PURIFICATION AND CHARACTERIZATION OF 2-(2'-HYDROXYPHENYL)BENEZENESULFINATE DESULFINASE

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TABLE OF CONTENTS

ILLUSTRATIONS

FIGURE

TABLE

ABSTRACT

PURIFICATION AND CHARACTERIZATION OF 2-(2'-HYDROXYPHENYL)-BENEZENESULFINATE DESULFINASE

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Dibenzothiophene (DBT) is representative of a broad range of organosulfur compounds that are commonly found in petroleum products. *Rhodococcus* sp. IGTS8 is able to desulfurize DBT to 2-hydroxyphenyl (HBP) and inorganic sulfur by utilizing a four-enzyme pathway. A desulfinase, 2-(2'-hydroxyphenyl)-benzenesulfinate desulfinase (HPBS desulfinase) catalyzes the desulfination of 2-(2'-hydroxyphenyl)benzenesulfinate to HBP and sulfite. It is the final and slowest enzyme in the pathway and is likely to be rate limiting *in vivo*. HPBS desulfinase was separated from a crude cell lysate by anion exchange, hydrophobic interaction, and gel filtration column chromatography. Isoelectric focusing was used to estimate a pI of 5.5. The temperature and pH optima were determined to be 35°C and pH 7.0 respectively. HPBS desulfinase has a K_m for HPBS of 0.90 μM and a V_{max} of 55.5 μM min^{-1}. Product inhibition studies were run at various concentrations of HBP, sulfite or product analogs. HBP and sulfite have no discernable effect on enzyme activity but 2,2'-dihydroxybiphenyl is inhibitory. The effect of metal ions and various chelators on activity indicate a susceptibility to Cu^{2+}, Zn^{2+}, with Ca^{2+} slightly enhancing the rate. The chelators 1,10 phenanthroline; 2,2 dipyridol; and 8-hydroxyquinoline were inhibitory while EDTA had no effect.

CHAPTER I

INTRODUCTION

Sulfur contamination of fossil fuels is a serious concern for the petroleum industry. The sulfur content of petroleum products can range from 0.025% to 11% by weight (1). Generally, sulfur is present as elemental sulfur, H_2S, and organosulfur compounds; including sulfides, mercaptans, and thiophenes. When combusted, these contaminants form sulfur oxides, a major source of pollution that can lead to acid rain. Stricter environmental guidelines regarding emissions has led the petroleum industry to seek out and develop more effective and efficient means of desulfurization.

Inorganic sulfur compounds are effectively removed through well-established physical methods. However, the desulfurization of organosulfur compounds has proven to be more problematic. The most common methods currently in use involve catalytic cracking and hydrodesulfurization (HDS). These processes utilize high temperatures and pressures in the presence of a metal catalyst to generate H_2S and hydrocarbon by-products (2). HDS is an expensive process that is not particularly effective with respect to thiophenic derivatives, which

generate H_2S and hydrocarbon by-products (2). HDS is an expensive process that is not particularly effective with respect to thiophenic derivatives, which comprise the largest percentage of organosulfur compounds present in crude oil. Also, as sulfur content increases so does the concentration of heavy metals, which can deactivate the catalyst required for HDS.

As an alternative to HDS, biodesulfurization using bacterial systems is being heavily investigated. Bacterial systems are advantageous because they can remove sulfur from a broader range of thiophenic compounds than HDS, under milder conditions, and without the resultant corrosive H_2S by-product. Species with demonstrated desulfurization ability include *Acinetobacter, Agrobacterium, Arthrobacter, Corynebacterium, Pseudomonas, Rhizobium,* and *Rhodococcus* (3-8).

Dibenzothiophene (DBT) (Figure 1) is the most prevalent of the thiophenic derivatives, accounting for up to 60% of sulfur contamination in some petroleum products (1); and is regarded as the model compound for studying enzymatic desulfurization pathways. Two pathways have been described for the degradation of DBT. In the first, one of the benzene rings of DBT is oxidized and then cleaved, eliminating the sulfur within water-soluble carbon fragments. This pathway utilizes DBT as a carbon source and will also degrade non-sulfur-containing compounds, resulting in a loss of carbon and thus reducing the value of the fuel. In the second pathway, the sulfur is oxidized to a sulfone then the carbon-sulfur bonds are cleaved, generating inorganic sulfite and hydroxy-

biphenyl (HBP) without the concurrent loss of carbon. It is through this sulfur-specific pathway that the bacteria *Rhodococcus* sp. IGTS8 degrades DBT

Rhodococcus sp. IGTS8 is a Gram-positive soil bacterium commonly found near fossil fuel deposits (1). The enzymatic pathway responsible for desulfurization consists of four proteins and is shown in Figure 2. The first enzyme of the pathway is dibenzothiophene monooxygenase (DszC) which catalyzes a sequential oxidation of DBT to DBT sulfone. The second enzyme, dibenzothiophene-5, 5-dioxide monooxygenase (DszA) converts DBT sulfone to 2-(2'-hydroxyphenyl)benzenesulfinate (HPBS) through oxidation and cleavage of one of the carbon-sulfur bonds. Both the DszC and DszA enzymes require flavins and an NADH-FMN oxidoreductase (DszD) for activity. The final enzyme, HPBS desulfinase (DszB) catalyzes the desulfination of HPBS to 2-hydroxy-biphenyl (HBP) and sulfite, without any apparent coenzyme requirement.

Recently, studies concerning the isolation and initial characterization of the proteins involved in the *Rhodococcus* sp. IGTS8 desulfurization pathway have begun to appear in the literature (9,10,11,12) These studies focused on the DszC (10), DszA (11), and the NADH-FMN oxidoreductase (12) enzymes; and provide little information concerning the DszB enzyme (9). The genes which code for these proteins are plasmid-borne and have been thoroughly investigated (13,14,15). Despite these developments, there is a lack of information concerning the structure of the active site of the individual enzymes or their catalytic mechanisms. A greater understanding of these properties will provide a

framework for improving the overall efficiency of the *Rhodococcus* sp. IGTS8 desulfurization system.

The research presented in this thesis focuses on the final enzyme in the pathway, HPBS desulfinase (DszB). This enzyme catalyzes the cleavage of the carbon-sulfur bond of HPBS to produce HBP and sulfite (Figure 2). Desulfinases in general, are poorly understood and little is known about HPBS desulfinase. It is believed to be the slowest enzyme in the pathway, making it the probable rate limiting step *in vivo* (9). It is a single substrate enzyme which does not appear to require a coenzyme, allowing for a more direct characterization of the mechanism. HPBS desulfinase is the only known aromatic desulfinase and does not share significant sequence homology with any other known protein.

The goal of this research was to purify HPBS desulfinase from *Rhodococcus* sp. IGTS8 and to determine basic physical and kinetic parameters. HPBS desulfinase was purified to homogeneity through anion exchange, hydrophobic interaction, hydroxyapatite and gel filtration chromatography. A fluorimetric assay was developed to quantify enzyme activity Polyacrylamide gel electrophoresis and the fluorimetric assay served to gauge the effectiveness of the purification process. N-terminal analysis was conducted to verify that the correct protein was indeed purified. HPBS desulfinase was purified 1600-fold and the kinetic parameters, K_m, V_{max} and k_{cat} were determined by measuring the appearance of product (HBP) over time. The physical properties determined include pH, temperature optima, and the pI.

One of the mechanisms proposed for HPBS desulfinase activity involves the use of a metal cofactor to withdraw electron density from the substrate, facilitating cleavage of the carbon-sulfur bond. Two studies were conducted to investigate this proposal. First, metal activation experiments were performed using various metal cations commonly found in biological systems. Second, HPBS desulfinase was incubated in solutions of different metal chelators and the effect on activity was determined.

Several experiments were conducted to gain some insight into the nature of the active site. Product inhibition studies were conducted by steadily increasing the concentration of HBP or sulfite and measuring the effect on enzyme rate. The structural and electronic characteristics required to enable compounds to bind to the active site were investigated through the use of isosteric and isoelectronic analogs of the substrate. Chemical modification experiments were used to tentatively identify amino acid residues that may play an important role in binding or catalysis.

CHAPTER II

MATERIALS

Freeze dried and lyophilized *Rhodococcus rhodochrous sp.* IGTS8 was purchased from the American Type Culture Center (ATCC), Manassas, Virginia. Biphenosultıne was provided by Dr Herbert L Holland from Brock University Difco Bactoagar (Lot #111787JF) and Dıfco Nutrient Broth (Lot #106724JE) were purchased from Dıfco Laboratories, Detroit, Michigan. The Q Sepharose™ Fast Flow anıon exchange media and the Superdex 75™ gel filtration media were purchased from Pharmacia, Piscataway, 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 were purchased from Bio-Rad Laboratories, Hercules, California. All other reagents used were reagent grade or better and were purchased from Aldrich Chemical

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

Novex NUPAGE pre-cast 4-12% Bis-Tris polyacyrlamide electrophoresis gels (No. NP0321), Novex electrophoresis reagents, the Novex SilverXpress Silver Staining kit (No. LC6100, Lot #80922), the Novex Colloidal Blue Staining Kit (No. LC6025, Lot #81005), the Novex pre-cast pH 3-10 IEF gels (No. EC6655A), Novex isoelectrofocusing buffers, Servalyt®Precotes® IEF Markers 3-10, and the Mark12 wide range molecular weight protein standards were purchased from Novex Experimental Technology, San Diego, California.

A Novex XCELL-11™ electrophoresis module was used for all polyacrylamide and isoelectric focusing gel electrophoreses. The Novex Mark 12™ molecular weight standard contains: myosin (200,000), β-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). Servalyt® Precotes® IEF Markers 3-10 contains: cytochrome c (10.7), ribonuclease A (9.5), lectin (8.3, 8.0, 7.8), myoglobin (7.4,6.9), carbonic anhydrase (6.0), β-lactoglobulin (5.3,5.2), trypsin inhibitor (4.5), glucose oxidase (4.2), amyloglucosidase (3.5).

A Beckman DU-7400 UV spectrophotometer was used for all ultraviolet-visible experiments. A Perkin Elmer Luminescence spectrophotometer LS50-B was used for all fluorimetric assays. A Gilson FPLC system consisting of a FC203B

fraction collector, two Minipuls3 peristaltic pumps, and a 112 UV/VIS detector was used for all column chromatography experiments.

CHAPTER III

METHODS

Rhodococcus sp. IGTS8 Growth Conditions

The methods described below were based on a procedure by Li (17). Basal Salt Media 2 (BMS2) contained the following per liter: KH_2PO_4, 4 g; NH_4NO_3, 3 g; $MgCl_2 \cdot 4H_2O$, 0.5 g; succinate, 1 g; glycerol, 10 g; ethanol, 8 g; $MnCl_2 \cdot 6H_2O$, 20 mg; $FeCl_2 \cdot 4H_2O$, 50 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.02 mg; $CuCl_2$, 0.02 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; $ZnCl_2$, 0.08 mg; $FeCl_3 \cdot 6H_2O$, 0.4 mg; $CaCl_2$, 20 mg; $MoCl_2$, 0.1 mg; and $2NiCO_3 \cdot 3Ni(OH)_2 \cdot 4H_2O$, 0.005 mg (pH adjusted to 7.3 with 2 M KOH). Minimal salt solution contained 4 g/L of KH_2PO_4 and 3 g/L of NH_4NO_3 with the pH adjusted to 7.3. All solutions were autoclaved or sterile filtered and stored at room temperature. The required nutrients and sulfur source (200 μM DBT) were added to the minimal salt solution just prior to inoculation.

Rhodococcus cells were maintained on nutrient agar at 30°C. Colonies were propagated on nutrient agar, re-streaking every two to three days. After incubation for 48 hours, cells were inoculated into overnight tubes containing 5 mL of nutrient media and incubated at 30°C with shaking for 48 hours, to stationary phase. The cells were then pelleted by centrifugation at 7000 x *g* for five minutes, washed twice with sterile minimal salt solution and inoculated into 250 mL of BMS2, with 200μM DBT serving as the sulfur source. The nutrient media with suspended cells was then incubated at 30°C with shaking for five days, until late log phase. The cells were harvested by centrifugation at 8000 rpm (Beckman J2-21) for one hour and washed twice with sterile minimal salts. The resuspended cells were centrifuged at 10,000 rpm for one hour. The pelleted *Rhodococcus* cells were stored at -70°C and the supernate was discarded.

Protein Measurements

Protein concentrations were determined by the A_{230}/A_{260} method developed by Kalb and Bernlohr (18). Absorbance at 230 nm and 260 nm were measured in 1 mL cuvettes on a Beckman DU 7400 UV-Vis spectrophotometer. Samples were diluted so that absorbances were less than 1.200 AU. Protein concentrations were then estimated using the following equation: μg/mL protein=$183(A_{230}) - 75.8(A_{260})$.

Substrate Synthesis

The synthesis of the substrate, HPBS, is based on the method provided by Gregory Mrachko of Energy Biosystems Corporation. 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 reacted overnight with stirring. The pH of the product, which is soluble, was adjusted to between eight and nine with HCl. The resulting 20 mM HPBS solution was then divided into 1 mL aliquots and stored at 4°C.

