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Full Length Research Paper

Purification and properties of *Rhizobial* DehL expressed in *Escherichia coli*

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The *Rhizobium* sp. DehL was produced by heterologous expression of the cloned gene in *Escherichia coli*. DehL enzyme was purified to homogeneity and characterized. The molecular weights were estimated to be 61 and 31 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), respectively, suggesting that the enzyme is a dimer. The purified enzyme was specific to the L-isomer monochloropropionate (L-2CP) and dichloroacetate (DCA). This protein was not able to act on 2,2-dichloropropionate (2,2DCP) and trichloroacetate (TCA). The estimated kinetic data indicated that this enzyme has high affinity to its specific substrates. By searching protein amino acid sequence database, the predicted amino acid sequence of DehL showed a high level of homology to those L-specific monochloropropionate (D,L-2CP) dehalogenase of *Rhizobium* sp. NHG3 with 53% sequence identity. The amino acid sequence of DehL showed low level sequence identity to those of Class 1D dehalogenases, suggesting DehL from *Rhizobium* sp. may belong to different group of dehalogenase classification preferably Class 1L dehalogenase.

Key words: Dehalogenase, DehL, *Rhizobial, dehL.*

INTRODUCTION

Halogenated organic compounds are found widely throughout the biosphere due to rapid developments in industry and agriculture. They can cause serious environmental pollution and health problems to the human population. Microbial catabolism of dehalogenase producing bacteria has been well studied (Kwok et al., 2007; Janssen et al*.,* 2001; Song et al., 2003; Park et al., 2003; Olaniran et al*.,* 2002; Olaniran et al*.,* 2004). A variety of halogenated compounds such as haloacids, which are produced by chemical industries, are degraded through dehalogenation by microbial dehalogenases. This involves carbon-halogen bond cleavage. The dehalogenation reactions have been classified into different types according to their substrate specificities (Slater et al*.,* 1997). Groups 1L and 1D are specific for the L- and D-isomers of 2-haloacids respectively, leading to the inversion of the product configuration.

Most of the dehalogenase producing bacteria contained more than one dehalogenases. The only organism so far reported to make all three forms of dehalogenase is a *Rhizobium* sp. (Leigh et al., 1988). DehL was shown to be specific for L-isomer monochloropropionate (L-2CP) and also acted on dichloroacetate (DCA) but not on 2,2-dichloropropionate (2,2DCP) or monochloroacetate (MCA). DehE was non-stereospecific dehalogenase acting on D,L-2CP, 2,2DCP, DCA, MCA and TCA (Huyop et al., 2004). DehD was shown to act only on D-isomer monochloropropionate (D-2CP) and MCA with no activity towards 2,2DCP or DCA. For each dehalogenase, the lactate produce from D,L-2CP has the opposite stereochemical form to that of the substrate (Leigh et al*.,* 1988). However, their structure and reaction mechanisms remain unclear. The present study deals with the purification and further characterisation of DehL specific dehalogenase. Accordingly, this dehalogenase is useful

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in degradation of toxic 2-haloalkanoic acids in the environment. Currently, it is far from clear why most dehalogenase producing organisms have more than one dehalogenases. An investigation of the kinetic properties of dehalogenase may shed light on this question.

MATERIALS AND METHODS

Source of DehL

The genes encoding DehL was originally isolated by Cairns (1994) from *Rhizobium* sp. chromosomal DNA as plasmid pSC2. Further subcloning of pSC2 into pUC18 resulted in pSC4 (*dehL*$^+$) which expressed DehL dehalogenase.

Bacterial strains, plasmids and growth conditions

Escherichia coli K-12 strain NM522 (Gough and Murray, 1983) was used as host for plasmid pSC4 (*dehL*$^+$). Cells were grown aerobically at 30^{o}C in mineral salts medium (Hareland et al*.,* 1975) containing D,L-2CP as carbon source supplemented with 0.05% (w/v) yeast extract. Isopropyl thio-β-D-galactoside (IPTG) (final concentration 0.3 mM) was added to the growth medium before incubating at 30^{o}C. Carbon sources and supplements were sterilised separately and added aseptically. Growth was followed by measurement of the absorbance at $A_{680\ nm}$.

