

PURIFICATION, CHARACTERIZATION, AND ACTIVE SITE STUDIES OF
2-(2'-HYDROXYPHENYL) BENZENESULFINATE DESULFINASE (DSZB)
FROM *NOCARDIA ASTEROIDES* SP. STRAIN A3H1

THESIS

Presented to the Graduate Council of
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San Marcos, Texas

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by

Jerusha Comoa Vaz

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DEDICATION

I dedicate this work to the memory of my late father, Herman Llewelyn Vaz.

ACKNOWLEDGEMENTS

First, I thank God for His Grace and Providence that have brought me this far.

I would like to express my heartfelt gratitude to Dr. Linette M. Watkins, my advisor, for granting me the opportunity to conduct this research. She has provided me with tremendous support and guidance even through difficult times and has been a great source of inspiration to me. I also thank the members of my committee, Dr. L. Kevin Lewis and Dr. Rachell E. Booth for always accommodating me whenever I had questions. To all my lab colleagues, especially Daniel Petty and Leona Martin, I will never forget how hard we worked together as a team, and the good times we shared.

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ABSTRACT

PURIFICATION, CHARACTERIZATION, AND ACTIVE SITE STUDIES OF 2-(2'-HYDROXYPHENYL) BENZENESULFINATE DESULFINASE (DSZB) FROM *NOCARDIA ASTEROIDES* SP. STRAIN A3H1

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Dibenzophene (DBT) and its complex heterocyclic derivatives comprise most of the sulfur that contaminates fossil fuels. The 2-(2'-hydroxyphenyl) benzenesulfinate desulfinate (DszB) enzyme metabolize sulfur from DBT compounds. DszB, one of the four enzymes of the desulfurizing pathway, catalyzes the carbon-sulfur bond cleavage in the last, and rate limiting step. The wildtype A3H1-DszB and the recombinant A3H1-R84Q-Y24F and A3H1-R84Q-C27S DszBs from *Nocardia asteroides* sp. strain A3H1 were purified and characterized. The A3H1-R84Q-C27S- DszB was inactive. The temperature and pH optima for wildtype and A3H1-R84Q-Y24F DszBs were 35 °C and 8.5, respectively; the K_m and k_{cat} were $3.15 \pm 0.74 \mu M$ and $1.24 \pm 0.054 \text{ min}^{-1}$, and $51.25 \pm 0.96 \mu M$ and $4.33 \pm 0.67 \text{ min}^{-1}$, respectively. Overall, the A3H1-DszB showed a slower turnover rate and a moderately lower specificity for substrate when compared to the other DszB homologs referenced in this study. The mutant A3H1-R84Q-Y24F-DszB revealed that the enzyme was more susceptible to inhibition than the wildtype. Both

DszBs were inhibited by 2,2-biphenol. Significant inhibition by a mixture of HBP and sulfite was observed in both enzymes.

CHAPTER 1

INTRODUCTION

Sulfoxides emitted into the atmosphere from the combustion of sulfur-containing fossil fuels have generated one of the most dangerous environmental hazards – acid precipitation. The effects of acid rain are high fish mortality, reduction of forest growth, and corrosion of structures, to name a few [1]. To reduce these effects, governments have intervened with stringent environmental regulations to reduce the allowable sulfur content in petroleum products to 15 ppm by 2010 [2, 3]. Satisfying this mandate, however, is proving to be a challenge as the traditional hydrodesulfurization (HDS) process currently utilized is inefficient in the processing of high-sulfur content fuel. With the depletion of low-sulfur fuel reserves, more of the high-sulfur content crude oil is being refined, necessitating even more efficient desulfurization. Biodesulfurization (BDS), a process that utilizes desulfurizing bacteria to generate higher yields of desulfurized petroleum product without losing calorific value, is being explored as a possible alternative since its development two decades ago [4].

BDS is conducted under milder conditions than HDS. HDS utilizes high temperature and pressure and inorganic catalysts to remove unhindered thiols from petroleum. Yet, this process is incapable of removing sulfur from the aromatic organosulfur compounds like dibenzothiophene (DBT) (Figure 1) and the alkylated DBTs

that comprise about 70% of the sulfur found in petroleum [5, 6]. Most diesel fuel has sulfur content of 0.01 – 0.5% by weight, with the middle distillate fraction containing 500 – 5000 ppm. This high-sulfur fraction is recalcitrant to HDS, destroys catalytic converters in motor vehicles, and upon combustion produces particulate emissions that contribute to acid rain [5, 7].

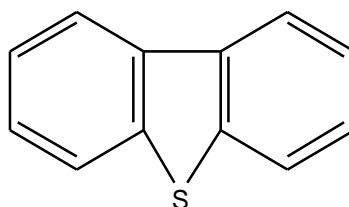


Figure 1. Structure of Dibenzothiophene (DBT).

The United States government intervened in June 2006, through the Environmental Protection Clean Air Act, to reduce the sulfur content of highway diesel fuel to 15 ppm [2]. Sulfur content for non-road applications was reduced from 3400 ppm to 500 ppm in 2007, and will be further reduced to 15 ppm by 2010 [3]. In response, the 2007 Highway Rule mandated that refineries produce ultra-low sulfur diesel. In addition to environmental applications, these mandates have drastic economic implications as future fuel specifications will be determined by new motor vehicles being manufactured [8]. This situation therefore, requires close collaboration between oil refiners and motor vehicle manufacturers in developing products that satisfy government mandates for cleaner air.

The BDS process involves desulfurizing DBT to form 2-hydroxybiphenyl (HBP) and sulfite using three enzymes: DBT-monooxygenase (DszC), DBT-5,5-dioxide

monooxygenase (DszA), and 2-(2'-hydroxyphenyl)benzenesulfinate (HPBS) desulfinase (DszB) (Figure 2). The sulfur in DBT is oxidized twice by DszC to form dibenzothiophene-5,5-dioxide (DBTO₂). Next, one of the sulfur bonds on DBTO₂ is oxidatively cleaved by DszA to form HPBS. Both DszC and DszA require a flavin reductase (DszD) to regenerate the necessary cofactors. In the final and rate limiting step, the last carbon-sulfur bond is cleaved by DszB to generate HBP and hydrogen sulfite [4]. This pathway is sulfur-specific and does not significantly lower the fuel value of the DBT.

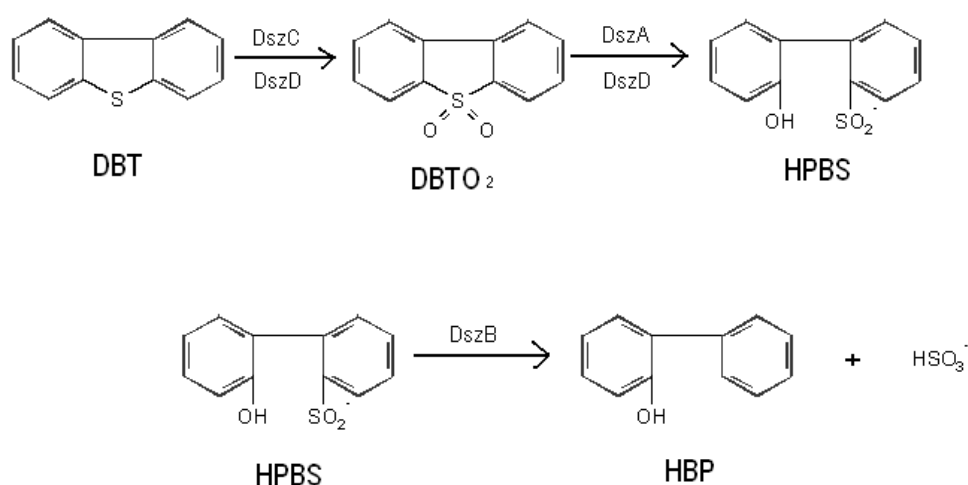


Figure 2. Pathway for desulfurization of DBT in petroleum [9].

The desulfurization pathways in *Rhodococcus erythropolis* strains have been studied most extensively. The genes involved in the dsz pathway from *R. erythropolis*, *dsza*, *dszb*, and *dszc*, encode enzymes containing 453, 365, and 417 amino acids, respectively. Initial kinetic characterization of all three of these enzymes from *R. erythropolis* sp strain IGTS8 (IGTS8) was carried out by Gray *et al.* [9]. The kinetics of the DszA and DszB enzymes were reported. The K_m and k_{cat} values for DszA are 1 μ M

and 1 s^{-1} , and the K_m and k_{cat} for DszB are $1 \text{ }\mu\text{M}$ and 2 min^{-1} , respectively. No k_{cat} value for DszC was determined, but a K_m of $<5 \text{ }\mu\text{M}$ was reported [9].

Since the original report of the *dsz* pathway in *Rhodococcus* [9], several strains of bacteria have been found to contain similar DBT desulfurizing enzymes. These include the mesophiles *Rhodococcus erythropolis* KA2-5-1 [10], *Corynebacterium* sp SY-1 [11], and *Nocardia asteroides* CYKS2 [12], and thermophilic bacteria such as *Paenicibacillus* sp A11-2 [13], *Mycobacterium phlei*, and *Bacillus subtilis* WU-S2B [14].

Initially, it was proposed that thermophiles would be beneficial for industrial purposes because of improved enzymatic rates and enzyme stability at the higher temperatures. However, the mesophiles have better specificity for organosulfur compounds [9, 18]. Both catalytic rate and substrate specificity will determine to what extent the BDS can be commercialized. Since the DszB enzyme is the slowest enzyme in the pathway, several approaches have been described that investigate factors influencing enzyme catalytic rate and specificity. In one recent genetic study of recombinant *R. erythropolis* DR-2, the overlap between the termination codon of the *dszA* and *dszB* genes within the *dsz* operon was removed, resulting in significantly increased enzymatic activity and heat stability when compared to the control [19]. Additional biochemical studies conducted so far on native and recombinant DszBs have provided important information about the active site, structure, and mechanism of the enzyme [10, 16].

First, metal activation and chelation studies revealed that the enzyme cleaves carbon-sulfur bonds without the aid of a metal cofactor. In the metal activation studies the addition of metals such as magnesium, manganese, iron or cobalt did not activate the enzyme. However, copper and zinc totally inhibited enzyme activity of native and

recombinant DszB *Rhodococcus* [10, 16]. Sulfur is known to be a ligand for copper or zinc, and these two metals associate with cysteine and either interfere with substrate binding or the catalysis. Metal chelators are known to form a complex with and/or sequester metals. Metal chelators were mixed with the enzyme to determine if there was a tightly bound metal present at the enzyme active site. In the chelation study, both chelating compounds and non-chelating analogs inhibited enzyme activity of the native DszB [10, 16]. This study revealed that the aromatic structure of the compounds, not their chelating ability, caused the observed inhibition. The results of metal activation and chelation studies indicate that the DszB enzyme does not use a metal cofactor to cleave the carbon-sulfur bond.

Second, chemical modification studies were performed on the native and recombinant DszBs to identify the amino acid residues at the active site. The enzyme lost all activity upon the addition of tryptophan, tyrosine, and cysteine modification reagents [16]. The presence of a competitive inhibitor protected against inactivation. These results indicate that these three amino acid residues are components of the active site and are important for binding and/or catalysis. In addition, the modified sole cysteine residue is completely conserved and is proposed to play a major role in binding and/or catalysis. In a site-directed mutagenesis experiment, DszB lost all activity when the cysteine was mutated to serine [10, 16].

Thirdly, inhibition studies were conducted to define the structural and electronic properties of an inhibitor of the native DszB from *R. erythropolis*. The best inhibitors of the native DszB from IGTS8 have a hydrogen bond donor on one aromatic ring and a hydrogen bond acceptor on the other aromatic ring. From these studies, it was proposed

that an H-bond acceptor and an H-bond donor on a planar aromatic compound were required for inhibition. These studies, coupled with homology studies and pH studies led to the theory that the tyrosine and cysteine residues are present at the active site and catalyze an acid-base reaction with the tyrosine acting as the acid and the cysteine acting as the base. (Figure 3) [16].

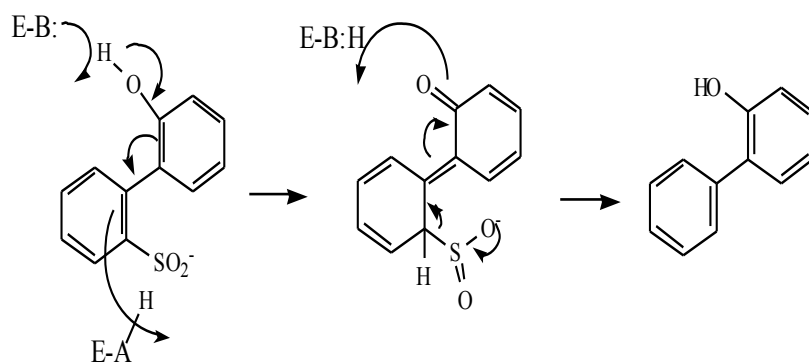


Figure 3. Mechanism for acid-base reaction at the active site of DszB proposed by Watkins *et al.* [16].

An alternative mechanism was proposed by Lee *et al.* (Figure 4) [17]. In this proposed mechanism, cysteine acts as a nucleophile cleaving the carbon-sulfur bond on HPBS in a nucleophilic addition reaction. Their conclusion was based on x-ray crystallography and site-directed mutagenesis of the recombinant *R. erythropolis* DszB.

