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A Further Characterization of 3-Chloropropionic Acid Dehalogenase from *Rhodococcus* sp. HJ1

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Abstract: The main aim of the present study is to further characterize a new dehalogenase enzyme found in the crude extracts from *Rhodococcus* sp. The ability of the enzyme to catalyze the dehalogenation of various halogen-substituted organic acids was investigated and the highest activity was found with 3-chloropropionic acid as a sole carbon source in the growth medium. The enzyme followed Michaelis-Menten kinetics and the Km for 3-chloropropionic acid was 0.2 mM. Maximum activity was found at pH 7.6 at 30°C. The enzyme activity in the cell-free extract was unaffected by diaminoethane tetraacetic acid (EDTA), dithiothreitol (DTT) or by Mn and Zn ions but was reduced by $HgCl_2$ (70%) and $Pb(NO_3)_2$ (80%). The enzyme removed the chlorine atom present on a number of 3- and 4-carbon alkanoic acids if the halogen was on the β-position.

Key words: 3-chloropropionic acid, dehalogenase, *Rhodococcus* sp.

INTRODUCTION

The halogenated organic compounds enter our environment due to industrial or agricultural activities and constitute a major group of pollutants. The recalcitrance and toxicity of some of these compounds has led to increasing public concern. The biological methods involve the use of microbial or enzymic biocatalysts as a process of bioremediation. Various microorganisms are able to utilize halogenated alkanoic acids as a sole carbon source and therefore, are assumed to have a significant role in their natural detoxification. The bacterial strains possess inducible enzyme capable of cleaving off carbon-halogen bond. Dehalogenation is the critical step in the degradation of chlorinated aliphatic acids and mineralization of halogenated compounds is attributed to a group of enzymes which are called dehalogenases. Various dehalogenase producing microorganisms have frequently been reported by Janssen *et al*. (2001), Song *et al*. (2003) and Park *et al*. (2003). Some of these microorganisms were originally isolated from contaminated soil and sewage oxidation ponds (Olaniran *et al*., 2001, 2004).

The 3-chloropropionic acid is classified as β-chloro substituted haloalkanoates. This compound can be considered as a possible chemical inclusion in certain herbicides and is carcinogenic and genotoxic to the animal and human. The study of β-chloro substituted haloalkanoates is considered important to that of well established α-chloro substituted alkanoates like 2,2-dichloropropionic acid (Marchesi and Weightman, 2003; Jing *et al*., 2008) which is an analogue isomer for 3-chloropropionic acid. Currently, very few literatures have been reported regarding degradation of β-chloro substituted

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haloalkanoic acids such as 3-chloropropionic. The dehalogenase investigated to date demonstrate specific substrate specificity. The batch culture method was used for the isolation of the microorganism which utilized 3-chloropropionic acid as sole source of carbon and energy. In this study we report the detection of a new dehalogenase with a substantially stable, in the cell-free extracts from *Rhodococcus* sp. HJ1 isolated from an agricultural area in University Technology Malaysia.

MATERIALS AND METHODS

Isolation and Identification of Organism

The bacterial isolate used in the present study was originally isolated from soil through 3-chloropropionic acid degradation. Samples of soil (5 g) were added into minimal salts medium (pH 6.8) containing 20 mM 3-chloropropionic as the sole carbon source.

After 7 days of incubation, through shaking samples were diluted in 0.1 M Tris-acetate buffer pH 7.6 and 0.1 mL portions were plated on the solidified minimal medium. A single strong colony on the plate was picked up and maintained as slant cultures.

Growth Conditions

The culture was grown at 30°C on a rotary shaker in 250 mL flasks containing 100 mL medium. The liquid PJC minimal media was prepared as 10x concentrated basal salts containing K_2HPO_4. $3H_2O$ (42.5 g L^{-1}), NaH_2PO_4. $2H_2O$ (10.0 g L^{-1}) and $(NH_4)_2SO_4$ (25.0 g L^{-1}). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g L^{-1}), $MgSO_4$(2.0 g L^{-1}), $FeSO_4$. $7H_2O$ (120.0 mg L^{-1}), $MnSO_4$. $4H_2O$ (30.0 mg L^{-1}), $ZnSO_4$. H_2O (30 mg L^{-1}) and $CoCl_2$ (10.0 mg L^{-1}) in distilled water (Hareland *et al*., 1975). Minimal media for growing bacteria contained 10 mL of 10x basal salts and 10 mL of 10x trace metal salts per 100 mL of distilled water and were autoclaved (121°C, for 15 min).

