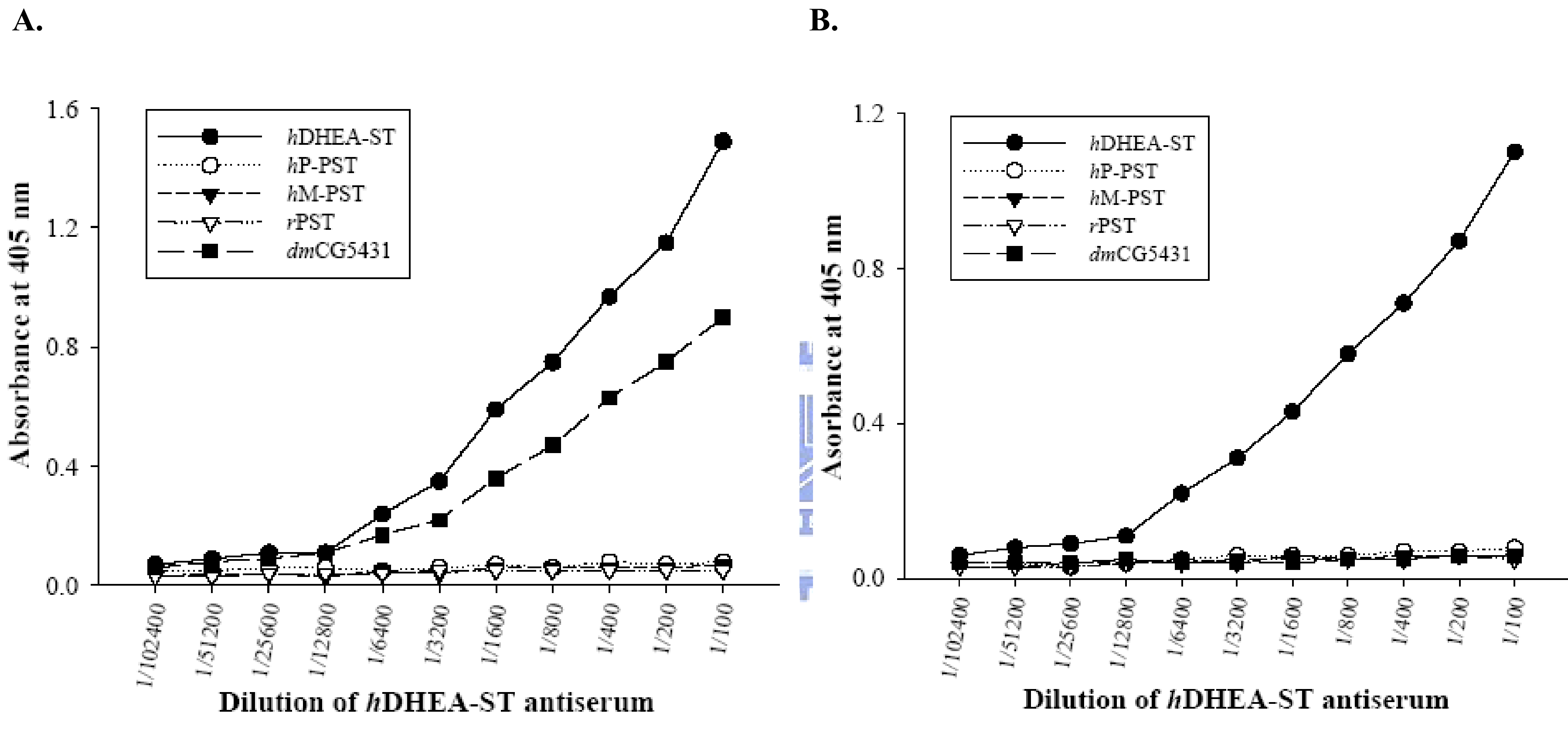


Figure 1. The *h*DHEA-ST antiserum titres determined by ELISA using recombinant ST isoforms and putative cytosolic ST of *Drosophila melanogaster*

The ELISA plates coated with about 0.5 µg purified STs and analyzed by serial dilutions of *h*DHEA-ST antiserum. A. Different cytosolic

STs, *h*DHEA-ST (●), *h*P-PST (○), *h*M-PST (▼), *r*PST (▽) and *dm*CG5431 (■), were used and analyzed by ELISA in natural conformation (without any treatment). B. The same cytosolic STs mentioned above were used but denatured by treatment with β-ME and heating, and then analyzed by ELISA. The data for *h*P-PST, *h*M-PST, and *r*PST overlap.

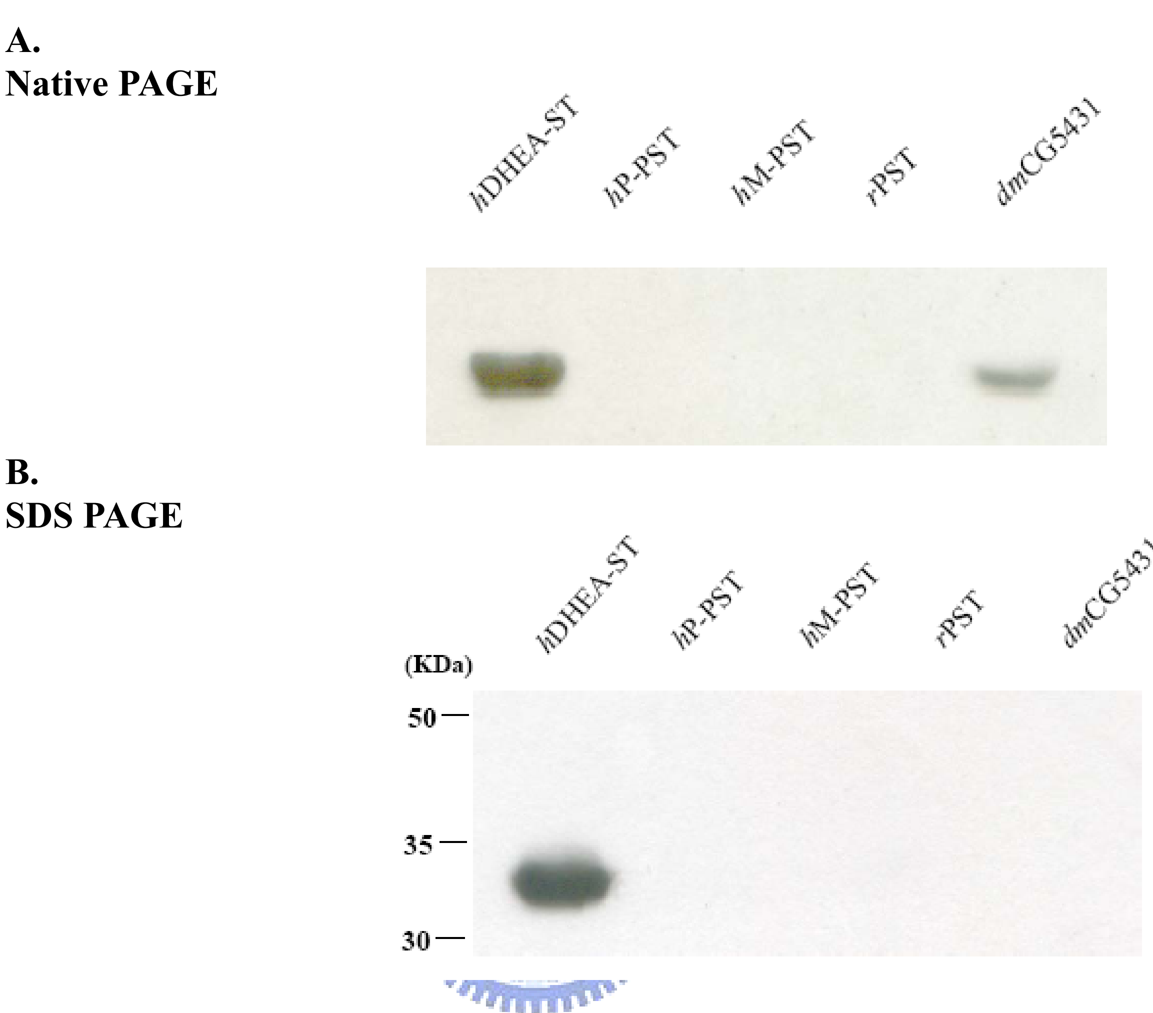


Figure 2. Conformation-dependent recognition of *h*DHEA-ST antiserum

The purified recombinant STs were loaded on 12% native PAGE and SDS PAGE, and then transferred onto nitrocellulose membrane. A. Native immunoblot of purified STs was analyzed by immersing with 1:5000 diluted *h*DHEA-ST antiserum. Result showed both *h*DHEA-ST and *dm*CG5431 were recognized. B. SDS PAGE coupled with immunoblot of denatured STs also analyzed by immersing with 1:5000 diluted *h*DHEA-ST antiserum showed only *h*DHEA-ST was recognized by using *h*DHEA-ST antiserum as a probe. Each lane was loaded approximately 5μg purified proteins. The standard molecular mass was indicated on the *left*.

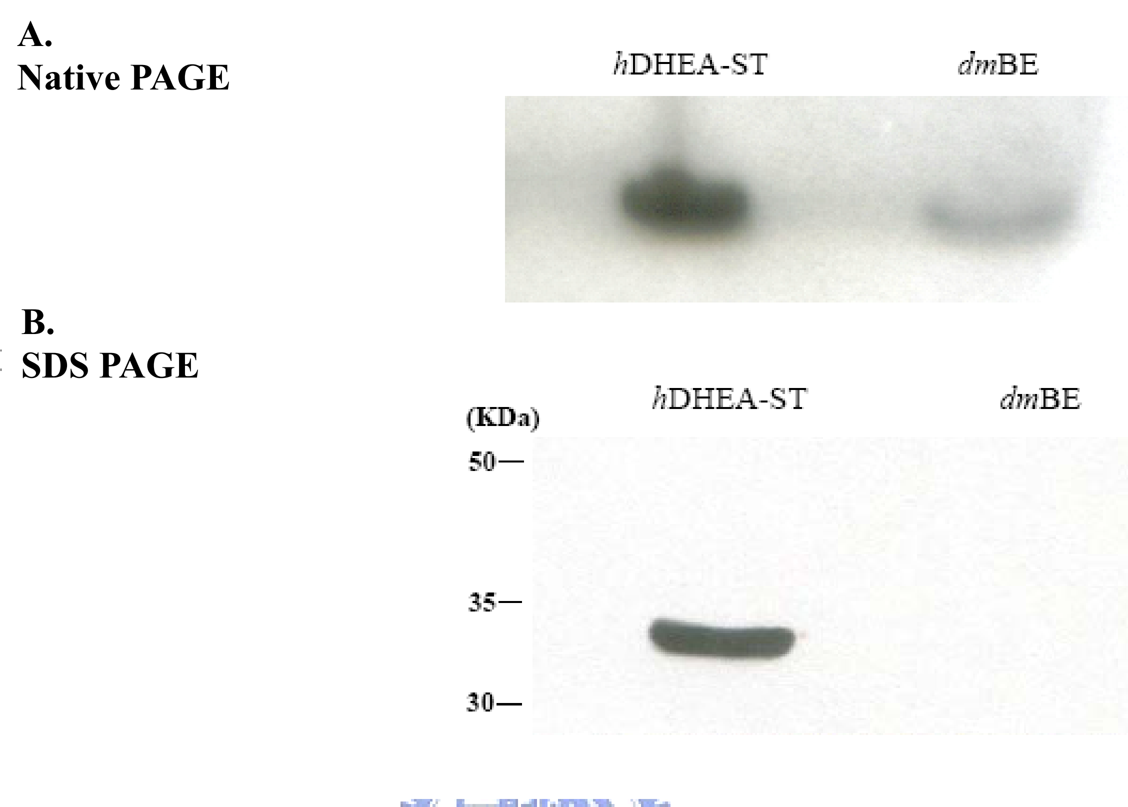
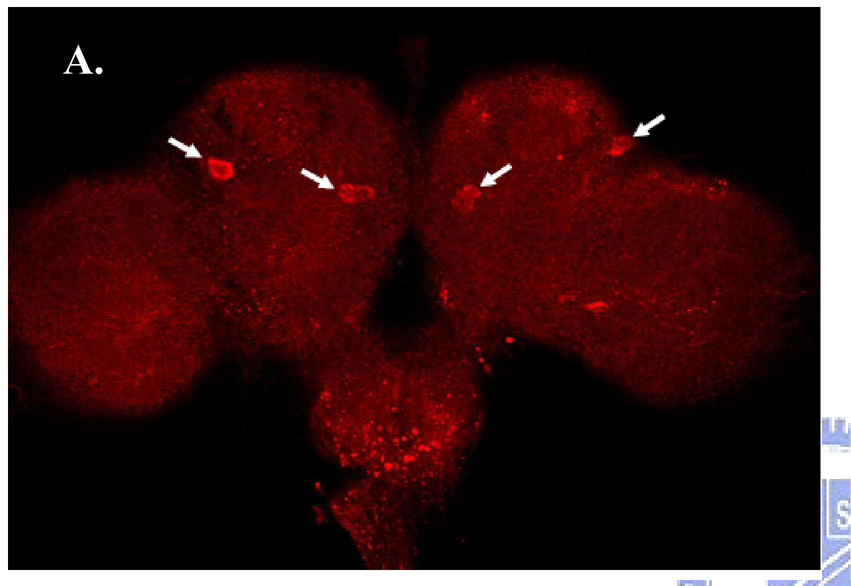
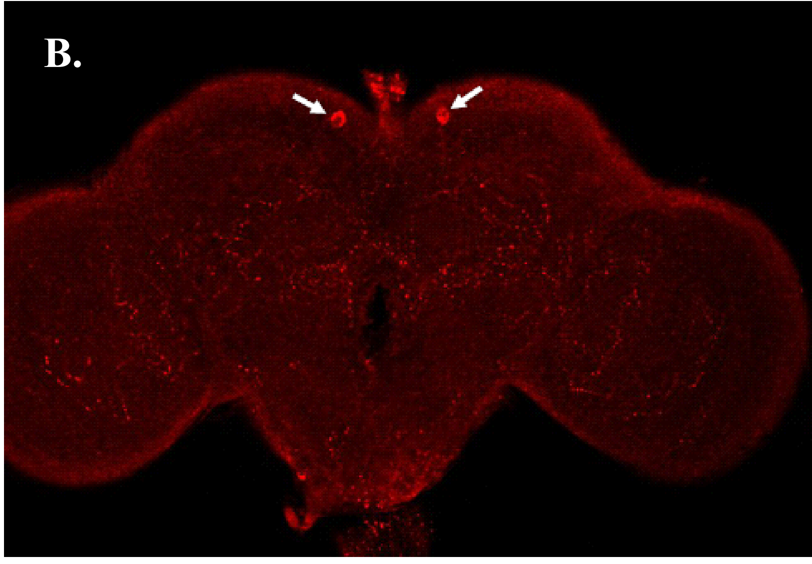


Figure 3. The expression of DHEA ST-like protein in *Drosophila* brain extracts

A. The native immunoblot of *Drosophila* brain extracts with *h*DHEA ST antibody. B. SDS-PAGE immunoblot of *Drosophila* brain extracts and purified *h*DHEA ST with *h*DHEA ST. *dm*BE represented *Drosophila* brain extracts.





