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# Purification and characterization of halohydrin hydrogen-halide lyase from a recombinant *Escherichia coli* containing the gene from a *Corynebacterium* sp.

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**Summary.** An enzyme catalysing the interconversion of 1,3-dichloro-2-propanol (DCP) to epichlorohydrin (ECH) was purified from *Escherichia coli* JM109/pST001, which carried the gene from *Corynebacterium* sp. N-1074. The enzyme was crystallized by the addition of ammonium sulphate. The enzyme had a relative molecular mass ($M_r$) of about 105 000 and consisted of four subunits identical in $M_r$ (approx. 28 000). The enzyme catalysed both the transformation of various halohydrins into the corresponding epoxides with liberation of halide and its reverse reaction. These facts indicated that the enzyme was halohydrin hydrogen-halide-lyase.

## Introduction

Many halogenated compounds are produced as herbicides and pesticides, and several of them cause severe environmental pollution. Several microorganisms growing on halogenated compounds have been isolated, and there have been many investigations focused on the enzymatic cleavage of the carbon-halogen bonds. Previously, an enzyme catalysing the conversion of halohydrins to the corresponding epoxides was found in a *Flavobacterium* sp. (Castro and Bartnicki 1968), and the stereochemistry of epoxide formation from halohydrin has been examined (Bartnicki and Castro 1969).

Epichlorohydrin and halohydrin-degrading bacterial strains from freshwater sediments have also been isolated by van den Wijingaard et al. (1989), who proposed routes for the degradation of epichlorohydrin involving the action of an epoxide-forming halohydrin dehalogenase.

Recently, we isolated bacterial strains that can transform 1,3-dichloro-2-propanol (DCP) into optically active 3-chloro-1,2-propanediol (MCP) (Nakamura et al. 1991a). One of these strains, *Corynebacterium* sp. N-1074, was examined in detail, and then it was suggested that this strain exhibited activities for the transformation of DCP into MCP via epichlorohydrin (ECH) as an intermediate. Furthermore, genes involved in the interconversion of DCP to ECH from this strain have been cloned and expressed in *Escherichia coli* (unpublished data).

In the present paper, we describe the purification and the properties of the enzyme catalysing the interconversion of DCP to ECH from the recombinant *E. coli*.

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## Materials and methods

### *Materials*

DEAE-Sephacel, phenyl-Sepharose CL-4B and octyl-Sepharose CL-4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, Mich., USA). Carrier Ampholyte (pH 3.5–10.0) was purchased from Pharmacia LKB Biotechnology (Bromma, Sweden). All other chemicals used were from commercial sources and were of reagent grade.

### *Microorganism and culture conditions*

*E. coli* JM109/pST001, in which the gene involved in the interconversion of DCP to ECH from *Corynebacterium* sp. N-1074 was cloned (unpublished data), was used in this study.

Subcultivation was carried out at 37° C for 12 h with reciprocal shaking in a test tube containing 4 ml LB medium consisting of 1.0% tryptone, 0.5% yeast extract, 0.5% NaCl (pH 7.0) supplemented with 50 mg/l of sodium ampicillin. Then, 4 ml of the subculture was inoculated into a 2-l shaking flask containing 800 ml LB medium supplemented with 50 mg/l sodium ampicillin and 1 mM isopropyl-β-D(−)-thiogalactopyranoside (IPTG). Cultivation was carried out at 37° C for 16 h with reciprocal shaking.

### *Enzyme assay*

Halohydrin hydrogen-halide-lyase was routinely assayed by determining the halide release from DCP. A standard reaction mixture (1.0 ml) containing 50 μmol DCP, 100 μmol TRIS-$H_2SO_4$ buf-

fer (pH 8.0) and an appropriate amount of enzyme was incubated at 20° C for 10 min. Halide formed was determined spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulphate as described by Iwasaki et al. (1952). One unit of enzyme activity was defined as the amount of the enzyme that catalysed the formation of 1 μmol halide/min. In some inhibition experiments, the ECH formed was measured by gas-liquid chromatography (GLC).

Protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford (1976) using a dye reagent supplied by Bio-Rad (Richmond, Calif., USA). For halohydrin hydrogen-halide-lyase from *E. coli* JM109/pST001, protein was determined from absorbance at 280 nm. The absorption coefficient was calculated to be 0.55 $mg^{-1} \cdot ml \cdot cm^{-1}$ by absorbance at 280 nm and dry-mass determination.

### *Purification of halohydrin hydrogen-halide-lyase*

All purification steps were performed at 0–5° C. TRIS-$H_2SO_4$ buffer (pH 8.0) was used throughout the purification steps unless otherwise specified.

*Step 1. Preparation of cell-free extracts.* Washed cells harvested from 18 l culture were suspended in about 600 ml of 0.1 M TRIS buffer and disrupted for 15 min with an ultrasonic oscillator (19 kHz, Insonator model 201M, Kubota, Tokyo, Japan). Cell debris was removed by centrifugation at 15000*g* for 1 h. The supernatant solution was used as the cell-free extract.

*Step 2. Ammonium sulphate fractionation.* Solid ammonium sulphate was added to the cell-free extract to 30% saturation with stirring, and the resulting precipitate was removed by centrifugation at 15000*g* for 1 h. The supernatant solution was further saturated with ammonium sulphate to 60%. The resulting precipitate was collected by centrifugation at 15000*g* for 1 h, dissolved in 10 mM TRIS buffer and dialysed against the same buffer.

*Step 3. First DEAE-Sephacel column chromatography.* The enzyme solution was applied to a DEAE-Sephacel column (3.4 × 30cm) equilibrated with 10 mM TRIS buffer. The column was washed with 1 l of 10 mM TRIS buffer and then with 600 ml of 10 mM TRIS buffer containing 0.1 M ammonium sulphate. The enzyme was eluted with 10 mM TRIS buffer containing 0.2 M ammonium sulphate. The active fractions were combined, and dialysed against 10 mM TRIS buffer.

*Step 4. Second DEAE-Sephacel column chromatography.* The enzyme solution from step 3 was applied to a DEAE-Sephacel column (2.6 × 25 cm) equilibrated with 10 mM TRIS buffer, and the column was washed with the same buffer. The enzyme was eluted with a 0–0.3 M linear gradient of ammonium sulphate in 10 mM TRIS buffer (total volume, 600 ml). The active fractions were combined, and dialysed against 10 mM TRIS buffer.

*Step 5. Ammonium sulphate fractionation.* The enzyme solution from step 4 was fractionated by the stepwise addition of solid ammonium sulphate to 30, 35, 40, 45, 50, 55 and 60% saturation. The precipitate was collected at each saturation point by centrifugation at 15000*g* for 1 h, and dissolved in 10 mM TRIS buffer. The active fractions (35–50% saturation precipitate) were combined, and dialysed against 10 mM TRIS buffer containing 20% saturated ammonium sulphate.

*Step 6. Phenyl-Sepharose CL-4B column chromatography.* The enzyme solution from step 5 was applied to a phenyl-Sepharose CL-4B column (1.8 × 25 cm) equilibrated with 10 mM TRIS buffer containing 20% saturated ammonium sulphate, and washed with 10 mM TRIS buffer containing 20% and 10% saturated ammonium sulphate. The enzyme was eluted by lowering the ionic strength of ammonium sulphate (5–2.5% saturated) in the same buffer. The active fractions were combined, and dialysed against 10 mM TRIS buffer containing 20% saturated ammonium sulphate.

*Step 7. Octyl-Sepharose CL-4B column chromatography.* The enzyme solution from step 6 was placed on an octyl-Sepharose CL-4B column (1.8 × 15 cm) equilibrated with 10 mM TRIS buffer containing 20% saturated ammonium sulphate, and eluted with the same buffer. The active fractions were combined, and solid ammonium sulphate was added to the enzyme solution to give 60% saturation.

