

Characterization of a Thermostable Esterase Activity from the Moderate Thermophile *Bacillus licheniformis*

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A new esterase activity from *Bacillus licheniformis* was characterized from an *Escherichia coli* recombinant strain. The protein was a single polypeptide chain with a molecular mass of 81 kDa. The optimum pH for esterase activity was 8–8.5 and it was stable in the range 7–8.5. The optimum temperature for activity was 45°C and the half-life was 1 h at 64°C. Maximum activity was observed on *p*-nitrophenyl caproate with little activity toward long-chain fatty acid esters. The enzyme had a K_M of 0.52 mM for *p*-nitrophenyl caproate hydrolysis at pH 8 and 37°C. The enzyme activity was not affected by either metal ions or sulfhydryl reagents. Surprisingly, the enzyme was only slightly inhibited by PMSF. These characteristics classified the new enzyme as a thermostable esterase that shared similarities with lipases. The esterase might be useful for biotechnological applications such as ester synthesis.

Key words: esterase; lipase; *Bacillus licheniformis*; specificity; caproate

From an industrial point of view, the more an enzyme is thermostable, the more interesting it is for the development of new applications. This statement comes from the observation that, compared to chemical catalysts, biocatalysts are usually less stable under operational conditions. Therefore, any increase of the enzyme stability will be an important improvement for process development.

Thus, the search for thermostable enzymes is still an active research field with the ultimate goal of obtaining suitable biocatalysts for biotransformations. The classical way of getting such thermostable enzymes is to screen thermophilic strains. As an alternative to this approach we have screened moderate thermophilic bacterial strains. Several thermotolerant strains have been isolated from soil samples by enrichment culture at 50°C.^{1–3} These strains appeared to be good candidates for the isolation of thermostable enzymes.^{2–4} From one of the isolated strains (LCB40), which has been identified as *Bacillus licheniformis*, an esterase gene has been cloned and partially characterized.⁵

The preliminary studies on the gene product showed quite intriguing properties. The primary structure of the protein, deduced from the nucleic acid sequence,

showed strong similarities with lipases but the activity on insoluble lipase substrates such as *p*-nitrophenyl palmitate or olive oil was very low.⁵ Thus, according to its molecular structure this enzyme resembles lipases while its activity is closer to that of an esterase. In this paper, we report a more detailed characterization of this new esterase activity in terms of molecular and enzymatic properties as an attempt to better understand its biological activity and biotechnological potentials.

Materials and Methods

Bacterial strain and culture conditions. *Escherichia coli* BL21(DE3) containing plasmid pBLE111 carrying an esterase gene from *Bacillus licheniformis* was used.⁵ Cells were grown in 2xTY medium⁶ containing ampicillin (50 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (240 mg/l) at 37°C under agitation.

Enzyme preparation. Cells from 1-litre of culture were harvested in stationary phase by centrifugation (6.000 *g*, 15 min, 4°C) and suspended in the same volume of 50 mM Tris-HCl buffer pH 8. The cells were again collected by centrifugation (6.000 *g*, 30 min, 4°C) and the pellet was suspended in a smaller volume (50 ml) of the same buffer. Phenylmethylsulfonyl fluoride (PMSF) was added in a final concentration of 1 mM to prevent proteolysis. Cells were disrupted in a French pressure cell (5.5 T/cm²) and the cell debris removed by low speed centrifugation (3.000 *g*, 30 min, 4°C). The supernatant was desalted by gel filtration chromatography onto a G25 Sephadex column (Pharmacia, Uppsala, Sweden). The elution buffer was 10 mM ammonium acetate, pH 8. The active fractions were pooled and directly lyophilized.