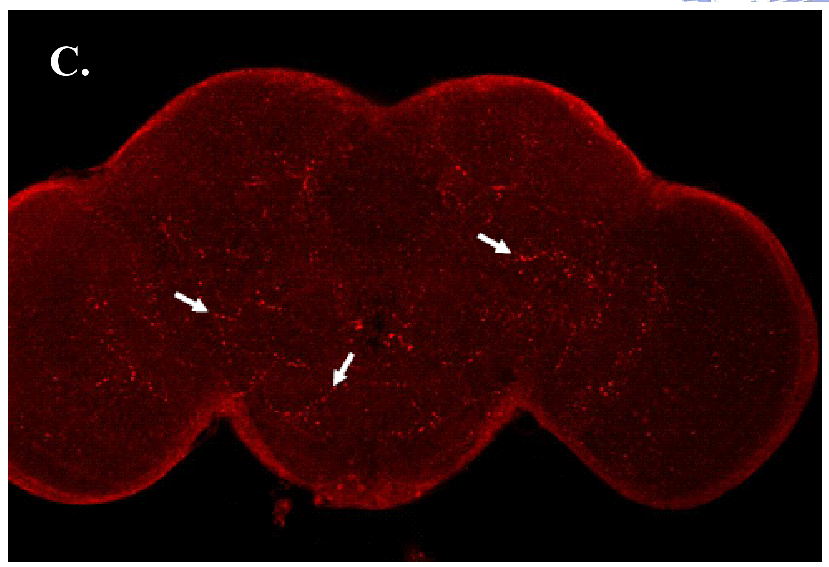


Figure 4. Distribution of the DHEA ST-like protein in *Drosophila* brain as illustrated by confocal laser scanning microscope

A. DHEA ST-like protein selectively expressed in posterior region of brain. B. DHEA ST-like protein expressed in dorsal part of the brain. C. Expression of DHEA ST-like protein in neural fibers throughout the entire *Drosophila* brain. Granular staining suggests fibers labeled. 200X magnification.

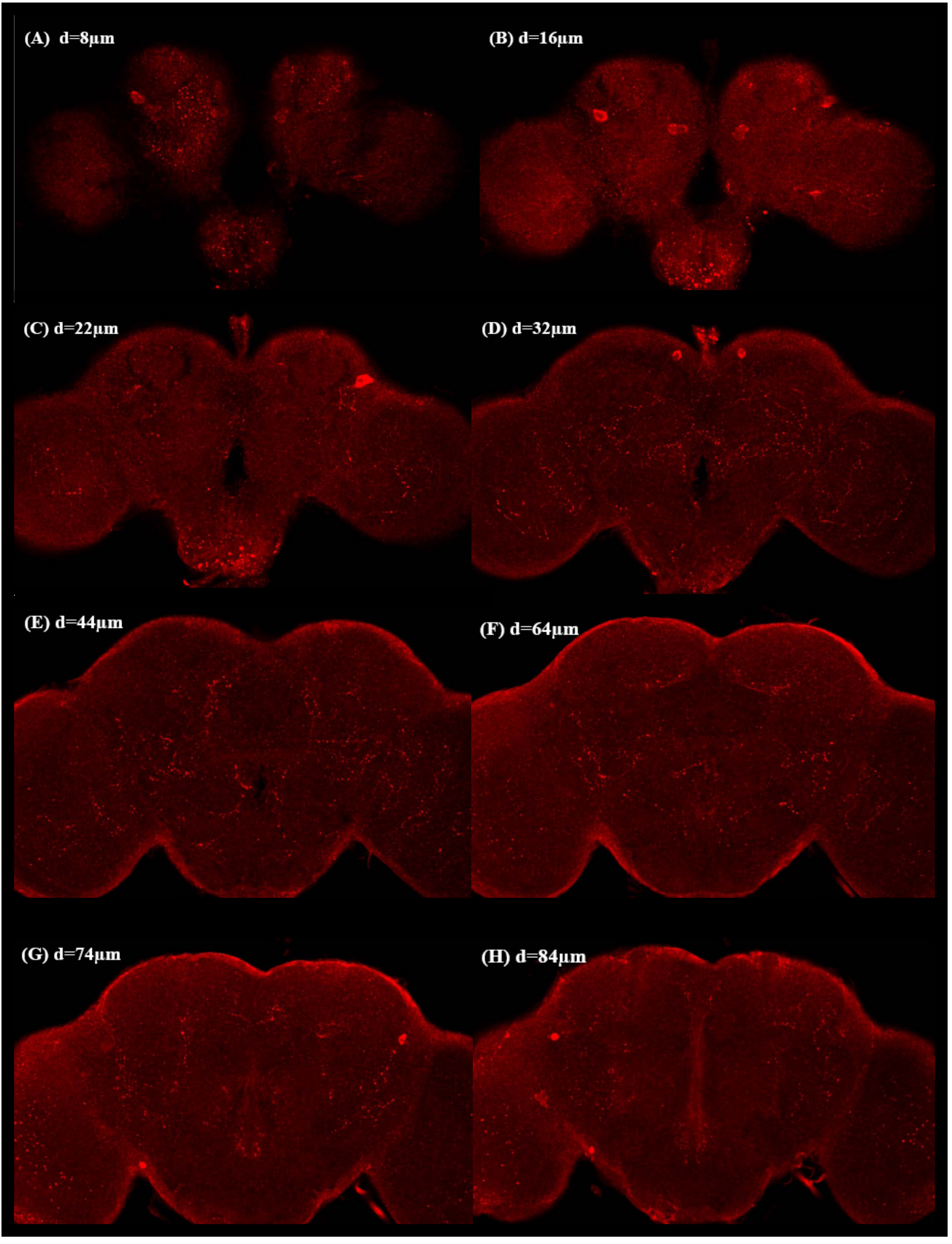
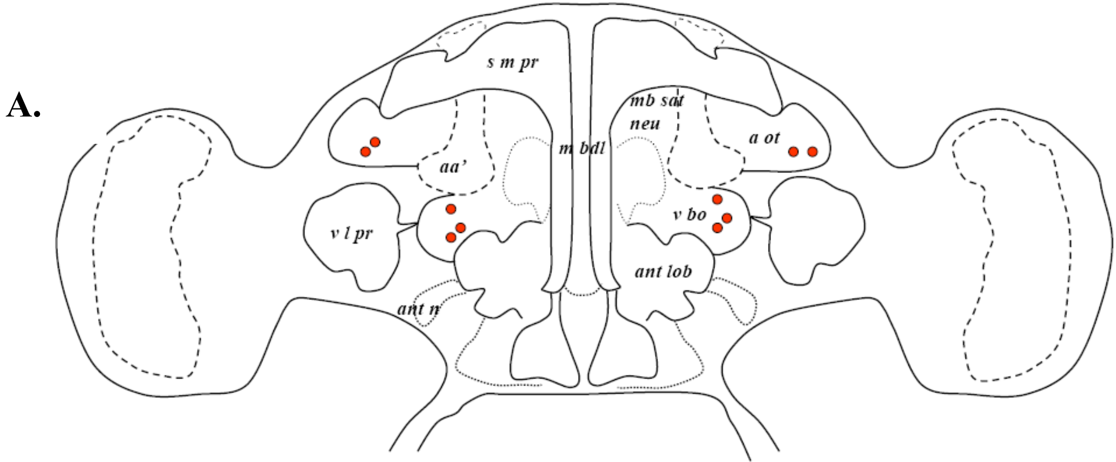


Figure 5. The continuous confocal images of *Drosophila* brain expressed DHEA ST-like protein

A-H. DHEA ST-like immunoreactive neurons and fibers were selectively present

throughout the entire *Drosophila* brain. The continuous confocal images were dissected by 2 μm interval.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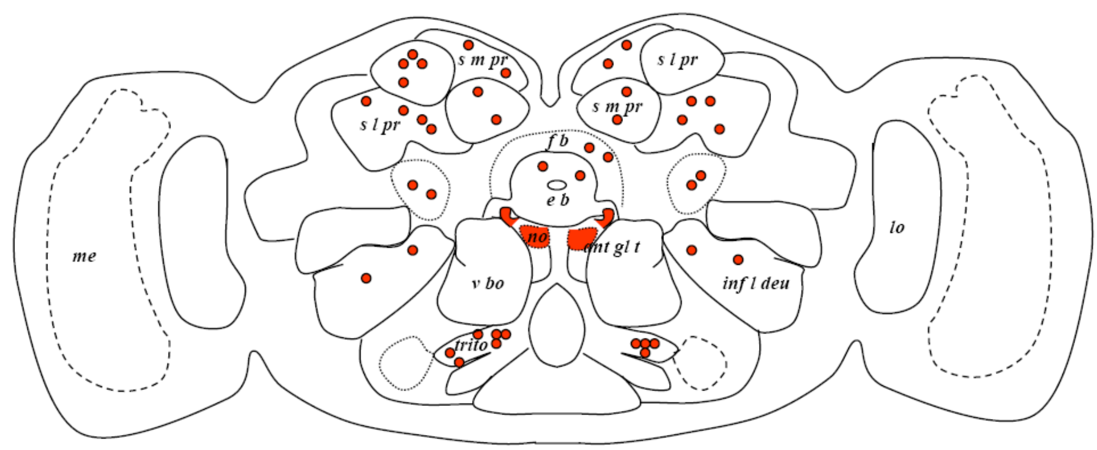
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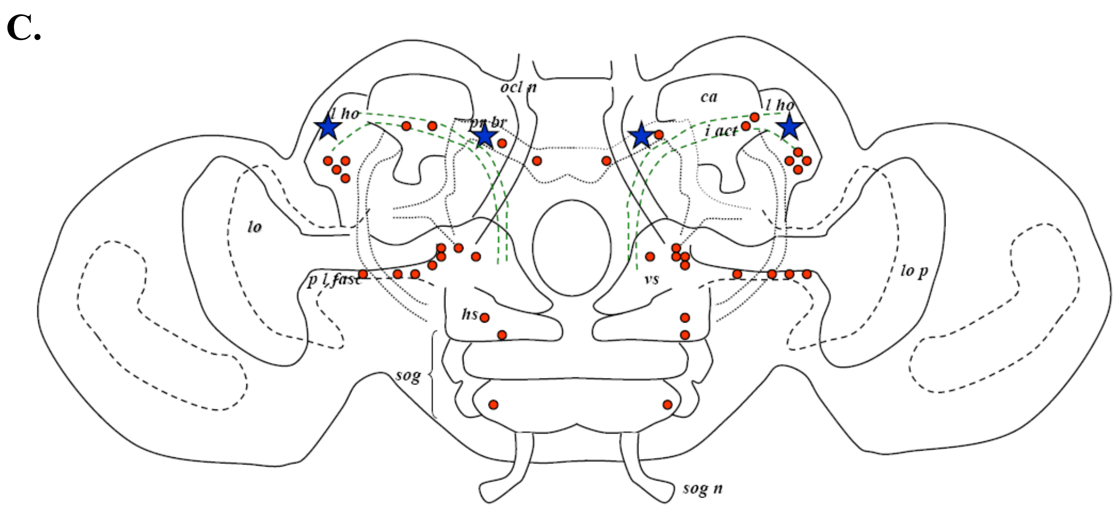
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Figure 6. Selective distribution of DHEA ST-like protein in *Drosophila*

Schematic frontal sections illustrating the distribution of DHEA ST-like immunoreactive cell bodies (★) and fibers (●) in *Drosophila* brain. The anatomical structures are designated on the hemisections according to the FlyBase database (http://flybase.bio.indiana.edu/). A. Anterior section of brain. B. Middle section of brain. C. Posterior section of brain. The density of the symbols is meant to be proportional to the relative density of the immunoreactive elements. Abbreviations are as in **Table 1**.

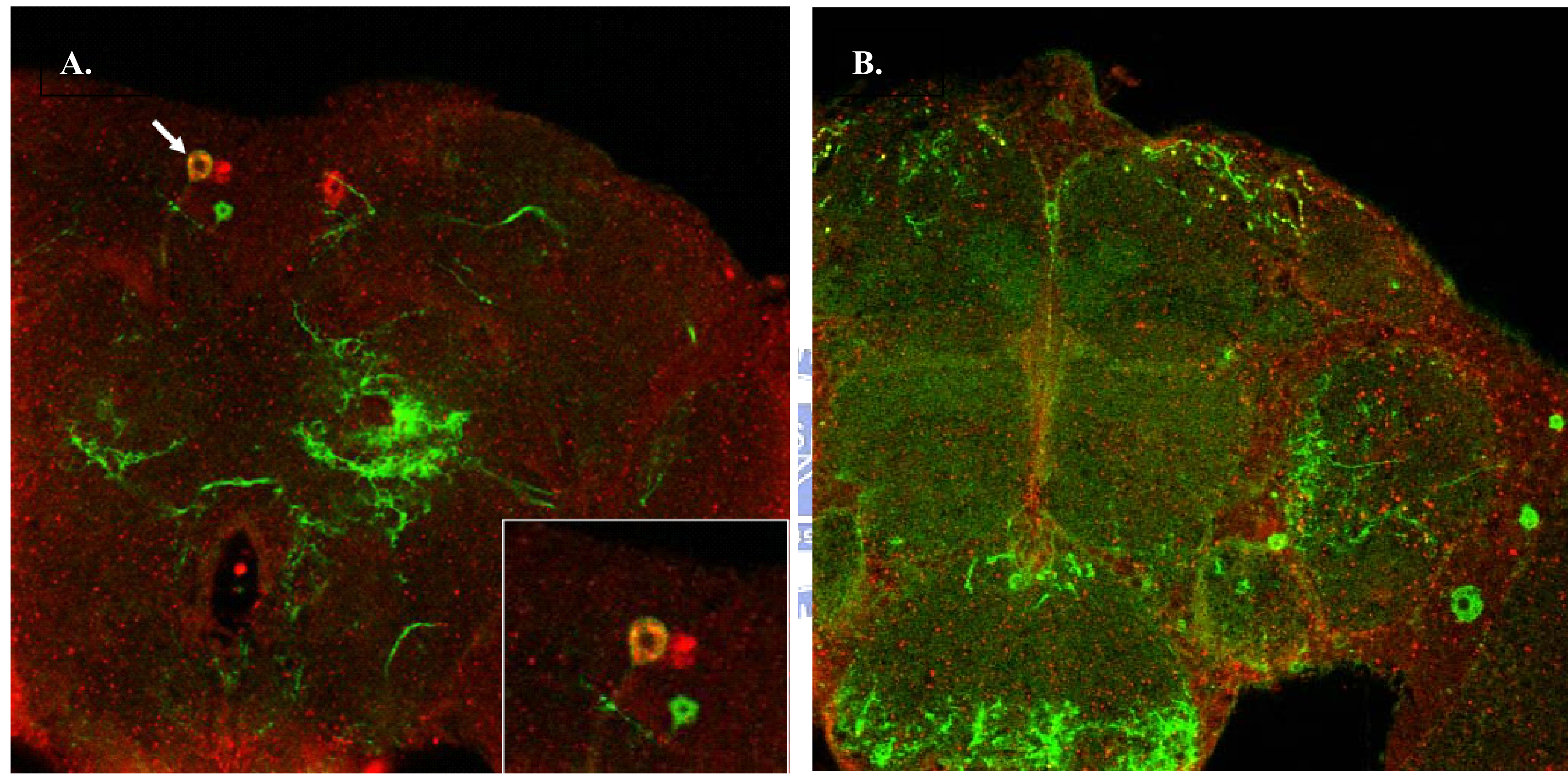

Figure 7. Colocalization of the DHEA ST-like protein expression and DPM neuron in *Drosophila* brain

A. The arrowhead indicates the colocalization (orange) of DHEA ST-like protein (red) and DPM neuron (green) (400X magnification). *Right*-*lower*: the magnification of the colocalized region of DHEA ST-like protein and DPM neuron. B. Control image by

non-immunoactive antiserum.

