

THE GENE CLONING, OVEREXPRESSION, PURIFICATION, AND CHARACTERIZATION OF DIBENZOTHIOPHENE MONOOXYGENASE AND DESULFINASE FROM *GORDONIA ALKANIVORANS* RIPI90A

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ABSTRACT

The biodesulfurization (BDS) of sulfur compounds in fossil fuels is a process to reduce sulfur dioxide emissions that cause environmental pollution. *Gordonia alkanivorans* RIPI90A is able to convert dibenzothiophene, an organic sulfur compound in petroleum, to 2-hydroxybiphenyl (2-HBP) in 4S pathway. In this study, (DBT), DszA and DszB, DBT sulfone monooxygenase, and desulfinate were respectively isolated from *G. alkanivorans* RIPI90A. PCR amplified fragments were obtained by using primers designed based on known sequences from *G. alkanivorans* RIPI90A. They are identified as *dszA* and *dszB* and have shown high similarity compared to *Rhodococcus erythropolis* IGTS8 (88% for *dszA* and 88% for *dszB*). Subsequently, *dszA* and *dszB* genes were expressed under the control of T7 promoter in *Escherichia coli*. The recombinant proteins were purified to achieve homogeneity using Ni-agarose column chromatography. The molecular mass of the purified DszA and DszB were determined to be 51.9 and 39.2 kDa respectively by using SDS-polyacrylamide gel electrophoresis. DszA showed a K_m of 0.14 ± 0.005 mM and a maximal velocity of 0.004 ± 0.0004 mM/min. DszB showed a wide substrate range in a way that all aromatic sulfonates compounds acted as its substrate; as it seemed the active site was suitable for the sulfonated aromatic rings. The K_m and V_{max} values of DszB were calculated to be 1.81 ± 0.02 mM and 6.55 ± 0.005 μ M/min respectively using 4-Amino-3-hydroxy-naphthalene-sulfonic acid as a substrate.

Keywords: DszA, DszB, Purification, *Gordonia alkanivorans* RIPI90A, Biodesulfurization

INTRODUCTION

The presence of sulfur in petroleum products is a serious concern for the petroleum industry. The combustion of sulfur-containing fuels leads to the atmospheric emission of sulfuroxides (SO_x), which is the major cause of acid rain and other health and environmental problems [1, 2].

Hydrodesulfurization (HDS) is the conventional method for removing non-volatile organic sulfur compounds from petroleum fractions. However, HDS is operated under high temperature and pressure and is not effective for the removal of DBT and alkylated DBT's, which constitute the main organosulfur compounds in fossil fuels [3, 4]. Microorganisms are considered to be

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potential catalysts for removing sulfur from the refractory organic sulfur compounds, and bio-desulfurization (BDS) has attracted much attention as an alternative and complementary method. The desulfurization pathway of DBT has extensively been studied with *Rhodococcus erythropolis* strain IGTS8 [1]. The pathway, also called 4S pathway, proceeds via two cytoplasmic monooxygenases (DszC and DszA) supported by flavin reductase (DszD) and a desulfinate (DszB). DBT monooxygenase (DszC) catalyzes the sequential oxidation of DBT to DBT-sulfoxide and DBT-sulfone. DBT-sulfone converts to 2-(2'-hydroxyphenyl) benzene sulfinate (HPBS) through the oxidative cleavage of one of the DBT-sulfone carbon-sulfur bonds by second enzyme, DBT-5, 5'-dioxide monooxygenase (DszA). The final enzyme in the pathway, HPBS desulfinate, catalyzes the desulfination of HPBS to produce 2-hydroxybiphenyl (2-HBP) and sulfite. The oxygen atom incorporated at each step of this desulfurization pathway is derived from molecular oxygen [5,6]. DszC and DszA do not use NADH directly, but use FMN₂ from a FMN:NADPH oxidoreductase (DszD) [7]. DszD couples the oxidation of NADH with substrate oxygenation by DszA and DszC. The DszA enzyme is widely studied and has been purified from different strains, namely *R. erythropolis* IGTS8, *R. erythropolis* D-1, and *Paenibacillus* sp. Strain A11-2 [8,9]. It is a homodimer with a subunit molecular weight of 50 kDa and a reaction rate of 5-10-fold higher than DszC [7]. A great deal of genetic studies have been done on the *dsz* operon but only a few reports are available on the purification and characterization of Dsz enzymes. A DBT-desulfurizing bacterial strain has been previously isolated and identified as *Gordonia alkanivorans* RIPI90A based on the 16S rDNA gene sequence (Gene-Bank accession no. DQ321498) [10]. In this study, we have isolated, cloned, and sequenced the DBT monooxygenase and desulfinate genes from *G. alkanivorans* RIPI90A. Subsequently,

