Journal of Microbiology and Biotechnology
Vol. 7. No. 1, 37-42, 1997

Purification and Partial Characterization of Thermostable Carboxyl Esterase from *Bacillus stearothermophilus* L1

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A bacterial strain L1 producing a thermostable esterase was isolated from soil taken near a hot spring and identified as *Bacillus stearothermophilus* by its microbiological properties. The isolated thermostable esterase was purified by ammonium sulfate fractionation, ion exchange and hydrophobic interaction chromatographies. The molecular weight of the purified enzyme was estimated to be 50,000 by SDS-PAGE. Its optimum temperature and pH for hydrolytic activity against PNP caprylate were 85°C and 9.0, respectively. The purified enzyme was stable up to 70°C and at a broad pH range of 4.0-11.5 in the presence of bovine serum albumin. The enzyme was inhibited by phenylmethylsulfonyl fluoride and diethyl *p*-nitrophenyl phosphate, indicating the enzyme is a serine esterase. The enzyme obeyed Michaelis-Menten kinetics in the hydrolysis of PNPEs and had maximum activity for PNP caproate (C_6) among PNPEs (C_2-C_{12}) tested.

Carboxyl esterases (EC 3.1.1.1) catalyzing the hydrolysis of carboxylic esters are ubiquitous enzymes that have been purified and studied from different sources, including animals, plants, and microorganisms. These enzymes generally have a broad substrate specificity and heterogeneity (10, 11). The molecular and catalytic properties of esterases from mammalian sources have been well studied, while limited investigations have been made on microbial esterase. Microbial esterases have been isolated and partially characterized from *Bacillus* species, *Pseudomonas species*, *Aspergillus niger*, and *Sulfolobus acidocaldarius*.

Recent interest in the potential applications of esterases in a number of valuable biotransformations has focussed upon (7): resolution of racemic mixtures, synthetic reactions, blocking or unblocking of catalytic groups in peptide chemistry, and the modification of sugars. For such applications, stability with respect to temperature, organic solvents, detergents and common protein denaturants is important. Thermophilic microorganisms are an obvious potential source of stable enzymes (4, 13). To date thermostable carboxylestrerases have been purified and characterized only from *S. acidocaldarius* (14), *B. stearothermophilus* (1, 9, 12, 15), and *B. acidocaldarius* (6).

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Key words: *Bacillus stearothermophilus*, thermostability

This work reports the purificatrion and functional characterization of a highly active esterase from a strain of *Bacillus stearothermophilus*, functionally distinct from those *B. stearothermophilus* esterases previously reported (1, 9, 12, 15).

MATERIALS AND METHODS

Materials

p-Nitrophenyl esters (PNPE), phenylmethylsulfonyl fluoride (PMSF), diethyl *p*-nitrophenyl phosphate (E600), iodoacetamide, tributyrin, tricaprylin, and molecular weight marker for SDS-PAGE were purchased from Sigma Chemical Co.. DEAE-Sepharose CL-6B was obtained from Pharmacia LKB. Bovine serum albumin was from USB Corp..

Isolation and Cultivation of Thermostable Esterase-producing Bacteria

Soil samples taken near a hot spring at Yusung were suspended in sterilized water and the suspension was spread onto a 1% (v/v) TBN agar plate (1.5% agar; pH 7.2) containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. A bacterial strain that formed a clear zone after 12 h incubation at 55°C was isolated. The strain was cultivated in a 5 liter jar fermentor using 3 liter of modified Y medium (1% polypeptone, 0.5% yeast extract, 0.2% beef extract, 0.2% glycerol, 0.3% NaCl, 0.2% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4$, pH 7.2) at 55°C with an agitation rate of 400 rpm and an aeration rate of 0.5 vvm.

Enzyme Assay

Activity was measured with *p*-nitrophenyl caprylate (PNPC) or other PNPEs as substrates. The reaction mixture consisted of 0.01 ml of 10 mM substrate in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM Tris-HCl buffer (pH 8) containing an appropriate amount of the enzyme. In the assay of revealing the biochemical properties of the purified enzyme, 0.05 mg/ml of BSA was added to reaction mixtures. The enzyme reaction was performed for 3 min at 55°C, unless otherwise specified. The amount of *p*-nitrophenol liberated during the reaction was measured by its optical density at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 mol of *p*-nitrophenol per min at 55°C, and the specific activity was expressed as the number of units per mg of protein.