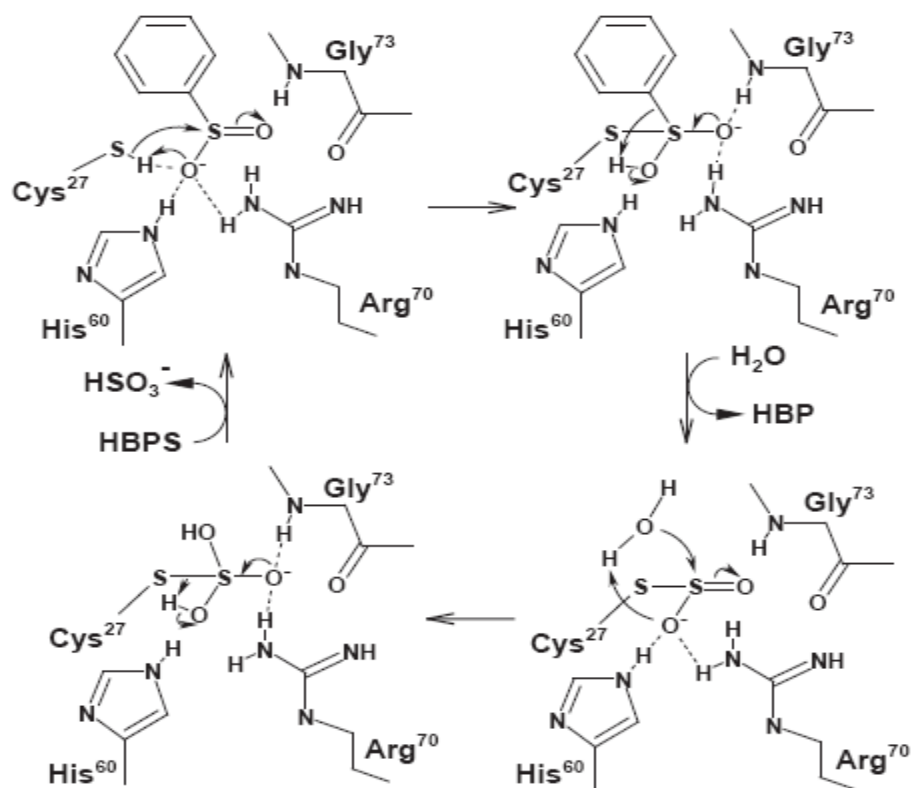


Figure 4. Mechanism for nucleophilic substitution reaction at the active site of DszB proposed by Lee *et al.*[17].

In support of the active site studies, the *dszb* gene sequence of two strains each of *Rhodococcus erythropolis* and *Nocardia asteroides* bacteria revealed that the tyrosine of position 24 and the sole cysteine residue at position 27 are conserved [15]. These residues are proposed to be components of the active site of the DszB enzyme [9, 10, 16]. Sequence alignment of the *dszb* gene from mesophilic and thermophilic bacteria with similar desulfurizing pathway also revealed conservation of the tyrosine and cysteine residues (Figure 5) [17].

DszB	20	DTLT Y SN CPV PNALLTASESGFLD
BdsB	9	DVLAY Y SN CPV PNALLTALESNLLA
TdsB	10	TRVAY Y SN CPV ANALLVASRTGKLE
Atu3432	6	SEIW Y TR CPV PTPVGLAAQLGYLG
SMa2087	8	SHIW Y TR CPV PTPVGLATQLGLLD
ACIAD1512	10	NTLW Y TR CPV PTGLGISIQGWFK
		★
		27

Figure 5. Sequence alignment of the DszB and DszB homologs from mesophilic and thermophilic bacteria [17]. The conserved cysteine residue at position 27 is the sole cysteine within the *dszb* gene sequence.

The studies previously described were performed on native or recombinant DszB from *Rhodococcus*. The purification methods used to obtain these enzymes required between four and seven chromatography steps in which a variety of columns were used to obtain relatively small quantities of pure DszB. A pGEX-4T-3 plasmid was engineered by Dr. Greg Sawyer at the University of Texas at Austin to overexpress and purify the DszB protein in a one-step purification process [20]. The plasmid contains a thrombin cleavage site flanked by Glutathione-S-Transferase (GST) on the N-terminal end and the *dszb* gene insert on the C-terminal end of the thrombin recognition site. The GST system facilitates the one-step purification by binding of the expressed GST-DszB fusion protein to the Glutathione (GSH) column. After the thrombin cleavage reaction, the DszB can be easily eluted from the column.

The recombinant *dszb* gene from IGTS8 was ligated into the plasmid pGEX-4T-3 creating the plasmid pGEX-4T-3/IGTS8. The plasmid was co-expressed with pREP4-GroESL in BL21(DE3) cells. The GroEL and GroES chaperone proteins facilitate proper folding of the DszB [20]. However, during purification, DszB was cleaved internally at the arginine at the 84th position. To eliminate the thrombin recognition site on the DszB, the arginine was mutated to glutamine using site-directed mutagenesis, and the resulting

pGEX-4T-3/IGTS8-R84Q plasmid was overexpressed, purified, and characterized in the Watkins Lab at Texas State [19]. The DszB from this construct however, was unstable and exhibited different properties than the native enzyme. In order to eliminate the need for the R84Q mutation and to generate a wildtype protein, a tobacco etch virus (TEV) cleavage site was inserted between the thrombin recognition site and the *dszb* gene within pGEX-4T-3/IGTS8 plasmid [20]. The TEV site was inserted between the *dszb* gene and the thrombin cleavage site. The pGEX-4T-3/IGTS8-TEV construct however, did not express the GST-DszB fusion protein in any detectable amount.

Another DszB enzyme studied in the Watkins lab is the DszB from *Nocardia asteroides* sp strain A3H1 (A3H1). A3H1 is of particular interest because it has a different specificity than IGTS8 [18]. Dr. Greg Sawyer, in collaboration with the Watkins Lab [16, 20] ligated the *dszb* gene into the pGEX-4T-3 vector. The resulting pGEX-4T-3/A3H1 construct also expressed an enzyme susceptible to internal thrombin cleavage. In response, two new plasmids were created. The pGEX-4T-3/A3H1-R84Q was created by site-directed mutagenesis and codes for the R84Q mutation. The other construct, pGEX-4T-3/A3H1-TEV, was created by inserting the TEV site between the thrombin cleavage site and the *dszb* gene [20].

The DszB from pGEX-4T-3/A3H1-R84Q co-expressed with the chaperone proteins GroES and GroEL was purified and characterized and found to be stable [21]. The A3H1-R84Q-DszB had a molecular weight of 40 kDa. The optimum temperature and pH were 30 °C and 8.0, respectively. The kinetic parameters K_m and k_{cat} were $22.9 \pm 8.5 \mu M$ and $13.2 \pm 1.65 \text{ min}^{-1}$, respectively. The activity of the enzyme was inhibited by copper and zinc metals as they are known to form ligand with the cysteine residue

proposed to be at the active site of the DszB, thereby inactivating the enzyme. In order to test the proposed acid-base mechanism of DszB, site-directed mutagenesis was used to create the pGEX-4T-3/A3H1-R84Q-Y24F by mutating the tyrosine at position 24 to phenylalanine, and the pGEX-4T-3/A3H1-R84Q-C27S by mutating the sole, conserved cysteine at position 27 to serine. The expression and characterization of these mutants is described in the research herein. The pGEX-4T-3/A3H1-TEV on the other hand, failed to express the DszB, the DszB-GST fusion protein in amounts detectable by SDS-PAGE or Western blot. Attempts to express the pGEX-4T-3/A3H1-TEV construct are described in the research herein.

The pTAC-MAT-Tag-2 vector with a 6X His tag (with nickel metal affinity) attached to the C-terminal end of the vector, was chosen as the expression vector for A3H1-DszB. The creation of a pTAC-MAT-Tag-2/A3H1 construct, and the expression, purification and characterization of the wild type A3H1-DszB was the primary goal of this project. To date, this is the first study conducted on the wild type HPBS desulfinate from A3H1. The desulfinate enzyme was compared to the native desulfinate enzyme from IGTS8, the recombinant desulfurizing enzyme from KA2-5-1, and the recombinant thermophilic desulfurizing enzyme from WU-S2B and A11-2. The recombinant HPBS desulfinases from A3H1-R84Q-Y24F and A3H1-R84Q-C27S were characterized and the effects of the active site amino acid substitution on the enzyme were measured. In sum, these studies will provide a better understanding of the DszB mechanism to improve the desulfurization process, and hopefully created groundwork for future experiments that contribute to the commercialization of BDS to obtain cleaner burning petroleum and a cleaner atmosphere.

CHAPTER II

MATERIALS AND METHODS

Materials

The pGEX-4T-3/A3H1-TEV and the pGEX-4T-3/A3H1-R84Q plasmids were obtained from Dr. Greg Sawyer in the Jon Robertus lab at the University of Texas at Austin (Table 1). The pTAC-MAT-Tag-2 vector was purchased from Sigma-Aldrich (St. Louis, MO). The pREP4-GroESL plasmid was provided by Dr. Dean Appling from the University of Texas at Austin. *Escherichia coli* (*E. coli*) Top10, XL1 Blue, and BL21 (DE3) cells were obtained from Invitrogen, Inc. (Carlsbad, CA). All the plasmids that were used in this study are described in Table 1.

Table 1. A description of the plasmids used in this thesis.

Name	Description	Reference
pTAC-MAT-Tag TM -2	The <i>E. coli</i> Expression vector with 6X Nickel Metal Affinity Tag (MAT) at the C-terminal (3' end).	Sigma-Aldrich
pTAC-MAT/A3H1	The pTAC-MAT-Tag TM -2 vector with the <i>dszb</i> gene from A3H1.	Described herein
pGEX-4T-3	The Expression vector with a GST gene fusion system and a thrombin cleavage site.	GE Healthcare Life Sciences
pGEX-4T-3/A3H1-TEV	The pGEX-4T-3 vector with the wild-type <i>dszb</i> gene from A3H1 and the Tobacco Etch Virus (TEV) cleavage site.	Robertus Lab
pGEX-4T-3/A3H1-R84Q	The pGEX-4T-3 vector with the mutant R84Q <i>dszb</i> gene from A3H1.	Robertus Lab
pGEX-4T-3/A3H1-R84Q-Y24F	The pGEX-4T-3 vector with the mutant R84Q and Y24F <i>dszb</i> gene from A3H1.	Watkins
pGEX-4T-3/A3H1-R84Q-C27S	The pGEX-4T-3vector with the mutant R84Q and C27S <i>dszb</i> gene from A3H1.	Watkins Lab
pREP4-GroESL	pREP4 vector with the genes encoding the GroES and GroEL chaperone proteins	Dr. Dean Appling, UT-Austin

The reagents used to prepare media for cell growth - BactoTM agar, BactoTM tryptone, and BactoTM yeast extract - were from Becton Dickinson (Sparks, MD). The antibiotics used in the media, kanamycin and ampicillin, were from SigmaTM- Aldrich (St. Louis, MO) and Shelton Scientific, Inc. (Shelton, CT), respectively. The Orbit Environ Shaker and Lab Rotator were from Lab-Line Instruments, Inc. (Melrose Park, IL). The Thermolyne Maxi Mix II vortex equipment was from Thermo Scientific (Waltham, MA). The Gyromax 747 Orbital Incubator Shaker and Hirayama HIClave

HV-50 autoclave were from Amerex Instruments, Inc. (Lafayette, CA). The Hermle Z180M centrifuge was from Labnet International, Inc. (Edison, NJ).

All plasmid DNA was isolated using the Plasmid Purification Mini Kit with QIAGEN-tip 20 or QIAprep Spin Miniprep Kit from QiagenTM (Valencia, CA). The reagents used to make agarose and polyacrylamide gels and the Western blot, the horizontal gel electrophoresis equipments, and the Smart SpecTM 3000 spectrometer were from Bio-Rad (Hercules, CA). The Ultra-PureTM agarose used to make gels for electrophoresis was from Invitrogen, Inc. (Carlsbad, CA). The standard heat block and the ACCU P'O'W'E'R model 300 for running the agarose gel electrophoresis were from VWR Scientific (West Chester, PA). The ethidium bromide used to stain the agarose gels was from EM Science (Lawrence, KS). The Novex EI 9001 – XCELL IITM Mini Cell polyacrylamide gels electrophoresis apparatus was purchased from Invitrogen (Carlsbad, CA). The anti-GST primary antibody and the anti-goat secondary antibody were from Rockland, Inc. (Gilbertsville, PA). The Kodak digital service image station 440 CF with Kodak ID 3.6 software used to image agarose and polyacrylamide gels was from Eastman (Rochester, NY). The primers used for sequencing the plasmid DNA were from Sigma Genosys (The Woodlands, TX). The AllegraTM 25R centrifuge was obtained from Beckman Coulter, Inc. (Fullerton, CA).

The pTAC-MAT-TagTM-2 vector was purchased from SigmaTM-Aldrich (St. Louis, MO). The primers used to sequence the *dszb* gene in the pTAC-MAT-Tag-2 vector were from Invitrogen, Inc. (Carlsbad, CA). The Wizard®SV Gel and PCR Cleanup System were from Fisher Scientific (Houston, TX). The Microfuge® 18 centrifuge from Beckman Coulter (Fullerton, CA) was used in the plasmid preparation of

the pTAC-MAT/A3H1 plasmid. The Jumpstart™ Taq DNA Polymerase was from Sigma™-Aldrich (St. Louis, MO). The 2720 Thermal Cycler used for the PCR reaction was purchased from Applied Biosystems (Foster City, CA). The FOTO/Phoresis UV black box used to visualize the PCR product on the agarose gel was from Fotodyne (Hartland, WI). The restriction enzymes used in the digests of the PCR and ligation reactions were obtained from New England Biolabs (Ipswich, MA). The T4 DNA Ligase, Alkaline Calf Intestine Phosphatase, and the buffers used in the ligation reactions were also obtained from New England Biolabs (Ipswich, MA).

Cell lysis was performed using a Sonics Vibra Cell™ Ultrasonic Processor from Sonics & Materials, Inc. (Newton, CT). The Glutathione-Agarose 10 mL lyophilized powder used in purification of A3H1-R84Q-Y24F-DszB and A3H1-R84Q-C27S-DszB was from Sigma™-Aldrich (St. Louis, MO). For purification of the A3H1-DszB, the ProBond Nickel Resin used was from Invitrogen, Inc. (Carlsbad, CA), and the imidazole was from Sigma™-Aldrich (St. Louis, MO). The thrombin protease and isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from VWR International, Inc. (St. Louis, MO). The pooled samples from the purified HPBS desulfase were concentrated using Centriplus®YM-10 Centrifugal filter unit from Millipore Corporation (Bedford, MA). The Bovine Serum Albumin (BSA) used in the Bradford assay was obtained from New England Biolabs (Ipswich, MA). Protein concentration using the Bradford assay was read with a Molecular Devices SpectraMAX 190 plate reader and Soft Max®Pro 4.7.1 software (Downingtown, PA). The ND 1000 Spectrophotometer V3.1 from Nano Drop Technologies (Wilmington, DE) was also used to measure protein concentration.

The fluorimetric assays were performed using a Perkin Luminiscence Spectrometer LS50B and data were collected using the FL WinLab software from the Perkin Elmer Corporation (Norwalk, CT) or a Varian Spectrophotometer with the Cary Eclipse software program from Varian, Inc. (Palo Alto, CA). The water bath equipment used in conducting the assays was purchased from VWR (Suwanee, GA), and the microplate heater was from Zyttron (Trenton, NJ). The kinetic parameters of the enzyme were analyzed using the VisualEnzymics program from Softzynamics, Inc. (Princeton, NJ).