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they were expressed in *E. coli* as a heterologous host. The enzymes were purified to near homogeneity and the kinetic parameters were determined.

MATERIALS AND METHODS

Chemicals

DBT sulfone was obtained from Aldrich (Milwaukee, USA). As stock solutions for culture media and reaction mixtures, DBT sulfone was dissolved in acetonitrile. Restriction endonucleases, *Pfu* DNA polymerases, T4 DNA ligase, and isopropyl-beta-D-thiogalactopyranoside (IPTG) were obtained from MBI Fermentas. NADH, NADPH, and FMN were from Fluka, as were all sulfonated substrates.

Bacterial Strains, Plasmids, and Culture Conditions

Gordonia alkanivorans RIPI90A was used as the source of genomic DNA for cloning of *dszA* and *dszB* genes. *E. coli* DH5 α was used as a host for general cloning and *E. coli* BL21 cells were used as a host for gene expression. Plasmid pUC19 (Fermentas) was used as a cloning vector and plasmid pET21a (Novagen) as an expression vector. *G. alkanivorans* RIPI90A was cultivated on Luria-Bertani (LB) medium at 30 °C.

General Recombinant DNA Techniques

Recombinant DNA techniques were performed as described by Sambrook and Russell [11] or according to manufacturer's protocols. Total DNA of *Gordonia alkanivorans* RIPI90A was prepared as previously described [12]. Plasmid DNA was prepared using high pure plasmid isolation kit (Roche Applied Sciences, Mannheim, Germany). Plasmids were introduced into *E. coli* by electroporation under standard conditions using Gene pulser X-cell electroporator (BioRad). All sequencing was carried out in both directions to eliminate reading errors by Applied Biosystems 3130XL Genetic Analyzer

instrument (USA).

Gene Cloning

To isolate the entire *dszA* and *dszB* open reading frames from *G. alkanivorans* RIPI90A genomic DNA, primers annealed to the 5' and 3' ends of the coding regions of *dszA* and *dszB* gene were designed based on the known sequences of *G. alkanivorans* RIPI90A *dsz* operon (GenBank accession number EU364831). PCR amplification was carried out using programmable thermal-cycler (Eppendorf). The reaction mixture contained 50 µl of 20 mM Tris-HCl (with a pH of 8.8 at 25 °C), 10 mM of (NH₄)₂SO₄, 10 mM of mM KCl, 0.1% (v/v) Triton X-100, 0.1 mg/ml of BSA, 2 mM of MgSO₄, 0.2 mM of (each) deoxynucleotide triphosphate, 50 µmol of each primer, 0.5 µg of RIPI90A genomic DNA, and 1.25 U of *pfu* DNA polymerase. The reaction was performed at 95 °C for 1 min, then at 61 °C for 30 s, and finally at 72 °C for 3 min for a total of 35 cycles within a 5-min initial denaturation step at 95 °C in the first cycle and a 5-min extension at 72 °C in the last cycle. The forward and reverse primers designed for *dszA* and *dszB* genes have the sequences of AF: 5'-AAGGATCCATGGCTCAACGGCGACAAC-3' and AR: 5'-AAAAAGCTTGTGTGTCGAGGATGCCGGTAT-3', BF: 5'-AAAGAATTCATGGCAGGCCGCTCAG-3' and BR: 5'-AAAAAGCTTTCGGTGACGGTTGAGGCTG-3' respectively. These primers were designed with *Bam*HI and *Hind*III recognition sites for *dszA* gene and *Eco*RI and *Hind*III recognition sites for *dszB* gene for the convenient in-frame cloning of the genes into the multiple cloning site of the pET21a vector. This placed the *dszA* and *dszB* genes under the control of strong bacteriophage T7 transcription and translation signals. The cloned genes were fully sequenced to confirm the fidelity of PCR amplification and the accuracy of reading frame.