Protein Measurement

The protein concentration was measured by the Bradford method (2) using dye reagent purchased from Bio-Rad. Bovine serum albumin was used as a standard protein.

Purification of Esterase

All procedures were performed at 4°C. The supernatant obtained by centrifuging a 3 liter culture broth was concentrated to 500 ml with an ultrafiltration kit (Microgon, Cole-Parmer Instrument Co.). Solid ammonium sulfate was added to the concentrated supernatant to 30% saturation and left overnight. The supernatant was collected by centrifugation and brought to 80% saturation in the same manner. The precipitate was collected by centrifugation and dissolved in 10 mM potassium phosphate buffer, pH 7.0 and dialyzed overnight against the same buffer. The dialysate was applied to DEAE-Sepharose CL-6B column (2.0×17 cm). Esterase activity was eluted with a 0 - 0.5 M linear gradient of KCl in 10 mM potassium phosphate buffer (pH 7.0). The fractions with esterase activity were concentrated by ultrafilitration kit using an Amicon PM 10 membrane. Ammonium sulfate was added to the concentrated solution to the final concentration of 0.5 M and enzyme solution was applied to a Phenyl-Superose HR 5/5 column equilibrated with 0.5 M ammonium sulfate in 10 mM potassium phosphate buffer (pH 7). After the column was washed with the same buffer and with a linear gradient of 0.5 to 0 M ammonium sulfate, the enzyme was eluted with 10 mM potassium phosphate buffer (pH 7). The active fractions were pooled and used as purified enzyme.

Polyacrylamide Gel Electrophoresis and Activity Assay

SDS-PAGE (12%) was performed using slab gels as described by Laemmli (5). Proteins on the gels were stained with Coomassie Brilliant Blue R-250. Renaturation of the enzyme after SDS-PAGE was done by soaking gels for 1 h in 50 mM Tris-HCl buffer (pH 8). Activity assay of the renatured gels was achieved by attaching it to tributyrin (or tricaprylin) agar plate and incubating at 55°C.

N-Terminal Amino Acid Sequencing

Purified esterase was subjected to SDS-PAGE and elctroblotted on polyvinylidene difloride membrane (Bio-Rad Trans-Blot Transfer Medium) (8). After staining with Coomassie Brilliant Blue R-250, protein bands were cut out. The N-terminal amino acid sequence was determined by the automated Edman degradation method using a gas phase protein sequencer (model 476A, Applied Biosystem Ins., Foster, CA, U.S.A.).

Effects of Temperature on Esterase Activity

The optimum temperature for enzyme activity was determined by assaying esterase activities at various temperatures. To test the effect of temperature on the stability of the enzyme, enzyme solutions were incubated in water baths at various temperatures in the presence of 1 mg/ml BSA. After 30 min, they were removed, rapidly centrifuged and the residual enzyme activity of the supernatant assayed with PNPC as a substrate.

Effects of pH on Esterase Activity

To determine pH stability of the enzyme, enzyme solution was added to 0.1 M sodium acetate (pH 4-6), potassium phosphate (pH 6-7.5), Tris-HCl (pH 7.5-9), glycine-KCl-KOH (pH 9-11), and K_2HPO_4-K_3PO_4 (pH 11-12.5) buffers. After incubation for one hour at room temperature in the presence of 1 mg/ml BSA, the remaining esterase activity was measured using PNPC as a substrate. To find the optimum pH of the enzyme, esterase activities were checked at various pHs.

Effects of Inhibitors, Metal Ions, and Detergents

To test enzyme stability for various inhibitors, metal ions, and detergents, enzyme solutions were assayed after preincubation for 10 min with each reagents in the presence of 1 mg/ml of BSA.

Kinetics

Kinetics were performed at 55°C, using a Beckman DU68 spectrophotometer equipped with Beckman Temperature Controller. Initial velocity versus substrate concentration data were fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation by weighted linear least-squares analysis.

RESULTS

Isolation and Taxonomical Characterization of Strain L1

Thermophilic bacterial strains capable of producing esterase were isolated from soil samples. Among them, an isolate, strain L1, showed highly thermostable esterase activity on the tributyrin agar plate when incubated at 55°C. Strain L1 was motile, rod-shaped, and spore-forming. The results of fermentative and biochemical tests for this strain done as described in Bergey's Manual of Systematic Bacteriology (3) are included in Table 1.

