EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT 2-(2'-HYDROXYPHENYL)BENZENESULFINATE DESULFINASE FROM *RHODOCOCCUS ERYTHROPOLIS* sp. IGTS8

THESIS

Presented to the Graduate Council of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Masters of SCIENCE

By

Leanne T. Harper, B.S.

San Marcos, Texas
August 2011

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ACKNOWLEDGEMENTS

I first would like to thank my family for all their support and encouragement, my husband Brian Harper for his patience and understanding of my persistence in completing my education, my sister Linda Gonzalez whom made returning to school financially possible, and my mother Delia Teneyuque for taking care of my children on those last minute moments I needed to be in the laboratory.

Secondly, I would like to thank Dr. Linette Watkins for accepting me in the graduate program when no one else would consider a full-time working student. She had trust in me to work hard and get this done; better late than never. Last but not least, I would like to give thanks to Desserae Sheptson for giving me the motivation to continue my education.

Most importantly, I am grateful for the serendipitous chance I was given to finish my research and submit my thesis for my graduate degree.

This manuscript was submitted July 1, 2011.

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ABSTRACT

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT 2-(2'-HYDROXYPHENYL)BENZENESULFINATE DESULFINASE FROM *RHODOCOCCUS ERYTHROPOLIS* sp. IGTS8

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Catalytic biodesulfurization uses microbes to remove the heterocyclic sulfur from dibenzothiophene-(DBT) without degrading the hydrocarbon fuel value. *Rhodococcus erythropolis* has been determined to degrade DBT to the final products 2-hydroxybiphenyl and sulfite using four enzymes. The last metabolic step in the pathway is performed by 2-(2'-hydroxyphenyl)benzenesulfinate desulfinase(DszB) and is the rate-limiting step. The gene coding for the DszB from *Rhodococcus erthropolis* IGTS8 was cloned into the expression vector pTAC-MAT-Tag-2 and then transformed into BL21(DE3) containing pREP4-GroESL. The enzyme was successfully expressed and purified using the ProBond^{TM} Nickel column. Quantification showed a yield of 0.10 mg of DszB from 500 g of wet cells. Fluorimetric studies showed optimal activity at 35 ºC and pH range 8-10. The temperature stability range was 25- 35 º C. Kinetic studies show a K_m of 3.75 ± 4.79μM and k_{cat} 0.68 ± 0.15 min^{-1}, respectively. The recombinant HPBS desulfinase showed similar optima temperature and stability as the native enzyme. The

kinetic parameters shared similar values with the native enzyme, within experimental error.

CHAPTER I

INTRODUCTION

Oil is an invaluable resource for the world. However scientists in countries around the globe have been investigating how to reduce the environmental impact of its usage. During the combustion of fossil fuels, emissions such as nitrogen oxides, carbon dioxides and sulfur oxides are a cause of concern. The hydrocarbons and nitrogen oxide gases are contributors to ground-level ozone and the sulfur oxide gases cause acid rain *(1)*. Environmental regulations have been set around the world to reduce the amount of emissions industries and commercial sources can release. Emission rights and operating permits are becoming more restricted and more of a priceless commodity among industrial entities *(2)*. Although emissions rights vary among states and countries, the main objective of all government entities is to reduce the amount of harmful emissions. The ultra-low emission regulations have increased the need of new emission control technologies *(1)*.

In the United States, the acceptable level of sulfur in diesel was first reduced from 2000 ppm to 500 ppm by the Clean Air Act of 1990. In the years that followed, the sulfur emission levels have been reduced even further to 15 ppm in 2006 *(3)*. In the past 20 years, there has been a push by the United States legislature to reduce the amount of

sulfur oxides that are released into the air due to usage of fossil fuels. The main focus has been setting limits on the amount of sulfur oxides released from burning petroleum in the combustion of gasoline in automobiles. Researchers have been developing ways to reduce pollutants when burning fossil fuels like coal and oil.

Hydrodesulfurization (HDS) during oil and gas refining is the conventional method for sulfur removal, but the process is not economically favorable and not energy efficient due to the high pressure and high temperature required *(4, 5)*. Also HDS has limitations since it does not desulfurize the complex polyaromatic sulfur heterocycles (PASHs) commonly found in fossil fuels *(4)*. Current research has looked towards alternative methods of removing sulfur from oil. Biodesulfurization (BDS) is one method under investigation. There are several benefits of using BDS. These benefits include 50 percent lower capital costs and 25 percent lower operating cost than HDS (3). In addition, BDS does not degrade octane in gasoline, and BDS does not require high temperature, pressure, or collateral processes. This results in significantly reduced energy requirements and improved environmental standards at a lower cost *(3)*. Many organisms are known to degrade the PASH dibenzothiophene (DBT), see Figure 1, a model compound found in petroleum products. However, many break down the PASH carbon backbone to carbon dioxide and thus degrade the fuel value *(5)*. In contrast, by using a microorganism like *Rhodococcus erythropolis*, sulfur can be selectively removed without degrading the fuel value. The product 2-hydroxybiphenyl inhibits any further catalytic breakdown of the hydrocarbon backbone *(5)*.

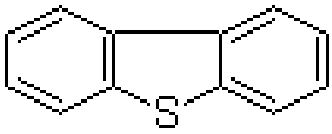

Figure 1. Structure of dibenzothiophene.

BDS research has been directed at 1) the characterization of bacterial strains that use sulfur in thiophenes and benzothiophenes as the primary energy source, 2) the isolation and expression of the biocatalysts that take part in the metabolic pathways, and 3) optimization of their enzymatic activities *(6)*. The first identified organism showed to selectively oxidize sulfur from PASHs was from the *Rhodococcus erythropolis* family. *Rhodococcus erythropolis* D-1(D-1) and *Rhodococcus erythropolis* IGTS8 (IGTS8) are naturally growing organism originally isolated in coal deposits and are the most extensively characterized organism capable of desulfurization. They both use the sulfur from PASHs as a primary source of metabolic sulfur. Both D-1 and IGTS8 use a series of enzymes to convert DBT to 2-hydroxybiphenyl *(7)*. The metabolic pathway has been identified (Figure 2).

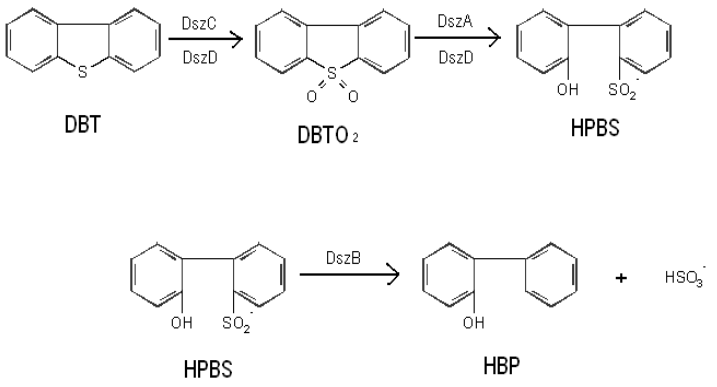

Figure 2. Pathway for oxidative desulfurization of DBT (8).

The first biocatalyst in the pathway is dibenzothiophene monooxygenase which is coded by the *dsz*C gene of D-1 and IGTS8. This enzyme catalyzes the oxidation of dibenzothiophene to form dibenzothiophene sulfone. The second step in the pathway is catalyzed by dibenzothiphene-5-dioxide sulfone monooxygenase, coded by the *dsz*A gene. In this step, DBT-sulfone is oxidized to 2-(2'-hydroxyphenyl)benzenesulfinate (HPBS). Both DszC and DszA require the cofactors FADH and FMN and DszD, an FADH-FMN oxidoreductase that regenerates the coenzymes for the monoxygenases. The last biocatalyst is 2-(2'-hydroxyphenyl)benzenesulfinate desulfinase (HPBS desulfinase) and is coded by the *dsz*B gene. It removes the sulfur from HPBS forming sulfite and 2-hydroxybiphenyl (HBP). HPBS desulfinase does not require cofactors and is considered to be the rate-limiting enzyme *(9)*.