Bacterial Growth and Induction of DszA and DszB Protein

Recombinant *E. coli* cells were cultivated at 37

°C in an LB medium containing 50 µg of ampicillin per milliliter. For the induction of the *dszA* and *dszB* genes under the control of the T7 promoter, 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the LB medium and the recombinant cells were cultivated at 25 °C and 200 rpm for 6 hrs. The estimation of induced DszA and DszB protein was carried out by SDS-PAGE according to the protocol described by Laemmli [13].

Protein Purification

The cells were harvested by centrifugation at 5000 rpm for 20 min, resuspended in a lysis buffer (50 mM of Tris with a pH of 8.0, 300 mM of NaCl, 2 mM of imidazole, 1 mM PMSF, 0.2 mg/mL of lysozyme, 2% Triton X-100, 0.02% NaN₃, and 10% glycerol), and finally sonicated on ice for 10 min for 10 times with an interval of 1 min. The total lysate was centrifuged at 12000 rpm and 4 °C for 20 min. Because of a high level of expression, large amounts of the proteins would appear in the pellet, which were not supernatant. Thus some additional processes were employed for purification; The pellet was dissolved in a denaturation buffer containing 50 mM Tris with a pH of 8.0, 300 mM of NaCl, 8M of urea, 30 mM of imidazole, and 10% glycerol (5 mL/1gr wet weight), and was then centrifuged at 12000 rpm for 20 min. The supernatant was shaken with 2 mL of Ni-agarose resin for 2 hours at room temperature. As the produced proteins were appended to a His-domain at the N-terminus, binding to the Ni-agarose was facilitated. A 1×10 cm column was packed with this protein-tagged resin. In the next step, the column was washed with eight volume of washing buffer containing a gradient of urea from 4 to 0 M (4 M of urea, 50 mM of Tris with a pH of 8.0, 300 mM of NaCl, 30 mM of imidazole, and 10% glycerol). The His-tagged proteins were eluted with four volumes of elution buffer containing 50 mM of Tris with a pH of 8.0, 300 mM of NaCl, 250 mM of

imidazole. The eluted protein was assayed for purity by SDS-PAGE and used for enzyme characterization.

Analytical Technique

For DszA activity measurement, a reaction mixture (with a total volume of 250 μL) containing 100 mM of potassium phosphate buffer (pH=7.0), 10 μM of FMN, 6 mM of NADH, 0.25 mM of DBT sulfone (in acetonitrile), 2 unit/mL of FMN-NAD(P)H oxidoreductase, and DszA was prepared. After 30 min of incubation at 35 $^{\circ}\text{C}$ and 175 rpm, the reaction was terminated by the addition of 12 μL of 12 N HCl followed by the extraction with 200 μL of ethyl acetate. After centrifugation at 13000 rpm for 20 min, the supernatant was injected into the HPLC for measuring the amount of residual DBT sulfone in the organic layer. 1 unit of enzyme activity was defined as the amount which produces 1 μmol of HBPSi per minute.

The HPLC was carried out with a C18-S5ODS1 column (250 mm \times 4.6 mm). A spectrophotometric UV detection was used ($\lambda = 280$ nm). The mobile phase contained methanol and 20 mM of KH_2PO_4 , which was adjusted to have a pH of 2.5 by using H_3PO_4 . The ratios of potassium phosphate solution to the methanol were 40:60 for the measurement of DszA activity (the retention times of 2'-hydroxybiphenyl-2-sulfinic acid (HBPSi) and DBT sulfone were 3.4 and 4.5 min respectively) at a flow rate of 1 mL/min. DszB activity was assayed with Ellman's reagent (5, 5'-dithio bis (2-nitrobenzoic acid)) which produced a bright yellow color due to the production of disulfide and TNB (2-nitro-5-thiobenzoic acid) upon reaction with sulfite produced by enzyme reaction with sulfonated substrates [14, 15].

