ends (3′-RACE) was performed using the Takara 3′-Full RACE core kit. First-strand cDNA was synthesized using AMV reverse transcriptase with zebrafish total RNA as the template in conjunction with an oligo dT-3 sites adaptor primer. Afterwards, a polymerase chain reaction (PCR) was carried out using an oligonucleotide (5′-CGTAGTGGCACCACGCTAATGC-3′) designed based on the sequence determined for the above-mentioned zebrafish TPST cDNA and a three sites adaptor primer as the primer pair with the first-strand cDNA as the template. Amplification conditions were 25 cycles of 30 s at 94 °C, 30 s at 59 °C, and 1 min at 72 °C. The reaction mixture was analyzed by agarose electrophoresis. A discrete PCR product detected was isolated and subcloned into pSTBlue-1 cloning vector and subjected to nucleotide sequencing (Sanger et al. 1977). The nucleotide sequences, as well as the deduced amino acid sequences, of the cDNA were analyzed using BLAST search for sequence homology to known TPSTs.

To amplify the full-length zebrafish TPST cDNA for subcloning into the pcDNA3.1 eukaryotic expression vector, a sense primer (5′-CCCTGACTTTTGCCCCCACCTGC-3′) corresponding to a region 65 residues upstream from the initiation codon of the open reading frame (see Fig. 1) and an antisense primer (5′-GGTAGTTGTGACTCCG-3′) corresponding to a region 116 residues downstream from the stop codon were synthesized. With these two oligonucleotides as primers, PCR in a 50-μL reaction mixture was carried out under the action of *Ex Taq* DNA polymerase using zebrafish first-strand cDNA as the template. Amplification conditions were 25 cycles of 40 s at 94 °C, 45 s at 47.5 °C, and 1 min and 20 s at 72 °C. The final reaction mixture was applied onto a 1.2% agarose gel and separated by electrophoresis. The discrete PCR product band, visualized upon ethidium bromide staining, was excised from the gel and the DNA fragment therein was purified by spin filtration. The purified zebrafish TPST cDNA, containing 3′ A-overhangs, was subcloned into the 3′ T-overhangs-containing pcDNA3.1 eukaryotic expression vector at a cloning site located between *Bst*X I and *Eco*RV restriction sites. To verify its authenticity and direction of cloning, the cDNA insert was subjected to nucleotide sequencing (Sanger et al. 1977).

Transient expression of the zebrafish TPST in COS-7 cells

COS-7 cells, routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, were used as the host cells for the expression of recombinant zebrafish TPST protein. Dishes (100 mm) of COS-7 cells were individually transfected with 10 μg of pcDNA3.1 vector only or pcDNA3.1 harboring the zebrafish TPST cDNA using the Lipofectamine 2000 mediated procedure. Transfection was for 18 h at 37 °C. Afterwards, the transfected cells were incubated at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. At the end of a 48-h incubation, the cells were rinsed twice with phosphate-buffered saline and lysed in an ice-cold lysis buffer containing 20 mM Hepes–NaOH (pH 7.0), 1% Triton X-100, and 10 μg aprotinin/mL. The lysate was subjected to centrifugation and the supernatant was used in the TPST assays described below. Lysates of untransfected COS-7 cells or COS-7 cells transfected with pcDNA 3.1 alone were prepared as controls.

Preparation of wild-type and mutated PSGL-1 N-terminal peptides as substrates for the zebrafish TPST

For the preparation of cDNA encoding the N-terminal region (ATEYEYLDYDFL) of PSGL-1, two complementary 42-mer oligonucleotides (sense strand 5′-GATCCGCCACCGAATATGAGTACCTAGATTATGATTTCCTGG-3′ and antisense strand 5′-AATTCCAGGAAATCATAATCTAGGTACTCATATTCGGTGGCG-3′) were synthesized with, respectively, *Bam*HI and *Eco*RI restriction sites incorporated at the ends. The annealed oligonucleotides were subcloned into the *Bam*HI/*Eco*RI site of pGEX-4T-1 and transformed into *E. coli* XL1-Blue MRF′. For the preparation of cDNA encoding mutated PSGL-1 N-terminal peptides, sense and antisense mutagenic primers with the TAT or TAC codon changed to TTT or TTC were synthesized and similarly processed and subcloned into pGEX-4T-1 and transformed into *E. coli* XL1-Blue MRF′. Figure 2 shows the amino acid sequences of the wild-type and mutated PSGL-1 N-terminal peptides. Competent *E. coli* BL21 cells transformed with pGEX-4T-1 harboring cDNA encoding the wild-type or mutated PSGL-1 N-terminal sequence were grown to A_{600nm} = 0.5 in 100 mL of LB medium supplemented with 100 μg ampicillin/mL. Upon induction with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 25 °C, the cells were collected by centrifugation at 1000*g* for 10 min at 4 °C and homogenized in 15 mL of an ice-cold lysis buffer (containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA) using an Aminco French press. The crude homogenate thus prepared was subjected to centrifugation at 10 000*g* for 20 min at 4 °C. The collected supernatant that includes the fusion protein was fractionated using 0.5 mL of glutathione–Sepharose for 1 h, and the Sepharose was washed three times with lysis buffer and twice with thrombin digestion buffer (containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 2.5 mM $CaCl_2$). Afterwards, the fusion protein bound to the Sepharose was treated with 1 mL of a thrombin digestion buffer containing 5 units thrombin/mL. After a 2-h incubation at room temperature, the preparation was subjected to centrifugation. The recombinant peptide released into the supernatant was purified using a Waters Sep-Pak Plus C18 cartridge according to the manufacturer's instructions. Purified peptide was used as substrate in the enzymatic assay.

Enzymatic assay

The standard assay for TPST activity was carried out in a reaction mixture (25 μL final volume) consisting of 50 mM MES (pH 5.75), 50 mM NaF, 20 mM $MnCl_2$, 0.1% Triton X-100, 40 μM peptide substrate, and 15 μM PAP[^{35}S]. The reaction was started by the addition of the enzyme, allowed to proceed for 30 min at 28 °C, and terminated by heating at 100 °C for 3 min. The precipitates formed were cleared by centrifugation, and the supernatants collected were analyzed for [^{35}S]sulfated product using a previously developed thin-layer chromatography 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, different buffers (50 mM MES at pH 5.5, 5.75, or 6.0; MOPS at pH 6.25, 6.5, or 7.0) instead of 50 mM MES (pH 5.75) were used in the reactions. For the kinetic studies on the sulfation of peptide substrates, varying

