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OVEREXPRESSION, PURIFICATION AND ANALYSIS OF DEHALOGENASE D OF *RHIZOBIUM SP.*

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ABSTRACT

Halogenated organic compounds are found widely throughout the environment and microbial catabolism can lead to their biodegradation. The action generally involves enzyme-catalysed carbon-halogen bond cleavage. Dehalogenase D enzyme act only on D-2-chloropropionic acid and D,L-2-chloropropionic acid. Other substrate for the enzyme included monochloroacetic acid and monobromoacetic acid but not 2,2-dichloropropionic acid. From 4 g (wet weight) of cells, 2.8 mg protein (4.3 U enzyme) was efficiently purified using Fast Protein Liquid Chromatography (FPLC). The cell-free extract was prepared in 0.01M Tris-acetate buffer pH7.6 and was applied to a FPLC Mono Q HR 5/5 anion-exchange column equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10%(mass/vol.) glycerol buffer, pH7.6 and eluted with sodium phosphate gradient to 100 mM. The *dehD* gene consisted of 798 bp which encoded a 266 amino acid protein with a predicted molecular mass of 29 386 Da. This value corresponds to the value of 29 000 Da estimated by SDS/PAGE. DehD protein has some similarity to the previously published sequence of several stereospecific dehalogenases with 59% identity to *Rhizobium sp*. NHG3 and 23% identity to *Pseudomonas putida* strain AJ1.

Keywords: Dehalogenase D, D,L-2-chloropropionic acid, *Rhizobium sp.*

INTRODUCTION

Halogenated organic compounds are found widely throughout the environment and microbial catabolism can lead to their biodegradation. The action generally involves enzyme-catalysed carbon-halogen bond cleavage. This process is known as '*dehalogenation reaction*'.

A bacterium isolated from soil by elective culture on 2,2-dichloropropionic acid and identified as *Rhizobium sp*. was found to produce three dehalogenases (Allison, *et al*., 1983). All three enzymes acted on 2-chloropropionic acid, with dehalogenase L (DehL) being stereospecific for L-2-chloropropionic acid, dehalogenase D (DehD) being stereospecific for D-2-chloropropionic acid and dehalogenase E shows non-stereospecific and can act on both isomers of D- and L-2-chloropropionic acid (Leigh *et al*., 1986).

The use of dehalogenases is important for industrial biocatalysis, for example in the manufacture of chiral intermediates. D-2-haloacid dehalogenase has found commercial application in the production of L-2-chloropropionic as a chiral feedstock chemical for the production of herbicides (ICI patent no. 179603) and anti-inflammatory agents (Swanson, 1999). A dehalogenase from the D-2-haloacid dehalogenase was studied in detail for use in industry for developing a bioreactor system where D-2-haloacid dehalogenase from *Pseudomonas putida* AJI/23 was immobilised. This resulted in increased temperature stability and ability to withstand mildly alkaline conditions (Parker and Colby, 1995). In the present paper, we describe the overexpression of the gene of a DehD enzyme of *Rhizobium sp*. and the purification of enzyme produced by the overexpression system. This overexpression system may shed light into the properties, structure and function of the enzyme in the future.

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MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

E.coli K-12 strain NM522 was used for plasmid pUC18. Cells were grown aerobically at 30°C in PJC minimal medium containing 20 mM D,L-2-chloropropionic acid as carbon source. Ampicillin (100 µg/ml) and isopropyl thio-β-D-galactoside (IPTG) were incorporated as appropriate. Growth was followed by measurement of the absorbance at A_{680nm}.

Enzyme purification

E.coli K-12 strain NM522 carrying plasmid pSC3 containing *dehD*$^+$ gene, was grown in a 10 ml LB/amp culture overnight at 37°C. Cells from 2 ml of the culture were washed with sterile PJC before inoculating into 20 mM D,L-2-chloropropionic minimal medium containing 0.05% yeast extract plus 0.3 mM IPTG. Cells were grown at 30°C and harvested at late logarithmic phase (A_{680nm} 0.7 to 0.8). The A_{680nm} readings were taken every two hours. The cells 4 g (wet weight) were harvested and