TNB was the colored species produced in this reaction and had the molar extinction coefficient of 14150 $\text{M}^{-1}\text{cm}^{-1}$ at 412 nm. The specific substrate of DszB in the desulfurization pathway

is HBPSi which converts to HBP and sulfite upon reaction. In this study, we measured the kinetic parameters of the enzyme by measuring the amount of sulfite produced from different sulfonated substrates such as 4-amino-3-hydroxy-naphthalene-sulfonic acid, 3-(N-morpholino) propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and 2-morpholino-ethane-sulfonic-acid. The reaction mixture (with a total volume of 200 μL) containing a 100 mM potassium phosphate buffer (with a pH of 7.0), 500 μM of sulfonated substrate, and an appropriate amount of DszB was prepared. The reaction was initiated by the addition of enzyme to the reaction mixture. After 30 min, at 35 $^{\circ}\text{C}$ and 175 rpm, the reaction was terminated by the addition of Ellman's reagent; colorimetric reaction was allowed to continue at room temperature for 2 min. Absorbance was measured at 412 nm. One unit of enzyme activity was defined as the amount which produced 1 μmol of sulfite per minute.

RESULTS AND DISCUSSION

Cloning and Sequencing of *dszA* and *dszB* Genes

Polymerase chain reaction (PCR) experiments were performed with oligonucleotides based on the known nucleotide sequences of *G. alkanivorans* RIPI90A *dsz* operon (GenBank accession number EU364831) and the total DNA from RIPI90A as template. The fragments containing *dszA* and *dszB* genes with lengths of 1.4 kb and 1.1 kb respectively were PCR-amplified and cloned into pET system and the nucleotide sequence of these products were determined. The overall nucleotide sequence of *dszA* and *dszB* genes of *G. alkanivorans* showed 83.1% homology to the corresponding genes of *Rhodococcus* sp. IGTS8 and 83.2% to those of *G. nitida*.

Expression and Purification of DszA and DszB Proteins

DszA and DszB were overproduced in *E. coli* BL21 (DE3) cells by using IPTG as an inducer. Cells were harvested after 6 hr induction, and then the supernatant and debris of sonicated cells were collected. A small amount of soluble enzyme detected in the supernatant. SDS-PAGE analysis revealed that overproduced proteins were present in the insoluble fraction indicating that the major part of the recombinant protein accumulated as inclusion bodies (Figure 1a and 1b). To decrease the extent of protein aggregation, the expression conditions such as induction time, temperature, and IPTG concentration were optimized and a very little activity of either enzyme was detected in the cell-free extracts (data are not presented). However, the main part of the recombinant protein still remained in the insoluble fraction. To refold the inactive proteins, a gradient of urea in the elution buffer was applied during the purification process. The purification of His6-N-tagged DszA and DszB proteins in a single-step affinity chromatography using Ni-agarose column resulted in almost homogenous protein preparations. The effectiveness of purification process was evaluated by SDS-PAGE analysis. The results of the electrophoretic analysis of DszA and DszB overexpression and purification are shown in Figure 1a and 1b respectively. The molecular mass of the DszA and DszB was 51.9 kDa and 39.2 kDa respectively and no extra bands were observed

Determination of Kinetic Parameters of DszA and DszB

Kinetic parameters of the purified refolded DszA were obtained at room temperature using different concentrations of DBT-sulfone for the depiction of standard curve. The Michaelis constant was 0.14 ± 0.005 mM and the maximal velocity was 0.004 ± 0.0004 mM/min. The Eadie-Hofstee plot is shown in Figure 2. Enzyme activity for DszB was studied in the presence of

different sulfonated substrates (Table 1) and finally kinetic parameters, namely K_m and V_{max} , were determined using different concentrations of 4-amino-3-hydroxy-naphtalen-sulfonic acid. Each value represented the mean of at least three independent sets of experiments. The Eadie-Hofstee plot is shown in Figure 3. The Michaelis constant was 1.81 ± 0.02 mM and maximal velocity was 6.55 ± 0.005 μ M/min.

