Isolation, partial purification, and characterization of a novel petromyzonol sulfotransferase from *Petromyzon marinus* (lamprey) larval liver

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Abstract   We have isolated, partially purified, and characterized the 5α–petromyzonol (5α-PZ), (5α-cholan- 3α, 7α, 12α, 24-tetrahydroxy-) sulfotransferase (PZ-SULT) from larval lamprey liver. Crude liver extracts exhibited a PZ-SULT activity of 0.9120 pmol/min/mg in juvenile and 12.62 pmol/min/mg in larvae. Using crude larval liver extracts and various 5 β-cholan substrates and allocholic acid there was negligible activity, however, with 5α-PZ and 3-keto-5α-PZ the SULT activity was 231.5 pmol/min/mg and 180.8 pmol/min/mg respectively. This established that the sulfotransferase of lamprey larval liver extracts prefers (5 α) substrates and it is selective for hydroxyl at C-24. PZ-SULT was purified through various chromatography procedures. Partially purified PZ-SULT exhibited a pH optimum of 8.0, a temperature optimum of 22°C, and activity was linear for 1h. PZ-SULT exhibited a K_m of 2.5 μM for PAPS and a K_m of 8 μM for PZ. The affinity purified peak PZ-SULT exhibited a specific activity of 2,038 pmol/min/mg. The peak protein upon SDS-PAGE, correlated to an Mw 47 kDa. Photoaffinity labeling with PAP^{35}S, specifically crosslinked the 47 kDa protein, further confirming the identity of PZ-SULT. Partial amino acid sequencing of the putative 47 kDa PZ-SULT protein yielded a peptide sequence (M)SISQAVDAAFXEI, which possessed an overall (∼35–40%) homology with mammalian SULT2B1a.—Venkatachalam, K. V., D. E. Llanos, K. J. Karami, and V. A. Malinovskii. **Isolation, partial purification, and characterization of a novel petromyzonol sulfotransferase from *Petromyzon marinus* (lamprey) larval liver.** *J. Lipid Res.* 2004. **45:** 486–495.

Supplementary key words   petromyzonol sulfate • chemoattractant • pheromone

In mammals, bile acids and salts are synthesized in the liver and stored in the gall bladder (1) and aid in solubilizing fats, which facilitates lipolysis (2). A substituent in cyclopentanoperhydrophenanthrene nucleus (3) that is above the plane is termed β, whereas a substituent that is below the plane is α oriented. The hydrogen attached to carbon-5 (C-5) can be either α or β oriented (4). The α hydrogen at C-5 results in *trans* fusion of the ring structure, yielding nearly a planar structure (5, 6), e.g., allocholic acid (ACA) (7). Higher (C-27) bile acids and bile alcohols (also called cholestanes) are found in many organisms. For example, in the small skate *Raja erinacea*, the major sulfated bile alcohol is scymnol sulfate [3α,7α,12α, 24ξ,26,27-hexahydroxy-5β-cholestane-26 (27) sulfate] (8). The partial purification and characterization of the enzyme that sulfonates 5β-scymnol from the liver of the shark *Heterodontus portusjackson* has been reported (9). In the coelacanth *Latimera chalumnae*, a 26-sulfate of latimerol (5α-cholestane-3β,7α,12α,26,27-pentol) and sulfate esters of 5α-cyprinol (5α-cholestane-3α,7α,12α,26,27-pentol) and 5α-bufol (5α-cholestane-3α,7α,12α,25,26-pentol) have been reported (10). The identification of cyprinol sulfate from grass carp bile and its toxic effects in rats have been reported (11). The West Indian manatee *Trichechus manatus latirostris* produces a sulfo-conjugate of 5α-cholestane-3α,6β,7α-25,26-pentol (12). The presence of various C-27 bile alcohols/salts in fish, amphibians, and mammals has been reported (13, 14).

Cholanes are 24-carbon (C-24) compounds, are similar to cholestanes (C-27), and can also possess hydrogen with an α or β orientation at position number 5, with the usual hydroxyls at 3, 7, and 12 (either α or β) and a carboxyl (bile acid) or hydroxyl (bile alcohol) group at position 24. 5α-PZ is a 5α-cholan-3α,7α,12α,24-tetrol (7). Petromyzonol (PZ) has been shown to be produced in copious amounts in *Petromyzon marinus* (lamprey), a jawless, boneless, primi-

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Abbreviations: 3-keto-PZ, 3-keto-petromyzonol; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PZ, petromyzonol; PZS, petromyzonol sulfate; PZ-SULT, petromyzonol sulfotransferase; SULT, sulfotransferase.

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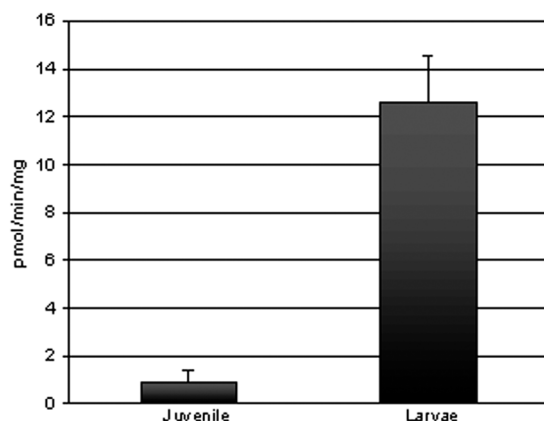   e-mail: venk@nova.edu

Fig. 1. Petromyzonol sulfotransferase (PZ-SULT) activity in juvenile and larval liver extracts. Data represent the mean values (n = 3), and error bars indicate ±SD.

tive fish that belongs to the class of Agnatha. The 24-sulfonated derivative of PZ commonly called petromyzonol sulfate, (PZS) (15, 16) and its derivative 5α-cholane-(7α,12α, dihydroxy)-3-one, 24-sulfate (3-keto-petromyzonol-sulfate, 3-keto-PZS, a more potent chemoattractant) have been shown to play a crucial roles as pheromones during the reproductive cycle of lampreys (17). The adult lamprey has been shown to rerurn to the same breeding ground for spawning by smelling the sulfonated derivatives of PZ produced by the larval lamprey. The sulfonate group at the C-24 is very crucial for its bioactivity as a chemoattractant

(15–17). The Great Lakes of North America are overpopulated with the vicious lamprey, which, as an adult, feeds on economically important teleosts (salmon, trout, etc.) by sucking the blood from these organisms. Thus, the predator lamprey is a menace to the fishing industry. One of the mechanisms for controlling the overpopulation of this organism is to use 5α-PZS and its derivative as the bait to trap adults. Knowledge of the biosynthesis and regulation of the 5α-PZS is very crucial to understanding the reproductive physiology of the lamprey so that eventually strategies can be sought to control the lamprey population. This paper is the first to report the isolation, partial purification, and characterization of a novel cholan-specific sulfotransferase (SULT) that is stereo selective (5α-cholan) and a regio-selective, C-24 hydroxyl-preferring enzyme from larval livers of lamprey.

