Purification, Properties and Reactivity of the Esterase from *Micrococcus* sp.

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The esterase from *Micrococcus* sp., which hydrolyzes *n*-propyl-2-fluorocyclopropanecarboxylate (3) enantioselectively, was highly purified by three types of chromatography. The purified enzyme was inactivated by Hg and diisopropyl fluorophosphate (DFP). It was a monomer with a molecular weight of about 35000. The enzyme exhibits esterase activity towards many aliphatic propyl esters. The enantioselectivity for substrate (3) using purified enzyme did not differ from that of crude enzyme.

Key words quinolone analogue; *n*-propyl-2-fluorocyclopropanecarboxylate; *Micrococcus* sp.; substrate specificity

7-[7(*S*)-7-Amino-5-azaspiro[2.4]heptan-5-yl]-8-chloro-6-fluoro-1-quinolinecarboxylic acid (1), a new-generation antibacterial quinolone carboxylic acid, has antibacterial activity with a broad spectrum, and was discovered by Hayakawa *et al.*¹⁾ A number of syntheses of the important key intermediate, (1*S*,2*S*)-2-fluorocyclopropanecarboxylic acid (2), have been reported (Chart 1).²⁾ In a previous paper,³⁾ we reported the enantioselective hydrolysis of compound 3, and 3 is the most reactive substrate among the aliphatic esters we examined (Chart 2).

We found that the lyophilized cells of *Micrococcus* sp. (DSC 4011), which was selected from soil, catalyzed the enantioselective hydrolysis of 3. In the microbial reaction, although compound 5, which has a desirable configuration, was obtained in good chemical yield, the *E* value⁴⁾ was not particularly high. Therefore, we have been interested in one or two esterases which catalyze the enantioselective reaction.

In this paper, we report the purification of the enzyme which catalyzes the hydrolysis of 3, and describe properties and the substrate specificity of this purified enzyme.

MATERIAL AND METHODS

Materials *n*-Propyl acetate was purchased from Kishida Co., Ltd. *n*-Propyl propionate, *n*-propyl *n*-butyrate, *n*-propyl isobutyrate and *n*-propyl benzoate were purchased from Tokyo Kasei Co., Ltd. *n*-Propyl *n*-varelate and *n*-propyl isovarelate were purchased from Wako Pure Chemical Co., Ltd. All chemicals used were of analytical grade.

Enzyme Assay The esterase activity was assayed by the rate of hydrolysis of substrate 3. One unit was defined as the amount of enzyme catalyzing the formation of carboxylic acid (4) per minute. The assay mixture (1 ml) consisted of 0.1 M sodium phosphate buffer (pH 7.0) and 5 mg of *n*-propyl ester 3, and was incubated for 30 min at 40 °C. After incubation, the propyl ester and the formed acid 4 were extracted by ethyl acetate at pH 2. The organic layer was separated, and diazomethane in ether was added to the solution. The propyl ester and the methyl ester were analyzed by GC.

Microorganism and Culture Conditions *Micrococcus* sp. (DSC 4011) was cultured aerobically in the medium of 1% peptone, 0.3% meat extract and 0.1% NaCl (pH 7.0). Cultivation was performed at 30 °C (150 rpm by reciprocal shaker) in a Sakaguchi flask containing 100 ml of the

medium. The cells were harvested at the beginning of the stationary phase by centrifugation and stored at –20 °C until further use.

Analytical Methods The enzyme purity was evaluated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by the method of Laemmli⁵⁾ on a 10% gel. Gels after electrophoresis were stained with Coomassie brilliant blue R250. The enzyme activity in the chromatographic fraction was assayed by measuring the rate of formation of 4 from substrate 3. Protein was assayed by the method of Lowry *et al.*⁶⁾ with bovine serum albumin (Sigma Co., Ltd.) as the standard. In the several steps of chromatography, the amount of protein was measured by the absorbance at 280 nm. The specific activity was expressed as the enzyme activity (unit) per 1 mg protein.

