Purification and Properties of Carboxylesterase from *Ochrobactrum anthropi*

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Carboxylesterase from *Ochrobactrum anthropi* was purified to homogeneity by a combination of DEAE-cellulose, Sephadex G-100, and hydroxyapatite column chromatographies. The molecular weight of the enzyme was calculated to be 58,000 by Sephadex G-75 gel filtration. SDS-polyacrylamide gel electrophoresis showed that the enzyme consisted of two identical subunits with a molecular weight of 30,000. The enzyme was inactivated by several organophosphates and sulfhydryl reagents. The esterase hydrolyzed only acetate and propionate esters. In the presence of Triton X-100, the esterase hydrolyzed *l*-menthyl acetate but not its optical isomer. This result suggests that the enzyme can be used for the optical resolution of *l*-menthol.

Carboxylesterase (EC 3.1.1.1) is widely distributed in animals, plants, and microorganisms, and catalyzes the hydrolysis of various short chain fatty acid esters.¹⁻⁶⁾ Optical resolutions by esterase and lipase have been reported.⁷⁻⁹⁾ Of these, that of menthol ester is of great practical value; *l*-menthol is a peppermint flavor and is an important perfume, but *d,l*-menthol is currently prepared chemically by hydrogenation of thymol, and four racemates (*d,l*-menthol, *d,l*-isomenthol, *d,l*-neo-menthol and *d,l*-iso-neo-menthol) are contained in the preparation. Thus, the isolation of *l*-menthol from a mixture of these racemates is very important industrially. The hydrolysis of *l*-menthol ester by *Rhodotorula mucilaginosa*,¹⁰⁾ *Rhodotorula minuta* var. *texensis*,¹¹⁾ and *Pseudomonas* sp.¹²⁾ were reported, but the relevant enzymes have not been purified.

We have found that *Ochrobactrum* (*O.*) *anthropi* produces a novel carboxylesterase which specifically hydrolyzes only *l*-menthyl acetate in the presence of Triton X-100. This paper describes the purification and some properties of carboxylesterase from *O. anthropi*.

Materials and Methods

Media and culture conditions. For the first screening of the esterase-producing microorganism, agar plates emulsified with polyvinyl alcohol (medium A) were used, which contained 0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.2% polyvinyl alcohol, 1% *l*-menthyl acetate, and 2% agar (pH 7.0). Medium B for the production of esterase contained 0.6% meat extract, 1% peptone, 0.5% NaCl, and 1% *l*-menthyl acetate (pH 7.0). The cultivation in test tubes was done in 2 ml of medium B at 28°C for 65 hr on a reciprocal shaker. The cultivation in a 30-liters jar fermentor was done in 18 liters of medium B at 28°C for 17 hr with constant stirring at 500 rpm and the aeration of 5 liters/min.

Detection of esterase-producing microorganism. Each colony grown on the agar plate of medium A was cultivated in a test tube containing 2 ml of medium B under the conditions described above. To the culture broth, 2 ml of diethyl ether was added and the ether layer was withdrawn. The resulting ether extracts were applied to a thin layer plate of silica gel 60 (Merck) and developed with chloroform. *l*-Menthol and *l*-menthyl acetate were detected by the method of Stahl *et al.*¹³⁾

Assay of esterase activity. The esterase activity was assayed by the method of Fukumoto *et al.*¹⁴⁾ The reaction mixture contained 0.5 ml of *l*-menthyl acetate, 5 ml of 20 mM Na-phosphate buffer (pH 7.0) containing 0.1% Triton X-100, and 0.5 ml of the enzyme solution, unless otherwise specified. The mixture was incubated at 30°C

for 1 hr with stirring at 500 rpm. Twenty milliliters of ethyl alcohol were then added and the amount of liberated acetic acid was titrated with 0.05 N KOH. One unit of enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of acetic acid per min.

Measurement of molecular weight. The purified enzyme was put on a Sephadex G-75 column (1.5 $\times$ 93 cm, Pharmacia) and eluted with 10 mM Na-phosphate buffer (pH 7.0) containing 0.1 M NaCl and 10 mM 2-mercaptoethanol. The Sephadex G-75 column was standardized with chymotrypsinogen A (M.W. 25,000), ovalbumin (45,000), and bovine serum albumin (67,000).

The subunit molecular weight was estimated by SDS polyacrylamide gel electrophoresis¹⁵⁾ with lysozyme (14,300), β -lactoglobulin (18,400), carbonic anhydrase (29,000), bovine serum albumin (68,000), and phosphor-ylase B (97,400) as standards.

