JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 7613–7619
0021-9193/92/237613-07$02.00/0

Vol. 174, No. 23

Resolution and Some Properties of Enzymes Involved in Enantioselective Transformation of 1,3-Dichloro-2-Propanol to (*R*)-3-Chloro-1,2-Propanediol by *Corynebacterium* sp. Strain N-1074

TETSUJI NAKAMURA,[1]* TORU NAGASAWA,[2] FUJIO YU,[1] ICHIRO WATANABE,[1] AND HIDEAKI YAMADA[3]†

Central Research Laboratory, Nitto Chemical Industry Company, Ltd., 10-1 Daikoku-cho, Tsurumi-ku, Yokohama 230,[1] Department of Food Science and Technology, Nagoya University, Chikusa-ku, Nagoya 464,[2] and Department of Agricultural Chemistry, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606,[3] Japan

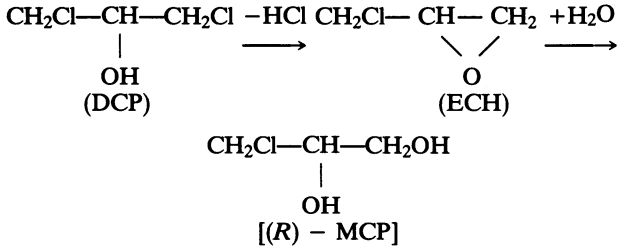
Received 19 May 1992/Accepted 23 September 1992

During the course of the transformation of 1,3-dichloro-2-propanol (DCP) into (*R*)-3-chloro-1,2-propanediol [(*R*)-MCP] with the cell extract of *Corynebacterium* sp. strain N-1074, epichlorohydrin (ECH) was transiently formed. The cell extract was fractionated into two DCP-dechlorinating activities (fractions I_a and I_b) and two ECH-hydrolyzing activities (fractions II_a and II_b) by TSKgel DEAE-5PW column chromatography. Fractions I_a and I_b catalyzed the interconversion of DCP to ECH, and fractions II_a and II_b catalyzed the transformation of ECH into MCP. Fractions I_a and II_a showed only low enantioselectivity for each reaction, whereas fractions I_b and II_b exhibited considerable enantioselectivity, yielding *R*-rich ECH and MCP, respectively. Enzymes I_a and I_b were isolated from fractions I_a and I_b, respectively. Enzyme I_a had a molecular mass of about 108 kDa and consisted of four subunits identical in molecular mass (about 28 kDa). Enzyme I_b was a protein of 115 kDa, composed of two different polypeptides (about 35 and 32 kDa). The specific activity of enzyme I_b for DCP was about 30-fold higher than that of enzyme I_a. Both enzymes catalyzed the transformation of several halohydrins into the corresponding epoxides with liberation of halides and its reverse reaction. Their substrate specificities and immunological properties differed from each other. Enzyme I_a seemed to be halohydrin hydrogen-halide-lyase which was already purified from *Escherichia coli* carrying a gene from *Corynebacterium* sp. strain N-1074.

Various halogenated compounds are synthesized and used as herbicides, pesticides, and their starting materials. It has been known that some of them cause severe environmental pollution. Some halogenated compounds are catabolized through enzymatic dehalogenation reactions by several bacteria (10–12, 25, 28, 31, 32, 36). Many investigations have been focused on the enzymatic cleavage of the carbon-halogen bonds (4, 6, 9, 14, 15, 17, 18, 26, 27, 29, 33).

The enzymatic production of optically active compounds by means of microbial dehalogenation reactions has also been reported. For example, both enantiomers of lactic acid have been prepared from DL-2-chloropropionic acid by L- and DL-2-haloacid dehalogenase (19). The production of optically active 3-chloro-1,2-propanediol (MCP), which is a useful chiral building reagent, from racemic MCP or 2,3-dichloro-1-propanol by degradative bacteria has been attempted elsewhere (12, 30). However, such an optical-resolution method of racemic substrates is not advantageous, because the molar conversion yield of the resulting desired enantiomer is less than 50% unless the residual substrate is racemized.

Recently, we found that optically active MCP was produced from a prochiral symmetric compound, 1,3-dichloro-2-propanol (DCP), by MCP- or epichlorohydrin (ECH)-utilizing bacteria (23). Further investigations with one of these bacteria, *Corynebacterium* sp. strain N-1074, suggested that (*R*)-MCP was formed from DCP via ECH:



In the present paper, we describe the resolution of enzymes involved in the transformation of DCP into (*R*)-MCP from the crude extract of *Corynebacterium* sp. strain N-1074 and their enzymatic properties.

MATERIALS AND METHODS

Materials. DCP, (*R,S*)-MCP, and ECH were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). (*R*)-MCP was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Polypeptone and yeast extract were purchased from Oriental Yeast (Tokyo, Japan). Meat extract was obtained from Mikuni (Tokyo, Japan). DEAE-Sephacel, phenyl-Sepharose CL-4B, and octyl-Sepharose CL-4B were obtained from Pharmacia LKB (Uppsala, Sweden). BIO-

* Corresponding author.
† Present address: Department of Biotechnology, Kansai University, Suita, Osaka 564, Japan.

GEL HT and TOYOPEARL HW-55S were obtained from Bio-Rad (Richmond, Calif.) and Tosoh Corporation (Tokyo, Japan), respectively. Marker proteins for molecular weight determination on high-performance liquid chromatography (HPLC) and an electrophoresis calibration kit were obtained from Oriental Yeast and Pharmacia LKB, respectively. Complete and incomplete Freund adjuvants and special Noble agar were purchased from Difco Laboratories (Detroit, Mich.). All other chemicals used were obtained from commercial sources.

