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# Extracellular lipolytic activity in *Phoma glomerata*

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**Summary**

Several properties of the lipolytic activity exhibited by the conidial fungus *Phoma glomerata* were studied. Lipolytic activity in an aqueous buffer medium was measured on triacylglycerol, phosphoglyceride and cholesterol ester under different experimental conditions. The effect of storage temperature on the stability of the hydrolytic activity, and optimal conditions of temperature and time of maximal activity were determined. The optimal conditions for maximal lipolytic activity were found to be 40–50 °C and 1 h. The activity released to the medium by 1 mg cells for 1 h at 40 °C was stated as the enzyme released unit (ERU). The protein fraction of MW >50 kDa obtained by ultrafiltration of the medium, was active on the three substrates assayed, and it showed a non-specific hydrolytic activity on both the 1- and 2-acyl esters either in the neutral glyceride or in the phosphoglyceride. A protein of $M_r$ approx. 75 kDa was the only one that showed esterase activity. The crude medium, stored at −15 °C, maintained its initial hydrolytic activity on triacylglycerol for at least 42 days, though when it was kept for 10 days at 4 °C, the activity fell to 50%. Kinetic parameters using substrates such as triolein (TO), dipalmitoyl phosphatidylcholine (DPPC) and cholesteryl oleate (ChoO), were comparatively evaluated. The activity of the enzyme in the hydrolysis of TO showed the highest values, whereas the maximal specific activities were less when the enzyme was assayed against DPPC and ChoO.

## Introduction

Lipases are powerful tools for catalysing not only hydrolysis, but also esterification and transesterification reactions involving water-insoluble esters. They can be used in the food industry for the development and enhancement of food flavours, and also in the pharmaceutical and agrochemical industries for the synthesis of structured lipids and for the production of health products (Ghandi 1997; Schmid & Verger 1998). Industry continues looking for economical sources of lipases with high activity, the need for novel lipases being obvious. Extracellular lipases are produced by many microorganisms, including fungi and bacteria. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, making them very attractive tools for industrial applications (Godtfredsen 1990). They may have an advantage over lipases from other origins for their availability and relative ease of purification.

The species here studied, *Phoma glomerata*, is a ubiquitous conidial fungus frequently found in plant materials, soil, cement, paint, wood, etc. (Domsch *et al.* 1993). It colonizes roots and seeds, and it causes mycoses and allergenic effects in man (Boerema *et al.* 1965). It can also cause leaf spotting (Hosford 1975) and soft-rot in wood (Duncan & Eslyn 1966). The hydrolytic activity of this fungus upon triolein (TO), using preparations of free and immobilized whole cells, has previously been evaluated (Pollero *et al.* 1997). It was concluded that two immobilized systems were appropriate for the repeated enzymatic hydrolysis of TO, because satisfactory activity yield and operational stabilities were achieved. In the present work the properties of the lipolytic activity of *P. glomerata* released to a simple aqueous medium were studied. The effect of storage temperature on the stability of enzyme activity and conditions of temperature and time for maximal activity were determined. Kinetic parameters of the reactions, using three different lipid substrates, were comparatively evaluated. The enzyme was partially isolated and characterized.

## Materials and methods

### *Chemicals*

[$^{14}$C]Triolein [(TO) (112.0 mCi/mmol)], [$^{14}$C]dipalmitoylphosphatidylcholine [(DPPC) (110.0 mCi/mmol)] and

[$^{14}$C]cholesteryl oleate [(ChoO) (59.5 mCi/mmol)] were purchased from New England Nuclear Corp. (Boston, MA). Cofactors used for enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade and obtained from commercial sources.

*Biological material*

*Phoma glomerata*, isolated and characterized as described previously (Pollero *et al.* 1997), was cultured in 500 ml flasks that contained 100 ml of corn meal medium at 25–27 °C for a week. Cells were separated from the cultures by centrifugation. Cells (about 80–90 mg wet wt.) were incubated in 1.5 ml of 50 mM buffer Tris–HCl pH 7.5, with shaking (90 strokes/min) at different times (15, 30, 60, 90 and 120 min) and different temperatures (10, 30, 40 and 50 °C ). Cells were separated by centrifugation. Enzyme released to the medium, 25 $\mu$g of proteins per mg cells in optimal conditions (1 h at 40 °C), was used for kinetic assays and characterization.

