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Physiology of lipase formation in *Penicillium candidum*

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Penicillium candidum **grew and produced lipase in a culture medium supplemented with 0.2% olive oil. Significant enzyme production required the presence of olive oil and was prevented by cycloheximide. Polyacrylamide gel electrophoresis of filtrates from olive oil fermentations gave a single band of lipase activity (MW 80 KDa). Among the olive oil components only oleate allowed significant lipase production. Other carboxylic and saturated fatty acids containing similar or lower numbers of carbon atoms, did not cause derepression of lipase formation.**

Keywords: lipases; *Penicillium candidum*; enzymes; regulation; oleic acid; derepression

Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) that hydrolyze long chain triacylglycerides are produced by a wide variety of microorganisms [13]. Although microbial lipases have several commercial applications, their larger markets have been either in cheese ripening acceleration [14] or as laundry detergent additives [2]. Future uses may include the synthesis or hydrolysis of peptides and other esters, biosurfactant production and triglyceride transesterifications, as well as the resolution of racemic mixtures [8,21]. Certain species of *Aspergillus, Mucor, Rhizopus, Geotrichum* and *Penicillium* represent common sources of commercially important lipases [12]. During our search for fungal lipases suitable for dairy flavor generation, *Penicillium candidum* was selected from among 15 fungal strains for further characterization. This mold produces an active extracellular lipase with accompanying low protease levels [20]. Even though fungal lipases are of industrial importance, little information is available with respect to the factors and conditions that control their biosyntheses. In this paper we report studies on enzyme formation in this fungus and describe some additional properties of the enzyme.

Materials and methods

Microorganism

A wild strain of *P. candidum* was obtained from the Instituto de Investigaciones Biomedicas Culture Collection, Mexico DF 04510. The microorganism was preserved and the inoculum was prepared as previousy reported [20].

Cultural conditions

All fermentations were performed at 29°C and 160 rpm in 250-ml Erlenmeyer flasks with 50 ml of 'D' medium [4], supplemented with 1% Casamino Acids (Difco, Detroit, MI, USA), as previously described [20]. For lipase production, 0.2% commercial olive oil was added to the medium.

Analytical methods

Lipase activity was measured as described by Menassa and Lamberet [16]. Buffer and substrate concentrations used were those reported by Hoffelman *et al* [9]. Samples of the fermentation broth (3 ml) were filtered through Whatman No. 540 filter paper, and the filtrates (0.5 ml) were assayed for lipase activity. One lipase unit (UL) was defined as the amount of enzyme that released 1 μmol of butyric acid per min at 37°C.

Biomass was estimated from the filtered mycelium by dry cell weight (DCW), after drying it at 60°C for 24 h.

Polyacrylamide gel electrophoresis (PAGE)

Before PAGE, 25-ml samples of filtrate containing 0.06 mg protein ml^{-1} were concentrated to 250 μl (100-fold) by centrifugation on Amicon Centriflo CF25 membranes (1000 × *g* at 4°C). Samples (15 μl) with protein levels ranging from 90–100 μg were applied to the gel tracks. PAGE was done under denaturing conditions according to the method of Laemmli [10] by use of a 10% (w/v) polyacrylamide running gel with 1.2 mg ml^{-1} bovine serum albumin and a 4% stacking gel in the presence of 3.5 mM sodium dodecyl sulfate (SDS). PAGE was carried out in tris-glycine buffer (0.025 and 0.19 M respectively) at pH 8.6. A Gibco 10-kDa Protein Ladder was used as MW standard reference. The electrophoresis was performed at 200 V for 1.5 h at 4°C. Proteins were stained with 0.25% (w/v) Coomassie Blue R250 prepared in methanol–acetic acid–water (45 : 10 : 45, v/v/v). Lipase activity was detected in the gel by means of the agar print technique described by Hofelmann *et al* [9] at 37°C, with the use of 0.086% (w/v) *p*-nitrophenyl laurate as substrate. Prior to this step, SDS was removed from the gel by submerging it for 30 min in 0.02 M succinic acid buffer (pH 7.2), in the presence of 5% Triton X-100 and then for 30 min in the same buffer with a lower detergent concentration (0.05%). A final gel treatment was carried out for 30 min in the succinate buffer alone.

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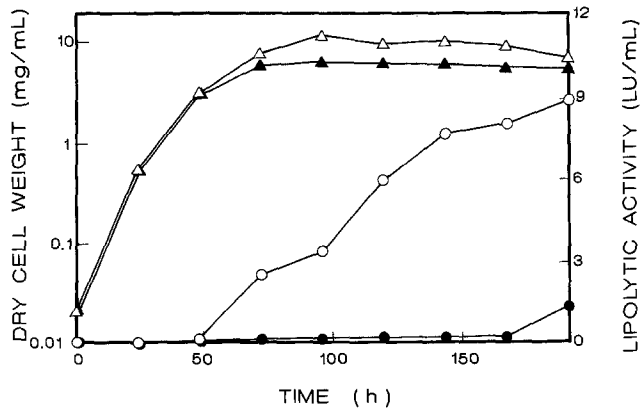

Figure 1 Time course of *Penicillium candidum* growth (△, ▲) and lipase activity (○, ●) in 'D' medium supplemented with 1% Casamino Acids in the presence (open symbols) or absence (closed symbols) of 2% commercial olive oil. Fermentations were performed at 29°C and 160 rpm in 250-ml Erlenmeyer flasks containing 50 ml culture medium.

