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# A NEW CATALYTIC FUNCTION OF HALOHYDRIN HYDROGEN-HALIDE-LYASE, SYNTHESIS OF β-HYDROXYNITRILES FROM EPOXIDES AND CYANIDE

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Summary: Halohydrin hydrogen-halide-lyase, which catalyzes the interconversion of halohydrins to epoxides, purified from a recombinant E. coli was found to catalyze the transformation of 1,2-epoxybutane into β-hydroxyvaleronitrile in the presence of cyanide. Chloride inhibited competitively the formation of β-hydroxyvaleronitrile. The enzyme also catalyzed the transformation of some other epoxides into the corresponding β-hydroxynitriles in the presence of cyanide. 

Recently, we isolated bacterial strains which could transform 1,3-dichloro-2-propanol into optically active 3-chloro-1,2-propanediol. We examined one of these strains, Corynebacterium sp. N-1074, in detail and then it was suggested that this strain has both activities for the interconversion of 1,3-dichloro-2-propanol to epichlorohydrin (equation I) and for the conversion of epichlorohydrin to 3-chloro-1,2-propanediol (equation II) (1). An enzyme catalyzing the interconversion of 1,3-dichloro-2-propanol to epichlorohydrin was purified from Escherichia

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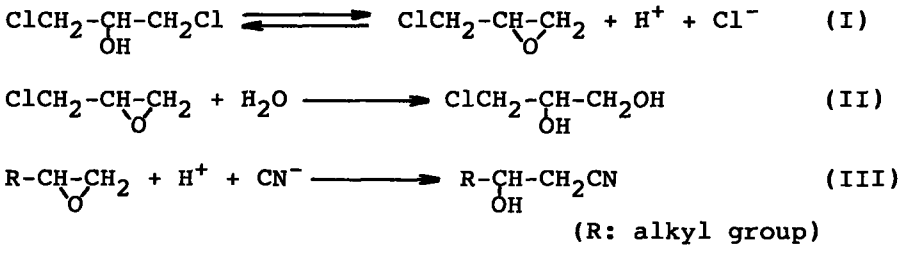
coli JM109/pST001 that carried its gene from Corynebacterium sp. N-1074, and then characterized (2). The enzyme was found to be a halohydrin hydrogen-halide-lyase which catalyzed the transformation of various halohydrins into the corresponding epoxides with liberation of halide and its reverse reaction. Further studies on the catalytic properties of the enzyme revealed that the enzyme also catalyzes the synthesis of β-hydroxynitriles from epoxides and cyanide (equation III).

This paper describes the new catalytic activity, the nucleophilic substitution reaction of epoxides by cyanide, catalyzed by halohydrin hydrogen-halide-lyase.




## MATERIALS AND METHODS

Chemicals: Epoxides used in this study were purchased from Nakalai Tesque (Kyoto, Japan). γ-Chloro-β-hydroxybutyronitrile was synthesized from 2-hydroxy-3-chloropropyl p-toluenesulfonate and potassium cyanide (3). 2-Hydroxy-3-chloropropyl p-toluenesulfonate was prepared from epichlorohydrin and p-toluenesulfonic acid (4). β-Hydroxybutyronitrile and β-hydroxyvaleronitrile were also synthesized by a similar method using 1,2-epoxypropane and 1,2-epoxybutane instead of epichlorohydrin, respectively. These β-hydroxynitriles were purified by distillation, physicochemically identified and used as standards.

Enzyme: Halohydrin hydrogen-halide-lyase purified from E. coli JM109/pST001 that carried its gene from Corynebacterium sp. N-1074 was used in this study (2).

Analytical methods: Epoxides and β-hydroxynitriles were determined by gas liquid chromatography (GLC). GLC was performed with 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). Cyanide was determined from the absorbance at 575 nm using N-chlorosuccinimide-succinimide reagent and

barbituric acid-pyridine reagent (5). The products of the enzyme reaction were isolated and identified by infrared, mass, $^{1}$H-NMR and $^{13}$C-NMR spectroscopy. Infrared and mass spectra were recorded with a Perkin Elmer 1710 FT-IR (Norwalk Conn., USA) and a Hitachi M-80 (Tokyo, Japan), respectively. $^{1}$H- and $^{13}$C-NMR spectra were recorded in $CDCl_3$ using tetramethylsilane as an internal standard with a Nihondenshi JNM GX-270 (Tokyo, Japan).

