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A POTENTIAL USE OF DEHALOGENASE D (DEHD) FROM *RHIZOBIUM SP.* FOR INDUSTRIAL PROCESS

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Abstract. The *Rhizobium sp.* DehL and DehD were produced by heterologous expression of the cloned gene in *E.coli* and both proteins were purified using anion-exchange column chromatography. DehL and DehD were characterised by kinetic analysis to determine their K_m, K_{cat} and the Specificity constant. The kinetic analysis showed that DehD from *Rhizobium sp.* has lower K_m value (0.04 mM with D,L-2-CP), higher K_{cat} (6.28 sec^{-1} for D,L-2-CP) and Specificity constant (1.46×10^5 $M^{-1}sec^{-1}$ for D,L-2-CP) compared to other D-specific dehalogenases from different organism suggesting DehD enzyme from *Rhizobium sp.* is a better catalyst. K_m, K_{cat} and Specificity constant for DehL were 0.12 mM, 25 s^{-1} and 2.08×10^5 $M^{-1}s^{-1}$, respectively, using D,L-2CP as the substrate. D-2-haloacid dehalogenase is important for industrial biocatalysis compared to the L-2-haloacid and the kinetic data of DehD hold promise for further development to be used in an industrial process.

Keywords: Dehalogenase, DehL, DehD, *Rhizobium*

Abstrak. DehL dan DehD dari *Rhizobium sp.* yang dihasilkan secara pengekspresan gen di dalam *E.coli* telah ditulenkan menggunakan kromatografi turus pertukar ion. Pencirian telah dilakukan terhadap DehL dan DehD melalui analisis kinetik untuk menentukan nilai K_m, K_{cat} dan pemalar tetap. Analisis kinetik menunjukkan DehD dari *Rhizobium sp.* mempunyai nilai K_m yang lebih rendah (0.04 mM dengan D,L-2-CP) dan nilai K_{cat} (6.28 $saat^{-1}$ bagi D,L-2-CP) dan pemalar tetap (1.46×10^5 M^{-1} sec^{-1} bagi D,L-2-CP) yang lebih tinggi berbanding dengan dehalogenase D-spesifik daripada organisma lain menunjukkan DehD dari *Rhizobium sp.* merupakan pemangkin yang lebih baik. Nilai K_m, K_{cat} dan pemalar tetap bagi DehL adalah 0.12 mM, 25 s^{-1} dan 2.08×10^5 $M^{-1}s^{-1}$ setiap satu, menggunakan D,L-2CP sebagai bahan tindak balas. D-2-haloasid adalah penting dalam industri bio-pemangkin berbanding dengan L-2-haloasid, dan data kinetik DehD menunjukkan potensi penggunaanya dalam proses industri sekiranya dimajukan.

Kata kunci: Dehalogenase, DehL, DehD, *Rhizobium*

Abbreviations: D,L-2-CP: D,L-2-chloropropionic acid; D,L-2-BP: D,L-2-bromopropionik acid; 2,2-DCP: 2,2-dichloropropionic acid; D,L-2,3-DCP: D,L-2,3-dichloropropionic acid; DCA: dichloroacetic acid; MCA: monochloroacetic acid; TCA: trichloroacetic acid; MBA: monobromoacetic acid; DBA: dibromoacetic acid; TBA: tribromoacetic acid; DehL: dehalogenase L; DehD: dehalogenase D, DehE: dehalogenase E; K_m: Michaelis constant; K_{cat}: turnover number; V_{max}: maximum velocity; sec^{-1}: per-second

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1.0 INTRODUCTION

Halogenated organic compounds are widely found throughout the biosphere and their microbial catabolism has been reviewed [1-4]. The microbial catabolic reaction, which catalyses cleavage of carbon-halogen bond is known as dehalogenation reaction. This reaction have been classified into three different types for example the enzymes that act on D,L-2-CP can be stereospecific for the D- or L- form or can act equally on both isomers. The only organism so far reported to synthesize all three forms of dehalogenases is a *Rhizobium sp*. [5].