In the Watkins lab, we have previously purified and characterized the aromatic desulfinase, 2-(2'-hydroxyphenyl)benzenesulfinate desulfinase in its native form from *Rhodococcus erythropolis*-IGTS8. This enzyme catalyzes the final step in the

desulfurization pathway of DBT. It was purified 1200-fold from *Rhodococcus erythropolis* sp. strain IGTS8 using 7 steps. The optimal temperature and optimal pH is 35 °C and 7.0. Its K_m is 0.9 ± 0.15 μM and its k_{cat} is 1.3 ± 0.07 min^{-1} *(8)*. Data show that this enzyme's activity is slow relative to the catalytic rate of the other enzymes in the desulfurization pathway. Ongoing research with this enzyme in the Watkins lab is directed at understanding the chemical mechanisms used to remove aromatic sulfur from DBT.

The mechanism of HPBS desulfinase is not known, however two separate mechanisms have been proposed. The first proposed mechanism describes the chemistry as a nucleophilic substitution reaction that results in cleavage of the sulfur-carbon bond and the second proposed mechanism is an acid-base reaction (Figure 4) *(10, 8)*.

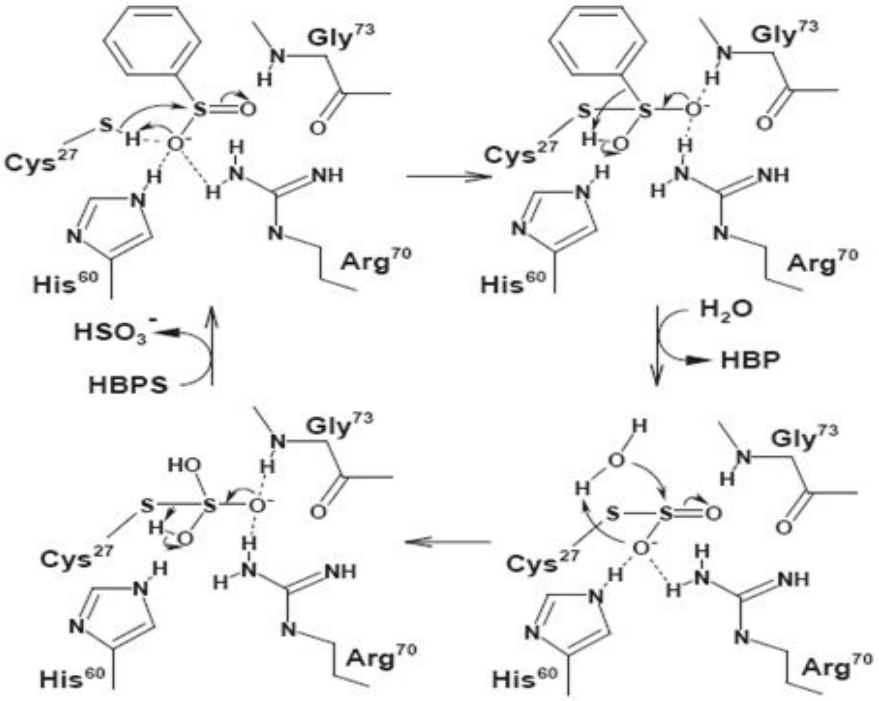

Figure 3. Mechanism for nucleophilic substitution reaction at the active site of HPBS desulfinase proposed by Lee et al. ***(7).***

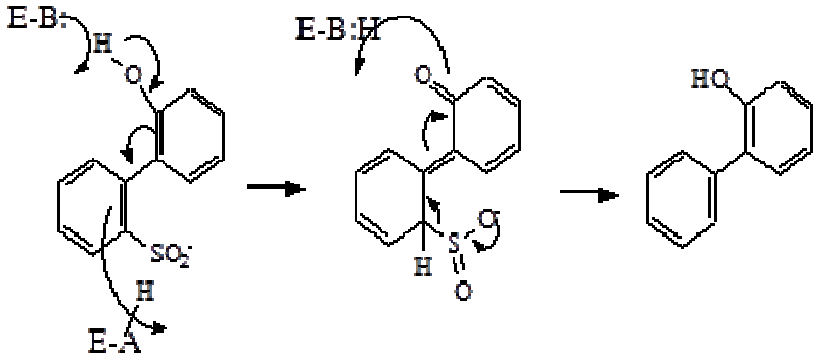

Figure 4. Mechanism for acid-base reaction at the active site of HPBS desulfinase proposed by Watkins et al. ***(8)***

The research presented in this thesis focuses on the expression and characterization of recombinant HPBS desulfinase from *Rhodococcus* strain IGTS8. All other studies of HPBS desulfinase have been carried out with enzymes purified from recombinant vectors expressed in *E. coli*. In all of these studies, the reported kinetic parameters were significantly different than the native HPBS desulfinase *(10, 11)*. For instance, studies on the KA2-5-1-recombinant enzyme reported a K_m of 8.2 μM which is nine times greater than the native value and a k_{cat} value of 7.38 which is a turnover rate that is seven times faster than that of the native *(10)*.

Our lab would like to compare the native HPBS desulfinase characteristics with our own engineered recombinant IGTS8 HPBS desulfinase to better understand the difference in kinetic parameters that have been observed and to establish whether recombinant systems can effectively mimic the native enzyme in mechanistic studies. First an expression system was developed to overexpress the recombinant enzyme in *Escherichia coli* BL21(DE3) cells. The primary reason for using a recombinant expression system is the low yield from the native organism. Earlier studies in our lab found that *R. erythropolis* grows very slowly and the yield of protein was minimal (1 mg /1 kg wet cells). The development of an overexpression system that would not interfere with the solubility, folding, and kinetic properties of the investigated protein, and would allow for a one-step purification of the enzyme was essential to continue our research of HPBS desulfinase.

Secondly, characterization of the recombinant enzyme was carried out to determine if the expression system interfered with or enhanced the HPBS desulfinase activity by comparing the data from the recombinant system to the native enzyme.

The body of research is being presented in order to better understand the catalysis of the HPBS desulfinase enzyme by developing an overexpression system for the enzyme that will provide sufficient quantities for further studies.

CHAPTER II

MATERIALS

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*) 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™-2	The *E. coli* Expression vector with 4X Nickel Metal Affinity Tag (MAT) at the C-terminal (3'end)	Sigma-Aldrich *(12)*
pTAC-MAT/IGTS8	The pTAC-MAT-Tag™-2 vector with the dszb gene from IGTS8	Described herein (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-Bacto™ agar, Bacto™ tryptone, and Bacto™ yeast extract-were from Becton Dickinson (Sparks, MD). The antibiotics used in the media, kanamycin and ampicillin, were from Sigma-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 Hirayama HICLAVE HV-50 autoclave was from Amerex Instruments, Inc. (Lafayette, CA). The Hermle Z180M centrifuge was from Labnet International, Inc. (Edison, NJ).

