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Identification of zebrafish steroid sulfatase and comparative analysis of the enzymatic properties with human steroid sulfatase

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Abstract

Steroid sulfatase (STS) plays an important role in the regulation of steroid hormones. Metabolism of steroid hormones in zebrafish has been investigated, but the action of steroid sulfatase remains unknown. In this study, a zebrafish *sts* was cloned, expressed, purified, and characterized in comparison with the orthologous human enzyme. Enzymatic assays demonstrated that similar to human STS, zebrafish Sts was most active in catalyzing the hydrolysis of estrone-sulfate and estradiol-sulfate, among five steroid sulfates tested as substrates. Kinetic analyses revealed that the K_m values of zebrafish Sts and human STS differed with respective substrates, but the catalytic efficiency as reflected by the V_{max}/K_m appeared comparable, except for DHEA-sulfate with which zebrafish Sts appeared less efficient. While zebrafish Sts was catalytically active at 28°C, the enzyme appeared more active at 37°C and with similar K_m values to those determined at 28°C. Assays performed in the presence of different divalent cations showed that the activities of both zebrafish and human STSs were stimulated by Ca^{2+}, Mg^{2+}, and Mn^{2+}, and inhibited by Zn^{+2} and Fe^{2+}. EMATE and STX64, two known mammalian steroid sulafatase inhibitors, were shown to be capable of inhibiting the activity of zebrafish Sts. Collectively, the results obtained indicated that zebrafish Sts exhibited enzymatic characteristics comparable to the human STS, suggesting that the physiological function of STS may be conserved between zebrafish and humans.

Graphical abstract

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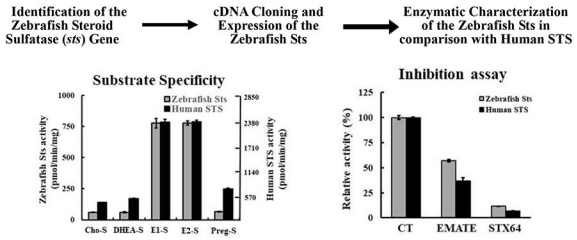

Keywords

Steroid sulfatase; steroid sulfates; zebrafish

1. Introduction

Sulfatases are enzymes responsible for the hydrolysis of sulfate esters of a variety of biomolecules, including steroid sulfates, sulfated glycosaminoglycans, and sulfolipids [1,2]. Sulfatases are known to constitute a gene family and to date, fifteen members have been identified in humans [3,4]. Of fifteen human sulfatases, arylsulfatase C has been shown to be responsible for the hydrolysis of sulfated steroids, and thus called the steroid sulfatase (STS) [5,6]. Together with estrogen- and hydroxysteroid-sulfotransferases (SULT1E1, SULT2A1, SULT2B1), STS plays an important role in the metabolism and homeostasis of steroids including cholesterol, androgen, and estrogen [7–9]. While defective STS has been shown to cause X-linked ichthyosis, a disorder characterized by the accumulation of cholesterol sulfate [10,11], excess activity of STS has been linked to hormone-dependent cancers including breast and prostate cancers [8,12]. STS has been shown to be upregulated in cancer cells, leading to high concentrations of estrogens and, consequently, accelerated cell growth. STS inhibitors have, therefore, been developed as a new class of anti-cancer drugs and a number of inhibitors have been reported [13].

STS has been shown to be ubiquitously expressed in mammalian tissues [14]. Interestingly, the expression was found to be much higher in placenta than in other tissues [14,15], implying that STS activity may play an important role in fetal development. STS has also been shown to be expressed in the liver, intestine, and brain [14,16]. Interestingly, the expression of STS in some fetal tissues, e.g., brain and intestine, was found to be stronger than that in corresponding adult tissues [14]. While the physiological relevance of STS in fetal development remains poorly understood, the importance of steroidogenesis during embryogenesis has been demonstrated using mouse and zebrafish models [17,18,19]. For instance, a *cypllal* gene knockdown experiment using zebrafish, suppressing the biosynthesis of pregnenolone from cholesterol, has demonstrated that steroids are essential for the normal epibolic embryo cell movement and gastrulation [20]. Moreover, a paralogous gene, *cyplla2*, has been shown to play an important role in the normal development of zebrafish larvae [21].

The zebrafish has become a valuable animal model for studying the physiological involvement of steroid hormones and associated diseases, especially in the early development [19,22]. Many mammalian steroidogenic enzymes, e.g., Cypllal and Cypl9al,

have been shown to be conserved in zebrafish and the steroidogenic pathway in zebrafish has been demonstrated to be similar to that in mammalian models [19,22]. In mammals, the action of steroid hormones is known to be regulated by reversible metabolic pathways, particularly the sulfation as mediated by cytosolic sulfotransferases (SULTs) SULT1E1, SULT2A1, and SULT2B1 and the desulfation as mediated by STS [9,23]. Zebrafish steroid-sulfating Sults have been well characterized, indicating that sulfation indeed plays an important role in the regulation of steroid hormones in zebrafish [24,25]. Zebrafish Sts, however, has not yet been investigated, leaving the question unanswered whether steroid hormones in zebrafish are regulated by reversible metabolic pathways.

In this communication, we report the identification, cloning, and expression of zebrafish Sts. Expressed zebrafish Sts was characterized in comparison with human STS with respect to substrate specificity, kinetic properties, inhibition by known steroid sulfatase inhibitors, as well as dependence of desulfating activity on temperature and divalent metal cations.

