GREAT LAKES FISHERY COMMISSION

2003 Project Completion Report[1]

Molecular cloning of petromyzonol sulfotransferase of *Petromyzon marinus* and enzymatic synthesis of petromyzonol sulfate

by:

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GLFC-Final Report (Year-2003)

We have isolated, partially purified and characterized the petromyzonol sulfotransferase (PZ-SULT) from the larval lamprey liver. The details of this work has been described, in the attached online published manuscript, which was accepted for publication in the Journal of Lipid Research (**JLR**). The print version of this article will appear sometime in march of next year (2004). JLR is a well reputed journal and the impact is even greater especially after it became part of the American Society of Biochemistry and Molecular Biology (**ASBMB**) journal, similar to the Journal of Biological Chemistry (JBC).

The exact title of the journal article is "**Isolation, Partial Purification and Characterization of a Novel Petromyzonol Sulfotransferase from Petromyzon marinus, Lamprey (Larval) Liver**[#]"

K. V. Venkatachalam, Domingo E. Llanos, Kristophe J. Karami, and Vladimir A. Malinovskii

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http://www.jlr.org/cgi/content/abstract/M300346-JLR200v1?ct

I have included the abstract of this accepted crucial JLR paper. For detailed information kindly refer to the attached full paper below.

***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 homogenates of liver extracts exhibited a PZ-SULT activity of 0.9120 pmol/min/mg in juvenile and 12.62 pmol/min/mg in larvae. Using concentrated crude larval liver extracts and 5 β-cholan substrates like cholic acid and its various derivatives; there was only background level of sulfonated products. The extracts were then tested for sulfotransferase activity using 5α-cholan substrates, 5α-PZ and 3-keto-5α-PZ which exhibited an activity of 231.5 pmol/min/mg and 180.8 pmol/min/mg respectively. With allocholic acid there was negligible sulfotransferase activity. This established that the sulfotransferase present in the lamprey larval liver extracts prefers (5 α) substrates and it is

selective for hydroxyl at the C-24, for sulfonation. Partially purified PZ-SULT exhibits a pH optimum of 8.0; a temperature optimum of 22^{0}C and the stability of the activity was linear for one hour. Using optimal conditions, PZ-SULT activity was then purified by DEAE ion exchange, gel filtration and PAP affinity column chromatography. PZ-SULT exhibited a Km of 2.5 μM for PAPS and a Km of 8 μM for PZ. The affinity purified peak PZ-SULT fraction exhibited a specific activity of 2038 pmol/min/mg. The peak activity fraction while subjected to SDS-PAGE, correlated to a protein with a molecular weight of 47 kDa. Photoaffinity labeling with PAP^{35}S cosubstrate, 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 of (M)SISQAVDAAFXEI, which possessed an overall ~ 35-40% homology with mammalian SULT2B1a.
Abbreviations: SULT, sulfotransferase; PZ, petromyzonol; 3-keto-PZ, 3-ketopetromyzonol

We have obtained tremendous amounts of information on the biosynthesis of the crucial lamprey pheromone PZ and the enzyme PZ-SULT, which catalyzes the formation of this compound, which will be of great value and asset to the GLFC. However our strategies on cloning the cDNA corresponding to PZ-SULT, has not been accomplished yet. Also now I feel that I might have been over ambitious in accomplishing everything to successful completion within the proposed time frame. Nevertheless I have briefly described our tireless efforts in isolating the cDNA corresponding to the PZ-SULT.

Degenerate oligonucleotide probes:

PAPS binding motif is highly conserved among various sulfotransferases (SULT) from different organisms. Using this amino acid information, oligonucleotide probes were designed to isolate the cDNA corresponding to the PZ-SULT by RT-PCR method. Subsequent molecular cloning and DNA sequencing revealed the identity of the RT-PCR products. This process yielded lamprey cDNA's that are unrelated to PZ-SULT. Though may not be relevant directly, perhaps at a later time these informations will be of use in understanding the molecular biology of the lamprey.

These cDNA informations were deposited into GenBank.

1. **Petromyzon marinus KIAA0542-like protein mRNA, partial sequence.**
   GenBank ACCESSION: AY090634

2. **Petromyzon marinus vitellogenin-like protein mRNA, partial cds.**
   GenBank ACCESSION: AY077582

Thus our attempts to isolate the PZ-SULT cDNA using degenerate primers, yielded only 2 other unrelated lamprey cDNA's. However during the early years of this project (year 2002) one of the undergraduate student won the ASBMB travel award, which is a feather on the cap for the GLFC undertakings.

Isolation of PZ-SULT cDNA using gene specific primers:

Using the amino terminal peptide sequence corresponding to the PZ-SULT protein (for exact sequence, please refer to the attached accepted JLR article), oligonucleotide primer was designed. Using this primer RT-PCR was performed. Though being gene specific primer it did not yield PCR products, perhaps due to improper PCR conditions. We are varying the annealing temperature, Mg_2^+ concentration etc., to obtain the PZ-SULT cDNA. Touchdown PCR is a another method that is being tested for obtaining specific products. We have also raised peptide antibodies, in hope for screening cDNA library. I have proposed all these in the forthcoming pre-proposal to make it a dream come true. With the available tools (gene specific primers, peptide antibodies) and better strategies proposed in my 2004 pre-proposal the chances of obtaining PZ-SULT cDNA is great. With the much wanted cDNA for PZ-SULT a lot can be done in the way of gene expression and regulation of PZ-SULT.

Again I am looking forward to explore more on this sea of biochemistry/molecular biology of lamprey world, in answering questions that are crucial to the GLFC, so that strategies are available to control lamprey over-population in the Great Lakes. With our knowledge on the biochemistry of PZ-SULT it is only going to be better for future successful paths in understanding the molecular biology of this pheromone synthesis.

Isolation, Partial Purification and Characterization of a Novel Petromyzonol Sulfotransferase from Petromyzon marinus, Lamprey (Larval) Liver[#]

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Running Title: Biosynthesis of a Chemoattractant Petromyzonol (5α-cholan-3α, 7α, 12α, trihydroxy) 24-sulfate.

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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 homogenates of liver extracts exhibited a PZ-SULT activity of 0.9120 pmol/min/mg in juvenile and 12.62 pmol/min/mg in larvae. Using concentrated crude larval liver extracts and 5β-cholan substrates like cholic acid and its various derivatives; there was only background level of sulfonated products. The extracts were then tested for sulfotransferase activity using 5α-cholan substrates, 5α-PZ and 3-keto-5α-PZ which exhibited an activity of 231.5 pmol/min/mg and 180.8 pmol/min/mg respectively. With allocholic acid there was negligible sulfotransferase activity. This established that the sulfotransferase present in the lamprey larval liver extracts prefers (5α) substrates and it is selective for hydroxyl at the C-24, for sulfonation. Partially purified PZ-SULT exhibits a pH optimum of 8.0; a temperature optimum of 22^{0}C and the stability of the activity was linear for one hour. Using optimal conditions, PZ-SULT activity was then purified by DEAE ion exchange, gel filtration and PAP affinity column chromatography. PZ-SULT exhibited a Km of 2.5 mM for PAPS and a Km of 8 mM for PZ. The affinity purified peak PZ-SULT fraction exhibited a specific activity of 2038 pmol/min/mg. The peak activity fraction while subjected to SDS-PAGE, correlated to a protein with a molecular weight of 47 kDa. Photoaffinity labeling with PAP^{35}S cosubstrate, 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 of (M)SISQAVDAAFXEI, which possessed an overall ~ 35-40% homology with mammalian SULT2B1a.

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

Supplementary key words: sulfotransferase, lamprey, petromyzonol sulfate, chemoattractant, pheromone.