Table 1. Microbiological characteristics of thermophilic strain L1.

	B. acido-caldarius	*B. stearoth-ermophilus*	Strain L1
Gram staining	+	+	ND
Rod-shaped	+	+	+
Endospore	+	+	+
Sporangium swollen	+	+	+
Catalase	ND	d	weak +
Voges-Proskauer test	–	–	
Growth at 7% NaCl	ND	–	
Acid from D-Glucose	ND	+	+
Hydrolysis of Starch	+	+	+
Casein	ND	d	–
Growth at pH 6.8	–	+	+
5.4	d	–	-
Growth at 30°C	–	-	–
40°C	-	+	+
50°C	+	+	+
60°C	+	+	+

Symbols: d, 11-89% of strains are positive; ND, no data available.

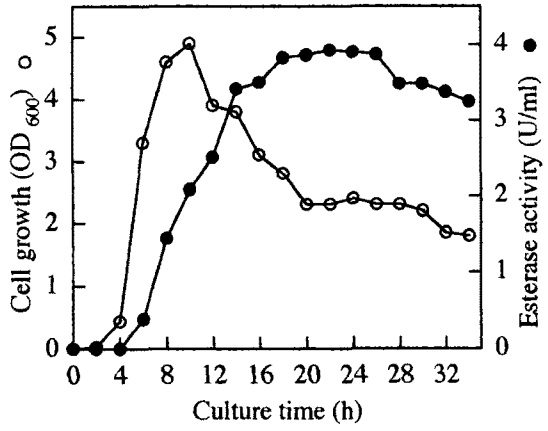

Fig. 1. Cell growth of *B. stearothermophilus* L1 and its extracellular esterase activity.

Most biochemical properties were similar to those of *B. acidocaldarius* and *B. stearothermophilus*. However, the lower limit of growth temperature for this strain was lower than that of *B. acidocaldarius* and the pH range of its optimum growth is neutral, whereas *B. acidocaldarius* grows well in acidic conditions. These results show that strain L1 is a strain of *Bacillus sterothermophilus* and was designated as *Bacillus sterothermophilus* L1.

Purification of L1 Esterase

When *B. stearothermophilus* L1 was cultivated at 55°C, esterase activity was detected in the extracellular medium. As shown in Fig. 1, the maximum extracellular esterase activity of the culture medium was about 4 units/ml, when PNPC was used as a substrate. However, since PNPC is a substrate for both esterase and lipase, we also

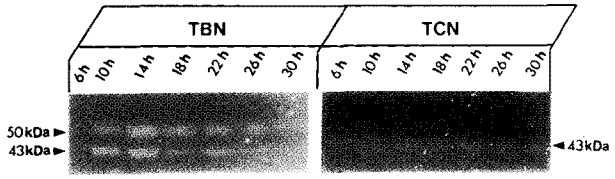

Fig. 2. Zymogram of *B. stearothermophilus* L1 extracellular culture medium.
Activity assay after SDS-PAGE for its extracellular culture medium obtained at 4-h intervals.

