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Characterization of Membrane-Bound Lipase from a Thermophilic *Rhizopus oryzae* Isolated from Palm Oil Mill Effluent

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ABSTRACT: The characteristics of the membrane-bound lipase from a thermophilic *Rhizopus oryzae* were studied. The pH and temperature optima for lipase activity were at 7.0 and 37°C, respectively. The enzyme was stable and acidic conditions, retaining more than 80% of its initial activity at pH 4.0 after 30 min incubation. It was stable up to 50°C with 70% of initial activity retained after 3 h incubation. The enzyme is 1,3 specific and exhibits substrate preference. Monoacid triglyceride substrates were hydrolyzed better than methyl esters, polyoxysorbitan and sorbitan substrates.

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KEY WORDS: Characterization, lipase, membrane-bound lipase, *Rhizopus oryzae.*

In view of their potential use in ester synthesis, interesterification reactions, and ester hydrolyses, lipases continue to receive great attention both academically and industrially. Among the sources of lipases, microbial lipases are more widely applied in industries due to their shorter generation time; ease of bulk production, which is further enhanced with advancement in fermentation technologies; and ease of manipulation, either genetically or environmentally.

Lipases from various microorganisms have been reported, and many of the lipases have been purified to homogeneity and their properties elucidated (1–3). However, most of the reports were mainly on extracellular lipases. Reports or work on intracellular or membrane-bound lipases is still lagging behind. Membrane-bound enzymes may have advantages over intracellular or extracellular counterparts since they can be regarded as natural immobilized enzymes, thus having the advantages of an immobilized enzyme system.

In our attempt to isolate new lipolytic microbes from our environment, we isolated a lipolytic thermophilic *Rhizopus oryzae* from palm oil mill effluent (POME). The fungus can grow at temperatures up to 50°C and produces both extracellular and membrane-bound lipases (4). We purified and characterized both of the enzymes to compare their properties. The characteristics of the extracellular enzyme were presented elsewhere (5). This paper describes the characteristics of membrane-bound lipase from the thermophilic *R. oryzae*.

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EXPERIMENTAL PROCEDURES

Microorganism. Rhizopus oryzae (thermophilic) was isolated from the effluent treatment pond of a palm oil mill. It is now deposited at the American Type Culture Collection (Rockville, MD), as *Rhizopus oryzae* S3, ATTC #96382. The fungus was able to grow at up to 50°C and produced a remarkable amount of extra- and intracellular (membrane-bound) lipase (4). The fungal identification was done by International Mycological Institute, Surrey, England.

Growth media and culture condition. The cultivation was carried out as described by Salleh *et al.* (4). The medium consisted of (g/L) tryptone, 50; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1; $NaNO_3$, 1; and glucose, 10. Initial pH of the medium was adjusted to 6.0. The fungus was grown in 100 mL media in a 250 mL conical flask at 37°C for 72 h at 150 rpm.

Enzyme activity. Lipase activity was determined according to Salleh *et al.* (4). The reaction mixture comprised 2.5 mL olive oil emulsion in water (1:1, vol/vol), 1% (wt/vol) polyvinyl alcohol, 0.02 mL 0.02 M $CaCl_2$, and 50 mg membrane-bound enzyme. The reaction was carried out for 30 min at 37°C with shaking at 200 rpm. The reaction was terminated by titration to pH 10.0 using a pH autotitrator (Radiometer ABU 90, Copenhagen, Denmark). A unit of activity is equivalent to one µmole of free fatty acid released per minute.

Extraction of membrane-bound lipase. Membrane-bound enzyme was extracted according to Nakashima *et al.* (6). Briefly, the mycelium was washed once with distilled water and twice with acetone, then rinsed with distilled water. The mycelium was then freeze-dried. The dried mycelium was cut into small pieces, ground, and its activity assayed.

Characterization of the enzyme. Effect of temperature on lipase activity. The effect of temperature on the lipase activity was measured at various temperatures ranging from 28 to 70°C. The highest activity of the enzyme was noted as 100% activity. For thermostability, the enzyme was incubated at 40, 50, 60, 70, 80, and 90°C for 30 min. After each period of incubation, the enzyme was immediately cooled in an ice bath for 15 min and the residual activity determined.