Purification of Enzyme All isolation and purification procedures were carried out at 0–4 °C. The standard buffer, 50 mM sodium phosphate buffer (pH 7.0), was used for purification procedures unless otherwise stated. The harvested cells (40 g) were suspended in 100 ml of the standard buffer, then disrupted by sonication at 4 °C. After sonication, the suspension was centrifuged and the supernatant solution was dialyzed against the standard buffer. The crude extract was brought to a saturated concentration of 40% with solid ammonium sulfate. The precipitate obtained by centrifugation was dissolved in and dialyzed against the standard buffer. The dialyzed solution was placed on a 250 ml (50×150 mm) Source Q column (Pharmacia Co., Ltd.) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.25 M NaCl. Proteins were eluted with a linear gradient of the same buffer

Chart 1

Chart 2

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containing NaCl from 0.25 to 0.5 M at a flow rate of 6 ml/min. The active fractions were collected, and the ammonium sulfate concentration was brought to about 1.5 M. The solution was placed on a 50 ml (26×100 mm) Source PHE column (Pharmacia Co., Ltd.) equilibrated with 100 mM phosphate buffer (pH 7.0) containing 1.5 M ammonium sulfate, then the proteins were eluted with a linear gradient of the same buffer containing ammonium sulfate from 1.5 to 0 M at a flow rate of 2 ml/min. The active fractions were collected and concentrated by ultrafiltration, and the solution was placed on a Superose 12 HR 10/30 column (Pharmacia Co., Ltd.). Proteins were eluted with 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl at a flow rate of 1 ml/min. The active fractions were pooled and stored at -20 °C until use.

Estimation of Molecular Weight The molecular weight of the native enzyme was measured by gel filtration through a TSK gel G-3000SW (7.5×300 mm, Toso Co., Ltd.) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The standard proteins used were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and ribonuclease A (14 kDa). The molecular weight of the denatured enzyme was estimated by SDS-PAGE. The relative mobility of the enzyme was compared with those of phosphorylase b (97 kDa), bovine serum albumin (67 kDa), aldolase (42 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa).

RESULTS AND DISCUSSION

Purification of the Enzyme by Column Chromatography The esterase, which hydrolyzes **3** enantioselectively, was purified from 1080 mg of the crude extract by ammonium sulfate fractionation, anion exchange chromatography, hydrophobic chromatography and gel filtration. The purification procedures are summarized in Table 1. The desired enzyme was obtained with about 106-fold purification in 70% yield after all steps. The purified enzyme exhibited a single band on SDS-PAGE.

Properties of the Purified Enzyme Some properties of the purified enzyme were examined. Based on gel filtration through TSKgel G-3000SW, the esterase was estimated to have a molecular weight of 35000. Results of SDS-PAGE showed that the molecular weight of the denatured enzyme was 33000. Since those values were similar, the enzyme appeared to be a monomer.

The enzyme showed maximum activity around 40 °C, and retained almost 90% of its activity after being incubated at 50 °C for 30 min. The enzyme lost most of its activity on incubation at 60 °C for 30 min. The enzyme was stable at pH 4.0–9.0, and its optimum pH was around 7.0 for substrate **3**. This finding agreed very closely with that for the crude enzyme.

The effects of a 1 mM solution of various metal ions and inhibitors on the esterase were tested (Table 2). The enzyme was strongly inactivated by Hg and diisopropyl fluorophosphate (DFP), but not by EDTA or *o*-phenanthroline. These findings suggest that no metal ions are present but that a serine residue exists in the active site of the enzyme.

Substrate Specificity The hydrolytic activities toward various *n*-propyl esters by purified esterase are shown in Table 3. The enzyme hydrolyzed many types of aliphatic es-

Table 1. Summary of Purification of the Esterase from *Micrococcus* sp.