Preparation of cell-free extracts and protein purification

Extracts were prepared from cells in the mid-exponential to late-exponential phase of growth (A_{680nm} 0.4-0.6). Bacteria were harvested by centrifugation at 10,000 g for 10 min at 4^{o}C. The cell suspension was sonicated at 0^{o}C for 30 s at an amplitude of 10 µm, using MSE soniprep 150 ultrasonicator. Unbroken cells and cell wall material were removed by centrifugation at 20,000 g for 15 min at 4^{o}C.

For purification of DehL, the cell-free extract was prepared in 0.1 M Tris-acetate buffer pH 7.6. Approximately 2.5 mg protein (4 U enzyme) was applied to a MonoQ HR 5/5 anion-exchange column equilibrated with 10 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (w/v) glycerol buffer, pH 7.6 and eluted with sodium phosphate gradient to 100 mM at a flow rate of 1 ml/min over 15 ml.

Molecular mass estimation analysis

Subunit molecular masses estimated by SDS/PAGE as described (Laemmli, 1970). The Fast Protein Liquid Chromatography (FPLC) gel filtration step was used to estimate the native molecular masses. Two columns of Superose 12 connected in series were equilibrated overnight using a buffer containing 20 mM Tris-acetate, 0.1 M sodium acetate pH 7.6. Samples (0.2 ml) from the MonoQ step were applied to the gel filtration column at approximately 0.5 mg of protein per run. The column was run at a flow rate of 0.4 ml/min. The columns were calibrated using molecular weight standards from SIGMA: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

Assay of dehalogenase activity

The enzyme reaction was carried out at 30^{o}C in a mixture of 5 ml 0.09 M Tris-acetate pH 7.5, 1 mM substrate and enzyme. Samples were removed at intervals, and the free halide was determined colorimetrically (Bergman and Sanik, 1957). The colour was allowed to develop for 10 min at room temperature and measured at $A_{460\ nm}$. Enzyme activity (1U) was defined as the amount of enzyme that catalyses the formation of 1 µmol halide ion/min.

Kinetic analysis

Standard 5 ml assays were prepared by the addition of varying amounts of purified enzyme to allow an accurate rate of reaction to be determined at several substrate concentrations. K_m was calculated using Michaelis-Menten plot, Microcal Origin version 6.0 Microsoft software. K_{cat} is equivalent to the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate:

$K_{cat} = V_{max}$ (µmol Cl^-/min/µmol enzyme) / 60 s

The catalytic efficiencies for different substrates were calculated as the ratio of K_{cat} and K_m. This parameter was known as Specificity Constant.

Sequence analysis

Sequence analysis was carried out using MultAlin software (Corpet, 1988). International databases were search using BLAST programme (Altschul et al*.,* 1990).

RESULTS

Expresssion of *dehL* in *E. coli*

The growth of *E. coli* NM522 [pSC4] (*dehL*$^+$) in D,L-2CP minimal media required IPTG, indicating that the expression of both genes is dependent on the *lac* promoter of the vector. Growth of *E. coli* carrying pSC4 plasmid was not detected in the absence of IPTG. The presence of IPTG led to a specific activity as in Table 1.

The stability of DehL in crude extract

The stability of DehL enzyme was examined. The crude preparation of dehalogenase was measured over 10 days storage at ice temperature (0^{o}C). Extracts were assayed for dehalogenase activity using a specific substrate of L-2CP. The extracts were examined from the day they were prepared (day 0) to day 10.

