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Purification and properties of a highly enantioselective L-menthyl acetate hydrolase from *Burkholderia cepacia*

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ABSTRACT

A highly enantioselective L-menthyl acetate esterase was purified to homogeneity from *Burkholderia cepacia* ATCC 25416, with a recovery of 4.8% and a fold purification of 22.7. The molecular weight of the esterase was found to be 37 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The N-terminal amino acid sequence was "MGARTDA", and there was no homology in contrast to other *Burkholderia* sp. esterases. This enzyme preferentially hydrolyzed short-chain fatty acid esters of menthol with high stereospecificity and high hydrolytic activity, while long-chain L-menthyl esters were poor substrates. Considered its substrate specificity and N-terminal sequence, this esterase was concluded as a new enzyme belonging to the carboxylesterase group (EC 3.1.1.1) of esterase family. The optimum temperature and pH for enzyme activity using racemic menthyl acetate as substrate were 30 °C and 7.0, respectively. The esterase was more stable in the pH range of 7.0–9.0 and temperature range of 30–40 °C. Hydrolytic activity was enhanced by Ca^{2+}, K^{+} and Mg^{2+}, but completely inhibited by Hg^{2+}, Cu^{2+}, ionic detergents and phenylmethylsulfonyl fluoride (PMSF) at 0.01 M concentration.

1. Introduction

Optically active L-menthol and its derivatives are widely used in pharmacy and food industry [1–3]. Menthol has 8 isomers and among them only L-menthol or (1*R*,2*S*,5*R*)-menthol exhibits pharmacological activity and pleasant taste and odor, while the other isomers not only are biologically inactive but also show unpleasant taste and odor, such as mouldy and bitter taste [4]. Nonetheless, the synthetic menthols are always produced as racemic mixtures. Therefore, the kinetic resolution of an optically pure L-enantiomer form is urgently required.

The kinetic resolution of racemic menthol has mainly been conducted by physical and chemical methods, such as the asymmetric synthesis of an L-enantiomer [5] or the use of chiral chromatography and stereoselective crystallization [6,7]. However, these methods entail expensive manufacturing processes and are complex for industrial application. The use of biochemical processes for the preparation of an optically active L-menthol from its corresponding racemic compound using biocatalysts from a microbial origin has recently drawn much attention. There have been a few studies on the lipase-catalyzed resolution of DL-menthol to give chiral L-menthol, such as Wu et al. [8] used *Candida cylindracea* lipase stereoselective esterification resolution racemic menthol in *n*-hexane using acid anhydrides as acylating agents, Vorlova et al. [9] obtained optically pure L-menthol by hydrolyzing DL-menthyl benzoate using *Candida rugosa* lipase. However, the enzymes used have limited substrate scope and operational stability, or the level of the enantiomeric excess was unsatisfactory for the demands of large-scale production.

Burkholderia cepacia esterase/lipase has proven to be useful as a biocatalyst for obtaining enantiomerically pure compounds, in particular primary and secondary alcohols and their esters [10–13]. In our previous work, a strain named *B. cepacia* ATCC 25416 had been isolated in our laboratory [14]. It could hydrolyze 0.05 M DL-menthyl acetate to L-menthol in aqueous reaction system with optical purity of 96% and conversion of 50% within 20 h. In addition, it also exhibited high reaction capacity and good operational stability and was shown to be a potentially useful biocatalyst for the preparation of L-menthol. So it is important to reveal and characterize the key hydrolase with the activity of enantioselective hydrolysis of racemic menthyl eaters. In general, bacterial hydrolases include esterase (EC 3.1.1.1) and lipase (EC 3.1.1.3) [15]. Moreover, the hydrolases (esterase/lipase) are

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usually very complicated in their molecular structure, and their properties are various according to their sources, so there are some difficulties in hydrolases purification. Many methods about extracellular esterases/lipases purification from *Burkholderia* sp. have been reported [16–20], but few were about intracellular esterases/lipases.

We here purified the key hydrolase from *B. cepacia* ATCC 25416 whole cells by ammonium sulfate precipitation, butyl-Sepharose, DEAE-Sepharose, and Superdex G-75 column chromatography. The catalytic properties of the enzyme were also investigated for the development of a practical enzymatic process.

