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# Identification of a novel estrogen-sulfating cytosolic SULT from zebrafish: Molecular cloning, expression, characterization, and ontogeny study ☆

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**Abstract**

By searching the expressed sequence tag database, a zebrafish cDNA encoding a putative cytosolic sulfotransferase (SULT) was identified. Sequence analysis indicated that this zebrafish SULT belongs to the SULT1 cytosolic SULT gene family. The recombinant form of this novel zebrafish SULT, expressed using the pGEX-2TK expression system and purified from transformed BL21 (DE3) *Escherichia coli* cells, displayed sulfating activities specifically for estrone and 17β-estradiol among various endogenous compounds tested as substrates. The enzyme also exhibited sulfating activities toward some xenobiotic phenolic compounds. This new zebrafish SULT showed dual pH optima, at 6.5 and 10–10.5, with estrone or *n*-propyl gallate as substrate. Kinetic constants of the sulfation of estrone, 17β-estradiol, and *n*-propyl gallate were determined. Developmental stage-dependent expression experiments revealed a significant level of expression of this novel zebrafish estrogen-sulfating SULT at the beginning of the hatching period during embryogenesis, which continued throughout the larval stage onto maturity.


*Keywords:* Sulfotransferase; Estrogen; Sulfation; Molecular cloning; Expression; Ontogeny; Zebrafish

In mammals and other vertebrates, the cytosolic sulfotransferases (SULTs) constitute a group of enzymes that catalyze the transfer of a sulfonate group from the active sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) [1], to substrate compounds containing hydroxyl or amino groups [2–5]. Such sulfation reactions are generally thought to serve for detoxification of xenobiotics, as well as biotransformation of endogenous compounds such as steroid and thyroid hormones, catecholamines, and bile acids [2–5]. In the case of endogenous estrogens, estrone and 17β-estradiol, sulfation has been shown to lead to their inactivation [6], and sulfated estrogens have been proposed to serve as a circulating pool of estrogens that may be desulfated to become active estrogens in peripheral target tissues in mammals [6,7]. In humans, the most significant SULTs involved in the sulfation of endogenous estrogens are the estrogen SULT (SULT1E1) and the thermostable phenol SULT (SULT1A1) [6]. How these enzymes function to regulate the metabolism and homeostasis of endogenous estrogens remains to be fully elucidated.

☆ *Abbreviations:* SULT, sulfotransferase; RT-PCR, reverse transcription-polymerase chain reaction; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Moreover, only fragmentary information is available concerning the cell type/tissue/organ-specific expression of these estrogen-sulfating SULTs, and very little is known with regard to the ontogeny of these enzymes. To resolve these outstanding issues, a suitable animal model is required.

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies [8,9]. Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size, availability of a relatively large number of eggs, rapid development externally of virtually transparent embryo, short generation time, etc. These unique characteristics make the zebrafish an excellent model for a systematic investigation on the ontogeny, cell type/tissue/organ-specific expression, and physiological involvement of individual cytosolic SULTs. A prerequisite for using the zebrafish in these studies, however, is the identification of the various cytosolic SULTs and their functional characterization. We have recently embarked on the molecular cloning of zebrafish cytosolic SULTs [10–13]. Sequence analysis via BLAST search revealed that the zebrafish cytosolic SULTs we have cloned [10–13] display sequence homology to mammalian cytosolic SULTs. Of the six zebrafish cytosolic SULTs that have been expressed and characterized, the SULT1 isoform 2 showed strong sulfating activities toward estrone and 17β-estradiol [11,14]. Like the human SULT1A1, the zebrafish SULT1 isoform 2 also exhibited sulfating activities toward several other endogenous compounds including thyroid hormones and L-3,4-dihydroxyphenylalanine (L-Dopa), as well as a variety of xenobiotic phenolic compounds [11,14]. Whether there is an additional SULT, equivalent to the human SULT1E1, more dedicated to the sulfation of endogenous estrogens in zebrafish remains to be clarified.

We report here the identification of a novel zebrafish estrogen-sulfating cytosolic SULT. Its enzymatic activities toward a variety of endogenous compounds and xenobiotics including some representative flavonoids and phenolic compounds were examined. Kinetic parameters of the enzyme with estrone and 17β-estradiol as substrates were determined. Moreover, its developmental stage-dependent expression was investigated.