2. Materials and Methods

2.1. Materials.

Cholesterol, dehydroepiandrosterone (DHEA), estrone (E_1), 17β-estradiol (E2), pregnenolone, adenosine 5'-triphosphate (ATP), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), and 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid (Hepes) were from Sigma Chemical Company. Zebrafish liver cell (ZFL) line (ATCC CRL-2643) and COS-7 African green monkey kidney fibroblast-like cell line (ATCC CRL-1651) were obtained from American Type Culture Collection. Recombinant SULT1E1, SULT2A1, and SULT2Blb were prepared using the pGEX-2TK expression and purification system as previously described [26,27]. Trizol and pcDNA4 mammalian expression vector were products of Invitrogen. Takara *Ex Taq* DNA polymerase was purchased from Clontech Laboratories, Inc. First-strand cDNA synthesis kit was from GE Healthcare Life Sciences. *Taq* DNA polymerase was a product of Promega Corporation. *Eco* RI, and *Xho* I restriction endonucleases and NEB 5-alpha competent cells were from New England Biolabs. Oligonucleotide primers were synthesized by MWG Biotech. Carrier-free sodium [^{35}S]sulfate was from American Radiolabeled Chemicals. [^{35}S]PAPS was synthesized from ATP and carrier-free [^{35}S]sulfate using the recombinant human bifunctional PAPS synthase as previously described [28]. Cellulose thin-layer chromatography (TLC) plates were from Merck Millipore. Ecolume scintillation cocktail was from MP Biomedicals. Estrone-3-*O*-sulfamate (EMATE) was a product of Toronto Research Chemicals. STX64 was from Santa Cruz Biotechnology. All other reagents were of the highest grades commercially available.

2.2. Molecular cloning of zebrafish *sts* and human *STS*.

Zebrafish *sts* cDNA was reverse-transcribed from the total RNA, isolated from ZFL cells, by RT-PCR. ZFL cells were routinely maintained, under a 5% CO2 atmosphere at 28°C, in LDF culture medium (50% Leibovitz's L-15, 35% DMEM and 15% Ham's F-12, and 10-8 M sodium selenite) supplemented with 5% fetal bovine serum, 0.5% trout serum, 0.1 mg/ml bovine insulin, 50 μg/ml streptomycin sulfate, and 30 μg/ml penicillin G. Total RNA was

isolated from ZFL cells using Trizol according to the manufacturer's procedure and used as the template for the first-strand cDNA synthesis, with oligo (dT) as a primer. PCR was then carried out under the action of PrimeSTAR Max DNA Polymerase using the prepared first-strand cDNA as the template in conjunction with sense (5'-CCGCTCGAGCGCTGTACAATGAAATCCTTTCAATGGATTC-3') and antisense (5'-CCGCTCGAGCGCTATTCTTTTTGAAGCCCGTCCTGTACCTTC-3') oligonucleotide primers, designed based on the nucleotide sequence of zebrafish *sts* (GenBank Accession # XM_005168397), with *Xho* I restriction site incorporated at the end. Amplification conditions were 1 min at 98°C and 35 cycles of 98°C for 10 sec, 55°C for 10 sec, and 72°C for 1 min 30 sec. The final reaction mixture was applied onto a 0.8% 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 restricted using *Xho* I, subcloned into pcDNA4 mammalian expression vector, and transformed into NEB 5-alpha competent *E. coli* cells for amplification and purification. A zebrafish *sts* variant (designated *sts_tv2*) cDNA (GenBank Accession no. BC049491) packaged in pME18S-FL3 and a human *STS* cDNA (BC075030) packaged in pCR4-TOPO (Clone ID: 5914856 and 30915417, respectively) were purchased from GE Dharmacon. The zebrafish *sts_tv2* cDNA and human *STS* cDNA were digested by *Xho I* and *Eco* RI, respectively, and subcloned into the corresponding restriction sites of pcDNA4A vector. Zebrafish *sts* and human *STS* cDNAs thus cloned were verified for authenticity by nucleotide sequencing.

2.3. Preparation of recombinant zebrafish and human steroid sulfatases.

Recombinant zebrafish Sts_tv1, Sts_tv2, and human STS were transiently expressed in COS-7 cells, routinely maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C and 5% CO_2. COS-7 cells, grown to 80% confluence in individual wells of a 6-well plate, were transfected with pcDNA4A, pcDNA4A-zf*sts_tv1*, pcDNA4A-zf*sts_tv2*, or pcDNA4A-hum*STS*, using Lipofectamine 2000, according to the procedure recommended by the manufacturer. After a 48-hour incubation at 37°C, the cells were washed with PBS and lysed in 200 μl of a lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail. Cell lysates thus prepared were subjected to centrifugation at 1,000 × g for 20 min at 4°C, and the supernatants collected were stored at −80°C until used for the enzyme assays.

2.4. Preparation of [^{35}S]-labeled steroid-sulfates.

[^{35}S]cholesterol-sulfate, [^{35}S]DHEA-sulfate, [^{35}S]El-sulfate, [^{35}S]E2-sulfate, and [^{35}S]pregnenolone-sulfate were prepared via SULT-mediated sulfation with [^{35}S]PAPS as the sulfonate group donor. Sulfation reactions were carried out under the following conditions. Each reaction mixture contained 50 mM Hepes buffer at pH 7.5, 14 μM [^{35}S]PAPS (15 Ci/mmol), 1 mM DTT, 50 μM substrate, and SULT enzyme (SULT1E1 for E_1 and E_2, SULT2A1 for DHEA and pregnenolone, SULT2Blb for cholesterol). The reactions were performed for 2 hours at 37°C and the final reaction mixtures were subjected to a two-step solid-phase extraction (SPE). Each reaction mixture was first loaded onto a Waters Sep-Pak C18 cartridge, washed with 20% methanol, and eluted into 80% methanol

solution. The eluate was dried using a SpeedVac concentrator, reconstituted in 20% methanol/2% formic acid solution, and loaded onto an Oasis WAX column. After sequential wash with 2% formic acid solution followed by 100% methanol, [^{35}S]steroid-sulfate was eluted into 1% NH_4OH/methanol. The eluate was dried using a SpeedVac concentrator, and reconstituted in DMSO. One-μl of the reconstituted [^{35}S]steroid-sulfate solution was spotted on a cellulose TLC plate and subjected to TLC with *n*-butanol/isopropanol/formic acid/H_2O (3:1:1:1; by volume) as the solvent system. After TLC, the radioactive spot corresponding to the [^{35}S]steroid-sulfate, as revealed by autoradiography, was cut out, eluted with H_2O, and counted for [^{35}S]radioactivity for the calculation of the concentration and specific radioactivity of the purified [^{35}S]steroid-sulfate.