2. Experimental parts

2.1. Materials

L-Menthol, DL-menthol, α-naphthyl acetate and Fast Blue B were purchased from Sigma Chemical Co. (St. Louis, MO) at 99% purity or higher. Butyl-Sepharose, DEAE-Sepharose, Superdex G-75 and standard proteins (*Mr* of 14,400, 20,100, 31,000, 43,000, 66,200 and 97,400) were purchased from Pharmacia (Shanghai, China). DL-Menthyl formate, DL-menthyl acetate, DL-menthyl propionate, DL-menthyl butyrate, DL-menthyl caprylate, and DL-menthyl benzoate were prepared in our lab with 99% GC purity or higher. The other chemicals used in this work were of analytical grade from local sources.

2.2. Microbial strain and cultivation

B. cepacia ATCC 25416 was cultivated aerobically at 30 °C for 48 h in an optimized medium containing 2.27% (w/v) glucose, 1.83% (w/v) peptone, 1.58% (w/v) beef extract, 0.42% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2$ and 0.5% NaCl (pH 7.0).

2.3. Purification of L-menthyl acetate hydrolase

(a) *Preparation of crude cell extract*. After incubation, the microorganism cells were collected by centrifugation at 10,000 × *g* for 15 min and washed twice with physiological saline (0.85% NaCl), then 10 g cells (wet weight) were suspended in 30 mL of buffer A (0.05 M potassium phosphate, pH 7.0, containing 0.1% Triton X-100). The addition of 0.1% Triton X-100 was essential to prevent activity loss and decrease hydrophobicity of the enzyme preparation [20,21]. All the purification steps were performed at 0–4 °C and potassium phosphate buffer was used as a standard buffer. The suspension was sonicated for 10 min at 4 °C to disrupt cells, and the cell debris was removed by centrifugation at 20,000 × *g* for 10 min. Solid ammonium sulfate was added with stirring to the supernatant to give 50% saturation. After 1 h, the active precipitate was collected by centrifugation at 20,000 × *g* for 10 min and dissolved in 30 mL of buffer A, and the solution was dialyzed overnight against a 30-fold volume of buffer B (0.05 M potassium phosphate, pH 7.0, containing 0.1% Triton X-100 and 0.8 M ammonium sulfate).

(b) *Butyl-Sepharose hydrophobic chromatography*. The dialyzed enzyme solution was applied to a Butyl-Sepharose (Pharmacia) column (1.6 cm × 20 cm) which had been pre-equilibrated with buffer B. The enzyme was eluted with a linear gradient of 0.8–0 M ammonium sulfate in buffer A, with a flow rate of 1.2 mL/min. Protein concentration was estimated by measuring the absorbance at 280 nm. Enzyme activity in eluted fractions was determined by the standard procedure as described below. Active fractions were pooled and concentrated by ultrafiltration (Millipore 8050, USA), then dialyzed against buffer A.

(c) *DEAE-Sepharose CL-6B column chromatography*. The concentrated solution from step (b) was applied to a DEAE-Sepharose CL-6B (Pharmacia) column (2.5 cm × 30 cm) pre-equilibrated with buffer A. The column was washed with two bed volumes of buffer A, and then the bound proteins were eluted with five bed volumes of each of buffer A containing 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl. Active fractions eluted at 0.2 M NaCl were pooled and concentrated by ultrafiltration (Millipore 8050, USA), then dialyzed against buffer A.

(d) *Superdex G-75 gel filtration chromatography*. The concentrated solution from step (c) was loaded on a Superdex G-75 (Pharmacia) column (1.6 cm × 60 cm) which had been pre-equilibrated with buffer A and proteins were eluted at a flow rate of 1.0 mL/min with detection at 280 nm.

2.4. Protein assays

Protein concentrations were determined by the Bradford [22] method using bovine serum albumin (BSA) as the standard. During the chromatographic purification, the protein content of fractions was routinely estimated by measuring the UV absorbance at 280 nm. The molecular mass of the enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on a slab gel containing 0.1% SDS under reduced conditions according to the method of Laemmli [23]. Standard proteins (*Mr* of 14,400, 20,100, 31,000, 43,000, 66,200 and 97,400) were used as markers. For enzyme activity staining, proteins were first renatured by incubation for 2 h in Triton X-100 solution (0.05 M potassium phosphate, pH 7.0, containing 1.0% Triton X-100). Next, the gel was incubated in a mixture of freshly prepared solutions of α-naphthyl acetate and Fast Blue B [24,25] (100 mL of 0.05 M potassium phosphate (pH 7.0) containing 1 mL of 60 mg/mL α-naphthyl acetate/acetone and 100 mg Fast Blue B). In the presence of hydrolytic (lipase or esterase) activity, released α-naphthol forms a violet complex with Fast Blue B. Staining was terminated by incubating in 20% methanol and 10% acetic acid for 15 min.