Fig. 1. Nucleotide and deduced amino acid sequences of the zebrafish TPST cDNA. Nucleotides are numbered in the 5′ to 3′ direction. The translation stop codon is indicated by an asterisk. The putative transmembrane segment and the residues involved in the binding of the 5′-phosphosulfate group and the 3′-phosphate group of PAPS are underlined.

```
   1  CTGTTGGCCTACTGGAGCGATTATTTACATACAGACAGAATTCACGAGAAATGTGCTCGC
  61  GGTGTCAGATTCTGAGTCTCCTGAGAGGTTTATAGCTCCATTTACACGATCTGTTCGTCT
 121  GCATTTCATCACGCGATTCTGGAGAATGTAGAATTCGAGGAGTTGTGCTTTCTGTACGGC
 181  ACAATGGTGCTGACAGAGAATATATGGCTTGATTTACCATGTCTGAGTTTATTGAAGCCT
 241  CAAGCAAAGCCCAGAAGACCTGCTGACTGAGGAAAAAAATCTCCCATCCCTTCCCTCCCC
 301  CCAAAAAAACTTTTACACTTCGCCTTTTACATTCCTTCACCCTGACGCTTTTTGGAAACA
 361  TTCACTCCGTCTTTCCCCTTCCCTGACTTTTGCCCCCACCTGCAAAATCTGTTTAACTCA
 421  TTTGTTATTTTTCCCCCTACAAGCAAAGTACTTCCCTAACTGTAAGTCATGAGAAAACAA
   1                                                  M  R  K  Q
 481  ACGTGCAATGTTCTCCTAGTCTGTGGGGTCATCAGCTCCATCACAGTATTTTATCTTGGC
   5  T  C  N  V  L  L  V  C  G  V  I  S  S  I  T  V  F  Y  L  G
 541  CTCAGTACGATAGAGTGTCCGAATGCCCGTTCTCGTGCATCACAGCATGGGTGGGTGGTA
  25  L  S  T  I  E  C  P  N  A  R  S  R  A  S  Q  H  G  W  V  V
 601  AACCTACACGCTGGCAGAAACCTTAGTGACCCATTACAGCTCCCTGAGGAGTACAATGAG
  45  N  L  H  A  G  R  N  L  S  D  P  L  Q  L  P  E  E  Y  N  E
 661  GAAACTCCTCTCATTTTTGTTGGCGGAGTCCCTCGTAGTGGCACCACGCTAATGCGGGCT
  65  E  T  P  L  I  F  V  G  G  V  P  R  S  G  T  T  L  M  R  A
 721  ATGTTAGATGCTCACCCTATCGTGCGGTGCGGAGAAGAGACCCGGGTCATCCCTCGGTTG
  85  M  L  D  A  H  P  I  V  R  C  G  E  E  T  R  V  I  P  R  L
 781  TTAGCCATGCAGGCAACCTGGAGTCACTCGGCACGAGAGCGGGTCCGTCTGGATGAGGCT
 105  L  A  M  Q  A  T  W  S  H  S  A  R  E  R  V  R  L  D  E  A
 841  GGTGTCACTGATGATGTTTTGGACTCTGCTGTACGTGCGTTTCTTTTGGAGATCATAGTA
 125  G  V  T  D  D  V  L  D  S  A  V  R  A  F  L  L  E  I  I  V
 901  GGGCATGGGGAGCCAGCGCCGAGGCTCTGCAACAAGGACCCATTTGCTCTGAAGTCCATG
 145  G  H  G  E  P  A  P  R  L  C  N  K  D  P  F  A  L  K  S  M
 961  TCCTACCTCTCAAAACTCTTCCCAAAGGCGAAGTTTATTCTCATGCTTCGTGATGGCAGG
 165  S  Y  L  S  K  L  F  P  K  A  K  F  I  L  M  L  R  D  G  R
1021  GCCACCGTTCACTCGATGATCTCCCGCAAGGTTACTATTACTGGGTTTGACTTGACAAGT
 185  A  T  V  H  S  M  I  S  R  K  V  T  I  T  G  F  D  L  T  S
1081  TACCGGGATTGTTTGGTAAAGTGGAACCGGGCGGTGGAAGTGATGTACGACCAGTGCCTG
 205  Y  R  D  C  L  V  K  W  N  R  A  V  E  V  M  Y  D  Q  C  L
1141  GCTGCAGTGGATGGCAACTGTCTGCCTGTCCATTATGAGCAGCTTGTGCTGCATCCTGAG
 225  A  A  V  D  G  N  C  L  P  V  H  Y  E  Q  L  V  L  H  P  E
1201  CGGGTGATGCGCAGGCTCCTTCAGTTCTTGGATCTGCCATGGGACACTGCTGTGCTGCAT
 245  R  V  M  R  R  L  L  Q  F  L  D  L  P  W  D  T  A  V  L  H
1261  CATGAACAGCTAATTGGGAAAGTCGGAGGAGTTTCGCTGTCAAAGGTGGAACTGTCAACA
 265  H  E  Q  L  I  G  K  V  G  G  V  S  L  S  K  V  E  L  S  T
1321  GATCAAGTAGTGAAGCCAGTGAATACAGAGGCTCTGTCTAAATGGGTGGGCAAGATTCCT
 285  D  Q  V  V  K  P  V  N  T  E  A  L  S  K  W  V  G  K  I  P
1381  GCTGATGTAGTGAAGGACATGCCCAGCCTTGCCCCCATGCTGAGTCGCTTGGGTTATGAC
 305  A  D  V  V  K  D  M  P  S  L  A  P  M  L  S  R  L  G  Y  D
1441  CCTCTGGCCAACCCACCAAACTACAACAAGCCTGATCTCTTATATCTGAACAACACAAAA
 325  P  L  A  N  P  P  N  Y  N  K  P  D  L  L  Y  L  N  N  T  K
1501  ATAGTAAGGCCGATGTAAAACTGAAAGGTCATCTCTGTGGATACAACCTGTGAATATTTG
 345  I  V  R  P  M  *
1561  GAAGCAATAAACAATGTGCATTTTTTGTAGCACGCAAAGTCTCTACTTGAATTAAGAACA
1621  TGTATTGACAAAACGGAGTCACAACTACCATTGTTTCATACAAAGTGAGTTCTAGTTTTA
1681  ATGTTGTATTTTAAGTCATTTCTCAATTAAGTGCATTTCAGATGTTTTGCTGTTTGTACC
1741  GATGTTATGATGTTGTTATTAATGTCAGTCCGATGACTATAAGGTGGCTTTTGTCTTGTG
1801  GTTCTATTTGTATGCATGCATGTCACAGTAAATGTGTCCTAAAGAGTGATTATCACACAG
1861  GAAACTATGTTTGTTGAAAGAGGAAGTCAAAGTTGCACAGAAATATCACTATTTTTCTCT
1921  GACCGCAGGAGAACTAAGTAAGTTCCATCTAC
```

concentrations of the substrate compounds and 50 mM MES at pH 5.75 were used.

Miscellaneous methods

PAP[^{35}S] (15 Ci/mmol (1 Ci = 37 GBq)) was synthesized from ATP and [^{35}S]sulfate using recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase as described previously (Yanagisawa et al. 1998). In vitro transcription/translation of the zebrafish TPST was performed using the TNT coupled reticulocyte lysate system according to the manufacturer's instructions. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–

Fig. 2. Amino acid sequences of the wild-type (wt) and mutated PSGL-1 N-terminal peptides. The three tyrosine residues and the mutated phenylalanine residues are boldfaced.

PSGL-1 wt	ATE**Y**E**Y**LD**Y**DFL
PSGL-1 1,2 Y	ATE**Y**E**Y**LD**F**DFL
PSGL-1 1,3 Y	ATE**Y**E**F**LD**Y**DFL
PSGL-1 2,3 Y	ATE**F**E**Y**LD**Y**DFL
PSGL-1 1Y	ATE**Y**E**F**LD**F**DFL
PSGL-1 2Y	ATE**F**E**Y**LD**F**DFL
PSGL-1 3Y	ATE**F**E**F**LD**Y**DFL
PSGL-1 F	ATE**F**E**F**LD**F**DFL

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.

Results and discussion

Although considerable progress has been made in recent years in the study of the TPST enzymes, some fundamental questions concerning their ontogeny, regulation, and physiological involvement remain unanswered. The present study was prompted by an attempt to develop a zebrafish model to address these important issues. As a first step toward achieving this goal, we have decided to clone, express, and characterize the TPST enzymes present in zebrafish.

Molecular cloning of the zebrafish TPST

The zebrafish TPST cDNA cloned by reverse transcription – PCR in conjunction with 3′-RACE was subjected to nucleotide sequencing, and the nucleotide sequence obtained was deposited at the GenBank database under accession No. AY263386. Figure 1 shows the nucleotide and deduced amino acid sequences of this zebrafish enzyme. The open reading frame encompasses 1047 nucleotides and encodes a 349 amino acid polypeptide with a calculated molecular mass of 39 030 Da (without considering posttranslational modifications). Sequence analysis based on BLAST search revealed that the deduced amino acid sequence of the zebrafish TPST displayed, respectively, 66% and 60% identity to those of human and mouse TPST-1 (Ouyang et al. 1998) and TPST-2 (Beisswanger et al. 1998; Ouyang and Moore 1998). It should be pointed out that although the cloned zebrafish TPST showed higher percent homology to human or mouse TPST-1, it may actually correspond to TPST-2. We have recently cloned and sequenced another zebrafish TPST (Liu et al., unpublished data), and sequence analysis revealed it to resemble more closely human or mouse TPST-1. Moreover, it is worthwhile mentioning that TPSTs have also been found to be present in lower animals such as *Caenorhabditis elegans* and *Drosophila melanogaster* but not in yeast (Moore 2003). The zebrafish TPST cloned in the present study, while displaying higher percent identity in amino acid sequence to human and mouse TPSTs, also exhibited significant homology to TPSTs from these latter animals. Hydropathy analysis revealed that, similar to human or mouse TPSTs, the zebrafish TPST cloned in the present study contains a putative transmembrane segment (as underlined) located near the N terminus. The zebrafish TPST therefore appears to be a type II transmembrane protein with a short N-terminal cytoplasmic tail with the bulk of this putative Golgi protein being present in the Golgi lumen. Previous studies employing X-ray crystallography in conjunction with sequence alignment have revealed that all cytosolic and Golgi sulfotransferases contain two conserved sequence elements (Negishi et al. 2001). These two sequence elements, designated the 5′-phosphosulfate binding motif and the 3′-phosphate binding motif, are responsible for binding to, respectively, the 5′-phosphosulfate group and the 3′-phosphate group of PAPS, a cosubstrate for sulfotransferase-catalyzed sulfation reactions (Lipmann 1958). Examination of the amino acid sequence of the zebrafish TPST also revealed residues 76–80 (RSGTT, underlined) that correspond to the 5′-phosphosulfate binding motif. And, similar to human and mouse TPSTs previously reported, the zebrafish TPST also contains two conserved residues (Arg^{181} and Ser^{189}, boldfaced and underlined) proposed to be involved in binding the 3′-phosphate group of PAPS (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998).

Expression of recombinant zebrafish TPST in COS-7 cells

The full-length zebrafish TPST cDNA packaged in pcDNA3.1, a eukaryotic expression vector, was used. To ensure that the cDNA can be used as a template for transcription followed by translation for the synthesis of TPST protein, an in vitro transcription/translation experiment was first carried out. As shown in Fig. 3, a 39-kDa protein band was detected in the reaction mixture upon SDS–PAGE, indicating the production of the TPST protein. pcDNA3.1 harboring the zebrafish TPST cDNA was then used to transfect COS-7 cells for the expression of the recombinant enzyme. Lysates of untransfected cells and cells transfected with pcDNA3.1 vector alone or pcDNA3.1 harboring the zebrafish TPST cDNA were then assayed for TPST activity using the wild-type PSGL-1 N-terminal peptide as substrate. As shown in Table 1, lysates of untransfected cells and cells transfected with pcDNA3.1 alone showed approximately the same level of TPST activity. This basal TPST activity is due to the endogenous TPST of COS-7 cells. In contrast, there was a six-fold increase in TPST activity in lysate of COS-7 cells transfected with pcDNA3.1 harboring the zebrafish TPST cDNA, indicating clearly the production of functionally active zebrafish TPST.