All plasmid DNAs were 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 polyacrylamide gels and the electrophoresis equipment was from Bio-Rad (Hercules, CA). The Standard heat block and the ACCUPOWER model 300 for running gel electrophoresis were from VWR Scientific (West Chester, PA). The pTAC-MAT-TagTM-2 vector was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lysis was performed using a Sonics Vibra Cell^{TM} Ultrasonic Processor from Sonics & Materials, Inc. (Newton, CT). The ProBond Nickel resin used was from Invitrogen, Inc. (Carlsbad, CA), and the imidazole was from Sigma-Aldrich (St. Louis, MO). Isopropyl β D-1-thiogalactopyranoside (IPTG) was purchased from VWR International, Inc. (St. Louis, MO). 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 SoftMaxRPro 4.7.1 software (Downingtown, PA).

The fluorimetric assays were performed using a Varian Spectrophotometer with the Cary Eclipse software program from Varian, Inc. (Palo Alto, CA). The microplate heater used in conducting the assays was from Zytron (Trenton, NJ). The kinetic parameters of the enzyme were analyzed using the VisualEnzymics program from Softzymics, Inc. (Princeton, NJ).

CHAPTER III

METHODS

Expression system of recombinant IGTS8-DszB

The *dsz*B gene from *R. erythropolis* IGTS8 was cloned into the pTAC-MAT-Tag-2 plasmid (figure 3) using a ligation reaction. The ligation reaction included a 4:1 ratio of dszB gene from IGTS8 and pTAC-MAT expression vector, T4 DNA ligase, and 10 X T4 ligation buffer *(13)*. The pTAC-MAT/IGTS8 plasmid was then transformed into competent cells BL21(DE3) already containing pREP4-GroESL. The pTAC/IGTS8 was sent to University of Texas-Austin ICMB core facilities for sequencing. Sequencing results show gene insertion was successful *(13).*

A 50 ml portion of LB broth containing 40 µg/ml kanamycin and 100 µg/ml ampicillin was inoculated with one colony of the *E. coli* BL21 (DE3) cells containing the pREP4-GroESL and pTAC-MAT-Tag-2/IGTS8. The inoculated culture was incubated overnight at 37 ºC with shaking at 175 rpm. 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, stored at -70 ºC.

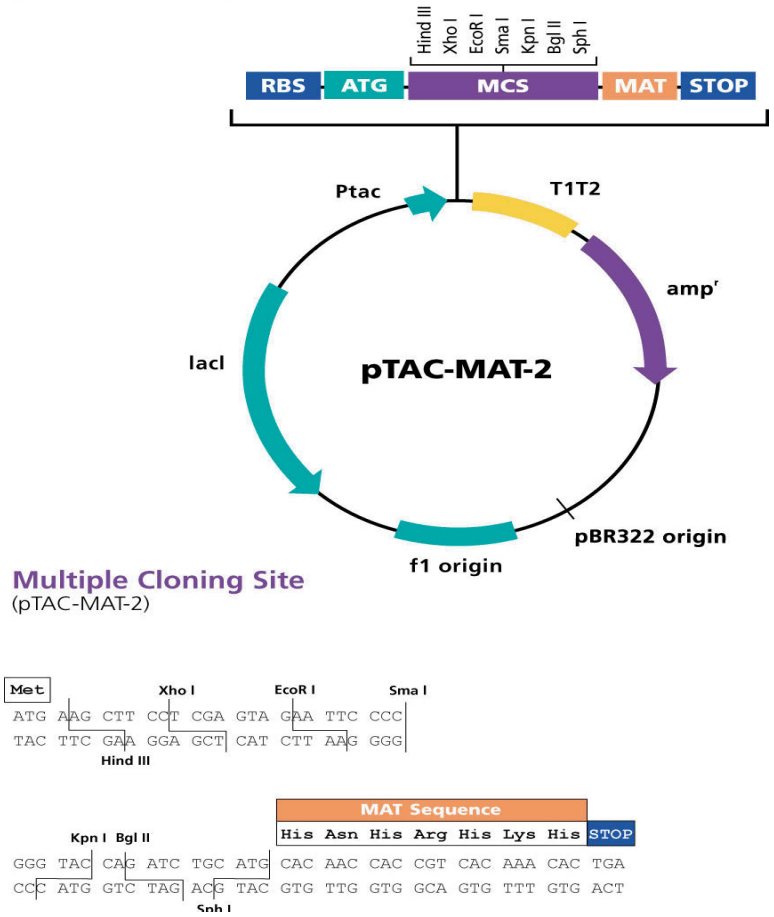

Figure 5. pTAC-MAT-2 purchased from Sigma-Aldrich. The d*sz*b gene of IGTS8 was cloned into the pTAC vector to allow one-step purification using the C-terminal His tag (MAT).

Expression and purification of HPBS desulfinase

Expression of dszb gene

A 50 ml portion of LB broth containing kanamycin (40 µg/ml) and ampicillin (100 µg/ml) was inoculated with one colony of BL21(DE3)/pREP4-GroESL cells transformed with pTAC-MAT-Tag-2/IGTS8. The inoculated culture was incubated overnight at 37 ºC with shaking at 175 rpms. A 5 ml portion of the incubation was used to inoculate 500 ml LB broth, to which 500ul of the 40 µg/ml kanamycin and 100 µg/ml of ampicillin were added. The mixture was then incubated on a rotary shaker at 37°C until the OD_{600} was between 0.800 and 0.870. Gene expression was induced by adding 500 µl of 1 M IPTG with shaking at 30 °C for 20 hours. The cells were collected by centrifugation at 4000 *x g* for 15 minutes at 4°C using an A-10 rotor. The supernatant was discarded and the pelleted cells were suspended in 10 ml of 1X Native Purification Buffer-ProBond Nickel™.

Cell lysis

Following resuspension of the induced cells in 10 ml of 1X Native Purification Buffer-ProBond Nickel™, approximately 20 µg of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was added. In addition to the PMSF, 20 µg of lysozyme was added. The mixture was shaken and then incubated on ice for 30 minutes. The cells were lysed by sonication at 50% amplitude with four 10-second bursts with 10 seconds rest (in ice) between bursts. The lysed cell pellet was obtained by centrifugation of lysate at 12000 *x g* for 30 minutes at 4 ºC. The cell debris was discarded. The volume was recorded and a 50 µl sample of the supernatant was taken for analysis and placed on ice. All samples saved were analyzed by SDS-PAGE.

Purification of HPBS desulfinase

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 *(14)*. The resin was suspended in the lysate solution and binding was initiated by gentle agitation on a rotary shaker for 60 minutes at room temperature (25-27 °C). The resin was then allowed to settle by gravity for 15 minutes at room temperature. The supernatant, which contains the unbound proteins, was gently aspirated using pipets. A 50 µl sample of the supernatant was saved for SDS-PAGE analysis. The column and resin was washed with 8 ml of Native Wash Buffer Nickel ProBond™ (50 mM NaH_2PO_4, 0.5 M NaC1, 50 mM imidazole, pH 6.0. The resin was then settled by gravity for 15 minutes at room temperature. The supernatant aspirated by pipetting and again a 50 µl sample was saved for SDS-PAGE analysis. This washing procedure was repeated three more times. The HPBS desulfinase was eluted off the resin using 10 ml of the Native Elution Buffer from Nickel ProBond Purification™ (50 mM NaH_2PO_4, 0.5 M NaCl, 250 mM imidazole, pH 8.0). The fractions were collected in 1 ml portions in sterile eppendorf tubes and were later analyzed using SDS-PAGE. The aliquots were stored at 4 °C without glycerol, 4 °C with 50% glycerol, -20 °C with 50% glycerol or fast freezed with dry ice and acetone and stored at -70 °C. The column was cleaned using 0.5 M sodium hydroxide and resin was stored with 20 percent ethanol.