N-Terminal amino acid sequence analysis was detected as follows: following SDS-PAGE, the purified proteins were transblotted onto a PVDF membrane and stained with 0.1% Coomassie Brilliant blue. The single band was cut out and subjected to N-terminal amino acid sequence analysis in Shanghai GeneCore BioTechnologies Co., Ltd.

2.5. Enzyme activity assays

The enzyme activity was usually measured using DL-menthyl acetate as substrate at 30 °C and pH 7.0. A typical reaction was described as follows: the enzyme solution (0.2 mL) was diluted with 0.8 mL of 0.05 M potassium phosphate buffer (pH 7.0). The reaction was started by adding DL-menthyl acetate to a final concentration of 0.05 M. The mixture was incubated at 30 °C for 30 min in a rotatory shaker at 200 rpm. At the end of reaction, both the alcohol produced and the residual ester were extracted into 1 mL of ethyl acetate and subjected to GC analysis. The specific activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-menthol per minute.

2.6. Substrate specificity of the purified enzyme

To investigate the substrate specificity of the enzyme, hydrolytic reactions of a series of racemic menthyl esters were performed in standard conditions. The enantioselective hydrolysis of various racemic menthyl esters was carried out in 1 mL purified enzyme solution with the substrate concentration of 0.05 M at 30 °C and

Table 1
Purification of L-menthyl ester hydrolase from *Burkholderia cepacia* ATCC 25416

Purification step	Total[a] activity (U)	Protein[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor (fold)
Crude extract	64.7	249	0.26	100	1
$(NH_4)_2SO_4$ precipitate	53.7	185	0.29	83	1.1
Butyl-Sepharose	20.8	21	0.99	32	3.8
DEAE-Sepharose	7.3	3.1	2.35	11	9.0
Superdex G-75	3.1	0.53	5.91	4.8	22.7

[a] 1 unit: 1 μmol of L-menthol released per minute using racemic menthyl acetate as substrate.
[b] Proteins were estimated by Bradford method as described in Section 2.4.

pH 7.0 for 20 h. The remaining substrate and the alcohol produced were analyzed by GC.

2.7. Effects of pH, temperature, metal ions and detergents or inhibitors

The optimum pH of enzyme was determined at 30 °C using four different buffers: glycin–NaOH (pH 9–10), Tris–HCl (pH 8–9), K_2HPO_4–KH_2PO_4 (pH 6–8) and CH_3COOH–CH_3COONa (pH 5–6). The pH stability was tested by preserving the enzyme in buffers of various pH values at 30 °C for 4 h and the residual activity was assayed (at pH 7.0) using 0.05 M racemic menthyl acetate as substrate. The activity determined immediately before incubation was defined as 100%.

The effects of temperature on activity and stereospecificity were examined using 0.05 M racemic menthyl acetate as substrate at different temperatures in the range 25–60 °C at pH 7.0. The thermal stability of enzyme was checked at 35, 40, 45, 50, 55 and 60 °C. The enzyme was held in a 0.05 M potassium phosphate buffer (pH 7.0) for 4 h at the specified temperature. After cooling, the remaining activity and the enantioselectivity were measured.

In order to study the effects of metal ions and detergents or inhibitors on enzyme activity and enantiospecificity, purified enzyme was pre-incubated with 1 or 10 mM of various metal ions or detergents or inhibitors for 1 h at 30 °C and pH 7.0. Potassium phosphate buffer was replaced by Tris–HCl buffer during these experiments to prevent the precipitation of some metal ions. The residual activity and the enantiospecificity were determined using 0.05 M DL-menthyl acetate as substrate.