CHAPTER 3

Identification and Characterization of Two Novel Cytosolic Sulfotransferases, SULT1 ST7 and SULT1 ST8, from Zebrafish

Cytosolic sulfotransferases (SULTs) constitute a family of Phase II detoxification enzymes that are involved in the protection against potentially harmful xenobiotics as well as the regulation and homeostasis of endogenous compounds. Compared with humans and rodents, the zebrafish serves as an excellent model for studying the role of SULTs in the detoxification of environmental pollutants including environmental estrogens. By searching the expressed sequence tag database, two zebrafish cDNAs encoding putative SULTs were identified. Sequence analysis indicated that these two putative zebrafish SULTs belong to the SULT1 gene family. The recombinant form of these two novel zebrafish SULTs, designated SULT1 ST7 and SULT1 ST8, were expressed using the pGEX-2TK glutathione *S*-transferase (GST) gene fusion system and purified from transformed BL21 (DE3) cells. Purified GST-fusion protein form of SULT1 ST7 and SULT1 ST8 exhibited strong sulfating activities toward environmental estrogens, particularly hydroxylated polychlorinated biphenyls (PCBs), among various endogenous and xenobiotic compounds tested as substrates. pH-dependence experiments showed that SULT1 ST7 and SULT1 ST8 displayed pH optima at 6.5 and 8.0, respectively. Kinetic parameters of the two enzymes in catalyzing the

sulfation of catechin and chlorogenic acid as well as 3-chloro-4-biphenylol were determined. Developmental expression experiments revealed distinct patterns of expression of SULT1 ST7 and SULT1 ST8 during embryonic development and throughout the larval stage onto maturity.

I. INTRODUCTION

Cytosolic sulfotransferases (SULTs) constitute a group of enzymes that catalyze the transfer of a sulfonate group from the universal sulfate donor, 3'-phosphoadenosine-5'-phophosulfate (PAPS), to acceptor substrate compounds containing hydroxyl or amino groups (Mulder and Jakoby, 1990; Falany and Roth, 1993; Weinshilboum and Otterness, 1994; Coughtrie, 2002). Such sulfation reactions, taking place in mammals and other vertebrate species, are generally thought to serve for the regulation of endogenous compounds such as steroid/thyroid hormones, catecholamine neurotransmitters, as well as the detoxification of dietary, therapeutic, and environmental xenobiotics (Mulder and Jakoby, 1990; Falany and Roth, 1993; Weinshilboum and Otterness, 1994; Coughtrie, 2002). In the latter case, sulfated products may become more water-soluble and can be more easily excreted from the body. From this perspective, the SULTs can be considered as a part of the chemical defense mechanism (Allali-Hassani et al., 2007; Gamage et al., 2006).

In recent years, environmental estrogens have been increasingly recognized as a potential hazardous factor for wildlife as well as humans (Roy et al., 1997). In general, they are able to bind to estrogen receptors and thereby mimicking estrogenic actions (Ridgway and Wiseman, 1998) or interfere with the action of enzymes that help regulate the level of

endogenous estrogens (Kester et al., 2000). Some examples of this diverse group of compounds are diethylstilbestrol, bisphenol A, alkyl phenolic compounds, polychlorinated biphenyls (PCBs), herbicides, polystyrenes and plasticizers (Danzo, 1998). These environmental estrogens have been implicated in the malfunctioning of the reproductive system and abnormal embryonic development of wildlife, and in a worldwide debate on a decline in sperm quality in men (Carlsen et al., 1992; Auger et al., 1995) and an increased incidence of human breast cancer (Falck et al., 1992; Rogan, 2007).

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies (Kari et al., 2007; Lieschke and Currie, 2007). Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of a large number of eggs, rapid development externally of virtually transparent embryos, and short generation time. These characteristics make the zebrafish an excellent model for a systematic investigation on the physiological involvement of the SULTs, including the sulfation of environmental estrogens. A prerequisite for using the zebrafish in these studies, however, is the identification of the various SULTs and their functional characterization. We have recently embarked on the molecular cloning of zebrafish SULTs. Sequence analysis via BLAST search revealed that the zebrafish SULTs we have cloned display sequence homology to mammalian SULTs. Of the ten zebrafish SULTs that have been cloned, six fall

within the SULT1 gene gamily (Sugahara et al., 2003a, 2003b; Liu et al., 2005; Yasuda et al., 2005a, 2005b); three belong to the SULT2 gene family (Sugahara et al., 2003c; Yasuda et al., 2006); and one (designated SULT X) appears to be independent from all known SULT gene families (Sugahara et al., 2003d). The zebrafish SULT1 enzymes previously cloned and expressed displayed differential sulfating activities toward endogenous compounds including L-Dopa, dopamine, 17β-estradiol, estrone, and thyroid hormones, as well as a variety of xenobiotic phenolic compounds.

We report here the identification of two new zebrafish SULT1 enzymes, designated SULT1 ST7 and SULT1 ST8. Their enzymatic activities toward a variety of endogenous compounds and xenobiotics were examined. Kinetic parameters of the two enzymes in catalyzing the sulfation of representative substrates were determined. Moreover, their developmental expression during embryogenesis onto maturity was investigated.

II. MATERIALS AND METHODS

Materials

3,3'5-triiodo-L-thyronine (L-T_3), L-thyroxine (L-T_4), 17β-estradiol, estrone, cholesterol, dehydroepiandrosterone (DHEA), D-Dopa, L-3,4-dihydroxyphenylalanine (L-Dopa), dopamine, allopregnanolone, chlorogenic acid, kaempferol, genistein, β-naphthol, catechin, caffeic acid, daidzein, gallic acid, butylated hydroxylanisole, butylated hydroxytoluene, quercetin, myricetin, *n*-propyl gallate, *p*-nitrophenol, β-naphthylamine, acetaminophen, epicatechin, epigallocatechin gallate, mestranol, minoxidil, bisphenol A, *n*-octylphenol, *n*-nonylphenol, diethylstilbestrol (DTT), 17α-ethynylestradiol, 17β-estradiol, aprotinin, adenosine 5'-triphosphate (ATP), sodium dodecyl sulfate (SDS), sodium acetate, 2-morpholinoethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-2-hydroxylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid (TAPS), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), reduced glutathione, and isopropyl β-D-thiogalactopyranoside (IPTG) were products of Sigma Chemical Company (St. Louis, MO). 3-Chloro-4-biphenylol and 3,3',5,5'-tetrachloro-4,4'-biphenyldiol, both with a minimum purity of 95%, were obtained from Ultra Scientific (N. Kingstown, RI). TRI Reagent was from Molecular Research

Center, Inc. (Cincinnati, OH). Unfertilized zebrafish eggs, embryos and larvae at different developmental stages were prepared by Scientific Hatcheries (Huntington Beach, CA). Total RNAs from zebrafish embryos and larvae at different developmental stages, as well as 3-month-old adult male or female fish, were isolated using the TRI Reagent, based on manufacturer's instructions. *Taq* DNA polymerase was a product of Promega Corporation (Madison, WI), and Takara *Ex Taq* DNA polymerase was from Fisher Scientific (Pittsburgh, PA). T_4 DNA ligase and *Bam* HI restriction endonuclease were from New England Biolab (Ipswich, MA). Oligonucleotide primers were synthesized by MWG Biotech (Huntsville, AL). pSTBlues-1 AccepTor Vector Kit and BL21 (DE3) competent cells were purchased from Novagen (Gibbstown, NJ). Prestained protein molecular mass standard was from Life Technologies (Gaithersburg, MD). First-strand cDNA Synthesis Kit, pGEX-2TK glutathione *S*-Transferase (GST) gene fusion vector, GEX-5'-and GEX-3' sequencing primers, and glutathione-Sepharose 4B were products of Amersham Biosciences (Piscataway, NJ). Recombinant human bifunctional ATP sulfurylase/adenosine-5'-phosphosulfate kinase was prepared as described previously (Yanagisawa et al., 1998). Cellulose thin-layer chromatography (TLC) plates were products of EMD Chemicals (Gibbstown, NJ). Carrier-free sodium [^{35}S]sulfate, Ecolume scintillation cocktail, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, progesterone, hydrocortisone, 4-androstene-3,17-dione, and corticosterone were from MP Biomedicals (Solon, OH). All

other reagents were of the highest grades commercially available.

Cloning, bacterial expression, and purification of recombinant zebrafish SULT1 ST7 and SULT1 ST8

By searching the GenBank database, two zebrafish sequences (GenBank Accession # XM_688954 (SULT1 ST7) and AI384974 (SULT1 ST8)) encoding putative SULTs were identified. The former is a full-length coding sequence derived from an annotated genomic sequence (GenBank Accession # NW_635013), and the latter is a partial sequence covering the 5'-region of the coding sequence. A full-length cDNA clone containing the latter sequence was purchased from RZPD Deutsches Ressourcenzentrum fur Genomforchung GmbH (Berlin, Germany), and amplified, purified, and subjected to nucleotide sequencing to obtain its complete coding sequence. To subclone these two cDNAs into the pGEX-2TK 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 either the total RNA of a 2-week-old zebrafish larvae (for SULT1 ST7) or the commercially obtained cDNA (for SULT1 ST8) as template. Amplification conditions were 2 min at 94°C and 20 cycles of 94°C for 35 s, 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-2TK vector, and verified for authenticity by nucleotide sequencing (Sanger et al., 1977). To express the recombinant zebrafish SULT1 ST7 and SULT1 ST8, competent BL21 (DE3) cells transformed with pGEX-2TK harboring the cDNA encoding SULT1 ST7 or ST8 were grown in 1 L LB medium supplemented with 60 μg/ml ampicillin. After the cell density reached 0.6 $OD_{600\ nm}$, IPTG (0.1 mM final concentration) was added to induce the production of recombinant zebrafish SULT1 ST. 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) 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 GST-SULT1 ST fusion protein was eluted with 3 ml of an elution solution containing 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione at 4°C. For the preparation of GST-free SULT1 ST, the GST-SULT1 ST fusion protein bound on glutathione Sepharose was treated with 3 ml of a thrombin digestion buffer (50 mM

Tris-HCl, pH 8.0, 150 mM NaCl, and 2.5 mM $CaCl_2$) 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 SULT1 ST released into the supernatant and the GST-SULT1 ST fusion protein prepared as described above were analyzed by SDS- polyacrylamide gel electrophoresis (SDS–PAGE) and subjected to enzymatic characterization.

Enzymatic assay

The sulfating activity of recombinant zebrafish SULT1 ST7 or ST8 was assayed using radioactive PAP[^{35}S] as the sulfate donor. The standard assay mixture, with a final volume of 25 μl, contained 50 mM Mops buffer at pH 7.0, 14 μM PAP[^{35}S] (15Ci/mmol), 1 mM DTT, and 50 μM substrate. Controls with DMSO or water, in place of substrate, were also prepared. The reaction was started by the addition of the enzyme, allowed to proceed for 5 min at 28°C, and terminated by heating at 100°C for 2 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to the analysis of [^{35}S]sulfated product using the previously developed TLC procedure (Liu and Lipmann, 1984), with *n*-butanol/isopropanol/88% formic acid/water (3:1:1:1; by volume) as the solvent system. To examine the pH-dependence of the sulfation of chlorogenic acid by SULT1 ST7 or ST8, different buffers (50 mM sodium acetate at 4.5, 5.0, or 5.5; Mes at 5.5, 6.0, or 6.5; Mops at

6.5, 7.0, or 7.5; Hepes at 7.0, 7.5, 8.0; Taps at 8.0, 8.5, 9.0; Ches at 9.0, 9.5. or 10.0; and Caps at 10.0, 10.5, 11.0, or 11.5), instead of 50 mM Mops (pH 7.0), were used in the reactions. For the kinetic studies on the sulfation of catechin (by SULT1 ST7), chlorogenic acid (by SULT1 ST8), and 3-chloro-4-biphenylol (by both SULT1 ST7 and ST8), varying concentrations of these substrate compounds and 50 mM Mops buffer at pH 7.0 were used. The reactions were also carried out for 5 min at 28°C, and terminated by heating at 100°C for 2 min. The protein concentrations of SULT1 ST7 and ST8 used in the final reaction mixtures in the kinetic studies were 0.03 mg/ml and 0.08 mg/ml, respectively.

Analysis of the developmental expression of the zebrafish SULT1 ST7 and SULT1 ST8

RT-PCR was employed to investigate the developmental stage-dependent expression of the zebrafish SULT1 ST7 and SULT1 ST8. Aliquots containing 5 µg each of the total RNAs isolated from zebrafish embryos and larvae at different developmental stages as well as 3-month-old adult male or female fish were used for the synthesis of the corresponding first-strand cDNAs using the First-Strand cDNA Synthesis Kit (Amersham Bioscience). One µl aliquots of the 33 µl first-strand cDNA solutions prepared were used as templates for the subsequent PCR amplification. PCR reactions were carried out in 25 µl reaction mixtures using *EX Taq* DNA polymerase, in conjunction with gene-specific sense and antisense oligonucleotide primers (see **Table 1**). Amplification conditions were 2 min at

94°C, followed by 40 cycles of 30 s at 94°C, 35 s at 56°C, and 65 sec at 72°C. The final reaction mixtures were applied onto a 0.9% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining.

