ACTIVE SITE INVESTIGATION AND AFFINITY PURIFICATION OF 2-(2'HYDROXYPHENYL)BENZENESULFINATE DESULFINASE

THESIS

Presented to the Graduate Council of

Southwest Texas State University

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

By

Melissa Kay Cody, B.S.

San Marcos, TX

May 2002

ACKNOWLEDGEMENTS

I would like to express tremendous thanks to Dr. Linette Watkins, my graduate advisor, for her unique support and understanding of my quirks and individual challenges. She has been a teacher, a mentor, and a disciplinarian.

I have no words to express my love and appreciation for my husband, Kyle, who has put up with many long nights alone while I was in the lab. He has listened to my frustrations and my successes with patience and understanding. I love you and thank you with all my heart. I couldn't have done it without you.

This manuscript was submitted on April 4, 2002.

TABLE OF CONTENTS

FIGURES

TABLES

CHAPTER I

INTRODUCTION

Enzymatic carbon-sulfur bond cleavage reactions are uncommon and not very well understood. 2-(2'-Hydroxyphenyl)benzenesulfinate desulfinase (HPBS desulfinase) is a bacterial enzyme which cleaves aromatic sulfur from petroleum products. The goal of initial characterization studies on HPBS desulfinase was ultimately to improve industrial biodesulfurization in petroleum products, yet, understanding of carbon-sulfur bond cleavage reactions has other applications including usage in both the pharmaceutical and agricultural industries. The focus of this project was to better understand the catalytic mechanism of HPBS desulfinase in order to use it as a model for future studies on desulfinase enzymes.

HPBS desulfinase is the third enzyme in the three step desulfurization pathway of *Rhodococcus erythropolis* sp. IGTS8, a Gram-positive bacteria originally found in soil near coal deposits. (1) There are two known methods of bacterial desulfurization. The first method degrades the carbon backbone utilizing both the carbon and the sulfur. The second method oxidizes the sulfur atom to form a sulfone and then cleaves both carbon-sulfur bonds, releasing

inorganic sulfite. In this sulfur specific degradation pathway, the microorganisms utilize sulfite for cell growth, but leave the caloric value of the petroleum substrates relatively unchanged. (2) *Rhodococcus erythropolis* sp. IGTS8 utilizes the second, sulfur specific, degradation pathway. Over sixty percent, by weight, of the sulfur found in crude oil is in the form of dibenzothiophene (DBT) (figure 1) and its derivatives. These heterocycles resist cleavage of sulfur by chemical methods such as hydrodesulfurization, so DBT is used as a model compound for the study of biodesulfurization processes. (3)

There are four proteins involved in the desulfurization of DBT by *Rhodococcus erythropolis* sp. IGTS8. The pathway is shown in figure 2. The first enzyme is dibenzothiophene monooxygenase coded by the dszC gene. This enzyme catalyzes the oxidation of DBT to DBT sulfone and it requires both an NADH-FMN oxidoreductase and flavins to maintain activity. (4) The second enzyme also requires these cofactors and converts the DBT sulfone to 2-(2'-hydroxyphenyl)benzenesulfinate (HPBS). This enzyme is dibenzothiophene-5, 5-dioxide sulfone monooxygenase and it is coded by the dszA gene. (5) HPBS desulfinase, coded by the dszB gene (figure 3), is the final enzyme in the pathway. It catalyzes the removal of sulfur from HPBS and the release of 2-hydroxybiphenyl (HBP). HPBS desulfinase does not require any cofactor for activity and is believed to be the rate limiting step in the pathway. The fourth enzyme, DszD is the NADH-FMN oxidoreductase required by the monooxygenases and it requires FMN for activity. (6)

Although enzymes catalyzing carbon-sulfur bond cleavage reactions are not well understood, there are a few that have been characterized with limited mechanistic studies. The majority of these enzymes require pyridoxal phosphate (PLP) as a cofactor. One dominant example of one of these enzymes is cysteine desulfurase(I). In 1994, Zheng et al described cysteine desulfurase(I), a NIFS gene product, which catalyzes the removal of sulfur from L-cysteine to produce L-alanine. The published cysteine desulfurase(I) mechanism proposes the formation of a cysteine-PLP ketimine adduct followed by nucleophilic attack on the cysteine in the substrate by the thiolate anion of cysteine 325 (7). Although several enzymes have proposed a role for an active site cysteine, this is the only published mechanism for these enzymes.

Enzymatic carbon-sulfur bond cleavage reactions have pharmaceutical, agricultural, and industrial applications. Allinase is an enzyme isolated from garlic which catalyzes the cleavage of the carbon sulfur bond in alliin to form allylsulfenic acid which spontaneously forms allicin (8). Allicin is believed to be the active ingredient in garlic for use in herbal treatment of infectious disease as well as for prevention of stroke and arteriosclerosis. These herbal treatments are being investigated for pharmaceutical applications. For garlic treatments to be effective there must be active allinase present (8). Studies are being conducted to ensure that allinase still has activity in processed garlic for bottling or long-term storage.

Cystine lyase, an enzyme widely studied for its agricultural applications, is responsible for off-aroma deterioration in fresh, unblanched broccoli. (9) The

enzyme catalyzes the elimination reaction of L-cystine to give thiocysteine, pyruvate, and ammonia. (10) This same activity has also been found in other members of the *Brassica* genus such as mustard and cabbage. It has been proposed to use cystine lyase as a blanching indicator to improve the quality of frozen vegetables. (11)

The initial characterization of HPBS desulfinase has been completed. The K_m and V_{max} are 0.90 μM and 2.6 μmol $hour^{-1}$ mg^{-1}. Isoelectric focusing was used to estimate a pI of 5.5. The optimum pH is 7.0 and optimum temperature is 35°C. Product inhibition studies were completed using HBP and sulfite as inhibitors; both showed no measurable inhibition. Product analogs were also analyzed. Only 2,2'-biphenyl (figure 4) showed significant inhibition with a K_i of 16.5 μM. Metal ions were also analyzed for their effect on enzyme activity. Cu^{2+} and Zn^{2+} showed an inhibitory effect, while Ca^{2+} slightly enhanced the rate. Several chemical chelating agents were also studied. The compounds 1,10-phenanthroline; 2,2 dipyridol; and 8-hydroxyquinoline acted as inhibitors while EDTA showed no effect on activity. The inhibition observed is believed to be due to the structure of the chelating agents and not their chelating ability because non chelating analogs of these chelators showed the same inhibitory affect.

The research presented in this thesis focuses on two areas of research involving HPBS desulfinase. The first involves an investigation into the active site structure and mechanism of the enzyme. The active site structure was investigated using chemical modification reagents specific for particular amino acid residues. (12) Earlier pH studies in our lab determined the pK_a of groups

essential for enzyme activity. At neutral pH, there is a basic group with a pK_a around 6 and an acid group with a pK_a around 9. An acid base mechanism, supported by this data, was developed for the enzyme. (figure 5) The basic residue abstracts a proton from the hydroxyl group on HPBS; electron density is disturbed throughout the ring system; and a proton is abstracted from the acidic residue. Inhibitor studies suggest that these catalytic residues are located on the same side of the enzyme active site. An alternative mechanism for HPBS desulfinase is a nucleophilic mechanism. (figure 6) Both mechanisms were also investigated in this study using solvent isotope effect studies.

The second portion of this research focuses on the development of an expression system for affinity purification and mutation of the enzyme. The wild type and site directed mutant genes were amplified using the polymerase chain reaction. Both chemical and enzymatic methods were employed to ligate the dszB gene into a cloning vector for expression in *E. coli.*

This body of research is being presented in order to better understand the mechanism of enzymatic carbon-sulfur bond cleavage reactions. Pharmaceuticals based on the herbal benefits of garlic and agricultural advances such as better frozen broccoli could also result from a better understanding of this class of enzyme. Industrial biodesulfurization of fossil fuels could also potentially be improved by increasing the rate of this reaction.

CHAPTER II

MATERIALS

Rhodococcus erythropolis sp. IGTS8 in a lyophilized form was purchased from the American Type Culture Center (ATCC) (Manassas, Virginia). Biphenosultine was provided by Dr. Herbert L. Holland from Brock University. Difco Bactoagar, nutrient broth, bacto-tryptone, and yeast extract were purchased from Difco Laboratories (Detroit, Michigan). Difco is now sold by Fisher Scientific (Pittsburg, Pennsylvania). The Q-Sepharose Fast Flow anion exchange media and the Superdex 75 gel filtration media were purchased from Pharmacia (Pisctaway, New Jersey). The Toyopearl-phenyl 650M hydrophobic interaction media was purchased from Supelco (Bellefonte, Pennsylvania). The P-6DG desalting/buffer exchange media, the Macro-Prep Type-1 ceramic hydroxyapatite media and all columns and fittings for FPLC were purchased from Bio-Rad Laboratories (Hercules, California). All other chemicals and reagents used were reagent grade or better and were purchased from Aldrich Chemical

Company (Milwaukee, Wisconsin), Fisher Scientific (Pittsburgh, Pennsylvania), or Sigma Chemical Company (St. Louis, Missouri).

Reagents were sterilized in a HICLAVE HV-50 autoclave from Amerex Instruments Inc. (Lafayette, California). Sterile conditions were provided for bench work by a Laminar Flow Hood model NU-201-430 from NUAIRE (Plymouth, Minnesota). Cells were both shaken and incubated in a Gyromax Orbital Incubator Shaker from Amerex Instruments Inc. (Lafayette, California). Enzyme and other reagents were concentrated and filtered using Centricon YM-10 and YM-30 centrifugal filters from Millipore Corporation (Bedford, Massachusetts).

All Novex gel electrophoresis reagents, gels, and equipment were purchased from Invitrogen (Carlsbad, California). A Novex XCELL-11 electrophoresis module was used for polyacrylamide gel electrophoresis. The Novex Mark 12 molecular weight standard contains: myosin (200, 000), beta-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,300), glutamic dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000) trypsin inhibitor (21,500), lysozyme (14,400), and aprotin (6,000).

Agarose gel electrophoresis was performed using agarose purchased from Invitrogen, Life Technologies (Carlsbad, California). The power supply for gels was an ACCU P O W E R model 300 power supply from VWR Scientific. Gels were horizontal and were run in a multi-sub cell GT apparatus from Bio-Rad Laboratories (Hercules, California). A λ DNA HIND III restriction digest was used

as a high molecular weight standard and a 100 base pair marker was used as a low molecular weight standard. Both were purchased from New England Biolabs (Beverly, Massachusetts).

A Beckman DU-7400 UV spectrophotometer was used for all ultraviolet-visible determinations. A Perkin Elmer Luminescence spectrophotometer LS50-B was used for all fluorimetric assays. A Gilson FPLC system consisting of a FC203B fraction collector, two Minipulus3 peristaltic pumps, and a 112 UV/VIS detector was used for all column chromatography experiments. A Beckman J2-21 centrifuge was used for all macroscale centrifugation. A HERMLE Z180 M centrifuge from National Labnet Co. (Woodbridge, NJ) was used for all microscale centrifugation. Lyophilization was done in a Freeze Dry System, Freezone model 4.5 from Labconco Co. (Kansas City, Missouri).

Restriction Enzymes, the IMPACT CN affinity purification system and cloning system, and *E. Coli* strain ER2566 were purchased from New England Biolabs (Beverly, Massachusetts). Wizard DNA purification kits were purchased from Promega (Madison, Wisconsin). Primers were purchased from Integrated DNA Technologies Inc. (Coralville, Indiana). The dszB gene was a gift from Enchira Biotechnology Corporation (The Woodlands, Texas). The gene was received in a pBADTOPO vector. pBADTOPO, TA, and pBAD-Thio/TOPO cloning and expression systems. Taq Polymerase, ProBond purification resin, and *E. coli* strains One Shot Top-10 and Bl-21 were purchased from Invitrogen (Carlsbad, California). PCR was performed in a Gene Amp PCR system model 2400 from Applied Biosystems (Foster City, California). Electroporation was

done in an Electro Cell Manipulator 600 from BTX, a division of Genetronics Inc. (San Diego, California). The cuvettes were Electroporation Cuvettes Plus model #620 also from BTX.

Software utilized for data analysis included Enzfitter version 2.014.0 from Biosoft (Cambridge, United Kingdom), and Microsoft Excel. Software used to run FPLC was Gilson Unipoint Software version 1.71. The FLWinLab program version 3.00 from Perkin Elmer was used to operate the spectrafluorimeter.

CHAPTER III

METHODS

Standard Fluorescence Assay

The standard assay was used for all activity measurements for HPBS desulfinase. Water,10x buffer A (250 mM NaH_2PO_4, 1 M NaCl, pH 7.4), and HPBS were mixed in a 96 well plate and incubated on a plate warmer to 35°C. The reaction was initiated with the addition of enzyme. Total volume, enzyme concentration and HPBS concentration were dependent on the particular study. Product formation was then monitored over time using a Perkin Elmer LS50B Luminescence spectrophotometer by measuring the increase in fluorescence, at 414 nM (λ_{exc} = 288 nM). A standard curve for HBP fluorescence was used to calculate initial velocity from fluorescence data. The specific activity was then calculated using velocity and enzyme concentration and reported in units/mg. A unit is defined as one μmol of HBP produced per hour. This assay was developed based on a procedure outlined by Gray. (1)

HBP Standard Curve

A 10 mM stock solution of HBP was prepared with 0.016 g of HBP and 10 mL of H_2O. The solution was heated gently while stirring to allow HBP to go into solution. Dilutions of HBP were prepared in a 96 well plate, total volume 200 μL, at the following concentrations: 50, 40, 30, 20, 10, 5, 3, 1, 0.1, and 0.05 μM. The dilutions were then measured for fluorescence using a Perkin Elmer LS50B Luminescence spectrophotometer at 414 nM (λ_{exc} = 288 nM). Values for intensity were plotted in a standard curve against HBP concentration and fit to a best fit line by Excel to give fluorescence = 21.1(μM HBP). (R^2 = 0.9921)

Substrate (HPBS) Synthesis

Biphenosultine (0.0432 g, 20 mM), a gift from Dr. Herbert L. Holland of Brock University, was suspended in 10 mL of 44 mM NaOH and stirred overnight. The soluble product was adjusted to pH between 8 and 9 with HCl. The resulting 20 mM HPBS solution was divided into 1 mL aliquots and stored at -20°C. The synthesis of HPBS is based on a method provided by Gregory Mrachko of Enchira Biotechnology, formerly Energy Biosystems Corporation.

