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Purification and Partial Characterization of a Lipase From *Bacillus coagulans* ZJU318

Tang Lianghua*[,1,2] and Xia Liming[1]

[1]Department of Chemical Engineering and Bioengineering, Zhejiang University, Hangzhou-310027, China, E-mail: biotlh@fjnu.edu.cn; and [2]College of Bioengineering, Fujian Normal University, Fuzhou-350007, China

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Abstract

An extracellular lipase was purified from the fermentation broth of *Bacillus coagulans* ZJU318 by CM-Sepharose chromatography, followed by Sephacryl S-200 chromatography. The lipase was purified 14.7-fold with 18% recovery and a specific activity of 141.1 U/mg. The molecular weight of the homogeneous enzyme was (32 kDa), determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The enzyme activity was maximum at pH 9.0 and was stable over a pH range of 7.0–10.0, and the optimum temperature for the enzyme reaction was 45°C. Little activity loss (6.2%) was observed after 1 h of incubation at 40°C. However, the stability of the lipase decreased sharply at 50 and 60°C. The enzyme activity was strongly inhibited by Ag^{+} and Cu^{2+}, whereas EDTA caused no inhibition. SDS, Brij 30, and Tween-80 inhibited lipase, whereas Triton X-100 did not significantly inhibit lipase activity.

Index Entries: Lipase; *Bacillus coagulans*; purification; characterization; stability.

Introduction

Lipases (triacylglycerol hydrolases; EC 3.1.1.3) are important enzymes that catalyze the hydrolysis or formation of lipids. They also exhibit other activities such as phospholipase and cholesterol esterase. Nowadays, lipases have immense applications in oils and fat detergents, baking, cheese making, chemical synthesis, and pharmaceutical industries *(1,2)*.

Lipases used for biotechnological purposes are mainly produced by microbes. Bacterial lipases specifically play an important role in commercial

*Author to whom all correspondence and reprint requests should be addressed.

applications. Some important lipase-producing bacterial genera include *Bacillus*, *Pseudomonas*, and *Burkholderia (3)*. Recently, a bacterial strain producing lipase was screened and identified as *Bacillus coagulans* ZJU318 in our laboratory. The purification and characterization of the alkaline lipase produced by this strain are reported herein.

Materials and Methods

Microorganism and Culture Conditions

Bacillus coagulans ZJU318 was screened in our laboratory and was maintained on Luria-Bertani agar. The liquid medium for lipase production consisted of 0.5% (w/v) bacto peptone, 0.5% (w/v) yeast extract, 0.4% (w/v) olive oil, and 0.05% (w/v) NaCl. The pH was adjusted to 7.0 with 1 *M* NaOH. Cultivation was performed in 250-mL flasks containing 25 mL of medium at 28°C using an inoculum of 2.5 mL of an overnight preculture (6×10^8 cells/mL). The flasks were incubated under shaking conditions (250 rpm) for 22 h. Cell-free supernatant was obtained by centrifuging at 9000*g* for 20 min at 4°C.

Enzyme Assay

The photometric assay substrate was prepared as described by Kordel et al. *(4)* with slight modifications. Solution A contained *p*-nitrophenylpalmitate (pNPP) or *p*-nitrophenylacetate (pNPA) dissolved in 10 mL of 2-propanol to concentrations of 16.5 and 50 m*M*, respectively. Solution B for the pNPP assay consisted of 100 m*M* Tris-HCl buffer (pH 8.0) containing 0.4% Triton X-100 and 0.1% gum arabic. For the pNPA assay, 200 m*M* sodium acetate buffer (pH 6.2) was used because pNPA was unstable at pH >7.0. The reaction mixture consisting of 1 part solution A and 9 parts solution B was prepared fresh before the assay. A 100-µL vol of an appropriate dilution of the enzyme solution was added to 3000 µL of the reaction mixture. The kinetics were detected at 410 nm and 37°C with an Ultraspec K photometer (Pharmacia LKB). Under the conditions used, the extinction coefficient (ε_{410}) of *p*-nitrophenol was $1.46 \times 10^5 cm^2/mol$.