INTRODUCTION

In mammals bile acids and salts are synthesized in the liver and stored in the gall bladder (1), which aids in solubilizing fats which facilitates lipolysis (2). A substituent in cyclopentanoperhydrophenanthrene nucleus (3) that is above the plane is termed beta (β) whereas a substituent that is below the plane is alpha (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 (7). Higher (C-27) bile acids and bile alcohols (also called cholestanes) are found in many organisms. For example in small skate *Raja erinacea,* the major sulfated bile alcohol is scymnol sulfate (Scys), [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 portusjacksoni* has been reported (9). In 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 has been reported (10). The identification of cyprinol sulfate from grass carp bile and its toxic effects in rats has 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 and similar to cholestanes (C-27), can also possess hydrogen with an α or β orientation at position number 5, with the usual hydroxyls at 3, 7, 12 (either α, or β) and a carboxyl (bile acids) or hydroxyl (bile alcohol) group at 24th position. 5α-PZ is a 5α -cholan- 3α, 7α, 12α, 24-tetrol (7). PZ has been shown to be produced in copious amounts in *Petromyzon marinus* (lamprey), a jawless, boneless primitive 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 role as a pheromone

during the reproductive life cycle of lamprey (17). The adult lamprey has been shown to come 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, 16, 17). The Great Lakes of North America is over populated 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 to control the over population of this organism is to use 5α-PZS and its derivative as the bait to trap adults. The biosynthesis and the regulation of the 5α-PZS is very crucial in 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 that is stereo selective (5α-cholan) and a regio selective (for hydroxyl at C-24) preferring enzyme from larval livers of lamprey.

MATERIALS AND METHODS

Materials

Radiochemicals [^{35}S] 3'-phosphoadenosine 5'-phosphosulfate (PAPS) for enzyme assays was purchased from NEN life science products or ARC Inc. Cholan substrates allocholic acid, 3-keto-PZ, PZS, nordesoxy cholic acid (NDC), 5α-PZ are purchased from Toronto Research Chemicals Inc (Toronto, Canada). 5α-PZ is also purchased from Cayman Co (Ann Arbor MI). 5β-24-ol, 5β-PZ, were purchased from Steraloids, Inc., (Newport, RI). Cholic acid (CA), lithocholic acid (LCA), cholesterol (Cholest), deoxylithocholic acids (DLCA) were purchased from Sigma-Aldrich Inc. DEAE- ion exchange matrix (Macro-prep DEAE support) was 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 pre-stained 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) column affinity matrix, Sephadex (G-

100-50) exclusion limit > 100,000 Mw, 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 3 µl of reaction buffer [150 mM 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. Typical reaction was carried out at 22^0C for 5 min and stopped by placing reaction tubes in boiling water for 5 min. The contents were briefly centrifuged and 1µl aliquots were transferred to silica gel thin-layer chromatography plates and chromatographed using chloroform:methanol:water (70:26:4) 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^oC-15^0C. Frozen larval livers ~1.39g (7-8 mg/larval liver) were ground in 3 ml of homogenization buffer [100 mM tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, protease inhibitor cocktail III from Calbiochem (San Diego, CA)], with a pinch of sand in a mortar and pestle. The mortar was rinsed with 2 ml of homogenization buffer and 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 x g. The resulting supernatant was then subjected to centrifugation at 10,000 x g. The supernatant was then subjected to 100,000 x g and the resulting soluble supernatant was used for PZ-SULT purification.

PZ-SULT Purification

The soluble extracts were applied onto 5 ml ion exchange (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 concentration of NaCl ranging from 0.05 M-0.4 M. ~10 ml per step gradient were collected. Protein contents in fractions were measured using the dye-binding method as described by (Bio Rad). 4 µl 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 on to 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, concentrated using Ultrafree-4 centrifugal filter unit, [Mw cut off 5 kDa, (Millipore)] to ~ 1.8 ml. A portion of the fraction (1.6 ml) was applied onto a 2 ml affinity column matrix, adenosine-3', 5'-diphosphate (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 (Carlsbad, CA).

Photoaffinity Labeling

Larval liver 100, 000 x g, supernatant was purified through DEAE ion exchange chromatography similar to the procedures before. 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 non-peak fraction was concentrated using, Ultrafree-4 centrifugal filter unit, [Mw cut off 5 kDa, (Millipore)]. Peak PZ-SULT active fraction and a non-peak, 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 cold chase experiment, the reaction contained an additional 0.36 mM non-radioactive 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/cm^2) at 25^{0}C using CL-1000 Ultraviolet crosslinker from UVP, Inc., (Upland, CA). The reaction was chilled on ice and stopped by adding 6 μL of SDS-PAGE loading buffer from Invitrogen (Carlsbad, CA). The proteins were then denatured by heating the samples at 95^{0}C for 5 min. A 35 μL aliquot was loaded on to 12% Bis-tris gels. Gels were stained with Silver-Express according to instructions provided by Invitrogen (Carlsbad, CA). For autoradiography the gels were dried and exposed to x-ray film Xomat AR from (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 and then treated with 100 μl of 2% CNBr 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% TFA for 12 h and 2 times with 20% acetonitrile without TFA. The supernatants containing the digested peptides were removed. The remaining membrane after acetonitrile/water/TFA extractions 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 Company; (Madison, WI). GenBank deposited mammalian sulfotransferases used in the analysis were, SULT2B1a (protein ID: AAC78553.1), SULT2B1b (protein ID: AAC78554.1), SULT2B1a (protein ID: AAC78498.1), SULT2A1 (protein ID: NP 003158.2).

RESULTS

Isolation of PZ-SULT

PZ-SULT activity in Juvenile versus Larval Liver

Liver tissue extracts (100 x g supernatant) from juvenile and larvae were tested for PZ-SULT activity. Tissue extracts from both juvenile and larvae, without the exogenously added 5α-PZ cosubstrate exhibited negligible activity. There was ten-fold higher PZ-SULT activity with 5α-PZ in larvae (12.62 pmol/min/mg) compared to juvenile activity of (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 sulfotransferase activity. The analogs tested included cholesterol (cholest) which is the precursor for all C-27 cholestane, 5α-cholan substrates: [5α-PZ, 5α-PZS, 3-keto-5α -PZ, allo cholic acid (ACA)]. 5β−cholan substrates: [5β-PZ, 5β-cholan-24ol (5β-24ol), nordesoxycholic acid (NDC), cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA). None of the common 5 β bile acid compounds (CA, NDC, DCA, LCA)] and 5β-24-ol a bile alcohol, formed any sulfonated products. 5β-PZ possessed a negligible activity of 25.3 pmol/min/mg. With 5α-PZ the extract contained a sulfotransferase activity of 231.5 pmol/min/mg. With 3-keto-5α-PZ, the activity was 180.8 pmol/min/mg. All other substrates including allocholic acid exhibited only background activity for sulfonation (Fig. 2). Allocholic acid is same as the 5α -PZ except at the 24^{th} position it has a carboxyl group instead of a hydroxyl group. The PZ-SULT activity exhibits a temperature optimum of 22 ^{0}C (Fig. 3A). The activity was stable and linear for 1 hour of incubation at 22 ^{0}C at pH 8.0 (Fig. 3B) and a pH optimum of (8.0) (Fig. 3C). The partially purified enzyme when tested with analogs (ACA, 5β-PZ, 5α-PZS) showed negligible activity similar to crude extracts (data not included).

PZ-SULT purification

The 100,000 x g, supernatant preparation containing soluble proteins exhibited a PZ-SULT activity of 7.68 pmol/min/mg was used for protein purification. Upon chromatography over DEAE-ion exchange column the PZ-SULT peak activity eluted at 0.3 M NaCl (Fig. 4). Peak fractions (30-35) about 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 ultrafree-4 centrifugal filtration unit (Mw cut off 5 kDa). About 1.6 ml of the concentrated PZ-SULT fractions were allowed to bind to PAP affinity column and the unbound proteins were removed by washing with buffer A containing 0.05 M NaCl. The bound proteins eluted with 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 2038 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, however it contained the higher molecular weight contaminants. (Fig. 7).