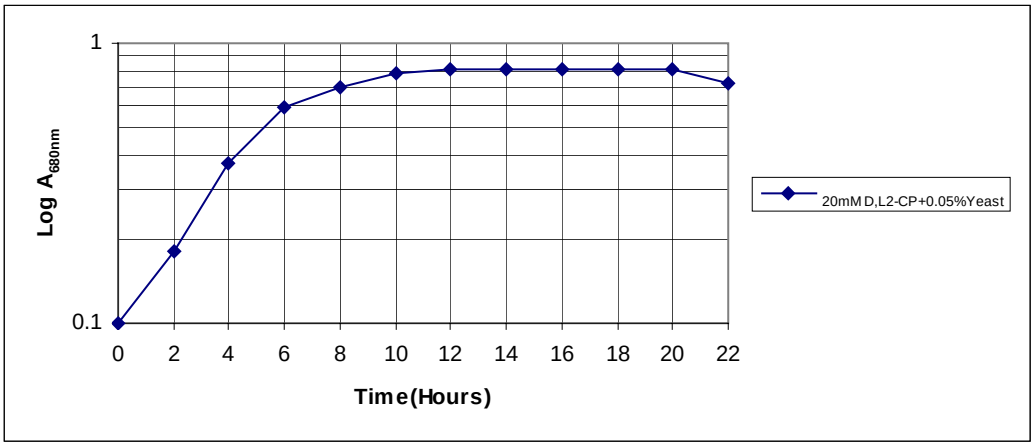

Fig. 1. Growth of *E.coli* NM522 [pSC3] (*dehD*$^+$) in D,L-2-chloropropionic minimal medium.

Table 1. Specific activity for DehD, using D-2-chloropropionic acid as substrates.

Growth condition	Specific activity (μmolCl$^-$/min/mg protein)
(20 mM D,L-2-chloropropionic acid + 0.05% yeast extract)	1.61

rinsed in 0.01 M Tris-acetate buffer pH7.6. The cell pellets resuspended in 20 ml of the same buffer was centrifuged at 10,000 g for 10 minutes at 4^{o}C. The cells were then resuspended in 4 ml of 0.01 M Tris-acetate buffer pH7.6 and maintained at 0^{o}C for ultrasonication in an MSE Soniprep 150W ultrasonic disintegrator at a peak amplitude λ=10 microns for 30 seconds. Unbroken cells and cell wall material were removed by centrifugation at 20,000 g for 15 minutes at 4^{o}C. The latter was centrifuged at 140,000 g for 60 minutes to give a soluble preparation. To further purify the enzyme, the 2.8 mg protein (4.3 U enzyme) was applied to a Mono Q HR 5/5 anion-exchange column equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (mass/vol.) glycerol buffer, pH 7.6 and eluted with sodium phosphate gradient to 100 mM.

Assay of dehalogenase activity

Cells were harvested at late log phase and extracts were assayed for dehalogenase activity using D-2-chloropropionic as substrate for DehD. Cell-free extract was prepared in 0.01 M Tris-acetate buffer pH7.6. The enzyme reaction was carried out at 30^{o}C in a mixture of 5 ml 0.09 M Tris-acetate (pH 7.5), substrate and enzyme. Samples were removed at 5 min intervals, and the free halide was determined colorimetrically (Bergman and Sanik, 1957). The colour was allowed to develop for 10 mins at room temperature and measured at A_{460nm}. Enzyme activity (1U) was defined as the amount of enzyme that catalyses the formation of 1 µmol halide ion/min. The assay was carried in duplicate.

RESULTS AND DISCUSSION

Growth analysis

E.coli NM522[pSC3] (*dehD*$^+$) were inoculated into minimal medium flasks containing (i) 20mM D,L-2-chloropropionic + 0.05% yeast extract. IPTG (final concentration 0.3 mM) was added to the growth medium before incubating at 30^{o}C. The A_{680nm} readings were taken every two hours. The growth curves and the maximum growth measured at A_{680nm} are shown in Fig. 1. From the curve, the doubling time, Td = 3 hours.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) analysis of DehD

The specific activity was calculated as in Table 1. SDS-PAGE analysis of the crude cell-free extracts that was carried out shown how much dehalogenase protein had been produced. SDS-PAGE for DehD showed the expected 29 kDa protein was expressed (Fig. 2). The amount of DehD was estimated visually to be about 20 – 30% of the total cellular proteins indicating that only 5-fold purification would be needed to obtain the pure enzymes.