Characterization of the recombinant zebrafish TPST

The wild-type and a series of mutated PSGL-1 N-terminal peptides (see Fig. 2) were prepared for the characterization of the recombinant zebrafish TPST.

Temperature dependence, pH optimum, and divalent cation requirement

We first examined the temperature dependence of the activity of the zebrafish TPST using the wild-type PSGL-1 N-terminal peptide as substrate. As shown in Fig. 4, the zebrafish TPST exhibited approximately the same level of activity at 28 and 37 °C. At 21 °C, there was a 50% decrease in the sulfating activity of the enzyme, and only a very low level of activity

Fig. 3. Production of the zebrafish TPST protein by in vitro transcription/translation. The figure shows the autoradiograph taken from the dried polyacrylamide gel used for the SDS–PAGE of the reaction mixtures. Samples analyzed: lane 1, in vitro transcription/translation using pcDNA3.1 vector only as the template; lane 2, in vitro transcription/translation using pcDNA3.1 harboring the zebrafish TPST cDNA as the template.

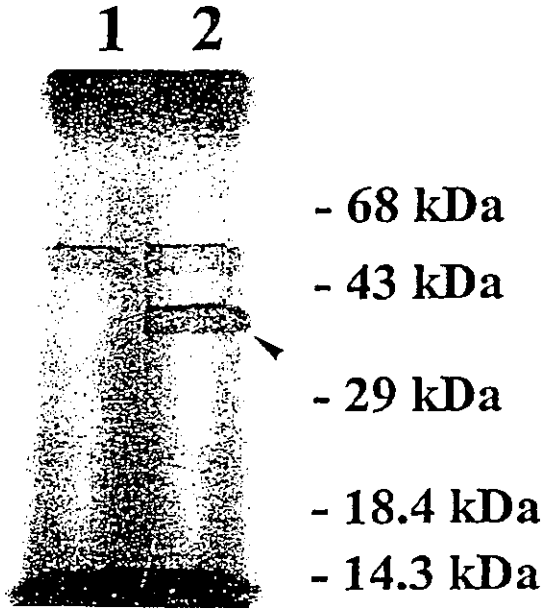


Table 1. Expression of recombinant zebrafish TPST in COS-7 cells.

	Specific activity ($pmol \cdot min^{-1} \cdot mg^{-1}$)	Relative activity (fold)
None	2.03±0.67	1.0
pcDNA3.1	2.07±0.38	1.0
pcDNA3.1-TPST-2 cDNA	11.7±0.20	5.8

Note: Values are means ± SD derived from three determinations.

was detected at 44 °C. As indicated in Table 1, the untransfected COS-7 cell lysate contained a low level of TPST activity due to the endogenous enzyme. It is likely that this endogenous TPST activity may account for the bulk of the activities detected at 37 and 44 °C. It is worthwhile mentioning that although zebrafish are subjected to fluctuation in body temperature in their natural habitat, they have been shown to be best maintained in aquaria heated to 28 °C (Westerfield 2000). Taking into consideration their optimal temperature of growth and the results from the temperature dependence study, we therefore decided to characterize the other enzymatic properties of the zebrafish TPST at 28 °C. Another important property is with regard to the pH optimum of the enzyme. In a pH dependence experiment, the zebrafish TPST displayed a pH optimum of 5.75 (Fig. 5). Previous studies have revealed TPSTs as Golgi enzymes with the catalytic domain being located in the Golgi lumen (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998). The acidic pH optimum determined for the zebrafish TPST, therefore, is compatible with the acidic environment in the

Fig. 4. Temperature dependence of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out at designated temperatures under standard assay conditions as described in Materials and methods. The data represent calculated mean values derived from three experiments.

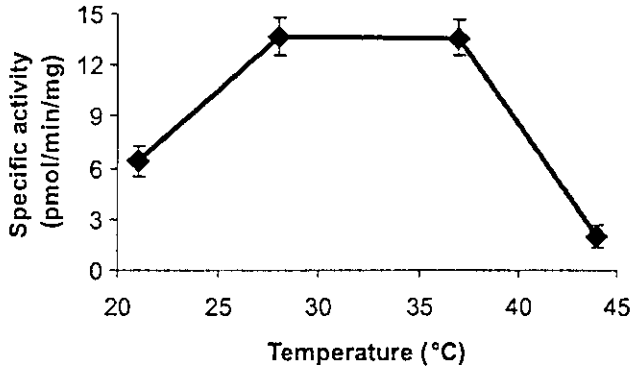
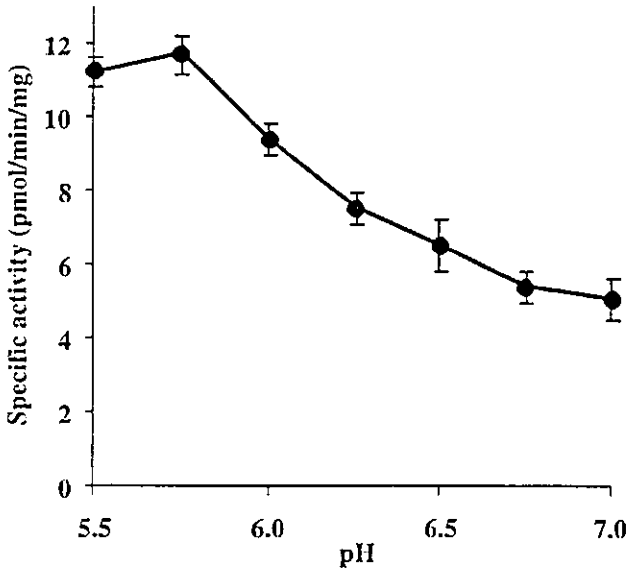
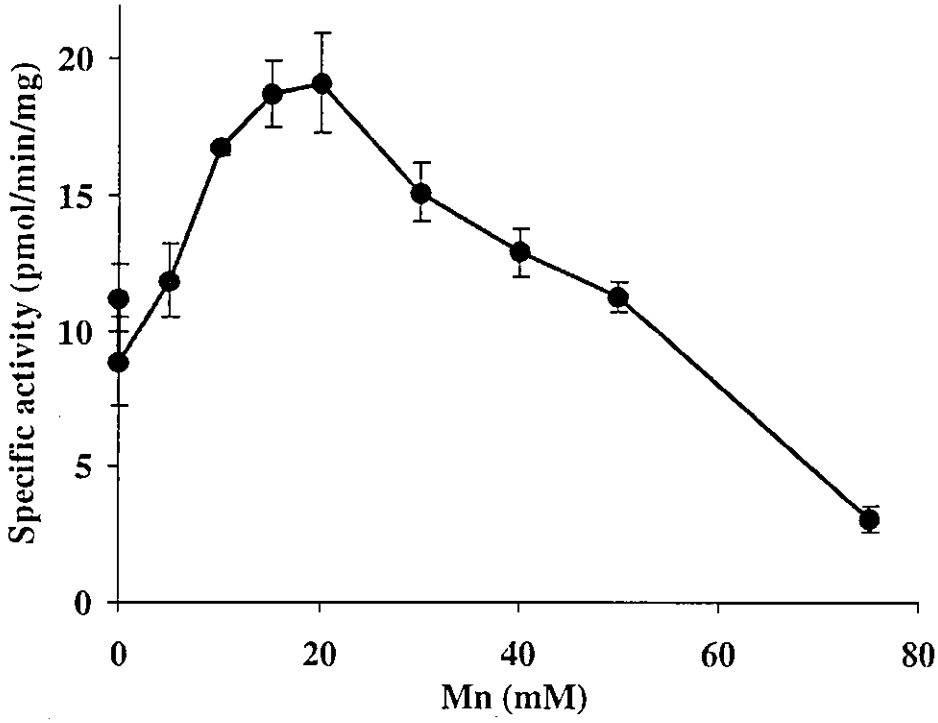


Fig. 5. pH dependency of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out under standard assay conditions, as described in Materials and methods, using different buffer systems. The data represent calculated mean values derived from three experiments.