As shown in Figure 1, the enzyme remained stable for 24 h but then the activity of DehL started to decrease. Storage at 0^{o}C resulted in a 33% loss for DehL over 10 days. Therefore, DehL was stable and all measurements and analysis were made on the same day the enzyme was prepared.

Table 1. Specific activity for DehL using L-2CP as substrate.

Growth of *E. coli* in 20 mM D,L-2CP + 0.05 % yeast extract + IPTG	Specific activity (µmolCl⁻/min/mg protein)
E. coli NM522 [pSC4] (*dehL*⁺)	3.22

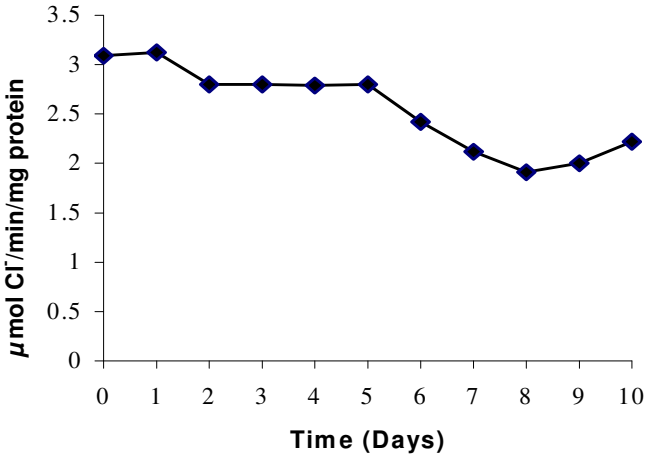

Figure 1. Stability of DehL in crude extracts from 0 to 10 days during storage at 0°C.

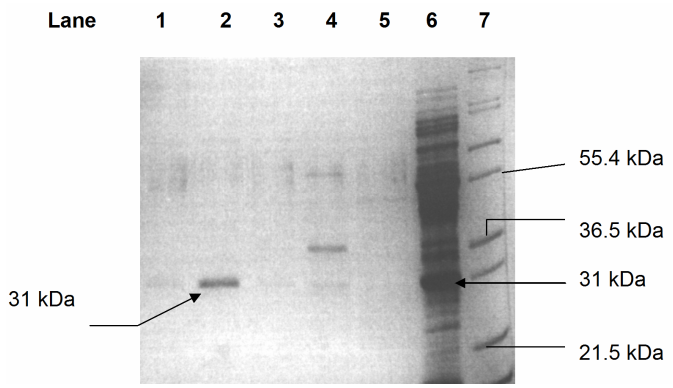

Figure 2. SDS-PAGE analysis of the purification of DehL (31 kDa). Lane 1: MonoQ fraction 10; Lane 2: MonoQ fraction 11 (8 µg protein); Lane 3: MonoQ fraction 12; Lane 4: MonoQ fraction 13; Lane 5: MonoQ fraction 14; Lane 6: crude extract of DehL; Lane 7: Molecular weight standards (kDa).

Purification of the DehL enzyme

DehL eluted in one fraction at approximately 46 mM sodium phosphate. The peak had 4 U enzyme and a specific activity of 20.0 U/mg with L-2CP as substrate with an estimated recovery of 100%. Analysis of the fraction by SDS-PAGE showed that at least 90% of the protein seen was accounted for by a 31 kDa band (Figure 2).

Table 2. Specific activity (µmolCl⁻/min/mg protein) of crude *Rhizobial* dehalogenase with their various substrates.

Substrate	DehL
2,2DCP	ND
L-2CP	5.00
D-2CP	ND
MCA	ND
DCA	1.82
TCA	ND

ND = Not detected.

Determination of the native molecular weight of DehL

To determine the native molecular masses of DehL, the enzyme was purified by the anion exchange and gel filtration procedures, using extracts from induced *E. coli* strain NM522 (pSC4). The molecular mass obtained from the gel filtration column was 61 kDa for DehL. The subunit size of DehL by SDS-PAGE analysis was 31 kDa, and the native form of this enzyme is a dimer of identical subunits.