## RESULTS AND DISCUSSION

The synthesis of β-hydroxyvaleronitrile proceeded as a function of enzyme concentration and incubation time (Figure 1A and 1B), when 1,2-epoxybutane and cyanide were incubated with halohydrin hydrogen-halide-lyase. Only weak spontaneous formation of β-hydroxyvaleronitrile was observed under the reaction conditions without the addition of the enzyme. The boiled enzyme did not catalyze the synthesis of β-hydroxyvaleronitrile. The enzyme had an optimal reactivity of around pH 8.0 for the synthesis of β-hydroxyvaleronitrile in 100 mM Tris-$H_2SO_4$ buffer (Figure 2). The enzyme followed Michaelis-Menten kinetics in the

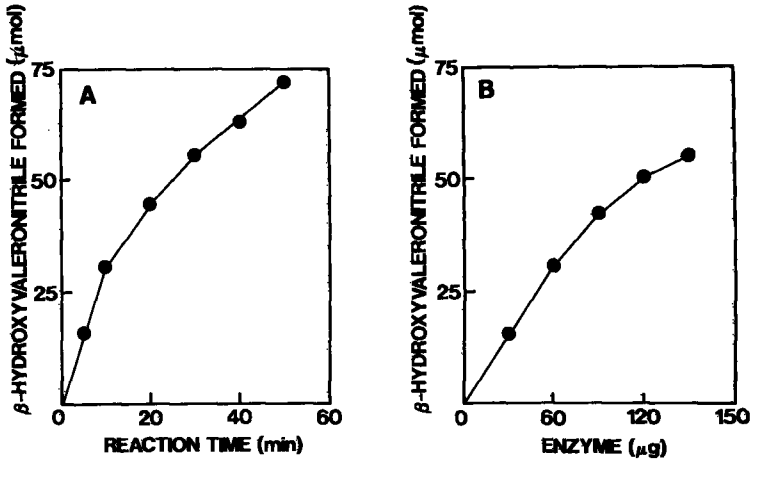
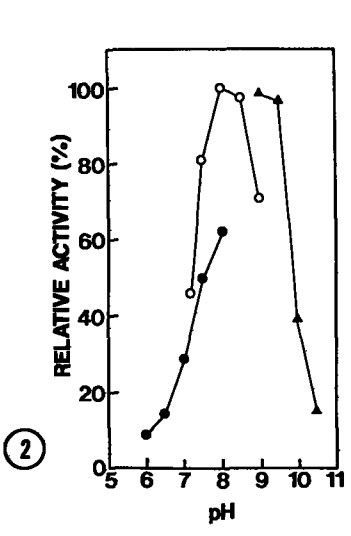



Figure 1. Synthesis of β-hydroxyvaleronitrile as a function of the enzyme concentration and incubation time. The reaction was carried out at 20°C in a reaction mixture (1 ml) containing 1,2-epoxybutane (200 μmol), potassium cyanide (200 μmol), Tris-$H_2SO_4$ buffer (100 μmol), pH 8.0, and the enzyme. In Figure 1A, 60 μg of enzyme protein was used and in Figure 1B, incubation was carried out for 10 min.