2.5. Enzymatic assay.

The sulfatase activity of recombinant STS enzymes expressed in COS-7 cells were assayed using [^{35}S]labeled substrate compounds, prepared as described above, under the following conditions. The standard assay mixture, in a final volume of 20 μl, contained 50 mM Hepes buffer at pH 7.0, 1 mM DTT, 10 mM Ca^{2+}, and 5 μM substrate. The reaction was started by the addition of 1.0 μg enzyme, allowed to proceed for 15 min at 28°C or 37°C, and terminated by heating at 100°C for 3 min. TLC analysis of the final reaction mixture was carried out based on the above-mentioned procedure and the radioactive spot corresponding to the freed [^{35}S]sulfate ion was cut out, eluted into 20 mM Tris-HCl, pH 8.0, and counted for [^{35}S]radioactivity. In the kinetic experiments, the assays were performed using varying concentrations of each of the substrates plus 10 mM Ca^{2+}. Kinetic parameters were calculated based on the Michaelis-Menten equation using GraphPad Prism5 software with non-linear regression. To analyze the effects of divalent metal cations, enzymatic assays were carried out using 1 μM [^{35}S]E_1-sulfate and 10 mM of the tested divalent metal cation (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, or Fe^{2+}) at 28°C (for zebrafish Sts) or 37°C (for human STS).

2.6. Sulfatase inhibition assay.

The inhibitory effect of EMATE and STX64, two known irreversible steroid sulfatase inhibitors, on the sulfatase activity of zebrafish Sts was examined using 5 μM E_1-sulfate as the substrate in the absence or presence of 0.1 μM tested inhibitor under above-mentioned reaction conditions (at 28°C for zebrafish Sts and 37°C for human STS). After a 15-min reaction, the reaction mixture was analyzed by HPLC using an Agilent 1220 Infinity LC System (Agilent Technologies). Desulfated E_1 was separated on a Capcell Core ADME column (2.7 μm, 4.6 mm ID × 100 mm; Shiseido) at 40°C using 0.05% TFA in 60% methanol under isocratic elution at a flow rate of 1 mL/min and detected at 201 nm.

3. Results

3.1. Molecular cloning of zebrafish *sts*.

By searching the GenBank database, two zebrafish cDNA sequences related to the zebrafish *sts* gene (Gene ID: 402795) were identified. One (GenBank Accession # XM_005168397; designated zf*sts_tv1*) appears to be a full-length cDNA. The other (GenBank Accession # BC049491; designated zf*sts_tv2*) encompasses the same coding sequence up to the codon for Asn474 (located in the middle portion of exon 10 of the zebrafish *sts* gene) and thereafter

carries an additional 267 nucleotides coding for 89 amino acid residues that shows little sequence homology to the zebrafish St_stv1 (Figure 1). A blast search revealed that the 3'-region of the latter cDNA appeared to have been derived from chromosome 6 (NC_007117.7), in contrast to the prior sequence, which is coded by the *sts* gene present in chromosome 1 (NC_007112.7) (data not shown). These results indicated that the zf*sts_tv2* clone, obtained as an IMAGE clone, might have been erroneously produced by chromosomal crossover between chromosome 1 and 6. Therefore, the full-length clone (*ste_tv1*;XM_005168397) was judged to be an authentic zf*sts* clone and the other one (BC049491; zf*sts_tv2*) was judged to be an aberrant cDNA. To obtain these two zebrafish *sts* cDNAs for further studies, the zf*sts_tv1* cDNA was RT-PCR-cloned using the total RNA isolated from ZFL cells as the template. The aberrant cDNA (zf*sts_tv2*) on the other hand, was obtained commercially (clone ID: 5914856), since it could not be amplified using ZFL total RNA. The nucleotide sequence determined for the zf*sts_tv1* cDNA contained seven mismatched nucleotides (A262G, G663C, C987T, A1035G, T1164G, C1309T, C1698T), compared with the sequence of the GenBank XM_005168397 clone. Of these seven variations, three, A262G, T1164G, and C1309T, were nonsynonymous, leading to amino acid changes I88V, N388K, and L437F in the coded zebrafish Sts enzyme. The zf*sts_tv1* cDNA cloned from the ZFL cells thus appears to be an allelic variant form in contrast the one deposited in the GenBank database. Figure 1 shows the amino acid sequence alignment of zfSts_tv1, zfSts_tv2, and human STS. zfSts_tv1 displays homology with human STS throughout the entire sequence with an amino acid identity of 59.6 %. zfSts_tv2, on the other hand, shares nearly identical amino acid sequence with zfSts_tv1 up to Asn474, and thereafter shows little amino acid homology to either zfSts_tv1 or human STS. Previous studies had revealed a number of important structural elements of STS, including the sulfatase signature sequence (C/S-X-P-X-R), active site catalytic residues, substrate recognition residues, and transmembrane domains [2,7,29]. As shown in Figure 1, both zfSts_tv1 and zfSts_tv2 contain the sulfatase signature sequence, C-X-P-X-R, and the conserved catalytic residues such as Lysl33 and His286 (corresponding to Lysl34 and His290 in human STS). Like human STS, both zfSts_tv1 and zfSts_tv2 carry two transmembrane domains (spanning, respectively, Ilel84 to Met204 and Arg209 to Val230) as predicted by the SOSUI analysis (data not shown). These results suggested that both zfSts_tv1 and zfSts_tv2 may possibly be functional STS enzymes.

3.2. Comparison of the sulfatase activities of zebrafish Sts and human STS.

Enzymatic activities of zfSts_tv1 and zfSts_tv2, expressed in COS-7 cells, were analyzed in comparison with human STS using representative STS substrates, including cholesterol-sulfate, DHEA-sulfate, E_1-sulfate, E_2-sulfate, and pregnenolone sulfate. Assays were performed at two temperatures, 28°C (a growth temperature for zebrafish) and 37°C (the normal body temperature for humans). As shown in Table 1, activity data obtained indicated that zfSts_tv1 displayed a similar activity profile to human STS, whereas zfSts_tv2 showed no activity toward any of the tested substrates. Interestingly, zfSts_tv1 exhibited stronger activities at 37°C than at 28°C toward all steroid-sulfates tested as substrates. Compared with human STS, zfSts_tv1 appeared to display higher substrate specificity toward E_1-sulfate and E_2-sulfate. Moreover, the activities of zfSts_tv1 toward cholesterol-sulfate, DHEA-sulfate, or pregnenolone-sulfate were much lower than those of human STS.