MATERIALS AND METHODS

Materials

The radiochemical $[^{35}S]3'$-phosphoadenosine 5'-phosphosulfate (PAPS) for enzyme assays was purchased from NEN Life Science Products or ARC, Inc. Cholan substrates ACA, 3-keto-PZ, PZS, nordesoxycholic acid (NDC), and 5α-PZ were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). 5α-PZ was also purchased from Cayman Co. (Ann Arbor, MI). 5β-24-ol, 5β-PZ was purchased from Steraloids, Inc. (Newport, RI). Cholic acid (CA), lithocholic acid (LCA), cholesterol, and deoxylithocholic acid (DLCA) were purchased from Sigma-Aldrich, Inc. DEAE ion exchange matrix (Macro-prep DEAE support) was
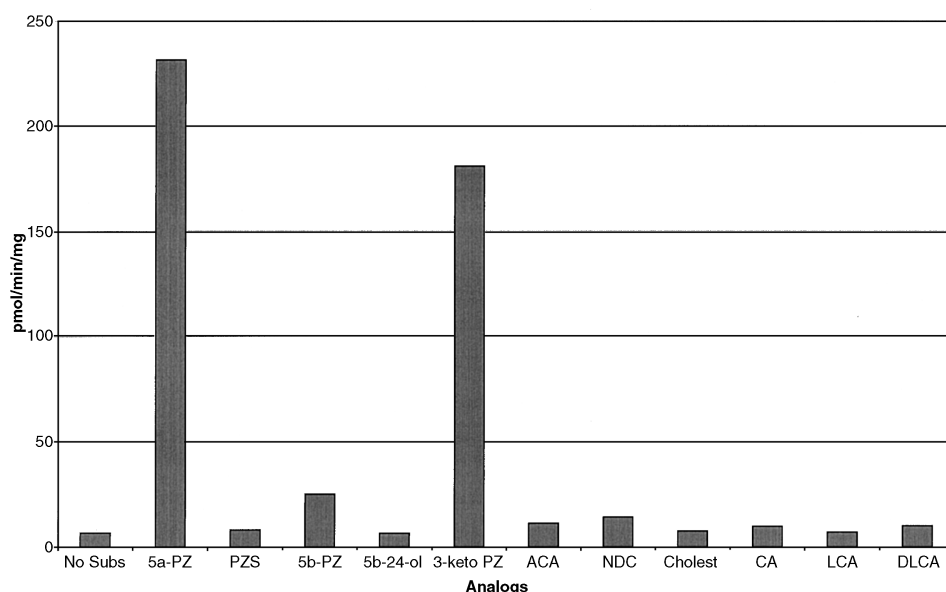

Fig. 2. Sulfotransferase (SULT) activity in crude larval liver extracts against various compounds. The compounds were dissolved in ethanol. The final concentrations of the substrates in 10 μl enzymatic reactions were: 0.768 mM, 5α-PZ; 1.4 mM, petromyzonol sulfate (PZS); 0.14 mM, PZ-24-SO$_4$; 0.423 mM, 5β-PZ; 0.58 mM, 5β-cholan-24-ol; 1.02 mM, 3-keto-PZ (3-keto-petromyzonol); 1.22 mM, allocholic acid (ACA); 1.32 mM, nordesoxycholic acid (NDC); 0.784 mM, cholesterol (Cholest); 0.742 mM, CA (cholic acid); 0.805 mM, lithocholic acid (LCA); and 0.772 mM, deoxylithocholic acid (DLCA). Plain ethanol was used in the no substrate (No subs) reaction. Data represent the mean values (n = 2).

purchased from Bio-Rad (Hercules, CA). Thin-layer chromatography PE silica gel G plates were obtained from Whatman (Clifton, NJ). Nu PAGE, 12% Bis-tris gels, and SDS-PAGE prestained protein molecular weight standards were obtained from Invitrogen (Carlsbad, CA). Larval and juvenile lamprey livers were shipped in dry ice periodically by Hammond Bay Biological Station, Millersburg, MI. ATP, 3′-phosphoadenosine 5′-phosphate (PAP) col-

umn affinity matrix, Sephadex (G-100-50) (exclusion limit >100,000 molecular mass) for gel filtration, and all other common biochemicals were also purchased from Sigma-Aldrich, Inc.