## Materials and methods

*Materials.* Estrone, 17β-estradiol, dehydroepiandrosterone, L-Dopa, hydrocortisone, progesterone, 3,3′,5-triiodo-L-thyronine (L-$T_3$), L-thyroxine (L-$T_4$), β-naphthol, *n*-propyl gallate, epigallocatechin gallate, myricetin, genistein, quercetin, kaempferol, daidzein, butylated hydroxy anisole, *p*-nitrophenol, chlorogenic acid, catechin, caffeic acid, epicatechin, gallic acid, aprotinin, thrombin, adenosine 5′-triphosphate (ATP), PAPS, sodium dodecyl sulfate (SDS), Trizma base, isopropyl-β-D-thiogalactopyranoside (IPTG), inorganic pyrophosphatase, dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical. Allopregnanolone was from Calbiochem. 4-Androstene-3,17-dione, corticosterone, 17α-hydroxy pregnenolone, 17α-hydroxy progesterone, pregnenolone, and carrier-free sodium [$^{35}$S]sulfate were products of ICN Biomedicals. Dopamine was from Pfaltz and Bauer, Inc. TRI Reagent was from Molecular Research Center. Unfertilized zebrafish eggs and zebrafish embryos and larvae at different developmental stages were prepared by Scientific Hatcheries. Total RNA from a 3-month-old zebrafish and the corresponding first-strand cDNA were prepared as described previously [11]. *Taq* DNA polymerase was a product of Promega Corporation. Takara *Ex Taq* DNA polymerase was purchased from PanVera Corporation. $T_4$ DNA ligase and *Bam*HI restriction endonuclease were from New England Biolabs. XL1-Blue MRF′ and BL21 (DE3) *Escherichia coli* host strains were purchased from Stratagene. Oligonucleotide primers were synthesized by MWG Biotech. pGEX-2TK glutathione *S*-transferase (GST) gene fusion vector, glutathione–Sepharose 4B, First-strand cDNA Synthesis Kit, and GEX-5′ and GEX-3′ sequencing primers were products of Amersham Biosciences. Recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase was prepared as described previously [15]. Cellulose thin-layer chromatography (TLC) plates were from EM Science. All other chemicals were of the highest grade commercially available.

*Molecular cloning, expression, and purification of the zebrafish estrogen-sulfating cytosolic SULT.* By searching the expressed sequence tag database, a zebrafish cDNA clone (GenBank Accession # BC075996) encoding a putative cytosolic SULT was identified. Using sense (5′-CGCGGATCCATGGAACAGGAACCACTGAGCTATGAAGAA-3′) and antisense (5′-CGCGGATCCTTAAATGTTTAAGCGGAAGGGTATGTCCAC-3′) oligonucleotide primers, designed based on reported 5′- and 3′-coding regions of the zebrafish SULT cDNA and with *Bam*HI sites incorporated at the ends, a PCR in a 100 μl reaction mixture was carried out under the action of *Ex Taq* DNA polymerase, with the zebrafish first-strand cDNA as the template. Amplification conditions were 2 min at 94 °C and 25 cycles of 94 °C for 35 s, 62 °C for 40 s, and 72 °C for 1 min. The final reaction mixture was applied onto a 1.2% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. The PCR product band detected was excised from the gel, and the DNA therein was isolated by spin filtration. Purified PCR product was subjected to *Bam*HI restriction, cloned into the *Bam*HI-restricted pGEX-2TK vector, and verified for authenticity by nucleotide sequencing [16].

To express the recombinant zebrafish SULT, competent *E. coli* BL21 (DE3) cells, transformed with pGEX-2TK harboring the zebrafish SULT cDNA, were grown in 1 L Luria–Bertani medium supplemented with 60 μg/ml ampicillin. After the cell density reached 0.6 $OD_{600nm}$, 0.1 mM IPTG was added to induce the production of recombinant zebrafish SULT. After an overnight induction at room temperature, the cells were collected by centrifugation and homogenized in 25 ml ice-cold lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using an Aminco French press. Twenty microliters of 10 mg/ml aprotinin (a protease inhibitor) was added to the crude homogenate, which was then subjected to centrifugation at 10,000*g* for 15 min at 4 °C. The supernatant was fractionated using 2.5 ml glutathione–Sepharose, and the bound GST fusion protein was eluted by an elution buffer (50 mM Tris–HCl, pH 8.0, plus 10 mM reduced glutathione) or 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 0.5 U/ml bovine thrombin. Following a 30-min incubation at room temperature with constant agitation, the preparation was subjected to centrifugation. The recombinant zebrafish SULT present in the supernatant collected was analyzed with respect to its enzymatic properties.

