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A new DL-2-haloacid dehalogenase acting on 2-haloacid amides: purification, characterization, and mechanism

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

DL-2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively. We have constructed an overproduction system for DL-2-haloacid dehalogenase from *Pseudomonas putida* PP3 (DL-DEX 312) and purified the enzyme to analyze the reaction mechanism. When a single turnover reaction of DL-DEX 312 was carried out in $H_2{}^{18}O$ by use of a large excess of the enzyme with D- or L-2-chloropropionate as a substrate, the lactate produced was labeled with ^{18}O. This indicates that the solvent water molecule directly attacked the substrate and that its oxygen atom was incorporated into the product. This reaction mechanism contrasts with that of L-2-haloacid dehalogenase, which has an active-site carboxylate group that attacks the substrate to displace the halogen atom. DL-DEX 312 resembles DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113 (DL-DEX 113) in that the reaction proceeds with a direct attack of a water molecule on the substrate. However, DL-DEX 312 is markedly different from DL-DEX 113 in its substrate specificity. We found that DL-DEX 312 catalyzes the hydrolytic dehalogenation of 2-chloropropionamide and 2-bromopropionamide, which do not serve as substrates for DL-DEX 113. DL-DEX 312 is the first enzyme that catalyzes the dehalogenation of 2-haloacid amides.

Keywords: DL-2-Haloacid dehalogenase; 2-Haloacid amide; Lactamide; Mass spectrometry

1. Introduction

Various organohalogen compounds have been synthesized and utilized as pesticides, herbicides, and solvents. Some of them are persistent, but some of them can be degraded by microorganisms. Many different types of microbial dehalogenases that catalyze the dehalogenation of these compounds have been isolated and characterized [1–4]. Dehalogenases attract a great deal of attention from the viewpoint of environmental technology [5]. They are also useful in industrial chemistry because they catalyze the specific conversion of various halogenated compounds [6,7].

2,2-Dichloropropionic acid (Dalapon, Dow Chemical Company) has been widely used as a herbicide. Senior et al. isolated a bacterial strain, *Pseudomonas putida* PP3, which degrades 2,2-dichloropropionic

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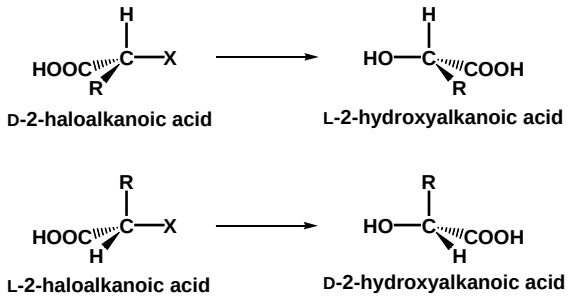

Fig. 1. Reactions catalyzed by DL-DEX 312.

acid [8]. The bacterium acquired the ability to assimilate haloalkanoic acids during a prolonged continuous flow culture of its parent strain in the presence of 2,2-dichloropropionic acid. The bacterium produces two 2-haloacid dehalogenases, which catalyze the hydrolytic dehalogenation of 2-haloalkanoic acids to produce the corresponding 2-hydroxyalkanoic acids [9]. These enzymes act on both D- and L-2-chloropropionic acids, and thus they are called DL-2-haloacid dehalogenases. In the present study, we constructed an overproduction system for one of the two dehalogenases, DL-DEX 312, which is encoded by the gene on an unusual mobile genetic element [10], and we purified the enzyme and characterized it. We clarified the stereospecificity of the enzyme reaction: the enzyme catalyzes the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively (Fig. 1). We further analyzed its reaction mechanism and examined its substrate specificity, finding that it catalyzes the hydrolytic dehalogenation of 2-chloropropionamide and 2-bromopropionamide. This is the first report of a dehalogenase that acts on haloacid amides.