Protein concentration

Bradford assay

The Bradford Assay was conducted to determine the protein concentration of the purified HPBS desulfinase. 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 standard concentrations used were 0.0476, 0.0381, 0.0286, 0.0195, 0.0143, 0.0095, and 0.008 mg/ml. A 25% (v/v) Bradford Reagent solution totaling 200 µl was incubated with either 10 µl of standard or protein sample at room temperature for 30 minutes. The absorbance was measured at 595 nm using a SpectraMAX 190 well plate reader from Molecular Devices, with Soft Max R 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 desulfinase samples.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 20 µl of each protein elution sample was thoroughly mixed with 2X SDS buffer (10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue) and1.5 µl of β-mercaptoethanol. The solution was heated at 95 ºC for five minutes, loaded onto a 10% SDS-PAGE gel, and separated by electrophoresis at 125 volts for 80 minutes. The gel was rinsed with a gel-fixing solution (50ml of 95% ethanol, 30 ml of water, 10 ml of acetic acid and adjusted volume to 100 ml) The gel was allowed to soak in the gel-fixing solution for one hour. The gel-fixing solution was then aspirated out by pipetting and replaced with gel-washing solution (30 ml of methanol and 10 ml of acetic acid, and water to a total volume of 100 ml). The gel was washed overnight with gentle agitation at room temperature. Gel washing solution

was aspirated and replaced with Coomassie stain (0.4 g of Coomassie Blue R350 in 200 ml of 40% methanol in water, 200 ml of 20% acetic acid in water) for 4 hours. The stain was aspirated and replaced with destaining solution (50ml of methanol to 30 ml of water, 10 ml of acetic acid, and total volume adjusted with water to 100 ml). The gel was destained for two hours with gentle agitation. The destain was removed by aspiration and the gel was equilibrated with storage solution (25 ml of acetic acid and 400ml of water) for 1 hour. The gel was visualized using the Alpha Innotech RED™ gel documentation system (San Leandro, CA).

HPBS substrate synthesis

The substrate HPBS was synthesized using the method provided by Gregory Mrachko of Energy Biosystems Corporation. Biphenosultine (0.0432 g, 20 mM) was suspended in 10 ml of 44 mM NaOH was reacted overnight with stirring at room temperature.

The pH of the soluble product, HPBS (20 mM), was adjusted to approximately 8.55 with 1 M HCl and stored at -20 °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 desulfinase immediately after elution. The standard assay was done using a 96 well plate with each well containing 1X buffer A (25 mM NaH_2PO_4/Na_2HPO_4, 100 mM NaCl, pH 7.4), 100 µM HPBS, and 0.34 µg of HPBS desulfinase in a total volume of 220 µl. The assay components, except for the substrate, were carefully mixed together and incubated

separately from substrate at 35 °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 2hydroxybiphenyl (HBP) was measured over time using Varian Spectrofluorimeter at 414 nm (excitation was at 280 nm) for 20 minutes measuring each sample every 15 seconds. 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: 0.5, 0.8, 1.0, 2.0, 3.3, 5.0, and 10 µM. The assay was conducted in a 96 well plate. Each well contained HBP, 1X Buffer A (25mM NaH_2PO_4/Na_2HPO_4, 100 mM NaCl, pH 7.4) and ddH_2O to a total volume of 220 µl. The fluorescence of the HBP product was measured using a Varian Spectrofluorimeter monitoring emissions at 414 nm (excitation wavelength was 280 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- $y = 0.1761x + 1.0068$; $R^2 = 0.9908$.

Kinetic studies

A fluorimetric assay was conducted with varying concentrations of the substrate HPBS to determine the kinetic parameters of HPBS desulfinase. The amount of HPBS desulfinase used per assay was 0.4 mg. The HPBS concentrations used were 5, 10, 50, 75, 100, and 125 µM. The assays were conducted in 96 well plates in triplicate. The activity of the HPBS desulfinase was measured using the fluorimetric assays described previously. One unit of activity was defined as 1.0 µM HBP formed per minute. The

concentration of HBP formed was calculated from the fluorescence intensity using the HBP standard curve. Samples were run in triplicates. The data were then fitted to the Michaelis-Menten graph using VisualEnzymatics software from Softzymics (Princeton, NJ).

Determination of temperature optima and stability

Temperature studies were conducted using a Varian Spectrofluorimeter and a Zytron plate heater for temperature control. HPBS desulfinase activity was monitored between 25 – 60 ºC in 5 degree increments. To determine the temperature optima, HPBS desulfinase, assay Buffer A and ddH_2O were mixed and allowed to equilibrate for five minutes at the respective temperature prior to initiation of the reaction with HPBS. To determine the temperature stability of the enzyme, the same reaction mixture was equilibrated at the respective temperature for 30 minutes prior to initiation of the reaction with HPBS. Enzyme activity was measured after addition of HPBS (100 μM). 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 desulfinase was determined by fluorimetric assay for pH values ranging 4.0 - 9.5 in 0.5 pH unit increments. A 10X MTEN buffer containing 500 mM 2-(N-Morpholino)ethanesulfonic acid (MES) hydrate, 250 mM Tris, 250 ethanolamine, and 1 M NaCl was prepared and divided in aliquots. The pH of each aliquot was then adjusted using NaOH and 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 (100 μM). Each assay was run in triplicate. The data

were collected by Varian Spectrofluorimeter. The activity of the enzyme at the respective pH values was plotted using Microsoft Excel.

Storage stability

The recombinant HPBS desulfinase enzyme was stored at various conditions and then periodically assayed to determine ideal storage conditions for optimal activity. The various storage conditions include 4°C, 4°C with 50% glycerol, -20°C with 50% glycerol and fast freezing with dry ice and acetone and storage at -70°C.

CHAPTER IV

RESULTS

Expression of pTAC-MAT/IGTS8 HPBS desulfinase

Several expression systems were developed and unsuccessful in obtaining the wild type recombinant HPBS desulfinase from *R. erythropolis* IGTS8. A pBAD-ThioTOPO expression vector was used but yielded insoluble protein. A second expression vector a pGEX-4T-3 Glutatione S-transferase (GST) fusion vector was also tried, but cleavage of the fusion protein resulted in proteolytically cleaved protein. Creation of a vector that replaced the thrombin cleavage site with a T.E.V. cleavage site resulted in non-expression of protein. A successful expression system was then created by ligating the *dsz*B gene into a pTAC-MAT-Tag-2 vector and co-expressing with the pREP4-GroESL plasmid.

The HPBS desulfinase was co-expressed with a plasmid that also coded for chaperone proteins GroES and GroEL (pREP4-GroESL). These chaperone proteins were necessary to insure correct folding of the recombinant enzyme. IPTG was added to the 500 ml LB stock culture to induce overexpression. Overexpression was observed by SDS-PAGE (Figures 4 and 5).

pTAC-MAT/IGTS8-DszB

The DszB desulfinase was purified using a one-step binding protocol and a ProBond^{TM} nickel metal column as described in the Methods section. Samples taken from two examples purifications (purification A and B) are shown in Figure 6 and 7, respectively. Lane 2 shows an overexpression band for GroEL (66 kDa) below 70 kDa indicating successful induction of cells. In the washings and elutions, lanes 4-10, the GroEL band is reduced. A band is seen in the elutions at approximately 40 kDa, indicating the presence of HPBS desulfinase. GroES (15 kDa) is also observed in Figure 7.