*Enzymatic assay.* The sulfating activity of the recombinant zebrafish cytosolic SULT was assayed using radioactive PAP[$^{35}$S] as the sulfate group donor. The standard assay mixture, in a final volume

25 μl, contained 50 mM of Mops buffer at pH 7.0, 1 mM DTT, and 14 μM PAP[$^{35}$S]. The substrate, dissolved in DMSO or water at 10 times the final concentration in the assay mixture, was added subsequent to Mops buffer and PAP[$^{35}$S]. Controls with DMSO or water alone were also prepared. The reaction was started by the addition of the enzyme (1.25 μg), allowed to proceed for 5 min at 28 °C, and stopped by placing the assay mixture-containing thin-walled tube on a heating block, pre-heated to 100 °C, for 2 min. The precipitates were cleared by centrifugation for 1 min, and the supernatant was subjected to the analysis of [$^{35}$S]sulfated product using the previously established TLC procedure [17]. The solvent system used was *n*-butanol/isopropanol/88% formic acid/water at a ratio of 3:1:1:1 (by volume) for most sulfated products, and 0.5:1.5:2:2 and 2:1:1:2 for sulfated myricetin and caffeic acid. To examine the pH-dependence, different buffers (50 mM Mes at 5.5, 6.0, or 6.5; Mops at 6.5, 7.0, or 7.5; Taps at 7.5, 8.0, 8.5 or 9.0; Ches at 9.0, 9.5, or 10.0; and Caps at 10.0, 10.5, or 11.0), instead of 50 mM Taps (pH 8.0), were used in the reactions. For the kinetic studies on the sulfation of estrone, 17β-estradiol, and *n*-propyl gallate, varying concentrations of these substrate compounds and 50 mM Mops buffer at pH 7.0 were used. Each assay was performed in triplicate, together with a control without enzyme. The results obtained were calculated and expressed in nanomoles of sulfated product formed/min/mg purified enzyme.

*Analysis of the developmental stage-dependent expression of the zebrafish cytosolic SULT*. RT-PCR was employed to investigate the developmental stage-dependent expression of the zebrafish cytosolic SULT. Total RNAs from zebrafish embryos and larvae at different developmental stages were isolated using the TRI Reagent based on manufacturer's instructions. First-strand cDNAs were reverse-transcribed from the total RNA samples using the First-strand cDNA Synthesis Kit (Amersham Biosciences). PCRs in 50 μl reaction mixtures were carried out using *Ex Taq* DNA polymerase with the first-strand cDNAs prepared as templates, in conjunction with gene-specific sense (5′-ACAGAATAAATTAATAATTCAAGCGAACAAAGTCGAA-3′) and antisense (5′-TCTGAAAGCAAATTGTTTGACATCTGATATAAGGCTA-3′) oligonucleotide primers. Amplification conditions were 2 min at 94 °C followed by 39 cycles of 35 s at 94 °C, 40 s at 60 °C, and 1 min at 72 °C. The final reaction mixtures were applied onto a 1.2% agarose gel, separated by electrophoresis, and visualized by ethidium bromide staining. As a control, PCR amplification of the sequence encoding zebrafish β-actin was concomitantly performed using the above-mentioned first-strand cDNAs as templates, in conjunction with gene-specific sense (5′-ATGGATGAGGAAATCGCTGCCCTGGTC-3′) and antisense (5′-TTAGAAGCACTTCCTGTGAACGATGGA-3′) oligonucleotide primers designed based on the reported zebrafish β-actin nucleotide sequence (GenBank Accession # AF057040).

*Miscellaneous methods*. PAP[$^{35}$S] was synthesized from ATP and carrier-free [$^{35}$S]sulfate using the bifunctional human ATP sulfurylase/adenosine 5′-phosphosulfate kinase and its purity was determined as previously described [18]. The PAP[$^{35}$S] synthesized was adjusted to the required concentration and specific activity of 15 Ci/mmol at 1.4 mM by the addition of cold PAPS. SDS–polyacrylamide gel electrophoresis was performed on 12% polyacrylamide gels using the method of Laemmli [19]. Protein determination was based on the method of Bradford with bovine serum albumin as the standard [20].