2. Experimental

2.1. Chemicals and bacterial strain

Escherichia coli JM107 harboring pAWT24 coding for DL-2-haloacid dehalogenase from *P. putida* PP3 was kindly provided by Prof. J.H. Slater (University of Wales) [11]. The accession number of the gene is AY138113 (GenBank), and the amino acid sequence of the protein is shown in Fig. 2. DL-2-Chloropropionamide and DL-2-bromopropionamide were purchased from Aldrich, and L- and D-2-chloropropionic acids were obtained from Nacalai Tesque (Kyoto, Japan). Butyl-Toyopearl 650M was from Tosoh (Tokyo, Japan). Cellulofine GCL2000 was from the Seikagaku Corporation (Tokyo, Japan). $H_2{}^{18}O$ (95–98%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and Nippon Sanso (Tokyo, Japan). Lysyl endopeptidase of *Achromobacter lyticus* M497-1 was from Wako Industry Co. Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

2.2. Enzyme and protein assay

The enzyme activity was determined by measuring chloride ions released from DL-2-chloropropionate. The standard assay mixture (0.2 ml) contained 25 mM DL-2-chloropropionate, a 100 mM Tris–H_2SO_4 buffer (pH 9.5), and enzyme. After incubation at 30 °C for 10 min, the reaction was terminated by addition of 10 μl of 3 M H_2SO_4. The chloride ions released were measured spectrophotometrically according to the method of Iwasaki et al. [12]. One unit of the enzyme was defined as the amount of the enzyme that catalyzes the dehalogenation of 1 μmol of substrate/min.

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MTNPAYFPQL SQLDVSGEME STYEDIRLTL RVPWVAFGCR  40
VLATFPGYLP LAWRRSAEAL ITRYAEQAAD ELRERSLLNI  80
GPLPNLKERL YAAGFDDGEI EKVRRVLYAF NYGNPKYLLL 120
ITALSESMQM RPVGGAEVSS ELRASIPKGH PKGMDPLLPL 160
VDATKASTEV QGLLKRVADL HYHHGPASDF QALANWPKVL 200
QIVTDEVLAP VARTEQYDAK SRELVTRAPE LVRGLPGSAG 240
VQRSELMSML TPNELAGLTG VLFMYQRFIA DITISIIHIT 280
ECLDGAEAAS KSPFPI
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Fig. 2. Amino acid sequence of DL-DEX 312.

The protein assay was done with a Bio-Rad protein assay kit.

2.3. Construction of a plasmid for the overproduction of DL-DEX 312

The gene coding for DL-DEX 312 was amplified by PCR with pAWT24 as a template. The primers used were: 5′-GGACCAAGCTTTTATGGATAGGAGA-3′ and 5′-GAGAAGCTTATACCCCAATAAGTTAGATAGGAA-3′. The underlined sequences are *Hin*dIII sites. The PCR product was digested with *Hin*dIII and inserted into the *Hin*dIII site of pUC119 to obtain an expression plasmid named pUCD312, in which the DL-DEX 312 gene was under the control of the *lac* promoter.

2.4. Purification of DL-DEX 312 from the recombinant E. coli cells

E. coli JM109 was transformed with pUCD312. The recombinant cells were cultivated at 37 °C for 16 h in an LB medium containing 100 μg/ml ampicillin and 0.2 mM IPTG. The cells were harvested by centrifugation, suspended in a 50 mM potassium phosphate buffer (pH 7.5), and disrupted by ultrasonic oscillation. The cell debris was removed by centrifugation. A freshly prepared 0.5% protamine sulfate solution (0.05 volume) was added slowly with stirring. After standing for 5 min, the supernatant solution was collected by centrifugation. The supernatant solution was brought to 40% saturation with ammonium sulfate, and the precipitate was removed by centrifugation. Ammonium sulfate was added to the resultant supernatant solution to 70% saturation, and the precipitate collected by centrifugation was dissolved in a 50 mM potassium phosphate buffer (pH 7.5). The enzyme solution was applied to a Butyl-Toyopearl 650M column, and elution was carried out with a linear gradient from 30 to 0% saturation of ammonium sulfate in a 50 mM potassium phosphate buffer (pH 7.5). The active fractions were applied to a Cellulofine GCL2000 column equilibrated with a 50 mM potassium phosphate buffer (pH 7.5), and elution was carried out with a 10 mM potassium phosphate buffer (pH 7.5). The final preparation of the enzyme was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis.