Dehalogenase specificity and kinetic analysis

The substrate specificities are shown in Table 2. The enzyme activity of DehL was tested with D-2CP, MCA, 2,2DCP and TCA and no chloride released was observed suggesting their specificities. The kinetic analysis of the dehalogenase is shown in Table 3.

Dehalogenase amino acid sequence comparison

The *Rhizobial* DehL and *Rhizobial* DehD were completely different with each other by amino acid sequence comparison (14% identity) (data not shown). Blast search (NCBI) with the amino acid sequence from DehL was conducted to determine if there is any other protein with a similar amino acid sequence present in the databases. The purified DehL showed high identity (53%) to sequences from L-specific monochloropropionoic acid dehalogenase from *Rhizobium* sp. NHG3 (Higgins et al., 2002) with three regions at N-terminus, middle region and C-terminus (Figure 3). However, DehL amino acid sequence did not show any sequence homology nor indi-

Table 3. K_{cat} and specificity constants for DehL enzyme.

Substrate	K_{cat} (sec^{-1})	K_m	Specificity constant ($M^{-1}sec^{-1}$)
L-2CP	20.00	1.50×10^{-4}M (0.15 mM)	1.33×10^{5}
D,L-2CP	25.00*	1.20×10^{-4}M (0.12 mM)*	2.08×10^{5}*
D,L-2,3DCP	03.28*	3.00×10^{-5}M (0.03 mM)*	1.05×10^{5}*
DCA	06.25	1.30×10^{-4}M (0.13 mM)	4.80×10^{4}

*Values corrected for L- isomer.

vidual region of particular significance to those of other dehalogenases in the same Class 1L (Slater et al*.,* 1997); for example L-DEX (*Pseudomonas putida* strain YL) with only 15% identity, Deh109 (*Pseudomonas putida* strain 109) with only 14% identity, HadL (*P. putida* strain AJ1) with only 13% identity, DehCII (*P. putida* strain CBS3) with only 15% identity, Deh2 (*Moraxella sp*. strain B) with only 13% identity, DhlB (*Xanthobacter autotrophicus* strain GJ10) with only 13% identity, DehCI (*P. putida* strain CBS3) with only 12% identity and HdIVa (*Pseudomonas cepacia* strain MBA4) with only 13% identity.

The sequence of DehL was also compared with those of haloalkane dehalogenase (DhlA) from *X. autotrophicus* GJ10 (Janssen et al*.,* 1989) and gave 14% sequence identity (data not shown). Lower sequence identity was expected from the sequence of non-related dehalogenases.

DISCUSSION

The DehL enzyme was purified and characterized. The amino acid sequence of *Rhizobial* DehL and DehD has little in common with only 14% amino acid sequence identity. This observation is comparable to the 20% identity reported for the equivalent dehalogenases (HadD and HadL) from *P. putida* AJ1, which were deemed to have different ancestral origins (Jones et al*.,* 1992). The present investigation using cloned dehalogenases confirms this stereospecificity as well as the inability of DehL to react with 2,2DCP. MCA and TCA were confirmed not to be substrates for DehL.

Class 1D dehalogenases are less common in nature than 1L enzymes. The D-specific monochloropropionic acid dehalogenases from the *Rhizobium* sp., *Rhizobium* sp. NHG3 (Higgins et al*.,* 2002) and *P. putida* AJ1 (Smith et al*.,* 1990) are the only such enzymes known.

Class 1L removed halide from L-2CP inverting the product configuration and react with sulfhydryl blocking reagents. This class also include halidohydrolases I and II of DCA-degrading *Pseudomonads* and the *Pseudomonas dehalogenans* NCIMB 9061 (Goldman et al., 1968; Little and Williams 1971). However, both these genes have not been sequenced. The native molecular mass of DehL from *Rhizobium* sp. was 61 kDa, whereas HadL from *P. putida* strain AJ1 had a native molecular mass of 79 kDa (Jones et al*.,* 1992). The subunit size of DehL and HadL were 31 and 26 kDa, respectively, suggesting DehL is a protein dimer and HadL is a trimer.

