PURIFICATION AND CHARACTERIZATION OF 2-(2′-HYDROXYPHENYL) BENZENESULFINATE DESULFINASE FROM *NOCARDIA ASTEROIDES* STRAIN SP. A3H1

THESIS

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CHAPTER I

INTRODUCTION

Organosulfur compounds are the major source of contamination in fossil fuels and petroleum. When emitted into the atmosphere, sulfur oxides are the major pollutants that contribute to acid rain. In order to lower the amount of these contaminants, legislation has implemented regulations restricting the amount of sulfur in petrochemicals (1, 2). Hydrodesulfurization (HDS) is the current method used to reduce the amount of sulfur in petroleum, but is not effective in removing sulfur from dibenzothiophene (DBT) (Figure 1) or alkylated DBTs, the major organosulfur components in crude oil.

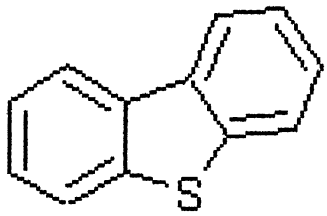


Figure 1. Structure of dibenzothiophene.

HDS is accomplished with a chemical catalyst under high pressure and high temperature (3). Since the abundance of low-sulfur crude oil has decreased, the refining of crude oil has become heavier and the sulfur content of crude oil is higher. In order to achieve low sulfur crude oil, refineries need to alter current HDS conditions including:

increasing the temperature and lowering space velocities. These changes will require new capital investments and the cost for operations will increase (4).

The original sulfur content in crude oil varies from 0.04% - 6% (w/w) and after distillation about 70% of the sulfur content in the middle-distillate fraction is made up of DBT and other alkylated DBTs (5). The concentration of the middle-distillate fraction utilized to create diesel fuel has a wide range from <500 to >5,000 parts per million (ppm). In 2005 the United States restricted the amount of the middle-distillate fraction or sulfur content to 50 ppm (4). This restriction was not low enough and the Environmental Protection Agency (EPA) implemented the 2007 Highway Rule, which caused diesel manufacturers to comply with the sulfur content restriction of 15 ppm. The 2007 Highway Rule requires refineries to create a new diesel fuel that is called Ultra-Low Sulfur Diesel (ULSD). ULSD is more expensive not only in production costs but also consumer cost, about 4 and 5 cents more per gallon (6).

Thus, a different method is needed to desulfurize DBT. Biodesulfurization (BDS) is a process that can remove organically bound sulfur from petroleum via microorganisms while maintaining the fuel value (7-10). *Brevibacterium*, *Arthrobacter*, *Pseudomonas*, *Beijerinckia*, and *Rhizobium* are microorganisms that are known to metabolize DBT. However, these microorganisms metabolize DBT via a ring breaking oxidative pathway. The pathway results in a net carbon loss and reduces the value of the fuel.

There is a sulfur specific, oxidative pathway. This pathway is known as the dsz pathway and does not destroy the carbon ring structure (Figure 2). The oxidation of DBT through the dsz pathway utilizes four different enzymes: DszA ($DBTO_2$-monooxygenase or $DBTO_2$-MO), DszB (2-(2′-hydroxyphenyl) benzenesulfinate desulfinase or HPBS

desulfinase), and DszC (DBT-monooxygenase or DBT-MO). In order for DBT-MO and $DBTO_2$-MO to have activity, flavins and DszD (NADH-FMN oxidoreductase) are required (10,12).

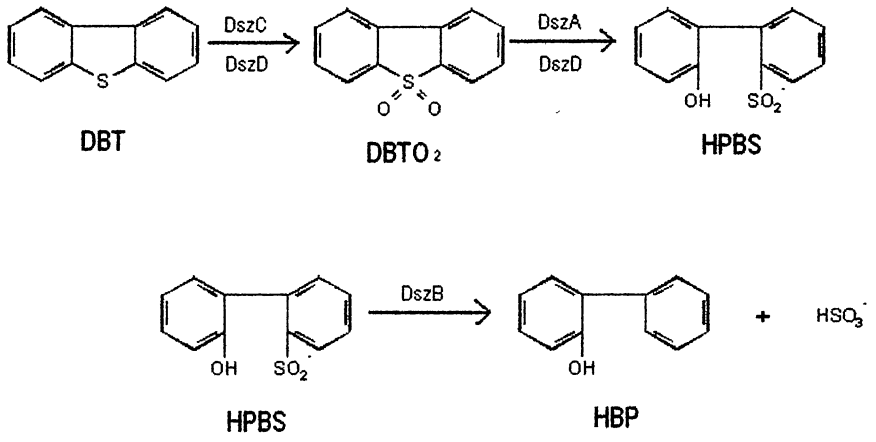


Figure 2. The dsz pathway for sulfur-specific desulfination of DBT.

DBT is oxidized by the first two enzymes DBT-MO (DszC) and an NADH-FMN oxidoreductase (DszD) to form DBT sulfone. $DBTO_2$-MO (DszA) and DszD, the next two enzymes, convert DBT sulfone to HPBS by the oxidative cleavage of one carbon-sulfur bond within $DBTO_2$. The desulfination of HPBS by the third enzyme, HPBS desulfinase (DszB), produces 2-hydroxybiphenyl (HBP) and sulfite (10). The HPBS desulfinase enzyme catalyzes the rate-limiting step of the dsz pathway *in vivo*, the final carbon-sulfur bond cleavage (11, 12).

There are several microorganisms including *Rhodococcus erythropolis* sp. strain IGTS8 (IGTS8), D-1, H-2, KA2-5-1, *Corynebacterium* sp. strain SY1, *Bacillus subtilis* WU-S2B, *Paenibacillus* sp. strain A11-2 and *Nocardia asteroides* strain sp. A3H1

(A3H1) that metabolize DBT through the dsz pathway (1, 3, 8-11,13,14). The microorganisms use the sulfur in their biomass as a nutrient source for cell growth (11).

The desulfinase enzyme has been purified and characterized from a few different bacteria: *Rhodococcus erythropolis* sp. strain IGTS8, *Bacillis subtilis* WU-S2B and *Paenibacillus* sp. strain A11-2. *Rhodococcus erythropolis* strain sp. IGTS8 has been extensively studied compared to the other bacterial strains. The three enzymes utilized in the dsz pathway; DszA, DszB, and DszC, have been purified and their kinetic parameters have been reported. The *dsz*a, *dsz*b and *dsz*c genes encode proteins of 453, 365, and 417 amino acids, respectively. The molecular weight of DszA is about 50 kDa, DszB is about 40 kDa and DszC is about 45 kDa. The K_m and k_{cat} for DszA, DszB, and DszC are: 1 μM and 1 s^{-1}, 1 μM and 2 min^{-1}, estimated < 5 μM, respectively and no k_{cat} was reported for DszC (11).

Further characterization experiments were conducted on the desulfinase enzyme from *Rhodococcus erythropolis* sp. strain IGTS8. The desulfinase enzyme was purified and characterized. The molecular weight, optimum pH, optimum temperature, and kinetic parameters were determined (10). The findings of the characterization experiment for the desulfinase enzyme from IGTS8 will be compared to characteristics found in the current study for the desulfinase enzyme from a novel bacterium, *Nocardia asteroides* sp. strain A3H1.

In recent work, the *dsz*b gene from A3H1 was cloned into the *E. coli* expression vector pGEX-4T-3 (15). Cloning into this vector will allow for one-step purification compared to the several steps utilized when purifying the native HPBS desulfinase from

IGTS8. The recombinant HPBS desulfinase enzyme from A3H1 will be purified and characterized.

Previous chemical modification studies completed on the native HPBS desulfinase enzyme from IGTS8 support the presence of tyrosine, tryptophan and cysteine residues in the active site (10). There is only one cysteine and one conserved tyrosine in the amino acid sequence of all known HPBS desulfinase enzyme (Figure 3).

Figure 3. Sequence alignment of *dsz*b from four different species.

There is no known mechanism for the HPBS desulfinase, but there are two proposed mechanisms. The mechanism to be addressed in the current studies is the one

proposed by Watkins *et al.* (Figure 4) (10). The Watkins group has proposed an acid-base chemistry mechanism. An alternative mechanism proposed by Lee *et al.* is a one-ring nucleophilic addition mechanism (16). After the chemical modification studies were completed on the desulfinase enzyme from IGTS8, the cysteine at position twenty-seven was proposed to be acting as the base and the tyrosine at position twenty-four was proposed to be acting as the acid.

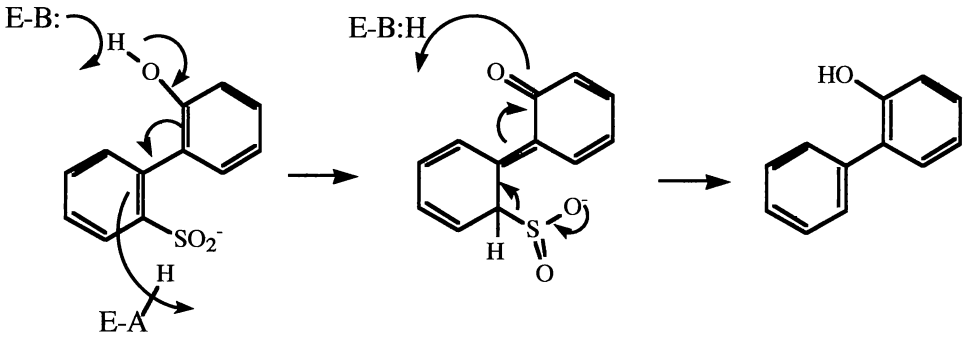


Figure 4. Proposed mechanism for HPBS desulfinase.

