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Overexpression and feasible purification of thermostable L-2-halo acid dehalogenase of *Pseudomonas* sp. YL

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Abstract

The gene encoding thermostable L-2-halo acid dehalogenase of *Pseudomonas* sp. YL was isolated, and its overexpression system was constructed. Gene library was prepared from *Sau*3AI fragments of total DNA from *Ps.* sp. YL, pUC118 as a vector and *Escherichia coli* JM109 as a host. The recombinant cells resistant to bromoacetate, a germicide, were isolated and shown to produce L-2-halo acid dehalogenase. Subsequently, subcloning was carried out with pKK223-3 as a vector, and the length of DNA inserted was reduced to 1.1 kbp. One of the subclones showed very high activity, and the amount of the dehalogenase produced corresponded to about 30% of the soluble protein. From 5 g (wet weight) of cells, 105 mg of dehalogenase was efficiently purified by heat treatment and DEAE-Toyopearl chromatography. This overexpression system provides a large amount of the thermostable enzyme to enable us to study the properties, structure and application of the enzyme.

Abbreviations: IPTG – isopropyl β-D-thiogalactopyranoside, KPB – potassium phosphate buffer, SDS – sodium dodecyl sulfate, X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

Many organic halogen compounds have been synthesized and widely used as pesticides, herbicides, solvents and so on. Some are toxic and persistent, and cause an environmental problem.

Various dehalogenases that catalyze the cleavage of halogen-carbon bonds have been found and characterized. These include halo acid dehalogenase (Jones et al. 1992; Thomas et al. 1992; van der Ploeg et al. 1991; Shneider et al. 1991), haloalkane dehalogenase (Janssen et al. 1989), 4-chlorobenzoate-coenzyme A dehalogenase (Babbitt et al. 1992), tetrachloro-*p*-hydroquinone reductive dehalogenase (Orser et al. 1993), and some others. Most detailed enzymological studies have been carried out on a haloalkane dehalogenase that catalyzes the hydrolytic dehalogenation of 1,2-dichloroethane. The reaction mechanism of the enzyme from *Xanthobacter autotrophicus* GJ10 has been elucidated based on its three dimensional structure (Verschueren et al. 1993). However, the structures and reaction mechanisms of other dehalogenases remain unclear.

2-Halo acid dehalogenases catalyze the hydrolytic dehalogenation of 2-halo alkanoic acids to produce 2-hydroxy alkanoic acids. They are classified into four groups based on their substrate specificities (Fig. 1). Type I enzymes act specifically on L-2-halo acids to produce the corresponding D-2-hydroxy acids (Motosugi et al. 1982a). Type II enzymes catalyze the conversion of D-2-halo acids into the L-2-hydroxy acids (Smith et al. 1990). Type III and IV enzymes act on both stereoisomers of 2-halo acids. Type III enzymes catalyze the dehalogenation of DL-2-halo acids with inversion of configuration (Motosugi et al. 1982b). Type IV enzyme reactions proceed with retention of

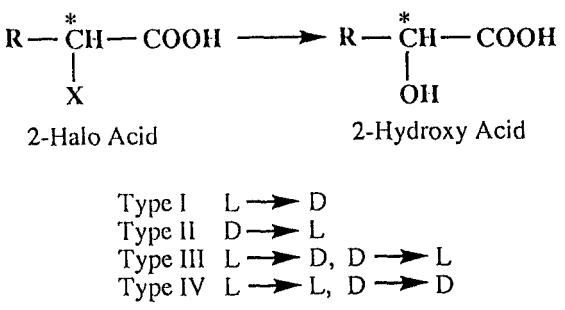

Fig. 1. The reactions catalyzed by 2-halo acid dehalogenases. Type I and Type II are called L-2-halo acid dehalogenase and D-2-halo acid dehalogenase, respectively. Both Type III and Type IV are called DL-2-halo acid dehalogenase. R, alkyl group; X, halogen atom.

configuration (Weightman et al. 1982). 2-Halo acid dehalogenases are useful for the production of optically active hydroxy acids, which are used for the synthesis of various pharmaceuticals and agrochemicals.

We have isolated several bacterial strains which effectively produce 2-halo acid dehalogenases. One of these, *Pseudomonas* sp. YL, produces two different types of dehalogenases: the thermostable Type I enzyme that is inducibly synthesized by 2-chloropropionate, and the Type III enzyme that is inducibly produced by 2-chloroacrylate (Liu et al. 1994).