Photoaffinity Labeling

For routine purification, gel filtration step was eliminated since 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 carry over of many contaminating proteins. When the peak fraction that had most PZ-SULT activity (10931.8 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 8 A). 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 PAP^{35}S and for subsequent autoradiography. From the purified PZ-SULT active fraction, the autoradiography revealed a photoaffinity labeled band corresponding to 47 kDa and no other labeled products were observed and from the fraction that did not contain PZ-SULT activity no

labeled products were observed (Fig 8 B). In addition, (data not included) when labeling was performed using PZ-SULT active fraction, in the presence of excess non-radioactive PAPS (0.36 mM), no radioactive band was detected confirming the specific labeling by the cosubstrate PAPS of the sulfotransferase.

Kinetic Analyses

Partially purified enzyme preparations from DEAE ion-exchange and PAP affinity column purified fractions which contained copious PZ-SULT activity were used for determining the kinetic parameter km for PAPS and PZ. With varying PZ concentration and a fixed concentration of PAPS (9 μM), the PZ-SULT exhibited a Km 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 measured. The PZ-SULT exhibited a km of 2.5 μM for PAPS (Fig. 9B).

Protein Sequencing

PZ-SULT protein is N-terminally blocked. Partial amino acid sequence yielded a sequence of (M)S I SQAVDAAFxE I. Partial amino acid comparison of PZ-SULT with mammalian sulfotransferases, SULT2B1a (GenBank protein ID: AAC78553.1), SULT2B1b (GenBank protein ID: AAC78554.1), SULT2B1a (GenBank protein ID: AAC78498.1), 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 sulfotransferase activity using lamprey larval liver extracts did not form any sulfonated products. Cholanes, similar to cholestanes, possess the usual tri-hydroxyl groups at positions 3, 7 and 12 and the carbon at 24^{th} position is carboxyl (bile acids) or hydroxyl (bile alcohol) (4). Certain primitive fish such as the lamprey and lung fish produces 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 the ability to serve as a pheromone during the reproductive life cycle. The sulfo-conjugate and not the free alcohol of 5α-PZ and its derivative (3-keto-5α-PZ) serve as chemoattractant (15, 16, 17). When larval and juvenile liver extracts were tested for PZ-SULT activity the larval liver extract contained about ten fold higher PZ-SULT activity. Perhaps this makes sense based on the reproductive life cycle of lamprey and the associated role of PZS as a chemoattractant. Sea lamprey belongs 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 on the bottom of the streams where they hatch and remain there, 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, 16, 17). The migration of lamprey is somewhat reminiscent of the well-known salmon runs. The lamprey return to spawn in the shallow streams which had supported spawning in the past. Lampreys recognize the species-specific odor compound of 5α-PZS derivative. Ovulating lamprey is attracted to 3-keto-5α-PZS produced by the male and uses this as a cue for spawning (16). The apparent reason for higher PZ-SULT activity levels in larvae could be speculated in that the larvae holds the key (the chemoattractant) to the home coming ovulating females since larvae spends the most amount of time in the same breeding grounds of adults.

When most active larval liver extracts were tested with various analogs for sulfonation none of the 5β substrates formed significant sulfonated products except 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 to 5β substrates. Among the 5α substrates tested, only 5α-petromyzonol and 3-keto-5α-PZ formed significant sulfonated products. 3-keto-5α-PZ is the same as 5α-PZ except that the 3-keto-PZ contains a keto group at the 3rd position. This clearly indicates that the substitution at position number three does not affect the sulfonating activity of SULT and the sulfonation is perhaps occurring at the other hydroxyl positions. The exact

biosynthetic sequence of the 5α-PZS and the more potent chemoattractant 3-keto-5α-PZ has 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). Based on the sulfotransferase activity levels between 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 first formed and then the 3α-dehydrogenase oxidizes the 5α-PZS into 3-keto-5α-PZS the potent chemoattractant. Allocholic acid is the same as the 5α-PZ except the 24^{th} position is carboxyl instead of a hydroxyl group. Allocholic acid 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, although 5α-PZ-24SO_4 which has three free unconjugated hydroxyl groups at positions (3, 7, 12) and has the potential to be sulfonated, it did not form any radioactive sulfonated products. Thus, we conclude that the PZ-SULT present in larval liver extract is stereo specific (5α-PZ preferring) and it is also regio selective (C-24 hydroxyl) preferring enzyme for sulfonation.

The affinity column purified PZ-SULT fraction exhibited a specific activity of 2038 pmol/min/mg and it 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 sulfotransferase from fish. The only other known partially purified and characterized sulfotransferase from fish is a cholestane (C-27) type, 5β-scymnol sulfotransferase, from shark (*Heterodontus portusjacksoni*). The 5β-scymnol sulfotransferase enzyme exhibits a molecular weight of 40-45 kDa (9).
The molecular weight is very close to the 5α-cholane type, petromyzonol sulfotransferase although evolutionarily lamprey (boneless) is far removed from sharks (bony fish). Various mammalian sulfotransferases ranged in Mw from 30-45 kDa (19).

The 14 amino acid peptide sequence (M)SISQAVDAAFxE I corresponding to putative PZ-SULT, when compared with mammalian SULT2A1, SULT2B1a and SULT2B1b, using Omiga DNA and protein software analysis program, the analysis yielded a remarkable overall homology of (~ 35-40%). It is particularly interesting that the amino acid residues SISxA of the PZ-SULT is highly conserved in mammalian SULT2b1a. The cytosolic sulfotransferases (SULT) superfamily is divided into five

families one of which (SULT2) is primarily engaged in the sulfoconjugation of neutral steroids and sterols. The mammalian hydroxysteroid sulfotransferases have sizes that range from 282-295 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 purified peak PZ-SULT activity fraction, photoaffinity labeling with $PAP^{35}S$ was performed to confirm the identity of PZ-SULT. Photoaffinity labeling crosslinked the 47kDa polypeptide, that further confirmed the isolation of a PZ-SULT polypeptide. Similar photoaffinity labeling of human cytosolic and recombinant sulfotransferases using 2-azidoadenosine 3', 5'-[5'-^{32}P] bisphosphate has been reported. (21)

5α-PZ-SULT exhibited a Km of 8 μM for PZ and a Km of 2.5 μM for PAPS. 5β-scymnol sulfotransferase, in comparison exhibited a Km of 4 μM for PAPS and 14 μM for 5β-scymnol (9). These values are very close although they are entirely different enzymes; one is a cholestane (C-27) sulfotransferase and the other is a cholane (C-24) sulfotransferase from two different fish. In this paper we have described first, 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 extremely crucial in understanding the reproductive life cycle and possible strategies for controlling the over population of lamprey in the Great Lakes.