The primary goal of this project was purification and characterization of the recombinant HPBS desulfinase enzyme from A3H1. The recombinant desulfinase enzyme from A3H1 was compared to the native desulfinase enzyme from IGTS8, recombinant desulfinase enzyme from KA2-5-1, and the desulfinase enzymes from WU-S2B and A11-2. The *dsz*b gene from A3H1 was altered by site-directed mutagenesis to provide a better understanding of the proposed acid/base mechanism involved in the removal of the sulfur from petroleum. Understanding the reaction mechanism will allow for further alterations of the HPBS desulfinase enzyme to speed up the reaction and improve the process of removing the sulfur in fossil fuels.

CHAPTER II

MATERIALS

The pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV plasmids were provided by Dr. Greg Sawyer from the University of Texas at Austin. The reagents used to make all media: Bacto™ tryptone, Bacto™ agar and Bacto™ yeast extract were from Becton Dickinson (Sparks, MD). The Gyromax 747 Orbital Incubator shaker and HICLAVE HV-50 autoclave was from Amerex Instruments Inc. (Lafayette, CA). The Hermle Z180M centrifuge was from Labnet International Inc. (Edison, NJ).

The QIAprep Spin Miniprep Kit used to isolate all plasmid DNA was from Qiagen (Valencia, CA). The Smart Spec™ 3000 used to quantitate plasmid DNA and all horizontal gel electrophoresis equipment and agarose was from Bio-Rad (Hercules, CA). The ethidium bromide used to stain the agarose gels was from EM Science (Lawrence, KS). The ACCU P·O·W·E·R model 300 power supply used for running agarose gel electrophoresis and polyacrylamide gel electrophoresis and the Standard heat block were from VWR Scientific (West Chester, PA). The polyacrylamide gel electrophoresis apparatus used was the Novex El9001 – Xcell II Mini Cell (San Diego, CA). All reagents and equipment used to make the polyacrylamide gels were purchased from Bio-Rad (Hercules, CA). The agarose and polyacrylamide gel pictures were taken using a Kodak

digital service image station 440 CF with Kodak 1D 3.6 software from Eastman (Rochester, NY).

Cell lysis was completed using a Sonics Vibra Cell™ Model VC 130 sonicator (Newton, CT). The cells were centrifuged using a Beckman Model J2-21 centrifuge from Beckman Coulter (Fullerton, CA). For protein purification, the glutathione resin and thrombin protease were purchased from Sigma-Aldrich (St. Louis, MO). Protein concentration was determined using BCA™ Protein Assay Kit purchased from Pierce (Rockford, IL). All fluorimetric assays utilized a Perkin Elmer Luminescence Spectrometer LS50B and data were collected with the FL Winlab software from Perkin Elmer Corporation (Norwalk, CT).

The QuikChange® II Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA). The primers used to introduce the mutations were from Sigma Genosys. The 2720 thermal cycler was purchased from Applied Biosystems (Foster City, CA).

CHAPTER II

METHODS

Preparation of Competent *Escherichia coli* (*E. coli*) Cells

All competent *E. coli* cells were prepared to transform plasmid DNA into cells already containing the plasmid pREP4_GroESL. BL21(DE3) cells containing the plasmid pREP4_GroESL was streaked onto a fresh LB (Luria-Bertani) agar plate and placed in a 37°C incubator overnight. A tube containing 10 mL of LB broth was inoculated with a single colony from the freshly streaked plate and let grow with overnight shaking at 250 rpm overnight in a 37°C incubator. A 1 mL aliquot from the overnight culture was used to inoculate 200 mL of LB broth and placed in 37°C incubator shaking at 250 rpm until the OD (optical density) A_{550} = 0.5. The light scattering at 550 nM was measured using a 1.5 mL disposable cuvette in a Smartspec 3000 spectrophotometer (Bio-Rad). Once the OD = 0.5, the cells were placed in an ice-water bath for 5 minutes. The cells were then centrifuged at 4,400 x *g* at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 80 mL of ice cold Buffer 1 (30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8). The resuspended pellet was placed in ice water for 5 minutes and then centrifuged at 4,400 x *g* for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 8 mL of ice cold Buffer 2 (10 mM MOPS/KOH, pH 6.5, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% (v/v)

glycerol). The resuspended pellet was placed in ice water for 15 minutes. Aliquots of 100 μL stored at -70°C.

Transformation of Plasmid DNA into Top 10 *E. coli* Cells

Plasmid DNA (Table 1) was transformed into Top 10 *E. coli* cells for manipulation and storage. Dr. Greg Sawyer from The University of Texas provided the plasmid DNA, pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV. Competent Top10 cells were placed on ice to thaw. Once thawed, 5-10 ng of plasmid DNA was added to 100 μL of competent Top10 *E. coli* cells and the sample was incubated on ice for 10 minutes. The cells were heat shocked at 42°C for 2 minutes using a heat block. A 1 mL sample of LB broth at 42°C was added to the sample. The cells were then placed at 37°C shaking at 250 rpm for 1 hour. Following incubation, a 250 μL aliquot of the transformation mixture was plated onto LB/AMP (100 μg/mL) plates and incubated overnight at 37°C. This same procedure was used to transform pGEX-4T-3/A3H1-R84Q or pGEX-4T-3/A3H1-TEV into the competent BL21/pREP4_GroESL cells.

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

Name	Description	Reference
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-R84Q	The pGEX-4T-3 vector with the *dsz*b gene that has the R84Q mutation from A3H1.	(15)
pGEX-4T-3/A3H1-TEV	The pGEX-4T-3 vector with the wild-type *dsz*b gene from A3H1 that has a TEV cleavage site.	(15)
pREP4_GroESL	The pREP4 vector with the genes encoding the GroES and GroEL chaperone proteins.	(Dr. Dean Appling, UT-Austin)

Plasmid Isolation

An overnight culture was prepared with 5 mL of LB broth with AMP (100 μg/mL) and one colony from a plate containing freshly transformed cells. The culture was incubated overnight at 37°C shaking at 250 rpm. Plasmid DNA was isolated following the QIAprep Spin Miniprep Kit protocol (Qiagen).

Horizontal Gel Electrophoresis

A 0.8% (w/v) agarose gel in 1X TAE Buffer (40 mM Tris-base, pH 8, 20 mM acetic acid, 1 mM EDTA) was prepared. A 100 ng sample of DNA was mixed with a 1 μL 6X dye buffer (40% (v/v) sucrose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) and diH_2O. The DNA samples were loaded into the gel, covered with 1X TAE Buffer containing ethidium bromide (0.2 mg/mL) and separated by electrophoresis for 1.5 hours at 85 volts. A Kodak Imaging System was used to document the gel.

DNA Sequencing

Isolated plasmid DNA, pGEX-4T-3/A3H1-R84Q or pGEX-4T-3/A3H1-TEV, were sent to The University of Texas at Austin DNA Sequencing ICMB Core Labs. The primers used to sequence pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV were the pGEX-4T-3 forward and reverse primers listed in Table 2. The sequences were then compared to the known sequences.

Table 2
Primers that were used for sequencing the pGEX-4T-3/A3H1-R84Q and pGEX-4T- 3/A3H1-TEV.

Name	Sequence	Direction	Description
pGEXFS	5′ GGCTGGCAAGCCACGTTTGGTG 3′	Forward	Starts sequencing at the 5′ end of the pGEX-4T-3 vector
pGEXRS	5′ CCGGGAGCTGCATGTGTCAGAGG 3′	Reverse	Starts sequencing at the 3′ end of the pGEX-4T-3 vector

HPBS desulfinase R84Q Expression and Purification

Co-expression of GroEL, GroES and HPBS desulfinase R84Q

LB broth (50mL) with kanamycin (30 μg/mL) and ampicillin (100 μg/mL) was inoculated with one colony of pGEX-4T-3/A3H1-R84Q in BL21/pREP4_GroESL and allowed to grow overnight at 37°C shaking at 250 rpm. A 5 mL aliquot of this culture was used to inoculate 500 mL of LB broth with kanamycin (30 μg/mL) and ampicillin (100 μg/mL). The culture was at 37°C shaking at 250 rpm until OD A_{600} = 0.6 – 0.8. Expression was induced with 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) at room temperature (23-25°C) overnight. The cells were collected by centrifugation at 3,000 x *g* for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10 mL of Purification Buffer 1 (50 mM K_2HPO_4, pH 7.0, 50 mM KCl).

Cell Lysis

After the cells were resuspended in 10 mL of Purification Buffer 1, approximately 10 μg of PMSF (phenylmethyl sulfonyl fluoride) was added. The cells were ruptured by

sonication with three ten second pulses at an amplitude of 50. A 50 μL sample was taken after the sonication and placed on ice. The volume was estimated and recorded. The ruptured cells were pelleted by centrifugation at 24,000 x *g* for 15 minutes at 4°C. A 50 μL sample of the supernatant was taken and placed on ice. The volume of the supernatant was measured and recorded. The pellet was discarded. The supernatant was then filtered using a 10 mL syringe and a 0.2 μM cellulose filter. A 50 μL sample was taken and placed on ice. The volume was estimated and recorded. All expression samples were later analyzed by SDS-PAGE gel.

