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A novel generation of optically active 1,2-diols from the racemates by using halohydrin dehydro-dehalogenase

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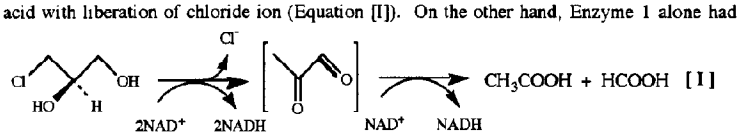
Abstract: A novel enzyme dehalogenating halohydrins, designated as halohydrin dehydo-dehalogenase (HDDase), was purified from *Alcaligenes* sp. DS-S-7G. The enzyme catalyzed oxidative dehalogenation of (*R*)-3-chloro-1,2-propanediol [monochlorohydrin (MCH)] to acetic acid and formaldehyde *via* hydroxyacetone stereoselectively by the addition of artificial electron acceptors. The dehalogenating activity was much higher in the presence of 2,6-dichlorophenolindophenol (DCIP) and phenazine methosulfate (PMS). The resulting stereoselective dehydro-dehalogenation was applicable to preparation of various optically active halohydrins and 1,2-diols so that the respective residual isomers had excellent enantiomeric excesses (ee) (60-99% ee).

Introduction: Optically active 1,2-diols are very important as chiral building blocks for syntheses of pharmaceuticals, agrochemicals and natural products. Also, optically active halogenated alcohols such as 2,3-dichloro-1-propanol [dichlorohydrin (DCH)] and 3-chloro-1,2-propanediol [monochlorohydrin (MCH)] were useful compounds as precursors of optically active epichlorohydrin (1-3) and glycidol (4-6), respectively. Therefore many biological, enzymatic and chemical methods for preparing above compounds were reported (7-12). Recently, Sharpless *et al.* reported the catalytic asymmetric dihydroxylation of olefins. This method was based on mirror-image alkaloid derivatives serving as highly enantioselective ligands for the osmium tetraoxide and was applicable to a wide range of olefinic substrates. The resulting diols were obtained in high yields (80-90%) and with a variety of enantiomeric excesses (ee) (65-99% ee). Nevertheless, terminal olefins were not good substrates for these ligands so that ee of diols prepared from the terminal olefins was below 90% ee level (13-16).

More recently, we found a novel dehalogenating system of MCH and purified the enzymes involved in the reaction from *Alcaligenes* sp. DS-S-7G (17, 18), which stereoselectively assimilates (*R*)-MCH from the racemate (5). The dehalogenation of (*R*)-MCH was catalyzed by two enzymes (Enzyme 1 and Enzyme 2) in the presence of NAD^+ as an electron acceptor so that (*R*)-MCH was oxidatively converted to acetic acid and formic acid with liberation of chloride ion (Equation [I]). On the other hand, Enzyme 1 alone had the dehydro-



dehalogenating activity of (*R*)-MCH under the aerobic condition, but Enzyme 2 had no activity. However, in the presence of NAD^+, the dehydro-dehalogenating activity by Enzyme 1 was 4.5 times higher with electron-transporting action of Enzyme 2 (17, 18). Moreover, we investigated the effects of other electron acceptors for the dehalogenation of (*R*)-MCH by Enzyme 1. 2,6-Dichlorophenolindophenol (DCIP) and phenazine methosulfate (PMS) were found to be utilized as electron acceptors instead of NAD^+ so that the activity was

increased to about one thousand-fold rate. From these results, we designated Enzyme 1 as a halohydrin dehydro-dehalogenase (HDDase). In addition, Enzyme 1 had broad substrate specificities for various halohydrins and 1,2-diols.

In this paper we describe the effects of various electron acceptors, properties of HDDase and its stereoselective applications for preparing optically active halohydrins and 1,2-diols.

Results and discussion

Effects of various electron acceptors for halohydrin dehydro-dehalogenase (HDDase) The effects of various electron acceptors for (*R*)-MCH by HDDase were examined. Enzyme 1 alone had the dehydro-dehalogenating activity in the presence of NAD^+ and O_2 as an electron acceptor, of which values were estimated to be 3.5 mU mg^{-1} and 4.8 mU mg^{-1}, respectively. Under the anaerobic condition (N_2 atmosphere), no activity was observed. In the presence of DCIP as an electron acceptor, however, HDDase had high dehydro-dehalogenating activity, which was about three hundred-fold in comparison with NAD^+ as an electron acceptor. Moreover, the enzymatic reaction rate was another three times higher when PMS was coupled as an intermediate electron carrier with DCIP. Other compounds such as methyl viologen, cytochrome C and 2-*p*-indophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT) were unsuitable for HDDase as electron acceptors. These facts suggested that mixture of PMS and DCIP may be most suitable electron acceptors for HDDase. Therefore, the activity was assayed by coupling PMS as an initial electron acceptor in the presence of DCIP as a terminal electron acceptor. The effects of pH on the activity were examined using Britton-Robinson buffer so that the optimal pH and stability of pH were estimated to be pH 7.4 and pH 6.0-8.0, respectively. Also, the effects of metals and inhibitors for the activity were examined. This dehydro-dehalogenation for (*R*)-MCH was completely inhibited by existence of 1 mM Hg^{2+} and 5 mM *p*-chloromercuribenzoic acid.