*Step 8. Crystallization.* The precipitate from step 7 was centrifuged at 15000*g* for 1 h, and dissolved in 10 mM TRIS buffer to obtain a protein concentration of about 30 mg/ml. Solid ammonium sulphate was added slowly to the enzyme solution at 0° C while stirring gently with a glass rod, until the induced turbidity remained upon stirring. The presence of crystallized materials was evident from a silky sheen that appeared upon stirring the mixture.

### *Relative molecular mass ($M_r$) determination*

The $M_r$ of the denaturated protein was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed in 12% polyacrylamide slab gels by the method of Laemmli (1970). The gels were stained for protein with Coomassie brilliant blue R-250 and destained in ethanol/acetic acid/$H_2O$ (3:1:6, v/v/v). The $M_r$ of the enzyme subunit was determined from the mobilites of standard proteins.

The $M_r$ of the native enzyme was estimated by HPLC on a TSK G3000SW column (0.75 × 60 cm, Tosoh, Tokyo, Japan) at a flow rate of 0.7 ml/min with elution of 0.1 M TRIS-$H_2SO_4$ buffer (pH 8.0) containing 0.2 M NaCl at room temperature. The absorbance of the effluent was recorded at 280 nm. The $M_r$ of the enzyme was calculated from the mobilities of the standard proteins, glutamate dehydrogenase ($M_r$ = 290000), lactate dehydrogenase (142000), enolase (67000), adenylate kinase (32000) and cytochrome *c* (12400) (Oriental Yeast Co., Osaka, Japan).

### *Other analytical measurements*

The isoelectric point of the enzyme was determined using the method of Winter and Karlson (1976).

The amount of halohydrins and epoxides was determined by GLC, using a Shimadzu GC-7A system (Kyoto, Japan) equipped with a flame ionization detector with a 15 m capillary column of ULBON HR-1 (Chromatopacking Center, Kyoto, Japan). The injection port and column temperatures were set at 250 and 100° C, respectively. Helium at a pressure of 0.2 $kg/cm^2$ was used as carrier gas.

Products of the enzymatic reaction were analysed and then identified by GC mass spectrometry (MS) (M-80, Hitachi, Tokyo, Japan).

Metal analysis of enzyme samples was performed with an inductively coupled radiofrequency plasma spectrophotometer, Shimadzu ICPV-1000 (27120 MHz).

## Results

### *Purification of halohydrin hydrogen-halide-lyase*

By using the purification procedures described in Materials and methods, the enzyme was purified tenfold with a yield of 50% from the cell-free extract. A pho-

tomicrograph of the crystallized enzyme is shown in Fig. 1. The activity of the crystallized enzyme was 2.75 units/mg protein under the standard reaction conditions. The results of the purification are summarized in Table 1. The purified enzyme was very stable, and was stored in 10 mM TRIS buffer at 0° C for 6 months without loss of activity.

*Molecular mass and subunit structure*

The purified enzyme showed only one band on SDS-PAGE (Fig. 2). The $M_r$ corresponding to the band was estimated to be about 28000 based on the mobility relative to those of the reference proteins. The $M_r$ of the enzyme was determined to be about 105000 by HPLC on a TSK G3000SW column. These results indicate that the enzyme is a tetrameric protein consisting of four subunits identical in $M_r$.

The 25 residues of the N-terminal amino acid sequence were determined, and it was confirmed that those were identical to the predicted amino acid sequences from the nucleotide sequence (data not shown). The isoelectric point of the enzyme was 4.8.

The qualitative analysis of metals in the enzyme solution was performed with an inductively coupled radiofrequency plasma spectrophotometer. However, the enzyme contained no metals.