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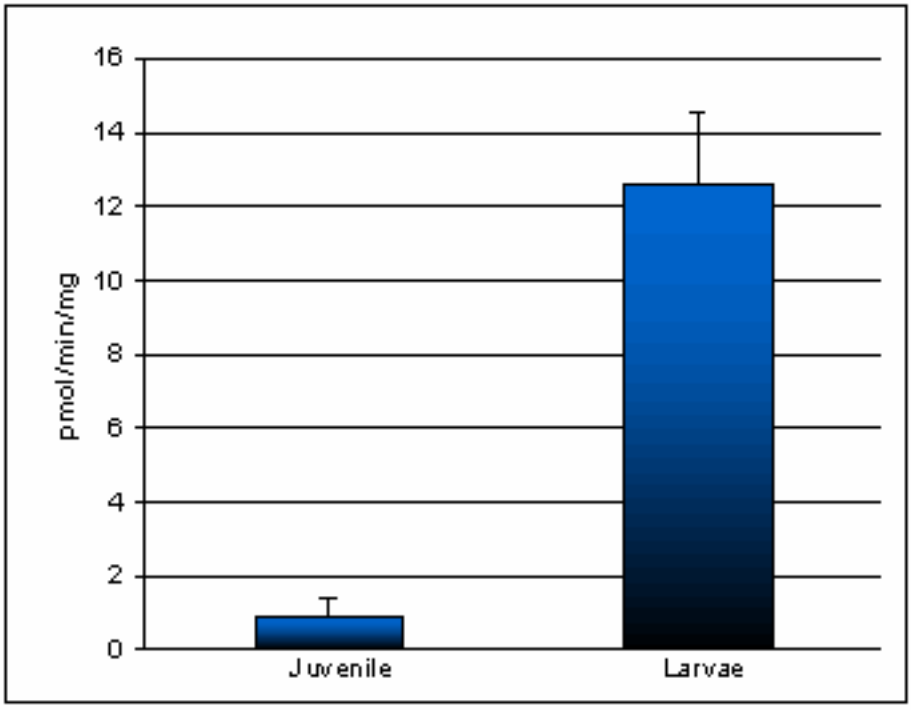

Figure 1. (B) PZ-SULT activity in juvenile and larval liver extracts. Data represent the mean values (n=3) and error bars indicate the standard deviation.

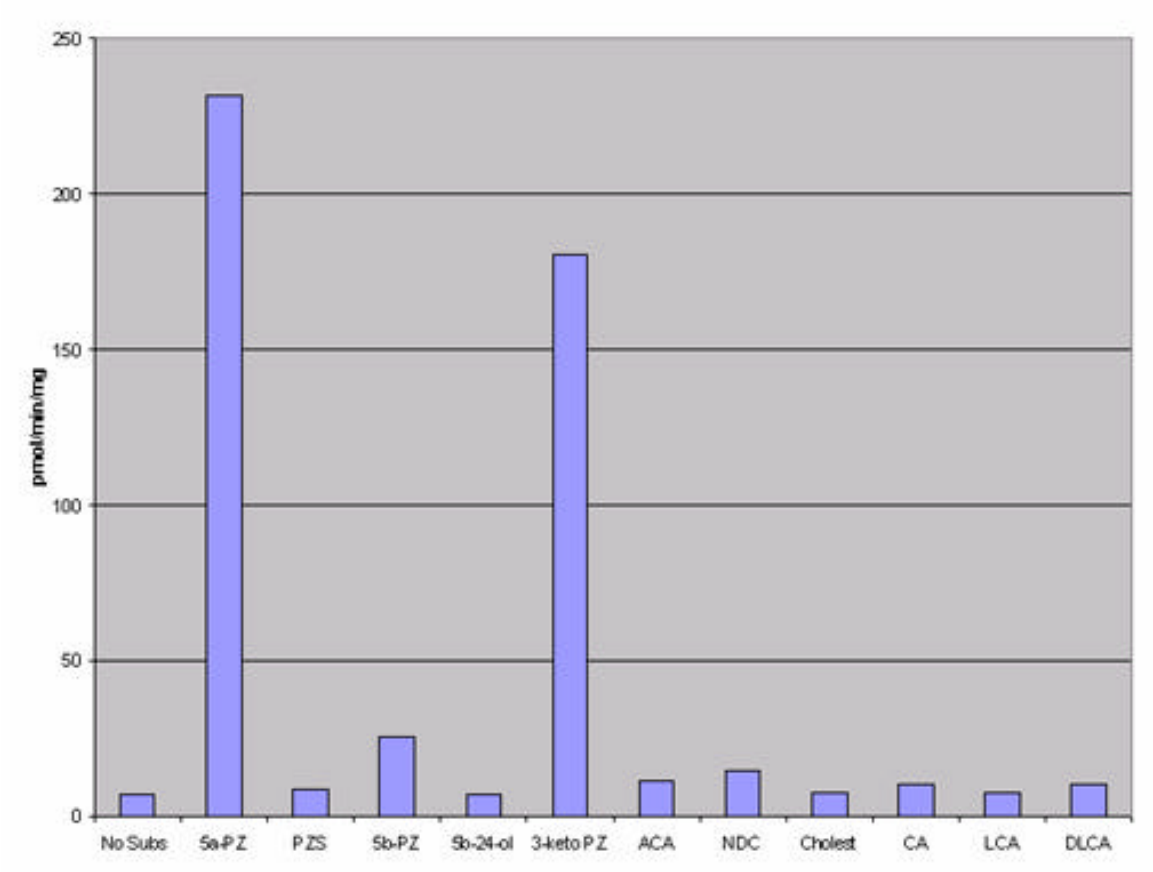

Figure 2: **Sulfotransferase activity in crude larval liver extracts against various compounds**. The compounds were dissolved in ethanol. The final concentration of the substrates in 10 µl enzymatic reaction ranged from 0.768 mM 5α-PZ, 0.14 mM PZ-24-SO_4, 0.423 mM 5β-PZ, 0.58 mM 5β-cholan-24-ol, 1.02 mM 3-keto-PZ, 1.22 mM ACA (allocholic acid), 1.32 mM NDC (nordesoxy cholic acid), 0.784 mM of cholest, (cholesterol) 0.742 mM of CA (cholic acid), 0.805 mM of LCA (lithocholic acid), 0.772 mM of DLCA (deoxy lithocholic acid). No subs reaction was substituted with plain ethanol. Data represent the mean values (n=2).

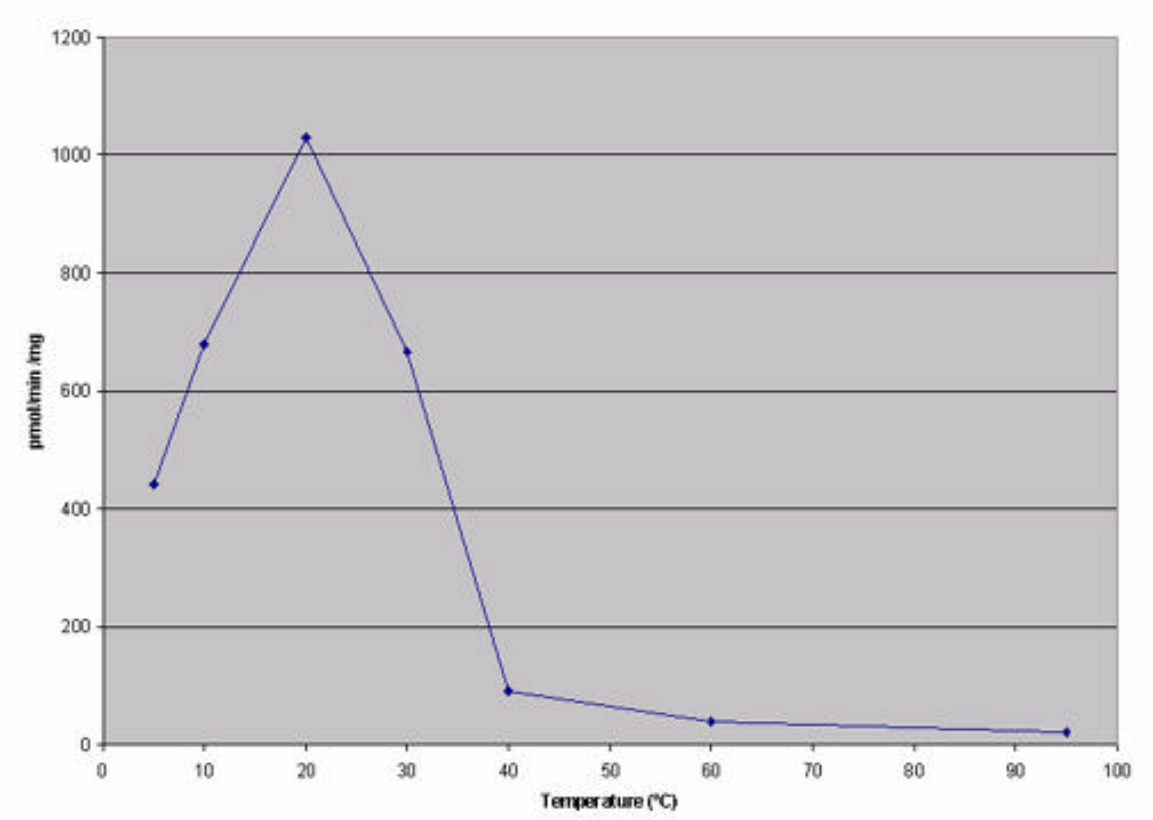

Figure 3A **Effect of Temperature on the PZ-SULT activity**. Reaction at each temperature was performed for 15 min at pH 8.0 according to the standard assay procedures described in the methods.

