African Journal of Microbiology Research. Vol.(2) pp. 187-191, July, 2008
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2008 Academic Journals

Full Length Research Paper

Purification and properties of a non-stereospecific dehalogenase enzyme E (DehE) from *Methylobacterium* sp. HJ1

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Accepted 12 June, 2008

The bacterial isolate HJ1, which was identified as a *Methylobacterium* sp., grew on 2, 2-dichloropropionic acid as the sole carbon source and produced a 2-haloalkanoic acid hydrolytic dehalogenase. This non-stereospecific dehalogenase E (DehE) catalysed the hydrolytic dechlorination of 2, 2-dichloropropionic acid and D, L-2-chloropropionic acid to produce pyruvate and lactate, respectively. The enzyme was purified to homogeneity and characterized. The molecular weight was 36 kDa by SDS-polyacrylamide gel electrophoresis and 72 kDa by gel filtration, suggesting that the enzyme is a protein dimer. The purified enzyme was only inhibited by $HgSO_4$ and was non-stereospecific to haloalkanoic acids. The K_m value for the hydrolysis of 2, 2-dichloropropionic acid was 0.25 mM. The enzyme removes chloride present on the α-position, but not on the β-position, of a number 2–carbon alkanoic acids.

Key words: haloalkanoic acid, dichloropropionate, 2, 2-dichloropropionic acid.

INTRODUCTION

Synthetic halogenated organic compounds are found widely throughout the biosphere due to their high consumption in modern industrial and agricultural processes. These xenobiotic compounds show high toxicity and persistence and cause serious environmental pollution. Microbial dehalogenases detoxify harmful halogenated compounds by cleaving their carbon-halogen bonds (Copley, 1998; Kurihara et al., 2000) and have received a great deal of attention (Fetzner and Lingens, 1994). Microorganisms are capable of metabolizing a wide range of monochlorinated, dichlorinated and aliphatic substrates because they possess one or more dehalogenases. Certain soil-borne microorganisms can readily attack 2, 2-dichloropropionic acid and use it as a carbon source with rapid liberation of chloride ions. Representative bacterial genera that have been reported to be effective in liberating chloride in haloalkanoic acids are *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Alcaligenes*, *Arthrobacter* and *Nocardia* (Janssen et al., 2001; Song et al., 2003; Park et al., 2003). Some of these microorganisms have been isolated originally from contaminated soil and sewage oxidation ponds (Olaniran et al., 2001; Olaniran et al., 2004). The study of 2, 2-dichloropropionic acid degradation is important as it can be compared to that of β-chloro-substituted alkanoates, for example, 3-chloropropionic acid, which is an analogue and isomer of 2, 2-dichloropropionic acid (Allison et al., 1983). Further interest in this subject was raised when it became apparent that α-chloroalkanoate-degrading microorganisms were unable to dechlorinate the β-substituted haloalkanoates, which differed only in chlorine substitution. In the present study, we demonstrate the existence of a single and novel non-stereospecific DehE type dehalogenase from *Methylobacterium* sp. HJ1 and also describe the properties of this enzyme.

MATERIALS AND METHODS

Materials

Halogenated compounds were obtained from Sigma Chemical Co. (USA). Other chemicals were of analytical grade.

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Bacterial strains and growth conditions

The bacterium, tentatively identified as *Methylobacterium* sp. HJ1, was isolated from agricultural soil for its ability to utilize 2, 2-dichloropropionic acid at a high rate (Jing and Huyop, 2008). The bacterium was grown aerobically in chloride-free minimal medium, as described earlier (Hareland et al., 1975), containing 2, 2-dichloropropionic acid (20 mM) as the sole carbon and energy source.

Preparation of cell-free extracts

Cell-free extracts were prepared from bacterial cells in the mid- to late-exponential phase of growth. Cells from a 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 7.2) 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 7.2) and maintained at 0°C for ultrasonication in an MSE Soniprep 150 W Ultrasonic disintegrator at peak amplitude (λ = 10 microns) for 30 s. Unbroken cells and cell wall material were removed by centrifugation at 20 000 × *g* for 15 min at 4°C. Cell-free extracts were ultracentrifuged at 120 000 × *g* for 90 min at 4°C for use in protein purification.