## Results and discussion

Sulfation of endogenous estrogens has been known for decades [2–5]. Previous studies have demonstrated that sulfation leads to the inactivation of estrone and 17β-estradiol [6], and sulfated estrogens have been proposed to serve as a circulating pool of estrogens that may be desulfated to become active estrogens in peripheral target tissues in mammals [6,7]. As part of an effort to develop a zebrafish model for investigating in greater detail the role of sulfation in the metabolism and homeostasis of endogenous estrogens, we identified and characterized a novel zebrafish estrogen-sulfating SULT in the present study.

### *Molecular cloning, expression, purification, and characterization of the zebrafish estrogen-sulfating cytosolic SULT1 isoform 6*

Using RT-PCR the open reading frame of the cDNA encoding a novel zebrafish cytosolic SULT was cloned and sequenced. The nucleotide sequence obtained was submitted to the GenBank database under Accession No. AY937249. Fig. 1 shows the nucleotide and deduced amino acid sequences of the newly cloned zebrafish SULT. The open reading frame encompasses 927 nucleotides and encodes a 308-amino acid polypeptide. Similar to other cytosolic SULTs, the new zebrafish SULT contains sequences resembling the so-called "signature sequences" (YPK(A/S)GTxW in the N-terminal region and RKGxxGDW(V/K)NxFT in the C-terminal region; as underlined) characteristic of SULT enzymes [21]. 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 [1], and thus designated the "5′-phosphosulfate binding (5′-PSB) motif" [22]. The cloned zebrafish SULT also contains the "3′-phosphate binding (3′-PB) motif" (amino acid residues 143–153; as underlined) responsible for the binding to the 3′-phosphate group of PAPS [22]. Sequence analysis based on BLAST search revealed that the deduced amino acid sequence of the new zebrafish SULT displays 56%, 52%, and 50% identity to mouse SULT1A2, rat SULT1D1, and dog SULT1B1, and lower % identity to other known mammalian SULTs. It is generally accepted that members of the same SULT gene family share at least 45% amino acid sequence identity, and members of subfamilies further divided in each SULT gene family are greater than 60% identical in amino acid sequence [21,23,24]. Based on these criteria, the newly cloned zebrafish SULT, while clearly belonging to the SULT1 gene family (and, therefore, tentatively designated the zebrafish SULT1 isoform 6), cannot be classified into any of the existing subfamilies within the SULT1 family (see the dendrogram shown in Fig. 2). Compared with known zebrafish SULTs, the newly cloned zebrafish SULT1 isoform 6 displays 45.2%, 48.2%, 47.5%, 45.7%, and 55.3% amino acid sequence identity to, respectively, the zebrafish SULT1 isoforms 1, 2, 3, 4, and 5 previously reported [10–13]. Based on the criteria for classification mentioned above, the zebrafish SULT1 isoform 6 may not be categorized