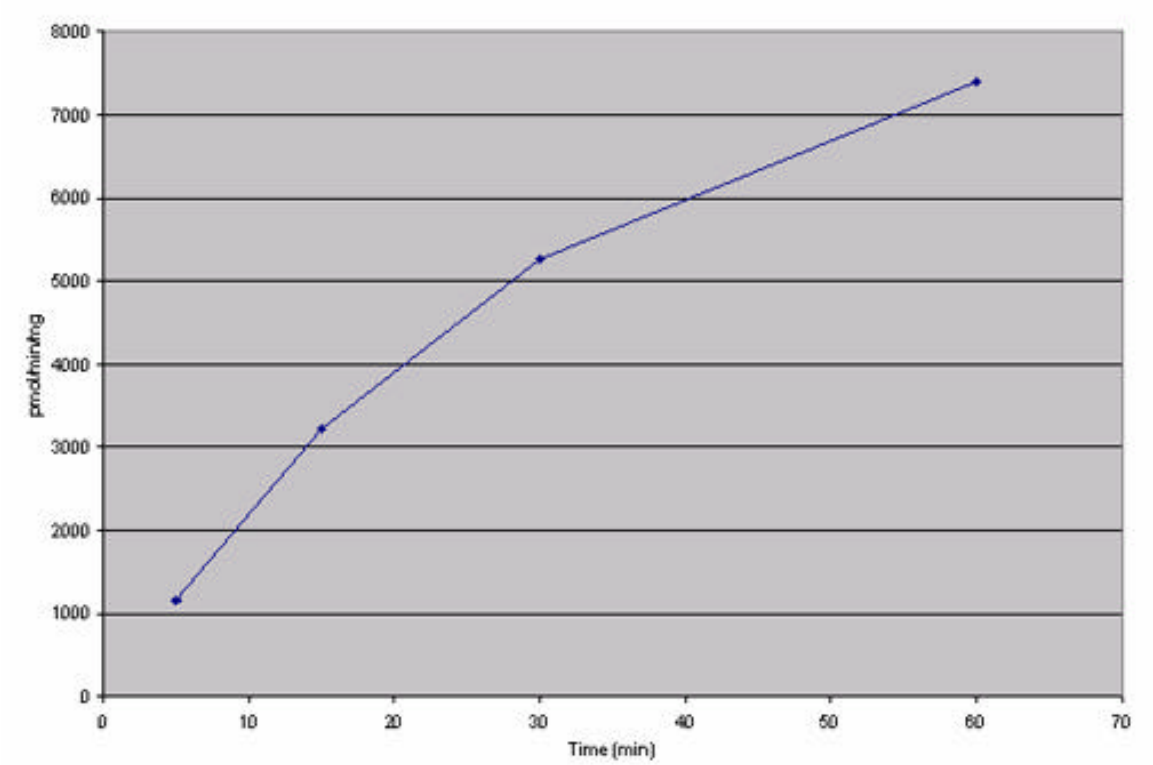

Figure 3B **Effect of time on the PZ-SULT activity**. Reactions at various time periods were performed at 22^{0}C, pH 8.0. The reactions were then stopped by heat inactivation by boiling at 95^{0}C, and the contents were processed, according to the standard assay procedures described in the methods.

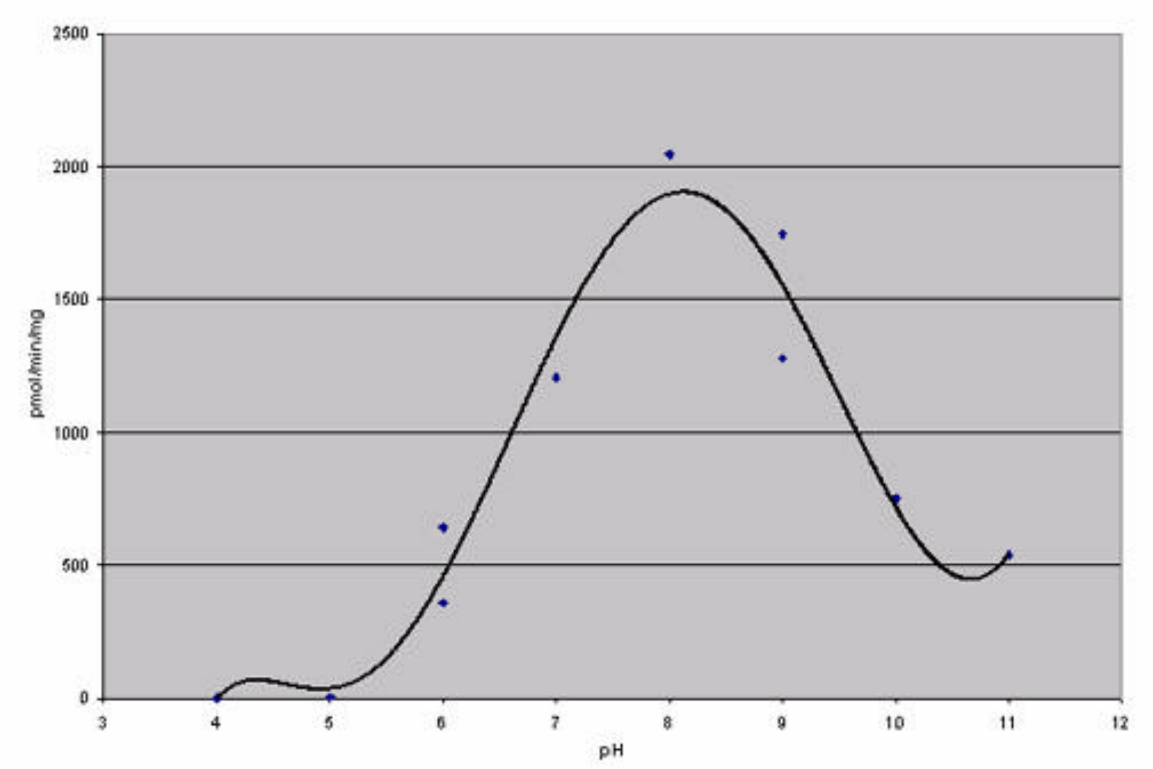

Figure 3C **Effect of pH on the PZ-SULT activity**. Reactions were performed for 15 min at 22^0C, using respective reaction buffers of various pH that contained all the ingredients as the standard assay described in the methods. [The following buffers were used for various pH: citrate reaction buffer (pH 4,5 and 6), phosphate buffer (pH 6 and 7), Tris-HCl (pH 8, 9) and glycine (pH 9, 10 and 11).]