Microorganism and culture conditions. *Corynebacterium* sp. strain N-1074 (23), which was previously isolated from a soil sample, was subcultivated at 28°C for 24 h with reciprocal shaking in a test tube containing 4 ml of a medium (pH 7.0) consisting of 5 g of polypeptone, 5 g of meat extract, 0.5 g of yeast extract, and 2 g of NaCl per liter of tap water. Then, 4 ml of the subculture was inoculated into a 2-liter shaking flask containing 800 ml of a medium (pH 7.2) consisting of 10 g of glycerol, 2 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 2.5 g of KH_2PO_4, 5 g of Na_2HPO_4, 2 g of yeast extract, and 2 g of MCP per liter of tap water. The cultivation was carried out at 28°C for 68 h with reciprocal shaking. The cells were harvested by continuous-flow centrifugation at 10,000 × *g* and then washed once with 50 mM Tris-H_2SO_4 buffer (pH 8.0).

Enzyme assay and analytical measurements. Enzyme activities were estimated in the range in which product formation versus incubation time and protein concentration is linear. The level of DCP-dechlorinating activity was assayed in a reaction mixture (1 ml) containing 50 μmol of DCP, 100 μmol of Tris-H_2SO_4 (pH 8.0), and an appropriate amount of enzyme. The level of the activity for the formation of MCP from ECH was assayed in a reaction mixture (1 ml) containing 50 μmol of ECH, 100 μmol of Tris-H_2SO_4 (pH 8.0), and an appropriate amount of enzyme. Each reaction was carried out at 20°C for 10 min. Halide formation was determined spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulfate as described by Iwasaki et al. (8). MCP formation was measured by gas-liquid chromatography. One unit of enzyme activity for the dechlorination of DCP and that for the formation of MCP from ECH were defined as the amount of enzyme which catalyzed the formation of chloride and MCP at a rate of 1 μmol/min under the standard assay conditions, respectively. All data were expressed as the means of triplicate experiments and were consistent within ±5% or less.

In the experiments for substrate specificity, halide formation was measured with various halohydrins as substrates. The reaction was carried out at 20°C for 10 min in a reaction mixture (1 ml) containing 50 μmol of substrate, 100 μmol of Tris-H_2SO_4 (pH 8.0), and an appropriate amount of enzyme, except that 25 μmol of 1,3-dibromo-2-propanol was used as a substrate. Products of the enzymatic reaction were analyzed and then identified by gas-chromatographic mass spectrometry (MS-80; Hitachi, Tokyo, Japan).

The amount of protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (3) by using a dye reagent supplied by Bio-Rad, with bovine serum albumin as standard.

The amounts of halohydrins and epoxides were determined by gas-liquid chromatography, with a Shimadzu (Kyoto, Japan) GC-7A system equipped with a flame ionization detector with a 30-m capillary column of ULBON HR-1701 (Chromatopacking Center, Kyoto, Japan). The injection port and column temperatures were set at 250 and 120°C, respectively. Helium at a pressure of 0.5 kg/cm^2 was used as carrier gas.

The formation ratio of optical isomers of MCP and ECH was determined by HPLC analysis of their *p*-toluenesulfonate derivatives (2-hydroxy-3-chloropropyl *p*-toluenesulfonate) (7). The *p*-toluenesulfonate derivatives of MCP and ECH were prepared by the reaction with *p*-toluenesulfonyl chloride in pyridine (5) and with *p*-toluenesulfonic acid in CH_2Cl_2 (7), respectively. HPLC was performed with a Shimadzu LC-5A system equipped with a Chiralcel OC column (4.6 by 250 mm; Daicel Industries Ltd., Tokyo, Japan) with hexane–2-propanol (98.5:1.5 [vol/vol]) as the eluent at a flow rate of 2.5 ml/min. The A_{235} was measured (7).

Preparation of cell extract. Washed cells harvested from 12 liters of culture were suspended in about 600 ml of 50 mM Tris-H_2SO_4 buffer (pH 8.0) and disrupted for 30 min with an ultrasonic oscillator (19 kHz; Insonator model 201M; Kubota, Tokyo, Japan). The cell debris was removed by centrifugation at 15,000 × *g* for 1 h. The supernatant solution was used as the cell extract.

Resolution and purification of the enzymes involved in the transformation of DCP into MCP. All procedures were performed at 0 to 5°C, and 10 mM Tris-H_2SO_4 buffer (pH 8.0) was used unless otherwise specified. The cell extract was subjected to ammonium sulfate fractionation and DEAE-Sephacel column chromatography prior to the resolution of each component involved in the transformation of DCP into MCP as follows. Solid ammonium sulfate was added to the cell extract (650 ml; about 4,700 mg of protein) to 30% saturation with stirring, and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 1 h. The supernatant solution was further saturated with ammonium sulfate to 70%. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 1 h, dissolved in Tris buffer, and dialyzed against the same buffer. The enzyme solution was applied to a DEAE-Sephacel column (3.5 by 30 cm) equilibrated with Tris buffer. After being washed with Tris buffer (1 liter) and then with Tris buffer containing 0.1 M ammonium sulfate (750 ml), the active fractions catalyzing the dechlorination of DCP and the formation of MCP from ECH were eluted with Tris buffer containing 0.2 and 0.3 M ammonium sulfate (each 750 ml). Both active fractions were combined, and the combination was dialyzed against Tris buffer. Each component involved in the transformation of DCP into MCP was resolved by HPLC on a TSKgel DEAE-5PW column (2.15 by 15 cm; Tosoh) with an increasing linear gradient of ammonium sulfate (0 to 0.3 M) at a flow rate of 3 ml/min (see Fig. 2). Four components were obtained, namely, fractions I_a and I_b catalyzing the dechlorination of DCP and fractions II_a and II_b catalyzing the formation of MCP from ECH.

