# ASSAY DEVELOPMENT AND PURIFICATION OF 2-(2′-HYDROXYPHENYL) BENZENESULFINATE (HPBS) DESULFINASE

THESIS

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Master of Science

By

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San Marcos, Texas
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For my father,
Norbert R. Schneider

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# ABSTRACT

## ASSAY DEVELOPMENT AND PURIFICATION OF 2-(2′-HYDROXYPHENYL) BENZENESULFINATE (HPBS) DESULFINASE

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May 2000

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All fossil fuels are composed primarily of carbon and hydrogen, but also contain sulfur and nitrogen. The presence of sulfur contributes to the corrosion of pipelines, production and refining equipment. When fossil fuels are combusted, they release toxic sulfur oxides that lead to the formation of acid rain; therefore desulfurization of fossil fuels has become of great interest.

Bacterial degradation is a newly developed method of desulfurization. A bacterium that is effective in desulfurization of fossil fuels is *Rhodococcus* sp. IGTS8. *Rhodococcus* sp. IGTS8 removes sulfur from the model organosulfur compound dibenzothiophene (DBT) without destroying the hydrocarbon backbone using a four enzyme pathway. The focus of this research is the final enzyme in this four enzyme pathway, 2-(2′-hydroxyphenyl) benzenesulfinate desulfinase (HPBS desulfinase). HPBS desulfinase is the slowest enzyme in the desulfurization of DBT. Since it is the slowest of the four enzymes, it is the probable rate-limiting enzyme *in vivo.* In this study, assays were developed and the partial purification of HPBS desulfinase from *Rhodococcus* sp. IGTS8 was examined. Use of an anion exchange column and a hydrophobic interaction column allowed partial purification of HPBS desulfinase to be accomplished. Gibb's assay, ultraviolet and visible spectroscopy, fluorescence spectroscopy, and polyacrylamide gel electrophoresis were employed to monitor the purification process.

# CHAPTER I

## INTRODUCTION

Fossil fuels are composed of carbon and hydrogen, but also contain heteroatoms such as sulfur and nitrogen. The sulfur contamination alone can be as high as 11%(1). The presence of sulfur adversely effects fossil fuel refinement contributing to the corrosion of pipelines, production and other expensive equipment. The desulfurization of fossil fuels is also an area of great environmental interest because combustion of fossil fuels releases toxic sulfur oxides into the atmosphere ultimately producing acid rain.

There is a demand for low sulfur and low nitrogen fossil fuels because of stringent regulatory standards for reduced levels of emissions. The amendments to the Clean Air Act of 1964 require compliance with emission standards and a reduced percentage of emissions. Exposing fuel to either pre- or post-combustion desulfurization results in a lower percentage of emissions.

Sulfur contamination is present in two forms, organic and inorganic. The inorganic sulfur, composed mainly of elemental sulfur, sulfate, thiosulfate, and sulfide, can be removed with physical methods. Organic sulfur, composed

mainly of substituted benzo- and dibenzothiophenes, thiols, and thiophenes, is much harder to remove because the sulfur is contained in the heteroaromatic compounds. Dibenzothiophene (DBT) (Figure1) is one of the model compounds used in desulfurization studies because it is an important constituent of high sulfur oils and is one of the most abundant sulfur compounds in petroleum distillates and residues (2). Approximately 60% of petroleum sulfur contamination can be composed of DBT (1).

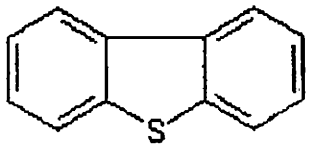


**Figure 1. Organosulfur model dibenzothiophene (DBT)**

The current method for desulfurization is hydrodesulfurization (HDS). HDS involves the reaction of petroleum fractions with an inorganic catalyst and hydrogen under conditions of high temperature and pressure to produce hydrogen sulfide and desulfurized product (3). However, HDS is not capable of lowering the percent sulfur of the fuel to meet the stringent EPA standards. Another drawback to HDS is that a corrosive product, hydrogen sulfide, is formed.

**Desulfurization through Bacterial Systems**

Methods for desulfurization that are currently being developed use bacterial enzymatic systems. Bacterial systems may offer many advantages to conventional desulfurization methods since bacterial desulfurization occurs under milder conditions (ambient temperatures and pressures) without producing corrosive hydrogen sulfide as a byproduct. Bacteria are also able to remove sulfur from a broader range of organosulfur compounds, and are self-regenerating and self-reproducing. Several strains of bacteria including *Pseudomonas*, *Acinetobacter*, *Rhizobium*, *Cornebacterium*, and *Rhodococcus* have been shown to degrade organosulfur compounds (4-7).

DBT, the organosulfur model compound, can be broken down in one of two known pathways. In the first pathway, DBT is used as a carbon source and degraded to produce carbon-sulfur fragments and water soluble carbon fragments. This pathway is not commercially useful for the desulfurization of fossil fuels because there is less carbon to combust as fuel, since the sulfur is lost as water soluble carbon fragments. Also, this pathway does not appear to be specific for organosulfur compounds.

In the second pathway DBT is used as a sulfur source and degraded to water-soluble sulfite and hydroxybiphenyl (HBP) (Figure 2). The second pathway is more useful for the desulfurization of fossil fuels because there is no loss of fuel value and the sulfur is removed as a non-toxic product.

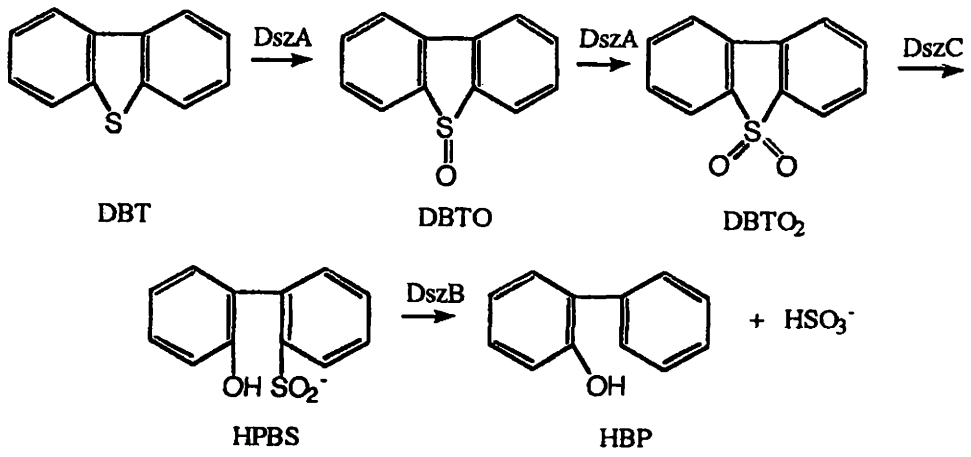


**Figure 2. Organosulfur specific dibenzothiophene degradation pathway.**

*Rhodococcus* sp. IGTS8 is a bacterium originally isolated near coal deposits. *Rhodococcus* sp. IGTS8 removes sulfur in crude oil and petroleum distillates through the second pathway. The sulfur specific DBT degradation pathway used by IGTS8 involves four proteins. The four genes that code for the enzymes have been isolated and characterized (8-10). Purification of the four enzymes from IGTS8 has been described in the literature (3). The first two enzymes involved are dibenzothiophene monooxygenase (DBT-MO) and dibenzothiophene-5,5-dioxide monooxygenase ($DBTO_2$-MO). DBT-MO is coded by *dsz* C and $DBTO_2$-MO is coded by *dsz* A. Both enzymes require flavins and an NADH-FMN oxidoreductase which is coded by *dsz* D for the oxidation of DBT

to 2-(2'-hydroxyphenyl) benzenesulfinate (HPBS). HPBS desulfinase, the third enzyme in the pathway, is coded by *dsz* B and apparently does not require a coenzyme to catalyze the desulfination reaction. Most available information detailing the enzymes and reaction products in the DBT pathway has been described using whole cell or cell-free systems. Thus, the active site structures and catalytic mechanisms of the individual enzymes are poorly understood. Understanding the specificity, structure, and mode of action of these enzymes will provide basic knowledge necessary to improve the commercial desulfurization of fossil fuels. For example, if a change was made in the active site of the enzyme, it may alter its specificity so it could work on different types of contaminants.