Chemicals. *l*-Menthyl acetate was purchased from Inoue Koryo. All the other reagents were of special grade.

Results

Isolation and identification of esterase-producing microorganism

The soil suspension was spread on a medium A plate and incubated at 30°C for 3 days. Each colony was cultivated in the medium B containing *l*-menthyl acetate at 28°C for 65 hr, and several strains that accumulated *l*-menthol in the culture broths were isolated. Among

them, a strain showing the highest esterase activity was used in the following experiments, and its characteristics were investigated. The strain was rod-shaped, aerobic, gram negative, spore negative, and motile, produced acid from several carbohydrates, and reduced both nitrate and nitrite (Table I). From these characteristics, the strain was identified as *Ochrobactrum anthropi*.¹⁶⁾

Purification of carboxylesterase

All steps for the purification were done below 15°C.

Preparation of crude extract, streptomycin treatment, and ammonium sulfate fractionation. The cells of *O. anthropi* (about 100 g, wet weight) were harvested from 18 liters of the culture broth and suspended in 400 ml of 50 mM Na-phosphate buffer (pH 7.0) containing 1% Triton X-100. The suspended cells were disrupted by sonication (9 kHz, Kubota's model 1200M Insonator) at 140 W for 20 min. The cell debris was removed by centrifugation (20,000 $\times g$, 40 min). The crude extract was treated with 20% streptomycin solution to a final concentration of 1%. The mixture was centrifuged after standing for 1 hr and the precipitate was removed by centrifugation (15,000 $\times g$, 15 min). The supernatant was brought to 80% saturation with solid ammonium sulfate. The resulting precipitate was collected by filtration, dissolved in a small amount of 20 mM Na-phosphate buffer (pH 7.5), and dialyzed overnight against the same buffer.

DEAE-cellulose column chromatography. The dialyzed solution was put on a DEAE-cellulose column (2.8 $\times$ 18 cm, Whatman) equilibrated with 20 mM Na-phosphate buffer (pH 7.5) and washed with the same buffer. The enzyme was eluted with a 450 ml linear gradient of zero to 0.6 M NaCl in 20 mM Na-phosphate buffer (pH 7.5). The active fractions were collected and concentrated by ultrafiltration.

Sephadex G-100 gel filtration. The concentrated enzyme solution was put on a Sephadex G-100 column (2.4 $\times$ 92 cm, Pharmacia) equilibrated with 10 mM Na-phosphate

Table I. CHARACTERIZATION OF A STRAIN PRODUCING ESTERASE

Morphological characteristics

Rod. Spore formation; negative. Gram stain; negative. Motility; positive.

Colony morphology on nutrient agar

Round, regular, entire, off-white, low convex, smooth, shiny, mucoid opaque.

Biochemical characteristics

O-F test; negative. Nitrate and nitrite reduction; positive. Indole production; negative. Catalase, oxidase, urease, cytochrome oxidase, and phenylalanine deaminase; positive. Arginine dehydrogenase, and β -galactosidase; negative. Acid production from erythritol; positive. Acid production from glucose; negative. Hydrolysis of gelatine and esculin; negative. Assimilation of glucose, arabinose, mannose, *N*-acetylglucosamine, maltose, caprate, malate, and citrate; positive. Assimilation of mannitol, glucomate, adipate, and phenylacetate; negative.

buffer (pH 7.0) containing 0.1 M NaCl and 10 mM 2-mercaptoethanol, and eluted with the same buffer. The active fractions were collected and dialyzed overnight against 1 mM Na-phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol.

Hydroxyapatite column chromatography. The dialyzed enzyme solution was put on a hydroxyapatite column (2.8 × 19.5 cm, Seikagaku Kogyo Co.) equilibrated with 1 mM Na-phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol and washed with the same buffer. The enzyme was eluted with a 500 ml linear gradient of 1 to 150 mM Na-phosphate buffer (pH 6.5) containing

10 mM 2-mercaptoethanol. The active fractions were collected and dialyzed overnight against 1 mM Na-phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol. The resulting enzyme solution was fractionated by rechromatography on a hydroxyapatite column under the same conditions except for eluting with a 500 ml linear gradient of 1 to 100 mM Na-phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol (Fig. 1). The active fractions were collected and concentrated by ultrafiltration.

The purification steps of the esterase are summarized in Table II. The purified enzyme was judged to be homogenous by disc polyacrylamide gel electrophoresis.¹⁷⁾

Molecular weight and isoelectric point

The molecular weight of the purified enzyme was estimated to be 58,000 by gel filtration on Sephadex G-75 and that of the subunit was estimated to be 30,000 by SDS polyacrylamide gel electrophoresis. These data indicated that the native enzyme was composed of two identical subunits.