DehL was shown to be specific for L-2-CP and also acted on DCA but not on 2,2-DCP or MCA. DehE was non-specific, acting on D- and L-2CP, 2,2-DCP, DCA and MCA. DehD was shown to act only on D-2CP and MCA, with no activity towards 2,2-DCP or DCA. For each dehalogenase, DehL, DehE and DehD the lactate produced from D,L-2-CP had the opposite stereochemical form to that of the substrate [6]. Therefore, it was curious that *Rhizobium sp*. had more than one dehalogenases with DehE showing non-stereospecificity and could act on all the identified substrates. A possible explanation was that DehE evolved from DehL and DehD.

Dehalogenases were known for their ability to degrade pollutants. However, the development of dehalogenating enzyme systems for chemical processing was not broad for industrial implementation due to a limited range of potential industrial targets [7]. Industrial biocatalysis may be conducted either as whole cell microbial catalyst or using an enzyme *ex vivo*. Only hydrolytic dehalogenases are utilised commercially or are in development for industrial use. The use of dehalogenases is important for the manufacture of chiral intermediates. D-2-haloacid dehalogenase from *Pseudomonas* was used in the production of L-2-CP as a chiral feedstock chemical for the production of herbicides (ICI patent no. 179603) and pharmaceutical products for example, anti-inflammatory agents [8].

In the present investigation, DehL and DehD were characterised and their kinetic data analysed in the hope that the kinetic values from *Rhizobium sp*. dehalogenase (DehD) might confer additional advantage over the current use of dehalogenase in industry.

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Growth Conditions

The genes encoding DehL and DehD were originally isolated from *Rhizobium sp.* chromosomal DNA as plasmid pSC2. Further subcloning of pSC2 into pUC18 resulted in pSC4 ($dehL^+$) and pSC3 ($dehD^+$), which expressed DehL and DehD, respectively [9]. *E.coli* K-12 strain NM522 was used as host for plasmid pSC4 ($dehL^+$) and/or pSC3 ($dehD^+$). Cells were grown aerobically at 30°C in minimal medium plus D,L-2-CP as carbon source supplemented with 0.05% (w/v) yeast extract. Isopropyl thio-(-D-galactoside (IPTG) (final concentration 0.3 mM) was added to the growth medium before incubating at 30°C. Carbon sources and supplements were sterilised separately and added aseptically. Growth pattern was recorded as absorbance at A_{680nm} (λ_{max}).

2.2 Preparation of Cell-free Extracts and Protein Purification

Extracts were prepared from cells harvested 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°C. The cell suspension was sonicated at 0°C for 30 s at an amplitude of 10 µm, using MSE soni prep 150 ultrasonicator. Unbroken cells and cell wall material were removed by centrifugation at 20 000 g for 15 min at 4°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 (4U 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 1ml/min over 15ml.

For purification of DehD the cell-free extract was prepared in 0.01 M Tris-acetate buffer pH 7.6 and 2.8 mg protein (4.3U enzyme) was applied to a MonoQ 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 at a flow rate of 1ml/min over 15ml.

2.3 Kinetic Analysis and Assay of Dehalogenase Activity

The enzyme reaction was carried out at 30°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 [10]. The colour was allowed to develop for 10 min 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.