Miscellaneous Methods

PAP[^{35}S] was synthesized from ATP and carrier-free [^{35}S]sulfate using the bifunctional human ATP sulfurylase/APS kinase and its purity determined as previously described (Yanagisawa et al., 1998; Lin and Yang, 2000). The PAP[^{35}S] synthesized was adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS-PAGE was performed on 12% polyacrylamide gels using the method of Laemmli (1970). Protein determination was based on the method of Bradford (1976) with bovine serum albumin as the standard.

III. RESULTS

Molecular cloning of the zebrafish SULT1 ST7 and SULT ST8

By searching the GenBank database, two zebrafish sequences (GenBank Accession # XM_688954 (SULT1 ST7) and AI384974 (SULT1 ST8)) encoding putative SULTs were identified. The cDNAs encoding these two putative SULTs were amplified by RT-PCR, cloned into the pGEX-2TK prokaryotic expression vector, and subjected to nucleotide sequencing for authenticity (Sanger et al., 1977). The nucleotide sequences obtained were submitted to the GenBank database under the Accession number EU502841 for SULT1 ST7 and EU502842 for SULT1 ST8. **Figure 1** shows the alignment of the deduced amino acid sequences of the two newly identified zebrafish SULT1 STs. The open reading frames of SULT1 ST7 and SULT1 ST8 both encompass 906 nucleotides and code for 301-amino acid polypeptides. Similar to other SULTs, these two new zebrafish SULT1 STs contain sequences resembling the so-called "signature sequences" (YPKSGTxW in the N-terminal region and RKGxxGDWKNxFT in the C-terminal region; as underlined) (Weinshilboum et al., 1997). Of these two sequences, YPKSGTxW has been demonstrated by X-ray crystallography to be responsible for binding to the 5'-phosphosulfate group of PAPS, a co-substrate for SULT-catalyzed sulfation reactions (Lipmann, 1958), and thus has been designated the "5'-phosphosulfate binding (5'-PSB) motif" (Negishi et al., 2001). The two

zebrafish SULT1 STs also contains the “3’-phosphate binding (3’-PB) motif” (amino acid residues 137-147; as underlined) that has been proposed to be responsible for the binding to the 3’-phosphate group of PAPS (Negishi et al., 2001). It is generally accepted that members of the same SULT gene family share at least 45% amino acid sequence identity, and members of subfamilies within each SULT gene family are greater than 60% identical in amino acid sequence (Weinshilboum et al., 1997; Nagata and Yamazoe, 2000; Blanchard et al., 2004). Sequence analysis based on a BLAST pairwise search revealed that the deduced amino acid sequence of the zebrafish SULT1 ST7 and SULT1 ST8 display 45-76% and 36-40% amino acid sequence identity to, respectively, the six zebrafish SULT1 STs (Sugahara et., 2003a; 2003b; Liu et al., 2005; Yasuda et al., 2005a; 2005b) and the three SULT2 STs (Sugahara et al., 2003c; Yasuda et al., 2006) previously reported. Between the two newly identified zebrafish SULTs identified, 83% amino acid sequence identity was observed. Based on these criteria, these two zebrafish SULTs appear to belong to the SULT1 gene family, and are therefore designated the zebrafish SULT1 ST7 and SULT1 ST8 in accordance with the nomenclature used in ZFIN database (cf. the dendrogram shown in **Figure 2**).

Expression, purification, and characterization of recombinant zebrafish SULT1 ST7 and SULT1 ST8

pGEX-2TK harboring zebrafish SULT1 ST7 or ST8 cDNA was transformed into BL21 (DE3) cells for the expression of the recombinant protein. As shown in **Figure 3**, the GST fusion protein form of recombinant zebrafish SULT1 ST7 or ST8, purified from the *E. coli* extract, migrated at ca. 60 kDa position upon SDS-PAGE. Upon thrombin digestion to cut out the GST moiety, the zebrafish SULT1 ST7 and ST8 both migrated as ~35 kD proteins. Preliminary experiments showed that the thrombin-digested SULT1 ST7 and ST8 exhibited lower and unstable sulfating activity in comparison with the GST-fusion protein form of these two enzymes (data not shown). Therefore, the GST-fusion protein form of both zebrafish SULT1 ST7 and ST8 was used for the enzymatic characterization. A pilot experiment first revealed that the SULT1 ST7 and ST8 exhibited strong activities toward chlorogenic acid. pH-dependence experiments subsequently performed showed that pH optima of SULT1 ST7 and SULT1 ST8 with chlorogenic acid as substrate were, respectively, 6.5 and 8.0 (**Figure 4**). A number of endogenous and xenobiotic compounds were tested as substrates for these two enzymes, and the activity data obtained are compiled in **Table 2**. Based on the molecular mass of the GST moiety (25,499 Daltons) and those of the GST-SULT1 fusion proteins (60,531 Daltons for ST7 and 60,892 Daltons for ST8), correction factors of 1.728 and 1.720 were used in the calculation of specific activities of SULT1 ST7 and ST8, respectively. Among the compounds we tested, the zebrafish SULT1 ST7 and ST8 displayed strongest sulfating activities toward catechin (at 9.89 ± 1.07

nmol/min/mg enzyme) and chlorogenic acid (at 1.59 ± 0.11 nmol/min/mg enzyme), respectively. In addition, SULT1 ST7 also showed sulfating activities toward kaempferol, genistein, β-naphthol, caffeic acid, daidzein, quercetin, *n*-propyl gallate, β-naphthylamine, acetaminophen, epigallocatechin gallate, chlorogenic acid; and SULT1 ST8 displayed activities toward kaempferol, genistein, β-naphthol, caffeic acid, daidzein, quercetin, *n*-propyl gallate, epicatechin, epigallocatechin gallate. Interestingly, neither SULT1 ST7 nor SULT1 ST8 exhibited detectable activities toward endogenous compounds including 3,3'5-triiodo-L-thyronine (L-T_3), L-thyroxine (L-T_4), D-Dopa, L-Dopa, dopamine, 17β-estradiol, estrone, 4-androstene-3, 17-dione, chlolesterol, corticosterone, DHEA, hydrocortisone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, progesterone, allopregnanolone, and other exogenous compounds including gallic acid, butylated hydroxylanisole, butylated hydroxytoluene, myricetin, *p*-nitrophenol, mestranol, and minoxidil. SULT1 ST7 and SULT1 ST8 were also assayed for sulfating activity towards some environmental estrogens including bisphenol A, *n*-octylphenol, *n*-nonylphenol, diethylstilbestrol, 17α-ethynylestradiol, 17β-estradiol and hydroxylated PCBs. The results compiled in **Table 3** indicated that both SULT1 ST7 and SULT1 ST8 exhibited the strongest sulfating activities toward the two representative hydroxylated PCBs (3-chloro-4-biphenylol and 3,3',5,5'-tetrachloro-4,4'-biphenyldiol) tested as substrates.

To further investigate the enzymatic characteristics of SULT1 ST7 and ST8, the kinetic parameters of these two enzymes in catalyzing the sulfation of an environmental estrogen, 3-chloro-4-biphenylol, and two xenobiotics compounds, catechin and chlorogenic acid were examined. In these experiments, varying concentrations of these compounds were used in the assays. Data obtained were processed using the SigmaPlot to generate the best fitting trend-lines for the Lineweaver-Burk double-reciprocal plots (**Figure 5**). The kinetic constants shown in the figure revealed that, while the V_{max} values of SULT1 ST7 and ST8 toward the substrates tested were comparable, the K_m values of SULT1 ST8 were an order of magnitude higher than those of SULT1 ST7 (**Table 4**). Based on calculated V_{max}/K_m values, both SULT1 ST7 and SULT1 ST8 appeared to be catalytically more efficient with 3-chloro-4-biphenylol as substrate than with catechin or chlorogenic acid as substrate.

Developmental stages-dependent expression of the zebrafish SULT1 ST7 and SULT1 ST8

The developmental stages-dependent expression of the zebrafish SULT1 ST7 and ST8 were examined. As shown in **Figure 6A**, the mRNA encoding SULT1 ST7 was not detected in unfertilized eggs and in embryos during the early phase of embryonic development. An initial expression of the SULT1 ST7 mRNA was observed at the hatching period (48 h) and selectively expressed in the larval stages (1- to 2-week-old

larvae). Throughout the post-larvae stage onto early maturity, however, no message encoding SULT1 ST7 could be detected. Interestingly, a significant level of its coding message was again expressed in adult female, but not male zebrafish. For SULT1 ST8, no expression was detected in unfertilized eggs and during entire embryogenesis. A significant level of expression appeared in 1-week-old larvae and, intriguingly, gradually decreased in 2- and 3-week-old larvae, and disappeared thereafter throughout the juvenile unto maturity. In contrast to the developmental stage-dependent expression of the SULT1 ST7 and ST8, β-actin, a house keeping protein, was found to be expressed throughout the entire developmental process (**Figure 6B**).

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IV. DISCUSSION

The current study is part of an overall effort to establish a zebrafish model for systematic studies on the ontogeny, cell type/tissue/organ-specific distribution, as well as the physiological involvement of the SULTs, a group of Phase II detoxifying enzymes commonly found among vertebrates (Nowell and Falany, 2006). A prerequisite for using zebrafish in these studies is the identification of the various SULTs and their biochemical characterization. We have recently embarked on the molecular cloning of zebrafish SULTs (Sugahara et al., 2003a, 2003b, 2003c, 2003d; Liu et al., 2005; Yasuda et al., 2005a , 2005b, 2006). Sequence analysis via BLAST search revealed that the zebrafish SULTs we have cloned display sequence homology to mammalian SULTs. Of the ten zebrafish SULTs that have been cloned, six fall within the SULT1 gene family (Sugahara et al., 2003a, 2003b; Liu et al., 2005; Yasuda et al., 2005a, 2005b), three belongs to the SULT2 gene family (Sugahara et al., 2003c; Yasuda et al., 2006), and one appears to be independent from all known SULT gene families (Sugahara et al., 2003d). In this study, we have identified two new SULT1 STs, SULT1 ST7 and SULT1 ST8, and demonstrated that both SULT1 ST7 and SULT1 ST8 displayed sulfating activities toward xenobiotic compounds, particularly hydroxylated PCBs and other environmental chemicals that have been reported to be capable of exerting estrogenic effects in humans as well as wildlife (Safe, 1994). In the enzymatic characterization, SULT1 ST7

and SULT1 ST8, with representative substrates, displayed pH optima at 6.5 and 8.0, respectively. Kinetic parameters of the two enzymes in catalyzing the sulfation of catechin and chlorogenic acid as well as 3-chloro-4-biphenylol were determined. It should be pointed out that SULT1-like enzymes have also been reported to be present in other fish species (Assem et al., 2006; Martin-Skilton et al., 2006; Wang and James, 2007). These SULT1-like enzymes exhibited differential sulfating activities toward various endogenous as well as xenobiotic compounds. For some of them, endocrine disruptors including PCBs have been shown to exert inhibitory effects on their sulfating activities (Martin-Skilton et al., 2006; Wang and James, 2007).

As mentioned in the **Introduction** section, environmental-estrogen-like chemicals have been implicated in the developmental abnormality of wildlife (Guillette et al., 1995; Fry, 1995), as well as pathophysiologic conditions of humans (Carlsen et al., 1992; Auger et al., 1995; Falck et al., 1992; Rogan, 2007). Prominent among these environmental estrogens are the PCBs (Ulbrich and Stahlmann, 2004). PCBs are a group of the halogenated aromatic hydrocarbons which are among the most persistent and widespread environmental estrogens (Safe, 1994; Pocar et al., 2006). Because of their chemical stability, lipophilic property and resistance to degradation, PCBs are making their way into all levels of food chain and preferentially bioaccumulate and biomagnify in wildlife as well as humans (McFarland and

Clarke, 1989; Buckman et al., 2006). Several studies have demonstrated that the accumulation of PCBs may affect the reproductive system in fish populations (Örn et al., 1998; Mac et al., 1993; Hansen et al., 1985). Exposure of high level of PCBs in aquatics have been shown to lead to decreased hatching success, impairment of egg development, a decline of the larvae survival, and inhibition of spermatogenesis and various testicular abnormalities (Freeman et al., 1982; Sangalang et al., 1981). Although the PCB-induced toxicity has been extensively investigated, both the metabolic mechanism and ultimate elimination of PCB and its metabolites in aquatic species remain poorly understood. It should be pointed out that PCBs have been shown to be subjected to biotransformation by cytochrome P-450-mediated hydroxylation in vertebrates (Borlakoglu and Wilkins, 1993; Henriksen et. Al., 2000; Buckman et al., 2006). Hydroxylated PCBs have also been found in a number of fish species and may be derived from the biotransformation of individual PCB congeners by the Phase I enzymes, particularly cytochrome P450 (Vega-López et al., 2007; Mortensen et al., 2007). In the present study, two new zebrafish SULTs, SULT1 ST7 and SULT1 ST8, were identified and shown to exhibit strong sulfating activities toward the two representative hydroxylated PCBs, 3-chloro-4-biphenylol and 3,3’,5,5’-tetrachloro-4,4’-biphenyldiol. That both enzymes displayed sulfating activities toward hydroxychlorobiphenyls may imply the utilization of sulfation as a means for the inactivation/disposal of hydroxylated PCBs in zebrafish. It is worthwhile pointing out that,

in our previous study, two other zebrafish SULTs, SULT1 ST1 and SULT1 ST2, also exhibited differential activities toward hydroxylated PCBs (Sugahara et al., 2003a). In that study, metabolic sulfation of representative hydroxylated PCBs was also demonstrated using cultured zebrafish liver cells (Sugahara et al., 2003a). Whether sulfation truly poses a physiological involvement with regard to the metabolic elimination of hydroxylated PCBs in zebrafish will be an interesting and important issue to clarify.

The adverse effects caused by the halogenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and PCBs may depend on the developmental stage of the affected animal (Elonen et al., 1998; Ankley and Johnson, 2004). These environmental contaminants pose particularly hazardous effects to fish, especially during the early stage of their development (Matta et al., 1997; Powell et al., 2000; Elonen et al., 1998; Toomey et al., 2001; Walker et al., 1991). Embryonic exposure to PCBs or complex mixtures of congeners had been shown to result in edema, hemorrhage, craniofacial deformity, pathological alterations, and significant mortality in newly hatched fish (Spitsbergen et al., 1991; Walker et al., 1991; Walker and Petersen, 1991; Walker et al., 1992; Toomey et al., 2001). An increasing volume of evidence has demonstrated that PCB-induced toxicity is highly dependent on the developmental stage and structural and functional maturation of fish species (Matta et al., 1997; Örn et al., 1998; Powell et al., 2000;

Mac et al., 1993; Monosson et al., 1994). Whether developing fish embryos or larvae are equipped with mechanisms, in particular detoxifying enzymes such as SULTs, for counteracting or eliminating PCBs, however, had remained unresolved. The developmental stage-dependent expression of zebrafish SULT1 ST7 and ST8 as revealed in this study may imply the use of sulfation as a mechanism for the inactivation and/or elimination of PCBs, following their cytochrome P-450-mediated hydroxylation, by developing zebrafish. An initial expression of SULT1 ST7 was observed during the hatching period when primary organogenesis including early maturation of organ rudiments, protrusion of mouth, and cartilage development in the jaw primordia are nearly complete. This suggested the capacity of hatched larvae in metabolizing PCBs before the exogenous feeding commences and before the gut and endodermal organs are fully developed (Falk-Petersen, 2005). During larval development, the prominent abundance of SULT1 ST7 and ST8 were observed, which then disappeared thereafter throughout the juvenile onto maturity. Interestingly, a significant level of SULT1 ST7-coding message was again expressed in adult female, but not male, zebrafish. The significance of this latter finding awaits further clarification. Collectively, these data may nevertheless imply the physiological involvement of SULT1 ST7 and ST8 in counteracting PCB-induced adverse effects in developing zebrafish embryo/larva and in maintaining the normal functioning of reproductive system of female zebrafish in adulthood (Danzo, 1998; Toppari, 2002).

