SPECIFICITY STUDIES OF RECOMBINANT 2-(2'-HYDROXYPHENYL) BENZENESULFINATE DESULFINASE (DszB) FROM NOCARDIA ASTERIODES A3H1 AND RHODOCOCCUS ERYTHROPOLIS IGTS8

By

Daniel N. Cromwell

A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biochemistry
December 2013

Committee Members:

Linette M. Watkins, Chair

Steve Whitten

Rachell E. Booth

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

ACKNOWLEDGEMENTS

I would like to thank Dr. Linette M. Watkins, Dr. Steve Whitten, Dr. Rachell Booth, Dr. Chang Ji, Dr. Debra Feaks, Chela Wendt-Weiland, Gustav Agbley, Watkin's lab, Whitten's Lab, and family members that have significantly help me conduct graduate level research.

TABLE OF CONTENTS

Page

LIST OF TABLES

Tables **Page**

LIST OF FIGURES

Figures **Page**

ABSTRACT

Biodesulfurization is a process that uses bacteria that contain enzymes capable of selectively removing sulfur from petroleum. The dsz pathway is well studied and capable of removing sulfur from dibenzothiophene (DBT). A desulfinating enzyme, 2-(hydroxyphenyl)benzenesulfinate desulfinase (DszB) catalyzes the final step of the dsz pathway, cleaving the sulfinate bond and releasing the sulfur without destroying the carbon backbone of the compound. The dsz genes from *Rhodococcus erythropolis* IGTS8 and *Nocardia asteroides* A3H1 were previously cloned into pTAC-MAT-Tag-2 and transformed into BL21(DE3) containing pREP4-GroESL. The plasmid pREP4-GroESL contains genes than encode the GroES and GroEL, chaperones that participate in the proper folding of DszB. Structural and bioinformatic studies of DszB were conducted on the sequence of amino acids from 180 to 210. Amino acids A195 and A200 were selected to be mutated to arginine and were hypothesized to be important in determining the enzyme specificity for *Nocardia asteroides* A3H1. Site-directed mutagenesis of the *dsz* gene was conducted to create DszB-A3H1-A200R. Recombinant enzymes were purified with nickel affinity chromatography, placed in a fixed timed assay with appropriate substrate, and coupled with sulfite oxidase to study enzyme specificity. The desulfinase enzyme DszB-A3H1 A200R was characterized with fluorescent studies that included kinetic, pH, temperature, and temperature stability. Fluorimetric studies show that the mutation increased the enzyme temperature stability and increased the ability

todesulfinate at higher pH values. The kinetic data showed a km of 3.71 ± 1.54 μM and kcat of 1.1 ± 0.10 min-1.The specificity of DszB-A3H1-A200R showed a change in specificity, preferring 2-(2'-hydroxyphenyl)enthen-1-sulfinate (HBES) over benzenesulfinate (BS).

CHAPTER 1

INTRODUCTION

The emission of sulfur from fossil fuels into the atmosphere as sulfur oxides is a topic of great importance. Preservation of the environment depends on the ability to produce cleaner burning fossil fuels by reducing sulfur levels in petroleum products [1]. Upon combustion of fossil fuels, sulfur oxide compounds are produced. Sulfur oxide will hydrate to sulfuric acid and form acid rain, a contributor to environmental change [2]. The need to reduce sulfur in petroleum and limit the impact of acid rain has increased with government and public awareness of the problem. Traditionally, hydrodesulfurization (HDS) has been employed to remove most sulfur from petroleum [3]. HDS requires high pressure, high temperature, and rare catalytic metals that remove sulfur compounds with hydrogen, forming hydrogen sulfide. This method is expensive and many compounds such dibenzothiophene (DBT) and its derivatives are resistant to such techniques [3, 4]. A new method is needed in order to lower cost of desulfurization while achieving the lowered sulfur levels required for emission regulations [4].

One alternative desulfurization method being explored is biodesulfurization (BDS) [5]. BDS is a process that uses an organism's naturally existing metabolic pathway to remove sulfur for incorporation to biomass. BDS, as a sulfur process, has the potential to be cleaner, cheaper, more efficient, with the capability of removing a wide range of organosulfur compounds than HDS [3, 4]. Specifically, the use of moderate pressures and temperature enables BDS to be a viable alternative form of HDS. More importantly, the ability of BDS to desulfurize DBT and DBT derivatives make it

particularly attractive.

The introduction of BDS as a viable method for removing sulfur from petroleum requires improvements in several areas [6]. BDS enzymes will need to work faster on a broader range of substrates in a two phase oil-water suspension. In addition, a mechanism for biocatalyst recovery and wastewater treatment must be elucidated. If BDS is to be considered as an alternative desulfurization method, a 500-fold increase in catalytic rate would be needed to compliment HDS [7]. Due to the slow rate of desulfurization, most research to date has been directed at improving catalytic rates through metabolic engineering. Methods to increase rates in the BDS pathway include removing inhibitory biocatalytic products, increasing enzyme concentrations through genetic manipulation, harmonizing desulfurizing enzymes with bioreactors, and optimization of bioreactor media [6, 8].

The primary BDS pathway of importance is the 4S pathway, which is capable capable of removing sulfur from DBT and DBT derivatives without degrading carbon-carbon bonds. The desulfurization of DBT to 2-hydroxybiphenyl (HBP) involves four enzymes: DBT-5,5-dioxide monooxygenase (DszA), 2-(2′-hydroxyphenyl)benzenesulfinate (DszB), DBT-monooxygenase (DszC) and flavin reductase (DszD) (Figure 1) [9]. The monooxygenase DszC oxidizes DBT twice, first forming dibenzothiophene sulfone (DBTO) and then dibenzothiophene-5,5-dioxide ($DBTO_2$). The first carbon-sulfur bond is then broken on $DBTO_2$ by DszA, forming 2-(2’-hydroxyphenyl)benzenesulfinate (HPBS). DszC and DszA reactions are coupled with DszD and reduced cofactor flavin mononucleotide ($FMNH_2$). $FMNH_2$ is oxidized to FMN in the reaction and regenerated by DszD using NADH. DszB carries out the final

and rate limiting and rate-limiting carbon-sulfur cleavage on HPBS forming HBP and hydrogen sulfite. Understanding DszB, its structure and mechanism, will ultimately result in improved rates of desulfurization for a broader range of organosulfur compounds.

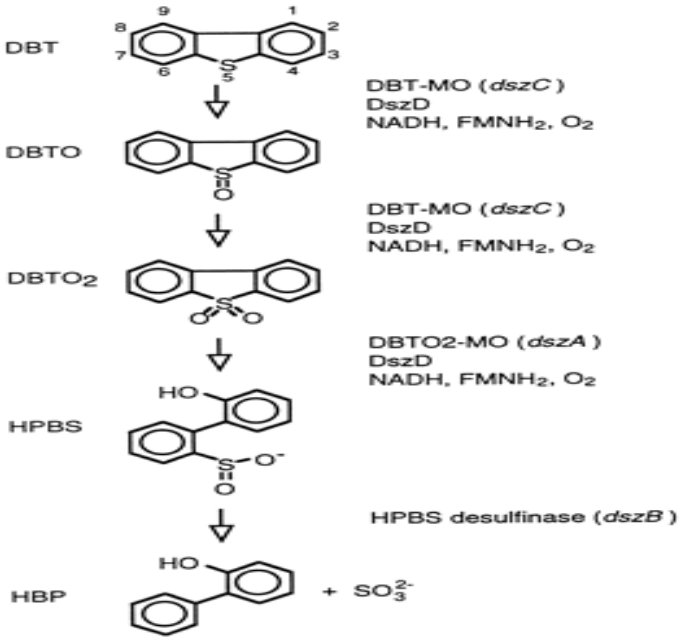


Figure 1. 4S desulfurization pathway [9].

DszB does not require co-factors, metals or proteinic components for catalysis [9]. The mechanism of DszB is shown to require a highly conserved cysteine residue at the active site [10]. Site directed mutagensis of the *R. erythropolis* KA2-5-1 mutant C27S showed complete loss of activity, while other cysteine modifying reagents severely lowered catalysis. There are several scientific studies that examine the mechanism; however, very few studies have looked at specificity [9, 10, 11].

There are over 350 thiophene derivatives found in crude oil, allowing specificity studies of the desulfurization pathways to be studied [12]. Thiophenic derivatives include

heterocyclic compounds with one to nine rings. However, specificity studies of DszB are hindered by the lack of commercially available compounds that contain partially processed thiophenes with sulfinate groups. Relatively few studies on purified DszB or DszB homologs regarding substrate specificity have been conducted. One notable study compared the specificity of 2-(2'-Hydroxyphenyl)benzene sulfinate desulfinase (TdsB) from *Paenibaccilus sp* All-2 to DszB from *R. erythropolis* using two different synthetic substrates [13]. TdsB is capable of converting HPBS to HBP, along with 2-(2'-hydroxyphenyl)ethen-1-sulfinate (HPES) to o'-hydroxystyrene (HS) (figure 2), while the DszB from *R. erythropolis* can only convert HPBS to HBP. The amino acid sequence of the two enzymes share a homology of 67% and a amino acid identity of 55 %, but their substrate specificities differ significantly [14]. Moreover, the DszB from KA2-5-1 is capable of removing sulfinate from biphenyl-2-sulfinic acid (BPS) without the need of a hydroxyl group [15]. The absence of a hydroxyl group suggests that it is not needed for catalysis.

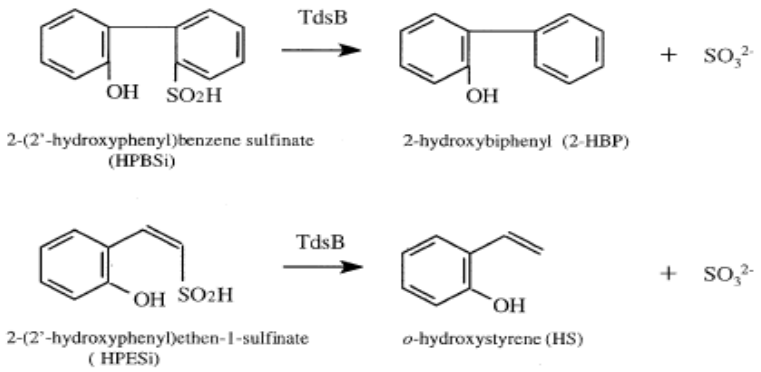
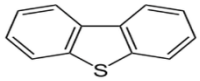
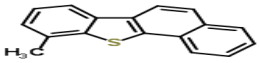
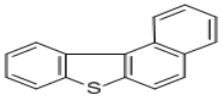
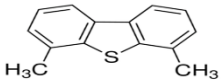
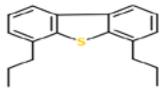
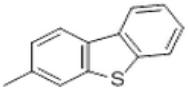
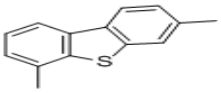
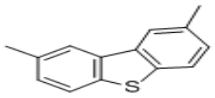


Figure 2. TdsB specificity toward HPBS and HPES [14].

The remainder of specificity studies were carried in whole cell culture assays where the specificity of DszB was inferred from the disappearance or build up of organic

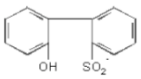
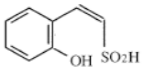
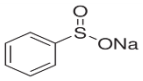
compounds in the dsz pathway. Studies showed *Myobacterium sp.* MR65 can desulfurize DBT, 10-methylbenzo[b]naphtho[2,1-d] thiophene (10-methyl BNT), Benzo[b]naphtho[2,1-d]thiophene (BNT), 4,6 Diethyl DBT, 4,6 Dipropyl DBT, 3-methyl-DBT, and 3,6 dimethyl DBT, see table 1. [16]. Different desulfurization rates were observed between the thiophenic compounds. Thiophenic compounds having alkyl groups near the sulfur atom had lower rates of desulfurization when compared to non alkylated thiophenic compounds. Other studies were conducted on *Bacillus subtilis* WU-S2B, a moderate thermophile, which is capable of removing sulfur from DBT and DBT analogs [17]. *Bacillus subtilis* WU-S2B can desulfurize DBT, BNT, 2,8 dimethyl DBT, and 4,6 dimethyl preferring DBT over other thiophenic compounds (table 1).