Enzyme assay. Esterase activity was assayed by following the rate of *p*-nitrophenyl caproate (pNPC6) hydrolysis (Sigma Chemical Co., St. Louis MO, USA) to yield *p*-nitrophenol.⁷ Unless stated otherwise, the reaction was done at 37°C in 50 mM Tris-HCl, pH 8. One enzyme unit corresponds to the amount of enzyme liberating 1 µmol of *p*-nitrophenol per min ($\epsilon = 12.75 \text{ cm}^2/\text{mol}$). Absorbance was measured continuously at 410 nm with a LKB-Ultrospec II spectrophotometer. For the measure-

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Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; pNPC6, *p*-nitrophenyl caproate; pHMB, *p*-hydroxymercuri benzoate; IPTG, isopropyl-β-D-thiogalactopyranoside

ment of specificity, *p*-nitrophenyl esters containing fatty acids with carbon chains from C2 to C16 were used. All other conditions for the assay were identical. For the thermal stability studies the enzyme was incubated in the assay buffer (without substrate) at various temperatures for 60 min. and the remaining activity was assayed on *p*-nitrophenyl caproate. For pH stability measurements, the enzyme was incubated at various pHs, between 6 and 9, in the same buffer for one hour at room temperature (21°C). Proteins were assayed by the method of Lowry⁸⁾ with crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard.

Electrophoresis and activity detection. SDS-PAGE was run according to Laemmli⁹⁾ with 10.5% (w/v) acrylamide gels. Samples of 10 or 100 µg of bacterial protein were used. The gels loaded with 10 µg of proteins were stained for proteins using Coomassie blue under standard conditions.⁹⁾ Molecular masses were measured by comparison of mobility with standard proteins (plot of log of molecular mass against relative mobility). The gels with 100 µg of proteins were used for activity measurement with the following modifications: the sample was dissolved in a buffer without β-mercaptoethanol and it was not heated at 100°C before being put on. After migration, the gel was washed under agitation for 30 min in 500 ml of a renaturation buffer (Tris-HCl 2.4 g/l, malic acid 2.32 g/l and NaOH 0.8 g/l) containing 25% (v/v) isopropanol, and for 15 min in 500 ml of the same buffer without isopropanol. Renatured gels were put onto a Petri dish containing 30 ml of a agar (20%) containing 20 mM Tris-HCl buffer, pH 8 with 10% of a 16.5 mM solution of pNPC6 in isopropanol. Then, the Petri dish was incubated over-night at 37°C. Appearance of a clear halo zone indicated esterase activity.

Gel filtration chromatography. The molecular mass of the native enzyme was estimated by FPLC using a Superose 12 column (Pharmacia, Uppsala, Sweden). The purified esterase preparation (1.9 mg) was dissolved in 0.2 ml of elution buffer (20 mM Tris-HCl, pH 8). The column was calibrated using Dextran blue, bovine serum albumin (M_r 67 kDa), ovalbumin (M_r 43 kDa), chymotrypsinogen A (M_r 25 kDa) and Ribonuclease I (M_r 13 kDa) from Bio-Rad.

Results

Enzyme preparation

The esterase was produced using the recombinant *E. coli* BL21 (pBLE111) strain that lacks the *lon* and *OmpT* esterases. The activity was retained on a DEAE Sepharose CL-6B column equilibrated with a 20 mM Tris-HCl pH 8 buffer and eluted with 0.35 M NaCl. The enzyme was purified 10-fold by this chromatography with 47% recovery of activity. The purified fractions showed two or three active bands (activity detected on pNPC6) after SDS-PAGE while only one band was found with the crude extract. This result suggested that proteolysis occurred during purification. Similar results were also observed upon storage at 4°C in the chromato-

graphic buffer. This proteolysis could be decreased, but not completely stopped, by addition of 1 mM PMSF to the buffers used during the preparation. However, this addition resulted in slow inhibition of the esterase activity (see below) and thus, could not be used routinely.

To obtain the protein without any degradation products, the preparation method simply included a G25 gel filtration chromatography and lyophilization as described in Materials and Methods. From a 1-liter culture, 1,116 enzyme units (pNPC6) were obtained with an activity yield of 67%. The dried enzyme preparation was stable at 4°C (84% of the original activity was recovered after 2 months).