Cell Growth

Rhodococcus cells were propagated on nutrient agar plates by re-streaking every two to three days and incubating plates at 30°C. Overnight tubes containing 5 mL of nutrient media were inoculated and incubated with shaking for 48 hours. The cells were pelleted by centrifugation at 7000 x g for 5 minutes.

They were then washed twice with a sterile minimal salt solution (4 g/L of KH_2PO_4 and 3 g/L of NH_4NO_3, pH 7.3) and re-pelleted. The cells were then inoculated into 250 mL of Basal Salt Media 2 (BMS2). BMS2 contained the following per liter: KH_2PO_4, 4 g; NH_4NO_3, 3 g; $MgCl_2\,4H_2O$, 50 mg; $Na_2B_4O_7\,10H_2O$, 0.02mg; $CuCl_2$, 0.02 mg; $CoCl_2\,6H_2O$, 1 mg; $ZnCl_2$, 0.08 mg; $FeCl_3\,6H_{2O}$, 0.4 mg; $CaCl_2$, 20 mg; $MoCl_2$, 0.1 mg; and $2NiCO_3.Ni(OH)_2\,4H_2O$, 0.005 mg (pH 7.3). BMS2 was prepared with nutrients and the sulfur source (200 μM DBT) just prior to inoculation. The media was suspended with cells and incubated with shaking (200 rpm) at 30°C for five days, until late log phase. The cells were harvested by centrifugation at 10,000 rpm in a Beckman J2-21 centrifuge for one hour, washed twice with minimal salts, and centrifuged again. The cell pellet was stored at -70°C All solutions and nutrients were either autoclaved or sterile filtered and stored at 4°C. The method described above was based on a procedure by Li (13).

Protein Measurements

UV

Protein concentrations were measured by the method developed by Kalb and Bernlohr using the following equation: μg/mL protein = $183(A_{230}) - 75.8(A_{260})$. (14) Absorbance at 230 and 260 nM was measured in 1 mL quartz cuvettes on a Beckman DU 7400 UV-Vis spectrophotometer. Samples were diluted until absorbances were less than 1.000 AU.

Fluorescamine

Protein concentrations were also measured using a fluorescamine assay. Bovine Serum Albumin (BSA) was serially diluted from 0 to 1000 μg/mL using 25 mM sodium-phosphate buffer pH 7.4. Aliquots (150 mL) of BSA solutions or unknown protein samples were placed into individual wells in a 96 well plate. Fluorescamine (50 μL of 1.08 μM) was added to each well and the plate was shaken for one minute. The fluorescence was then measured in the Perkin Elmer Luminescence spectraphotometer at 460 mM. (λexc. = 400 nm) A standard curve was constructed from the BSA standard data and fit to a best fit line using Excel, and the unknown protein concentration was determined from that standard curve.

HPBS Desulfinase Purification

Cell Lysis

Rhodococcus cells stored at -70°C were thawed at 4°C and resuspended in ice cold Buffer A containing: 0.2 mg/mL DNAse, 0.5 mM dithiothreitol (DTT), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). The resulting solution was passed through a Stansted Cell Disrupter. The cell lysis was carried out at 16-18,000 psi. The lysis solution was collected on ice and transferred to a centrifuge where cell debris and unbroken cells were removed by centrifugation

at 10,000 rpm for 90 minutes at 4°C. The supernate was collected, labeled "cell lyse", and immediately transferred to the first column. The pellet was discarded.

FPLC

The FPLC procedures were performed on a Gilson FPLC system at 4°C. All column eluants were monitored continuously using a Gilson UV-Vis detector at 280 nm. After each column was completed collected fractions were assayed for activity using the standard assay with 100 μL of enzyme fraction and 91 μM HPBS. Fractions showing activity were pooled to apply to the next column. After each group of fractions was pooled and mixed, a 1.0 mL sample was saved for further analysis.

Anion Exchange Chromatography

A 300 mL Q-sepharose Fast Flow anion exchange column was equilibrated with 10 column volumes of Buffer A. The crude cell extract was loaded at 4.5 mL /minute. After non-binding protein was washed through, bound protein bond protein was eluted using a salt gradient from 100 mM to 450 mM NaCl. Fractions were collected every 1.5 minutes during the gradient. The column was washed of any bound protein with 1.5 M NaCl and re-equilibrated with Buffer A.

Desalting Chromatography

A 150 mL P-6DG desalting column was equilibrated with 10 column volumes of 25 mM sodium phosphate buffer pH 7.4. Pooled Q-sepharose fractions were loaded at a flow rate of 1.5 mL/minute. The column was washed with 25 mM sodium phosphate buffer pH 7.4 and fractions were collected every 2 minutes until the protein absorbance on the chromatogram returned to baseline. The column was re-equilibrated with 25 mM sodium phosphate buffer pH 7.4.

Hydroxyapatite Chromatography

A 25 mL Macro-Prep Type 1 ceramic hydroxyapatite column was equilibrated with five column volumes of 25 mM sodium phosphate buffer pH 7. Pooled P-6DG fractions were loaded at a flow rate of 2.0 ml /minute. The column was washed with 25 mM sodium phosphate buffer pH 7 and fractions were collected every 2 minutes until the protein absorbance on the chromatogram returned to baseline. The column was washed free of any bound protein with 400 mM potassium phosphate buffer pH 7.4 and re-equilibrated with 25 mM sodium phosphate buffer pH 7.4.

Hydrophobic Interaction Chromatography

A 50 mL Toyopearl-phenyl650M column was equilibrated with 10 column volumes of 1.7 M $(NH_4)_2SO_4$ (pH 6.6). Pooled hydroxyapatite fractions were brought to 1.7 M $(NH_4)_2SO_4$ and loaded at a flow rate of 3.0 mL/minute. After non-binding protein was washed through, bound protein was eluted using a

decreasing salt gradient from 1.7 M $(NH_4)_2SO_4$ to 0 M $(NH_4)_2SO_4$, Buffer A. Fractions were collected every minute during the gradient. The column was washed with Buffer A and re-equilibrated with 1.7 M $(NH_4)_2SO_4$ (pH 6.6).

Sample Concentration

Pooled fractions from the Toyopearl column were concentrated in 2 mL Centricon YM-10 centrifuge filter devices. The sample was centrifuged at 3,500 rpm at 4°C until sample volume was below 3.0 mL. Both the filtrate and retentate were assayed for activity and the retentate was applied to the next column.

Size Exclusion Chromatography

A 300 mL Superdex 75 gel filtration column was equilibrated with 10 column volumes of buffer B (25 mM sodium phosphate, 200 mM NaCl, pH 7.4). The concentrated protein sample was loaded onto the top of the gel bed with a pipette. The sample was allowed to gravity load onto the column. An equal volume of buffer was also gravity loaded onto the column. Buffer was slowly returned to the column and it was attached to the FPLC system. The column was run with buffer B beginning with a flow rate of 2.0 mL/minute and stepping down over 30.0 minutes until a flow rate of 0.4 mL/minute was achieved. Fractions were collected every two minutes over the next 570 minutes. The column was re-equilibrated with buffer B.

Protein Storage

Superdex fractions showing activity were pooled and allowed to equilibrate. The sample was then divided into 0.5, 1.0, and 2.0 mL aliquots and frozen at -70°C. Each vial was labeled with the volume of protein, the protein concentration, and the preparation date.

Gel Electrophoresis

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were prepared using ~10 μg of protein, 4x lithium dodecylsulfate (LDS) sample buffer (1.09 M glycerol, 141 mM Tris-Base, 106 mM Tris-HCl, 73 mM LDS, 0.51 mM EDTA, 0.22 mM Serva Blue G-250, and 0.175 mM phenol red), and 2% β-mercaptoethanol. The samples were then denatured by heating at 90°C for 10 minutes. The samples and a Mark12 protein molecular weight standard were loaded onto a pre-cast NuPAGE 4-12% bis-Tris polyacrylamide gel. The gel was run at 200 volts constant voltage for 35 minutes in MES-SDS running buffer (500 mM 2-(*N*-morpholino)ethane sulfonic acid, 50 mM Tris-base, 3.5 mm SDS, 1 mM EDTA, pH 7.3). The gel was removed from the cartridge and fixed with 50% methanol, 7% acetic acid solution for 10 minutes. The gel was then stained overnight using Colloidal Blue stain. The stain was removed and the gel was de-stained with water.

Agarose Gel Electrophoresis

A 50 mL, 0.8% agarose gel was prepared using 0.4 g agarose and 50 mL 1x TAE buffer. 50x TAE buffer prepared using: 242 g Tris-base, 57.1 mL glacial Acetic Acid, 100 mL 0.5 M EDTA (pH 8.0), and 84 mL H_2O and diluted with H_2O to 1x. (15) The gel solution was heated in a microwave on high setting for 30 seconds three times, swirling after each. The gel was then poured into a mold and allowed to set. The mold was then placed into a horizontal gel electrophoresis apparatus and surrounded by 1xTAE buffer. The gel was loaded with samples prepared in 6x dye buffer (40% sucrose, 0.25% bromophenyl blue, 0.25% xylene cyanol FF, and H_2O to 10 mL) (15). A λ DNA – *Hind* III digest (3 μL digest, 5 μL 6x dye, and 21 μL TE buffer) was used as a molecular weight standard for 2,027 to 23,130 base pairs. TE buffer contains: 0.12 g Tris, 0.03 g EDTA, H2O to 100 mL, pH 8.0 (15). A 100 base pair marker (3 μL marker, 5 μL 6x dye, and 21 μL TE buffer) was used as a molecular weight standard for 100 to 2,200 base pairs. The gel was electrophoresed at 70 volts for 30 to 90 minutes. After electrophoresis the gel was stained with an ethidium bromide solution (25 uL of 10 mM ethidium bromide in 250 mL of H_2O).

Kinetic Studies

The kinetic parameters K_m and V_{max} and K_{cat} for HPBS desulfinase were determined by running the standard fluorescence assay using varied concentrations of substrate, HPBS. The following concentrations of HPBS were evaluated: 0.25 μM, 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM, and

128 μM. HPBS desulfinase concentration in each assay was 268 μg/mL. Data was averaged over three trials and fitted to substrate inhibition equations using EnzFitter version 2.014.0.

Chemical Modification Studies

Cysteine Modification by 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB)

A 10 mM stock solution of DTNB was prepared using 0.0396 g of DTNB in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to prepare 120.0 μM, 12.0 μM, 1.2 μM, 0.12μM, and 0.012 μM solutions for use in the modification reactions. HPBS desulfinase (90 μg/mL) was concentrated using Centricon YM-10 centrifugal filter devices in a Beckman centrifuge. Concentrated enzyme (60 μL) was mixed gently with 5 μL of H_2O and warmed to 35°C. DTNB (5 μL) was added to the reaction to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 5 or 10 minute time intervals and remaining activity monitored using the standard fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. The reaction was repeated with DTNB reaction concentrations of 10 μM, 1.0 μM, 0.1 μM, 0.01 μM, and 0.001 μM. Final enzyme concentration in the assay varied from 0.214 to 0.429 μg/mL. Relative activity for HPBS desulfinase was determined from the average initial velocity over several trials. The relative activity and DTNB reaction concentrations were used to determine k_i for DTNB modification.

Tryptophan Modification by N-bromosuccinimide (NBS)

A 10 mM stock solution of NBS was prepared using 0.178 g of NBS in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to prepare 120.0 μM, 12.0 μM, 1.2 μM, 0.12 μM, and 0.012 μM solutions for use in the modification reactions. Enzyme (60 μL of 90 μg/mL) was mixed gently with 5 μL of H_2O and incubated at 35°C. NBS (5 μL) was added to the reaction tube to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 5 or 10 minute time intervals and remaining activity monitored using the standard fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. The reaction was repeated with NBS reaction concentrations of 10 μM, 1.0 μM, 0.1 μM, 0.01 μM, and 0.001 μM. Final enzyme concentration in the assay was 0.107 μg/mL. Relative activity for HPBS desulfinase was determined from the average initial velocity over several trials. The relative activity and NBS reaction concentrations were used to determine k_i for NBS modification.

Tyrosine Modification by tetranitromethane (TNM)

A 10 mM stock solution of TNM was prepared using 9 μL of TNM in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to prepare 120.0 μM, 12.0 μM, 1.2 μM, 0.12 μM, and 0.012 μM solutions for use in the modification reactions. Enzyme (60 μL of 90 μg/mL) was gently mixed with 5 μL of H_2O and incubated at 35°C. TNM (5 μL) was added to the reaction tube to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 5 or 10 minute time intervals and remaining activity monitored using the standard

fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. The reaction was repeated with TNM reaction concentrations of 10 μM, 1.0 μM, 0.1 μM, 0.01 μM, and 0.001 μM. Final enzyme concentration in the assay was 0.107 μg/mL. Relative activity for HPBS desulfinase was determined from the average initial velocity over several trials. The relative activity and TNM reaction concentrations were used to determine k_i for TNM modification.

Chemical Modification Protection Studies

2,2'-biphenol Protection of Cysteine Modification

A 10 mM stock solution of 2,2'-biphenol was prepared using 0.0186 g of 2,2'-biphenol in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to a concentration of 600 μM. Enzyme (60 μL of 90 μg/mL) was mixed gently with 5 μL of 600 μM 2,2'-biphenol and incubated at 35°C. After one minute 5 μL of 12.0 μM DTNB was added to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 10 minute time intervals and remaining activity monitored using the standard fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. Final enzyme concentration in the assay was 0.107 μg/mL. Relative activity for HPBS desulfinase was determined from the average initial velocity over several trials. These values were used to determine k_{obs} for protected DTNB modification at 1.0 μM.