Purification of the DehD enzyme

Cell-free extract was prepared in 0.01 M Tris-acetate buffer pH7.6 and 2.8 mg protein (4.3U enzyme) was applied to a Mono Q HR 5/5 anion-exchange column equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10%(mass/vol.) glycerol buffer, pH7.6 and eluted with sodium phosphate gradient to 100 mM. DehD eluted in four fractions between approximately 30 mM and 40 mM sodium phosphate. Each fraction had 1.3 U, 1.0 U, 1.15 U and 0.5 U enzyme, respectively and the enzyme recovered was estimated to be 92%. Analysis of the fractions by SDS-PAGE is shown in Fig. 3 In Fraction 11, there was slight contamination from host cellular protein. Specific activity of one of the fractions was measured (Fraction 10) which gave 14.4U/mg with D-2-chloropropionic as substrate. Since more than one fraction had DehD, possibly there are different forms of DehD protein. The Km value for each

fraction was determined using D-2-chloropropionic as substrate. All showed similar Km values of approximately 0.064 mM, suggesting that they are the same DehD protein. A possible reason for the protein being eluted at different ionic strengths is that a host protease may act on the dehalogenase protein. This problem may be avoided by expressing the gene in a protease-negative host cell.

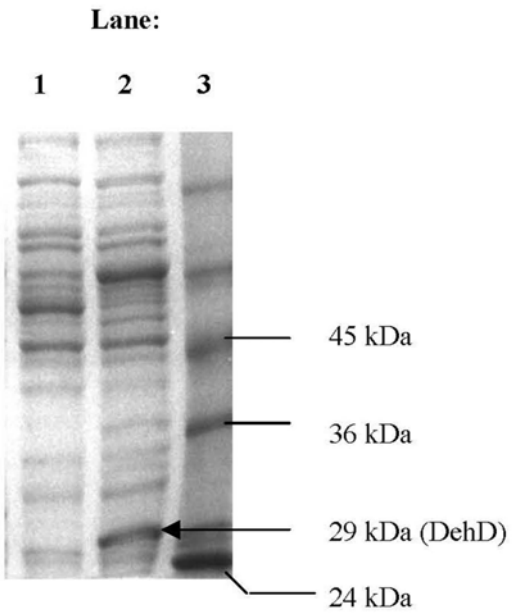

Fig. 2. SDS-PAGE analysis of crude extracts from *E.coli* NM522 [pSC3] (*dehD*$^+$) grown on D,L-2-chloropropionic acid minimal medium.

Lane 1: NM522[pUC18](8µg protein)
Lane 2: Crude extract from NM522[pSC3] (*dehD*$^+$) (8µg protein)
Lane 3: SDS VII marker proteins

Determination of Km values for dehalogenase enzymes with various substrates

A range of substrates was used to obtain Km values for DehD enzyme. The overall results are shown in Table 2. Compounds that are not a substrate for an enzyme are indicated by a dash. Using DehD, the Km for D-2-chloropropionic was 0.067 mM. When D,L-2-chloropropionic with equimolar amounts of the two isomers was used the Km values were very similar to those with the single enantiomer. This indicates that under these conditions, the presence of the non-substrate enantiomer has little or no effect on Km. From all this information on Km values, the actual substrate concentration to use for enzyme assay to ensure that the enzyme was saturated with substrate could be determined.

Molecular analysis

From the complete dehalogenase D gene, the amino acid sequence was determined. The initiation codon was ATG (methionine). The deduced amino acid sequence of this open-reading frame corresponded to a 29 kDa protein (consisted of 798 bp, encoded by a 266 amino acids protein with a calculated subunit molecular weight of approximately of 29 386 Da (Table 3). The calculated protein size agreed to the SDS-PAGE analysis that was carried out as shown in Fig. 3.