2.4 K_m and K_{cat} Determination

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 using 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 using the equation:-

$$K_{cat} = \frac{V_{max}(\mu\text{mol C1}^- / \text{min}/\mu\text{mol enzyme})}{60\ \text{s}}$$

3.0 RESULTS AND DISCUSSION

DehD and DehL were stereospecific for D- and L-2-CP, respectively as reported earlier [11]. The present investigation confirms the stereospecificity as well as the inability of DehD and DehL to react with 2,2-DCP, TCA and TBA as shown in Table 1. DCA and MCA as well as DBA and MBA were confirmed not to be the substrates for DehD and DehL, respectively (Table 1). Both dehalogenases were able to act on D,L-2,3-DCP. The analysis indicated that only chloride from one position was released, presumably from carbon 2 because dehalogenase from *Rhizobium sp.* did not react with 3CP therefore, chloride at carbon 3 was not attacked [12]. The possible product of dehalogenation was proposed to be 2-hydroxy-3-chloropropionate. The total chloride released using DehL or DehD enzyme also indicated that D,L-2,3-DCP had equimolar L- or D- isomers similar to D,L-2-CP.

The K_m values for various substrates of crude and purified dehalogenases did not show any major difference between them (data not shown). However, purification of

Table 1 K_m values for different substrates using pure dehalogenases

	K_m (mM)	
Halogenated Compound	**DehL**	**DehD**
D-2-CP	-	0.06±0.01
D-2-BP	-	0.48±0.09
L-2-CP	0.15±0.02	-
L-2-BP	0.11±0.01	-
D,L-2-CP	0.12±0.01*	0.04±0.01*
D,L-2-BP	0.10±0.01*	0.40±0.04*
2,2-DCP	-	-
D,L-2,3-DCP	0.03±0.01*	0.38±0.11*
MCA	-	0.25±0.04
DCA	0.13±0.01	-
TCA	-	-
MBA	-	0.67±0.17
DBA	0.27±0.09	-
TBA	-	-

(Values are the means of tri plicate determinations)
Note: (-): not a substrate for enzyme
(*): Km values corrected for L- or D- isomer

both dehalogenases was carried out to determine the K_{cat} values as shown in Table 2 and 3. The K_m determination in Table 3 showed DehD had the lowest K_m values of 0.06 mM and 0.04 mM, for both D-2-CP and D,L-2-CP respectively, whereas DehL (Table 2), gave 0.15 mM and 0.11 mM for both L-2-CP and D,L-2-CP respectively. However, using the same substrate for DehE gave K_m values four times higher as reported earlier [13]. Lower K_m values suggests DehD and DehL are better enzymes for D,L-2-CP. The low K_m values also indicated the possibility that the *Rhizobium sp.* might grow at low concentration of halogenated compounds. For the commercial application the enzyme has high affinity towards the substrate and this might reduce cost of raw materials used in an industry.

Table 2 K_{cat}, K_m and specificity constants for DehL enzyme

Substrate	$K_{cat}(sec^{-1})$	K_m (mM)	Specificity Constant ($M^{-1}sec^{-1}$)
L-2-CP	20.00	0.15	1.33×10^5
L-2-BP	20.00	0.11	1.81×10^5
D,L-2-CP	25.00*	0.12*	2.08×10^5*
D,L-2-BP	17.40*	0.10*	1.74×10^5*
D,L-2,3-DCP	3.28*	0.03*	1.05×10^5*
DCA	6.25	0.13	4.80×10^4
DBA	81.16	0.27	3.04×10^5

(*) : values corrected for L- isomer

Table 3 K_{cat}, K_m and specificity constants for DehD enzyme

Substrate	$K_{cat}(sec^{-1})$	K_m (mM)	Specificity Constant ($M^{-1}sec^{-1}$)
D-2-CP	7.45	0.06	1.12×10^5
D-2-BP	187.53	0.48	3.90×10^5
D,L-2-CP	6.28*	0.04*	1.46×10^5*
D,L-2-BP	193.33*	0.40*	4.83×10^5*
D,L-2,3-DCP	29.58*	0.38*	7.78×10^4*
MCA	4.25	0.25	1.70×10^4
MBA	362.50	0.67	5.41×10^5