HPBS desulfinase R84Q Purification

The filtered supernatant from the cell lysis was loaded onto a 10 mL glutathione column. The flow-through was captured and later analyzed by SDS-PAGE. The column was then washed overnight with 1 liter of Purification Buffer 1 (50 mM K_2HPO_4, pH 7.0, 50 mM KCl). A 25 μL aliquot of the resin was taken to be analyzed by SDS-PAGE to determine if the GST-HPBS desulfinase R84Q fusion protein was bound to the column. The beads were resuspended in Purification Buffer 1 to a final volume of 18 mL and mixed with 2 mL of thrombin cleavage buffer (200 mM Tris-HCl, pH 8, 25 mM $CaCl_2$) and 50 units of thrombin protease. The cleavage reaction was incubated on a rotary shaker at 4°C overnight. A sample was taken to check the cleavage efficiency by SDS-PAGE. The cleaved protein solution was then transferred back to a clean column and the resin was allowed to settle. The HPBS desulfinase was captured in fractions that immediately washed off the column. Additional enzyme was removed from the column using 30 mL of Purification Buffer 1. The GST is retained on the column, which was

removed using the procedure described by Sigma. The purification scheme can be seen in Figure 5.

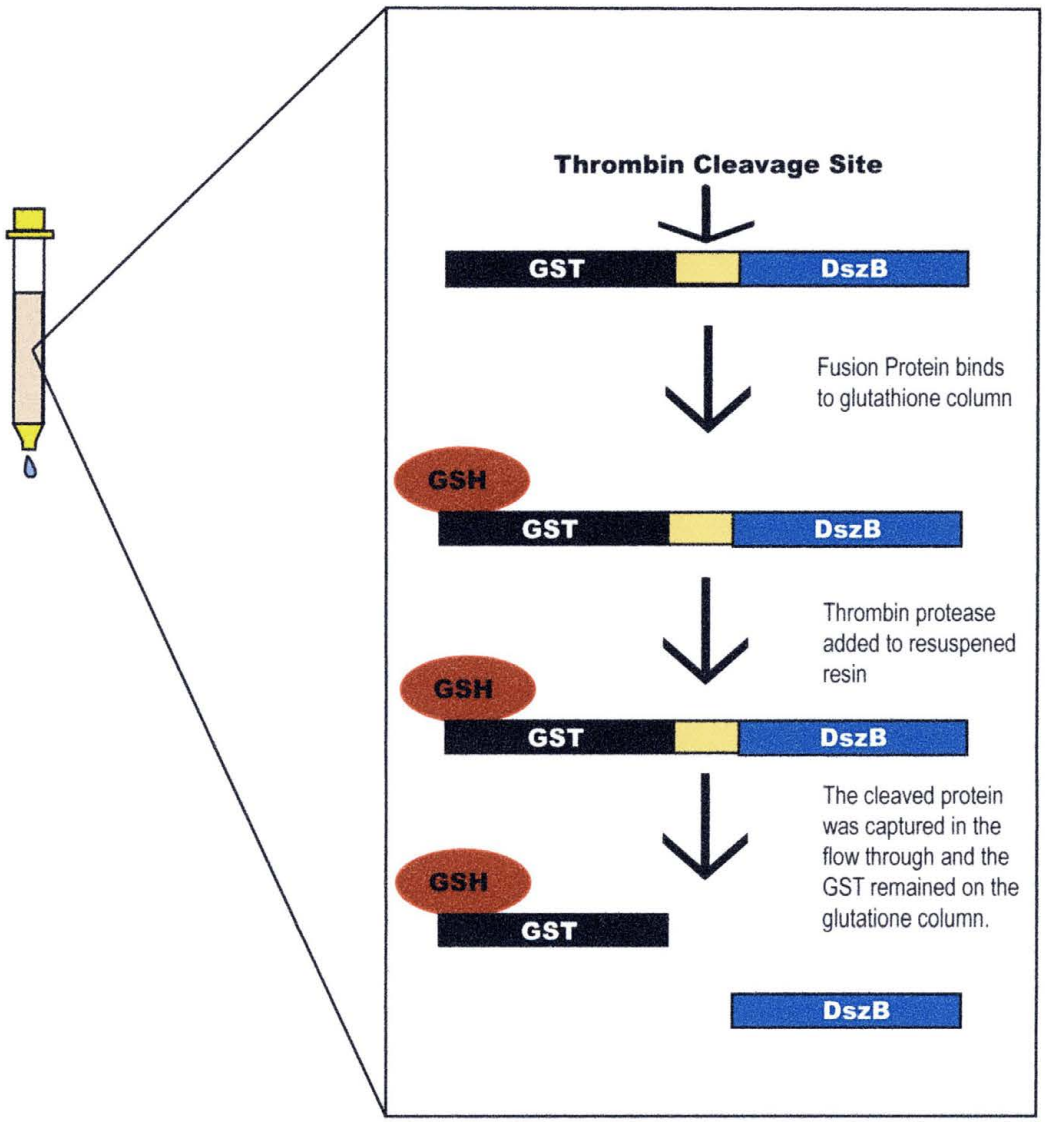


Figure 5. Purification Scheme for HBPS desulfinase R84Q.

BCA (bicinchoninic acid) Assay

The BCA assay was used to determine the protein concentration of HPBS desulfinase. The following reagents were added to a 96-well microtiter plate: ddH_2O, Bovine Serum Albumin (BSA), HPBS desulfinase, BCA reagents A and B. The BSA standards were at the following concetrations: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025, and 0 mg/mL. The HPBS desulfinase samples were diluted 1/10 in ddH_2O. The BCA reagents used were 50 parts BCA reagent A to 1 part reagent B and 200 μL of the BCA reagent mixture was incubated with 25 μL of standard or sample at room temperature for a minimum of 15 minutes. The absorbance was measured at 562 nm using a SpectraMax 190 plate reader with Softmax Pro 4.7.1 software. A standard curve was created using Microsoft Excel. The best-fit line from the standard curve was used to calculate the concentration of protein in the purified HPBS desulfinase samples.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Approximately 1 μg of protein was mixed with 1X SDS Buffer (10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.0025% (w/v) Bromophenol blue, 5% (v/v) β-mercaptoethanol), heated at 95°C for five minutes, loaded onto a 10% gel and separated by electrophoresis at 150 volts for 1.5 hours. The gel was rinsed three times with diH_2O and then stained with a 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 washed again three times with diH_2O and destained overnight with a Coomassie destain solution (7.5% (v/v) glacial acetic acid, 10% (v/v) ethanol).

Fluorimetric Assays

Standard Activity Assay

A fluorimetric assay was used to measure the activity of HPBS desulfinase. The assay was run in a 96-well plate and contained diH_2O, 1X Buffer A (25 mM NaH_2PO_4, 100 mM NaCl, pH 7.4), HPBS desulfinase and 100 μM 2-(2′-hydroxyphenyl)benzene-sulfinate (HPBS) in a 200 μL total volume. The assay components, excluding the enzyme, and the enzyme were incubated in separate wells at 37°C for five minutes on a Zytron plate heater. The enzyme was then added and fluorescence due to hydroxy-biphenol (HBP) product formation was measured over time using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds. FL WinLab software was used to collect the data and the initial velocity was determined using Microsoft Excel.

HBP Standard Curve Assay

A standard HBP assay was run to determine the fluorescence intensity of 1 μM HBP. Assays were run in 96-well plates and contained several different concentrations of HBP (50, 40, 30, 20, 10, 5, 3, 1, 0.1, 0.05, 0.01 and 0 μM), 7 X Buffer A (175 mM NaH_2PO_4, 700 mM NaCl, pH 7.4) and diH_2O in 200 μL total volume. The fluorescence of HBP was measured using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds. A standard curve was created with fluorescence intensity on the y-axis and HBP concentration on the x-axis. Microsoft Excel was used to obtain a best-fit line. The slope corresponded to the

fluorescence/μM of HBP. This value was used to calculate the V_{max} and k_{cat} of HPBS desulfinase.

Kinetics Assay

A standard activity assay was run to determine the kinetic parameters, K_m, V_{max}, and k_{cat}, of the purified HPBS desulfinase R84Q. The assay was run in a 96-well plate that contained 1X Buffer A (25 mM NaH_2PO_4, 100 mM NaCl, pH 7.4), 0.0016 μg of HPBS desulfinase R84Q, diH_2O, and varied substrate (HPBS) concentrations (5, 10, 20, 40, 60, 80, and 100 μM) in a total volume of 200 μL. The assay components, excluding the enzyme and Buffer A, were incubated at 37°C for five minutes on a Zytron plate heater. The enzyme and Buffer A, incubated separately, were added and fluorescence due to HBP product formation was measured over time using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds. FL WinLab software was used to collect the data and the initial velocity was determined using Microsoft Excel. The data from Microsoft Excel was averaged for the two trials and EnzFitter version 2.014.0 was used to fit the data to the Michaelis-Menten equation.