The carbon source 3-chloropropionic acid was neutralized with 1 M NaOH and sterilized by filtration and added to the autoclaved salts medium to a final concentration of 20 mM. The extent of growth determined by measuring the absorbance at $A_{600\ nm}$ and the release of chloride at $A_{460\ nm}$ (Iwasaki *et al*., 1956). The bacteria were harvested by centrifugation during the late-logarithmic phase and the cells were washed three times in 0.1 M Tris-acetate buffer pH 7.6. These cells could be stored for several months in the frozen state with no appreciable loss of dehalogenating activity.

Preparation of Cell Free Extracts

Cell-free extracts were prepared from bacterial cells in late-exponential phase of growth. Cells from 100 mL culture were harvested by centrifugation at 10,000 g for 10 min at 4°C. The cell pellets were resuspended in 20 mL of 0.1 M Tris-acetate buffer pH 7.6 and centrifuged at 10,000 g for 10 min at 4°C. The cells were then resuspended in 4 mL of 0.1 M Tris-acetate buffer pH 7.6 and maintained at 0°C for ultrasonication in an MSE Soniprep 150 W ultrasonic disintegrator at a peak amplitude λ = 10 microns for 30 sec. Unbroken cells and cell wall material were removed by centrifugation at 20,000 g for 15 min at 4°C.

Enzyme Assay

Activity of the enzyme was measured by determining the release of chloride indicated by a colorimetric method employing mercuric thiocyanate (Bergman and Sanik, 1957). For the enzyme assay, dehalogenase activity was determined as total chloride released at 30°C in an incubation mixture containing 0.1 M Tris-acetate buffer (pH 7.6) (4700 μL), 0.1 M halogenated aliphatic acid (50 μL) and distilled water and enzyme to a final volume of 5 mL. After 5 min equilibration at 30°C, the reaction was initiated by adding cell-free extract. Samples (1.0 mL) were removed at appropriate intervals and

assayed for halide ions. Protein was determined by the biuret procedure with crystalline egg albumin as a standard (Gornall *et al*., 1949). Specific activity is defined as the μmole of chloride liberated per milligram protein in 10 min under the stated conditions.

Kinetic Studies

The effect of substrate concentration on the cell-free extracts was determined for concentration of 3-chloropropionate between 0.1 and 5 mM. The assay was made in 0.1 M Tris-acetate buffer, (pH 7.6).

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co. (USA) and Merck-Schuchardt (Germany), 2,2,3-trichloropropionic, 3-chlorobutyric, 2,2-dichlorobutyric and 2,2,3-trichlorobutyric acids were provided by Dow Chemical Co. (USA).

Determination of the Temperature and pH Optimum

For the determination of the temperature optimum enzyme extracts were incubated for 15 min at various temperature and 1 mL sample were removed at 5 min interval for the enzyme assay at 30°C as described above. To determine pH optimum, crude extract were mixed with equal amounts of 100 mM buffer of various pH. The reaction was started by addition of the substrate and after 10 min incubation chloride ions released was determined as described. Control experiment, lacking enzyme preparation was included in each set of assay to detect spontaneous halogen released.

Determination of Effect of Co-Factors

The assay mixtures constituted as described contained inhibitors: $Pb(NO_3)_2$; $HgCl_2$; $MnSO_4$ and $ZnSO_4$ at a final concentration of 1 mM. After incubation, at 30°C for 10 min the reaction was stopped and the chloride release was determined.

Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

In non-denaturing PAGE, the enzyme extract remains active. Gels were prepared based on the method of Hardman and Slater (1981). Resolving gels containing 12% bis-Acrylamide in 375 mM Tris.SO_4 pH 8.8 were polymerized by the addition of 0.05% ammonium persulphate and 0.05% TEMED. Stacking gels were formed from 4% bis-Acrylamide in 125 mM Tris.SO_4 pH 6.8.

Gels were left overnight at 4°C before being used to allow the ammonium persulphate to decompose completely. Gels were run using a Mini-Protean II gel system from Biorad in 25 mM Tris, 19 mM glycine buffer (pH 8.3) at a constant voltage of 200 V and a temperature 4°C for 1 h. Samples were prepared as for cell extracts and mixed with 0.1 volumes of sample buffer (0.1% bromophenol blue; 10% glycerol; 100 mM DTT in 50 mM Tris-acetate pH 6.8). The gel was run until the dye front reached the bottom of the gel. Gels were then stained for dehalogenase activity.

The gel was incubated in 50 mM Cl^- free halogenated substrate for 15 min at 30°C. Substrate was carefully removed and the gel was then placed in a 0.1 M $AgNO_3$ solution and incubated in the dark until bands appeared on the gel due to the precipitation of AgCl. The gel was then washed with distilled water to remove the $AgNO_3$ and fixed by washing in 5% acetic acid for 10 min.