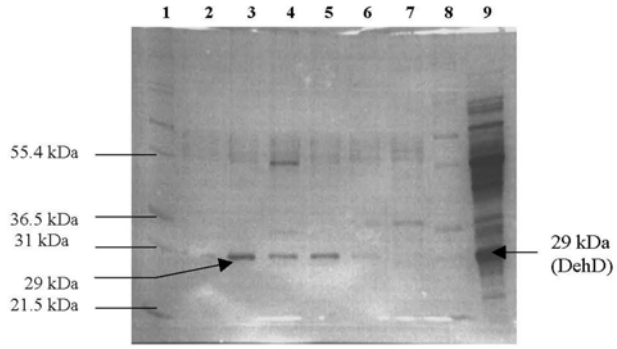

Fig. 3. SDS-PAGE analysis of the purification of DehD

Lane 1: Protein markers.
Lane 2: MonoQ fraction 9
Lane 3: MonoQ fraction 10 (4µg protein)
Lane 4: MonoQ fraction 11 (4µg protein)
Lane 5: MonoQ fraction 12 (3µg protein)
Lane 6: MonoQ fraction 13 (1µg protein)
Lane 7: MonoQ fraction 14
Lane 8: Protein markers
Lane 9: Crude extract of DehD

Bioinformatic analysis

The *Rhizobial* DehL and DehD were completely different with each other by amino acid sequence comparison (18% identity). The deduced amino acid sequence of DehE also showed little identity to DehD (14%) and DehL (16%). It is of interest to compare DehD amino acid sequence by Blast search (NCBI) programme to see if there is any other protein with a similar amino acid sequence now present in the databases. Sequence from DehD showed slight identity to D,L-DEX enzyme (18% identity) derived from *Pseudomonas sp*. strain 113 (Nardi-Dei *et al.,* 1997). However, the DehD protein has higher sequence similarity to the previously published sequence of several stereospecific dehalogenases for example 59% identity to *Rhizobium sp*. NHG3 (Higgins *et al*., 2005) and 23% to *Pseudomonas putida* strain AJ1 (Barth *et al.,* 1992).

CONCLUSIONS

Dehalogenase D gene was very efficiently expressed in the recombinant cells. The amount of the enzyme produced correspond to about 20 – 30% of the total cellular proteins judging from the specific activities of the crude extract of 1.61 µmolCl^-/min/mg protein. The purified enzyme from each fraction had 1.3, 1.0, 1.15 and 0.5 U/mg enzyme, respectively and the enzyme recovered was estimated to be 92%. The *dehD* gene encoded a 266

Table 2. Km values (mM) for different substrates using crude dehalogenase D (DehD) preparations.

Substrates	Km values (mM) for DehD
D-2-CP (D-2-chloropropionic acid)	0.067±0.014
L-2-CP (L-2-chloropropionic acid)	-
D,L-2-CP (D,L-2-chloropropionic acid)	0.089±0.015
2,2DCP (2,2-dichloropropionic acid)	-
D,L-2,3DCP (D,L-2,3-dichloropropionic acid)	0.53±0.063
MCA (monochloroacetic acid)	1.02±0.34
DCA (dichloroacetic acid)	-
TCA (trichloroacetic acid)	-
MBA (monobromoacetic acid)	0.62±0.11
DBA (dibromoacetic acid)	-
TBA (tribromoacetic acid)	-

amino acid protein with a predicted molecular mass of 29 386 Da. This value corresponds to the value of 29 kDa estimated by SDS/PAGE. This procedure is very useful for purification of a large amount of enzyme for characterization and application.

Table 3. Predicted amino acid composition of DehD from *Rhizobium sp*.

Amino Acids	DehD (Residue Per–Subunit)
Alanine (A)	27
Cysteine (C)	05
Aspartic acid (D)	12
Glutamic acid ((E)	11
Phenyalanine (F)	10
Glycine (G)	16
Histidine (H)	09
Isoleucine (I)	19
Lysine (K)	03
Leucine (L)	26
Methionine (M)	05
Asparagine (N)	01
Proline (P)	18
Glutamine (Q)	10
Arginine (R)	30
Serine (S)	26
Threonine(T)	17
Valine(V)	12
Tryptophan (W)	04
Tyrosine (Y)	04
Stop (Z)	1
Number of amino acids	266
Calculated molecular weight	29 386 Da

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