Temperature Study

The temperature optima and temperature stability of HPBS desulfinase was determined by incubating the enzyme at different temperatures and running a standard activity assay. The temperature optima was determined by incubating 1X Buffer A, 0.03 μg HPBS desulfinase, and 10 μM HPBS in a 96-well microtiter plate at temperatures

ranging from 20-60°C in increments of 5°C for 5 minutes. The HPBS was incubated separately prior to addition to the rest of the assay mixture. The fluorescence of HBP was measured using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds. For the temperature stability of HPBS desulfinase, 10X Buffer A, 10 μM HPBS, and 0.03 μg HPBS desulfinase were mixed in a 96-well microtiter plate and incubated at temperatures ranging from 25-60°C in increments of 5°C for 30 minutes and the fluorescence was measured as stated previously.

pH Study

The optimum pH for HPBS desulfinase R84Q was determined using the standard fluorimetric assay and the MTEN adjusted to different pH values ranging from pH 3.0-9.0. A 10X MTEN solution (250 mM Tris, 250 mM Ethanolamine, 500 mM MES, 1 M NaCl). was diluted to 5X and buffered to the desired pH values. Individual wells in a 96-well microtiter plate contained 1X MTEN Buffer at the different pH values, 200 μM HPBS, 0.0016 μg HPBS desulfinase and ddH_2O in a final volume of 200 μL. The fluorescence of product formation was measured using a Perkin Elmer Luminescence Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds. The data was collected by the FL WinLab software and the initial velocity was determined using Microsoft Excel.

Metal Activation Study

The relative activity of HPBS desulfinase R84Q was measured in the presence of different metals. Chloride salt solutions (100 μM) of the following metals were used: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. A 96-well microtiter plate was used and each well contained 1X Buffer A, ddH_2O, 100 μM HPBS, 0.029 μg HPBS desulfinase R84Q and 100 μM of each metal. The relative activity was measured by a fluorimetric assay using a Perkin Elmer Luminescene Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds.

Inhibition Study

Inhibition studies were conducted using different substrate analogs. The substrate analogs used were: 4-hydroxyl-4-biphenyl carboxylic acid, 2-biphenylcarboxylic acid, p-xylene-2-sulfinic acid, 4-cyclohexylphenol, 1,8-naphthosultam, 2,2′- bipyridine-3,3′-diol, 1-napthalene sulfonic acid, and diphenic acid. Fluorescence assays were done using a 96-well microtiter plate containing 1X Buffer A, ddH_2O, 100 μM HPBS, 30 μg HPBS desulfinase R84Q and 100 μM inhibitor. The relative activity was measured by a fluorimetric assay using a Perkin Elmer Luminescene Spectrometer LS50B at 414 nm (λ_{exc} = 288 nm) for 20 cycles; each cycle was 60 seconds.

Polymerase Chain Reaction (PCR)

Mutant Primer Design

Mutant primers were designed to mutate the cysteine at position twenty-seven to a serine and an alanine and the tyrosine at position twenty-four to a phenylalanine. The primers were designed using a one base change in the three-letter code for the amino acid of interest. The mutation from cysteine to alanine is a two base change so the primer was made based on the mutation from cysteine to serine, therefore allowing for a one base change to mutate from a serine to an alanine. The mutant primers used are listed in Table 3. The melting temperature, GC content, and secondary structure were calculated using the IDT SciTools Oligoanalyzer, www.idtdna.com and DNA Calculator from Sigma Genosys, www.sigma-genosys.com. The primers were used to make mutants from the recombinant pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV. The mutations were made using PCR.

Table 3
Primers used for introducing three different mutations into the A3H1-R84Q/pGEX-4T-3 and A3H1-TEV/pGEX-4T-3.

Name	Sequence	Direction	Description
CGC27S-F-05	5′ CACTGACCTACAGCAACTCCCCGATACCCAACGCGC 3′	Forward	Introduces the C27S mutation into the *dsz*b gene on the 3′ to 5′ plasmid DNA strand.
CGC27S-C-05	5′ GCGCGTTGGGTATCGGGGAGTTGCTGTAGGTCAGTG 3′	Reverse	Introduces the C27S mutation into the *dsz*b gene on the 5′ to 3′ plasmid DNA strand.
CGY24F-F-05	5′ TCCTCGACACACTGACCTTCAGCAACTGCCCGATACC 3′	Forward	Introduces the Y24F mutation into the *dsz*b gene on the 3′ to 5′ plasmid DNA strand.
CGY24F-C-05	5′ GGTATCGGGCAGTTGCTGAAGGTCAGTGTGTCGAGGA 3′	Reverse	Introduces the Y24F mutation into the *dsz*b gene on the 5′ to 3′ plasmid DNA strand.
CGC27A-F-05	5′ CACTGACCTACAGCAACGCCCCGATACCCAACGCGC 3′	Forward	Introduces the C27A mutation into the *dsz*b gene on the 3′ to 5′ plasmid DNA strand.
CGC27A-C-05	5′ GCGCGTTGGTATCGGGGCGTTGCTGTAGGTCAGTG 3′	Reverse	Introduces the C27A mutation into the *dsz*b gene on the 3′ to 5′ plasmid DNA strand.

PCR

A QuikChange® II Site-Directed Mutagenesis Kit from Stratagene was used to introduce the mutations in pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV. The control reaction contained 1X reaction buffer, 10 ng pWhitescript, 125 ng control primer #1 and #2, 0.8 mM of dNTP mix, 2.5 U of *PfuUltra* HF DNA polymerase and ddH_2O in a total volume of 50 μL. The reactions to create the mutant plasmids containing the C27S, C27A and Y24F mutants contained 1X reaction buffer, 50 ng of dsDNA template (pGEX-4T-3/A3H1-R84Q or pGEX-4T-3/A3H1-TEV), 125 ng of mutant forward and reverse primer, 0.8 mM dNTP mix, 2.5 U *PfuUltra* HF DNA polymerase, and ddH_2O in a total volume of 50 μL. The reaction was run on a 2720 Thermal Cycler (Applied Biosystems) using the following program. The first segment was set for 95°C for 30 seconds, the second segment has three parts; denature for 30 seconds at 95°C, anneal for 1 minute at 55°C, elongate for 6 minutes at 68°C. The second segment was repeated in 16 cycles. The final segment cooled the sample to 4°C until the reaction was pulled from the thermal cycler.

***Dpn* I Digest of the PCR Amplification Product**

The *Dpn* I digest was carried out to degrade the parental dsDNA template. The restriction enzyme *Dpn* I (10 units) was added to each control and sample amplification reaction and gently mixed. The reaction was then centrifuged for 1 minute and immediately at 37°C for 1 hour using a standard heat block from VWR Scientific Products.

Transformation of *Dpn* I-treated DNA into XL1-Blue Cells

The XL1-Blue cells were thawed on ice and 1 μL of the *Dpn* I-treated DNA, control and sample, was added to 50 μL of the XL1-Blue cells. The sample was placed on ice for 30 minutes, heat shocked at 42°C for 45 seconds and then placed on ice for 2 minutes. A 0.5 mL sample of preheated LB broth was then added to each transformation mixture and the transformation mixtures were incubated at 37°C with shaking at 250 rpm for 1 hour. A 200 μL aliquot of the mutagenesis transformation control was plated onto LB/AMP (100 μg/mL) plates containing 80 μg/mL X-gal and 20 mM IPTG and a 250 μL aliquot of the sample mutagenesis transformation was plated onto LB/AMP (100 μg/mL) plates. The plates were incubated at 37°C for >16 hours. The mutant dsDNA plasmids were isolated following the same protocol as described previously under Plasmid Isolation.

CHAPTER IV

RESULTS

The recombinant HPBS desulfinase R84Q enzyme was purified and characterized. The *dsz*b gene sequence from A3H1 was altered by site-directed mutagenesis to provide a better understanding of the amino acids that are proposed to play a role at the active site of the enzyme. Understanding the role of the amino acids at the active site will provide insight into the desulfurization mechanism.

The plasmid DNA pGEX-4T-3/A3H1-R84Q or pGEX-4T-3/A3H1-TEV was transformed into Top10 cells. The R84Q mutation was introduced into the *dsz*b gene from A3H1 to prevent internal cleavage by thrombin protease during the purification. Both plasmids were isolated and quantitated. These plasmids were then transformed into BL21 cells containing pREP4_GroESL. Expression and purification of HPBS desulfinase R84Q was completed and fluorimetric assays were conducted to characterize the enzyme. The two plasmids pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV were mutated using a QuikChange® II Site-Directed Mutagenesis Kit. The pGEX-4T-3/A3H1-R84Q had two different mutations introduced. The cysteine at position twenty-seven was mutated to a serine and the tyrosine at position twenty-four was mutated to a phenylalanine. The pGEX-4T-3/A3H1-TEV had the same two mutations introduced at the same positions as pGEX-4T-3/A3H1-R84Q with an additional mutation where

the serine at position twenty-seven was mutated to an alanine. The mutant plasmids were then transformed into BL21/pREP4_GroESL cells for expression and purification. The mutations were introduced into the *dsz*b gene from A3H1 in order to investigate the role of the amino acid residues proposed to be at the active site of the HPBS desulfinase enzyme.

Co-expression of GroEL, GroES and HPBS desulfinase R84Q

The HPBS desulfinase R84Q was co-expressed with the chaperone proteins GroEL and GroES. The presence of the chaperone proteins helps to facilitate proper folding of the HPBS desulfinase R84Q. Expression of GroEL, GroES and HPBS desulfinase was induced with 1 mM IPTG. An SDS-PAGE gel was run to confirm over-expression of the fusion protein glutathione S-transferase (GST) and HPBS desulfinase R84Q. The fusion protein has a molecular weight of 69 kDa. The GST alone has a molecular weight of 26 kDa and the molecular weight of HPBS desulfinase R84Q is about 40 kDa. The chaperone protein GroEL has a molecular weight of about 66 kDa. Figure 6 shows the over-expression of the fusion protein GST-HPBS desulfinase R84Q and the first steps of the purification of HPBS desulfinase R84Q. Lane 1 contains the protein ladder, lane 2 is a sample of the cell lysate, lane 3 is a sample after sonication of the cell lysate, lane 4 is the supernatant after pelleting the cell debris, and lane 5 is the filtered supernatant after pelleting the cell debris.