Methods

Transformation of Plasmid DNA into *E. coli* Cells

To create plasmid stocks, the pGEX-4T-3/A3H1-TEV plasmid was transformed into competent *E. coli* Top10 cells using a standard heat shock method. Top10 cells and plasmid DNA were placed on ice to thaw, after which 5-10 ng of plasmid DNA was added to 100 μ L of competent *E. coli* Top10 cells. This sample was then incubated on ice for 10 minutes, and heat shock was performed at 42 °C for 2 minutes using a heat block. A 1 mL portion of LB broth at 42 °C was added to the sample and the cells were incubated at 37 °C with shaking at 250 rpm for 1 hour. A 300 μ L aliquot of this transformation mixture was plated onto LB plates to which ampicillin (100 μ g/mL) was added, and incubated overnight at 37 °C. For the control, 5 - 10 ng pUC19 DNA was transformed into Top10 cells.

For protein expression, the pTAC-MAT-Tag-2/A3H1, pGEX-4T-3/A3H1-R84Q-Y24F, or pGEX-4T3/A3H1-R84Q-C27S plasmids were co-transformed into competent

BL21 (DE3)/pREP4_GroESL cells using the above-mentioned procedure and plated on media that contained ampicillin (100 µg/ml) and kanamycin (30 µg/mL).

Plasmid DNA Isolation

One colony from each of the plates of the *E. coli* Top10 cells transformed with either pGEX-4T-3/A3H1-TEV or pTAC-MAT-TAG-2/A3H1 was used to make the respective overnight culture with 5 mL of LB broth and ampicillin (100 µg/mL). Each culture was incubated overnight at 37 °C with shaking at 250 rpm. The plasmid DNA was isolated using the QiagenTM Plasmid Purification Mini Kit with QIAGEN or QIAprep Spin Miniprep Kit. Isolated plasmids were stored in nuclease-free ddH₂O at -20 °C.

DNA Quantitative Analyses

UV Spectroscopy

DNA concentration was determined by UV spectroscopy using the Smart SpecTM 3000 spectrometer. A 100 µL sample (1 µL DNA and 99 µL ddH₂O) was read at 260 nm and the DNA concentration was calculated using the equation: Concentration = A_{260} (AU) x 50 (ng/µL x AU) x 100 (dilution factor).

Nanodrop Spectroscopy

DNA concentration was determined using ND1000 spectrophotometer with software version Nanodrop 3.1.2. The A_{260} of a 2 µL sample was measured and the concentration of the DNA samples was calculated with the formula previously described in UV Spectroscopy, omitting the dilution factor.

Horizontal Gel Electrophoresis

DNA was visualized using agarose gel electrophoresis. A 40 mL 0.8% (w/v) agarose gel was made for gel electrophoresis. The 50X TAE buffer, pH 8.5 (tris(hydroxymethyl) amino methane (Tris) base (2 M), glacial acetic acid (5.71% (v/v)), Na₂EDTA·2H₂O (100 mM), and ddH₂O added to make 1 liter solution) was diluted with ddH₂O to make 1X TAE buffer. This mixture was heated in the microwave for two 30-second intervals with swirling between intervals. After cooling at room temperature until warm, the solution was poured into a gel mold and allowed to set for 40 minutes. The mold was then inserted into the gel electrophoresis apparatus and covered with 1X TAE buffer. The 6X dye buffer that was included in the DNA samples consisted of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (v/v) sucrose. Samples consisted of 235 – 305 ng/μL of DNA, 2 μL 6X dye, and ddH₂O added to make a total volume of 6 μL. A 1 kb ladder (10% (w/v) ladder, 6X dye, ddH₂O) along with the samples were loaded onto the gel and separated by electrophoresis for 75 minutes at 85 volts. The gel was then stained with ethidium bromide (0.2 mg/mL) for 15 minutes and visualized using a Kodak digital imaging system.

DNA plasmid Sequencing

A 12 μL sample containing 500 ng of plasmid DNA and 10 pmol of either forward or reverse primers was submitted for sequencing analysis to the DNA Sequencing ICMB Core Labs at the University of Texas at Austin. The forward and reverse primers used to sequence pGEX-4T-3/A3H1, pGEX-4T-3/A3H1-R84Q-Y24F, pGEX-4T-3/A3H1-R84Q-C27S, and pTAC-MAT-Tag-2/A3H1, are listed in Table 2. The sequences were then compared to that of the A3H1 *dszB* gene.

Table 2. Description of the primers that were used for sequencing the pGEX-4T-3/A3H1-TEV, pGEX-4T-3/A3H1-R84Q-Y24F, pGEX-4T-3/A3H1-R84Q-C27S, pTAC-MAT/A3H1 DNA plasmids.

Primer	Sequence	Direction	Utility
C27S-C-05	5' GCGCGTTGGGTATCGGGGAGTTGCTGTAGGTCAGTG 3'	Reverse	Used to sequence the entire GST gene and promoter region
S154A-C	5' CGCGCCTCCCATGCGCCCAGCGCTAC 3'	Reverse	Used to sequence cleavage sites and GST promoter region
pGEXFS	5' GGCTGGCAAGCCACGTTTGGTG 3'	Forward	Sequences from the 5' to 3' end of pGEX-4T-3 vector
pGEXRS	5' CCTCTGACACATGCAGCTCCCGG 3'	Reverse	Sequences from the 3' to 5' end of pGEX-4T-3 vector
pTACF	5'CCGGGAGCTGCATGTGTCAGAGG 3'	Forward	Sequences the multiple cloning site of the pTAC-MAT-Tag-2 vector from the N-terminus (5' end).
pTACR	5' CTGTATCAGGCTGAAAATCTTCTC 3'	Reverse	Sequences the multiple cloning site of the pTAC-MAT-Tag-2 vector from the C-terminus (3' end).

Ligation of *dszb* Gene into pTAC-MAT-Tag-2 Vector

Primer Design

The forward and reverse primers made by IDT were complementary to the 5' and 3' terminus of the sequence of the A3H1 *dszb* gene. A description of the primers used is shown in Table 3. The primer GC content, melting temperature, and acceptable secondary structure were evaluated using the IDT SciTools Oligoanalyzer at www.idtdna.com and the DNA Calculator from Sigma genosys at www.sigma-genosys.com. The *dszb* gene was obtained from the template pGEX-4T-3/A3H1-TEV plasmid using PCR.

Table 3. Description of the primers that were used for the polymerase chain reaction (PCR) for ligation of the *dszb* gene into the pTAC-MAT-Tag-2 vector.

Primer	Sequence	Direction	Utility
A3H1F	5' GCGAATTCATGGCAGGCCGCCTCAG 3'	Forward	Sequences complementary to the <i>dszb</i> gene from the 5' to 3' end
A3H1R	5'GCAGATCTATCGGTGACGGTTGAGGCTG 3'	Reverse	Sequences complementary to the <i>dszb</i> gene sequence from the 3' to 5' end

Polymerase Chain Reaction (PCR)

The forward primer (A3H1F) and the reverse primer (A3H1R) were each diluted with 1X TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA·Na₂) to make a 100 µM stock solution. The 50 µL reaction mixture for PCR contained the following: 34 µL ddH₂O, PCR buffer (1X), filtered Dimethyl Sulfoxide (DMSO, 0.42 M), MgCl₂ (2.5 mM), dNTPs (0.2 mM), Forward and Reverse primers (0.2 µM each), 0.5 µL of Jumpstart Taq Polymerase (2.5 U/µL), and 1 µL of 50 ng template DNA (pGEX-4T-3/A3H1-TEV). The reactions were mixed thoroughly by gently pipetting up and down. The template DNA was then added, followed by the JumpStart Taq Polymerase. The reaction was placed within the 2720 Thermal Cycler (Applied Biosystems) and the PCR was set to proceed as follows: 94 °C for 30 seconds to allow denaturation of the DNA, 55 °C for 45 seconds to effect primer annealing to the template strand, and 72 °C for 90 seconds for elongation of the DNA strand. This reaction constituted one cycle. After 35 cycles, the reaction was run at 72°C for 7 minutes for final extension of the DNA. The mixture was incubated at 4°C until analysis.

Digestion of pTAC-MAT-Tag-2 Vector and dszb DNA with EcoRI and BglII

The pTAC-MAT-Tag-2 vector was transformed into XL1Blue cells as previously described, and plated on LB plates with ampicillin (100µg/mL). The plasmid DNA was isolated and quantified as previously described. Both of the purified DNA products were visualized by horizontal agarose gels.

A double enzyme digest was done to prepare the pTAC vector for insertion of the *dszb* gene into the pTAC-MAT-Tag-2 vector. A double digest of the vector and the *dszb*

gene insert was prepared as follows: pTAC-MAT-Tag-2 plasmid DNA (10.25 ng), 5 μ L 10X RB-3 (reaction buffer), EcoRI (400 U/mL), BglII, (80 U/mL) diluted to 50 μ L with sterile ddH₂O. A negative control was run that contained no restriction enzyme. The ddH₂O, pTAC-MAT-Tag-2 vector, and PCR buffer were mixed together and briefly centrifuged. The restriction enzymes were added and the reaction was gently mixed. The mixture was incubated in a water bath for one hour at 37 °C. The digestion reaction was terminated by incubation at -20 °C for thirty minutes.

The amplified *dszb* gene from the PCR reaction was visualized on a 0.8% (w/v) agarose gel. The DNA band was excised from the gel and purified using the Wizard SV Gel and PCR Clean-up System from Promega. A double digest of the clean PCR gene product was prepared: 10.25 μ g of DNA, 5 μ L of 10X Reaction Buffer 3, EcoRI (400 U/mL) and BglII (80 U/mL), diluted to 50 μ L with sterile ddH₂O. A negative control was run without restriction enzymes. The digestion reaction proceeded as previously described. Both digested products were visualized on horizontal gels.

Ligation of the dszb Gene into the pTAC-MAT-Tag-2 Vector

The EcoRI/BglII digested pTAC-MAT-Tag-2 vector was treated with 2 units Calf Intestine Phosphatase (CIP) and incubated at 37 °C for one hour. The PCR product, the CIP- treated pTAC-MAT-Tag-2 vector, and an untreated sample of the pTAC-MAT-Tag-2 vector, were run on horizontal agarose gels, excised, and purified using the Wizard SV Gel and PCR Clean-up system from Promega. The purified DNA was quantified as described previously.

The ligation of the insert and vector was carried out using a 3:1 molar ratio of insert to vector – 9 μ L of pTAC-MAT-Tag-2 vector (5 ng), 7 μ L *dszb* gene insert (3.2 ng). These were added to 1 μ L T4 ligase (0.20 U/ μ L), and 2 μ L 10X ligase buffer diluted to 20 μ L with nuclease-free H₂O. The reaction was mixed by vortexing and brief centrifugation for 3 to 5 seconds. The ligation reaction proceeded overnight at 16 °C.

Screening of Ligation Products

The ligation reaction was transformed into competent XL1Blue cells. The transformation procedure that is outlined in the Methods section was used, except that the ligation reaction was incubated for 30 minutes with the XL1Blue cells prior to the heat shock treatment. The plasmid DNA was isolated using the methods previously described.

To screen plasmids for the correct pTAC-MAT-Tag-2/A3H1 construct, both single and double cuts of the isolated plasmid were performed. Both digestion reactions were carried out as previously described, but on a qualitative scale using 6 μ L of ligated DNA (8.20 ng/ μ L) and 1 μ L (0.20 – 2 U/ μ L) of restriction enzyme in 20 μ L total volume. The plasmids were single cut with BssSI or HindIII, and double cut with EcoRI and BglII or SphI and HindIII. The digestion reactions were incubated at -20 °C for 30 minutes prior to running the 0.8% (w/v) agarose gels for visualization.

Preparation of Frozen Permanents

A 50 mL portion of LB broth containing 30 μ g/ml kanamycin and 100 μ g/mL ampicillin was inoculated with one colony of BL21 (DE3)/pREP4-GroESL cells transformed with pGEX-4T-3/A3H1-TEV, pGEX-4T-3/A3H1-R84Q-Y24F, pGEX-4T-3/A3H1-R84Q-C27S, or pTAC-MAT-Tag-2/A3H1. The transformation mixture was

incubated overnight at 37°C with shaking. To create a stock for future experiments, frozen permanents were prepared as follows: 0.8 mL sterile 25% (w/v) glycerol was gently mixed with 0.6 mL cells from the 50 mL broth culture, and stored at -70 °C.

Expression and Purification of HPBS desulfinate

Expression of dszb genes

A 50 mL portion of LB broth containing kanamycin (30 µg/mL) and ampicillin (100 µg/mL) was inoculated with one colony of BL21 (DE3)/pREP4-GroESL cells transformed with pGEX-4T-3/A3H1-TEV, pGEX-4T-3/A3H1-R84Q-Y24F, pGEX-4T-3/A3H1-R84Q-C27S, or pTAC-MAT-Tag-2/A3H1. The transformation mixture was incubated overnight at 37°C with shaking. A 5 mL portion of the incubation was used to inoculate 500 mL LB broth, to which kanamycin (30 µg/mL) and ampicillin (100 µg/mL) were added. The mixture was then incubated in rotary shaker at 37 °C until the OD₆₀₀ was between 0.6 and 0.8. Gene expression was induced by adding 1 mM IPTG with shaking at room temperature (23 – 25 °C) overnight. A 50 µL sample of the mixture was collected for analysis and placed on ice. The cells were collected by centrifugation at 4000 x g for 15 minutes at 4 °C. A 50 µL sample from both the supernatant and the induced cells were collected for analyses and placed on ice. The supernatant was discarded and the pelleted cells were suspended in 15 mL of Purification Buffer A (50 mM KH₂PO₄/K₂HPO₄, pH 7.0, 50 mM KCl).

Cell Lysis

BL21 (DE3) Cells Containing pGEX-A3H1-TEV, pGEX-A3H1-R84Q-Y24F and pGEX-A3H1-R84Q-C27S

Following resuspension of the induced cells in 15 mL of Purification Buffer A, approximately 20 µg of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was added. The mixture was then incubated on ice for 30 minutes. The cells were lysed by sonication on ice at 50 % amplitude with three 10-second bursts with 10 seconds rest between bursts. A 50 µL sample was saved for analysis and placed on ice. The lysed cell pellet was obtained by centrifugation at $12000 \times g$ for 30 minutes at 4 °C. The cell debris was discarded. The supernatant was filtered using a 0.2 µm cellulose syringe filter. The volume was recorded and a 50 µL sample of the supernatant was taken for analyses and placed on ice. All samples saved for analysis were analyzed by SDS-PAGE.