One unit of lipase activity was defined as the amount of lipase that liberated 1 µmol of *p*-nitrophenol from pNPP or pNPA, respectively, per minute.

Determination of Protein

Protein was assayed by the method of Lowry et al. *(5)*.

Purification of Lipase

Ion-Exchange Chromatography

Cell-free supernatant (1000 mL) was adjusted to pH 6.7 with 1 *M* HCl. An ion-exchange chromatograph was used with a glass column (16×200 mm) filled with 30 mL of CM-Sepharose. After the column was equilibrated with

a buffer consisting of 20 m*M* phosphate (pH 6.7), the supernatant was loaded onto the column. Then the column was washed with 120 mL of starting buffer at a flow rate of 1 mL/min. The lipase was finally eluted at a flow rate of 1 mL/min with 60 mL of 20 m*M* phosphate buffer containing 500 m*M* NaCl.

Gel Filtration

The combined active fractions from the CM-Sepharose chromatography were freeze-dried and resuspended in 1 mL of 0.1 *M* phosphate buffer (pH 7.0). The precipitate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was loaded onto a Sephacryl S-200 column (16 × 600 mm). Then the column was washed with 200 mL of 0.1 *M* phosphate buffer (pH 7.0) at a flow rate of 1 mL/min. The fractions were assayed by the pNPA method.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) was performed according to Laemmli *(6)*. Gels were stained with Coomassie Brilliant Blue R-250.

Effects of Temperature, pH, Detergents, and Agents on Lipase Activity

The optimum temperature for activity was determined by increasing the temperature in steps of 10°C from 25 to 65°C. During the temperature optima and stability experiments, the pH was kept at 8.0.

For determination of pH dependence on enzyme activity, the following buffers (200 m*M*) were used: sodium acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0), glycine-NaOH (pH 8.0–11.0). The reaction temperature was kept at 37°C. The effects of pH on enzyme stability were analyzed by the photometric assay after preincubation in buffered solutions for 24 h at 20°C.

The effects of detergents on the enzyme activity were analyzed in the presence of up to 0.1 and 0.5% of various detergents in 20 m*M* glycine-NaOH buffer (pH 9.0) at 37°C.

The effects of various agents on lipase activity were analyzed in the presence of 1 m*M* various agents.

Results and Discussion

Production of Enzyme

B. coagulans ZJU318 produced an extracellular lipase in a medium (pH 7.0) consisting of 0.5% (w/v) bacto peptone, 0.5% (w/v) yeast extract, 0.4% (w/v) olive oil, and 0.05% (w/v) NaCl. Maximum enzyme activity (24.7 U/mL) was found after 22 h under shaking conditions (250 rpm) at 28°C.

Purification of Enzyme

When the cell-free supernatant was loaded on a CM-Sepharose column, most of the protein, including 60% of the lipase activity, respectively, passed the column without binding to CM-Sepharose. An additional 4.9%

Table 1
Purification of a Lipase From *B. coagulans* ZJU318

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Cell-free supernatant	2570	24,700	9.6	1	100
CM-Sepharose	125.4	9880	78.8	8.2	40
Sephacryl S-200	31.5	4446	141.1	14.7	18

of the protein, including 40% of the lipase activity, respectively, was eluted with the phosphate buffer containing 500 m*M* NaCl. An 8.2-fold increase in specific activity was obtained by the CM-Sepharose chromatography.

When the concentrated active fractions from CM-Sepharose chromatography passed through a Sephacryl S-200 column, 18% of the total lipase activity was obtained. The enzyme, in the form of homogeneous lipase, exhibited a 14.7-fold increase in specific activity compared with the cell-free supernatant.

The extracellular lipase from *B. coagulans* ZJU318 was purified by employing a two-step procedure. Table 1 provides a summary of the purification procedure. The lipase was purified 14.7-fold with 18% recovery and a specific activity of about 141 U/mg. Generally, the yield of the lipase purification procedure is relatively low, between 2 and 20% *(7)*.