Effect of pH. The effect of pH on the enzyme activity and stability was measured at pH ranges of 4.0 to 10.0. For pH optimum determination, the lipase activity was measured in various buffers (pH 4–10). For pH stability, the enzyme was incubated in 1 mL of various buffers at 37°C for 30 min. After incubation, the residual lipase activity was assayed. The activity was expressed as a percentage, with the highest activity as 100%. Buffer systems used were 0.05 M acetate buffer (pH 4–5), 0.05 M phosphate buffer (pH 6–7), and 0.1 M Clark and Lubs buffer (pH 8–10)

Substrate and positional specificity. The substrate specificity was determined following the method by Okumura *et al.* (3). Various substrates (0.5 g) were incubated with 50 mg membrane-bound enzyme, 2.0 mL phosphate buffer pH 6.0, and 0.02 mL 0.02 M $CaCl_2$. The mixture was shaken at 200 rpm for 2 h at 37°C. The reaction was terminated by addition of 5.0 mL ethanol/acetone (1:1 vol/vol) and fatty acids liberated were titrated with 0.05 M NaOH to pH 10.0 using a pH autotitrator (Radiometer ABU 90).

For positional specificity, the hydrolysis of triolein was monitored with time. The reaction mixture, containing 0.1 mL triolein, 4.0 mL 0.05 M phosphate buffer pH 5.0, and 50 mg membrane-bound lipase, was incubated with constant shaking at 37°C. Samples were taken at 2, 24, and 48 h and spotted onto silica gel thin-layer chromatography (TLC) plates (60F–254, 0.25 mm, Merck, Darmstadt, Germany). The solvent system used for TLC comprised petroleum ether/diethylether/acetic acid (80:30:1, vol/vol/vol). The dried plate was spread with concentrated sulfuric acid using a spray gun (CAMAG, Muttenz, Switzerland). The plates were dried at 80–110°C until spots were visible.

RESULTS AND DISCUSSION

Effect of pH on lipase activity. Figure 1 shows that the activity of the membrane-bound lipase of *R. oryzae* was optimal at pH 6.0. This conforms to the characteristics of most fungal lipases which exhibit activity in the acidic range. Among them are *R. oryzae* and *R. rhizodiformis* (pH 6.0) (5), *Aspergillus niger* (pH 5.6) (7), *R. delemar* (pH 5.6) (8), and *Geotrichum candidum* (pH 6.3) (9). However, in contrast to most fungal lipases reported, which were only active in acidic conditions, this enzyme was active in both acidic and alkaline conditions. At pH 4.0 and 8.0, the enzyme still exhibited more than 80% activity. Compared to extracellular lipase from the same fungi, the membrane-bound lipase was active over a broader pH range. The extracellular lipase displayed only 7 and 0% activities at pH 9 and 10, respectively (5), while the membrane-bound lipase still exhibited 50 and 32% activities.

The pH stability result (Fig. 2) again showed that the enzyme was stable at both acidic and alkaline pH. Even at pH as high as 10.0 and as low as 4.0, the enzyme still retained more than 70% of its initial activity for 30 min. The alkaline stability of the membrane-bound lipase of this fungus is quite encouraging, since most fungal lipases are not stable at alkaline pH.

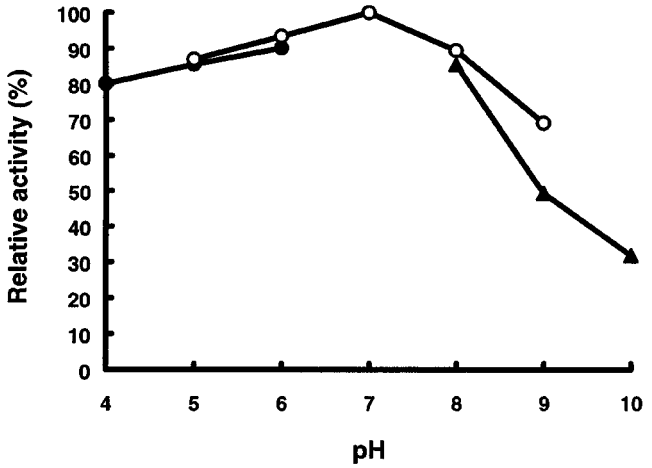

FIG. 1. pH profile of the membrane-bound lipase. The buffers used were McIlvine (4–6, ●), phosphate (5-9, ○), and Clark & Lubs (8–10, (▲). The activity at optimal pH was taken as 100%.

