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Dibenzothiophene Desulfurizing Enzymes from Moderately Thermophilic Bacterium *Bacillus subtilis* WU-S2B: Purification, Characterization and Overexpression

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The moderately thermophilic bacterium *Bacillus subtilis* WU-S2B desulfurized dibenzothiophene (DBT) at 50°C through the selective cleavage of carbon-sulfur bonds. In this study, three enzymes involved in the microbial DBT desulfurization were purified and characterized. The first two enzymes, DBT monooxygenase (BdsC) and DBT sulfone monooxygenase (BdsA), were purified from the wild-type strain, and the last one, 2′-hydroxybiphenyl 2-sulfinic acid desulfinase (BdsB), was purified from the recombinant *Escherichia coli* overexpressing the gene, *bdsB*, with chaperonin genes, *groEL/ES*. The genes of BdsC and BdsA were also overexpressed. The molecular weights of BdsC and BdsA were determined to be 200 and 174 kDa, respectively, by gel filtration chromatography, suggesting that both enzymes had four identical subunits. BdsB had a monomeric structure of 40 kDa. The three enzymes were characterized and compared with the corresponding enzymes (DszC, DszA, and DszB) of mesophilic desulfurization bacteria. The specific activities of BdsC, BdsA, and BdsB were 84.2, 855, and 280 units/mg, respectively, and the latter two activities were higher than those of DszA and DszB. The heat stability and optimum temperature of BdsC, BdsA, and BdsB were higher than those of DszC, DszA, and DszB. Other enzymatic properties were investigated in detail.

[**Key words:** dibenzothiophene, desulfurization, *Bacillus*, monooxygenase, desulfinase]

Sulfur compounds in fossil fuels cause worldwide environmental problems. Hydrodesulfurization (HDS) is the current method for sulfur removal, but heterocyclic sulfur compounds are not completely removed. Regulations to reduce the sulfur content of fuels for motor vehicles will undoubtedly become increasingly stringent in the future. In these circumstances, biological desulfurization (BDS) has attracted attention as an alternative and complementary method of treating recalcitrant organic sulfur compounds (1, 2).

Dibenzothiophene (DBT) and its derivatives are widely recognized as model target compounds in BDS research, and many kinds of DBT-desulfurizing microorganisms have been isolated; for example, the *Rhodococcus erythropolis* strains IGTS8 (3), D-1 (4), and H-2 (5), the *Corynebacterium* sp. strain SY-1 (6), and the *Gordona* sp. strain CYKS1 (7). Particularly, the *R. erythropolis* strain IGTS8 has been extensively studied, and the genes involved in DBT desulfurization (*dszABC*) have been characterized (8). DBT is desulfurized to yield 2-hydroxybiphenyl (2-HBP) by these DBT-desulfurizing microorganisms without degradation of the carbon skeleton, meaning that they accomplish the desulfurization of petroleum without a decrease in calorific value. This property affords a great advantage when these microorganisms are applied to industrial processes. Most of the DBT-desulfurizing organisms so far reported are mesophilic, so that they have difficulty in DBT desulfurization at temperatures higher than 30°C. It is presumed that most sulfur compounds will be desulfurized by the HDS process in petroleum refining, after which BDS process will be applied to desulfurize the more recalcitrant sulfur compounds. Since HDS requires a high temperature (300–350°C), cooling is essential for the practical use of the desulfurizing microorganisms. For this reason, thermophilic microorganisms would be more advantageous than mesophilic ones. In addition, contamination by undesirable microorganisms which affect the BDS process would be avoided at high temperatures.

