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Full Length Research Paper

Purification and properties of a new dehalogenase enzyme from *Pseudomonas* sp. B6P grow in 3-chloropropionate (3CP)

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Halogenated compounds are widely used in agriculture and industries and have been associated with environmental pollution. Degradation of 3-chloropropionate (3CP) by microorganism has been established and this enzyme could only remove halogen atom at the β- position of 3-carbon alkanoic acids. *Pseudomonas* sp. B6P was originally isolated from paddy field which was able to degrade 3CP therefore, suggesting it may have some desirable properties. The enzyme was purified from cell-free extracts having a monomer of 56,000 Da. It was found to be stable between pH 5 to 8 and its optimal pH was 8. Its activity was not affected by metal ions such as Mn^{2+}, Fe^{3+} and Mg^{2+}, but was inhibited by Hg^{2+} and Ag^{2+}. The enzyme is specific for 3CP, and the Km value (0.20 mM ± 0.05).

Key words: Biodegradation, 3-chloropropionic acid, dehalogenase, bioremediation, haloalkanoic acid, *Pseudomonas* sp.B6P.

INTRODUCTION

Halogenated aliphatic compounds are widely used in agriculture and industries. These compounds have widespread applications as herbicides, fungicides, insecticides, solvents and hydraulic or transfer fluids and consequently, these compounds are now accumulating in the environment (Fetzner and Lingens, 1994; Bhatt et al., 2007). These substances can be decontaminated using non-biological or microbiological degradation methods which transforms the xenobiotic substances into harmless products. However, the latter are favored because they are economical, safer and environmentally friendly.

Currently, dehalogenase enzymes that catalyze the cleavage of carbon-halogen bonds receive greater attention because of their potential use in biotechnological applications in the bioremediation of halogenated environmental pollutants. Biodegradation of 2-haloacid or α-chloro substituted alkanoates were well documented; for example 2,2-dichloropropionic acid (2,2DCP) and D,L2-chloropropionic acid (D,L2CP) (Motosugi et al., 1982; Allison et al., 1983; Asmara et al., 1993; Barth et al., 1992; Thasif et al., 2009; Jing et al., 2008), whereas the degradation of 3-chloropropionate (3CP) or β–chloro substituted alkanoates used as chemical inclusion of certain herbicides was not well established. The understanding of its degradation pathways was limited. This research is important because α-chloro alkanoate degrading bacteria were unable to dechlorinate the β–chloro substituted

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alkanoates which differed only in chlorine substitution (Allison, et al., 1981; Jing and Huyop, 2008).

Recently, a species of *Pseudomonas* strain B6P that utilized 3CP as a sole carbon source was isolated and suggested the occurrence of a new dehalogenase which can act only on 3CP (Mesri et al., 2009). In this work, the purification of this enzyme from bacterial cells and the enzymological properties of its purified form were described. This enzyme may also have potential applications in the detoxification of contaminated water or soil.

MATERIALS AND METHODS

Chemicals

Various halogenated compounds of analytical grade were purchased from Sigma-Aldrich Chemical Co. (USA).

Culture conditions

Batch cultures of *Pseudomonas* sp. B6P were grown in 2 L conical flasks with 500 ml of PJC mineral medium as described by Hareland et al. (1975) that contained a final concentration of 3CP of 20 mM with shaking at 200 rpm.

Preparation of cell free extract, enzyme assay and associated analytical methods

Harvested cells were suspended in 0.1 M Tris-acetate, 1 mM EDTA, 10% (mass/volume) glycerol, pH 7.5 and centrifuged at 10000 xg for 10 min at 4°C. The cells were then resuspended in 4 ml of the same buffer, and maintained at 0°C for sonication in an MSE Soniprep 150 W ultrasonic disintegrator at a peak amplitude (λ = 10 microns) for 30 s. Unbroken cells and cell wall materials were removed by centrifugation at 40000 xg for 30 min at 4°C to remove cell debris and supernatant was decanted and kept at 4°C. Unless otherwise indicated, the dehalogenase activity was determined by measuring the chloride ion released using a colorimetric method that included mercuric thiocyanate (Bergman and Sanik, 1957). Dehalogenase activity was determined as the total chloride released at 30°C in a reaction that contained 0.1 M Tris-acetate buffer (pH 7.5) (4700 µl), 1 mM halogenated aliphatic acid (50 µl of a 0.1 M stock), distilled water and purified enzyme added to a final volume of 5 ml. All reaction components except the enzyme were combined and allowed to equilibrate for 5 min at 30°C and then the reaction was initiated by addition of the purified enzyme. Samples (1.0 ml) were removed at appropriate intervals and halide ions were assayed. Protein concentration was determined by the biuret procedure with crystalline egg albumin as a standard (Gornall et al., 1949). Specific activity was defined as the micromoles of chloride liberated per milligram protein in 10 min under the stated conditions.

