

**Partial Purification And Characterization Of The DCSA (3, 6-Dichloro
Salicylic Acid) Converting Enzyme In *Pseudomonas xanthomonas*
maltophilia (PXM)**

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Summary

The DCSA converting enzyme investigated in this study is probably a monooxygenase enzyme system that converts 3,6-dichlorosalicylic acid (DCSA) to 3,6-dichloro 2,5-dihydroxysalicylic acid (DCHSA). The DCSA converting enzyme was purified by a two step procedure that utilized ammonium sulfate fractionation (AS) and gel filtration chromatography. NADH and Mg^{++} stimulated the activity of the purified enzyme. An estimated MW of 82,000 kD for the enzyme was obtained by gel filtration.

Key Words: DCSA, dicamba, herbicides, plasmids, aromatic compounds

Özet

Bu çalışmada 3,6- di kloro salisilik asidi DCSA) 3, 6-dididroksi salisilik aside (DCHSA) dönüştüren muhtemel monooksijenaz enzimi üzerinde çalışıldı. DCSA yi dönüştüren enzim iki farklı adımda amonyum sülfat ve jel filtrasyonu yoluyla saflaştırıldı. NADH ve Mg kısmi olarak saflaştırılan enzimin aktivitesini artırdı. Enzimin molekül ağırlığı yaklaşık olarak 82000 kD olarak hesaplandı.

Anahtar Kelimeler: DCSA, dicamba, herbisitler, plazmidler, aromatic bileşikler

INTRODUCTION

Herbicide usage has benefited modern life by improving the quality and the quantity of the world's food supply. Most of the commercial herbicides available are chloroaromatic compounds. Some of these compounds contain a chlorine substituent(s) attached to a benzoic acid structure.

The physical and chemical properties of these chloroaromatic compounds are important in the understanding of their biohazardous effects on the environment. Chlorine substitution makes the compound more resistant to biodegradation via bacterial metabolism [2]. A large amount of research has shown that soil, water, air, and biota have become the reservoir of herbicides and their degradation products. One of these herbicides with potential biohazard is 3,6-dichloro-2-methoxy benzoic acid (dicamba).

Dicamba is widely used as a herbicide for control of broadleaf weeds and several grassy weeds [3]. Although the short term toxicity of dicamba is relatively low (LD=1.04 mg/kg orally in rats), there is concern about the environmental persistence of this herbicide [9]. Generally, chlorinated compounds are more stable than non-halogenated aromatic compounds. Therefore, these chlorinated aromatic compounds tend to remain in the environment. For example, polychlorinated biphenyls (PCB's) cause significant environmental problems because of their unusual chemical stability and toxicity. Dicamba, because of its chloroaromatic structure is persistent in soil and resistant to volatilization, ultraviolet photolysis oxidation and hydrolysis [4]. Dicamba and other chloroaromatic compounds are a health concern because they may be harmful to animals and humans. 3,6-dichlorosalicylic acid (DCSA) is an intermediate compound of dicamba degradation.

Many microorganisms have been found to be capable of degrading chlorinated aromatic compounds. Surface soil harbors a large microbial population. Bacteria of the genus *Pseudomonas* are involved in many cases of microbial degradation of xenobiotic chloroaromatic compounds.

The aromatic ring is cleaved through ortho or meta pathways and metabolic breakdown products are utilized through the TCA cycle [11]. The biological dissolution of aromatic compounds has been used as a model for the biodegradation process of haloaromatic

compounds. In *Pseudomonas*, the biodegradation of benzoic acid and other aromatic compounds involves ring cleavage through ortho or meta pathways. The majority of herbicides contain a benzene ring [10]. Common substituents attached to aromatic molecules make the molecule more reactive to electrophilic attack. These include amino and hydroxyl groups. Substituent electrophilic substitution occurs mainly at the ortho and para positions. Hydroxylation of the benzene ring is the first step of biodegradation and addition of hydroxyl group(s) to the aromatic ring occurs before ring cleavage is observed [8]. The pathway of ring cleavage of benzoic acid has been proposed by Johnson and Stainer [7]. A pathway for ring cleavage of chlorinated benzoic acid by *Pseudomonas sp.* has been proposed by [6]. Monooxygenase enzymes system catalyzes the biodegradation of dicamba. This reaction requires molecular oxygen.