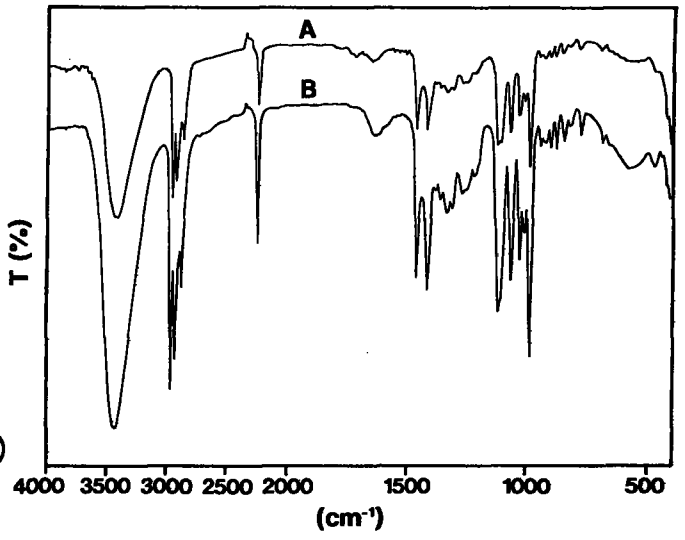




Figure 2. Effects of pH on synthesis of β-hydroxyvaleronitrile by the enzyme. The reaction was carried out at 20°C for 10 min in a reaction mixture (1 ml) containing 1,2-epoxybutane (50 μmol), potassium cyanide (50 μmol), an appropriate amount of the enzyme and following buffer: Potassium phosphate (●), Tris-$H_2SO_4$ (○), $(NH_4)_2SO_4$-$NH_4Cl$ (▲).

Figure 3. IR spectra of authentic (A) and isolated (B) β-hydroxyvaleronitrile.

reaction. The apparent $K_m$ values were calculated to be 330 mM for 1,2-epoxybutane and 135 mM for cyanide. The maximum velocity, $V_{max}$, of the synthesis of β-hydroxyvaleronitrile was calculated to be 90.9 μmol/min/mg. The enzymatically synthesized β-hydroxyvaleronitrile was isolated from a large scale reaction mixture. Incubation was carried out at 20°C for 2 h in a reaction mixture containing 1,2-epoxybutane (10 mmol), potassium cyanide (10 mmol), the enzyme (3 mg) and Tris-$H_2SO_4$ buffer (5 mmol), pH 8.0, in a total volume of 50 ml. The product was extracted from the reaction mixture with diethylether (50 ml x 3). The extract was evaporated to remove the solvent and the residual 1,2-epoxybutane after drying by sodium sulfate anhydrous. The infrared spectrum of the product thus obtained was in good agreement with that of authentic β-hydroxyvaleronitrile (Figure 3). Identification by $^1$H-NMR, $^{13}$C-NMR and mass

Table 1. The enzymatic formation of β-hydroxynitriles from some epoxides in the presence of cyanide

| Substrate | Product[1] | Relative activity[2] (%) |
|---|---|---|
| 1,2-Epoxybutane | β-Hydroxyvaleronitrile | 100 |
| 1,2-Epoxypropane | β-Hydroxybutyronitrile | 36.2 |
| Epichlorohydrin | γ-Chloro-β-hydroxy-butyronitrile | 67.1 |

The reaction was carried out at 20°C in a reaction mixture (50 ml) consisting of 100 mM Tris-$H_2SO_4$ buffer (pH 8.0), 50 mM substrate and potassium cyanide with an appropriate amount of the enzyme.
[1] Products formed were identified by $^1$H-NMR and $^{13}$C-NMR: β-hydroxybutyronitrile, $^1$H-NMR ($CDCl_3$): 1.32 (3H, d), 2.52 (2H, m), 3.56 (1H, s), 4.14 (1H, m) ppm; $^{13}$C-NMR ($CDCl_3$): 22.5, 27.4, 63.8, 118 ppm. γ-chloro-β-hydroxybutyronitrile, $^1$H-NMR ($CDCl_3$):2.73 (2H, m), 3.50 (1H, s), 3.65 (2H, d), 4.20 (1H, m) ppm; $^{13}$C-NMR ($CDCl_3$): 23.3, 47.3, 61.6, 98.4 ppm. The analytical data of β-hydroxyvaleronitrile are described in the text.
[2] The activity toward 1,2-epoxybutane, corresponding to 14.0 μmol/min/mg protein, was taken as 100%.