2,2'-biphenol Protection of Tryptophan Modification

A 10 mM stock solution of 2,2'-biphenol was prepared using 0.0186 g of 2,2'-biphenol in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to a concentration of 600 uM. Enzyme (60 μL of 90 μg/mL was mixed gently with 5 μL of 600 μM 2,2'-biphenol and incubated to 35°C. After one minute 5 μL of 12.0 μM NBS was added to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 10 minute time intervals and remaining activity monitored using the standard fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. Final enzyme concentration in the assay was 0.107 μg/mL. Relative activity for HPBS desulfinase was determined from the average initial velocity over several trials. These values were used to determine k_{obs} for protected NBS modification at 1.0 μM.

2,2'-biphenol Protection of Tyrosine Modification

A 10 mM stock solution of 2,2'-biphenol was prepared using 0.0186 g of 2,2'-biphenol in 10 mL of ethanol. The stock solution was diluted with distilled H_2O to a concentration of 600 uM. Enzyme (60 μL of 90 μg/mL) was mixed gently with 5 μL of 600 μM 2,2'-biphenol and incubated to 35°C. After one minute 5 μL of 12.0 μM TNM was added to initiate modification. Aliquots (10 μL) of the reaction mixture were removed at 10 minute time intervals and remaining activity monitored using the standard fluorescence assay with 8 μM HPBS in a total assay volume of 200 μL. Final enzyme concentration in the assay was 0.107 μg/mL. Relative activity for HPBS desulfinase was determined from the

average initial velocity over several trials. These values were used to determine k_{obs} for protected TNM modification at 0.1 μM.

Solvent Isotope Effects

A 1 mL sample of HPBS desulfinase (90 μg/mL) and a 1.5 mL sample of 100 μM HPBS were frozen at -70°C. The samples were then lyophilized in a Labconco freeze dry system Freezone 4.5. The enzyme was re-suspended in 500 μL of deuterium oxide (D_2O) for a final concentration of 180 μg/mL. The HPBS was re-suspended in 500 μL of D_2O for a final concentration of 300 μM. Activity at three enzyme concentrations, 0.226, 0.643, and 1.29 μM in D_2O was monitored using the standard fluorescence assay with 10x D_2O buffer A (pH 7.0, pD 7.4) (16), 15 μM HPBS in D_2O, and a total assay volume of 200 μL. A duplicate standard assay was run using H_2O and unexchanged enzyme and substrate in the same concentrations. The exchange process was repeated twice, each time re-suspending the enzyme and substrate in enough D_2O to match the sample concentrations in the first exchange. The standard assay was repeated after the second and third exchange. Specific activity was calculated from the initial velocity.

Primer Design

The published dszB gene sequence (figure 3) was used to design PCR primers for cloning and mutagenesis. Three forward primers were designed. One to produce a native gene with no tags or changes, one with a SAPI

restriction site incorporated onto the 5' end, and one with the cysteine residue at amino acid position 27 changed to an alanine and the KPN I restriction site at base pair 88 removed. Two reverse primers were designed. One with an *ECO RI* restriction site incorporated onto the 3'end and one to produce a native gene with no tags or changes. The primers were checked on Oligoanalyzer (web address: http://207.32.43.43.248/oligocalc.asp, web site: www.IDTDNA.com) for the GC content and melting temperature to ensure that the primers would be compatible under identical PCR conditions.

PCR

The PCR for dszB gene was optimized to the following conditions. The following reagents were mixed on ice: 50 ng template, 1.5 μL of DMSO, 1 μL of 10 mM dNTP mix, 1.25 μL of 10 μM forward primer, 1.25 μL of 10 μM reverse primer, 0.5 μL of Taq polymerase, and 5 μL of 10x PCR buffer (supplied with Taq). These reagents were heated to 94°C in a PCR machine and incubated for 5 minutes, a "hot start". The run was paused and the following reagents added: 5 μL of 25 mM MgCl and H_2O to 50 μL. The run was then continued for 35 cycles: 94°C for 1 minute, 45°C to 65°C (dependent on the appropriate primer melting temperature) for 1 minute, and 72°C for 1 minute. The reaction was then held at 72°C for 10 minutes to finish extention on any incomplete DNA chains. The reaction products were analyzed by agarose gel electrophoresis and stored at -20°C.

Restriction Digests

All restriction digests were performed under similar conditions. Each reaction contained: restriction enzyme, 10x buffer (appropriate to the enzyme), DNA, and sterile H_2O. The reactions were incubated in a H_2O bath for 1 hour at 37°C. The reaction products were analyzed by agarose gel electrophoresis.

Cloning of DszB for Affinity Purification

IMPACT CN

The IMPACT vector pTYB11 (17) (figure 7) was double digested using both *Sap* I and *Eco* RI restriction enzymes. The digested product was separated from the reaction mixture by agarose gel electrophoresis followed by purification using a Wizard DNA prep kit. The PCR product of the dszB gene (insert) was produced using primers LMW3 and LMW4 designed to incorporate a *SAP* I site onto the 5' end and an *Eco* RI site onto the 3' end of the gene. This product was digested using both *Sap* I and *Eco* RI restriction enzymes and purified directly from the reaction using a Wizard DNA prep kit. The vector and insert were mixed in ratios of 1:1, 3:1, and 1:3 with 1 μL ligase and H_2O and incubated at 4°C overnight. The ligation reaction product (2 μL) was mixed with *E. coli* ER2566 cells (40 μL) and transformed with 2.5 kV of electricity in an electroporation cuvette. SOC media (200 mL) was added to the cells and they were incubated at 37oC for 1 hour. SOC media was prepared using: 950 mL H_2O, 20 g bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, and 10 mL of 250 mM KCl, pH to 7.0,

volume to 1 L with H_2O, autoclave, add 2 mL of sterile 1 M Glucose (15). The cells were then plated onto Luria-Bertani Media (LB) plates containing 100 μg/mL ampicillin. LB (1 liter) contains: 950 mL H2O, 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 10 g NaCl, pH to 7.0 with 3 M NaOH (15). Colonies were chosen and grown in culture tubes containing 5 mL LB broth and 100 μg/mL ampicillin. DNA was isolated using a Wizard DNA prep kit and analyzed by agarose gel electrophoresis.

pBADTHIO-TOPO Cloning

The PCR product was ligated into the pBadThio-Topo vector (18) (figure 9) using the Topo cloning reaction (19) (figure 8). 4 μL PCR product, 1 μL Topo salt solution (1.2 M NaCl, and 0.06 M $MgCl_2$) and 1μL Topo vector were mixed and incubated at room temperature for 5 minutes. The reaction mixture was then placed on ice until the transformation reaction was performed. The ligated vector and insert were transformed into One Shot TOP10 *E. coli* by gently mixing 2 μL of the Topo cloning reaction product with one vial of cells and incubating on ice for 30 minutes. The cells were then heat shocked for 30 seconds in a 42°C H_2O bath. The tubes were transferred back to ice and 250 μL of room temperature SOC media added. The tubes were then shaken (200 rpm) horizontally at 37°C for one hour. Cells were then plated on a LB plate containing 100 μg/mL ampicillin. Colonies were chosen and grown in culture tubes containing 5 mL LB broth and 100 μg/mL ampicillin. DNA was isolated using a Wizard DNA prep kit and analyzed by agarose gel electrophoresis, PCR, and restriction digests.

ProBond Affinity Purification

E. coli cells containing Topo cloned plasmids were grown for three days in 250 mL cultures of LB media containing 100 μg/mL ampicillin. Cells were harvested by centrifugation, resuspended in 15 mL of native buffer (50 mM sodium phosphate, 0.5 M NaCl pH 8.0), and lysed in a Stansted cell disruptor at 4-6,000 psi. The lysate was centrifuged at 3,000 rpm for 15 minutes. The supernatant was loaded onto a 5 mL ProBond nickel chelating column (20). The column was gently shaken at 4°C for one hour to allow for protein binding. The column was connected to the FPLC system and monitored by UV as before. The column was then washed with 200 mL of native wash buffer (0.02 M Imidazole, 50 mM sodium phosphate, 0.5 M NaCl pH 8.0). Native wash buffer was replaced with native elution buffer (0.25 M Imidazole, 50 mM sodium phosphate, 0.5 M NaCl pH 8.0). The column was washed with 75 mL of native elution buffer. Fractions were collected during the wash every five minutes and every 2 minutes during the elution. Fractions collected during elution were assayed for activity using the standard assay.

CHAPTER IV

RESULTS

HPBS protein purification

A typical run of the purification protocol resulted in a 300 to 500 fold purification with a specific activity of 2-2.5 U/mg. A purification table for HPBS desulfinase from 109.8 g of wet cells is shown in table 1. A SDS gel of protein fractions from purification steps is shown in figure 10. The figure clearly shows that the product of the final gel filtration column produces one intense band on the gel indicating that it is pure.

Kinetic Studies

Data was fit to the substrate inhibition equation using Enzfitter. A Michelis-Menton plot is shown in figure 11. K_m was calculated to be 0.75 μM. V_{max} was calculated to be 2.61 U/mg. The k_{cat} was calculated to be 1.83/minute. These values, along with error and R^2 values, are shown in table 2.

Chemical Modification Studies

Cysteine Modification by 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB)

The natural log of residual activity was plotted at each DTNB reaction concentration. Using equation 1 (table 3), the k_{obs} was calculated (12). The data is shown in figure 12. The values for k_{obs} were 0.1054/min, 0.3307/min, 0.3598/min, 0.4536/min, and 0.839/min for 0.001 μM, 0.01 μM, 0.1 μM, 1.0 μM, and 10.0 μM DTNB, respectively. Using equation 2 (table 3), the k_i for DTNB was calculated to be 0.0481 μM^{-1} min^{-1} (12) (figure 13).

Tryptophan Modification by N-bromosuccinimide (NBS)

The natural log of residual activity was plotted at each NBS reaction concentration. Using equation 1, the k_{obs} was calculated. The data is shown in figure 14. The values for k_{obs} were 0.1034/min, 0.1512/min, 0.1784/min, 0.2634/min, and 0.4945/min for 0.001 μM, 0.01 μM, 0.1 μM, 1.0 μM, and 10.0 μM NBS, respectively. Using equation 2, k_i for NBS was calculated to be 0.0352 μM^{-1} min^{-1} (figure 15).

Tyrosine Modification by tetranitromethane (TNM)

The natural log of residual activity was plotted at each TNM reaction concentration. Using equation 1, the k_{obs} was calculated. (figure 16) The values for k_{obs} were 0.0861/min, 0.4195/min, 0.5191/min, 1.7618/min, and 8.7076/min

for 0.001 μM, 0.01 μM, 0.1 μM. 1.0 μM, and 10.0 μM TNM, respectively. Using equation 2, the k_i for TNM was calculated to be 0.8301 μM^{-1} min^{-1} (figure 17).

Chemical Modification Protection Studies

2,2'-Biphenol Protection of Cysteine Modification

The natural log of residual activity was plotted against time for the protected reaction and unprotected reaction at 1.0 μM DTNB (figure 18). Using equation 1, the k_{obs} was calculated. The k_{obs} for the protected DTNB modification reaction was 0.2372/min. The k_{obs} for the unprotected DTNB modification reaction was 0.4536/min.

2,2'-Biphenol Protection of Tryptophan Modification

The natural log of residual activity was plotted against time for the protected reaction and unprotected reaction at 1.0 μM NBS (figure 19). Using equation 1, the k_{obs} was calculated. The k_{obs} for the protected NBS modification reaction was 0.1371/min. The k_{obs} for the unprotected NBS modification reaction was 0.2634/min.

2,2'-Biphenol Protection of Tyrosine Modification

The natural log of residual activity was plotted against time for the protected reaction and unprotected reaction at 1.0 μM TNM (figure 20). Using equation 1, the k_{obs} was calculated. The k_{obs} for the protected TNM modification

reaction was 0.1918/min. The k_{obs} for the unprotected TNM modification reaction was 0.5191/min.

Solvent Isotope Effects

Initial velocity data for both native enzyme and enzyme exchanged into D_2O was plotted on the same graph with specific activities indicated for each line (figure 21). The specific activity values were 2.001 U/mg, 0.451 U/mg, 0.339 U/mg, and 0.181 U/mg for native, first exchange, second exchange, and third exchange reactions respectively. The V^H/V^D was 4.43 (21). (calculated using native and first exchange initial velocity values)

Primers

Forward primer LMW3 and reverse primer LMW4 were designed with a *SAP I* site in the forward direction and an *ECO RI* site in the reverse direction for use with the IMPACT CN cloning system. The plasmid produced when the PCR product from these two primers was ligated into a pBAD-THIO/TOPO vector was named pThioTag(clone number). All primers are shown in Table 4.

Forward primer LMWMC1 and reverse primer LMWMC2 were designed with a mutation in the underlined position (Table 4) to mutate cysteine 27 to an alanine. There is also a silent mutation (in red-Table 4) at base 84, changing a G to an A to remove the *KPN I* restriction site at base pair 88. The plasmid produced when the PCR product from these two primers was ligated into a pBAD-THIO/TOPO vector was named pC27A(clone number).

Forward primer LMW6F and reverse primer LMWMC3 were designed to produce a native dszB gene with no changes or flanking sequences. The plasmid produced when the PCR product from these two primers was ligated into a pBAD-THIO/TOPO vector was named pBThio(clone number).

PCR

PCR reactions were successful when 2% and 3% DMSO was added to the reactions and the reactions were initiated with a hot start. The products were analyzed by agarose gel electrophoresis and a ~1,100 base pair band was seen in the 2% DMSO reaction lane as well as the 3% DMSO reaction lane, however more product was formed with 3% DMSO (figure 22). Magnesium concentration variations from 1.5 mM to 2.5 mM did not show any significant variation in product formation.

IMPACT CN cloning

Moderate numbers of colonies (thirty to fifty per plate) were produced from the plated transformed cells. Over four trials, 93 colonies were screened and none contained a plasmid that consisted of vector and insert. Positive controls were run using whole uncut pTYB11 under identical reaction conditions. The plated cells produced large numbers of colonies almost indistinguishable from each other. Negative controls were also run by leaving the insert out of the ligation reaction. The plated cells produced no colonies in the first two trials, and three to five colonies per plate in the last two trials.

pBAD-THIO/TOPO cloning

Colonies (12) of *E. coli* containing pBThio plasmids were screened using agarose gel electrophoresis. pBThio4, pBThio7, pBThio11, and pBThio12 all appeared on the gel to be ~5,500 base pairs. These four clones were screened for directionality by digestion with restriction enzymes *Nco* I and *Kpn* I. The agarose gel showed that pBThio4, pBThio11, and pBThio12 were in the vector in the correct orientation, while pBThio7 was in backwards (figure 23). The clones were also screened by PCR. The gel of the PCR products showed that pBThio4 and pBThio12 did, in fact, contain the dszB gene (figure 24).