Fluorimetric Assay

The fluorimetric assay described below was based on a procedure outlined by Gray (9). HPBS desulfinase activity was determined by monitoring the formation of HBP using a Perkin Elmer Luminescence spectrophotometer LS50-B. One unit of activity was defined as the amount of protein, in mg, that catalyzed the formation of 1 μM of HBP per hour. To determine HBP concentration a standard curve from 0.1 μM to 10 μM HBP was generated to give the linear equation: y =75.05x + 92.04, r^2= 0.9997. The assays were performed in a 96 well plate. Each well contained 10-50 μL of HPBS desulfinase (15 μM), 20 μL of 1 mM HPBS (14 μM), and diluted with Buffer A (25 mM NaH_2PO_4, 100 mM NaCl, pH 7.4) to give a total volume of 220 μL. The emission of HBP was monitored at 410 nm (λ_{EX} = 288 nm) for 30 minutes. The concentration of HBP formed was determined from the standard curve.

Purification Scheme

Cell Lysis

Frozen *Rhodococcus* cells were thawed and resuspended in cold Buffer A, with 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), and 0.2 mg/mL DNAse. The suspension was kept on ice and passed through a Stansted Cell Disrupter Model A0512/116 (Energy Service Company, Washington D.C.) at 13-14,000 psi. Cell debris and unbroken cells were removed by centrifugation at 12,000 rpm for 1 hour at 4°C. The pellet was discarded; the supernate was labeled "crude cell extract" and used immediately.

FPLC

All FPLC procedures were performed at 4°C on a Gilson FPLC system. Eluted protein was detected continuously with a Gilson 112 UV-Vis detector and monitored at a wavelength of 280 nm. After each column the eluant was assayed for activity. The fractions with HPBS desulfinase activity were pooled for application the next column. From each group of pooled fractions a 0.5 mL sample was taken for further analysis.

Anion Exchange Chromatography

A Q Sepharose™ Fast Flow anion exchange column (Pharmacia, Piscataway, New Jersey) was equilibrated with 5 column volumes of Buffer A or until the eluant pH ≈7.4. The crude cell extract was loaded at 3.0 mL/minute. The bound

protein was eluted with a gradient of 100 mM to 450 mM NaCl. Fractions, one every two minutes, collected during the gradient were assayed for activity using the fluorimetric assay previously described. Those fractions showing activity were pooled and applied to the following column. The Q Sepharose™ column was washed with one liter of 1.5 M NaCl and regenerated with two liters of Buffer A.

Desalting/Buffer Exchange Chromatography

A P-6DG desalting column (Bio-Rad, Hercules, California) was equilibrated with five column volumes of 25 mM NaH_2PO_4 (pH 7.4). The pooled fractions from the Q Sepharose™ column were loaded at 1.5 mL/minute. The column was washed with 25 mM NaH_2PO_4 and 3 mL fractions were collected until the protein concentration of the eluent returned to baseline. The fractions were assayed and those demonstrating activity were pooled and applied to the following column. The P-6DG column was re-equilibrated with 500 mL of 25 mM NaH_2PO_4.

Hydroxyapatite chromatography

A Macro-Prep® Type 1 ceramic hydroxyapatite column (Bio-Rad, Hercules, CA) was equilibrated with five column volumes of 25 mM NaH_2PO_4 pH 7.4. The pooled fractions from the P-6DG column were loaded at 2.0 mL/min. Fractions were collected every two minutes until the protein of interest eluted. The fractions were assayed, and the fractions demonstrating activity were pooled for

application to the following column. The remaining bound protein was eluted with 200 mL of 400 mM KH_2PO_4, pH 7.4. The column was then re-equilibrated with 500 mL of 25 mM NaH_2PO_4.

Hydrophobic Interaction Chromatography

The pooled fractions from the hydroxyapatite column were brought to 1.7 M $(NH_4)_2SO_4$. The Toyopearl-phenyl650M column (Supelco, Bellefonte, PA) was equilibrated with five column volumes of 1.7 M $(NH_4)_2SO_4$ at pH 6.6. The sample was loaded at 3.0 mL/min. Bound protein was eluted with a decreasing salt gradient from 1.7 M NH_4SO_4 to 0 M NH_4SO_4, Buffer A. During the gradient fractions were collected every minute. The fractions with activity were pooled and applied to the following column. The Toyopearl-phenyl 650M column was washed with Buffer A and re-equilibrated with 1.7 M NH_4SO_4, pH 6.6.

Concentrating Sample

The pooled sample from the hydrophobic interaction column was concentrated via a Centriplus® YM-10 Centrifugal filter unit (Millipore Corp, Bedford, MA). The sample was centrifuged at 6,000 rpm for 60 minutes at 4°C. The retentate and filtrate were assayed for activity. The retentate was saved for application to the following column.

Gel Filtration Chromatography

A Superdex 75™ gel filtration column (Pharmacia, Piscataway, NJ) was equilibrated with five column volumes of buffer (25 mM NaH_2PO_4, 200 mM NaCl, pH 7.4). The concentrated sample was loaded at 0.3 mL/min. Fractions were collected every five minutes for 100 minutes, then every minute for the duration of the run. The fractions were assayed for activity. Those with activity were pooled and allowed to equilibrate at 4°C. The pooled sample was divided in 500 μL aliquots and stored at -70°C for further analysis. The column was re-equilibrated with two column volumes of buffer.

Gel Electrophoresis

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were prepared in reduced 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, 0.175 mM phenol red] and denatured by heating at 90°C for 10 minutes. The samples and the Mark12™ molecular weight standard were loaded into the wells of a NuPAGE™ pre-cast 4-12% bis-Tris polyacrylamide gel. The samples were electrophoresed with 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] for 30 to 40 minutes at 200 volts constant voltage.

Isoelectrofocusing Gel (IEF)

A 250 μL protein aliquot was injected into a Slide-A-Lyzer® 10K dialysis cassette (Pierce, Rockford,IL) which had been hydrated in buffer [25 mM NaH_2PO_4, 20 mM NaCl, pH 7.4] for 5 minutes. The protein was allowed to dialysize overnight. Samples were prepared by adding one part protein sample to one part NOVEX IEF Sample Buffer (2x) [20% 10x Cathode Buffer (3-7), 30% glycerol, 50% distilled water]. The buffers were prepared accordingly: one part NOVEX IEF Cathode Buffer (10x) [lysine (free base), 400 mM, pH 2.4] to nine parts distilled water, and one part NOVEX IEF Anode Buffer (50x) [phosphoric acid, 500 mM, pH 10.1] to 49 parts distilled water. Both buffers were de-gassed for 10 minutes under vacuum. The samples and the SERVA IEF pH standards were loaded into the wells of a pre-cast NOVEX pH 3-10 IEF gel. The samples were electrophoresed for one hour at 100 volts constant voltage, one hour at 200 volts constant voltage, 20 minutes at 300 volts constant voltage, 20 minutes at 400 volts constant voltage, and 20 minutes at 500 volts constant voltage.

N-Terminal Sequence Analysis

A 500 μL protein sample was thawed and injected into a Slide-A-Lyzer® cassette, which had been hydrated in distilled water. The sample was allowed to dialysize overnight against distilled water. The protein concentration was

determined by the A_{230}/A_{260} method (18). The sample was then made 1 mM in DTT, frozen and transported to the University of Texas at Austin Protein Microanalysis Facility (Austin, TX) for analysis.

Enzyme Characterization

Determination of Temperature Optima and Stability

A Perkin Elmer Luminescence spectrophotometer LS50B was equipped with a Microplate Heater (American Scientific Products, model # AGG-000-013) for temperature control. Temperature effects on protein activity were monitored over a range of 25°C to 60°C in 5°C increments. The reaction mixture, HPBS desulfinase and Buffer A, was allowed to equilibrate at the designated temperature for five minutes in the "initial rate" study and for 30 minutes in the "temperature stability" study. After incubation the substrate, 20 μL of 1 mM HPBS, was added to initiate the assay. Activity was monitored using the fluorimetric assay previously described. The relative activity for each temperature was then determined and used to gauge the optimal temperature.

Determination of pH Optima and Stability

The buffers used in the study were 20 mM citric acid–Na_2HPO_4 (pH range 3.5 – 6.0), 20 mM NaH_2PO_4 (pH range 6.0 – 8.0), 20 mM Tris-HCl (pH range 7.0 – 9.0), and 20 mM glycine-NaOH (pH range 8.0 – 10.5). All buffers were prepared

by dissolving the appropriate amount of reagent in distilled, deionized water; then titrating to the desired pH with 0.1 M HCl or 0.1 M NaOH. The pH of each reaction mixture was measured with an Orion Micro-Probe 9802 microelectrode (Beverly, MA). The reaction mixtures, consisting of HPBS desulfinase and buffer, were incubated for five minutes for the pH optima study and 30 minutes for the pH stability study. Each assay was initiated by the addition of substrate, 20 μL of 1.0 mM HPBS. Activity was monitored using the fluorimetric assay previously described. The relative activity at each designated pH was then used to determine the optimal pH.

Kinetic Studies

The kinetic parameters for HPBS desulfinase were determined by varying the concentration of the substrate HPBS. The concentrations that were evaluated were 0.5 μM, 0.8 μM, 1.0 μM, 2.0 μM, 3.33 μM, 5.0 μM, and 10 μM. Total protein concentration for each assay was 0.28 μM. Activity was monitored using the fluorimetric assay previously described. Fluorescence readings were taken every 15 seconds over a five minute period. Each assay was repeated three to five times. Data was fitted to Michaelis-Menten and Lineweaver-Burk parameters using EnzFitter version 2.014.0 (BIOSOFT®, Cambridge, UK).

Inhibition Studies

Product inhibition assays were used to determine if increasing concentrations of HBP or sulfite had any effect on HPBS desulfinase activity. A 10 mM stock

solution of HBP in ethanol and a 1 mM aqueous solution of Na_2SO_4 were prepared. These were then diluted to 1 mM in distilled water. Concentrations were varied from 10 μM to 100 μM and the effects were monitored by the fluorimetric assay.

The protein response to substrate and product analogs was also studied. Stock solutions were prepared accordingly: xanthene (0.0186 g), 2-hydroxydiphenylmethane (0.0184 g), 2-methoxybiphenyl (18 μL), phenylhydroquinone (0.0186 g), 2-biphenyl carboxylic acid (0.0198 g), 1-naphthalene sulfonic acid (0.0230 g), 2-amino-4-phenylphenol (0.0185 g), diphenic acid (0.0242 g), 4'-hydroxy-4-biphenyl carboxylic acid (0.0214 g), 1,8- naphthosultam (0.0205 g), and 2,2'-biphenol (0.0186 g) were each dissolved in 10 mL of ethanol to give a concentration of 10 mM. All reagents were then diluted to 1 mM with distilled water, except for 2-methoxybiphenyl which was diluted with ethanol. Reaction mixtures contained 10 to 20 μL of HPBS desulfinase (approximately 0.3 μM), 20 μL of 1 mM HPBS (90 μM), 22 μLof 1 mM analog (100 μM), and were diluted to a total volume of 220 μL with Buffer A, pH 7.4. Activity was monitored for 30 to 60 minutes using the fluorimetric assay described earlier. Rates were calculated relative to the assay run in the absence of inhibitor.

Metal Activation Studies

Aqueous 1 mM stock solutions for each of the following metal compounds was prepared as follows: $CaCl_2 \cdot 2\ H_2O$ (0.0015 g), $CoCl_2 \cdot 6\ H_2O$ (0.0023 g), $CuCl_2$

(0.0013 g), $FeCl_2 \cdot 4\ H_2O$ (0.0020 g), $MgCl_2 \cdot 6\ H_2O$ (0.0020 g), $MnCl_2 \cdot 4\ H_2O$ (0.0020 g), and $ZnCl_2$ (0.0014 g) were each dissolved in distilled water. Reaction mixtures consisted of 20 μL of 1 mM HPBS (90 μM), 10 μL of enzyme (0.15 μM), 30μL of 0.1 mM metal solution (14 μM), and were diluted to a volume of 220 μL with Buffer A, pH 7.4.

Chelating Agents

Stock solutions of the chelators and some nonchelating analogs were prepared accordingly: 1,10-phenanthroline (0.0180 g), 4,7-phenanthroline (0.0180 g), 2,2'-dipyridyl (0.0136 g), 4,4'-dipyridyl (0.0136 g), and 8-hydroxyquinoline (0.0145 g) were dissolved in 10 mL of ethanol to give a final concentration of 10 mM. A 10 mM aqueous solution of ethylenediamine tetra-acetic acid (EDTA, 0.0380g) was also prepared. An aliquot of each stock solution was diluted to 1 mM with distilled water. Reaction mixtures contained 10 to 20μL of enzyme (0.20μM), 20 μL of 1 mM HPBS (90 μM), 22 μL of the chelating reagent (100 μM), and were diluted to a volume of 220 μL with Buffer A, pH 7.4. Activity was monitored using the fluorimetric assay discussed previously.

Chemical Modification Studies

The chemical modification reagents were prepared accordingly: phenyl-glyoxalhydrate (0.0134 g), pyridoxal-5-phosphate (0.0265 g), N-

bromosuccinimide (0.0178 g), 5,5'-dithiobis(2-nitrobenzoic acid) (0.0396 g), and tetranitromethane (9 μL) were dissolved in 10 ml of ethanol. Each reagent was further diluted to 1 mM with distilled water. Reaction mixtures contained 300 nM HPBS desulfinase, Buffer A, and 10-100 μM chemical modification reagent. Each reaction mixture was divided into two equal sets. The first set of samples was made 100 μM in HPBS (excess substrate) and immediately assayed for activity. The second set was allowed to equilibrate for 45 minutes before the assay was initiated by the addition of substrate (100 μM HPBS). Relative activity of HPBS desulfinase with the chemical modification reagents in the presence or absence of excess substrate was determined.