spectroscopy was also performed. $^1$H-NMR ($CDCl_3$): 0.95 (3H, t), 1.59 (2H, m), 2.54 (2H, m), 3.59 (1H, s), 3.85 (1H, m) ppm; $^{13}$C-NMR ($CDCl_3$): 9.92, 25.5, 29.8, 68.9, 119 ppm; CI(i-butane)-MS: m/z 100 $(M+H)^+$. The enzymatic formation of β-hydroxynitriles using some epoxides was also examined (Table 1). 1,2-Epoxypropane and epichlorohydrin were also transformed into β-hydroxybutyronitrile and γ-chloro-β-hydroxybutyronitrile, respectively, in the presence of potassium cyanide by the enzyme. Its reverse reaction, liberation of cyanide from these β-hydroxynitriles was also examined. Then these β-hydroxynitriles (50 mM) were incubated with the enzyme in 100 mM Tris-$H_2SO_4$ buffer (pH 8.0) at 20°C, but no formation of cyanide was observed. Thus once formed β-hydroxynitriles were not attacked by the enzyme.

The effect of chloride on the formation of β-hydroxyvaleronitrile by the enzyme was examined. The presence of chloride in the reaction mixture caused an

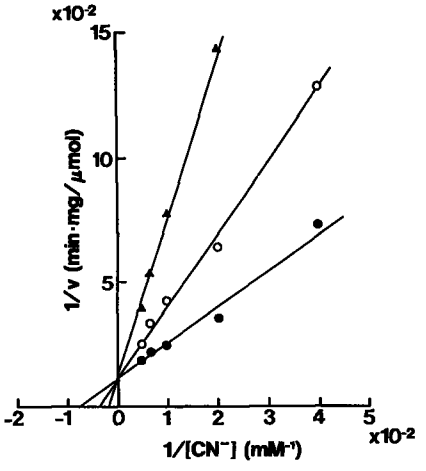



Figure 4. Lineweaver-Burk plots for the formation of β-hydroxyvaleronitrile. The initial velocity was measured at 20°C in a reaction mixture (1 ml, pH 8.0) containing Tris-$H_2SO_4$ buffer (100 µmol), 1,2-epoxybutane (200 µmol), an appropriate amount of the enzyme and various concentrations of potassium cyanide in the presence of 0 mM (●), 25 mM (○), 50 mM (▲) potassium chloride.

inhibition for the formation of β-hydroxyvaleronitrile, and the inhibition was found to be competitive with cyanide as shown in Figure 4. The $K_m$ value for cyanide and the $K_i$ value for chloride were calculated to be 135 mM and 17 mM, respectively. Cyanide also inhibited competitively the enzymatic formation of 1-chloro-2-butanol from 1,2-epoxybutane and chloride (data not shown). These facts suggest that both reactions are catalyzed at the same active site of the enzyme although the affinity for each ion for the enzyme is fairly different.

An enzyme, which catalyzes the transformation of halohydrins into the corresponding epoxides with liberation of halide and its reverse reaction, was previously found in a Flavobacterium sp. by Castro and Bartnicki, and then partially purified (6). van den Wijngaard et al. also purified and characterized a similar kind of enzyme from Arthrobacter sp. AD2 (7). We also purified a similar enzyme from a recombinant E. coli JM109/pST001 that carried its

gene from Corynebacterium sp. N-1074 (2). Then we crystallized and characterized the enzyme, and designated it "halohydrin hydrogen-halide-lyase". Further studies on the application of the enzyme for production of useful compounds revealed a new catalytic function of the halohydrin hydrogen-halide-lyase, the transformation of epoxides into β-hydroxynitriles in the presence of cyanide. Both formations of β-hydroxynitriles and halohydrins from epoxides seemed to be catalyzed at the same active site of the enzyme. This suggests that reactions with epoxides and some other nucleophilic reagents also might be catalyzed by the enzyme. These reactions by the enzyme might follow $SN_2$ type nucleophilic substitution like a chemical reaction, but the difference in the affinity of the enzyme for chloride and cyanide strongly suggests that the interaction between the enzyme and these ions affects the reactions. Studies on the enantioselectivity for the formation of β-hydroxynitriles by the enzyme are under way.

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