**Purification of the HPBS desulfinase**

The goal of this thesis was to partially purify and monitor activity of HPBS desulfinase, the final enzyme in the pathway. HPBS desulfinase catalyzes cleavage of the carbon-sulfur bond to form HBP and sulfite. This enzyme was chosen for research for several reasons. First, the enzyme is unique because cleavage of a carbon-sulfur bond is rare in enzyme systems and the enzyme does not share significant sequence homology with any known enzyme. Second, the apparent lack of coenzyme requirement may allow for a more direct characterization of the enzyme mechanism. Lastly, purified HPBS desulfinase is the slowest enzyme in the DBT pathway, and this is likely to be rate-limiting enzyme *in vivo*. HPBS desulfinase was extracted and partially purified from

*Rhodococcus* sp. IGTS8 and activity assays were developed. HPBS desulfinase was partially purified with anion exchange and hydrophobic interaction chromatography. A spectrofluorimetric assay (3) and Gibb's assay (7) were the main assays used to monitor activity. Ultraviolet and visible spectroscopy was used to determine protein concentrations. Polyacrylamide gel electrophoresis monitored the overall purification process. The HPBS desulfinase was purified 18 fold and a spectrofluorimetric assay was found to be the most effective assay of enzymatic activity.

# CHAPTER II

## MATERIALS

Novex NUPAGE™ pre-cast 4-12% Bis-Tris polyacrylamide electrophoresis gels (No. NP0321), Novex electrophoresis reagents, the Novex SilverXpress™ Silver Staining Kit (No. LC6100, Lot# 80922), the Novex Collodial Blue Staining Kit (No. LC6025, Lot# 81005), and the Novex Mark 12™ wide range molecular weight protein standards were purchased from Novel Experimental Technology, San Diego, California. Difco Nutrient Broth (Lot # 106724JE) and Difco Bactoagar (Lot# 111787JF) were purchased from Difco Laboratories, Detroit, Michigan. Freeze dried and lyophilized *Rhodococcus rhodochrous sp.* IGTS8 was purchased from the American Type Culture Center (ATCC), Manassas, Virginia. Bio-Rad Protein Assay Kit (No. 500-001, Lot# 59163A, Lot# 58935A) was purchased from Bio-Rad Laboratories, Hercules, California. Biphenosultine was provided by Dr. Herbert L. Holland from Brock University. Chicken egg white lysozyme with activity of 50,000 units/mg of protein was purchased from Sigma Chemical Co, St. Louis, Missouri. Y-Per reagent was purchased from Promega, Madison, Wisconsin. The Q Sepharose and Source 30 Q anion exchange columns were purchased from Pharmacia, Piscata Way, New Jersey.

A Green dye-binding column was purchased from Amicon, Inc., Beverly, Massachusetts. A P-6 DG desalting column was purchased from Bio-Rad Laboratories, Hercules, California. A Toyopearl-phenyl hydrophobic interaction column was purchased from Supelco, Bellefonte, Pennsylvania. All other reagents were reagent grade or better and were purchased from Fisher, Pittsburgh, Pennsylvania or Sigma Chemical Co., St. Louis, Missouri.

A Novex XCell-II™ electrophoresis module was used for all polyacrylamide gel electrophoretic analyses. The Novex Mark 12™ molecular weight standards contain: 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).

A Beckman DU-7400 UV spectrophotometer or a Molecular Devices SpectraMax 190 spectrophotometer was used in all ultraviolet and visible experiments. A Perkin Elmer Luminescence spectrophotometer LC50B or a Perkin Elmer HTS 7000 Plus were used in all fluorimetry experiments. A Vibracell sonicator with a microtip was used in all sonication experiments. A Kontes bomb cavitation device was used for all bomb cavitation experiments.

# CHAPTER III

# METHODS

## *Rhodococcus* Growth Conditions

The growth conditions described below were based on a procedure outlined by Li (11). Basal Salt Media 2 (BSM2) contained the following per liter: $KH_2PO_4$, 4 g; $NH_4NO_3$, 3 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; succinate, 1 g; glycerol, 10 g; ethanol, 8 g; $MnCl_2 \cdot 4\ H_2O$, 20 mg; $FeCl_2 \cdot 4\ H_2O$, 50 mg; $Na_2B_4O_7 \cdot 10\ H_2O$, 0.02 mg; $CuCl_2$, 0.02 mg; $CoCl_2 \cdot 6\ H_2O$, 1 mg; $ZnCl_2$, 0.08 mg; $FeCl_3 \cdot 6H_2O$, 0.4 mg; $CaCl_2$, 20 mg; $MoCl_5$, 0.1 mg; and $2NiCO_3 \cdot 3Ni(OH)_2 \cdot 4H_2O$ 5 μg (adjusted to pH 7.3 with NaOH). The $KH_2PO_4$ and $NH_4NO_3$ (minimal salts) solution was prepared in a flask and autoclaved. All solutions were autoclaved or sterile filtered and stored at room temperature. The required nutrients and a sulfur source were added to the minimal salt solution just prior to inoculation.

*Rhodococcus* cells were maintained on nutrient agar. Sterile plates containing nutrient agar were streaked with *Rhodococcus* cells every four to five days. The *Rhodococcus* cells were inoculated into overnight tubes containing 5

mL of nutrient media and grown to stationary phase. The *Rhodococcus* cells were pelleted by centrifugation at 7,000 *g* for five minutes, washed twice with sterile minimal salts, and inoculated into BSM2. BSM2 without added sulfur was used as a negative control, while BSM2 with 200 μM sodium sulfite added was used as a positive control. All other BSM2 flasks contained 1 mM DBT or 200 μM Dimethylsulfoxide (DMSO) as a sulfur source. Only cells grown in the presence of 1 mM or 200 μM DBT were used in the purification. After the *Rhodococcus* cells were grown to late log phase, the cells were centrifuged at 8,000 *g* for twenty minutes and washed twice with sterile minimal salts. The pelleted *Rhodococcus* cells were stored at -70° C. The supernatant was used immediately or stored at 4° C.

**Cell Lysis**

***Brief Sonication***

Frozen *Rhodococcus* cells were thawed and resuspended in Buffer A [25 mM $NaP_I$, 100 mM NaCl, 0.5 mM dithiothreitol (DTT), pH 7.4] including 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/mL DNAse. The suspension was sonicated for two seconds three times at 20 kHz. The sonicated cell lysate was centrifuged at 14,000 *g* for ten minutes and the supernatant used immediately.

### *Thirty Minute Sonication*

Frozen *Rhodococcus* cells were thawed and resuspended in Buffer A. The suspension was sonicated (two seconds on, and one second off) for thirty minutes at 20 kHz on ice. The sonicated cell lysate was centrifuged at 14,000 *g* for ten minutes and the supernatant used immediately.

### *Detergent*

The detergent used for lysis was the Y-Per reagent. The cell lysis procedure described below was based on literature (Promega, Madison, Wisconsin). Frozen *Rhodococcus* cells were thawed and resuspended in Buffer A. The suspension contained a homogenous mixture of *Rhodococcus* cells and 5 mL of Y-Per reagent/gram of *Rhodococcus* cells. The solution was incubated at 30°C for thirty minutes with shaking then was centrifuged at 14,000 *g* for ten minutes and the supernatant used immediately.

### *Bomb Cavitation*

Frozen *Rhodococcus* cells were thawed and resuspended in Buffer A. The homogenous mixture was placed in a bomb cavitation cell for one hour under nitrogen at 180 psi. After one hour, the chamber was opened and the cells and cell debris were centrifuged at 14,000 *g* for fifteen minutes and the supernatant used immediately.

***Lysozyme***

The cell lysis procedure described below was based on the protocol described by Consevage (12) and from the literature of Sigma (St. Louis, Missouri). Frozen *Rhodococcus* cells were thawed and resuspended in 50 mM Tris-HCl, pH 8. The reaction mixture contained a homogenous suspension of *Rhodococcus* cells and 5 mg/mL of lysozyme. An aliquot was taken immediately after lysozyme was added. The solution was incubated at 37°C for thirty minutes. Following incubation, 0.25 M Ethylenediaminetetraacetic acid (EDTA), pH 8 was added to the reaction mixture to produce a final concentration of 62 mM EDTA. The suspension was incubated at 37°C for an additional thirty minutes. The mixture was centrifuged at 14,000 *g* for ten minutes and the supernatant used immediately.

***French Press***

The cell lysis procedure described below was based on the procedure outlined by Gray (3). Frozen *Rhodococcus* cells were suspended in Buffer A [25 mM $NaP_i$, 100 mM NaCl, 0.5 mM DTT, pH 7.4] including 1 mM PMSF and 1 mg/mL DNAse. The suspension was passed through an ice cold Aminco French pressure cell at 20,000 psi two times. Cell debris and unbroken cells were removed by ultracentrifugation at 40,000 rpm for forty-five minutes. The supernatant was filtered through 0.45-micron cellulose acetate filters (Gelman

Science) before the supernatant was applied to a column. The supernatant was labeled "crude cell extract" and used immediately or stored at 4°C.