*Transformation of DCP into ECH and its reverse reaction by the enzyme*

A time course of the enzymatic reaction was examined using purified enzyme. ECH and chloride was formed with DCP as the substrate, and DCP was formed with ECH as the substrate in the presence of KCl (Fig. 3A, B). Both reactions were almost stoichiometric. No formation of DCP was detected when ECH was incubated without the addition of KCl. The enzyme followed Michaelis-Menten kinetics in both reactions. The $K_m$ value for DCP on the transformation of DCP into ECH was calculated to be 2.44 mM, and the $K_m$ values for ECH and chloride on the transformation of ECH into DCP in the presence of KCl were 10.6 and 350 mM, respectively. The $V_{max}$ values for the transformation of DCP into ECH and that of ECH to DCP in the presence of KCl were calculated to be 3.13 and 35.0 μmol/min per milligram protein, respectively. Thus, the enzyme catalyses the transformation of DCP into ECH with liberation of chloride and its reverse reaction.

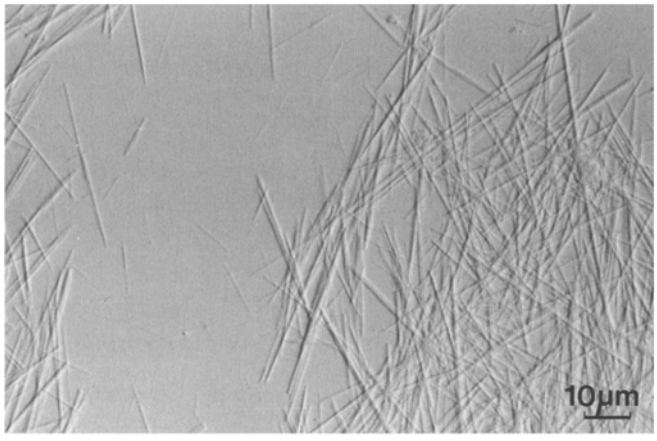


**Fig. 1.** Photomicrograph of crystalline halohydrin hydrogen-halide-lyase

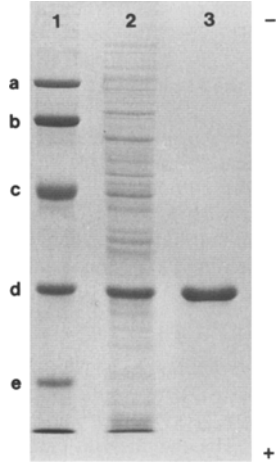


**Fig. 2.** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of halohydrin hydrogen-halide-lyase: *lane 1*, marker proteins: *a*, phosphorylase b (94 kDa); *b*, bovine serum albumin (67 kDa); *c* ovalbumin (43 kDa); *d*, carbonic anhydrase (30 kDa); *e*, soybean trypsin inhibitor (20.1 kDa); *lane 2*, cell-free extract of *Escherichia coli* JM109/pST001 (50 μg); *lane 3*, the enzyme (10 μg)

**Table 1.** Purification of halohydrin hydrogen-halide-lyase from *Escherichia coli* JM109/pST001

| Step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| 1. Cell-free extract | 4125 | 1093 | 0.265 | 100 |
| 2. $(NH_4)_2SO_4$ (0.3–0.6) | 2387 | 993 | 0.416 | 90.9 |
| 3. DEAE-Sephacel (1st) | 1214 | 894 | 0.736 | 81.8 |
| 4. DEAE-Sephacel (2nd) | 710 | 734 | 1.03 | 67.2 |
| 5. $(NH_4)_2SO_4$ (0.35–0.50) | 437 | 717 | 1.64 | 65.6 |
| 6. Phenyl-Sepharose CL-4B | 257 | 679 | 2.64 | 62.1 |
| 7. Octyl-Sepharose CL-4B | 222 | 612 | 2.76 | 56.0 |
| 8. Crystallization | 199 | 547 | 2.75 | 50.0 |

Protein was determined according to the method of Bradford (1976) in steps 1 and 2, and by absorbance at 280 nm using the absorption coefficient of 0.55 $mg^{-1} \cdot ml \cdot cm^{-1}$ in steps 3–8