Characterization of Enzyme

A molecular mass of approx 32 kDa was determined for the purified lipase by SDS-PAGE (Fig. 1). The molecular mass of *B. coagulans* ZJU318 lipase was larger than those of *Bacillus pumilus* B26 *(8)*, *Bacillus* sp. H-257 *(9)*, *Bacillus thermocatenulatus* DSM730 *(10)*, *Bacillus subtilis* 168 *(11)*, *Bacillus* sp. *(12)*, but smaller than those from *Bacillus stearothermophilus* MC7 *(7)*, *Bacillus* sp. *(13)*, *B. stearothermophilus* P1 *(14)*, *Bacillus* sp. J33 *(15)*, *Bacillus thermoleovorans* ID-1 *(16)*, *Bacillus* sp. THL027 *(17)*, *B. stearothermophilus* L1 *(18)*, and *B. thermocatenulatus (19)*.

Effects of pH and Temperature on Lipase Activity and Stability

Optimum activity was observed at pH 9.0 (Fig. 2A). Between pH 7.0 and 10.0 the lipase was stable, but the stability was low at acidic pH (Fig. 2B).

B. coagulans ZJU318 lipase could tolerate a pH range of 7.0–10.0 (Fig. 2B). It was stable in this range for 24 h and showed an optimum pH of 9.0. Even at pH 11.0, 56% residual activity was obtained. High pH optima for lipase activity have been reported in *Bacillus alkalophilus (20)*, *Penicillium expansum* DSM1994 *(21)*, *Rhizopus japonicus (22)*, *Humicola lanuginosa (23)*, and *Aspergillus terreus (24)*. The highest stability of the enzyme was observed at pH 8.0–10.0 (Fig. 2B). The remarkable stability of *B. coagulans* ZJU318 lipase in this range has proved it to be a potential alkaline lipase.

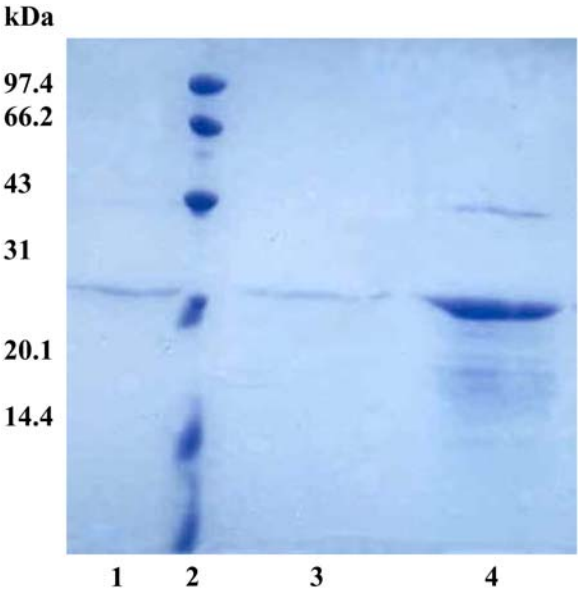

Fig. 1. Separation of lipase-containing fractions by SDS-PAGE. Lanes 1 and 3, Sephacryl S-200 eluate fraction; lane 4, CM-Sepharose eluate fraction; lane 2, low molecular weight marker proteins: from top to bottom, rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), and hen egg-white lysozyme (14.4 kDa).

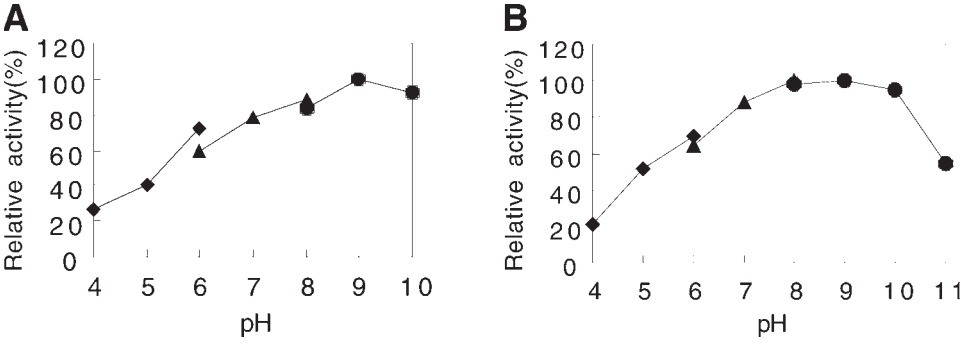

Fig. 2. Effect of pH on **(A)** lipase activity and **(B)** stability: (◆) sodium acetate buffer (pH 4.0–6.0); (▲) phosphate buffer (pH 6.0–8.0); (●) glycine-NaOH (pH 8.0–11.0).