Table 1. DBT analogs used in cell free assays.

Compound (Abbreviation)	Structure
DBT	
10-methyl BNT	
BNT	
4,6 Diethyl DBT	
4,6 Dipropyl DBT	
3-methyl DBT	
3,6 dimethyl DBT	
2,8 dimethyl DBT	

The primary goal of this research project is the study and understanding of DszB enzyme specificity and the characterization of DszB-A3H1 mutants from *Nocardia asteroides* A3H1. The specificity of DszB-A3H1, DszB-A3H1-A200R, and DszB-IGTS8 were studied with the sulfinate containing substrates HPBS, benzenesulfinate (BS), and 2-(2'-hydroxyphenyl)ethen-1-sulfinate (HPES) (table 2) in fixed timed assays. Fixed timed assays were coupled with sulfite oxidase to determine the presence of the product sulfite.

Table 2. HPBS analogs.

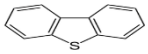
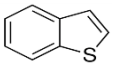
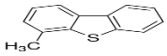
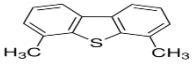
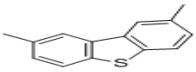
Compound	Abbreviation	Structure
2-(2'-hydroxyphenyl)benzenesulfinate	HPBS	
2-(2'-hydroxyphenyl)ethen-1-sulfinate	HPES	
Benzenesulfinate	BS	

A series of computer programs were used to visualize, compare and identify critical amino acids involved in DszB specificity. PyMol molecular graphics system, version 1.3.0.0 Schrödinger, LLC was used to visualize changes in the amino acid sequence and structure of DszB upon substrate binding. The crystal structure of DszB from *R. erythropolis* KA2-5-1 (2DE2) was superimposed on crystal structure of DszB from *R. erythropolis* KA2-5-1-C27S (2DE3) mutant in complex with HPBS [10]. The two crystal structures were traced and threaded with the DszB-A3H1 amino acid sequence. Distinctive structural changes between the two crystal structures were isolated and applied to DszB-A3H1 homology studies. DszB amino acid sequences from *R. erythropolis* IGTS8, *N. asteroides* A3H1, *Paenibaccilus sp.* A11-2, *B. subtilis* WU-S2B, and *Mycobacterium sp.* G3 were compared in Weblogo version 2.8.2 (2005-09-08), identifying important and highly conserved amino acids. The purposed mutations were designed to decrease the size of the catalytic pocket to incorporate smaller substrates such as BS and HPES. HPES was synthesized from 1,4-dihydro-2,3-benzoxathiin-3-oxide in two one-step reactions from two outlined protocols [18, 19].

A secondary goal in this research project includes the separation, purification, and collection of HPBS and HPBS analogs from cell free assays using HPLC. Cell free

assays were conducted with lysate from *R. erythropolis* IGTS8, using the thiophenic compounds DBT, benzothiophene (BT), 2,8-dimethyl-DBT, 4-methyl-DBT, and 4,6-methyl-DBT as shown in table 3. Individual compounds, DBT, $DBTO_2$ HPBS and HBP were mixed together and successfully separated using HPLC. The separation of DBT, $DBTO_2$, HPBS and HBP served as a standard when deriving compounds from the cell free assay.

Table 3. DBT analogs.

Compound	Abbreviation	Structure
Dibenzothiophene	DBT	
Benzothiophene	BT	
4-methyl-dibenzothiophene	4-methyl-DBT	
4,6-methyl-dibenzothiophene	4,6-methyl-DBT	
2,8-dimethyl-dibenzothiophene	2,8-dimethyl-DBT	

CHAPTER 2

METHODS

Culture Growth, Expression and Lysate Preparation of *Rhodococcus erythropolis* IGTS8

Rhodococcus erythropolis IGTS8 was purchased from American Type Culture Center (ATCC No.53968). All solutions, glassware, and media were autoclaved or sterile filtered to prevent cross contamination during culture growth and expression of *Rhodococcus erythropolis* IGTS8. Cells were inoculated from frozen permanents and cultured in 200 mL of LB nutrient broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 1 mM NaOH). Cells were cultured for 48 h at 30 ^{o}C at 200 rpm, using a Gyromax 747 Orbital Incubator Shaker. Cultured cells were pelleted with a Beckman Coulter 25R centrifuge at 7,000 × *g* for 20 min. LB was decanted and pelleted cells were washed twice with minimal salt solution (4.0 g/L KH_2PO_4, 3.0 g /L NH_4NO_3, pH 7.3). After washing pelleted, cells were suspended in 5 mL of minimal salt solution and used for inoculating DszB induction media (4.0 g/L KH_2PO_4, 3.0 g/L NH_4NO_3, 0.5 g/L $MgCl_2$•$6H_2O$, 1.0 g/L succinate, 10.0 g/L glycerol, 8.0 g/L ethanol, 20.0 mg/L $MnCl_2$•$4H_2O$, 50.0 mg/L $FeCl_2$•$4H_2O$, 0.02 mg/L $Na_2B_4O_7$•$10H_2O$, 0.02 mg/L $CuCl_2$, 1.0 mg/L $CoCl_2$, 0.08 mg/L $ZnCl_2$, 0.4 mg/L $FeCl_3$•$6H_2O$, 20.0 mg/L $CaCl_2$, 0.1 mg/L $MoCl_5$, 5.0 µg/L $2NiCO_3$•$3Ni(OH)_2$•$4H_2O$, adjusted to pH 7.3 with NaOH), containing 200 µM DBT to promote expression of *dszb* genes [20]. One mL of suspended cells was transferred to each flask containing 250 mL of expression media and cultured for 7 days at 30 ^{o}C, shaking at 200 rpm. After incubation, cells were pelleted at 12,000 × *g* at 4 ^{o}C for 1 h and suspended in 2-10 mL of mineral salt solution. The thickened cells were

passed through a 1 mL eppendorf pipette and frozen into small pellets using liquid nitrogen. Frozen cells where crushed into a fine powder with five 3 min cycles at 30 beats/sec, using a Retsch MM 400 bead-beater. Between each cycle, the containers were cooled with liquid nitrogen to keep the crushed lysate from thawing during each cycle. Ruptured cells were stored at -70 ^{o}C or used immediately. A sample of 0.5-1.0 g of frozen powdered lysate was transferred into a microcentrifuge tube. Lysate was thawed on ice for 20 min and suspended in 1 mL of cold lysis buffer (25 mM NaH_2PO_4, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), 0.2 mg/mL DNAse, pH 7.4). Lysate was placed in a water bath and incubated at 37 ^{o}C for 20 min. Lysate was pelleted at 12,000 × *g* for 1 h at 4 ^{o}C and filtered with a 0.2 μ cellulose filter. Filtered lysate was used to conduct the cell free assay.

Substrate Purification using HPLC

Cell free assays were conducted with and without DszB inhibitor to maximize product formation of HPBS and HPBS analogs. The cell free assay was composed of 100 μL of lysate, 10 μM FMN, 6 mM NADH, 25 mM DBT or DBT analog, and 100 mM phosphate buffer pH 7.2, with or without 100 μM $CuCl_2$ (inhibitor) [9]. The reaction was shaken at 300 rpm for 12 h at 30 ^{o}C using an Eppendorf Thermomixer R. The reaction was stopped with 0.01 mL of 12 M HCl and 250 μL of ethyl acetate was added to extract organic molecules. The mixture was centrifuged at 15,682 × *g* for 10 min using a Hermle Z180M microcentrifuge to facilitate the pelleting of protein precipitation and separation of organic and aqueous phases. The organic phase was removed and filtered by centrifugation at 15,682 × *g* for 2 min, using a Quick Spin 0.2 μ nylon filter from

Nalgene. The filtrate was placed in a Savant Speed Vac SC110 to facilitate the removal of ethyl acetate. Analytes were suspended in 200 μL acetonitrile or buffer A (25 mM NaH_2PO_4/$NaHPO_4$, 100 mM NaCl, pH 7.4) for kinetic studies.

Substrates synthesized in cell free assay were purified by HPLC using a Supelco C18 (3.9 x 300 mm, 15-particle size column) attached to an Agilent 1200 series HPLC with a flow rate of 1 mL/min. A gradient mobile phase of acetonitrile and water was used to separate metabolites from the dsz pathway, see table 4 [21]. Fractions were collected and the absorbance at 280 nm was measured on a ND1000 spectrophotometer 3.8.0 to determine which fractions contained HPBS and HPBS analogs. Fractions were pooled and analyte was concentrated with a rotary evaporator (SpeedVac) to increase dryness and removal of acetonitrile through evaporation. The resulting analyte was suspended in 100 μL of buffer A for kinetic studies.

Table 4. Metabolite separation using a mobile phase gradient. A flow rate of 1 mL/min using a mobile phase gradient of acetonitrile and water was used to separate metabolites from the dsz pathway.

Time (min)	Gradient acetonitrile:water
0.0	50:50
1.0	50:50
2.0	50:50
3.0	30:70
4.0	20:80
5.0	30:70
6.0	50:50
6.5	70:30
7.0	90:10

Cloning into Plasmid Vector pTAC-MAT-Tag-2

The *dszb* gene from *Nocardia asteroides* A3H1 and *Rhodococcus erythropolis* IGTS8 were previously cloned into pTAC-MAT-Tag®-2 vector from Sigma-Aldrich by Jerusha Vaz and Leanne Harper in Watkins lab to create pTAC-A3H1 and pTAC-IGTS8, respectively. See figure 3 for pTAC-MAT-Tag®-2 expression vector and table 5 for cloned vectors and mutants used in this study. The pTAC-A3H1 plasmid was used as a PCR template used to create mutants. Mutant plasmids derived from pTAC-A3H1 were co-transformed into BL21(DE3) with plasmid pREP4-GroESL.

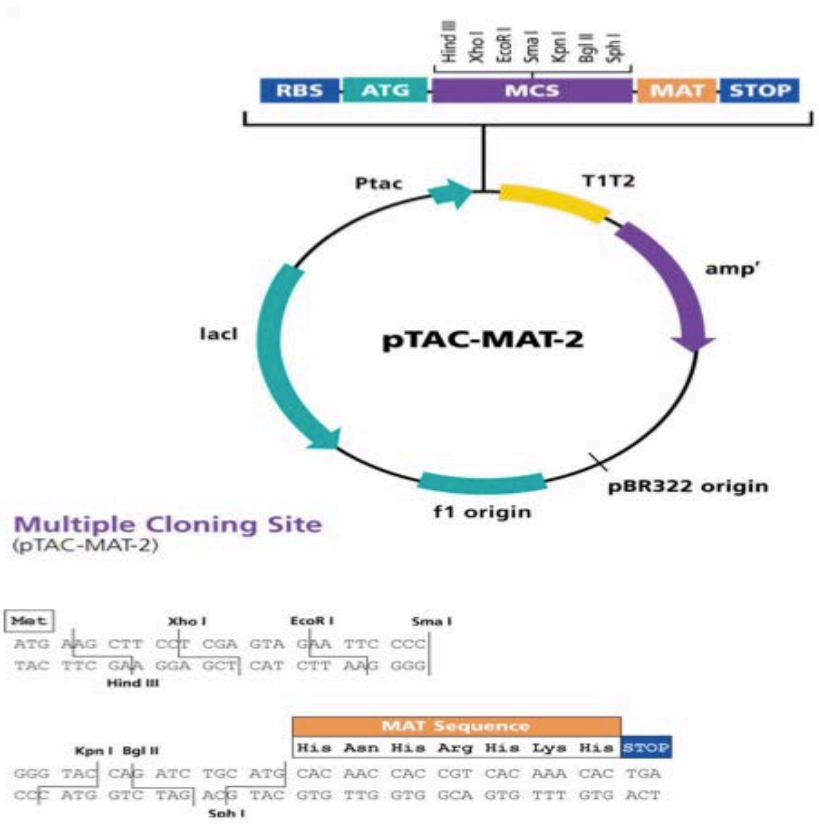


Figure 3. pTAC-MAT-Tag-2 expression vector from Sigma-Aldrich. The *dszb* gene from A3H1 and IGTS8 were cloned into pTAC-MAT-Tag®-2 expression vector, allowing a one-step purification of C-terminal His tag (MAT) with nickel binding resin.