3.3 Kinetic properties of the zebrafish Sts and human STS.

To further characterize zfSts, the kinetic parameters with each of the five substrates were analyzed at 28°C and 37°C. The concentration dependence analysis showed typical hyperbolic curves for all five substrates tested, indicating that zfSts_tv1 catalyzes the hydrolysis of these substrates in accord with the Michaelis-Menten kinetics (Figure 2). This mechanism was also confirmed by linear Eadie-Hofstee plots (data not shown). Like zfSts_tv1, human STS also exhibited the Michaelis-Menten kinetics with all substrates tested (data not shown). K_m values of zfSts_tv1 at 28°C were comparable with those at 37°C for all substrates tested, while Vmax values at 28°C were considerably lower than those determined at 37°C (Table 2). At both reaction temperatures, zfSts_tv1 exhibited the lowest K_m value with pregnenolone-sulfate and higher catalytic efficiency (V_{max}/K_m) toward E_2-sulfate and pregnenolone-sulfate. Compared with zfSts_tv1, the V_{max} values determined for human STS were significantly higher for all substrates (Table 3). The K_m values, however, varied with the substrates (Table 2 and 3). The K_m values with E_1-sulfate and E_2-sulfate were comparable between zfSts_tv1 and human STS. Interestingly, with cholesterol-sulfate and pregnenolone-sulfate, zfSts_tv1 exhibited 10 times lower K_m values than human STS. Conversely, the K_m values of zfSts_tv1 with DHEA-sulfate were more than five times higher than that of human STS. These results indicated that zfSts displayed catalytic properties more comparable with E_1-sulfate and E_2-sulfate to human STS than with other three substrates.

3.4. Effect of divalent cations on the sulfatase activities of zebrafish Sts and human STS.

To investigate the effect of divalent cations on the catalytic activity of zfSts, enzymatic assay was carried out using E_1-sulfate as substrate in the presence of different divalent cations. Of the five divalent cations tested, Ca^{2+}, Mg^{2+}, and Mn^{2+} were found to exert stimulatory effects on the activity of zfSts_tv1, whereas Fe^{2+} appeared to be inhibitory for zfSts_tv1. Significantly, Zn^{2+} was found to abolish completely its activity (Figure 3). Interestingly, these five cations showed a similar pattern of stimulatory/inhibitory effects on the activity of human STS. These observations indicated that divalent cations may play an important role in influencing the catalytic action of zfSts as well as human STS.

3.5. Inhibitory effect of steroid sulfatase inhibitors on the sulfatase activity of zebrafish Sts.

Sulfatase inhibition assays using EMATE and STX64, two known irreversible steroid sulfatase inhibitors [13], were performed in order to explore the inhibitory effect of the steroid sulfatase inhibitors on the sulfatase activity of zebrafish Sts in comparison with that of human STS. As shown in Figure 4, STX64, at a concentration of 0.1 μM, exerted 88.3% and 93% inhibition on the sulfatase activity of, respectively, zebrafish Sts_tv1 and human STS. EMATE also inhibited the activity zebrafish Sts_tv1 and human STS, albeit to a lesser degree than did STX64. Both EMATE and STX64, therefore, were capable of inhibiting the sulfatase activity of not only human STS, but also zebrafish Sts,

4. Discussion

STS has been detected in organisms ranging from marine invertebrates to mammals [30,31]. Although mammalian STSs have been cloned, expressed, and characterized [5,6], the homologous enzymes from non-mammalian vertebrates have not been studied. By searching the NCBI database, the zebrafish *sts* gene (Gene ID: 402795) and two transcripts (GenBank Accession # XM_005168397 and # BC049491) were identified. In this study, a zfSts cDNA corresponding to the GenBank Accession # XM_005168397 sequence (*sts_tv1*) was cloned from the ZFL cells, and was shown to contain the complete ORF of the zebrafish *sts* gene (Figure 1). Another cDNA (designated zf*sts_tv2*) corresponding to the GenBank Accession # BC049491 sequence was obtained commercially and was shown to contain a highly homologous 5'-STS coding region and a short unrelated 3'-region, which appears to originate from a different gene (NC_007117.7) present on chromosome 6.

Previous studies have shown that in human STS, two polypeptide segments, Thr484 through Phe488 and Trp550 through Trp553, form, respectively, a region capable of recognizing the steroid backbone and a hydrophobic tunnel leading the substrate to the active site of the enzyme [29]. These regions, corresponding to Thr480 through Phe484 and Pro546 through Trp549 in zfSts_tv1, appear not conserved in zfSts_tv2 (Figure 1). These differences may underscore the lack of STS activity of zfSts_tv2. Regarding the authentic zfSts (Sts_tv1), the activity data obtained were comparable to those determined for human STS, implying that the physiological function of STS may be conserved in humans and zebrafish in terms of their enzymatic characteristics. It is noted, however, although the authentic zfSts showed higher steroid sulfatase activity and catalytic ability toward E_1-sulfate and E_2-sulfate than human STS (Tables 1–3), the catalytic properties of zfSts with other substrates, including cholesterol-sulfate, DHEA-sulfate, and pregnelone-sulfate, appeared to be slightly different. It is possible that how steroid side-chains are recognized may differ between human and zebrafish STSs. Based on the crystal structure of human STS, six amino acid residues seem to be involved in the recognition of the side-chain of steroid substrates [29]. Three of these six residues appeared not conserved; Phe490 vs. Ile486, Tyr493 vs. Phe489, and Trp550 vs. Phe546, in zfSts (Figure 1). These variations may be the reason for the differential substrate specificity found between zebrafish and human STSs. Human STS has been known to require divalent cations, especially Ca^{2+}, for its catalytic action [7]. Interestingly, enzymatic assays performed in the current study showed similar effects of divalent cations on the sulfatase activity of zebrafish Sts and human STS toward E_1-sulfate (Figure 3), suggesting that the divalent cations may play a similar role in the catalytic actions of both zebrafish Sts and human STS. Indeed, all residues (Asp34, Asp35, Asp338, and Gln339) previously shown to be involved in the Ca^{2+} recognition by human STS, are conserved in zebrafish Sts.