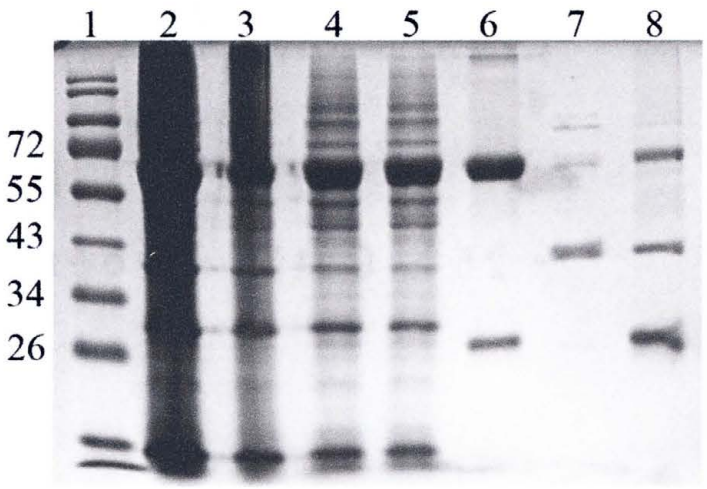


Figure 6. 10% SDS-PAGE of over-expressed fusion protein and purification of HPBS desulfinase R84Q. Lane 1 Fermentas protein ladder, lane 1 over-expressed fusion protein GST-HPBS desulfinase R84Q, lane 3 after cells were sonicated, lane 4 supernatant after centrifugation, lane 5 filtered supernatant, lane 6 glutathione resin after 1 L overnight wash, lane 7 supernatant from thrombin cleavage reaction, lane 8 resin from thrombin cleavage reaction. The gel was stained with Coomassie blue.

Purification of HPBS desulfinase R84Q

HPBS desulfinase was purified using a GST tag, which allowed for one-step purification. The cell lysate was applied to the glutathione column and the column was washed with 1 liter of purification buffer 1 overnight. The bound fusion protein resulted in a band at 69 kDa and can be seen in lane 6 (Figure 6). The desulfinase was then chemically cleaved from the GST tag using thrombin protease. The cleaved desulfinase resulted in a band at about 40 kDa, which can be seen in lane 7 (Figure 6). A sample of the glutathione resin was taken after the cleavage reaction to verify that there was no fusion protein still bound to the column. GST resulted in a band at 26 kDa, the desulfinase resulted in a band at about 40 kDa and can be seen in lane 8 of Figure 6. HPBS desulfinase R84Q was eluted from the column after the cleavage reaction. The

total amount of protein isolated was approximately 1 mg/L. An SDS-PAGE (Figure 7) was run to analyze the purity of the desulfinase. Purified HPBS desulfinase R84Q resulted in a single band around 40 kDa. The chaperone protein GroEL has a molecular weight of about 66 kDa.

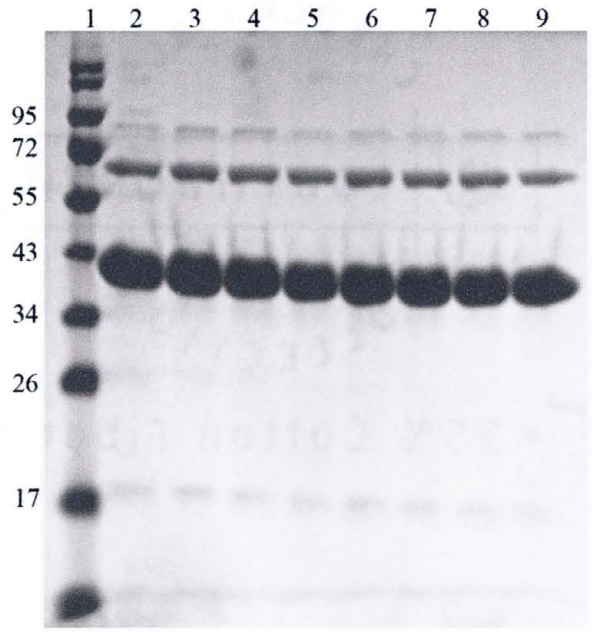


Figure 7. 10% SDS-PAGE of the fractions collected from the purification of HPBS desulfinase R84Q. Lane 1 Fermentas protein ladder, lanes 2-7 are aliquots 1-6 of the purified HPBS desulfinase R84Q, lane 8-9 are aliquots taken from the 30 mL purification buffer 1 wash. All the enzyme aliquots taken were about 2 mL each and the wash aliquots were 15 mL each. The gel was stained with Coomassie blue.

Fluorimetric Assays

Kinetics Assay

The kinetic parameters of HPBS desulfinase R84Q were determined by running a

fluorimetric assay. The assay contained various HPBS substrate concentrations ranging from 1 μM to 100 μM and was run at 37°C at a pH of 7.4. The product formation was monitored over time using fluorescence. The first seven minutes of data was analyzed. EnzFitter was used to fit the data to Michaelis-Menten equation and a Michaelis-Menten graph was made using Microsoft Excel (Figure 8). The V_{max}, K_m, and k_{cat} of HPBS desulfinase R84Q were determined to be 0.16 ± 0.02 U/mg, 22.9 ± 8.5 μM and 13.2 min^{-1}.

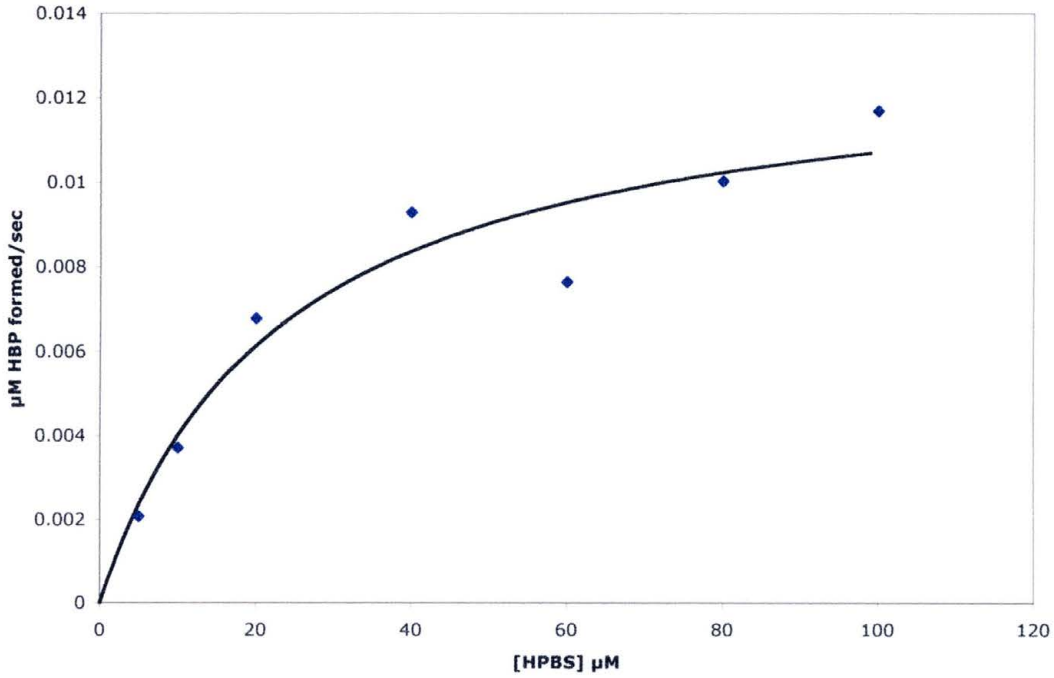


Figure 8. Michaelis-Menten Graph for HPBS desulfinase R84Q. Samples of HPBSdesulfinase R84Q were incubated with varied substrate concentrations from 5 to 100 μM. A fluorimetric assay was run and the data was analyzed and fit to the Michaelis-Menten equation using EnzFitter version 2.014.0.

Temperature Study

The activity of HPBS desulfinase R84Q was monitored and measured after incubating for five minutes at temperatures ranging between 20 and 60°C. The temperature optimum observed for HPBS desulfinase R84Q was 30°C. As the temperature increased the activity of HPBS desulfinase R84Q decreased and the same was seen at lower temperatures from the optimum (Figure 9).

