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Novel long-term storage cholesterol esterase isolated from thermotolerant *Pseudomonas aeruginosa* strain RE24.3

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ABSTRACT: Cholesterol esterase (CHE; EC 3.1.1.13) plays an important role in the hydrolysis of cholesterol ester to cholesterol and free fatty acids. It is used for clinical detection of cholesterol. Herein, the CHE producing microorganisms were isolated from oil contaminated soil samples at 45 °C by spreading the sample on nutritive agar. *Pseudomonas aeruginosa* strain RE 24.3 was selected since it produced the highest CHE activity. The purified enzyme, namely CHEPaRE24.3, had high activity between 30 and 45 °C at the optimum pH of 7.0. The substrate specificity test revealed that CHEPaRE24.3 hydrolysed a variety of cholesterol esters. Inhibitor examination found that Hg^{2+}, β-mercaptoethanol, and DTT could inhibit the enzyme activity at 99.38%, 100%, and 43.2%, respectively. It is remarkable that PMSF showed no effect on enzyme activity. The stability test indicated that the CHEPaRE24.3 enzyme remained at 70% activity after 28 days storage at 4 °C, 29 °C, or 37 °C. The long-term storage stability and thermotolerance of CHEPaRE24.3 as well as its resistance to PMSF suggests that it could be applicable for use in a cholesterol biosensor.

KEYWORDS: biochemical characterization, bacteria, enzyme

INTRODUCTION

Cholesterol esterase (CHE; EC 3.1.1.13) is a glycoprotein that belongs to the lipase/esterase family [1–3]. Triacylglycerols, cholesteryl esters, phosphoglycerides, esters of vitamins A and D, and monoacylglycerols are also among the physiological substrates of the enzyme [4]. Most experimental studies have been done on mammalian CHEs because of their importance in cholesterol absorption and metabolism [5,6]. CHE has been extensively examined in a number of mammalian tissues such as pancreas [7], small intestine [7], brain [8], liver [9], and placenta [10]. The enzyme also has a widespread distribution in bacteria, fungi, and yeasts such as *Pseudomonas fluorescens* [11], *P. aeruginosa* [12], *Fusarium oxysporum* [13], *Candida cylindracea* [14], *Saccharomyces cerevisiae* [15], *Staphylococcus aureus* [16] and *Streptomyces lavendulae* [17]. The practical use of these enzymes is limited to the measurement of total cholesterol in combination with cholesterol oxidase (EC 1.1.3.6) in blood. Some commercially available enzymes have been isolated and purified from microorganisms.

Methods for cholesterol detection can be classified into two categories, enzymatic and non-enzymatic methods. The measurement of enzymatic reaction is generally performed by colorimetric [18,19] or fluorometric techniques [20,21]. Enzymatic reactions have been applied to detect cholesterol by using biosensor technology. Cholesterol biosensors such as carbon nanotube [22], acrylamine glass [23], and oxygen electrode [24] were developed to determine the total cholesterol in blood and serum. These tools were developed based on chemical detection by using CHE and cholesterol oxidase. Hence, the development of the novel CHE is crucial for its application in cholesterol biosensors.

In this study, we explored and characterized the novel CHE from thermotolerant *P. aeruginosa* isolated from oil-contaminated soil samples. The enzyme was characterized for its substrate specificity, storage time, optimal pH, optimal temperature, thermal stability, as well as the effect of metal ions, inhibitors, organic solvents, and detergents on its activity.

MATERIALS AND METHODS

Samples, cultures, and medium

The lipid contaminated soil samples were collected from Rachaburi province, Thailand. Microorganisms were collected by filtration according to the protocol of Entcheva[25]. Each sample was crushed and dissolved in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0.) 1:1.5 (g/ml), 1% Triton X-100, and shaken at 37 °C for a few hours. The supernatants were collected and transferred for bacterial cultivation in minimum medium (1% cholesteryl (*CL*) oleate, 0.5% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, and 0.05% KCl) with shaking at 37 °C for 72 h. The bacterial culture was collected by centrifugation at 8650*g* for 15 min. The pellet was resuspended in TE buffer and spread for single-colony isolation in rich-medium agar (2% polypeptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar) prior to incubation at 37 °C for 12–14 h.