Methods

PZ-SULT assay. The assay was performed in a total volume of 10 μl. The assay consisted of 3 μl of reaction buffer [150 mM
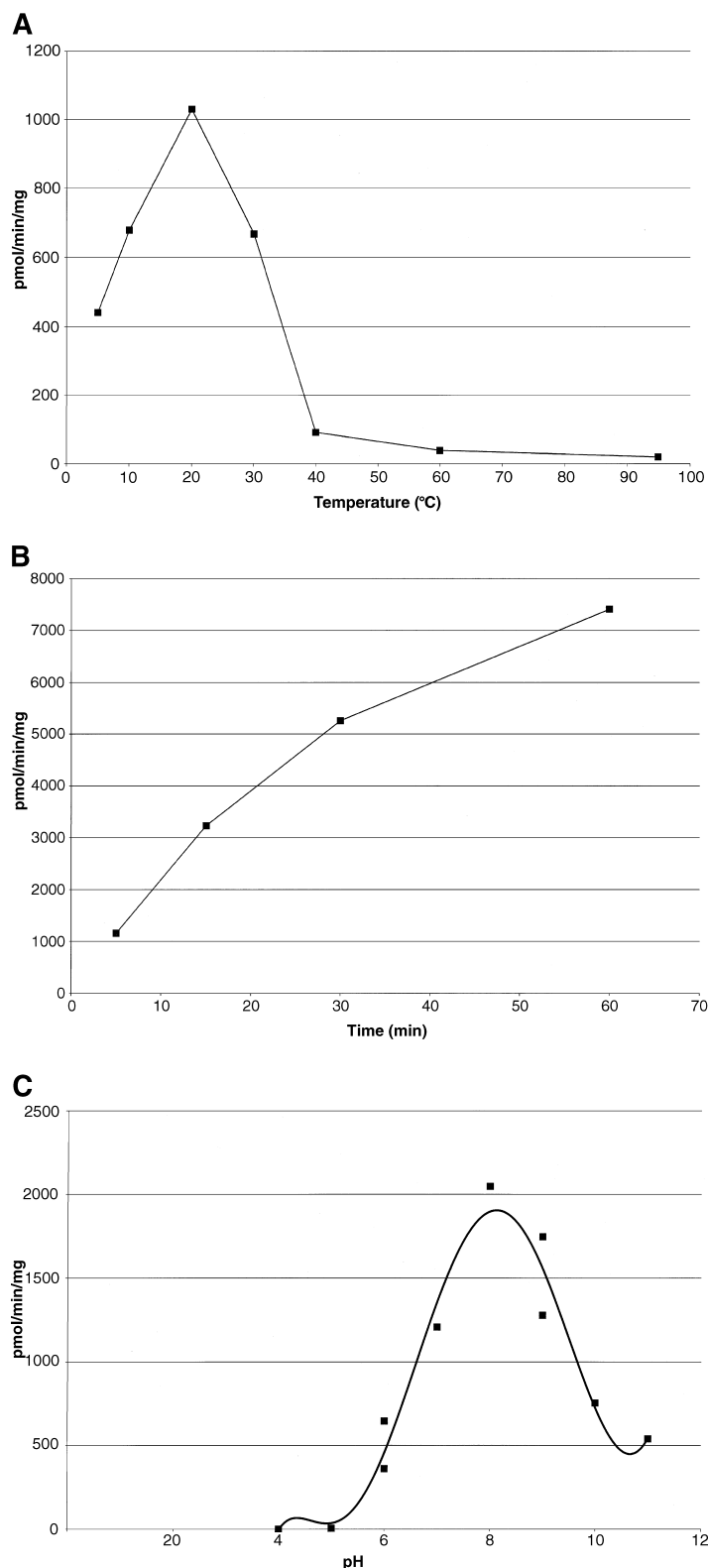

Fig. 3. A: Effect of temperature on PZ-SULT activity. Reaction at each temperature was performed for 15 min at pH 8.0 according to the standard assay procedures described in Materials and Methods. B: Effect of time on PZ-SULT activity. Reactions at various time periods were performed at 22°C, pH 8.0. The reactions were then stopped by heat inactivation by boiling at 95°C, and the contents were processed according to the standard assay procedures described in Materials and Methods. C: Effect of pH on the PZ-SULT activity. Reactions were performed for 15 min at 22°C, using reaction buffers of various pHs that contained, respectively, ingredients used in the standard assays described in Materials and Methods. [The following buffers were used for various pHs: citrate reaction buffer (pH 4.0, 5.0, and 6.0); phosphate buffer (pH 6.0 and 7.0); Tris-HCl (pH 8.0 and 9.0), and glycine (pH 9.0, 10.0, and 11.0).]
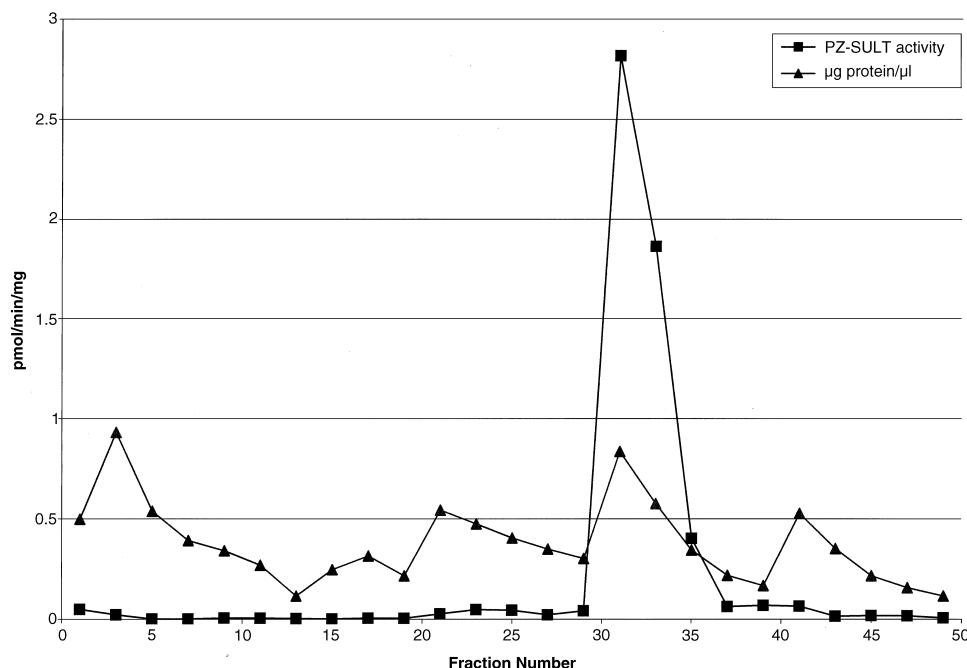
Fig. 4. DEAE–macro prep elution profile of PZ-SULT. Crude larval liver 100,000 *g* supernatant was purified through DEAE ion exchange columns. The columns were washed with buffer A with no salt to remove unbound proteins and then eluted with buffer A containing NaCl (0.05 M to 0.4 M) consisting of 10 ml step gradients. Approximately 1 ml fractions were collected, and a 4 μl aliquot was assayed for PZ-SULT activity. Protein amounts were determined by Bio-Rad dye binding assay. The protein contents per μl are represented (as described in Materials and Methods). Peak PZ-SULT activity eluted at 0.3 M NaCl concentration.

Tris-HCl (pH 8.0), 50 mM KCl, 15 mM MgCl$_2$, 3 mM EDTA, 45 mM dithiothreitol (DTT)], 4 μl enzyme preparation, 1 μl 50 mM ATP, 1 μl PAP^{35}S (0.16 μCi/0.09 nMol), and 1 μl of 7.68 mM PZ. The typical reaction was carried out at 22°C for 5 min and stopped by placing reaction tubes in boiling water for 5 min. The contents were briefly centrifuged, and 1 μl aliquots were transferred to silica gel thin-layer chromatography plates and chromatographed using chloroform-methanol-water (70:26:4; v/v/v) as the solvent system. Following chromatography, the thin-layer chromatography plates were dried and exposed overnight to X-ray film (Eastman Kodak Co.). The respective PZS spots were cut out and the radioactivity determined by liquid scintillation.