```
10         20         30         40         50         60
ATGGAACAGGAACCACTGAGCTATGAAGAAGCCATTACAAGGGCAGCAGATGCTATTCAG
M  E  Q  E  P  L  S  Y  E  E  A  I  T  R  A  A  D  A  I  Q
         70         80         90        100        110        120
CGATTTCCTCTAAAAGATGTTCAGGGTGTTCCTCTCATGAGCACAATTGCTGACAACTGG
R  F  P  L  K  D  V  Q  G  V  P  L  M  S  T  I  A  D  N  W
       130        140        150        160        170        180
AAATCCATTTCAGAATTCTGCCCTGACCCATCAGACCTACTGATCTCTACTTACCCTAAA
K  S  I  S  E  F  C  P  D  P  S  D  L  L  I  S  T  Y  P  K
       190        200        210        220        230        240
GCAGGCACTACCTGGACTCAAGAGATAGTGGATCTTCTGCTAAACAATGGAGATGCTCAG
A  G  T  T  W  T  Q  E  I  V  D  L  L  L  N  N  G  D  A  Q
       250        260        270        280        290        300
GTGTGCAAGAGAGCACCAACAGCTGTCCGTATTCCCTTTCTGGAAATTTGTGCTCCTCCG
V  C  K  R  A  P  T  A  V  R  I  P  F  L  E  I  C  A  P  P
       310        320        330        340        350        360
CCCATACCTTCAGGGCTTGAATTGCTTAAACAGATGAAACCACCTAGAGTCATTAAAACT
P  I  P  S  G  L  E  L  L  K  Q  M  K  P  P  R  V  I  K  T
       370        380        390        400        410        420
CACCTGCCCATTCAACTAGTGCCTGTAGGATTCTGGCAAAATAAATGCAAGGTCATTTAT
H  L  P  I  Q  L  V  P  V  G  F  W  Q  N  K  C  K  V  I  Y
       430        440        450        460        470        480
ATGGCACGGAATGCGAAGGACAACCTTGTAAGTTACTTCCACTTTGATCGTATGAATCTT
M  A  R  N  A  K  D  N  L  V  S  Y  F  H  F  D  R  M  N  L
       490        500        510        520        530        540
ACCCAGCCTGAGCCAGGACCTTGGGATGGATACATCCACAAGTTCATGAAAGGACAACTA
T  Q  P  E  P  G  P  W  D  G  Y  I  H  K  F  M  K  G  Q  L
       550        560        570        580        590        600
GGTTGGGGCTCTTGGTATGACCACGTAAAAGGTTACTGGAAGGAATCCAAGGAAAGGAAT
G  W  G  S  W  Y  D  H  V  K  G  Y  W  K  E  S  K  E  R  N
       610        620        630        640        650        660
ATTCTCTACATACTCTATGAGGATATGAAAGAGAGCCCTTCTAGAGAAATTAAGAGGATC
I  L  Y  I  L  Y  E  D  M  K  E  S  P  S  R  E  I  K  R  I
       670        680        690        700        710        720
ATGCACTATTTGGACCTGTCTGTTTCTGAGGATGTCATAAATAAGATTGTGCAGCTGACA
M  H  Y  L  D  L  S  V  S  E  D  V  I  N  K  I  V  Q  L  T
       730        740        750        760        770        780
TCTTTCCATGTCATGAAGGACAATCCAATGGCTAACTACTCATACATCCCCAAAGCTGTG
S  F  H  V  M  K  D  N  P  M  A  N  Y  S  Y  I  P  K  A  V
       790        800        810        820        830        840
TTTGATCAGTCCATTTCTGCCTTCATGAGAAAAGGAGAGGTTGGTGACTGGGTAAACCAT
F  D  Q  S  I  S  A  F  M  R  K  G  E  V  G  D  W  V  N  H
       850        860        870        880        890        900
TTCACCCCAGCTCAGTCCAAGATGTTTGATGAAGACTATACAAACCAGATGAAAGATGTG
F  T  P  A  Q  S  K  M  F  D  E  D  Y  T  N  Q  M  K  D  V
       910        920        930
GACATACCCTTCCGCTTAAACATTTAA
D  I  P  F  R  L  N  I  *
```