2.5. Identification of lactamide produced from 2-chloropropionamide and 2-bromopropionamide

The enzyme reaction was carried out at 30 °C for 5 min in a mixture containing 1 ml of 100 mM DL-2-chloropropionamide or DL-2-bromopropionamide, 300 μl of 1.5 M Tris–H_2SO_4 (pH 9.5), and 43 μg of lyophilized enzyme. The reaction mixtures were applied to a cation exchange column (Dowex-50H^+). The flow-through fraction was lyophilized and dissolved with D_2O for ^{1}H-NMR analyses. The ^{1}H-NMR spectra were obtained with a Varian VXR 200 spectrometer (200 MHz).

2.6. Single turnover reaction of DL-DEX 312 in $H_2{}^{18}O$

For a single turnover experiment, 200 nmol of DL-DEX 312 in 50 μl of a 400 mM Tris–H_2SO_4 buffer (pH 9.5) was lyophilized. The reaction was initiated by dissolving the dried enzyme in 50 μl of $H_2{}^{18}O$ containing 20 nmol of D- or L-2-chloropropionate (neutralized with NaOH), and the mixture was incubated at 30 °C for 24 h. The reaction mixtures were ultrafiltrated, diluted 10-fold with 50% acetonitrile/H_2O (1/1), and then introduced into the mass spectrometer using a Harvard Apparatus syringe infusion pump operating at 2 μl/min. The molecular mass of the produced lactate was measured with a PE-Sciex API III triple quadrupole mass spectrometer equipped with an ionspray ion source in the negative ion mode (Sciex, Thornhill, Ont., Canada).

2.7. Digestion of DL-DEX 312 with lysyl endopeptidase

To digest the enzyme in $H_2{}^{18}O$, 10 nmol of DL-DEX 312, 1.0 μmol of D- or L-2-chloropropionate (neutralized with NaOH), and 20 μmol of Tris–H_2SO_4 (pH 9.5) were mixed in 50 μl of $H_2{}^{18}O$ and incubated at 30 °C for 24 h. Twenty microliters of aliquot was lyophilized and dissolved with 20 μl of $H_2{}^{18}O$ containing 8 M urea. After incubation at 37 °C for 1 h, 30 μl of 1 M Tris–H_2SO_4 (pH 9.0) in $H_2{}^{18}O$ and 10 μl of 33 μM lysyl endopeptidase in $H_2{}^{18}O$ were added to this solution, and incubation was carried out at 37 °C for 12 h.

2.8. LC/MS analysis of the proteolytic digests

The proteolytic digests of the enzyme were loaded onto a YMC-PackC4-AP column (100 mm × 1.0 mm i.d.) (YMC Co., Kyoto, Japan) connected to a mass spectrometer and then eluted with a linear gradient of 0–80% acetonitrile in 0.06% trifluoroacetic acid over 60 min at a flow rate of 40 μl/min. The total ion current chromatogram was recorded in the single quadrupole mode with a PE-Sciex API III mass spectrometer equipped with an ionspray ion source. The quadrupole was scanned from 300 to 2000 amu with a step size of 0.25 amu and a 0.5 ms dwell time per step. The ion spray voltage was set at 5 kV, and the orifice potential was 80 V. The molecular mass of each peptide was calculated with MacSpec software supplied by Sciex.

3. Results and discussion

3.1. Purification of DL-DEX 312

DL-DEX 312 was purified to homogeneity as described in Section 2.4. The results are summarized in Table 1.

3.2. Stereospecificity of the DL-DEX 312 reaction

We found that the reaction catalyzed by DL-DEX 312 proceeds with inversion of the C-2 configuration of the substrates. HPLC analysis with a chiral separation column indicated that D- and L-2-chloropropionates were converted into L- and D-lactate, respectively, by the action of DL-DEX 312. The configurations of the products were confirmed by examining whether they served as substrates for L- and D-lactate dehydrogenase.