ProBond Purification

The chromatograph shown in figure 25 was produced from the UV absorbance data from the column. Fractions 6 – 13 in the second tray of fractions showed activity and were pooled for a total volume of 8 mL. These fractions correspond to minute 412 through 428 on the chromatograph.

CHAPTER V

DISCUSSION

Substrate Inhibition

The velocity of HPBS desulfinase drops off significantly at high substrate concentrations. Since neither reaction product is an inhibitor (22), substrate inhibition was examined. When kinetic data for high and low substrate concentrations was fitted to a substrate inhibition equation, the K_m and V_{max} values for the enzyme did not change significantly from the values obtained from kinetic assays from data collected for only low substrate concentrations.

In 1999, Villadsen published an article dealing with microorganisms used to treat toxic waste in chemical factories. These organisms show substrate-inhibited kinetics when exposed to these chemicals. At too high substrate concentrations the organisms are unable to break down the chemical and they die. (23) This is applicable to HPBS desulfinase because it also shows substrate inhibited kinetics. At very high substrate concentrations the rate of HPBS desulfinase slows down so much that it essentially is no longer catalytically viable.

It is believed that the substrate HPBS is held in the active site of HPBS desulfinase by hydrophobic and π-π stacking type interactions with tryptophan and possibly tyrosine residues in the protein. The kinetic data supports the idea that at high substrate concentrations HPBS acts as an inhibitor. While substrate inhibition of a monomer is un-described, it is possible that HPBS is able to bind into the active site of the enzyme "backwards" placing the catalytic residues too far away to do chemistry allowing HPBS to simultaneously act as both a substrate and an inhibitor. This could also be achieved if the basic sulfur residue on the enzyme does a nucleophilic attack on the keytone intermediate in the acid-base mechanism forming a covalent complex and therefore a "suicide inhibitor."

Chemical Modification Studies

Cysteine Modification by 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB)

DTNB reacts with SH groups to form 2-nitro-5-thiobenzoic acid (24) (figure 26). This reaction will inactivate proteins that have a chemically essential cysteine residue by modifying the sulfur residue. DTNB modifies HPBS desulfinase with a k_i of 0.481/μM. Since the modification of cysteine has a significant effect on the catalytic efficiency of the enzyme, it can be concluded that there is a cysteine residue involved in either binding or catalysis. HPBS desulfinase has only one cysteine residue in the published sequence at position 27. Initial pH studies on the enzyme indicate that a catalytically essential group

has a pKa at 6 (22). This pKa could be consistent with a catalytic cysteine residue acting as a base at neutral pH. The proposed acid-base mechanism for HPBS desulfinase is supported by the involvement of a cysteine residue as a base.

Tryptophan Modification by N-bromosuccinimide (NBS)

NBS oxidizes the indole ring of tryptophan residues to an oxindole derivative (25). The reaction is shown in figure 27 (21). This reaction will inactivate enzymes with a tryptophan residue involved in either binding or catalysis. NBS modifies HPBS desulfinase with a k_i of 0.0352/μM. Since tryptophan modification by NBS produces an effect on the catalytic efficiency of the enzyme, it can be concluded that there is one or more tryptophan residues involved in either binding or catalysis. The structure of the reaction substrate HPBS indicates that hydrophobic or π-π stacking type interactions could be involved in the binding of the substrate. This is supported by the involvement of tryptophan in the reaction. Both the hydrophobic nature of tryptophan and the presence of π electrons implicate tryptophan residues in the binding of HPBS to HPBS desulfinase. There are seven tryptophan residues in the protein.

Tyrosine Modification by tetranitromethane (TNM)

The reaction of tetranitromethane with tyrosine nitrates the phenol ring ortho to the hydroxyl producing 3-nitrotyrosine (26) (figure 28). This reaction will inactivate tyrosine residues making them unavailable for binding or catalysis.

TNM modifies HPBS desulfinase with a k_i of 0.8301/μM. Since tyrosine modification by TNM has a significant effect on the catalytic efficiency of the enzyme it can be concluded that there is a tyrosine residue involved in either binding or catalysis. HPBS desulfinase has only three tyrosine residues in the published sequence and one is at position 24, two residues away from the cysteine residue. Initial pH studies on the enzyme indicate that a catalytically essential group has a pKa at 9 (22). This pKa could corresponed to a catalytic tyrosine residue acting as an acid at neutral pH. The proposed acid base mechanism for HPBS desulfinase is supported by the involvement of a tyrosine residue as a base. Tyrosine residues can also be involved in the binding of HPBS due to π-π stacking type interactions.

Chemical Modification Protection Studies

2,2'-biphenol Protection of Cysteine Modification

The use of 2,2'-biphenol as an active site protection agent shows a 1.91 fold decrease in the k_{obs} for the inactivation reaction (figure 18). This supports the earlier conclusion that a cysteine residue is located in the enzyme active site. The protection agent bound to the active site blocks DTNB from reacting with the active site cysteine residue to inactivate the protein.

2,2'-biphenol Protection of Tryptophan Modification

The use of 2,2'-biphenol as an active site protection agent shows a 1.92 fold decrease in the k_{obs} for the inactivation reaction (figure 19). This supports the earlier conclusion that one or more tryptophan residues are located in the enzyme active site. The protection agent bound to the active site blocks NBS from reacting with the active site tryptophan residues to inactivate the protein.

2,2'-biphenol Protection of Tyrosine Modification

The use of 2,2'-biphenol as an active site protection agent shows a 2.71 fold decrease in the k_{obs} for the inactivation reaction (figure 20). This supports the earlier conclusion that one or more tyrosine residues are located in the enzyme active site. The protection agent bound to the active site blocks TNM from nitrating the active site tyrosine residues to inactivate the protein.

Active Site Model

Chemical modification studies of HBPS desulfinase indicate the presence of cysteine, tyrosine, and tryptophan in the active site. It is proposed that the catalytic acid and base in the active site are tyrosine 24 and cysteine 27. A search of other dszB desulfurase enzymes was performed on GenBank. Four other enzymes were identified and each contained the YXXC motif in the first thirty five amino acid residues. *Agrobacterium tumefaciens* str. C58 (Cereon) contains this sequence at residues 33 and 35. (27) *Sphingomonas paucimobilis* contains this sequence at residues 22 and 24. (28) *Paenibacillus* sp. A11-2

contains this sequence at residues 14 and 16. (29) *Sinorhizobium meliloti* strain 1021 contains this sequence at residues 11 and 13. (30) All of these enzymes will desulfurize dibenzothiophene. This appears to be the catalytic unit for this class of enzyme.

IMPACT CN cloning

Both the inability to perform a sticky end ligation on the dszB gene and the difficulty encountered during PCR of the gene, suggest that the DNA for the dszB gene is folding into some kind of secondary structure not usually seen with this small of a DNA segment. The gene also contains a high GC content that could be responsible for the difficulties encountered in both PCR and enzymatic ligations.

Affinity purification

The dszB gene was successfully ligated via topoisomerase cloning reaction into a cloning vector. This vector was screened using both PCR and restriction digests and the fusion protein shows activity. For these reasons it is believed that the pBThio12 plasmid clone gene product is a fusion protein containing the dszB gene. The DNA will be submitted to DavisSequencing for final sequence analysis.

The affinity purification was successful. HPBS desulfinase fusion protein was expressed in a soluble fashion in *E. coli*. The fusion protein bound to the Nickel chelating ProBond resin and eluted in a small number of fractions. Further

analysis of the fusion protein will be completed along with refinement of the purification scheme in order to produce large amounts of recombinant HPBS desulfinase. The fusion tag also needs to be cleaved off using enterokinase. When this is completed three non-native residues will remain on the N-terminus of the protein. Characterization of the recombinant HPBS desulfinase will be completed before using it for further mechanistic studies.

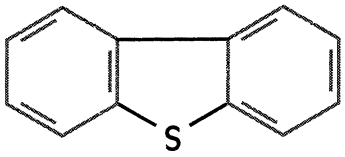

Figure 1: Structure of Dibenzothiophene.

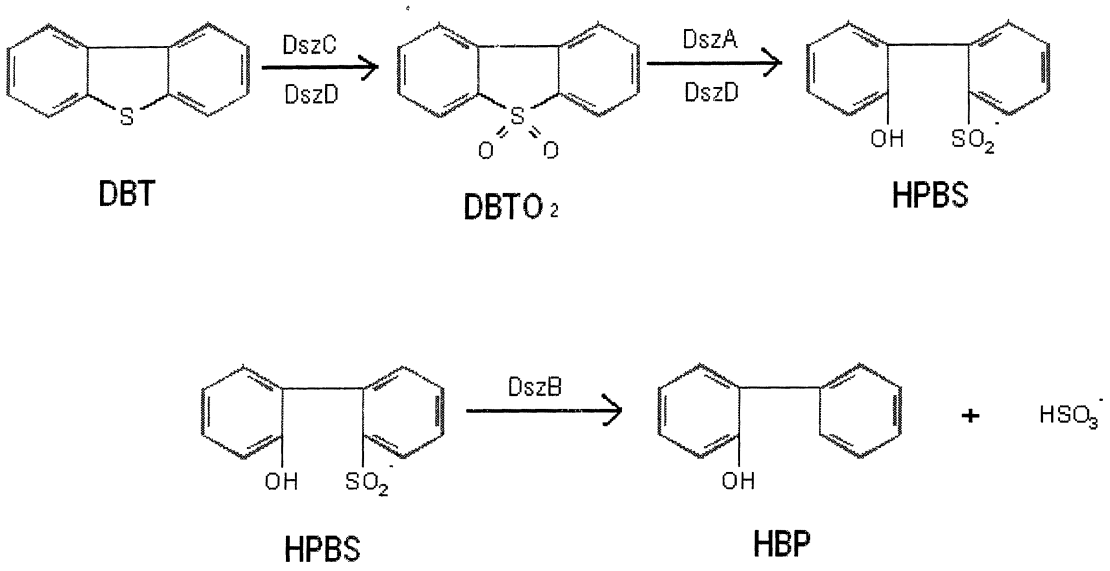

Figure 2: Rhodococcus sp. IGTS8 four enzyme, three step desulfurization pathway for DBT.

```
          5' ATGACAAG GCGCGTCGAC CCCGCAAACC CCGGTTCAGA ACTCGATTCC
GCCATCCGCG ACACACTGAC CTACAGCAAC TGCCCGGTAC CCAACGCTCT GCTCACGGCA
TCGGAATCGG GCTTCCTCGA CGCCGCCGGC ATCGAACTCG ACGTCCTCAG CGGCCAGCAG
GGCACGGTTC ATTTCACCTA CGACCAGCCT GCCTACACCC GTTTTGGGGG TGAGATCCCG
CCACTGCTCA GCGAGGGGTT GCGGGCACCT GGGCGCACGC GTCTACTCGG CATCACCCCG
CTCTTGGGGC GCCAGGGCTT CTTTGTCCGC GACGACAGCC CGATCACAGC GGCCGCCGAC
CTTGCCGGAC GTCGAATCGG CGTCTCGGCC TCGGCAATTC GCATCCTGCG CGGCCAGCTG
GGCGACTACC TCGAGTTGGA TCCCTGGCGG CAAACGCTGG TAGCGCTGGG CTCGTGGAGC
GCGCGCGCCT TGTTGCACAC CCTTGAGCAC GGTGAACTGG GTGTGGACGA CGTCGAGCTG
GTGCCGATCA GCAGTCCTGG TGTCGATGTT CCCGCTGAGC AGCTCGAAGA ATCGGCGACC
GTCAAGGGTG CGGACCTCTT TCCCGATGTC GCCCGCGGTC AGGCCGCGGT GTTGGCCAGC
GGAGACGTTG ACGCCCTGTA CAGTTGGCTG CCCTGGGCCG GGGAGTTGCA AGCCACCGGG
CGGGCGGGAG TGGTGGATCT CGGCCTCGAT GAGCGCAATG CCTACGCCAG TGTGTGGACG
GTCAGCAGCG GGCTGGTTCG CCAGCGACCT GGCCTTGTTC AACGACTGGT CGACGCGGCC
GTCGACGCCG GGCTGTGGGC ACGCGATCAT TCCGACGCGG TGACCAGCCT GCACGCCGCG
AACCTGGGCG TATCGACCGG AGCAGTAGGC CAGGGCTTCG GCGCCGACTT CCAGCAGCGT
CTGGTTCCAC GCCTGGATCA CGACGCCCTC GCCCTCCGTT AGCGCACACA GCAATTCCTG
CTCACCAACA ACTTGCTGCA GGAACCCGTC GCCCTCGATC AGTGGGCGGC TCCGGAATTT
CTGAACAACA GCCTCAATCG CCACCGATAG 3'
```

Figure 3: DNA sequence of the gene from Rhodococcus erythropolis sp. IGTS8 that codes for HPBS desulfinase (dszB).

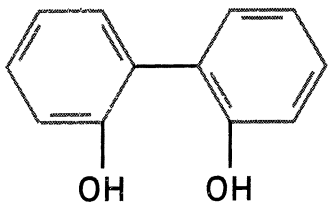

Figure 4: Structure of 2-2'-biphenol, an inhibitor of HPBS desulfinase with a Ki of 16.5 μM.