*Assays for enzyme activities*

Extracellular hydrolytic activities were determined using 1 $\mu$Ci of labelled substrate per tube. The standard assay mixture, containing 100 mM Tris–HCl (pH 7.5), 0.03% Triton X-100, either 30 $\mu$g unlabelled TO, 20 $\mu$g DPPC or 10 $\mu$g ChoO, were incubated at 40 °C with aliquots of the enzyme released in a total volume of 700 $\mu$l. Enzyme released unit (ERU) was defined as the amount of enzyme released to the aqueous medium by 1 mg of cells (wet wt.) at 40 °C for 1 h (equivalent to 25 $\mu$g proteins). The reactions were performed in test tubes, at 40 °C in a thermostatically-controlled shaker at 90 strokes/min for various times. Incubations were stopped by immersing the tubes in a boiling water bath for 5 min. The products of hydrolysis and remaining substrates were extracted with chloroform:methanol 2:1 v/v) by the method of Bligh & Dyer (1959), and separated by thin layer chromatography (TLC) on high-performance TLC Silicagel 60 plates (Merck, Darmstadt) with hexane/ether/acetic acid (80:20:1.5 v/v) and chloroform/methanol/acetic acid/water (65:35:4:4 v/v). Radioactivity in different lipid classes (substrates and hydrolysis products) was detected and quantified on the chromatographic plates by scanning proportional counting with a Berthold LB 2723 Dunnschicht Scanner II apparatus (Germany). Appropriate standards were run simultaneously. Assay mixtures without enzyme preparations were incubated and processed simultaneously as controls. The hydrolytic activities were expressed as ng substrate hydrolysed per ERU.

*Partial isolation and characterization*

Total proteins released to the aqueous medium were quantified colorimetrically. They were fractionated by ultrafiltration, using Centricon concentrators (Amicon Inc., Beverly, MA) cut-off 10, 30 and 50 kDa. The hydrolytic capacity of each fraction on TO, DPPC and ChoO was separately checked. The protein fraction of molecular weight higher than 50 kDa was analysed by native polyacrylamide gel electrophoresis (PAGE) in a mini-slab electrophoresis unit. Molecular-weight standards (Pharmacia, Uppsala, and Sigma Chemical Co., St. Louis, MO) ranging from 67 to 669 kDa, were run in parallel lines. Proteins were visualized in one gel by staining with Coomassie brillant blue. A similar gel was subjected to hydrolytic activity assay using naphthyl acetate as substrate, following the technique described by Goullet (1975).

## Results and discussion

*Conditions of enzyme release*

Optimal conditions of temperature and time for the release of lipolytic activity from the cells to the buffer medium, were determined. Lipolysis was assayed by measuring the products of TO hydrolysis after incubation of the free cell buffer-medium with the substrate. The temperature dependency of the lipolytic activity released by the cells is illustrated in Figure 1. Lipase activity release was observed over the range 10–50 °C, and it was found that it reached its maximum at 40 °C. The time dependency of the enzyme activity release by the cells was measured over the range 15–120 min. Results in Figure 2 show an optimal time of release at 60 min. At longer times (90 min) the activity declined, probably due to an enzyme inactivation under these experimental conditions. The induction effect of several lipids which enhance fungal lipase secretion are known