Table 5. Plasmids.

Plasmid	Description	Source
pTAC-MAT-Tag®-2	Expression vector containing tac promoter and 6-His-tag	Sigma-Aldrich
pTAC-A3H1	pTAC-MAT-Tag-2 vector containing *dszb* gene from *N. asteroides* A3H1	Jerusha Vaz [31]
pTAC-A3H1-A200R	pTAC-MAT-Tag-2 vector containing *dszb* gene from *N. asteroides* A3H1 was mutated.	Herein
pTAC-IGTS8	pTAC-MAT-Tag-2 vector containing *dszb* gene from *R. erythropolis* IGTS8	Leanne Harper [32]
pREP4-GroESL	Plasmid containing genes for Gro-EL and Gro-ES chaperone proteins.	Dr. Dean Appling [22]

Mutagenesis of pTAC-A3H1

The molecular graphic system PyMol version 1.3.0.0 Schrödinger, LLC was used to examine published crystal structure of DszB-KA2-5-1 that contained substrate bound and unbound [10]. The crystal structure 2DE2 of DszB-KA2-5-1 contains no HPBS, while crystal structure 2DE3 of DszB-KA2-5-1-C27S has HPBS complexed with DszB. The two crystal structures were superimposed and threaded with DszB-A3H1 sequence to determine substrate binding interactions and to identify amino acids for site-directed mutagenesis.

Site directed mutagenesis of pTAC-A3H1 was conducted using a Stratagene Quick Change Site Directed Mutagenesis Kit. A website program from Stratagene was used to design primers (www.genomics.agilent.com), see table 6. Primers were ordered from Integrated DNA Technologies (Coralville, IA) and suspended in sterile water to make 100 μM stock solutions and stored at -20 ^{o}C. Standard protocols from Stratagene for site directed mutagenesis and transformation were followed [23]. PCR samples were prepared on ice and contained 1X PCR reaction buffer, 50 ng pTAC-A3H1 plasmid, 0.2 μM of forward and reverse primers, 200 μM dNTPs and 1 U of *Pfu* Turbo polymerase in 50 μL of total volume. The sample was placed in a 2720 thermocycler from Applied Biosystems and the amplification program from Stratagene was followed. First segment, initial denature for 30 sec at 95 ^{o}C; Second segment followed three parts: denaturation for 30 sec at 95^{o}C, annealed for 1 min at 55 ^{o}C and elongated for 6 min at 68 ^{o}C . After PCR, the reaction was stopped with 10 U of DpnI and incubated at 37 ^{o}C for 1 h on a VWR heat block. Reaction products were analyzed by agarose gel electrophoresis.

Table 6. Mutagenic and sequencing primers for pTAC-A3H1.

Primer	Sequence	Description
A3H1-A195R-F	5'-CAGTTGGAAGACGCCAGGACCCTCAAGGGTGC-3'	Forward A195R
A3H1-A195-R	5'-GCACCCTTGAGGGTCCTGGCGTCTTCCAACTG-3'	Reverse A195R
A3H1-A200R-F	5'-GCCGCGACCCTCAAGGGTAGGGACC-3'	Forward A200R
A3H1-A200R-R	5'-AAACAGGTCCCTACCCTTGAGGGTCG-3	Reverse A200R
A3H1-F	5'-GCGAATTCATGGCAGGCCGCCTCAG-3'	Forward Sequencing
A3H1-R	5'-GCAGATCTATCGGTGACGGTTGAGGCTG-3'	Reverse Sequencing

Horizontal Agarose Electrophoresis

A 0.8% (w/v) agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) was used to analyze the PCR reaction. DNA samples contained a 5-9 μL reaction mixture and 1X dye (0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 4% (v/v) sucrose and sterile water) in 10 μL of total volume. A 1 kb plus ladder (Invitrogen) was used as the standard. The gel was run for 45-60 min at 90 volts. The gel was stained with ethidium bromide (0.2 mg/mL) for 20 min and destained overnight with sterile water. The gel was imaged and documented with a Alpha Innotech Red.

Transformation of XL1 Blue and BL21(DE3)

XL1-Blue competent cells from Stratagene were used in transformation [23]. XL1-Blue cells (200 µL per tube) were thawed on ice for 10 min and pTAC-A3H1-A200R was added at a final concentration of 0.1 ng/mL. The mixture was placed on ice for an additional 10 min. Samples were heat shocked for 90 sec at 42 ^{o}C and immediately placed on ice for 2 min. The mixture was incubated at 37 ^{o}C for 45 min and plated on selective media containing the appropriate antibiotic. Co-transformation of pTAC-A3H1-A200R and pREP4-GroESL followed the protocol described in the BL21 (DE3) competent cells instruction manual [24]. Frozen permanents were made from transformed BL21(DE3) cells. Aliquots of 0.6 mL of overnight cultures were added to 0.8 mL of sterile 25% glycerol and frozen at -70 ^{o}C.

DNA Isolation and Extraction

All plasmids were purified from XL1-Blue cells using a Qiaprep Spin Mini-prep Kit. Single colonies were used to inoculate 10 mL of LB, containing either kanamycin (30 µg/mL) or ampicillin (100 µg/mL). Cultures were grown overnight at 37 ^{o}C, shaking at 250 rpm. Cells were pelleted at 15,682 × *g* for 2 min. Plasmids were purified using the low copy protocol of Qiaprep Spin Mini-prep Kit [25]. DNA was quantitated using a ND1000 spectrophotometer with software version Nanodrop 3.1.2. Samples were submitted for sequencing at ICMB DNA Sequencing Facility at the University of Texas at Austin.

Culture Growth, Expression, and Purification of Recombinant *E.coli* Systems

Cells were cultured in a 250 mL Erlenmeyer flask containing 50 mL of LB and both kanamycin (30 μg/mL) and ampicillin (100 μg/mL). Cells were grown at room temperature overnight and shaken with a lab-line shaker. A 5 mL aliquot of cultured cells was transferred to a 1 L flask containing 500 mL of LB. Cells were grown to a OD_{550} between 0.6-0.8, using a Beckman DU730 UV/Vis spectrophotometer to measure growth of culture. Expression of protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and shaken at room temperature for an additional 16-20 hours. Cells were pelleted by centrifugation at 4,000 × *g* for 15 min at 4 ^{o}C and suspended in 8-10 mL of chilled native binding buffer (50 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole, pH 8.0) [26]. After suspension, 10 μg of PMSF and 0.5 mg/mL lysozyme were added and the solution was incubated on ice for 30 min. The cells were lysed on ice with 4-5 rounds of 10 sec sonications at 50 % amplitude, followed by a 10 sec rest using a Branson Sonifier 450. Lysed cells were centrifuged at 12,000 × *g* for 30 min at 4 ^{o}C and filtered with a 0.2 μ cellulose filter. Aliquots were collected before and after filtration and saved for SDS-PAGE analysis.

Purification of Recombinant His-tag Proteins using Nickel Resin

G Bioscience nickel resin and Probond nickel resin were used in purifying recombinant His-tag proteins. Recharging, regeneration and storage of resins each

followed the manufacturer's protocols [26, 27]. A 4 mL slurry of nickel resin was transferred into a 10 mL column, creating a 2 mL resin bed. Resin was settled and the aqueous phase was siphoned off. The resin was washed with two 8 mL water washes, suspended with 16 column volumes of native binding buffer, and allowed to settle. The aqueous phase was siphoned off.

An 8 mL aliquot of filtered lysate was prepared and loaded onto a nickel column. The suspension was gently mixed for 30-60 min, and allowed to settled before the remaining lysate was siphoned off [26]. The resin was rinsed 2-4 times with wash buffer (50 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 20 mM imidazole, pH 8.0) and protein was eluted with 8 mL of elution buffer (50 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl, 250 mM imidazole, pH 8.0). Eluted samples were tested for concentration and activity. Aliquots of the enzyme, DszB-A3H1, DszB-A3H1-A200R and sulfite oxidase were stored either at 4 ^{o}C or -20 ^{o}C with 30% glycerol. The enzyme, DszB-IGTS8 was stored at 4 ^{o}C. All purifications were conducted at 4 ^{o}C, following the Probond purification protocol. At each step of the purification process aliquots were saved for SDS-PAGE analysis.

SDS-PAGE for Purification Analysis

A 4% stacking and 10% resolving gel was used to monitor purification of DszB-A3H1, DszB-A3H1-A200R, DszB-IGTS8 and sulfite oxidase. Samples contained of 1X SDS dye (10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate, 0.0025% (w/v) bromophenol blue, 5.0% β-mercaptoethanol) and 15 µL (1.05 to 12.75 µg) of purified elution. Samples were mixed and heated at 95 ^{o}C for 5 min. The

gel was submerged in 1X SDS running buffer (3.00 g/L Tris base, 14.4 g/L glycine, and 1.0 g/L sodium dodecyl sulfate that had a pH of 8.3) and loaded with 15 μL of sample. Samples were run in a BioRad Mini Protean Tetra cell at 125 volts for 80 min. The gel was stained with Coomassie blue (0.3 mM Coomassie Brilliant Blue, 7.5% (v/v) glacial acetic acid, 50% (v/v) ethanol), for 1 h and destained overnight with destain solution (7.5% (v/v) glacial acetic acid, and 10% (v/v) ethanol). Imaging and gel documentation was done with a Android HTC 7.2 mega pixel camera or Alpha Innotech red.

Determination of Protein Concentration

Protein concentrations were determined using nanodrop spectroscopy. A sample volume of 1-2 μL was added to a ND1000 spectrophotometer with Nanodrop 3.1.2 software. The absorbance of eluted samples from the nickel column were measured at A_{280} , displaying the concentration of protein as mg/mL.

Characterization Studies

Kinetic, temperature, stability, and pH studies of HPBS desulfinase were conducted with fluorescence assays, using a Cary Eclipse Fluorescence Spectrophotometer from Varian. The product HBP excites at 288 nm while emitting energy at a lower wavelength of 414 nm. All studies were run in triplicates and conducted in a 96 well plate.

Kinetic Studies

The kinetic parameters of HPBS desulfinase were determined by measuring the rate of product formation at varying HPBS concentrations. The fluorescent product formed from HPBS was monitored to determine initial desulfination rates. HPBS concentrations of 0.1, 0.5, 0.025, 0.01, 0.005 and 0.0025 mM were used to determine kinetic parameters of DszB-A3H1-A200R and DszB-A3H1. The reaction contained 6.2 μg of DszB-A3H1-A200R or 6.8 μg of DszB-A3H1, buffer A (25 mM NaH_2PO_4/Na_2HPO_4, 100 mM NaCl, pH 7.4) and substrate, in a total reaction volume of 200 μL. The reaction was incubated at 35 ^oC for 5 min and initiated with the addition of HPBS. A unit of enzyme activity was defined as 1.0 μM HBP formed /min. Kinetic data were fitted with the Michaelis-Menten equation, using VisualEnzymics from Softzymics.