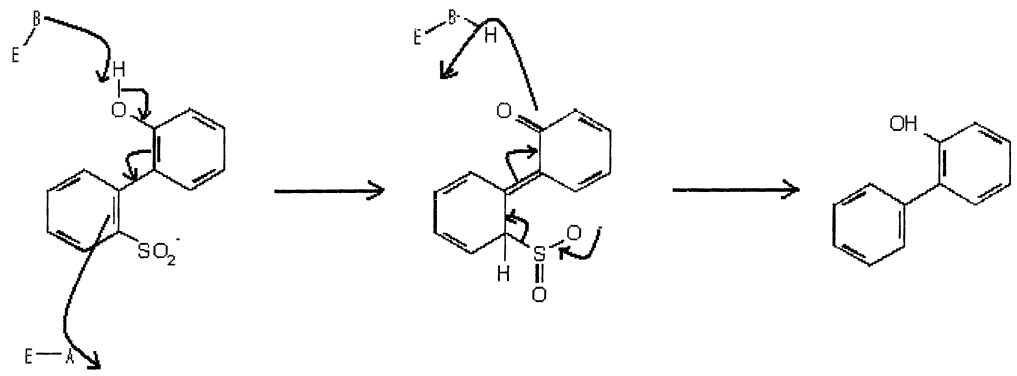

Figure 5: Proposed acid-base mechanism of HPBS desulfinase.

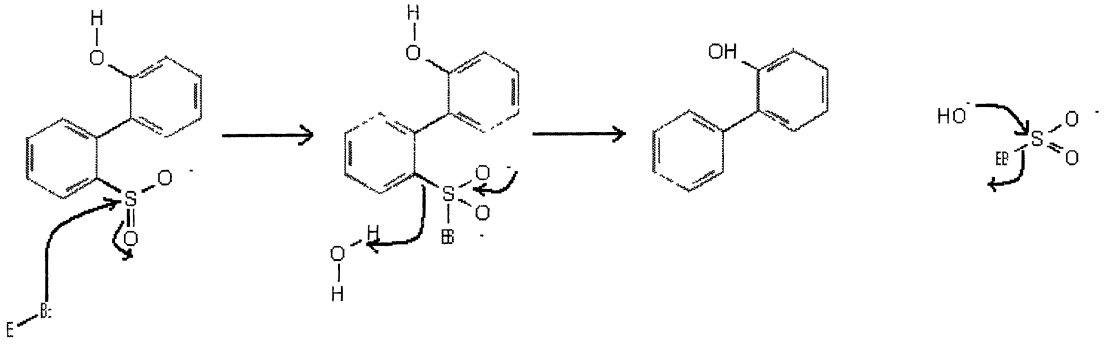

Figure 6: Proposed nucleophilic attack mechanism of HPBS desulfinase.

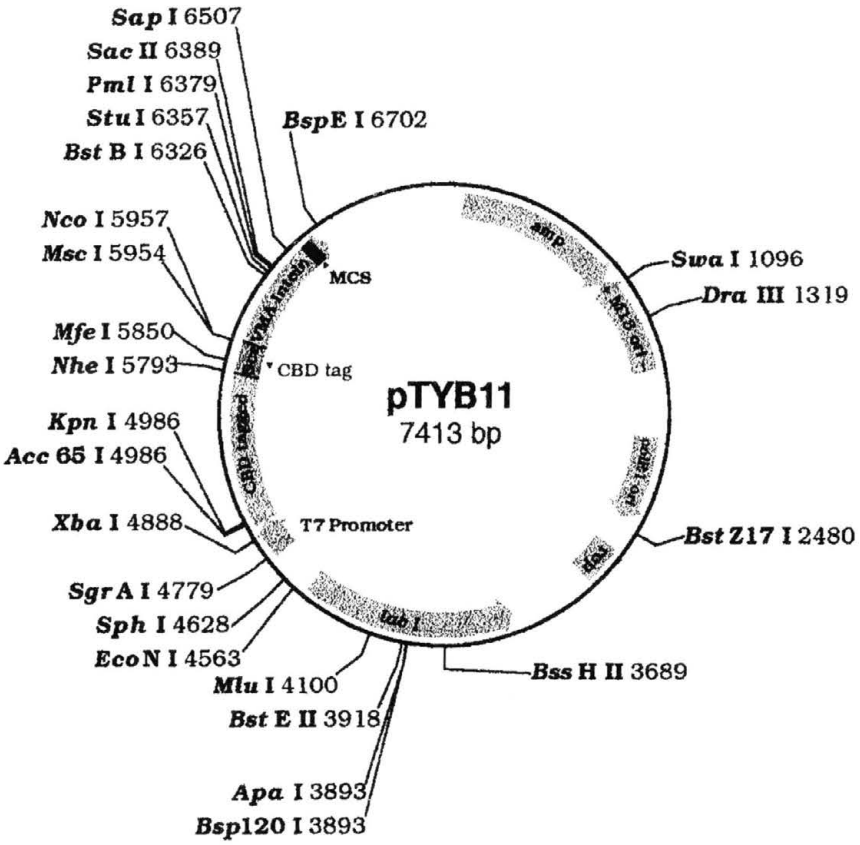

Figure 7: Map of the IMPACT CN cloning vector pTYB11. (14A)

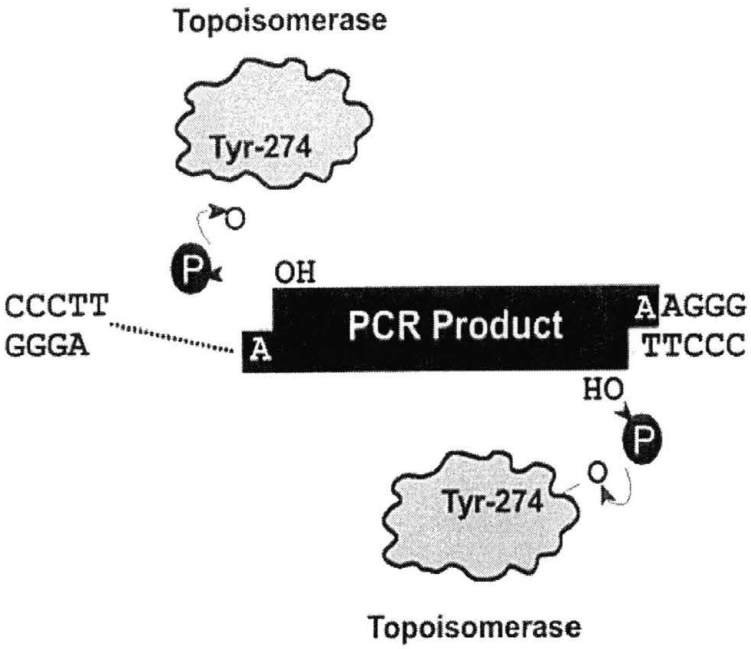

Figure 8: Schematic of the Topoisomerase chemical cloning reaction (14B)

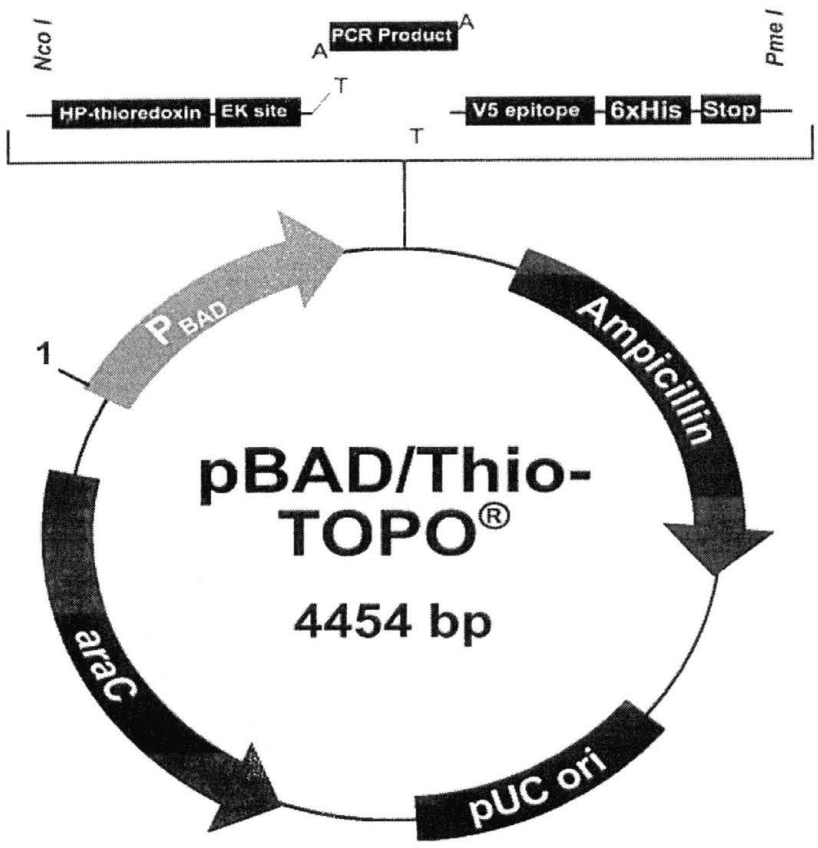

Figure 9: Map of pBAD/THIO-TOPO cloning vector. Vector includes and arabinose expression induction gene and an ampicillin resistance gene. The inclusion of the HP-Thioredoxin peptide on the N-terminus of the fusion protein will allow for one step affinity purification of the protein (14C).

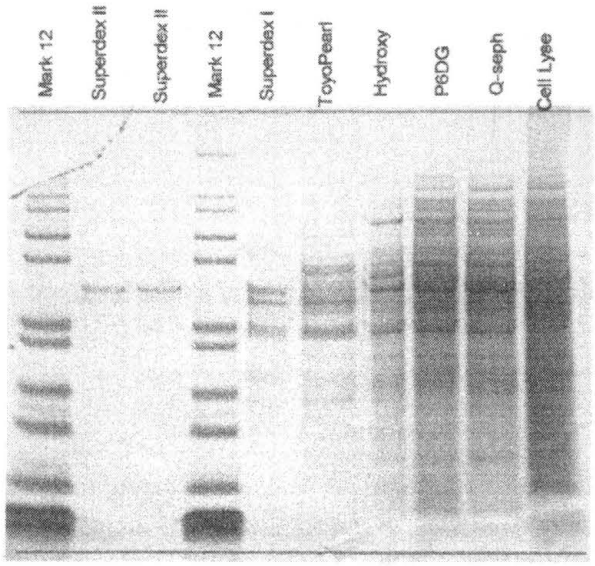

Figure 10: SDS Page gel of protein fractions from a HPBS desulfinase purification. The superdex column was repeated twice to guarantee very pure enzyme.

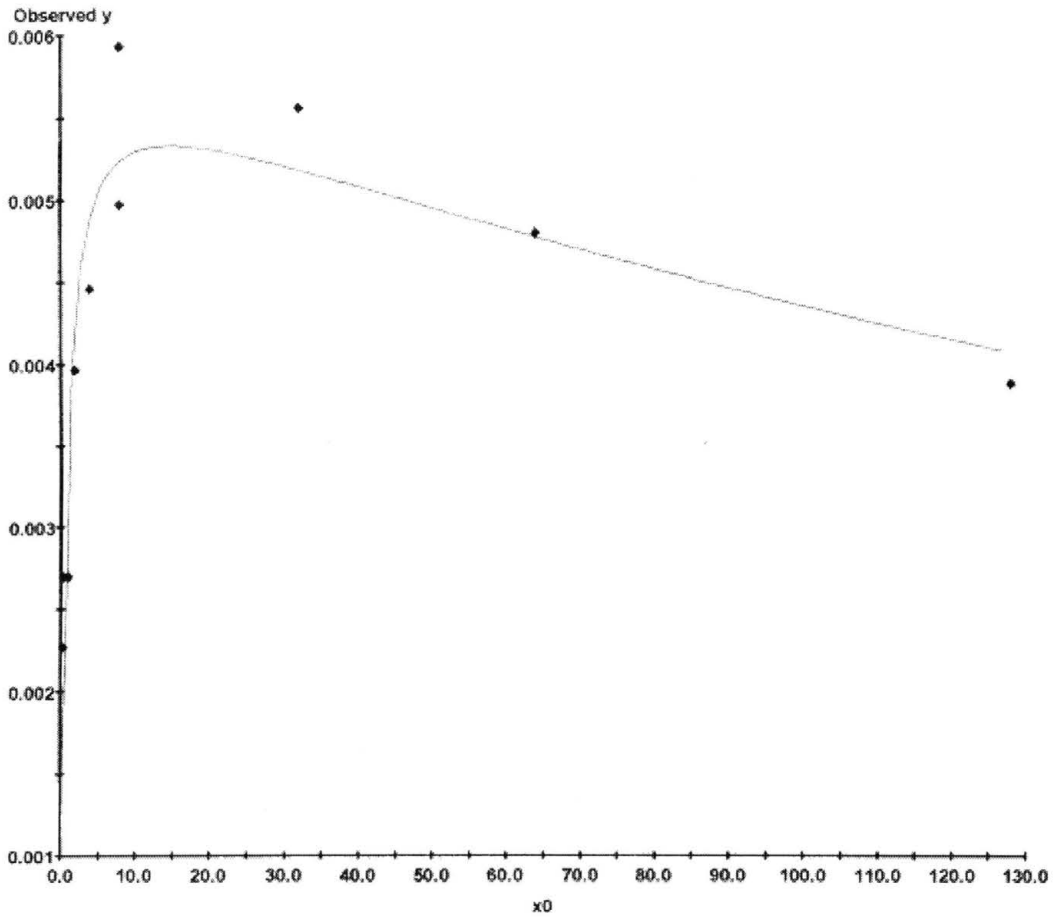

Figure 11: Plot of kinetic data fit to substrate inhibition equation.