Monooxygenases comprise a class of oxygenases. Oxygenases are enzymes that incorporate oxygen into substrates. Dioxygenases incorporate both atoms of oxygen into one substrate. Monooxygenases incorporate one atom of oxygen into a product; the other atom is reduced to water. Oxygenases and oxidases require different cofactors. Oxidase enzymes catalyze the oxidation of a substrate by oxygen without incorporation of oxygen into the product. Most oxidases utilize a metal or a flavin coenzyme. The general reaction catalyzed by oxidase enzyme is as follows: $FADH_2 + O_2 \rightarrow FAD + H_2O_2$

The general reaction catalyzed by oxygenases is as follows:

$AH + BH_2 + O_2 \rightarrow A-OH + B + H_2O$ Oxygenases, like oxidases, have required cofactors. For example, oxygenases require NADH, $FADH_2$, or NADPH as well as Mg^{2+} or Fe^{2+} [12].

MATERIAL AND METHODS

Culture Maintenance and Growth

For preparation of cells as a source of DCSA degrading activity strain PXM was grown on plates containing 2000 ppm dicamba and chlorine reduced medium (see below) for 48-72 hours. A single colony was inoculated into 500 ml of medium containing 2000 ppm dicamba and chlorine reduced medium. The dicamba containing liquid culture was grown at 30 °C and 200 rpm for 48-72 hours. Whenever cells reached a stationary phase,

they were harvested and kept at -20°C until used for enzyme preparation. The dicamba and chlorine reduced medium and their combination were prepared as follows. A purified 10,000 ppm stock solution of dicamba was prepared. 10 grams of dicamba were added to 900 ml of deionized water. Since dicamba is not terribly soluble in water, 2 ml 5 mM NaOH was added to increase the solubility. The pH was adjusted to 7.0 by adding 5 M NaOH drop by drop and the total volume adjusted to 1000 ml. Since dicamba cannot be autoclaved, this solution was aseptically filtered through a 45 micron millipore filter before adding it into the medium. A 2000 ppm dicamba concentration was used to maintain PXM. The composition of the reduced chlorine free medium was as follows: 1.39 g KHPO_4 , 0.87 g KH_2PO_4 , 0.66 g $(\text{NH}_4)_2\text{PO}_4$, 0.097 g MgSO_4 , 0.025 g MnSO_4 , 0.005 g $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ and 0.001 g CaSO_4 per 1000 ml deionized distilled water with the pH adjusted to 7.0 with 5 M NaOH. The Complete medium was prepared by mixing the 10,000 ppm dicamba stock and the reduced chlorine medium in a proportion of 20:80 for both plates and broth. This medium was solidified (for plates) with 0.08 % gel-rite and 0.001 % $\text{MgSO}_4 \cdot \text{H}_2\text{O}$.

Preparation of Cells as a Source of DCSA Converting Activity for Purification

PXM were grown on 2000 ppm dicamba containing medium. After cells reached a stationary phase, they were harvested by centrifugation at 10,000 rpm for 10 minutes in a Sorvall GSA rotor and stored at -20°C until used. 10 grams of frozen cells were resuspended in 5 ml of 50 mM potassium phosphate buffer (pH 7.0). After resuspending the cells, lysozyme was added (1mg/ml) to the cell suspension, which was stirred on ice for 3 hours. Cells were then sonicated by a pulsed sonifier cell disrupter for 2 minutes followed by adding 10 microgram/ml of DNase I to the homogenate and incubation on ice for 10 minutes. The homogenate was centrifuged at 15,000 rpm for 1 hour in a Sorvall GSA rotor. After centrifugation, the supernatant was separated from the pellet. The supernatant was assayed for enzyme activity (as described above) as well as for protein content by UV absorbance at 280 nm.

Ammonium Sulfate Fractionation

All procedures were performed at 5°C . Solid ammonium sulfate was slowly added to a 7 ml volume of cleared cell lysate to 40 % (wt/vol) with constant stirring. After 15

minutes of additional stirring, the mixture was centrifuged for 15 minutes at 15,000 rpm in a Sorvall GSA rotor and the pellet was discarded. Additional solid ammonium sulfate was added to 70 % (wt/vol) with constant stirring of the supernatant. After 15 additional minutes of stirring, the mixture was centrifuged and the supernatant was discarded. The pellet was dissolved in 7 ml of 50 mM potassium phosphate buffer (pH 7.0) and 5 mM MgCl₂.