BL21 (DE3) Cells containing pTAC-MAT-Tag-2/A3H1

The pTAC-MAT-Tag-2/A3H1 cells were ruptured using the above-mentioned procedure. However, the induced cells were incubated with lysozyme prior to sonication according to the protocol from ProBond™ purification system from Invitrogen [22], and the supernatant was not filtered.

Purification of HPBS desulfinase

HPBS desulfinase from pGEX-A3H1-TEV, pGEX-A3H1-R84Q-Y24F and pGEX-A3H1-R84Q-C27S

The filtered supernatant was loaded onto a 10 mL glutathione-agarose column equilibrated using the procedure from Sigma-Aldrich [23]. A 50 µL aliquot of the flow

through was analyzed by SDS-Page to determine if the fusion protein was captured by the column. The column was then washed overnight with 1 L of Purification Buffer A (50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.0, 50 mM KCl). A 50 μL portion of the initial flow through was collected for analysis by SDS-PAGE to check the purity of the captured fusion protein. The column resin was then resuspended in Purification Buffer A to a total volume of 18 mL. A 30 μL aliquot was taken for analysis and placed on ice. To initiate the cleavage of the HPBS desulfonase from the GST, 2 mL of thrombin cleavage buffer (200 mM Tris, 25 mM CaCl_2 , pH 8.0) and 50 units of thrombin protease were added to the suspension. The cleavage reaction was incubated overnight on a rotary shaker at 4 °C. A 50 μL sample was taken to determine the cleavage efficiency by SDS-PAGE. The resin within the suspension was allowed to settle. The cleaved HPBS desulfonase was eluted from the column and collected in 1 mL fractions. The column was then washed with 25 mL of Purification Buffer A to remove the remaining HPBS desulfonase from the column. The wash was also collected in 1 mL fractions and stored at -70 °C. The GST that was bound to the resin was removed and the column regenerated using a procedure from Sigma-Aldrich [23].

HPBS desulfonase from pTAC- A3H1

An 8 mL portion of the lysate collected after pelleting of the cell debris was loaded onto a Nickel ProBond™ Purification column previously prepared and equilibrated following a procedure from Invitrogen [22]. The resin was suspended in the lysate solution and binding was initiated by gentle agitation on a rotary shaker for 30 to 60 minutes. The resin was then settled by gravity or low speed centrifugation (800 $\times g$) for 1 minute. The supernatant, which contains the unbound protein, was gently aspirated

or removed by pipetting. A 100 μ L portion of the supernatant was saved at 4 °C for analysis by SDS-PAGE. To ensure the purity of the HPBS desulfinate, the column was washed with 8 mL of Native Wash Buffer (50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 0.5 M NaCl, 50 mM imidazole, pH 6.0). The resin was then settled by gravity or low speed centrifugation and the supernatant aspirated or removed by pipetting. A 100 μ L sample of the supernatant was saved at 4 °C for analysis. This procedure was repeated three more times. The HPBS desulfinate was eluted using 8 - 12 mL of Native Elution Buffer (50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 0.5 M NaCl, 250 mM imidazole, pH 8.0). The fractions were collected in 1 mL portions and were later analyzed using SDS-PAGE. The aliquots were stored at 4 °C or in 30% glycerol at -20 °C. The column was cleaned and recharged using the procedure outlined in ProBond™ purification system from Invitrogen [22].

Protein Concentration

After elution from the respective columns, the HPBS desulfinate fractions were pooled and concentrated. Protein concentration was carried out by centrifugation using a Centriplus®YM-10 Centrifugal filter unit at 3000 \times g at 4 °C until 5 mL of the retentate remained. The retentate was removed and the filter was washed with 1 mL of the respective buffer. The concentrated HPBS desulfinate and the filter wash were assayed for activity. The concentrated HPBS desulfinate was used in subsequent assays.

Sample Analyses

Bradford Assay

The Bradford Assay was conducted to determine the protein concentration of the purified HPBS desulfinate. The Protein Assay solution with dye reagent concentrate

(Bio-Rad) and Bovine Serum Albumin (BSA) were added to a 96-well microtiter plate. The BSA standards concentrations used were 0.05, 0.04, 0.03, 0.02, 0.014, 0.01, 0.005, and 0 mg/mL. A 25 % (v/v) of Protein Assay solution totaling 200 μ L was incubated with either 10 μ L of standard or sample at room temperature for a minimum of 10 minutes. The absorbance was measured at 595 nm using a SpectraMAX 190 well plate reader from Molecular Devices, with Soft Max® Pro 4.7.1 software. The equation of the best fitting line of the standard curve generated by Microsoft Excel was used to calculate the protein concentration of the purified HPBS desulfonase samples.

Nanodrop Spectroscopy

Protein concentration was determined using ND1000 spectrophotometer with software version Nanodrop 3.1.2. The A_{280} of 2 μ L samples was measured and the concentration of the protein was obtained from the A_{280} reading in mg/mL and compared to the concentration determined in the Bradford assay to calculate the extinction coefficient, ϵ .

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Approximately 6.5 μ L of each protein sample was thoroughly mixed with 1X SDS Buffer (10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 1% (w/v) Bromophenol blue, 5% (v/v) β -mercaptoethanol). The solution was heated at 95 °C for three to five minutes, loaded onto a 10% SDS-PAGE gel, and separated by electrophoresis at 125 volts for 110 minutes. The gel was rinsed three times with ddH₂O and stained with gentle agitation with Coomassie blue stain (0.3 mM Coomassie Brilliant Blue, 7.5% (v/v) glacial acetic acid, 50% (v/v) ethanol) for 1 hour. The gel was rinsed

three times with ddH₂O and destained overnight with Coomassie blue destain solution (7.5 % (v/v) glacial acetic acid, 10% (v/v) ethanol). The gel was visualized using a Kodak digital imaging system.

Western Blot

Samples of lysed BL21 (DE3) cells containing pGEX-4T3/A3H1-R84Q-Y24F which were induced with 1 mM of IPTG were run on a 10% SDS-PAGE gel according to the protocol for SDS-PAGE described above. The gel was not stained with Coomassie Blue stain. The gel-nitrocellulose sandwich was placed in a blotting apparatus with chilled 1X Transfer buffer (Tris 0.3 % (w/v), glycine 1.44 % (w/v), and methanol 20% (v/v)) and separated by electrophoresis at 100 volts for 45 minutes at 4 °C. The nitrocellulose was then washed for 30 minutes with gentle agitation in blocking solution (milk powder 5% (w/v), TBS solution 0.60% (w/v), Tris 0.88% (w/v), and NaCl, pH 7.5). A 1/1000 portion of anti-GST primary antibody (from goat) was added and incubated overnight at 4 °C. The blot was washed three times for 5 minutes with TBST (100 mL TBS solution, Tween-20 0.1% (v/v)). A 1/20000 portion of the anti-goat secondary antibody (from donkey) was added and incubated at room temperature for 1 hour with gentle agitation. The blot was washed twice with TBST and once with TBS, each time for 5 minutes and with gentle agitation. The nitrocellulose was washed with a 10 mL solution containing 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (0.15 mg/mL), 4-nitro blue tetrazolium (NBT) (0.30 mg/mL), 1 mM Tris, and 0.05 mM MgCl₂, and removed when sufficient color had developed. The blot was allowed to dry at room temperature after a rinse with ddH₂O.

HPBS Substrate Synthesis

The substrate HPBS was synthesized from the method provided by Gregory Mrachko of Energy Biosystems Corporation. Biphenosultine (0.0432g, 20 mM) suspended in 10 mL of 44 mM NaOH was reacted overnight with stirring at room temperature (23 – 25 °C). The pH of the soluble product, HPBS (20 mM), was adjusted to approximately 8.55 with 1 M HCl and stored at -70 °C in 1 mL aliquots. The Biphenosultine was a gift from Dr. Herbert L. Holland of Brock University.

Fluorimetric Assays

Standard Activity Assay

A fluorimetric assay was used to measure the activity of HPBS desulfinate. The standard assay was done using a 96 well plate. Each well contained 1X Buffer A (25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 100 mM NaCl, pH 7.4), 100 μM HPBS, and 10 – 45 μg of HPBS desulfinate in a total volume of 200 μL . The assay components, except for the substrate, were carefully mixed together. The mixture and the substrate were incubated separately at 37 °C for five minutes on a Zytron plate heater. The HPBS was then added to the wells to initiate the assay. The fluorescence generated by formation of the product hydroxybiphenyl (HBP) was measured over time using a Perkin Elmer Luminescence Spectrometer LS50B monitoring at 414 nm ($\lambda_{\text{ex}} = 288 \text{ nm}$) for 20 cycles of 60 seconds each. The data was collected using the FL WinLab software. The initial velocity was determined using Microsoft Excel.

HBP Standard Curve

The fluorescence of the HBP as a function of HBP concentration was measured. The HBP standard curve was generated using the following HBP concentrations: 0, 1, 5, 10, 20, 50, and 75 μM . The assay was conducted in a 96 well plate. Each well contained HBP, 1X Buffer A (25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 100 mM NaCl, pH 7.4) and ddH₂O to a total volume of 200 μL . The fluorescence of the HBP product was measured using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm ($\lambda_{\text{ex}}=288$ nm). The standard curve of fluorescence intensity versus HBP concentration was plotted using Microsoft Excel and the equation for the best fit line was determined.

Characterization of HPBS desulfonase

Kinetic Studies

A fluorimetric assay was conducted with varying concentrations of substrate HPBS to determine the kinetic parameters of HPBS desulfonase. For the A3H1-DszB, the HPBS concentrations that were studied were 5, 10, 50, 75, and 100 μM . The concentration of HPBS desulfonase used was 1.51 μM . For the A3H1-R84Q-Y24F enzyme, the HPBS concentrations used were 5, 10, 20, 25, 50, 75, 100, and 125 μM , and the concentration of HPBS desulfonase was 1.61 μM . The assays were conducted on 96 well plates in triplicate. The activity of the HPBS desulfonase was measured using the fluorimetric assays described previously. One unit of activity was defined as 1 μM HBP formed per minute. The concentration of HBP formed was calculated from the fluorescence intensity using the HBP standard curve ($F_{\text{HBP}}: y=3.4888x + 2.9245$,

$R^2 = 0.9919$). The data were then fitted to the Michaelis-Menten graph using VisualEnzymatics software from Softzynamics (Princeton, NJ).

Determination of Temperature Optima and Stability

Temperature studies were conducted using Perkin Luminiscence Spectrometer LS50B to which a Zytron plate heater was attached for temperature control. HPBS desulfonase activity was monitored between 25 °C and 60 °C in 5 °C increments. To determine the temperature optima, HPBS desulfonase, assay Buffer A and ddH₂O were mixed and allowed to equilibrate for five minutes at the respective temperature prior to initiation of the reaction mixture with HPBS. To determine the temperature stability of the enzyme, the same reaction mixture was equilibrated at the respective temperature for 30 minutes, and then at room temperature for 10 minutes prior to initiation of the reaction with HPBS. Enzyme activity was measured after addition of HPBS (75 µM for A3H1-DszB or 100 µM for A3H1-R84Q-Y24F-DszB). Each assay was run in triplicate. From the data collected, the relative activity of the enzyme for a specific temperature was plotted using Microsoft Excel.

Determination of pH Optima

The effect of pH on HPBS desulfonase was determined by fluorimetric assay for pH values ranging between 4.0 and 9.5 in 0.5 increments. A 10X MTEN buffer containing 500 mM 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, 250 mM TRIS, 250 mM ethanolamine, and 1 M NaCl was prepared and divided in aliquots. The pH of each aliquot was then adjusted using NaOH or HCl to the required pH values. The enzyme was equilibrated for five minutes in the 1X buffer at each pH value. Enzyme

activity was measured after addition of HPBS (75 μ M for A3H1- DszB or 100 μ M for A3H1-R84Q-Y24F- DszB). Each assay was run in triplicate. The data were collected by the FL WinLab software of the Perkin Luminiscence Spectrometer LS50B spectrometer. The relative activity of the enzyme at the respective pH values was plotted using Microsoft Excel.

Inhibition Study

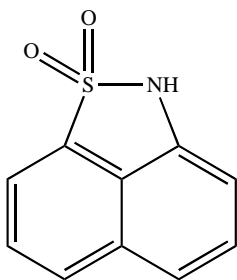
Product Inhibition Studies

The activity of HPBS desulfinate was monitored in the presence of varying concentrations of the sulfite or HBP. A 20 mM aqueous stock solution of sodium sulfite and a 1 mM solution of HBP were prepared. The concentrations that were evaluated for both compounds were 10, 20, 50, 75, 100, 250, and 500 μ M. The assay contained sulfite or HBP, assay Buffer A, HPBS desulfinate, and HPBS (20 μ M for A3H1- DszB or 100 μ M for A3H1-R84Q-Y24F- DszB) in a total volume of 200 μ L. The fluorimetric assay was run using a Varian Spectrophotometer with Cary Eclipse software. The relative activity was calculated by comparing the rate of the enzyme in the presence and absence of HBP or sulfite.

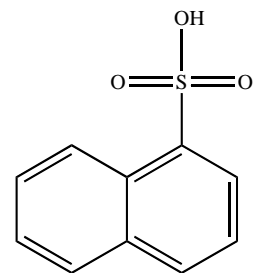
Inhibition Studies

The effects of various potential inhibitor of HPBS desulfinate were measured using a Varian Spectrophotometer with the Cary Eclipse software. A 10 mM solution of each of the following analogs was prepared (Figure 6). The product analog: 2,2'-biphenol (0.0186 g); or substrate analogs: 4'-hydroxy-4-biphenyl carboxylic acid (0.0214 g), diphenic acid (0.0242 g), 1-naphthalene sulfonic acid (0.0230 g), 1,8-naphthosultone

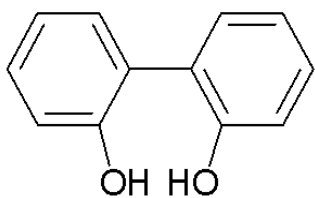
(0.0206 g), and 1,8-naphthosultam (0.0205 g) were dissolved in 10 mL ethanol. Each was then diluted to 1 mM with sterile ddH₂O with the exception of 4'-hydroxy-4-biphenyl carboxylic acid and 1, 8-naphthosultone which were diluted to 1 mM with ethanol. The assay was run in a 96 well plate. The reaction mixture contained HPBS and analog (both were 20 μ M for A3H1- DszB, 230 μ M for A3H1-R84Q-Y24F- DszB), and HPBS desulfonase, and assay Buffer A, to a total volume of 200 μ L. Controls were run with no inhibitor, no HPBS, or no enzyme. A control was also run to determine the effects of 10% and 100% ethanol on enzyme activity. The relative activity of the enzyme in the presence of the analogs was calculated by comparison to the assay without analog, but with the same concentration of ethanol.