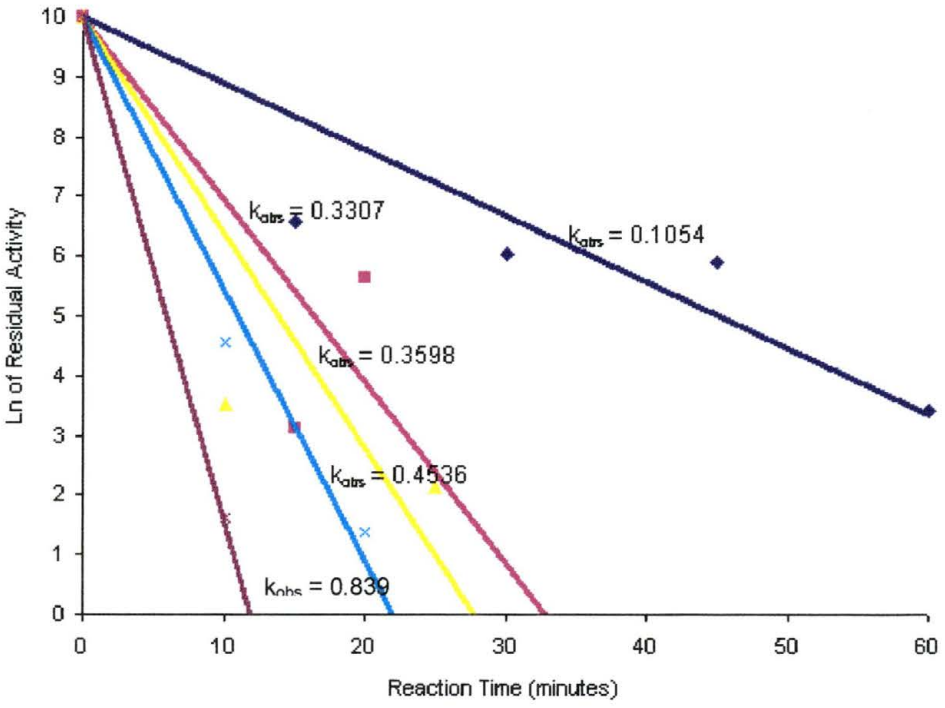

Figure 12: DTNB Cysteine Modification – Residual Activity vs Time

Plot of activity loss for HPBS desulfinase modified with DTNB for 0 to 60 minutes. (blue-0.001 μM DTNB, pink-0.01 μM DTNB, yellow-0.1 μM DTNB, aqua-1.0 μM DTNB, and violet-10.0 μM DTNB) Negative slope of lines is equal to the k_{obs} for each DTNB modification reaction. Data was fit to a best fit line using Excel.

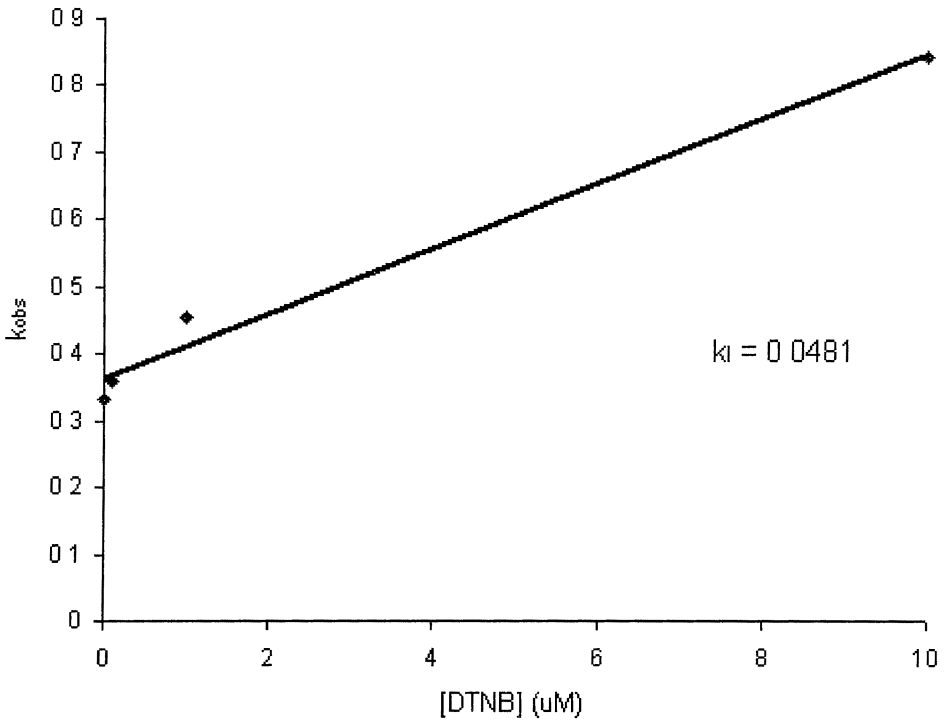

Figure 13: k_I for DTNB Modification of HPBS Desulfinase

Plot of k_{obs} values from DTNB modification reactions vs DTNB concentrations (μM). Slope of line is equal to the k_I (1/μM) for DTNB modification. Data was fit to a best fit line using Excel.

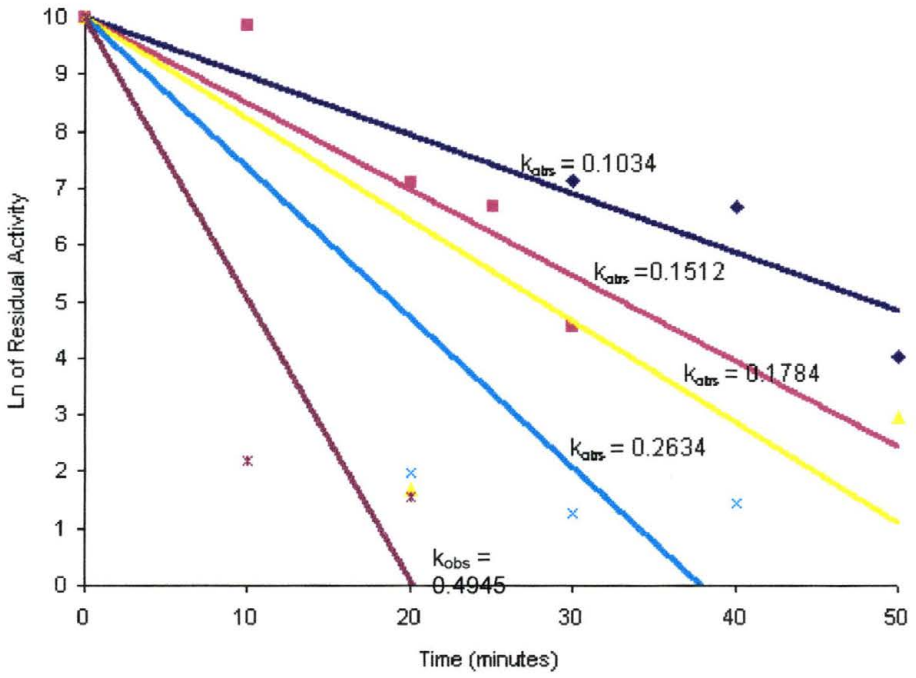

Figure 14: NBS Tryptophan Modification – Residual Activity vs Time

Plot of activity loss for HPBS desulfinase modified with NBS for 0 to 50 minutes. (blue-0.001 μM NBS, pink-0.01 μM NBS, yellow-0.1 μM NBS, aqua-1.0 μM NBS, and violet-10.0 μM NBS) Negative slope of lines is equal to the k_{obs} for each NBS modification reaction. Data was fit to a best fit line using Excel.

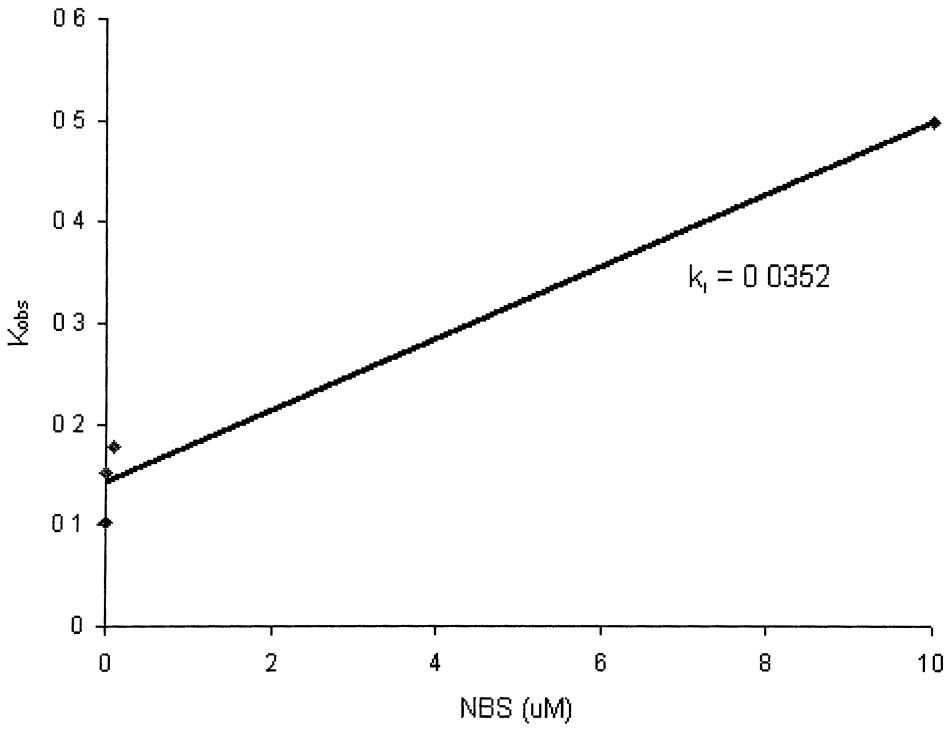

Figure 15: k_I for NBS Modification of HPBS Desulfinase

Plot of k_{obs} values from NBS modification reactions vs NBS concentrations (μM). Slope of line is equal to the k_I (1/μM) for NBS modification. Data was fit to a best fit line using Excel.

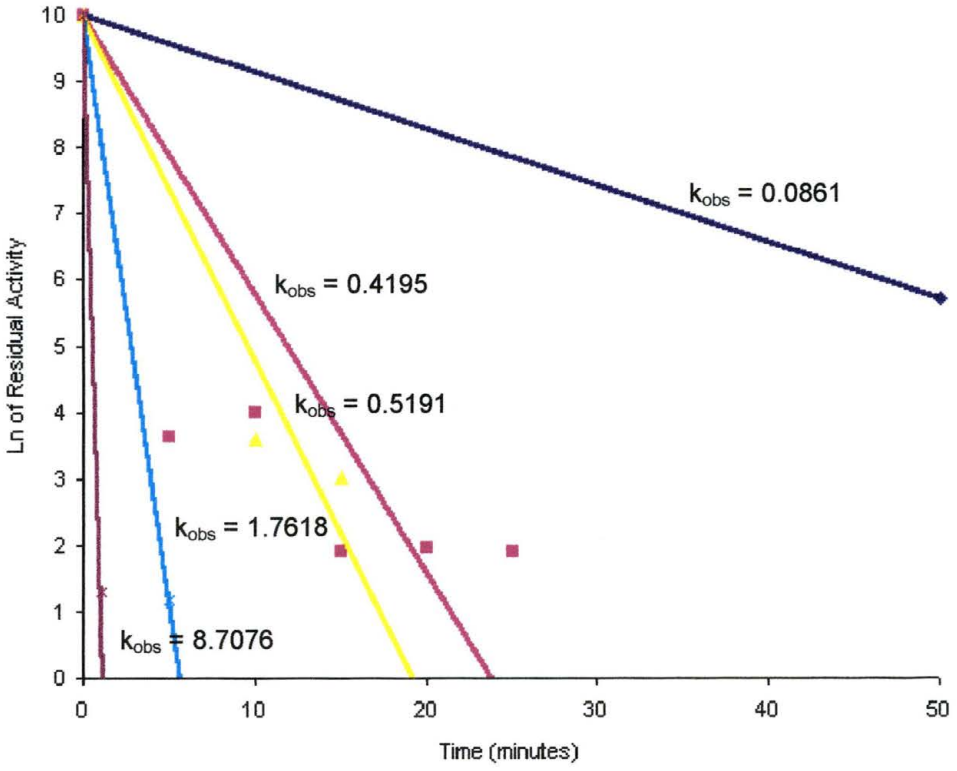

Figure 16: TNM Tyrosine Modification – Residual Activity vs Time

Plot of activity loss for HPBS desulfinase modified with TNM for 0 to 50 minutes. (blue-0.001 μM TNM, pink-0.01 μM TNM, yellow-0.1 μM TNM, aqua-1.0 μM TNM, and violet-10.0 μM TNM) Negative slope of lines is equal to the k_{obs} for each TNM modification reaction. Data was fit to a best fit line using Excel.

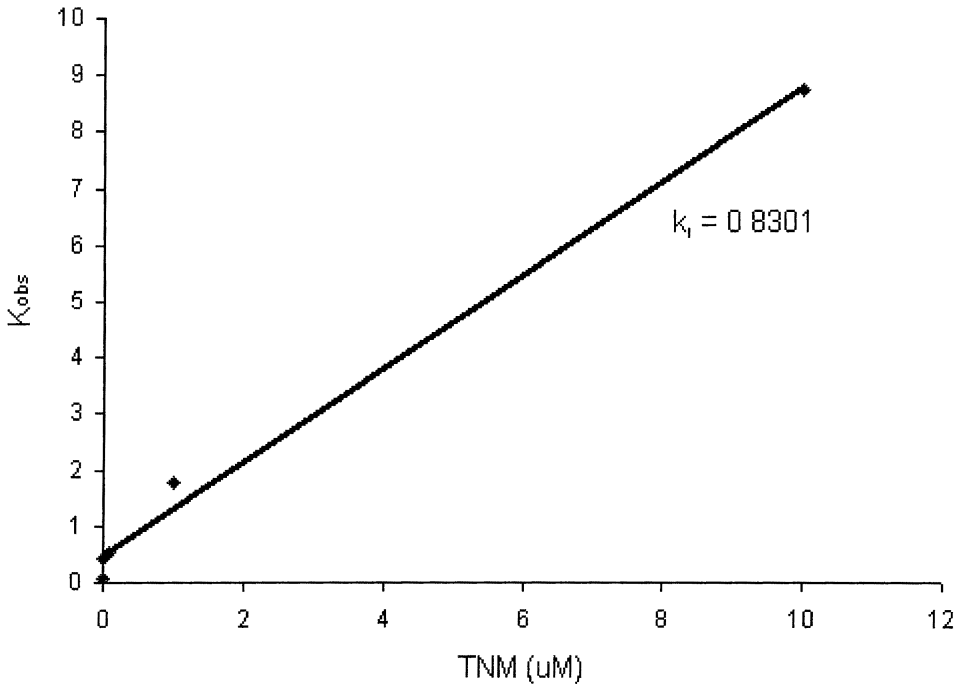

Figure 17: k_I for TNM Modification of HPBS Desulfinase

Plot of k_{obs} values from TNM modification reactions vs TNM concentrations (μM). Slope of line is equal to the k_I (1/μM) for TNM modification. Data was fit to a best fit line using Excel.