Molecular mass

The molecular mass (M_r) of the esterase (activity detection using pNPC6) after SDS-PAGE showed one single band of M_r 81.3 kDa with the crude *E. coli* extract. With the purified powder, a major band of 81.3 kDa was revealed with a minor one at 67.5 kDa. With enzyme fractions that have been stored in a liquid form for several days at 0°C, or with the fractions purified on the DEAE Sepharose column (see above), the intensity of the band at 81.3 kDa decreased and that at 67.5 kDa increased. Upon prolonged incubation, activity was also associated with proteins of smaller size. Therefore, it was concluded that the protein of 81.3 kDa was the native enzyme and the others degradation products by proteolysis. This value is in good agreement with that (81.5 kDa) deduced from the amino acid sequence.⁵⁾ Gel filtration chromatography of the purified esterase preparation on a Superose 12 column showed a major activity peak (M_r 75 kDa) and a minor one (M_r 60 kDa) which demonstrated the monomeric nature of the esterase.

Substrate specificity

Esterase specificity for fatty acids was tested by measuring the rate of hydrolysis of *p*-nitrophenyl esters with fatty acid chain length from C₂ to C₁₈ (see Materials and Methods). The enzyme was active (Fig. 1) on short chain fatty acids esters containing 2 to 8 carbon atoms with a maximum activity for *p*-nitrophenyl caproate (pNPC6). With substrate with longer chain length, pNPC10 to pNPC18, the activity was very low (less than 3% of maximum). These results fully confirmed our first conclusion that the new enzyme showed an esterase-like specificity pattern. However, this pattern is quite different from that of most of the known esterases, which usually show major activity on pNPC2. Specificity for mid chain length fatty acid has also been reported for the esterase of *Bacillus stearothermophilus*¹⁰⁾ and *Bacillus coagulans*.³⁾

Effects of temperature on activity and stability

Esterase activity was assayed at temperatures ranging from 21 to 60°C (Fig 2A). Activity increased rapidly between 21 and 40 °C with maximal activity around 45°C. Activity remained nearly maximal in a broad range of temperature (40–55°C) and as much as 67% of maximum activity was retained at the highest (60°C) temperature tested suggesting high thermostability. The Ar-

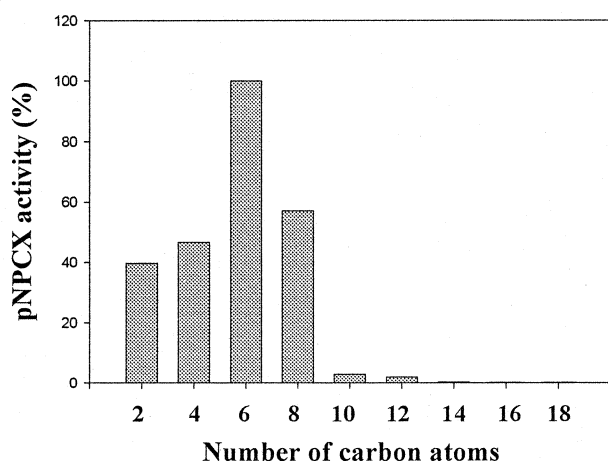


Fig. 1. Effects of Substrate Chain-length of Fatty Acids *p*-Nitrophenyl Esters on Esterase Activity.

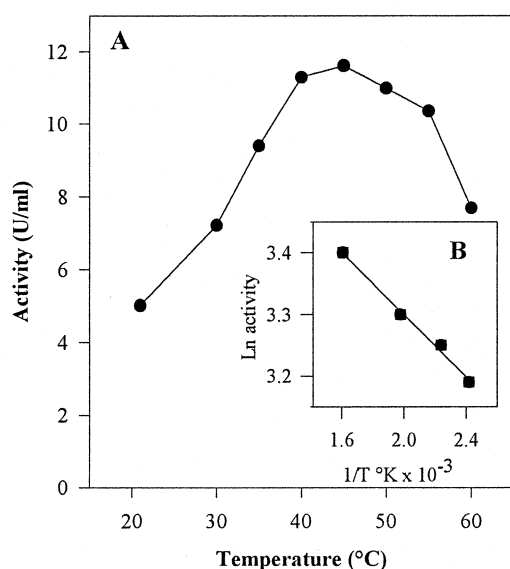


Fig. 2. A: Effects of Temperature on Esterase Activity (pNPC6). B: Arrhenius Plot of Data in Part A.