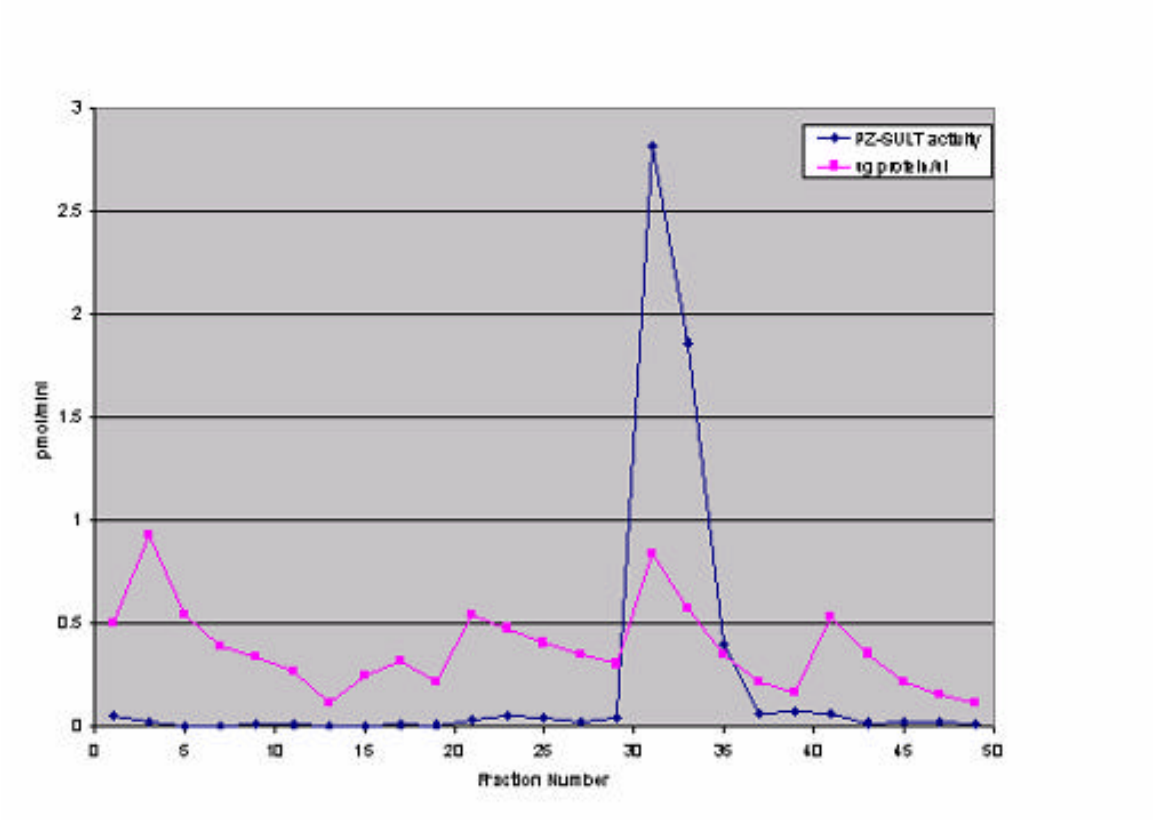

Figure 4 **DEAE –Macro prep elution profile of PZ-SULT**. Crude larval liver 100,000 x g supernatant was purified through DEAE ion exchange columns. The column was washed with buffer A with no salt to remove unbound proteins and then eluted with Buffer A containing NaCl (0.05 M to 0.4 M) consisting of 10 ml step gradients. About 1 ml fractions were collected and a 4 µl aliquot was assayed for PZ-SULT activity and protein amounts were determined by Bio Rad dye binding assay and the protein contents per µl are represented (as described in Experimental Procedures). Peak PZ-SULT activity eluted at 0.3 M NaCl concentration.

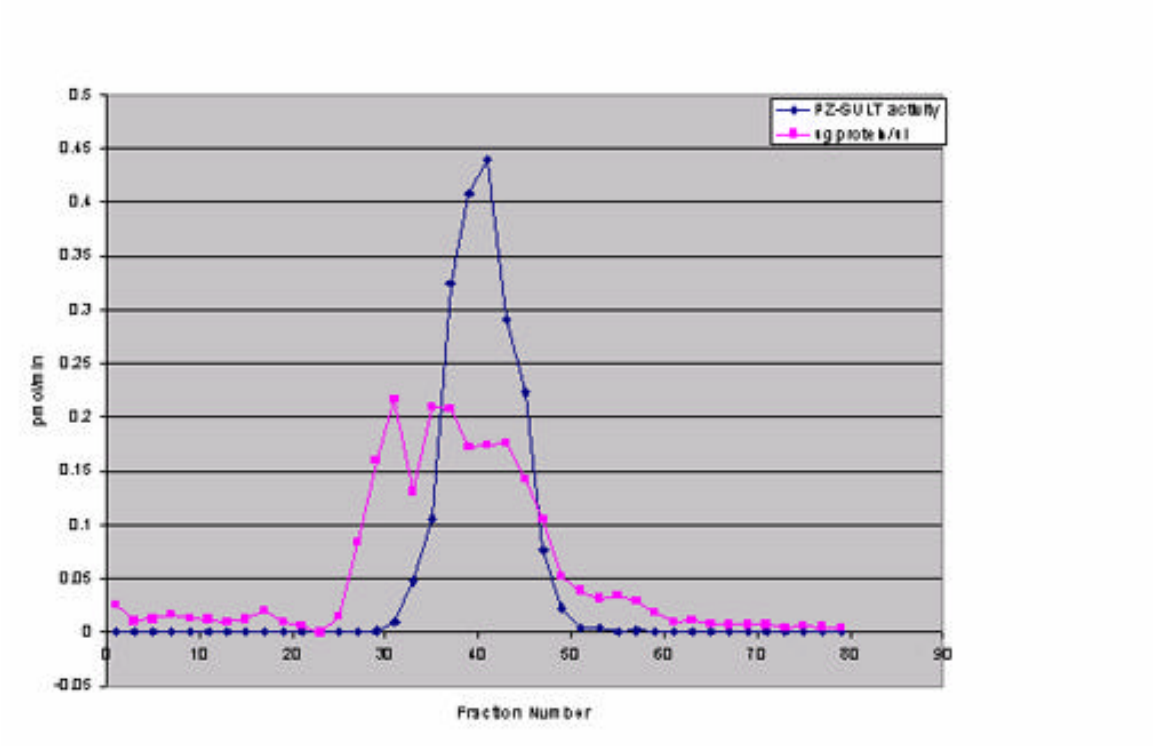

Figure 5 **Sephadex G-100 gel filtration chromatography purification of PZ-SULT**. DEAE fractions 30-35 that contained PZ-SULT activity was pooled and loaded on to the gel filtration column. The column was eluted with buffer A containing 0.05 M NaCl at a rate of 1 ml/12 min. PZ-SULT activity in 4 µl aliquot from 1 ml fraction was measured and protein per 1 µl is represented.