The Type I enzyme from *Ps.* sp. YL shows high thermostability, wide substrate specificity, and strict enantioselectivity. It is not inactivated at all by heat-treatment at 60° C for 30 min, and is active in organic solvents such as *n*-heptane to dehalogenate L-2-halo acids with carbon chain length of 3–16 to form the corresponding D-2-hydroxy acids. The enzyme is useful for the production of various D-2-hydroxy acids.

In the present paper, we describe the cloning and expression of the gene of a thermostable Type I enzyme, L-2-halo acid dehalogenase, of *Ps.* sp. YL and the purification of enzyme produced by the over-expression system.

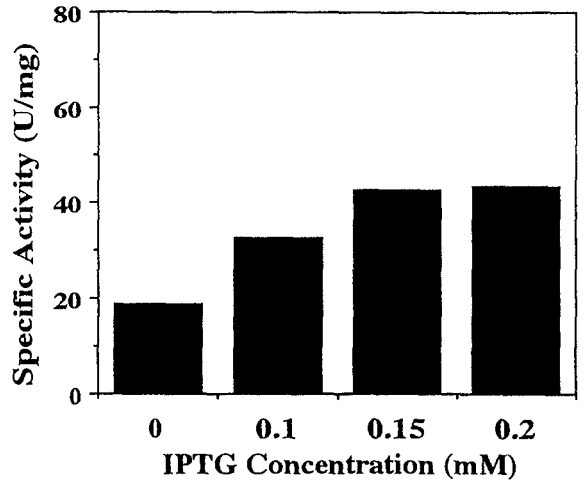

Fig. 2. Effect of IPTG on the production of L-2-halo acid dehalogenase in the recombinant *E. coli* cells. Cultivation was carried out at 37° C for 12 h in the LB medium containing 0.1 mg/ml ampicillin and 0, 0.1, 0.15, or 0.2 mM IPTG. Specific activities of dehalogenase in each cell extracts were measured with DL-2-chloropropionate as a substrate.

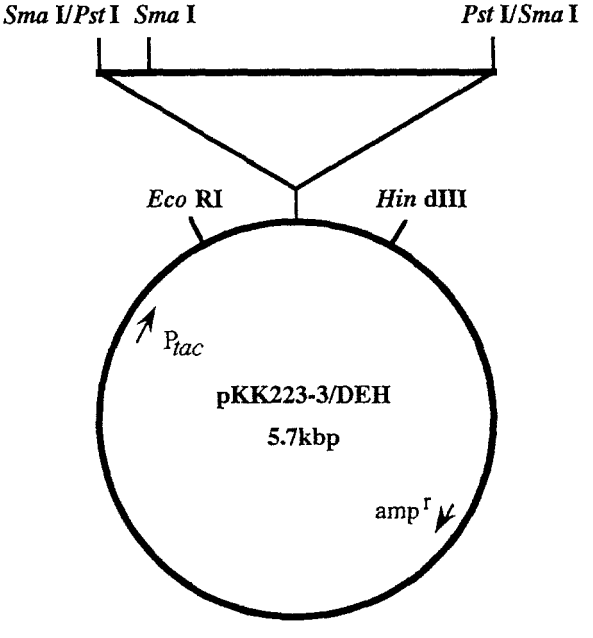

Fig. 3. pKK223-3/DEH encoding the thermostable L-2-halo acid dehalogenase of *Ps.* sp. YL.

Materials and methods

Organisms

Cells of *Pseudomonas* sp. YL isolated from soil were used as a source of DNA encoding the thermostable L-2-halo acid dehalogenase. *Escherichia coli* JM109 was used as a host cell for the DNA recombination experiments.