CHAPTER IV

RESULTS AND DISCUSSION

Purification of HPBS desulfinase

The purification protocol outlined in the Methods section was a modification of the procedure reported by Gray (9). In Gray's procedure the crude cell extract was first applied to a Q-Sepharose™ anion exchange column and bound protein was eluted by an increasing salt gradient. The active fractions were dialyzed overnight, and then applied to dye ligand column connected in line with a Source30Q® (Pharmacia, Piscataway, NJ) anion exchange column. Fractions with activity were desalted by various means and applied to a Toyopearl-phenyl 650M hydrophobic interaction column for final purification. Numerous attempts using this procedure failed to yield purified HPBS desulfinase. Ohshiro *et al.,* who successfully purified both the DszC and DszA proteins, also reported difficulties with Gray's protocol (10,11).

Gray's procedure was originally developed to purify all four of the proteins in the desulfurization pathway while the goal of this study was to isolate HPBS

desulfinase, so some modifications were expected. Those modifications and the evolution of the purification procedure utilized in this research are described as follows. Cell lysis was achieved by means of a Stansted cell disrupter instead of a French pressure cell. The cell disrupter allows for a continuous flow which saves time and minimizes sample handling. The cell disrupter also provides a more homogenous lysate.

The dye ligand column was determined to be superfluous and was subsequently eliminated from the protocol. The sole purpose for originally employing a dye ligand column was to bind the DszC protein and separate it from the other proteins in the pathway. However results indicate the majority of DszC can be effectively separated from HPBS desulfinase during the gradient of the Q-Sepharose anion exchange column. Figure 3 represents a typical elution profile for the Q-Sepharose™ column. Fractions containing DszC and DszA generally elute at higher salt concentrations although there is usually some overlap of the peaks

All desalting and buffer exchanges were accomplished by means of a P-6DG column instead of dialysis. This change was made to shorten the preparative time and to reduce sample dilution. P-6DG media achieves desalting and buffer exchange by separating macromolecules from smaller compounds (less than 6,000 Da) through gel filtration. Figure 4 represents a typical elution profile for the P-6DG column. After HPBS desulfinase elution, a sharp peak and significant tailing was observed. These fractions contained a negligible amount of desulfinase activity and were not collected into the P-6DG pooled sample.

Source30Q is a refined (30μm beads), highly uniform anion exchange media usually applied towards the end of purification for finer resolution. Preliminary results indicated that while the Source30Q column was effective at concentrating the protein sample very little purification was achieved. Greater success resulted from the use of a hydroxyapatite column, which consistently produced a 40-60 fold increase in purification and replaced the Source30Q in the protocol. Figure 5 represents a typical elution profile for the Macro-Prep® Type 1 ceramic hydroxyapatite column. HPBS desulfinase does not bind to the hydroxyapatite media and is eluted in the low, broad peak near the beginning of the profile, typically between 5 and 45 minutes. The protein sample collected from the P-6DG column tends to be an orange-buff color. The hydroxyapatite column effectively removes most chromophores and the fractions that contain activity are colorless. The sharp peak towards the end of the profile represents the bound chromophores, which are removed from the column with 400 mM KH_2PO_4.

The Toyopearl-phenyl 650M hydrophobic interaction column was used as described by Gray (9) and was quite effective, generally resulting in 200 to 300 fold increases in purification. However, there were some persistent problems with reproducibility. These were eliminated by reducing the pH of the 1.7 M AMS buffer to 6.6 and gaining better control of the gradient through use of a FPLC system. Figure 6 represents a typical elution profile for the Toyopearl-phenyl column. Despite the increase in purification the sample was still not pure according to SDS-PAGE (Figure 7). A final purification step was necessary.

Several methods were considered but gel filtration via a Superdex 75 column was the first method attempted and proved to be quite successful. Figure 8 represents a typical elution profile for the Superdex 75™ column. Typically there is close correspondence between the number of bands in the SDS-PAGE gel of the pooled fractions from the Toyopearl-phenyl 650M and the number of peaks in the Superdex 75 elution profile. HPBS desulfinase was purified to homogeneity as demonstrated by SDS-PAGE (Figure 9). A summary of the purification procedures is given in Table 1.

The purified HPBS desulfinase appeared as a single band on the SDS-PAGE gel with an estimated molecular mass of 40 kDa which corresponds with the mass derived from the reported amino acid sequence (9). The N-terminal sequence of the first 12 amino acids was determined to be Thr-Ser-Arg-Val-Asp-Pro-Ala-Asn-Pro-Gly-Ser-Glu which agrees completely with the amino acid sequence deduced from the *DszB* gene sequence (13). The protein was colorless when isolated indicating that there were no tightly bound chromophores. IEF gel electrophoresis suggests an approximate pI of 5.6 (Figure 10).

Determination of Temperature Optima and Stability

The effect of incremental increases in temperature on HPBS desulfinase activity was monitored over time. The reaction mixtures were prepared according to the procedure outlined in the Methods section. Activity was

determined by means of the fluorimetric assay previously described. Results of the "initial incubation" study and the "Temperature Stability" study are shown in Figures 11 and 12 respectively. The optimum temperature was determined to be 35°C and the protein is stable at this temperature for at least 30 minutes. This is identical to the optimum temperature of DszA and the NADH-FMN oxido-reductase (10,11). The DszC protein has optimum temperature of 40°C (12). All subsequent assays were run at 35°C.

Determination of pH Optima and Stability

The buffers used in this study and the reaction parameters are described in detail in the Methods section. Protein activity was monitored over a range of pH 3.5 to pH 9.7 for 30 minutes using the fluorimetric assay previously described. The first set of experiments were designated the "initial rates" pH optima study and the results are shown in Figure 13. The second set of experiments were designed to determine the pH stability of HPBS desulfinase. The assays were equilibrated for 30 minutes in each of the buffers and the results are shown in Figure 14. The optimum pH was determined to be pH 7.5. The optimum pH for DszC, DszA, and NADH-FMN oxidoreductase were reported to be 8.0, 7.5, and 6.0 respectively (10,11,12).

Kinetics Analysis

Kinetic parameters were determined by data collected during initial rate studies. Assays of varying concentrations of substrate (HPBS) were monitored by the fluorimetric assay. A detailed description of the assays is provided in the Methods section. Initial velocity was calculated by linear regression analysis of the data points collected in the first 90 seconds of each assay. Initial velocity data for all replications of each substrate concentration is presented in Table 2 Data were fit to Michaelis-Menten parameters and plotted by EnzFitter version 2.014.0 (BIOSOFT®, Cambridge, UK). Observed velocity versus substrate concentration, [S], is shown in Figure 15. Predicted velocity versus substrate concentration is shown in Figure 16. The K_m and V_{max} of HPBS desulfinase were determined to be 0.904 ± 0.15 μM and 55.5 ± 2.33 μmole min^{-1} mg^{-1} (333 μM min^{-1}) respectively, resulting in a k_{cat} of 198 min^{-1}.

Gray reports the HPBS desulfinase kinetic parameters as follows: K_m = 1 μM, V_{max} = 0.7 μM min^{-1}, and k_{cat} = 2 min^{-1} (9). The K_m values are statistically equivalent but there are significant differences between the velocity and turnover rates. As noted previously, Gray concludes the purification with the Toyopearl-phenyl 650M hydrophobic interaction column. Activity assays of Toyopearl-phenyl 650M fractions showed significant inhibition originally thought to be due to the build up of the HBP product. This inhibition disappears upon further purification by the Superdex 75™ column.

In neutral solution HPBS can be converted, non-enzymatically, to biphenyl-2-sulfinic acid (BPSi) and further to biphenyl-2-sulfonic acid (BPSO) (10). It has

been demonstrated that DszA, in the presence of NADH-FMN oxidoreductase, can utilize either BPSi or BPSO to generate 2,2-dihydroxybiphenol (DHBP) (10,19). This alternative pathway is shown in Figure 17. DHBP has been shown to be an inhibitor of HPBS desulfinase, see Table 4. The molecular mass DszA and the NADH-FMN oxidoreductase have been reported as 50 kDa and 25 kDa, respectively (9). The SDS-PAGE of the Toyopearl-phenyl 650M column fractions indicate bands approximating these molecular masses (Figure 7). It is conceivable that enough NADH is present in the reaction mixture to activate this alternative pathway for DszA, producing DHBP, and thus inhibiting HPBS desulfinase activity. Perhaps Gray's reduced kinetic values are due to this inhibition phenomenon.

Statistical data for K_m and V_{max} determination is provided in Table 3. A large difference between the observed and predicted velocities at the lower substrate concentrations was noted. At these decreasing substrate concentrations the production of HBP is approaching the limit of detection of the LS50B Perkin-Elmer Luminescence spectrophotometer.

Product Inhibition Studies

As noted previously, HPBS desulfinase catalyzes the desulfination of HPBS to produce HBP and inorganic sulfite (Figure 2). The effects of increasing concentrations of each of the products were studied to determine if product inhibition is a factor in HPBS desulfinase activity *in vitro*. Solutions of HBP and

Na_2SO_3 were prepared as described in the Methods section. The concentration range for each product was 10 μM to 100 μM, well in excess of the protein concentration. Activity was monitored using the fluorimetric assay described earlier. The results of this study, presented in Table 4, indicate that these concentrations of HBP and Na_2SO_3 have no apparent inhibitory effect on protein activity.

The Effects of Various Analogs on Activity

Isosteric analogs of HPBS and /or HBP were used to determine the structural elements important for substrate binding. All analogs surveyed consisted of two phenyl rings with various substitution patterns. The compounds were divided into three groups: HBP analogs, with substitution on only one of the phenyl rings; HPBS analogs, with a functional group substituted on each ring; and organosulfur analogs. The structures of each of the compounds are shown in Figures 18-20 and the results are discussed below.

Product Analogs

Several analogs of HBP were tested for their ability to act as inhibitors. These analogs included xanthene, 2-hydroxydiphenylmethane, 2-methoxybiphenyl, phenylhydroquinone, 2-biphenyl carboxylic acid, and 2-amino-4-phenylphenol. The structures of each compound are shown in Figure 18.

Stock solutions of the HBP analogs were prepared as described in the Methods section. Activity was monitored by way of the fluorimetric assay described earlier. Results are presented in Table 4.

The only HBP analog to display inhibitory behavior was 2-methoxybiphenyl. This was determined to be caused by the solvent rather than any interaction between the compound and the protein. All analogs were initially made 10 mM in ethanol and subsequently diluted to 1 mM with distilled water, except for 2-methoxybiphenyl which is completely insoluble in water and was diluted in ethanol. Further investigation revealed that while a reaction mixture containing 1% ethanol has no effect on activity; a reaction that is 10% ethanol inhibits activity significantly.

Substrate Analogs

Several analogs of HPBS were tested for their ability to bind to the active site. These substrate analogs included 2,2'-biphenol, 4'-hydroxy-4-biphenyl carboxylic acid, and diphenic acid. The structures of each compound are presented in Figure 19. Stock solutions of the HPBS analogs were prepared as described in the Methods section. Activity was determined by the fluorimetric assay described previously. Relative activity for each analog is shown in Table 4.

Results indicate that compounds with a hydrogen bond acceptor on each of the phenyl rings can bind effectively to the active site. The pattern of substitution does not appear to be a factor. Interestingly, these effects were also demonstrated by the chelating agents discussed later.

Organosulfur Analogs

Several sulfur containing compounds were tested for their ability to inhibit enzyme activity. The analogs included 1-naphthalene sulfonic acid, 6-hydroxy-2-naphthalene sulfonic acid, 1,8-naphthosultone, and 1,8-naphthosultam The structures of each compound are shown in Figure 20. Stock solutions were prepared as described in the Methods section. Activity was determined by the fluorimetric assay described previously. Relative activity for each compound is shown in Table 4.

Three of the four compounds studied inhibited HPBS desulfinase activity. The exception being 1,8-naphthosultone. Structurally 1,8-naphthosultone is very similar to 1,8-naphthosultam, see Figure 20. The reason for this demonstrated inhibitory effect is not clearly understood at this point. Interestingly, 1,8-naphthosultone (BPSO) is an alternate substrate for the DszA enzyme (10), Figure 17.

The inhibitory effect of 1-naphthalene sulfonic acid was also somewhat puzzling. It has been demonstrated that compounds with substitution on only one of the rings were not effect inhibitors. Perhaps the rigidity of the naphthalene structure allows the oxygens on the sulfonic acid functionality to interact sufficiently with the binding site to result in inhibition.

Metal Activation Studies

Proteins that cleave carbon-sulfur bonds are poorly understood. Proposed mechanisms for those that are known are generally modeled on either lyase enzymes, which cleave carbon-carbon or carbon-nitrogen bonds. In both of these models bond cleavage is facilitated through the use of a cofactor capable of stabilizing the transition-state through electrostatic interaction. Metal cofactors are commonly associated with phosphoryl-transfer enzymes. The effect of various ions, routinely found in biological systems, on HPBS desulfinase activity was studied so that a better understanding of the mechanism might be established.