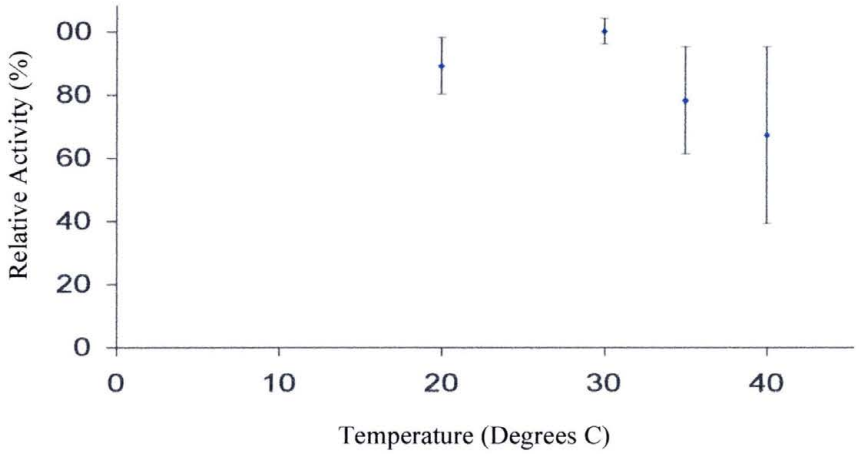


Figure 9. Temperature optima for HPBS desulfinase R84Q. Samples of HPBS desulfinase R84Q were incubated at different temperatures for five minutes. The standard fluorescence activity assay was run to monitor product formation. Activity was measured relative to the standard fluorescence assay run Buffer A pH 7.4, 30°C.

pH Study

The optimum pH for HPBS desulfinase R84Q was determined by incubating the enzyme in MTEN buffer at different pH values ranging from 3.0 to 9.0. The activity of the enzyme was measured by the fluorescence assay and a pH profile was created (Figure 10). The activity of the enzyme steadily increased between pH 4.0 and 8.0 and beyond

pH 8.0, the activity decreased. The enzyme had the highest relative activity at pH 8.0 and therefore was the optimum pH for HPBS desulfinase R84Q.

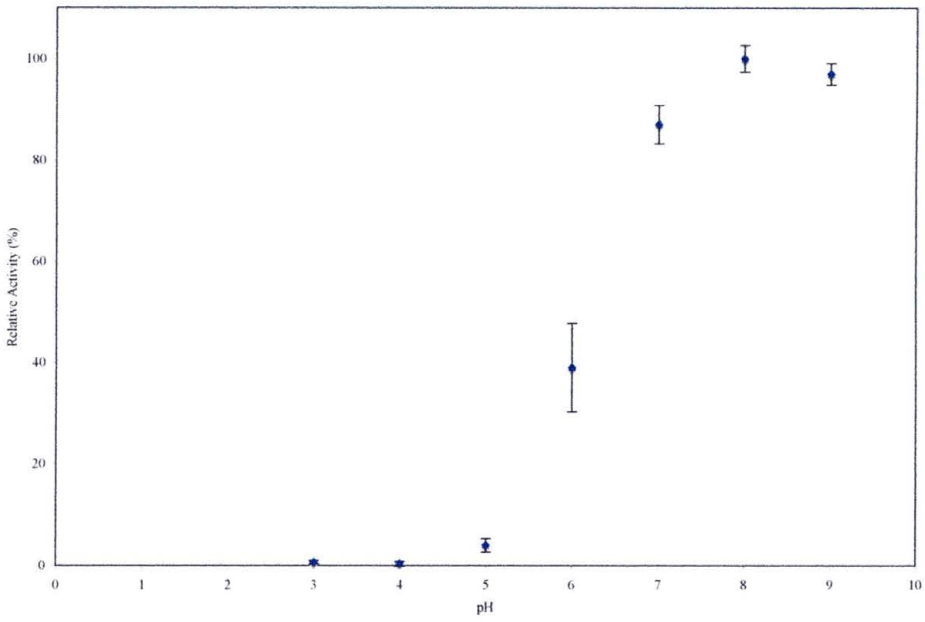


Figure 10. pH profile for HPBS desulfinase R84Q. Samples of HPBS desulfinase R84Q were incubated in MTEN buffer for five minutes at a different pH ranging from 3.0 to 9.0. A fluorescence assay was done to monitor enzyme activity.

Metal Activation Study

HPBS desulfinase R84Q activity was measured in the presence of seven different divalent metals and was compared to activity in the absence of metal (Table 4). Cobalt increased enzyme activity slightly. Calcium increased enzyme activity by 30%. Iron, magnesium and manganese increased enzyme activity by 40%. Zinc on the other hand decreased activity by 58%. Copper drastically decreased enzyme activity by 83%. The high error was due to the metal precipitating out of solution.

Table 4
Metal activation study for HPBS desulfinase R84Q.

Metal	Relative Activity (%)
$CaCl_2$	130 ± 55
$CoCl_2$	110 ± 53
$CuCl_2$	17 ± 8.5
$FeCl_2$	140 ± 98
$MgCl_2$	140 ± 70
$MnCl_2$	140 ± 49
$ZnCl_2$	42 ± 17

Samples of HPBS desulfinase R84Q (0.029μg) were incubated with 100 μM of each metal compound. Enzyme activity was measured using the fluorescence assay with the control where no metal compound was present.

Inhibition Study

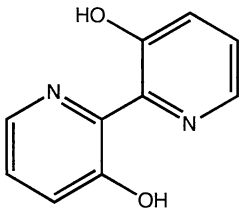
Different substrate analogs were tested to determine the effect of HPBS desulfinase R84Q activity. The fluorescence assay was used to measure enzyme activity. The substrate analogs used were 4′-hydroxy-4-biphenyl carboxylic acid, 2-biphenyl carboxylic acid, *p*-xylene-2-sulfonic acid, 4-cyclohexyl phenol, 2,2′-bipyridine-3,3′-diol, 1,8-naphthosultam, 1-naphthalene sulfonic acid, and diphenic acid. The structures of the substrate analogs can be seen in Figure 11. The relative activity of the enzyme can be seen in Table 5. The following compounds slightly inhibited enzyme activity: 2-biphenyl carboxylic acid, *p*-xylene-2-sulfonic acid, 4-cyclohexyl phenol and 1-naphthalene sulfonic acid. 4′-hydroxy-4-biphenyl carboxylic acid and 1,8-naphthosultam inhibited

enzyme activity by 65% and 55%, respectively. 2,2′-bipyridine-3,3′-diol drastically decreased enzyme activity. Diphenic acid increased enzyme activity by 9%.

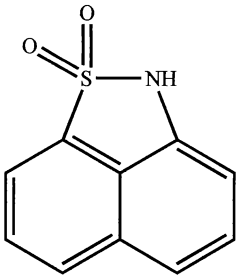
Table 5
Inhibition study of HPBS desulfinase R84Q.

Inhibitor	Relative Activity (%)
4′-hydroxy-4-biphenyl carboxylic acid	35
2-biphenylcarboxylic acid	76
p-xylene-2-sulfonic acid	84
4-cyclohexyl phenol	84
2,2′-bipyridine-3,3′-diol	0.5
1,8-naphthosultam	45
1-naphthalene sulfonic acid	82
diphenic acid	109

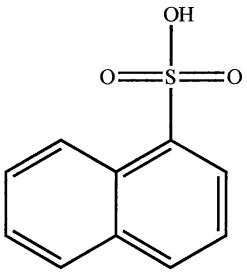
Samples of HPBS desulfinase R84Q were incubated with 100 μM of each inhibitior, 100 μM HPBS substrate and 1X Buffer A. The fluorescence assay was used to measure enzyme activity.



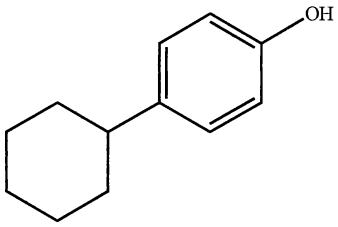
2-2′-bipyridine-3,3′-diol



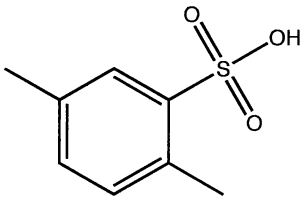
1,8-naphthosultam



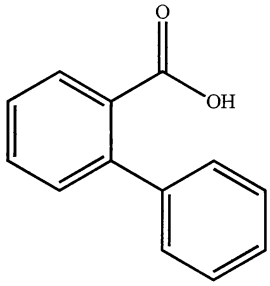
1-naphthalene sulfonic acid



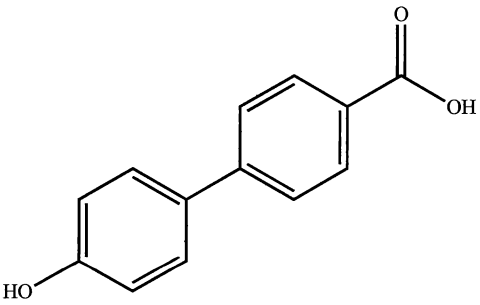
4-cyclohexyl phenol



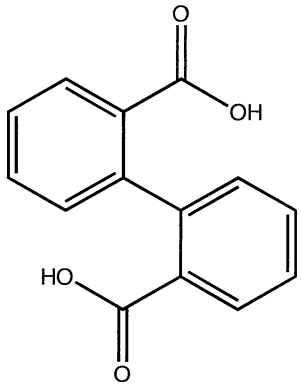
p-xylene-2-sulfonic acid



2-biphenyl carboxylic acid



4′-hydroxy-4-biphenyl carboxylic acid



diphenic acid

Figure 11. Substrate Analogs used in the Inhibition Study.