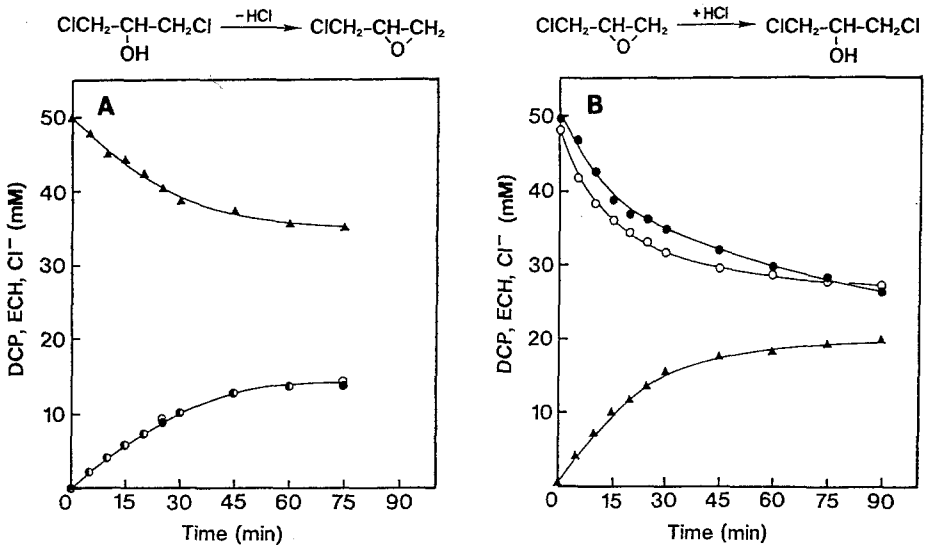


**Fig. 3.** Time course of the transformation of 1,3-dichloro-2-propanol (DCP) into epichlorohydrin (ECH) (**A**) and its reverse reaction (**B**). The reactions were performed at 20° C in a reaction mixture (50 ml) containing 2.5 mmol DCP, 5 mmol TRIS-$H_2SO_4$ (pH 8.0), and 250 µg of the enzyme (**A**), or 2.5 mmol ECH, 2.5 mmol KCl, 5 mmol TRIS-$H_2SO_4$ (pH 7.2), and 250 µg of the enzyme (**B**): ●, ECH; ○, $Cl^-$; ▲, DCP

*Effects of temperature and pH*

The effects of temperature and pH on the activity of the enzyme were examined. The optimum pH was determined to be 8.0–9.0 for the transformation of DCP into ECH, and 7.2–7.5 for its reverse reaction. Maximal activity was observed at 55° C with DCP as the substrate in 100 mM TRIS-$H_2SO_4$ buffer (pH 8.0). The enzyme retained the following activities after incubation in 100 mM TRIS-$H_2SO_4$ buffer (pH 8.0) at different temperatures for 10 min: 45° C, 100%; 50° C, 91.2%; 55° C, 26.4% and 60° C, 5.6%.

*Inhibitors*

The inhibitory effect of various compounds on the enzyme activity was examined in a reaction mixture containing 1 mM inhibitors under the standard reaction conditions. $AgNO_3$ and $HgCl_2$ inhibited 53% and 26%, respectively. However, no significant inhibition was caused by other thiol reagents such as *N*-ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoic acid) and *p*-chloromercuribenzoate. Carbonyl reagents such as hydroxylamine, phenylhydrazine and semicarbazide were also inert.

*Substrate specificity*

The enzyme activity toward various halohydrins was examined, and it was found that the enzyme could catalyse the release of halide from several substrates (Table 2). The activity toward 1,3-dibromo-2-propanol was more than tenfold that with DCP, but the affinity for 1,3-dibromo-2-propanol was lower than that for DCP. The activity toward 2-bromoethanol was also much higher than that towards the chlorinated analogue, 2-chloroethanol. These products were identified by GC-MS and it was found that halohydrins were converted to the corresponding epoxides by the enzyme. These epoxides were also transformed into the corresponding 1-chloro-2-ol compounds in the presence of KCl by the enzymatic reaction (data not shown). These results show that the enzyme is "halohydrin hydrogen-halide-lyase", catalysing the transformation of halohydrins into the corresponding epoxides with liberation of halide, and its reverse reaction.