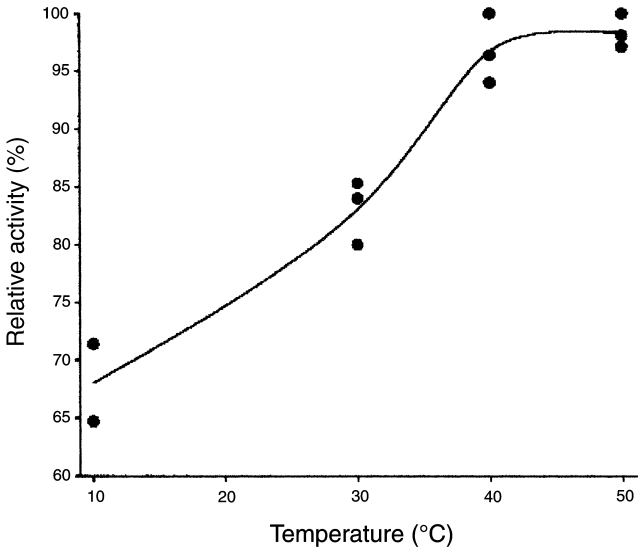


*Figure 1.* Effect of temperature on the enzyme release. Cells were incubated in 50 mM buffer Tris–HCl pH 7.5, with shaking, at the specified temperatures for 1 h. They were removed and the lipolytic activity of the medium was assayed using TO as substrate.

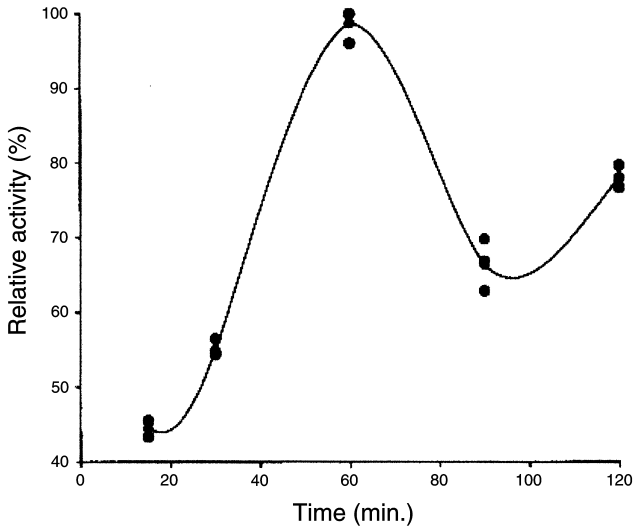


*Figure 2.* Time-course for the enzyme release. Cells were incubated in buffer medium, with shaking, at 40 °C for the specified times. Lipolytic activity of the cell free medium on TO was assayed.

(Rapp 1995; Gaspar *et al.* 1999). Also it was suggested that, in the absence of inductors, lipase activity secretion is possibly associated for a short time to the cell wall (Rapp 1995). In this regard, it must be remarked that no addition was done to the diluted buffer medium, and that, in spite of these simple conditions, considerable lipolytic activity was released from *P. glomerata* cells to the medium. Repeated incubations of the same pool of cells in the buffer medium were done. It was found that, after five consecutive assays for a 7-day period, cells maintained 85% of their original enzyme activity release. Crude enzyme preparations used in all the following assays were obtained at the already mentioned optimal conditions of time and temperature.

*Optimization of reaction conditions and stability of the stored enzyme*

The hydrolytic activity on TO as a function of amount of enzyme, as well as the optimal temperature and pH, were determined. It was found that up to a maximum of 40 ERU was the amount of enzyme admitted for proportional response in the hydrolysis reaction. Optimal activities were found at 45 °C and pH 7.5. In order to determine the stability of the stored enzyme, samples of crude enzyme released to the medium were maintained at 4 °C and −15 °C, respectively. Their remaining lipolytic activities were measured at different storage times. It was found that preparations stored at −15 °C retained the original lipase activity for at least 6 weeks. In contrast, non-frozen preparations showed a significant loss of activity, falling to about 20% after 20-day storage.

*Partial purification and characterization*

By ultrafiltration using different MW cut-off membranes, total proteins released by the cells to the medium were separated into four fractions. Enzyme activities in each fraction on TO, DPPC and ChoO were examined. It was found that the fraction of MW higher than 50 kDa was the only active one, and that such hydrolytic activity occurred on all three substrates. Proteins from the active fraction were separated by PAGE under native conditions, and the enzyme was detected on the gels by activity staining. Those results obtained from the electrophoresis analysis are shown in Figure 3. It is observed that the fraction contains several protein bands, but only one of them, with a MW of approximately 75 kDa, has hydrolytic activity. Results suggest