Polymerase Chain Reaction (PCR)

PCR

PCR was used to introduce two different mutations into pGEX-4T-3/A3H1-R84Q and three different mutations into the pGEX-4T-3/A3H1-TEV plasmids. The two mutations introduced into pGEX-4T-3/A3H1-R84Q were C27S and Y24F. The same two mutations that were introduced into pGEX-4T-3/A3H1-R84Q were also introduced into pGEX-4T-3/A3H1-TEV along with an additional mutation where the serine at position twenty-seven was mutated to an alanine. A QuikChange® II Site-Directed Mutagenesis Kit was utilized to create the mutant plasmids. Horizontal gel electrophoresis was completed using a 0.8% (w/v) agarose gel to verify PCR products. The plasmid DNA, pGEX-4T-3/A3H1-R84Q, is about 6,000 bp in length. The bands at about 4,500 bp indicate supercoiled plasmid DNA PCR product and can be seen in Figure 12 for the pGEX-4T-3/A3H1-R84Q C27S and Y24F mutants. The pGEX-4T-3/A3H1-R84Q containing the C27S and Y24F mutations were transformed into competent XL1-Blue cells and the mutant plasmids were isolated and sequenced. Sequencing verified that the C27S and Y24F mutations were introduced into the plasmid DNA.

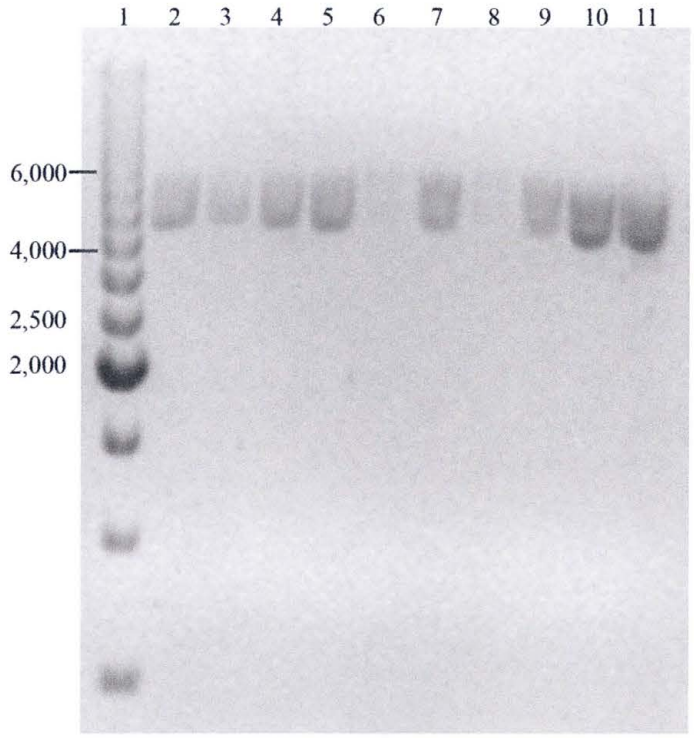


Figure 12. Horizontal gel electrophoresis of pGEX-4T-3/A3H1-R84Q C27S and Y24F mutant PCR products. A 0.8% (w/v) agarose gel was loaded with 10 μL of PCR product and and separated by electrophoresis at 85 volts for 1.5 hours. Lane 1 – 500 bp ladder from Invitrogen, lanes 2-6 – pGEX-4T-3/A3H1-R84Q C27S mutant PCR product and lanes 7-11 – pGEX-4T-2/A3H1-R84Q Y24F mutant PCR product.

The PCR products for the pGEX-4T-3/A3H1-TEV C27S mutants can be seen in Figure 13. The linear plasmid DNA is about 6,000 bp and the supercoiled plasmid DNA will run lower at about 4,000 bp. The PCR products were then transformed into competent XL1-Blue cells. The mutant plasmid DNA was isolated and sequenced. The sequencing verified that the C27S mutation was introduced into three out of the four plasmids.

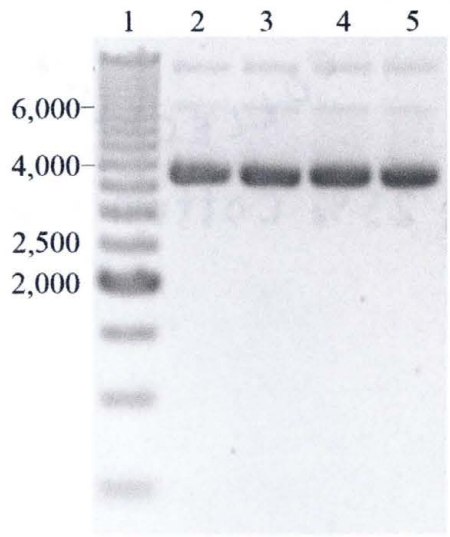


Figure 13. Horizontal gel electrophoresis of pGEX-4T-3/A3H1-TEV C27S mutant PCR products. A 0.8% (w/v) agarose gel was loaded with 10μL of PCR product and 1X DNA buffer. Elecetrophoresis was at 85 volts for 1.5 hours. Lane 1 – 500 bp ladder from Invitrogen, lane 2 – pGEX-4T-3/A3H1-TEV C27S mutant, lane 3 – pGEX-4T-3/A3H1-TEV C27S mutant, lane 4 – pGEX-4T-3/A3H1-TEV wild-type and lane 5 – pGEX-4T-3/A3H1-TEV C27Smutant.

The other two mutations introduced into pGEX-4T-3/A3H1-TEV resulted in the same PCR products as seen in Figures 12 and 13. The plasmids were transformed into XL1-Blue cells and isolated. Sequencing of the samples verified that both the Y24F and C27A mutations were introduced into the plasmid DNA.

CHAPTER V

DISCUSSION

Hydrodesulfurization (HDS) is the current method employed to remove sulfur in fossil fuels. This method uses high temperature (300 to 350°C) and high pressure and is not efficient in removing the sulfur from many organosulfur compounds found as major contaminants in fossil fuel (9,11). Therefore, a new method of desulfurization needs to be implemented to remove the sulfur from these organosulfur contaminants. Biodesulfurization (BDS) is a method that uses enzymes found in natural occurring bacteria to desulfurize the organosulfur compounds. The current research has described purification of an enzyme in the desulfurization pathway from the novel bacteria *Nocardia asteroides* sp. strain A3H1. This pathway has four enzymes: DszA, DszB, DszC and DszD. The enzyme of interest for this project was DszB (HPBS desulfinase), the rate-limiting enzyme in the pathway. This enzyme was placed in a recombinant system and purified, characterized and mutated. The purification, characterization and mutation of HPBS desulfinase R84Q will provide information about the enzyme from the bacterium and also give insight into the mechanism of the desulfinase enzyme.

Co-expression of GroEL, GroES and HPBS desulfinase R84Q and Purification of HPBS desulfinase R84Q

The *dsz*b gene from A3H1 was cloned into the vector, pGEX-4T-3, creating the plasmid pGEX-4T-3/A3H1. The pGEX-4T-3/A3H1 plasmid was transformed into competent BL21/pREP4_GroESL and the gene expression was induced with IPTG. The GST-HPBS desulfinase fusion protein was cleaved by a thrombin protease, which cleaves after the arginine within the thrombin cleavage site (Figure 14).

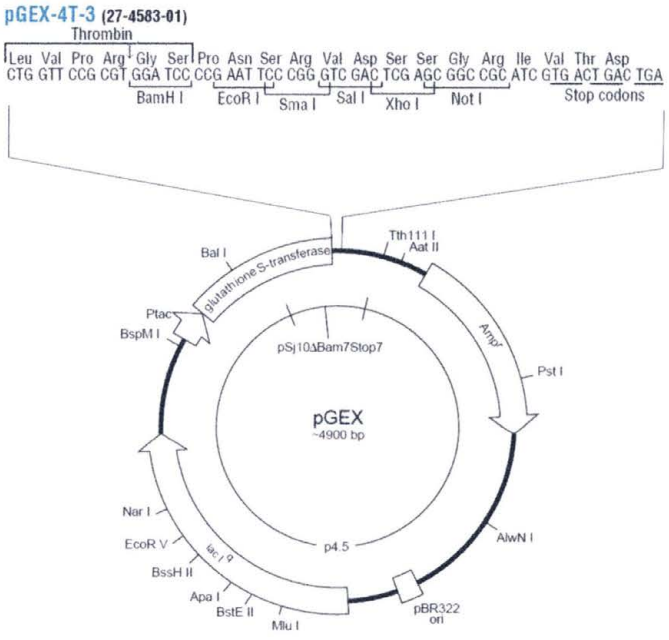


Figure 14. The pGEX-4T-3 Schematic with the thrombin cleavage site.

After the initial purification of the wild-type HPBS desulfinase, the cleavage of the soluble fusion protein resulted in an internal cleavage of HPBS desulfinase. In order

to determine the cleavage site, N-terminal sequencing was used to identify at what position cleavage occurred. Based on the results from the N-terminal sequencing, cleavage occurred after the arginine at the 84th position. Therefore the arginine was mutated to a glutamine using the QuikChange® II Site-Directed Mutagenesis Kit. The R84Q *dsz*b gene from A3H1 was cloned into the pGEX-4T-3 vector creating the pGEX-4T-3/A3H1-R84Q plasmid. The R84Q mutation within the gene sequence later showed resistance to thrombin cleavage and the pGEX-4T-3/A3H1-R84Q plasmid was used in all further experiments (15).

The *dsz*b gene from A3H1 without the R84Q mutation was cloned into the pGEX-4T-3 vector and a new cleavage site was engineered to prevent internal cleavage by the use of the thrombin protease. A TEV cleavage site was engineered into the plasmid construct resulting into the pGEX-4T-3/A3H1-TEV. All the cloning and R84Q mutation experiments were done by Dr. Greg Sawyer from the University of Texas at Austin.