Fig. 1. Nucleotide and deduced amino acid sequences of the zebrafish SULT1 isoform 6 cDNA. Nucleotides are numbered in the 5′ to 3′ direction. Two "signature sequences," respectively, located in the N-terminal and C-terminal regions, as well as a conserved sequence in the middle region are underlined. The translation stop codon is indicated by an asterisk.

within the same SULT1 subfamily with any of the five known zebrafish SULT1 isoforms.

The coding region of the zebrafish SULT1 isoform 6 cDNA was subcloned into pGEX-2TK, a prokaryotic expression vector, for the expression of recombinant enzyme in *E. coli*. As shown in Fig. 3, the GST fusion protein form of the recombinant zebrafish SULT1 isoform 6, purified from the *E. coli* extract, migrated at ca. 62 kDa position upon SDS–PAGE. Upon thrombin digestion, the zebrafish SULT1 isoform 6 moiety, however, migrated as a 30 kDa protein. This latter size is considerably smaller than the molecular weight (35,611) calculated based on its deduced amino acid sequence. It was subsequently noted that the throm-

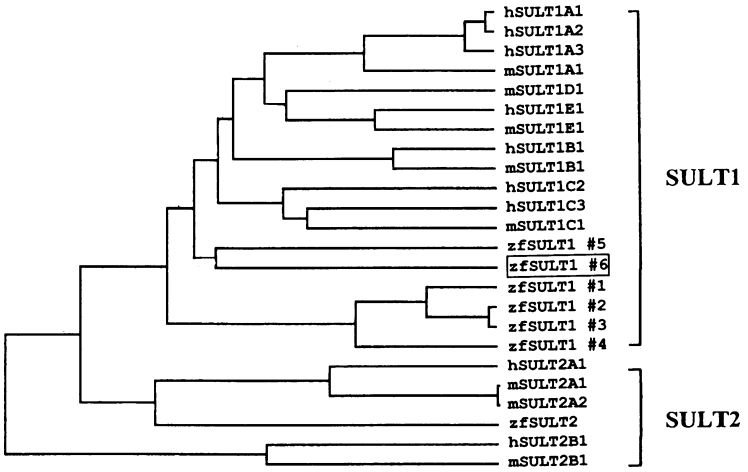


Fig. 2. Classification of the zebrafish SULT1 isoform 6 on the basis of deduced amino acid sequence. The dendrogram shows the degree of amino acid sequence homology among cytosolic SULTs. For references for individual SULTs, see the review by Weinshilboum et al. [21]. *h*, human; *m*, mouse; *zf*, zebrafish.

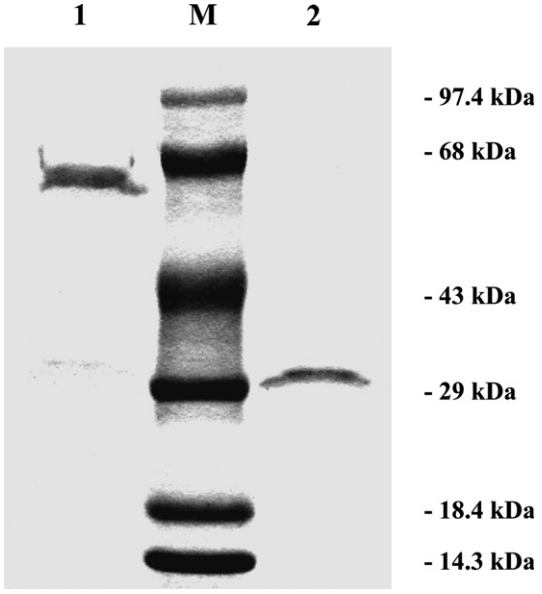


Fig. 3. SDS gel electrophoretic pattern of the purified recombinant zebrafish SULT1 isoform 6. Purified zebrafish SULT samples were subjected to SDS–PAGE on a 12% gel, followed by Coomassie blue staining. Samples analyzed in lanes 1 and 2 were, respectively, the GST fusion protein and thrombin-digested forms of the enzyme. Protein molecular weight markers co-electrophoresed in the middle lane are: lysozyme ($M_r = 14{,}300$), β-lactoglobulin ($M_r = 18{,}400$), carbonic anhydrase ($M_r = 29{,}000$), ovalbumin ($M_r = 43{,}000$), bovine serum albumin ($M_r = 68{,}000$), and phosphorylase b ($M_r = 97{,}400$).

bin-digested zebrafish SULT1 isoform 6 exhibited much lower and unstable sulfating activity in comparison with the fusion protein form of the enzyme. The fusion protein form of zebrafish SULT1 isoform 6, therefore, was used for the characterization of its enzymatic properties. An initial experiment showed that the enzyme exhibited strong sulfating activities toward estrone and *n*-propyl gallate. Intriguingly, a pH-dependence experiment subsequently performed revealed the enzyme to exhibit two pH optima, at 6.5 and 10–10.5, with either estrone or *n*-propyl gallate as substrate (Fig. 4). A number of endogenous and xenobiotic compounds were tested as substrates for the enzyme, and the activity data obtained are compiled in Table 1. (The specific activities determined were corrected for the molecular mass of the GST moiety in the fusion protein form of the enzyme.) Interestingly, among the endogenous substrates, the zebrafish SULT1 isoform 6 showed sulfating activities toward only the two endogenous estrogens, estrone and 17β-estradiol. The enzyme also exhibited activities toward some of the xenobiotic compounds tested, including β-naphthol, *n*-propyl gallate, epigallocatechin gallate, myricetin, genistein, quercetin, kaempferol, daidzein, butylated hydroxy anisole, *p*-nitrophenol, chlorogenic acid, catechin, and caffeic acid. These latter activities are in line with this new enzyme being a member of the SULT1 (phenol SULT) gene family. It should also be pointed out that, of the five zebrafish SULT1 isoforms previously reported [10–12], the SULT1 isoform 2 also exhibited strong activities toward estrone and 17β-estradiol [10,14]. Unlike the SULT1 isoform 6 identified in the present study, however, the zebrafish SULT1 isoform 2 was also found to be active toward several other endogenous compounds including thyroid hormones, $T_3$ and $T_4$, dopamine, L-Dopa, and dehydroepiandrosterone [10,14]. The SULT1 isoform 6, therefore, appears to be the only zebrafish enzyme known to date that displays substrate specificity exclusively for endogenous estrogens. It will be interesting to investigate whether the SULT1 isoform 6 plays a unique and important role in the metabolism and homeostasis of endogenous estrogens in vivo.