Bacterial screening and genetic identification

Each bacterial colony isolated from soil was cultivated in 5 ml of rich-medium at 37 °C with agitation at 200 rpm for 5 days. Culture broth was centrifuged for 15 min at 8650*g* and supernatant was collected and examined for CHE activity based on Gallo's method[1]. Bacterial isolates producing CHE were identified by using PCR amplification of 16 S rDNA gene employing bacterial universal primers Bac8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and Bac1492R (5′-ACGGTTACCTTGTTACGACTT-3′)[26]. Reaction mixtures were manipulated in 100 µl volumes containing 100 ng of DNA samples, 1 × PCR buffer, 20 µl of each primer, 10 mM dNTP, 1.5 mM $MgCl_2$, and 1.5 units of *Taq* DNA polymerase. The PCR amplification was performed by using a Thermal Cycler (MJ Research, PTC-200) for 30 cycles. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. PCR products were electrophoresed in 1.2% agarose gel at 110 V for 35 min prior to staining in ethidium bromide solution and observed under UV-light. PCR products were ligated to pGEM-T Easy Vector (Promega) prior to analysis by using the dideoxy DNA sequencing method (Macrogen, Korea).

Enzyme assay

CHE activity was determined based on Gallo's method[1] by using micellar cholesteryl linoleate as the substrate. The standard assay was developed in 200 µl microplate volume. For enzyme measurement, 150 µl of substrate solution (100 mM sodium phosphate buffer, pH 7.0), 4.5 mM *CL*-linoleate, 0.33% Triton X-100, and 0.68% isopropanol was added to 29 µl of enzyme solution (supernatant of each sample) and incubated at room temperature (29 ± 2 °C) for 30 min. The reaction was stopped by heating at 95 °C for 5 min. Then, 21 µl of developing solution (0.25% saturated phenol, 1.5 purpurogallin units of horseradish peroxidase, 1.5 mM 4-aminoantipyrin, and 0.1 unit of cholesterol oxidase) was added before measuring absorbance at 500 nm. The reaction was recorded for 30 min on an Anthos 2010 microplate reader (Biochrom). One unit of enzyme was defined as the amount of enzyme required to produce 1 µmol of quinoneimine dye (molar extinction coefficient $\lambda = 1.36 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$ at 500 nm) per min from the reduction of 4-aminoantipyrin.

Protein assay

Protein was determined according to Bradford protein assay kit (Fermentas) by using bovine serum albumin as the standard. The Coomassie Brilliant Blue G-250 products were measured by determining the absorbance at 595 nm.

Enzyme purification

P. aeruginosa isolated from soil was cultivated in 3 l rich medium at 37 °C for 5 days and shaken at 200 rpm for 5 days. The culture broth was centrifuged for 15 min at 8650*g*. The supernatant was fractionated by a salting-out technique using $(NH_4)_2SO_4$ at 20–80% saturation. Each step was centrifuged at 12 000*g* for 15 min at 4 °C. The pellet containing enzyme was further dissolved in a minimum volume of 20 mM phosphate buffer (pH 7.0). The crude enzyme was dialysed against 10 mM Tris-HCl (pH 8.0) using a cellulose membrane with a molecular cutoff at 12 kDa. The CHE enzyme was then purified by DEAE-52 ion-exchange chromatography (Whatman). Protein was eluted by using 20 mM phosphate buffer (pH 7.0) containing semi-gradient 0–1 M NaCl at flow rate of 0.5 ml/min. The elution buffer concentration was increased by 0.1 M NaCl for every step of purification. Every eluted fraction was assayed for CHE activity and analysed in SDS-PAGE gel (12.5%).

Molecular mass analysis

The molecular mass of the purified CHE was determined by the SDS-PAGE method[27] using Page Ruler protein ladder (Fermentas) as standard molecular weights. Before electrophoresis, samples were boiled for 10 min in the presence of 2 × sample buffer containing β-mercaptoethanol. The gels were stained by using the standard Coomassie Brilliant

Blue R250 method[28]. The periodic acid Schiff (PAS) staining method was used to identify any glycoprotein compartments after SDS-PAGE[29].