In conclusion, we have identified two novel xenobiotic-sulfating SULT1 STs, SULT1 ST7 and ST8, which may play a role in the metabolism of environmental estrogens including hydroxylated PCBs. This study is part of an overall effort to obtain a complete repertoire of the SULT enzymes present in zebrafish. As pointed out earlier, the identification of the various SULTs and their biochemical characterization is a prerequisite for using the zebrafish as a model for a systematic investigation on the physiological relevance of SULTs in the detoxification of environmental xenobiotics. More work is warranted in order to achieve this goal.

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Table 1. Oligonucleotide primers used in the cloning and the RT-PCR analysis of zebrafish SULT1 ST7 and SULT1 ST8

Target Sequence		Sense and Antisense Oligonucleotide Primers
SULT1 ST7	Sense:	5'-CGCGGATCC**ATG**GATCTCCCAGACATATCCTCTATTAAA-3'
	Antisense:	5'-CGCGGATCC**TTA**AATCTTAGTGCGGAAATTGAGAGTGGT-3'
SULT1 ST8	Sense:	5'-CGCGGATCC**ATG**GCAAACCAAGACAAATCCTCTATTGAATTA-3'
	Antisense:	5'-CGCGGATCC**TTA**AATAATCACACAGAAGTATTAAATCTCAGT-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 zebrafish SULT1 STs or β-actin nucleotide sequence.

Table 2. Specific activities of zebrafish SULT1 ST7 and ST8 with endogenous and xenobiotics as substrates

Endogenous compounds	Specific activity[a]		Xenobiotics	Specific activity	
	SULT1 ST7	SULT1 ST8		SULT1 ST7	SULT1 ST8
3,3'5-Triiodo-L-thyronine (L-T_3)	ND[b]	ND	Chlorogenic acid	1.50 ± 0.27	1.59 ± 0.11
L-Thyroxine (L-T_4)	ND	ND	Kaempferol	0.74 ± 0.12	0.79 ± 0.08
17β-Estradiol	ND	ND	Gallic acid	ND	ND
Estrone	ND	ND	Genistein	0.71 ± 0.04	0.16 ± 0.01
4-Androstene-3, 17-dione	ND	ND	β-Naphthol	1.38 ± 0.27	0.17 ± 0.02
Chlolesterol	ND	ND	Catechin	9.89 ± 1.07	0.65 ± 0.1
Corticosterone	ND	ND	Caffeic acid	0.46 ± 0.04	ND
Dehydroepiandrosterone	ND	ND	Daidzein	0.99 ± 0.03	0.11 ± 0.03
D-Dopa	ND	ND	Butylated hydroxylanisole	ND	ND
L-Dopa	ND	ND	Butylated hydroxytoluene	ND	ND
Dopamine	ND	ND	Quercetin	1.50 ± 0.27	0.78 ± 0.06
Hydrocortisone	ND	ND	Myricetin	ND	ND
17α-hydroxypregnenolone	ND	ND	*n*-Propyl gallate	4.55 ± 0.11	0.16 ± 0.01
17α-hydroxyprogesterone	ND	ND	*p*-Nitrophenol	ND	ND
Pregnenolone	ND	ND	β-Naphthylamine	0.12 ± 0.01	ND
Progesterone	ND	ND	Acetaminophen	1.09 ± 0.02	ND
Allopregnanolone	ND	ND	Epicatechin	ND	0.51 ± 0.10
			Epigallocatechin gallate	2.17 ± 0.88	1.32 ± 0.09
			Mestranol	ND	ND
			Minoxidil	ND	ND

[a]Specific activity refers to nmol substrate sulfated·min^{-1} ·mg^{-1} purified enzyme. Data represent means ± SD derived from three experiments.

[b]ND, specific activity determined is lower than the detection limit (estimated to be ≈0.01 $nmol \cdot min^{-1} \cdot mg$ protein).

Table 3. Specific activities of the zebrafish SULT1 ST7 and zebrafish SULT1 ST8 with environmental estrogens as substrates

Environmental estrogen	Specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$) [a]	
	SULT1 ST7	SULT1 ST8
Bisphenol A	ND [b]	ND
n-Octylphenol	0.20 ± 0.06	ND
n-Nonylphenol	ND	ND
Diethylstilbestrol	ND	ND
17α-Ethynylestradiol	ND	ND
3-Chloro-4-biphenylol	1.85 ± 0.21	3.65 ± 0.14
3,3’,5,5’-Tetrachloro-4,4’-biphenyldiol	1.82 ± 0.04	2.81 ± 0.04
17β-Estradiol	ND	ND
Dehydroepiandrosterone	ND	ND

[a] Data represent means ± SD derived from three experiments.

[b] ND, specific activity determined is lower than the detection limit (estimated to be ≈0.01 $nmol \cdot min^{-1} \cdot mg$ $protein^{-1}$).

Table 4. Kinetics constants of the zebrafish SULT1 ST7 and ST8 with xenobiotics and environmental estrogens as substrates[a]

	Substrate	V_{max}	K_m (μM)	V_{max} / K_m
SULT1 ST 7	Catechin	16.51 ± 1.07	9.06 ± 0.91	1.82
	3-Chloro-4-biphenylol	16.09 ± 1.27	0.78 ± 0.09	20.63
SULT1 ST 8	Chlorogenic acid	5.14 ± 0.32	192.18 ± 15.28	0.03
	3-Chloro-4-biphenylol	24.18 ± 3.78	94.98 ± 17.65	0.26

[a]Data shown represent means ± SD derived form three experiments.

```
SULT1 ST7    1   MDLPDISSIKLPSRPKIFEFEGISMISYFTDNWEKLKNFQARPDDILIATYPKAGTTWVS    60
SULT1 ST8    1   MANQDKSSIELPGRPEIFEFEGILMISCFTDNWENVKNFQARPDDILIATHPKAGTTWVS    60

SULT1 ST7   61   YILDLLYFGKVEPNGQSSLPIYMRVPFLESCFPGMPSGTELADNLPNSPRLIKTHLPVQL   120
SULT1 ST8   61   YILDLLYFGKEDPKHQTKLPIYKRVPFLESCFPVMPSGTEQADNLPTSPRLIKTHLPVQL   120

SULT1 ST7  121   VPKSFWGQNSKVVYVARNAKDNVVSFFHFDRMNHGQPEPGDWDTFLQAFIKGERVFGSWF   180
SULT1 ST8  121   IPKSFWEQNSRVVYVARNAKDTVVSYFHFTRMNMAQPEPGDWNIFLEDFIKGQRVFGSWF   180

SULT1 ST7  181   DHVCGWWEKKKTYPNLHYMFYEDIAKDINGEVESLCTFLKLSRSDEEKEKIINGVQFDAM   240
SULT1 ST8  181   DHVCGWWEKKKTYPNLHYMFYEDMAKDINCELESLCTFLKLSRSDEEKEKIINDVQFDAM   240

SULT1 ST7  241   KQNVMTNYSTIPTMDFTISPFMRKGKVGDWKNHFTVAQNEQFDEDYKEKMKNTTLNFRTK   300
SULT1 ST8  241   KQNKMTNYSTVPVMDCTISPFMREGKVGDWKNYFTVAQNEHFDKDYKQKMKNTTLKFCTE   300

SULT1 ST7  301   I                                                              301
SULT1 ST8  301   I                                                              301
```

Figure 1. Alignment of deduced amino acid sequences of the zebrafish SULT1 ST7 and SULT1 ST8

Residues conserved among these two SULT1 STs enzymes are in shaded boxes. Two “signature sequences” located, respectively, in the N-terminal and C-terminal regions, as well as a conserved sequence in the middle region are underlined.

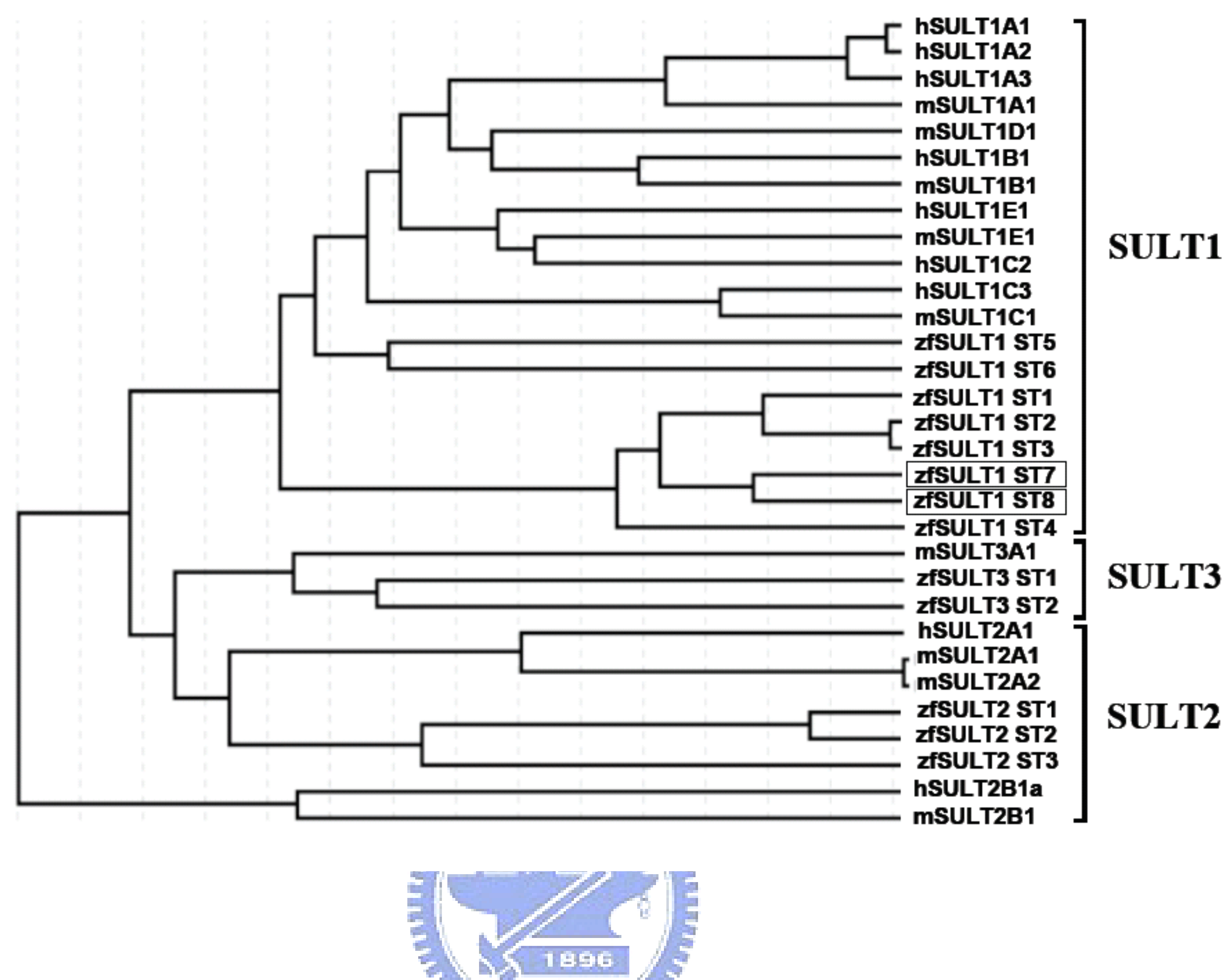


Figure 2. Classification of the zebrafish SULT1 ST7 and SULT1 ST8 on the basis of their amino acid sequences

The dendrogram shows the degree of amino acid sequence homology among different SULTs. For references for individual SULTs, see the review by Blanchard et al. (2004). h, human; m, mouse; and zf, zebrafish. The dendrogram was generated based on Greedy algorithm (Brodskii et al., 1995; Nikolaev et al., 1997).

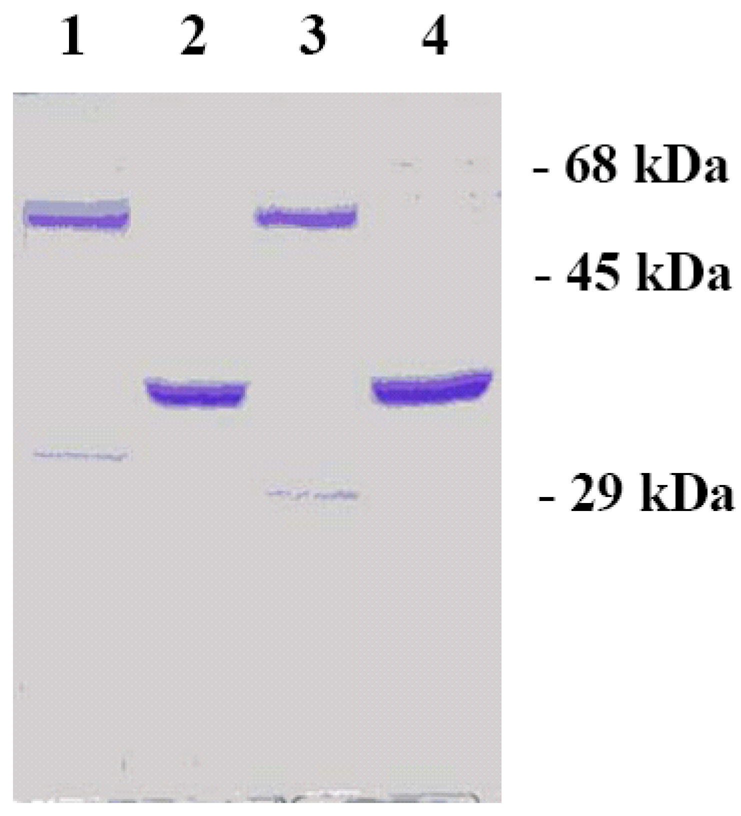


Figure 3. SDS gel electrophoretic pattern of the purified recombinant zebrafish SULT1 ST7 and ST8

Purified zebrafish SULT samples were subjected to SDS-PAGE on a 12% gel, followed by Coomassie blue staining. Samples analyzed in lane 1 and 2 were, respectively, GST-fusion protein and thrombin-digested forms of SULT1 ST7. Samples in lane 3 and 4 were GST-fusion protein and thrombin-digested forms of SULT1 ST8. Positions of the protein molecular weight markers co-electrophoresed were: carbonic anhydrase (*M*r = 29,000), ovalbumin (*M*r = 45,000), and bovine serum albumin (*M*r =68,000).