Isolation of PZ-SULT. All operations were carried out at 4°C–15°C. Frozen larval livers (∼1.39 g; 7–8 mg/larval liver) were ground in 3 ml of homogenization buffer [100 mM Tris-HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA, protease inhibitor cocktail III from Calbiochem (San Diego, CA)] with a pinch of sand using a mortar and pestle. The mortar was rinsed with 2 ml of homogenization buffer, and the liquid contents were pooled with the rest of the homogenates. Centrifugation was performed in a Beckman ultracentrifuge (model L7-65) using a Ti-70.1 rotor. The homogenate was first centrifuged at 100 *g*. The resulting supernatant was then subjected to centrifugation at 10,000 *g*. The supernatant was then subjected to 100,000 *g*, and the resulting soluble supernatant was used for PZ-SULT purification.

PZ-SULT purification. The soluble extracts were applied onto a 5 ml ion exchange matrix (Macro-prep, DEAE support) and then washed with buffer A [20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT]. The column was eluted at a rate of 1 ml/min with a step gradient of buffer A containing increasing concentrations of NaCl ranging from 0.05 M to 0.4 M. Approximately 10 ml of buffer eluate per step gradient was collected. Protein contents in fractions were measured using the dye binding method de-

scribed by Bio-Rad. Four microliters of each fraction was assayed for PZ-SULT activity, as described previously. PZ-SULT activity fractions (30–35) (total of ∼6 ml) were pooled and applied to a Sephadex G-100 gel filtration column (80 ml bed volume). The column was eluted with buffer A, and ∼1 ml fractions were collected at a rate of 12 min per fraction. PZ-SULT activity fractions (33–47) were further pooled and concentrated using an Ultrafree-4 centrifugal filter unit [molecular mass cutoff 5 kDa, (Millipore)] to ∼1.8 ml. A portion of the fraction (1.6 ml) was applied onto a 2 ml affinity column matrix, PAP immobilized on cross-linked 4% beaded agarose (Sigma-Aldrich). The enzyme preparation was passed through at least five times, and after the final pass, the proteins were allowed to bind to the matrix for >60 min. The column was then eluted with buffer A containing a salt step gradient, 5 ml each of 0.05 M to 0.4 M NaCl. PZ-SULT-active fractions from the PAP column were resolved by SDS-PAGE using 12% Bis-tris gels. Gels were stained with Silver-Express according to instructions provided by Invitrogen.

Photoaffinity labeling. Larval liver 100,000 *g* supernatant was purified through DEAE ion exchange chromatography, in a procedure similar to those described earlier. For subsequent PAP affinity column purification, only the very peak fraction was included, and the rest of the peak was eliminated. This avoided carry-over of many contaminating proteins. The very peak fraction from the PAP affinity column-purified enzyme fraction and a nonpeak fraction were concentrated using an Ultrafree-4 centrifugal filter unit [molecular mass cutoff 5 kDa (Millipore)]. A peak PZ-SULT-active fraction and a nonpeak PZ-SULT-inactive fraction were used for photoaffinity labeling. The reaction was performed in a total volume of 50 μl. The reaction consisted of 45 μl affinity-purified proteins in buffer containing 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, ∼0.20 M NaCl, and 5 μl of PAP^{35}S [6.76 μCi (specific activity of 3.00 Ci/mmol)]. In a
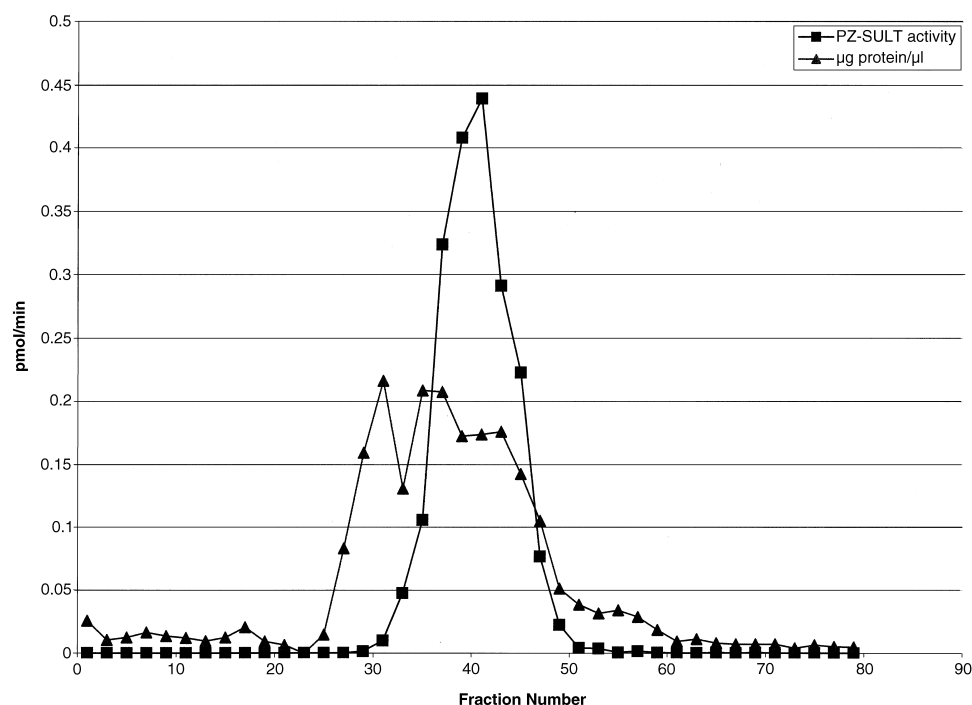
Fig. 5. Sephadex G-100 gel filtration chromatography purification of PZ-SULT. DEAE fractions 30–35 that contained PZ-SULT activity were pooled and loaded onto the gel filtration column. The column was eluted with buffer A containing 0.05 M NaCl at a rate of 1 ml/12 min. PZ-SULT activity in a 4 μl aliquot from 1 ml fraction was measured. Protein per 1 μl is represented.

cold chase experiment, the reaction contained an additional 0.36 mM nonradioactive PAPS. Photoaffinity labeling was performed using UltraVette UV cuvettes from BrandTech Scientific, Inc., (Essex, CT). The samples were irradiated for 10 min (672,000 μJ/cm²) at 25°C using CL-1000 UV cross-linker from UVP, Inc., (Upland, CA). The reaction was chilled on ice and stopped by adding 6 μl of SDS-PAGE loading buffer from Invitrogen. The proteins were then denatured by heating the samples at 95°C for 5 min. A 35 μl aliquot was loaded onto 12% Bis-tris gels. Gels were stained with Silver-Express according to instructions provided by Invitrogen. For autoradiography, the gels were dried and exposed to X-ray film (Xomat AR, Eastman Kodak Co.) for 6 days.