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude extract	1080	313	0.29	100
(NH ₄) ₂ SO ₄ precipitation	415	249	0.60	80
Resource Q (ion exchange)	22.4	180	8.0	58
Resource Phe (hydrophobic)	4.9	130	26.5	42
Superose 12 (gel filtration)	3.1	98	0.6	31

Table 2. Effects of Metal Ions and Chemical Reagents on Esterase Activity

Additive	Residual activity ^{c)} (%)
None	100
Hg ²⁺	<2
Mg ²⁺	84
Co ²⁺	94
Ca ²⁺	104
Fe ²⁺	83
Mn ²⁺	81
Cu ²⁺	80
Zn ²⁺	86
Ni ²⁺	89
EDTA	92
Phenanthroline	63
MIA ^{a)}	86
DFP ^{b)}	3.1
Eserine	82

a) MIA: monoiodoacetic acid. b) DFP: diisopropyl fluorophosphate. c) Enzyme activity was measured in 0.1 M phosphate buffer (pH 7.0) containing metal ions or chemical reagents. Activity is shown as a percentage of the activity with no additions.

Table 3. Hydrolytic Activity toward *n*-Propyl Esters by Purified Esterase

Run	Substrate ^{a)}	Relative activity (%) ^{b)}
1	CH ₃ -CO ₂ <i>n</i> -Pr	29.2
2	CH ₃ CH ₂ -CO ₂ <i>n</i> -Pr	180
3	CH ₃ CH ₂ CH ₂ -CO ₂ <i>n</i> -Pr	416
4	(CH ₃) ₂ CH ₂ -CO ₂ <i>n</i> -Pr	97.0
5	CH ₃ (CH ₂) ₂ CH ₂ -CO ₂ <i>n</i> -Pr	260
6	(CH ₃) ₂ CH ₂ CH ₂ -CO ₂ <i>n</i> -Pr	72
7	C ₃ H ₅ -CO ₂ <i>n</i> -Pr	29.2
8	C ₃ H ₄ F-CO ₂ <i>n</i> -Pr (3)	100
9	C ₆ H ₅ -CO ₂ <i>n</i> -Pr	3.4

a) Reaction conditions: 0.03 mmol of substrate, 0.1 unit of purified enzyme, 0.1 M phosphate buffer pH 7.0 (1 ml), 40 °C, 30 min. b) Activity is expressed as a percentage of substrate toward **3**.

Table 4. Enantioselectivity toward **3** by Crude and Purified Enzyme

Enzyme	Conv. ^{a)} (%)	Acid (4)		Ester (5)		<i>E</i> ⁴⁾ value
		<i>ee</i> (%) ^{b)}	Config.	<i>ee</i> (%) ^{b)}	Config.	
Crude enzyme	55	78	(1 <i>R</i> ,2 <i>R</i>)	98	(1 <i>S</i> ,2 <i>S</i>)	35
Purified enzyme	56	72	(1 <i>R</i> ,2 <i>R</i>)	98	(1 <i>S</i> ,2 <i>S</i>)	36

a) Reaction conditions: 0.03 mmol of substrate, ca. 0.1 unit of enzyme, 0.1 M phosphate buffer pH 7.0 (1 ml), 40 °C. The reaction was stopped after an appropriate degree of conversion (0.55) was reached. b) The enantiomeric excesses are measured by the method described previously.³⁾

ters (runs 1—7) in addition to substrate **3**, but it had low activity toward the aromatic ester (run 7). The rate of hydrolysis was higher for esters with straight chains than for esters with branched chains. The activity was highest toward *n*-propyl *n*-butyrate (run 3). The enantioselectivity toward **3** by purified esterase is shown in Table 4. The *E* values of the hydrolysis reaction were similar for crude extract and purified enzyme. We thus found that one enzyme catalyzed the hydrolysis of both enantiomers of substrate **3**. Only a few enzymes catalyze the enantioselective hydrolysis of monofluoro compounds. The authors are planning to study the distribution of the esterase activity in various microorganisms, and to compare the amino acid sequence of the purified enzyme with that of other esterases.

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