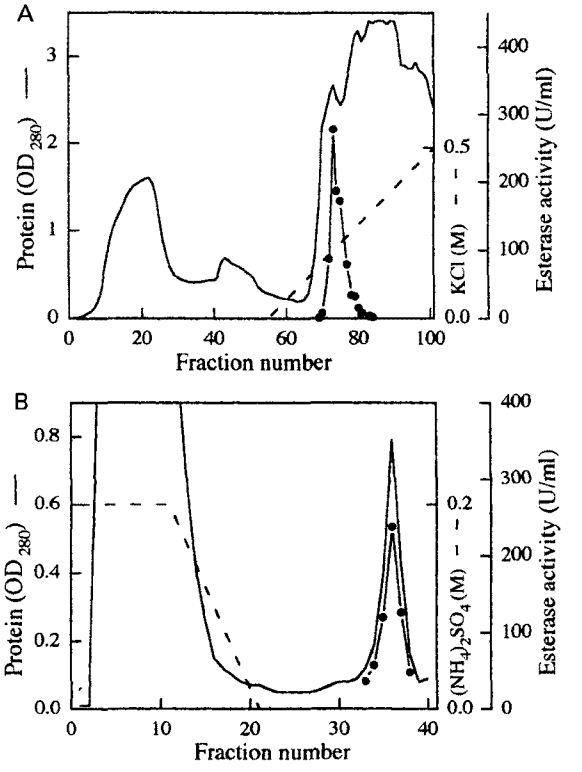

Fig. 3. Chromatography of *B. stearothermophilus* esterase.
A, Chromatography of crude enzyme on DEAE-Sepharose CL-6B column. The enzyme was eluted with a linear gradient of KCl in a phosphate buffer, at a flow rete of 60 ml/h. —●—, esterase activity. B, Phenyl Superose chromatography of active fractions from DEAE-Sepharose CL-6B column. The enzyme was eluted with a decreasing gradient of phosphate buffer containing ammonium sulfate at a folw rate of 0.5 ml/min. —●—, esterase activity.

tested whether the activity is due to esterase or lipase as follows: after SDS-PAGE was performed for extracellular medium, activity assay of the gel was performed using tributyrin (C_4) and tricaprylin (C_8) as substrates. Esterase can not hydrolyze tricaprylin, whereas lipase can hydrolyze both tributyrin and tricaprylin. Two bands (50 and 43 kDa) on the gel showed hydrolytic activites for tributyrin (Fig. 2). The 43-kDa protein also showed hydrolytic activity for tricaprylin, so that suggested it

was a lipase (In fact, it hydrolyzed triolein, a long chain triacylglycerol.). On the other hand, the 50-kDa protein was considered to be a genuine esterase from substrate specificity.

The 50-kDa esterase was purified by DEAE-Sepharose CL-6B and Phenyl-Superose column chromatographies (Fig. 3). By DEAE-Sepharose column, the 50-kDa esterase was separated from most other proteins including the 43-kDa lipase, which was eluted by a buffer containing above 0.45 M KCl. The 50-kDa esterase was purified to homogeniety on SDS-polyacrylamide gel by passing it through a Phenyl-Superose column (Fig. 4). As the enzyme interacted strongly with the resin, it was eluted last. The final purified enzyme (40 μg/ml) was used to test its biochemical properties.

A summary of the purification procedure is given in Table 2. Purification ratio was 690 fold and 31% of enzyme activity was recovered. The purified enzyme showed a specific activity of 2400 units/mg protein when assayed at 55°C with PNPC as a substrate.

Effects of Temperature on Esterase Activity

The effect of temperature on the esterase activity of L1 esterase was examined at various temperatures varing from 40 to 95°C. As shown in Fig. 5, the enzyme showed maximum activity at 85°C. The thermostability of the enzyme was drastically decreased above 70°C.

Effects of pH on Esterase Activity

The enzyme was stable at a broad pH range of pH 4.0-11.5 (Fig. 5). Its optimum pH for esterase activity was 9.0.

Effects of Inhibitors, Ions, and Detergents

The 50-kDa esterase activity was inhibited by PMSF and diethyl *p*-nitrophenyl phosphate (E600), the serine protease inhibitors (Table 3). This result shows the enzyme has serine residue in its active site as with other serine type-proteases. Most metal ions tested have no serious effect on enzyme stability.

Substrate Specificity and Kinetic Parameters

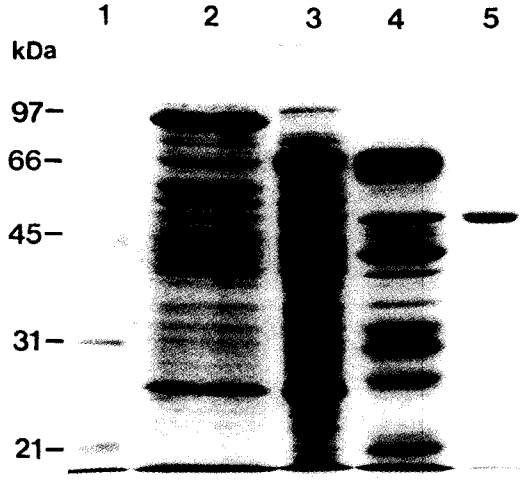

Fig. 4. SDS-PAGE of *B. stearothermophilus* esterase.
Lane 1, size marker; lane 2, culture supernatant; lane 3, after ammonium sulfate precipitation; lane 4, after DEAE-Sepharose CL-6B chromatography; lane 5, after phenyl-Superose chromatography.