Arrhenius plot of the first data was linear (Fig 2B) and a low activation energy of 32.6 kJ/mol was estimated. This value was similar (31.5 kJ/mol) to that of esterase 4a from *Brevibacterium* sp R312¹¹⁾ and that (35.4 kJ/mol) of the *Bacillus stearothermophilus* G18A7 esterase.¹²⁾ Esterase thermostability was studied by measuring residual activity after a 1-h incubation at temperatures ranging from 21 to 70°C. The enzyme was fully active until 50°C and the temperature for 50% inactivation was 64°C (Fig 3). This value is similar to that for the *B. stearothermophilus* esterase: 65°C estimated from the results published by Matsunaga *et al.*¹⁰⁾ However, the *B. licheniformis* esterase was more thermostable than those from *Pseudomonas* sp. KWI-56,¹³⁾ *B. subtilis* NRRL 3654,¹⁴⁾ *Nocardia mediterranei*,¹⁵⁾ and the 3 esterases (2, 4a and 4b) from *Brevibacterium* sp. R312.¹¹⁾ Only the esterases from *B. acidocaldarius*,¹⁶⁾ *S. acidocaldarius*,^{17,18)} and *B. stearothermophilus* G18A7¹²⁾

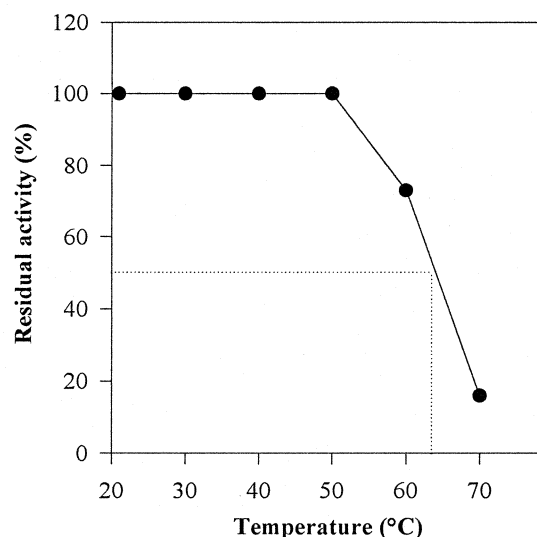


Fig. 3. Effects of Temperature on Esterase Stability. The residual activity was assayed (pNPC6) after 1-h of incubation at the indicated temperature.

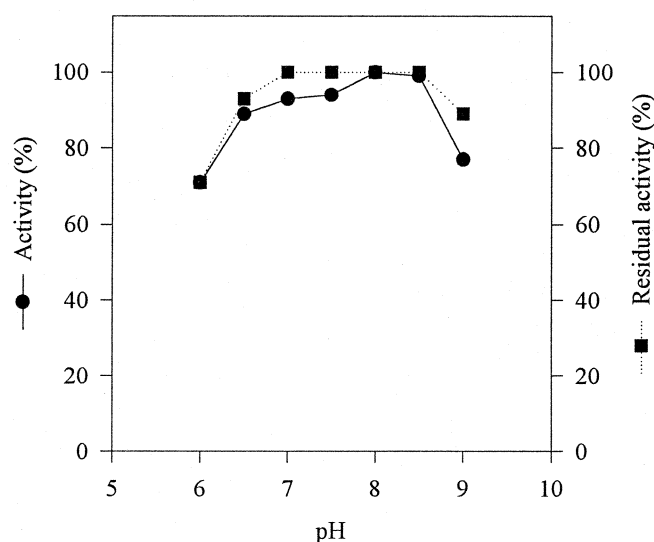


Fig. 4. Effects of pH on Esterase Activity and Stability. Activity (pNPC6) was assayed at different pHs under standard conditions. Residual activity was assayed (pNPC6) after a 1-h incubation at 21°C at the indicated pH.

are more thermostable.