Temperature and Stability Studies

Temperature effects on DszB-A3H1-A200R were conducted over temperature ranges from 25 to 60 ^oC at 5 ^oC increments. The assay was composed of buffer A, 6.0 μg of enzyme, 0.1 mM HBPS and incubated for 5 min before starting the reaction. Temperature stability studies involved a 30 min incubation from temperature ranges from 25 to 55 ^oC at 5 ^oC increments. After incubation, the reaction was incubated at room temperature for 10 min before HPBS was added to the reaction. Product formed from HPBS by HPBS desulfinase was monitored fluorometrically and initial velocities were calculated in Excel to determine the relative activity of DszB-A3H1-A200R.

pH Studies

The desulfinase activity of DszB-A3H1-A200R was monitored between pH values of 5.5 and 9.5, at 0.5 pH unit increments. A 10X MTEN buffer (500 mM 2-(N-morpholino)ethansulfonic acid (MES) hydrate, 250 mM Tris, 250 mM ethanolamine) was diluted to a 5X MTEN buffer and the pH was adjusted to the required value using HCl or NaOH. The assay to monitor pH effects consisted of 1X MTEN, 6.0 μg of enzyme, and 0.1 mM HPBS. The assay was incubated at 35 ^{o}C for 5 min and initiated with the addition of HPBS. HBP product formation was monitored fluorometrically and initial velocities were calculated in Excel to determine the relative activity of DszB-A3H1-A200R.

HBP Standard Curve

The HBP standard curve was generated to determine unknown concentrations of HBP. Varied concentrations of HBP were excited, allowing HBP to fluorescence. The fluorescent intensities were plotted against HBP concentrations, producing a standard curve. The standard curve was preformed in a 96 well plate with buffer A and HBP concentrations between 0 and 75 μM. A Cary Eclipse Fluorescence Spectrophotometer was used to monitor fluorescent intensities at 414 nm (λ_{ex}=288 nm). Fluorescence intensities were plotted against HBP concentrations using Excel. Formula y = 0.48x+2.07 was generated from a best fit line, using linear regression.

Synthesis of 1,4-dihydro-2,3-benzoxathiin-3-oxide

The synthesis of the sultine 1,4-dihydro-2,3-benzoxathiin-3-oxide was carried out using the published protocol from Micheal D. Hoey and Donald C. Dittmer [18]. A suspension of sodium hydroxymethanesulfinate (3 g) and 20 mL of N,N-dimethylformamide was stirred for 10 min in a beaker. The reaction was started with the addition of a,a'-dichloro-o-xylene (1.75 g) and tetrabutylammonium bromide (0.64 g). The reaction sat with stirring at 25 ^{o}C for 18 h. The reaction was diluted to 150 mL with water, and then vacuum filtered. Organic compounds from the filtrate were extracted with 50 mL of diethyl ether. Two additional 50 mL diethyl ether extractions were conducted on the aqueous phase. The organic phase was dried with anhydrous magnesium sulfate and vacuum filtrated. The solvent was removed through evaporation on a vacuum pump, yielding a yellowish-orangery sultine 1,4-dihydro-2,3-benzoxathiin-3-oxide. NMR was conducted with $CDCl_3$ to determine the purity of the sultine.

An alternative synthesis of 1,4-dihydro-2,3-benzoxathiin-3-oxide was outlined and followed from patent US 8,119,820 B2 [28]. A suspension of sodium hydroxymethanesulfinate (9 g) and 20 mL of dimethyl sulfoxide was stirred for 10 min. The reaction was started with the addition of a,a'-dichloro-o-xylene (5.1 g), potassium carbonate (6.07 g), sodium iodide (0.055 g), 5.6 mL of DMSO and sat for 3 h with stirring at 25 ^{o}C. Organic compounds from the reaction were extracted with 20 mL of methanol/ethyl acetate (20:80). The suspension was filtered and transferred to a separatory funnel with 50 mL of a 3.5% brine solution. Additional ordered extractions were preformed on the aqueous phase with 10, 7.5, and 12.5 mL of methanol/ethyl acetate (20:80). The organic solvent was dried with anhydrous magnesium sulfate,

vacuum filtered, and removed through evaporation on a vacuum pumped. Purity of sultine was determined through NMR using $CDCl_3$.

2-(2'-hydroxyphenyl)ethen-1-sulfinate Synthesis

The synthesis of 2-(2'-hydroxyphenyl)ethen-1-sulfinate (HPES) followed published work from Tadashi Okuyama [19]. A ring opening hydrolysis reaction of sulfinate esters was used to synthesize 20 mM HPES from 1,4-dihydro-2,3-benzoxathiin-3-oxide. Compound, 1,4-dihydro-2,3-benzoxathiin-3-oxide (0.030 g) was suspended in 10 mL of 44 mM NaOH. The suspension was stirred overnight at 25 ^{o}C, filtered with a 0.45 μ nylon filter, and stored at 4 ^{o}C.

NMR

NMR was conducted to verify the formation of 1,4-dihydro-2,3-benzoxathiin-3-oxide after each synthesis reaction. The sample was loaded into a NMR tube and diluted with $CDCl_3$ to the proper volume using a depth gauge. Spectra were obtained using a 400 MHz Bruker NMR spectrometer for proton NMR.

Specificity Assay

The specificity of DszB-A3H1, DszB-A3H1-A200R, and DszB-IGTS8 was measured in a coupled sulfite oxidase assay follow in a protocol from Sigma-Aldrich.

[29] A fixed timed assay contained buffer A, 0.1mM of substrate and 8.1 µg (DszB-A3H1), 8.5 µg (DszB-A3H1-A200R) or 0.7 µg of (DszB-IGTS8) HPBS desulfinase. The reaction was mixed overnight at 30 ^{o}C on an orbital shaker, rotating at 300 rpm. The sulfite oxidase assay was conducted in a 1 mL cuvette, composed of 1X buffer B (47.5 mM NaH_2PO_4 , 15 mM $NaHPO_4$, 250 mM NaCl, pH 7.4), 60 µM cytochrome c, 5.3-8.35 µg of sulfite oxidase and 100 µL of incubated fixed timed assay. A change in absorbance at 550 nm, corresponding to cytochrome c reduction was measured over 10 min to determine production if sulfite was produced from the fixed timed assay.

Sulfite Standard Curve

A sulfite standard curve was generated by varying sulfite concentrations of 0.02, 0.01, 0.005, 0.002, and 0.001 mM sodium sulfite. Assays were conducted in a 1 mL cuvette containing 0.4 mM of sodium sulfite, 1X buffer B, 60 µM cytochrome c, and 5.3 µg of sulfite oxidase [29]. A change in absorbance at 550 nm corresponding to cytochrome c reduction was measured over 10 min and the standard curve was plotted in Exel.

CHAPTER 3

RESULTS AND DICUSSION

Sequence Analysis

Percent Identity and Homology of Desulfinase Enzymes

The sequence for DszB enzymes from *R. erythropolis* IGTS8 (DszB), *Paenibaccilus sp.* A11-2 (TdsB), *B. subtilis* WU-S2B (BdsB), and *Mycobacterium sp.* G3 (DszB) were compared to *N. asteroides* A3H1 (DszB) for amino acid identity and homology.

Table 7. Percent amino acid identity and homology of *N. asteroides* A3H1 (DszB).

Organism Containing Desulfinase	Amino Acid Identity (%)	Homology (%)
R. erythropolis IGTS8 (DszB)	87	92
Mycobacterium sp. G3 (DszB)	67	78
B. subtilis WU-S2B (BdsB)	67	78
Paenibaccilus sp. A11-2 (TdsB)	54	68

PyMol

A structural analysis of DszB-KA2-5-1 desulfinase from R. erythropolis was conducted by comparing crystal structures with (2DE2) and without substrate bound (2DE3)[10]. The crystal structures of DszB-KA2-5-1 desulfinase were visualized with PyMol molecular graphics system, version 1.3.0.0 Schrödinger, LLC. Crystal structures were obtained from the RCSB protein data bank and visually traced onto each other with PyMol (figure 4). The crystal structure 2DE2 of DszB-KA2-5-1 does not contain the substrate HPBS, while crystal structure 2DE3 of DszB-KA2-5-1-C27S is in complex with HPBS. The crystal structures of DszB-KA2-5-1 desulfinase show structural changes upon substrate binding. Amino acids from 50-65 and 180-210 undergo structural changes when HPBS is in complex with DszB-KA2-5-1. The two variable amino acid sequences change their structural motif from a loop into an alpha helix. Amino acids 50-65 form an alpha helix that rotates toward the active site, while amino acids 180-210 form an alpha helix that rotates away from the active site. The variable sequences were isolated and threaded with the DszB-A3H1 amino acid sequence to visualize structural changes upon substrate binding to DszB-A3H1. Most research regarding DszB has been concerned with mutating active site amino acid to either elucidate the catalytic mechanism or improve desulfination rates. Little attention has been focused on specificity or the non-active site amino acids of DszB. The amino acid sequence 180-210 are non active site amino acids, but are hypothesized to be important in determining specificity by forming the size and shape of the active site pocket.

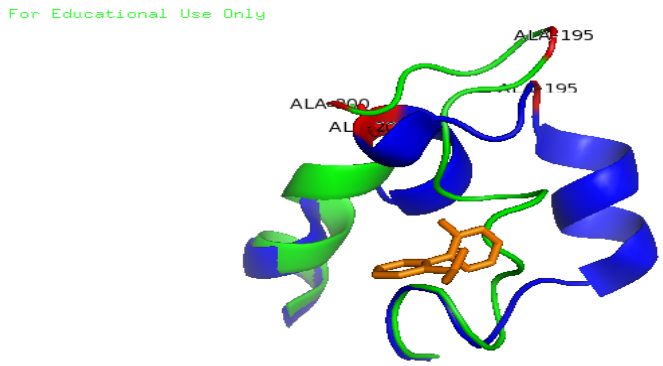


Figure 4. PyMol image of DszB from *R. erythropolis* KA2-5-1 amino acids 180 to 210.. 2DE2 (green) overlaying 2DE3 (Blue). Amino acids 180-210 with proposed mutations on A195(Red) and A200 (Red).

The amino acid sequence 180 to 210, from DszB-A3H1, was compared to other desulfinating enzymes capable of desulfinating HPBS or HPBS analogs. A desulfinase enzyme of particular interest, TdsB from thermophilic *Paenibaccilus sp.* A11-2, is capable of desulfinating HPES and HPBS, while mesophilic desulfinases from *R. erythropolis* can only desulfinate HPBS [13]. The amino acid sequence 180-210 found in the desulfinating enzymes *R. erythropolis* IGTS8 (DszB), *N. asteroides* A3H1 (DszB), *Paenibaccilus sp.* A11-2 (TdsB), *B. subtilis* WU-S2B (BdsB), and *Mycobacterium sp.* G3 (DszB), were compared for similarities and visualized with Weblogo (figure 5.). Weblogo version 2.8.2 (2005-09-08) is a web based program used to visualize amino acids or nucleic acids when comparing multiple sequence alignments for reoccurring similarities. In mesophiles, the amino acid sequence from 180 to 210 has more alanines, while thermophiles consist of more basic amino acids. Another homology study between DszB, BdsB, and TdsB enzymes reported that the thermophilic desulfinase contained

nine non active site acid-base amino acids that DszB does not have [30]. Site-directed mutagenesis of non polar amino acids A195 and A200 to basic amino acid arginine in DszB-A3H1, was hypothesized to change enzyme specificity. The non-polar amino acid mutation to a basic amino acid was supported by homology and crystallography studies.