Human placental STS has been used as a prototype enzyme for examining the biochemical characteristics of STS [32–34]. Kinetic studies performed using purified placental STS have demonstrated a range of K_m values: 6.7 μM with cholesterol-sulfate, 3.0 μM with DHEA-sulfate, and 50.6 μM with E_1-sulfate with [32–34]. For STS isolated from fibroblasts, a smaller K_m value, 5 μM, with E_1-sulfate has been reported [35]. Moreover, gonadal STS steroid has been shown to exhibit the K_m values of 3.85 μM and 0.73 μM, respectively, with DHEA-sulfate and pregnenolone-sulfate [36]. The K_m values with representative steroid

sulfatase substrates determined in our study using recombinant enzymes therefore appeared relatively consistent with those determined using tissues-derived steroid sulfatase enzymes.

A major difference between zebrafish Sts and human STS, in terms of the environmental influence on the enzymatic activity, is the temperature at which these two enzymes function *in vivo*. In contrast to humans whose normal body temperature is maintained at 37°C, zebrafish are tropical fish and their regular habitant water temperatures may fluctuate between 25°C and 38°C [37]. Enzymatic assays demonstrated that the enzymatic activity of zfSts at 37°C was approximately 1.5-2 times higher than that determined at 28°C, a temperature usually used for laboratory maintenance of zebrafish (cf. Table 1 and 2). Interestingly, assays performed at both temperatures appeared to have little effects on the affinity toward the substrates tested. It is thus possible that the STS of zebrafish living at higher temperature may be more active than those at lower temperature.

A number of STS inhibitors have been developed for use in cancer therapy, especially in the treatment of breast cancer [13]. EMATE has been reported to be a potent irreversible STS inhibitor, and most of the inhibitors subsequently developed were designed based on EMATE [13,38]. It is noted that although EMATE is fully capable of inhibiting the steroid sulfatase, it has been reported to exert estrogenic effects [39]. An arylsulfamate compound, STX64, is now considered a more promising STS inhibitor due to its weaker estrogenic activity and highly potent STS inhibitory effect [40,41]. STX64 has been subjected to the Phase I/II clinical trials for treating the postmenopausal women with breast cancer [42,43]. Although the monotherapy clinical trial was discontinued, the combination trial has demonstrated that the combination of STX64 and an aromatase inhibitor, Al, has the clinical benefit for treating the breast cancer without severe side effects [44]. Whether the STS inhibitors are capable of inhibiting the zebrafish Sts activity therefore is an important issue in order to develop the zebrafish as an animal model for the *in vivo* screening and toxicity test of STS inhibitors, in addition to the study on the physiological function of STS. Our results clearly indicated that EMATE and STX64 effectively inhibited the activity of zebrafish Sts to the similar degree as with human STS. These results suggested that the zebrafish can serve as a useful model animal for the development of steroid sulfatase inhibitors.

Another important consideration in regard to the establishment of the zebrafish model is related to the transporters for sulfated steroids. Homeostasis of sulfated steroids is regulated by not only SULTs and STS but also transporters. Since sulfated steroid due to the negatively charged sulfate group are too highly hydrophilic to pass though the plasma membrane, transporters for sulfated steroid play an important role in their uptake into and efflux from cells [45,46]. Uptake of sulfated steroids is mediated by solute carrier transporters (SLCs), particularly the sodium-dependent organic anion transporters (SOAT; SLC10A6) and organic anion transporting polypeptides (OATPs). Efflux of sulfated steroids, on the other hand, is mediated by the ATP binding cassette transporters (ABCs), particularly the multi drug resistance-related proteins (MRPs). A number of *oatps* and *mrps* have been detected in zebrafish, while *soat* has not been detected even in the zebrafish genome database [47–49]. Although zebrafish (sulfated) steroid transporters have not been well studied, a previous study has demonstrated that zebrafish Oatp1d1 is capable of transporting

E_1-sulfate and other conjugated steroids [50]. It thus appears that steroid circulation system may be conserved in the zebrafish.

5. Conclusion

In this study, an active zebrafish steroid sulfatase (Sts) was identified. The enzymatic characteristics of zfSts appeared comparable to the orthologous human STS. These results suggested that the main physiological function of STS, i.e., the regulation of the homeostasis of steroid hormones, may be conserved between zebrafish and humans. In view of the rapid development and ease of maintenance, the zebrafish may serve as an excellent model for investigating the physiological relevance of STS in embryogenesis, organ development, as well as early body development.

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Abbreviations:

STS	steroid sulfatase
SULTs	cytosolic sulfotransferases
PAPS	3’- phosphoadenosine-5’- phosphosulfate

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Highlights

- Zebrafish steroid sulfatase (zfSts) was identified in this study.
- zfSts was capable of hydrolyzing steroid sulfate including estrone-sulfate.
- Catalytic properties of zfSts were comparable to those of human STS.