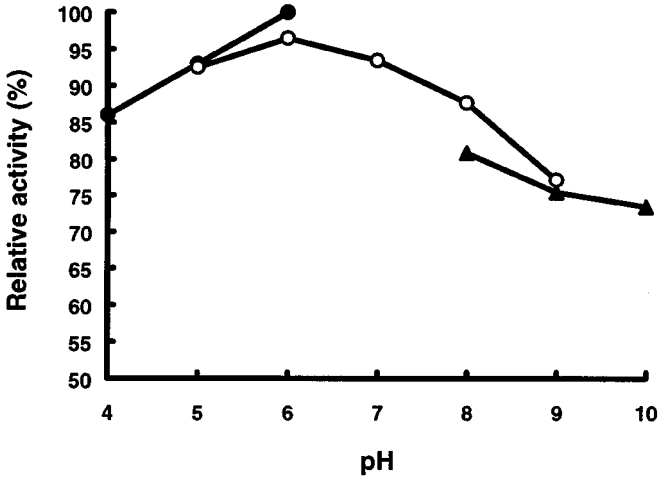

FIG. 2. pH stability of the membrane-bound lipase. The enzyme was incubated for 30 min in different buffers. The buffers used were McIlvine (4–6, ●), phosphate (5–9, ○), and Clark & Lubs (8–10, ▲). The pH was adjusted to pH 7 before assay. The optimal activity was taken as 100%

Effect of temperature on lipase activity. The effect of temperature on the enzyme activity was studied from 28 to 70°C. The optimal temperature for the membrane-bound lipase was 37°C (Fig. 3). Most microbial lipases exhibit optimal temperatures in the range of 25 to 40°C, as shown by lipase from *A. niger* (7) which had an optimal temperature at 25°C, while the optima of *Synephalastrum racemosum* (10) and *Mucor javanicus* (11) were at 37°C. Lipases from *G. candidum* (9), *R. japonicus* (12), and *R. japonicus* NR 400 (13) exhibited optimal activities at 40°C. Temperature stability studies showed that this membrane-bound lipase was stable at 50°C, retaining more than 90% of its initial activity after 30 min (Fig. 4). In fact, when incubated at 50°C for 24 h, the membrane-bound lipase still retained about 55% of its activity (Fig. 5). Under the same conditions, the activity of the extracellular lipase from the same fungus was reduced to 24% (5). The greater stability of the membrane-bound lipase could be at-

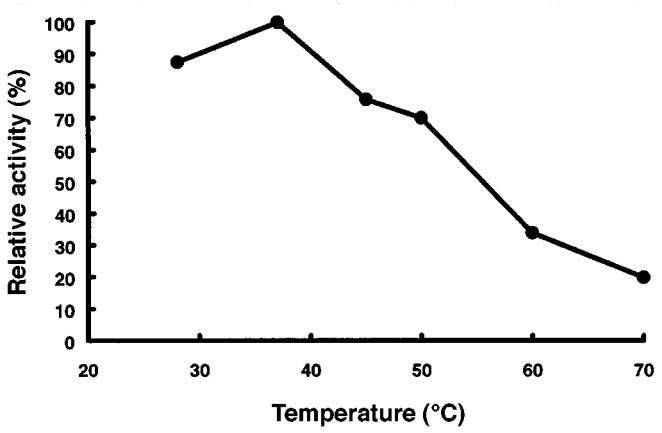

FIG. 3. Temperature profile of the membrane-bound lipase. The maximal activity was taken as 100%.