Paenibacillus sp. A11-2 has been reported as a thermophilic DBT-desulfurizing bacterium (9), and the genes, *tdsABC*, corresponding to *dszABC*, were cloned and characterized (10). Then, two thermophilic desulfurization bacteria, *Bacillus subtilis* WU-S2B (11) and *Mycobacterium phlei* WU-F1 (12), were isolated, and the desulfurizing genes *bdsABC* were cloned from both strains (13). All known bacteria with the ability of DBT desulfurization possess three

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desulfurization enzymes. First, DBT is oxidized to DBT sulfone via DBT sulfoxide by DBT monooxygenase (DszC/TdsC/BdsC) coupled with flavin reductase. Second, DBT sulfone is converted to 2′-hydroxybiphenyl 2-sulfinic acid (HBPSi) by DBT sulfone monooxygenase (DszA/TdsA/BdsA) coupled with flavin reductase. Finally, HBPSi is desulfurized to 2-HBP with the release of the sulfur atom as sulfite by HBPSi desulfinase (DszB/TdsB/BdsB). DszC, DszA, and DszB, and flavin reductase from the *R. erythropolis* strains IGTS8 (14) and D-1 (15–18) were purified and characterized. In addition, Konishi *et al*. purified and characterized TdsC, TdsA, and TdsB, and flavin reductase from *Paenibacillus* sp. A11-2 (19–21). DszB was also purified from the desulfurization strain, *R. erythropolis* IGTS8 (22). Recently, the X-ray analysis of DszB was conducted for the first time as an enzyme involved in the DBT desulfurization (23).

To date, the genes involved in microbial DBT desulfurization have been isolated from some bacterial strains. However, the enzymes have been characterized from only two species, *Rhodococcus* (DszABC) and *Paenibacillus* (TdsABC), and it has been demonstrated that the properties of TdsABC enzymes are not always superior to those of DszABC enzymes as follows. Certainly, the heat stability of Tds enzymes was higher than that of Dsz enzymes, but the specific activities of the purified Tds enzymes were considerably lower than those of Dsz enzymes. Consequently, Bds enzymes from thermophilic *Bacillus* species should be worth characterizing. It was confirmed that the growing or resting cells of *B. subtilis* WU-S2B (11) and the recombinant *E. coli* cells harboring *bds*ABC genes (13) exhibited DBT desulfurizing activity at 50°C. However, we could not rule out the possibility that the properties of the cells made a greater contribution to the activity at high temperatures than those of the enzymes. In this report, we describe the purification and characterization of BdsC, BdsA, and BdsB from *B. subtilis* WU-S2B and a recombinant *E. coli* strain. Then, we clarify that the desulfurizing activity of the strain at high temperature is due to the properties of its desulfurizing enzymes.

MATERIALS AND METHODS

Materials DBT sulfone was purchased from Aldrich (Milwaukee, WI, USA). Dibenz(*c*,*e*)[1,2]oxathiin 6-oxide (BPSi) and sodium 2-phenylbenzene sulfinate were gifts from the Japan Co-operation Center, Petroleum (Shizuoka). 2,2′-Dihydroxybiphenyl (2,2′-DHBP) was obtained from Tokyo Kasei (Tokyo). Q-Sepharose Fast Flow, MonoQ HR 10/10, Superdex 200HR 10/30, and low molecular weight markers for SDS–PAGE were purchased from Amersham Biosciences (Piscataway, NJ, USA). Gigapite, a kind of hydroxyapatite resin, was obtained from Seikagaku (Tokyo). The ultrafiltration apparatus (model 8200) and its membranes (YM-10) were purchased from Millipore (Billerica, MA, USA). The plasmid for overexpression, pET21-a, was purchased from Novagen (Madison, WI, USA), and the plasmid, pKY206 (24), having the chaperonin genes, *groEL/groES*, was a gift from Dr. Yasushi Kawata of Tottori University. The other chemicals used were obtained from Wako Chemical Industries (Osaka).