The isoelectric point of the purified enzyme was determined to be 4.4 by isoelectric focusing.¹⁸⁾

Effects of Triton X-100 on activity

Since a surfactant such as Triton X-100 was found necessary for the expression of enzyme activity during the course of the purification,

Fig. 1. The Second Column Chromatography of *O. anthropi* Esterase on Hydroxyapatite.

The experimental details were described in the text. ○—○, esterase activity; ●—●, protein (absorbance at 280 nm); — — —, Na-phosphate buffer (pH 6.5).

Table II. PURIFICATION OF ESTERASE FROM *O. anthropi*

The activity was measured under the conditions described in Materials and Methods except for using 50 mM acetate buffer (pH 5.6) instead of 20 mM Na-phosphate buffer (pH 7.0).

Steps	Total protein ^a (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	ND ^b	775	ND ^b	100
Streptomycin	ND ^b	747	ND ^b	96
Ammonium sulfate	ND ^b	537	ND ^b	69
DEAE-cellulose	435	420	0.96	54
Sephadex G-100	159	354	2.20	46
Hydroxyapatite (1st)	10.4	225	21.6	29
Hydroxyapatite (2nd)	3.6	105	29.2	14

^a The protein content was estimated from the absorbance at 280 nm with Hammarsten milk casein (Merck) as a standard.

^b ND, not determined. Can not be determined because of the presence of Triton X-100.

Fig. 2. Effects of Triton X-100 on Activity.

The effects of Triton X-100 on the activity were measured under the conditions described in Materials and Methods except for the concentration of Triton X-100.

Fig. 3. Effects of pH on Activity (○) and Stability (●).

The effects of pH on the activity were measured under the conditions described in Materials and Methods except for using Britton–Robinson's buffer (pH 5.0–9.0) instead of 20 mM Na-phosphate buffer (pH 7.0). The pH stability of the enzyme was examined by the measurement of the residual activity after the incubation in Britton–Robinson's buffer (pH 2.5–11.0) at 30°C for 17 hr.

the effects of Triton X-100 on the activity were investigated. The activity for *l*-menthyl acetate reached the maximum in the presence of 0.1% Triton X-100 and did not decrease even at the concentration of 1.0% (Fig. 2).

Effects of pH and temperature

The enzyme was the most active at pH 6.5 and was stable from pH 6.0 to 8.0 during 17 hr of incubation at 30°C (Fig. 3). The optimum temperature for the activity was 35°C and the enzyme was stable below 40°C at pH 7.0 for

Fig. 4. Effects of Temperature on Activity (○) and Stability (●).

The effects of temperature on the activity were measured at various temperatures. The thermal stability of the enzyme was examined by the measurement of the residual activity after its treatment in 20 mM Na-phosphate buffer (pH 7.0) for 15 min at various temperatures.

Table III. EFFECTS OF INHIBITOR ON ESTERASE ACTIVITY

Esterase (1.8 U) was incubated with the various inhibitors in 2 ml of total volume at 30°C for 1 hr and the remaining activity was measured.

Inhibitors	Concn. (mM)	Residual activity (%)
None	—	100
Eserine	1	97
DFP ^a	1	17
Paraoxon ^b	1	0
PMSF ^c	1	87
PCMB ^d	1	0
5'5-Dithiobis-(2-nitrobenzoate)	1	100
EDTA ^e	1	100
NaF	10	38

^a DFP, diisopropyl fluorophosphate.

^b Paraoxon, diethyl *p*-nitrophenyl phosphate.

^c PMSF, phenylmethanesulfonyl fluoride.

^d PCMB, *p*-chloromercuribenzoate.

^e EDTA, ethylenediaminetetraacetic acid.

15 min (Fig. 4).

Effects of metal ions and inhibitors

The purified enzyme was incubated with 1 mM of each metal ion at 30°C for 1 hr. The enzyme activity was measured in the reaction mixture described in Materials and Methods except that 20 mM Tris–HCl buffer (pH 7.2)

Table IV. SUBSTRATE SPECIFICITY OF THE PURIFIED ENZYME

The reaction was done with or without 0.1% Triton X-100 using 0.5 ml of various esters. The activity was indicated as percentage of the value for *l*-methyl acetate in the presence of 0.1% Triton X-100.