3.3. Single turnover reaction of DL-DEX 312 in $H_2{}^{18}O$

Two different reaction mechanisms have been proposed for 2-haloacid dehalogenases (Fig. 3). In the mechanism shown in Fig. 3A, the solvent water molecule directly attacks the substrate to displace the halide ion. In the mechanism shown in Fig. 3B, the active-site carboxylate group nucleophilically attacks the substrate to displace the halide ion, which results in the formation of the ester intermediate. The intermediate is subsequently hydrolyzed by the solvent water molecule.

To clarify which mechanism the DL-DEX 312 reaction proceeds through, a single turnover reaction of DL-DEX 312 was carried out in $H_2{}^{18}O$ with D- or L-2-chloropropionate as a substrate and an excess amount of the enzyme. A majority of the lactate produced was labeled with ^{18}O (Fig. 4), which indicates that an oxygen atom of the solvent water was directly incorporated into the product. This supports the mechanism shown in Fig. 3A, but does not support the mechanism shown in Fig. 3B.

3.4. Multiple turnover reaction of DL-DEX 312 in $H_2{}^{18}O$

We carried out a multiple turnover reaction of DL-DEX 312 in $H_2{}^{18}O$ with D- or L-2-chloropropionate as a substrate. After completion of the reaction, the enzyme was digested with lysyl endopeptidase in $H_2{}^{18}O$, and the resulting peptide fragments were separated on a reversed phase column interfaced with an ionspray mass spectrometer as a detector. If the reaction proceeded through the mechanism shown in Fig. 3B, ^{18}O would be incorporated into the active-site carboxylate group. The total ion current chromatogram was obtained with the mass spectrometer

Table 1
Purification of DL-DEX 312 from recombinant *E. coli* cells

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude extract	1300	7500	5.8	100	1
Protamine sulfate	970	7400	7.6	99	1.3
Ammonium sulfate	270	6900	26	92	4.5
Butyl-Toyopearl 650M	94	4900	52	65	9
Cellulofine GCL2000	27	1800	67	24	12

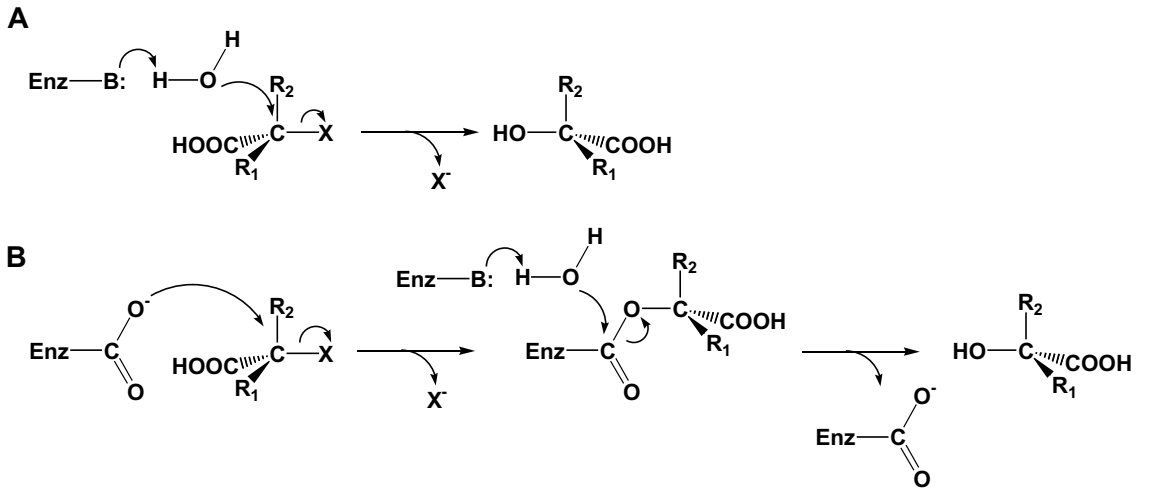

Fig. 3. Reaction mechanisms of 2-haloacid dehalogenases: (A) a general base catalytic mechanism; (B) nucleophilic attack by an acidic amino acid residue followed by hydrolysis of the ester intermediate.