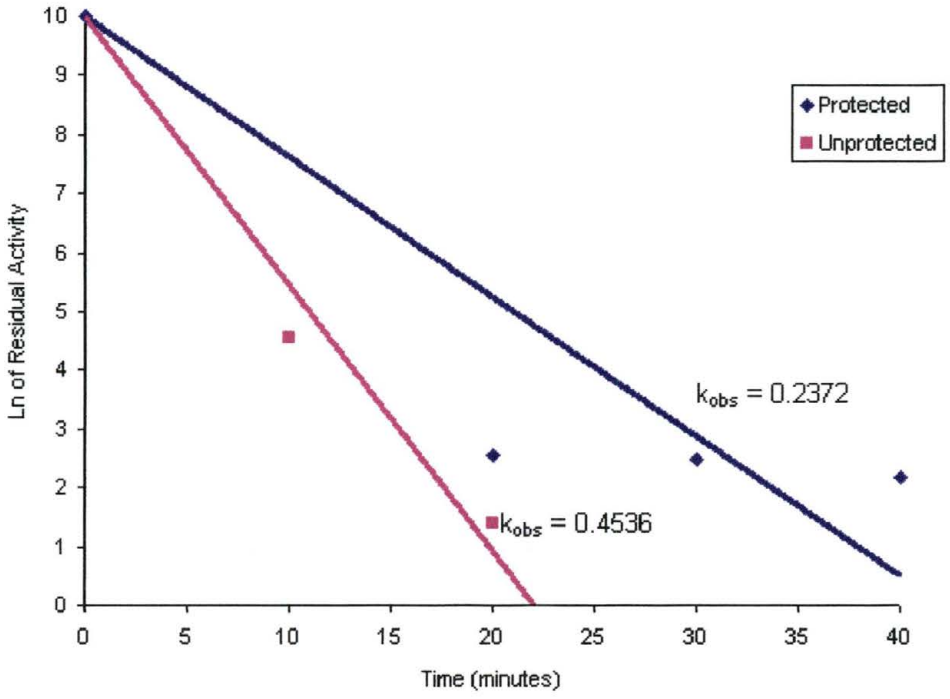

Figure 18: 2,2'-biphenol Protection from DTNB Modification

Plot of activity loss of HPBS desulfinase modified with 1.0 μM DTNB with 2,2'-biphenol as an active site protection agent and without a protection agent. The negative slope of the lines is equal to the k_{obs} for each DTNB modification reaction. Data was fit to a best fit line using Excel.

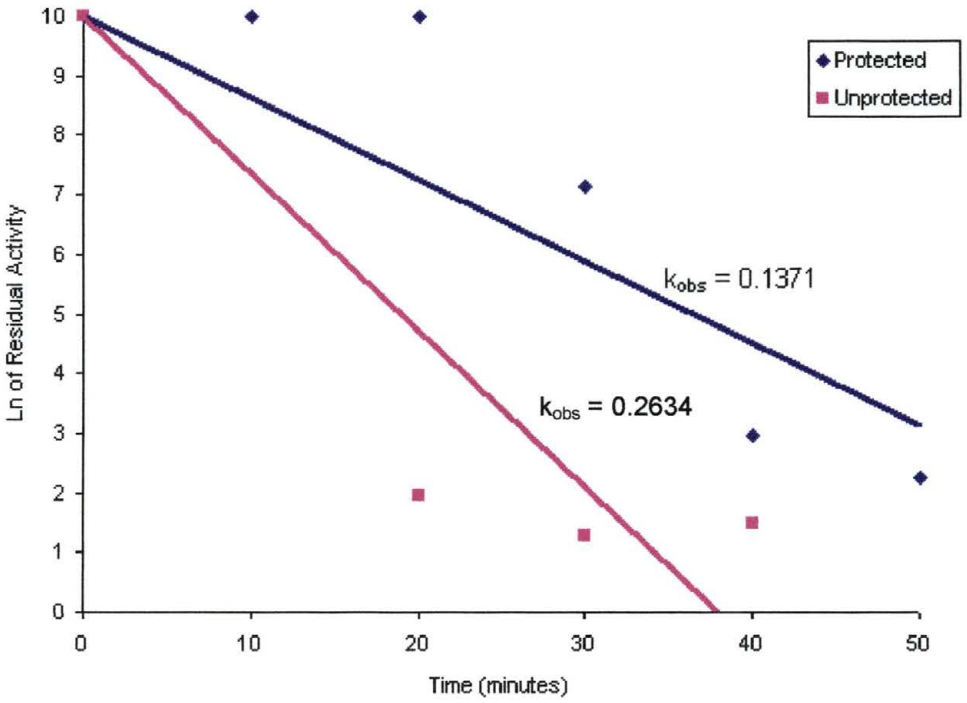

Figure 19: 2,2'-biphenol Protection from NBS Modification

Plot of activity loss of HPBS desulfinase modified with 1.0 μM NBS with 2,2'-biphenol as an active site protection agent and without a protection agent. The negative slope of the lines is equal to the k_{obs} for each NBS modification reaction. Data was fit to a best fit line using Excel.

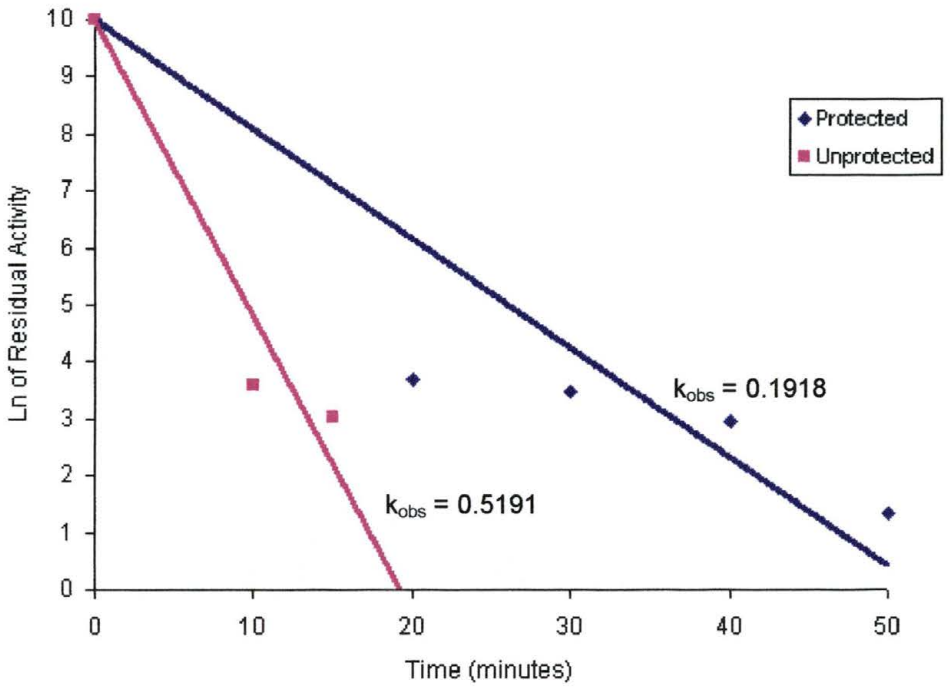

Figure 20: 2,2'-biphenol Protection from TNM Modification

Plot of activity loss of HPBS desulfinase modified with 0.1 μM TNM with 2,2'-biphenol as an active site protection agent and without a protection agent. The negative slope of the lines is equal to the k_{obs} for each TNM modification reaction. Data was fit to a best fit line using Excel.

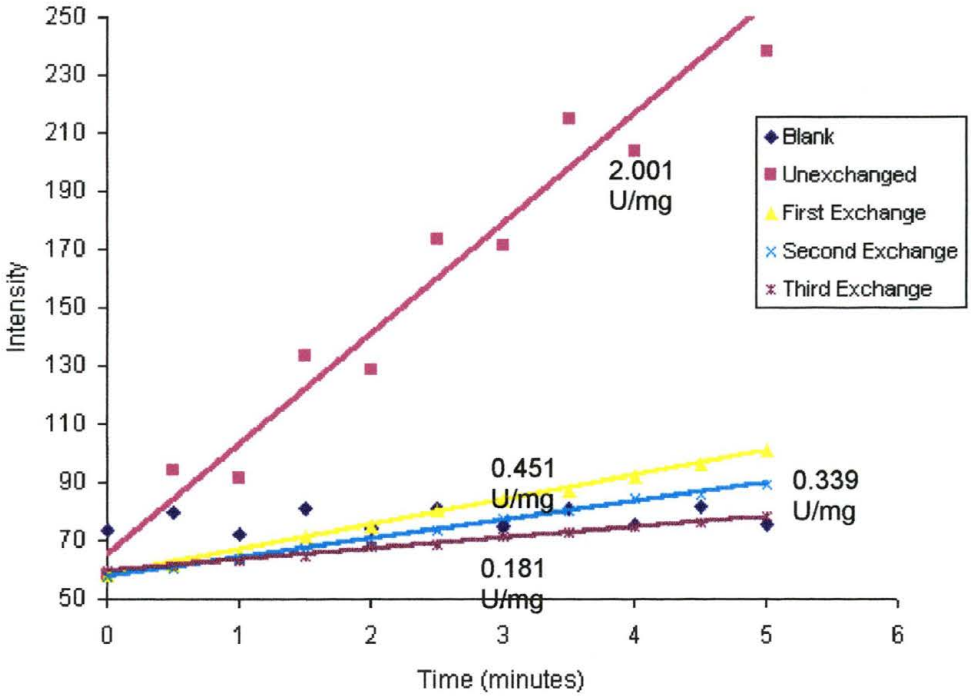

Figure 21: Solvent Isotope Effects

Velocity plot for Solvent Exchange reactions. Specific activity (U/mg) was calculated from the slope of the line and the standard curve for HBP (y = 21.1x). Data was fit to a best fit line using Excel.

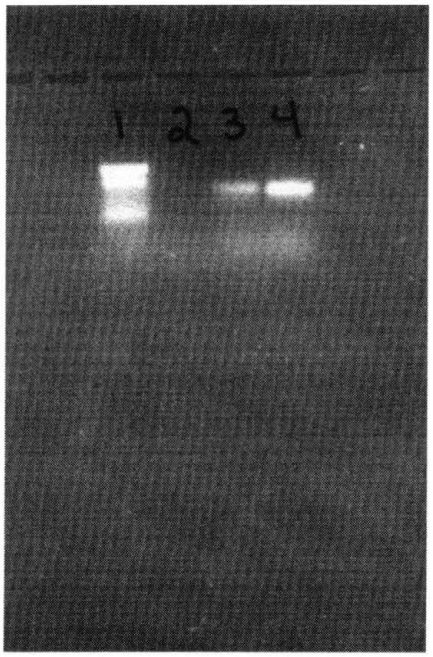

Figure 22: Agarose gel of PCR products using primers LMW3 and LMW4 stained with ethidium bromide. Lane 1: 100 base pair marker; Lane 2: 0 DMSO in the reaction; Lane 3: 2% DMSO in the reaction; Lane 4: 3% DMSO in the reaction.

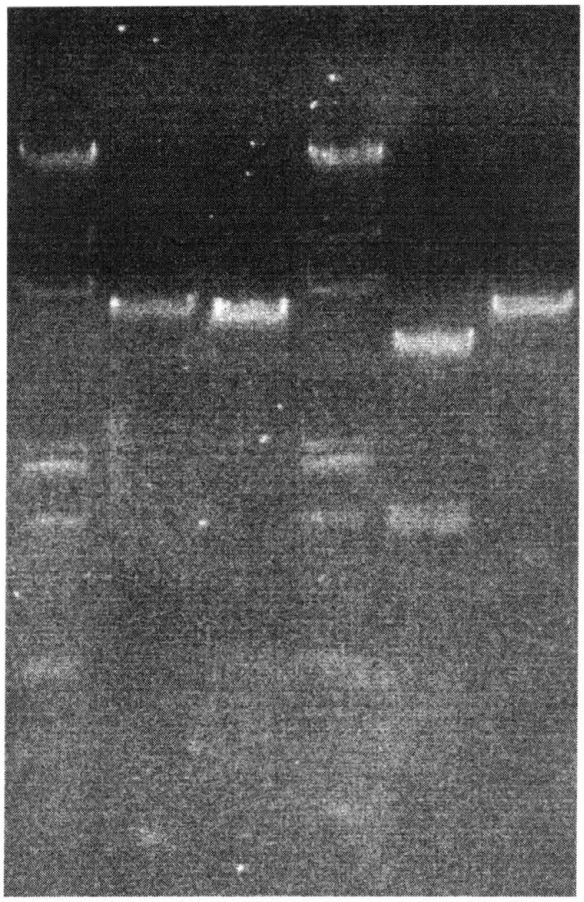

Figure 23: Agarose gel of restriction digest of pBThio clones. Lane 1: standards; Lane 2: pBThio12 digest; Lane3: pBThio11 digest; Lane 4: standards; Lane 5: pBThio7 digest; Lane 6: pBThio 4 digest.

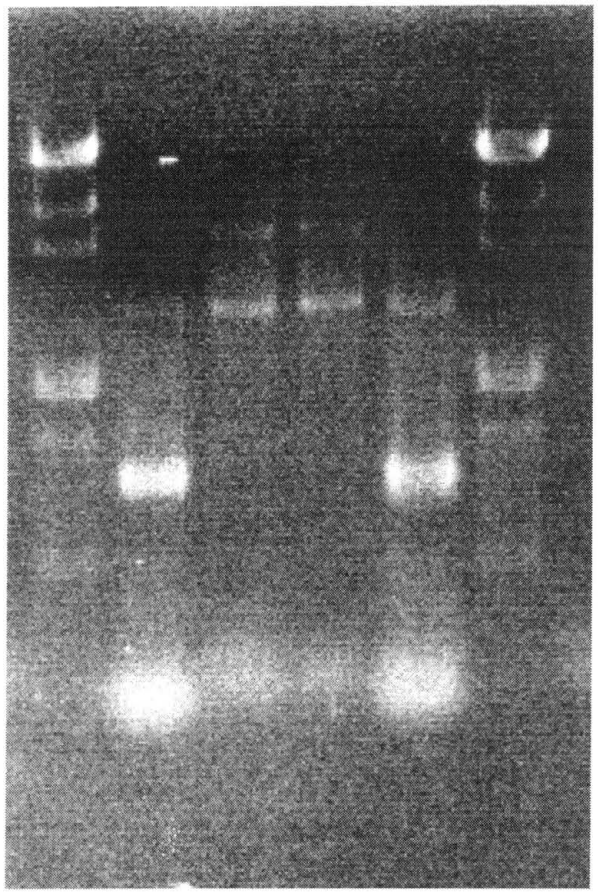

Figure 24: Agarose gel of pBThio clones after PCR reactions. Lane 1: standards; Lane 2: pBThio12 PCR product; Lane 3: pBThio 11 PCR product; Lane 4: pBThio7 PCR product; Lane 5: pBThio4 PCR product; Lane 6: standards.