Optimum pH

The pH optimum was evaluated by using the standard assay protocol described above with substitution of the following appropriate buffers: 100 mM glycine-HCl at pH 2 and 3; 100 mM sodium acetate at pH 3, 4, and 5; 100 mM NaH_2PO_4 at pH 5, 6, and 7; 100 mM Tris-HCl at pH 7, 8, and 9; 100 mM glycine-NaOH at pH 9, 10, and 11. The cholesterol substrate was prepared in the appropriate buffers.

Optimum temperature

The enzyme optimum temperature was determined by using the standard activity assay at temperatures ranging from 25–80 °C at the optimum pH.

Enzyme stability and storage time

The enzyme stability of purified CHE was performed at optimum pH at 4 °C, room temperature, 37 °C, 45 °C, and 60 °C for 2 days. The storage time was analysed at 4 °C, room temperature, and 37 °C every 7 days for 28 days by using standard condition and assay.

Substrate specificity

Substrate specificity of the enzyme was determined by using a standard enzyme assay method with a variety of cholesteryl esters with a chain length between C2 and C18:2.

Effects of detergents, metal ions and inhibitors

The effects of detergents, metal ions, and inhibitors on the CHE activity were determined by using standard conditions. The enzyme was incubated with 20 mM phosphate buffer (pH 7.0) containing 3% of detergents or 1 mM of metal ions and inhibitors for 1 h at room temperature. After treatment, the reaction mixtures were incubated at room temperature for another 30 min followed by heating at 95 °C for 5 min. The CHE activity was determined by using standard conditions.

RESULTS

Bacterial isolation and identification

After soil sample screening, the *P. aeruginosa* strain 24.3 was purified and its DNA was identified by using PCR amplification of 16 S rDNA. Its nucleotide sequences were compared against those of other organisms reported in GenBank. *P. aeruginosa* strain 24.3 was classified as *P. aeruginosa* with 99% similarity.

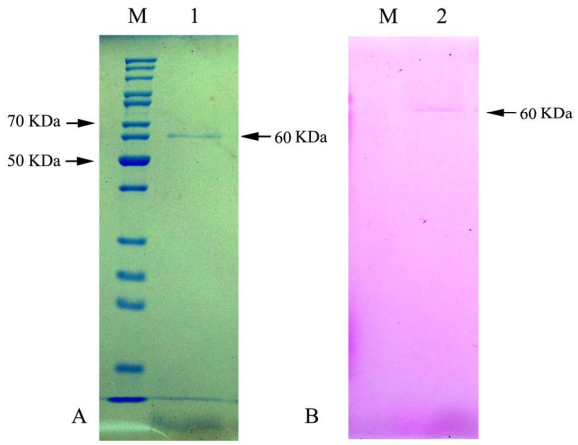

Fig. 1 SDS-PAGE of CHEPaRE24.3 after using purification using semi-gradient gel column chromatography DEAE-52. Sample containing 2.5 mU of CHEPaRE24.3 was electrophoresed on the 12.5% SDS-PAGE and stained with (A) Coomassie Brilliant Blue R250 and (B) PAS staining. Lane M: molecular marker; lanes 1 and 2: purified CHEPaRE24.3 after DEAE-52 column chromatography.

Enzyme purification

Purification of CHE from *P. aeruginosa* strain RE24.3 was achieved when $(NH_4)_2SO_4$ at 40% saturation was used. After DEAE-50 ion exchange chromatography, a single band of 60 kDa molecular weight (Fig. 1A) was observed with 1.98% recovery and 51 purification fold. According to PAS staining, CHEPaRE24.3 exhibited a clear red band indicating the presence of a carbohydrate chain (Fig. 1B). The specific activity of enzyme using *CL*-linoleate as a substrate was 1.173 μmol min^{-1} mg^{-1} which was comparable to that of commercial CHE (Wako, Japan) (Table 1).