Dialysis

Dialysis was done in tubing with a 12,000-14,000 molecular weight cutoff. The purpose for dialyzing was to remove ammonium sulfate from the redissolved sample. The volume of sample to buffer was 1:100 (v/v). Dialysis was for 15 hours at 5 °C changing the buffer twice.

DCSA Converting Enzyme Purification by Gel Filtration

The dialyzed enzyme pool (4 ml) concentrated from the ammonium sulfate fractionation was applied to a Sephadex G-100 column (2.5 x 50 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The column was eluted with the equilibration buffer at a flow rate of 0.2 ml/min. Fractions of 2.5 ml were collected and assayed for enzyme activity as well as protein content. Enzyme assays were at 30°C in 5 ml total volume using 1 ml of each fraction. Cofactors were 0.5 mM NADH, 0.2 mM FAD, and 10 mM MgCl₂. Protein content was measured by UV at A_{280nm}. The enzymatically active fractions were pooled for further assays. 1 mM DCSA was used as a substrate. All column chromatographs were run at 5 °C.

Native Molecular Weight Determination by Gel Filtration

The native molecular weight of the DCSA converting enzyme was estimated from the gel filtration results discussed above using the parameter $K_{av} = (V_e - V_o)/(V_t - V_o)$. K_{av} is the coefficient which defines the proportion of pores that can be occupied by a particular molecule. V_e is the elution volume of that molecule, V_o is the void volume of the column and V_t is the total volume of the column. V_o was determined from chromatography of blue dextran on the same column used for enzyme purification. V_t was determined from the column dimensions. A standard curve was determined by chromatographing bovine serum

albumin, trypsin inhibitor and chymotrypsinogen A, calculating K_{av} for each, and plotting native MW vs. K_{av} .

Determination of Cofactor Requirements for Partially Purified DCSA Converting Enzyme

Cofactor requirements for DCSA converting enzyme were tested with partially purified enzyme. The following cofactors were used (concentrations given are final concentrations in the reaction mixtures): 0.5 mM NADH, 0.2 mM FAD, 10 mM $MgCl_2$ and 0.5 mM NADPH. 1 ml of Sephadex G-100 active fraction pool was used for further assays.

RESULTS

To purify the DCSA converting enzyme, ammonium sulfate fractionation was conducted as described in Materials and Methods. The purification factor of 1.5 by this step yielded a specific activity of 3.4×10^{-4} mM/min/mg protein (Table 1). A standard curve for DCSA was established (Fig.1) to calculate amount of degraded DCSA during enzyme assay.

Purification of the DCSA converting enzyme by gel filtration on Sephadex G-100 yielded an overall 4.3-fold purification (Table 1). The specific activity was 1×10^{-3} mM/min/mg protein at this point.

Bovine serum albumin, chymotrypsinogen A, and trypsin inhibitor were used as size standards (Fig. 2). Their molecular weights are 66,000, 25,000, and 20,100, respectively. A single Sephadex G-100 column (50x2.5 cm) was used for all proteins, including the three standards, blue dextran (Fig. 2) and DCSA converting enzyme (Fig.3). The standard curve of K_{av} against log molecular weight (Fig. 4) predicted a molecular weight of about 82,000 kD for the DCSA converting enzyme. The enzyme system required NADH, FAD and $MgCl_2$ for maximal activity (Table 2). Compared with controls NADH and Mg^{++} enhanced enzyme activity by 5.8 times.

