

Dehalogenation of chlorinated aliphatic acid by *Rhodococcus* sp.

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Abstract. Environmental pollution from industrial chemicals and herbicide/pesticide from agricultural activities cause a considerable problem to the society. 3-chloropropionic acid is synthetic halogenated compounds used in herbicides. An enzyme preparation derived from a soil microorganism, identified as a strain of *Rhodococcus* sp. by biochemical and partial 16S rRNA sequencing method, acted upon several chlorinated aliphatic acids. The enzyme system was induced by growth of the bacterium on 3-chloropropionic or 3-chlorobutyric acids. The responsible enzyme removed the chlorides ions present on a number of 3- and 4-carbon alkanic acids if the halogen was on the β - but not on the α -position. No evidence was obtained for 3-hydroxypropionic acid serving as an intermediate in the metabolism of β -chloropropionic acid.

Keywords. 3-chloropropionic acid, dehalogenase, *Rhodococcus* sp.

INTRODUCTION

Many soils microorganisms are capable of utilizing halogen-substituted organic acids as their sole carbon sources for growth, and the organically bound halogen is liberated as the halide ion (Fetzner *et al.*, 1994; Olaniran *et al.*, 2001; Olaniran *et al.*, 2004). Since many of these compounds are potent herbicides, studies of the dehalogenation reactions and the processes by which the molecules are detoxified assumes considerable importance.

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 chemical structure was shown in Figure 1.

Currently, very few literatures have been reported regarding degradation of β -chloro substituted haloalkanoic acids such as 3-chloropropionic. This study was hence considered important since degradation of 3-chloropropionic acid was poorly understood compared to well studied α -chloro substituted haloalkanoic acids such as 2,2-dichloropropionic and D,L-2-chloropropionic (Schwarze, *et al.*, 1997). In addition, further interest was generated by this subject, when it became apparent that α -chloroalkanoate degrading microorganisms were unable to dechlorinate the β -substituted haloalkanoic acid which differed only in chlorine substitution (Hardman and Slater 1981).

The present investigation was designed to characterized the enzymatic mechanism of dehalogenation of β -chloro aliphatic acids.

MATERIALS AND METHODS

Isolation and identification of organisms. A bacterium capable of utilizing 3-chloropropionic acid as a sole carbon source was isolated from Universiti Teknologi Malaysia agricultural soil area by enrichment culture technique. The enrichment medium contained the inorganic salts as listed below. The bacteria characteristics were identified using a standard microbiological Gram staining technique and 16S rRNA gene sequencing. Other biochemical characterization of the isolate was carried out using the standard procedures (Cappuccino and Sherman, 2002). The polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA gene and the universal primers used were Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3'). The amplification reactions contained in 50 μ l with 300ng template DNA, 20pmol forward primer (Fd1), 20 pmol of reverse primer (rP1), 25 μ l (2x) PCR master mix (Fermentas Inc. USA) and deionized water. PCR cycle was set as: initial denaturation 94°C for 5 min, followed by cooling, denaturation 94°C, annealing, 55°C for 1 min; extension, 74°C for 4 min and final extension, 74°C for 10 min. The PCR product was electrophoresed on a 0.8% agarose gel. For sequencing reaction, the PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) prior sending for sequencing (1st Base Laboratory,

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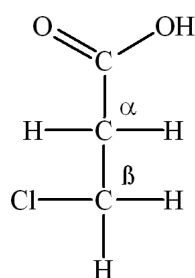


Figure 1. Chemical structure of 3-chloropropionic acid.

Biosyntech, Selangor). The sequences were compared to the sequence in the public databases using BLAST search program on the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>).

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 $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (42.5g/l), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (10.0 g/l) and $(\text{NH}_4)_2\text{SO}_4$ (25.0 g/l). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g/l), MgSO_4 (2.0 g/l), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (120.0 mg/l), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (30.0 mg/l), $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ (30 mg/l) and CoCl_2 (10.0 mg/l) in distilled water (Hareland *et al.*, 1975). Minimal media for growing bacteria contained 10ml of 10x basal salts and 10ml of 10x trace metal salts per 100ml of distilled water and were autoclaved (121°C, for 15 minutes).