To investigate in more detail the sulfation of endogenous estrogens, the kinetics of sulfation of these com-

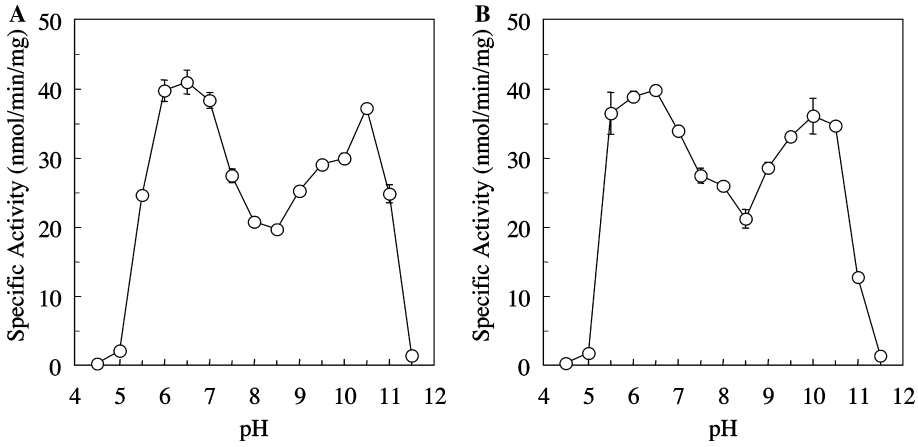


Fig. 4. pH dependency of the sulfating activity of the zebrafish SULT1 isoform 6 with (A) estrone and (B) *n*-propyl gallate as substrates. The enzymatic assays with 50 μM of each substrate were carried out under standard assay conditions as described under Materials and methods, using different buffer systems as indicated. The data represent calculated mean values ± standard deviation derived from three experiments.

Table 1
Specific activities of the zebrafish SULT1 with endogenous and xenobiotic compounds as substrates[a]

| Endogenous compounds | Specific activity (nmol/min/mg) | Xenobiotic compounds | Specific activity (nmol/min/mg) |
|---|---|---|---|
| Estrone | 30.2 ± 1.1 | β-Naphthol | 38.0 ± 0.5 |
| 17β-Estradiol | 19.1 ± 0.6 | *n*-Propyl gallate | 34.5 ± 0.9 |
| Allopregnanolone | ND[b] | Epigallocatechin gallate | 28.3 ± 0.6 |
| 4-Androstene-3,17-dione | ND | Myricetin | 26.5 ± 0.7 |
| Corticosterone | ND | Genistein | 18.4 ± 0.5 |
| Dehydroepiandrosterone | ND | Quercetin | 17.5 ± 0.2 |
| L-Dopa | ND | Kaempferol | 16.5 ± 1.0 |
| Dopamine | ND | Daidzein | 15.4 ± 1.0 |
| Hydrocortisone | ND | Butylated hydroxy anisole | 10.8 ± 0.2 |
| 17α-Hydroxy progesterone | ND | *p*-Nitrophenol | 6.6 ± 0.1 |
| 17α-Hydroxy pregnenolone | ND | Chlorogenic acid | 5.9 ± 0.2 |
| Pregnenolone | ND | Catechin | 4.6 ± 0.3 |
| Progesterone | ND | Caffeic acid | 4.1 ± 0.3 |
| 3,3′,5-Triiodo-L-thyronine (L-$T_3$) | ND | Epicatechin | ND |
| L-Thyroxine (L-$T_4$) | ND | Gallic acid | ND |

[a] Specific activity refers to nmol substrate sulfated/min/mg purified enzyme. Data represent means ± SD derived from three experiments.
[b] Activity not detected.

pounds by the zebrafish SULT1 isoform 6 was examined. Data obtained were processed using the Excel program to generate the best fitting trendlines for the Lineweaver–Burk double-reciprocal plots. Table 2 shows the kinetic constants determined for the sulfation of estrone and 17β-estradiol, as well as that of *n*-propyl gallate. It appeared that the enzyme displayed lower $K_m$ for 17β-estradiol and higher $V_{max}$ for estrone. Catalytic efficiency of the enzyme, as reflected by $V_{max}/K_m$, appeared to be comparable with estrone or 17β-estradiol as substrate. With *n*-propyl gallate as substrate, the enzyme showed $V_{max}$ and $K_m$ values comparable to those determined for estrone. Collectively, these data indicate that the zebrafish SULT1 isoform 6, while showing some variations, exhibited rather comparable activities in catalyzing the sulfation of the two endogenous estrogens and *n*-propyl gallate.