Purification of HPBS desulfinase R84Q was done at 4°C using a glutathione column. The GST-HPBS desulfinase R84Q fusion protein was applied to the column and the column was washed with 1 L of purification buffer 1 overnight. The fusion protein was cleaved with thrombin protease and the cleaved protein was captured in aliquots. The use of the pGEX-4T-3 vector allowed for a one step purification. In all previous studies on desulfinase enzymes, the purification process consisted of several different chromatography steps. For the purification of HPBS desulfinase from the native IGTS8, *Rhodococcus erythropolis* KA2-5-1, *Bacillus subtilis* WU-S2B, and *Paenibacillus* sp. strain A11-2, four to seven different chromatography steps were needed (8, 9, 10). The protein yield for the purification of HPBS desulfinase R84Q was about 1 mg/L. The

protein yield of the DszB enzyme from KA2-5-1 was about 29 mg/L, for the native IGTS8 the protein yield was about 0.08 mg/L (8, 10). The purity of the protein after one chromatography step can be seen in Figure 7. The gel indicated that the protein was relatively pure with the exception of the presence of some bound GroEL and GroES. The small amount of GroEL and GroES still attached to the protein should have little effect on overall enzyme activity, but helps with protein stability. GroEL and GroES are not covalently attached to the protein because the chaperone proteins migrated independently from the HPBS desulfinase R84Q. HPBS desulfinase R84Q requires the presence of the chaperone proteins to facilitate proper folding. In recent studies, when the *dsz*b gene was expressed the protein was insoluble, which resulted in inclusion bodies (10). The *dsz*b gene from KA2-5-1 and WU-S2B was therefore expressed in the presence of the GroEL and GroES chaperone proteins.

Kinetics Assay

The kinetic parameters for HPBS desulfinase R84Q were determined by running a fluorimetric assay that varied the substrate (HPBS) concentration and kept the enzyme concentration fixed. The K_m, V_{max}, and k_{cat} values were determined to be 22.9 ± 8.5 μM, 0.16 ± 0.02 U/mg, and 13.2 min^{-1}, respectively. The native desulfinase enzyme purified from IGTS8 reported by Watkins *et al.* (10) had a K_m, V_{max}, and k_{cat} values of 0.9 ± 0.15 μM, 0.53 ± 0.03 U/mg, and 1.3 ± 0.07 min^{-1}, respectively. Gray *et al.* (11) reported K_m and k_{cat} values of 1 μM and 2 min^{-1}, respectively, for the native IGTS8. The K_m and k_{cat} reported by Nakayama *et al.* for the recombinant desulfinase from KA2-5-1 were 8.2 μM and 0.123 s^{-1}, respectively (8). Konishi and Maruhashi reported K_m and k_{cat} values of 0.33

mM and 0.32 s^{-1}, respectively for the desulfinase from A11-2 (3). The assay conditions for the kinetic assays for Watkins *et al.* and Gray *et al.* were identical to each other and the assay conditions used by Nakayama *et al.* and Konishi and Maruhashi were similar to each other. However to measure HBP formation Nakayama *et al.* used HPLC and Konishi and Maruhasi used GC (3,8,10,11). The assay conditions used to determine the kinetic parameters for HPBS desulfinase R84Q were similar to Watkins *et al.* and Gray *et al.* (10,11). The results indicate that the desulfinase from A3H1 has a much slower turnover rate with the substrate HPBS and a lower affinity for HPBS. This results in a k_{cat}/K_m that was four orders of magnitude smaller for the A3H1-R84Q desulfinase. The recombinant enzymes from *Rhodococcus* have k_{cat}/K_m values between 10^{-2} and 10^{-6} μM/sec.

Temperature Study

The optimum temperature of HPBS desulfinase R84Q was determined to be 30°C. The activity of the enzyme was lower at temperatures below and above the optimum temperature. The temperature optimum reported for the native IGTS8 and KA2-5-1 was 35°C. The optimum temperature for WU-S2B was slightly higher at 37°C and in the Konishi and Maruhashi study the optimum temperature was 55°C. The higher temperature optimum in the Konishi and Maruhashi study was because the desulfinase enzyme was from a thermophilic bacterium, *Paenibacillus* sp. strain A11-2. The desulfinase enzyme in the other studies was from mesophilic bacteria with the exception of WU-S2B, which was also from a thermophilic bacterium.

pH Study

The optimum pH for HPBS desulfinase R84Q was narrow with the optimal determined to be pH 8.0. At pH values lower and higher than the optimum pH the activity of the enzyme was lower. A 1X MTEN buffer was used in the activity assay to determine the optimum pH. The advantage to using one buffer was different pH values could be utilized and buffer effect could be negated. The pH range for the MTEN buffer was from pH 3.0 to pH 9.0 and when a pH above 9.0 was used buffer effects were seen. In previous studies, different buffers were used to achieve the various pH values. Watkins *et al.* used four different buffers each with overlapping buffering ranges. The optimum pH reported in the Watkins *et al.* study for the native IGTS8 was between pH 6.0 to 7.5. The HPBS desulfinase from *Rhodococcus erythropolis* KA2-5-1 had an optimum pH at pH 7.5 (8). The A11-2 HPBS desulfinase had an optimum pH of 8.0 (3) and the WU-S2B HPBS desulfinase has an optimum pH between pH 6.0 – 9.0.

Metal Activation Study

The activity of HPBS desulfinase R84Q was measured in the presence of seven different divalent metals. Colbalt slightly increased enzyme activity by 10%. Calcium, iron, magnesium and manganese significantly increased enzyme activity. Calcium has been reported to help with the stabilization of enzyme structure (10). The increase in enzyme activity could be attributed to the calcium ions associating with the enzyme to aid in stabilizing the enzyme structure. Calcium was also reported to increase enzyme activity in HPBS desulfinase IGTS8 and A11-2 (3, 10). In the Watkins *et al.* study, magnesium and manganese had little or no effect on enzyme activity, while iron

decreased activity. Similar results were reported with magnesium and iron with HPBS desulfinase A11-2. However, manganese inhibited enzyme activity in A11-2. A main trend was reported with copper, which drastically inhibited enzyme activity in HPBS desulfinase R84Q, IGTS8, KA2-5-1, WU-S2B, and A11-2. Only one cysteine residue was found in the amino acid sequence of all HPBS desulfinases. Sulfur has been reported to be a favorable ligand for copper and zinc (10). The dramatic decrease in enzyme activity in the presence of these two metals could indicate the metals are binding to the sulfur of the cysteine residue. Previous chemical modification and protection studies have concluded that the cysteine residue is located at the active site of HPBS desulfinase, therefore resulting in loss of enzyme activity in the presence of copper and zinc (10).

Inhibition Study

HPBS desulfinase R84Q activity was tested in the presence of different substrate analogs along with the substrate (HPBS). HPBS desulfinase R84Q shared no inhibition in the presence of diphenic acid. Some inhibition was measured with *p*-xylene-2-sulfonic acid, 4-cyclohexyl phenol, 1-napthalene sulfonic acid and 2-biphenylcarboxylic acid. For HPBS desulfinase IGTS8, addition of diphenic acid resulted in inhibition and 2-biphenyl carboxylic acid had no inhibition. For both HPBS desulfinase R84Q and IGTS8, addition of 1-napthalene sulfonic acid and 4-hydroxy-4-biphenyl carboxylic acid resulted in inhibition. One substrate analog completely inhibited enzyme activity in A3H1-R84Q, 2,2′-bipyridine-3,3′-diol.

The inhibition of enzyme activity for A3H1-R84Q by 2,2′-bipyridine-3,3′-diol also has some mechanistic implications. This substrate analog has two proton donors. Our

proposed mechanism for HPBS desulfinase is an acid-base mechanism (Figure 4). A cysteine is proposed to act as the base and a tyrosine is proposed to act as the acid. Chemical modification studies and protection studies previously done concluded that there was evidence to support a cysteine, tyrosine, and tryptophan present at the active site (10). The results of the inhibition study using 2-2′-bipyridine-3-3′-diol support the tyrosine acting as the acid due to the complete inhibition of enzyme activity. In a recent study, a crystal structure for HPBS desulfinase from *Rhodococcs erythropolis* was completed and, based on the crystal structure, the cysteine was proposed to act as a nucleophile (16).

Summary

The purification and characterization of HPBS desulfinase R84Q from *Nocardia asteroides* sp. strain A3H1 was completed. The protein was purified in one step on a glutathione column using a GST tag. The molecular weight of the protein was 40 kDa. The K_m, V_{max}, and k_{cat} values were determined to be 22.9 ± 8.5 μM, 0.16 ± 0.02 U/mg, and 13.2 min^{-1}, respectively. The optimum pH and temperature were 8 and 30°C, respectively. The enzyme activity was inhibited in the presence of two metals, copper and zinc. The substrate analog 2,2′-bipyridine-3,3′-diol completely inhibited enzyme activity. Two different mutations, C27S and Y24F, were introduced into the *dsz*b gene sequence of pGEX-4T-3/A3H1-R84Q and pGEX-4T-3/A3H1-TEV, with one additional mutation, C27A, in the latter. The purification and characterization of the mutant proteins will provide a better understanding of the role the cysteine and tyrosine play in the active site mechanism.

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