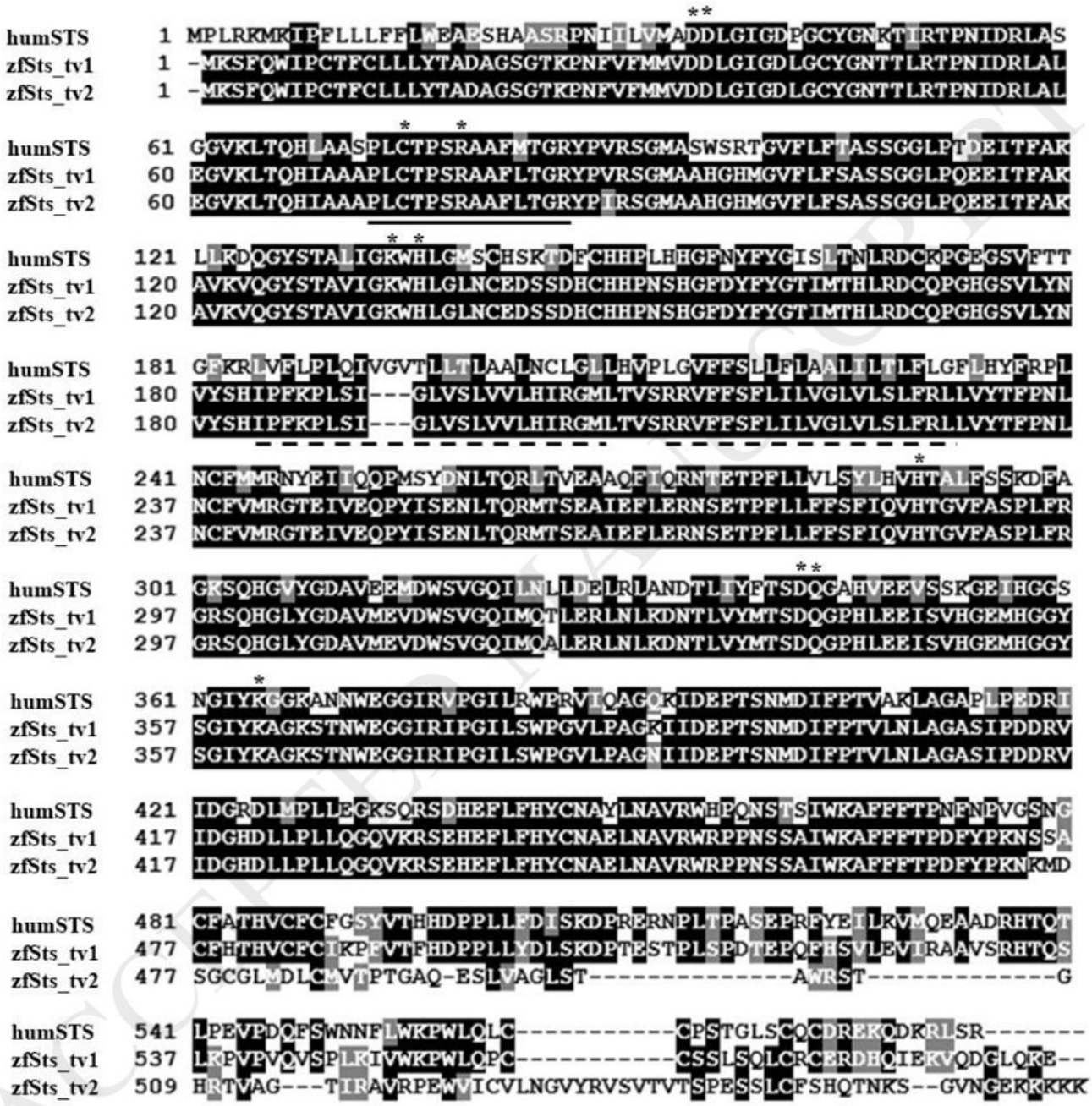

Figure 1.

Amino acid sequence alignment analysis of the zebrafish Sts and human STS. The deduced amino acid sequences of Sts/STS were referred to the following NCBI Reference Sequence number; hum STS (**BC075030**), zfSts_tv1 (GenBank ID **XM_00516839**), zfSts_tv2 (GenBank ID **BC049491**). The alignment was performed using Clustal Omega program in EMBL-EBI. The underlines represent the sulfatase signature sequence-containing core sequence, C/S-x-P-x-R [2,7]. The asterisks and dashed lines indicate conserved catalytic residues and trans-membrane domains, respectively [29].

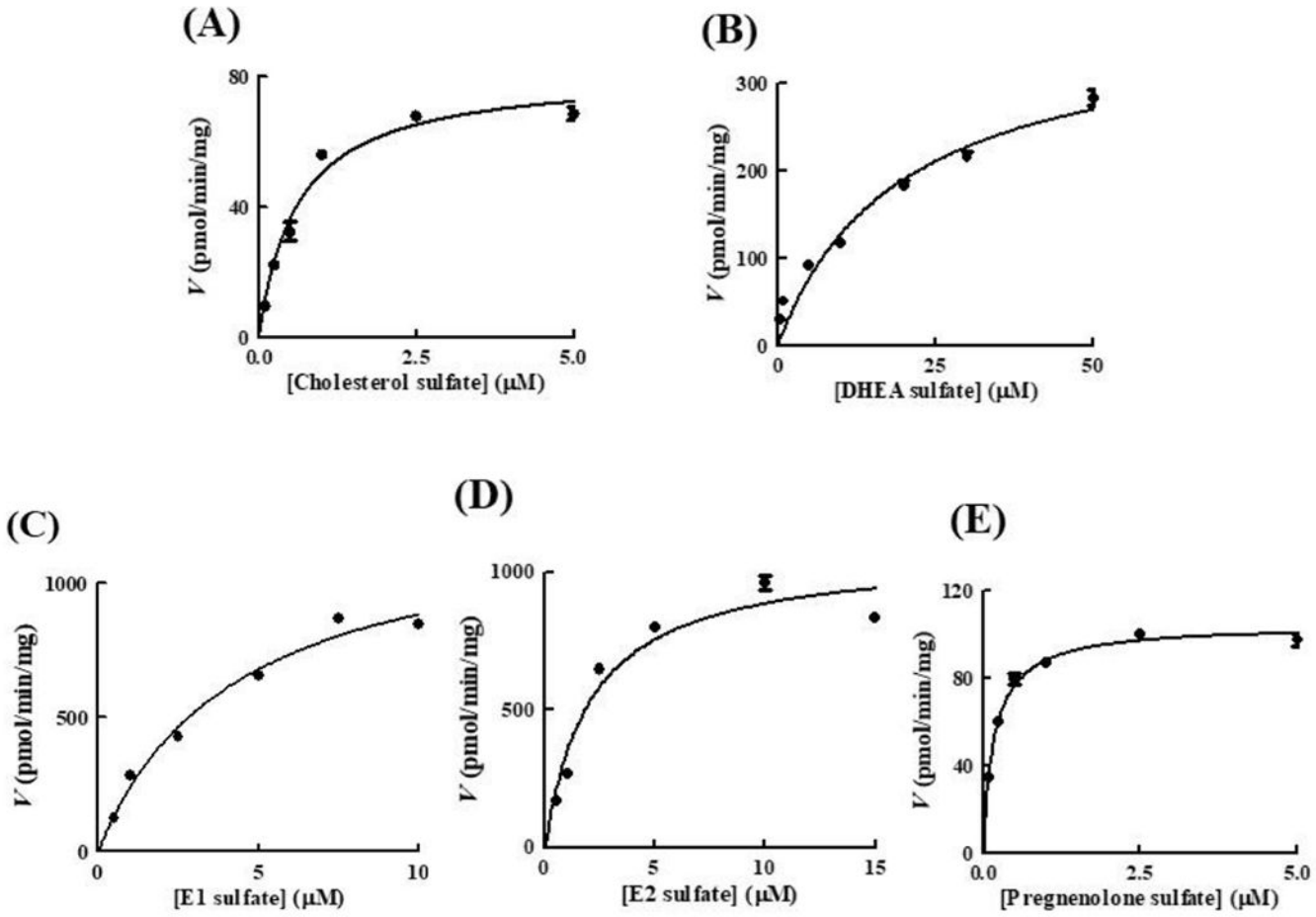

Figure 2.