Table 2. Summary of purification of *B. stearothermophilus* esterase.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture supernatant	1100	3900	3.5	1	100
$(NH_4)_2SO_4$	470	3300	7.0	2	85
DEAE-Sepharose	34	2600	76	22	67
Phenyl-Superose	0.50	1200	2400	690	31

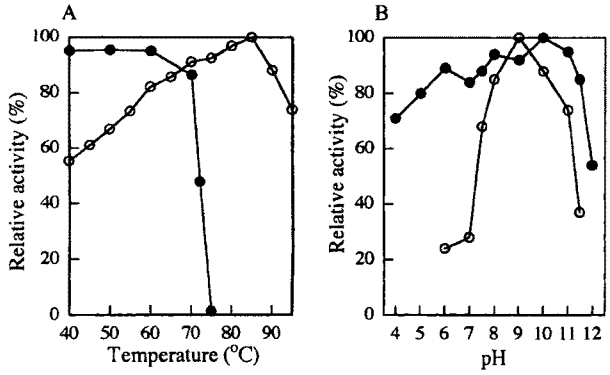

Fig. 5. Effects of temperature (A) and pH (B) on enzyme activity and stability.
The purified 50 kDa-esterase was assayed at various temperatures (○; A) and pH values (○; B). For the stability test, the enzyme was assayed after incubation at various temperatures (●; A) for 30 min and pH values (●; B) for 1 h.

Table 3. Effects of various enzyme inhibitors, metal ions, and detergents on the *B. stearothermophilus* esterase.

Reagents	Concentration	Relative activity (%)
None		100
PMSF	1 mM	30
E600	1 mM	6.9
EDTA	1 mM	95
Iodoacetamide	1 mM	84
$CaCl_2$	1 mM	107
$CoCl_2$	1 mM	100
$CuCl_2$	1 mM	91
$FeSO_4$	1 mM	110
$HgCl_2$	1 mM	100
$MgCl_2$	1 mM	107
$MnCl_2$	1 mM	100
$ZnCl_2$	1 mM	90
β-Mercaptoethanol	1% (v/v)	107
Triton X-100	1% (v/v)	97
Sodium deoxycholate	1% (w/v)	80
SDS	1% (w/v)	8

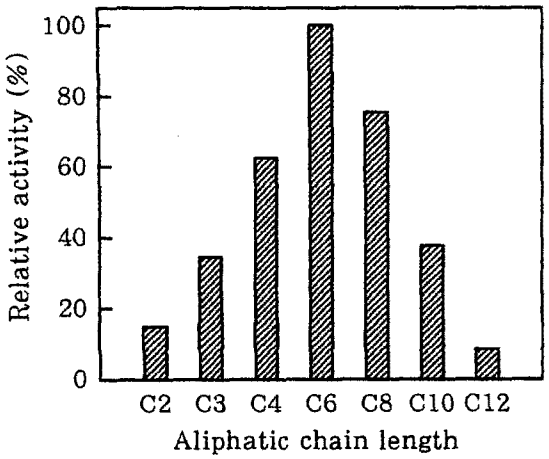

Fig. 6. Relative activity for hydrolysis of PNPEs by the purified 50 kDa-esterase.
Each substrate was used with final 0.1 mM concentration. Its specific hydrolytic activity for PNP caproate is 3200 U/mg protein.

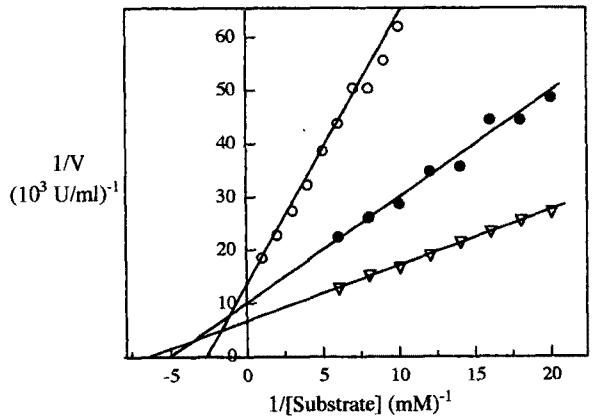

Fig. 7. Lineweaver-Burk plot of the purified 50 kDa-esterase.
Its hydrolytic activities for PNPA (○), PNPP (●), and PNPB (▽) were assayed in the presence of BSA (0.05 mg/ml). The protein concentration used in the kinetic assay was 40 μg/ml.