Optimum temperature, pH optimum, and stability test

The CHEPaRE24.3 enzyme showed highest activity at pH 7.0 in 20 mM phosphate buffer (Fig. 2). The cholesterol esterase activity remained high in a broad range of temperatures (30–45 °C, Fig. 3). The enzyme retained more than 70% of its initial activity after 24 h, which gradually decreased to 24% after 48 h at 60 °C. The storage time of enzyme revealed that it could be kept up to 28 days at 4 °C, room temperature, or 37 °C with remaining activity of 82%, 72%, and 68%, respectively (Fig. 4).

Table 1 Summary of CHEPaRE24.3 purification.

Fractions	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Recovery (%)	Purification (fold)
Culture filtration	4311	102	0.023	100	1
Ammonium sulphate ppt	428.1	11.7	0.027	11.47	1.17
DEAE-52	1.17	2.025	1.173	11.985	51

CHE activity was determined in 100 mM phosphate buffer (pH 7.0) at room temperature using *CL*-linoleate as the substrate.

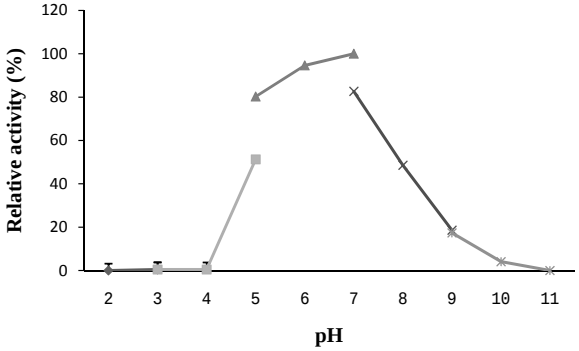

Fig. 2 Effect of pH on CHEPaRE24.3 activity assayed at various pH at room temperature. The buffer systems (0.1 M) were glycine-HCl (pH 2–3, ♦), sodium acetate (pH 3–5, ■), sodium phosphate (pH 5–7, ▲), Tris-HCl (pH 7–9, ×), and glycine (pH 9–11, ∗).

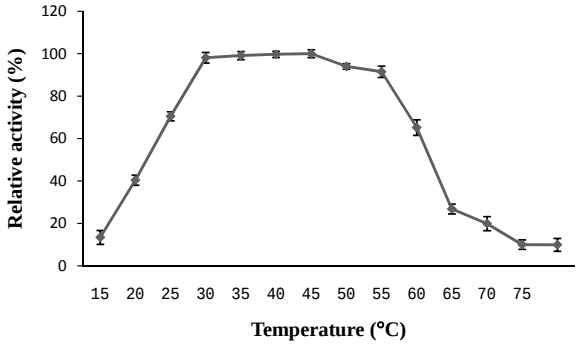

Fig. 3 Effect of temperature on CHEPaRE24.3 enzyme activity. (♦) represents variation of temperature after incubation at each temperature for 30 min. The standard error was less than 5% when triplicate relations were tested.

Substrate specificity

CHEPaRE24.3 enzyme could hydrolyse *CL*-linoleate, *CL*-oleate, *CL*-myristate, *CL*-n-decanoate, *CL*-stearate, *CL*-palmitate, and *CL*-dodecanoate with relative activities of 100%, 28.4%, 25.8%, 17.4%, 13.4%, 13.2%, and 9.2%, respectively (Fig. 5).

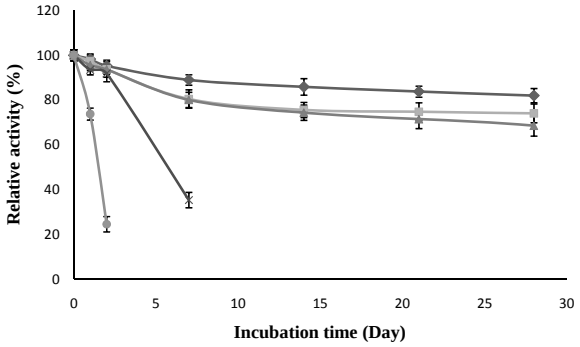

Fig. 4 Stability test and storage time of CHEPaRE24.3 enzyme. The activity was assayed in 100 mM sodium phosphate buffer (pH 7.0) at 4 °C (♦), room temperature (■), 37 °C (▲), 45 °C (×), and 60 °C (●) for 28 days. The standard error was less than 5% when triplicate reactions were tested.

