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Overexpression and Rapid Purification of L-2-Halo Acid Dehalogenase of *Pseudomonas putida* No. 109

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An overexpression system for L-2-halo acid dehalogenase (L-DEX) of *Pseudomonas putida* No. 109 was constructed. The 0.9-kbp *Bss*HII-*Nru*I fragment of pBR-EJ encoding L-DEX of *Ps. putida* No. 109 was inserted into pBA5, a pUC19 derivative containing both *lac* promoter and the promoter of L-DEX of *Pseudomonas* sp. YL. The resultant plasmid, named pYB1, was introduced into *Escherichia coli* JM109. This recombinant *E. coli* showed very high L-DEX activity, and the amount of L-DEX corresponded to 16% of the total soluble proteins. From 55 g (wet weight) of cells, 190 mg of L-DEX was efficiently purified by Butyl-Toyopearl and Gigapite chromatographies. This overexpression system provides us with a large amount of L-DEX, and enables us to study their properties, structures and application.

KEY EORDS: L-2-Halo acid dehalogenase/ Overexpression/ *Pseudomonas*

1. INTRODUCTION

Halogenated organic compounds have been widely used as pesticides, herbicides, solvents, and so on. Since some of them are toxic and persistent, they cause an environmental pollution. Therefore, microbial dehalogenation system attracts a great deal of attention.

2-Halo acid dehalogenases catalyze the hydrolytic dehalogenation of 2-halo acids to produce the corresponding 2-hydroxy acids.[1] They are classified into four groups based on their substrate specificities as follows. Type I enzyme acts specifically on L-enantiomers of the substrates, and produces D-2-hydroxy acids.[2,3] Type II enzyme catalyzes the conversion of D-2-halo acids into the corresponding L-2-hydroxy acids.[4] Type III and Type IV enzymes act on both L- and D-enantiomers of the substrates. The reactions of Type III and Type IV enzymes proceed with inversion and retention of C2-configuration of the substrates, respectively.[2,5-7] These are invaluable not only to the enzymological study of halogen-carbon cleavage, but also to the production of optically active hydroxy acids which are useful for the chemical industry.

We have purified two Type I enzymes (L-2-halo acid dehalogenases, L-DEXs) from two species of soil bacteria, *Pseudomonas* sp. YL[2] and *Pseudomonas putida* No. 109.[3] The properties of these L-DEXs are summarized in Table 1. There are notable differences in their subunit structures, thermostabilities and kinetic constants. L-DEX of *Ps.* sp. YL has a dimeric form, whereas the other has a monomeric form. L-DEX of *Ps.* sp. YL is much more thermostable than L-DEX of *Ps. putida* No. 109, and has a higher optimum temperature. The *K*m value of L-DEX

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Table 1. Properties of L-DEX from *Ps.* sp. YL and *Ps. putida* No. 109.

	Ps. sp. YL	*Ps. putida* No. 109
Molecular weight		
native enzyme	54,000	25,000
subunit	27,000	25,000
Optimum pH	9.5	10.5
Optimum temperature	65°C	45°C
Thermostability[a]		
60°C, 30 min	100%	25%
70°C, 15 min	76%	0%
*K*m (mM)		
chloroacetate	1.1	1.0
L-2-chloropropionate	0.37	3.8

[a] Remaining activities after heat treatment are shown.

of *Ps.* sp. YL for L-2-chloropropionate is about ten times lower than that of L-DEX of *Ps. putida* No. 109.

The genes encoding these L-DEXs have already been isolated and sequenced.[8,9] The overexpression system for L-DEX of *Ps.* sp. YL has been constructed. In the present study, we constructed an overexpression system for L-DEX of *Ps. putida* No. 109. This system provides us with a large amount of the enzyme, and enables us to study structures and properties of two different L-DEXs comparatively.

2. MATERIALS AND METHODS

2.1 *Materials*

Plasmid pBR-EJ encoding L-DEX of *Ps. putida* No. 109 was provided by Dr. H. Kawasaki (University of Osaka Prefecture).[8] Expression vector pKK223-3 was purchased from Pharmacia (Uppsala, Sweden). *Escherichia coli* JM109, pUC118, pUC119, restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan). Butyl Toyopearl and Gigapite were from Tosoh (Tokyo, Japan) and Toagosei Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. All other chemicals were of analytical grade.