L1 esterase hydrolyzed a broad range of PNP substrates between PNP acetate (PNPA) and PNP laurate (Fig. 6). The enzyme had maximum activity for PNP caproate. When the acyl chain length of the substrate was increased above C_6 or decreased below C_6, there was a gradual decrease in enzyme activity.

L1 esterase obeyed Michaelis-Menten kinetics in the hydrolysis of PNPEs. This is indicated by linear Lineweaver-Burk plot with PNPA, PNP propionate (PNPP), and PNP butyrate (PNPB) as substrates (Fig. 7). The Michaelis constant (K_m) values of L1 esterase for PNPA, PNPP, and PNPB were 0.35 mM, 0.18 mM, and 0.16 mM, respectively. The maximum catalytic rate (k_{cat}) of L1 esterase for PNPA, PNPP, and PNPB at 55°C were 1540, 2000, and 3200 s^{-1}, respectively. The specificity constant k_{cat}/K_m values of L1 esterase for those substrates were similar with those of Tok19A1 esterase (15), a well characterized esterase produced from *B. stearothermophilus* Tok19A1, although both the K_m and k_{cat} values of L1 esterase were rather higher than those of Tok19A1 esterase.

DISCUSSION

We isolated a strain of *B. stearothermophilus* able to hydrolyze both tributyrin and tricaprylin. It produced two different kinds of enzymes with a molecular mass of 43 kDa and 50 kDa, repectively. The 43-kDa protein is able to hydrolyze tricaprylin and triolein as well as tributyrin, whereas the 50-kDa protein hydrolyzed tributyrin only. These results clearly show that the 43-kDa protein is a lipase and the 50-kDa protein is a genuine esterase. Since to date there has been no report about a *B. stearothermophilus* strain producing both esterase and lipase at the same time, this is the first report about a *B. stearothermophilus* strain able to produce both enzymes.

In this work, the 50-kDa esterase was purified to homogeneity to examine its chemical and kinetic properties by ion exchange and hydrophobic interaction chromatographies. The N-terminal amino acid sequence of the purified 50-kDa esterase was determined: NH_2-Gln-Glu-Gln-Thr-Val-Val-Glu-Thr-Lys(or Leu)-Tyr-(Glu or Tyr)-Arg-Leu(?)-. To date several intra- and extracellular esterases from different strains of *B. stearothermophilus* have been purified and partially characterized (1, 9, 12, 15). Among them, the apparent molecular mass of Tok 19A1 esterase was similar to that of L1 esterase in this research. However, the N-terminal amino acid sequence of L1 esterase is quite different from that of Tok19A1 enzyme. So the esterase from *B. stearothermophilus* L1 is thought to be different from all other known esterases from *B. stearothermophilus* strains.

The purified L1 esterase is unstable when the enzyme concentration is less than about 40 μg/ml. Enzyme stability was improved by the addition of bovine serum albumin (50 μg/ml) to enzyme dilution buffer and enzyme reaction mixtures. In the case of Tok19A1 esterase, the thermostability of the enzyme was also increased sevenfold by the addition of 1 mg/ml BSA. This increase of thermostability was explained by heterogeneous protein-protein interactions (15).

The enzyme was strongly inhibited by diethyl *p*-nitrophenyl phosphate, an organophosphorous compound, indicating it is a serine esterase as with other esterases (9).

In the presence of bovine serum albumin, the 50-kDa esterase was stable up to 70°C and at a broad pH range (pH 4-11). Since structural stability of the enzyme at high temperatures and at broad pHs is important in

valuable biotransformation processes, the 50-kDa esterase could be used satisfactorily as a biocatalyst in those unusual conditions.

We are currently studying its gene cloning to reveal the primary structure of the 50-kDa esterse as well as the detailed physicochemical and enzymological properties. These results will be reported shortly.

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(Received September 10, 1996)