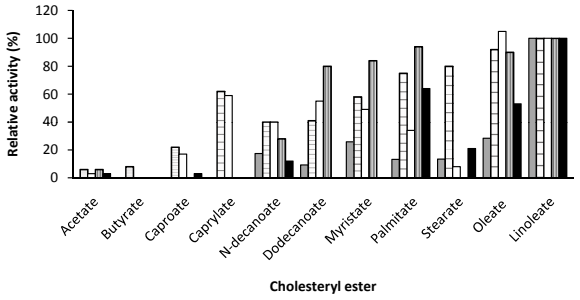

Fig. 5 Substrate specificity of microbial cholesterol esterases. The activity was assayed in 100 mM sodium phosphate buffer (pH 7.0) at 4 °C. The enzyme activities were compared among CHEPaRE24.3 (grey bars), CHE-A (horizontal striped bars) [12], COE-311 (white bars) (*Pseudomonas* sp., strain COE-311 in Toyobo Enzyme Manual [12]), CHE-1 (vertical striped bars) (*Pseudomonas* sp., strain CHE-1 in Amano Enzyme Manual [12]), and *P. fluorescens* (black bars) [11]. The standard error was less than 5% when triplicate reactions were tested.

Effect of detergents

Study on the effect of various detergents indicated that cholic acid, Tween 20, deoxycolic acid, Triton

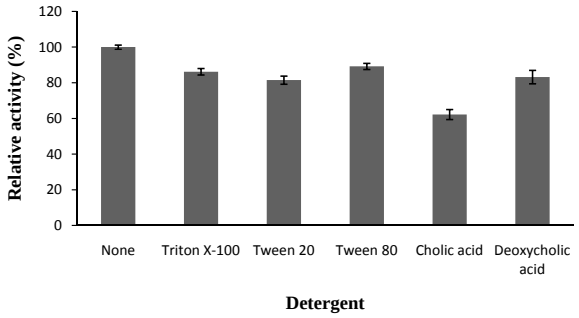

Fig. 6 Effect of detergents on cholesterol esterase activity. CHE was incubated with each compound (0.3% or 1 mM) for 1 h at room temperature (29 ± 0.5 °C) in 100 mM Phosphate buffer (pH 7.0) using *CL*-linoleate as the substrate. The standard error was less than 5% when triplicate reactions were tested.

X-100, and Tween 80 could inhibit CHEPaRE24.3 activity at 37.8%, 18.5%, 16.8%, 13.8%, and 10.9%, respectively (Fig. 6).

Effect of metal ions and inhibitors

The effects of metal ions and inhibitors on the catalytic activity of CHEPaRE24.3 were compared to that of CHE-A, *Streptomyces* sp. X9, and *S. avermitilis*[30]. Most metal ions showed no effect on CHEPaRE24.3 except Hg^{2+}, which totally inhibited the enzyme activity as well as that of CHE of *S. avermitilis*. Reducing agents, such as DTT and β-mercaptoethanol suppressed the CHEPaRE24.3 activity by 43% and 100%, respectively (Table 2).

Table 2 Effects of metal ions and inhibitors on CHE. SX9 = *Streptomyces* sp. X9; SA = *S. avermitilis*

Inhibitors	Relative activity (%)			
	CHEPa-RE24.3	CHE-A	SX9	SA
None	100	100	100	100
NaCl	114.5	ND	100	100
KCl	93.4	ND	90	100
$CaCl_2$	100.7	104	100	100
$ZnSO_4$	88.4	ND	100	100
$FeCl_2$	114.8	99	80	90
$CuSO_4$	98.1	92	85	120
$MnSO_4$	92.7	ND	ND	ND
$HgCl_2$	0.6	ND	50	0
$CdSO_4$	124.9	ND	ND	ND
EDTA	85.4	97	100	93
EGTA	96.8	ND	100	94
Iodoacetic acid	97	92	100	100
O-Phenanthroline	101	ND	100	95
TPCK	125	ND	93	85
b-mercaptoethanol	0	ND	94	77
DTT	56.8	ND	0	0
PMSF	97.9	6	100	100
PCMB	98.5	ND	78	75

ND: No data. CHE was incubated each compound (1 mM) for 1 h at room temperature in 100 mM phosphate buffer (pH 7.0). The activity was assayed with *CL*-linoleate as the substrate. EDTA: ethylenediaminetetraacetic acid; EGTA: ethylene glycol tetraacetic acid; TPCK: *N*-tosyl-L-phenylalanyl clomethylketone; DTT: dithiothreitol; PMSF: Phenylmethanesulfonyl; PCMB: *p*-chloromercuribenzoic acid. The standard errors of the means are below 5% (3 experiments).