2.8. GC analysis

The amounts of the produced menthol and the remaining menthyl esters were analyzed by CP3900 gas chromatography (Varian, Inc., USA) equipped with a flame-ionization detector and a CP-Chirasil-Dex CB column (25 m length, 0.25 mm i.d.) using H_2 as a carrier gas. The injector and detector were set at 270 °C, respectively. The column temperature was kept at 100 °C for 8 min, then raised to 200 °C at a rate of 4 °C per min and finally kept at 200 °C for 10 min. Enantiomeric excesses of substrate (ee_s) and product (ee_p), enantiomeric ratio (*E*-value) and conversion based on the GC analyses were calculated as described by Chen et al. [26].

3. Results and discussion

3.1. Purification of the key esterase

The result of purification was summarized in Table 1. During the purification, the key esterase was found to have a strong hydrophobicity, and detergent (Triton X-100) had no obvious influence on the activity of enzyme. Jaganathan and Boopathy [21] had reported that Triton X-100 could prevent the esterase inactivation by interacting with the acyl pocket hydrophobic region. Fernandez-Lorente et al. [27] adopted Triton X-100 to reduce the hydrophobicity of *Aspergillus niger* lipase on hydrophobic chromatographic column during enzyme purification. Therefore, 0.1% Triton X-100 was adopted to prevent activity loss and decrease hydrophobicity in the enzyme preparation. The highest hydrolytic activity was found in the 50% ammonium sulfate fraction. Gupta et al. have reviewed that most of hydrolases are hydrophobic in nature, and the purification of hydrolases may best be achieved by opting for hydrophobic interaction chromatography [28]. Considering the overall enzyme yields and purity, we employed a butyl-hydrophobic chromatography after ammonium sulfate precipitation. In the Butyl-Sepharose hydrophobic chromatography, the enzyme was eluted out at 0.2 M ammonium sulfate. Following ion exchange and gel filtration chromatography, the specific activity of this enzyme was greatly enhanced and 0.53 mg esterase was obtained from 249 mg of crude extract. An overall 22.7-fold purification was achieved, with an activity recovery of 4.8% from the crude cell extract after four steps. The specific activity of the purified esterase was 5.91 units mg^{-1} protein with racemic menthyl acetate as substrate.

After four steps, the purified enzyme seemed to be homogeneous because it migrated as a single band on SDS-PAGE. Then the

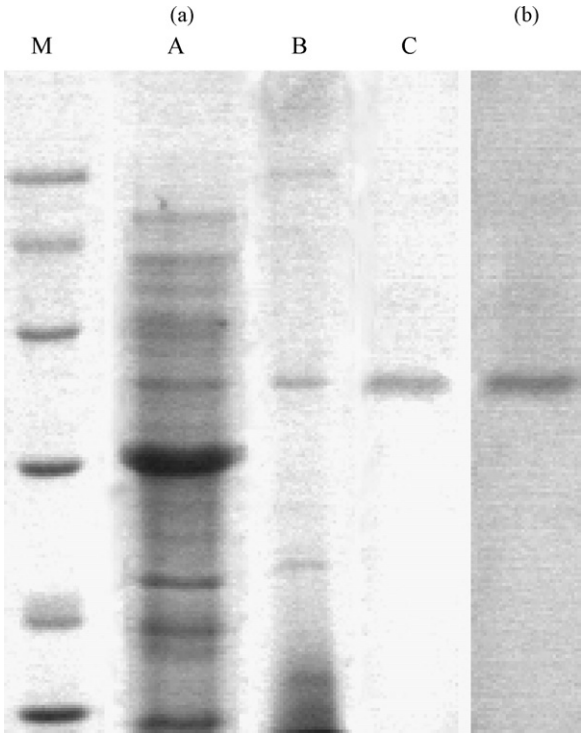


Fig. 1. (a) SDS-PAGE profiles with Coomassie Brilliant blue stain of different stages of purification. M: protein marker, 14, 31, 43, 66 and 97 kDa; (A) after butyl-Sepharose; (B) after DEAE-Sepharose; (C) after Superdex G-75 and (b) activity staining of the renatured enzyme after SDS-PAGE.