Results

Growth and lipase formation

P. candidum grew and produced lipolytic activity in 'D' medium with 2% olive oil. Enzyme production started after the second day of incubation (at the end of the exponential growth phase) and increased linearly up to the seventh day of fermentation (Figure 1). In the absence of olive oil the cultures produced only basal lipase levels which increased at the end of the fermentation. Olive oil concentrations ranging from 0.01 to 2% were tested in order to evaluate the effect of the substrate concentration on enzyme formation. As shown in Figure 2, although an increase in the volumetric lipolytic activity with up to 0.2% was observed, the minimum substrate concentration which allowed maximum stimulation of specific enzyme activity levels was 0.02%. As shown in the same figure, fungal growth was also stimulated by the oil amount in the culture media with a maximum effect at a concentration of 2%.

Differential rate of lipase synthesis

To ascertain the basis of the stimulation, *Penicillium* cultures, previously grown for 48 h in 'D' medium, were exposed to olive oil in the presence or absence of a protein synthesis inhibitor [18,22] and lipase activity was plotted *versus* DCW at different times during the fermentation (Figure 3). A linear increase in DCW and lipolytic activity was observed when the cultures were supplemented with 2% olive oil after 48 h fermentation. Under these conditions, enzyme levels were 34-fold higher than those obtained in the absence of olive oil. As shown in the same figure, this increase in lipolytic activity was prevented or discontinued when cycloheximide (100 μg ml^{-1}) was added to the cultures at 48 or 96 h. Therefore a derepression rather than an activation was considered as the cause of stimulation by olive oil.

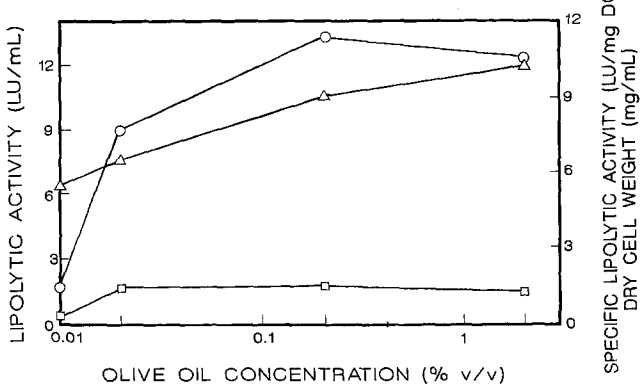

Figure 2 Effect of the olive oil concentration on maximum growth (△), volumetric (○) and specific lipase production (□). Fermentations were performed for 120 h as described in Figure 1.

Some properties of the enzyme

In order to characterize the filtrates with lipase activity, polyacrylamide gel electrophoresis was carried out on concentrated filtrates. As shown in Figure 4, when the enzyme was assayed on the gel its activity appeared as a single band but only in those filtrates coming from fermentations containing olive oil. The molecular weight for this enzyme was approximately 80 kDa. As a control, a filtrate from *Mucor miehei* with lipase activity was treated and tested in the same way, and its molecular weight was estimated to be 39 kDa (not shown). This value was in agreement with previous reports [3].

Effect of olive oil components on lipase synthesis

Considering that, in addition to glycerol, olive oil contains 80% oleate, 10% palmitate and 7% stearate [23], the effect of these compounds on enzyme production was investigated. Among the olive oil components tested, enzyme synthesis was stimulated only in the presence of oleate (Figure 5). With the exception of triolein, other carboxylic and fatty acids like acetic, butyric, citric, lauric, and myristic, did not derepress lipase formation. Unsaturated compounds other than oleate and triolein, were not examined in this work.

Discussion

P. candidum produced and excreted lipase when grown in the presence of olive oil. The finding that this nutrient was

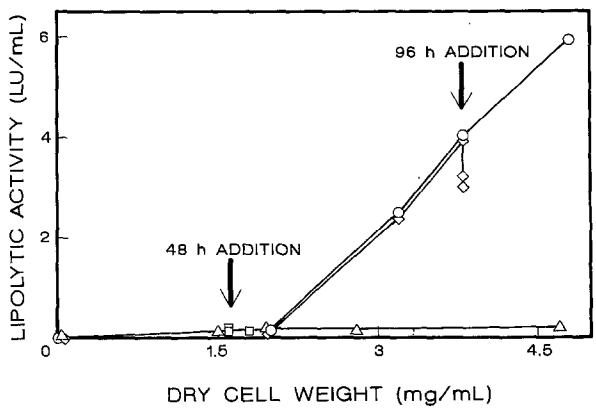

Figure 3 Effect of olive oil on lipase formation and its prevention by cycloheximide. Cultures were grown in 'D' medium and at 48 h exposed to the effect of 0.2% olive oil (○, ◇, □). Cycloheximide (100 μg ml^{-1}) was added to cultures after 48 (□) or 96 h (◇). Control without olive oil or cycloheximide (△).