Enzyme assay and associated analytical methods

The enzyme activity was measured in a reaction mixture (5 ml) containing: 0.09 M Tris-acetate (pH 7.5), 1 mM halogenated alkanoic acid (substrate) and an appropriate amount of enzyme. Before initiation of the reaction and in the absence of the enzyme, the reaction mixture was equilibrated at 30°C in a water bath for 10 min. The reaction was then initiated by the addition of enzyme and 1 ml samples were removed at intervals, after which the free halide was determined colorimetrically (Bergman and Sanik, 1957). The color was allowed to develop for 10 min at room temperature and measured at Absorbance 460 nm. Enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 µmol halide ion/minute.

To examine the effect of the temperature, enzyme extracts were incubated for 15 min at various temperatures (between 25 – 50°C) and 1 ml samples were removed at 5 min intervals for the enzyme assay at 30°C, as described above. To determine the pH optimum, crude extracts were mixed with equal amounts of 100 mM buffer of various pHs. The reaction was started by addition of the substrate and after incubation for 10 min the chloride ions released was determined as described. A control reaction mixture, lacking the enzyme preparation, was included in each set of assays to detect spontaneously released halogen.

To determine the effect of inhibitors, as listed in Table 4, the assay mixtures comprised of inhibitors at a final concentration of 1 mM. After incubation at 30°C for 10 min, the reaction was stopped and the chloride ions released was determined.

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.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Methods were based on the protocol of Laemmli (1970), using the Mini-Protean II electrophoresis apparatus (BioRad, USA).

Kinetic studies

The effect of substrate concentration on the cell-free extracts was determined at concentrations between 0.1 and 5 mM of 2, 2-dichloropropionate. The assay was performed in 0.1 M Tris-acetate buffer (pH 7.2).

Diethylaminoethyl (DEAE) cellulose chromatography

The enzyme was partially purified on a DEAE-cellulose column. Approximately 20 mg protein was applied on to a 12 ml column. Initially, the extract was washed through the column 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.

Fast protein liquid chromatography (FPLC)

FPLC was carried out using a Pharmacia system with a MonoQ HR 5/5 column (anion exchange chromatography). Anion exchange chromatography was performed using a linearly increasing gradient of phosphate ions. Buffers are required during purification process, one containing a low concentration of salt (Buffer A) and the other containing a high concentration of salt (Buffer B). Depending on the dehalogenase, buffer A contained 5 or 20 mM phosphate buffer (pH 7.8), 1 mM EDTA, 10% Glycerol, 1 mM DTT, and buffer B contained 100 or 200 mM phosphate buffer, 1 mM EDTA, 10% Glycerol, 1 mM DTT. These buffers were then mixed via a gradient controller to yield a linear gradient of 5 to 100 mM or 20 to 200 mM of phosphate. Samples for purification were prepared as cell-free extracts and approximately 5 mg of protein were applied to the MonoQ column per run. The column was run at a flow rate of 1 ml/min. Fractions (usually 1 ml) were collected and then assayed to determine which contained maximal dehalogenase activity. These fractions were stored on ice until use.

For molecular weight determination, two Superose 12 HR 10/30 (size exclusion chromatography) columns were used in series for greater resolution. The columns were equilibrated overnight using a buffer containing 20 mM Tris-acetate, 0.1 M sodium acetate pH 7.5. The sodium acetate was used to increase the ionic strength and so stop any interaction with the gel matrix. Samples (0.2 ml) from the MonoQ step were applied to the gel filtration column at approximately 0.5 - 0.8 mg of protein per run. The column was run at a flow rate of 0.4 ml/min. For molecular weight determination the columns were calibrated using molecular weight standards from Sigma, the relative molecular weights of these being: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

Measurement of lactate and pyruvate

Lactate was assayed using a Sigma diagnostic kit (826UV) together with lactate dehydrogenase, whereas pyruvate was assayed using the Pyruvate Assay Kit (BioVision-USA).

RESULTS

Enzyme purification

Table 1 summarizes the results of purification of a single dehalogenase from *Methylobacterium* sp. HJ1. The enzyme was purified 11-fold with 45% recovery (Table 1). The purified enzyme showed a single protein band on SDS-PAGE (Figure 1), and its activity was detected by

Table 1. Purification of DehE from *Methylobacterium* sp. HJ1.

