

## Partial purification and characterization of a lipolytic enzyme from spores of the arbuscular mycorrhizal fungus *Glomus versiforme*

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**Abstract:** The lipolytic activity of extracts from *G. versiforme* spores was detected and measured in vitro assays using triolein as a substrate. Among the different subcellular fractions assayed, a membrane-rich one showed the greatest lipolytic activity. Like other membrane-bound enzymes, *G. versiforme* lipase was well extracted in presence of detergent. The time-course of triolein hydrolysis by several lipase preparations was studied. The partial purification of the lipase from the total homogenate revealed increasing enzyme specific activities in the following order: homogenate < sediment at 13 000 g for 30 min < acetone powder < fraction obtained by gel permeation chromatography. The molecular mass of the enzyme was estimated to be 30 kDa as determined by gel filtration column chromatography.

**Key Words:** acetone powder, lipase activity, spore homogenates, triacylglycerol hydrolysis

phosphate dehydrogenase, succinate dehydrogenase, glucose-6-phosphate dehydrogenase, ATPase (Smith and Gianinazzi-Pearson, 1988; Jakobsen, 1991; Gianinazzi-Pearson et al., 1991). García-Romera et al., (1990) reported the presence of pectinases, cellulases and hemicellulases in extracts of spores of *Glomus mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe. García-Garrido et al., (1992) studied the production of endoglucanases during the process of penetration and development of *Glomus fasciculatum* (Taxter sensu Gerdemann) Gerdemann and Trappe in roots of lettuce. However, there is no biochemical study on the lipolytic activity, despite the high content of triacylglycerols (substrates of these enzymes) found in AMF.

Triacylglycerols are the predominant reserve compounds in spores of the AMF *Glomus versiforme* (Gaspar et al., 1994a). During germination they decrease continuously providing energy-rich material and carbon sources for the synthesis of other lipids like phospholipids (Gaspar et al., 1994b). Such degradation implies the action of hydrolytic enzymes of the lipase type (triacylglycerol acylhydrolase, EC 3.1.1.3) which catalyzes the breakdown of the triacylglycerols into glycerol and free fatty acids (Derewenda et al. 1993).

This work reports the hydrolytic activity on triolein of *G. versiforme* spores and it describes several purification steps of a protein fraction with lipase activity obtained from total homogenates of these spores. The purpose was to obtain basic information on the lipase activity with the aim of contributing to the knowledge about the role of lipids in this fungus as well as some properties of the enzymes involved in their metabolism.

### INTRODUCTION

The arbuscular mycorrhizal fungi (AMF) cannot, as yet, be grown in pure culture for long periods of time. Therefore knowledge on the physiology and biochemistry of these fungi has mainly been obtained either from spores or from the fungal structures associated with roots, where the fungus is influenced by symbiotic interactions with the plant. Several enzyme activities from AMF have been reported, cytochemical studies revealed the presence and localization of various enzymes such as glyceraldehyde-3-

### MATERIALS AND METHODS

**Biological material and growth conditions.**—A pure culture of *Glomus versiforme* (Karsten) Berch (LPS strain N° 80) was used in these experiments. It was maintained on *Medicago sativa* L. and *Sorghum vulgare* L. Plants were grown in 500 mL open pots of 9:1 sand : soil steamed mixture. *M. sativa* and *S. vulgare* seeds were sown in moistened sand, and after two wk the seedlings were transplanted to the pots containing 5 g of the AMF inoculum and grown under greenhouse conditions. Plants were watered from below using a capillary system.

After three mo spores were collected from the pot cultures by wet sieving and decanting the soil (Gerdemann and Nicolson, 1963). They were picked up under a dissecting microscope (20–70 X) and removed from soil detritus with the aid of thin forceps. Spores were disinfected with 2% chloramine T plus 200 ppm streptomycin for 20 min, drained, and the spores were rinsed with sterile distilled water. Batches of spores were processed and analyzed in triplicate.