Table 2
Kinetic constants of the zebrafish SULT1 isoform 6 with estrone, 17β-estradiol, and *n*-propyl gallate as substrates[a]

| Substrate | $V_{max}$ (nmol/min/mg) | $K_m$ (μM) | $V_{max}/K_m$ |
|---|---|---|---|
| Estrone | 93.0 ± 2.4 | 23.5 ± 1.1 | 3.96 |
| 17β-Estradiol | 19.8 ± 2.4 | 9.1 ± 0.8 | 2.18 |
| *n*-Propyl gallate | 109.8 ± 4.0 | 28.3 ± 1.1 | 3.88 |

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

### *Developmental stage-dependent expression of the zebrafish estrogen-sulfating cytosolic SULT1 isoform 6*

In view of its estrogen-sulfating activity, an important question is whether the expression of the newly identified SULT1 isoform 6 correlates with the development of estrogen-related endocrine systems of the zebrafish. To

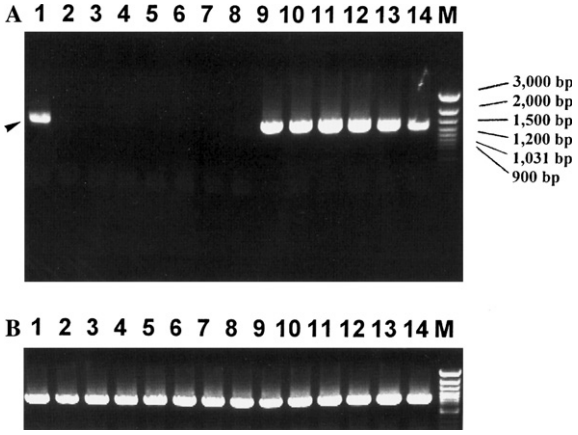


Fig. 5. (A) RT-PCR analysis of the developmental stage-dependent expression of the zebrafish SULT1 isoform 6. Final PCR mixtures were subjected to 2% agarose electrophoresis. Samples analyzed in lanes 1–14 correspond to unfertilized zebrafish eggs, 0-, 1-, 3-, 6-, 12-, 24-, 48-, and 72-h zebrafish embryos, 1-, 2-, 3-, and 4-week-old zebrafish larvae, and 3-month-old zebrafish. (B) RT-PCR analysis of the expression of the zebrafish β-actin at the same developmental stages as those described in (A).

gain insight into this issue, RT-PCR was employed to examine the expression of mRNA encoding the estrogen-sulfating SULT1 isoform 6 at different developmental stages. As shown in Fig. 5A, a significant level of expression was detected in unfertilized eggs indicating the presence of maternal transcript of the SULT1 isoform 6 gene. During the early phase of embryonic development, however, no message encoding the SULT1 isoform 6 could be detected. A significant level of expression of the zebrafish SULT1 isoform 6 was observed later at the beginning of the hatching period during embryogenesis, which continued throughout the larval stage onto maturity. Interestingly, previous studies have revealed that it is during the hatching period when primary organs including those of the endocrine system are formed [25]. In contrast to the developmental stage-dependent expression of the SULT1 isoform 6, β-actin, a housekeeping protein, was found to be expressed throughout the entire developmental process (Fig. 5B).

In conclusion, we have identified in the present study a novel estrogen-sulfating cytosolic SULT that may play an important role in the metabolism and homeostasis of endogenous estrogens in zebrafish. This study is part of an overall effort to obtain a complete repertoire of the cytosolic SULT enzymes present in zebrafish. As pointed out earlier, the identification of the various cytosolic SULTs and their biochemical characterization is a prerequisite for using the zebrafish as a model for a systematic investigation on fundamental issues regarding cytosolic SULTs. More work is warranted in order to achieve this goal.

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