Purification Step	Total protein (mg)	Total units (µmol/min)	Specific activity (Unit/mg)	Recovery	Purification (fold)
Crude extract	3	0.4	0.04	100%	1
DEAE-Cellulose Chromatography	0.3	0.18	0.26	60%	3.5
Mono-Q Chromatography	0.4	0.2	4.4	45%	11

Table 2. Substrate specific activity of the purified dehalogenase enzyme.

Substrate	Specific activity (µmolCl^-/min/mg protein)
Monochloroacetate	6
Dichloroacetate	12
Trichloroacetate	2
D,L-2-chloropropionate	11
3-chloropropionate	0
2,2-dichloropropionate	40
2,2,3-trichloropropionate	0
2,3-dichloroisobutyrate	0
2,2-dichlorobutyrate	10

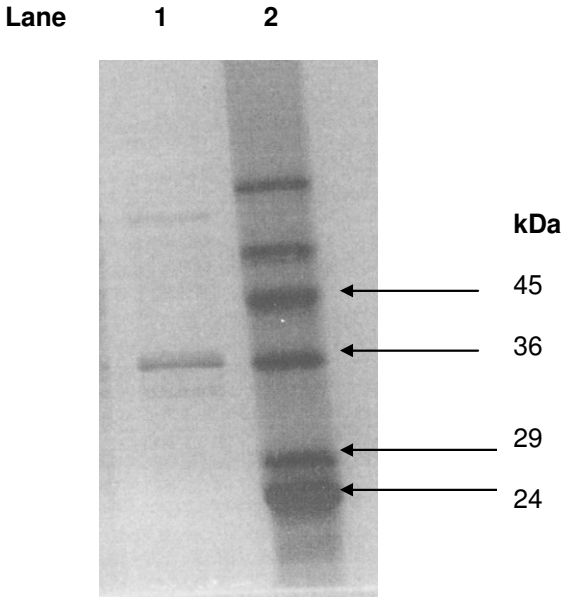

Figure 1. Electrophoresis of DehE from *Methylobacterium* sp. HJ1. SDS-PAGE of purified DehE (Lane 1, 3 µg). The molecular weight markers (Lane 2) are indicated in kDa to the right of the figure.

zymography using the standard procedure of Hardman and Slater (1981) (Figure 2). Only one dehalogenase band was present when incubated with 2, 2-dichloro-propionate and/or D, L-2-chloropropionate, respectively, and the band migrated at the same position in both instances. The purified enzyme retained more than 95% activity after storage for 3 months at -20°C in 25 mM potassium phosphate buffer (pH 7.5) containing 50% (w/v) glycerol. The enzymes were routinely stored in this manner.

Molecular weight and subunit structures

The purified protein was applied to two Superose 12 columns (Pharmacia), connected in series. The native molecular weight of the protein was found to be 72 kDa. The subunit structure was studied by SDS-PAGE. The molecular weight was 36 kDa for this protein, suggesting that DehE was a protein dimer.

Effects of pH and temperature

The maximum activity of DehE was found at pH 7.2. The effect of temperature was also examined. The optimal activity was observed at 35°C. The enzyme lost its full activity upon heating above 50°C for 15 min.

Substrate specificity

The substrate specificities of DehE is shown in Table 2. The enzyme was inactive on β-chloro-substituted aliphatic acids. Acetic and propionic acids were dehalogenated effectively. The Michaelis-Menten constant of this enzyme for 2, 2-dichloropropionic acid was 0.25 mM.

Table 3. Specificities of the purified dehalogenase enzyme (DehE).

Enzyme	Substrate	Chloride release (mM)	Product (mM)
DehE	D, L-2-chloropropionic acid	23	Lactate (24 mM)
DehE	2, 2-dichloropropionic acid	24	Pyruvate (24 mM)

Table 4. Effects of inhibitors on enzyme activity.