2.2 *Construction of plasmids for expression of L-DEX*

pSNK223. The *Bss*HII-*Nru*I DNA fragment (0.9 kbp) of pBR-EJ encoding L-DEX of *Ps. putida* No. 109 was isolated by agarose gel electrophoresis and electroelution, and both ends were made blunt. This fragment, consisting of 96-bp 5′-upstream, 672-bp coding and 142-bp 3′-downstream regions, was inserted into the *Sma*I site of pKK223-3. The direction of transcription was identical to that of the *tac* promoter of pKK223-3. The resultant plasmid was named pSNK223.

pYB1. pBA5, from which L-DEX of *Ps.* sp. YL is highly expressed, was digested with *Sma*I.[9] The resultant fragment consisting of pUC19, 159-bp 5′-upstream region of L-DEX gene of *Ps.* sp.

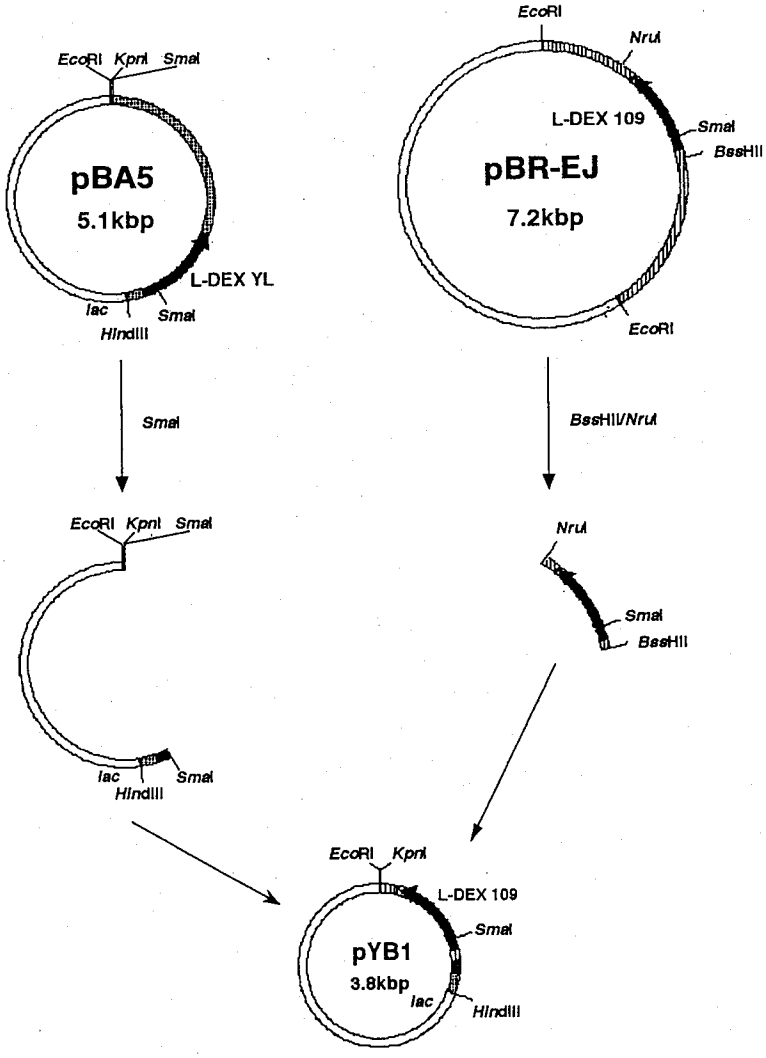

Fig. 1. Construction of pYB1. Detailed procedures for construction are described in Materials and Methods.

YL, and 89-bp region encoding an N-terminal part of the enzyme, was ligated with the blunted *Bss*HII-*Nru*I fragment (0.9 kbp) of pBR-EJ. The direction of transcription was identical to those of the *lac* promoter of pUC19 and the promoter of L-DEX gene of *Ps.* sp. YL (Fig. 1). *pYB118, pYB119.* *Hin*dIII-*Kpn*I fragment of pYB1 (1.2 kpb) was inserted into pUC118 or pUC119 digested with *Hin*dIII and *Kpn*I. The resultant plasmids were named pYB118 and pYB119, respectively.