Aqueous solutions of the metal ions studied were prepared as described in the Methods section. Activity was monitored using the fluorimetric assay previously described. Relative activity for each metal compound is shown in Table 5.

The alkaline earth metals are represented by Ca^{2+} and Mg^{2+}. Magnesium had no noticeable effect, while calcium consistently enhanced activity slightly. This is the opposite of what was expected for an intercellular protein. Magnesium is transported into the cytosol in exchange for calcium, which is transported into the extra-cellular matrix. Therefore most proteins activated but calcium tend to be extra-cellular. In these proteins the calcium ions aid in maintaining the required enzymatic structure, perhaps this stabilizing effect explains the increase in catalytic activity exhibited by HPBS desulfinase.

In biological systems, the concentrations of transition metals are quite low due to their toxicity. Higher concentrations can lead to the development of reactive oxidative species that can be very destructive to cellular processes (20) The concentration of metals in this study was approximately 14 μM and the results are summarized as follows: manganese had no apparent effect, iron was shown to be slightly inhibitory, cobalt had no initial effect but after 40 minutes activity began to decline, zinc reduced activity by 50% and copper completely inhibited the protein (Figure 21).

Metal activation studies were also conducted on DszA by Ohshiro *et al.* (10). DszA is the second enzyme in the pathway and catalyzes the conversion of DBT sulfone to HPBS. Comparatively, both proteins responded in a similar fashion to the presence of Fe^{2+}, Co^{2+}, Cu^{2+} and Mg^{2+} ions. The effects of Mn^{2+}, Ca^{2+} and Zn^{2+} on DszA were the opposite of those demonstrated by HPBS desulfinase.

The Effects of Various Chelating Agents

It has been demonstrated the HPBS desulfinase activation does not require the addition of a metal cofactor and this is consistent with what has been reported with regards to the other proteins in the pathway (10,11,12). However, to insure that HPBS desulfinase is not a metalloenzyme, further studies were required. Metalloenzymes tend to bind their metal cofactors so strongly that they are retained by the protein even after purification. The chelators 2,2'-dipyridyl, 8-hydroxyquiniline, 1,10-phenanthroline and ethylenediamine tetra-acetic acid

(EDTA) are capable of inhibiting metalloenzymes by either removing the metal or by forming a complex with the metal *in situ* (21). The structures of the chelators and some nonchelating analogs used in this study are shown in Figure 22.

Solutions of the chelating reagents were prepared according to the procedure outlined in the Methods section. Activity was determined by means of the fluorimetric assay described previously. Results are presented in Table 6.

The chelators 2,2'-dipyridyl, 8-hydroxyquiniline and 1,10-phenanthroline all demonstrated a considerable inhibitory effect. Structurally and electronically these compounds resemble the HPBS analogs discussed previously (Figure 19). In order to determine if the inhibition was due to their chelating capacity or simply a result of their structural properties, nonchelating analogs were studied (Figure 22). Results (Table 6) indicate that the chelators tested and their analogs have similar inhibitory effects on HPBS desulfinase activity. This suggests that the observed inhibition is related to their structure and not to their chelating ability.

Incubation with EDTA had no discernible effect on HPBS desulfinase activity. Based on this and the studies previously discussed it has been determined that HPBS desulfinase does not require a metal cofactor for catalysis. Gray reports that EDTA also did not inhibit either DszC or DszA (9). This data is disputed by Ohshiro (10) whose results indicate that EDTA decreases the activity of DszA by 50%. No explanation for this discrepancy was presented.

Chemical Modification

Chemical modifiers react with specific amino acid side chains resulting in a

loss of protein catalytic activity. However, it is difficult to determine if this loss is due to the modification of an amino acid in the active site or other amino acids involved in maintaining protein structure. To distinguish between these two situations, reactions were carried out in the presence of excess amounts of substrate (HPBS) and compared to reactions incubated without substrate. The excess substrate serves to protect the amino acid residues in the binding site from the modifying agent. If the protein retains activity while the active site is protected, but loses activity when the active site is not protected by excess substrate; it is an indication that the modified amino acid maybe present in the active site.

The chemical modifiers used in this study were prepared as described in the Methods section. Activity was monitored by the fluorimetric assay described earlier. The modifying agents, the amino acid residues they affect and the Results are presented in Table 7.

Initially various concentrations of modifiers were reacted with the enzyme in the presence of excess HPBS to gauge their effectiveness. Phenylglyoxal hydrate (an arginine modifier) and pyridoxal-5-phosphate (a lysine modifier) showed minimal inhibitory effect up to 100μM. 5,5-dithiobis(2-nitrobenzoic acid) (DTNB-a cysteine modifier), N-bromosuccinimide (a tryptophan modifier) and tetranitromethane (a tyrosine modifier) produced significant inhibition down to a concentration of 1 μM.

Assays were conducted using 100 μM and 10 μM of each modifier. In the first set of assays HPBS was added immediately to the reaction mixture to initiate the

assay. These reaction rates were compared to the rates of assays which were allowed to incubated with the modifying agent for 45 minutes. Preliminary results indicate an active site residue susceptible to PLP and phenylgloxal hydrate. This suggests that lysine, cysteine and/or arginine may be present in the substrate binding site. However, the experiment as conducted was not conclusive and the possibility of tyrosine or tryptophan being present remains. Furthur investigation is required.

DBT

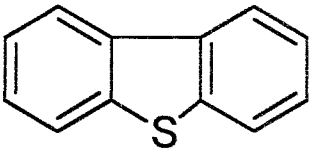


Figure 1. Dibenzothiophene

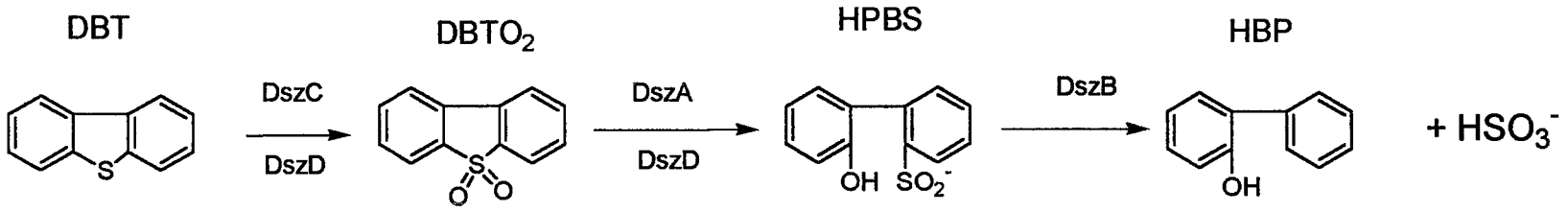


Figure 2. Dibenzothiophene (DBT) sulfur-specific degradation pathway.

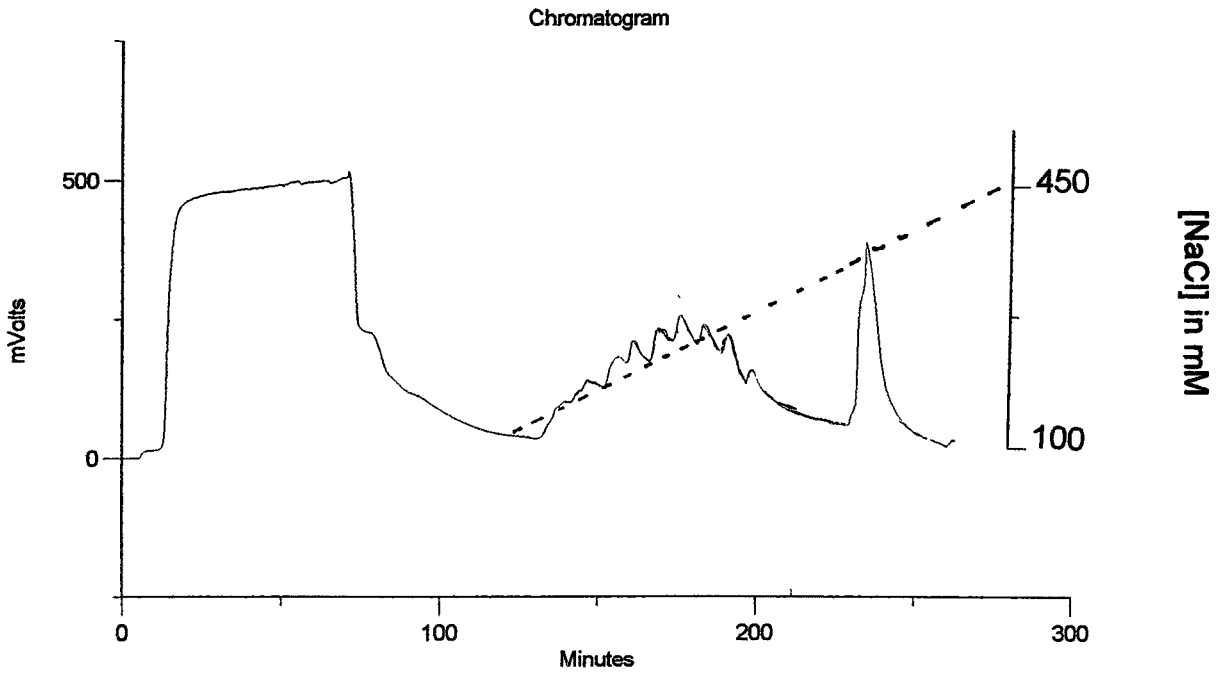


Figure 3. Q Sepharose™ anion-exchange column of crude cell extract. The Pharmacia Q Sepharose™ column was equilibrated with 10 column volumes of 25 mM sodium phosphate, 100 mM NaCl, pH 7.4 (Buffer A). The crude cell extract, 450 mL, was loaded at flow rate of 3.5 mL/minute and eluted with a linear 100-450 mM NaCl gradient. The protein concentration of the eluant was monitored at A_{280}. Fractions of 7 mL were collected every two minutes from the start of the gradient. The collected fractions were assayed for HPBS desulfinase activity. Fractions 22-29 (168-174 minutes) were pooled. The solid line represents the column profile. The dashed line represents the NaCl gradient.

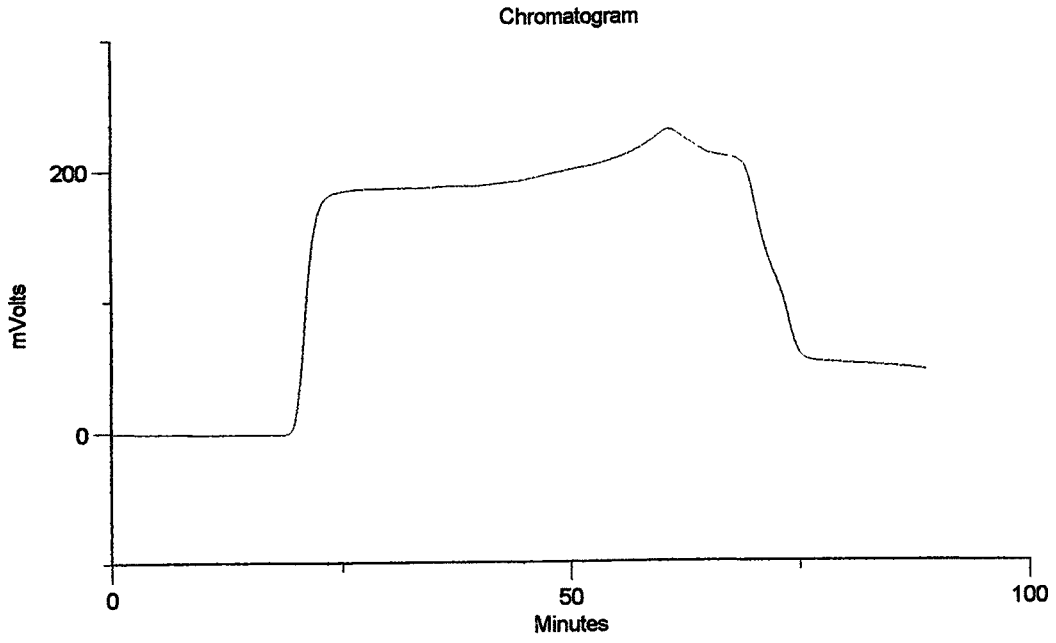


Figure 4. Desalting/Buffer exchange of Q Sepharose™ pooled sample. The pooled protein sample was loaded onto a Bio-Rad P-6DG column equilibrated with 5 column volumes of 25 mM sodium phosphate, pH 7.4, at a flow rate of 1.5 mL/minute. Fractions of 3 mL were collected every two minutes. The protein concentration of the eluant was monitored at A_{280}. Fractions collected between 25-67 minutes were pooled.