Reagent	Concentration (mM)	Remaining activity (%)
None		100
EDTA	1	95
$HgSO_4$	1	0
N-ethylmaleimide	1	97
p-chloromercuriphenylsulfonic acid	1	98
Iodoacetamide	1	100

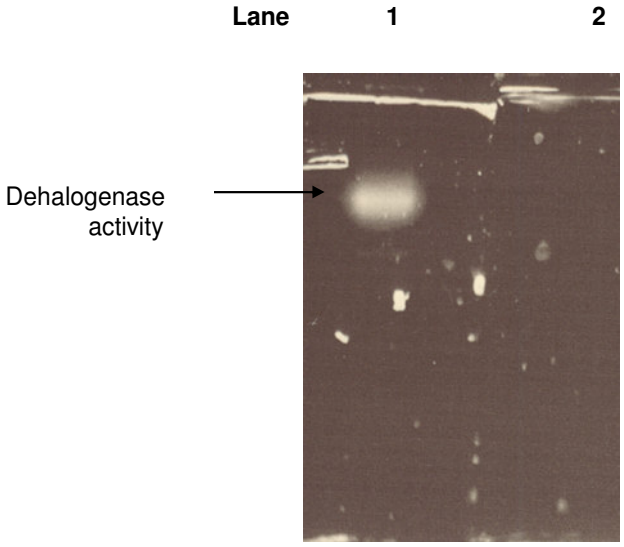

Figure 2. Activity staining of the purified DehE (5 µg) (Lane 1), while a control reaction, without DehE enzyme, is indicated in Lane 2.

Reaction products

The stoichiometry of the enzymatic dehalogenation of D,L-2-chloropropionic acid was investigated. As shown in Table 3, the DehE dehalogenase completely converted D, L-2-chloropropionic acid to lactate and 2,2-dichloropropionic acid to pyruvate.

Inhibitors

As shown in Table 4, the enzyme was not inactivated by incubation with EDTA, but was inhibited by $HgSO_4$. The enzyme was not inhibited by *N*-ethylmaleimide, *p*-chloromercuriphenylsulfonic acid and iodoacetamide.

DISCUSSION

A dehalogenase enzyme has been purified from *Methylobacterium* sp. HJ1 and characterized. The DehE enzyme was induced by 2, 2-dichloropropionic acid and acted on both D- and L-isomers of 2-haloacids forming lactate, whereas activity on 2, 2-dichloropropionic acid produced pyruvate. This enzyme is therefore similar to previously reported dehalogenases (Motosugi et al., 1982; Leigh, 1986; Liu et al., 1994; Nardi-Dei, 1997)

DehE from *Methylobacterium* sp. HJ1 resembles DehE from *Rhizobium* sp. (Stringfellow et al., 1997) in stereospecificity, subunit molecular weight (DehE, 32 kDa) and optimum pH (DehE, 7.6), but is distinct in subunit structure to the *Pseudomonas* sp. YL DL-DEX (Liu et al., 1994) that has a monomer structure.

The specificities of DehE (Table 2) indicated that only chloride from one position was released, presumably from carbon 2, whereas dehalogenase from *Rhizobium* sp. did not react with 3-chloropropionic acid and based on this information chloride at carbon 3 was not attacked (Allison et al., 1983).

For the D,L-DEX enzyme of *Pseudomonas* sp. YL, an equivalent enzyme to DehE from *Rhizobium* sp., it has been reported that an aspartic or glutamic acid residue may play an important role in catalysis. Moreover, the presence of *N*-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetamide did not cause inactivation of the enzyme, thus suggesting that cysteine was not involved in the enzyme catalysis (Nardi-Dei, 1997). It is too early to know whether the current isolated enzyme may have similar amino acid residues involved in its enzyme catalysis and further investigation needs to be carried out.

Previously, *Rhizobium* sp. was isolated using 2, 2-dichloropropionic acid and stereospecific dehalogenases (DehD and DehL) were also detected in this organism. Moreover, these enzymes were confirmed not to

act on 2, 2-dichloropropionic acid (Leigh, 1986). One possible reason might be that the commercially available 2, 2-dichloropropionic acid was not pure and contained D, L-2-chloropropionic acid. A second explanation is possibly that DehL and DehD were chromosomally located and the gene encoding DehE was obtained separately via plasmid transfer. Since the organism used in the present study produced only one dehalogenase, it was curious that other dehalogenase-producing organisms reported so far had more than one dehalogenases. Therefore, extensive study on dehalogenase gene regulation is required in the future.

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. RA Cooper (Leicester, UK) for previous guidance on dehalogenase work. Thanks are also due to Dr. Aidil's group (UKM) for advice on protein purification work. NHJ and FHS thank to the Malaysian Government for financial support under Fundamental Research Grant Vot numbers 74190 (IRPA-RM8) and 79073(e-Science Fund).

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