Substrates	Relative activity (with Triton X-100) (%)	Relative activity (without Triton X-100) (%)
<i>l</i> -Menthyl acetate	100	0
<i>l</i> -Menthyl propionate	30	0
Triacetin	86	81
Tripropionin	65	7
Tributyrin	4	0
Tricaproin	0	0
Tricaprylin	0	0
Tricaprin	0	0
Triolein	0	0
Methyl acetate	85	90
Methyl propionate	22	25
Methyl butyrate	0	0
Methyl caproate	7	0
Methyl caprylate	7	0
Methyl caprate	7	0
Methyl oleate	0	0
Phenyl acetate	69	0
Benzyl acetate	52	0

was used instead of 20 mM Na-phosphate buffer (pH 7.0). The enzyme activity was completely inhibited by Hg^{2+} , Zn^{2+} , Cd^{2+} and Cu^{2+} . Pb^{2+} and Fe^{3+} showed 20% inhibition, and other metal ions did not show any effect on the enzyme activity.

The effects of paraoxon and several organic inhibitors on the enzyme activity were investigated and the results are shown in Table III. The enzyme was strongly inactivated by paraoxon, diisopropyl fluorophosphate, and *p*-chloromercuribenzoate, but not by eserine, 5,5'-dithiobis(2-nitrobenzoate), or EDTA.

Substrate specificity

The hydrolytic activities of the enzyme toward various esters were investigated (Table IV). The enzyme hydrolyzed acetate esters very well and propionate esters slowly, but hardly did the esters of C4 and longer fatty acids. The activities on terpene and aromatic alcohol esters were expressed only in the presence of

Fig. 5. Optical Specificity for *d,l*-Menthyl Acetate.

A reaction mixture composed of 1 ml of *d,l*-menthyl acetate, 5 ml of 20 mM Na-phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and 2 U of enzyme was incubated at 30°C with stirring at 500 rpm, and then *d,l*-menthyl acetate and *d,l*-menthol were extracted with diethyl ether. The extracts were dissolved in 2 ml of pyridine, followed by addition of 100 μ l phenyl isocyanate and incubated at room temperature for 1 hr. The carbamated *d,l*-menthol was analyzed using a Chiralcell OD column (0.6 $\times$ 15 cm, Daicell Chemical Ltd.) connected to a Shimadzu HPLC system by eluting with hexane-2-propanol (9:1, v/v). The effluents were monitored by the absorption at 230 nm. $\circ$, *l*-menthol; $\bullet$, *d*-menthol.

0.1% Triton X-100, and the acetate esters were hydrolyzed very well. Among the various esters tested, *l*-menthyl acetate was the best substrate.

Optical specificity

The optical specificity of the enzyme was examined using *d,l*-menthyl acetate. As shown in Fig. 5, the enzyme hydrolyzed only *l*-menthyl acetate, but not *d*-menthyl acetate. Incubation of the same racemic substrate with 10 U of the enzyme for 8 hr resulted in 50% hydrolysis, leaving *d*-menthyl acetate unhydrolyzed. Thus, the enantiomeric excess of *l*-menthol was considered nearly 100%.

Discussion

Recently, enzymes attracted our attention after they were shown to be useful for oxydoreduction,¹⁹⁾ ester synthesis,²⁰⁾ and optical resolution⁷⁻⁹⁾ on the process of the organic synthesis. Since *l*-menthol is of great practical value as a peppermint flavor, we

searched for an enzyme that could be used for optical resolution and found a novel esterase produced by *O. anthropi*.

The esterase was purified from the intracellular fraction of *O. anthropi* to electrophoretic homogeneity. The purified enzyme acted nonspecifically on the alcohol moiety of the ester, but specifically on its acid moiety: it hydrolyzed only acetate and propionate esters (Table IV). The most interesting property of this enzyme was its optical specificity: it hydrolyzed *l*-menthyl acetate, but not *d*-menthyl acetate (Fig. 5). This result suggested this enzyme could be used industrially for optical resolution of racemates such as menthyl acetate.

Triton X-100 was necessary for the hydrolysis of highly hydrophobic substrates such as *l*-menthyl acetate, *l*-menthyl propionate, tripropionin, phenyl acetate, and benzyl acetate (Table IV). The role of Triton X-100 might be to change insoluble substrates to soluble ones, because esterases generally act on monomer substrates but not micellar substrates.

This enzyme hydrolyzed only acetate and propionate esters (Table IV), and was strongly inactivated by diisopropyl fluorophosphate and paraoxon (Table III). These results showed that the esterase of *O. anthropi* was a carboxylesterase (EC 3.1.1.1).²¹⁾ The fact that this enzyme was completely inactivated by PCMB (Table IV) and the metal ion (Cu^{2+} , Hg^{2+} , Cd^{2+} or Zn^{2+}) suggested that the sulfhydryl group was important for the expression of the enzyme activity.

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