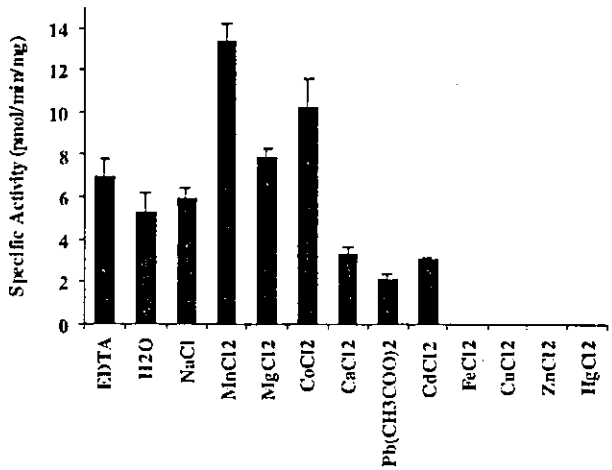
Golgi lumen (Mellman et al. 1986). Previous studies have revealed stimulatory effects of manganese on the activity of some mammalian TPSTs (Lin et al. 1994). We were interested in examining whether manganese is also capable of stimulating the activity of the zebrafish TPST. In a concentration dependence experiment (Fig. 6), it was found that in the presence of 20 mM $MnCl_2$, the sulfating activity of the zebrafish TPST was more than 2.5 times that determined in the absence of $MnCl_2$. At concentrations higher than 50 mM,

Fig. 6. Manganese dependence of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out in the presence of different concentrations of $MnCl_2$ under standard assay conditions as described in Materials and methods. The data represent calculated mean values derived from three experiments.



however, manganese became inhibitory. The mechanism underlying the stimulatory effects of manganese remains to be clarified. We further tested nine other divalent cations for their stimulatory/inhibitory effects on the activity of the zebrafish TPST. As shown in Fig. 7, at a 10 mM concentration, Co^{2+} showed a significant stimulatory effect, while Ca^{2+}, Pb^{2+}, and Cd^{2+} exerted some inhibitory effects. In contrast, the other four divalent cations, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}, inhibited completely the sulfating activity of the zebrafish TPST.

Fig. 7. Effects of divalent metal cations on the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The sulfating activity of the zebrafish TPST was assayed in the presence of different divalent metal cations or NaCl (as a control for the counter ion Cl^-) under standard conditions as described in Materials and methods. The concentration of the divalent metal cations tested was 10 mM, and the concentration of NaCl tested was 20 mM.



Substrate specificity

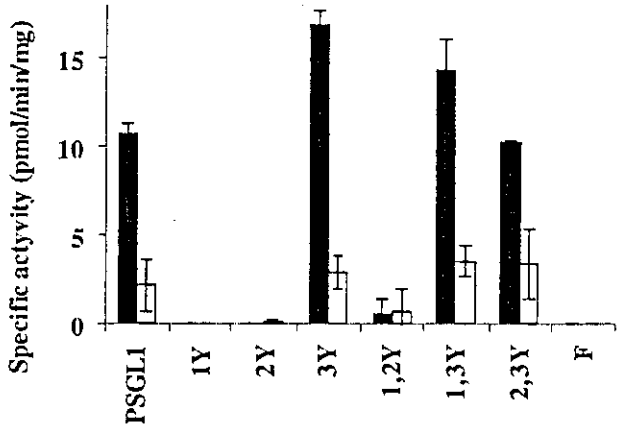
Previous studies have led to a consensus that a key feature of the tyrosine sulfation site is the presence of acidic residues on the N-terminal side of the sulfatable tyrosine (Huttner 1984; Hortin et al. 1986). As shown in Fig. 2, the wild-type PSGL-1 N-terminal peptide contains three tyrosine residues and all of them fulfill such a requirement for sulfation. We were interested in finding out whether the three tyrosine residues of the wild-type PSGL-1 N-terminal peptide are equally or differentially sulfated by the zebrafish TPST. Mutated peptides with one or two of the three tyrosine residues being replaced by phenylalanine were prepared and used as substrates. As shown in Fig. 8, the zebrafish TPST appeared to be most active toward mutated peptides that retained the C-terminal tyrosine residue. In contrast, mutated peptides where this C-terminal tyrosine residue was mutated served as very poor substrates for the zebrafish enzyme even when the N-terminal and (or) middle tyrosine residue(s) were intact. These results indicated clearly the specificity of the zebrafish TPST for the C-terminal tyrosine residue in the wild-type PSGL-1 N-terminal peptide. Kinetic constants for the wild-type and the three mutated PSGL-1 N-terminal peptide that have the C-terminal tyrosine residue intact were determined. Table 2 shows the differential K_m

Table 2. Kinetic constants of zebrafish TPST with wild-type and mutant PSGL1 N-terminal peptides as substrates.

Substrate	K_m (μM)	V_{max} (pmol·min^{-1}·mg^{-1})	V_{max}/K_m (pmol·min^{-1}·mg^{-1}/μM)
PSGL-1 (wild type)	35.9	4.1	0.11
3Y	31.5	42.2	1.34
1,3Y	5.2	17.5	3.37
2,3Y	8.5	10.0	1.18

Note: Values are means ± SD derived from three determinations.

Fig. 8. Substrate specificity of the zebrafish TPST with the wild-type and mutated PSGL-1 N-terminal peptides as substrates. The enzymatic assays were carried out under standard assay conditions as described in Experimental procedures. The data represent calculated mean values derived from three experiments. The solid bars correspond to the activities detected using the zebrafish TPST-expressing COS-7 cell lysate, and the open bars correspond to the activities detected using the control COS-7 cell lysate.



and V_{max} values of these peptide substrates. Interestingly, in the absence of both the N-terminal and the middle tyrosine residues, the C-terminal tyrosine residue was sulfated 10 times faster than in the presence of those two tyrosine residues.

To summarize, we have cloned a zebrafish TPST and expressed and characterized the recombinant enzyme. The recombinant zebrafish TPST exhibited some properties, including acidic pH optimum and stimulation by manganese, that are similar to those previously determined for mammalian TPSTs. Further studies concerning the ontogeny, regulation, and physiological involvement of the zebrafish TPST are now in progress.

Acknowledgments

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Current Topics

蕎麦焼酎揮発性成分の抗変異原性と抗酸化作用

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Abstract

We produced of buckwheat shochu to investigate the functional materials such as volatile compounds included it. Obtained Buckwheat shochu was determined about identification and quantification of volatile compounds by GC and GC/MS. Volatile compounds of buckwheat shochu were examined to investigate whether that have antimutagenic and/or antioxidant activity or not. Investigation was carried out by Ames test, modified Ames test and DPPH radical scavenger test. We identified several volatile compounds with antimutagenicity or antioxidant activity. Those results suggested that Buckwheat shochu may possess potential of functional volatile compounds.

Key words : Buckwheat Shochu, antimutagenicity, antioxidant activity, recombinant sulfotransferase

はじめに

本格焼酎は麹，酵母を用いた並行複発酵により得た醪（もろみ）*1を単式蒸留することで製造される日本古来の蒸留酒である。これまでは，主に南九州を中心に製造，消費されていた。しかしながら，近年の本格焼酎ブームで全国的なアルコール飲料として多くのファンを獲得するに至った。この焼酎ブームは，二日酔いしにくい等の消費者の健康志向に上手く乗った形で，従来のアルコール飲料とは異なり，本格焼酎の健康的なイメージが大きく貢献していると考えられる。現在，健康志向の高まりを受け食品の機能性について数多くの報告が見られる。アルコール飲料も例外ではなく，赤ワインのポリフェノールの機能性等様々な報告が見られるようになってきた。しかしながら，本格焼酎は蒸留酒であるため，圧搾工程で得られるワインより含有成分が少ないこと等の理由でその機能性に注目された研究はあまり報告されていない。そこでわれわれは蕎麦*2焼酎の揮発性成分を詳細に検討し，これら揮発性成分の抗変異原作用などの機能性を評価することで本格焼酎の機能性の開拓を試みた。

1. 方法

1-1. 焼酎製造

一次仕込みは河内菌白麹（*Aspergillus kawachii*）を用いた麦麹原料800g，汲み水960mlに協会焼酎酵母2号（SH-2）を添加した。二次仕込みは蕎麦グリッツ1600g，汲み水2880mlとした。発酵条件は一次25℃，6日間，二次30℃，14日間で行った。醪はロータリーエバポレーターで蒸留し，得られた焼酎を濃縮サンプルとした。以上のように本格焼酎製造は定法で行った。

1-2. 蕎麦焼酎揮発性成分の同定

アルコール濃度を10%に希釈したソバ焼酎をポー

*1 醪
酵母，麹，水，主発酵原料（本論文では蕎麦）から成る。

*2 蕎麦
タデ科の一年草の草本作物。焼酎原料として使用する場合は，殻（果皮）を除去して使用する。

Antimutagenicity and antioxidant activity of volatile compounds from Buckwheat shochu.