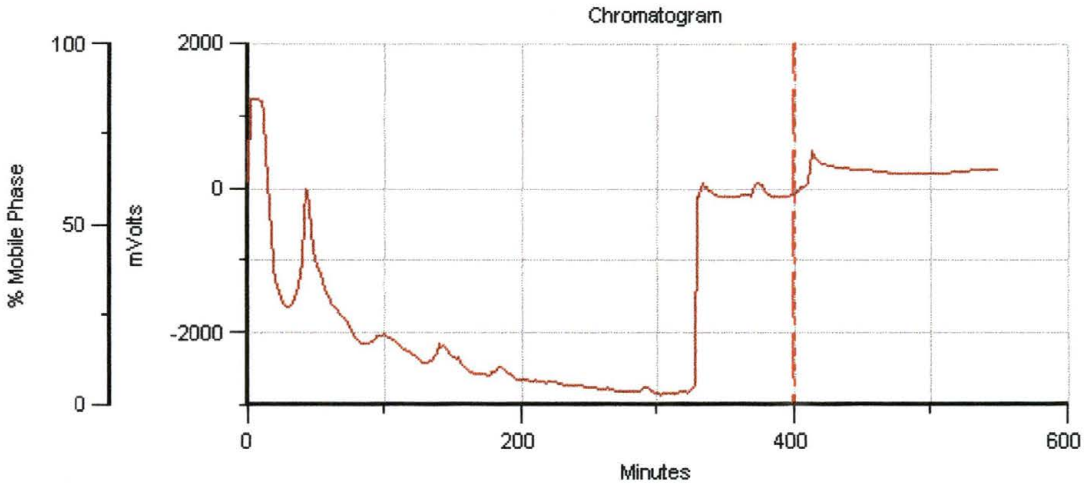

Figure 25: Probond Purification chromatograph. HPBS desulfinase fusion protein with activity corresponds to the peak at 412-428 minutes.

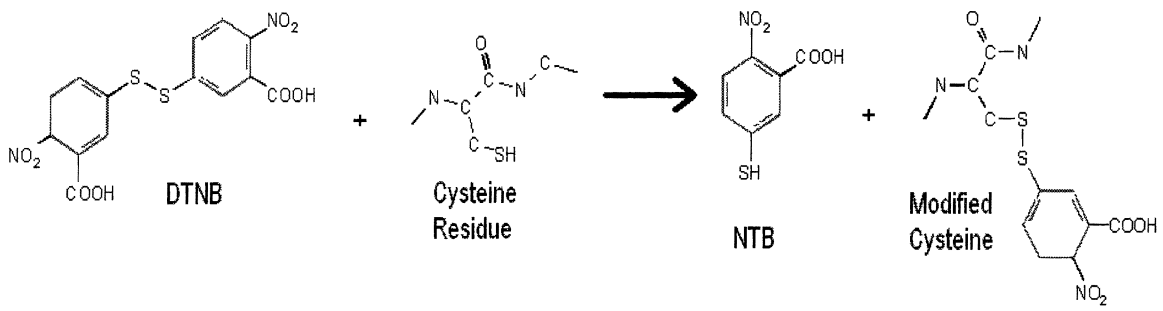

Figure 26: Reaction of cysteine residues with DTNB to produce NTB and modified, inactivated cysteine (16).

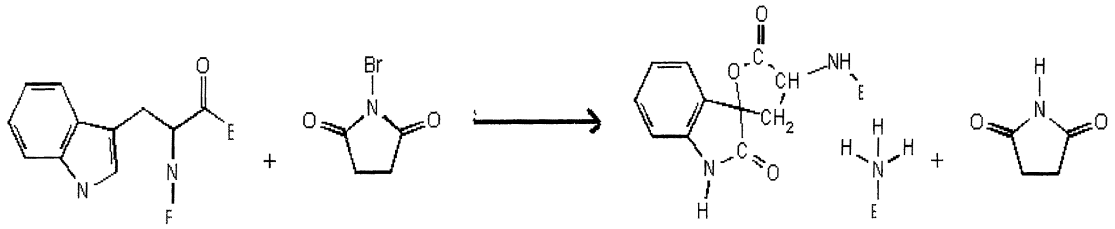

Figure 27: Reaction of tryptophan residues with NBS to produce fragmented protein and an oxidized tryptophan residue (17A).

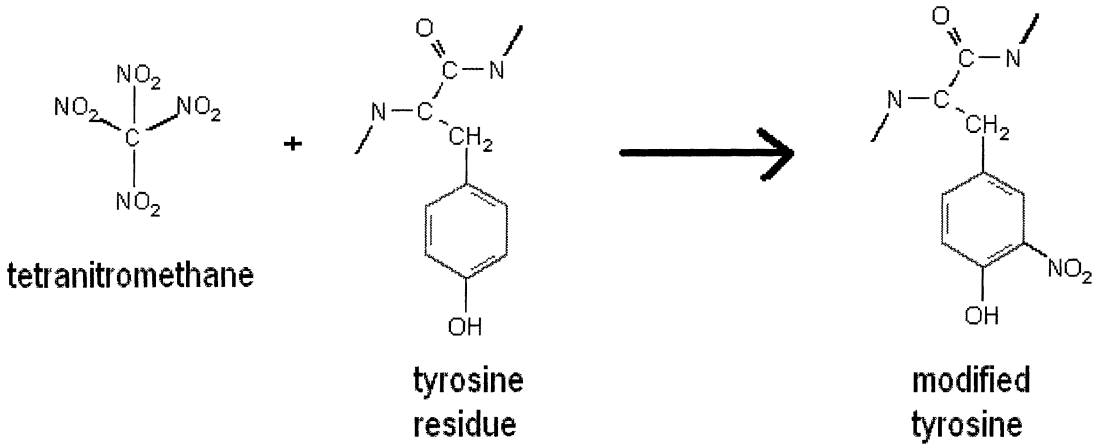

Figure 28: Reaction of tyrosine residues with tetranitromethane to produce a nitrated, inactivated tyrosine residue (18).

Table 1: Purification Profile

Purification Step	Volume	Protein Conc. (mg/mL)	Total Protein (mg)	Activity (U/mL)	Total Units	Specific Activity (U/mg)	Yield %	Fold Purification
Cell Lyse	423	4.1	1734.30	0.023	9.86	0.006		
Q-seph	135	0.85	114.75	0.072	9.72	0.085	99	15
P6DG	128	0.76	97.28	0.063	8.06	0.083	82	15
Hydroxy	155	0.56	86.80	0.045	6.98	0.080	71	14
Toyo	23	0.16	3.66	0.152	3.50	0.958	36	169
Superdex	19	0.09	1.71	0.189	3.59	2.1	36	370

*A Unit (U) of activity is defined as one μmol of HBP formed per hour.

Table 2: Statistical analysis of kinetic data

Fitted Parameters

Parameter	Value	Standard Error	Probability
V_{max}	2 61 U/mg	0 216	3 032
K_m	0 75 μM	0 238	0 008

Analysis of Variance

R^2 = 0 8459

Calculated Value

Kcat = 1 83/minute

95% Confidence Limits Covariance Matrix

Name	Lower	Upper
V_{max}	2 081	2 825
K_m	0 166	1 331

Table 3: Equations used in calculation of k_{obs} and k_I values for chemical modification studies.

Equation 1	$\text{Ln}\ (E_{at}/E_{a0}) = -\ k_{obs}t$
Equation 2	$k_{obs} = k_I[\text{TNM}]$

Table 4: Primer sequences and descriptions.

Name	Sequence	Direction	Description	Plasmid name
LMW3	5' GGTGGTTGCTCTTCCAACATGACAAG CCGCGTCGACCCCGC 3'	Forward	Incorporates a *SAP I* site onto the 5' end of the gene.	pThioTag(clone#)
LMW4	5' GGTGGTGAATTCCTATCGGTGGCGAT TGAGGCTGTTGTTCAGAAATTCC 3'	Reverse	Incorporates an *ECO RI* site onto the 3' end of the gene.	pThioTag(clone#)
LMWMC1	5' ATGACAAGCCGCGTCGACCCCGCAAACCCC GGTTCAGAACTCGATTCCGCCATCCGCGACAC ACTGACCTACAGCAACGCCCCAGTACCCAACGC3'	Forward	Incorporates a mutation in Cys27 to Ala27 and a silent removal of the *KPN I* site at base pair 88.	pC27A(clone#)
LMWMC2	5'CTATCGGTGGCGATTGAGGCTGTTGTTCAGAAA TTCCGGAGCCGCCCACTGATCGAGGGCGACGGG3'	Reverse	Produces a native 3' end, long to match LMWMC1 PCR conditions.	pC27A(clone#)
LMW6F	5'ATGACAAGCCGCGTCGACCCCGC3'	Forward	Native 5' end, no changes.	pBThio(clone#)
LMWMC3	5'CTATCGGTGGCGATTGAGGCTGTTGTTCAGAA ATTCGCG3'	Reverse	Native 3' end, no changes.	pBThio(clone#)

REFERENCES

1. Gray, K.A., Pogrebinsky, O.S., Mrachko, G. T., Xi, L., Monticello, D.J., Squires, C.H. (1996) *Nature Biotech.*, **14**, 1705.
2. Denome, S.A., Oldfield, C., Nash, L.J., Young, K.D. (1993) *Appl. Environ. Microbiol.*, **59**, 2837.
3. Xi, L., Squires, C.J., Monticello, D.J., Childs, J.D. (1994) *Biochem. Biophys. Res. Comm.*, **230**, 73.
4. Oshiro, T., Suzuki, K., Izumi, Y. (1997) *J.* Ferm. Bioeng., **83**, 233.
5. Ohshiro, T., Kojima, T., Torii, K., Kawasoe, H., Izumi, Y. (1999) *J. Biosci. Bioeng.*, **88**, 610.
6. Ohshiro, T., Kanbayashi, Y., Hine, Y., Izumi, Y. (1995) *Biosci. Biotech. Biochem.*, **59**, 1349.
7. Zheng, L., White, R.H., Cash, V.L., Dean, D.R. (1994) *Biochem.*, **33**, 4714.
8. Krest, I., Keusgen, M. (1999) *Planta Medica.*, **65**, 139.
9. Ramirez, E.C., Whitaker, J.R. (1999) *J. Agric. Food Chem.*, **47**, 2218.
10. Ukai, K., Sekiya, J. (1999) *Phytochem.*, **51**, 853.
11. Ramirez, E.C., Whitaker, J.R. (1998) *Ital. J. Food Sci.*, **10**, 171
12. Eyzaguirre, J., (1987) Chemical Modification of Proteins, 170.
13. Li, M.Z., Squires, C.H., Monticello, D.J., Childs, J.D. (1996) *J.Bacter.*, **178**, 6409.
14. Kalb, V.F., Bernlohr, R.W. (1977) *Anal. Biochem.*, **82**, 362.

15. Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) Molecular Cloning second edition

16. Methods in Enzymology. (1982) **87**, 551.

17. Impact CN Instruction Manual, Version 1.2, Catalogue Number 6900. New England Biolabs Inc., Beverly, Massachusetts.

18. pBAD/TOPO ThioFusion Expression Kit Instruction Manual, Version F, Catalogue Number K370-01. Invitrogen Life Technologies Carlsbad, California.

19. pBADTOPO TA Expression Kit Instruction Manual, Version K, Catalogue Number K4300-01. Invitrogen Life Technologies Carlsbad, California.

20. ProBond Purification System Instruction Manual, Version I, Catalogue Number K850-01. Invitrogen Life Technologies Carlsbad, California.

21. Copeland, R.A., (1996) Enzymes. 180

22. Watkins, L., Rodriguez, R., (2002) Personal Communication

23. Villadsen, J. (1999) *Ind. Eng. Cham. Res.*, **38**, 660.

24. Bazaes, S., Beytia, E., Jabalquinto, A.M., Solis de Ovando, F., Gomez, I., Eyzaguirre, J. (1980) *Biochem.* **19,** 2305.

25. Warwick, P.E., D'Souza, L., Freisheim, J.H. (1972) *Biochem.*, **11**, 3775.

26. Riordan, J.F. (1979) *Mol. Cell. Biochem.*, **26**, 71.

27. Hinkle, G., Slater, S.C., Goodner, B. (2001) Unpublished sequence from NCBI database. www.NCBI.NLM.NIH.gov.

28. Coco, W.M. et al. (2001) Unpublished sequence from NCBI database. www.NCBI.NLM.NIH.gov.

29. Ishii, Y., Konishi, J., Okada, H., Hirasawa, K., Onaka, T., Suzuki, M. (2000) *Biochem. Biophys. Res. Comm.*, **270**, 181.

30. Barnett, M.J. et al. (2001) *Proc. Natl. Acad. Sci.*, **98,** 9883.

VITA

Melissa Kay Cody was born on January 16, 1977, the daughter of Anthony Raymond Benitez and Sandra Kay Hahn Benitez. After completing her work at San Marcos High School, San Marcos, TX, in 1994, she attended several universities before completing her Bachelor of Science at Southwest Texas State University, San Marcos, TX, in Biology. In September of 2000 she entered the Graduate School of Southwest Texas State University, San Marcos, TX.

During this time, Melissa married Kyle Wayne Cody and they have two children: Blair Jordan Cody and Jacob Michael Cody.

Permanent Address: 1350 Wonder World Dr. # 602
San Marcos, TX 78666

This thesis was typed by Melissa Kay Cody.