## Protein Measurements

### *$A_{230}/A_{260}$ Method*

Protein concentration was determined by the $A_{230}/A_{260}$ method of Kalb and Bernlohr (14). Absorbances at 230 nm and 260 nm were measured. Protein concentrations were estimated using the equation: μg/mL total protein = 183 $A_{230} - 75.8\ A_{260}$.

### *Bradford Assay*

Protein concentration was also determined using the Bradford assay (Bio-Rad, Hercules, California). A standard curve from 2 to 10 μg/mL bovine serum albumin (BSA) was generated. Concentrated Bio–Rad dye (200 μL) was added to the BSA solution. The solution was mixed and incubated at least five minutes but not longer than one hour. Absorbances were measured at 595 nm with a Beckman Model DU-7400 spectrophotometer. Protein concentrations were estimated from the standard curve.

## Substrate Synthesis

The synthesis of the substrate, HPBS, described below is based on a method provided by Gregory Mrachko at Energy Biosystems Corporation. A 20 mM solution of HPBS in water was made from the starting material, biphenosultine, provided by Dr. Herbert L. Holland at Brock University. The sultine was converted to HPBS in an overnight reaction with 2.2 moleq of NaOH. The insoluble sultine was converted to the soluble HPBS. The pH was adjusted to between eight and nine with hydrochloric acid.

## Activity Assays

### *Gibb's Assay*

The Gibb's assay described below was based on the procedure outlined by Kayser (15). Gibb's reagent (2,6-dichloroquinone-4-chlorimide) (Sigma, St. Louis, Missouri) reacts with aromatic hydroxyl groups at pH 8.0 to produce a blue colored product with a $\lambda_{max}$ of 610 nm. Gibb's assay was used to determine the HBP concentration in crude cell extracts and supernatants from whole cells and to monitor HBP formation by HPBS desulfinase.

To measure the HBP concentration in crude cell extract or supernatant sample (200 μL) was mixed with 790 μL of Gibb's reaction buffer [4 g/L $KH_2PO_4$, 3 g/L of $NH_4NO_3$ pH 8.0 adjusted with 0.94 M $Na_2CO_3$] and 10 μL of 10 mg/mL Gibb's reagent in ethanol. The solution was incubated at room temperature for

thirty minutes, and the product detected via absorbance measured at 610 nm with the use of a Beckman Model DU-7400 spectrophotometer. Absorbance values were compared to a standard curve generated with 5 μM to 35 μM HBP. The background level of phenolic compounds in the crude cell extract was determined with a sample that contained only Gibb's reaction buffer and crude cell extract, while the background levels of phenolic compounds in the supernatant of whole cells was determined with a sample that contained the supernatant from the cells grown in the absence of a sulfur source or in the presence of 200 μM DMSO.

HBP formation by HPBS desulfinase was monitored with the use of Gibb's assay after the addition of the substrate, HPBS, to the assay mixture. The crude cell extract sample (200 μL) was mixed with 790 μL of Gibb's reaction buffer [4 g/L $KH_2PO_4$, 3 g/L of $NH_4NO_3$ pH 8.0 adjusted with 0.94 M $Na_2CO_3$] and 2 μL of 10 mM HPBS then incubated at room temperature for thirty minutes followed by 10 μL of 10 mg/mL Gibb's reagent (in ethanol). The solution was incubated at room temperature for an additional thirty minutes, and the product detected via absorbance measured at 610 nm with the use of a Beckman Model DU-7400 spectrophotometer. The absorbance values were compared to a standard 5 μM to 35 μM HBP curve. The background level of phenolic compounds from the crude cell extract was determined with a sample that contained only Gibb's reaction buffer and crude cell extract. The background level of HPBS was determined with a sample that contained only Gibb's reaction buffer and 0.1 mM HPBS.

***Microplate Gibb's Assay***

A microscale Gibb's assay was performed in a similar manner as Gibb's assay (above) with the exception that all volumes were decreased by one-fifth and absorbances were monitored with a Biotek Instruments Ceres UV900C plate reader.

***Red Assay***

The red assay described below was based on a procedure outlined by Wang (6). The red assay qualitatively detects the presence of phenolic groups.

The red assay was performed in a microplate and the assay volume was 210 μL. The following was added to 100 μL of crude cell extract in order: 10 μL of 5% $K_3Fe(CN)_6$ and 100 μL of 0.25% 4-aminoantipyrene. The plate was incubated at room temperature overnight, and the formation of a red color indicated the presence of phenolic compounds.

***Cytochrome c Assay***

The cytochrome c assay (cyt c) described below was based on the procedure outlined by Gray (3). Sulfite was detected with the use of sulfite oxidase (Sigma, St. Louis, Missouri) in a coupled enzyme assay. Cell culture supernatant (250 μL) was mixed with cyt c reaction buffer [50 mM $NaP_I$, pH 7.5,

100 mM NaCl, 60 μM cyt c]. The solution was mixed and measured spectrophotometrically at 549 nm and 560 nm. The enzyme, sulfite oxidase, (0.04 mg/mL) was added and the solution mixed. The absorbances at 549 nm and 561 nm were measured spectrophotometrically a second time, and the concentration of the sulfite was calculated by converting the $\Delta A_{549-561}$ to cyt c concentration ($\varepsilon$= 19 mM/cm) using the stoichiometry of two cyt c reduced per sulfite oxidized to sulfate.

***Fluorimetric Assay***

The fluorimetric assay described below was based on a procedure outlined by Gray (3). HBP formation was used to detect HPBS desulfinase activity by an increase in fluorescence using a Perkin Elmer Luminescence spectrophotometer LC50B or a Perkin Elmer HTS 7000 Plus.

The Perkin Elmer HTS 7000 Plus fluorimetric assay was performed in a microplate. Each well contained 200 μL of crude cell extract or column fraction. The background fluorescence for Buffer A and crude cell extract in the absence of substrate, HPBS, was monitored for the length of the assay. HPBS was added to a final concentration of 0.1 mM to the sample wells. The emission was monitored at 410 nm ($\lambda_{ex}$ = 280 nm) for at least one hour. The bandwidth of each filter was 10 nm.

The Perkin Elmer Luminescence spectrophotometer LC50B fluorimetric assay was performed in a cuvette. Samples of crude cell extract or column fractions from 10 μL to 100 μL were taken. Buffer A was added to make the total volume 1998 μL. The background fluorescence for Buffer A and crude cell extract in the absence of substrate, HPBS, was monitored for at least ten minutes. HPBS was added to a final concentration of 0.1 mM to the cuvette. The assay was monitored at 414 nm ($\lambda_{ex}$ = 288 nm) for at least ten minutes.

**Purification Scheme**

The purification protocol described below was based on a procedure outlined by Gray (3). All columns were equilibrated with Buffer A except for the Toyopearl-phenyl and Source 30Q columns. All column profiles from the purification scheme were generated from $A_{280}$ measurements. The filtered crude cell extract was loaded on a Q Sepharose anion exchange column (Pharmacia, Piscata Way, New Jersey) at a flow rate of 3.0 mL/minute. The bound protein was eluted from the column with a linear 100-450 mM NaCl gradient. The fractions collected were monitored spectrophotometrically at 280 nm. The enzyme fractions that showed activity were pooled and dialyzed overnight against Buffer A. The dialysate was loaded onto a Green dye-binding column (Amicon, Beverly, Massachusetts) at a flow rate of 0.5 mL/minute and the void volume containing the enzyme was collected. A Source 30Q anion exchange column (Pharmacia, Piscata Way, New Jersey) was equilibrated with 25 mM

$NaP_i$ pH 7.4. The enzyme fraction from the green dye-binding column was loaded onto the column at flow rate of 3.0 mL/minute. The bound protein was eluted from the column with a linear gradient from 0-500 mM NaCl. The fractions collected were monitored spectrophotometrically at 280 nm. The enzyme fractions that showed activity were pooled and loaded onto a P-6 DG desalting column (Bio-Rad, Hercules, California) at a flow rate of 1.0 mL/minute. The void volume containing the enzyme was collected and ammonium sulfate was added to a final concentration of 1.7 M. A Toyopearl-phenyl hydrophobic interaction column (Supelco, Bellefonte, Pennsylvania) was equilibrated with 1.7 M ammonium sulfate (AMS) pH 6.8. The enzyme fraction from the P-6 DG column was loaded onto the column at a flow rate of 3.0 mL/minute. The bound proteins were eluted from the column with a linear gradient from 1.7 M AMS to 0 M AMS. The fractions collected were monitored spectrophotometrically at 280 nm. The enzyme fractions that showed activity were pooled.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

The SDS-Polyacrylamide gel electrophoresis procedure described below was based on suggested protocols provided by Novex (San Diego, California) and modifications of the methods of Laemmli (13). Samples were prepared in reduced lithium dodecyl sulfate (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 for 10 minutes at 90°C.