Effects of pH on esterase activity and stability

Activity was assayed in pH range from 6 to 9 in 50 mM Tris-HCl buffer. This pH range was selected since measurements at lower pHs were not possible due to the lack of absorbance of *p*-nitrophenol at pH lower than 6. On the alkaline, i.e. pH higher than 9, the chemical hydrolysis of the substrate increased significantly, which made the enzymatic assay inaccurate. The esterase showed an optimum pH between 8–8.5 (Fig. 4). Activity at pH 6 and 9 was approximately 71 and 77% of maximum. The slightly basic optimum pH (8–8.5) is similar to that of several esterases. However, the *B. stearothermophilus*,¹⁰⁾ *Pseu-*

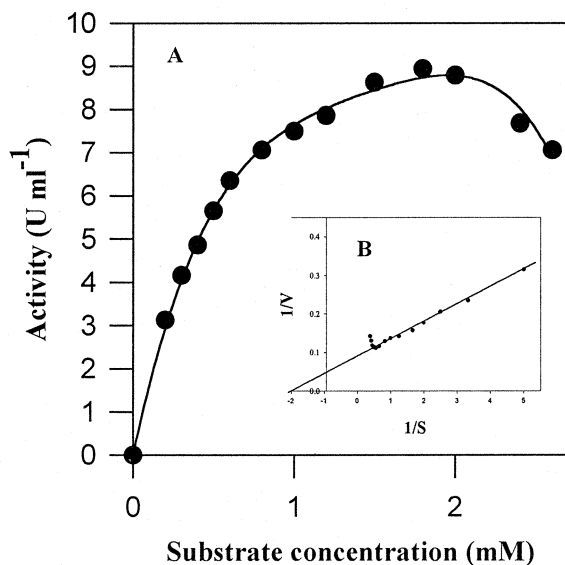


Fig. 5. A: Effects of pNPC6 Concentration on Esterase Activity; B: Lineweaver-Burk Plot of Data in Part A.

domonas sp. KWI-56,¹³⁾ and *N. mediterranei*,¹⁵⁾ esterases and esterase 2 from *Brevibacterium* sp. R312¹¹⁾ have a more neutral optimum pH. Stability was checked in the same pH range after 1-h of incubation (Fig 4). The stability was very high at all pHs tested. Similar results have been reported for the *B. stearrowthermophilus* esterase.¹⁰⁾

Effects of substrate concentration

The esterase activity was assayed in the range of substrate concentration from 0.3 to 2.7 mM using pNPC6 (Fig 5A). From the Lineweaver-Burk plot (Fig 5B) the Michaelis constant (K_M) was deduced from non-linear regression (SigmaPlot, Jandel Scientific), a value of 0.52 ± 0.05 mM was estimated. This value is similar to that of esterases a and b from *B. subtilis*,¹⁴⁾ esterases 4b, 2, and 4a from *Brevibacterium* sp. R312,¹¹⁾ and *B. stearrowthermophilus* G18A7.¹²⁾