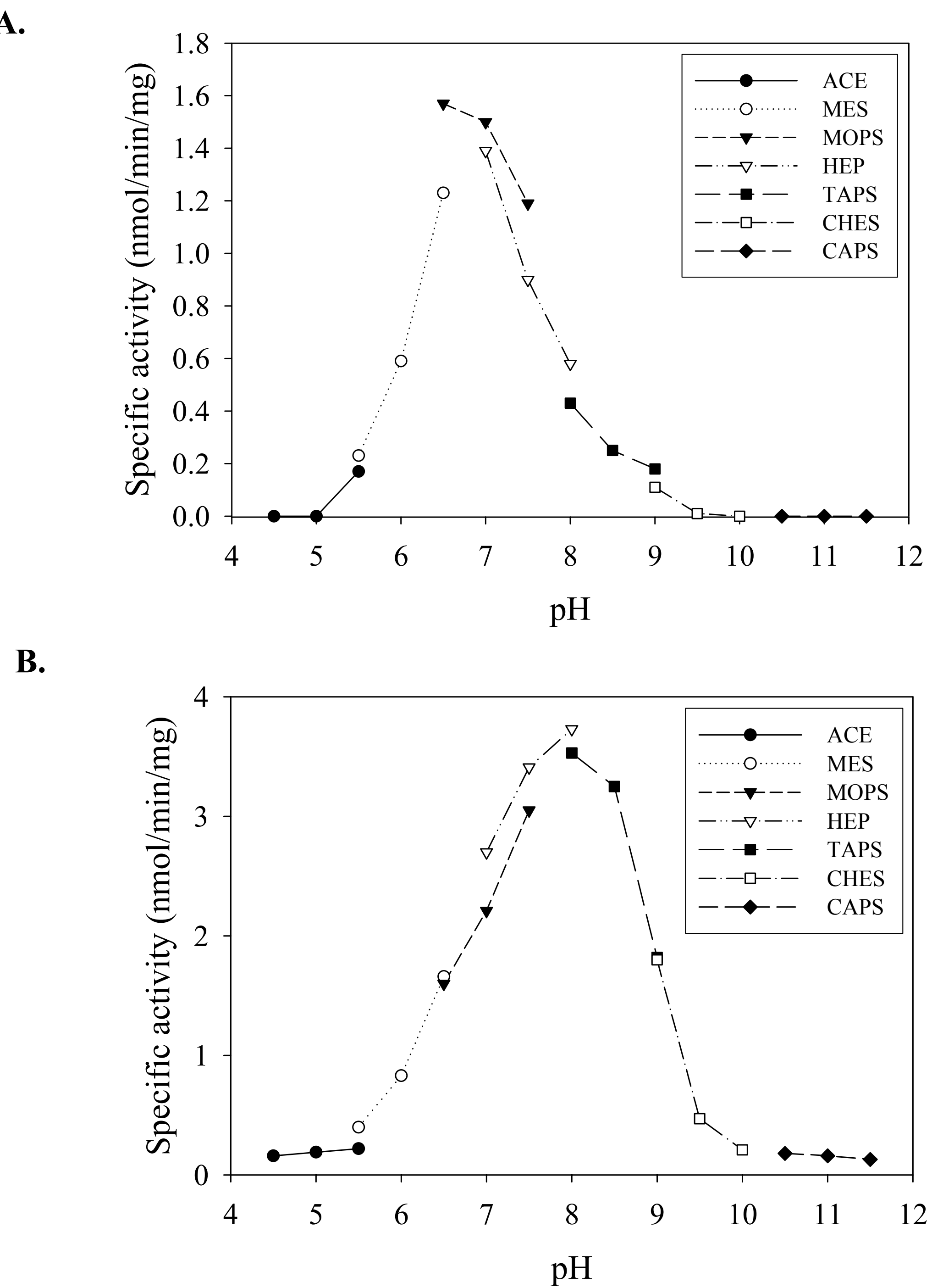
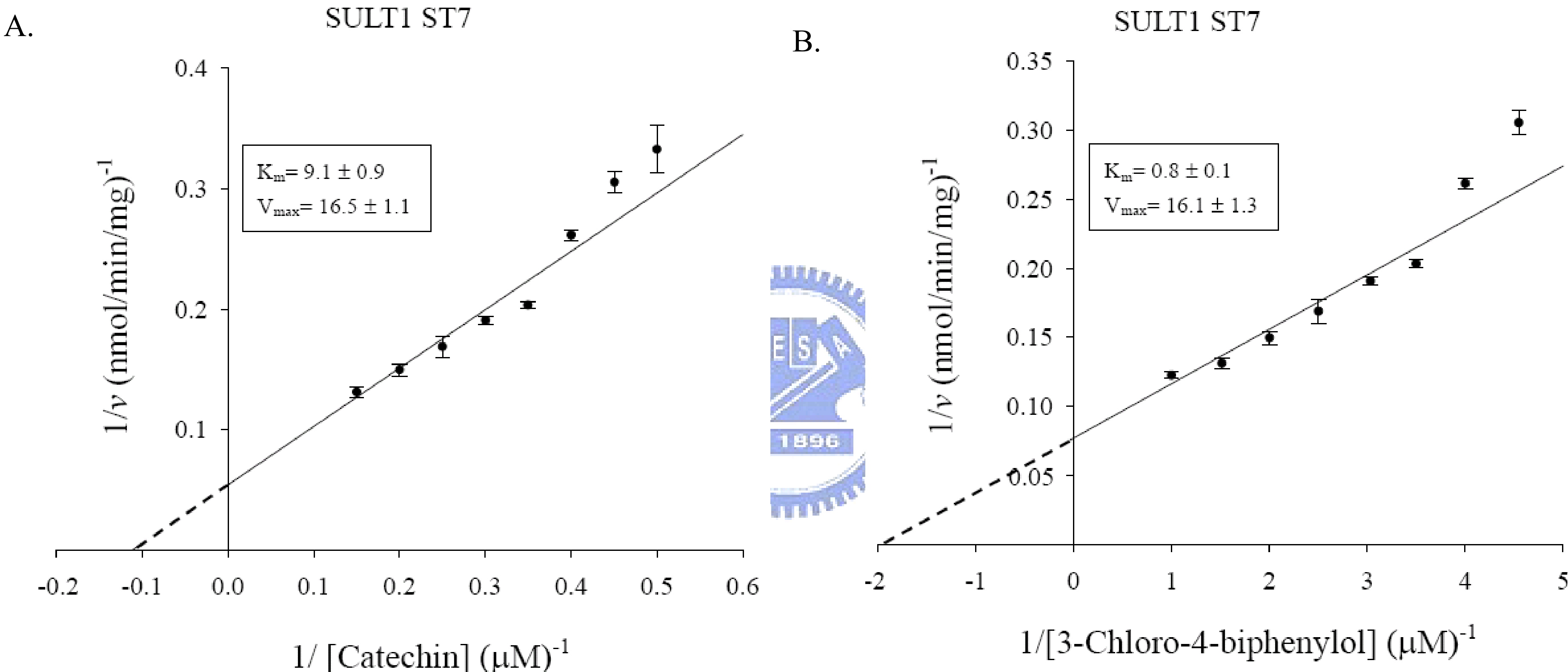


Figure 4. pH dependency of the sulfating activity of the zebrafish SULT1 ST7 (A) and SULT1 ST8 with chlorogenic acid (B) as substrates

The enzymatic assays with 50μM of each substrate were carried out under standard assay conditions as described under *Materials and Methods*, using different buffer systems as indicated. The data represent calculated mean values derived from three experiments.


A.
SULT1 ST7
K_m= 9.1 ± 0.9
V_{max}= 16.5 ± 1.1
1/v (nmol/min/mg)-1
1/ [Catechin] (μM)-1
B.
SULT1 ST7
K_m= 0.8 ± 0.1
V_{max}= 16.1 ± 1.3
1/v (nmol/min/mg)-1
1/[3-Chloro-4-biphenylol] (μM)-1

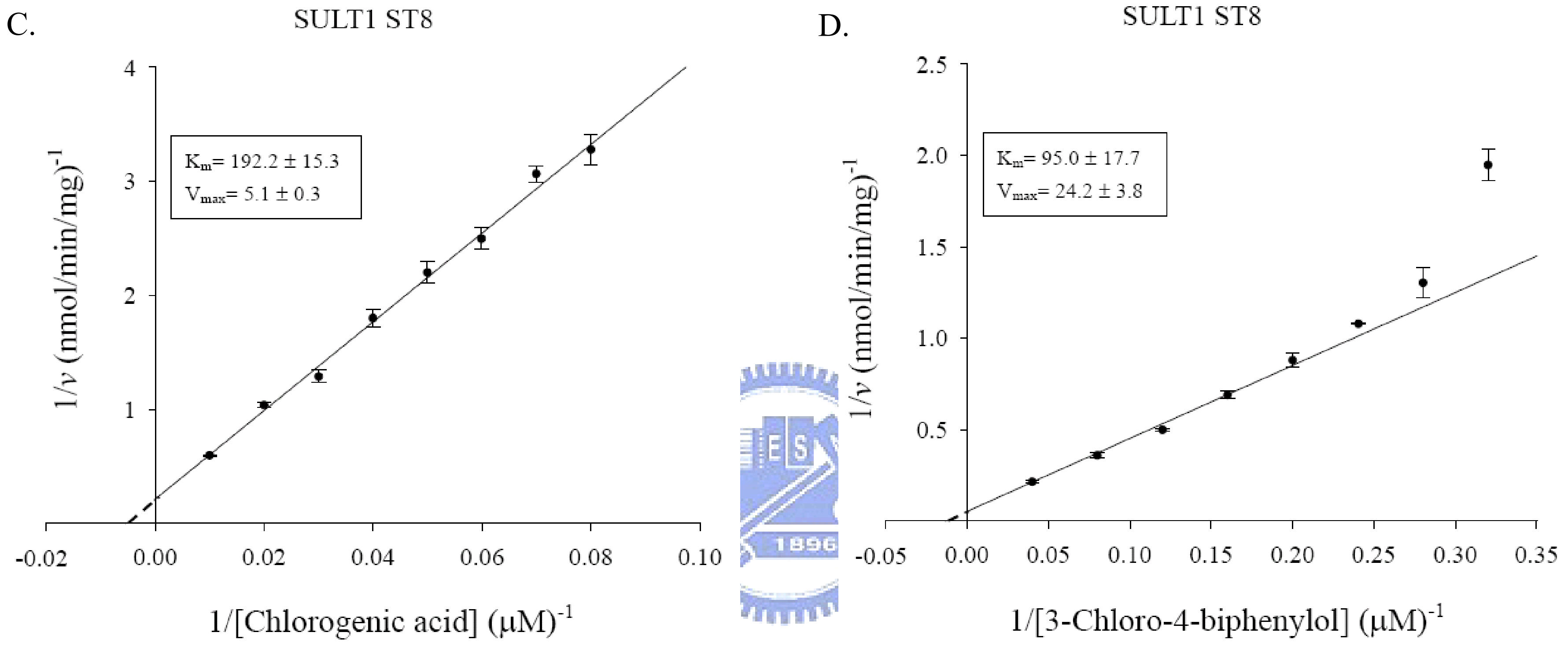


Figure 5. Kinetic analysis of SULT1 ST7 and SULT1 ST8

Lineweaver-Burk double-reciprocal plots of A) the sulfation of catechin by SULT1 ST7, B) the sulfation of 3-chloro-4-biphenyl by SULT1 ST7, C) the sulfation of chlorogenic acid by SULT1 ST8, and D) the sulfation of 3-chloro-4-biphenyl by SULT1 ST8. Concentrations of the substrates used are expressed in mM and velocities are expressed as nmol of product formed/min/mg enzyme. Each data point

represents the mean value derived from three determinations. The concentrations of substrates used for the kinetic analysis of SULT1 ST7 and ST8 were described in *Enzymatic assay*.

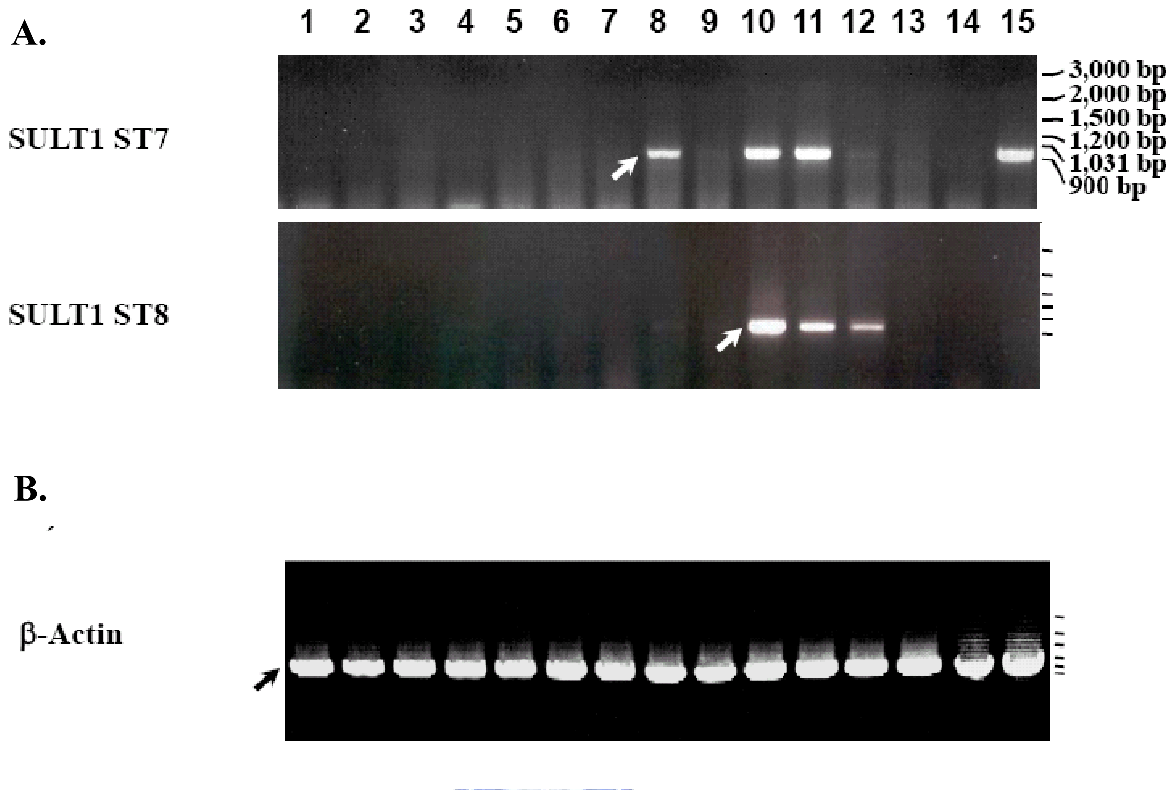


Figure 6. Developmental stage-dependent expression of zebrafish SULTs

RT-PCR analysis of the expression of mRNAs encoding SULT1 ST7 and SULT1 ST8 (A) at different stages during embryogenesis and larval development onto maturity. Final PCR mixtures were subjected to 2% agarose electrophoresis. Samples analyzed correspond to unfertilized zebrafish eggs (lane 1), zebrafish embryos during the zygote period (0-hour post-fertilization (pf); lane 2), cleavage period (1-hour pf; lane 3), blastula period (3-hour pf; lane 4), gastrula period (6-hour pf; lane 5), neurula/segmentaion period (12-hour pf; lane 6), pharyngula period (24-hour pf; lane 7), and hatching period (48- and 72-hour pf; lane 8, 9), 1, 2, 3, 4-week-old zebrafish larvae (lane 10, 11, and 12, 13), and

3-month-old adult male or female zebrafish (lane 14, 15), and DNA size markers. The PCR products corresponding to different zebrafish SULT1 cDNAs, visualized by ethidium bromide staining, are marked by arrows. B. RT-PCR analysis of the expression of the zebrafish β-actin at the same developmental stages as those described in (A). The figure is illustrative of three independent repetitions.