Figure 5. Weblogo of IGTS8, A3H1, A11-2, WU-S2B, and G3.

PCR and Site-directed Mutagenesis

Creation of pTAC-A3H1 Mutants

Site directed mutagenesis of pTAC-A3H1-A195R and A200R was carried out as described in methods. Mutagenesis of pTAC-A3H1 was difficult due to the high GC, increasing the resistance towards site-directed mutagenesis for genes that encode for amino acids 180 to 210. Primer-dimer formations, an indication that replication of DNA was unsuccessful, were observed with primers containing 18-22 bp. Polymerase, DMSO, enhancer buffers, salts, and annealing temperatures were varied in an attempt to obtain PCR product; however, PCR was unsuccessful. A primer containing 32 bp was used to successfully mutate pTAC-A3H1-A200R, providing a PCR product of 6,260 bp as shown in figure 6. A primer length of 40-65 bp was suggested by Stratagene to mutate pTAC-

A3H1-A195R. In conclusion, primer length was an important factor when mutating DNA with enriched GC content (66.2%). The PCR product, pTAC-A3H1-A200R, was sequenced to determine DNA integrity and successful mutation of pTAC-A3H1-A200R.

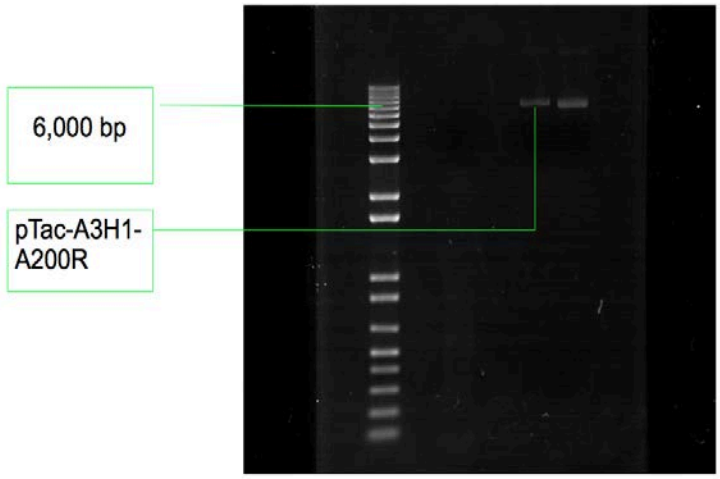


Figure 6. Horizontal gel of mutant pTAC-A3H1-A200R. Lane 1 1 kb plus latter, lane 2-3 pTac-A3H1-A195R, lane 4-5 pTAC-A3H1-A200R with 5 and 9 μL of sample.

Protein Purification

Purification of Recombinant Desulfinases and Sulfite Oxidase

The purification of recombinant DszB-A3H1, DszB-A3H1-A200R, DszB-IGTS8, and sulfite oxidase from *E.coli* has many advantages over expression and purification from native organisms that produce desulfinating enzymes. Cellular lysis of *E. coli* using sonication provided an effective method for releasing cellular components without compromising enzyme activity. Expression of DszB was initiated with IPTG by binding to the *lacI* repressor and allowing the transcription of genes. His-tagged recombinant enzymes were purified with nickel affinity chromatography in a one step purification method. Proteins eluted from the nickel column had concentrations of 0.60-0.81 mg/mL

for DszB-A3H1 and DszB-A3H1-A200R, 0.53-0.835 mg/mL for sulfite oxidase and 0.07 mg/mL for DszB-IGTS8.

The DszB enzyme is a monomeric protein that has a molecular weight of 40 kDa. DszB enzymes were co-expressed with Gro-ES and Gro-EL chaperones that have molecular weights of 15 kDa and 65 kDa, respectively. The purified DszB enzymes and chaperones were analyzed with SDS-PAGE. Concentrated bands at 40 kDa, 15 kDa, and 65 kDa were observed under SDS-PAGE analysis (figures 7. and 8.). The SDS-PAGE analysis matched current and past purifications of DszB, Gro-ES, and Gro-EL proteins [31, 32, 35]. Another SDS-PAGE analysis was performed after the purification of sulfite oxidase. Purified sulfite oxidase has a distinctive reddish color due to the metallocofactor molybdopterin. Sulfite oxidase is a homodimer with two matching 40 kDa subunits. The SDS-PAGE analysis of purified sulfite oxidase had a band at its approximate molecular weight of 40 kDa (figure 8.).

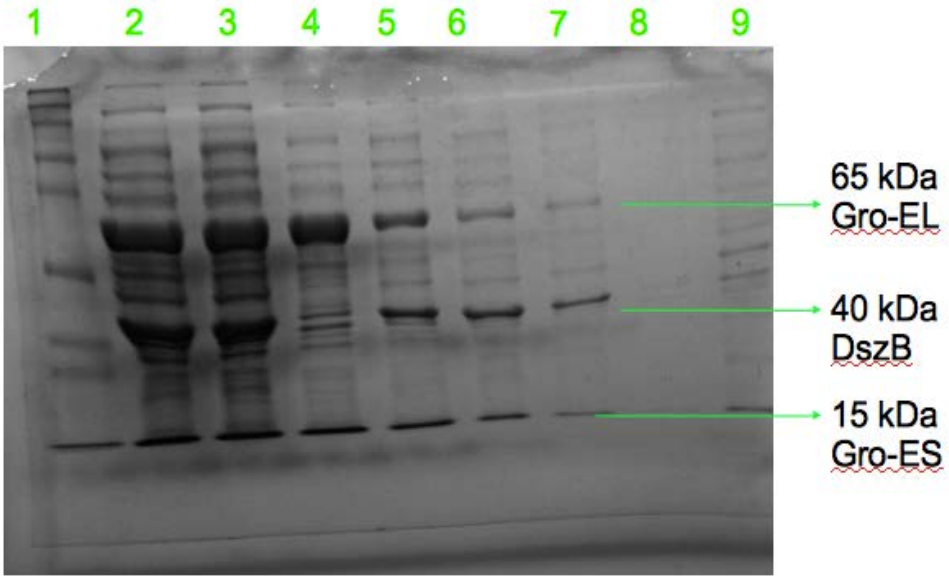


Figure 7. Purification of DszB-A3H1. Lane 1 full range rainbow marker, Lane 2 filtered lysate, Lane 3 incubation, Lane 4 wash 1, lane 5 wash 2, lane 6 wash 3, lane 7 elution 1, lane 8 elution 2, lane 9 full range rainbow ladder. A 10% resolving and 4 % stacking SDS gel was used and stained with coomassie blue.

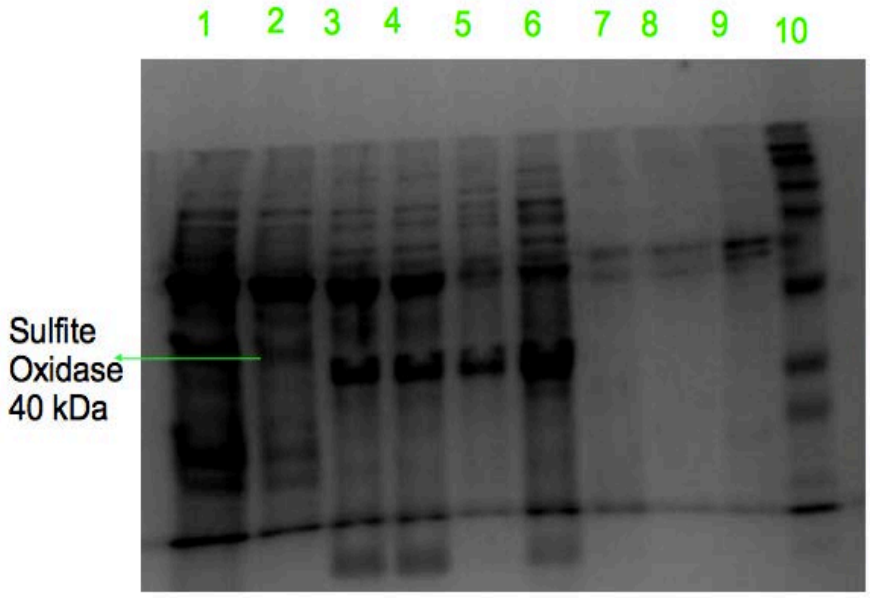


Figure 8. Purification of DszB-A3H1, DszB-A3H1-A200R, DszB-IGTS8 and SO. Lane 1 Full range rainbow marker, Lane 2-4 DszB-IGTS8, Lane 5-6 DszB-A3H1-A200R, Lane 7-8 DszB-A3H1, Lane 9-10 S0.

Characterization of DszB-A3H1-A200R

Stability and Storage Studies of Purified Recombinant Enzymes

All recombinant enzymes, after purification, were stored at 4 ^{o}C. Desulfinases DszB-A3H1 and DzsB-A3H1-A200R retained activity that lasted from 2 to 4 weeks. Desulfinase DszB-IGTS8 was less stable than DszB-A3H1 and DzsB-A3H1-A200R, with activity lasting 1 to 6 days. Sulfite oxidase retained active that lasted 4 to 6 weeks.

Determination of Temperature Optima and Temperature Stability of DszB-A3H1-A200R

Temperature optima studies were conducted with temperatures ranging from 25 ^{o}C to 60 ^{o}C to identify the optimum temperature for desulfination by DszB-A3H1-A200R, as described in methods. Results are shown in figure 9. The enzyme was active at temperatures between 25 ^{o}C to 45 ^{o}C, with peak activity at 35 ^{o}C. Enzyme activity was drastically diminished above or below its optimum temperature, producing a sharply peaked bell shaped curve. The optimum temperature for DszB-A3H1-A200R is 35 ^{o}C, matching the temperature optimum of desulfinating enzymes from *R. erythropolis* IGTS8, *R. erythropolis* KA2-5-1, recombinant *N. asteroides* A3H1, recombinant mutant DszB-A3H1-R84Q-Y24F, and recombinant DszB-IGTS8 [9, 10, 31, 32]. The A200R mutation of DszB-A3H1 had no effect on the temperature optima.

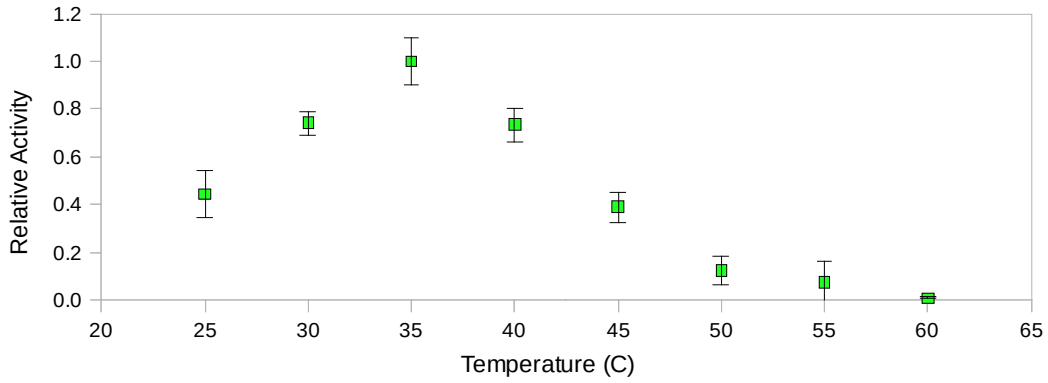


Figure 9. Temperature optimum assays. Temperature optimum assays were conducted fluorometrically, monitoring HBP fluorescence. Each reaction was incubated in triplicate at a given temperature for 5 min at pH 7.2 before the assay was started. The first three min of kinetic data was collected and plotted in Excel.