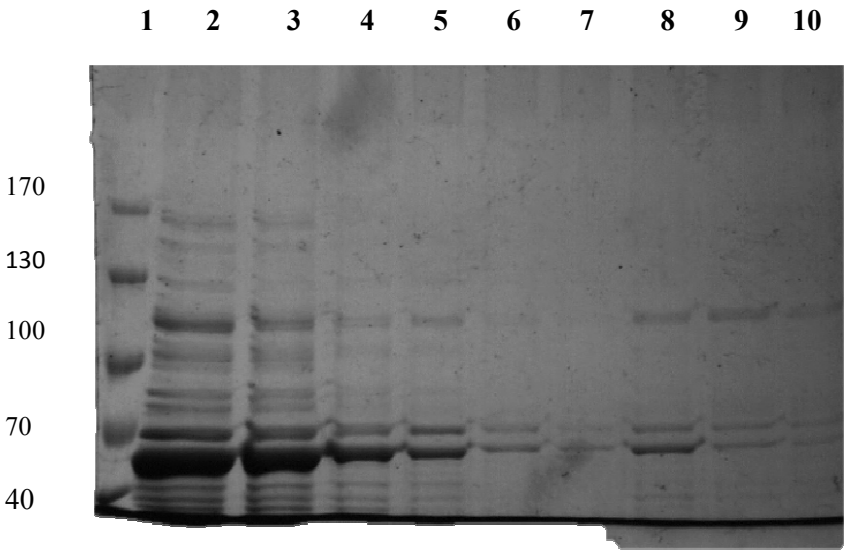

Figure 6. SDS-PAGE analysis of purification A samples of pTAC-MAT/IGTS8-DszB. Reagents and protocols explained in Methods section. Lane 1, PageRuler prestained ladder. Lane 2, protein in cell lysate after sonication of IGTS8 cells. Lane 3 nickel resin with bound lysate. Lane 4, wash of resin column after binding. Lanes 5-10, elutions of protein in 1 ml fraction aliquots.

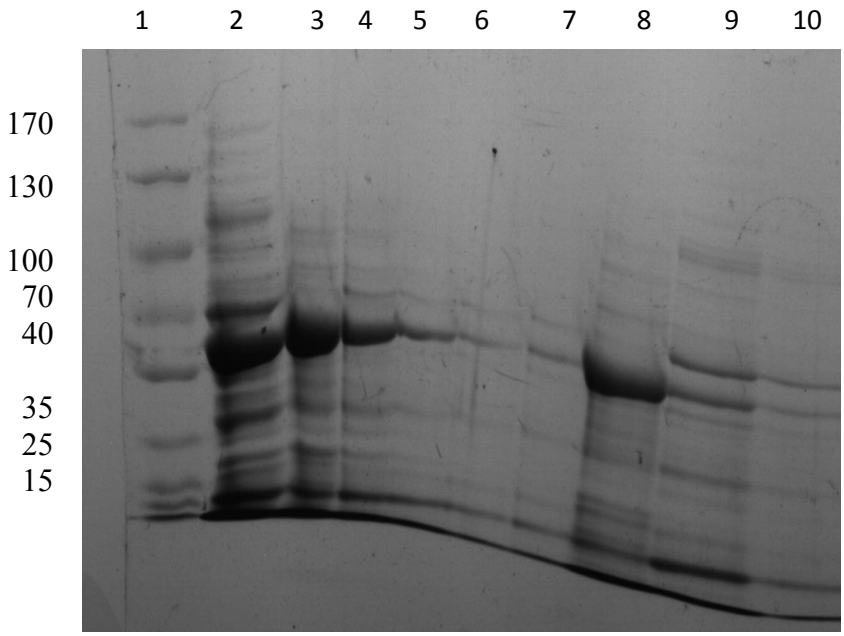

Figure 7. SDS-PAGE analysis of purification B samples of pTAC-MAT/IGTS8-DszB. Reagents and protocols explained in Methods section. Lane 1, PageRuler prestained ladder. Lane 2, protein in cell lysate after sonication of IGTS8 cells. Lane 3, nickel resin with bound lysate. Lanes 4-7, wash of resin column after binding. Lanes 8-10, elutions of protein in 1 ml fraction aliquots.

Protein concentration

Bradford assay

The Bradford assay was used to determine the protein concentration in the elution fractions from purification B. Out of the 500 ml stock culture of *E. coli* BL21 expressing pTAC-MAT/IGTS8, 0.120 mg of HPBS desulfinase was successfully obtained.

Table 2. **Concentration per aliquot of elutions from ProBond™ Nickel column**. Chart showing concentration of recombinant enzyme DszB after purification. Each elution was measured in triplicate. Data in chart represent average protein concentration with standard deviation.

Elution Number Aliquot 1 ml	Concentration mg/ml
1	0.034 ± 0.001
2	0.024± 0.001
3	0.021± 0.002
4	0.017± 0.001
5	0.008± 0.003
6	0.005± 0.002
7-10	≤0.003± 0.001

Fluorimetric assays

Standard activity assay

The enzyme activities of the recombinant IGTS8-DszB preparations were measured from purifications A and B. The standard activity was run according to the procedure previously outlined in the Methods section. The HPBS desulfinase from purification A showed no change in fluorescence over time indicating no formation of HBP (Figure 8A). The HPBS desulfinase from purification B showed an increase in fluorescence over time (ΔF/min) indicating formation of HBP (Figure 8B). Control experiments showed no change in Fluorescence in the absence of substrate or enzyme (Figure 8C).

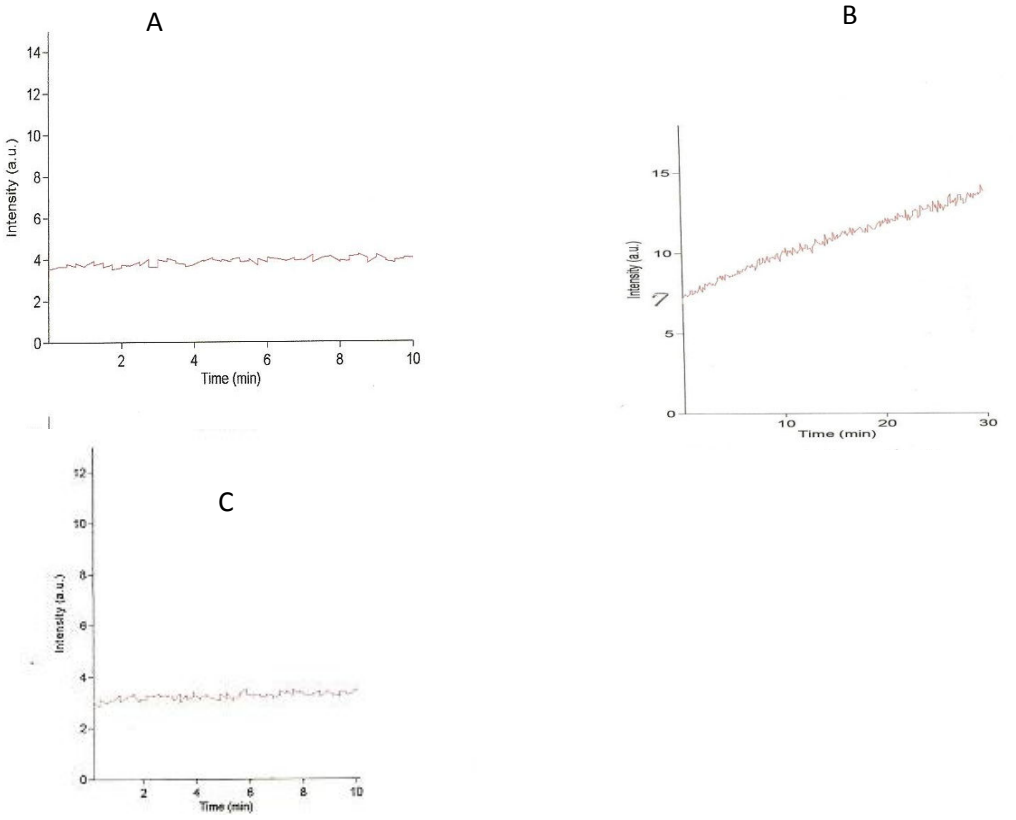

Figure 8. Standard assay graphs of activity of pTAC-MAT/IGTS8-DszB. (A) Standard assay elution from purification A showed no increase intensity over time. (B) Standard assay of elution from purification B showed increased intensity over time. (C) Negative control, no substrate.