Table 2
The N-terminal amino acid sequence of different *Burkholderia* sp. esterase/lipase

Esterase source	N-terminal amino acid sequence	Function
Burkholderia cepacia ATCC 25416 esterase	MGARTDA	Hydrolysis of L-menthyl acetate
Burkholderia sp. YY62 [20,29]	MDFYDANETRHPEQR	Hydrolysis of *tert*-butyl esters
Burkholderia gladioli [30,31]	MTAASLDP	Stereoselective hydrolysis of linalyl acetate
B. cepacia ST-200 [32]	APADNYAATR	Hydrolysis long-chain fatty acid esters of cholesterol
B. cepacia DSM 3959 LipA [33]	AAGYAATRYPIILVHGLSGTDK	*n.s.*
Burkholderia sp. 383 [34]	MTRPTFNGTE	Esterase/lipase/thioesterase
Burkholderia sp. 383 [34]	MTEPTPAVFI	hydrolase, α/β family
Burkholderia cenocepacia HI2424 [35]	MNHTDPHTVA	hydrolase, α/β family
Burkholderia cenocepacia AU 1054 [36]	MIQPTPVVFI	hydrolase, α/β family
Burkholderia pseudomallei 1710b [37]	MLAPLAFAAL	chlorogenate esterase

n.s.: not specified.

proteins in the gel were renatured by incubation for 2 h in Triton X-100 solution (0.05 M potassium phosphate, pH 7.0, containing 1.0% Triton X-100). The key enzyme which had hydrolytic ability for L-menthyl acetate was judged by activity staining with α-naphthyl acetate as substrate. The molecular weight of the purified hydrolase was estimated to be 37 kDa by SDS-PAGE (Fig. 1). The first 7 N-terminal residues of this hydrolase were identified as NH_2-M-G-A-R-T-D-A by N-terminal amino acid sequence analysis. There was no homology with other *Burkholderia* sp. esterase/lipase (shown in Table 2). In addition, the source of the purified esterase was very different from those of hitherto known L-menthyl ester hydrolases (Table 3). Therefore, the esterase purified in this work may be a new enzyme for L-menthyl ester hydrolysis considering the microbial source and the N-terminal sequence.

3.2. Substrate specificity of the purified esterase

Hydrolytic activities of the purified esterase were checked towards 0.05 M various fatty acid esters of menthol at 30 °C and pH 7.0 (Table 4). As shown in Table 4, with the increase of the carbon chain length of the fatty acids, the specific activity decreased, and only 8.7% conversion was detected with DL-menthyl caprylate. The highest conversion was measured on racemic menthyl acetate. The stereospecificity for racemic menthyl esters was L-enantiomer form under the experiment conditions. There was a clear decrease for the stereospecificity with the increase of the number of carbon atoms. In the comparison, the substrates in this work all have very low solubility in aqueous system. The enzyme hydrolysis rate was mainly influenced by oil–water interfacial area between enzyme and water. Compared with this, the physical state of the substrates may be minor factors. These results suggested that the esterase purified from *B. cepacia* ATCC 25416 is a short-chain fatty acid esters hydrolase, and belongs to the carboxylesterase group (EC 3.1.1.1).

Table 3
The hitherto known esterase/lipase acting on L-menthyl ester

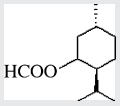
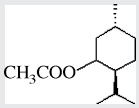
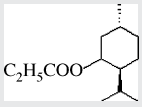
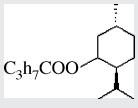
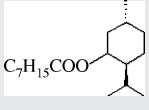
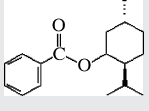
Enzyme source	Substrate	ee_p	Reference
Burkholderia cepacia ATCC 25416 esterases	DL-Menthyl acetate	>99%	
Gloeophyllum sepiarium IFO 6267 esterases	DL-Menthyl acetate	*n.s.*	Yamada et al. [38]
Rhodotorula-Minuta Var *Texensis* esterases	DL-Menthyl succinate	>99%	Omata et al. [39]
Ochrobactrum anthropi esterases	DL-Menthyl acetate	*n.s.*	Murase et al. [40]
Recombinant *Candida rugosa* lipase LIP1	DL-Menthyl benzoate	>99%	Vorlova et al. [9]

n.s.: not specified.