Substrates specificity of HDDase and its use in preparation of various optically active halohydrins and 1,2-diols Dehydro-dehalogenating activity of HDDase for various substrates such as halohydrins, 1,2-diols, alcohols and acids were examined (Table 1). This enzyme showed broad substrate specificities for alcohols, but not for acids. Particularly, halohydrins such as (*R*)-3-halogeno-1,2-propanediol and (*S*)-2,3-dihalogeno-1-propanol were catalyzed with high dehydro-dehalogenating activities. (*R*)-MCH, (*S*)-DCH and 1,3-dichloro-2-propanol were converted into acetic acid and formaldehyde, 3-chloro-propionaldehyde and 3-chloro-propionic acid, and monochloroacetone, respectively, with liberation of chloride ion. 3-Chloro-1-propanol, however, was catalyzed with little dehydro-dehalogenating activity, indicating that structure with vicinal halogen atom and -OH group was essential for high substrate specificity of HDDase (Equations [II] and [III]). In the case of (*R*)-MCH, it was converted to hydroxyacetone under the aerobic condition, that is, in the presence of O_2 alone as an electron acceptor (17, 18). These results indicate that HDDase catalyze (*R*)-MCH to acetic acid and formaldehyde *via* hydroxyacetone in the presence of PMS and DCIP (Equation [IV]). Also, 1,2-diols such as 1,2-propanediol, 1,2-butanediol, 1,2-pentanediol, 1,2-hexanediol and 3-phenyl-1,2-propanediol were catalyzed with dehydrogenating activities so that alkyl- and aromatic-1,2-propanediols were converted into the corresponding aldehydes and formic acid *via* the corresponding 2-hydroxy-1-aldehydes (Equation [V]). These results suggest that HDDase may have dehydrogenating activity for non-halogenated alcohols, as well as for halogenated alcohols. On the other hand, this HDDase had high stereoselectivity for MCH and DCH. (*R*)-MCH and (*S*)-DCH were dehydro-dehalogenated, but not (*S*)-MCH and (*R*)-DCH, respectively. Propylene chlorohydrin, butylene chlorohydrin and 3-phenyl-1,2-propanediol were dehydrogenated, but not with high stereoselectivity.

Table 1. Substrate specificity for various compounds by HDDase

Substrate	Relative activity (%)	Substrate	Relative activity (%)
(*R*)-3-Chloro-1,2-Propanediol	100	Methanol	< 5
(*S*)-3-Chloro-1,2-propanediol	< 5	Ethanol	< 5
3-Bromo-1,2-propanediol	50	*n*-Propanol	7
(*R*)-2,3-Dichloro-1-propanol	< 5	*sec*-Propanol	< 5
(*S*)-2,3-Dichloro-1-propanol	57	Ethylene glycol	11
2,3-Dibromo-1-propanol	94	1,2-Propanediol	31
1,3-Dichloro-2-propanol	19	1,3-Propanediol	< 5
3-Chloro-1-propanol	8	1,2-Butanediol	36
1-Chloro-2,3-diacetoxypropane	< 5	2,3-Butanediol	7
Ethylene chlorohydrin	< 5	1,2-Pentanediol	32
Propylene chlorohydrin	14	1,2-Hexanediol	43
Butylene chlorohydrin	65	1,2-Dihydroxy-3-butene	16
4-Chloro-3-hydroxybutyronitrile	11	1,2-Dihydroxy-5-hexene	60
1-Chloropropane	0	1-Phenyl-1,2-ethanediol	8
α-Chloropropionic acid	0	3-Phenyl-1,2-propanediol	13
β-Chloropropionic acid	0	3-Phenoxy-1,2-propanediol	< 5
Chloroacetone	0	Lactate	< 5
1,3-Dichloroacetone	0	Hydroxyacetone	48
Epichlorohydrin	< 5	Glycidol	< 5
		Glycerol	7
		Formaldehyde	6
		Acetic acid	0

The specific activity for (*R*)-MCH corresponding to 3.18 U mg^{-1} was taken to be 100%.

