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A three-component dicamba *O*-demethylase from *Pseudomonas maltophilia*, strain DI-6: Purification and characterization ☆

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Abstract

Dicamba *O*-demethylase is a multicomponent enzyme that catalyzes the conversion of the herbicide 2-methoxy-3,6-dichlorobenzoic acid (dicamba) to 3,6-dichlorosalicylic acid (DCSA). The three components of the enzyme were purified and characterized. $\text{Oxygenase}_{\text{DIC}}$ is a homotrimer $(\alpha)_3$ with a subunit molecular mass of approximately 40 kDa. $\text{Ferredoxin}_{\text{DIC}}$ and $\text{reductase}_{\text{DIC}}$ are monomers with molecular weights of approximately 14 and 45 kDa, respectively. EPR spectroscopic analysis suggested the presence of a single $[2Fe–2S]^{(2+/1+)}$ cluster in $\text{ferredoxin}_{\text{DIC}}$ and a single Rieske $[2Fe–2S]^{(2+;\,1+)}$ cluster within $\text{oxygenase}_{\text{DIC}}$. Consistent with the presence of a Rieske iron–sulfur cluster, $\text{oxygenase}_{\text{DIC}}$ displayed a high reduction potential of $E_{m,7.0} = -21$ mV whereas $\text{ferredoxin}_{\text{DIC}}$ exhibited a reduction potential of approximately $E_{m,7.0} = -171$ mV. Optimal $\text{oxygenase}_{\text{DIC}}$ activity in vitro depended on the addition of Fe^{2+}. The identification of formaldehyde and DCSA as reaction products demonstrated that dicamba *O*-demethylase acts as a monooxygenase. Taken together, these data suggest that $\text{oxygenase}_{\text{DIC}}$ is an important new member of the Rieske non-heme iron family of oxygenases.
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Keywords: Dicamba; *O*-Demethylase; Oxygenase; Ferredoxin; Reductase; Multicomponent enzyme; *Pseudomonas maltophilia*; Rieske non-heme oxygenase

The biodegradation of aromatic compounds in soil and groundwater is frequently a consequence of microbial activity [1]. Indeed, such activity is essential in preventing the build up of many pesticides in the environment and useful in the bioremediation of harmful pollutants [2]. Consequently, there has been an intensive effort to understand the structure and function of the enzymes that catalyze the degradation of several xenobiotic aromatic compounds [3]. Nonetheless, several chemicals used in large quantities over several years as pesticides in agricultural settings have received minimal attention. 2-methoxy-3,6-dichlorobenzoic acid (dicamba)[4] represents one such molecule. First introduced

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[4] *Abbreviations used:* Dicamba, 2-methoxy-3,6-dichlorobenzoic acid; DCSA, 3,6-dichlorosalicylic acid; TLC, thin layer chromatography; RCL, reduced-chloride media; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; IEF, isoelectric focusing.

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commercially in 1965, this herbicide has been used extensively to control broadleaf weeds in crops such as corn and wheat. Field studies have shown microbial degradation of dicamba under both aerobic and anaerobic conditions. However, the only evaluation of the metabolism and enzymatic degradation of dicamba has been limited to a single bacterium, *Pseudomonas maltophilia*, strain DI-6 [4–6]. This soil bacterium can completely mineralize dicamba to CO_2, H_2O, and Cl^- and utilize dicamba as a sole carbon and energy source [4]. Initial identification of 3,6-dichlorosalicylic acid (DCSA) as the first breakdown product of dicamba by strain DI-6 [4] has been confirmed by both in vivo and in vitro analyses [5]. Nonetheless, only preliminary studies of the enzyme responsible for this first-step reaction, dicamba *O*-demethylase, have been conducted [6].