Temperature stability studies were carried out as described in the method with temperatures ranging from 25 ^{o}C to 55 ^{o}C. Reactions were incubated for 30 minute before the reaction was initiated, results are shown in figure 10. A bell shaped curve was observed for temperature stability curve of DszB-A3H1-A200R, with the optimum temperature stability at 30 ^{o}C. A variation in the temperature stabilities of DszB was observed in recombinant, native, and mutant DszB enzymes (table 7.) [9, 13, 31, 32, 33, 34, 35].

The temperature stability of the desulfinase DszB-A3H1-A200R increased by 5^{o}C when compared to DszB-A3H1 and A3H1-R84Q-Y24F [31]. No significant difference in activity was observed when DszB-A3H1-A200R was compared to recombinant DszB-IGST8, however a 5 ^{o}C decrease in stability was observed when compared to native DszB-IGTS8 and DszB-KA2-5-1 [9, 32, 33,]. Site directed mutagenesis of DszB-A3H1-A200R had a minor role in stabilizing enzyme activity at elevated temperatures. Determining what critical amino acids are important in temperature stability is difficult

without the identification of more thermophilic enzymes. However, a particular study of interest involved a point mutation of DszB-KA2-5-1-Q65H [34]. The mutation increased temperature stability to 45 ^{o}C, an increase of 10 ^{o}C when compared to the native DszB-KA2-5-1. Amino acid substitution of glutamine to histidine is more basic.

Table 8. Temperature stability of DszB enzymes.

DszB	Temperature Stability ^{o}C
A3H1-R84Q-Y24F recombinant [31]	25
A3H1-R84Q recombinant [35]	-
A3H1-recombinant [31]	25
IGTS8 [9]	35
IGST8 recombinant [32]	30
KA2-5-1 [33]	28
KA2-5-1-Q65H recombinant [34]	45
A11-2 recombinant [13]	55

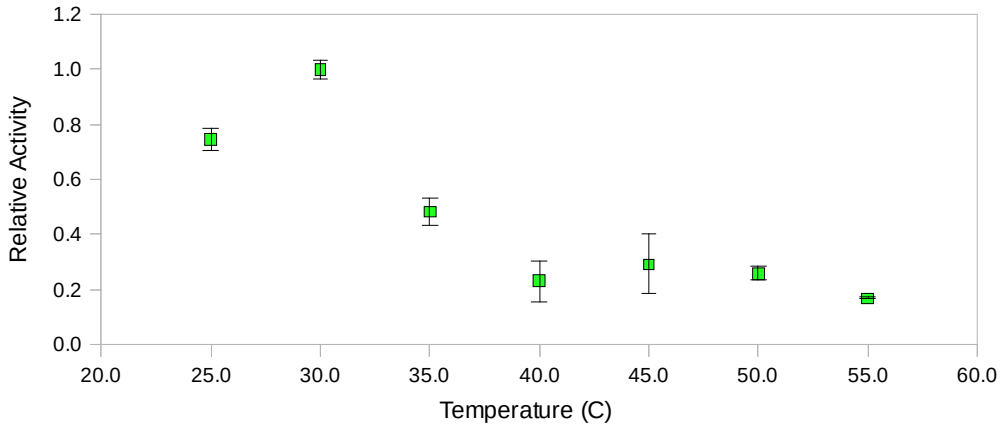


Figure 10. Temperatue stability assay. Temperature stability assays were conducted fluorometrically, monitoring HBP fluorescence. Each reaction was incubated in triplicate at it given temperature for 30 min and allowed to cool for 10 min at 25 C before the assay was started. Assay was conduct at pH 7.2. The first three min of kinetic data were collected and plotted in Excel.

pH Studies of DszB-A3H1-A200R

The effect of pH on DszB-A3H1-A200R was measured over pH values 5.5 to 9.5 as described in the methods. The enzyme was active between the pH values of 6.0 to 9.5. A steady linear increase in enzyme activity was observed from 6.0 to 9.0, and results are shown in figure 11. The optimum pH for DszB-A3H1-A200R was 9.0, and activity decreased slightly above its pH optimum. A variation of the pH optima from recombinant, native, and mutant DszB enzymes are shown in table 8 [9, 13, 31, 32, 33, 35]. The DszB-A3H1-A200R mutation shifted the pH activity profile to higher pH values. The optimum pH increased of 0.5 units from the 8.5 observed in DszB-A3H1.

Table 9. pH optimum values for DszB enzymes.

DszB	pH
A3H1 recombinant [31]	8.5
A3H1-R84Q-Y24F recombinant [31]	8.5
A3H1-R84Q recombinant [35]	8.0
KA2-5-1 [33]	7.5
IGTS8 recombinant [32]	undetermined
IGTS8 [9]	7.0
A11-2 recombinant [13]	8.0

A similar study of interest was conducted with the site-directed mutagenesis study of DszB-A3H1-R84Q from pGEX-4T-3/A3H1 [35]. The site-directed mutagenesis of DszB-A3H1-R84Q mutated the amino acid arginine to glutamine,and lowered the pH optimum to 8.0. The DszB-A3H1-R84Q mutation is a non active site mutation that directly lowered the pH optimum. Studies on the desulfinase, DszB-A3H1-A200R and DszB-A3H1-R84Q, suggests that basic amino acid mutation involving arginines on variable loops 50-65 and 180-210 assist in maintaining enzyme activity at elevated pH values.

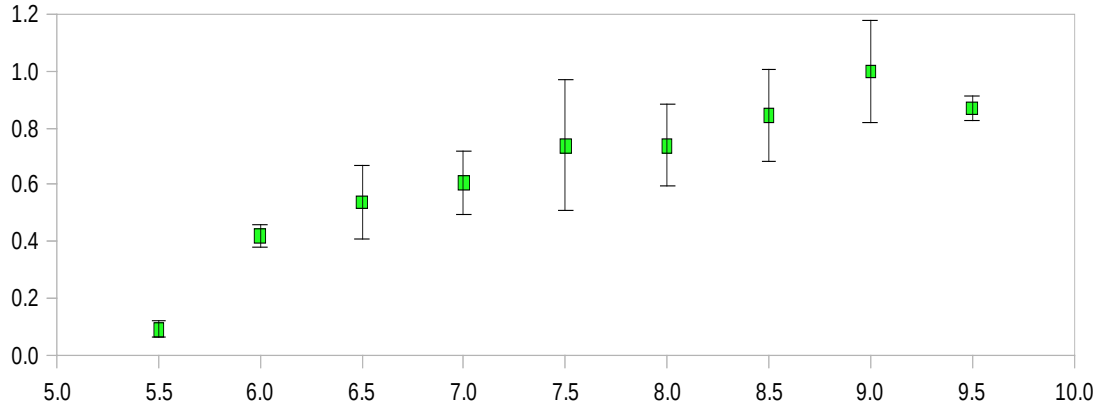


Figure 11. pH optimum assay. pH studies were conducted fluorometrically, monitoring HBP fluorescence. Each reaction was incubated in triplicate at it given temperature for 5 min at varied pH values with MTEN buffer. Data were collected and plotted in Excel.

Enzyme Kinetics of DszB-A3H1-A200R

The kinetic parameters of DszB-A3H1-A200R were measured as described in the method. A fixed concentration of enzyme was plotted against varying concentrations of HPBS to determine the initial rate of the reaction. Product formation of HBP was measured and data were collected from the initial rate period of each assay. The initial velocities of each data point was plotted against HPBS concentrations as shown in figure 12. Data were fitted to the Michaelis-Menten equation for kinetic analysis of DszB-A3H1-A200R. The K_m and k_{cat} of DszB-A3H1-A200R were determined to be 3.71 ± 1.54 μM and 1.10 ± 0.10 min^{-1}. The site-directed mutagenesis of DszB-A3H1-A200R showed no significant change in k_m or k_{cat} when compared to recombinant DszB-A3H1. Comparing DszB-A3H1-A200R to other mesophilic desulfinases there is relativly little

change in K_m or k_{cat} values, as shown in table 9. [9, 11, 13, 31, 32, 33, 35].

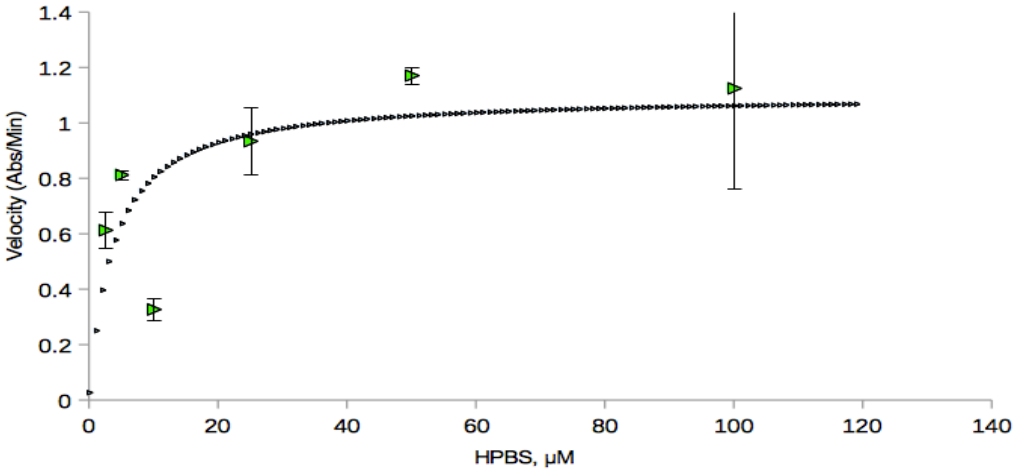


Figure 12. Kinetic study of DszB-A3H1-A200R. HBP formation was monitored through fluorescence. Data were fitted with VisualEnzymics for K_m and k_{cat} values.

Table 10. Comparison of kinetic data from DszB enzymes.

DszB	k_{cat} (min^{-1})	K_m (μM)
A3H1 recombinant [31]	1.24 ± 0.054	3.15 ± 0.74
A3H1-R84Q-Y24F – recombinant [31]	4.33 ± 0.67	51.25 ± 0.96
A3H1-R84Q – recombinant [35]	13.1 ± 1.3	22.9 ± 8.9
IGTS8 – native [11]	1.3 ± 0.07	0.90 ± 0.15
IGTS8 – native [9]	2	1
IGTS8-recombinant [32]	3.75 ± 4.79	0.68 ± 0.15
KA2-5-1 [33]	7.38	8.2
A11-2 – recombinant [13]	19.2	0.33

Substrate Synthesis

Synthesis of HPES

The synthesis of HPES was a two step synthesis, as described in the methods [18,19, 28]. The sultine 1,4-dihydro-2,3-benzoxathiin-3-oxide was synthesized in two separate one step reactions with different reagents, solvents, and extraction procedures. Compound HPES was derived from 1,4-dihydro-2,3-benzoxathiin-3-oxide in a ring opening base hydrolysis reaction. The synthesis of 1,4-dihydro-2,3-benzoxathiin-3-oxide was analyzed with proton NMR. The NMR contained peaks at 3.50, 4.31, 4.88, 5.23 and 7-7.42 confirmed product formation [36]. Results are shown in figure 13.

The synthesis of the sultine 1,4-dihydro-2,3-benzoxathiin-3-oxide in DMSO was significantly faster than DMF. DMSO was unable to be fully removed from the reaction,

and was observed via proton NMR, with a peaks at 2.53. However, the synthesis of 1,4-dihydro-2,3-benzoxathiin-3-oxide in DMSO was used in further experiments, including the ring opening base hydrolysis reaction and fixed timed assays. The synthesis 1,4-dihydro-2,3-benzoxathiin-3-oxide in DMF was unable to go to completion as determined by the presence of starting reagent a-a'-dichloro-o-xylene, observed by proton NMR with peaks at 4.66 and 7.11-7.32. Each reaction mixture was purified with a 20 g silica column and a cleaner product was obtained in lower yields. A column size of 2.5-5.0 g was recommended to increase purity and recovery of sample.