Fractions I_a and I_b were subjected to further purification. Enzyme I_a, which catalyzes the dechlorination of DCP, was purified from fraction I_a as follows. Solid ammonium sulfate was added to fraction I_a (40.5 ml; 79.4 mg of protein) to 35% saturation with stirring, and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 1 h. The supernatant solution was further saturated with ammonium sulfate to 50%. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 1 h, dissolved in Tris buffer containing 20%-saturated ammonium sulfate, and dialyzed against the same buffer. The enzyme solution was applied to an octyl-Sepharose CL-4B column (1.2 by 20 cm) equilibrated with Tris buffer containing 20%-saturated ammonium sulfate and eluted with the same buffer. The active fractions

were combined and applied to a phenyl-Sepharose CL-4B column (1 by 20 cm) equilibrated with Tris buffer containing 20%-saturated ammonium sulfate. The column was washed with the equilibration solution and Tris buffer containing 10%-saturated ammonium sulfate. The enzyme was eluted with Tris buffer containing 5%-saturated ammonium sulfate. The active fractions were combined and dialyzed against Tris buffer.

Enzyme I_b, which also catalyzes the dechlorination of DCP, was purified from fraction I_b as follows. Solid ammonium sulfate was added to fraction I_b (60.5 ml; 194 mg of protein) to 50% saturation with stirring, and the resulting precipitate was removed by centrifugation at 10,000 × *g* for 1 h. The supernatant was further saturated with ammonium sulfate to 70%. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 1 h, dissolved in Tris buffer containing 20%-saturated ammonium sulfate, and dialyzed against the same buffer. The enzyme solution was applied to an octyl-Sepharose CL-4B column (1.2 by 20 cm) equilibrated with Tris buffer containing 20%-saturated ammonium sulfate and eluted with the same buffer. The active fractions were combined and then applied to a phenyl-Sepharose CL-4B column (1 by 18 cm) equilibrated with Tris buffer containing 20%-saturated ammonium sulfate. The column was washed with the equilibration solution and Tris buffer containing 15%-saturated ammonium sulfate. Stepwise elution was carried out with 7.5- and 5%-saturated ammonium sulfate in Tris buffer. The active fractions were combined and dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The enzyme solution was applied to a BIO-GEL HT column (0.8 by 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) and washed with the same buffer. The enzyme was eluted with a 10 to 150 mM linear gradient of potassium phosphate buffer (pH 7.5). The active fractions were combined and concentrated with a Centriprep-30 (Amicon, Danvers, Mass.) to a volume of about 2 ml. The enzyme solution was run on a TOYOPEARL HW-55S column (2.5 by 110 cm) equilibrated with 50 mM Tris buffer containing 0.1 M ammonium sulfate. The rate of sample loading and elution was maintained at 50 ml/h with a peristaltic pump. The active fractions were combined.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% polyacrylamide slab gels by the method of Laemmli (16). The gels were stained for protein with Coomassie brilliant blue R-250 and destained in ethanol-acetic acid-H_2O (3:1:6 [vol/vol/vol]). The relative molecular weights (M_r) of the enzyme subunits were determined from the mobilities of standard proteins. The amounts of polypeptides in slab gels were measured for convenience with a dual-wavelength thin-layer chromatography Scanner CS-930 system (Shimadzu), with scanning at 650 nm.

PAGE under nondenaturing conditions was performed in 8% polyacrylamide slab gels by the method of Weightman and Slater (34). The gels were stained for protein with Coomassie brilliant blue R-250 or for dechlorination activity with DCP as a substrate. Activity staining was performed as follows. The gels were incubated in 0.1 M Tris-H_2SO_4 buffer (pH 8.0) containing 50 mM DCP at 20°C for 30 min. The incubated gels were washed with distilled water and placed in a 0.1 M $AgNO_3$ solution. The dechlorination activity was visualized by the formation of AgCl precipitation bands.

Two-dimensional electrophoresis to analyze the polypeptides of enzyme I_b was performed, with PAGE under nondenaturing (for first dimension) and SDS-denaturing (for second dimension) conditions as described above. A lane of the first-dimensional gel-loaded enzyme I_b was cut (about 5 mm wide), and the gel was incubated in 62.5 mM Tris-HCl buffer (pH 6.8) containing 1% SDS and 2% 2-mercaptoethanol for 2 h at room temperature. The incubated first-dimensional gel was placed on top of the second-dimensional gel and sealed in position with a solution of 0.5% agarose in the stacking-gel buffer. After electrophoresis in the second dimension, the gel was stained for protein.

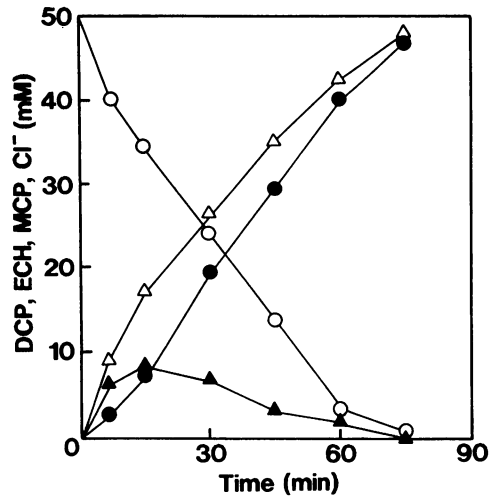


FIG. 1. Course of the transformation of DCP into (*R*)-MCP by a cell extract of *Corynebacterium* sp. strain N-1074. The reaction was carried out at 20°C in a reaction mixture (100 ml) containing 5 mmol of DCP, 50 mmol of Tris-H_2SO_4 buffer (pH 8.0), and cell extract (570 mg of protein). ○, DCP; ●, MCP; △, Cl^-; ▲, ECH.