The prepared samples and molecular weight marker (Novex) were electrophoresed at 200 Volts (constant) through NuPAGE™ precast 4-12% Bis-Tris polyacrylamide gels with the use of MES-SDS pH 7.3 running buffer [ 500 mM 2-(*N*-morpholino)ethane sulfonic acid, 50 mM Tris-Base, 3.5 mM SDS, 1 mM EDTA].

The protein bands were detected with the use of Novex Colloidal Blue Staining or Novex SilverXpress™ Silver Staining Reagent kits. The stained gels were dried between cellophane sheets in a Hoefer Scientific Instruments Model SE1140 Slab Gel Dryer. Digital images of gels were produced with Adobe® Photo Deluxe Software and a Kodak digital science™ image station 440CF and the Kodak digital science 1DV version 3.0.1 software. The molecular weight values of the protein bands were reported based on $R_f$ values.

# CHAPTER IV

# RESULTS AND DISCUSSION

## Growth of *Rhodococcus*

*Rhodococcus* cells were maintained on nutrient agar plates at 30° C. Cells were streaked on sterile nutrient agar from frozen permanents or from previously streaked plates. Shiny, pink-orange colored colonies are observed after thirty-six hours growth. *Rhodococcus* cells from nutrient agar plates were grown in nutrient media prior to inoculation in BSM2. A single colony stab was used to inoculated sterile nutrient media followed by incubation at 30° C, with shaking, for thirty-six to forty-eight hours. The cells were then pelleted, washed, and resuspended in sterile minimal salts. The resuspended cells were inoculated into sterile BSM2 media with an appropriate sulfur source and shaken at 30° C.

Growth was monitored by absorbance at 600 nm and cells were harvested in late log phase. Typical incubation times were five days for a 250 mL culture and ten to fourteen days for a 1000 mL culture. Cells harvested via centrifugation were pink-orange in color unless the cells were grown in the

presence of 1 mM DBT. These cells were beige colored. The average yield was 10 g *Rhodococcus* cells/L of culture (wet pellet weight).

**Expression of HPBS desulfinase Activity**

Experiments were performed to assure the *Rhodococcus* cells were not obtaining sulfur through another source, for example, the water. Cells were inoculated into sterile BSM2 media that did not contain a sulfur source. These cells, when incubated with shaking at 30° C, did not produce viable colonies. The same cells when inoculated in 1 mM DBT grew as detailed above. Thus, the *Rhodococcus* cells were unable to obtain sulfur from the BSM2 media or the water used in preparing the media.

Further experiments were conducted to show that the *Rhodococcus* cells expressed desulfinase activity. Cells inoculated into BSM2 media that contained either 200 μM DMSO or 1 mM DBT were grown to late log phase. The cells were pelleted and the concentration of phenolic compounds in the supernatant measured using Gibb's assay. BSM2 media grown in the absence of sulfur was used as a blank. The supernatant of cells grown in the presence of DMSO contained 25 μM phenolic compounds while the supernatant of cells grown in the presence of DBT contained 145 μM phenolic compounds. The sixfold increase in concentration was attributed to the production of the HBP, the phenolic product of the DBT degradation pathway. The DBT experiment provided evidence the

*Rhodococcus* cells expressed a desulfinase activity while the DMSO experiment provided an accurate estimate of the background levels of phenolic compounds in *Rhodococcus* cells that express desulfinase activity.

**Cell Lysis**

Significant effort was expended toward optimization of *Rhodococcus* cell lysis. Early attempts at purification of HPBS desulfinase were unsuccessful, and this was attributed to incomplete cell lysis since lysis. Several procedures were evaluated including: longer sonication times, enzymatic digestion with lysozyme, detergent lysis, bomb cavitation, and a French pressure cell. The effectiveness of the different cell lysis procedures was monitored by Kalb and Bernlohr (14) or Bradford (Bio-Rad, Hercules, California) methods.

The initial cell lysis methods tested were thirty minute sonication, detergent, and bomb cavitation. Frozen *Rhodococcus* cells were treated with thirty minutes of sonication as described in the methods section. There was little change in the protein concentrations before and after sonication. Another concern with sonication was the heat produced by the sonicator may denature the protein. Frozen *Rhodococcus* cells were treated with a detergent, Y-per reagent, as described in the methods section. There was negligible change in the protein concentrations before and after Y-per reagent addition. Frozen *Rhodococcus* cells were treated with bomb cavitation as described in the methods section. Inappreciable change in the protein concentrations before and

after bomb cavitation was observed. The initial methods tested were determined to be ineffective in the lysis of *Rhodococcus* cells.

The cell lysis method with lysozyme was particularly effective at the lysis of *Rhodococcus* cells. Frozen *Rhodococcus* cells were lysed with lysozyme as described in the methods section. There was a sevenfold increase in the protein concentrations before and after incubation with lysozyme. The method was modified in several different ways to attempt to obtain better total protein yield. Incubation time, the presence of 62 mM EDTA, the buffer system, and cell storage conditions were adjusted. Thirty minutes was the optimal time for each incubation. EDTA was required for efficient cell lysis as the removal of the EDTA from the incubation mixture lowered the protein concentrations significantly. When the buffer system was changed from 50 mM Tris-HCl to Buffer A, there was little change in protein concentrations. Freshly harvested and frozen *Rhodococcus* cells yielded similar protein concentrations. However, when the crude cell extract was applied to the first column (Pharmacia, Piscata Way, New Jersey) in the protein purification scheme, a previously unobserved peak coeluted with HPBS desulfinase. The peak could possibly be ovalbumin (OVA) or another protein derived from the chicken egg white, the source of the lysozyme. This impurity complicated the purification scheme, and another method of cell lysis was evaluated.

Cell lysis with the French pressure cell was the most effective method for *Rhodococcus* cells. Frozen *Rhodococcus* cells were lysed with French pressure cell as described in the methods section. There was a fivefold increase in the

protein concentrations before and after French pressure cell, and this method did not introduce any new contaminants.

**Characterization of HBP**

HBP was characterized with the use of ultraviolet, visible, and fluorescence spectroscopy. HBP solutions [20 mM in ethanol], at neutral, acidic, and basic pH were prepared as described in the methods section. Absorbance spectra were determined from 190 to 700 nm. A single peak was observed for all spectra. The $\lambda_{max}$ for the acidic and neutral 20 mM HBP solutions was 313 nm. The $\lambda_{max}$ for the basic 20 mM HBP solution was 353 nm. A red shift is typical for the deprotonated form of phenolic compounds. For fluorescence studies, the HBP solution was diluted with Buffer A. The optimal excitation and emission wavelengths have been previously described (Gray 3). Fluorescence emission was monitored from 0.1 μM to 10 μM HBP. Over this concentration range, the fluorescence emission was linear with HBP concentration.

**Characterization of HPBS**

HPBS was characterized using ultraviolet, visible, and fluorescence spectroscopy. A 20 mM HPBS solution was prepared as described in the methods section. The absorbance spectrum of the 20 mM HPBS solution was measured from 250 nm to 400 nm. HPBS has a $\lambda_{max}$ at 280 nm. The molar

extinction coefficient of HPBS solution at 280 nm is 1.65 /(mM*cm). HPBS solutions for fluorescence studies were diluted into Buffer A. A slight increase in emission at 414 nm ($\lambda_{exc}$ = 288 nm) was observed upon the addition of HPBS to Buffer A. The change was less than 10% of that observed with an identical concentration of HBP.

The effect of pH change on the absorbance spectra of HPBS was measured. Fresh 0.23 mM HPBS solutions at acidic (pH 2) and basic pH (pH 14) were prepared. A 0.23 mM HPBS solution at acidic pH solution was also prepared and allowed to stand for one week. All three samples were scanned from 250 to 700 nm and the absorbance spectra determined. The fresh sample of 0.23 mM HPBS solution at pH 2 showed a typical peak at 280 nm. The week old HPBS sample at acidic pH showed peaks at 265 nm and 297 nm. When the pH of this HPBS solution was adjusted back to pH 8, the absorbance spectrum was not affected, and the two peaks remained. The freshly prepared 0.23 mM HPBS solution at pH 14 showed a peak at 305 nm. The red shift from 280 nm to 305 nm that was seen at high pH is typical of the deprotonated form of phenolic compounds and the effect is reversible. HPBS and HBP have distinct absorbance spectra, and this study showed that pH extremes would not convert HPBS to HBP.