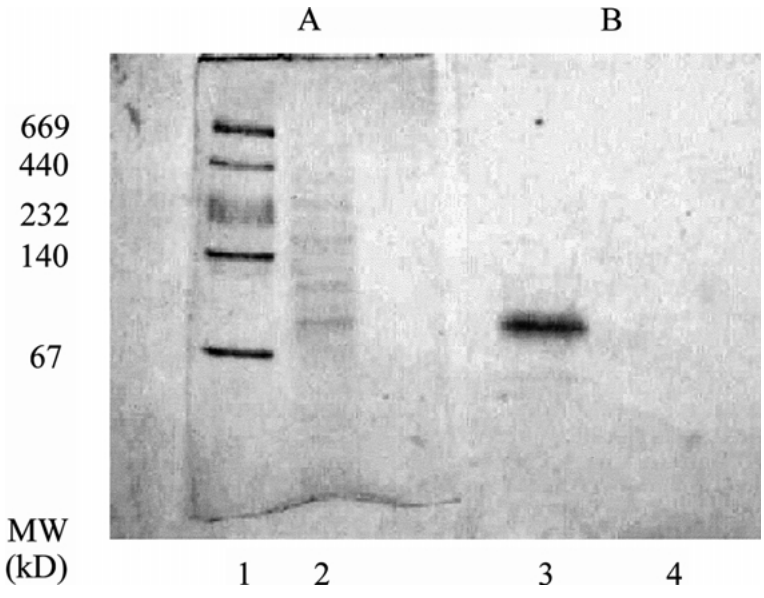


*Figure 3.* Electrophoresis of the protein fraction with lipolytic activity. Total proteins released by *P. glomerata* cells were fractionated by ultrafiltration. Fraction with MW higher than 50 kDa was analysed by PAGE under native conditions. Gel A – proteins revealed by Coomassie blue staining; Gel B – gel subjected to hydrolytic activity assay; 1 and 4: Molecular weight standards; 2 and 3: Sample.

that a single protein released by *P. glomerata* is responsible for the hydrolysis of the three fatty acid esters here assayed. Microbial lipases generally have molecular weights ranging from 20 to 60 kDa and di- and tetra merization has been observed (Stocklein *et al.* 1993). Such aggregates can be dissociated at high ionic strengths (Macrae 1983). It must be taken into account that purification of *P. glomerata* lipolytic enzyme was merely partial, and that the enzyme was not analysed under dissociating conditions. Consequently, the relatively high molecular weight found when compared with other microbial lipases, may result from either self-association of lower-molecular-weight polypeptides, or from the lack of large-aggregate dissociation due to the low ionic strengths used.

*Kinetic parameters*

The time-course for the hydrolysis of TO, DPPC and ChoO by the lipolytic enzyme, is shown in Figure 4. The hydrolysis of TO was linear with time up to approximately 30 min and, thereafter, it increased gradually to reach the maximum at 60 min. Using DPPC as substrate, linearity of the hydrolysis was maintained up to around 20 min. On ChoO, the hydrolysis was proportional to time up to 60 min. Measurements of the degree of hydrolysis were made using the crude enzyme under saturating conditions of substrates. The activity of the enzyme in the hydrolysis of TO showed the highest values with a $V_{max}$ of 30 ng/min ERU, whereas the maximal specific activities were lesser when the enzyme was assayed against DPPC ($V_{max} = 19$ ng/min ERU) and ChoO ($V_{max} = 13$ ng/min ERU).