The optimum activity of the enzyme was observed at 45°C (Fig. 3A). Little activity loss (6.2%) was observed after 1 h of incubation at 40°C in 0.2 *M* phosphate buffer (pH 8.0). However, the stability of the lipase decreased sharply after 1 h of incubation at 50 and 60°C (Fig. 3B). This indicates that *B. coagulans* ZJU318 lipase is a mesophilic enzyme.

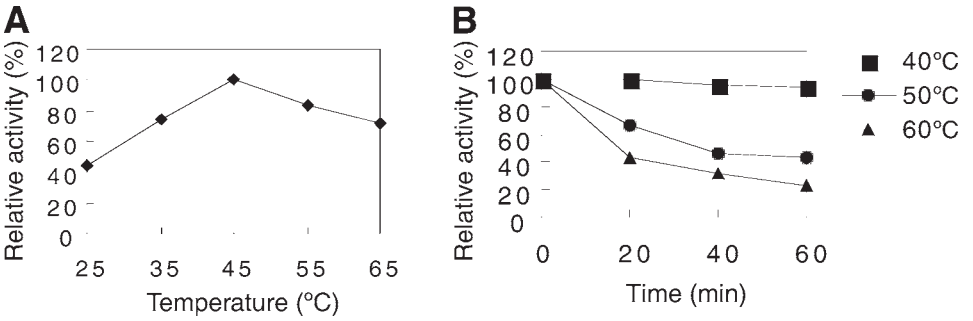

Fig. 3. Effect of temperature on **(A)** lipase activity and **(B)** stability. In (B) the enzyme dissolved in 0.2 *M* glycine-NaOH buffer (pH 8.0) was incubated at various temperatures, and then the remaining activity was measured every 20 min in the standard assay system.

Table 2
Effects of Detergents on Lipase Activity

	Relative activity with detergent in assay (%)	
Detergent	0.1% Concentration	0.5% Concentration
SDS	71	28
Tween-80	76	34
Brij 30	96	32
Triton X-100	102	81
None	100	100

Effects of Detergents on Lipase Activity

Table 2 presents the effects of various detergents on enzyme activity. At a concentration up to 0.5%, SDS, Brij 30, and Tween-80 inhibited lipase. However, Triton X-100 caused no significant inhibition in lipase activity, in agreement with the data of Stocklein et al. *(21)*, who found that most of the bacterial lipases were not affected by Triton.

Effects of Various Agents on Lipase Activity

Various compounds were studied for their effects on lipase activity (Table 3). Lipase from *B. coagulans* ZJU318 proved to be insensitive to some metal ions, i.e., Ca^{2+} and Mn^{2+}. This indicates an independence of the lipase on Ca^{2+}. Among the heavy metal ions, both Ag^{+} and Cu^{2+} caused significant inhibition (63 and 55%, respectively) at 1 m*M*. However, EDTA had no significant effect on the enzyme activity at either 1 or 10 m*M*, suggesting the absence of the requirement for a cofactor for lipase activity, which was in agreement with the findings of previous studies *(24–27)*.

Table 3
Effects of Various Agents on Lipase Activity

Substance	Concentration (1 m*M*)	Relative activity during assay (%)
$CaCl_2$	1	97
EDTA	1	94
EDTA	10	104
$MgSO_4$	1	106
$FeCl_3$	1	105
$MnSO_4$	1	105
KCl	1	109
$(NH_4)_2SO_4$	1	106
$FeSO_4$	1	94
NaCl	1	89
$AgNO_3$	1	37
$CuSO_4$	1	45
None	—	100

Conclusion

The results obtained in our study show that *B. coagulans* ZJU318 lipase is a potential alkaline lipase. This lipase is attractive for such biotechnological application as in oils and fat detergents.

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