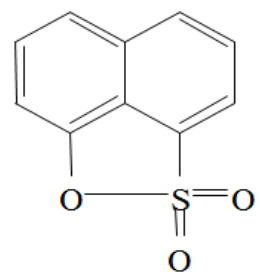
1,8-naphthosultam



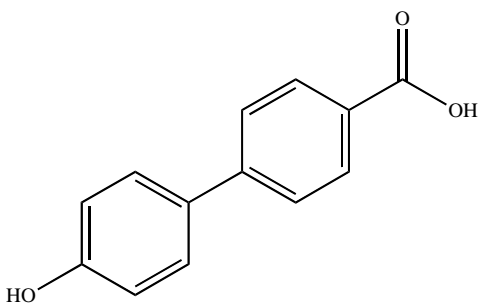
1-naphthalene sulfonic acid



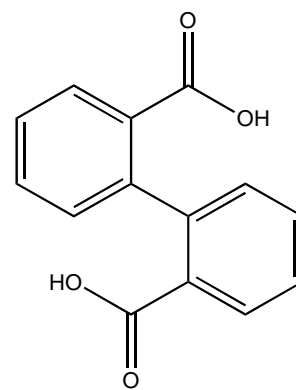
2,2'-biphenol



1,8 - naphthosultone



4'-hydroxy-4-biphenyl carboxylic acid



diphenic acid

Figure 6. Substrate Analogs used in the Inhibition Study.

CHAPTER III

RESULTS

Ligation of the *dszb* gene into the pTAC-MAT-Tag-2 Vector

PCR

The *dszb* DNA was obtained by PCR amplification from the pGEX 4T-3/A3H1-TEV plasmid. The reaction mixture was prepared and the PCR procedure conducted as described in the Methods section. The primers that were designed from the *dszb* gene for the amplification of the DNA are described in Table 3. The PCR product was gel purified and the DNA quantified. The PCR product was visualized on a horizontal agarose gel (Figure 7). The *dszb* gene is 1,098 base pairs. The DNA of the purified PCR product that is the *dszb* gene was observed at approximately 1.1 kb.

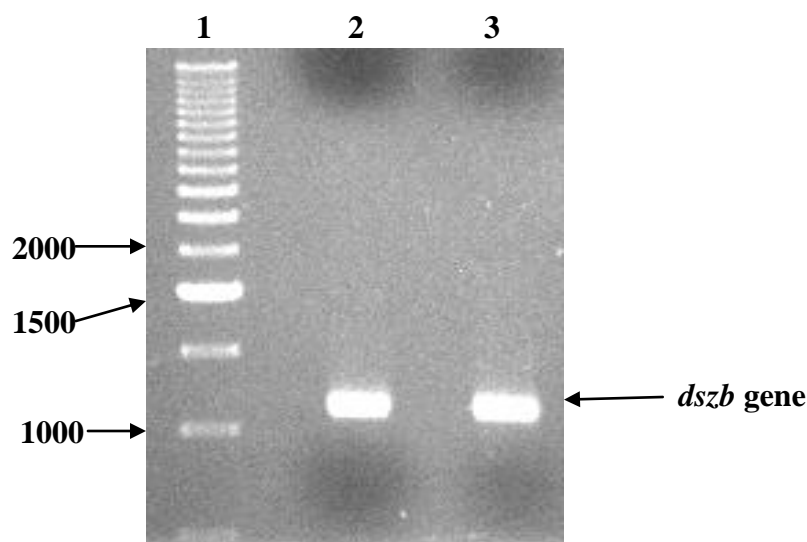


Figure 7. Horizontal Gel of the purified PCR of *dszb* gene. The gene was obtained from the pGEX-4T-3/A3H1 plasmid, gel purified and quantified. Lane 1, 500 bp ladder from Invitrogen; lane 3, 8 μ L PCR product in 10X DNA buffer. The gel was visualized using a UV Kodak imager.

Screening of Ligated Plasmids

The digested PCR product and the digested and CIP-treated pTAC-MAT-Tag-2 vector were ligated together as described in Methods. The ligated mixture was transformed into Top 10 *E. coli* cells and plasmid DNA was isolated from single colonies. The correct insertion of the *dszb* gene was verified by restriction digestion of the isolated plasmids. The negative and positive controls were the uncut vector and uncut plasmid. The digestion of the DNA with the restriction enzymes for the cutting reaction and the preparation of the reagents were previously outlined within the Methods section. The plasmid was cut with BssSI or BglII. The agarose gel is shown in Figure 8.

The resulting plasmid ran at approximately 6,260 bp. The singly cut plasmid in lanes 3 to 6 ran at approximately 6,300 bp on the horizontal gel where the plasmid with the gene insert was expected to be seen (Figure 8).

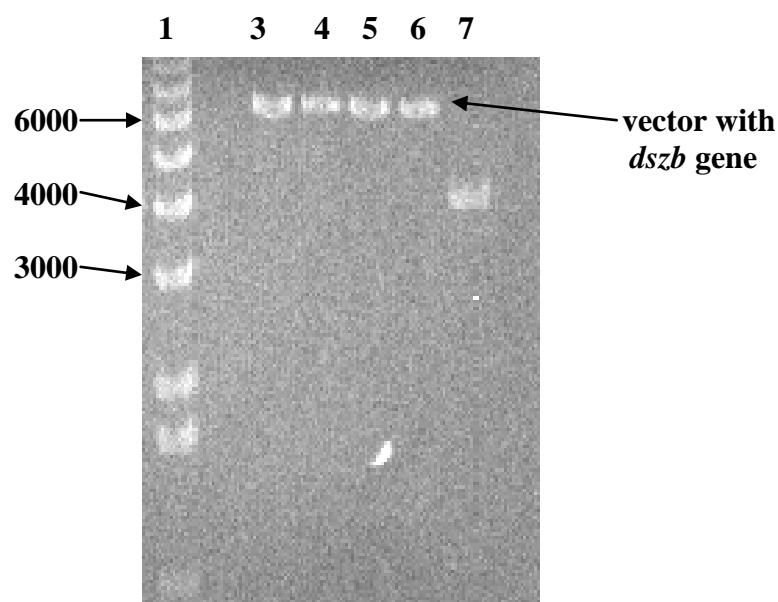


Figure 8. Horizontal Gel of single cut plasmid pTAC-MAT-Tag-2/A3H1. The pTAC-MAT-Tag-2 vector with the *dszb* gene is approximately 6260 bp. Lane 1, 1kb ladder from Invitrogen; Lane 3 to 6, single cut samples of the plasmid; Lane 7, uncut plasmid. The gel was visualized using a UV Kodak imager.

The double cut plasmid in lanes 2 to 8 ran at 5200 bp and 1100 bp, verifying that the *dszb* gene was successfully ligated into the pTAC-MAT-Tag-2 vector. Incomplete digestion of the plasmid resulted in a single cut plasmid that ran at 6300 bp (Figure 9).

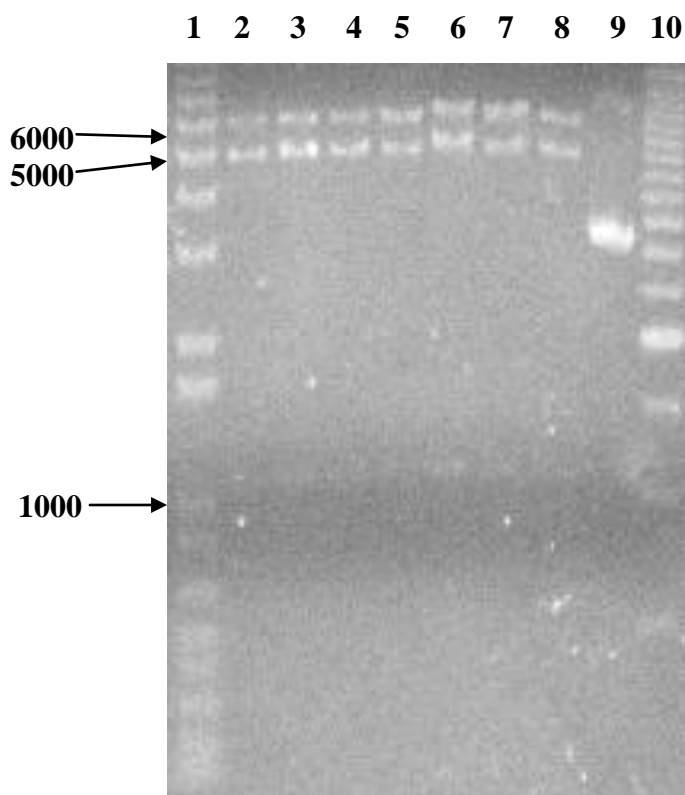


Figure 9. Horizontal Gel of double cut plasmid pTAC-MAT-Tag-2/A3H1. The pTAC-MAT-Tag-2 vector with the dszb gene is approximately 6,260 bp. Lane 1, 1 kb ladder from Invitrogen; lanes 2 – 8, double cut pTAC/A3H1 plasmid; lane 9, uncut pTAC-4T-3 vector; lane 10, 500 bp ladder from Invitrogen. The gel was visualized using UV Kodak imager.

Co-expression of Gro-ES and Gro-EL with HPBS desulfinate

The HPBS desulfinate from the four plasmids (pGEX-4T-3/A3H1-TEV, pGEX-4T-3/A3H1-R84Q-Y24F, pGEX-4T-3/A3H1-R84Q-C27S, and pTAC-MAT-TAG-2/A3H1) were each co-expressed with GroES and GroEL chaperone proteins. The chaperonins assist in folding of the HPBS desulfinate. The overexpression of the proteins was induced with 1 mM IPTG. SDS-PAGE was used to visualize protein overexpression. Overexpression of the HPBS desulfinate and the co-expression of the chaperone proteins GroES and GroEL were successful in all the plasmids except for the

pGEX-4T-3/A3H1-TEV. This plasmid did not overexpress the HPBS desulfinate as only the GroEL and GroEs chaperone proteins were visualized at 66 kDa and 15 kDa, respectively.

Overexpression of the A3H1-R84Q-Y24F-DszB was visualized using Western blot at 40 kDa in lane 2 (Figure 10). The A3H1-R84Q-DszB which was run as a positive control was also visualized at 40 kDa in lane 3 and 4.

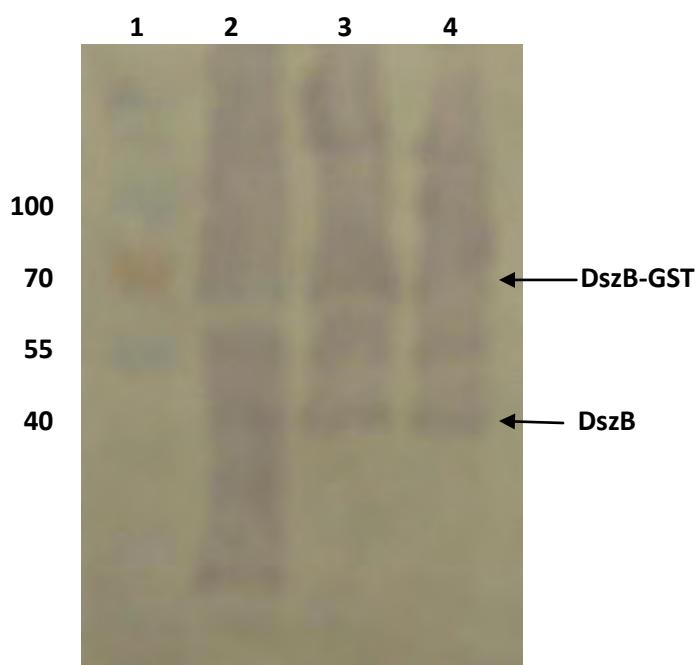


Figure 10. Western blot of HPBS desulfinate. The Western blot procedure was run on 10% SDS-PAGE gel. Lane 1, PageRuler prestained protein ladder; lane 2, induced cells from A3H1-R84Q-Y24F-DszB; lanes 3 and 4, induced cells from A3H1-R84Q-DszB. The anti-GST (goat) primary antibody and the anti-goat (donkey) secondary antibody were used to identify the protein by the GST-fusion protein.

Expression and purification of the HPBS desulfonases A3H1-R84Q-Y24F-DszB, A3H1-R84Q-C27S-DszB, and A3H1-DszB, are visualized in Figures 11, 12, and 13, respectively. Distinct bands on each gel are identified as follows. The DszB-Glutathione-S-Transferase (GST) fusion (69 kDa) is observed in lanes 2, 3, 6-10 in Figure 11, and lanes 4-9 in Figure 12. The isolated DszB (40 kDa) is seen in lanes 11 and 9 in Figures 11 and 12, respectively. The isolated DszB is visualized in lanes 11 -14 from the pTAC-MAT-Tag-2/A3H1 plasmid in Figure 13. The GroEL (66 kDa) is observed in all the figures and the GroES (15 kDa) in all the lanes in Figure 12. The GST protein has a molecular weight of 26 kDa and can be observed in lanes 8 and 9 in Figure 12.