in the single quadrupole mode, and peptides 1–87, 88–102, 103–116, 117–148, 149–152, 153–165, 166–175, 176–198, 199–220, 221–291, and 292–296 were isolated. The molecular masses of all proteolytic peptides, except for the one derived from the C-terminal region of the enzyme, were approximately 4 Da higher than the predicted molecular masses whether D- or L-2-chloropropionate was used as a substrate (Table 2). These data show that ^{18}O of the solvent water was incorporated into the C-terminal α-carboxylate group of each peptide by the action of lysyl endopeptidase, but not into the side-chain carboxylate group in the course of the dehalogenation reaction. This result also supports the above conclusion that the DL-DEX 312 reaction proceeds through the mechanism shown in Fig. 3A.

The reactions catalyzed by other dehalogenases, such as L-2-haloacid dehalogenase from *Pseudomonas* sp. YL [13–15] and fluoroacetate dehalogenase from *Moraxella* sp. strain B [16,17], proceeded through the mechanism shown in Fig. 3B. The only exception was DL-DEX 113, whose reaction proceeded through the mechanism shown in Fig. 3A [18]. DL-DEX 312 is the second example of an enzyme whose reaction does not involve the ester intermediate. It is likely that the reactions catalyzed by DL-2-haloacid dehalogenases generally proceed through the mechanism shown in Fig. 3A.

We previously showed that Thr65, Glu69, and Asp194 of DL-DEX 113 are essential for the catalysis. DL-DEX 312 shares sequence similarity with DL-DEX 113 (39% identity), and these residues are

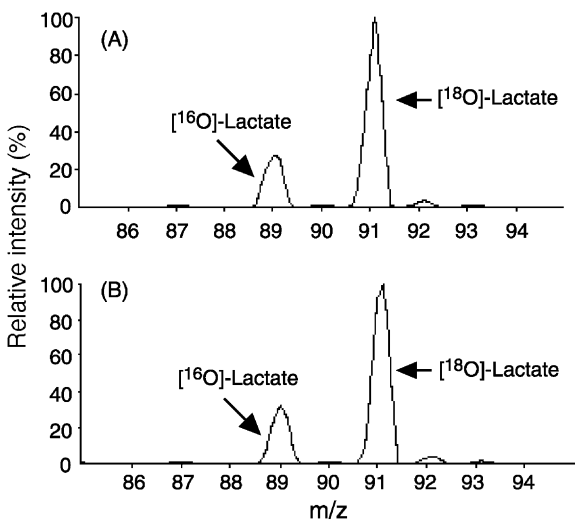

Fig. 4. Ion spray mass spectra of lactate produced by a single turnover reaction of DL-DEX 312 in $H_2{}^{18}O$. D-2-Chloropropionate was used as a substrate in (A) and L-2-chloropropionate was used in (B). The spectra were obtained between 86 and 94 amu. The step size was 0.1 amu, and the dwell time was 10 ms per step. The ion spray voltage was set at −3.5 kV, and the orifice potential was −50 V.

Table 2
Molecular masses of proteolytic fragments of DL-DEX 312 incubated with substrates in $H_2{}^{18}O$

Peak	Fragment	$[M + H]^+$		
		Predicted (average mass)	Measured	
			L-CPA[a]	D-CPA[a]
1	1–87	9900.42	9904.1	9904.5
2	88–102	1713.84	1717.5	1717.9
3	103–116	1697.98	1701.7	1701.4
4	117–148	3449.08	3453.4	3452.7
5	149–152	438.51	442.3	442.4
6	153–165	1370.66	1374.1	1374.2
7	166–175	1046.21	1049.8	1049.8
8	176–198	2745.08	2749.0	2749.1
9	199–220	2459.81	2463.7	2463.7
10	221–291	7647.92	7652.0	7652.0
11	292–296	560.67	561.0	561.0

[a] DL-DEX 312 was incubated with L-2-chloropropionate (L-CPA) or D-2-chloropropionate (D-CPA) in $H_2{}^{18}O$ and digested with lysyl endopeptidase.

conserved as Thr62, Glu66, and Asp189, respectively, in DL-DEX 312. In contrast, DL-DEX 312 did not show any sequence similarity with L-2-haloacid dehalogenase and fluoroacetate dehalogenase.