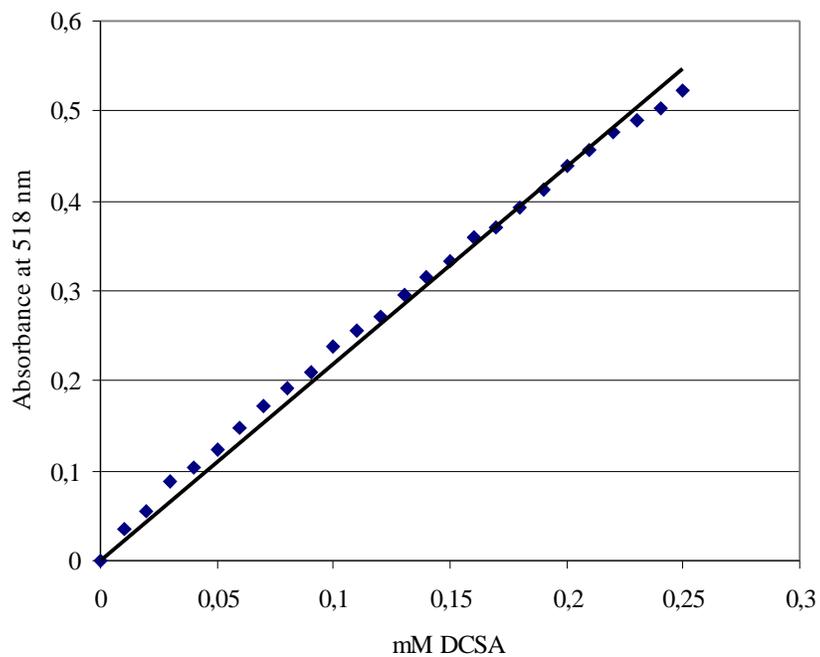


Figure 1. Standard Curve for DCSA. Line has been fit "Linear Regression by Method of Least Squares"

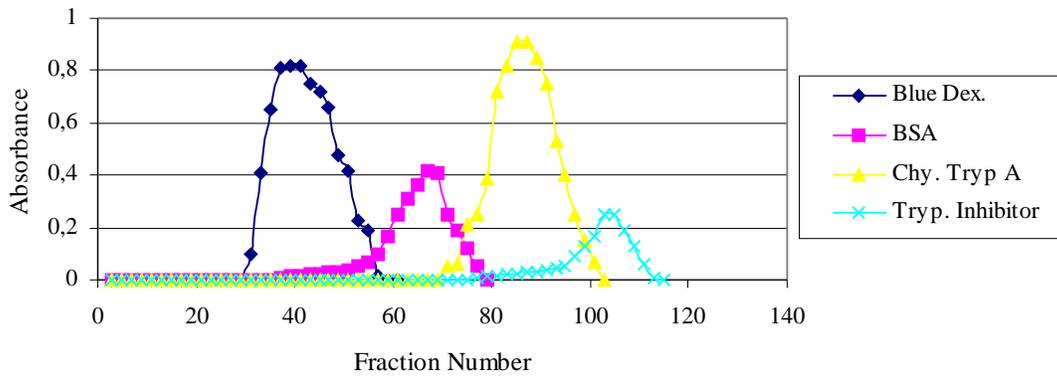


Figure 2. Gel Filtration of Three MW Standards on the Sephadex G-100 Column Used to Purify DCSA Converting Enzyme as Detected by A280 and Blue Dextran Measured by A 600

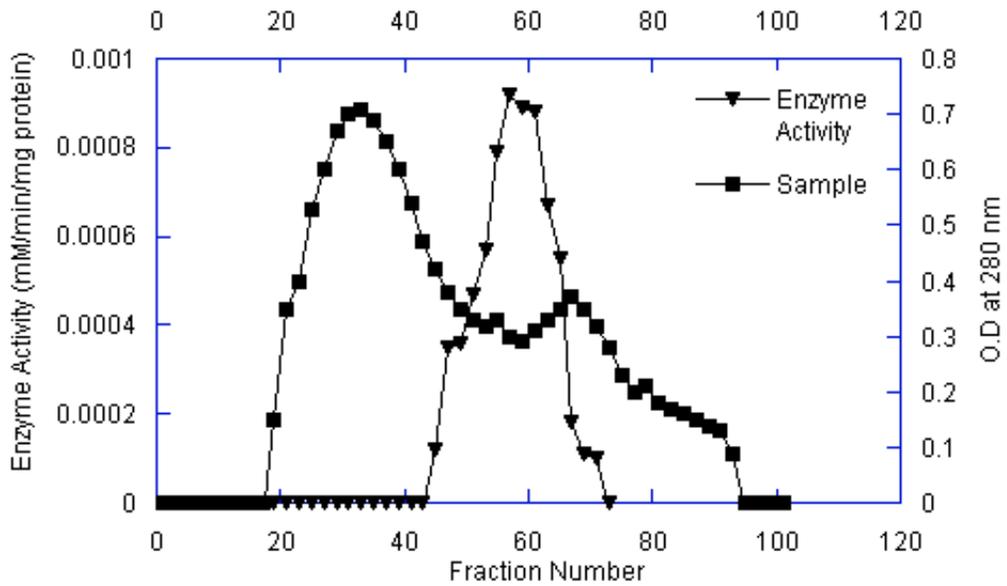


Figure 3. DCSA converting enzyme purification using gel filtration and purified DCSA Converting enzyme