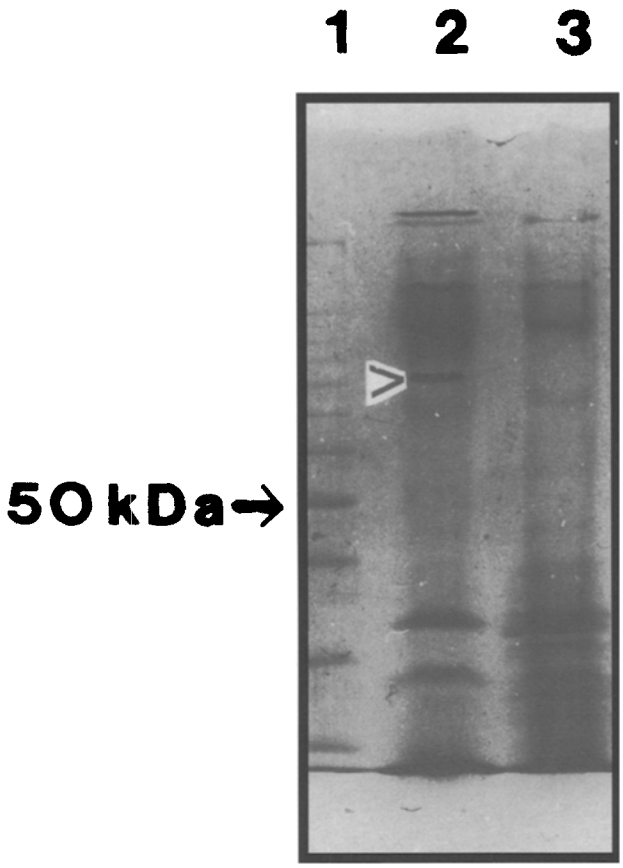

Figure 4 SDS polyacrylamide gel electrophoresis of *P. candidum* filtrates obtained from cells grown for 100 h in the presence (lane 2) or absence (lane 3) of 0.2% olive oil. The arrow indicates the position of lipase activity as detected by an agar print technique using 0.086% (w/v) *p*-nitrophenyl laurate as substrate. Lane 1 contains molecular size markers. Fermentations were performed as described in Figure 1.

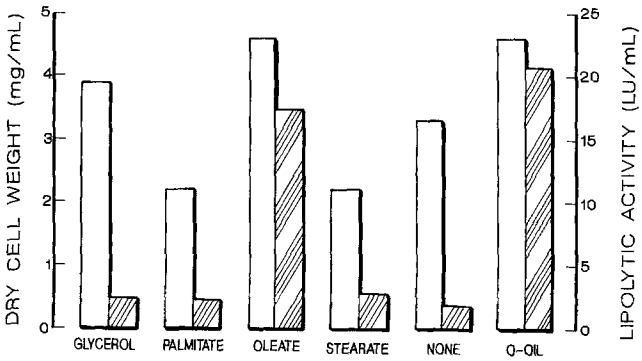

Figure 5 Effect of olive oil components (0.2%) on growth (□) and lipase production (▨) by *P. candidum*. Fermentations were performed for 196 h as described in Figure 1.

necessary to obtain high lipase levels and that this effect was prevented or discontinued by cycloheximide, suggested a derepressive action of this oil on enzyme formation. Among the olive oil components, stimulation of enzyme synthesis was observed only in the presence of oleate, suggesting that this group was responsible for lipase formation. The synthesis of other fungal lipases in the presence of olive oil has shown a variety of responses. For instance, an inductive influence by this substrate on enzyme synthesis has been reported for *Aspergillus niger* [19]. *Rhizopus delemar* [7] and *P. citrinum* [15] produced significant amounts of lipase in the absence of olive oil, although production was further stimulated by the nutrient. By contrast, enzyme production by *R. oligosporus* [17] and *P. roqueforti* [6] was reduced by the presence of this substrate.

Lipases having commercial interest are produced by a wide variety of microbial systems, which synthesize the activity either as a single protein or as isoenzymes. *P. candidum* produced a single protein with lipase activity, which had a molecular weight of 80 kDa under denaturing conditions. This value was four-fold higher than those reported for such other *Penicillium* species as *P. camemberti* and *P. caseicolum* analyzed under similar conditions [1,11], but resembled that reported for *Botrytis cinerea* [5].

Acknowledgements

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