3.3. Effects of pH and temperature on activity, enantioselectivity and stability of the purified esterase

Temperature and pH have some serious effects on the activity and stability of the enzyme. The purified enzyme was stable between pH 7.0 and 9.0 during incubation at 30 °C for 4 h, and lost most of its activity and stereospecificity at pH values lower than 6.0 or higher than 10.0. The optimum pH was 7.0 at which the enzyme was stable and the *E*-value was high (Fig. 2). The optimum pH range of the purified esterase was slightly wider than that of *B. cepacia* ATCC 25416 whole cells [14]. In whole cells, the results of DL-menthyl acetate hydrolysis were determined by the interactions of many enzymes. The enzymes in whole cells were complicated compared to the purified enzyme, and a small change of pH might cause great effects on the activity and the enantioselectivity of some enzymes besides the key esterase. In addition, the enzyme showed the maximum activity on racemic menthyl acetate at 30 °C, and retained 50% of its maximum activity after incuba-

Table 4
Hydrolysis of various racemic menthyl esters by the purified enzyme

Substrate	Primary structure	ee_p (%)	Conversion (%)	*E*-value
DL-Menthyl formate		97.7	48.4	281
DL-Menthyl acetate		97.2	49.7	280
DL-Menthyl propionate		96.5	48.6	182
DL-Menthyl butyrate		90.5	29.3	29
DL-Menthyl caprylate		77.8	8.7	9
DL-Menthyl benzoate		90.4	32.8	31

The reactions were conducted in 1 mL potassium phosphate (0.05 M, pH 7.0) buffer at 30 °C for 20 h with 0.05 M corresponding substrate. All assays were performed in duplicates and average values were taken.

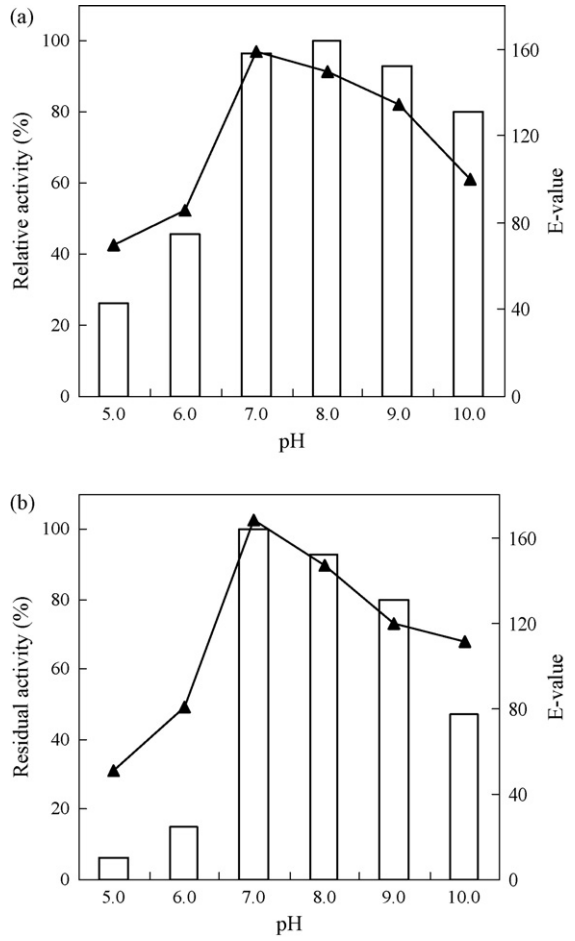


Fig. 2. Effects of pH on the enzyme activity (a) and stability (b) as determined with DL-menthyl acetate at 30 °C. (a) Relative enzyme activity (□) and enantioselectivity (▲) were assayed under various pH conditions. (b) Enzyme solution was incubated for 4 h under various pH conditions, and the residual activity (□) and enantioselectivity (▲) were determined.

tion for 4 h at 40 °C and pH 7.0. Most activity was lost when the enzyme was incubated for 4 h at 45 °C. Moreover, the enzyme was completely inactivated after 4 h incubation at 55 °C. This showed that this enzyme was quite heat-labile. The enantioselectivity of the purified esterase changed little at the temperature range of 25–60 °C (Fig. 3), while the enantioselectivity of *B. cepacia* ATCC 25416 whole cells was more temperature sensitive [14] because of the complexity of the enzymes in whole cells.