Gel permeation HPLC. The M_r of the native enzyme was estimated by HPLC with a TSKgel G3000SW column (0.75 by 60 cm; Tosoh) at a flow rate of 0.7 ml/min with elution of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.2 M NaCl at room temperature. The A_{280} of the effluent was recorded. The M_r of the enzyme was calculated from the mobilities of the standard proteins, which were glutamate dehydrogenase (M_r, 290,000), lactate dehydrogenase (M_r, 142,000), enolase (M_r, 67,000), adenylate kinase (M_r, 32,000), and cytochrome *c* (M_r, 12,400).

Antiserum preparation. Antibodies were produced by injecting 10 mg of halohydrin hydrogen-halide-lyase, which was previously purified from *Escherichia coli* JM109/pST001 carrying a gene from *Corynebacterium* sp. strain N-1074 (21), into young white male rabbits. The antigen was homogenized with an equal volume of complete Freund adjuvant and injected into multiple subcutaneous sites on the back. The rabbits received a booster injection at 3 weeks. The booster injection was of 1 mg of antigen homogenized with an equal volume of incomplete Freund adjuvant. On the 10th day after the booster injection, blood from each rabbit was collected from an ear vein and allowed to clot. The serum samples were centrifuged at 5000 × *g* for 10 min, and the supernatant was stored at −20°C. Ouchterlony plates (24) were made with a 1% special Noble agar in 10 mM Tris-H_2SO_4 buffer (pH 8.0) containing 0.01% sodium azide.

RESULTS

The transformation of DCP into (*R*)-MCP by cell extract. By using the cell extract of *Corynebacterium* sp. strain N-1074, the transformation of DCP into (*R*)-MCP through

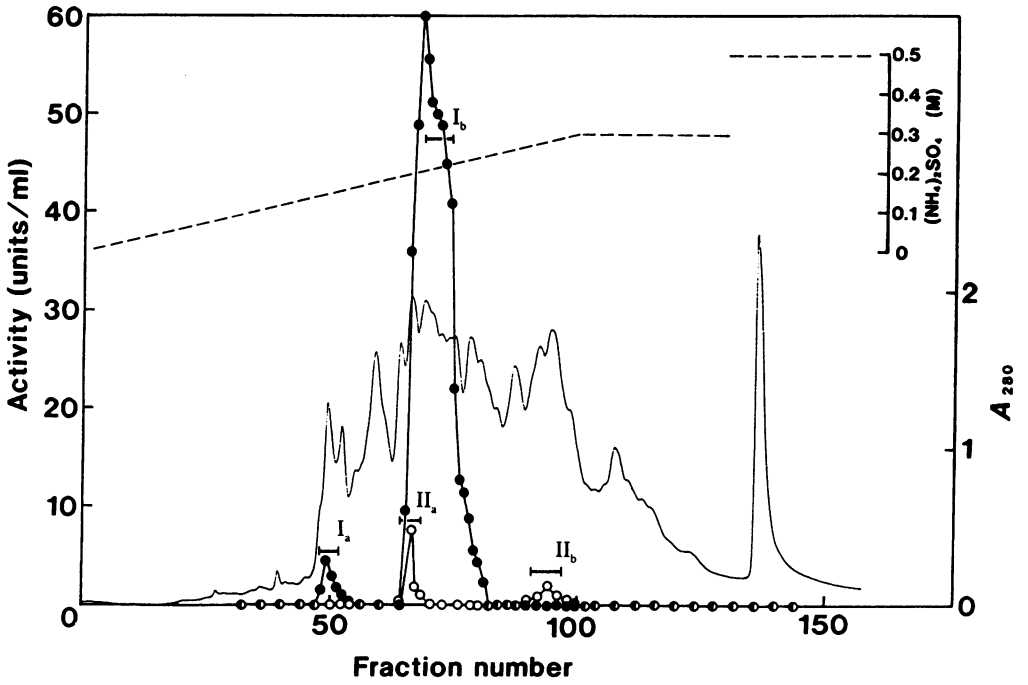


FIG. 2. An elution profile of the enzymes involved in the transformation of DCP into (*R*)-MCP by HPLC on a TSKgel DEAE-5PW column. Proteins were eluted with 10 mM Tris-H_2SO_4 buffer (pH 8.0) by a linear gradient of ammonium sulfate at a flow rate of 3 ml/min. ●, activity for DCP dechlorination; ○, activity for the formation of MCP from ECH; – – –, concentration of ammonium sulfate; ——, A_{280}; ▬, fraction pooled.

time was monitored (Fig. 1). The reaction was carried out at 20°C with shaking in a reaction mixture (100 ml) containing DCP (5 mmol), Tris-H_2SO_4 buffer (50 mmol [pH 8.0]), and the cell extract (570 mg of protein). During the course of the reaction, ECH was transiently formed and disappeared after prolonged incubation. Almost the same amounts of MCP and chloride were formed in accordance with the decrease in DCP after a 75-min reaction. This fact suggested that the transformation of DCP into MCP was catalyzed by at least two enzymes. The resulting MCP was extracted from the reaction mixture with ethyl acetate, and its optical purity was determined to be 35.9% enantiomeric excess by HPLC analysis as described in Materials and Methods.

Resolution of enzymes involved in the transformation of DCP into (*R*)-MCP. Enzymes involved in the transformation of DCP into (*R*)-MCP were partially purified from the cell extract of *Corynebacterium* sp. strain N-1074 by ammonium sulfate fractionation and DEAE-Sephacel column chromatography. Partial purification resulted in different yields of activities for the dechlorination of DCP and for the formation of MCP from ECH, and 87.1 and 42.9% of the enzyme activities from the initial level of the cell extract, respectively, were obtained. These results also indicated that the transformation of DCP into (*R*)-MCP proceeds via ECH by at least two kinds of enzyme.