The carbon source 3-chloropropionic acid was neutralized with 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 $A_{600\text{nm}}$ and the release of chloride. The bacteria were harvested by centrifugation during the mid-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 mid- to late-exponential phase of growth. Cells from 100 ml culture were harvested by centrifugation at 10,000 g for 10 minutes 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 minutes at 4°C. The cells were then resuspended in 4 ml of 0.1M Tris-acetate buffer pH7.6 and maintained at 0°C for ultrasonication in an MSE Soniprep 150W ultrasonic disintegrator at a peak amplitude $\lambda=10$ microns for 30 seconds. Unbroken cells and cell wall material were removed by centrifugation at 20,000 g for 15 minutes at 4°C. The latter was centrifuged at 140,000 g

Table 1. Specific activity for dehalogenase using various substrates.

Substrate	Chloride release ($\mu\text{mol Cl}$)
Two-carbon acids	
Chloroacetic acid	0
Dichloroacetic acid	0
Trichloroacetic acid	0
Three-carbon acids	
2-Chloropropionic acid	0
3-Chloropropionic acid	4.28
3-Chlorolactic acid	3.00
2,2-Dichloropropionic acid	0
2,3-Dichloropropionic acid	4.12
2,2,3-Trichloropropionic acid	0
Four carbon acids	
2-Chlorobutyric acid	0
3-Chlorobutyric acid	3.9
2,2,3-Trichlorobutyric acid	1.5
2,2-Dichlorobutyric acid	0

for 60 minutes to give a soluble preparation. To further purify the enzyme, the soluble preparation was treated with ammonium sulphate to 30, 50 and 80% of saturation. The resulting precipitates were dissolved in water and the solution was freed of ammonium sulfate by dialysis overnight at 0°C in 0.1M Tris-acetate buffer pH7.6.

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 (pH7.6) (4700 μl), 0.1 M halogenated aliphatic acid (50 μl) and distilled water and enzyme to a final volume of 5000 μl . After 5 minutes 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 (Gornall *et al.*, 1949) with crystalline egg albumin as a standard. Specific activity is defined as the μmole of chloride liberated per milligram protein in 10 min under the stated conditions.

Chemicals. All chemicals were obtained from Sigma-Aldrich Chemical Co. (USA). 2,2-dichloropropionic acid (2,2DCP), 2,2,3-trichloropropionic, 3-chlorobutyric, 2,2-dichlorobutyric and 2,2,3-trichlorobutyric acids were provided by Dow Chemical Co. (USA).

HPLC analysis. Samples of growth medium were analyzed using HPLC in order to monitor disappearance of 3-chloro-

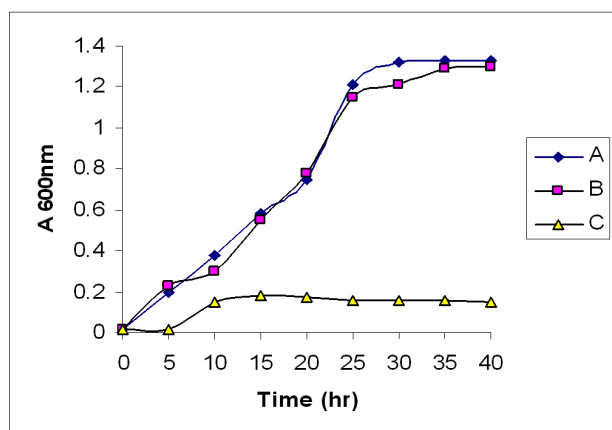


Figure 2. The growth of *Rhodococcus* sp. on 20 mM of A: 3-chloropropionic acid; B: Acrylic acid; and C: 3 hydroxy propionic acid.

propionic acid. Samples were filtered through nitrocellulose 0.2 μ m filters (Sartorius AG, Germany) to remove bacteria cells and particles. Samples were separated using an isocratic elution with a mobile phase containing potassium sulphate (20 mM): acetonitrile (60:40) in deionized water. Samples were detected with a UV detector equipped with a Supelco C-18 column (250 \times 4.6 mm, particle size of 5 μ m) using a flow rate of 2 ml/min.