Kinetic analyses of zfSts. Figure panels show the kinetic assays with cholesterol-sulfate (**A**), DHEA-sulfate (**B**), E1-sulfate (**C**), E2-sulfate (**D**), and Pregnenolone-sulfate (**E**). The fitting curves were generated using non-linear regression of Michaelis-Menten kinetics. Data shown represent calculated mean ± SD derived from three experiments.

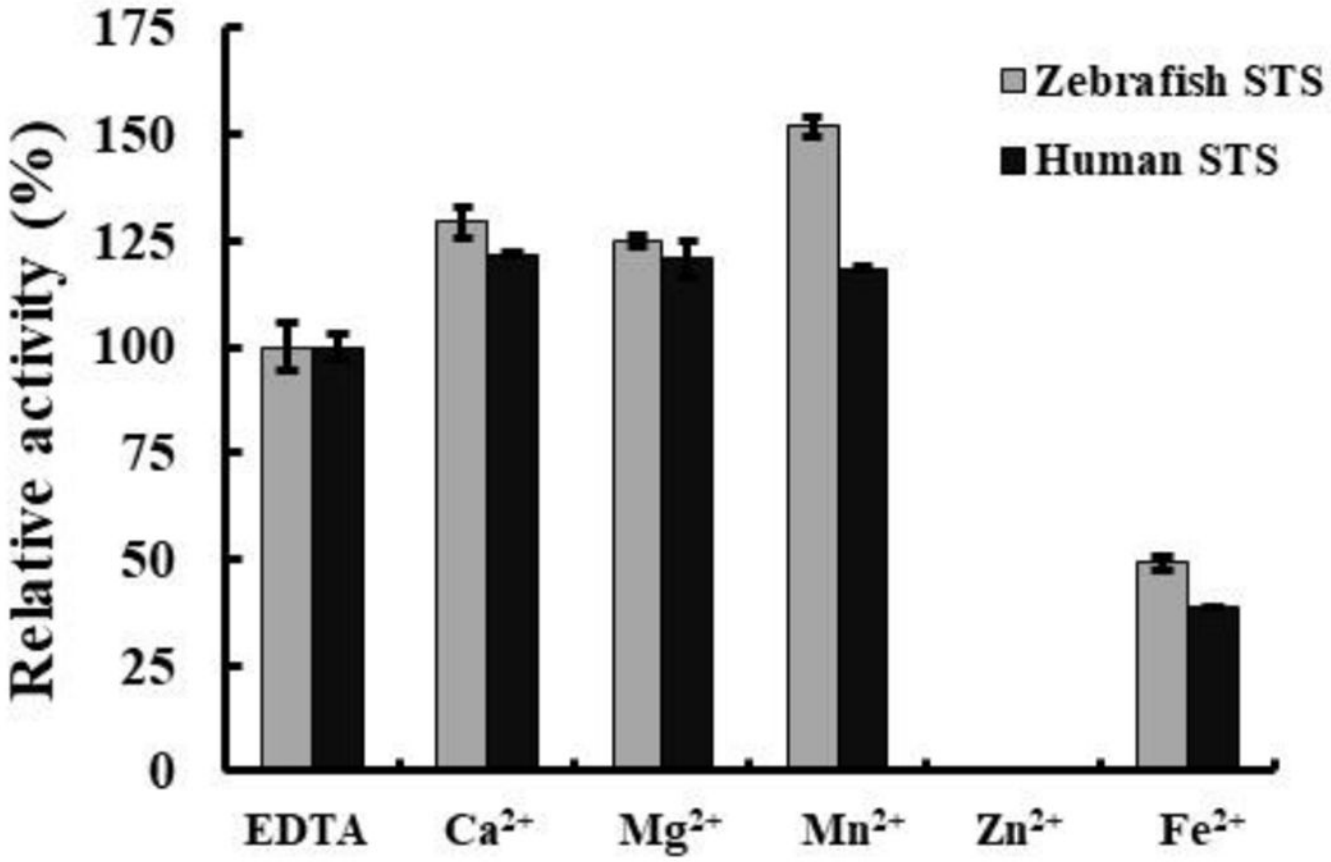

Figure 3.

Effect of divalent cation on the sulfatase activities of zebrafish Sts and human STS. Sulfatase assays were performed in the presence of the different divalent cations, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}. The relative activity (%) was expressed against the activity in the presence of EDTA. Data shown represent calculated mean ± SD derived from three experiments.