The partially purified enzyme was resolved with HPLC on a TSKgel DEAE-5PW column. An elution profile of the enzymes is shown in Fig. 2. Two DCP-dechlorinating activities and two ECH-forming activities were fractionated (Fig. 2). The four fractions (I_a, I_b, II_a, and II_b [shown in Fig. 2]) were collected and subjected to further investigation. Fractions I_a and I_b catalyzed not only the dechlorination of DCP but also the reversible transformation of DCP into ECH and chloride (Table 1). The resulting ECH from DCP catalyzed by fraction I_a was almost racemate, whereas the formation of ECH by fraction I_b was found to be considerably enantioselective and gave *R*-rich ECH. Only a little formation of MCP from ECH with fractions I_a and I_b was observed. *R*-rich MCP was formed from racemic ECH with fraction II_b, but the resulting MCP with fraction II_a was almost racemate.

Purification of enzymes I_a and I_b. Two enzymes which catalyze the interconversion of DCP to ECH, enzymes I_a and I_b, were purified from fractions I_a and I_b, respectively. The results of the purification are summarized in Table 2. Enzymes I_a and I_b catalyzed the dechlorination reaction of DCP at the rates of 3.27 and 103 μmol/min/mg of protein, respectively. Purified enzyme I_a migrated as one band on

TABLE 1. Catalytic properties of the fractions separated by HPLC[a]

Fraction[b]	Reaction mixture	Reaction time (min)	Product (mM)	*R/S*[c]
I_a (0.1)	A	60	ECH (11.3) Cl^- (11.1)	53.1/46.9
I_a (0.1)	B	60	DCP (14.3)	
I_b (0.01)	A	60	ECH (6.7) Cl^- (6.9)	75.9/24.1
I_b (0.025)	B	60	DCP (4.8)	
II_a (0.048)	C	90	MCP (13.4)	53.8/46.2
II_b (0.1)	C	90	MCP (6.4)	78.9/21.1

[a] Fractions separated by HPLC on a TSKgel DEAE-5PW column were incubated at 20°C in 100 mM Tris-H_2SO_4 buffer (pH 8.0; 100 ml) containing 50 mM DCP (A), 50 mM ECH and 50 mM KCl (B), or 50 mM ECH (C).

[b] The amounts of fraction added to each reaction mixture (in milliliters of fraction per milliliter of reaction mixture) are shown in parentheses.

[c] The formation ratios of optical isomers of ECH and MCP were determined by HPLC analysis of their *p*-toluenesulfonate derivatives.

TABLE 2. Purification of enzyme I_a and enzyme I_b from *Corynebacterium* sp. strain N-1074

Enzyme and step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
I_a					
TSKgel DEAE-5PW	79.4	100	1.26	100	
$(NH_4)_2SO_4$[a]	49.1	100	2.04	100	1.6
Octyl-Sepharose CL-4B	33.9	97.6	2.88	97.6	2.3
Phenyl-Sepharose CL-4B	18.1	59.2	3.27	59.2	2.6
II_b					
TSKgel DEAE-5PW	194	5,385	27.8	100	
$(NH_4)_2SO_4$[b]	96.6	3,124	32.3	58.0	1.2
Octyl-Sepharose CL-4B	50.6	1,954	38.6	36.3	1.4
Phenyl-Sepharose CL-4B	20.4	1,100	53.9	20.4	1.9
BIO-GEL HT	6.6	568	86.1	10.5	3.1
TOYOPEARL HW-55S	3.1	319	103	5.9	3.7

[a] The compound was 35 to 50% saturated.
[b] The compound was 50 to 70% saturated.

SDS-PAGE, and its molecular weight was estimated to be about 28,000 on the basis of its mobility relative to those of the marker proteins (Fig. 3). Purified enzyme I_a also migrated as a single band on PAGE under nondenaturing conditions, and it exhibited dechlorination activity (Fig. 4). Purified enzyme I_b migrated as two protein bands on SDS-PAGE, with molecular weights of about 35,000 and 32,000, and the ratio of the amounts of protein was calculated to be 3:1 on the basis of the intensities of the stained bands (Fig. 3). PAGE of purified enzyme I_b under nondenaturing conditions gave four protein bands. All four bands exhibited dechlorination activity, and a faint band under these bands was also observed (Fig. 4). Judging from two-dimensional electrophoresis (Fig. 5), the protein corresponding to each band seemed to be composed of four polypeptides mixed with different ratios of 35- and 32-kDa polypeptides (namely, 35-to-32-kDa polypeptide ratios of 4:0, 3:1, 2:2, 1:3, and 0:4). The protein composed of four 32-kDa polypeptides was barely visible with Coomassie brilliant blue-staining gel, probably because of its small amount.

Comparison of substrate specificities. The substrate specificities of purified enzymes I_a and I_b were compared (Table 3). With DCP as a substrate, the specific activity of enzyme I_b was about 30-fold higher than that of enzyme I_a. Both enzymes acted on various halohydrins and produced the corresponding epoxides, which were identified by gas-chromatographic mass spectrometry, with liberation of halide. Their substrate specificities, especially for MCP, 1,3-dibromo-2-propanol, and bromoethanol, differed from each other. The reverse reaction, transformation of epoxides into the corresponding halohydrins in the presence of halide, was also catalyzed by each enzyme (data not shown).