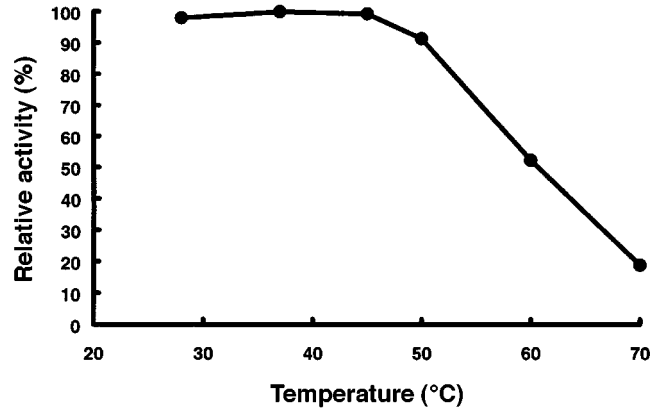

FIG. 4. Temperature stability of the membrane-bound lipase. The enzyme was incubated for 30 min at various temperatures, cooled, and assayed at 37°C. The activity before temperature exposure was taken as 100%.

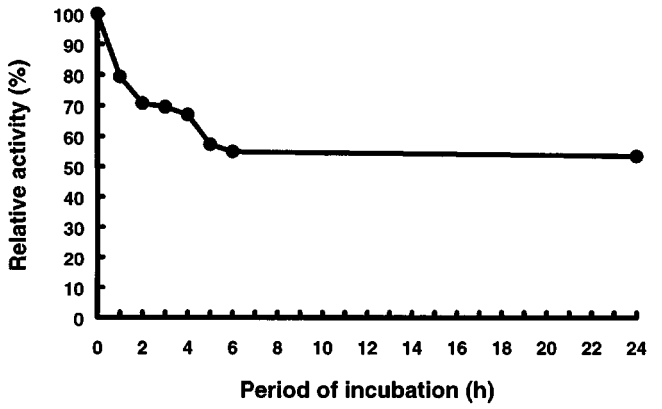

FIG. 5. Stability of the membrane-bound lipase at 50°C. The enzyme was incubated for various periods, cooled, and assayed at 37°C. The activity before temperature exposure was taken as 100%.

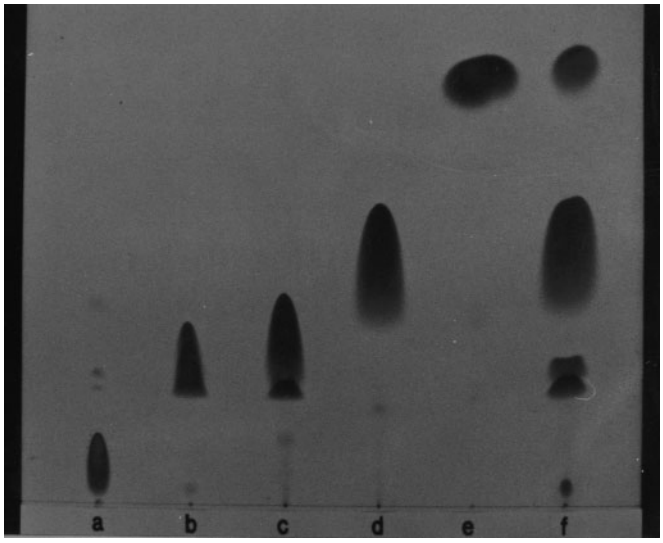

FIG. 6. Specificity of the membrane-bound lipase. The lines are monoolein (a), 1,2-diolein (b), 1,3-diolein (c), oleic acid (d), triolein (e), and triolein hydrolysate after 48 h reaction (f).

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TABLE 1
Substrate Specificity of the Membrane-Bound Lipase from *R. oryzae* (thermophillic)

Substrates	Relative hydrolysis (%)[a]
Monoacid triglycerides	
Triolein (16C)	100
Triacetin (2C)	107.5
Tributyrin (4C)	379.6
Tricaproin (6C)	271.4
Tricaprylin (8C)	300.0
Polyoxysorbitan	
Tween 20	60.2
Tween 40	78.5
Tween 60	69.0
Tween 80	46.2
Tween 85	63.4
Sorbitan	
Span 20	105.4
Methyl ester	
Methyl oleate	50.9
Methyl palmitate	81.8
Methyl myristate	62.7
Methyl stearate	48.2
Methyl caprylate	51.8
Methyl laurate	34.5

[a]Reaction rate with triolein under condition of assay was taken as 100%.

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