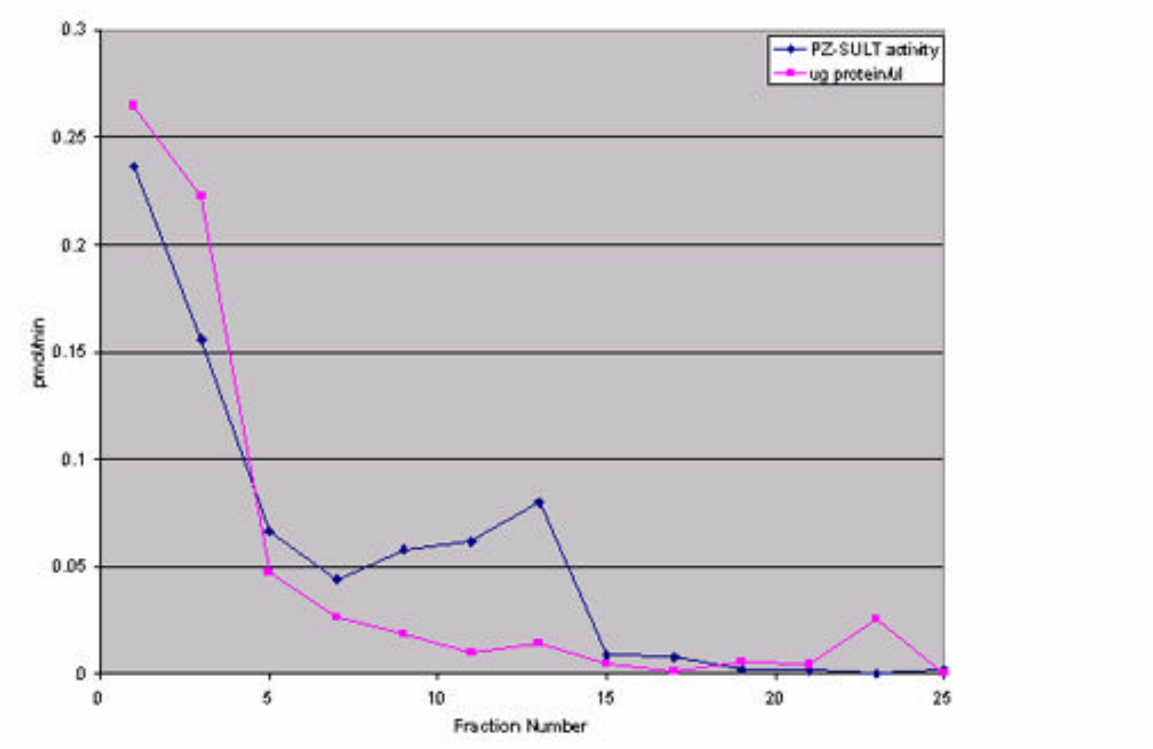

Figure 6 **3'-phosphoadenosine 5'-phosphate (PAP) affinity column chromatography purification of PZ-SULT**. PZ-SULT fractions (33-47) from gel filtration chromatography was pooled and concentrated using Amicon ultra filtration unit (Mw cut off ~5 kDa) and a 1.6 ml concentrate was passed through PAP column five times and 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 –0.4 M) step gradient. The activity that did not bind to the column eluted in buffer containing low salt wash of 0.05 M NaCl. The bound PZ-SULT activity eluted in buffer containing 0.2 M NaCl.

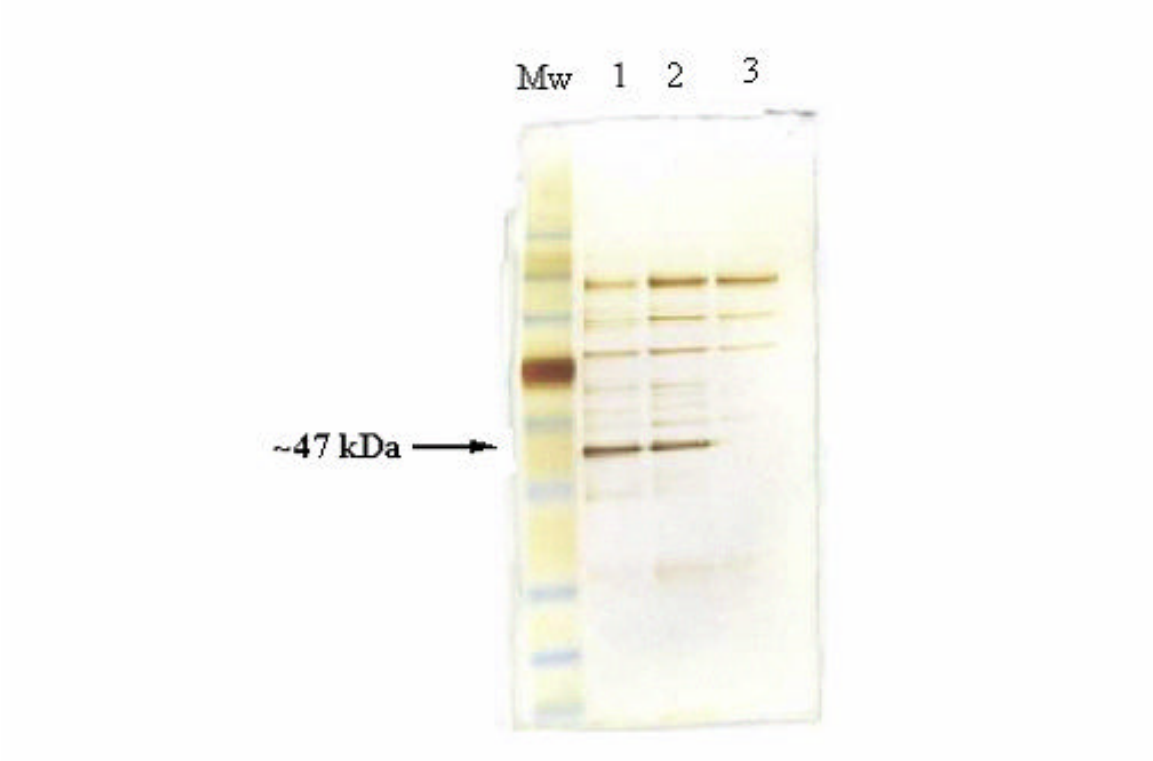

Figure 7 **SDS-PAGE analysis of PAP affinity column purified PZ-SULT**. Fractions 13 and 14 which contained PZ-SULT activity and fraction15 that contained no PZ-SULT activity were analyzed by 12% Bis-tris SDS-PAGE gels and stained with silver. Prestained Mw marker from Invitrogen followed by, lane 1 and 2 corresponds to PZ-SULT active fractions that contained, the major protein (47 kDa), which is indicated by the arrow. Lane 3 that contained no PZ-SULT activity lacks the 47 kDa band.