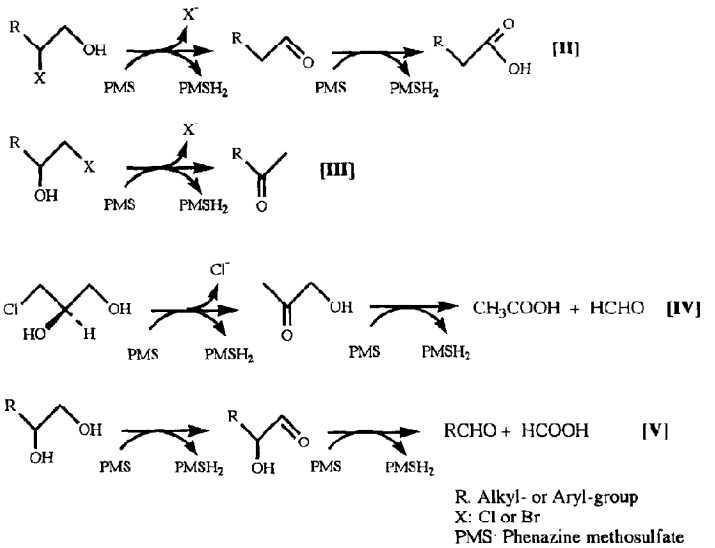


Table 2. Preparation of optically active halohydrins and 1,2-diols by enzymatic resolution

Substrate [a]	% ee	Residual substrate (%) [b]
1,2-Propanediol	60.0 (*R*)	42.3
1,2-Butanediol	97.5 (*R*)	48.2
1,2-Pentanediol	98.2 (*R*)	50.2
1,2-Hexanediol	98.2 (*R*)	50.0
1,2-Dihydroxy-3-butene	98.0 (*R*)	49.1
1,2-Dihydroxy-5-hexene	98.3 (*R*)	40.1
1-Phenyl-1,2-ethanediol	95.1 (*R*)	39.5
3-Chloro-1,2-propanediol	98.5 (*S*)	50.2
3-Bromo-1,2-propanediol	98.5 (*S*)	49.3
2,3-Dichloro-1-propanol	99.0 (*R*)	46.2
2,3-Dibromo-1-propanol	99.0 (*R*)	48.1

a) Racemic forms were used as a substrates.

b) At initial time, each reaction solution contained 0.2% (v/v) of substrate.

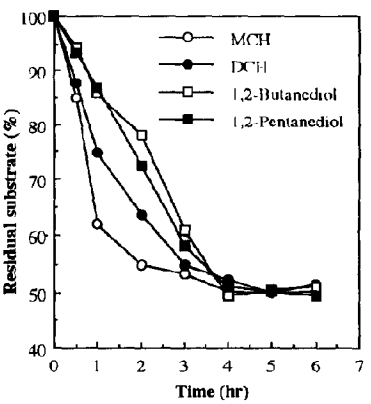
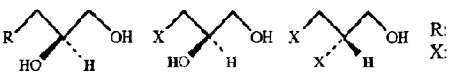


Fig. 1 Enzymatic resolution of various halohydrins and 1,2-diols by HDDase

Production of generating optically active halohydrins and 1,2-diols using Enzyme 1 were investigated. The results of enzymatic resolutions for various substrates tested were summarized in Table 2. Figure 1 shows degradation patterns for racemic MCH, DCP, 1,2-butanediols and 1,2-pentanediol by HDDase in the presence of PMS and DCIP as electron acceptors. The respective degradation reactions were stopped in 4 hr, at which ratios of residual substrates were about 50%. After isolation, syrups of MCH, DCP, 1,2-butanediols and 1,2-pentanediol were given in the average yield of 42% and estimated to be 98.5% ee [(*S*)-form], 99.0% ee [(*R*)-form], 97.5% ee [(*R*)-form] and 98.2% ee [(*R*)-form], respectively. As shown in Table 2, other obtained isomers also had excellent optical purities except for 1,2-propanediol, respectively.