Protein sequencing. The proteins from SDS-PAGE were electroblotted onto Immobilon-P membranes and stained with Ponceau S. A 47 kDa band was cut and amino terminally sequenced by the Edman degradation method using a Procise 491 protein sequencer (Applied Biosystems, Inc.). No sequence was obtained, perhaps due to NH_2-terminal blocking. The membranes were cut into small pieces and wetted with methanol, then treated with 100 μl of 2% cyanogen bromide in 70% formic acid under nitrogen for 24 h in the dark. The membranes were then extracted four times with 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 12 h and two times with 20% acetonitrile without TFA. The supernatants containing the digested peptides were removed. The membrane remaining after acetonitrile-water-TFA extraction was used for sequencing.

Homology analysis. The 14-amino acid PZ-SULT peptide was aligned with SULT using DNA and protein sequence analysis software OMIGA 2.0 (Oxford Molecular Co., Madison, WI). GenBank-deposited mammalian SULTs used in the analysis were SULT2B1a (protein ID, AAC78553.1), SULT2B1b (protein ID, AAC78554.1), SULT2B1a (protein ID, AAC78498.1), and SULT2A1 (protein ID, NP 003158.2).

RESULTS

Isolation of PZ-SULT

PZ-SULT activity in juvenile versus larval liver. Liver tissue extracts (100 *g* supernatant) from juveniles and larvae were tested for PZ-SULT activity. Tissue extracts from both juveniles and larvae, without the exogenously added 5α-PZ cosubstrate, exhibited negligible activity. There was 10-fold higher PZ-SULT activity with 5α-PZ in larvae (12.62 pmol/min/mg) compared with juvenile activity (0.9120 pmol/min/mg) (**Fig. 1**). Using enzymatically concentrated preparations (based on PZ-SULT activity) of larval liver tissue extracts, various analogs were tested for SULT activity. The analogs tested included cholesterol, which is the precursor of all C-27 cholestane, 5α-cholan substrates (5α-PZ, 5α-PZS, 3-keto-5α-PZ, and ACA), and 5β−cholan substrates [5β-PZ, 5β-cholan-24ol (5β-24ol), NDC, CA, DLCA, and LCA]. Neither the common 5β bile acid compounds (CA, NDC, DLCA, and LCA) nor 5β-24-ol, a bile alcohol, formed sulfonated products. 5β-PZ possessed a negligible activity of 25.3 pmol/min/mg. With 5α-PZ, the extract contained a SULT activity of 231.5 pmol/min/mg. With 3-keto-5α-PZ, the activity was 180.8 pmol/min/mg. All other substrates, including ACA, exhibited only background activity for sulfonation (**Fig. 2**). ACA is the same as the 5α-PZ, except that at position 24, it has a carboxyl group instead of a hydroxyl group. The PZ-SULT activity exhibits a temperature optimum of 22°C (**Fig. 3A**). The activity was stable and linear for 1 h of incubation at 22°C at pH 8.0 (Fig. 3B) and a pH optimum of 8.0 (Fig. 3C).
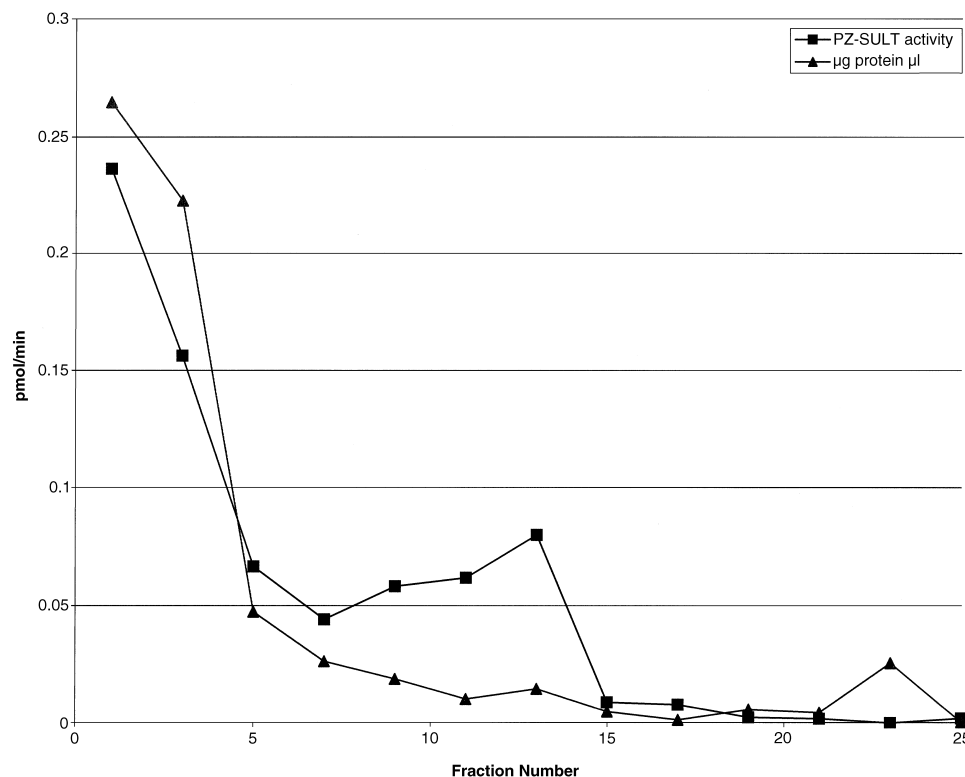
Fig. 6. 3′-Phosphoadenosine 5′-phosphate (PAP) affinity column chromatography purification of PZ-SULT. PZ-SULT fractions (33–47) from gel filtration chromatography were pooled and concentrated using an Amicon ultra filtration unit (molecular mass cutoff ∼5 kDa), and a 1.6 ml concentrate was passed through the PAP column five times. The final pass through was allowed to bind for >60 min. The column was washed and eluted with 5 ml each of buffer A with NaCl (0.05 M to 0.4 M) step gradient. The activity that did not bind to the column eluted in buffer containing a low-salt wash of 0.05 M NaCl. The bound PZ-SULT activity eluted in buffer containing 0.2 M NaCl.