3.4. Effects of metal ions or chelator on activity and enantioselectivity of the purified esterase

Many hydrolases are known to require metal ions [41,42]. The effects of some typical metal ions on esterase activity and enantioselectivity were investigated at pH 7.0 and 30 °C. As shown in Table 5, Ca^{2+}, K^{+} and Mg^{2+} had a stimulating effect on the esterase activity, and with the rise of Ca^{2+}, K^{+} or Mg^{2+} concentration, the esterase activity increased. Especially, Ca^{2+} had almost 150% enhancing effect on the activity of the enzyme at the concentration of 0.01 M. Ca^{2+} is known to promote the hydrolytic activity of some kinds of esterases/lipases [43,44]. Similarly, the esterase activity could be increased to almost 1.4 times or 1.2 times when 0.01 M K^{+} or Mg^{2+} was used. However, the activity of purified esterase was inhibited markedly by some metal ions such as Ba^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+}, no hydrolytic activity was detected when 0.01 M Cu^{2+} or Hg^{2+} was used. In general, esterases/lipases are strongly inhibited by Hg^{2+} (a thiol group inhibitor), which is due to the proximity of the SH group to the catalytic and interfacial binding site but spatially remote from the catalytic site [45]. The chelating agent EDTA had no clearly inhibition on the enzyme activity, which indicated this esterase was not a metalloenzyme. Little influence was observed for most metal ions or EDTA on the enantioselectivity toward racemic menthyl acetate in the case of the enzyme activity existence. The results indicated that the metal ions could influence the active site but not change the conformation of the enzyme.

3.5. Effects of detergents or inhibitors on activity and enantioselectivity of the purified esterase

Detergents or inhibitors can influence esterase/lipase activity and enantiospecificity by changing the conformation or altering interface characters between esterase/lipase and substrate [21,46]. The effects of different detergents and inhibitors on the hydrolysis activity and enantioselectivity of the purified esterase were systematically investigated using racemic menthyl acetate as substrate at 30 °C and pH 7.0 (Table 6). The enzyme was strongly inhibited (100% inhibition for menthyl acetate hydrolysis) by 0.01 M ionic detergents such as cetyltrimethyl ammonium bromide (CTAB), sodium deoxycholate (SDC) and sodium dodecyl sulfate (SDS). On the other

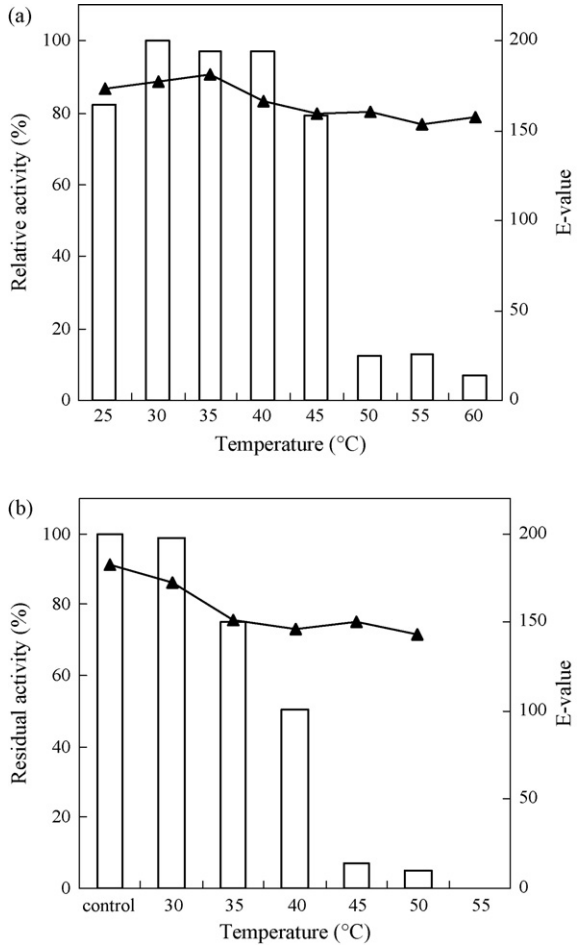


Fig. 3. Effects of temperature on the enzyme activity (a) and stability (b) as determined with DL-menthyl acetate at pH 7.0. (a) Relative enzyme activity (□) and enantioselectivity (▲) were assayed at the temperatures indicated. (b) Enzyme solution was incubated for 4 h at the temperatures indicated, and the residual activity (□) and enantioselectivity (▲) were determined at 30 °C.