In conclusion, HDDase had unique substrate specificities and high stereoselectivities for halohydrins and 1,2-diols. The facts suggest that the following structures [**1**]-[**3**] are essential for the high stereoselectivity of HDDase. First, structure [**1**] had -OH groups at C1 and C2 positions, that is, 1,2-diol. Second, when halogen existed at C3 position of the 1,2-diol (structure [**2**]), the activity was getting higher. Interestingly, dihalogeno alcohols such as 2,3-dihalogeno-1-propanol (structure [**3**]) was catalyzed with high stereoselectivity



[1] **[2]** **[3]**

although monohalogenated alcohols such as propylene chlorohydrin and butylene chlorohydrin could not be dehydro-dehalogenated stereoselectively. With respect to biological preparation of alkane-1,2-diols, formerly, asymmetric reduction of 1-hydroxy-2-ketones by baker's yeast have been researched (19, 20). Recently, Lee and Whitesides reported stereoselective reduction of 1-hydroxy-2-propanone and 1-hydroxy-2-butanone to the corresponding (*R*)-1,2-diols by glycerol dehydrogenase from *Enterobacter aerogenes* and *Cellulomonas* sp. (20). These methods were based on asymmetric reduction of prochiral compounds to chiral ones, but not oxidation. This feature seems to be different from ours. Also, Preparation of (*S*)-1,2-diols from the corresponding racemates by interaction of NAD^+-linked (*R*)-specific alcohol dehydrogenase and NADPH-linked (*S*)-specific 2-keto-1-alcohol reductase were known (21). This enzyme-interaction system gave the (*S*)-1,2 diols in high yield (60-100%), however, was unable to apply for preparation of optically active halogenated alcohols. From the points of substrate specificity, HDDase was considered to be a new type of dehalogenase. In addition we developed in preparation of highly optically active halohydrins and 1,2-diols by using HDDase (Table 2). Now, investigation of details between structures and functions of HDDase are in progress and will be published elsewhere in near future.

Experimental

Chemicals: Racemic 1-phenyl-1,2-propanediol, 1,2-dihydroxy-3-buten, 1,2-dihydroxy-5-hexene and 3-phenoxy-1,2-propanediol were prepared from the corresponding epoxides in the presence of H_2SO_4 as a catalyst (22). Butylene chlorohydrin was prepared by the addition of HCl (23). (*R*)- and (*S*)-MCH, and (*R*)- and (*S*)-DCH were obtained by our microbial resolution methods (1-6). Other chemicals were purchased from Aldrich Chemicals Co., Inc..

Microorganism and enzymes: *Alcaligenes* sp. DS-S-7G which grows on (*R*)-MCH as a source of carbon was isolated from soil by enrichment culture (5). The cultivation for production of HDDase was carried out in a 50-*l* jar fermentor (Model KMJ-501MGU, Mitsuwa Rikagaku Co., Ltd., Osaka, Japan) with 35 *l* of a synthetic medium containing 1.5% (v/v) racemic MCH, 0.5% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) K_2HPO_4, 0.1%

(w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.2% (w/v) $NaH_2PO_4 \cdot 2H_2O$, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.0001% (w/v) $CuSO_4 \cdot 5H_2O$ and 0.0001% (w/v) $MnSO_4 \cdot 5H_2O$ (17, 18). The conditions were as follows: agitation, 320 rpm; aeration, 10 l/min; temperature, 30 °C; pH, controlled at pH 6.5 with 7M NaOH (5).

Purification of HDDase was done with several column chromatographic methods (17, 18). Assay of HDDase was done in 3 ml of 20 mM phosphate buffer-2 mM $MgSO_4$, pH 7.2, (PM-buffer) containing 0.1% (v/v) substrates, 250 μM electron acceptors and an appropriate amount of purified HDDase. The activity was determined by following the change in optical absorbance of various electron acceptors. When phenazine methosulfate (PMS) was used as an electron acceptor, the assay was coupled with another electron acceptor, which was used as a terminal one (24, 25). The ε values of electron acceptors were as follows: NAD^+, 6.22 x 10^3 $molar^{-1}cm^{-1}$ (340 nm); DCIP, 21.0 x 10^3 $molar^{-1}cm^{-1}$ (600 nm) (24); methyl viologen, 11.8 x 10^3 $molar^{-1}cm^{-1}$ (600 nm) (26); cytochrome C, 19.1 x 10^3 $molar^{-1}cm^{-1}$ (550 nm) (27); INT, 19.3 x 10^3 $molar^{-1}cm^{-1}$ (503 nm) (25). The one unit of enzyme activity was defined as the amount of enzyme which reduced 1 μmol of electron acceptors per min.

Preparation of optically active halohydrins and 1,2-diols Preparative reaction was carried out in 50 ml of 20 mM PM-buffer, pH 7.2, containing 0.2% (v/v) various substrates, 5-10 mg of HDDase and 500 μM each of PMS and DCIP at 30 °C with vigorous agitation using a static stirrer. After the reaction, the mixture was centrifuged to remove precipitate, concentrated by evaporation, extracted with ethyl acetate and dried with anhydrous $MgSO_4$. The solvent was removed by evaporation and syrups of residual halohydrins and 1,2-diols were obtained.