Comparison of immunological properties. The immunological properties of enzymes I_a and I_b were analyzed by

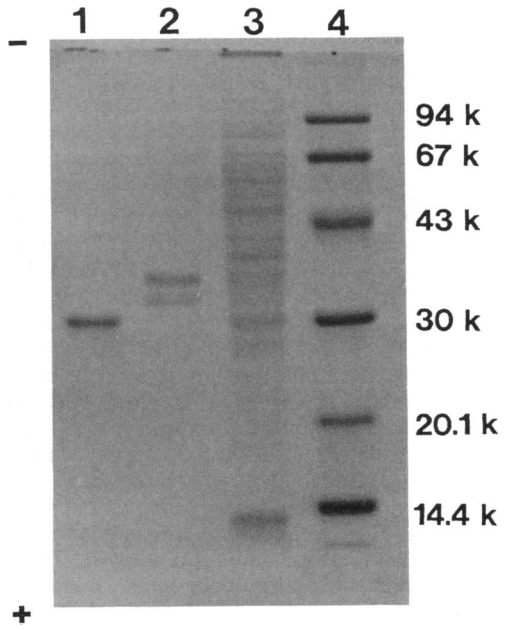


FIG. 3. SDS-PAGE of enzymes I_a and I_b. Lanes: 1, purified enzyme I_a (5 μg); 2, purified enzyme I_b (5 μg); 3, cell extract of *Corynebacterium* sp. strain N-1074 (50 μg); 4, marker proteins (Pharmacia-LKB). k, kilodaltons.

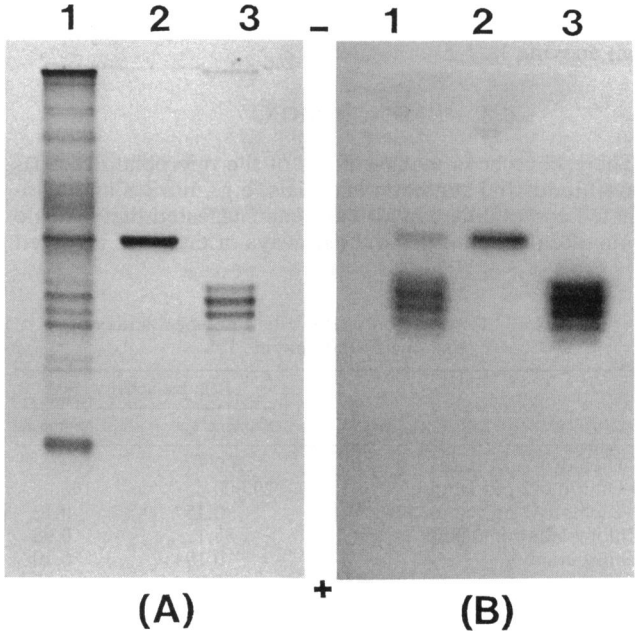


FIG. 4. Electrophoresis of enzymes I_a and I_b under nondenaturing conditions. The gels were stained for protein (A) or for dechlorination activity (B). Lanes: 1, cell extract of *Corynebacterium* sp. strain N-1074 (100 μg); 2, purified enzyme I_a (10 μg); 3, purified enzyme I_b (10 μg).

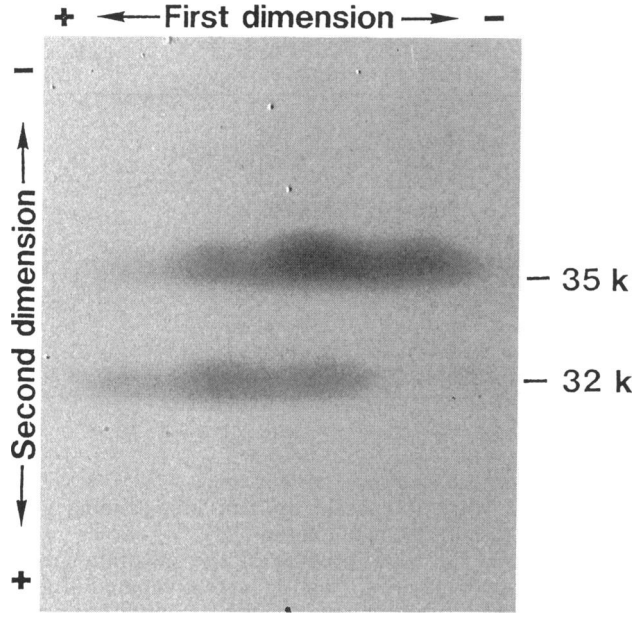


FIG. 5. Two-dimensional gel electrophoresis of enzyme I_b. After purified enzyme I_b (40 μg) was separated in the first-dimensional gel, the first-dimensional gel was loaded onto the second-dimensional gel, as described in Materials and Methods. k, kilodaltons.

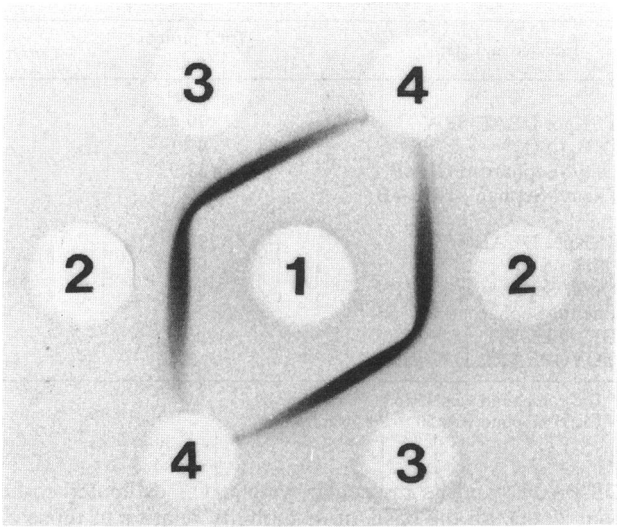


FIG. 6. Ouchterlony double-diffusion analysis. Wells: 1, antiserum against halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 (4 μl); 2, halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 (1.5 μg); 3, enzyme I_a (1.5 μg); 4, enzyme I_b (1.5 μg).