Kinetic studies

The kinetic parameters of recombinant HPBS desulfinase were obtained by conducting a fluorimetric assay as described in the Methods section. Fluorescence intensity was measured as a function of time at varying HPBS concentrations. VisualEnzymics from Softzymics, Inc. (Princeton, New Jersey) was used to fit data to the Michaelis-Menten equation.

The K_m and k_{cat} of the recombinant enzyme was determined to be 3.75 ± 4.79µM and 0.68 ± 0.15 min-1, respectively (Figure 9). The kinetic parameters share similarity with other recombinant systems expressing HPBS desulfinase described in other studies, see Table 3.

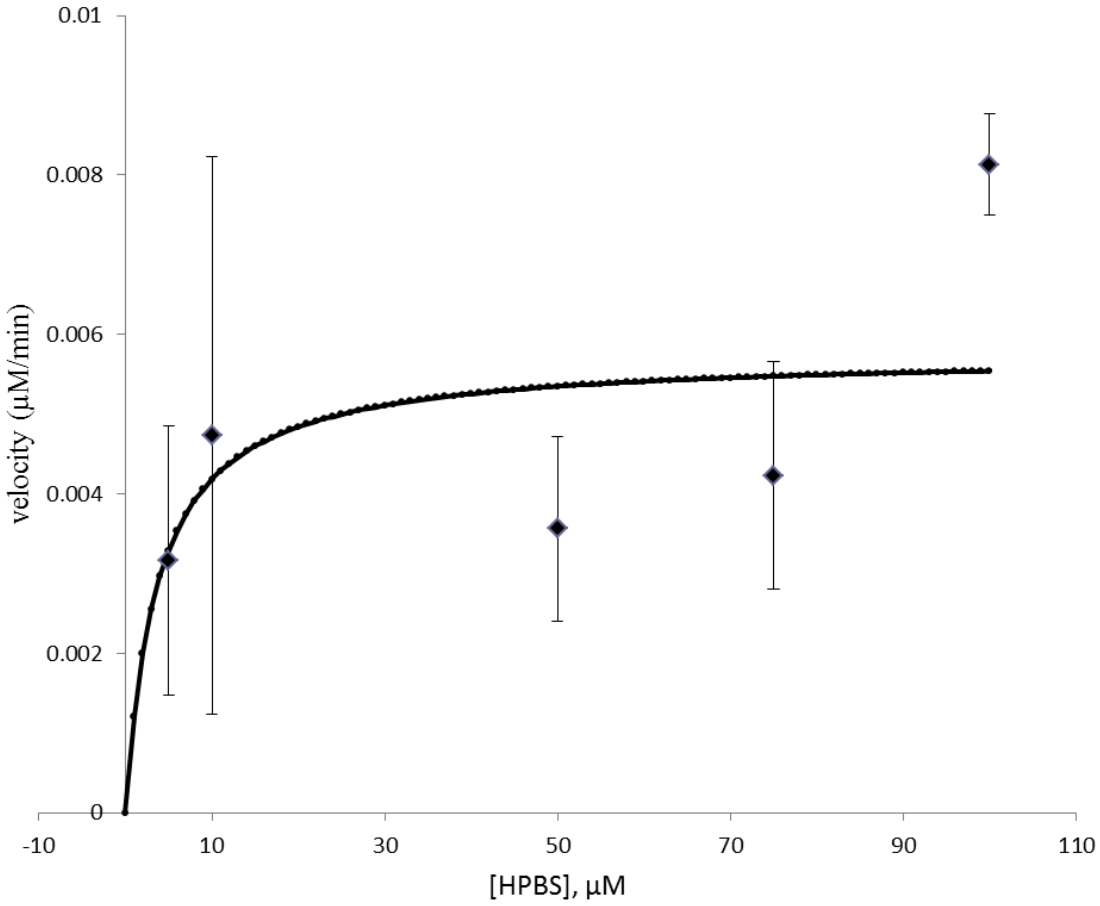

Figure 9. Michaelis-Menten graph for pTAC-MAT/IGTS8-DszB. Formation of HBP per minute at varied substrate HPBS concentration. Data were analyzed and fit to the Michaelis-Menten equation using Visualenzymics (solid line).

Table 3. Comparison of HPBS desulfinase in other studies. A comparison between the kinetic parameters of native DszB and the DszB homologs used in other studies *(15)*.

HPBS Source	**k_{cat} (min^{-1})**	**K_m (µM)**
A3H1- wildtype *(15)*	1.24 ± 0.054	3.15 ± 0.74
A3H1-R84Q-Y24F – recombinant *(15)*	4.33 ± 0.67	51.25 ± 0.96
A3H1-R84Q – recombinant *(16)*	13.1 ± 1.3	22.9 ± 8.9
IGTS8 – native *(8)*	1.3 ± 0.07	0.90 ± 0.15
IGTS8 – native *(9)*	2	1
KA2-5-1- recombinant *(7)*	7.38	8.2
WU-S2B – recombinant *(17)*	-	164
A11-2 – recombinant *(10)*	19.2	0.33

Determination of temperature optima and stability

The HPBS desulfinase activity as a function of temperature was evaluated using the fluorescence assay. The temperature studies were conducted at temperatures ranging from 25 to 60 º C in 5 degree increments. The temperature optimum for recombinant HPBS desulfinase from IGTS8 was 35 º C (Figure 10).

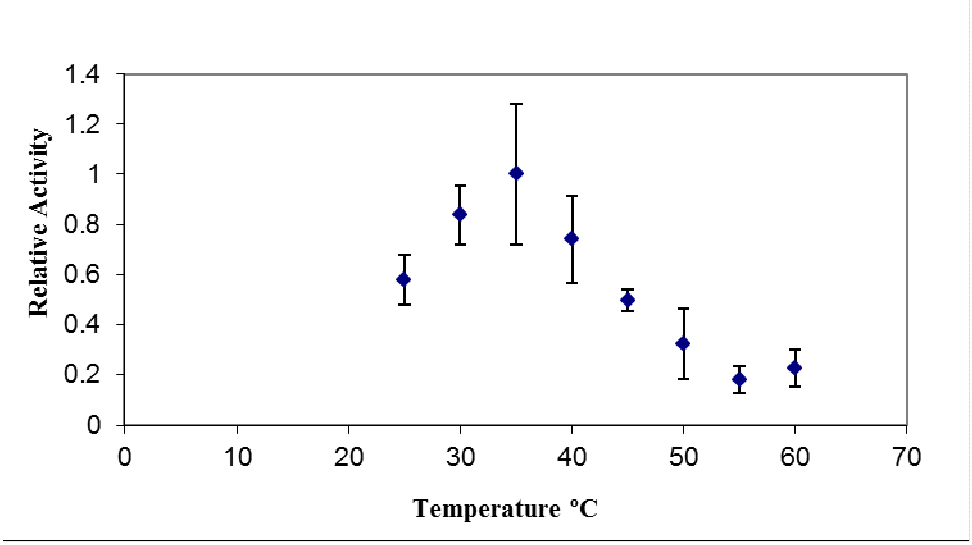

Figure 10. Temperature optimum for pTAC-MAT/IGTS8-DszB. Twenty µl samples of the enzyme were incubated for 5 minutes at the designated temperature. The fluorimetric assay was used to monitor HBP formation from 100 µM HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 35 ° C. Samples were run in triplicate and error bars represent standard deviations.