3.5. Effect of pH on enzyme activity

The pH dependence of the dehalogenation of D- and L-2-chloropropionates catalyzed by DL-DEX 312 was studied. The pH-activity profiles for both the isomers were closely similar, and the maximum activity was observed at pH 9.5 (Fig. 5A). This value is similar to the pH optimum for the dehalogenation catalyzed by DL-DEX 113 [19].

3.6. Thermal stability and effect of temperature on enzyme activity

The maximum activity of DL-DEX 312 was observed at 30–40 °C when the reaction was carried out at pH 9.5 (Fig. 6). DL-DEX 312 completely lost its activity by incubation at 40 °C for 35 min.

3.7. Substrate specificity of DL-DEX 312

The substrate specificity of DL-DEX 312 was examined (Table 3). All the monohaloacetates tested, except for monofluoroacetate, were efficiently dehalogenated. The halogen must be at the C-2 position of a compound to be a substrate: 3-chloropropionate, 3-bromopropionate, 3-chloro-*n*-butyric acid, and 4-chloro-*n*-butyric acid were not substrates. DL-DEX 312 resembles DL-DEX 113 in these respects [19]. However, DL-DEX 312 is markedly different from DL-DEX 113 in that DL-DEX 312 catalyzes the dehalogenation of 2-haloacid amides, such as DL-2-chloropropionamide and DL-2-bromopropionamide. The Michaelis constants for the substrates were

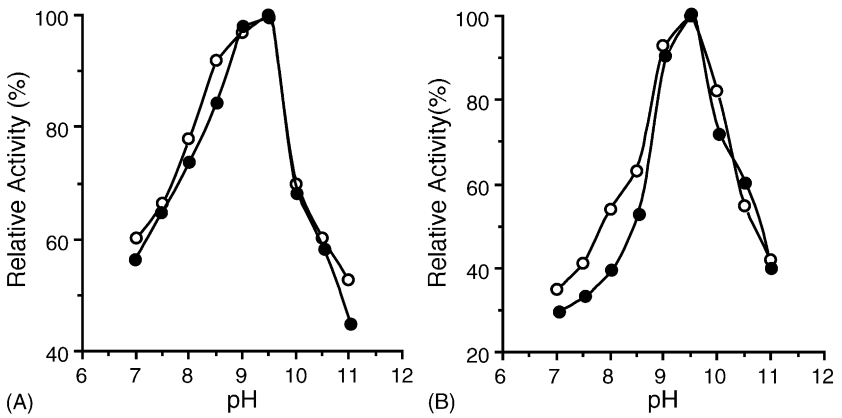

Fig. 5. Effect of pH on the DL-DEX 312 activity. pH dependence was assayed with D-2-chloropropionate (A, closed circles), L-2-chloropropionate (A, open circles), DL-2-chloropropionamide (B, closed circles), and DL-2-bromopropionamide (B, open circles) as substrates. The assay was carried out as described in Section 2 with a potassium phosphate buffer, a Tris–H_2SO_4 buffer, and a glycine–NaOH buffer in the pH ranges of 7.0–8.0, 8.0–10.0, and 10.0–11.0, respectively.