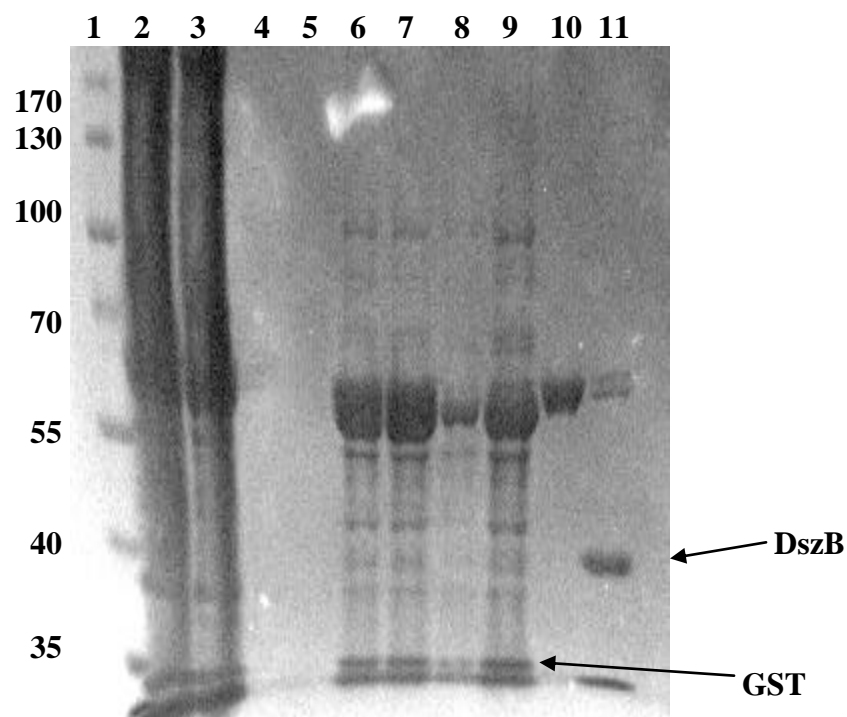


Figure 11. SDS-PAGE of the overexpression and purification samples of A3H1-R84Q-Y24F-DszB. The reagents and procedures were outlined in the Methods section. Lane 1, PageRuler prestained protein ladder, lanes 2 and 3, induced cells denatured by SDS and heat; lanes 4 and 5, growth media supernatant after cell pelleting; lane 6, post-sonication lysate; lane 7, filtered post-sonication lysate; lane 8, flow through glutathione-agarose column; lane 9, overnight wash of glutathione-agarose; lane 10, resuspended resin glutathione-agarose; lane 11, thrombin cleavage of GST-DszB fusion protein. The 10 % SDS gel was stained with Coomassie blue.

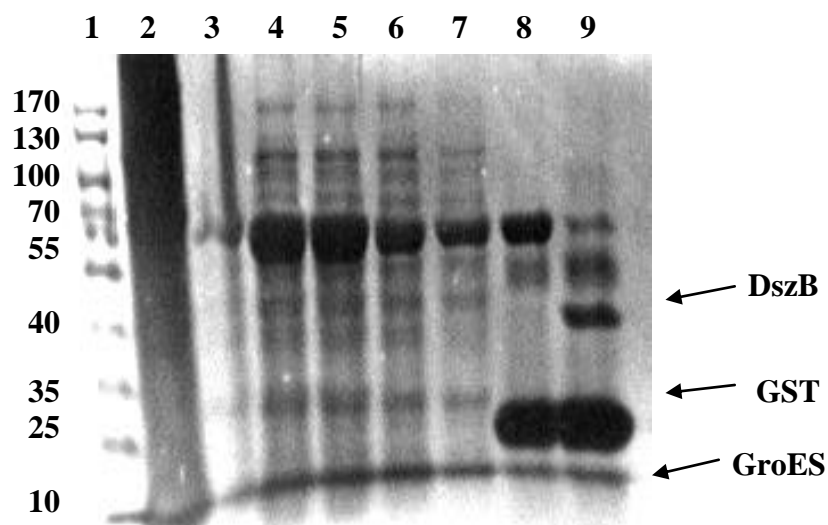


Figure 12. SDS-PAGE of the overexpression and purification samples of A3H1-R84Q-C27S-DszB. The reagents and procedures are outlined in the Methods section. Lane 1, PageRuler prestained protein ladder; lane 2, induced cells denatured by SDS and heat; lane 3, growth media supernatant after cell pelleting; lane 4, lysate; lane 5, filtered lysate; lane 6, flow through glutathione-agarose column; lane 7, overnight wash of glutathione-agarose; lane 8, resuspended resin glutathione-agarose; lane 9, thrombin cleavage of GST-DszB fusion protein. The 10 % SDS gel was stained with Coomassie blue.

The DszB protein from pTAC-MAT-Tag-2/A3H1 does not express as a GST fusion protein. The chaperone proteins GroEL and GroES, as well as the DszB are observed in the SDS-PAGE at 66, 15, and 40 kDa, respectively (Figure 13).

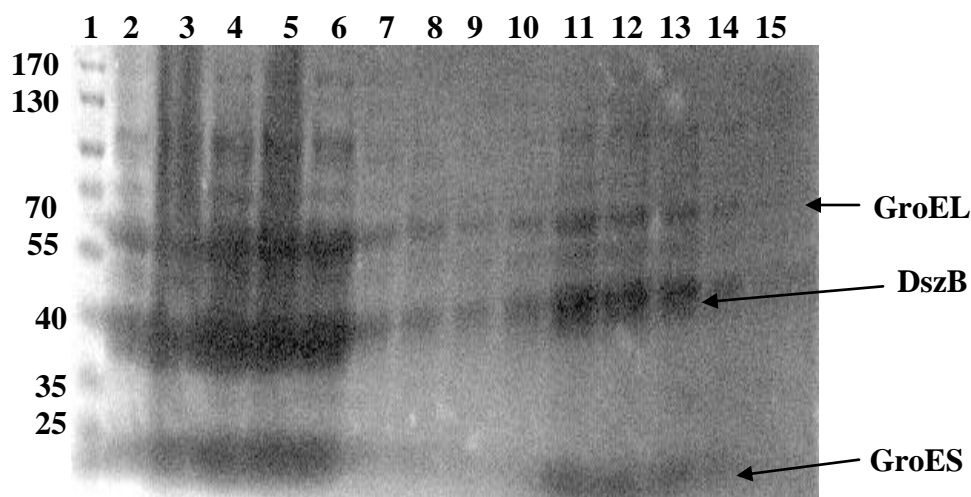


Figure 13. SDS-PAGE of the overexpression and purification samples of A3H1-DszB. The reagents and procedure are describes in the Methods section. Lane 1, PageRuler prestained protein ladder; lane 2, induced cells denatured by SDS and heat; lane 3, sonicated cells; lanes 4 and 5, post-sonicated lysate; lane 6, nickel resin with bound lysate; lanes 7 to 10, wash fractions 1-4; lanes 11 to 15, eluted DszB . The 10 % SDS gel was stained with Coomassie blue.

Purification of HPBS desulfinase

A3H1-R84Q-Y24F-DszB and A3H1-R84Q-C27S-DszB

Recombinant HPBS desulfinase (DszB), A3H1-R84Q-Y24F-DszB and A3H1-R84Q-C27S-DszB was purified on a glutathione (GSH)-agarose column. The GST tag attached to the carboxyl end of DszB facilitated a one-step purification process. The induced cell lysate was loaded onto the GSH column. The column was washed with 1 L Purification Buffer A to ensure protein purity and to rid the column of contaminants. The GST fusion protein with the DszB attached can be visualized in lanes 9 and 7 in Figures 12 and 13, respectively. The GST that still remained bound on the column was cleaved from the DszB with thrombin protease. To verify the efficiency of the cleavage reaction, a sample of the GSH resin was analyzed by SDS-PAGE. The DszB band can be seen at 40 kDa in lanes 11 and 9 in Figures 11 and 12, respectively. The GST fusion protein band was visualized at 26 kDa in lane 9 (Figure 12). The DszB was eluted from the

column following the cleavage reaction with thrombin protease. The total amount of protein collected in one purification was 10.27 mg for A3H1-R84Q-C27S-DszB, and 21.52 mg for A3H1-R84Q-Y24F-DszB, each from a volume of 500 mL of culture. The purity of the DszB protein was analyzed by SDS-PAGE (Figure 14). The purified HPBS desulfinase, A3H1-R84Q-Y24F DszB (lanes 2-8) and A3H1-R84Q-C27S-DszB (lanes 10-15) was visualized at 40 kDa. The chaperone protein GroEL ran at approximately 66 kDa.

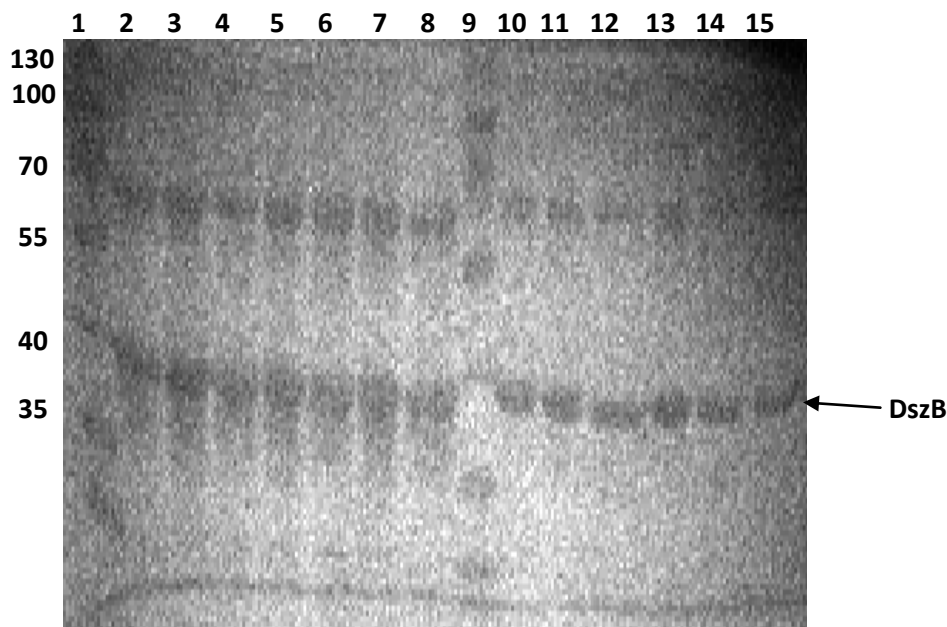


Figure 14. SDS-PAGE of eluted purified fractions of A3H1-R84Q-Y24F and A3H1-R84Q-C27S DszBs. The reagents were prepared and the electrophoresis reaction performed outlined in the Methods section. Lanes 1 and 9, PageRuler prestained protein ladder; for R84Q-Y24F-DszB: lanes 2 to 5, fractions 1-4 of flow through of loading of glutathione-agarose column; lanes 6 to 8, fractions 1-3 of glutathione-agarose column wash; for R84Q-C27S-DszB: lanes 10 to 12, fractions 1-3 of flow through of loading of glutathione-agarose column; lanes 13 to 15, fractions 1-3 of glutathione-agarose column wash. The 10% SDS gel was stained with Coomassie blue.

DszB-A3H1

The DszB from pTAC-4T-3/A3H1 plasmid was purified on a ProBond™ nickel metal column as described in the Methods section (Figure 13). Bands at 66 and 15 kDa, corresponding to the GroEL and the GroES chaperone proteins, respectively, were observed in lanes 2 - 14. The total amount of protein isolated from pTAC-4T-3/A3H1 was 4.28 mg from 500 mL of culture. Single distinct bands corresponding to DszB in the first 5 eluted fractions were observed at 40 kDa.

Fluorimetric Assays

Standard Activity Assay

The enzyme activities of the A3H1-DszB, A3H1-R84Q-Y24F-DszB, and A3H1-R84Q-C27S-DszB proteins were measured and compared with that of the A3H1-R84Q-DszB. The standard activity was run according to the procedure previously outlined in the Methods section. All the DszBs showed activity except for the A3H1-R84Q-C27S-DszB protein.

Kinetics Studies

The kinetic parameters of HPBS desulfinate (DszB) were obtained by conducting a fluorimetric assay as described in Methods. The VisualEnzymatics software from Softzymatics was used to fit the data to the Michaelis-Menten equation.

The K_m and k_{cat} of HPBS desulfinase for A3H1-DszB were determined to be 3.15 ± 0.74 μM and 1.24 ± 0.054 min^{-1} , respectively (Figure 15).

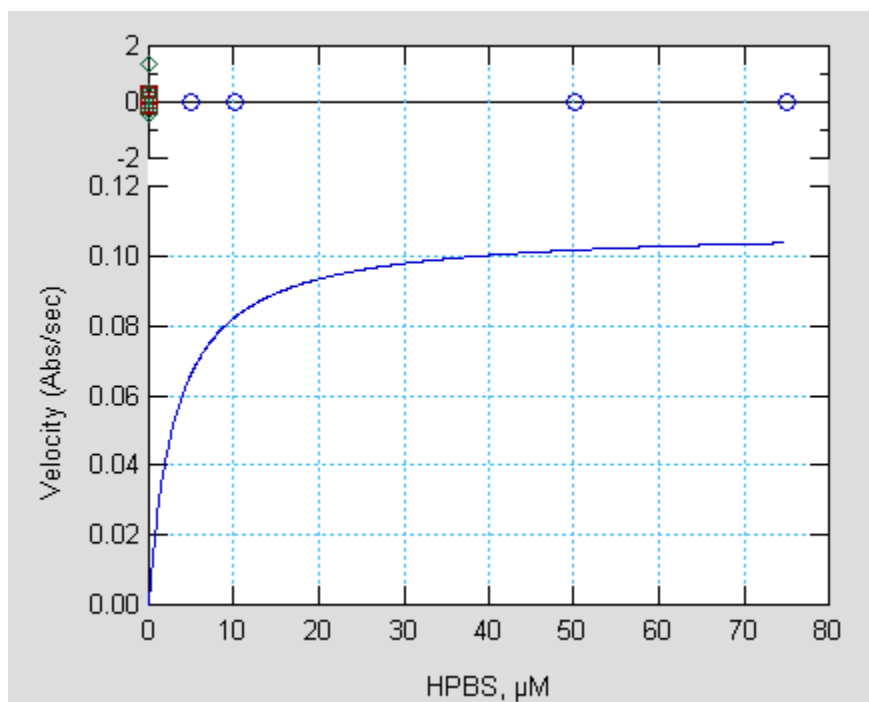


Figure 15. Michaelis-Menten graph for A3H1-DszB. Formation of micromolar of HBP formed per second at varied substrate HPBS concentration. Data was analyzed and fit to Michaelis-Menten equation using VisualEnzymatics.

The K_m and k_{cat} of HPBS desulfinase for A3H1-R84Q-Y24F-DszB were determined to be $51.25 \pm 0.96 \mu\text{M}$ and $4.33 \pm 0.67 \text{ min}^{-1}$, respectively (Figure 16).