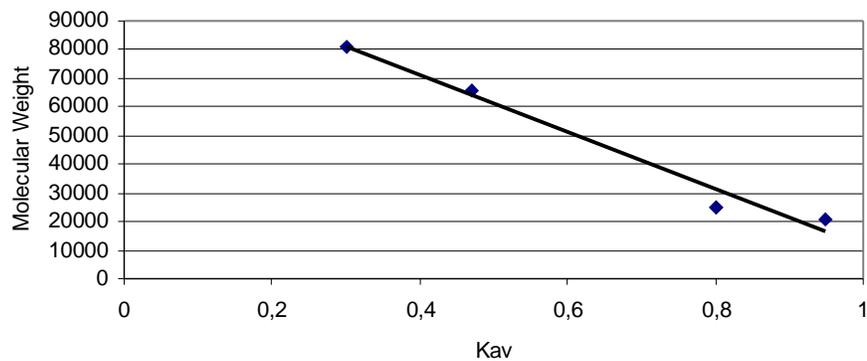


Figure 4. Kav Calculations From Data in Figure 2 and Their use in Determination of DCSA Converting Enzyme Molecular Weight.

Table 1. Purification Table for the DCSA Converting Enzyme from PXM

Step	Total Protein (mg)	Total activity mM/min	Specific Activity mM/min/mg protein	Purification Fold
Crude	550	1.3×10^{-1}	2.3×10^{-4}	1
AS	210	7.2×10^{-2}	3.4×10^{-4}	1.5
G-100	28	2.8×10^{-2}	1×10^{-3}	4.3

Table 2. Effects of Various Cofactors on G-100 Purified Enzyme.
Control contained no cofactors.

Cofactors	Activity (%)
Control	100
NADH	520
NADH, Mg ⁺⁺	580
FAD	150
FAD, Mg ⁺⁺	320
NADPH	170
NADPH, Mg ⁺⁺	250
NADH, FAD	455
Mg ⁺⁺	310

DISCUSSIONS

The DCSA converting enzyme was able to be purified through at least two steps, ammonium sulfate fractionation and Sephadex G-100 chromatography. Although the overall purification factor (4.3x) was modest, this does indicate that larger enzyme preparations may be able to support lengthier and more successful purification schemes.

The molecular weight of for DCSA converting enzyme was estimated by gel filtration to be 82, 000 (Fig. 4). This correlates fairly well with MW of 2, 4-DNT dioxygenase (100,000) [1].

The G-100 purified enzyme does as shown in Table 2 require some cofactors for maximal activity. The maximal activity was observed when Mg⁺⁺ and NADH were added together to the reactions. NADPH alone also enhanced the enzyme activity. On the other hand, adding both NADH and FAD to the reaction decreased the activity compared with NADH alone. NADPH did not enhance the activity by a significant amount. Previous studies also show that oxygenase enzymes require NADH and Mg⁺⁺ for their maximal

activities. For example dicamba O-demethylase [12] and 2-halobenzoate 1,2- dioxygenase [5].

It is possible to conclude that DCSA converting enzyme is probably a monooxygenase with MW of about 82,000 kD. It is apparent that DCSA converting enzyme like most of the oxygenases requires NADH and Mg^{++} for maximal activity.

Dicamba is a herbicide which is toxic to plants, animals and humans. Consequently, accumulation of dicamba in the environment may harm to plants, animals and humans. Better understanding of the degradation of DCSA, which is an intermediate compound dicamba degradation pathway could help to optimize dicamba degradation in such environments. It is also apparent that understanding of such toxic compound degradation may help to produce genetically engineered dicamba resistant plants.

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