In order to confirm the HPBS solution contained only the sulfinic acid, HPBS was oxidized to the sulfonic acid. The oxidation was forced with the addition of hydrogen peroxide. Solutions of 10% hydrogen peroxide, 5% hydrogen peroxide, and a 1:1 mixture of 0.23 mM HPBS and 10% hydrogen

peroxide were prepared. Scans from 190 nm to 800 nm were performed on all three samples. The oxidation of HPBS produced a new peak at 345 nm, a red shift. To ensure the shift in wavelength was not due to a change in pH, the experiment was repeated with a 5% hydrogen peroxide solution adjusted to pH 14. The 1:1 mixture of HPBS and hydrogen peroxide had a $\lambda_{max}$ higher than either solution alone, and the shift was not observed in any other experiments, therefore oxidation of HPBS is probable. The HPBS solution was scanned before each use to check for absorbance changes. A shift in the $\lambda_{max}$ had not been observed for the HPBS solutions in the absence of hydrogen peroxide; therefore, air oxidation of HPBS in solution does not appear to be problematic. These results have recently been confirmed through HPLC analyses.

## Activity Assays

### *Colorimetric*

Colorimetric assays were used to monitor HPBS desulfinase activity. The production of HBP, a phenolic compound, was monitored with the use of Gibb's assay or red assay. Sulfite production was monitored with the use of the cytochrome c assay. The effectiveness of all these assays was limited by low sensitivity due to high background absorbance.

The level of HBP present inside the *Rhodococcus* cells was determined with the use of Gibb's assay. *Rhodococcus* cells were grown in BSM2 media with 1 mM DBT as the sole sulfur source. The *Rhodococcus* cells were pelleted

and resuspended in Buffer A. One half of these cells were pelleted and washed one time with minimal salts before resuspending in Buffer A. Both washed and unwashed cells were treated with brief sonication as described in the methods section. Gibb's assay was performed on both crude cell extracts. The concentration of HBP from the washed *Rhodococcus* cells was 23 μM while the concentration from unwashed *Rhodococcus* cells was 103 μM. The unwashed *Rhodococcus* cells had a fivefold higher background level of HBP than the washed *Rhodococcus* cells. To lower the HBP background and to increase the sensitivity of all assays, the *Rhodococcus* cells were washed twice with minimal salts before any experiments were performed.

Experiments were conducted to determine if the substrate, HPBS, would interfere with the sensitivity of Gibb's assay. *Rhodococcus* cells were grown in BSM2 with 1 mM DBT as the sole sulfur source. The *Rhodococcus* cells were washed twice with minimal salts and treated with brief sonication as described in the methods section. The crude extract sample was divided into two samples, and 0.1 mM HPBS was added to one of the samples. Both samples and a blank which contained Gibb's reaction buffer and HPBS were allowed to incubate for thirty minutes at room temperature. At the end of the incubation period, Gibb's reagent was added and the assay was performed on all samples as described in the methods section. In samples with low level of enzyme activity, it was not possible to detect HBP formation over the background. The experiment was repeated with a control, *Rhodococcus* cells grown in nutrient media. The crude cell extract had less than 1 μM of phenolic compounds.

Gibb's assay was modified in several ways to obtain better sensitivity. Buffer system, incubation time, and cell storage conditions were independently adjusted. The buffer system was changed from minimal salts at pH 8 to1 M Tris-(hydroxymethyl) aminomethane HCl (Tris-HCl) at pH 8.0. No buffer effect was observed and the HBP concentrations measured with the different buffer system were within 1 standard deviation of the points on the standard curve. The incubation time was varied from fifteen minutes to forty-eight hours. Incubation time was not as important as enzyme concentration for HBP detection. Freshly harvested and frozen *Rhodococcus* cells grown in BSM2 media with 1 mM DBT as the sole sulfur source were treated with brief sonication. Both crude cell extracts were detected for HBP formation with the use of Gibb's assay. Similar results were observed for both crude cell extracts. The effectiveness of the cell lysis appeared to be more important than cell storage conditions.

The red assay is a dye-binding assay, which is similar to Gibb's assay, but the red assay uses a different dye. Experiments were performed to determine whether HPBS interferes with the red assay. Frozen *Rhodococcus* cells were grown in BSM2 media with 1 mM DBT as the sole sulfur source, and treated with brief sonication as described in the methods section. The red assay was performed as described the methods section with time points taken at 0, 45, and 90 minutes. The HPBS background level was too high for the red assay to be effective alternative to Gibb's assay.

The cytochrome c assay was used to determine the concentration of the reaction product sulfite. Sulfite was oxidized by sulfite oxidase, and cytochrome

c was the electron acceptor. The oxidation state of the cytochrome c was monitored. Experiments were conducted to determine if the other reductase enzymes would interfere with the sensitivity of the cytochrome c assay. *Rhodococcus* cells were grown in BSM2 with 1 mM DBT as the sole sulfur source. The *Rhodococcus* cells were washed twice with minimal salts and treated with brief sonication as described in the methods section. The crude extract sample was divided into two samples, and 0.1 mM HPBS was added to one of the samples. The cytochrome c assay was performed on both samples after an incubation period of thirty minutes at room temperature as described in the methods section. The results showed little change in the sulfite concentration, and other reductase enzymes interfered with the sulfite oxidase reaction. The experiment was repeated with a control, *Rhodococcus* cells grown in nutrient media. The crude cell extract had no significant change in the sulfite concentration.

The incubation time of the cytochrome c assay was modified to attempt to obtain better sensitivity. The incubation time was varied from one minute to twenty minutes. Incubation time did not appear to be as important as enzyme concentration for sulfite detection.

***Spectrofluorimetric***

The spectrofluorimetric assay was used to monitor HPBS desulfinase activity through the production of HBP, the product. HBP was monitored at the

emission wavelength of 414 nm. The assay was effective due to the high sensitivity since interference of other compounds, proteins, or enzymes with this assay are limited.

Experiments were performed to determine if HPBS, the substrate, interferes with the spectrofluorimetric assay. *Rhodococcus* cells were grown in BSM2 with 200 μM DBT as the sole sulfur source. The *Rhodococcus* cells were washed twice with minimal salts and lysed with the French press described in the methods section. The fluorescence assay was performed on the crude cell extract as described in the methods section. The HPBS sample showed no increase in fluorescence. Increases in fluorescence were observed when the crude cell extract or a fraction that contained HPBS desulfinase, the enzyme, was in the presence of HPBS, the substrate. When the absorbance of a sample at 280 nm with the use of ultraviolet spectroscopy was above 0.1, then an inner filter quenching effect decreased the fluorescence emission. Overall, the spectrofluorimetric assay was the most effective assay to monitor HPBS desulfinase activity.

**Purification of HPBS desulfinase**

Initial attempts at HPBS desulfinase purification were ineffective due to poor cell lysis, which resulted in low total protein. For the first purification scheme, 2.5 g of *Rhodococcus* cells grown in 1 mM DBT were treated with brief sonication as described in the methods section. Gibb's assay was used to monitor enzyme activity throughout the purification. The crude cell extract was

applied to the Q Sepharose column. Fractions that contained activity were pooled and desalted on the P-6 DG column. The desalted enzyme was applied to the phenyl column. HPBS desulfinase activity was not detected on any of the fractions from the phenyl columns as the amount of HBP formed was below the limit of detection for the Gibb's assay. SDS-PAGE analysis with silver staining was performed on the phenyl column fractions. No detectable protein band around 42,000 Da, the molecular weight of HPBS desulfinase, was detected in any of the lanes that contained fractions from the phenyl column.

The purification scheme was not effective due to poor cell lysis and the limited ability of Gibb's assay to qualitatively detect HPBS desulfinase activity. The low total protein yield was evidenced by the phenyl $A_{280}$ column profiles with peak absorbances of around 0.05. The purification scheme was modified in an attempt to better purify the HPBS desulfinase. A second Q Sepharose with a shallower gradient was added after the desalting column. This modification did not result in enhancement of the purification, and purification scheme was still ineffective.

A new cell lysis method was used on the purification scheme. Total protein yield was enhanced with lysozyme as the cell lysis method. Ten grams of *Rhodococcus* cells grown with 200 μM DBT were lysed with lysozyme as described in the methods section. Enzyme activity was monitored with Gibb's assay. The crude cell extract was applied to the Q Sepharose column. A new peak was observed that coeluted with the HPBS desulfinase (Figure 3).