Effects of various reagents on esterase activity

The effect of various reagents on esterase activity was tested by incubating the enzyme at 21°C for 1 h in the presence of 1 mM of each compound. None of the metal ions tested (ZnCl₂, CoCl₃, FeCl₃, MgCl₂, CaCl₂, CuCl₂, SnCl₂) stimulated or inhibited esterase activity. EDTA had no effect on the activity. Contrary to most of the known esterases, the *B. licheniformis* esterase activity was not inhibited by sulfhydryl reagents such as PHMB and HgCl₂. This result is in agreement with the lack of cystein residue in the amino acid sequence of the esterase.⁹⁾ Inhibition by PMSF was tested at two different concentrations (1 and 5 mM). This well-known covalent inhibitor of serine hydrolases¹⁹⁾ showed a slight inhibitory effect on the *Bacillus licheniformis* esterase (Fig. 6). After one hour, 18% inhibition was observed for both concentrations tested, and 61% of the activity remained after 5 h of incubation. This pattern is quite unusual for esterases, which are usually completely inhibited in less

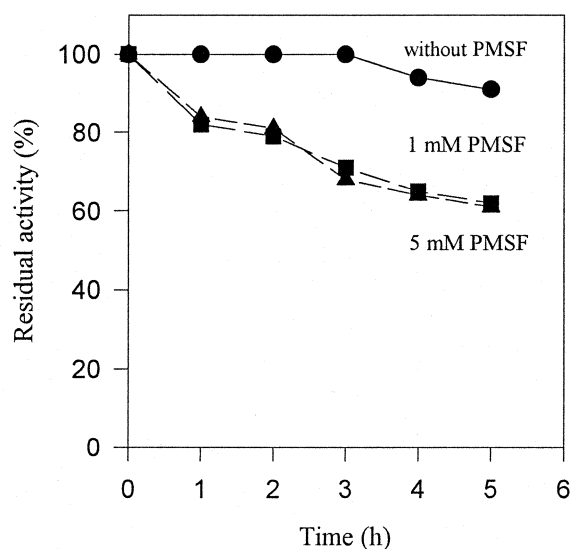


Fig. 6. Effects of PMSF on Esterase Activity.

than 60 min by PMSF at the concentration tested.

Discussion

The *B. licheniformis* esterase activity produced in *E. coli* BL21 (pBLE111) was characterized. Activity was linked to a single monomeric protein of M_r 81.3 kDa in a cell-free extract. Upon prolonged incubation in buffer at neutral pH or enzyme chromatography onto DEAE Sepharose, new active molecular species with lower M_r appeared, most probably due to proteolysis of the native enzyme. A similar situation has been described for the lipases from *Staphylococcus aureus*,²⁰⁾ and *Staphylococcus hyicus*.²¹⁾ These enzymes are synthesized as proteins of 82 and 86 kDa, respectively. However, the proteins purified from the extracellular medium have M_r of 45 and 46 kDa. This degradation has been interpreted as a maturation process although its exact significance was not clear. Most of the known esterases have M_r in the range of 30–50 kDa (Table 1). Thus the *B. licheniformis* esterase M_r is in the upper range of the usual M_r for esterases.

Although the two values were not the highest, the *B. licheniformis* esterase showed a high temperature for maximal activity (45°C) and high thermal stability (the temperature for 50% inactivation was 64°C) compared to other esterases (Table 1). Therefore, the new esterase can be classified as thermostable. The optimal pH for activity was slightly alkaline (8–8.5) which is in the upper range of the known esterases (Table 1).

The esterase specificity for pNPC6 was quite different from other esterases, which are mainly specific for short chain fatty acids from C2 to C3. Only the enzymes from *B. stearrowthermophilus*,¹⁰⁾ and to a lesser extent *B. acidocaldarius*,¹⁶⁾ and *S. acidocaldarius*^{17,18)} showed similar specificity patterns (Table 1). Therefore, the new esterase is a good candidate for uses in organic media for the synthesis of mid-chain length fatty acids esters.