**Table 2.** Substrate specificity of halohydrin hydrogen-halide-lyase from *E. coli* JM109/pST001

| Substrate | Relative activity (%) | $K_m$ (mM) |
|---|---|---|
| 1,3-Dichloro-2-propanol (DCP) | 100 | 2.44 |
| 1,3-Dibromo-2-propanol | 12500 | 7.00 |
| 2,3-Dichloro-1-propanol | 0.30 | |
| 3-Chloro-1,2-propanediol | 37.5 | 16.7 |
| 1-Chloro-2-propanol | 22.7 | |
| 2-Chloroethanol | 0.26 | |
| 2-Bromoethanol | 57.4 | |
| 4-Chloro-3-hydroxybutyronitrile | 14.0 | 0.40 |

The reaction was performed at 20° C in the standard mixture except that various halohydrins were used as substrates. The activity toward DCP, corresponding to 2.75 µmol/min per milligram protein, was taken as 100%

## Discussion

Halohydrin epoxidase, which catalyses the conversion of halohydrins to the corresponding epoxides, has been partially purified from a *Flavobacterium* sp. by Castro and Bartnicki (1968). A similar enzyme activity in bacterial cultures isolated from freshwater sediment was also found by van den Wijingaard et al. (1989). However, only a few studied have been performed on the properties of the enzyme.

We embarked on research attempting to manufacture optically active glycerol derivatives by the methods of enantioselective microbial transformation of prochi-

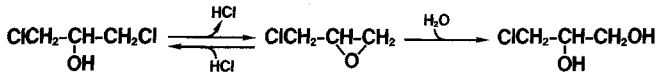


**Fig. 4.** A possible pathway for the transformation of DCP into 3-chloro-1,2-propanediol (MCP) by *Corynebacterium* sp. N-1074

ral starting materials, then found the bacterial strain *Corynebacterium* sp. N-1074 that could transform DCP into (*R*)-MCP (Nakamura et al. 1991a). It was suggested that this strain had enzyme activities for interconverting DCP to ECH and hydrolysing ECH (Fig. 4). Genes involved in the interconversion of DCP to ECH have been cloned and expressed in *E. coli* (unpublished data). Here we purified the enzme from one of the recombinants, *E. coli* JM109/pST001, because an enormous amount of the enzyme was inducibly formed in the cell by IPTG.

The properties of the enzyme were examined in detail, which indicated that the enzyme was halohydrin hydrogen-halide-lyase. To our knowledge this is the first time that halohydrin hydrogen-halide-lyase has been isolated in a crystalline form. At the same time, van den Wijingaard et al. (1991) also purified an enzyme catalysing the transformation of halohydrins into epoxides and its reverse reaction from *Arthrobacter* sp. AD2. They designated the enzyme "haloalcohol dehalogenase". The N-terminal amino acid sequence of their haloalcohol dehalogenase was quite similar to that of the enzyme from *E. coli* JM109/pST001 (unpublished data). The enzymatic properties of the haloalcohol dehalogenase were also similar to those of the enzyme from *E. coli* JM109/pST001, except for the $M_r$ of the native enzyme and the affinity for some substrates. Their "haloalcohol dehalogenase" and our "halohydrin hydrogen-halide-lyase" seem to be similar.

In addition, we found a new catalytic function of halohydrin hydrogen-halide-lyase, the transformation of epoxides into the corresponding $\beta$-hydroxynitrile compounds in the presence of cyanide (Nakamura et al. 1991b). We are presently attempting to apply the enzyme to the production of useful compounds.

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