The temperature stability values were obtained after the enzyme was incubated for 30 minutes at the designated temperature prior to assay initiation. The wild type recombinant HPBS desulfinase was most stable at 30 º C. Enzyme showed activity between 25 and 35 º C. At 40 º C, the enzyme showed a dramatic decrease in activity (Figure 11).

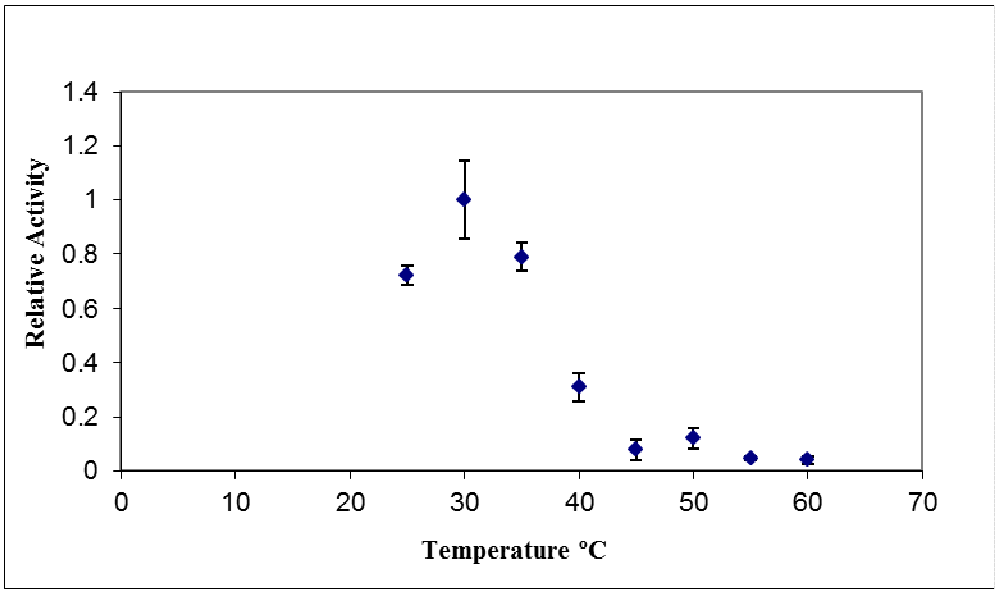

Figure 11. Temperature stability of pTAC-MAT/IGTS8-DszB. Twenty µl samples of enzyme were incubated for 30 minutes at the designated temperature prior to reaction initiation. The fluorimetric assay was used to monitor HBP formation from 100 µM HPBS. The enzyme activity is reported relative to the standard fluorimetric assay with Buffer A at pH 7.4 at 35° C. Samples were run in triplicates. Error bars represent standard deviations.

Determination of pH optima

A determination of the optimum pH for recombinant HPBS desulfinase was attempted 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 pH unit increments. The relative activity of the enzyme at each value was plotted using Microsoft Excel. Data showed a trend for increased activity between pH 8 and 10, but due to high standard deviation no optima pH could be determined (Figure 12).

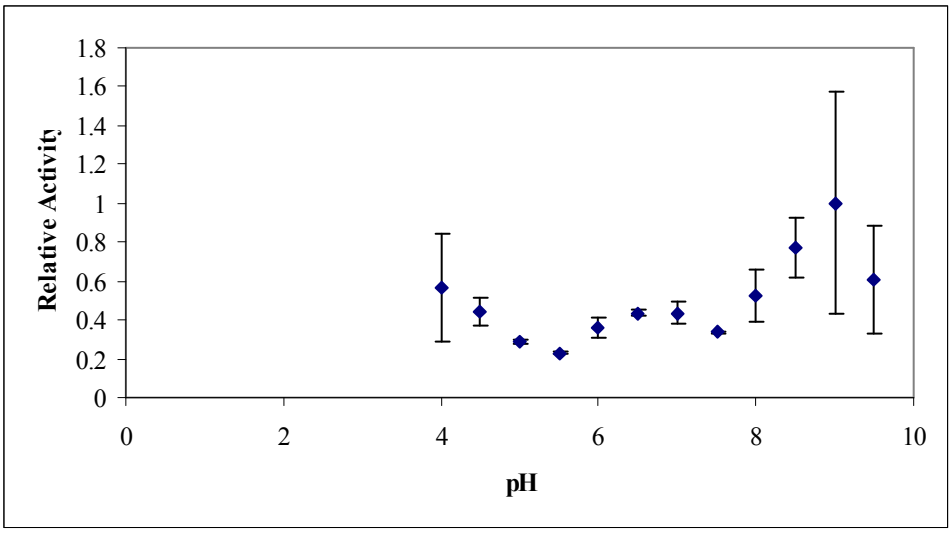

Figure 12. The pH optima for pTAC-MAT/IGTS8-DszB. The enzyme was incubated in MTEN buffer for 5 minutes at a designated pH value ranging from 4 to 9.5. The initial activity of each pH value was obtained by fluorescence and values obtained using Microsoft Excel. Samples were run in triplicates. Error bars represent standard deviations.

Storage stability of pTAC-MAT/IGTS8-DszB

The ability of HPBS desulfinase to retain activity during storage was dependent on storage conditions. Standard assays were performed using parameters described in the methods section to test the activity of the enzyme at various storage conditions every 24 hours. The addition of the 50% sterile glycerol to the recombinant IGTS8-HPBS desulfinase decreased the enzyme activity by half under all conditions (Figure 13). The storage condition that showed optimal retention of activity was 4 °C without glycerol. The remaining activity decreased significantly upon storage for one day at 4 °C with glycerol, at -20 °C with glycerol and -70 °C storage conditions (Table 3).

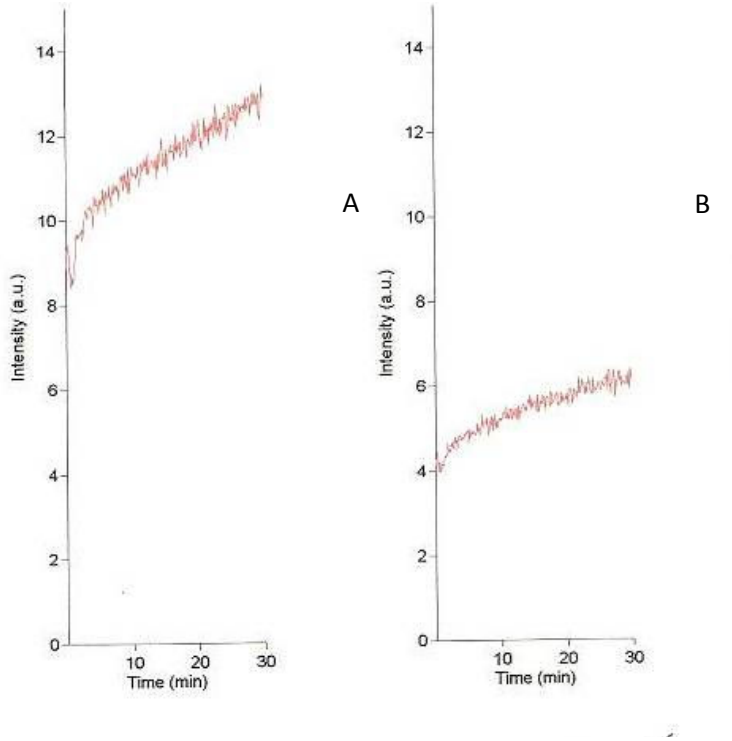

Figure 13. pTAC-MAT/IGTS8-DszB activity from standard assays. (A) Enzyme activity immediately after elution. (B) Enzyme activity immediately after elution with 50% glycerol.