The presence of this contaminant was confirmed through SDS-PAGE analysis (Figure 4). The new protein was approximately the same molecular weight as HPBS desulfinase (42,000 Da). The peak could possibly be ovalbumin (46,000 Da) or another protein from the chicken egg white, the source of the lysozyme. For the reasons stated, the lysozyme was shown to be an ineffective method of cell lysis to be used for purification of HPBS desulfinase. All further purification protocols used the French press.

The French press greatly increased the total protein yield over brief sonication, and no new contaminants were introduced. The *Rhodococcus* cells were grown in 200 μM DBT and 20 g were lysed with French pressure cell. The purification scheme was performed as described in the methods section. Each step of the purification was monitored with the HTS 7000 Plus fluorimetry assay. Any fractions that showed activity were pooled and applied to the next step in the purification. The purification chart is shown in Table 1. The crude cell extract was applied to the Q Sepharose column and the column profile is shown in Figure 5. Fractions 68 through 79 had activity and were pooled. The pooled fractions were dialysized overnight to remove the salt. The desalted enzyme was applied to the Green column and the proteins that did not bind to the column were applied to the Source 30Q column. A single peak eluted and was pooled. The pooled fractions were applied to the P-6 DG column to remove the salt. The void volume that contained enzyme was pooled, and brought to 1.7 M AMS. The pH of the phenyl running buffer was changed from pH 6.7 to 7.0 to elute the HPBS desulfinase in the middle of the gradient. The pooled enzyme was applied

to the phenyl column. Fractions 58 through 62 had activity and were pooled. The phenyl column profile is shown in Figure 6.

The purification scheme resulted in less than a twofold purification of HPBS desulfinase. An SDS-PAGE analysis showed multiple bands in the lane that contained the phenyl column pool indicating the HPBS desulfinase was not pure (Figure 7). The exact reason for this is unknown. It is possible that the HPBS desulfinase concentration was too low, and it interacted more strongly with other enzymes in solution than the column. The low protein levels were evident in the phenyl column profile with the peak absorbance of 0.2. The continuous loss of activity seen in the purification scheme was attributed to the length of time required for the multiple steps. The green and Source 30Q columns did not appear to have any significant effect on the purification of HPBS desulfinase, and were dropped from the purification scheme.

A modified purification scheme was performed with *Rhodococcus* cells that were grown in 200 μM DBT, and 14 g were lysed with French pressure cell. The modified purification scheme included only two columns, the Q Sepharose and phenyl column. Due to the stability of HPBS desulfinase in the early steps of the purification, dialysis was chosen over the P-6 DG column for removing the sodium chloride from the pooled enzyme fraction. Each step was monitored for activity with the HTS 7000 Plus fluorimetry assay. The purification chart is shown in Table 2. The crude cell extract was applied to the Q Sepharose column. Fractions 68 through 82 had activity and were pooled. The absorbance at $A_{280}$, gradient, and activity for the Q Sepharose column fractions are shown in Figure

8. The pooled enzyme fractions were dialysized overnight. The dialysized enzyme was applied to the phenyl column. Fractions 112 through 115 had activity and were pooled. The absorbance at $A_{280}$, gradient, and activity for the phenyl column fractions are shown in Figure 9. Shortly after the enzyme fraction from the phenyl column was pooled the enzyme lost activity. The protein concentration after the phenyl column was low. The loss of activity could be due to low protein concentrations at the end of the purification. SDS-PAGE analysis on this purification scheme showed the lane that contained the pooled fractions from the phenyl column had multiple bands, which indicated that the HPBS desulfinase was not pure (Figure 10). The mass of cells used in the purification was too low to yield a significant amount of HPBS desulfinase and the desulfinase was unstable at low concentrations.

Two final modifications to the purification scheme were performed. The first modification was to replace the dialysis step with a P-6 DG column in order to retain the highest possible enzyme activity by decreasing the amount of time required for the purification. The second modification was to lyse five times more *Rhodococcus* cells to achieve higher total protein and to keep the HPBS desulfinase more stable. A twentyfold increase in the purification was observed with this purification scheme (Table 3). The P-6 DG column assisted in the retention of total units which decreased with dialysis. The increased mass of *Rhodococcus* cells resulted in a higher yield of overall protein and HPBS desulfinase. With the higher amount of HPBS desulfinase, enzyme activity was retained longer.

## SUMMARY

The best cell lysis method in these studies for *Rhodococcus* cells used in the protein purification was the French press method. Even though the enzymatic cell lysis method resulted in a higher yield of total protein, an unobserved peak coeluted with the HPBS desulfinase. Sonication, detergent, and a bomb cavitation cell lysis methods were ineffective in opening *Rhodococcus* cells.

In these studies, the best assay for quantitative detection of HBP in crude cell extract was the spectrofluorimetric assay. The background phenolic groups did not interfere with the fluorimetry assay and the excitation and emission wavelengths were specific for HBP, which made it the most sensitive assay. In these studies, Gibb's assay was not sensitive enough for use in crude cell extracts, but was effective for the detection of HBP in supernatants of whole *Rhodococcus* cells. This agrees with the results observed by Kilbane (7). The increased mass of *Rhodococcus* cells and an effective assay to monitor enzyme activity, the greatest enhancement of purity was obtained.

The best purification scheme developed in these studies involved the Q Sepharose, P-6 DG, and phenyl columns. This scheme was significantly modified from the scheme developed by Gray (3). The Gray scheme purified all four enzymes and was not optimized for the purification of HPBS desulfinase on a lab scale. The Green column was removed from the purification scheme because; it binds DBT-MO tightly and had no effect on HPBS desulfinase

purification. The P-6 DG column was chosen over dialysis because the HPBS desulfinase lost activity rapidly during the phenyl column analysis, and it was observed that when the pooled enzyme fractions were left overnight during dialysis, activity was lost. The increased mass of *Rhodococcus* cells resulted in a higher yield of overall protein and retention HPBS desulfinase activity. The higher concentration of enzyme may have prevented rapid loss of enzyme activity.

The partially purified HPBS desulfinase can be used to perform some initial studies on the enzyme. Kinetic parameters such as $K_m$ and $V_{max}$ can be determined. In addition, product inhibition and substrate specificity studies can be performed. Other experiments await the complete purification of HPBS desulfinase, which can be achieved with the use of different columns.

**Table 1. Purification Profile I.**

| Purification Step | Volume (mL) | Protein Concentration (μg/mL) [a] | Total Protein (μg) | Activity (U/mL) [b] $*10^{-3}$ | Total Units $*10^{-2}$ | Specific Activity (U/mg) $*10^{-3}$ | Yield (%) | Fold Purification |
|---|---|---|---|---|---|---|---|---|
| Crude cell lyse | 41 | 2400 | 98400 | 158 | 650 | 66 | ------ | ---------- |
| Q Sepharose | 74 | 630 | 46700 | 23 | 170 | 36 | 26.1 | 0.55 |
| After dialysis | 62.5 | 420 | 25000 | 9 | 56 | 23 | 8.6 | 0.35 |
| Green | 43 | 236 | 10100 | 8 | 34.4 | 34.4 | 5.3 | 0.52 |
| Source 30Q | 7 | 782 | 5500 | 8 | 56 | 11 | 0.9 | 0.17 |
| P-6 DG | 13 | 240 | 3100 | nd [c] | nd | nd | nd | nd |
| Phenyl | 5 | 72 | 360 | 6.3 | 31.5 | 90 | 0.5 | 1.4 |

[a] Protein concentration was monitored by the Kalb and Bernlohr method.
[b] A Unit (U) of activity is defined as μmole of HBP formed per hour. Enzyme fractions were monitored by the HTS 700 Plus fluorimetry assay.
[c] Not Determined

**Table 2. Purification Profile II.**

| Purification Step | Volume (mL) | Protein Concentration (mg/mL) [a] | Total Protein (mg) | Activity (U/mL) [b] $*10^{-2}$ | Total Units $*10^{-2}$ | Specific Activity (U/mg) $*10^{-3}$ | Yield (%) | Fold Purification |
|---|---|---|---|---|---|---|---|---|
| Crude cell lyse | 16 | 10.6 | 170 | 3.2 | 51 | 3 | ------ | ---------- |
| Q Sepharose | 109 | 0.413 | 45 | 1.2 | 130 | 29 | 100 [c] | 9.6 |
| After dialysis | 110 | 0.234 | 26 | 0.7 | 77 | 30 | 59 | 10 |
| Phenyl | 6.5 | 0.264 | 1.7 | 1.44 | 9.36 | 55 | 7.2 | 18.3 |

[a] Protein concentration was monitored by the Kalb and Bernlohr method.
[b] A Unit (U) of activity is defined as μmole of HBP formed per hour. Enzyme fractions were monitored by the HTS 700 Plus fluorimetry assay.
[c] Crude cell lyse total units was inaccurate.