All the above plasmids were introduced into *E. coli* JM109.

2.3 *Cultivation of the recombinant cells*

The recombinant *E. coli* JM109 cells were grown aerobically at 37°C for 18 h in Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing

0.2 mg/ml ampicillin and various concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) (0~0.2 mM).

2.4 *Enzyme purification*

Step 1. Transformed *E. coli* carrying pYB1 was cultivated for 18 h in 12 *l* of the LB medium containing 0.2 mg/ml ampicillin and 0.2 mM IPTG. The cells (55 g wet weight) were harvested and washed with 50 mM potassium phosphate buffer (KPB) (pH 7.5). After suspended in 60 ml of the same buffer, the cells were disrupted by ultrasonic oscillation at 4°C for 25 min with a Seiko Instruments model 7,500 ultrasonic disintegrator. The cell debris was removed by centrifugation.

Step 2. The supernatant solution was brought to 30% saturation with ammonium sulfate, and the precipitate was removed by centrifugation at 17,000×g for 30 min. Ammonium sulfate was added to the resultant supernatant solution to 45% saturation, and the precipitate collected by centrifugation was dissolved in 50 mM KPB (pH 7.5) containing 20% ammonium sulfate.

Step 3. The enzyme solution was applied to a Butyl-Toyopearl 650 M column (4×40 cm). After washing of the column with 2 *l* of 50 mM KPB (pH 7.5) containing 30% ammonium sulfate, the enzyme was eluted with a 100 ml linear gradient of 30~0% saturated ammonium sulfate in 50 mM KPB (pH 7.5). The active fractions were concentrated by ultrafiltration and dialyzed against 1,000 volumes of 10 mM KPB (pH 7.5).

Step 4. The dialyzed solution was applied to a Gigapite column (4×50 cm) equilibrated with 10 mM KPB (pH 7.5). After washing of the column with 3 *l* of 10 mM KPB (pH 7.5), the elution was carried out with a 6 *l* linear gradient of 10~100 mM KPB (pH 7.5). The active fractions were concentrated by ultrafiltration.

2.5 *Enzyme and protein assay*

The standard assay mixture (0.2 ml) contained 5 μmol of DL-2-chloropropionate, 20 μmol of Tris-sulfate buffer (pH 9.5), and enzyme. After incubation at 30°C for 10 min, the reaction was terminated by addition of 20 μl of 1.5 M H_2SO_4. The chloride ions released were determined spectrophotometrically with mercuric thiocyanate and ferric ammonium sulfate as described by Iwasaki *et al.*.[10] One unit of the enzyme activity was defined as the amount of the enzyme that catalyzes the dehalogenation of 1 μmol of a substrate per min. The protein concentrations were measured with a Bio-Rad protein assay kit (Hercules, CA).

3. RESULTS AND DISCUSSION

3.1 *Overexpression of L-DEX gene in E. coli*

The amount of L-DEX of *Ps. putida* No. 109 expressed from pSNK223 was much higher than that from pBR-EJ (Table 2). The amount of L-DEX produced was increased by addition of IPTG to the medium. This suggests that L-DEX is produced at least partially under the control of *tac* promoter of pKK223-3. L-DEX was produced even in the absence of IPTG. This is probably due to the incomplete repression of the *tac* promoter and the function of the intrinsic promoter of L-DEX gene of *Ps. putida* No. 109.

In the plasmid, pYB1, the gene for L-DEX of *Ps. putida* No. 109 was placed downstream of both *tac* promoter of pUC19 and the promoter of L-DEX gene of *Ps.* sp. YL. The promoter of L-DEX gene of *Ps.* sp. YL has been shown to work very efficiently in *E. coli*, and the amount of

Table 2. Dehalogenase activities of the extracts of recombinant *E. coli* cells.