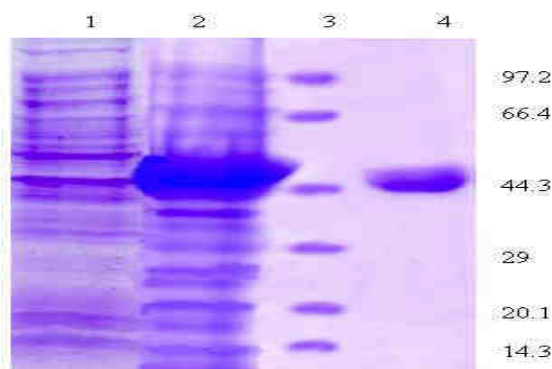


Figure 1a: SDS-PAGE analysis (12.5% polyacrylamide gel) of overexpression and purification.

Proteins were detected by coomassie brilliant blue; lane 1: crude extract before induction with IPTG, lane 2: crude extract after induction, lane 3: molecular size markers (kDa) 97.2, 66.4, 44.3, 29.0, 20.1, 14.3, lane 4: purified enzyme (DszA).

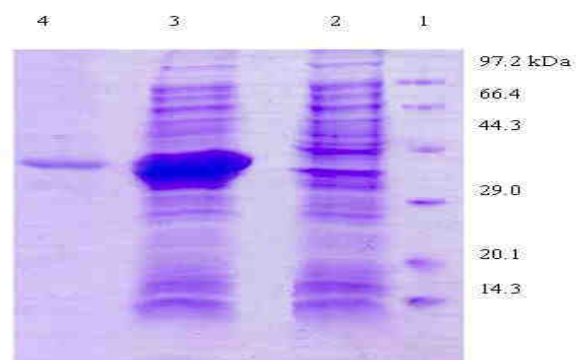


Figure 1b: lane 1: molecular size markers, lane 2: crude extract before induction with IPTG, lane 3: crude extract after induction, lane 4: purified enzyme (DszB).

With reference to activity, the refolding yield of enzymes is often estimated. Our results indicated that the refolding yield under refolding conditions was 37% and 40% for DszA and DszB, respectively (Table 2).

In 2007, Alves et al. isolated new desulfurization genes from *G. alkanivorans* strain 1B and successfully expressed into *E. coli* in the presence of inorganic sulfur. Therefore, it was strongly suggested that alternative culture media containing cheaper carbon sources, like those obtained from lignocellulosic or agro-industrial by-products should further be used [16].

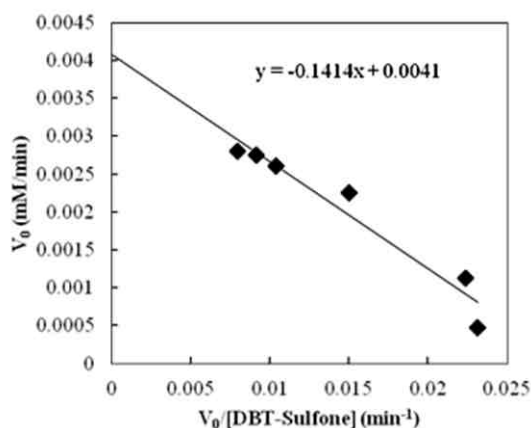


Figure 2: The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of DszA calculated by Eadie-Hofstee plot using DBT-sulfone as the substrate

Table 1: Enzyme activity of 2'-hydroxybiphenyl-2-sulfinate desulfinate (DszB) in the presence of different sulfonated substrates at 35 °C

Sulfonated substrates (1 mM)	V_{max} ($\mu\text{M}/\text{min}$)
MOPS	5.80 ± 0.004
HEPES	5.10 ± 0.005
MES (2-Morpholinoethane sulfonic acid)	2.55 ± 0.004
AHNS (4-amino-3-hydroxy-naphthalene-1-sulfonic acid)	6.55 ± 0.005

Table 2: Comparison of kinetic parameters between soluble and refolded forms of DszA and DszB

Protein	K_m (mM)	K_{cat} ($V_{max}/\mu\text{M}$)	Refolding Yield
Soluble DszA	0.14	35	100%
Refolded DszA	0.14	13	37%
Soluble DszB	1.83	3	100%
Refolded DszB	1.81	1.1	40%

G. alkanivorans RIPI90A has been previously isolated and reported as an efficient DBT-desulfurizing bacterium with unique advantages for the BDS process; however, as other natural desulfurizing bacteria, the expression of Dsz enzymes are strongly repressed in the presence of sulfate; nevertheless, we have successfully expressed the methionine and cysteine [10, 17]. Therefore, it is impossible to obtain high desulfurization activity in the presence of sulfur compounds in culture media. Accordingly, we decided to clone the dsz genes under the control of a sulfate non-repressible promoter.