RESULTS

Bacteria Morphology, Staining and Identification

The bacteria colony was observed as a rough surface, a smooth margin and a raised elevation. It formed milky-orange colonies on nutrient agar and 3-chloropropionic acid containing medium. It was

gram-positive rod in chains. The cells were acid-fast with no spores were demonstrated by malachite green staining. It also demonstrated its ability in utilizing lactose, gelatin liquefaction, producing catalase and grew on citrate. However, the isolates could not produce oxidase and was non-motile. The overall biochemical characteristics were matched to the genus *Rhodococcus* sp. as indicated in Bergeys Manual of Systematic Bacteriology (Holt *et al*., 1994). Using 16S rRNA technique the sequence was submitted to the gene bank under accession number, AM231909. The bacterial strain was designated as a *Rhodococcus* sp. HJ1.

Substrate Specificity

Rhodococcus sp. HJ1 was grown in a minimal medium containing 3-chloropropionic acid with cells doubling time of 10 to 11 h. The resulting cell suspension utilized 3-chloropropionic acid and several other chlorinated aliphatic acids rapidly. However, cells cultured in nutrient broth released chloride from these acids only after a lag period.

Further evidence for the inducibility of the dehalogenase was obtained by comparing the activities of cell extracts derived from cultures grown in nutrient broth and in a medium with 3-chloropropionic acid as sole carbon source. Thus, no halide liberation was detected in 20 h by extracts of bacteria grown in nutrient broth, whereas the 3-chloropropionic acid grown organism released 75% of the chloride in the same period of time. The specificity of the dehalogenase in the soluble form for chlorinated aliphatic acids is shown in Table 1. The reaction mixture contained 0.1 mM substrate and 2 mg of extract protein in 5 mL total reaction mixture. The enzyme failed to dehalogenate chlorinated acetic acids and the 3-and 4-carbon acids. There were only dechlorinated if the halide was in the β-position. The sole anomaly is 2,2,3-trichloropropionic acid, which was not metabolized though it has a chlorine on the β-carbon. The enzyme was inactive on all aliphatic acids with halogens solely on the α-carbon. With 3-chloropropionic acid as substrate, the dehalogenase enzyme activity followed Michaelis-Menten kinetics over the substrate range 0.1 and 5 mM revealed a Km of 0.2 mM.

Effect of Temperature and pH

The initial velocities for the first 10 min were measured with 3-chloropropionic acid at different temperature (at pH 7.6) (Fig. 1). The highest reaction rate was observed at 30°C. The enzyme was pre-incubated at different temperature for 15 min and retained the following activities when assayed at 30°C; 30°C 100%, at 50°C 60%, at 60°C 37% and at 80°C 0%. Optimum pH of the dehalogenase was found to be about pH 7.6 although the conversion still proceeded rapidly at pH 6.0 and 8.0.

Table 1: Specific activity for dehalogenase enzyme using various substrates

Substrate	Chloride release (μmol Cl^-)
2-carbon acids	
Monochloroacetie acid	0
Dichloroacetic acid	0
Trichloroacetic acid	NT
3-carbon acids	
D,L-2-chloropropionic acid	0
3-chloropropionic acid	4.45
3-chlorolactic acid	3.25
2,2-dichloropropionic acid	0
2,3-dichloropropionic acid	3.98
2,2,3-trichloropropionic acid	0
4-carbon acids	
2-chlorobutyric acid	0
3-chlorobutyric acid	3.45
2,2,3-trichlorobutyric acid	1.10
2,2-dichlorobutyric acid	NT