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ラスポリマー樹脂に通し，ジエチルエーテルで揮発性成分を抽出したものを濃縮サンプルとした。このサンプルを窒素気流下で500μlまで濃縮後GC-FID（水素炎イオン化検出器），GC/MSで同定分析した。GC条件はキャリアガス圧150kpa，カラム温度を70℃から240℃まで3℃/minで昇温後34分間保持した。分離カラムはDB-WAXetr（60m×0.32mmi. d.，膜厚0.25μm）を使用した。インジェクション，検出器の温度は260℃，スプリット比は10：1で行った。また，GC/MSはインジェクション部をスプリットレスにした以外は全て同条件で実施した。

1-3. Ames法による抗変異原性の検討

サルモネラ菌（*Salmonella typhimurium*）TA98株を用いた抗変異原試験を行った。間接変異原物質としてTrp-P-1（酢酸塩）をプレート1枚あたり1.5ng使用した。DMSOに溶解した試料（揮発性成分），ラット肝臓由来S-9に補酵素溶液を添加したS-9mixを滅菌小試験管で混合した。試料の終濃度は1mMとした。ここに；前培養したTA98菌株懸濁液を加え，37℃，20分間振とうした。振とう後，小試験管にトップアガーを加え最少グルコース培地に重層した。シャーレは37℃48時間暗所で倒置培養し生じたヒスチジン非要求性復帰変異コロニー数を計数した。抗変異原性（%）は下式により求めた。陽性対照試験として直接変異原物質である4-nitroquinoline-1-oxideを用いた場合は，S-9mixの代わりに滅菌水を用いた。また，抗変異原物質のコントロールとして，(−)-epigallocatechin gallate（EGCG）を終濃度500μMで用いた。

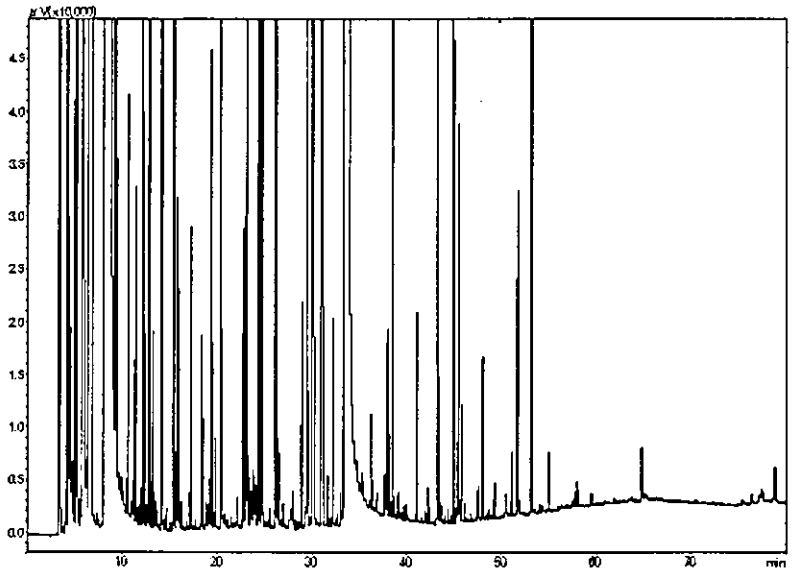

図1 濃縮した蕎麦焼酎のガスクロマトグラム

Trp-P-1のみ添加した時のプレートコロニー数は141±13コロニーであった。

抗変異原性（%）＝（1-（A-B）/（C-B））×100

A：試料とTrp-P-1添加時のコロニー数

B：添加物無しのコロニー数（自然復帰）

C：Trp-P-1のみ添加時のコロニー数

1-4. リコンビナント硫酸転移酵素を用いたAmes変法

測定法はGlatt et alらの方法を改変して行った[1]。まず，活性硫酸である3'-phosphoadenosine-5'-phosphosulfate（PAPS）を16.7μMになるようにBuffer B（150mM KCl，15mM Na_2SO_4，15mM $MgCl_2$，10mM Phosphate Buffer，pH7.4）で調製した。次にヒト肝臓由来のdehydroepiandrosterone硫酸転移酵素（hDHEA ST）の酵素液を60μg/mlになるようにBufferC（150mM KCl，0.5mg/ml BSA，10mM Phosphate Buffer，pH7.4）で調製した。さらに前培養したTA98菌株懸濁液を加えた。間接変異原物質として9-hydroxymethylanthraceneをプレートあたり104.1ng使用した。最後に，終濃度が1mMになるようDMSOに溶解した試料（揮発性成分）を小試験管で混合した。以下，方法3と同様に行った。陽性対照試験および抗変異原性（%）も方法3と同様に実施した。9-hydroxymethylanthraceneのみ添加した時のプレートのコロニー数は180±5コロニーであった。

1-5. 1,1-diphenyl-2-picrylhydrazyl（DPPH）ラジカル消去活性法

35%エタノールに溶解した各試料2.1mlを500mM Acetate Buffer（pH5.5）300μl，99.5%エタノール300μl，0.25mM DPPHエタノール溶液300μlとともに試験管に加えた後よく撹拌し，暗所で室温，1時間反応させて，517nmでの吸光度を測定した。試料の終濃度は1mMとした。試料の代わりに35%エタノールを対照として，下式のようにして求めた。また陽性対照試験として終濃度2.5μM EGCGを用いて行った。

DPPHラジカル消去率（%）＝（A-B）/A×100

A：ブランクの吸光度

B：試料添加時の吸光度

表1 蕎麦焼酎に含まれる揮発性成分

Peak no.	Compound name	mg/l	Peak no.	Compound name	mg/l
1	isopropyl alcohol*	0.02	35	n-octanol	0.02
2	ethyl alcohol	a)	36	isobutyric acid*	0.45
3	ethyl isobutyrate*	0.01	37	ethyl caprate	0.41
4	allyl formate*	0.01	38	1-nonanal	0.02
5	isobutyl acetate*	0.09	39	isovaleric acid	1.35
6	n-propyl alcohol	1.58	40	diethyl succinate	0.51
7	ethyl butyrate*	0.98	41	methionol	0.48
8	butyl acetate*	0.01	42	valeric acid	0.01
9	isobutyl alcohol	32.7	43	2-ethylbutyric acid*	0.01
10	isoamyl acetate	10.66	44	ethyl phenylacetate	0.05
11	1-butanol	0.62	45	nerol	0.01
12	1-ethoxy-2-propanol*	0.01	46	β-phenethyl acetate	5.77
13	3-methyl-1-butanol (I. A. OH)	551.83	47	caproic acid	0.37
14	ethyl caproate	0.61	48	2-methyl-hexanoic acid*	0.01
15	1-pentanol*	tr	49	benzyl alcohol	0.04
16	3-methyl-3-buten-1-ol*	0.02	50	phenethyl alcohol	103.32
17	3-hydroxy-2-butanone*	0.13	51	ethyl myristate	0.02
18	4-methyl-1-pentanol*	0.01	52	caprylic acid	0.3
19	2-heptanol	0.01	53	1-methyl-4-hydroxybenzene	0.01
20	3-methyl-2-buten-1-ol*	0.03	54	ethyl cinnamate	0.05
21	3-methyl-1-pentanol	0.05	55	ethyl pentadecanoate	0.01
22	ethyl lactate	tr	56	ethyl palmitate	0.11
23	1-hexanol	0.07	57	capric acid	0.1
24	3-ethoxy-1-propanol*	0.03	58	trans, trans-farnesol	0.04
25	2-ethylhexyl acetate*	0.01	59	ethyl stearate	0.01
26	ethyl caprylate	1.2	60	ethyl oleate	0.12
27	1-octen-3-ol	0.02	61	ethyl linoleate	0.21
28	1-heptanol	0.07	62	ethyl nonadecanoate*	0.01
29	acetic acid	0.1	63	myristic acid	0.01
30	ethylhexanol	0.05	64	dibutyl phthalate	0.01
31	2-nonanol	n. d.	65	nerolidol (cis-& trans-mixture)	0.09
32	ethyl DL-3-hydroxybutyrate*	0.07	66	palmitic acid	0.03
33	ethyl n-nonanoate*	0.01	67	oleic acid	0.01
34	linalool	0.09	68	bis (2-methoxyethyl) phthalate*	0.02

n. d. : not detected, a) Concentrations of ethanol was adjusted to 10% by material method., RI : retention index, tr : less than 0.01 ppm, * : newly identified

2. 結果および考察

2-1. 本格焼酎由来の揮発性成分

単式蒸留により得られる本格焼酎は図1に示したように，非常に多様な成分から構成され，それらが豊かな味と香りを生み出している。われわれは，蕎麦焼酎の揮発性成分の網羅的な分析を行い，表1に示したように68種類の成分を同定，定量し報告した[2]。その結果，本格焼酎の香気成分は，アルコール類の他に脂肪酸やそのエステル類など非常に多様な化合物が含まれていることが判明した。健康志向の消費者の支持もあって，本格焼酎は体によいと考えられているが，科学的な根拠に乏しく，それらを裏付ける研究報告もあまり知られていない。そこで，われわれは蕎麦焼酎より同定した68種類の香気成分に関して網羅的にその機能性を検討した。今回注目した機能性は抗変異原作用と抗酸化作用であり，それぞれAmes法およびリコンビナント硫酸転移酵素を用いた抗変異原試験とDPPHラジカル消去活性法により検討した。

2-2. 抗変異原性試験

Ames法による抗変異原性試験は，突然変異原性物質やガン原性物質としての可能性のある物質の第一次スクリーニング法として優れていることから，食品科学の分野でも広く実施されている[3,4]。S-9を用いたAmes法では，9サンプルが30%以上の抗変異原性を示した（表2)。その中で7サンプルがエステル化合物であり，さらに6サンプルがエチルエステル化合物で

