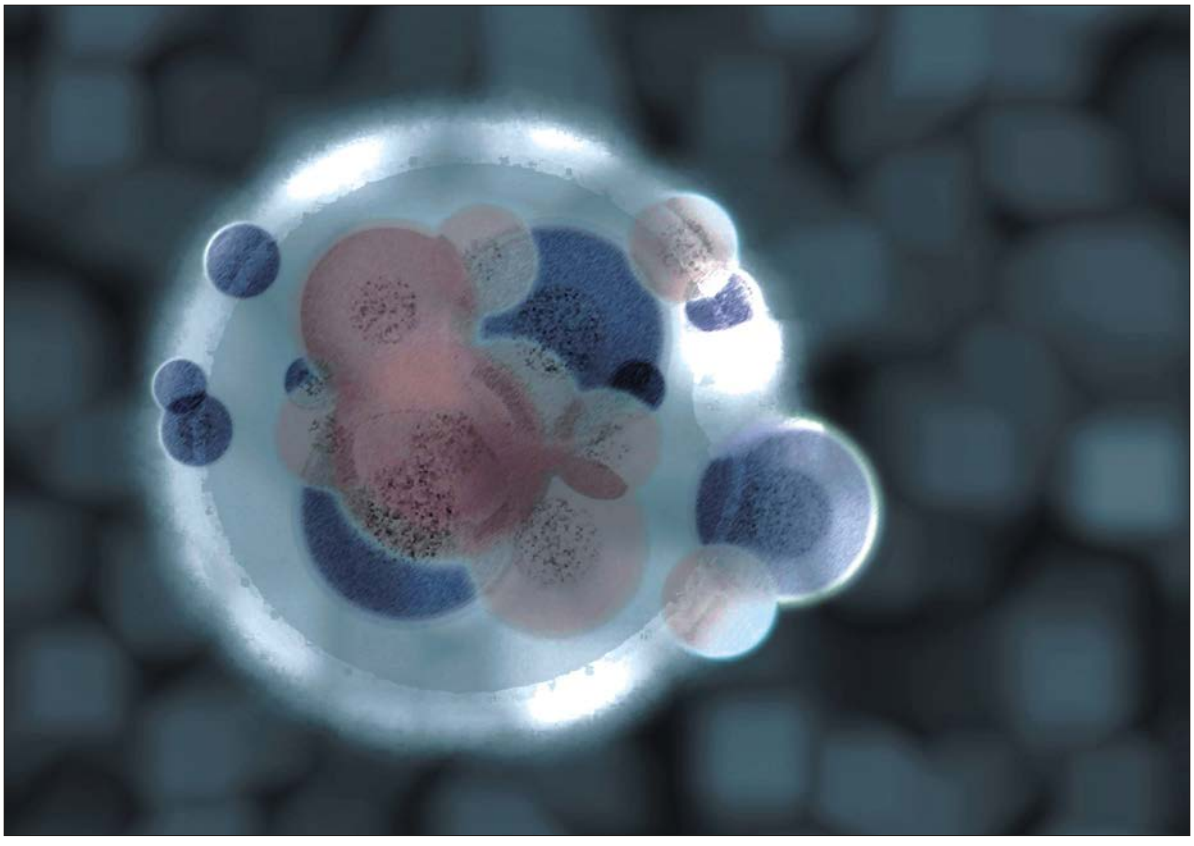

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 8 (1): 233-235, 2008
ISSN 1727-3048

Enzymatic Dehalogenation of 2,2-Dichloropropionic Acid by Locally Isolated *Methylobacterium* sp. HJ1

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Abstract: Synthetic halogenated organic compounds are found widely throughout the biosphere due to modern industrial and agricultural processes. Various soils microorganisms are able to utilize halogenated alkanoic acids as a sole carbon source. An active dehalogenase enzyme was demonstrated in the crude extracts of partially purified enzyme from *Methylobacterium* sp. HJ1. The ability of the enzyme to catalyze the dehalogenation of various halogen-substituted organic acids was investigated and the highest activity was found with 2,2-dichloropropionic acid with maximum activity in phosphate buffer pH 6.8. The partially purified enzyme was unaffected by Co^+, Mg^+ or Mn^+ ions or glutathione. The enzyme removed chlorides ions present on a number of 2-carbon alkanoic acids if the halogen was on the α but not on the β-position. The putative product of dehalogenation was pyruvate using a standard assay system and at the same time 2,2-dichloropropionic acid depletion was detected in growth medium by High Performance Liquid Chromatography (HPLC).