NT: Not Tested

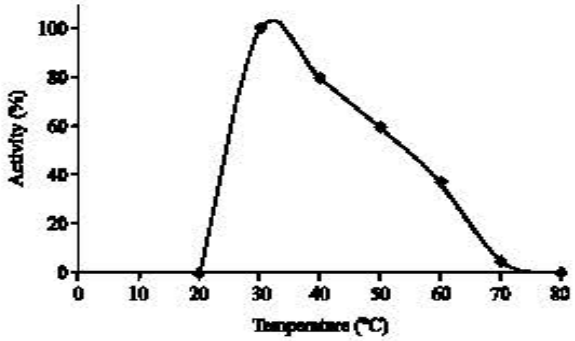

Fig. 1: Effect of temperature on enzyme activity

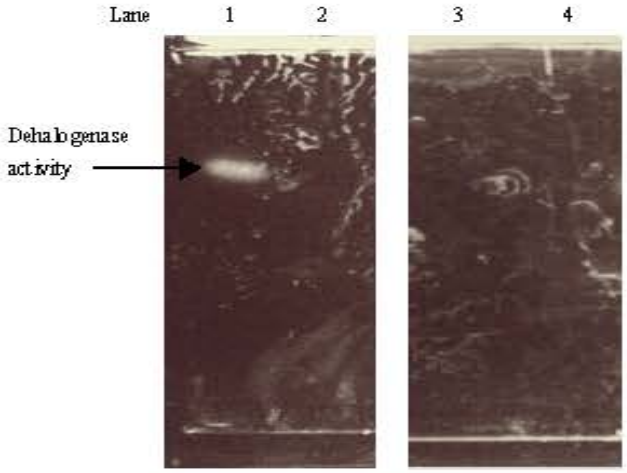

Fig. 2: Non-denaturing polyacrylamide gel electrophoresis analysis of dehalogenase activity. Dehalogenase activity towards 3-chloropropionic acid (Lane 1); Control: distilled water towards 3-chloropropionic acid (Lane 2); Dehalogenase activity towards 2,2-dichloropropionic acid (Lane 3); Control: distilled water towards 2,2-dichloropropionic acid (Lane 4)

Effect of Co-Factors

The effects of metals on the crude enzyme preparation is shown in Table 2. The enzyme was not activated by any of the metals tested but was markedly inhibited by $HgCl_2$ (70%) and $Pb(NO_3)_2$ (80%).

Stability of the Enzyme

Rhodococcus sp. HJ1 dehalogenase appeared to be stable in 1 mM DTT and EDTA, retaining its activity almost completely at room temperature for 3 h and at -20°C for almost 2 weeks. However, in the absence of DTT and EDTA, under the same storage conditions at -20°C, the activity of the extracts was lost by 60%. Enzyme was totally inactivated after boiling for more than 1 min.

Localisation of Dehalogenase on Non-Denaturing Polyacrylamide Gel Electrophoresis

The enzyme electrophoresed with the crude enzyme preparations yielded one dehalogenase band when incubated with 3-chloropropionic acid as substrate. No band was detected when incubated with 2,2-dichloropropionic acid (Fig. 2).

Table 2: Specific activity for dehalogenase using various substrates

Metals substrate	Remaining activity (%)
$Pb(NO_3)_2$	20
$HgCl_2$	30
$MnSO_4$	80
$Zn\ SO_4$	80

DISCUSSION

The results provide further evidence for the importance of the position of the halogen substituent in governing the susceptibility of chlorinated aliphatic acids to microbial attack. In current finding, *Rhodococcus* sp. was specific for the β-substituted halogenated aliphatic acids. This was assumed considerable importance as it can be compared to that of well documented α-chloro substituted haloalkanoic acid for example 2,2-dichloropropionic acid, D,L-2chloropropionic acid and monochloroacetate (Schwarze *et al*., 1997; Weightman *et al*., 1982; Torz and Beschkov, 2005). Results from the assay activity (Table 1) showed that HJ1 had been specifically adapted for β-chloroalkanoate dehalogenation. Its failure to dehalogenate α-chloroalkanoate revealed the specific adaptation of enzyme to dehalogenate the corresponding substituted alkanoic acid. This adaptation suggested that current enzyme was under the inductive control of the substrate in which the organism had been cultured. Enzyme inducibility was common feature of α- and β- haloalkanoate dehalogenases. The majority of the reported α-haloalkanoate dehalogenases showing this characteristic. Kerr and Marchesi (2006) reported that, a novel bacteria able to degrade α-halocarboxylic acids can be obtained using a variety culturing strategies, whereas our current investigation shows that this microorganism grow specifically on 3-chloropropionic acid only. Researchers who use enrichment technique to isolate bacteria to degrade compound of interest need to be aware that these microbes may or may not perform the task *in situ*. The proposed metabolism of chlorinated aliphatic acids by microorganisms might be catalyzed by a hydrolytic dechlorination. Most studies on microbial dehalogenation suggest that the reaction involves the replacement of the halogen with a hydroxyl group (Van Pee and Unversucht, 2003).

Rhodococcus sp. HJ1 enzyme described in this study has an average heat stability. The enzyme is neither inhibited by EDTA nor it is activated by metals. On non-denaturing gel electrophoresis the activity appears to be due to a single protein. The current enzyme is inducible, since nutrient broth grown cells of *Rhodococcus* sp. HJ1 exhibited no dehalogenase activity. Previous studies have shown that some dehalogenases acting on halogenated alkanoic acid are inducible (Motosugi *et al*., 1982) and some are constitutive (Kawasaki *et al*., 1981).

It was concluded that dehalogenase of the present study proved inducible and specific for catalyzing the removal of only β-substituted dehalogenase. It also suggested that the enzyme mechanisms of dehalogenase are assumed to be very specific.

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