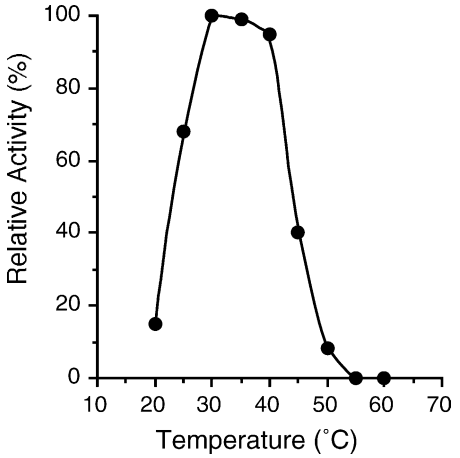

Fig. 6. Temperature-activity profile of the dehalogenation of DL-2-chloropropionate catalyzed by DL-DEX 312. The enzyme activity was measured at pH 9.5 for the first 10 min.

as follows: L-2-chloropropionate, 2.7 mM; D-2-chloropropionate, 8.1 mM; DL-2-chloropropionamide, 0.87 mM; and DL-2-bromopropionamide, 1.8 mM. We studied the pH dependence of the dehalogenation of DL-2-chloropropionamide and DL-2-bromopropionamide and found that the pH-activity profiles for these substrates are similar to those for D- and L-2-chloropropionates (Fig. 5B). The ^{1}H-NMR analyses revealed that the products from DL-2-chloropropionamide and DL-2-bromopropionamide were lactamide. This result indicates that the halide ion was released by the dehalogenation of 2-haloacid amides and not by the dehalogenation of 2-chloropropionate formed by possible amidase activity. DL-DEX 312 is the first enzyme that catalyzes the hydrolytic dehalogenation of 2-haloacid amides.

DL-DEX 312 and DL-DEX 113 are similar to each other in their primary structures [20], but they exhibit significantly different substrate specificities. The structural comparison between DL-DEX 312 and DL-DEX 113 would provide information about how the enzyme distinguishes between a carboxylate group and an amido group of the substrate.

3.8. Possible application of DL-DEX 312

In the reactions catalyzed by L-2-haloacid dehalogenase and fluoroacetate dehalogenase, the solvent water molecule does not directly attacks the substrate [13,16]. Therefore, it is impossible to employ a nucleophile other than a water molecule to displace the halogen atom. Indeed, hydroxylamine and ammonia added to the reaction mixture were not incorporated into the product, but they modified the active-site carboxylate groups to inactivate the enzyme [14,17]. In contrast, in the DL-DEX 312 reaction, the solvent water molecule nucleophilically attacks the substrates to displace the halogen atom. This raises the possibility that the enzyme might be used for the production of compounds other than 2-hydroxyalkanoic acids by employing a nucleophile other than a water molecule. The production of amino acids from 2-haloalkanoic acids with DL-DEX 312 is now under investigation.

Table 3
Substrate specificity of DL-DEX 312 and DL-DEX 113

Substrate[a]	Relative activity (%)[b]	
	DL-DEX 312	DL-DEX 113
Monochloroacetate	20	33
Monobromoacetate	332	280
Monoiodoacetate	91	96
Dichloroacetate	5	8
Trichloroacetate	6	3
D-2-Chloropropionate	76	84
L-2-Chloropropionate	88	118
DL-2-Chloropropionate	100	100
DL-2-Bromopropionate	415	380
2,2-Dichloropropionate	27	42
DL-2-Chloro-*n*-butyrate	20	18
DL-2-Bromo-*n*-butyrate	246	220
DL-2-Chloropropionamide	48	0
DL-2-Bromopropionamide	175	0

[a] The following substrates were inert: monofluoroacetate, chloroacetaldehyde, chloroacetonitrile, 2-chloroacrylonitrile, 3-chloropropionate, 3-bromopropionate, 2-chloroisobutyrate, 2-chloro-*n*-caproate, 3-chloro-*n*-butyrate, 4-chloro-*n*-butyrate, benzyl chloride, allyl chloride, isopropyl chloride, *sec*-butyl chloride, and cyclopropyl bromide.

[b] The initial velocity was measured by measuring the halide ions released and is expressed as the relative activity compared with DL-2-chloropropionate. Each reaction mixture (0.2 ml) contained a 100 mM Tris–H_2SO_4 buffer (pH 9.5), 25 mM substrate, and 0.5 U of the enzyme.

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