To examine the effect of the temperature, enzyme was incubated for 10 min at various temperatures (from 25 to 55°C) and 1 ml samples were removed at 5 min intervals for the enzyme assay at 30°C. To determine the optimum pH, enzyme was mixed with equal amounts of 100 mM buffer of various pHs. The reaction was started by the 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, the assay mixtures comprised 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 purification and characterization

Finely powdered ammonium sulphate (300 mg/ml) was added slowly to 30 ml cell free extract to give 45% saturation. The mixture was gently stirred until all ammonium sulphate had dissolved, and the precipitate was removed by centrifugation at 40000 g for 30 min and discarded. Further ammonium sulphate was added to the supernatant liquid (123 mg/ml) to give 65% saturation, and the precipitate was removed by centrifugation and redissolved in 0.1 M Tris-acetate buffer at pH 7.5 (6 ml). The latter solution was dialysed overnight against 20 mM Tris-acetate buffer at pH 7.5 (200 ml) to remove residual ammonium sulphate and adjust the ionic strength of the solution to a level suitable for ion-exchange chromatography.

Further enzyme purification was conducted on a AKTA prime chromatography system (Amersham Biosciences). All steps of the purification were conducted at 4°C. SDS–PAGE gels were prepared according to the method of Laemmli (1970). Samples of fractions were electrophoresed together with a pre-stained broad-range protein marker (Mark12 protein standard). Characterisation of the enzyme was conducted using protein that had been purified by anion exchange chromatography.

RESULTS

Purification of the enzyme

The purification of the 3CP dehalogenase by ammonium sulphate precipitation and chromatographic separation is summarized in Table 1. The purity of the enzyme was confirmed using SDS-PAGE (Figure 1), which also determined the subunit molecular weight. A single band was visible, revealing a pure enzyme. The molecular weight of the purified enzyme was 56,000 Da by SDS-PAGE.

Table 1. Purification of 3CP dehalogenase from *Pseudomonas* sp. B6P.

Purification Step	Total protein (mg)	Total unit (µmol/min)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Cell extract	5	0.4	0.08	100	1
$(NH_4)_2SO_4$ precipitation	0.2	0.2	1	50	50
Ion exchange (Mono-Q chromatography)	0.08	0.1	1.25	25	63