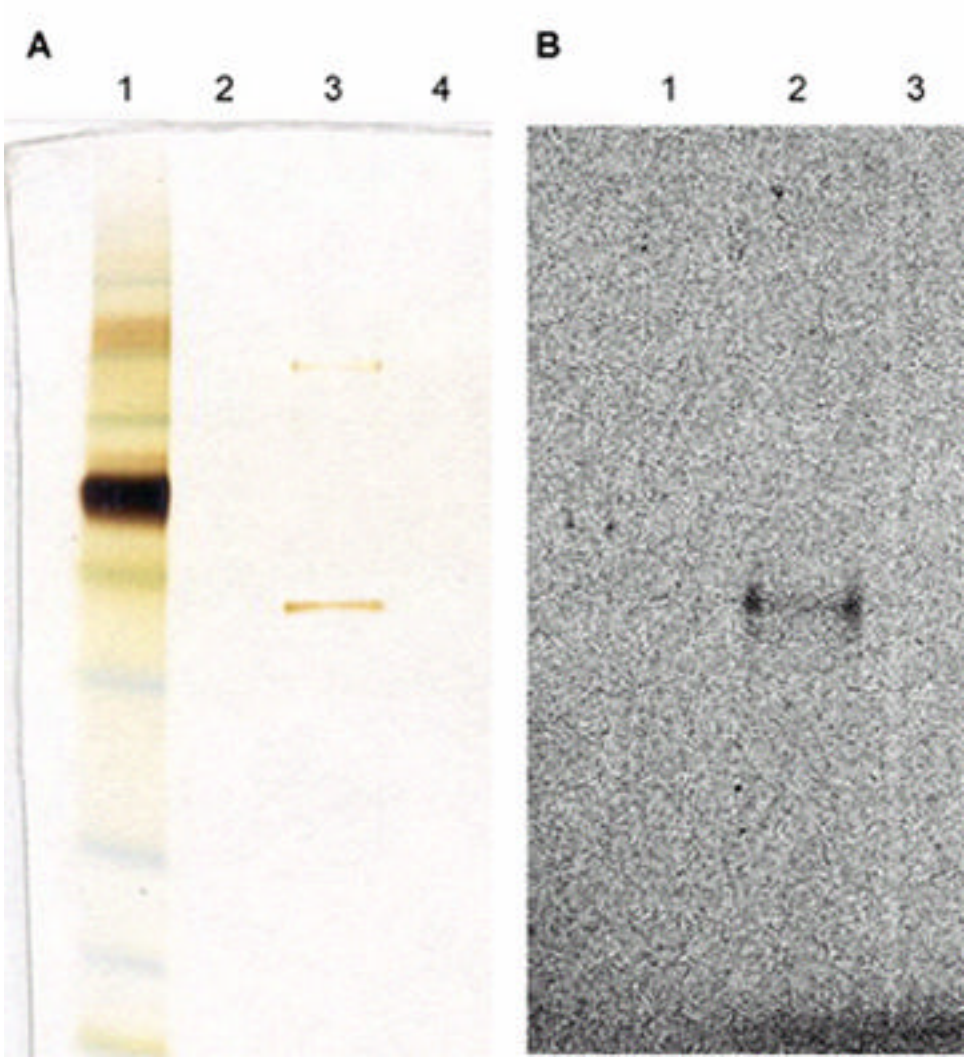

Figure 8. **Photoaffinity Labeling of peak PZ-SULT activity protein**. (A) SDS-PAGE analysis for purity of the PZ-SULT preparation used for photoaffinity labeling. 1. Mw marker, 2. empty lane, 3. PZ-SULT peak fraction (0.67 μg), 4) fraction that contained no PZ-SULT activity (0.25 μg). (B) Autoradiograph of the photoaffinity labeling. 1. empty lane, 2. Peak PZ-SULT fraction showing the crosslinked, labeled 47kDa protein. 3. Lane corresponding to fraction that contained no PZ-SULT activity.

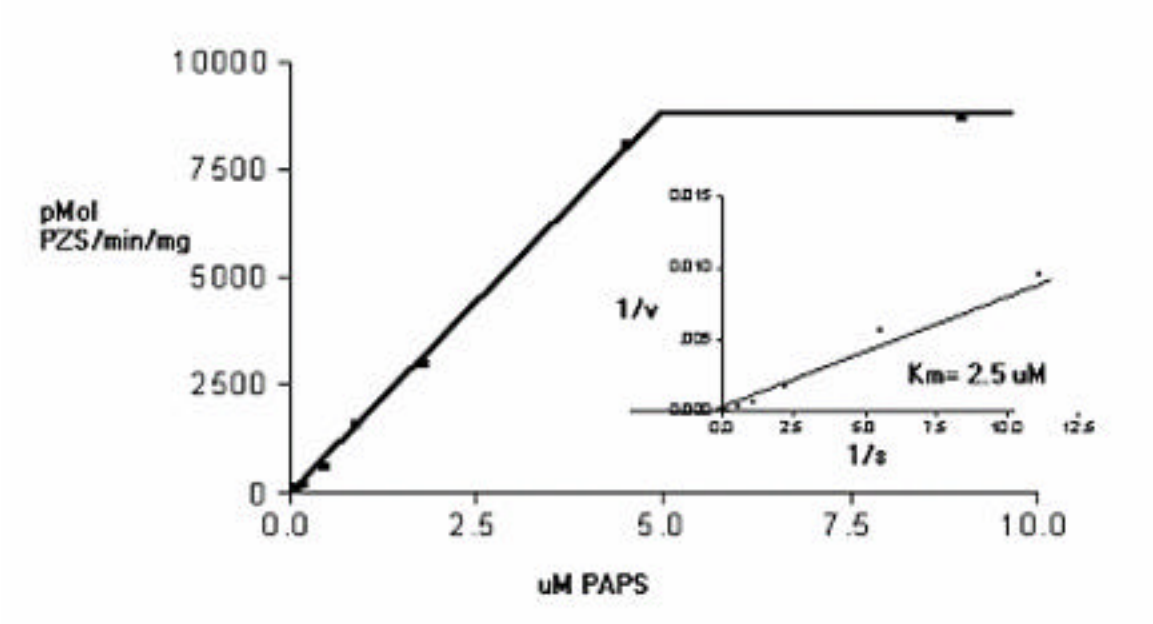

Figure 9A. **PZ-SULT kinetic analysis against PAPS as substrate**. DEAE chromatography followed by PAP affinity column, purified enzyme was assayed for PZ-SULT against various concentrations of PAPS as mentioned under "Experimental Procedures". Data points represent the average of two independent experiments. Double-reciprocal transformations are shown in the *insets.*

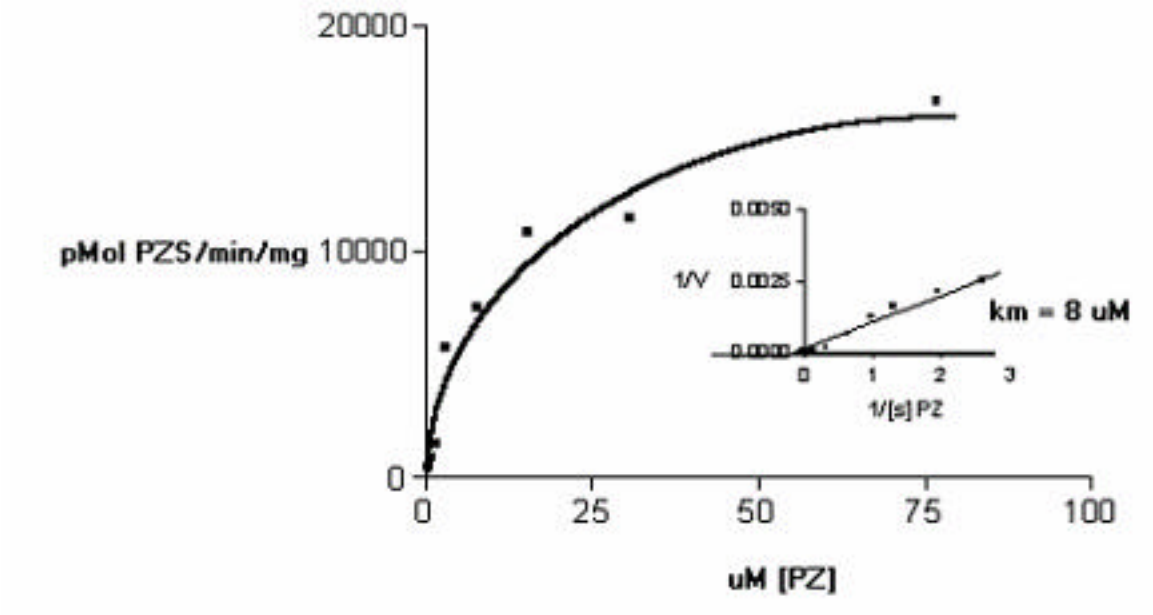

Figure 9B. **PZ-SULT kinetic analysis against PZ as substrate**. DEAE chromatography followed by PAP affinity column, partially purified enzyme was assayed for PZ-SULT against various concentration of PZ as mentioned under “Experimental Procedures”. Data points represent the average of two independent experiments. Double-reciprocal transformations are shown in the *insets.*

Figure 10. **Amino acid homology of lamprey PZ-SULT with mammalian SULT**. lamprey petromyzonol sulfotransferase (PZ-SULT), SULT2B1a (aa residues 31-45) (protein id: AAC78553.1 or AAC78498.1), SULT2B1b (aa residues 46-60) (protein id: AAC78554.1), SULT2A1 (aa residues 20-34) (protein id: NP003158.2).