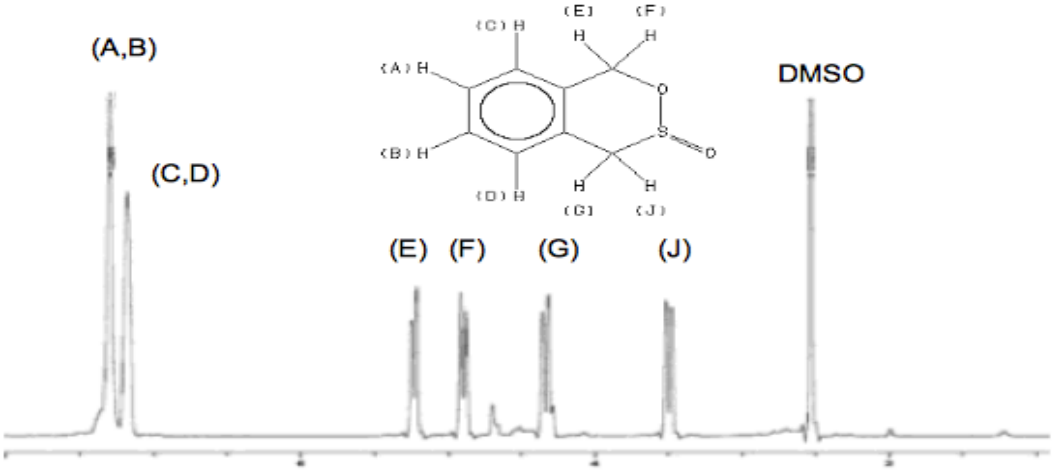


Figure 13. Proton NMR of sultine 1,4- dihydro-2,3-benzoxathiin-3-oxide with solvent DMSO. Sultine peaks include 3.44-3.57, 4.35, 4.90, 5.17, and 7.17-7.29. DMSO peak 2.49-2.77.

Substrate Synthesis using the Cell Free Assays

The synthesis of HPBS and HPBS analogs using the cell free assay was conducted as described in the methods. *R. erythropolis* IGTS8 cells were started in LB media and transferred into a expression media containing DBT. The cells were incubated in expression media for 7 days, turning the white media into a pinkish-salmon color. The cells were removed with centrifugation and prepared for lysing. Cultured cells were flash frozen and lysed with a bead beater, an efficient method for lysing *R. erythropolis* IGTS8 cells. Cell lysate was prepared and placed into the cell free assays with the appropriate thiophenic compounds. Organic compounds were extracted after the cell free assay was stopped and acidified with HCl. Compounds $DBTO_2$, HPBS, HBP, and DBT were mixed together and separated with HPLC to serve as standards for observing separation of derived compounds from the cell free assays' organic extract. The separation of DBT, $DBTO_2$ HPBS and HBP is shown in figure 14. Extracted organic compounds were purified with HPLC and placed in a fixed timed assay. Aliquots from the fixed timed assays were coupled with sulfite oxidase to detect sulfite formation.

The isolation and positive identification of HPBS from the cell free assay was unsuccessful. Peaks isolated by comparison to HPLC standards were collected and conformation of identity was attempted. However, the purification of HPBS from the cell free assay was difficult due to the number of compound's in the mixture and available techniques for identifying HPBS. Identification of HPBS was attempted with GC-MS, but proved unsuccessful. The chemical properties of HPBS make identifying HPBS difficult, due to the compounds high vaporization point and low mass to charge ratio. HPBS has a vaporization point above 500 ^{o}C, making detection of HPBS difficult with

the equipment at Texas State. Papers in the literature widely used GC-MS to identify DBT and HBP due to their low vaporization point and the ramp time needed to eluted the two compounds. Identification of DBTO and HPBS is not often reported in the literature due to the chemical properties of HPBS and high conversion rate of DBTO into $DBTO_2$ by DszD. The separation and elution of DBT, $DBTO_2$, HPBS, and HBP using GC-MS requires a temperature range of 275 to 800 ^{o}C [37]. However, DBT and HBP were successfully identified and separated with GC-MS using a 40 min ramp time at 270 ^{o}C (data not shown).

Aliquots from HPLC that eluted at a time consistent with HPBS elution were placed in a fixed timed assay that was coupled with sulfite oxidase. The sulfite oxidase assay was inconsistent, with false negatives and false positives were observed when using DBT as a starting substrate in the cell free assay. The negative and false positives observed from the sulfite oxidase assay is further explained in the substrate specificity section. Due to the several unsuccessful attempts isolate sulfinate compounds from other components in the organic extract, attention was redirected to the synthesis of HPBS analogs.

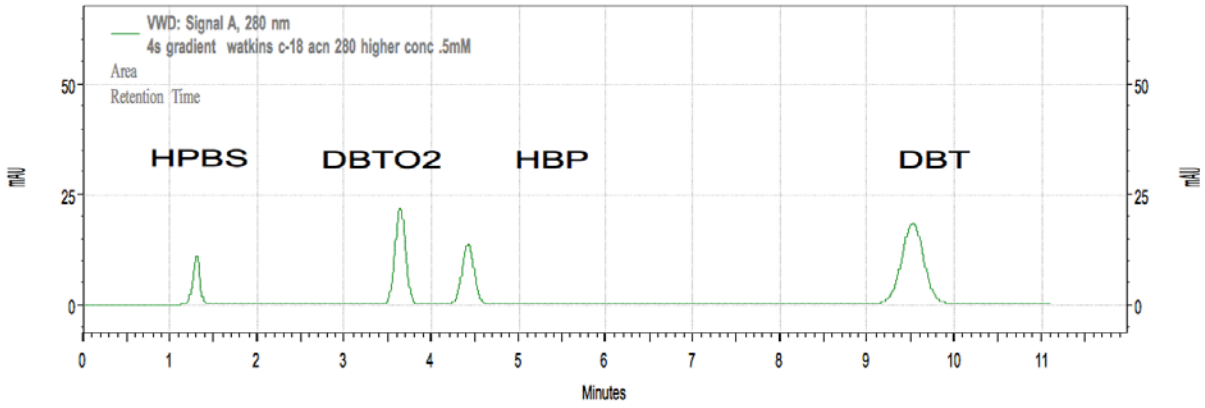


Figure 14. HPLC separation of HPBS, $DBTO_2$, HBP, and DBT. Separation DBT, $DBTO_2$, HPBS, and HBP were suspended in ACN, at a final concentration of 0.1mM. All four compounds were separated with a C18 column, using a gradient mobile phase as described in the methods [21].

Substrate Specificity

Substrate Specificity of DszB-A3H1, DszB-A3H1-A200R, and DszB-IGTS8

Sulfite oxidase is an enzyme that oxidizes sulfite to sulfate through the reduction of cytochrome c [38,39]. Sulfite oxidase is a metallo-enzyme that contains a molybdenum co-factor and heme group used in transferring electrons from sulfite onto cytochrome c. The oxidation state of cytochrome c is easily monitored through UV-Vis spectrometry.

Sulfinated compounds BS, HPES, HPBS were placed in a fixed timed assay containing either DszB-A3H1, DszB-A3H1-A200R, or DszB-IGTS8 (figure 15). At fixed times the aliquot of assay sample was removed and sulfite oxidase was added to detect the presence of the product sulfite. The activity of DszB in the prescence of all the substrate analogs is indirectly measured through the oxidation of sulfite and by

cytochrome c. The spectial difference between oxidized and reduced cytochrome c can be used to measure sulfite concentrations. The sulfite oxidase assay was performed as described in the methods.

Sulfite oxidase detected sulfite from HPBS when DszB-A3H1, DszB-A200R, and DszB-IGTS8 was used in the free cell assay. In the sulfite oxidase assay, the desulfinase enzymes DszB-A3H1, DszB-A200R, and DszB-IGTS8, had a change in absorbance of 0.073, 0.061, and 0.010, respectively. The desulfinase DszB-A3H1-A200R was able to desulfinate HPES with a change in absorbance of 0.012, which is significantly lower than the desulfination of HPBS. A change in absorbance of 0.009 was observed for DszB-A3H1 and BS. The desulfinase DszB-IGTS8 was unable to desulfinate BS or HPES, with a low rate of desulfination with HPBS. The low rate of desulfination could have been attributed to the high concentration of HPBS, inhibiting DszB-IGTS8.

Due to the redox potential of cytochrome c, a series of positive and negative controls were conducted to rule out false positives. Cytochrome c can act as a biocatalyst by accepting electrons and oxidizing various compounds that can donate electrons [40]. DBT, a compound of particular interest, can donate its electrons and reduce cytochrome c. Cytochrome c can participate in reactions that include hydroxylation, n-demethylation, expoxiation, s-oxidation, and aromatic oxidation [40]. Negative controls were run that included DMSO, substrates, and DszB desulfinase. A kinetic assay with HPBS and 0.1 mM DMSO was tested with DszB-A3H1-A200R and DszB-A3H1 to determine if DMSO had negative effects in the enzyme catalysis. DMSO at 0.1mM had no effect in the ability of DszB-A3H1-A200R or DszB-A3H1 to desulfinate, however DszB-IGTS8 was

affected by DMSO and the concentration was lowered to 0.01 mM DMSO in order to regain activity.

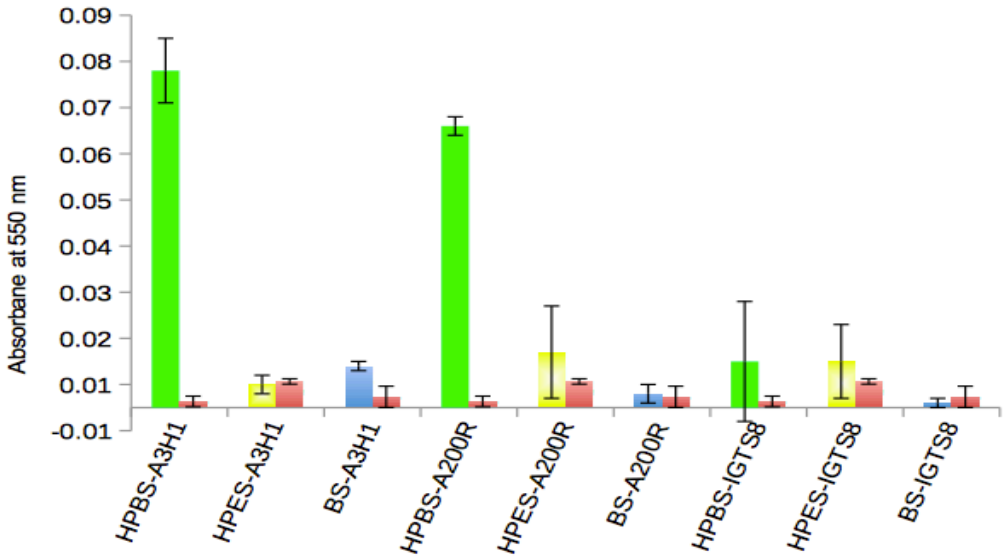


Figure 15. Sulfite Oxidase assay. Fixed time assay was conducted with DszB-A3H1, A3H1-A200R, and DszB-IGTS8. A final concentration of 0.1mM. HPBS (green), BS (blue), and HPES (yellow) was used in the SO assay. Controls were preformed with no enzyme (red). Data were collected and plotted with Excel.