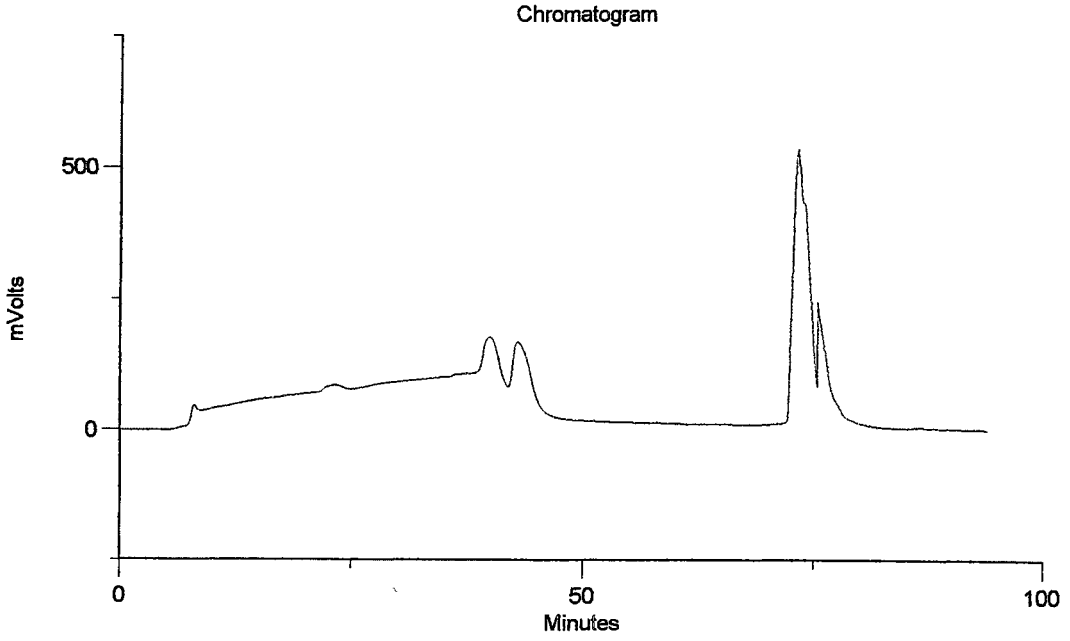


Figure 5. Hydroxyapatite column of P-6DG pooled sample. The pooled sample collected from the P-6DG column was loaded at 2.0 mL/minute onto a Bio-Rad Macro-Prep® Type 1 hydroxyapatite column which had been equilibrated with 5 column volumes of 25 mM sodium phosphate pH 7.4. The protein concentration of the eluant was monitored at A_{280}. Fractions of 4.0 mL were collected every two minutes and assayed for HPBS desulfinase activity. Fractions collected between 7-36 minutes were pooled. The column was washed with 400 mM potassium phosphate, pH 7.4, to remove remaining bound protein.

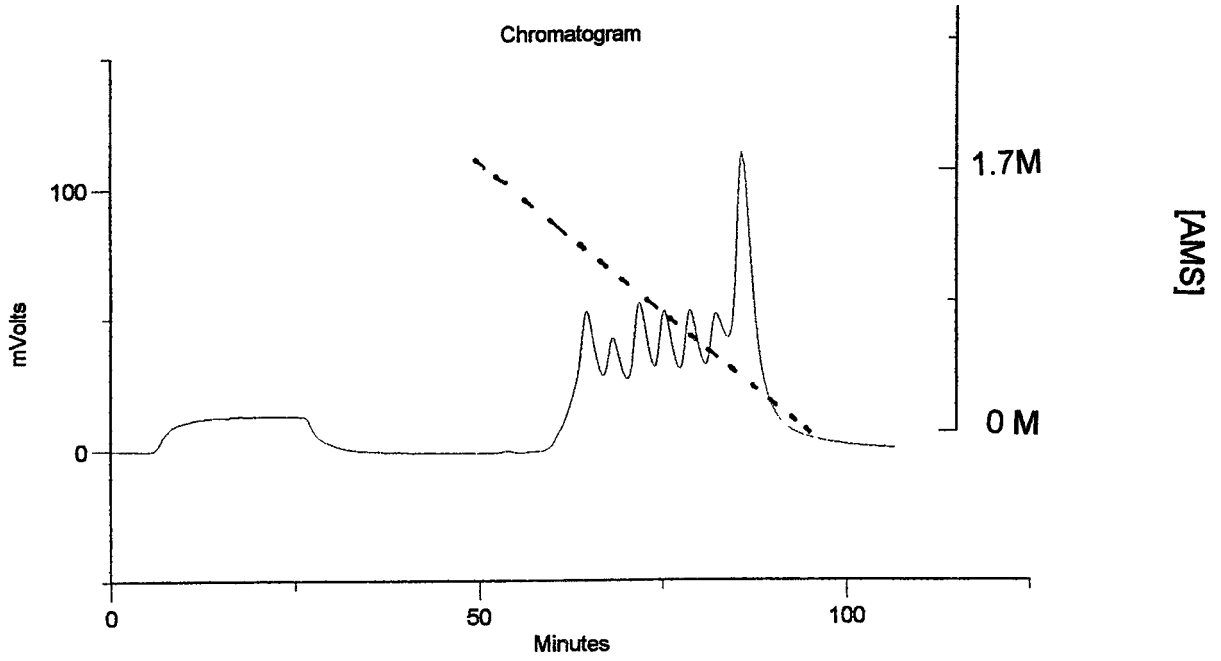


Figure 6. Hydrophobic interaction column of hydroxyapatite pooled sample. The pooled sample from the Macro-Prep® Type 1 column was load onto a Pharmacia Toyopearl-phenyl 650M column equilibrated with 5 column volumes of 1.7 M ammonium sulfate (AMS), pH 6.5. The protein concentration of the eluant was monitored at A_{280}. The column was eluted with a linear 1.7-0 M AMS gradient at a flow rate of 3.0 mL/minute. Fractions of 3.0 mL were collected every minute. The fractions were assayed for HPBS desulfinase activity. Fractions 27-32 (72-77 minutes) were pooled. The solid line represents the column profile; and the dashed line is the AMS gradient.

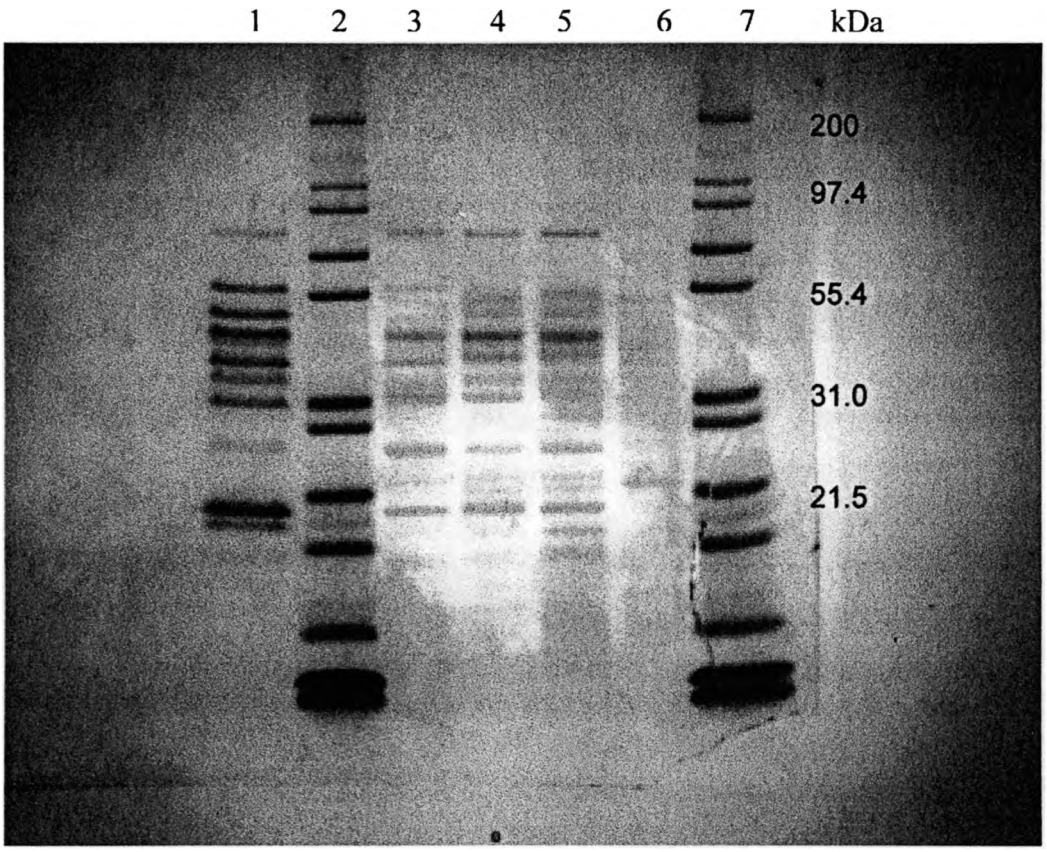


Figure 7. SDS-polyacrylamide gel electrophoresis of an earlier protein purification protocol. Aliquots of the collected fractions for each column were diluted in reduced SDS-PAGE sample buffer. The samples were electrophoresed and the protein bands were detected by colloidal Coomassie staining. Lane 1, Toyopearl-phenyl 650M fraction (1.4 μg); Lane 2, Mark 12® (Novex) molecular weight standard; Lane 3, hydroxyapatite fraction (1.7 μg); Lane 4, P-6DG fraction (2.5 μg); Lane 5, Q-sepharose fraction (2.5 μg); Lane 6, crude cell lyse (2.5 μg); Lane 7, Mark 12® (Novex) molecular weight standards.

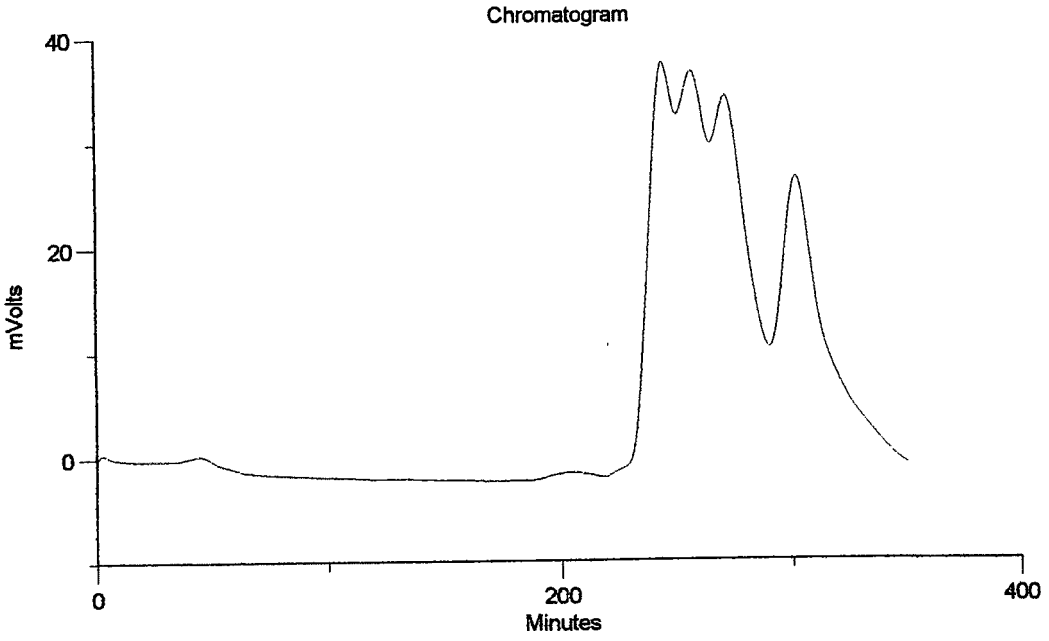


Figure 8. Gel Filtration of Toyopearl-phenyl 650M pooled sample. The pooled sample from the Pharmacia Toyopearl-phenyl 650M column was concentrated to 4.7 mL and loaded onto a Superdex 75 gel filtration column equilibrated with 5 column volumes of 25 mM sodium phosphate, 200 mM NaCl pH 7.4. The protein concentration of the eluant was monitored at A_{280}. Proteins were eluted at a flow rate of 0.5 mL/minute with 2.0 mL fractions being collected every four minutes. The fractions were assayed for HPBS desulfinase activity. Fractions 75-78 (297-313 minutes) were pooled, labeled DszB, dated and stored at -70°C.