CHAPTER 4

Identification of Tyrosine-sulfated Hemostatic Proteins by a Target-specific Chromatography

Tyrosine sulfation is emerging as a widespread post-translational modification (PTM) which can profoundly affect the protein properties and molecular interactions. Although a number of tyrosine-sulfated proteins have been identified, the majority of them still remain at large. A target-specific chromatography employed in the study to identify new tyrosine-sulfated hemostatic proteins involves first performing a sequence analysis of members of the three distinct hemostatic pathways by Sulfinator, followed by [^{35}S]sulfate-labeling of HepG2 human hepatoma cells, immunoprecipitation of targeted [^{35}S]sulfate-labeled hemostatic proteins, and tyrosine *O*-[^{35}S]sulfate analysis of immunoprecipitated proteins. Three new tyrosine-sulfated hemostatic proteins, protein S, prekallikrein and plasminogen, were identified in this study. Such a target-specific approach will allow for investigation of sulfated-tyrosine proteins of other biochemical/physiological pathways/processes and contribute to a better understating of PTM by tyrosine sulfation.

I. INTRODUCTION

Tyrosine sulfation is a ubiquitous post-translational modification (PTM) that takes place among secretory and membrane-bound proteins whose biosynthetic transport proceeds through the trans Golgi network (Nicolas et al., 1999). The enzyme that generate the modification, tyrosylprotein sulfotransferase (TPST), catalyzes the transfer of a sulfuryl group from the universal sulfate donor 3'-phophoadenosine 5'-phophosulfate (PAPS) to a variety of endogenous and exogenous compounds (**Figure 1**). PTM by tyrosine sulfation has been shown to exert regulatory effects on a number of proteins including coagulation factors VIII (Pittman et al., 1992) and IX (Arruda et al., 1990), complement C4 (Hortin et al., 1988), platelet membrane protein GP Ibα (Dong et al., 1994), as well as chemokine receptors and anticoagulatant biopharmaceuticals (Colvin et al., 2006). The modifications prominently affect the protein characteristics and behaviors, including protein-protein interactions, cellular trafficking, and proteolytic activation of extracellular proteins (Moore, 2003; Kehoe and Bertozzi, 2000). It has been estimated that as much as 1% of the total protein of an organism may be tyrosine-sulfated (Veldkamp et al., 2006). Tyrosine sulfation therefore is the most common PTM taking place on tyrosine residues of proteins (Seibert et al., 2006). Despite that a good number of proteins subjected to tyrosine sulfation have been identified to date, however, the majority of them still remain unknown.

Prediction of PTM of proteins is becoming an important subject in the field of computational biology (Stone and Hofsteenge, 1986). There are several well-established bioinformatics tools that can effectively distinguish the modified proteins from unmodified ones (Baeuerle and Huttner, 1985). A particular one that has been developed for the identification of potentially sulfatable tyrosine residues of proteins is the ExPASy Sulfinator prediction algorithm available at the Swiss-Prot website (http://www.expasy.org/tools/sulfinator/) (Huttner, 1988). However, while Sulfinator may enable users to conveniently identify potential tyrosine sulfation sites in proteins, it is not a guarantor for the proteins identified to be actually sulfated in cells. Therefore, in this study, we attempted to identify and verify new tyrosine-sulfated proteins by a newly established target-specific chromatography (**Figure 2**).

II. MATERIALS AND METHODS

Materials

Purified human plasminogen, prekellikrein and protein S antibodies all purchased from Cedarlane.laboratories (Eugene, OR). Carrier-free sodium [^{35}S]sulfate was from ICN Biomedicals Inc. (Irvine, CA). Cellulose thin-layer chromatography (TLC) plates were from EMD Chem. Inc. (Gibbstown, NJ), and 3-morpholinosydnonimine (SIN-1) was from Calbiochem Co. (San Diego, CA). Complete Miniprotease inihibitor cocktail was from Roche Diagnostics GmbH (Mannheim, Germany), and Ultrafree-MC 5000 NMWL filter units were products of Millipore Co. (Bedford, MA). HepG2 human hepatoma cell line (ATCC HB 8065) was from American Type Culture Collection (Manassas, VA). All other chemicals were of the highest grade commercially available.

Metabolic labeling of HepG2 human hepatoma cells

The experimental procedures of metabolic labeling of HepG2 cell were modified from Yasuda et al. (2005). HepG2 cells were routinely maintained, under a 5% CO_2 atmosphere at 37°C, in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin G (30 μg/ml), and streptomycin sulfate (50 μg/ml). Confluent HepG2 cells grown in individual wells of a 24-well culture plate, preincubated in sulfate-free (prepared by omitting

streptomycin sulfate and replacing magnesium sulfate with magnesium chloride) MEM for 4 h, were labeled with 0.2-ml aliquots of the same medium containing [^{35}S]sulfate (0.3 mCi/ml; 1Ci=37GBq) without serum. At the end of an 18-h labeling, the media were collected and a protease inhibitor cocktail was immediately added to prevent protein degradation.

Immunoprecipitation and gradient SDS electrophoresis

For immunoprecipitation, aliquots of the labeling medium were incubated individually with antibodies against, respectively, fibrinogen, Factor V, heparin cofactor II, plasminogen, prekallikrein, and protein S. After an overnight incubation on ice, Protein G-Sepharose CL-4B was added to each sample and the mixture was agitated by rotation at 4°C for 30 min. Protein G-Sepharose bound with the immune complex was subsequently brought down by centrifugation, washed three times with phosphate-buffered saline, and subjected to electrophoresis in a SDS/5.5-16% polyacrylamide gradient gel (Liu and Lipman, 1984). After the electrophoresis, the gel was stained with Commassie blue in 50% methanol/10% acetic acid (vol/vol) and destained with 25% methanol/ 7.5% acetic acid solution. The gel was dried under reduced pressure at room temperature and subjected to autoradiography.

Tyrosine sulfation analysis

The experimental procedures were modified form Liu and Lipman (1984). To determine

the sulfate content of the hemostatic proteins, the gel pieces were cut individually along with the molecular weights of each protein. The gel pieces were further sliced into small pieces and incubated with 50mM ammonium bicarbonate solution containing Pronase at 150 $\mu M \cdot ml^{-1}$. To improve the digestibility of the gel pieces, the preparation was incubated at 37°C with shaking to allow the proteins in the gel to be hydrolyzed and eluted. After 24 hr incubation, Pronase at 1.5 $mg \cdot ml^{-1}$ in 50mM ammonium bicarbonate solution was added and the digestion was continued for an additional 24 hr. For quantification, the resulting gel eluate was precipitated by centrifugation and add with nonradioactive Tyr(SO_3) standard solution (1mg/ml). The mixed solution was spotted on the TLC plate and the plate was subjected to high voltage electrophoresis (500V) in 7.8% (vol/vol) acetic acid/2.5% formic acid, pH 1.9 as electrophoresis buffer. For two-dimentional separation, the air-dried plate was subsequently developed with 1-butanol/formic acid/2-propanol/H_2O (3:1:1:1; by volume) as the solvent system. For the identification of Tyr(SO_3), the air-dried plate sprayed with ninhydrin solution (0.5% in acetone) and detected by the autoradiography.

III. RESULTS

Potential tyrosine sulfation sites of the plasma proteins identified using Sulfinator

Several blood coagulation factors have been shown to be tyrosine-sulfated (Walsh and Jefferis, 2006). It is possible other members of the blood coagulation pathway, as well as those involved in anticoagulation and fibrinolysis, may also be subjected to tyrosine sulfation. We first performed an amino acid sequence analysis of members of the three hemostatic pathways using the Sulfinator prediction algorithm. **Table 1** shows the potential tyrosine-sulfation sites identified for three previously reported tyrosine-sulfated hemostatic proteins, (fibrinogen, Factor V, and heparin cofactor II) and three unknown but potentially tyrosine-sulfated proteins (plasminogen, prekallikrein, and protein S).

Immunoprecipitation of known and potentially tyrosine-sulfated hemostatic proteins

To further examine the chemical nature of the bound [^{35}S]sulfate, the radioactive bands corresponding to the three potentially tyrosine-sulfated proteins, plasminogen, prekallikrein, and Protein S, were located by autoradiograph and excised from the dried gel, subjected to Pronase hydrolysis, followed by a two-dimensional thin-layer separation combining high-voltage electrophoresis and TLC, based on the procedure previously established (Liu and Lipman, 1984). **Figure 3** shows the autoradiograph taken from the

dried SDS gradient gel. All six proteins, as indicated by arrow heads on their respective electrophoretic lanes, were found to be [^{35}S]sulfated. The radioactive bands corresponding to these proteins were assigned based on their molecular weights: fibrinogen (M_r of Bβ subunit 55 000), Factor V (M_r 330 000), Heparin cofactor II (M_r 78 000), prekallikrein (M_r 88 000), protein S (M_r 70 000), and plasminogen (M_r 106 900). It is noted that nonspecific radioactive bands were also observed on different electrophoretic lanes (as shown in **Figure 3**). These could be due to proteins that interacted with specific proteins being immunoprecipitated or protein G-Sepharose gel beads.

Identification of tyrosine sulfation in hemostatic proteins

To further examine the chemical nature of the bound [^{35}S]sulfate, the radioactive bands corresponding to the three potentially tyrosine-sulfated proteins, plasminogen, prekallikrein, and Protein S, were located by autoradiograph and excised from the dried gel, subjected to Pronase hydrolysis, followed by a two-dimensional thin-layer separation combining high-voltage electrophoresis. As shown in **Figure 4**, the autoradiographs taken from the TLC plates used for the two-dimensional separation of the Pronase hydrolysates of [^{35}S]sulfate-labeled plasminogen, prekallikrein, and protein S clearly revealed their identity as tyrosine-sulfated proteins. The additional radioactive spots detected on the three autoradiographs are likely due to the carbohydrate-bound [^{35}S]sulfate also present in these

three proteins.

IV. DISCUSSION

Plasminogen is a central component in the fibrinolytic system, which is produced by the liver and is present in plasma and most extravascular fluids. It is a zymogen which, upon partial cleavage by the tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), is converted to an active form, plasmin (Cesarman-Maus and Hajjar, 2005). Plasminogen has also been shown to undergo PTM by *O*-glycosylation and N-glycosylation (Rudd et al., 1995). Prekallikrein is a precursor of kellikrein, which acts as a plasma hydrolase that cleaves urokinase-type plasminogen activator (uPA) kininogen to generate bradykinin and activates several coagulation factors such as Factors XII and VII (Alvin and Schmaier, 2000). The PTM by N-glycosylation of prekallikrein has previously been reported (Lu et al., 1996). For protein S, γ-carboxylation of glutamic acid residue and β-hydroxylation of asparagine residue have been demonstrated (Amstel et al., 1987). Our results showing the tyrosine sulfation of plasminogen, prekallikrein, and protein S imply that the different PTMs may comprehensively participate in the functioning of these hemostatic proteins.

In conclusion, three tyrosine-sulfated hemostatic proteins, plasminogen, prekallikrein, and protein S were firstly identified. Additionally, a simple methodology that allows for the

identification of new tyrosine-sulfated proteins was established. The same approach may be employed to identify sulfated tyrosine residues involved in other physiological pathways/processes.

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Table 1. Potential tyrosine sulfation sites of the plasma proteins identified using Sulfinator[a]

SWISS-Prot Name	Description	Site(s) [b]	Sequence Surrounding Sulfatable Tyrosine Residue
FIBA_HUMAN	Fibrinogen alpha chain [precursor]	ND[c]	NONE
FIBB_HUMAN	Fibrinogen beta chain [precursor]	255	ETSEM**Y**LIQPDSSVKPY
FIBG_HUMAN	Fibrinogen gamma chain [precursor]	300	PEADK**Y**RLTYAYFAGGD
		306	RLTYA**Y**FAGGDAGDAFD
		444	PAETE**Y**DSLYPEDDL--[d]
FA5_HUMAN	Coagulation factor V [Precursor]	693	DDEDS**Y**EIFEPPESTVM
		724	ESDAD**Y**DYQNRLAAALG
		726	DADYD**Y**QNRLAAALGIR
		1522	KDGTD**Y**IEIIPKEEVQS
		1538	SSEDD**Y**AEIDYVPYDDP
		1543	YAEID**Y**VPYDDPYKTDV
		1593	EISWD**Y**SEFVQRETDIE
HEP2_HUMAN	Heparin cofactor II [Precursor]	79	EEDDD**Y**LDLEKIFSEDD
		92	SEDDD**Y**IDIVDSLSVSP
PLMN_HUMAN	Plasminogen [precursor]	173	DPEKR**Y**DYCDILECEEE
		175	EKRYD**Y**CDILECEEECM
		323	NLDEN**Y**CRNPDGKRAPW
		554	RKLYD**Y**CDVPQCAAPSF
KLKB1_HUMAN	Plasma kallikrein [precursor]	40	DVASM**Y**TPNAQYCQMRC
		46	TPNAQ**Y**CQMRCTFHPRC
PROS_HUMAN	Vitamin-K-dependent protein S	297	NLDTK**Y**ELLYLAEQFAG

[a]The Sulfinator was published by F. Monigatti et al. (2002). Additional details can be found at the Sulfinator website: (http://www.expasy.org/tools/sulfinator/)

[b]The potential tyrosine sulfation site refers to the location of the potential sulfatable tyrosine residue in the amino acid sequence stored in the SWISS-PROT database.