The partially purified enzyme, when tested with analogs ACA, 5β-PZ, and 5α-PZS, showed negligible activity, similar to that shown by crude extracts (data not shown).

PZ-SULT purification

The 100,000 *g* supernatant preparation containing soluble proteins and exhibiting a PZ-SULT activity of 7.68 pmol/min/mg was used for protein purification. Upon chromatography over a DEAE ion exchange column, the PZ-SULT peak activity eluted at 0.3 M NaCl (**Fig. 4**). Peak fractions (30–35; ∼6 ml) were pooled and subjected to further purification by gel filtration column chromatography. PZ-SULT activity eluted between fractions 31 to 49 (**Fig. 5**). The gel filtration column fractions were then concentrated using an Ultrafree-4 centrifugal filtration unit (molecular mass cutoff 5 kDa). Approximately 1.6 ml of the concentrated PZ-SULT fractions was allowed to bind to a PAP affinity column, and the unbound proteins were removed by washing with buffer A containing 0.05 M NaCl. The bound proteins eluted with a high salt concentration (0.2 M NaCl) exhibited peak PZ-SULT activity (**Fig. 6**). The specific activity of the affinity column-purified PZ-SULT was found to be 2,038 pmol/min/mg. The peak PZ-SULT fraction contained a prominent 47 kDa protein and other contaminants. The fraction that did not contain PZ-SULT activity lacked the 47 kDa protein; how-

ever it contained the higher molecular weight contaminants (**Fig. 7**).

Photoaffinity labeling. For routine purification, the gel filtration step was eliminated, because it yielded no significant increase in purification and often resulted in loss of activity. Therefore, from the DEAE ion exchange chromatography, the highest activity fractions from the peak were pooled and further purified using PAP affinity column chromatography. This eliminated the carryover of many contaminating proteins. When the peak fraction that had the most PZ-SULT activity (10,931.8 pmol/min/mg) was tested for purity, as judged by SDS-PAGE and subsequent silver staining, a 47 kDa PZ-SULT activity-associated protein and a higher molecular weight contaminant were visualized (**Fig. 8A**). This PZ-SULT fraction and a fraction that did not contain PZ-SULT activity, with no visible 47 kDa band, were used for photoaffinity labeling with the cosubstrate PAP³⁵S and for subsequent autoradiography. From the purified PZ-SULT-active fraction, the autoradiography revealed a photoaffinity-labeled band corresponding to 47 kDa. No other labeled products were observed, and from the fraction that did not contain PZ-SULT activity, no labeled products were observed (Fig. 8B). In addition, (data not shown) when labeling was performed using the PZ-SULT-active fraction in the presence of excess nonradioactive PAPS (0.36 mM), no radioactive band
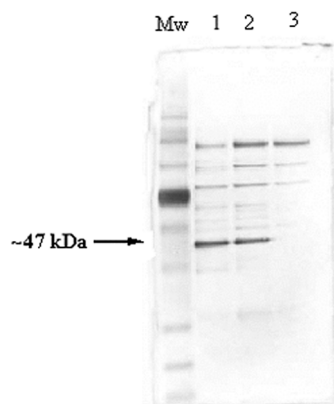
Fig. 7. SDS-PAGE analysis of PAP affinity column-purified PZ-SULT. Fractions 13 and 14, which contained PZ-SULT activity, and fraction 15, which contained no PZ-SULT activity, were analyzed by 12% Bis-tris SDS-PAGE gels and stained with silver. Prestained molecular mass marker from Invitrogen, followed by lanes 1 and 2 corresponding to PZ-SULT-active fractions containing the major protein (47 kDa), indicated by the arrow. Lane 3, which contained no PZ-SULT activity, lacks the 47 kDa band.

Fig. 8. Photoaffinity labeling of peak PZ-SULT-activity protein. A: SDS-PAGE analysis for purity of the PZ-SULT preparation used for photoaffinity labeling: 1, molecular mass marker; 2, empty lane; 3, PZ-SULT peak fraction (0.67 μg); 4, fraction containing no PZ-SULT activity (0.25 μg). B: Autoradiograph of the photoaffinity labeling: 1, empty lane; 2, peak PZ-SULT fraction showing the cross-linked, labeled 47 kDa protein; 3, corresponds to the fraction containing no PZ-SULT activity.

was detected, confirming the specific labeling by the co-substrate PAPS of the SULT.

Kinetic analyses. Partially purified enzyme preparations from DEAE ion exchange and PAP affinity column-purified fractions that contained high levels of PZ-SULT activity were used for determining the kinetic parameter K_m for PAPS and PZ. With varying PZ concentrations and a fixed concentration of PAPS (9 μM), the PZ-SULT exhibited a K_m of 8 μM for PZ (**Fig. 9A**). Similarly, at a fixed concentration of 75 μM PZ, the PAPS concentration was varied, and the formation of PZS was measured. The PZ-SULT exhibited a K_m of 2.5 μM for PAPS (Fig. 9B).

Protein sequencing. PZ-SULT protein is N-terminally blocked. Partial amino acid sequencing yielded a sequence of (M)SISQAVDAAFXEI. Partial amino acid comparison of PZ-SULT with mammalian SULTs, [SULT2B1a (GenBank protein ID, AAC78553.1), SULT2B1b (GenBank protein ID, AAC78554.1), SULT2B1a (GenBank protein ID, AAC78498.1), and SULT2A1 (GenBank protein ID, NP003158.2)] revealed an overall similarity of ∼35–40% to SULT2B1a. Notably, the pentapeptide sequence SISxA found in PZ-SULT is highly conserved in two of the mammalian SULT2B1a isoforms (**Fig. 10**).