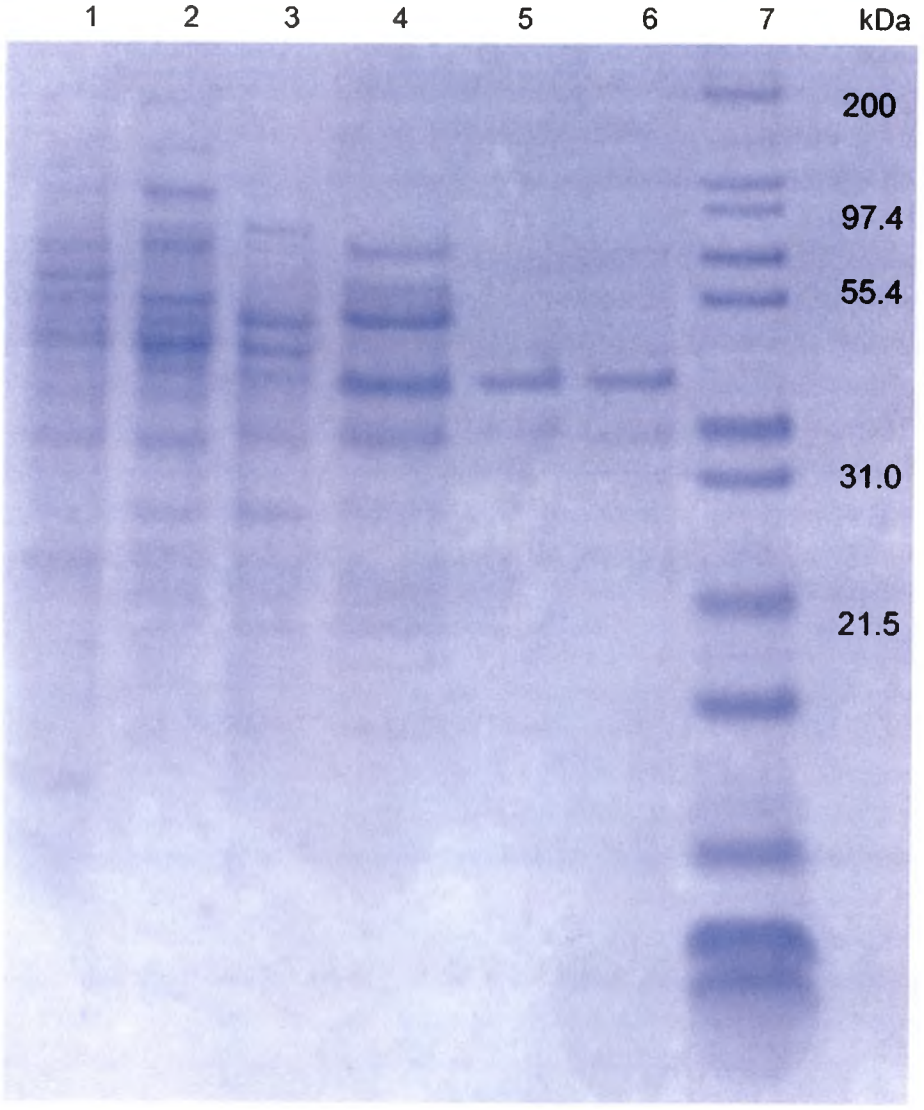


Figure 9. SDS-polyacrylamide gel electrophoresis of the refined protein purification protocol. Aliquots of the collected fractions for each column were diluted in reduced SDS-PAGE sample buffer. The samples were electrophoresed and the protein bands were detected by colloidal Coomassie staining. Lane 1, crude cell lyse (2.5 μg); Lane 2, Q-sepharose fraction (2.5 μg); Lane 3, hydroxyapatite fraction (2.5 μg); Lane 4, Toyopearl-phenyl 650M (1.9 μg); Lane 5, Superdex75 fraction B (1.2μg), Lane 6, Superdex75 fraction A (1.3 μg); Lane 7, Mark 12® (Novex) molecular weight standards.

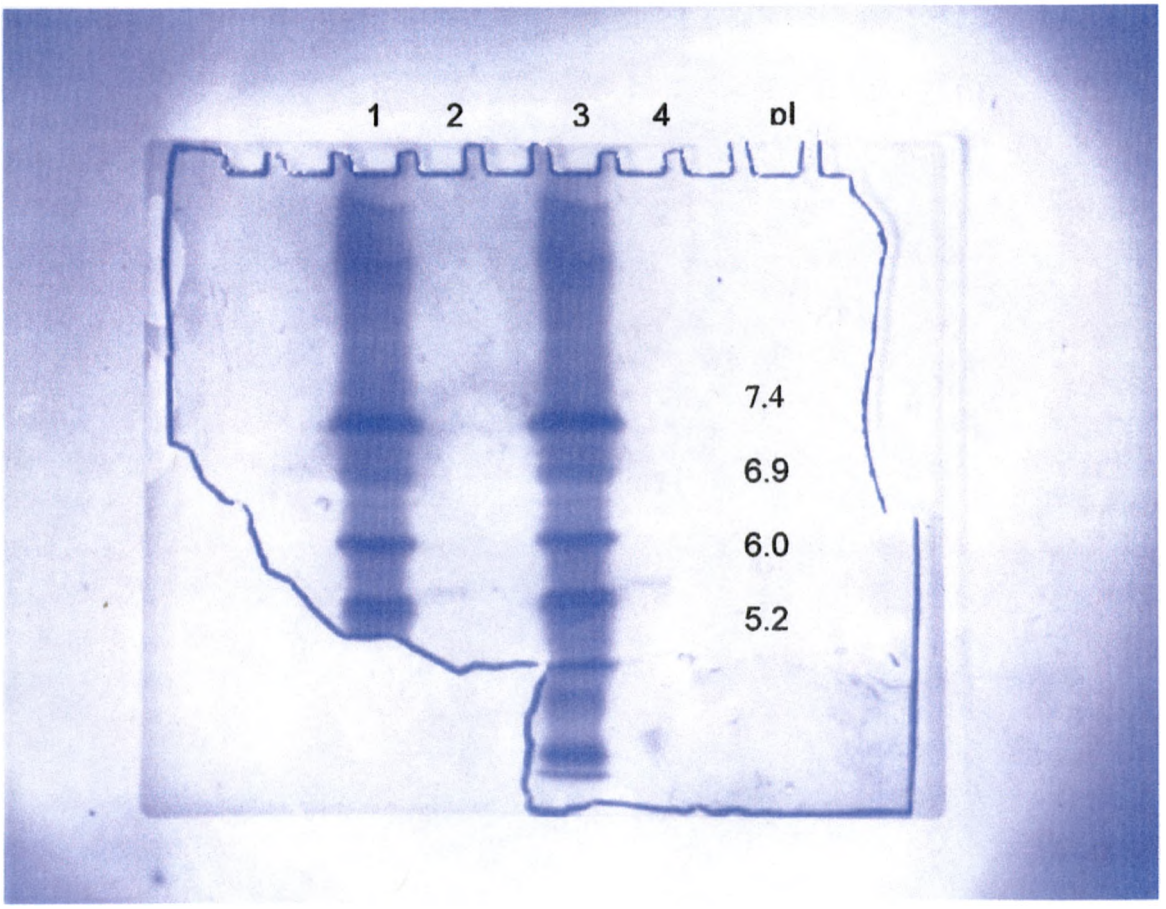


Figure 10. Isoelectric focusing-polyacrylamide gel electrophoresis of purified HPBS desulfinase. Samples of purified HPBS desulfinase diluted in IEF sampling buffer. The samples were electrophoresed through an IEF-PAGE gel and the protein bands were detected by colloidal Coomassie staining. Lane 1, pI 3-10 standards (Novex); Lane 2, purified HPBS desulfinase (5μg); Lane 3, pI 3-10 standards (Novex); Lane 4, purified HPBS desulfinase (5μg).

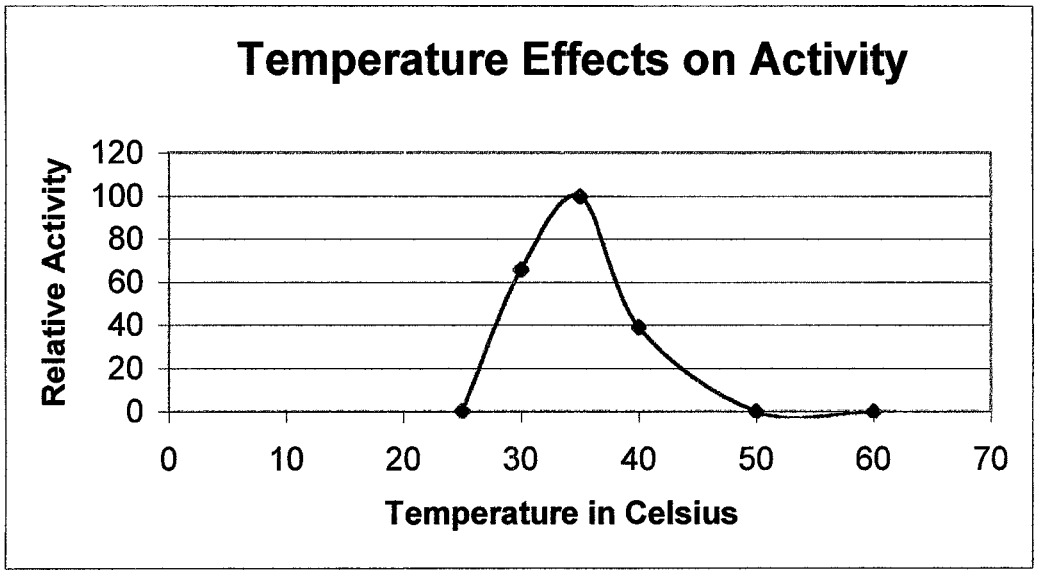


Figure 11. Temperature optima study of HPBS desulfinase. Samples of HPBS desulfinase were diluted to 150 nM in Buffer A. Each plate was incubated at the designated temperature for five minutes before the initiation of the assay. The temperatures ranged from 25-60°C in 5°C increments. Activity was monitored by the fluorimetric assay described in the Methods section. 100% Relative activity is equal to 3.24 Units/mg.

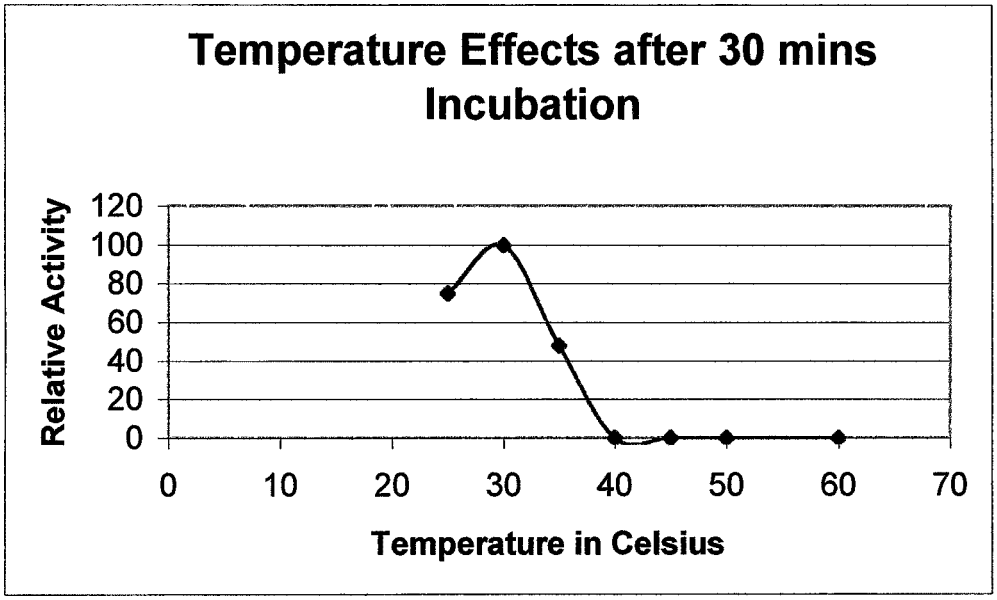


Figure 12. Temperature stability study of HPBS desulfinase. Samples of HPBS desulfinase were diluted to 150 nM in Buffer A. Each plate was incubated at the designated temperature for 30 minutes before the initiation of the assay. The temperatures ranged from 25-60°C in 5°C increments. Activity was monitored by the fluorimetric assay described in the Methods section. 100% Relative activity is equal to 1.74 Units/mg

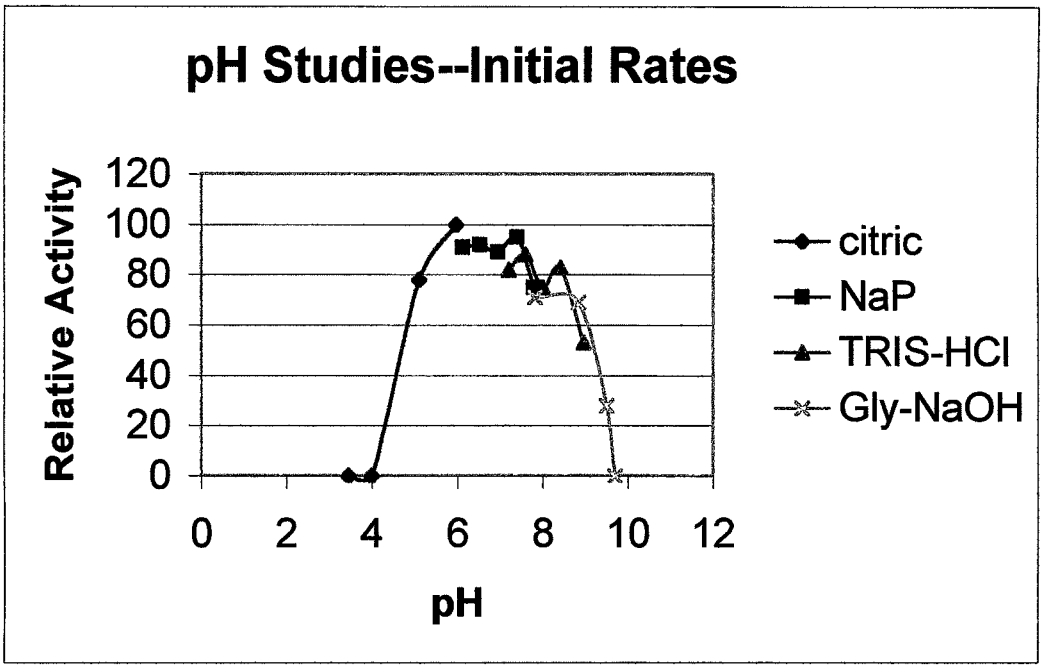


Figure 13. pH optima determination for HPBS desulfinase. Samples of HPBS desulfinase were diluted to 150 nM in a series of buffers to give equal concentrations of enzyme at designated pHs. The buffers used included 20 mM citric acid (pH 3.5-6), 20 mM sodium phosphate (pH 5-8), 20 mM TRIS-HCl (pH 7-9.5), 20 mM glycine-NaOH (pH 8-10). Each reaction was allowed to equilibrate for five minutes before initiation of the assay. Activity was measured by using the fluorimetric assay described in the Methods section. 100% Relative activity is equal to 2.45 Units/mg.