Ouchterlony double-diffusion tests (Fig. 6). The antiserum against halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 formed an immunoprecipitin line with enzyme I_a, and the immunoprecipitin lines for enzyme I_a and halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 were completely fused. On the other hand, the antiserum formed no immunoprecipitin line with enzyme I_b. These facts indicate that enzyme I_a was immunologically identical to halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 and different from enzyme I_b.

DISCUSSION

There have been many studies of the microbial degradation of industrial synthetic materials, e.g., nitriles and halogenated compounds, and it has been suggested that a single strain often contains several pathways or enzymes involved in their degradation. For example, nitriles can be converted to the corresponding acids by two pathways: the hydrolysis of nitriles directly to acids catalyzed by nitrilase and the transformation of nitriles into acids via amides catalyzed by nitrile hydratase and amidase (1, 2). All three enzymes involved in both pathways are produced in *Rhodococcus rhodochrous* J1 cells (20, 22). In the case of microbial degradation of 2-haloacids, two different dehalogenases in each of the bacteria that degrade them have often been found (6, 9, 13, 28).

Here, we investigated the pathways of DCP transformation into (*R*)-MCP by *Corynebacterium* sp. strain N-1074 and found that interconversion of DCP to ECH and hydration of ECH to MCP were involved in the transformation of DCP into MCP. We purified the two enzymes which catalyze the interconversion of DCP to ECH, and their enzymatic properties were compared. Both enzymes, enzyme I_a and enzyme I_b, seemed to be halohydrin hydrogen-halide-lyase, since they catalyze the conversion of several halohydrins to the corresponding epoxides with liberation of hydrogen-halide and its reverse reaction. The two enzymes differed with respect to enantioselectivity for DCP conversion to ECH, molecular mass, substrate specificity, and immunological properties. These facts indicate that each halohydrin hydrogen-halide-lyase of *Corynebacterium* sp. strain N-1074 is phylogenetically different, but not as a result of enzyme evolution by mutation of a gene. Enzyme I_a was immunologically identical to halohydrin hydrogen-halide-lyase purified from *E. coli* JM109/pST001 carrying a gene from *Corynebacterium* sp. strain N-1074 (21); also, their molecular masses and substrate specificities were almost the same. Furthermore, the N-terminal amino acid sequences of both enzymes were also identical (data not shown). These facts indicate that the halohydrin hydrogen-halide-lyase of *E. coli* JM109/pST001 is the enzyme I_a of *Corynebacterium* sp. strain N-1074.

Recently, another halohydrin hydrogen-halide-lyase gene from *Corynebacterium* sp. strain N-1074 was cloned and highly expressed in *E. coli* (unpublished data). Its enzymatic

TABLE 3. Comparison of the substrate specificities of enzyme I_a and enzyme I_b[a]

Substrate	Relative activity (%)	
	Enzyme I_a	Enzyme I_b
1,3-Dichloro-2-propanol	100[b]	100[b]
1,3-Dibromo-2-propanol	20,000	166
2,3-Dichloro-1-propanol	0.15	0.12
3-Chloro-1,2-propanediol	35.1	0.98
2-Chloroethanol	0.19	0.10
2-Bromoethanol	55.8	9.15
4-Chloro-3-hydroxybutyronitrile	12.8	3.33

[a] Dehalogenation activities were assayed by the method described in Materials and Methods. Products of the reaction were identified by gas-chromatographic mass spectroscopy as described in the text.

[b] The formation rates of halide from DCP, corresponding to 3.27 μmol/min/mg of protein (enzyme I_a) and 103 μmol/min/mg of protein (enzyme I_b), were taken as 100%.

properties were almost the same as those of enzyme I_b from *Corynebacterium* sp. strain N-1074. Enzyme I_b is of interest with regard to prochiral stereospecificity, since it converts DCP into *R*-rich ECH. To our knowledge, this is the first carbon-halogen cleavage enzyme with prochiral stereospecificity. Further studies investigating the detailed properties of enzyme I_b are under way.

Two kinds of ECH-hydrolyzing enzyme activity were also found in crude extracts of *Corynebacterium* sp. strain N-1074, and the two enzymes also differed with respect to enantioselectivity for the conversion of ECH to MCP. Further studies of these enzymes, which seem to be epoxide hydrolase, are also in progress. Thus, it can be concluded that the optical purity of (*R*)-MCP formed from DCP may reflect a conjugated system of at least four enzymes involved in the transformation of DCP into MCP.

ACKNOWLEDGMENT

We thank J. Imanishi, Kyoto Prefectural University of Medicine, for antibody preparation and helpful advice.