DISCUSSION

A novel thermotolerant long-term storage cholesterol esterase was purified from *P. aeruginosa* strain RE24.3, characterized, and compared to the CHE of other microorganisms (Table 3). According to PAS staining test, CHEPaRE24.3 carried a 60 kDa oligosaccharide, which is a similar finding to CHE in *P. aeruginosa*[12]. Referring to optimum pH and temperature data, the enzyme showed similar features to those of *Streptomyces* sp. X9[35] and *S. avermitilis*[30]. Actually, thermotolerant *P. aeruginosa* strain RE24.3 could grow at 60 °C but the best temperature for production of CHE was 37 °C. According to the optimum temperature test, CHEPaRE24.3 exhibited a hydrolytic activity in a wide range of temperatures (30–45 °C) which was different from other CHE. This characteristic was relevant to the use of enzyme when variation of temperature occurred. According to the stability test, approximately 70% of enzyme activity remained after long-term storage for 28 days. In contrast, the CHE from *Pseudomonas* sp. (Amano enzyme) retains approximately 80% of its activity after 5 days storage, decreasing to 20% after 12 days[35]. It is notable that the shelf-life of CHEPaRE24.3 was comparable to that of CHE from *Streptomyces* sp. X9[35].

The substrate specificity test indicated that CHEPaRE24.3 hydrolysed a variety of cholesterol esters. These data were similar to those of *P. aeruginosa* (CHE-A)[12], *Streptomyces* sp. X9[35], and *S. avermitilis*[30]. It was found that the long chain fatty acid CL-linoleate was a specific substrate for most of the tested CHE enzymes.

Upon inhibitor testing, the data revealed that reducing agents such as β-mercaptoethanol and DTT could inhibit CHEPaRE24.3 activity via hydrolysis of the disulphide bridge in the structure. It was also found that Hg^{2+} suppressed the CHEPaRE24.3 activity whereas it activated the CHE activity from

Table 3 Comparison of CHEPaRE24.3 to CHE of other microorganisms.

Enzymes	Temp range	Optimum temp	pH	Optimum pH	Storage time at room temp	Specific substrate cholesterol ester
CHEPaRE24.3	30–55 °C	30–45 °C	5–8	7	> 28 days	linoleate > oleate > myristate
CHE-A	40–70 °C	52 °C	5–10	5.5–9.5	ND	linoleate > oleate > sterate
Streptomyces sp. X9	35–50 °C	40 °C	5.5–7	6	> 28 days	linoleate > palmetate > oleate
S. avermitilis	35–45 °C	40 °C	5–7	6	ND	linoleate > myristate > palmetate

ND: No data.

S. avermitilis[30]. In general, Hg^{2+} strongly inhibited lipase and esterase activities via binding to the OH group at the catalytic site[36,37]. The catalytic triad consists of Ser, His, and Glu or Asp[38,39]. Thus the bulky Hg^{2+} group may cause the steric hindrance for the substrate approach to the active site.

PMSF did not show any effect on the CHEPaRE24.3 activity. Basically, PMSF is a specific inhibitor for serine protease that binds specifically to serine residue at the active site of serine proteases but not to any other serine residues in the protein. In this study, PMSF was tested for its inhibition on any contaminated serine proteases that may affect the CHEPaRE24.3 activity in the reaction.

In conclusion, the long-term storage property of thermotolerant CHEPaRE24.3 could make it suitable for use in a cholesterol biosensor. Further studies on nucleotide sequences and gene expression to enhance the production of cholesterol esterase will be needed.

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