Lipolytic Activity in Free and Immobilized Cells of *Phoma glomerata*

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ABSTRACT: The lipolytic activity of free and immobilized whole cells of the pathogenic fungus *Phoma glomerata* was demonstrated, and several properties of the lipase involved were determined. Free fungal cells and small pieces of immobilized cells, prepared by spontaneous colonization on a solid surface or entrapped in calcium alginate, were incubated with triolein in buffered medium. Different incubation conditions were assayed to optimize the reaction, to determine the effects of heating and time on stability of the immobilized preparations and the time course of the reactions. Although the enzyme cleaves all ester bonds of triolein, it shows some preference for the outer bonds. An optimal pH of 7.5–8.0, optimal temperature of 45°C for free and 50°C for immobilized cell preparations, the necessity for substrate emulsifiers, and reaction independence from calcium and magnesium were demonstrated. Results suggest that immobilized whole cells of *P. glomerata* would be a suitable tool to study its lipid physiology and to explore further the possible biotechnological use of its lipase activity.

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KEY WORDS: Fungal lipase, immobilized cells, lipase, lipids, lipolytic activity, microbial enzyme, *Phoma,* triacylglycerol hydrolysis, triolein degradation, whole cells.

Lipases have a wide distribution in animals (1), plants (2), and microorganisms (3). Microbial lipases are more diverse in their enzymatic properties and substrate specificities than those in plants and animals, making them attractive for industrial applications. Although reports on fungal lipases are abundant, to our knowledge there are no papers that describe the production and characteristics of lipases from *Phoma glomerata*, a ubiquitous fungus that is frequently found in plant materials, soil, cement, paint, wood, etc. (4). It colonizes roots and seeds (5), and it can also cause severe leaf spotting (6) and soft-rot in wood (7), as well as mycoses and allergenic effects in man (5).

This work reports the hydrolytic activity on triolein of free and immobilized cells of *P. glomerata* and describes some characteristics of this enzymatic system. The purpose was to obtain basic information on the lipase activity, to contribute to the knowledge about the role of lipids in this fungus and the properties of the enzyme involved in the hydrolytic process. The possibility of reusing these immobilized cell systems for lipolytic reactions was specially explored. Information is important for understanding the physiology of this pathogen fungus and for possible use of its lipase in biotechnology.

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MATERIALS AND METHODS

Chemicals. ^{14}C-Triacylglycerol (112 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Sodium alginate and pancreatic lipase were obtained from Sigma Chemical Company (St. Louis, MO). Other chemicals were of analytical grade and obtained from commercial sources.

Biological materials. *Phoma glomerata* was isolated from cement. Small pieces of the material were washed with sterile tap water; cells were suspended, cultured on agar-malta in Petri dishes, and characterized. Subcultures were done in 500-mL flasks that contained 100 mL of corn meal medium, incubated at 25–27°C for 6 d. Cells were separated from the cultures by centrifugation.

Three types of cell preparations were used to study lipolytic activity: (i) free cells (F-cells); (ii) immobilized cells attached to the surface of a solid support (a thin layer of the synthetic sealing material Fastix (Akapol S.A., Buenos Aires, Argentina), primarily composed of silicon rubber, by allowing spontaneous cellular colonization of the surface in a warm and wet chamber until an approximate concentration of 1 mg of cells per 25 mm^2 of surface was reached (C-cells); (iii) cells immobilized by entrapment in calcium alginate gel according to the procedure described by Bailliez *et al*. (8) (E-cells).

Assays for lipase activity. Cellular preparations of *P. glomerata* were incubated in buffer solutions with emulsified radioactive triolein. The standard assay mixture contained 100 mM Tris-HCl (pH 8.0), 0.03 % Triton X-100, and variable amounts of cell preparations and substrate in a total volume of 700 μL. The reactions were performed in test tubes in a thermostated shaker at 90 strokes per min for 20 min. Suspensions of F-cells, pieces of C-cells or beads of E-cells,

heated in a boiling water bath for 10 min, were used as controls. Control reactions with pancreatic lipase (specific for 1- and 3-positions) were also performed under the same incubation conditions. After incubation, cell preparations were separated by centrifugation, and total lipids from the reaction mixture were extracted with Cl_3CH–MeOH according to the procedure of Bligh and Dyer (9). Total lipids were analyzed by thin-layer chromatography, coupled to a flame-ionization detector, in an Iatroscan apparatus (Iatron Laboratories, Tokyo, Japan). Labeled products of the hydrolysis [mono- and diacylglycerols (DG), free fatty acids, and the remaining triacylglycerols] were detected on the plates by scanning proportional counting with a Berthold LB 2723 (Wildbad, Germany) apparatus; lipolytic activity was expressed as amount of triolein degraded. The procedures for analysis and radioassay of lipids have been described in previous works (10,11). The numerical values presented are the result of averaging of at least three determinations.