Hydrolysis of pNPC6 followed Michaelis-Menten kinetics with a high value of K_M compared to other

Table 1. Comparison of Molecular and Enzymatic Properties of Several Bacterial Esterases

Micro-organism	Molecular mass (kDa)	Optimum pH	Maximal temperature (°C)	Thermostability ^a %	Fatty acid specificity	K _M (mM)	Inhibition by effectors ^b			References
							EDTA	Sulphydryl reagents	PMSF	
<i>Bacillus licheniformis</i>	81.3	8–8.5	40–50	73/60°C	C6	0.52	–	–	–	This work
<i>Bacillus acidocaldarius</i>	36.5 ± 2.5	8	70	100/60°C/90 min	C5	0.08	– (5 min)	ND	+ (5 min)	16
<i>Brevibacterium</i> sp. R312										
Esterase 4b	38	6–8	43	60/40°C	C3	0.37	–	+	+ (5 mM)	11
Esterase 2	45	7.6	36	80/40°C	C4	0.32	–	–	+ (5 mM)	
Esterase 4a	56	8	30	25/40°C	C4	0.16	–	+	+ (5 mM)	
<i>Pseudomonas fluorescens</i>	29.5	9	70	ND	ND	0.092	ND	ND	ND	31
<i>Sulpholobus acidocaldarius</i>	117–128	7.5–8.5	ND	100/80°C	C5	0.152	– (10 min)	– (10 min, 10 mM)	+ (10 min, 0.1 mM)	17, 18
<i>Bacillus stearothermophilus</i>	47	7	65	70/65°C	C6	0.016	ND	+ (20 min, 0.1 mM)	ND	10
<i>Bacillus subtilis</i> NRRL 3654										
Esterase a	36	8.0	ND	50/40°C	C2	0.91	ND	+	–	14
Esterase b	105–110	8.0		50/40°C	C2	0.67	ND	+	–	
<i>Nocardia mediterranei</i>	68	7.5	35	0/60°C/10 min	C2	ND	– (2 h)	+ (2 h)	+ (2 h)	15
<i>Bacillus stearothermophilus</i> G18A7	38–45	9.5	ND	90/105°C/150 min	C2	0.76	ND	–	+	12
<i>Pseudomonas</i> sp. KW1-56	50	7.5	22	10/50°C/30 min	C3	ND	ND	+	+	13

a: residual activity after 1 h incubation (unless indicated) at indicated temperature.

b: residual activity: + if >70%; – if <70% after 1 h incubation with 1 mM effector (unless indicated).

esterases. The effects of metals ions, EDTA, and SH-reagents on the enzyme activity indicate that the new esterase contains neither metal nor cystein residues essential for catalysis.

The new esterase is slowly inhibited by PMSF, a pattern different from that of other esterases (Table 1). Generally only esterases of the ‘‘A’’ class according to Walker,²²⁾ are not inhibited by PMSF. This result is quite surprising since the *B. licheniformis* esterase contained the pentapeptide Gly-X-Ser-X-Gly, a well-conserved sequence in both esterase and lipases.²³⁾ Furthermore, the first glycine residue of the pentapeptide is replaced by an alanine as in the *B. subtilis*,²⁴⁾ *B. pumilus*,²⁵⁾ and *Galactomyces candidum* lipases.²⁶⁾ The serine of this pentapeptide is well known as the active catalytic residue that is modified by PMSF.

The slow inhibition observed with the *Bacillus licheniformis* esterase is similar to that observed with lipases.^{7,27,28)} Lipases are only slightly inhibited by PMSF because of the ‘‘lid’’ structure covering the serine residue of the active site, which becomes inaccessible to the reagent. The *B. licheniformis* esterase showed some similarity with the lid region of the human pancreatic lipase.⁵⁾ Occurrence of such a lid would explain the slow inhibition of this esterase by PMSF. However, this hypothesis does not agree with the facts that: a) so far, the presence of a lid structure had not been described for an esterase; b) esterases are enzymes that cannot bind to water-lipid interfaces like lipases; c) esterases do not show interfacial activation like lipases.^{29,30)} Therefore, the role of a lid structure on an esterase would not

be clear.

Thus, the present study confirmed our preliminary conclusion that the new enzyme is an esterase (mainly based on its low activity on insoluble/emulsified substrates) but that it shared some properties with lipases (such as lack of PMSF inhibition and sequence homologies).

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