表2 Ames法および硫酸転移酵素を組み込んだAmes変法を用いた抗変異原性試験結果

Peak No.	Compound name	Inhibition (%)		Peak No.	Compound name	Inhibition (%)	
		A	B			A	B
3	ethyl isobutyrate	n. d.	34.7±3.7	46	β-phenethyl acetate	34.1±6.8	28.1±2.6
4	allyl formate	n. d.	23.6±5.2	47	caproic acid	n. d.	40.1±1.7
5	isobutyl acetate	n. d.	14.0±1.5	48	2-methyl-hexanoic acid	n. d.	28.6±1.8
6	1-propanol	12±4.3	15.8±3.6	49	benzyl alcohol	14.7±4.1	20.2±4.7
10	isoamyl acetate	n. d.	31.0±7.6	50	phenethyl alcohol	n. d.	28.3±3.6
11	1-butanol	n. d.	28.6±0.8	51	ethyl myristate	35.3±3.1	n. d.
13	3-methyl-1-butanol	n. d.	39.4±1.1	53	1-methyl-4-hydroxybenzene	30.9±3.5	n. d.
19	2-heptanol	n. d.	30.5±5.8	55	ethyl pentadecanoate	30.6±0.2	n. d.
32	ethyl DL-3-hydroxybutyrate	36.5±5.1	8.1±1.2	60	ethyl oleate	31.9±0.7	2.6±0.8
33	ethyl n-nonanoate	33.3±7.0	n. d.	61	ethyl linoleate	53.7±0.5	n. d.
35	n-octanol	n. d.	25.4±4.1	67	oleic acid	46.1±0.4	n. d.
42	valeric acid	n. d.	20.7±1.5		EGCG*)	76.2±6.2	86.1±3.0

A：Ames test. B：Modified Ames test using sulfotransferase. n. d.：not detected. Value showed means±S. D. (N=3) *)：Final concentration of EGCG was 500μM.

あった。不飽和脂肪酸およびそのエチルエステル体であるno. 61/ethyl linoleate (53.7±0.5%), no. 67/oleic acid (46.1±0.4%) において40%以上の抗変異原性が認められた。

リコンビナント硫酸転移酵素を用いたAmes変法では，40%以上の抗変異原性がno. 47/caproic acid (40.1±1.7%) で認められた。また，20%以上の抗変異原性を示すサンプルは，従来のAmes法で9サンプルであるのに対して，硫酸転移酵素を用いたAmes変法では13サンプル確認された（表2)。特にno. 46/β-phenetyl acetate (28.1±2.6%), no. 49/benzyl alcohol (20.2±4.7%), no. 50/phenetyl alcohol (28.3±3.6%) 等のフェニル化合物で抗変異原性が多く認められた。従来のAmes法では低沸点揮発性成分で活性が認められなかったのに対して，硫酸転移酵素を用いたAmes変法では20%以上の抗変異原性が4サンプル認められ，no. 11/1-butanol (28.6±0.8%), no. 13/3-methyl-1-butanol (39.4±1.1%) のアルコール類で高い値を示した。

2種類の抗変異原性試験でいずれも20%以上の活性のある試料はno. 46/β-phenethyl acetateのみであった。これは，間接変異原物質を直接変異原物質に代謝活性化する異化代謝経路が，S-9mixの酸化還元反応を利用しているAmes法に対して，硫酸転移酵素による極性の高い物質との硫酸抱合反応を利用したAmes変法の違いによると推測される。このように2種の抗変異原試験を併用することで，反応機構の異なる抗変異原作用を検討することが可能となる。

2-3. DPPHラジカル消去活性法

DPPHラジカル消去活性法の結果を表3に示した。No. 53/1-methyl-4-hydroxybenzene (74.4±0.9%) で高い抗酸化性が認められたが，フェニル化合物であるbenzyl alcohol, β-phenylethyl isobutyrate, phenethyl alcohol, β-phenethyl acetate, ethyl cinnamateにおいて活性は見られなかった。アントシアニジンのB環に水酸基が増えると抗酸化活性も高まることから[5,6]，芳香環の水酸基すなわちフェノール基の存在が重要であると推測される。1-methyl-4-hydroxybenzeneの活性はEGCG (71.0±0.0%) と同程度であるが，測定に使用した濃度が1-methyl-4-hydroxybenzeneの1mMに対してEGCGの2.5μMと40倍も高いことから，EGCGの抗酸化性には及ばない結果となった。

1-methyl-4-hydroxybenzene (30.9±3.5%) は従来のAmes法においても抗変異原活性が確認されたが，硫酸転移酵素を用いたAmes変法において活性は認められなかった（表2)。また，今回の実験で抗変異原性試験および抗酸化性試験で共に活性のあるサンプルは認められなかった。

おわりに

本格焼酎の機能性成分については，本誌において血栓溶解活性の存在が報告されているが，それ以外には

*3 甘藷
サツマイモのこと。焼酎用原料としては，澱粉含量の高いコガネセンガン（農林31号）が主流。

表3 DPPHを用いた抗酸化活性試験の結果

Peak No.	Compound name	Radical scavending activity (%)
41	methionol	3.9±1.3
48	2-methyl-hexanoic acid	3.4±1.5
53	1-methyl-4-hydroxybenzene	74.4±0.9
	EGCG*)	71.0±0.0

Value showed means±S. D. (N=3)
*): Final concentration of EGCG was 2.5 μM.

ほとんど報告されていない[7]。今回，2通りの抗変異原性試験を実施したところ，従来のAmes法では高沸点揮発性成分で多く活性が認められ，硫酸転位酵素を用いたAmes変法では低沸点揮発性成分で多く活性が認められるという結果になった。また，甲類焼酎と本格焼酎の抗酸化性については僅かではあるが，本格焼酎の活性が高いことからも本格焼酎は抗酸化活性などの機能性を有する可能性が考えられた（データ未掲載）。これらの結果より，本格焼酎は抗変異原作用や抗酸化作用をもつ多様な香気成分が含まれ，これらの作用が組み合わさることで相乗的に高い機能性を示す可能性が考えられた。現在，これらの香気成分の構造と機能との関係について詳細に検討を進めている。また，蕎麦以外に甘藷*3，麦，米などを原料とした本格焼酎に関しても分析を進め広く本格焼酎に秘められた機能性を明らかにしていきたいと考えている。

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Biocides, tributyltin and triphenyltin, as possible inhibitors of the human sulfotransferase involved in the estrogen homeostasis

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Abstract

This work using purified recombinant human estrogen sulfotransferase (hSULT1E1) aimed to investigate the mechanism of the inhibition of estrogen sulfation by organotin compounds. Tributyltin (TBT) inhibited the sulfation of estrone (E1) and 17 β-estradiol (E2) by hSULT1E1 competitively, with IC_{50} values of, respectively, 3 and 12 μM. The sulfation of E1 and E2 was also inhibited competitively by triphenyltin (TPT), with IC_{50} of, respectively, 10 and 5 μM. These data strongly suggested that His^{107} residue might act as a ligand to establish a coordination bond with organotins at estrogen binding site in hSULT1E1. A more surprising finding was that TBT competed with 3′-phosphoadenosine 5′-phosphosulfate (PAPS), indicating that TBT may coordinate with certain amino acid residue such as Lys^{47} at the PAPS binding site of hSULT1E1. Taken together, these data provided clear evidence that TBT and TPT have a capacity to disrupt endocrine-mediated events by inhibiting hSULT1E1 involved in the metabolism of sex steroids.

Keywords: Organotin; Endocrine disruptor; 3′-Phosphoadenosine 5′-phosphosulfate; PAPS; Estrogen; Estradiol

1. Introduction

Organotins have been widely used as biocides like fungicides and antifouling agents primarily due to their capacity to inhibit the biosynthesis of ATP [1]. As these organotins accumulated in a

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wide range of marine organisms [2], numerous species of gastropod have developed a sub-lethal genital disorder known as imposex in correlation with the concentrations of these compounds. The recent identification of environmental chemicals capable of disrupting endocrine function in wildlife has raised concern that they may also interfere with human reproductive health and/or stimulate steroid hormone-dependent cancers.

Although the phenomenon of imposex has been explained by the hypothesis that organotins act as the inhibitors of aromatase (CTP 19A) [3,4], which converts androgen to estrogen, there has been no precise molecular mechanism underlying their inhibition. Even more, there was no significant decrease in aromatase activity found in gastropods exhibiting imposex [5,6]. Instead of the aromatase inhibition hypothesis, inhibition of sulfo conjugation of testosterone by tributyltin (TBT) has been proposed as a major cause of TBT-induced imposex phenomena [5]. However, the mechanism underlying the inhibition of sulfo conjugation has not been studied yet. In addition to the possible involvement in imposex in marine organisms, sulfation does play a key role in regulating human hormonal and neuronal homeostasis [7–10], as well as in biological events such as cell growth and differentiation in humans [11,12]. Furthermore, sulfation may facilitate the removal of xenobiotics from the human body [13,14]. Moreover, it has been reported that significant amounts of tins were found in organs such as brain, kidney, and liver from juvenile rats after oral administration of organotins [15]. Therefore, it is one of the important issues to understand how organotins inhibit the cytosolic sulfotransferases (STs) in order to have perspective on human reproductive health, though it is still difficult to assess whether there are true harmful events that occur in human.