Table 5
Effects of different metal ions on activity and enantioselectivity of the purified enzyme

Metal ion	Concentration (mM)	Residual activity (%)	*E*-value
Control		100	>100
Na^+	1	98	>100
	10	106	>100
K^+	1	117	>100
	10	136	>100
Mn^{2+}	1	93	>100
	10	92	>100
Ca^{2+}	1	123	>100
	10	148	>100
Hg^{2+}	1	N.D.	N.D.
	10	N.D.	N.D.
Cu^{2+}	1	39	>100
	10	N.D.	N.D.
Zn^{2+}	1	49	>100
	10	17	>100
Mg^{2+}	1	112	>100
	10	116	>100
Ba^{2+}	1	79	>100
	10	25	>100
Co^{2+}	1	46	>100
	10	9	>100
Fe^{3+}	1	61	>100
	10	11	>100
Al^{3+}	1	100	>100
	10	98	>100
EDTA	1	103	69
	10	101	41

Residual activity (%) was the DL-menthyl acetate hydrolytic activity obtained after incubating the enzyme with various metal ions at 30 °C for 1 h compared to the control. N.D.: not detected. All assays were performed in duplicates and average values were taken.

Table 6
Effects of detergents and inhibitors on activity and enantioselectivity of the purified enzyme

Detergents or inhibitors	Concentration (mM)	Residual activity (%)	*E*-value
Control	0	100	>100
Triton X-100	1	99	78
	10	102	65
Tween 80	1	101	49
	10	97	43
Cetyltrimethyl ammonium bromide	1	64	60
	10	N.D.	N.D.
Sodium dodecyl sulfate	1	79	33
	10	N.D.	N.D.
Sodium deoxycholate	1	86	47
	10	N.D.	N.D.
Phenylmethylsulfonyl fluoride	1	17	19
	10	N.D.	N.D.
β-Mercaptoethanol	1	106	>100
	10	103	>100
Dithiothreitol	1	97	>100
	10	101	85

Residual activity (%) was the DL-menthyl acetate hydrolytic activity obtained after incubating the enzyme with various inhibitors or detergents at 30 °C for 1 h compared to the control. N.D.: not detected. All assays were performed in duplicates and average values were taken.

hand, a serine enzyme inhibitor, phenylmethylsulfonyl fluoride (PMSF) completely inhibited the enzyme activity at 0.01 M concentration, which suggested that this esterase belongs to the class of serine hydrolases. This was similar to the findings of Maqbool et al. [47]. The nonionic surfactants (Triton X-100 and Tween 80) and the reducing agent like dithiothreitol (DTT) and β-mercaptoethanol had no significant inhibiting or stimulating effect on specific activity. There was some descent with the *E*-values when the inhibitors or detergents were used, except DTT and β-mercaptoethanol at low concentration.

4. Conclusions

We had purified a highly enantioselective L-menthyl ester hydrolase from *B. cepacia* ATCC 25416. This esterase was an intracellular enzyme. Its molecular weight was 37 kDa. The N-terminal sequence was "MGARTDA", which showed no homology with other *Burkholderia* sp. esterase/lipase. The enzyme specifically liberated short chain fatty acids from menthyl esters. The optimum temperature and pH were 30 °C and 7.0, respectively. This esterase was characteristically stable at 30–40 °C and pH 7.0–9.0. Enzyme activity was stimulated by Ca^{2+}, K^+ and Mg^{2+}, and inhibited by Ba^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+}. Additionally, the enzyme was strongly inhibited by ionic detergents and PMSF but not affected by nonionic surfactant and reducing agent. The enantioselectivity was not influenced by metal ions but decreased in the presence of inhibitors or detergents. The effects of metal ions and detergents/inhibitors on enzyme activity were the same as those of most reported esterases/lipases. The PMSF inhibition showed that the key enzyme was a serine hydrolase. Considered the characteristics and N-terminal sequence and the microbial source, the purified enzyme might be a new carboxylesterase (EC 3.1.1.1) for stereospecific hydrolysis of short acyl chain DL-menthyl esters to prepare optically pure L-menthol or its derivatives.

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