Key words: Dehalogenase enzyme, dichloropropionate, haloalkanoic acid, dehalogenation, 2,2-dichloropropionic acid

INTRODUCTION

Synthetic halogenated organic compounds are found widely throughout the biosphere due to high consumption in modern industrial and agricultural processes. These xenobiotic compounds show high toxicity and persistence and cause the contamination of soils and ground waters. The microbial catabolism of these compounds have been reviewed extensively (Hardman, 1991; Leisinger and Bader, 1993). The herbicide 2,2-dichloropropionic acid can be used to control specific annual and perennial grasses, such as quick grass, Bermuda grass, Johnson grass as well as cattails and rushes (Gangstad, 1986). It is toxic not only to plants but also to humans. Skin and inhalation exposure could be of significance to herbicide production workers, pesticide applicators and some agricultural workers (Doyle, 1984). Plants do not readily decompose 2,2-dichloropropionic acid or trichloroacetate. These two herbicides in general are quite stable in higher plants and animals but degraded rapidly in soil (Ashton and Crafts, 1981). Natural microorganisms capable of the metabolism of a wide range of monochlorinated, dichlorinated and aliphatic substrates (Hardman, 1991). Certain species of soil microorganism can readily attack 2,2-dichloropropionic acid and use this as a carbon source with rapid liberation of chloride ions. Some properties of cell free extract preparation of locally isolated strain, *Methylobacterium* sp. HJ1 capable of dehalogenating 2,2-dichloropropionic acid are examined in the present study.

MATERIALS AND METHODS

Pure culture isolation of adapted micro-organisms responsible for the decomposition of 2,2-dichloropropionic acid was accomplished via the plate streaking technique. Representatives of the genera were effective in liberating chloride from 2,2-dichloropropionic acid are *Pseudomonas*, *Agrobacterium*, *Rhizobium* sp., *Alcaligenes*, *Arthrobacter* and *Nocardia*. Several of these genera had been reported earlier by Jensen (1957), Hirsch and Alexander (1960), Berry *et al*. (1979) and Olaniran *et al*. (2001). Previously, *Methylobacterium dichloromethanicum* DM4 was reported for its ability to degrade chloromethane or dichloromethane as the sole carbon and energy source (Galli and Leisinger, 1985). However, this is the first reported *Methylobacterium* sp. to show the ability to degrade 2,2-dichloropropionic acid. It was selected for study because of its ability to utilise 2,2-dichloropropionic acid at higher rate of the chlorinated aliphatic acid.

Routinely, cells were grown in PJC chloride-free minimal media. Stock solution was prepared as 10x concentration of basal salts containing $K_2HPO_4.3H_2O$

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(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 concentrated stock that contained nitriloacetic acid $C_6H_9NO_6$ (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 $CoCl_2.6H_2O$ (10 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 at 15 psi). Carbon sources (1M, 2,2-dichloropropionate) was sterilised separately and added aseptically to the media to the desired final concentration. Liquid minimal cultures were supplemented with yeast extract to a final concentration of 0.05% (w/v). In order to prepare solid medium, Oxoid bacteriological agar (1.5% w/v) was added prior to sterilisation. Samples were removed periodically and growth determined by measuring turbidity at Absorbance 680 nm and chloride ion liberation at Absorbance 460 nm (Iwasaki *et al*., 1956).

Harvested cells were washed twice with ice cold distilled water to remove any excess chloride ion. Resuspended cells were ruptured in a ultrasonication (Vibra Cells ™) for 5 min. Sonicated cells were centrifuged at 10,000 g for 10 min and the cell debris discarded. All manipulations with the cell free system were carried out at 0°C.