**Table 3. Purification Profile III.**

| Purification Step | Volume (mL) | Protein Concentration (mg/mL) [a] | Total Protein (mg) | Activity (U/mL) [b] $*10^{-2}$ | Total Units $*10^{-2}$ | Specific Activity (U/mg) $*10^{-3}$ | Yield (%) | Fold Purification |
|---|---|---|---|---|---|---|---|---|
| Crude cell lyse | 75 | 9.0 | 675 | 1.65 | 120 | 1.8 | ------ | ---------- |
| Q Sepharose | 94 | 1.37 | 129 | 0.69 | 65 | 5 | 54.2 | 2.8 |
| P-6 DG | 80 | 1.2 | 96 | 0.74 | 60 | 6.3 | 50.0 | 3.5 |
| Phenyl | 16 | 4.4 | 71 | 15.8 | 250 | 36 | 167 [c] | 20 |

[a] Protein concentration was monitored by the Kalb and Bernlohr method.
[b] A Unit (U) of activity is defined as μmole of HBP formed per hour. Enzyme fractions were monitored by the HTS 700 Plus fluorimetry assay.
[c] Phenyl column was estimated from the enzyme fractions.

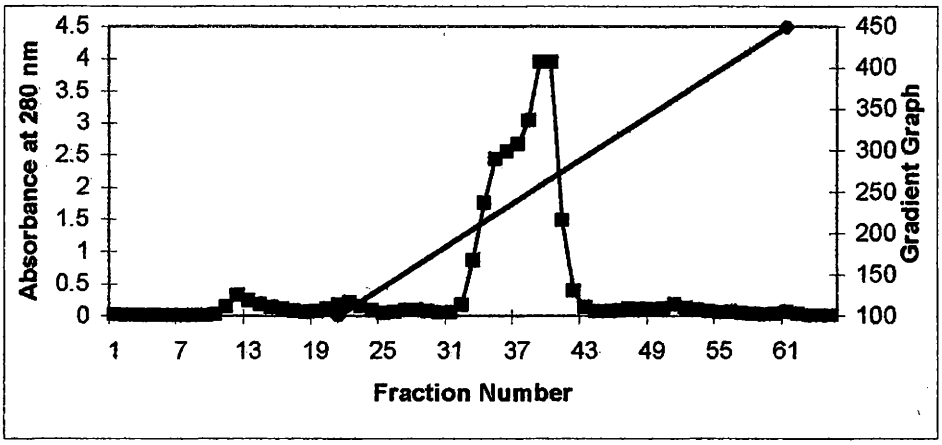


**Figure 3. Column Profile from a Q Sepharose anion exchange column with the enzymatic cell lysis method.** The (■) represents the absorbance at 280 nm. The (◆) represents the gradient from 100 to 450 mM NaCl. The column was equilibrated with Buffer A at a flow rate 3.0 mL/minute. Crude cell extract was loaded onto the column and eluted with a 100-450 mM NaCl gradient. Another peak was coeluted with the HPBS desulfinase peak. Fraction size was 5.9 mL. Fractions 33 through 42 were pooled.

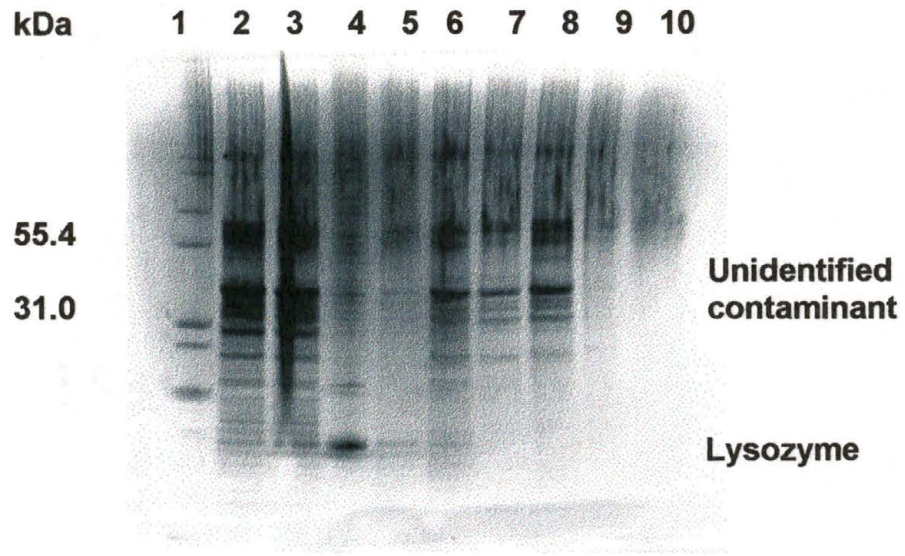


**Figure 4. SDS-polyacrylamide gel electrophoresis analysis of protein purification with the enzymatic cell lysis method.** Cell were lysed with the lysozyme as described in the methods section. The pooled samples were electrophoresed and the protein bands were detected by silver staining. Lane 1 Molecular weight standards (Novex); Lane 2 Q Sepharose a from an earlier prep (0.3 μg); Lane 3 after dialysis from an earlier prep (0.1 μg); Lane 4 crude cell lyse (0.5 μg); Lane 5 pool from Q Sepharose a (2.5 μg); Lane 6 pool from After Dialysis (1015 μg); Lane 7 pool from Green (0.85 μg); Lane 8 pool from Q Sepharose b (1.0 μg); Lanes 9 pooled from P6DG (0.7 μg); Lane 10 pooled from Toyopearl (0.7 μg).

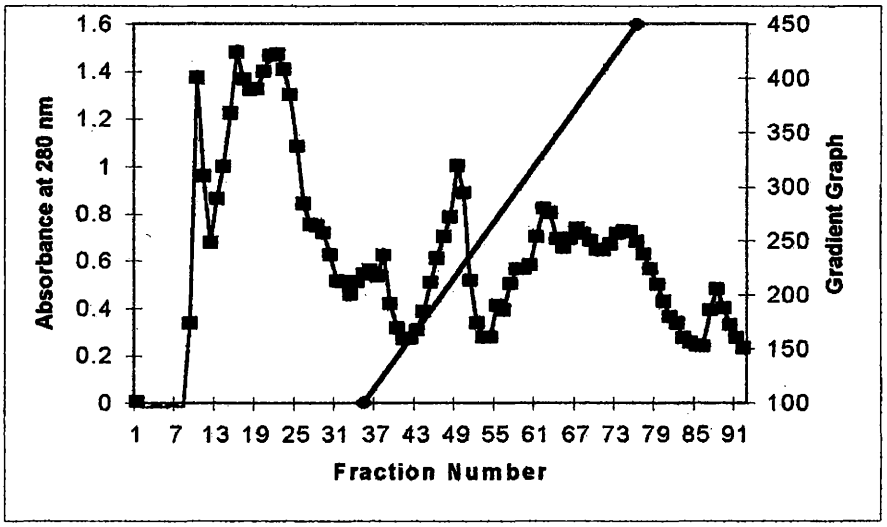


**Figure 5. Column Profile from a Q Sepharose anion exchange column with the high pressure cell lysis method.** The (■) represents the absorbance at 280 nm. The (◆) represents the gradient from 100 to 450 mM NaCl. The column was equilibrated with Buffer A at a flow rate 3.0 mL/minute. Crude cell extract was loaded onto the column and eluted with a 100-450 mM NaCl gradient. Fraction size was 6.9 mL. Fraction 68 through 79 were pooled.