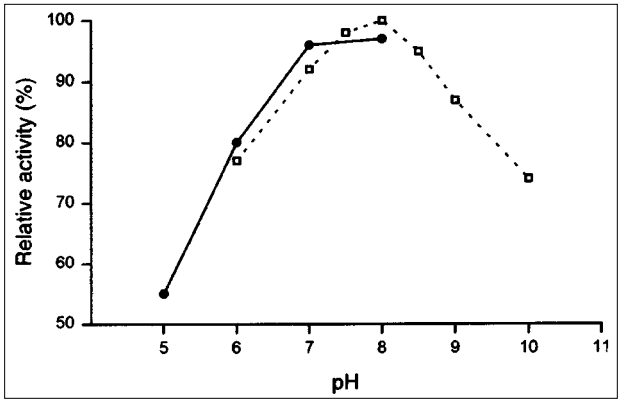

FIG. 1. Effect of buffer pH on the lipolytic activity of free cells of *Phoma glomerata*; (●) phosphate buffer; (□) Tris-HCl buffer; 6 mg of F-cells were incubated at 40°C for 30 min in 100 mM buffer (final concentration in the reaction mixture) with 20 µg triolein emulsified in the reaction mixture.

RESULTS

Cell preparations were incubated with ^{14}C-triolein, and labeled free oleic acid and minor proportions of both diolein isomers (1,2-DG and 1,3-DG), as well as monolein, were detected. Analysis showed that the amount of 1,2-DG formed was about three times that of 1,3-DG. Wild-strain and cultured cells, either free or immobilized, gave rise to similar results concerning hydrolysis products (results not shown). In contrast, when pancreatic lipase was used in control reactions, only 1,2-diolein and monolein were the products from the partial hydrolysis of triolein.

Optimization of reaction conditions. Optimal conditions for the lipolytic reaction were determined by incubating F-cells with triolein and by measuring the products of triolein hydrolysis and the remaining substrate in the incubation medium. The pH dependency of the lipase activity from *P. glomerata* free cells is illustrated in Figure 1. Enzyme activity was observed over the range pH 5–10 in the incubation mixture; optimal activity was at pH values between 7.5 and 8.0.

Various compounds were studied for their effects on lipase activity. Table 1 illustrates the relative activities when different buffers, substrate emulsifiers, and ions were added to the assay mixture. Except for propyleneglycol, significant effects were observed in the degree of hydrolysis when emulsifiers were added to the basic reaction mixture. Activity was not further affected when Ca^{++}, Mg^{++}, or a chelator was added in the presence of an emulsifier.

The effect of temperature of the reaction mixture on lipolytic activity of *P. glomerata* whole cells was measured (Fig. 2). Lipase activity of F-cells was detected up to 50°C, and the optimal temperature was 45°C . With immobilized cell preparations, enzyme activity was observed up to 60°C ; both C-cells and E-cells showed the same optimal temperatures of about 50°C.

Effect of heating on lipase stability of immobilized cells. Remaining activities were measured after exposing C-cell and E-cell preparations to different temperatures for 20 min in buffer solution. Figure 3 shows that both preparations retained nearly all lipase activity after heating at 60°C. At 70°C, activities decreased to 37 and 20% compared to the original values of C-cells and E-cells, respectively. At 80°C, no lipolytic activity was detected.

Stability of immobilized cells to repeated use. The proportion of the initial activity that remained after reuse of *P. glomerata* cell preparations in consecutive batch reactions is illustrated in Figure 4. Repeated incubations of F-cells, separated from the incubation medium by centrifugation and washing after each reuse (results not shown), showed a significant loss of activity (about 50%) by the last of three consecutive batch reactions. Immobilized cells were far more stable. More than 60% of the original activity of C-cells remained after seven batch uses (operation period, 10 d). E-cells maintained their original activity after five consecutive batch reactions on the

TABLE 1
Effect of Various Agents on the Lipase Activity of F-Cells of *Phoma glomerata*

Substance added	Concentration in the assay mixture	Relative activity (%)[a]
None[b]	—	68 ± 8
Triton X-100	0.03%	100 ± 4
Deoxycholate	1.0 mM	95 ± 4
Taurocholate	1.0 mM	90 ± 5
Propyleneglycol	2.5%	79 ± 7
$CaCl_2$[c]	5.0 mM	95 ± 3
$CaCl_2$[c]	1.0 mM	93 (2)
$MgCl_2$[c]	5.0 mM	100 ± 4
$MgCl_2$[c]	1.0 mM	95 (3)
EDTA[c]	5.0 mM	96 ± 5

[a]Average of three determinations ± SD or average of two determinations (extreme deviation of the mean).
[b]Basic reaction mixture consisted of: 0.1 M (final concentration) Tris-HCl, pH 8.0, and 3.0 µg triolein, sonicated in a total volume of 700 µL. Incubations were performed by shaking (90 strokes/min) at 40°C for 30 min.
[c]0.03% Triton X-100 was added.