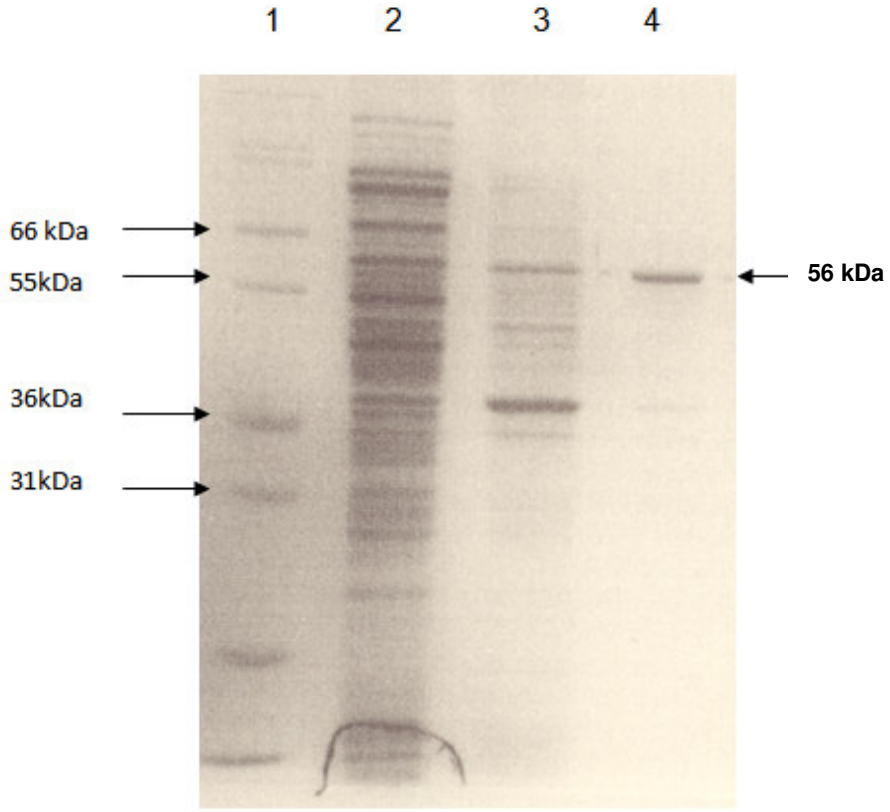

Figure 1. SDS-PAGE showing purification steps of dehalogenase enzyme from *Pseudomonas* sp. B6P. SDS VII marker (Lane 1); crude cell extract (Lane 2); $(NH_4)_2SO_4$ purification (Lane 3); MonoQ fraction (Lane 4).

Characteristics of the enzyme

The enzyme's characteristics were determined using purified enzyme from the anion exchange step. The optimum pH of the dehalogenase, as assayed at 30°C, was determined to be pH 8. However, the enzyme activity decreased dramatically outside these pH values between 5 and 9 (Figure 2).

The effect of temperature on enzyme activity was determined by assaying the 3CP dehalogenase at a range of temperatures between 20 and 55°C. The optimum temperature for the enzyme activity was observed at 30°C but the activity rapidly decreased above the temperature and less activity was measured above 40°C (Figure 3). The thermostability of the dehalogenase was assessed by incubating the enzyme for 10 min from 25 to 55°C prior to assaying. The residual activity fell dramatically at temperatures above 35°C, with less than 6% residual activity remaining when the enzyme was incubated at 40°C.

The inhibitory/stimulatory effect of several compounds was tested on the enzyme activity by pre-incubating the enzyme for 10 min with the compound of interest before assaying. Based on this analysis, the enzyme was inhibited dramatically by 1 mM Ag^{2+} and Hg^{2+}, with less than 1% of the enzyme residual activity. Interestingly, there was no inhibitory effect in the presence of 1 mM Mn^{2+}, Fe^{3+} and Mg^{2+}. Kinetic parameters of the

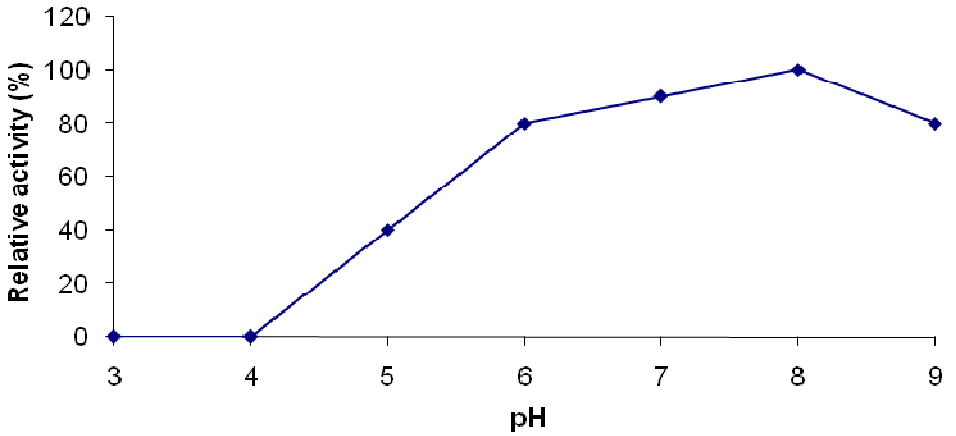

Figure 2. Effect of pH on the enzyme activity of the *Pseudomonas* sp. B6P dehalogenase.