Table 1. Purification of thermostable L-2-halo acid dehalogenase from *E. coli* pKK223-3/DEH.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude	18,000	500	36	1	100
Heat-treatment	18,000	360	50	1.4	100
DEAE-Toyopearl	12,400	105	118	3.3	69

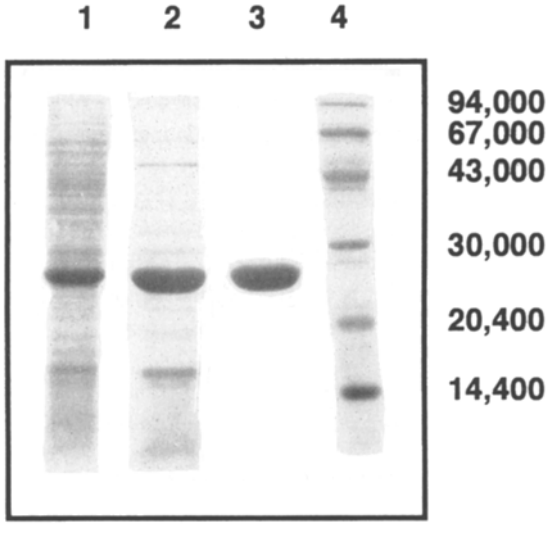

Fig. 4. SDS-polyacrylamide gel electrophoresis of thermostable L-2-halo acid dehalogenase. Lane 1, the soluble fraction of *E. coli* pKK223-3/DEH cells; lane 2, enzyme after heat treatment; lane 3, the purified enzyme after DEAE-Toyopearl chromatography; lane 4, molecular weight markers. The positions of molecular weight markers are indicated.

Culture conditions

Ps. sp. YL was cultivated aerobically at 28° C in the medium (pH 7.0) containing DL-2-chloropropionate (1.0 g/l), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (2.0), K_2HPO_4 (2.0), $MgCl_2.6H_2O$ (0.5), NaCl (1.0), and yeast extract (1.0).

The recombinant *E. coli* JM109 cells were grown aerobically at 37° C in Luria Bertani's (LB) medium (1% polypeptone, 0.5% yeast extract, and 1% NaCl, pH 7.5) containing 0.1 mg/ml ampicillin.

Library construction, gene cloning, and subcloning

Total DNA was isolated from *Ps.* sp. YL cells as described previously (Saito & Miura 1963). The DNA (0.6 μg/μl) was partially digested with 0.025 U/μl *Sau*3AI at 37° C for 20 min, and the fragments were electrophoresed in 0.7% agarose gel. DNA fragments of 3–10 kbp were electroeluted and ligated to a plasmid pUC118 digested with *Bam*HI and dephosphorylated by bacterial alkaline phosphatase. The resultant plasmids were introduced into *E. coli* JM109. General procedures for DNA manipulations were the same as described previously (Sambrook et al. 1989).

To isolate the dehalogenase gene, the gene library containing 5,400 recombinant *E. coli* clones were spread on the LB agar plates containing 0.1 mg/ml ampicillin, 40 μg/ml X-gal, 0.1 mM IPTG, and 0.2 mg/ml bromoacetate. After incubation at 37° C for 24 h, the colonies were isolated and cultivated in the LB medium containing 0.1 mg/ml ampicillin and 0.1 mM IPTG, and the dehalogenase activity was determined.

For subcloning, the plasmid recovered from the clone cells was digested with *Eco*RI and *Pst*I, and the resultant DNA fragments were blunted and ligated to *Sma*I digested pKK223-3 (prokaryotic expression vector) treated with bacterial alkaline phosphatase. The plasmids thus prepared were introduced into *E. coli* JM109 cells. Screening was carried out on the agar plates containing bromoacetate as described above.

Enzyme purification

E. coli pKK223-3/DEH cells were grown aerobically at 37° C for 14 h in 1 l of the LB medium containing 0.1 mg/ml ampicillin and 0.1 mM IPTG. The cells (5 g wet weight) were harvested and rinsed with 50 mM KPB (pH 7.5). After suspended in 20 ml of the same buffer, the cells were disrupted by ultrasonic oscillation

at 4° C for 25 min with a Seiko Instruments model 7500 ultrasonic disintegrator. The cell debris was removed by centrifugation. The supernatant solution was heated at 60° C for 30 min, and the denatured proteins were removed by centrifugation. The supernatant solution was dialyzed against 50 mM KPB (pH 7.5) and applied to a DEAE-Toyopearl 650 M column (4 × 40 cm). After washing of the column with 2 l of 50 mM KPB (pH 7.5), the elution was carried out with a 5 l linear gradient of 50–300 mM KPB (pH 7.5).