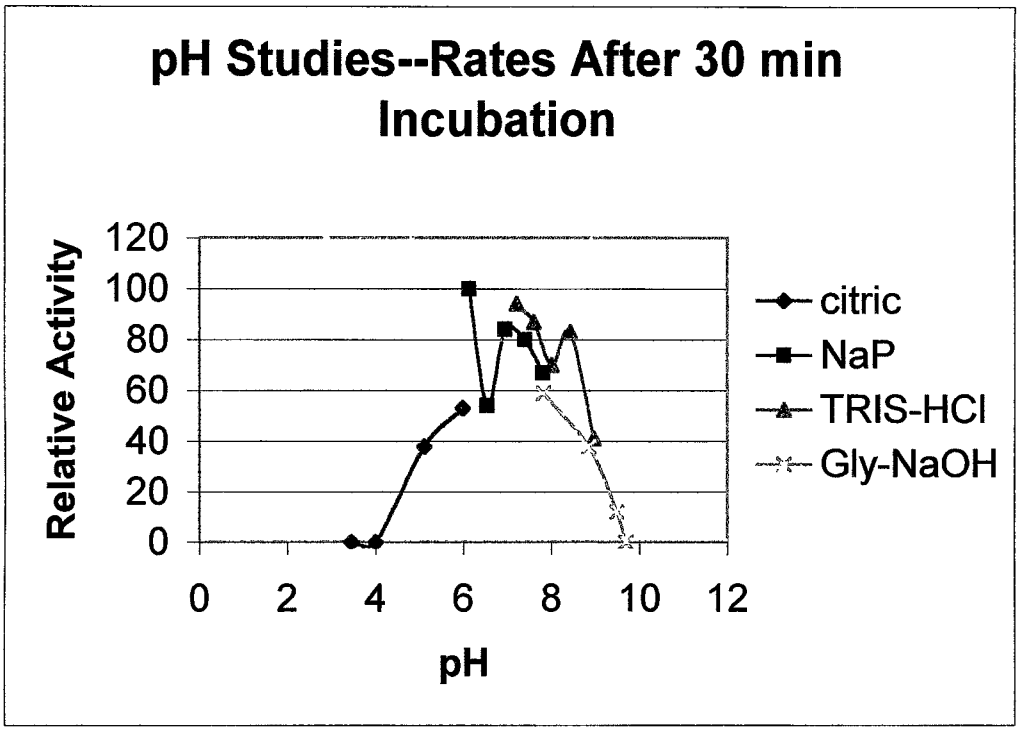


Figure 14. pH optima determination for HPBS desulfinase. Samples of HPBS desulfinase were diluted to 150 nM in a series of buffers to give equal concentrations of enzyme at designated pHs. The buffers used included 20 mM citric acid (pH 3.5-6), 20 mM sodium phosphate (pH 5-8), 20 mM TRIS-HCl (pH 7-9.5), 20 mM glycine-NaOH (pH 8-10). Each reaction was allowed to equilibrate for 30 minutes before initiation of the assay. Activity was measured by using the fluorimetric assay described in the Methods section. 100% Relative activity is equal to 1.59 Units/mg.

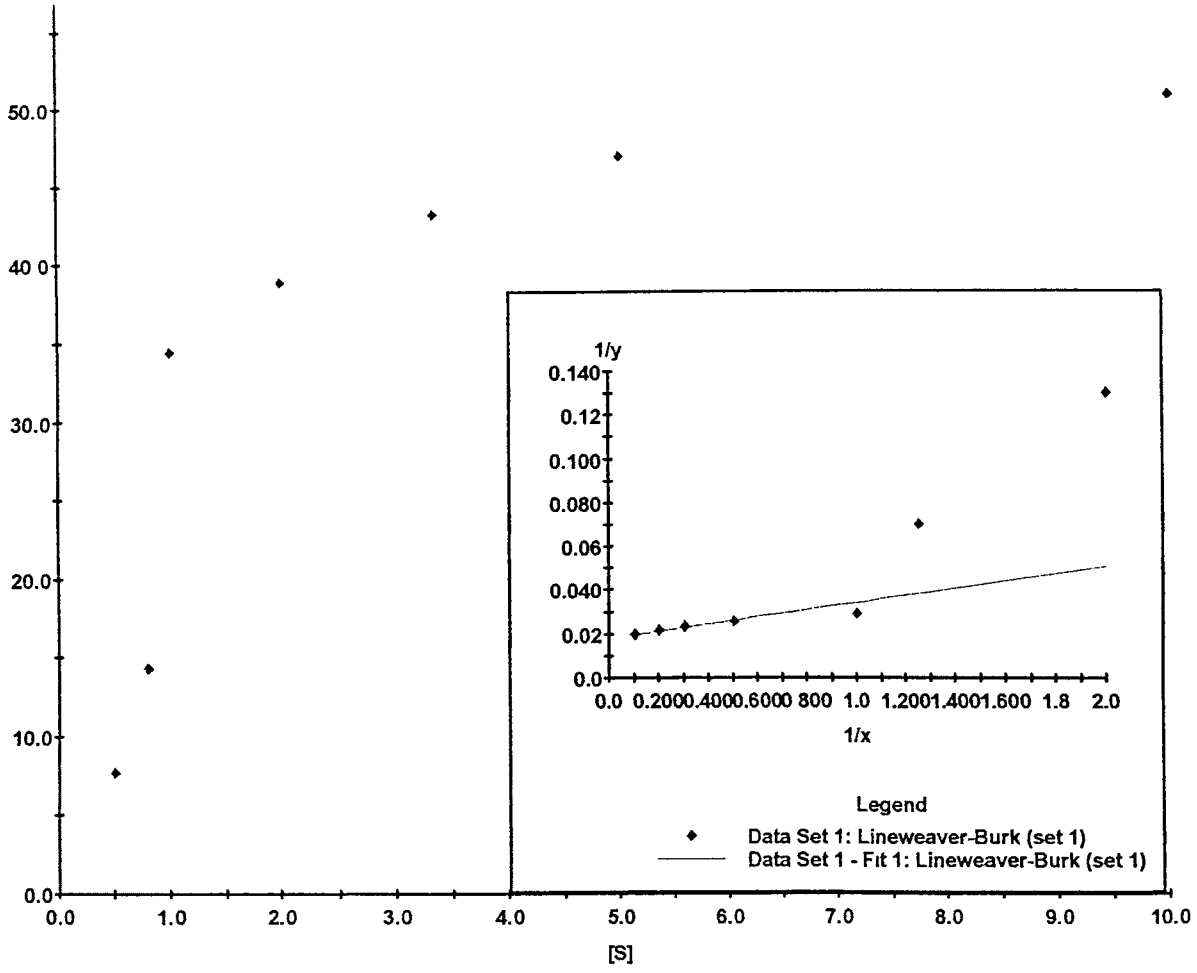


Figure 15. Michaelis-Menten transformation of kinetics data. Samples contained Buffer a, 0.15 μM HPBS desulfinase and varying concentrations of HPBS ([S]). Activity was monitored using the fluorimetric assay described in the Methods section. Data was plotted by EnzFitter (BIOSOFT).

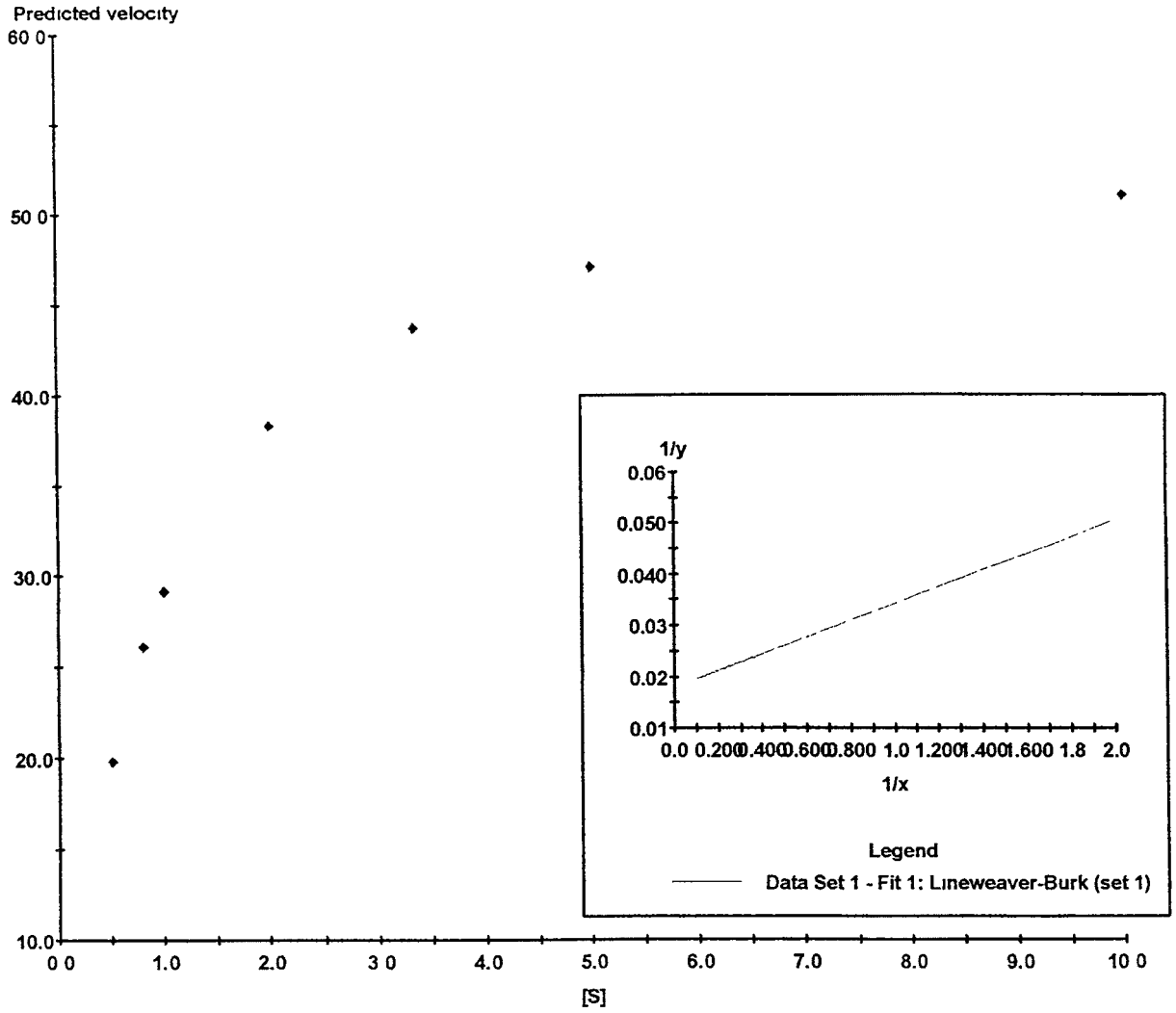


Figure 16. Michaelis-Menten transformation of fitted kinetics data. Samples contained Buffer a, 0.15 μM HPBS desulfinase and varying concentrations of HPBS ([S]). Activity was monitored using the fluorimetric assay described in the Methods section. Data was fitted and plotted by EnzFitter (BIOSOFT).