In the present study we describe the purification of dicamba *O*-demethylase and its biochemical and physical characterization. In so doing, we provide corroborating evidence that dicamba *O*-demethylase activity requires three separate components (reductase$_{DIC}$, ferredoxin$_{DIC}$, oxygenase$_{DIC}$) and that these components provide for efficient conversion of dicamba to 3,6-dichlorosalicylic acid. Our demonstration that the enzyme is a monooxygenase that requires Fe(II) for full activity and that the homotrimeric $(\alpha)_3$ oxygenase component contains Rieske [2Fe–2S] clusters lead us to suggest that oxygenase$_{DIC}$ is a member of the large and diverse family of Rieske non-heme iron oxygenases [2].

Methods and materials

Chemicals

Dicamba, DCSA, and [^{14}C]dicamba were supplied by Sandoz Agro (Des Plaines, IL). Chemicals were purchased from Sigma Chemical (St. Louis, MO). All columns and packing materials were purchased from Pharmacia. HPLC column and accessories were purchased from Alltech. Thin layer chromatography (TLC) plates were made of silica gel containing a UV 254 indicator and were purchased from J.T. Baker Chemical.

Bacterial strain and culture conditions

Pseudomonas maltophilia, strain DI-6, was grown in reduced-chloride media (RCL) [4] with glucose (2 mg/ml) and casamino acids (2 mg/ml) as the carbon sources. Cells were grown to an OD_{600} ranging from 1.5 to 2.0 by incubation at 30 °C on an orbital shaker at 225 rpm for 24–48 h. For enzyme purification, 6 liters of cells were harvested by centrifugation in a Beckman Avanti J-25I centrifuge at 4000*g* for 20 min. Pelleted cells were stored at −80 °C.

Preparation of cleared cell lysates

Frozen pelleted cells (∼25.0 g wet weight) were suspended in 40 ml of 100 mM $MgCl_2$, centrifuged, and resuspended in 50 ml of sonication buffer (100 mM Mops (pH 7.2), 1 mM dithiothreitol, and 5% glycerol). Lysozyme (0.4% final conc.), Protease Inhibitor Cocktail for Bacterial extracts (Sigma; P 8465; 5 ml/20 g of cells), and phenylmethylsulfonyl fluoride (PMSF; 0.5 mM final conc.) were added in succession. Cells on ice were subjected to sonication with a Sonics & Materials sonicator, Model VCX 600, at an amplitude of 50% in 9.0 s bursts with 3.0 s resting periods for 30 min. Lysed cells were centrifuged at 56,000*g* for 75 min at 4 °C. The supernatant (cleared cell lysate) was decanted and glycerol was added to a final concentration of 15% prior to storage at −80 °C.

Enzyme purification

All purification steps were carried out at 4 °C. After each step, active fractions were determined by a rapid enzyme assay [6] described below. After each purification step, protein concentrations in the fractions were determined by the Bradford method using reagents from Bio-Rad and bovine serum albumin as the protein standard. Enzymes were stored at −80 °C for future characterizations. Modifications of a protein purification scheme described by Small and Ensign [7] were employed for purification of the three components of dicamba *O*-demethylase.

Fractionation of dicamba O-demethylase components

Crude cell extract (∼2000 mg of protein) was loaded on a 26 × 200 mm DEAE–Sepharose Fast Flow column (Pharmacia XK; 25 ml bed volume) using a BioCAD Perfusion Chromatography Workstation. The column was equilibrated with 50 mM Mops (pH 7.2), 1 mM dithiothreitol, and 15% (v/v) glycerol (buffer A) and run at a flow rate of 5.0 ml/min. After the column was loaded, it was washed with buffer A until the 280 nm reading was <0.1 A. All three components were bound to the DEAE column under these conditions. The column was developed with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing ferredoxin$_{DIC}$ eluted at 400 mM NaCl and the reductase$_{DIC}$ and oxygenase$_{DIC}$ components co-eluted at 250 mM NaCl.

Purification of ferredoxin$_{DIC}$

DEAE–Sepharose column fractions containing ferredoxin$_{DIC}$ were pooled, buffer exchanged into 50 mM Mops (pH 7.2), 5% glycerol (v/v), and 200 mM NaCl (buffer B), and concentrated to approximately 2 ml using an Amicon Cell Concentrator with a YM10 membrane

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