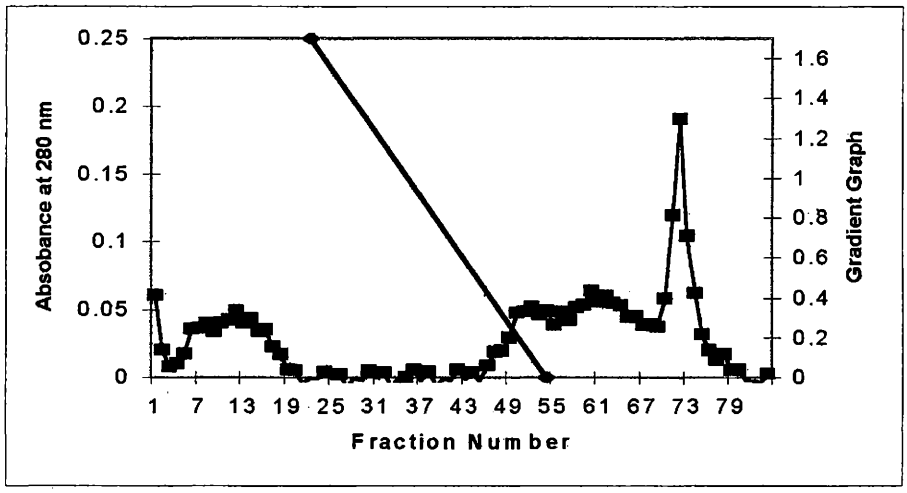


**Figure 6. Column Profile from the phenyl hydrophobic interaction column at pH 7.0.** The (■) represents the absorbance at 280 nm. The (◆) represents the gradient from 1.7 to 0 M AMS. The column was equilibrated with 1.7 M AMS, pH 7.0 at a flow rate 2.5 mL/minute. Pooled void volume from P6DG was loaded onto the column and eluted with a 1.7 M- 0 M AMS gradient. The enzyme fractions were below the limit of detection for Gibb's assay. Fraction size was 1.4 mL. Fractions 58 through 62 were pooled.

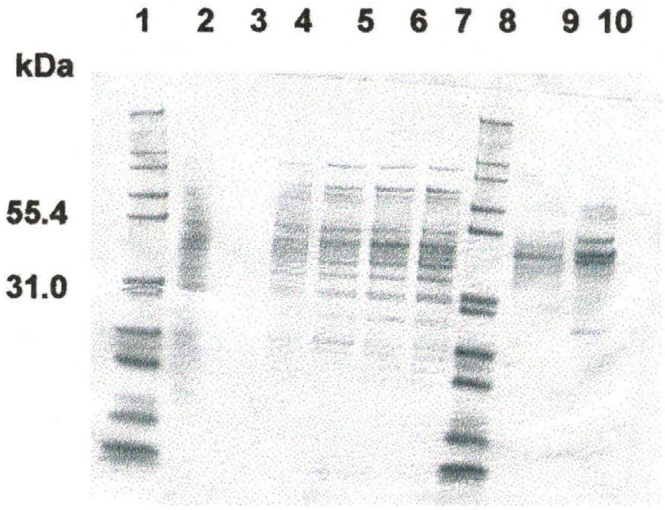


**Figure 7. SDS-polyacrylamide gel electrophoresis analysis of protein purification with high pressure cell lysis.** The pooled samples were electrophoresed and the protein bands were detected by Collidal blue staining. Lanes 1 and 8, Molecular weight standards (Novex); Lane 2 crude cell lyse (1.0 μg); Lane 3 Q Sepharose a (1.5 μg); Lane 4 after dialysis (2.5 μg); Lane 5 Green column (2.5 μg); Lane 6 Source 30Q (2.5 μg); Lane 7 P6DG (2.5 μg); Lane 9 Toyopearl 58-62 (1.3 μg); Lanes 10 Toyopearl 71-73 (1.0 μg).

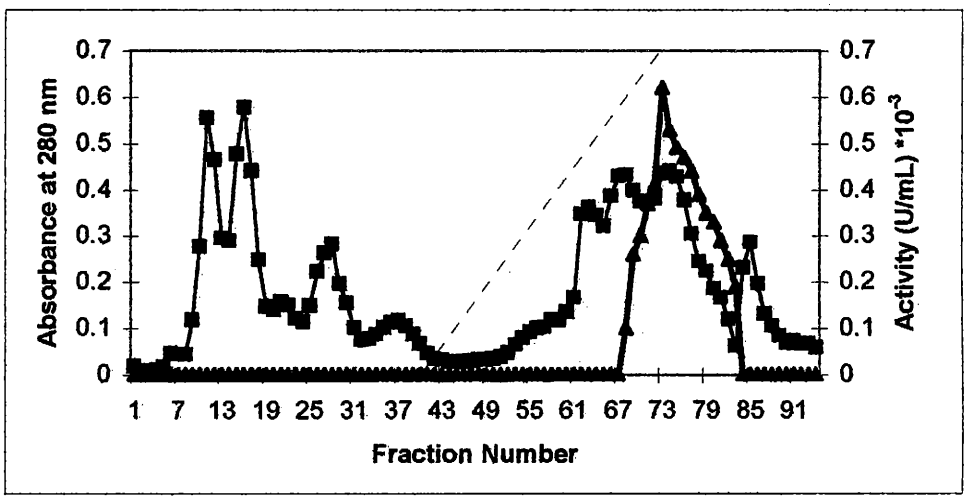


**Figure 8. Column Profile from a Q Sepharose anion exchange column with the high pressure cell lysis method.** The (■) represents the absorbance at 280 nm. The (▲) represents the enzyme activity. The dashed line represents the gradient from 100 to 450 mM NaCl. The column was equilibrated with Buffer A at a flow rate 3.1 mL/minute. Crude cell extract was loaded onto the column and eluted with a 100-450 mM NaCl gradient. Fraction size was 8.0 mL. Fractions 68 through 82 showed activity with the HTS 7000 Plus fluorimetry assay and were pooled.

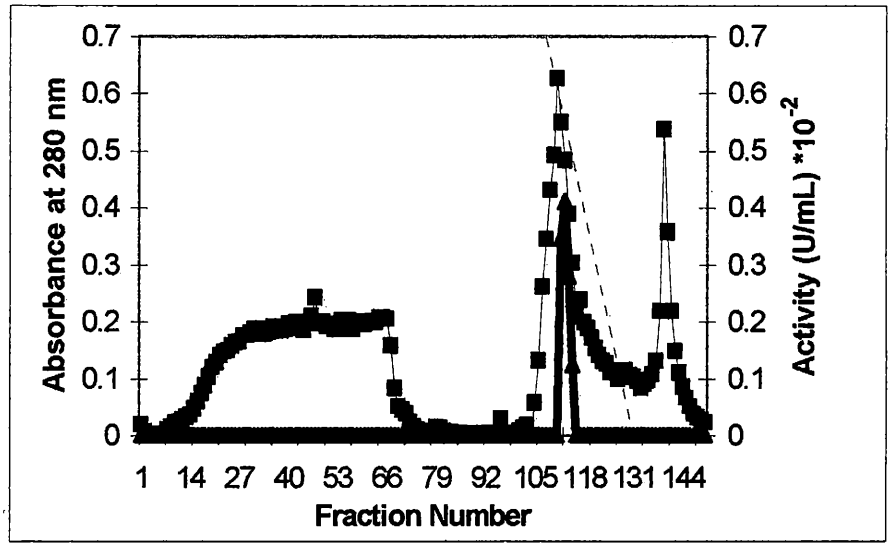


**Figure 9. Column Profile from the phenyl hydrophobic interaction column at pH 6.77.** The (■) represents the absorbance at 280 nm. The (▲) represents the enzyme activity. The dashed line represents the gradient from 1.7 to 0 M AMS. The column was equilibrated with 1.7 M AMS, pH 6.77 at a flow rate of 2.5 mL/minute. Fraction size was 1.5 mL. The dialysate was loaded onto the column, and eluted with a 1.7 M- 0 M AMS gradient. The enzyme fractions showed activity in fraction 112 through115 with the HTS 700 Plus fluorimetry assay and were pooled.

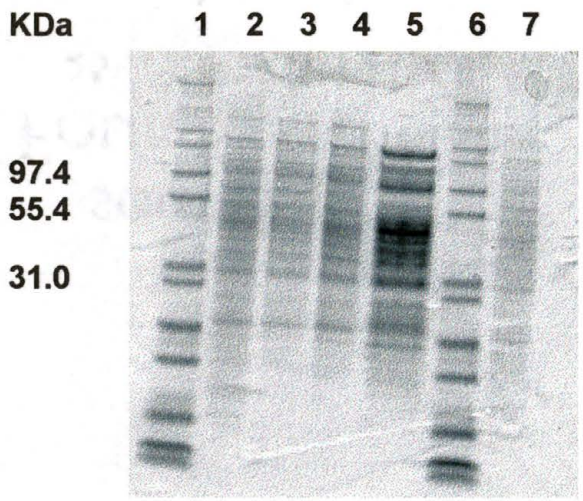


**Figure 10. SDS-polyacrylamide gel electrophoresis analysis of protein purification with the high pressure cell lysis method.** The pooled samples were electrophoresed and the protein bands were detected by collidal blue staining. Lanes 1 and 6, Molecular weight standards (Novex); Lanes 2 and 7 Crude Cell Extract (7.5 μg, 3.0 μg); Lane 3 Q Sepharose a (2.5 μg ); Lane 4 After dialysis (2.5 μg); Lane 5 Toyopearl (4.8 μg).

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