DISCUSSION

Cholesterol, the precursor for all C-27 cholestanes, when tested for SULT activity using lamprey larval liver extracts, did not form any sulfonated products. Cholanes, similar to cholestanes, possess the usual trihydroxyl groups at positions 3, 7, and 12, and the carbon at position 24 is carboxyl (bile acid) or hydroxyl (bile alcohol) (4). Certain primitive fish, such as the lamprey and the lung fish, produce 5α-petromyzonol (13). The sulfo-conjugate (bile salt) of PZ has been shown to serve as a chemoattractant
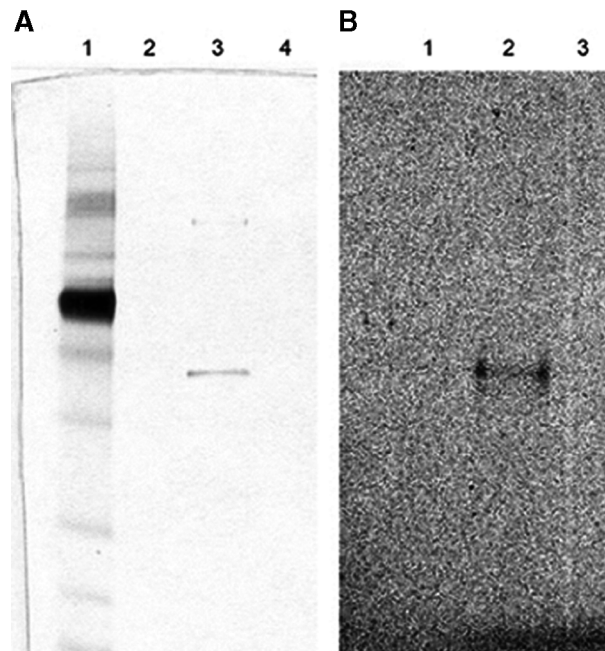
for the spawning adult lamprey (15). Thus, 5α-PZ can be categorized under zoo steroids, owing to its 5α structure and its ability to serve as a pheromone during the reproductive cycle. The sulfo-conjugate, and not the free alcohol, of 5α-PZ and its derivative, 3-keto-5α-PZ, serves as a chemoattractant (15–17). When larval and juvenile liver extracts were tested for PZ-SULT activity, the larval liver extract contained about 10-fold higher PZ-SULT activity. Perhaps this makes sense, based on the reproductive life cycle of lampreys and the associated role of PZS as a chemoattractant. Sea lampreys belong to a very primitive group of jawless fish known as the agnatha. They lack both a jaw and a backbone, and their complex life cycle includes a worm-like larval stage. Sea lamprey larvae bury themselves in the mud at the bottom of streams, where they hatch and remain, feeding and maturing slowly, for up to fifteen years. They can undergo radical metamorphosis, which transforms them into an eel-like, parasitic juvenile animal with a hook-studded sucker for a mouth and a strong appetite for trout and salmon. Thus, lamprey overpopulation is a major threat to the Great Lakes ecosystem (15–17). The migration of lampreys is somewhat reminiscent of the well-known salmon runs. The lampreys return to spawn in the shallow streams that have supported spawning in the past. Lampreys recognize the species-specific odor compound of 5α-PZS derivative. Ovulating lampreys are attracted to 3-keto-5α-PZS produced by the male and use this as a cue for spawning (16). The rea-
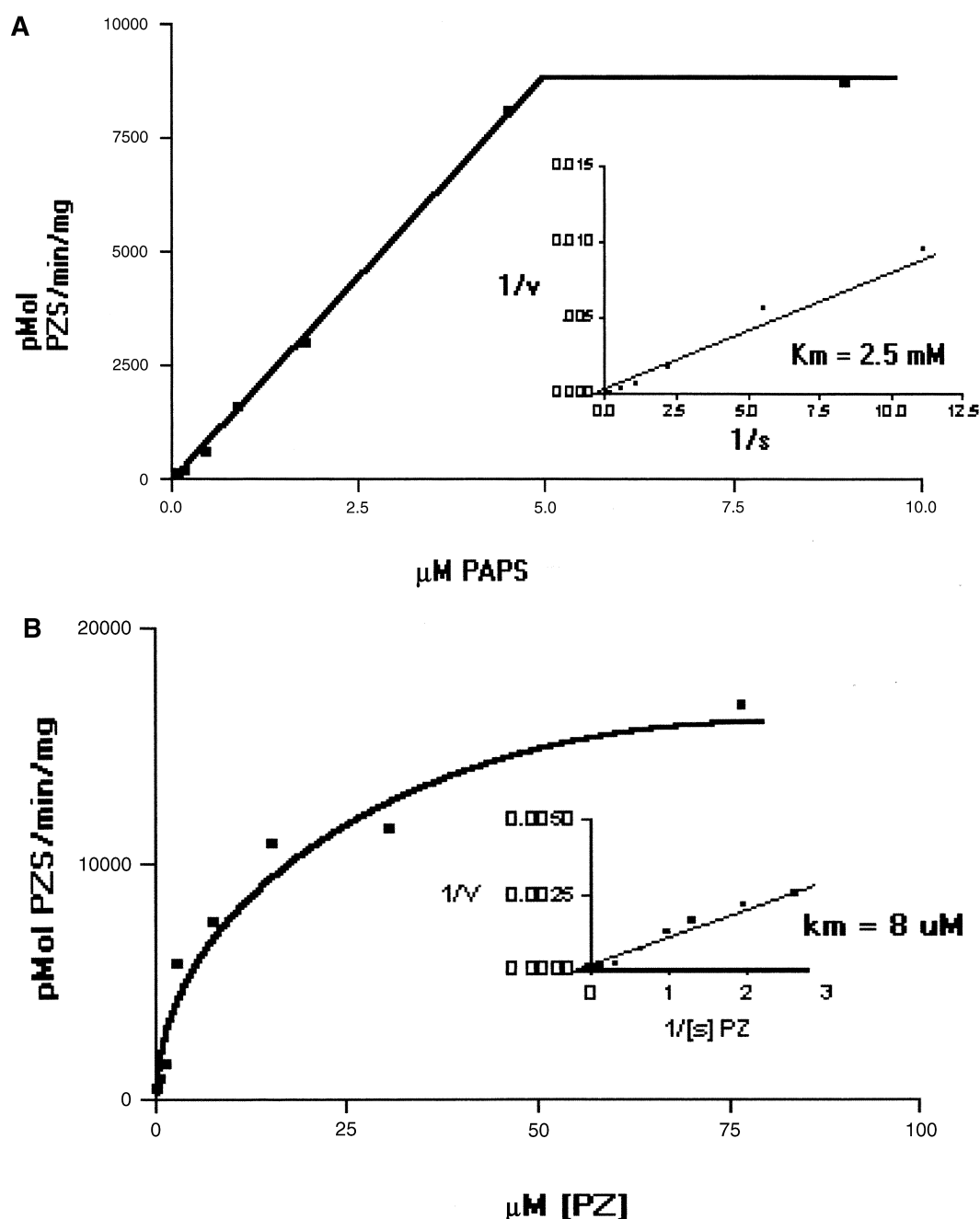
Fig. 9. A: PZ-SULT kinetic analysis against 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as substrate. DEAE chromatography followed by PAP affinity column. Purified enzyme was assayed for PZ-SULT against various concentrations of PAPS, as described in Materials and Methods. Data points represent the average of two independent experiments. Double-reciprocal transformations are shown in the insets. B: PZ-SULT kinetic analysis against petromyzonol (PZ) as substrate. DEAE chromatography followed by PAP affinity column. Partially purified enzyme was assayed for PZ-SULT against various concentration of PZ, as described in Materials and Methods. Data points represent the average of two independent experiments. Double-reciprocal transformations are shown in the insets.