CHAPTER IV

CONCLUSION

The determination of DszB enzyme specificity was conducted with three recombinant DszB enzymes. DszB-A3H1, DszB-A3H1-A200R, and DszB-IGTS8 were assayed against substrates BS, HPBS, and HPES. Site directed mutagenesis of DszB-A3H1-A200R showed a decrease in substrate specificity towards BS when compared to DszB-A3H1. Additional conclusion regarding change in DszB specificity could not be conclusively proven due to the overlap in experimental error. The characterization study of DszB-A3H1-A200R showed the temperature stability increased from 25 ^{o}C to 30 ^{o}C, and the pH optimum increased from 8.5 to 9.0. Small, but significant changes to stability and pH were influenced by the A200R mutation in DszB, while the enzyme's rate of catalysis and K_m was not affected. In conclusion, I believe that both the A195R and A200R mutations in DzsB-A3H1 would be needed in order to change enzyme specificity. More site-directed mutagenesis studies would be needed to determine the elements of etating specificity of DszB. Moreover, I believe future site-directed mutagenesis studies regarding both kinetics and enzyme specificity should be focused on amino acids E189, E191, and D193 in DszB-A3H1.

REFERENCES

1. Kilbane, J. Desulfurization of coals: the microbial solution. *Biotechnol.* **1989**, *7*, 97-101.

2. Shriver, L.; Carrere, D.; Schriver, A.; Jaeger, K. Matrix-isolation photolysis of SO_2, O_3 and H_2O: evidence for H2O:SO3 complex. *Chem. Phys.* **1991**, *181*, 505-511.

3. Takashi, O.; Izumi, I. Microbial desulfurization of organic sulfur compound in petroleum. *Biotechnol.* **1999**, *63*, 1-9.

4. Monticello, D. J.; Finnerty, W. R. Microbial Desulfurization of Fossil Fuels. *Microbiol.* **1985**, *39*, 371.

5. Gallagher, J.R.; Olson, E.S.; Stanley. D.C. Microbiol desulfurization of dibenzothiophene: a sulfur specific pathway. *Microbiol Lett.* **1993**, *107*, 31-5.

6. Ghasemali, M.; Andrew, S.B. Biocatalyst desulfurization (BDS) of petrodiesel fuels. *Mircobio.* **2008**, *154*, 2169-2183.

7. Kilbane, J.J. Microbial biocatalyst development to upgrade fossil fuels. *Curr. Opin. Biotechnol*. **2006**, *17*, 305-314.

8. Pacheco, M.; Lange, E.; Pienkos, P.; Yu, L.; Rouse, M.; Lin, Q.; Linguist, L., Recent advances in biodesulfurization of diesel fuel. AnnualMeeting NPRA: 1999 March 21–23; San Antonio, Texas.Washington, DC: National Petrochemical and Refiners Association:1999:AM-99-27.

9. Gray, K. A.; Pogrebinsky, O. S.; Mrachko, G. T.; Xi, L.; Monticello, D. J.; Squires, C. H. Molecular mechanism of biocatalytic desulfurization of fossil fuels. *N. Biotechn.* **1996**, *14*, 1705-1709.

10. Lee, W. L.; Ohshiro, T.; Matsubara, T.; Y. Isumi, Y.; Tanokura, M. Crystal Structure and Desulfurization Mechanism of 2'-hydroxybiphenyl-2sulfinic Acid Desulfinase. *J. Bio. Chem.* **2006**, *281*, 32534-32539

11. Watkins, L. M.; Rodrquez, R.; Schneider, D.; Broderick, R.; Cruz, M.; Chambers, R.; Ruckman, E.; Cody, M.; Mrachko, G. T. Purification and characterization of the aromatic desulfinase, 2-(2'-hydroxyphenyl)benzenesulfinate desulfinase. *Biochem. Biophys. Acta.* **2003**, *415*, 14-23.

12. Bressler, D. C.; Norman, J. A.; Fedorak, P. M. Ring Cleavage of sulfur heterocycles: How does it happen. *Biodegrad.* **1998**, *8*, 297-311.

13. Konishi, J.; Maruhashi, K. 2-(2'Hydroxyphenyl)benzene sulfinate desulfinase from the thermophilic desulfurization bacterium Paenibacillus sp. strain A11: purification and characterization. *Microbiol. Biotechnol.* **2003**, *62*, 356-361.

14. Ishii, Y.; Konishi, J.; Okada, H.; Hirasawa, K.; Onaka, T.; Suzuki, M. Operon structure and functional analysis of the genes encoding thermophilic desulfurizing enzymes of *Paenicacillus* Paenibacillus sp. A11-2. *Biochem. Biophys. Res. Commun*. **2000**, *270*, 81-88.

15. Gandour, R. D. *Biooorg. Chem.* **1981**, *10*, 169-176.

16. Kimiko, W.; Ken-ichi, N.; Maruhashi, M. K. Selective cleavage of 10-methyl benzo[b]naphtho[2,1-d]thiophene by recombinant Mycobacterium sp. strain. *Biotech. L.* **2003**, *25*, 797-803.

17. Kirimura, K.; Furuya, T.; Nishii, Y.; Ishii, Y.; Kino, K.; Usami, S. Biodesulfurization of Dibenzothiophene and its Derivatives through the Selective Cleavage of Carbon-Sulfur Bonds by a Moderatly Thermophilic Bacterium Bacillus subtilis WU-S2B. *J. Biosci. Bioeng.* **2001**, *91*, 262-266.

18. Hoey, M. D.; Dittmer, D. C. Convenient Synthesis of 1,4-Dihydro-2,3-Benzoxathiin-3-oxide, a Useful Precursor of 0-quinodimethane. *J. Org. Chem.* **1991**, *56*, 1947-1948.

19. Okuyama, T.; Takano, H.; Ohnishi, K.; Nagase, S. Ring Opening and Closure and Oxygen Isotope Exchange of Cyclic Sulfinate Esters. *J. Org. Chem.* **1994**, *59*, 472-476.

20. Li, M.Z; Squires, C.H.; Monticello, D.J.; Childs, J.D. Genetic Analysis of the *dsz* Promoter and Associated Regulatory Regions of *Rhodococcus erythropolis* IGTS8. *J. Bacter.* **1996**, *178*, 6409-6418.

21. Alcon, A.; Santos, V. E.; Martin, A. B.; Yustos, P.; Garcia-Ochoa, F. Biodesulphurization of DBT with Pseudomonas putida CECT5279: Influence of cell growth time on reducing equivalent concentration and HpaC activity. *Biochem.* **2005**, 26, 168-175.

22. Dale, G.E.; Schonfeld, H.J.; Langen, H.; Stieger, M. Increased solubility of trimethoprim-resistant type S1 DHFR from *Staphlococcus aureus* in *Esherichia coli* cells overproducing the chaperone GroEL and GroES. *Protein Eng*. **1994**, *7*, 925-31.

23. Stratagene. QuikChange® Site-Directed Mutagenesis Kit Instruction Manual. QuikChange® Site-Directed Mutagenesis Kit Instruction manual. http://www.tufts.edu/~mcourt01/Documents/Stratagene%20Quikchange%20mutagenesis.pdf (accessed Aug 6, 2012).

24. Stratagene. BL21(DE3) Competent Cells, BL21(DE3)pLysS Competent Cells, and BL21 Competent Cells. http://www.molecularinfo.com/MTM/A/A3/A3-1.pdf (accessed Aug 6, 2012).

25. Qiagen. QIAprep® Miniprep Handbook. http://www.qiagen.com/selectlocation.aspx?redirect=%2fliterature%2frender.aspx%3fid%3d370 (accessed Aug 6, 2012).

26. Invitrogen. ProBondTM Purification System For purification of polyhistidine-containing recombinant proteins. http://aquacul4.fish.washington.edu/Protocols:Information%20Sheets/Commercial%20Protocols:Manuals/Invitrogen%20-%20ProBond%20Purification%20System.pdf (accessed Aug 6, 2012).

27. G Biosciences. Nickel Chelating Resin. http://www.gbiosciences.com/634030510774687628.pdf (accessed Aug 6, 2012).

28. Indusegaram, S.; Charlotte, S.; Ridley, D. D. METHODS FOR PREPARING SULTINES. U.S. Patent 8,119,820 B2, February 21, 2012

29. Sigma. Enzymatic Assay of Sulfite Oxidase (EC 1.8.3.1) http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/sulfiteoxidase.Par.0001.File.tmp/sulfiteoxidase.pdf. (Accessed Aug 6, 2012).

30. Ohshiro, T.; Ohkita, R.; Takikawa, T.; Manabe, M.; Lee, W. C.; Tankokura, M.; Izumi, Y. Improvement of 2'-Hydroxybiphenyl-2-sulfinate Desulfinase, an enzyme involved in Dibenzothiophene Desulfurization Pathway, from Rhodococcus erythropolis KA2-5-1 by Site-Directed Mutagenesis. *Biosci, Biotechnol. Biochem.* **2007**, *71*, 2815-2821.

31. Vaz, J. C. Purification, Characterization, and Active Site Studies of 2-(2'-hydroxyphenyl) Benzensulfinate Desulfinase (DszB) From *Nocardia asteroides* sp. Strain A3H1. Thesis, Chemistry Department, Texas State, Tx, 2009.

32. Harper, L.T. Expression and characterization of recombinant 2-(2'-hydroxyphenyl)benzenesulfinate and desulfinase form *Rhodococcus erythropolis sp*. IGTS8. Thesis, Chemistry Department, Texas State, Tx, 2011.

33. Nakayama, N.; Matsubara, T.; Ohshiro, T.; Moroto, Y.; Kawata, Y.; Koizumi, K.; Hirakawa, Y.; Suzuki, M.; Maruhashi, K.; Izumi, Y.; Kurane, R. A novel enzyme, 2â€²-hydroxybiphenyl-2-sulfinate desulfinase (DszB), from a dibenzothiophene-desulfurizing bacterium Rhodococcus erythropolis KA2-5-1: gene overexpression and enzyme characterization. *Protein. Proteom.* **2002**, *1598*, 122-130.

34. Ohshiro, T.; Ohkita, R.; Takikawa, T.; Manabe, M.; Lee, W. C.; Tankokura, M.; Izumi, Y. Improvement of 2'-Hydroxybiphenyl-2-sulfinate Desulfinase, an enzyme involved in Dibenzothiophene Desulfurization Pathway, from Rhodococcus erythropolis KA2-5-1 by Site-Directed Mutagenesis. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 2815-2821.

35. Gonzales, C. Purification and Characterization of Recombinant 2-(2’ − hydroxyphenyl) Benzenesulfinate Desulfinase from *Norcardia asteroides* strain. A3H1.Thesis, Chemistry Department, Texas State, Tx, 2007.

36. Pirkle, W. H.; Hoekstra, M. S. Chiral Nuclear Magnetic Resonance Solvating Agent. Resolution, Determining of Enantiomeric Purity, and Assignment of Absolute Configuration of Cyclic and Acyclic Sulfinate Esters. *J. Am. Chem. Soc*. **1976**, 98, 1832-1839.

37. Papizadeh, M.; Roayaei, M.; Ardakani, R.; Motamedi, H.; Rasouli, I.; Zarei, M. C-S Targeted Biodegradation of Dibenzothiophene by Stenotrophomonas sp. NISOC-04. *Appl Biochem. Biotechnol* **2011**, *165*, 938-948.

38. Kessler, D. L.; Rajagopalan, K. V. Purification and Properties of Sulfite Oxidase from Chicken Liver. *J. Bio. Chem.* **1972**, *247 (20)*, 6566-6573.

39. Stryer, L. In *Oxidative Phosphorlation;* Biochemistry; W.H. Freeman and Company New York: USA, 1995; Vol. 4; 539-542.

40. Vazquez-Duhalt, R. Cytochrome c as a biocatalyst. *J. Mol. Cata. B: Enzymatic* **1999**, 241-249.