Enzyme and protein assay

The enzyme activity was determined by measurement of chloride ions released from 2-chloropropionate. The standard assay mixture (0.2 ml) contained 5 μmol of 2-chloropropionate (L-, D-, or DL-), 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 in (Iwasaki et al. 1956).

Chemicals

DNA restriction enzymes and DNA modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan). Plasmids, pUC118 and pKK223–3, were from Takara Shuzo and Pharmacia (Uppsala, Sweden), respectively. DEAE-Toyopearl was from Tosoh (Tokyo, Japan). D- and L-2-Chloropropionate were purchased from Sigma (St. Louis, MO, USA), and DL-2-chloropropionate was from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Results

Gene cloning and expression of thermostable L-2-Halo acid dehalogenase of Pseudomonas sp. YL

We obtained four colonies producing 2-halo acid dehalogenase from 5,400 recombinant *E. coli* transformants by bromoacetate screening. The cell extracts of the four clones were shown to act on L-2-chloropropionate, but not on D-2-chloropropionate; L-2-halo acid dehalogenase (Type I enzyme) was produced, but the Type III enzyme was not, which is produced by *Ps.* sp. YL grown on 2-chloroacrylate. This was also confirmed by western blot analysis with the antiserum raised against L-2-halo acid dehalogenase purified from *Ps.* sp. YL cells.

We examined the effect of IPTG concentrations on the enzyme production with the recombinant *E. coli* cells which showed the highest dehalogenase activity (Fig. 2). The dehalogenase was inducibly produced by IPTG, but was expressed even in the absence of IPTG.

Subcloning was carried out with the plasmid isolated from the recombinant *E. coli* cells showing the highest dehalogenase activity. The 9.5 kbp insert was digested, and the fragments were ligated to pKK223-3 as described in Materials and methods. Three colonies were isolated and shown to contain plasmids with 1.1 kbp insert (Fig. 3). This plasmid and the corresponding recombinant *E. coli* were named pKK223-3/DEH and *E. coli* pKK223-3/DEH, respectively. The specific activity of dehalogenase of the *E. coli* pKK223-3/DEH extract was 36 U/mg.

Rapid purification of thermostable L-2-halo acid dehalogenase from recombinant Escherichia coli cells

We purified the enzyme from the cell extract of *E. coli* pKK223-3/DEH by heat treatment and DEAE-Toyopearl 650 M column chromatography as described above. The results are summarized in Table 1. The enzyme was purified about 3.3-fold with a 69% yield. The purified enzyme was found to be homogeneous by SDS polyacrylamide gel electrophoresis (Fig. 4). The molecular weights of the native enzyme and the subunit were 54,000 and 27,000 respectively. They were in good agreement with those of the enzyme from the original strain, *Ps.* sp. YL. About 105 mg of the purified enzyme was obtained from 5 g of wet cells.

Discussion

The host *E. coli* cells used cannot grow on the bromoacetate-containing medium, and the dehalogenase can dehalogenate bromoacetate. We isolated the gene encoding L-2- halo acid dehalogenase based on this finding. The coding region was shown to be located in 1.1 kbp insert of pKK223-3/DEH, which is long enough to encode this enzyme whose subunit molecular weight is about 27,000. IPTG induced the synthesis of the enzyme, and accordingly the gene is probably

under the control of the *tac* promoter of the vector. However, the dehalogenase gene was also effectively expressed in the absence of IPTG. This result indicates that the inherent promoter of dehalogenase gene is operative in *E. coli*. The sequence analysis of the coding region and upstream region are currently under investigation.

We could not obtain the recombinant harboring the Type III enzyme gene by the bromoacetate screening method probably due to the poor gene expression or the low stability of this enzyme in *E. coli*.

L-2-Halo acid dehalogenase gene was very efficiently expressed in the recombinant cells. The amount of the enzyme produced corresponds to about 30% of the total soluble protein of the clone cells judging from the specific activities of the crude extract (36 U/mg) and the purified enzyme (118 U/mg). The activity of the extract is much higher than that of the original strain, *Ps.* sp. YL (0.82 U/mg). We have developed a simple and efficient purification method of the enzyme taking advantage of the high thermostability of enzyme; more than 100 mg of the purified enzyme was obtained from the recombinant cells grown in 1 l of the medium. This procedure is very useful for purification of a large amount of enzyme to characterize the enzyme, and application of the enzyme.

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