Analytical measurements Assay of enzymatic reaction was done with a gas chromatograph (Model GC-9A, Shimadzu, Kyoto, Japan) equipped with a PEG 20M-HP (5%, 60/80 mesh, GL Science Co., Ltd., Tokyo, Japan) column (3.2 mm in diameter and 1 m in length). The conditions of the gas chromatography were as follows: sample size, 1 μl; injection temperature, 240 °C; column temperature, 180 °C; carrier gas, nitrogen; flow rate, 50 ml min^{-1}; detector, flame ionization detector (FID).

Identification of products by enzymatic reaction was done using a gas chromatograph (Model GC-14A, Shimadzu, Kyoto, Japan) equipped with a capillary column (25 m x 0.25 mm, CBP20-M25-025, Shimadzu, Kyoto, Japan). The analytical conditions were as follows: sample size, 1 μl; injection temperature, 200 °C, column temperature, 100 to 250 °C at a rate of 10 °C min^{-1}; carrier gas, nitrogen; flow rate, 1 ml min^{-1}; split ratio, 100:1, detector, FID.

GC mass spectrometry (MS) was done using the electron ionization method. The instrument (Model JMS-AX505W, JEOL, Tokyo, Japan) with a capillary column (30 m X 0.25 mm, DBWAX, J & W Scientific, CA, USA) was operated at an electron beam energy of 70 eV. The conditions were as follows: sample size, 1 μl; injection temperature, 250 °C, column temperature, 40 °C for 3 min to 250 °C followed by 10 °C min^{-1}; carrier gas, helium gas; flow rate, 1 ml min^{-1}; split ratio, 60:1.

Determination of the ee and configuration for 1,2-diols such as 1,2-propanediol, 1,2-butanediol, 1,2-pentanediol, 1,2-hexanediol, 1,2-dihydroxy-3-buten, 1,2-dihydroxy-5-hexene and 3-halogeno-1,2-propanediol was done by gas chromatography analysis (Model G-3000, Hitachi, Tokyo, Japan) of the corresponding trifluoroacetic derivatives. The conditions were as follows: sample size, 1 μl of ethanol solution; column, CHIRALDEX^R-G-TA capillary column (0.25 mm in diameter, 30 m in length, Astec Inc., NJ, USA); column temperature, 70 °C; injection and detector temperature, 150 °C; detector, FID; carrier gas, nitrogen; split ratio, 1:100. The retention time corresponding to (*R*)- and (*S*)-enantiomers of 1,2-propanediol, 1,2-butanediol, 1,2-

pentanediol, 1,2-hexanediol, 1,2-dihydroxy-3-butene and 1,2-dihydroxy-5-hexene were 10.8 and 14.0 min, 14.2 and 16.1 min, 22.3 and 24.4 min, 39.1 and 43.2 min, 14.7 and 16.3 min and 39.8 and 43.8 min, respectively. In the case of 3-halogeno-1,2-propanediol, the ee was determined with gas chromatograph equipped with CHIRALDEX^{R}-A-PH capillary column (0.25 mm in diameter, 30 m in length, Astec Inc., NJ, USA) after conversion to glycidol with aqueous NaOH solution (5). The conditions were as follows: sample size, 1 μl of isopropanol solution; column temperature, 45 °C; injection and detector temperature, 150 °C; detector, FID; carrier gas, nitrogen; split ratio, 1:100. The ee of 2,3-dihalogeno-1-propanol was determined by the complexation gas chromatography after conversion to the corresponding 3-halogeno-1,2-epoxide with aqueous NaOH solution (2). The conditions were as follows: sample size, 1 μl of hexane solution; column, capillary column coated with bis-3-(heptafluorobutyryl)-1R-camphorates of cobalt (II) in SE-54 by dynamic method (0.25 mm in diameter, 30 m in length); column temperature, 40 °C; injection and detector temperature, 150 °C; detector, FID; carrier gas, nitrogen; split ratio, 1:50. For 1-phenyl-1,2-ethanediol, the ee was determined by high performance liquid chromatography (HPLC) analysis using a Hitachi HPLC system L-6000 (Tokyo, Japan) (21). The conditions were as follows: column, Chiral CEL OC (0.46 cm in diameter, 25 cm in length, Daicel Chemical Industries, Ltd., Tokyo, Japan); sample, 1 μl of ethanol solution; developing solution, hexane/2-propanol = 30:1 (v/v); flow rate, 1 ml min^{-1}; detection, UV at 235 nm; temperature, 25 °C.

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