[c]No potential sulfated-tyrosine sites were detected.

[d]End of the sequence.

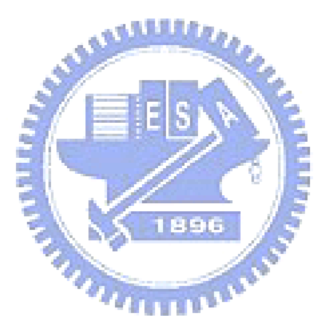

Figure 1. The tyrosylprotein sulfotransferase (TPST) reaction.

TPST catalyzes the transfer of sulfate group from the universal sulfate donor, PAPS, to the hydroxyl group of a luminally oriented peptidyltyrosine residue to form a tyrosine O^4-sulfate ester and 3',5'-ADP.

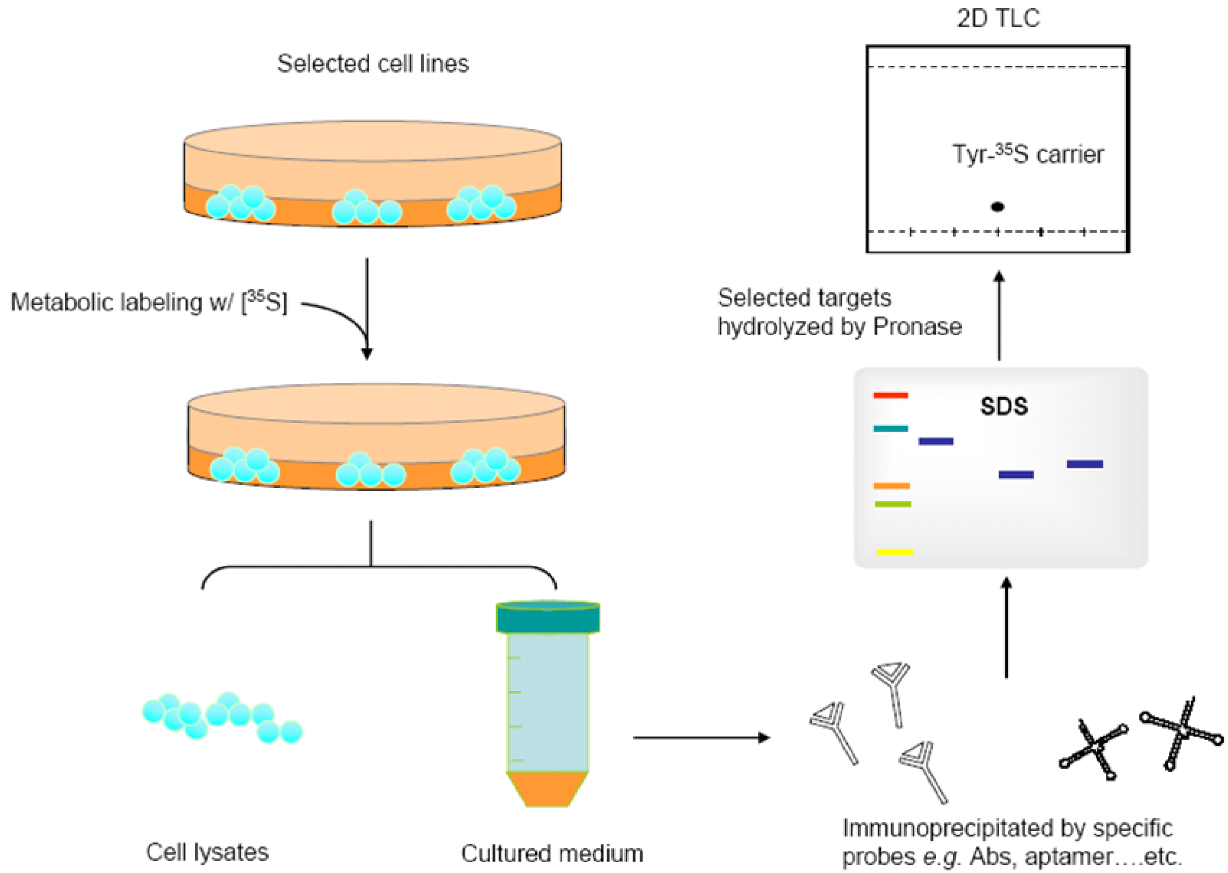

Figure 2. Principle of the target-specific chromatography for tyrosine sulfation.

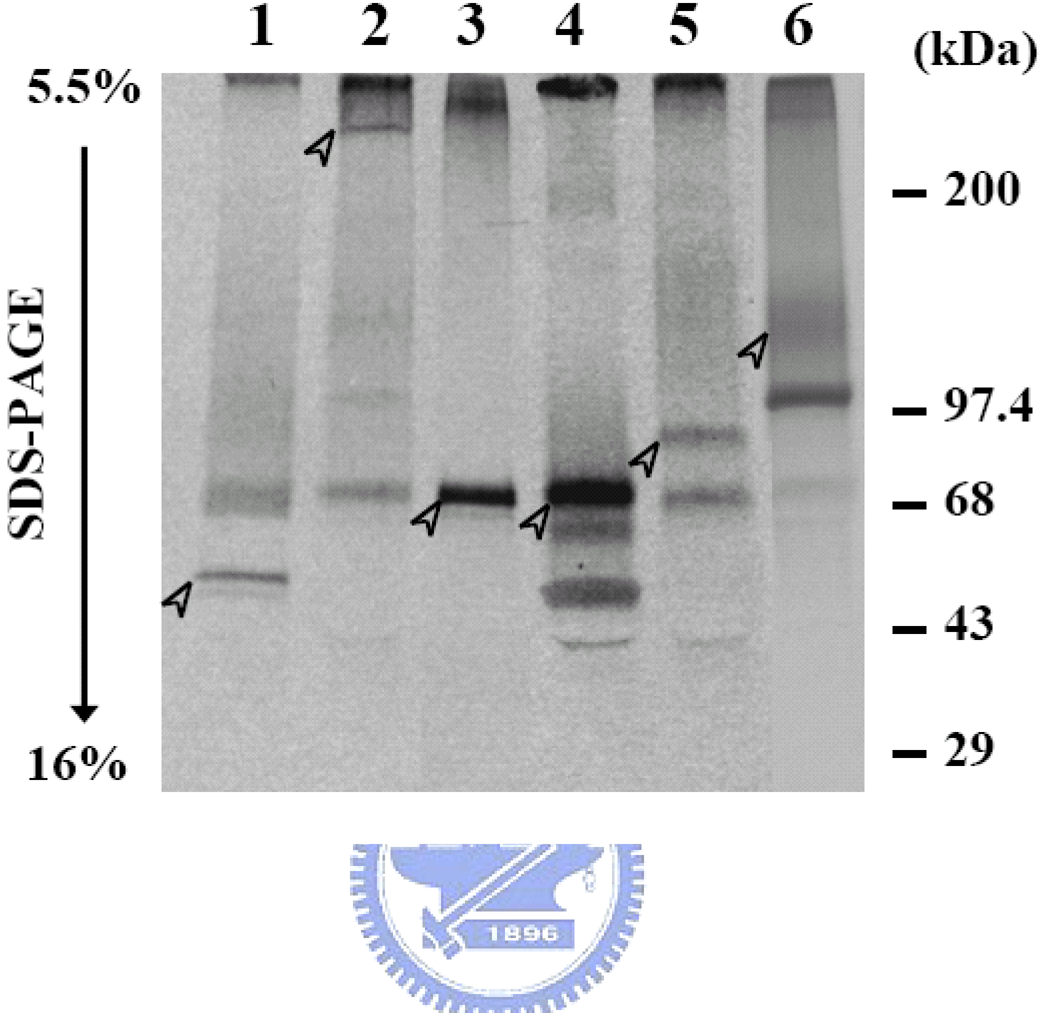

Figure 3. Immunoprecipitation of known and potentially tyrosine-sulfated hemostatic proteins.

The figure shows an autoradiograph taken from the dried SDS-PAGE gel used for the electrophoretic separation of proteins immunoprecipitated from spent medium of HepG2 cells metabolically labeled with [^{35}S]sulfate. Samples analyzed in lanes 1 through 6 were: fibrinogen, factor V, heparin cofactor II, protein S, prekallikrein, and plasminogen. Arrow heads indicated radioactive bands corresponding to the immunoprecipitated proteins.

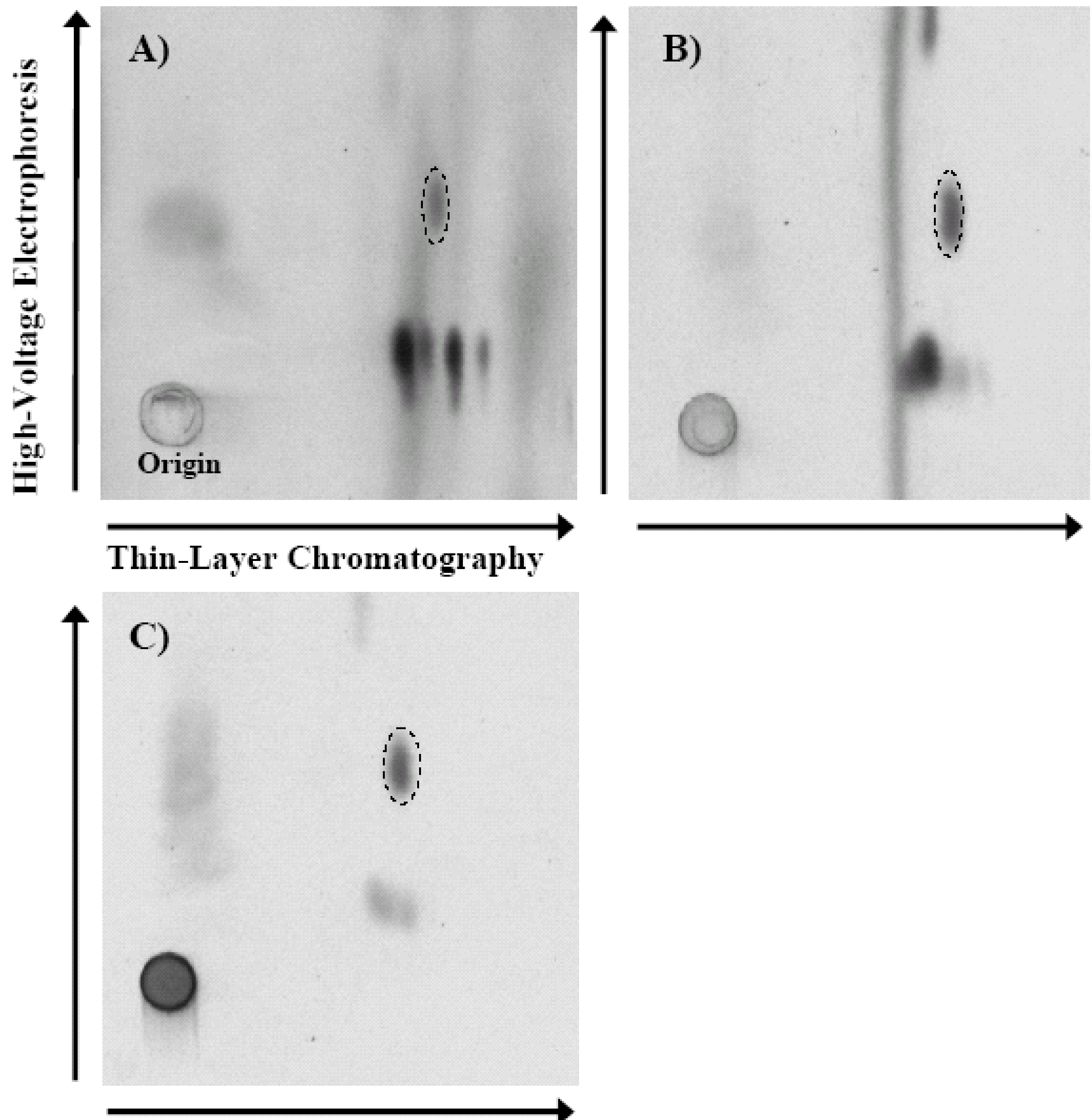

Figure 4. The isotope-labeled Tyrosine-sulfation in three homeostatic proteins

Two-dimensional thin layer analysis of the Pronase Hydrolysates of [^{35}S]sulfate -labeled A) protein S; B) plasminogen; C) prekallikrein. The figure shows the autoradiographs taken from TLC plates used for the two-dimensional thin-layer separation of the Pronase hydrolysate samples. The dashed-line circles correspond to the positions of synthetic tyrosine *O*-sulfate as revealed by ninhydrin staining.

APPENDIX

Table 1. Summary of the sulfated compounds catalyzed by SULTs and the physical significance in biology

Substrates	Physical significance
A. *p* –Nitrophenol	A. Detoxication; inactive form in circulation.
B. 2-Naphthol	B. Detoxication; inactive form in circulation.
C. Dopamine	C. Dominant Inactive form in circulation.
D. Thyroid hormone[a] (T_4 → ORD / IRD → T_3 → IRD / ORD → DIT)	D. Crucial step in regulating irreversible inactivation of thyroid hormones.
E. Estrone	E. Possible role in regulating the interaction of unconjugted steroids and receptors.
F. Kaempferol	F. Potent neuroexcitatory agent on $GABA_A$ receptor.
G. PREG(S)	G. Antagonist action on $GABA_A$ receptor.
H. DHEA(S)	H. Various neuroregulation, antagonist action on $GABA_A$ receptor

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Table 2. Summary of the physiological effects of sulfate metabolites

Sulfated compounds	Biological significance	Sulfated compounds	Biological significance
p-Nitrophenol sulfate	Detoxication; inactive form in circulation	Estrodiol sulfate	Possible role in regulating the interaction of unconjugted steroids and receptors
2-Naphthol sulfate	Detoxication; inactive form in circulation	Estrone sulfate	Possible role in regulating the interaction of unconjugted steroids and receptors
Dopamine sulfate	Dominant Inactive form in circulation	PREGS	potent neuro-excitatory agent on $GABA_A$ receptor
T4 sulfate	crucial step in regulating irreversible inactivation of thyroid hormones	DHEAS	antagonist action on $GABA_A$ receptor
Kaempferol sulfate	Regulation of flavonoids (chemo-preventive agent)	Allopregnanolone sulfate	as negative allosteric modulator of NMDA receptors
Daidzein sulfate	Regulation of flavonoids (chemo-preventive agent)	Corticosterone sulfate	Possible role in regulating the interaction of unconjugted steroids and receptors