REFERENCES

1. **Asano, Y., K. Fujishiro, Y. Tani, and H. Yamada.** 1982. Aliphatic nitrile hydratase from *Arthrobacter* sp. J-1. Purification and characterization. Agric. Biol. Chem. **46**:1165–1174.
2. **Asano, Y., Y. Tani, and H. Yamada.** 1980. A new enzyme "nitrile hydratase" which degrades acetonitrile in combination with amidase. Agric. Biol. Chem. **44**:2251–2252.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Castro, C. E., and E. W. Bartnicki.** 1968. Biodehalogenation, epoxidation of halohydrins, epoxide opening, and transhalogenation by a *Flavobacterium* sp. Biochemistry **7**:3213–3218.
5. **Fiorini, M., and C. Valentini.** November 1983. U.S. patent 4,413,142.
6. **Goldman, P., G. W. A. Milne, and D. B. Keister.** 1968. Carbon-halogen bond cleavage. III. Studies on bacterial halidohydrolases. J. Biol. Chem. **243**:428–434.
7. **Hamaguchi, S., T. Ohashi, and K. Watanabe.** 1986. Lipase-catalyzed stereoselective hydrolysis of 2-acyloxy-3-chloropropyl *p*-toluenesulfonate. Agric. Biol. Chem. **50**:375–380.
8. **Iwasaki, I., S. Utsumi, and T. Ozawa.** 1952. New colorimeric determination of chloride using mercuric thiocyanate and ferric ion. Bull. Chem. Soc. Jpn. **25**:226.
9. **Janssen, D. B., J. Gerritse, J. Brackman, C. Kalk, D. Jager, and B. Witholt.** 1988. Purification and characterization of a bacterial dehalogenase with activity toward halogenated alkanes, alcohols and ethers. Eur. J. Biochem. **171**:67–72.
10. **Janssen, D. B., D. Jager, and B. Witholt.** 1987. Degradation of *n*-haloalkanes and α,ω-dihaloalkanes by wild-type and mutants of *Arthrobacter* sp. strain GJ70. Appl. Environ. Microbiol. **53**:561–566.
11. **Janssen, D. B., A. Scheper, L. Dijkhuizen, and B. Witholt.** 1985. Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. **49**:673–677.
12. **Kasai, N., K. Tsujimura, K. Unoura, and T. Suzuki.** 1990. Degradation of 2,3-dichloro-1-propanol by a *Pseudomonas* sp. Agric. Biol. Chem. **54**:3185–3190.
13. **Kawasaki, H., N. Tone, and K. Tonomura.** 1981. Plasmid-determined dehalogenation of haloacetates in *Moraxella* species. Agric. Biol. Chem. **45**:29–34.
14. **Kawasaki, H., N. Tone, and K. Tonomura.** 1981. Purification and properties of haloacetate halidohydrolase specified by a plasmid from *Moraxella* sp. strain B. Agric. Biol. Chem. **45**:35–42.
15. **Keuning, S., D. B. Janssen, and B. Witholt.** 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. **163**:635–639.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Little, M., and P. A. Williams.** 1971. A bacterial halidohydrolase. Its purification, some properties and its modification by specific amino acid reagents. Eur. J. Biochem. **21**:99–109.
18. **Motosugi, K., N. Esaki, and K. Soda.** 1982. Purification and properties of a new enzyme, DL-2-haloacid dehalogenase, from *Pseudomonas* sp. J. Bacteriol. **150**:522–527.
19. **Motosugi, K., N. Esaki, and K. Soda.** 1984. Enzymatic preparation of D- and L-lactic acid from racemic 2-chloropropionic acid. Biotechnol. Bioeng. **26**:805–806.
20. **Nagasawa, T., M. Kobayashi, and H. Yamada.** 1988. Optimum culture conditions for the production of benzonitrilase by *Rhodococcus rhodochrous* J1. Arch. Microbiol. **150**:89–94.
21. **Nagasawa, T., T. Nakamura, F. Yu, I. Watanabe, and H. Yamada.** 1992. Purification and characterization of halohydrin hydrogen-halide-lyase from a recombinant *Escherichia coli* containing the gene from a *Corynebacterium* sp. Appl. Microbiol. Biotechnol. **36**:478–482.
22. **Nagasawa, T., K. Takeuchi, V. Nardi-Dei, Y. Mihara, and H. Yamada.** 1991. Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1. Appl. Microbiol. Biotechnol. **34**:783–788.
23. **Nakamura, T., F. Yu, W. Mizunashi, and I. Watanabe.** 1991. Microbial transformation of prochiral 1,3-dichloro-2-propanol into optically active 3-chloro-1,2-propanediol. Agric. Biol. Chem. **55**:1931–1933.
24. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
25. **Ruisinger, S., U. Klages, and F. Lingens.** 1976. Degradation of 4-chlorobenzoic acid by an *Arthrobacter* species. Arch. Microbiol. **110**:253–256.
26. **Scholten, J. D., K.-H. Chang, P. C. Babbitt, H. Charest, M. Sylvestre, and D. Dunaway-Mariano.** 1991. Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. Science **253**:182–185.
27. **Scholtz, R., T. Leisinger, F. Suter, and A. M. Cook.** 1987. Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. J. Bacteriol. **169**:5016–5021.
28. **Slater, J. H., D. Lovatt, A. J. Weightman, E. Senior, and A. T. Bull.** 1979. The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. J. Gen. Microbiol. **114**:125–136.
29. **Smith, J. M., K. Harrison, and J. Colby.** 1990. Purification and characterization of D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23. J. Gen. Microbiol. **136**:881–886.
30. **Takahashi, H., Y. Nakamura, M. Ogura, Y. Shimada, and K. Watanabe.** June 1987. European patent 224,246.
31. **Thiele, J., R. Muller, and F. Lingens.** 1987. Initial characterization of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. FEMS Microbiol. Lett. **41**:115–119.
32. **van den Wijngarrd, A. J., D. B. Janssen, and B. Witholt.** 1989. Degradation of epichlorohydrin and halohydrins by bacterial cultures isolated from freshwater sediment. J. Gen. Microbiol. **135**:2199–2208.
33. **van den Wijngarrd, A. J., P. T. W. Reuvekamp, and D. B. Janssen.** 1991. Purification and characterization of haloalcohol dehalogenase from *Arthrobacter* sp. AD2. J. Bacteriol. **173**:124–129.
34. **Weightman, A. J., and J. H. Slater.** 1980. Selection of *Pseudomonas putida* strains with elevated dehalogenase activities by continuous culture growth on chlorinated alkanoic acid. J. Gen. Microbiol. **121**:187–193.
35. **Weightman, A. J., A. L. Weightman, and J. H. Slater.** 1982. Stereospecificity of 2-monochloropropionate dehalogenation by the two dehalogenases of *Pseudomonas putida* PP3: evidence for two different dehalogenation mechanisms. J. Gen. Microbiol. **128**:1755–1762.
36. **Yokota, T., H. Fuse, T. Omori, and Y. Minoda.** 1986. Microbial dehalogenation of haloalkanes mediated by oxygenase or halidohydrolase. Agric. Biol. Chem. **50**:453–460.