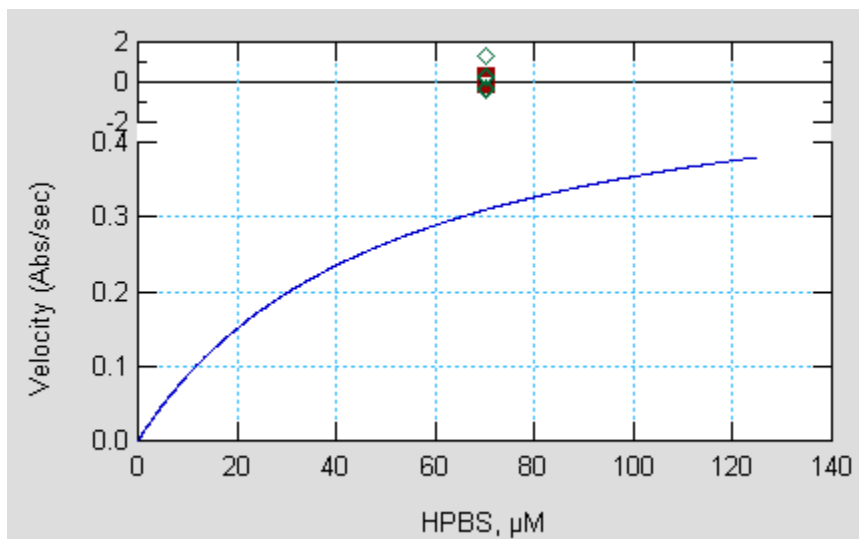


Figure 16. Michaelis-Menten graph for A3H1-R84Q-Y24F-DszB. Formation of micromolar of HBP formed per second at varied substrate HPBS concentration. Data was analyzed and fit to Michaelis-Menten equation using VisualEnzymatics.

Determination of Temperature Optima and Stability

The HPBS desulfinase activity as a function of temperature was evaluated using fluorescence. The temperature studies were conducted at temperatures ranging from 25 to 60 °C in 5 °C increments. The temperature optimum for A3H1-DszB (Figure 17) and A3H1-R84Q-Y24F-DszB (Figure 18) was 35 °C.

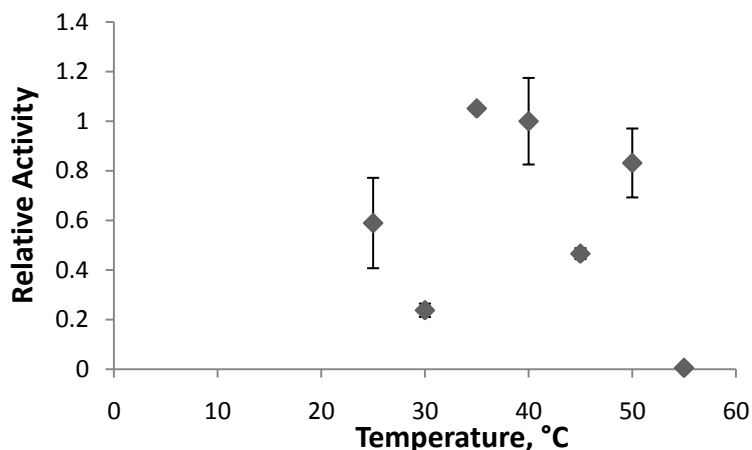


Figure 17. Temperature Optima for A3H1-DszB. Samples of the enzyme were incubated for 5 minutes at the designated temperature. The fluorimetric assay was used to monitor HBP formation from 75 μ M HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 25 °C.

The wild type A3H1-DszB was fairly active over a wide range, not losing all activity until 55 °C. The A3H1-R84Q-Y24F-DszB shows a narrow optimum, rapidly losing activity at temperatures lower and higher than 35 °C.

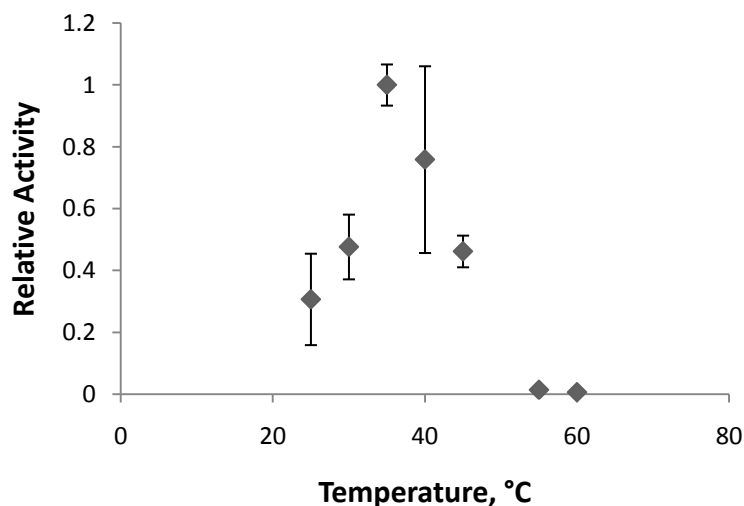


Figure 18. Temperature Optima for A3H1-R84Q-Y24F-DszB. Samples of the enzyme were incubated for 5 minutes at the designated temperature. The fluorimetric assay was used to monitor HBP formation from 100 μ M HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 25 °C.

The temperature stability values were obtained after the enzyme was incubated for 30 minutes at the designated temperature and then allowed to incubate at room temperature for 10 minutes prior to assay initiation. The wildtype HPBS desulfinate A3H1-DszB (Figure 19) was most stable for 30 minutes at 25 °C, the lowest temperature examined. Enzyme stability decreased drastically with increasing temperature. Enzyme activity was totally lost after incubation for 30 minutes at 45 °C.

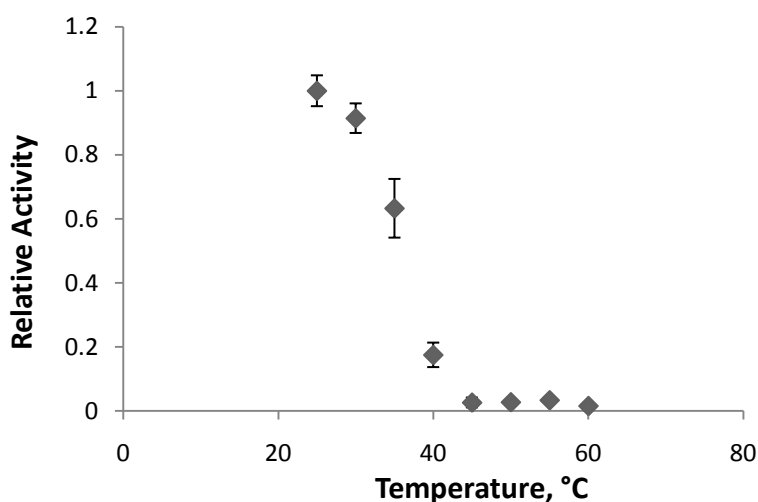


Figure 19. Temperature Stability of A3H1-DszB. Samples of the enzyme were incubated for 30 minutes at the designated temperature and then for 10 minutes at room temperature. The fluorimetric assay was used to monitor HBP formation from 75 μ M HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 25 °C.

The HPBS desulfinate A3H1-R84Q-Y24F (Figure 20) was most stable after incubation between 25 °C and 30 °C. There was also no significant difference between activity at 35, 40 and 45 °C. The enzyme slowly lost activity as temperature increased.

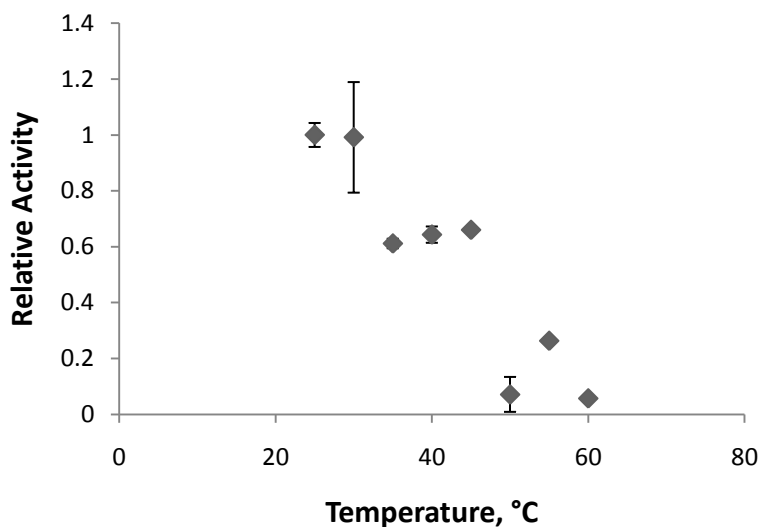


Figure 20. Temperature Stability of HPBS desulfinate A3H1-R84Q-Y24F. Samples of the enzyme were incubated for 30 minutes at the designated temperature and 10 minutes at room temperature. The fluorimetric assay was used to monitor the HBP product formation from 100 μ M HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 25 °C.

Determination of pH Optima

The optimum pH for HPBS desulfinate was determined using fluorimetric assays. The enzyme was incubated in MTEN buffer and the pH was adjusted to each designated pH value from 4 to 9.5 in 0.5 increments. The initial activity of the enzyme at each value was plotted using Microsoft Excel. The activity of HPBS desulfinate A3H1-DszB (Figure 21) gradually increased from pH 4.0 to 8.5 optimum, then slowly decreased.

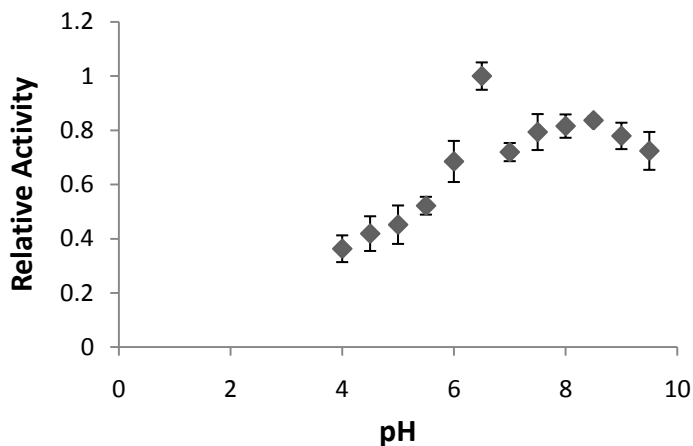


Figure 21. The pH Optima for A3H1 DszB. The enzyme was incubated in MTEN buffer for 5 minutes at a designated pH value ranging from 4 to 9.5. The initial activity of each pH value was obtained by fluorescence and values obtained using Microsoft Excel.

The optimum pH for HPBS desulfonase A3H1-R84Q-Y24F-DszB was 8.5 (Figure 22). The enzyme showed no activity between pH 4 and 5.5, but had a narrow optimum, rapidly losing activity at pH values lower and higher than 8.5.

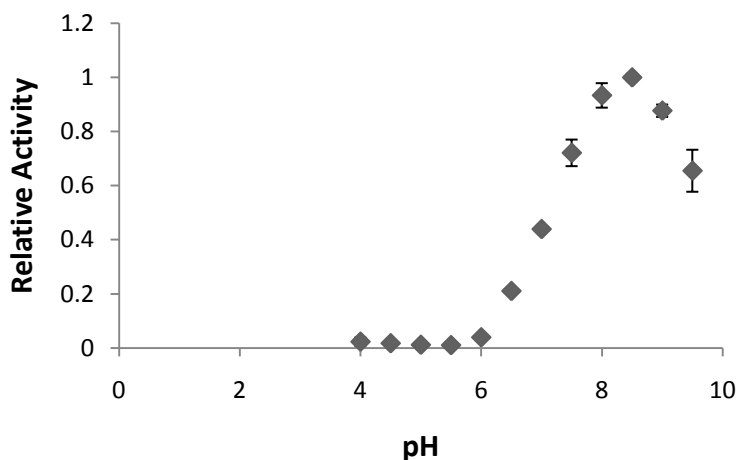


Figure 22. The pH Optima for A3H1-R84Q-Y24F-DszB. The enzyme was incubated in MTEN buffer for 5 minutes at a designated pH value ranging from 4 to 9.5. The initial activity of each pH value was obtained by fluorescence and values obtained using Microsoft Excel.

Inhibition Studies

Product Inhibition

The effects of varying concentrations of sulfite or HBP on HPBS desulfinate was measured using the fluorimetric assay as described in Methods. The activity of A3H1-DszB was not inhibited up to 500 μ M of HBP. The A3H1-R84Q-Y24F-DszB was inhibited at 100 μ M HBP. Activity of both enzymes was totally inhibited at 500 μ M sulfite. The activity of A3H1-R84Q-Y24F-DszB was totally inhibited by 100 μ M HBP/sulfite, while A3H1-DszB was inhibited by 68% at 250 μ M HBP/sulfite.

The effects of various substrate analogs on HPBS desulfinate activity were evaluated using the fluorimetric assay. The reagents were prepared and the assay conducted as previously outlined in the Methods section. The analogs used in the assays were as follows: 2-2'-biphenol, 4'-hydroxy-4-biphenyl carboxylic acid, diphenic acid, 1-naphthalene sulfonic acid, 1,8 – naphthosultone, and 1,8 – naphthosultam. The relative activity of the enzymes in the presence of these inhibitors is listed in Tables 4 and 5. The 2, 2-biphenol totally inhibited the activity of A3H1-DszB enzyme (Tables 4). The A3H1-DszB enzyme was inhibited significantly (75%) by 4'-hydroxy-4-biphenyl carboxylic acid, while inhibited the least (25%) by 1-naphthalene sulfonic acid (Table 4).

Table 4. Results of Inhibition* on HPBS desulfinate Activity from A3H1-DszB.

	Analogs	Relative Activity %
Product	2, 2'-biphenol	0
Substrate	diphenic acid	73
	1-naphthalene sulfonic acid	75
	1, 8-naphthosultam	72
	4'-hydroxy-4-biphenyl carboxylic acid	25
	1, 8-naphthosultone	33

*The reaction mixtures contained 20 μ M of analog, 20 μ M of HPBS, HPBS desulfinate, and Buffer A at pH 7.4. The enzyme activity was monitored using the fluorimetric assay described in the Methods section.

The A3H1-R84Q-Y24F-DszB enzyme activity was totally inhibited by 2-2'-biphenol and 4'-hydroxy-4-biphenyl carboxylic acid. The enzyme was least inhibited (87%) by diphenic acid, and lost significant activity with 1-naphthalene sulfonic acid (94%), 1, 8 – naphthosultam (99%), and 1, 8-naphthosultone (93%), (Table 5). The A3H1-R84Q-Y24F-DszB enzyme showed significant inhibition when compared to the A3H1-DszB enzyme.

Table 5. Results of Inhibition* on HPBS desulfinate Activity from DszB-A3H1-R84Q-Y24F.

	Analogs	Relative Activity %
Product	2-2'-biphenol	0
Substrate	diphenic acid	13
	1-naphthalene sulfonic acid	6
	1, 8 - naphthosultam	1
	4'-hydroxy-4-biphenyl carboxylic acid	0
	1, 8-naphthosultone	7

*The reaction mixtures contained 230 μ M of analog, 230 μ M of HPBS, HPBS desulfinate, and Buffer A at pH 7.4. The enzyme activity was monitored using the fluorimetric assay described in the Methods section.