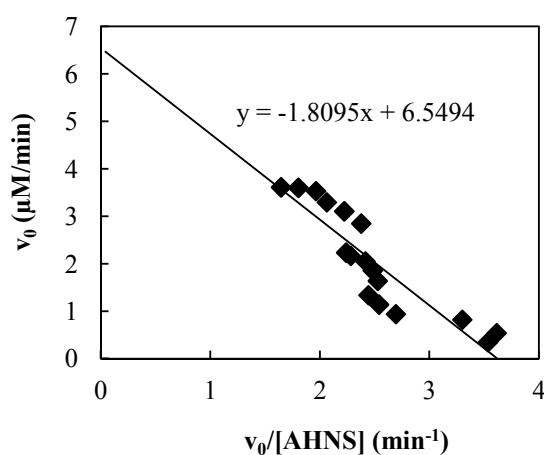


Figure 3: The Michaelis–Menten constant (K_m) and maximum velocity (v_{max}) of DszB calculated by Eadie-Hofstee plot using 4-amino-3-hydroxy-naphthalene-1-sulfonic acid as the substrate

In the previous study, we successfully expressed the *G. alkanivorans* RIPI90A dsz operon under the control of an inducible lac promoter. In this study, the T7 promoter was selected for the strong expression of recombinant dszA and dszB genes separately to obtain a high level of active enzyme. Subsequently, recombinant construct was made to place the dsz genes under the control of T7 promoter and transferred to *E. coli* BL21 as heterologous host, but the expression of the Dsz enzymes in recombinant strain resulted in a great amount of insoluble enzymes even after lowering the induction temperature and IPTG concentration. Therefore, the development of a strategy for the efficient refolding of the <http://jpst.ripi.ir>

proteins from inclusion bodies has been considered as an important issue in the recovery of soluble active proteins for functional studies. In the present work, the refolding of insoluble proteins with the gradient of urea during the purification process resulted in a marked increase in the amount of active enzymes. The efficiency of refolding was obtained, with reference to activity, to be 37% and 40% for DszA and DszB respectively. In order to obtain the maximum refolding yields, the refolding conditions were optimized by statistical methods.

In this study, the activity of DszB was studied in presence of different sulfonated substrates, and according to the results, AHNS was selected to determine kinetic parameters and DBT-sulfone for DszA. DszA, the sulfone monooxygenase, is the second enzyme in microbial DBT desulfurization metabolism and has a great significance in DBT desulfurization metabolism, because it cleaves one of the two C-S bonds of the DBT molecule. DszB is a unique enzyme capable of cleaving carbon-sulfur of 2-hydroxybiphenyl-2-sulfinate (HBPSi) in the final step in the microbial DBT desulfurization and produce 2-HBP [18].

CONCLUSIONS

In conclusion, we reported the expression, purification and refolding of DszA and DszB of Dsz enzymes of *Gordonia alkanivorans* RIPI90A. It was shown that *E. coli*-derived enzymes could effectively act on sulfonated substrates in vitro. The effective strategies for DszA and DszB production could be suggestive for a further study on the structure and function of the enzymes.

NOMENCLATURE

2-HBP : 2-hydroxybiphenyl
 BDS : Biodesulfurization
 DBT : Dibenzothiophene
 DszA : DBT-5, 5'-dioxide monooxygenase
 dszA : Gene encoding DszA

DszB : 2-(2'-hydroxyphenyl) benzene sulfinate desulfinase
 dszB : Gene encoding DszB
 DszC : DBT monooxygenase
 dszC : Gene encoding DszC
 FMN : Flavin mononucleotide
 HDS : Hydrodesulphurization
 NADH : Nicotinamide adenine dinucleotide
 NADPH : Nicotinamide adenine dinucleotide phosphate

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