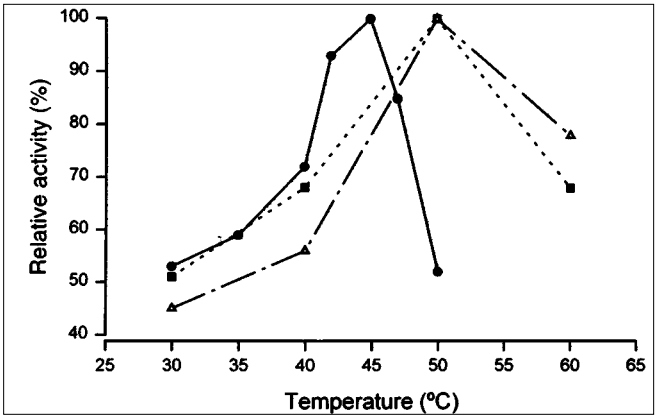

FIG. 2. Effect of temperature of the reaction mixture on relative lipolytic activity of *Phoma glomerata* whole-cell preparations; 4 mg of F-cells (●), 2 mg of C-cells (■), or 10 mg of E-cells (△) were incubated for 30 min with 20 µg of triolein in 100 mM Tris-HCl buffer, pH 8.0.

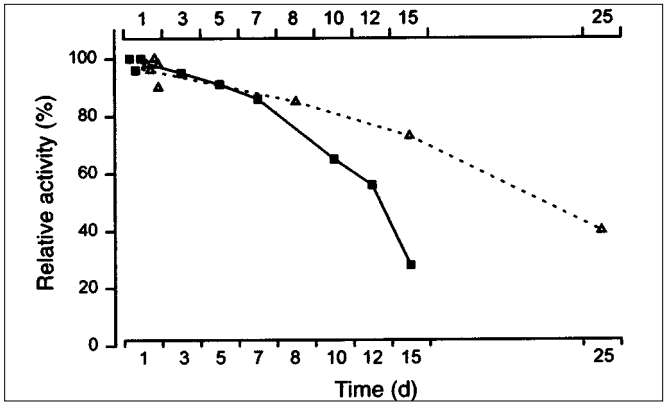

FIG. 4. Repeated use of cell preparations for hydrolysis of triolein. The cell preparations were used in consecutive batch reactions, and the activities remaining after each reaction were measured at 45°C under the same conditions as indicated in Figure 2. Results are expressed as a percentage proportion of the maximal rate. The between-use cell preparations were maintained at 4°C, C-cells (■) in a wet chamber and E-cells (△) in a 5-mM $CaCl_2$ solution.

first day, and 40% of activity remained even after 25 d. Maintenance of lipase activity by both immobilized cell systems is evident. E-cell beads, stored at 4°C in $CaCl_2$ solution, retained their full lipase activity for at least one month and exhibited a catalyst half-life that was twofold longer than that of C-cells.

Time course of hydrolysis. The time course of hydrolysis of triolein by free and immobilized cells, under the optimal conditions mentioned above, is shown in Figure 5. The hydrolysis of triolein by F-cells was linear with time up to approximately 40 min. Thereafter, it increased gradually to reach the maximum at 80 min (not shown in the figure). For immobilized cells, the degree of hydrolysis increased, along with reaction time, up to about 20 min and 40 min for C-cells and E-cells, respectively. The reaction rates in Figure 5 show that the lipolytic activity of C-cells was superior to that of free cells; E-cells were the least active among the three whole-cell preparations.

DISCUSSION

Lipase activity was detected in several cell preparations of *P. glomerata*, and some properties of this enzymatic system were determined. When F-cells were assayed, the products of triolein hydrolysis were found in the incubation medium, a fact that suggested an extracellular state of the lipolytic system. The hydrolytic reaction gives rise to both diolein isomers with a clear predominance of 1,2 diolein, indicating that, although the enzyme cleaves all ester bonds of the triacylglycerol molecule, cleavage of the outer ester bonds is faster than that of the inner ester bond of triolein. The possibility of spontaneous acyl migration (12) should be discarded because it was not evidenced when performing the control reaction with pancreatic lipase.