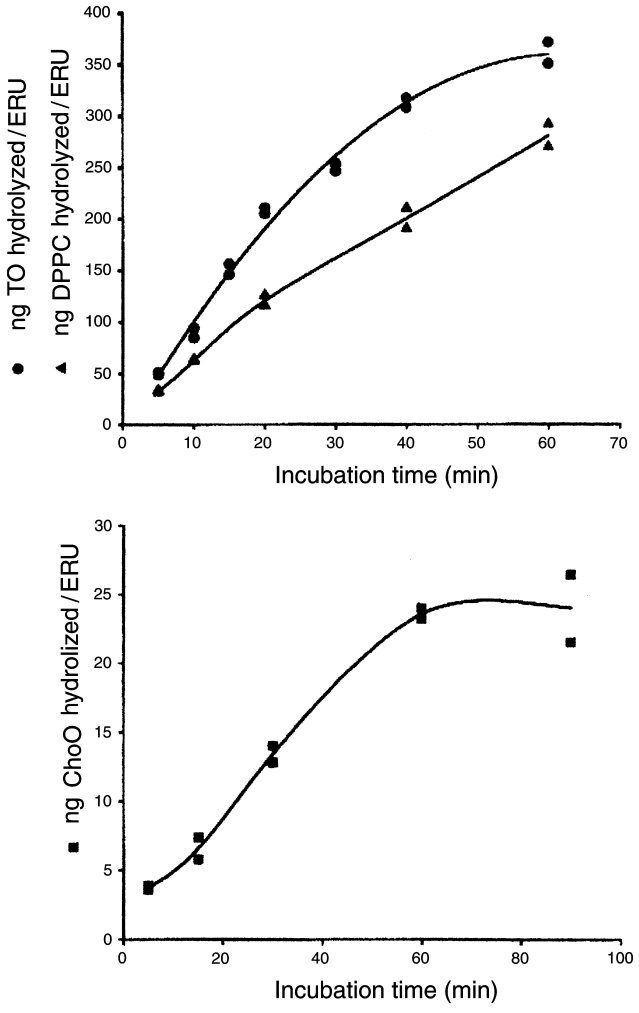


*Figure 4*. Time-courses for the hydrolysis of TO, DPPC and ChoO. Twenty ERU were incubated at the specified times with 30 $\mu$g of TO, 20 $\mu$g of DPPC or 10 $\mu$g of ChoO, emulsioned with Triton X-100 in buffer Tris–HCl.

*Substrate specificity*

When enzyme released to the aqueous medium was incubated with [$^{14}$C]triolein, free oleic acid and 1,2-diolein, followed by small proportions of 1,3-diolein and mono-olein, were detected. On the basis of their positional specificity, microbial lipases can be divided into two groups. One of them is 1,3-positional specific, and it releases free fatty acids, 1,2-diacylglycerols and monoacylglycerols, but not 1,3-diacylglycerols. The other group is non-specific, and it releases all the above mentioned products (Sonnet & Gazzillo 1991). Most lipases belong to the first group. In a screening of 25 active yeast lipases with respect to their positional specificity against triacylglycerol, Hou (1997) found that 19 showed 1,3-positional specificity and six showed random specificity. The lipolytic activity from *P. glomerata* shows random specificity, although cleavage of the outer ester bonds is markedly faster than that of the inner ester bond of TO.

Enzyme was also active in the hydrolysis of DPPC. Free palmitic acid was the only radioactive product of the reactions, and labelled lysophosphatidylcholine was not detected. A number of microbial lipases also catalyse the hydrolysis of phospholipids. However, most of them possess 1,3-positional specificity, and consequently they produce 2-monoacyl phosphoglycerides. In our case, the non-detection of this lysophospholipid when the lipolytic enzyme from *P. glomerata* was assayed on DPPC, would suggest a non-specific hydrolytic activity on both the 1- and 2-acyl esters of the phospholipid. Results from the assays with ChoO as substrate demonstrated that the enzyme also catalyses the hydrolysis of this ester, although the rate of reaction is slow compared with that obtained with TO or DPPC. Consequently, lipolytic activity released from *P. glomerata* cells, would be considered as a rather non-specific esterase activity.

The relatively rapid release of the enzyme to a simple medium as it is a diluted buffer solution without inducers, the continuous production of active crude extracts by the whole cells and the versatility of its hydrolytic action against different esters, may be attractive properties of *P. glomerata* lipolytic activity. On the other hand, there is a demand for enzymes which can be used in the production of structured lipids working in

microaqueous systems. As known lipases catalyse reversible reactions, so it may be speculated that the low selectivity of *P. glomerata* lipolytic enzyme observed in the hydrolysis, would be the same during the synthesis. This observation deserves further study to explore the practical uses of this enzyme as catalyst in organic synthesis.

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