Table 4. Percentage of activity of HPBS desufinase after storage. The enzyme stored at 4 ºC without glycerol showed the highest activity through day 7 after elution. Enzymes stored at other temperatures with glycerol showed significant decrease in activity after day 1. Enzyme stored at -70 ºC showed the lowest activity.

Storage condition	Day 1	Day 5	Day 7	Day 10
4 ºC	90%	70%	40%	<10%
4 ºC + 50% glycerol	60%	30%	30%	<10%
-20 ºC + 50% glycerol	50%	30%	30%	<10%
-70 ºC	30%	<10%	<10%	<10%

CHAPTER IV

DISCUSSION

Expression of pTAC-MAT/IGTS8-DszB

Low yield of the native HPBS desulfinase from *R. erythropolis* IGTS8 has severly limited the number of structural and mechanistic studies possible with the enzyme (8). Thus, the expression of HPBS desulfinase from IGTS8 in a recombinant system is crucial for further studies of desulfurization of DBT. Native HPBS desulfinase from IGTS8 was expressed using pTAC-MAT-Tag-2 plasmid with the IGTS8 *dsz*B gene inserted in the multiple cloning site. The vector contains a polyHis Tag (MAT) at the carboxy terminus to facilitate purification. The enzyme was successfully co-expressed with the pREP4-GroESL plasmid in BL21(DE3). The co-expressed chaperonin proteins Gro EL/ES were necessary for correct folding of the protein and keeping the protein soluble during expression and purification *(10)*. Previous studies done in the Watkins lab and at Energy Biosystems, Inc. have shown difficulty in obtaining soluble protein in absence of chaperonins *(13)*.

Purification of pTAC-MAT/IGTS8-DszB

The ProBond Nickel^{TM} resin column purification system was used to successfully purify the protein in a single step from lysed cells. From one 500 ml culture of cells,

approximately 100 µg of protein was successfully purified using this one-step purification system. In all purifications, SDS-PAGE analysis indicates the presence of three relevant bands in the elution lanes (see Figure 6 and 7). One band, slightly below 70 kDa represents GroEL (66 kDa), one at 40 kDa represents HPBS desulfinase (40 kDa) and one at 15 kDa that represents the GroES (15 kDa) chaperone protein. Analysis of both SDS-PAGE gels provides evidence that the recombinant IGTS8 HPBS desulfinase was co-purified with the chaperonins, GroES and GroEL. This suggest that HPBS desulfinase is unstable and requires the chaperonin proteins to maintain its three dimensional shape. This is supported by data from earlier attempts of purification, where the enzyme was inactive after affinity chromatography. Active purified enzyme was obtained only after careful adjustment of sonication conditions and temperature control during the purification. The presence of GroES and GroEL proteins after purification indicates that the protein mixture may need further processing to isolate the HPBS desulfinase from the chaperonins.

Fluorimetric assays

Standard assays

Fluorimetric assays done on earlier purification attempts including the one shown in Figure 6 indicated that purified enzyme had no catalytic activity. The fluorimetric assays done with the later purification as shown in Figure 7 showed dramatic enzymatic activity, confirming the production of the active HPBS desulfinase from the pTAC/IGTS8 expression vector.

pH studies

A single optimum pH for recombinant HPBS desulfinase could not be determined. MTEN buffer was used in the pH studies to diminish buffer effects seen in other studies with pH variance. The enzymatic activity increased between the pH range of 8 and 10. The enzyme's affinity for the HPBS substrate increased in a more basic environment. The native HPBS desulfinase has a pH optimum of 7 and has a bell shaped pH curve consistent with either an acid-base or nucleophilic substitution mechanism. The basic environment increases both the basicity and nucleophilicity of active site groups including the reactivity of the substrate hydroxide, the active site thiol and any active site water molecule.

Temperature studies

The optimum temperature for the recombinant HPBS desulfinase was 35°C. The enzyme showed relatively high activity between 25ºC and 40°C. Temperatures above 40 °C resulted in decreased activity. The enzyme showed relatively stable up to 40°C with 30° C being the temperature that resulted in optimal activity. Temperatures at 40° and above showed no activity indicating enzyme denaturation and loss stability. The temperature optimum and stability results are similar to those observed for native HPBS desulfinase and other mesophilic HPBS desulfinases purified in recombinant systems *(8, 10)*.

Kinetic studies

The kinetic parameters of the recombinant HPBS desulfinase were performed using fluorimetric assays with substrate HPBS concentration variance and a fixed enzyme

concentration. The substrate concentrations ranged from 5 μM to 125 μM. After fitting the data to the Michaelis-Menten equation using Visualenzymics, the K_m and k_{cat} values were determined to be 3.75 ± 4.79 μM and 0.68 ± 0.15 min^{-1}. The K_m values between the native and the recombinant HPBS desulfinase was similar to that of the native enzyme. The recombinant enzyme Km value of 3.75 is the same as the native value (0.9 μM), within experimental error. The difference in turnover number may be due to the enhanced stability resulting from the presence of the GroES protein or the folding of the recombinant enzyme might have changed the affinity or accessibility of the active site.

Storage stability of pTAC-MAT/IGTS8-DszB

The optimal storage condition for the recombinant IGTS8 HPBS desulfinase was found to be 4°C without glycerol. Glycerol and sucrose are most commonly used for stabilization of enzymes, especially in freezing and thawing samples, but the glycerol used during the HPBS desulfinase storage decreased the enzymatic activity by half. The enzyme stored at 4ºC in the absence of glycerol retained activity for two weeks while all other storage conditions resulted in inactive enzyme in less than 5 days. The glycerol is a polar molecule with hydroxyl groups that pull electrons towards its more electronegative oxygen. The glycerol may be acting as a competitive inhibitor of the substrate or the increased viscosity of the glycerol solution may be inhibiting catalysis or destabilizing the enzyme by interfering with the chaperone interactions.

Conclusion

Expression, purification and characterization of wild type recombinant IGTS8 HPBS desulfinase were accomplished. The data show that the recombinant IGTS8 HPBS

desulfinase shares similar characteristics to the native IGTS8, optimal temperature at 35°C and substrate turnover rate of 0.68 min^{-1}. However the co-expression of the chaperonin GroEL/ES may have affected the strerics surrounding the proposed active site and also the necessity of a basic environment. In short, the characterization studies identified in this study are helpful in future enzymatic studies to understand DszB mechanics. The mechanism for HPBS desulfinase is largely unknown with proposed catalytic mechanisms in question. Site-directed mutagenesis studies with the HPBS desulfinase enzyme can shed more light on the active site residues and help identify the chemistry.

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VITA

Leanne T. Harper was born in San Marcos, Texas on September 8, 1975, the daughter of Clemente and Delia Teneyuque. After completing high school at San Marcos High School, she attended Texas State University-San Marcos in 1994 and graduated with a Bachelor of Science in Microbiology and secondary teaching certification in 1999. The next ten years, Ms. Harper taught science in a variety of education institutions such as San Marcos High School, San Marcos Baptist Academy, New Braunfels Nancy Ney charter School, and lectured for the department of Biology at Texas State University. In 2003, she served as a science academic advisor for Texas State University-San Marcos. In 2005, she entered the biochemistry graduate program at Texas State. Two years later, Ms. Harper left the graduate program to raise her family and re-entered the program in the spring of 2011. Ms. Harper will continue to pursue a career in science education and plans to apply and attend the University of Texas Graduate School of Biochemical Science at Houston and enter the graduate program in molecular carcinogenesis.

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