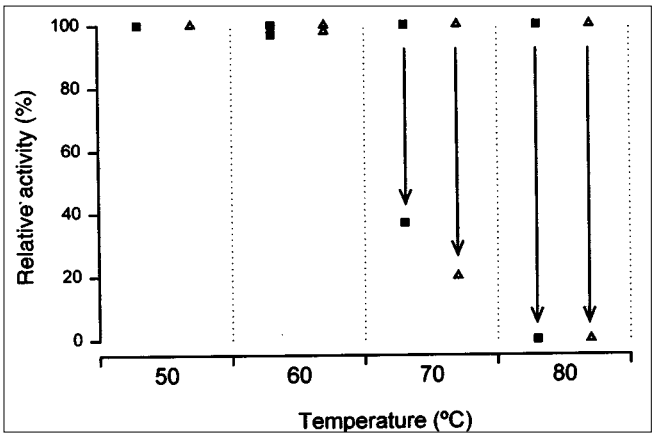

FIG. 3. Effect of heating on the stability of lipase activity from immobilized whole cells of *Phoma glomerata*. Remaining activity was measured at 45°C after exposing cell preparations to the specified temperature for 20 min in 0.1 M buffer Tris-HCl pH 8.0 (■) C-cells; (△) E-cells.

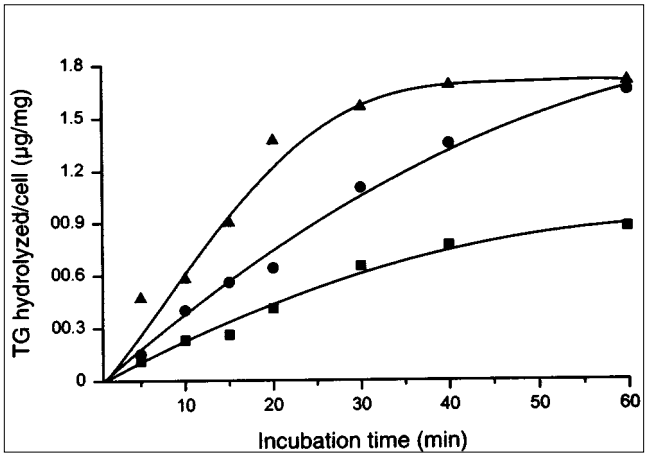

FIG. 5. Time course of the hydrolysis of triolein by *Phoma glomerata* whole cells. Cell preparations were incubated with 20 µg of triolein at the specified times under the optimal conditions mentioned above. (●) F-cells; (▲) C-cells; (■) E-cells; TG, triacylglycerol.

Optimal lipase activity was found at pH 7.5–8.0, though the enzyme was fairly active (more than 80% of the optimal) at pH 6–9 and 55% of the total activity was still observed at pH 5. Most extracellular lipases have acid or neutral pH optima (13). Nevertheless, alkaline pH optima has been reported for lipases of several species of filamentous fungi (14). The presence of a substrate emulsifier was proven to be necessary. Among the emulsifiers assayed, Triton X-100 was a little more effective than deoxycholate; only propyleneglycol did not increase the lipolytic reaction, perhaps due to a more deficient emulsion. Calcium, magnesium, and EDTA evidenced no significant effect on enzyme activity when an emulsifier was added to the reaction mixture, indicating nondependence of the lipase from *P. glomerata* on these ions.

The lipolytic activity of *P. glomerata* whole cells varied with temperature of the reactant medium. Lipase activity was influenced by the physical properties of the substrate, particularly the melting point, which affects substrate emulsification (15). Consequently, the optimal temperatures found are only applicable to the action of this particular enzyme system against triolein.

Immobilized preparations were rather stable to heat exposure. At the same pH and heating time conditions, similar values of thermostability were reported for an isolated extracellular lipase of the thermophilic fungus *Humicola lanuginosa* (16). Thus, immobilized *P. glomerata* lipase behaves similar to that of a thermophilic fungus. This is an advantage in reducing possible contamination by other microorganisms when cellular preparations are used.

Those methods used for cellular immobilization in this work were found to be suitable for repeated utilization of *P. glomerata* whole cells in triacylglycerol hydrolysis. Despite the spontaneous nature of the cell attachment to the surface of the sealing material, without any covalent binding or crosslinking, which requires a relatively long period of cellular colonization, the resulting material offers several advantages. It is physically resistant to washout caused by the substrate, by the reaction mixture components or by shaking; it can be easily cut into small pieces or used as large-sized thin layers. Cellular viability is not an obstacle because there is no leakage to the medium as happens with most entrapped cells.

The classical fashion for entrapping microbial cells in calcium alginate gel beads was also carried out (17). This cell preparation evidenced comparatively less activity than C-cells, probably due to slower diffusion of the substrate within the beads. Nevertheless, it was suitable to maintain the activity of the *P. glomerata* lipolytic system.

We conclude that both immobilized systems of *P. glomerata* cells tested here are appropriate for the repeated enzymatic hydrolysis of triolein because satisfactory activity yields and operational stabilities were achieved. The results obtained are interesting for further experimental design. These cellular immobilized systems should be useful to elucidate lipid physiology in *P. glomerata* as well as for technological use of lipases. Knowledge about the positional hydrolytic action, substrate specificity, and enzyme isolation deserves future investigations.

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