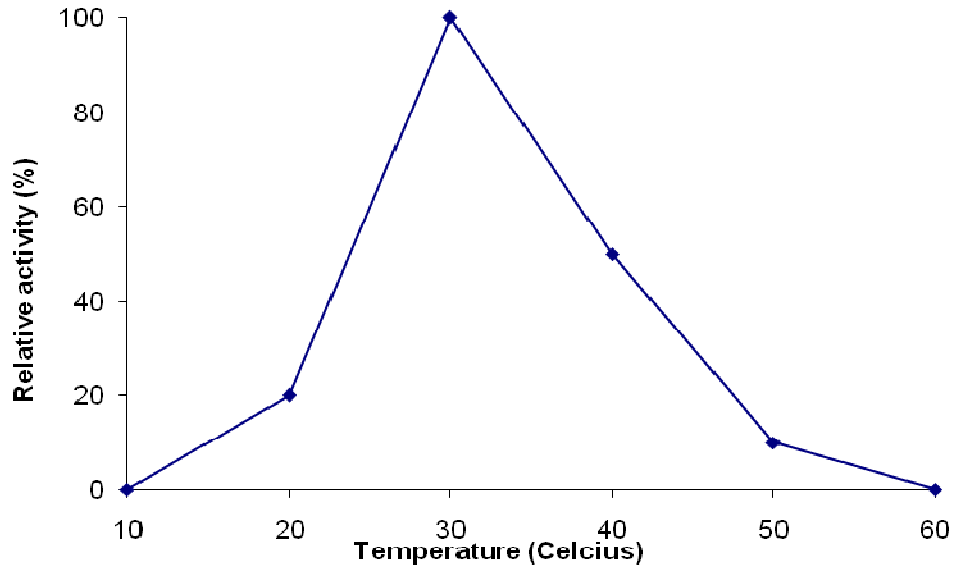

Figure 3. Effect of temperature (°C) on the enzyme activity of the *Pseudomonas* sp. B6P dehalogenase.

Pseudomonas sp. B6P dehalogenase using 3CP, was also determined. The apparent Km value was derived from computed Michaelis-Menten plot with a Km of 0.2 ± 0.05 mM.

DISCUSSION

The study of 3CP dehalogenase enzyme is important because of the position of the halogen substituent in governing the susceptibility of halogenated aliphatic acids to degradation by microbial enzyme since this enzyme is capable of hydrolysing the stable carbon-chloride at β-position. The findings suggested that *Pseudomonas* sp. B6P dehalogenase was specific for β-substituted halogenated aliphatic acids due to the fact that the strain could not grow in 2-haloalkanoic acids (2HAAs) as reported earlier. In addition, the 3CP dehalogenase enzyme was unable to dehalogenate α-halogenated substrates (Mesri et al., 2009).

The optimum pH for known bacterial dehalogenase enzymes is approximately 9, while for the enzyme isolated in this study it was pH 8, possibly useful for any potential application requiring a pH close to neutral. However, the enzyme described here is very sensitive to temperatures above 35°C. Many 2HAAs degrading enzymes are sensitive to thiol reagents such as Ag^{2+} and Hg^{2+}; similar to the current 3CP dehalogenase enzyme. However, other metal ions were found to have no effect on this enzyme.

Pseudomonas sp. B6P was only able to grow in 3CP and not in other halogenated substrate tested. In 3CP, it had a low Km (0.2 mM), possibly due to the high affinity of the enzyme to the substrate. This may explain the faster growth of the organism in 3CP minimal medium as reported earlier (Mesri et al., 2009). Other 2HAAs degrading enzyme also exhibited low Km values for their substrates, for example DehE of *Rhizobium* sp. on L2-CP (L2-chloropropionic acid) (0.56 mM), D2-CP (D2-chloropropionic acid) (0.85 mM) and 2,2DCP (0.19 mM) (Huyop et al., 2004). Likewise, the stereospecific DehD dehalogenase from *Rhizobium* sp. also demonstrated low Km values on D,L2CP (0.04 mM) and D,L2BP (D,L2-bromopropionic acid) (0.4mM), and similarly for DehL from the same organism on substrates D,L2CP and D,L2BP with apparent Km of 0.12 and 0.1 mM, respectively (Huyop and Cooper, 2003). The relatively low Kms might explain rapid growth rate of *Rhizobium* sp. in the degradation of 2,2DCP and D,L2CP using batch culture as reported by Allison et al. (1983) and Berry et al. (1979).

In conclusion, the 3CP dehalogenase from *Pseudomonas* sp. B6P is unique for its substrate specificities as compared with other known dehalogenase enzymes and for this reason, it suggest variability of dehalogenases in nature, which is important for the detoxification of contaminated environments.

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