son that the larvae show higher PZ-SULT activity levels could be that they hold the key (the chemoattractant) to the home-coming ovulating females, inasmuch as larvae spend considerable time in the breeding grounds.

When most active larval liver extracts were tested with various analogs for sulfonation, none of the 5β substrates formed significant sulfonated products, except for 5β-PZ, which had slightly above background (25.3 pmol/min/mg), which is about ten times less than 5α-PZ (231.5 pmol/min/mg). This clearly established that the larval liver extracts contained a stereo-specific (5α)-preferring enzyme compared with 5β substrates. Among the 5α substrates tested, only 5α-petromyzonol and 3-keto-5α-PZ formed significant sulfonated products. 3-Keto-5α-PZ is

Fig. 10. Amino acid homology of lamprey PZ-SULT with mammalian SULT. PZ-SULT, SULT2B1a (aa residues 31–45; protein ID, AAC78553.1 or AAC78498.1), SULT2B1b (aa residues 46–60; protein ID, AAC78554.1), and SULT2A1 (aa residues 20–34; protein ID, NP003158.2).

the same as 5α-PZ, except that the 3-keto-PZ contains a keto group at the third position. This clearly indicates that the substitution at position 3 does not affect the sulfonating activity of SULT and that the sulfonation is perhaps occurring at the other hydroxyl positions. The exact biosynthetic sequence of the 5α-PZS and of the more potent chemoattractant 3-keto-5α-PZ have not been elucidated. The pathway for the biosynthesis of C-27 5α-cyprinol has been proposed, and some of the crucial dehydrogenases have been identified (18). On the basis of the SULT activity levels of the two substrates, 5α-PZ (231.5 pmol/min/mg) and 3-keto-PZ (180.8 pmol/min/mg), one can speculate that 5α-PZS is formed first and then the 3α-dehydrogenase oxidizes the 5α-PZS into 3-keto-5α-PZS, the potent chemoattractant. ACA is the same as the 5α-PZ, except that position 24 is carboxyl instead of a hydroxyl group. ACA did not form any sulfonated products, which clearly established that the PZ-SULT prefers the hydroxyl group at the C-24 position for sulfonation. In addition, 5α-PZ-24SO₄, which has three free unconjugated hydroxyl groups at positions 3, 7, and 12 and has the potential to be sulfonated, did not form any radioactive sulfonated products. Thus, we conclude that the PZ-SULT present in larval liver extract is a stereo-specific (5α-PZ-preferring) and also regio-selective (C-24 hydroxyl-preferring) enzyme for sulfonation.

The affinity column-purified PZ-SULT fraction exhibited a specific activity of 2,038 pmol/min/mg and correlated with a band of 47 kDa analyzed by SDS-PAGE. This is the first report on the isolation, purification, and characterization of a novel 5α-cholane (C-24)-specific SULT from fish. The only other known partially purified and characterized SULT from fish is a cholestane (C-27) type, 5β-scymnol SULT, from shark (*Heterodontus portusjacksoni*). The 5β-scymnol SULT enzyme exhibits a molecular mass of 40–45 kDa (9).

The molecular mass is very close to that of the 5α-cholane type, petromyzonol SULT, although evolutionarily, lampreys (boneless) are far removed from sharks (bony fish). Various mammalian SULTs range in molecular mass from 30 to 45 kDa (19).

When the 14-amino acid peptide sequence (M)SIS-QAVDAAFXEI corresponding to putative PZ-SULT was compared with mammalian SULT2A1, SULT2B1a, and SULT2B1b using Omiga DNA and a protein software analysis program, the analysis yielded a remarkable overall

homology of ∼35–40%. It is particularly interesting that the amino acid residue SISxA of the PZ-SULT is highly conserved in mammalian SULT2B1a. The cytosolic SULT superfamily is divided into five families, one of which (SULT2) is primarily engaged in the sulfoconjugation of neutral steroids and sterols. The mammalian hydroxy-steroid SULTs have sizes that range from 282 to 295 amino acids, whereas SULT2B1a and SULT2B1b consist of 350 and 365 amino acids, respectively. Overall, the SULT2A1 and SULT2B1 isozymes are ∼37% identical at the amino acid level (20). Using a purified peak PZ-SULT activity fraction, photoaffinity labeling with PAP³⁵S was performed to confirm the identity of PZ-SULT. Photoaffinity labeling cross-linked the 47 kDa polypeptide, further confirming the isolation of the PZ-SULT polypeptide. Similar photoaffinity labeling of human cytosolic and recombinant SULTs using 2-azidoadenosine 3′, 5′-[5′-³²P]bisphosphate has been reported. (21) 5α-PZ-SULT exhibited a K_m of 8 µM for PZ and 2.5 µM for PAPS.

5β-Scymnol SULT, in comparison, exhibited a K_m of 4 µM for PAPS and 14 µM for 5β-scymnol (9). These values are very close, although they are entirely different enzymes from two different fish: one is a cholestane (C-27) SULT and the other is a cholane (C-24) SULT. In this paper, we have described a novel 5α-cholane-specific SULT-preferring hydroxyl at the C-24 and have reported the characterization of the biochemical properties. Understanding the overall biosynthesis of 5α-PZS is critical to understanding the reproductive cycle of lampreys and to arriving at possible strategies for controlling the overpopulation of lampreys in the Great Lakes.▮▮

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