Cultivation *B. subtilis* WU-S2B was grown in medium A-2 (11) supplemented with 100 mg/*l* DBT as the ethanol solution. Cultivation of the strain WU-S2B was done at 40°C for 48 h in a 30-*l* jar fermenter (Micros; New Branswick Scientific, Edison, NJ, USA) containing 20 *l* of medium with agitation at 200 rpm. Co-expression experiments with *groEL/groES* genes were performed according to the procedure reported previously (25). The *E. coli* cells expressing *bdsA* or *bdsB* and *groEL/groES* genes were cultivated in 2-*l* flasks containing 500 ml of LB medium with 100 μg/ml ampicillin and 10 μg/ml tetracycline at 25°C for 15 h. The *E. coli* cells for BdsC production were cultured in 500 ml of LB medium with 100 μg/ml ampicillin at 25°C for 24 h with the addition of 0.2 mM isopropyl-1-thio-β-D-galactoside (IPTG) at 12 h during the cultivation. To measure BdsC and BdsA activities, flavin reductase (TdsD) from the thermophilic strain *Paenibacillus* sp. A11-2 was used. The cultivation of the *E. coli* cells producing TdsD was done in 2-*l* flasks containing 500 ml of LB medium with 100 μg/ml ampicillin at 37°C for 7 h. IPTG was added at 3 h. The recombinant *E. coli* strains were cultured with reciprocal shaking at 120 strokes/min.

Purification of enzymes The cells were harvested by centrifugation at 7500×*g* and stored at −20°C. The frozen cells for BdsC, BdsA and TdsD production were thawed and suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM dithiothreitol (DTT) and 10% glycerol (basal buffer I), and then disrupted with an ultraoscillator at 20 kHz (Sonifier 450; Branson Instruments, Danbury, CT, USA). The cell debris was removed by centrifugation at 7500×*g* for 30 min. The cells for BdsB production were disrupted in a similar manner in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 10% glycerol (basal buffer II). Before the enzyme solution was subjected to column chromatography, it was dialyzed thoroughly against the equilibrating buffer. After the active fractions were eluted from the column, they were combined and concentrated by ultrafiltration. The MonoQ HR 10/10 and Superdex HR 10/30 chromatographies were controlled by an AKTA system, and glycerol was not added to the buffer. The flow rates for the MonoQ and Superdex columns were 2 and 0.25 ml/min, respectively, and 0.15 M NaCl was added to the buffer for the Superdex column.

TdsD from the recombinant E. coli strain Enzyme purification was done using wet cells weighing 10.8 g (from 3 *l* of culture broth). The amount of the protein and the specific activity of the cell extract were 673 mg and 12.6 units/mg, respectively. Table 1 shows a summary of the purification steps. TdsD was purified essentially to homogeneity (7.6-fold, 95.8 units/mg), and 29.2 mg of the purified enzyme was obtained with 33% yield.

BdsC from B. subtilis WU-S2B Enzyme purification was done from the cells with a wet weight of 326 g (from 60 *l* of culture broth). The amount of the protein and the specific activity of the cell extract were 8230 mg and 4.3 units/mg, respectively. The purification steps are summarized in Table 2. BdsC was purified essentially to homogeneity (19.6-fold, 84.2 units/mg), and 32.2 mg of the purified enzyme was obtained with 7.7% yield.

BdsA from B. subtilis WU-S2B The purification of BdsA was performed using the same cells as those for BdsC purification. The amount of the protein and the specific activity of the cell extract were 8230 mg and 10.4 units/mg, respectively. At the purification step of BdsC by Q-Sepharose column chromatography, BdsA was eluted separately from BdsC. Table 3 shows a summary of the purification steps. BdsA was purified essentially to homogeneity (82.6-fold, 859 units/mg), and 17.0 mg of the purified enzyme was obtained with 17% yield.

BdsB from the recombinant E. coli The enzyme purification was done from the recombinant *E. coli* cells with a wet weight of 96 g (from 18 *l* of culture broth). The amount of the protein and the specific activity of the cell extract were 9680 mg and 7.3 units/mg, respectively. The purification steps are summarized in Table 4. BdsB was purified essentially to homogeneity (28.9-fold, 211