Protein concentration was determined by the biuret procedure with serum albumin as a standard (Gornall *et al*., 1949). Crude and purified enzyme preparations were assayed in a mixture containing an enzyme (1-3 mg protein), 1 mL 0.2M phosphate buffer and 2,2-dichloropropionate as substrate (final concentration 1 mM) in a total volume of 5 mL. The reaction was initiated by adding the enzyme to the substrate and buffer, maintained in a water bath at 30°C. The reaction was terminated by pipetting 1 mL aliquote into 100 µL 9 M nitric acid and then followed by 100 µL of Hg (SCN) for the measurement of chloride ion. Specific activity is defined as the number of µg of chloride liberated per mg protein per 10 min. Phosphate buffer or tris were used to ascertain the pH optimum for cell free system. Since phosphate buffer gave slightly higher specific activities it was used in all subsequent studies. A sharp optimum was noted at pH 6.8. All values are corrected against boiled enzymes controls, where no chloride liberation above reagent levels was noted.

The enzymatic nature of the dehalogenation was indicated by a linear increase in velocity as the enzyme concentration was increased from 1 to 3 mg of protein. The specific activity of the crude enzyme was 0.4 µmol Cl^- per min per mg protein per 10 min. The crude enzyme was completely inactivated in less than 15 min at 60°C. Cell free preparations and partially purified fractions were stable when stored in a freezer at -5°C.

The enzyme was partially purified by DEAE-cellulose column. Approximately 27 mg protein was applied on to a 12 mL column. Initially the extract was washed through with 10 mM sodium phosphate buffer pH 6.8 containing 0.2 M NaCl. The eluent was collected in 6 mL fractions until the protein concentration fell to 1/10 of the highest value at which point the salt concentration was raised to 0.3 M. The higher salt concentration allowed the dehalogenase enzyme to be eluted from the column. No increase in activity was noted when Co^+, Mg^+ or Mn^+ ions or glutathione were added at the concentrations up to 10^{-3} M.

RESULTS AND DISCUSSION

The enzyme shows greatest activity on 2,2-dichloropropionate with less activity on 2-chloropropionate, dichloroacatate and 2,2-dichlorobutyrate (Table 1). The enzyme was inactive on any β-chloro substituted aliphatic acid. In the partially purified enzyme system, pyruvate was assayed using a standard pyruvate assay system. Degradation of 2,2-dichloropropionate was further confirmed by detection of 2,2-dichloropropionate depletion in growth medium by High Performance Liquid Chromatography (HPLC) and pyruvate as the product.

Mechanistic studies of enzymic reaction showed that dehalogenases cleave hydrolytically the carbon halogen bond, resulting in the formation of hydroxyalkanoic acids from monosubstituted compounds and oxoalkanoic acids (pyruvate) from disubstituted compounds (Foy, 1975). Kearney *et al.* (1965) determine degradation pathway for 2,2-dichloropropionate acid by an *Arthrobacter* sp. suggested that cleavage of the first chlorine substituent was catalyzed enzymatically, while the second was removed spontaneously from an unstable intermediate, forming pyruvate. Two possible mechanisms were proposed for the first enzymic dehalogenation. The first involved direct substitution with a nucleophilic attack at

Table 1: Substrate specificity of the partially purified enzyme

Substrates	Specific activity
Dichloroacetate	12
Trichloroacetate	0
2-chloropropionate	11
3-chloropropionate	0
2,2-dichloropropionate	38
2,2,3-trichloropropionate	0
2,3-dichlroisobutyrate	0
2,2-dichlorobutyrate	10
2,3,3-trichlorobutyrate	0

the α-carbon position by -OH. The second involved an oxidative mechanism involving 2-chloroacrylate. This pathway would not explain the dechlorination of other α-substituted alkanoic acids. The above mechanism would be inconsistent with the observed activity on dichloroacetate but this activity appears to be unrelated to the enzyme study since decrease in chloride liberation from this substrate was noted after partial purification.

ACKNOWLEDGMENT

The author thanks to Malaysian Government for sponsoring this work under VOT 79073 (MOSTI).

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