RESULTS

Bacteria morphology, staining, biochemical and 16S rRNA characterization. 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 *Bergey's Manual of Systematic Bacteriology* (Holt *et al.*, 1994). Partial 16S rRNA gene sequencing also matched to the sequence belongs *Rhodococcus* sp.

Degradation of halogenated compound. *Rhodococcus* sp. was grown in a minimal medium containing 3-chloropropionic acid, the resulting cell suspension metabolized 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

Table 2. The relationship between 3-chloropropionic acid consumed and turbidity of *Rhodococcus* growth medium.

Time (hour)	Amount of 3-chloropropionic acid left	Bacteria Growth (A600nm)
0	100 %	0.172
24	20 %	1.14
48	10 %	1.363

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 hour 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.

Under the conditions of the enzyme assay, cell free extracts was prepared from bacteria grown on 20 mM 3-chloropropionic acid minimal medium. The utilization of 3-chloropropionic acid was also confirmed by detection of 3-chloropropionic acid depletion in the growth medium using HPLC technique. The chloride formation by the crude and soluble preparations proceeded rapidly for 2 hour by which time 85-90% of the organic chlorine in 0.5 mM 3-chloropropionic acid was released. The specific activities were 0.013 and 0.018 μ molCl⁻/min/mg for the crude and soluble preparations, respectively. Dialysis overnight did not reduce the activity of either preparation.

Since not all the chlorine was released in any of the assays with the crude extract and the soluble fraction, the possibility of an inhibition by possible intermediates was investigated. Addition to the soluble fraction of 1 or 3 μ mol of acrylic acid inhibited chloride release from 3-chloropropionic acid by 10 and 15 %, respectively. Propionic acid (1 μ mol) inhibited the conversion 15 %, whereas 1 μ mol 3-hydroxypropionic acid had no effect.

To establish the pH optimum for the dechlorination of 3-chloropropionate, 0.1M Tris-acetate buffer - NaOH were used as buffers. In a 2 hour incubation, the maximum activity was observed at about pH 7.6, although the conversion still proceeded rapidly at pH 6.0 and 9.0. Dehalogenation did not occur if the incubation was performed under anaerobic

condition or if the enzyme was heated to 100°C for more than 1 minutes.

Addition of 1.0 μmol NADP to the soluble fraction (2.35 mg/ml) stimulated its dehalogenation of 3-chloropropionic acid by about one-third. There was no increase in activity noted upon the addition of L-cysteine or glutathione (0.01M). Enzyme preparation stored for 6 months at at -15°C did not release chloride, but the activity could be restored if NADP was supplied to reaction mixtures containing these aged preparations.

The cells were also grown in minimal media supplied with two possible intermediates in 3-chloropropionic acid metabolism (Figure 2). It is evident that acrylic acid and 3-chloropropionic acid were utilized readily and at similar rate. 3-hydroxypropionic was somewhat not degraded than other test substrates.

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 and D,L-2chloropropionic acid (Slater *et al.*, 1979; Weightman *et al.*, 1982; Schwarze *et al.*, 1997).

Jensen (1957) proposed that the 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 (Castro and Bartnicki, 1965). However, the failure to find growth in 3-hydroxypropionic acid minimal medium and the apparent formation of acrylic acid suggest it was far from prove that the chlorine is removed by dehydrodehalogenation. Addition of co-factor was necessary in order to achieve efficient degradation of 3-chloropropionic acid and stimulatory effect of NADP may be associated with its role in this reaction.

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