At present, the accumulating structural knowledge of STs through the recent crystal structure studies [16–19] provides an opportunity to clarify the inhibition mechanism of organotins. Moreover, the crystal structure of the human estrogen sulfotransferase (hSULT1E1)–PAPS complex provided a detailed view of the catalytic reaction mechanism [20,21]. Therefore, this study is focused on the effect of organotins on the activity of recombinant hSULT1E1 with β-estradiol (E2) and estrone (E1) as substrates, based on the known crystal structure and catalytic mechanism of STs.

This is the first report showing the evidence for molecular mechanism underlying the inhibition of hSULT1E1 by organotins.

2. Materials and methods

2.1. Expression and purification of recombinant human estrogen sulfotransferase

Recombinant human estrogen sulfotransferase (hSULT1E1) expressed using pET23c prokaryotic expression system was prepared as previously described [22]. Transformed BL21 (DE3) cells were grown in 100 ml of LB broth containing 50 mg/ml ampicillin. After the cell density reached to 0.6 $OD_{600\ nm}$, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the production of recombinant hSULT1E1. After 4-h induction at 25 °C, cells were collected by centrifugation and homogenized in 10 ml of a lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) using Aminco French press. The crude homogenates were centrifuged at 10,000*g* for 20 min at 4 °C on two times to down particulate fraction. The supernatant was applied onto an anion-exchange column (Resource-Q) and Fast Protein Liquid Chromatography (FPLC) system was used to get purified hSULT1E1. Obtained eluted fractions containing estrogen sulfotransferase activity were used as enzyme source to study.

2.1.1. Enzymatic assay

The sulfotransferase activity of recombinant hSULT1E1 was assayed using 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) as the sulfonate donor. The standard mixture, with a final volume of 25 μl, contained 50 mM Hepes–NaOH (pH 7.0), 0.5 μM [^{35}S]PAPS (45 Ci/mmol), and 100 μM of the substrate tested. All assays were started by the addition of the purified enzyme preparation, allowed to proceed for 20 min at 37 °C, and terminated by heating at 100 °C for 3 min. After centrifugation at 10,000*g* for 5 min at 4 °C, resultant supernatants were subjected to

the analysis of [^{35}S]sulfated product by TLC. Radioactivities of the sulfate products identified on the TLC plates were quantified using an image analyzer FLA 3000. Results presented in this study represent mean values derived from at least three separate experiments.

2.1.2. Synthesis of 3′-phosphoadenosine 5′-phospho [^{35}S]sulfate

[^{35}S]PAPS (45 Ci/mmol) was synthesized from ATP and [^{35}S]sulfate using the bifunctional human ATP sulfurylase/adenosine 5′-phosphosulfate kinase (PAPS-synthase 1) as described previously [22]. Protein determination was based on the method of Bradford [23] with bovine serum albumin as standard. The analysis of [^{35}S]sulfated products generated during the enzymatic assays was based on the TLC separation using ethyl acetate/*n*-butanol (2:1; by volume) as the solvent system [24].

3. Results and discussion

In spite of the fact that the mechanism underlying imposex in marine snails induced by organotins remains unclear, the inhibition of sulfo conjugation of testosterone by, for example, TBT has been proposed as a major cause [5]. Additional concerns about the safety of human exposure to organotins have been raised based on the results of experiments addressing the quantitative assessment of total tin in various tissues of juvenile rats after oral administration of organotins. When tributyltin acetate (1 mg Sn/kg) and triphenyltin acetate (0.87 mg Sn/kg) were administered orally to neonatal rats from day 2 to day 29 of age and sacrificed on day 30, total tin contents of liver were 610 and 348 ng Sn/g tissue, respectively [15]. Therefore, it is one of the important potential problems for endocrine disruption issues in human to determine whether and/or how organotins inhibit the cytosolic sulfotransferases (STs), although data are sparse regarding the effect of organotins on human reproductive health.

The recent crystallographic analysis had revealed the key amino acid residues involved in the sulfonate transfer reaction and PAPS hydrolysis. β-estradiol (E2) is buried deeply in the hydrophobic substrate pocket and the conserved His^{107} residue serves as a catalytic base facing the 3-hydroxy group of the E2 [20,21]. Notably, the imidazole ring of His^{107} plays an essential role in the charge relay mechanism [20,21,25]. Since the 3-hydroxy group of E2 is less nucleophilic to react with PAPS, this His^{107} residue may form a low-barrier hydrogen bond to increase the reactivity of the 3-hydroxy group of the E2, similar to the reaction mechanism of serine proteases [26]. Based on the chemical structure of organotins and the crystal structure of the STs, we hypothesized that hydrophobic moieties of trisubstituted organotins may interact with the hydrophobic regions of STs and tin atom with unoccupied orbital will form a coordination bond with the lone pair electrons of the nitrogen atom in the imidazole ring of the His^{107} residue. If this working hypothesis is correct in thinking, the catalytic reaction of STs is competitively inhibited by organotins. The obtained results should give important clues to understanding the mechanism based inhibition of STs by organotins and may be the first step for elucidating the true harmful events that occur in human by such the xenobiotics as organotins.

3.1. Inhibition of human recombinant SULT1E1 by TBT and TPT

To determine what extent TBT and TPT inhibit the sulfation caused by hSULT1E1, a range of these compounds were incubated with recombinant hSULT1E1. A preliminary experiment showed that TBT clearly inhibited the sulfation of E1 and E2. The IC_{50} values of the inhibition by TBT were then determined and calculated to be 3 and 12 μM, respectively (Fig. 1A). TPT also inhibited the sulfation of both E1 and E2 with IC_{50} values of 10 and 5 μM, respectively (Fig. 1B). These obtained IC_{50} values are very similar to the concentrations of the ATP synthesis inhibition in mitochondria by TBT as a potent ATP synthase inhibitor [1].

To determine the effect of TBT on the kinetic properties of hSULT1E1, the linear regression and extrapolation of data in the Lineweaver–Burk plot gave a series of lines crossing each other in the proximity of the ordinate indicating that TBT served as a competitive inhibitor of hSULT1E1 (Fig. 2).

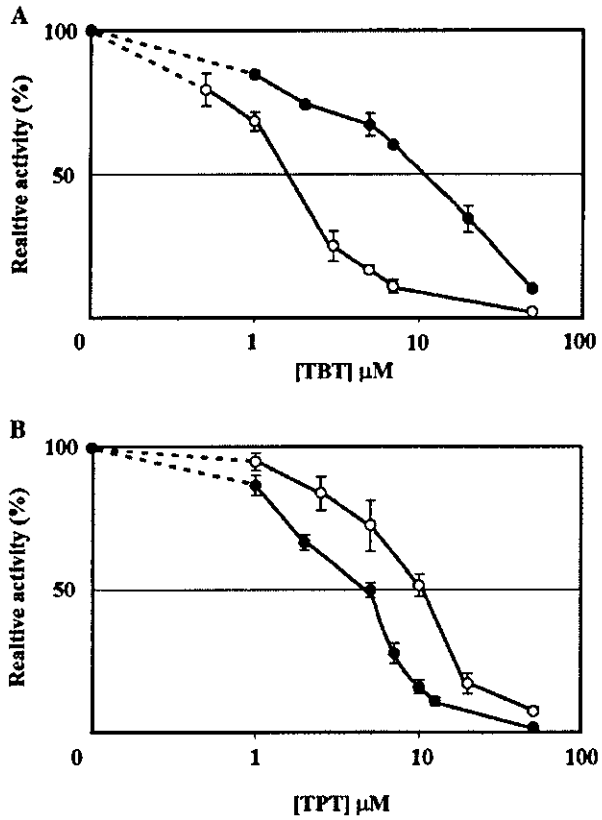

Fig. 1. (A) Effect of tributyltin (TBT) on recombinant human estrogen sulfotransferase, hSULT1E1. Dose-dependent inhibition by TBT of hSULT1E1 activity toward estrone (E1, 0.1 μM; ○) and β-estradiol (E2, 0.1 μM; ●). The IC_{50} values for E1 and E2 were calculated to be 3 and 12 μM, respectively. (B) Effect of triphenyltin (TPT) on recombinant human estrogen sulfotransferase, hSULT1E1. Dose-dependent inhibition by TPT of hSULT1E1 activity toward estrone (E1, 0.1 μM; ○) and β-estradiol (E2, 0.1 μM; ●). The IC_{50} values both for E1 and E2 were calculated to be 10 and 5 μM, respectively.