Pseudomonas species CBS3 that grows on 4-chlorobenzoate synthesized two dehalogenases, DehCI and DehCII (Klages et al., 1983; Morsberger et al., 1991). Both enzymes dehalogenated L-2CP but not D-2CP. Protein analysis showed both enzymes are dimeric proteins with overall molecular masses of 41 and 64 kDa and subunit molecular masses of 28 and 29 kDa, respectively. When DehCI and DehCII were compared, there was 45% nucleotide sequence homology. This corresponded to 38% amino acid sequence identity and over 70% amino-acid similarity. There appeared to be a close evolutionary relationship between the *dehCI* and *dehCII* gene suggesting a common origin from an ancestral gene.

P. cepacia MBA4 that grows on MBA (monobromoacetate) synthesizes two dehalogenases, III and IVa (Tsang et al*.,* 1988). The DehIII have not been studied in detail. However, DehIVa specific for L-2CP was studied further with predicted 231 amino acids residues and molecular mass of 25.9 kDa. This value corresponded to protein observed by SDS-PAGE (23kDa). The analysis by gel filtration gave 45 kDa suggesting protein dimer. Dehalogenase DehIVa and DehCI were compared and found to have 67% amino-acid identity and 81% similarity, and for DehIVa and DehCII the corresponding values were 37 and 56% amino-acid identity and similarity, respectively. *Pseudomonas* sp. 109 produces Deh109 which is a dimer with a slightly smaller native molecular mass (34 kDa) (Kawasaki et al., 1994). *Pseudomonas* sp. YL also produces a L-specific enzyme (L-DEX) with native molecular mass 54 kDa.

Moraxella sp. B produce Deh-2 was included in Class 1L on the basis of amino acid sequence information since it showed between 40 to 50% amino-acid identity with the members of the Class 1L (Kawasaki et al., 1992). However, the response to stereo-specific compounds is not known. This is an example of difficulty of classification on the basis of substrate specificity. However, the sequence data strongly suggested that this enzyme should be in Class 1L.

The deduced amino acid sequence of the *Rhizobium*

Figure 3. Sequence alignment of DehL from *Rhizobium sp.* with other dehalogenase proteins. The conserved residues are in Red. The numbers indicate the residual number of each amino acid sequence. Alignment with DehL from *Rhizobium sp.* NHG3 [53%], L-DEX (*Pseudomonas putida* strain YL) [15%], Deh109 (*Pseudomonas putida* no. 109) [14%], HadL (*Pseudomonas putida* strain AJ1) [13%], DehCII (*Pseudomonas putida* strain CBS3) [15%], Deh2 (*Moraxella sp.* strain B) [13%], DhlB (*Xanthobacter autotrophicus* strain GJ10) [13%], DehCI (*Pseudomonas putida* strain CBS3) [12%], and HdlIVa (*Pseudomonas cepacia* strain MBA4) [13%].

sp. *dehL* gene showed little identity (less than 15% identity) to the biochemically equivalent L-2CP specific dehalogenases as shown in Figure 3. This finding was unexpected; however, it suggests DehL from *Rhizobium* sp. might belong to a different group of L-2CP specific dehalogenase enzymes.

There is very little information regarding dehalogenase Km values. Some of the reported values were very high (Smith et al., 1990) compared to the current investigation (less than 0.5 mM). Generally, the kinetic analysis for DehL suggested that this enzyme is a better catalyst. This dehalogenase was also able to act on D,L-2,3DCP. Previous analysis indicated that only chloride from one position was released, presumably from carbon-2 because dehalogenase from *Rhizobium* sp. did not react with 3CP (Allison, 1981).

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