Plasmid	IPTG (mM)	Sp. activity (U/mg)
None	0.2	0
pBR-EJ	0.2	0.1
pSNK223	0	1.3
	0.2	2.4
pYB1	0	8.1
	0.1	8.5
	0.2	9.2
pYB119	0.2	8.5
pYB118	0.2	8.5

L-DEX of *Ps.* sp. YL reached about 50% of the total soluble proteins of the recombinant *E. coli* cells.[9] Judging from the specific activity of the cell extract of *E. coli* carrying pYB1 (8.1～9.2 U/mg), the amount of L-DEX of *Ps. putida* No. 109 corresponded to about 14～16% of the total soluble proteins (Table 2). The specific activity of the extract was only slightly increased by addition of IPTG to the medium.

Next, we examined the expression from pYB118 and pYB119. The direction of transcription was identical to that of *lac* promoter in pYB119, but opposite in pYB118. As shown in Table 2, L-DEX was produced from these plasmids with equal efficiency. This shows that *lac* promoter is not required for the overexpression of L-DEX, and that the high productivity is mainly due to the function of the promoter of L-DEX genes of *Ps.* sp. YL and *Ps. putida* No. 109.

Rapid purification of L-DEX from recombinant E. coli cells

We purified L-DEX of *Ps. putida* No. 109 from the cell extract of *E. coli* carrying pYB1 as described in Materials and Methods. The results are summarized in Table 3. The enzyme was purified about 11-fold with a 32% yield. The purified enzyme was found to be homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 2). We obtained 190 mg of the purified enzyme from 55 g of wet cells. The properties of this enzyme such as molecular weight, optimum pH, optimum temperature, and substrate specificity were essentially identical to those of the enzyme purified from *Ps. putida* No. 109.

The overexpression system and the rapid purification procedure established here provide us with a large amount of the enzyme, and are useful for the detailed studies on the structure and function of L-DEX.

Table 3. Purification of L-DEX from recombinant *E. coli* carrying pYB1.

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Cell extract	34,000	6,400	5.3	1	100
30～45% $(NH_4)_2SO_4$	33,000	2,200	15	2.8	97
Butyl-Toyopearl	20,000	500	40	7.5	59
Gigapite	11,000	190	58	11	32

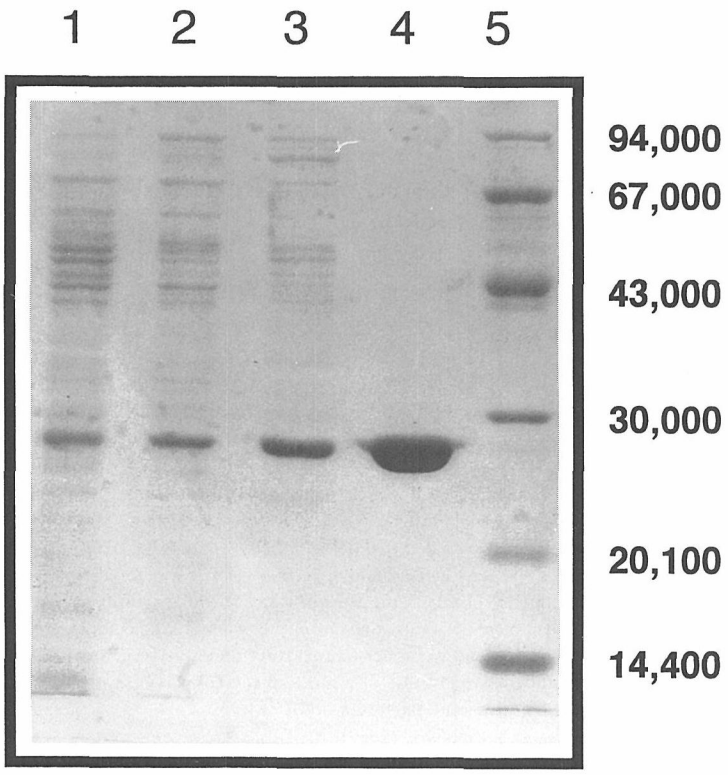

Fig. 2. SDS-polyacrylamide gel electrophoresis of L-DEX of *Ps. putida* No. 109. Lane 1, cell extract of *E. coli* carrying pYB1; lane 2, proteins after ammonium sulfate fractionation; lane 3, proteins after Butyl-Toyopearl chromatography; lane 4, the purified L-DEX after Gigapite chromatography; lane 5, molecular weight markers. The molecular weights of marker proteins are indicated. Each lane contains 15 μg proteins.

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