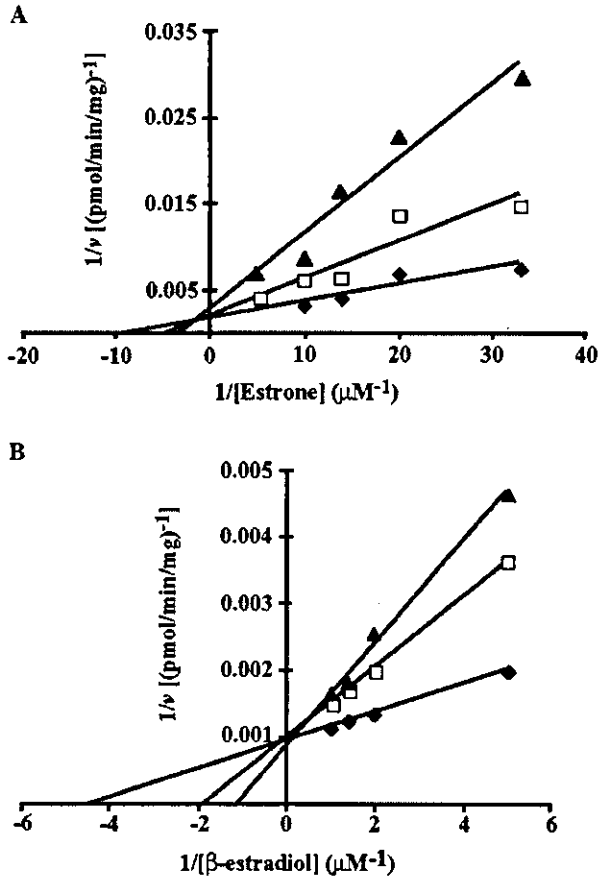

Fig. 2. Influence of TBT on the kinetic properties of hSULT1E1. The data were subjected to Lineweaver–Burk analysis of the inhibition of the sulfation of E1 and E2 at various TBT concentrations. hSULT1E1 activities on E1 in the range from 30 to 200 nM were determined in the presence (□, 2 μM; ▲, 7 μM) or absence (♦) of TBT (A). hSULT1E1 activities on E2 in the range from 0.2 to 1 μM were determined in the presence (□, 2 μM; ▲, 7 μM) or absence (♦) of TBT (B).

In the case of TPT (Fig. 3), TPT was also defined as a competitive inhibitor of hSULT1E1 based on this analysis. These results mean that TBT and TPT bind to the same catalytic site in hSULT1E1 as natural substrates like estrogens do. The fact thus fully supported our hypothesis that hydrophobic moieties of trisubstituted organotins may interact with the hydrophobic regions of STs and tin atom with unoccupied orbital may form a coordination bond with the lone pair electrons of the nitrogen atom in the imidazole ring of the His^{107} residue (Fig. 5). As a result, target estrogens could not reach the substrate binding site and His^{107} could not form a low-barrier hydrogen bond to increase the reactivity of the 3-hydroxy group of the target estrogens. These results imply that organotins may have an impact on human hormone metabolism.

3.2. Influence TBT on the binding of PAPS

Since sulfotransferases including hSULT1E1 contain two binding sites, that is, one for the acceptor substrate and the other for PAPS that serves as the sulfonate donor, the inhibitory mechanism of TBT on the binding of PAPS was characterized

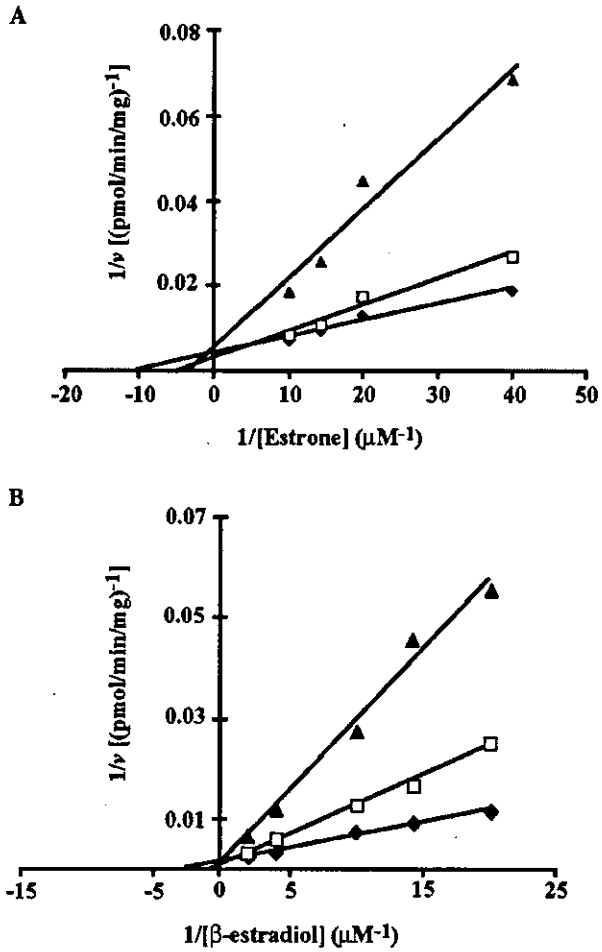

Fig. 3. Influence of TPT on the kinetic properties of hSULT1E1. The data were subjected to Lineweaver–Burk analysis of the inhibition of the sulfation of E1 and E2 at various TPT concentrations. hSULT1E1 activities on E1 in the range from 25 to 100 nM were determined in the presence (□, 5 μM; ▲, 15 μM) or absence (♦) of TPT (A). hSULT1E1 activities on E2 in the range from 50 to 500 nM were determined in the presence (□, 5 μM; ▲, 15 μM) or absence (♦) of TPT (B).

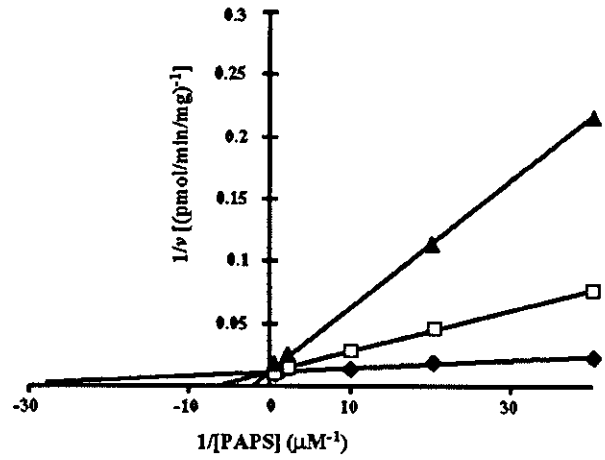

Fig. 4. The interaction activity of TBT with PAPS on the kinetic properties of hSULT1E1 activity. The data were subjected to Lineweaver-Burk analysis of the inhibition of the sulfation of E1 at various TBT concentrations. PAPS at concentrations ranging from 0.025 to 2.5 μM with E1 (25 nM) were used in the presence of different concentrations of TBT.

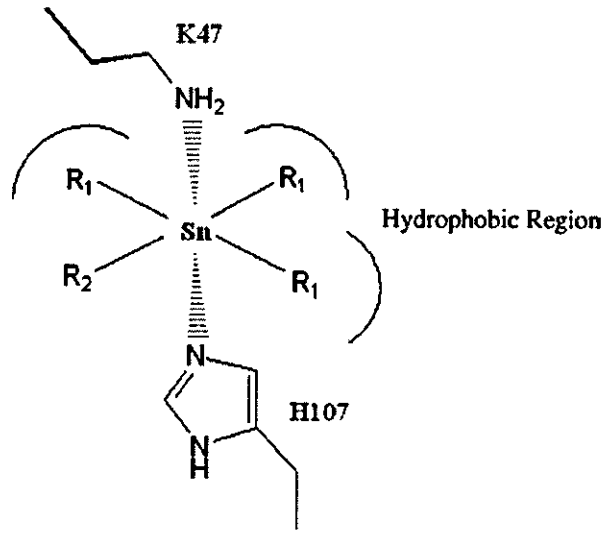

Fig. 5. The proposed inhibition mechanism of organotins on sulfotransferases. Residue numbers are those of SULT1E1 [20,21].

based on the Lineweaver–Burk plot analysis. As expected, based on the information of the crystal structure of hSULT1E1, the type of inhibition mechanism of hSULT1E1 by TBT was that of a competitive nature (Fig. 4). This type of inhibition occurs at the same concentration range as those of substrates. This suggests that TBT shares the same binding site with PAPS in hSULT1E1 to coordinate with the critical amino acids residues such as Lys^{47} for the binding of PAPS to the enzyme.

As conclusions, our data demonstrated that organotins such as TBT competitively inhibited the binding of both estrogens to the substrate binding site and PAPS to the catalytic site, leading to the decreased sulfation activity. The most likely explanation of the results obtained in the present study is that the tin atom could form a

coordination bond with the important conserved amino acid residues in all STs, i.e., His^{107} and Lys^{47}, in the catalytic triad as illustrated in our mechanism based hypothesis (Fig. 5). The inhibition of STs leading to reduced sulfation of estrogens and bioactive chemicals may have important implications in the abnormal sexual development in animals as well as increased incidence of hormone-dependent tumors. More studies on the interaction between the structure favoring the enzyme catalysis and the inhibitors are warranted in order to fully understand the involvement of different sulfotransferases including brain neurosteroid ST, dehydroepiandrosterone ST, and the Golgi-membrane heparan ST.

This is the first report showing the evidence for molecular mechanism underlying the inhibition of ST by organotins. Therefore, the inhibition of STs may cause an adverse effect not only in marine organisms but also in humans if sufficient amount of organotins is accumulated by food chains. Moreover, the idea based on our hypothesis may in the future aid in useful drug design to control the activity of STs.

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生物が獲得した無機硫酸の賢い利用法：硫酸転移酵素による解毒代謝機構

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