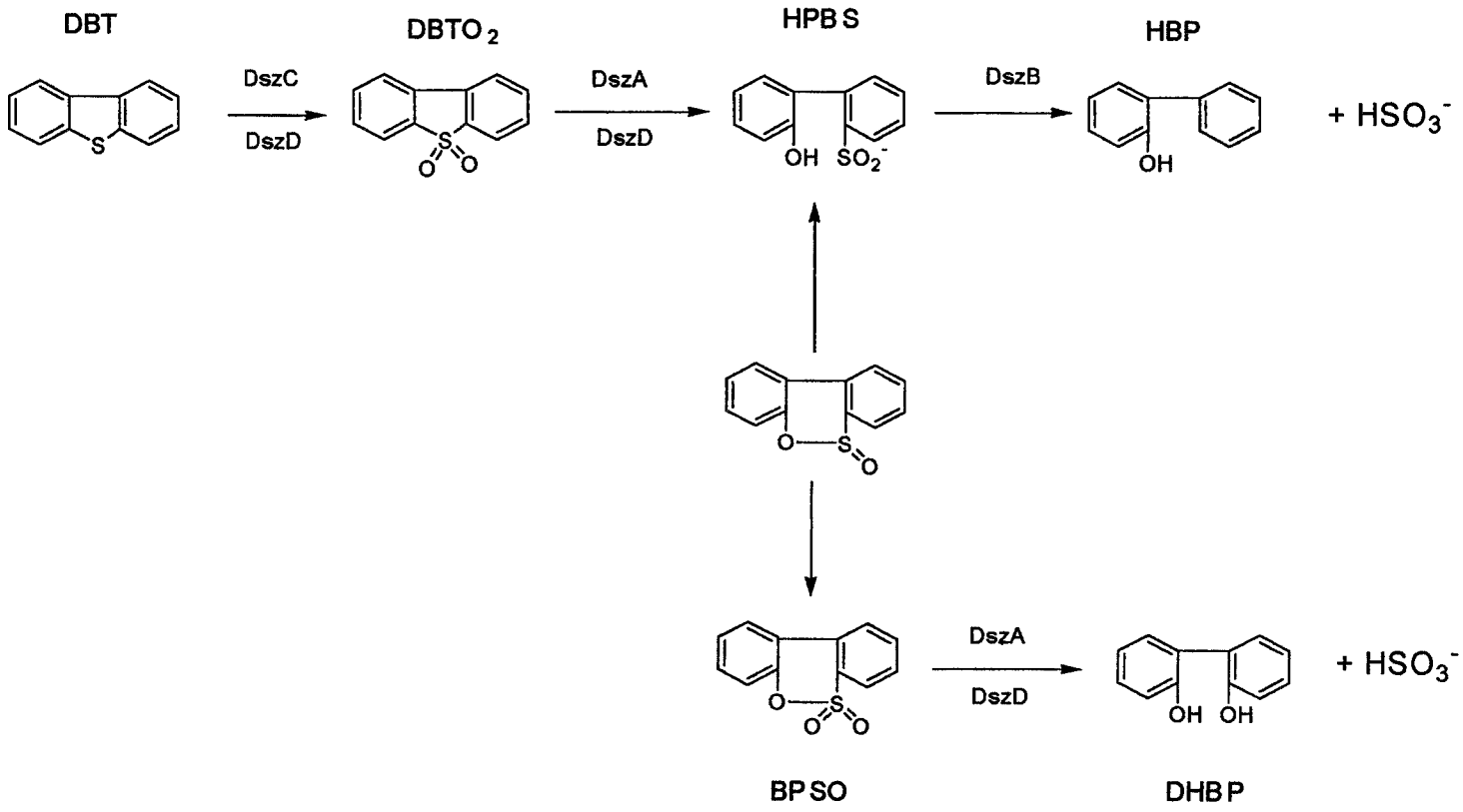
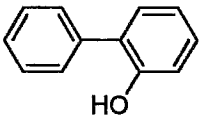
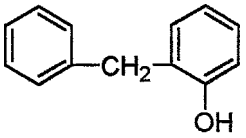


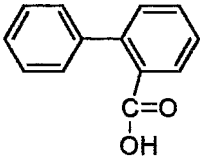
Figure 17. Modified dibenzothiophene degradation pathway.



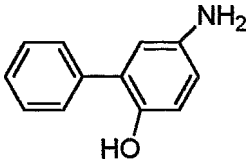
2-HYDROXYBIPHENOL
HBP



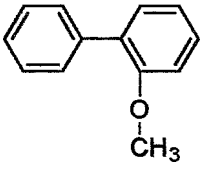
2-HYDROXYDIPHENYLMETHANE
HDP



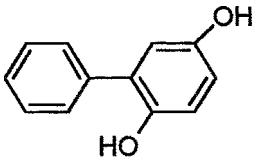
2-BIPHENYLCARBOXYLIC ACID
2-BPC



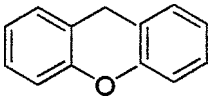
2-AMINO-4-PHENYLPHENOL



2-METHOXYBIPHENYL
MOB

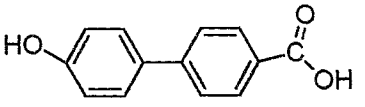


PHENYLHYDROQUINONE

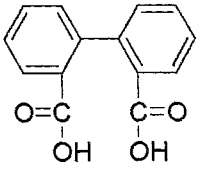


XANTHENE

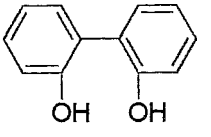
Figure 18. HBP analogs



4'-HYDROXY-4-BIPHENOL
CARBOXYLIC ACID

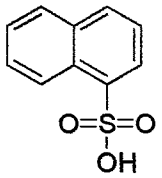


DIPHENIC ACID
DPA

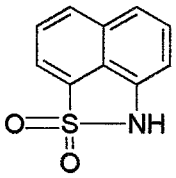


2-2'-BIPHENOL

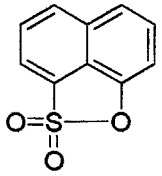
Figure 19. HPBS analogs



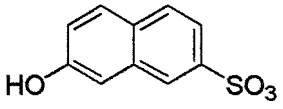
1-NAPHTHALENE SULFONIC ACID



1,8 NAPHTHOSULTAM



1,8 NAPHTHOSULTONE



6-HYDROXY-2-NAPHTHALENE SULFONIC ACID

Figure 20. Organosulfur analogs

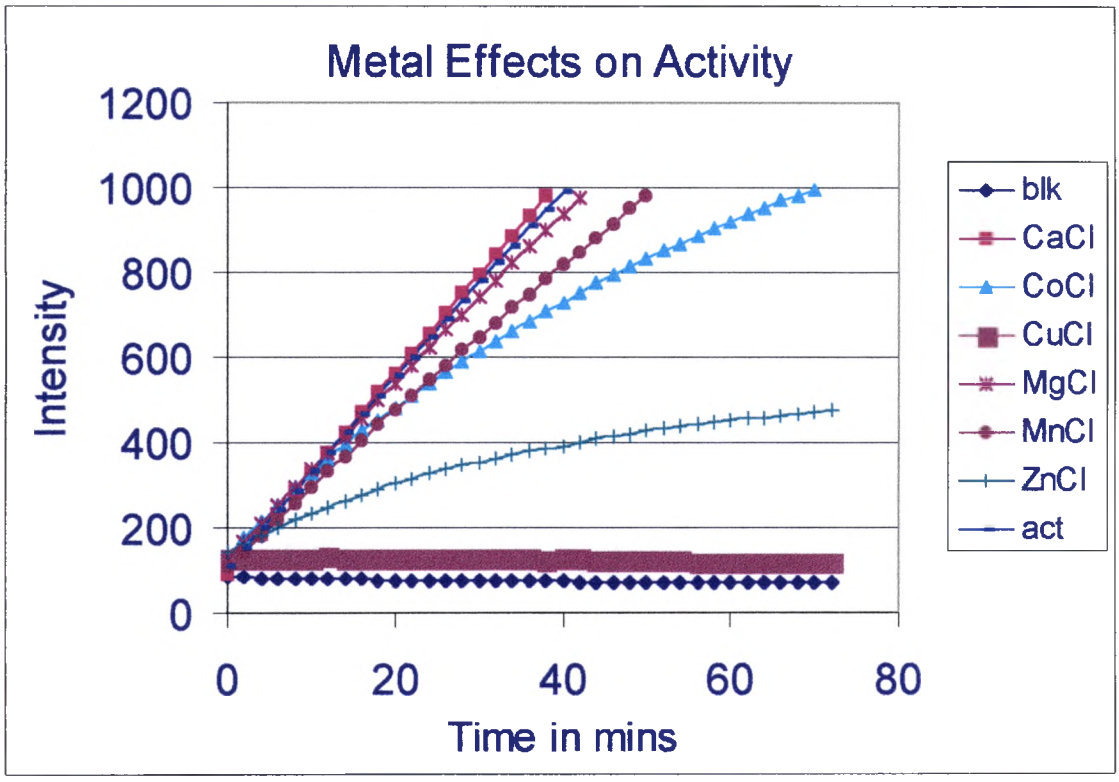
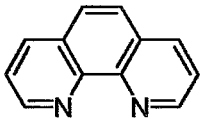
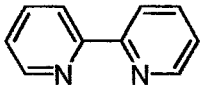


Figure 21. Metal activation study. HPBS desulfinase was diluted to 150 nM in Buffer A. Each reaction was made 100 μM in an individual metal solution. Activity was monitored using the fluorimetric assay described in the methods section.

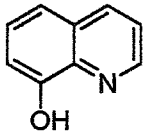
CHELATORS



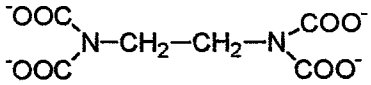
1, 10 PHENANTHROLINE



2,2 DIPYRIDYL

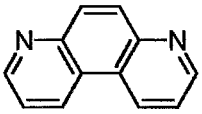


8-HYDROXYQUINOLINE

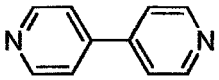


EDTA

NON-CHELATING ANALOGS



4, 7 PHENANTHROLINE



4,4 DIPYRIDYL

Figure 22. Chelators and non-chelating analogs.

Table 1. Purification Profile

Purification Step	Total protein (mg)	Total Units[a]	Specific Activity (U/mg)	Yield (%)	Fold purification
Cell lysate	3900	5.1	0.0013	----	----
Q-Sepharose pool	532	7.4	0.014	145	11
P-6DG pool	456	5.8	0.013	110	10
Hydroxyapatite pool	114	11.2	0.098	220	75
Toyopearl-phenyl	22	11.1	0.50	220	382
Superdex75 pool	1.2	2.5	2.1	49	1600

[a] A Unit (U) of activity is defined as 1 μM of HBP formed per hour. Enzyme fractions were monitored by the fluorimetric assay described in the Methods section.

Table 2. Data for determining kinetics constants

[HPBS] in μM	Velocity	Avg.
0.5	5.0 8.4 9.7 6.5 7.9 8.7	7.70 ± 1.8
0.8	12.3 10.4 16.6 14.6 12.8 13.5	13.4 ± 1.9
1.0	35.7 32.4 35.2	34.4 ± 1.4
2.0	41.9 36.7 38.2	38.9 ± 2.2
3.33	44.1 41.5 43.9	43.2 ± 1.2
5.0	48.5 48.7 43.4	46.9 ± 2.3
10.0	53.9 51.2 47.6	50.9 ± 2.6

Table 3. Statistical analysis of kinetics data[a]

Fitted Parameters

Name	Std Value	Error	Probability
V_{max}	55.56	2.338	1.229
K_m	0.9042	0.1500	9.040×10^{-4}

Analysis of Variance

$R^2 = 0.9175$

95% Confidence Limits Covariance matrix

Name	Lower	Upper
V_{max}	49.07	62.05
K_m	0.4878	1.321

[a] Data compiled by EnzFitter (BIOSOFT)

Table 4. Results of inhibition studies[a]

	ANALOGS	RELATIVE ACTIVITY
Product	2-Hydroxybiphenol	100%
	2-Biphenylcarboxylic acid	94%
	Phenylhydroquinone	93%
	2-Methoxybiphenyl	29%
	2-Amino-4-phenyphenol	98%
	Xanthene	92%
	2-Hydroxydiphenylmethane	98%
Organosulfur	1-Naphthalene sulfonic acid	56%
	6-Hydroxy-2-naphthalene sulfonic acid	68%
	1,8-Naphthosultone	93%
	1,8-Naphthosultam	58%
Substrate	2,2'-Biphenol	37%
	4'-Hydroxy-4-biphenol carboxylic acid	60%
	Diphenic acid	81%

[a] Reaction mixtures contained 100 μM in the analog, 100 μM HPBS, 150 nM HPBS desufinase, and Buffer A. Activity was monitored using the fluorimetric assay.

Table 5. Metal activation studies[a]

Metals	Relative Activity			Avg	Std Dev
Ca^{2+}	112	111	112	112	0.47
Co^{2+}	98	99	82	93	7.8
Cu^{2+}	5	6	5	5	0.47
Fe^{2+}	97	77	89	88	8.2
Mg^{2+}	110	101	95	102	6.2
Mn^{2+}	80	101	101	94	9.9
Zn^{2+}	50	51	50	51	0.47

[a] Reaction mixtures contained 100 μM metal compounds, 150 nM HPBS desulfinase, 100μM HPBS, and Buffer A. Activity was monitored using the fluorimetric assay.

Table 6. Effect of chelators and non-chelating analogs[a]

COMPOUNDS	RELATIVE ACTIVITY
1,10-Phenanthroline	35%
4,7-Phenanthroline	27%
2,2'-Dipyridyl	----
4,4'-Dipyridyl	58%
8-Hydroxyquinone	66%
EDTA	100%

[a] Reaction mixtures contained 100 μM chelator, 150 nM HPBS desulfinase, 100μM HPBS, and Buffer A. Activity was monitored using the fluorimetric assay.

Table 7. Chemical modification agents[a]

	Relative Activity	
	Initial	**45 minutes**
Phenylgloxal hydrate [Arg]		
100 μM	100%	43%
10 μM	81%	51%
Pyridoxal-5-phosphate [Lys]		
100 μM	88%	37%
10 μM	62%	18%
N-Bromosuccinimide [Trp]		
100 μM	0%	0%
10 μM	0%	0%
Tetranitromethane [Tyr]		
100 μM	0%	0%
10 μM	0%	0%
5,5'-Dithiobis(2-nitrobenzoic acid) [Cys]		
100 μM	0%	0%
10 μM	35%	0%

[a] Reaction mixtures contained indicated concentrations of chemical modification agents, 100 μM HPBS, 300 nM HPBS desulfinase and Buffer A. Activity was monitored using the fluorimetric assay.

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