(*) : values corrected for D- isomer

Thermostability test was not carried out using cloned DehD and DehL. However, in previous analysis using crude cell free extract prepared from *Rhizobium sp*. grown on 2,2-DCP showed exposure of crude extract to 40°C resulted in a decrease in dehalogenase activity using 2,2-DCP, D,L-2-CP, MCA and DCA as substrates [12]. Temperature stability test was carried out using the D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1/23 for use in industry using a continuous bioreactor model system for the conversion of racemic D,L-2-CP to the L-isomer [14]. The D-2-haloacid dehalogenase from *Pseudomonas putida* AJI/23 was immobilised. The enzyme was stable at 30°C in aqueous solution. However, the half life of the immobilised enzyme at 30°C was greater compared with a soluble enzyme indicating that immobilisation had significant stabilising effect on D-2-CP dehalogenase to temperature inactivation.

Dehalogenase of opposite stereospecificity, a thermostable L-2-haloacid dehalogenase (L-DEX) enzyme from *Pseudomonas* and L-2-haloacid dehalogenase from Azotobacter strain RC26 have been characterised in terms of their better thermostability and resistance to enzyme inhibitors [15, 16]. However, enzyme of this kind is less important than D-2-haloacid dehalogenase.

There is very little information in the current literature regarding dehalogenase K_m values. Some of the reported values were very high compared to the current investigation.

Pseudomonas AJ1/23, was reported to have two dehalogenase enzymes, which act specifically on the D- and L-isomer of 2-CP similar to DehD and DehL of *Rhizobium sp*. Had-D, which was specific for D-isomer was purified and its kinetic properties studied. The K_m value for MCA and D,L-2-BP was 27.5 mM and 1.99 mM, respectively [17]. These values were apparently much higher than those from *Rhizobium sp*. with the corresponding values for DehD enzyme for MCA of 0.25 mM and for D,L-2-BP at 0.4 mM. K_{cat} values and the Specificity constants were calculated for each DehL and DehD using different substrates are shown in Table 2 and Table 3. The Specificity constant values have an upper limit of 10^8 to 10^9 $M^{-1}sec^{-1}$ [18]. Some enzymes were reported to have values near to the upper limit such as fumarase, 1.6×10^8 $M^{-1}sec^{-1}$ [19]. However, DehL and DehD like many enzymes of metabolism have slightly lower values in the range of 10^4 to 10^5 $M^{-1}sec^{-1}$. The Specificity constants for D,L-2-CP of DehL, and DehD were 2.08×105 $M^{-1}sec^{-1}$, and 1.46×105 $M^{-1}sec^{-1}$, respectively, suggesting that both DehL and DehD were better catalysts.

Rhizobium sp. was originally isolated using 2,2-DCP and DehL and DehD were also present since these enzymes were confirmed not to act on 2,2-DCP. One possible reason might be the commercially available 2,2-DCP was not pure and contained D,L-2-CP.

4.0 CONCLUSIONS

The *Rhizobium sp*. dehalogenases were confirmed to be stereospecific for the D- and L-CP. The kinetic study showed that DehD had higher affinity for D-2-CP and D,L-2-CP as shown by low K_m values of 0.06 mM and 0.04 mM respectively, and significantly higher K_{cat} and Specificity constants as shown in Table 2 and Table 3. These data suggested that both DehD and DehL were better catalysts for D,L-2-CP. Since it has low K_m values it is of particular interest to investigate the growth of *Rhizobium sp*. at lower substrate concentration because if the microorganism could only remove high concentrations of pollutants there still will be low concentrations of pollutants in the environment that is considered harmful.

The challenge at the moment to apply enzymes in industry is the kinetic properties for instance low K_{cat}, high K_m and also product inhibition which often limit the productivity of these enzymes. However, since D-2-specific dehalogenase was important in an industry compared to the L-2-haloacid dehalogenase for herbicide and pharmaceutical products, the attractive K_m values and K_{cat} values for DehD from *Rhizobium sp*. might suggests that this enzyme may have merit in an industrial process.

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