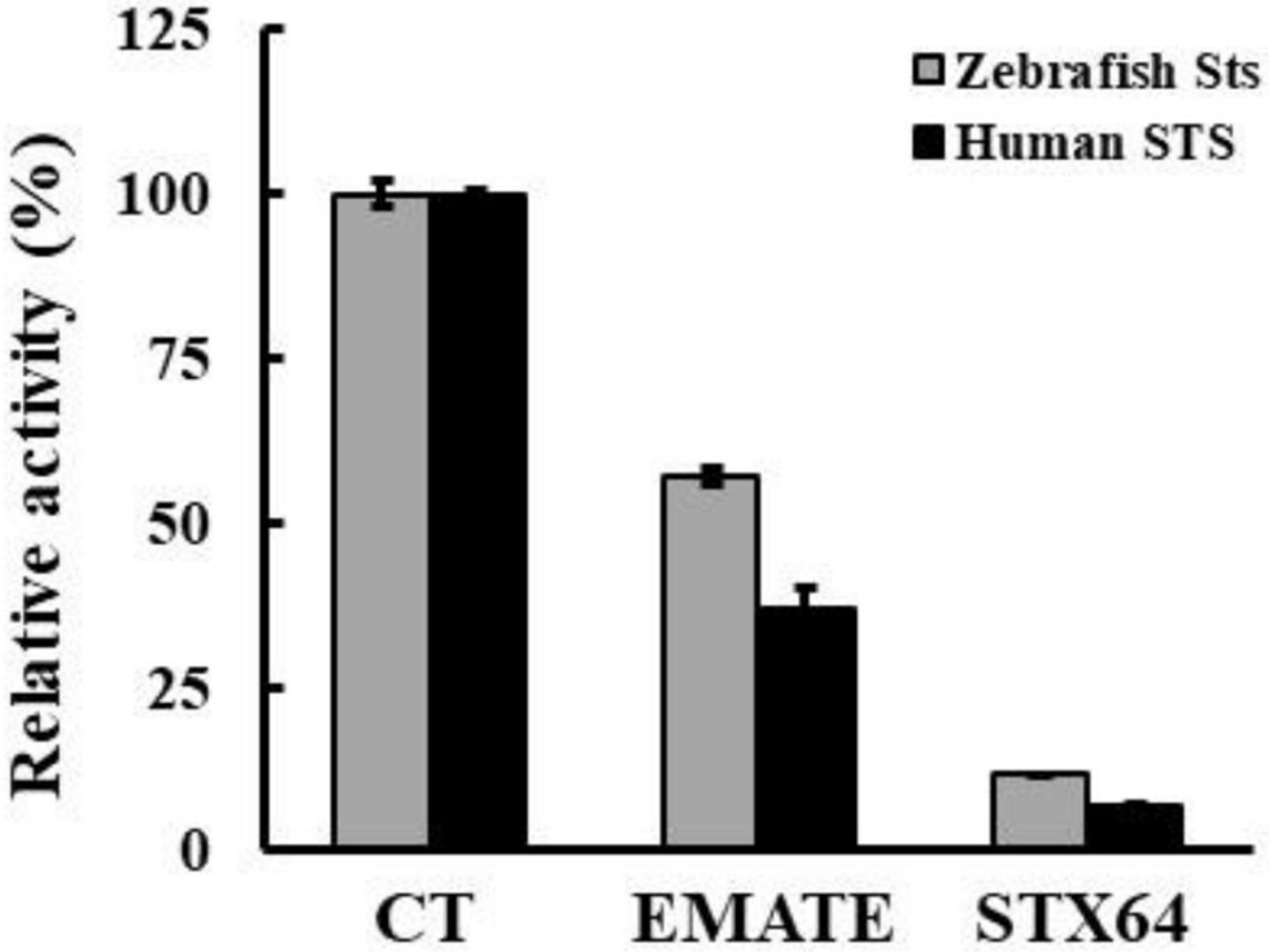

Figure 4.

Inhibitory effect of EMATE and STX64 on the sulfatase activities of zebrafish Sts and human STS. Sulfatase assays were performed in the absence or presence of 0.1 μM EMATE or STX64. The relative sulfatase activity (%) is expressed against the sulfatase activity detected in the absence of inhibitors. Data shown represent calculated mean ± SD derived from three independent experiments.

Table 1.

Specific activities of the zebrafish Sts and human STS[a]

	Specific activity (pmol/min/mg)[a]					
	28°C[b]			37°C[b]		
Substrate	**Mock**	**zfSts**	**humSTS**	**Mock**	**zfSts**	**humSTS**
Cho-sulfate	N.D.[c]	59.6 ± 2.1	234.5 ± 6.3	N.D.	107.3 ± 4.1	416.8 ± 4.5
DHEA-sulfate	N.D.	59.2 ± 5.0	293.4 ± 17.3	N.D.	101.5 ± 10.1	511.2 ± 9.6
E1-sulfate	25.4 ± 18.7	775.6 ± 38.2	1895.4 ± 72.1	52.9 ± 11.7	1165.0 ± 38.1	2352.4 ± 68.7
E2-sulfate	11.5 ± 1.2	777.3 ± 15.7	1630.4 ± 58.8	19.6 ± 2.8	1155.0 ± 32.6	2358.4 ± 44.5
Preg-sulfate	4.5 ± 1.9	65.3 ± 3.5	426.6 ± 4.5	7.5 ± 1.5	124.1 ± 9.7	741.7 ± 25.4

[a] Specific activity corresponds to pmol desulfated product formed/min/mg protein. Results shown represent mean ± standard deviation derived from three separate assays.

[b] Reaction was carried out under the incubation temperature 28°C or 37°C.

[c] ND refers to activity not detected. The detection limit of this assay was estimated to be 1.0 pmol desulfated product formed /min/mg protein.

Table 2.

Kinetic parameters of zfSts determined at different reaction temperatures[a]

	at 28°C			at 37°C		
	V_{max} (pmol/min/mg)	K_m (μM)	V_{max}/K_m	V_{max} (pmol/min/mg)	K_m (μM)	V_{max}/K_m
Cho-sulfate	81.25 ± 2.4	0.62 ± 0.06	131.7	141.8 ± 6.7	0.96 ± 0.13	148.0
DHEA-sulfate	374.3 ± 25.4	19.38 ± 3.07	19.3	453.8 ± 22.6	15.72 ± 1.98	28.9
E1-sulfate	1262.0 ± 60.8	4.32 ± 0.49	292.3	1766.0 ± 35.2	3.39 ± 0.18	521.4
E2-sulfate	1070.0 ± 44.7	2.07 ± 0.29	516.9	1647.0 ± 73.8	1.93 ± 0.30	855.6
Preg-sulfate	104.1 ± 1.2	0.18 ± 0.01	571.7	185.4 ± 3.2	0.25 ± 0.01	741.6

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

[b]Kinetic parameters were determined based on the equation for Michaelis-Menten kinetics.

Table 3.

Kinetic parameters of the human STS[a]

	V_{max} (pmol/min/mg)	K_m (μM)	V_{max}/K_m
Cho-sulfate	998.7 ± 44.8	5.73 ± 0.84	174.4
DHEA-sulfate	883.1 ± 14.1	2.85 ± 0.23	310.0
E1-sulfate	5538.0 ± 193.8	5.05 ± 0.39	1095.8
E2-sulfate	5715.0 ± 280.6	4.31 ± 0.47	1326.0
Preg-sulfate	1188.0 ± 14.8	1.75 ± 0.04	680.4

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

[b]Kinetic parameters were determined based on the equation for Michaelis-Menten kinetics.