CHAPTER IV

DISCUSSION

The research in this thesis describes the purification and characterization of wild type HPBS desulfinate from *Nocardia asteroides* A3H1. To date, this is the first study of the wild type DszB enzyme from *Nocardia asteroides* A3H1. Pure HPBS desulfinate was isolated using an efficient one-step purification process. Results obtained from the characterization of the wild type A3H1 desulfinate were compared to desulfinates obtained from other organisms.

Coexpression and Purification of HPBS desulfinate

The wild type *dszB* gene in the construct pTAC-MAT-Tag-2/A3H1 was overexpressed and purified, and the kinetic parameters and inhibition studies were performed. The mutant *dszB* genes in the pGEX-4T-3/A3H1-R84Q-C27S and pGEX-4T3/A3H1-R84Q-Y24F constructs were also overexpressed, purified, and characterized. The plasmids coexpressed the chaperone proteins GroEL and GroES in the BL21 (DE3)/pREP4-GroESL. Expression was induced with IPTG.

Protein purification using the nickel metal column or the glutathione-agarose column was a one-step process. This process contrasted significantly with previous studies that utilized between four to seven chromatography steps to obtain DszB enzyme [10, 14, 16]. These purification procedures generated pure enzyme, although some GroEL and GroES chaperone proteins were also included. The GroEL and GroES

chaperone proteins are co-expressed with HPBS desulfhinase to facilitate proper folding of the protein. Previous studies have employed the use of chaperone proteins. In these studies, no inherent inhibition of enzymatic activity was noted and protein stability was increased. Enzyme expressed without the chaperone proteins was insoluble [10].

Protein purification using the nickel column generated highly concentrated pure protein in a small volume thereby eliminating the need for multiple steps or protein concentration. The distinct bands at 40 kDa can be visualized in lanes 11-14 in Figure 13. Protein purification using the glutathione-agarose column generates less concentrated pure protein. However, use of less Purification Buffer A during the thrombin cleavage step would potentially generate more concentrated fractions.

HPBS desulfhinases A3H1-R84Q-Y24F and A3H1-R84Q-C27S

The mutant *dszb* genes in the pGEX-4T-3/A3H1-R84Q-C27S and pGEX-4T-3/A3H1-R84Q-Y24F constructs that were created in the Watkins lab to explore active site studies, were overexpressed with IPTG, purified on glutathione columns, and characterized. Results from fluorimetric assays revealed that A3H1-R84Q-C27S-DszB showed no activity, while A3H1-R84Q-Y24F-DszB had enzyme activity.

The effects of the C27S substitution confirmed earlier reports that this sole, conserved cysteine at position 27 is a component of the active site and is required for enzyme catalysis [10, 16]. This is consistent with its role as either an active site base or an active site nucleophile [16, 17].

The A3H1-R84Q-Y24F-DszB protein showed activity. Compared with A3H1-R84Q-DszB, the K_m for the pGEX-A3H1-R84Q-Y24F was twice as large, indicating that the enzyme is less specific for substrate. The A3H1-R84Q-Y24F-DszB is slower than the

pGEX-A3H1-R84Q-DszB because the k_{cat} for the pGEX-A3H1-R84Q-DszB is four times larger than that of A3H1-R84Q-Y24F-DszB. In addition, the results from the inhibition studies indicate that, except for diphenic acid, the A3H1-R84Q-Y24F-DszB is significantly inhibited by the analogs used in the studies. It was also inhibited by a smaller concentration of the products of HBP and sulfite as compared to A3H1-DszB. Two aspects to be noted are that the substitution of the tyrosine at position 24 to phenylalanine affects the substrate specificity of the enzyme, as well as rendering it more susceptible to inhibition. The conclusion could therefore be drawn that tyrosine is indeed a component of the active site of the DszB enzyme, although it does not support its role as an essential active site base.

HPBS desulfinase A3H1

The pGEX-4T-3/A3H1-TEV plasmid that was created by Dr. Sawyer did not express the A3H1 DszB protein. In this study, causes for lack of expression of the *dszb* gene in the pGEX-A3H1-TEV construct were explored. Sequencing data provided verification that the construct was in frame and no mutations had occurred. In addition, the *lac* promoter site was intact, and the insertion points of the TEV site and the *dszb* gene were properly aligned within the vector. Cell cultures were then grown in appropriate media, and expression was induced with IPTG. However, neither the DszB protein, the GST, nor the GST-DszB fusion protein was expressed in amounts detectable by SDS-PAGE or Western blot analyses.

Consequently, a different vector, pTAC-MAT-Tag-2 was chosen to overexpress the DszB protein. Instead of using a fusion protein system, the pTAC-MAT-Tag-2 vector has a 6X His tag preceded by the multiple cloning site (MCS), and utilizes a nickel metal

affinity column. The *dszB* gene was obtained from the pGEX-A3H1-TEV construct using PCR, and ligated into the MCS of the pTAC-MAT-TAG-2 vector. The recombinant pTAC-A3H1 plasmid was then purified and sequenced. Following inducement with IPTG, the pTAC-A3H1 plasmid successfully overexpressed the A3H1-DszB as indicated by SDS-PAGE analyses.

Protein Analyses

The SDS-PAGE revealed pure A3H1-DszB at 40 kDa (Figures 11, 12, 13). Also visualized were the chaperone proteins GroEL and GroES which have molecular weights of 66 kDa and 15 kDa, respectively. The SDS-PAGE indicated that the GroEL and the GroES migrated as two separate entities. The GST-DszB fusion protein was visualized at 69 kDa (Figures 11, 12, 13). The chaperone proteins are necessary for proper folding of the protein and stability of enzyme.

pH Studies

The optimum pH of 8.5 was reported for A3H1-DszB and A3H1-R84Q-Y24F-DszB. The activity of the enzyme decreased at pH values lower or higher than the optimum pH value. The MTEN buffer system was used in creating the pH profile. This method diminishes the buffer effects as seen in assays that utilized buffers with different pH values [21]. The optimum pH reported for R84Q was 8 [21]. The native IGTS8-DszB was reported to have pH optimum of 7 [16] and the KA2-5-1-DszB a pH of 7.5 [10]. An optimum pH of 8 was reported for the recombinant DszB from thermophilic bacterium *Paenibacillus* sp. strain A11-2 [13].

Temperature Studies

The optimum temperature for wild type A3H1-DszB and A3H1-R84Q-Y24F-DszB was 35 °C. The activity of the enzyme slowed significantly at temperatures that were lower or higher than the optimal temperature. The optimum temperature reported for the native IGTS8-DszB and KA2-5-1-DszB was 35 °C [10, 16]. The temperature optimum reported for the moderately thermophilic WU-S2B-DszB was slightly higher at 37 °C [14]. The thermophilic *Paenibacillus* sp strain A11-2 had optimum temperature at 55 °C [13]. The wild type A3H1-DszB and A3H1-R84Q-Y24F-DszB were most stable at 25 °C after incubation for 30 minutes at that temperature. The enzyme lost activity at higher temperatures. The A3H1-R84Q-Y24F-DszB is more stable than A3H1-DszB at higher temperatures.

Kinetic Studies

The kinetic parameters of HPBS desulfinatease were obtained using fluorimetric assays with varied substrate HPBS concentrations while the enzyme concentration remained fixed. The HPBS concentrations evaluated varied between 5 and 125 μM . For the A3H1-DszB, the K_m and k_{cat} values were $3.15 \pm 0.74 \mu\text{M}$ and $1.24 \pm 0.054 \text{ min}^{-1}$, while the values for the A3H1-R84Q-Y24F-DszB were $51.25 \pm 0.96 \mu\text{M}$ and $4.33 \pm 0.67 \text{ min}^{-1}$, respectively (Figures 15 and 16). A comparison of the kinetic parameters between DszB and some DszB homologs was made in Table 6, and described as follows.

Table 6. A comparison between the kinetic parameters of DszB and the DszB homologs referenced in this study.

DszB	k_{cat} (min^{-1})	K_m (μM)
A3H1- wildtype	1.24 ± 0.054	3.15 ± 0.74
A3H1-R84Q-Y24F - recombinant	4.33 ± 0.67	51.25 ± 0.96
A3H1-R84Q - recombinant (Watkins Lab)	13.1 ± 1.3	22.9 ± 8.9
IGTS8 - native (Watkins <i>et al.</i>)	1.3 ± 0.07	0.90 ± 0.15
IGTS8 - native (Gray <i>et al.</i>)	2	1
KA2-5-1- recombinant (Nakayama <i>et al.</i>)	7.38	8.2
WU-S2B - recombinant (Ohshiro <i>et al.</i>)	-	164
A11-2 - recombinant (Konishi <i>et al.</i>)	19.2	0.33

The k_{cat} values revealed that the A3H1-DszB enzyme had a similar rate of substrate turnover compared to the DszBs from the native IGTS8 DszB. The rate of substrate turnover of the thermophilic A11-2-DszB from *Paenibacillus* was 15 times faster than that of A3H1-DszB. The mutant DszB from A3H1-R84Q-Y24F showed a faster substrate turnover rate than the DszB enzymes from the wildtype A3H1 and the native IGTS8.

The K_m values revealed that the wildtype A3H1-DszB enzyme specificity for substrate was 52 times higher than that of WU-S2B-DszB, and two times higher than KA2-5-1-DszB. However, the DszB enzymes from the native IGTS8 and the

recombinant A11-2 were more substrate specific than the A3H1-DszB. The A3H1-R84Q-Y24F-DszB was sixteen times less specific for substrate than the A3H1-DszB, two times less than the A3H1-R84Q-DszB, and significantly less than the other DszB homologs under comparison. The A3H1-R84Q-Y24F-DszB, however, was three times more specific for substrate than the mildly thermophilic recombinant WU-S2B-DszB.

In general, the kinetic values show that the A3H1-DszB had a slower turnover rate and a moderate substrate specificity in comparison to the DszB homologs. The A3H1-R84Q-Y24F-DszB showed less specificity for substrate and lower substrate turnover than A3H1-R84Q-DszB. The hydroxyl group on the tyrosine residue at position 24 might play an important role in substrate binding.

Inhibition Studies

The enzyme activity of HPBS desulfinate was evaluated with varying concentrations of 2-HBP or sulfite, while enzyme and HPBS concentrations were held fixed. Both the wildtype and recombinant enzymes were inhibited by 500 μ M sulfite. The wildtype DszB enzyme was not inhibited by HBP at concentrations up to 500 μ M, while the recombinant DszB was inhibited at 100 μ M HBP. The wildtype DszB was inhibited by a mixture of 250 μ M sulfite and HBP, while the recombinant DszB was inhibited with a mixture of 100 μ M sulfite and HBP. Nakayama reported that the KA2-5-1-DszB enzyme was inhibited by HBP of more than 500 μ M, and Konishi reported weak inhibitory effects by HBP on the A11-2-DszB enzyme [10, 13]. The extent to which HBP and/or sulfite inhibit the DszB enzyme is another factor to be considered for the successful commercialization of BDS.

The activity of HPBS desulfinase was measured in the presence of product and substrate analogs with HPBS. The activities of the A3H1-DszB and A3H1-R84Q-Y24F-DszB were measured using 20 μ M and 230 μ M of analog, respectively. Both the wildtype A3H1-DszB and recombinant A3H1-R84Q-Y24F-DszB were totally inhibited by 2, 2'-biphenol. The wildtype A3H1-DszB was also totally inhibited by 4-hydroxy-4-biphenyl carboxylic acid and 93% by 1, 8-naphthosultone. The A3H1-DszB was inhibited the least (25%) by 1-naphthalene sulfonic acid, while the A3H1-R84Q-Y24F-DszB showed the most activity (13%) with diphenic acid. Compared to A3H1-DszB, the recombinant A3H1-R84Q-Y24F-DszB was significantly inhibited by all analogs used in the study.

The analog studies confirmed the findings that inhibitors have a hydrogen bond acceptor on one aromatic ring, and a hydrogen bond donor on the other aromatic ring [16]. The structures of the inhibitor 4'-hydroxy-4-biphenyl carboxylic acid and the non-inhibitor diphenic acid revealed that the former has both functional groups on either aromatic ring, while the latter has both functional groups on both rings (Figure 6). The A3H1-R84Q-Y24F-DszB was significantly inhibited when compared to the A3H1-DszB enzyme. The hydroxyl group on the tyrosine residue may also play an important role in binding.

Storage Stability of HPBS desulfinate

In this study it was found that the optimum storage condition for A3H1-DszB is at 4 °C, or at -20 °C in glycerol. Lee *et al.* reported that a glycerol molecule was located at the active site of the DszB and it was proposed that the glycerol may be necessary for keeping the enzyme stable [17]. The enzyme remained relatively stable at 20 °C when stored in 30% glycerol. A standard activity assay was conducted on the enzyme in glycerol after being stored for six months. The enzyme fraction in 30 % glycerol showed optimum activity. The protein became insoluble when stored at higher than 4°C without a cryoprotector and lost enzymatic activity. The A3H1-R84Q-Y24F-DszB was stored at -20 °C, and long term at -70 °C. The enzyme lost activity when fractions stored at -70 C without a cryoprotectant were thawed more than once.

Conclusions

The purification and characterization of HPBS desulfinate from the wildtype *Norcardia asteroides* sp. strain A3H1 was achieved. This study describes the first time the wild type A3H1-DszB enzyme has been characterized. The recombinant A3H1-R84Q-Y24F-DszB enzyme was also purified and characterized. The experiments in this thesis generated three findings. First, A3H1-DszB showed relatively similar substrate specificity as the A3H1-R84Q-DszB, but the enzyme is slower. Second, the results from A3H1-R84Q-C27S-DszB showing that the enzyme lost activity when cysteine was mutated to serine verifies that cysteine plays an important role as a component at the active site of the enzyme. The cysteine residue is essential for binding and/or catalysis [16]. The tyrosine is believed to be one of the amino acid residues at the active site of the

DszB enzyme [16]. Mutation of the tyrosine at position 24 resulted in slower rate of substrate turnover than the A3H1-R84Q-DszB, and lower specificity for substrate and greater susceptibility to inhibition of enzyme activity than the A3H1-DszB enzyme. The hydroxyl group on the tyrosine residue is therefore important for substrate binding and for binding in general. Future experiments on HPBS desulfinate could further explore the possibility of increasing the substrate specificity and rate of activity of the enzyme.

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VITA

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