**Chemicals.**—[1-<sup>14</sup>C] Triolein (112.0 mCi / mmol, 99% radiochemically pure) was purchased from New England Nuclear Corp. (Boston, MA). Cofactors used for enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, MO). All chemicals and solvents were of analytical grade.

**Preparation of total spore homogenate.**—The isolation and purification of the lipolytic system were carried out at 4 C. *G. versiforme* spores (0.1–0.2 mg wet cells) were homogenized in a solution containing 400.0 mM sucrose, 0.1 mM EDTA, 1.0 mM MgCl<sub>2</sub>, 0.1 M CaCl<sub>2</sub>, 10.0 mM NaHCO<sub>3</sub>, 2.0 mM DTT and 20.0 mM Tris/HCl (pH 8.5), using a 0.1-mL glass homogenizer (Wheaton U. S. A.). This total homogenate was used immediately for the assay of lipase activity. The effects of pH and emulsifiers (sodium desoxycholate and Triton X-100) and the necessity of protease inhibitors (Aprotinin and phenylmethylsulfonylfluoride PMSF) on lipase activity, were determined. In other assays the total spore homogenate was processed to obtain cellular subfractions.

In order to determine the location of lipase in the cell, the crude spore homogenate preparations (400 μL) were centrifuged at 5000 g for 10 min and at 13 000 g for 30 min and the resulting supernatants and pellets were assayed for lipase activity. Aliquots of the fraction pelleted at 13 000 g for 30 min were treated with 0.3 % v/v Triton X-100 in 10 mM Tris-HCl (pH 8) and used for enzyme purification by gel permeation chromatography.

**Analytical gel permeation.**—Proteins from the fraction pelleted at 13 000 g for 30 min from the spore homogenates, previously treated with Triton X-100 as described above, were separated by HPLC on a Merck-Hitachi liquid chromatograph equipped with a L-6,200 pump. Detection was performed by absorption at 280 nm using a L-4,200 UV-VIS Detector. The samples were filtered through a 0.45-μm pore-membrane and applied in aliquots of 200 μL to a Superdex 200 HR 10/30 (Pharmacia, Uppsala, Sweden) gel permeation column (10 300–310 mm). To estimate the molecular weight of chromatographic subfractions, bovine serum albumin (67 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and

ribonuclease A (13.7 kDa) were used as marker proteins. Subfractions were eluted with 10 mM Tris/HCl (pH 8) and 0.05% Triton X-100 at a flow rate of 0.7 mL/min. They were concentrated either by lyophilization on a 5 SL Virtis Lyophilizator (New York) or by ultrafiltration through PM-10 membranes (Amicon Co.), and used immediately for the assays of lipase activities.

**Acetone powder preparation.**—Aliquots of total spore homogenates were used to prepared the acetone powder according to Hassanien and Mukherjee (1986). The spores (0.2 mg) were washed three times with distilled water and homogenized in a 0.1-mL glass homogenizer (Wheaton U. S. A.) with 100 μL acetone. The acetone extract was separated from the residue by centrifugation and the residue reextracted 3 times at 4 C with 100 μL acetone each time. The residue was dried until the acetone powder was obtained and used immediately for the assay of lipase activity.

**Assay of enzyme activity.**—Lipase activity was determined using [1-<sup>14</sup>C] labeled triolein (0.1 μCi/tube). The basic assay mixture contained 30 μg unlabeled triacylglycerols, 0.3 % v/v Triton X-100, and 20 mM Tris-HCl; the reaction mixtures were emulsified by sonication using a Heat Systems-Ultrasonics, Inc. model: W220-F sonicator at 4 C for 1 min. Emulsions were always prepared immediately before use. The assay mixtures for lipase activity contained 130 μL substrate emulsion, 200 μL enzyme preparation and bidistilled water which made up to a final volume of 600 μL. They were incubated at 30 C with continuous stirring at different times. The incubations were stopped by immersing the tubes in a boiling water bath for 10 min. The products of hydrolysis and the remaining substrate were extracted with chloroform : methanol (2:1 v/v) by the method of Folch et al. (1957) and separated by thin-layer chromatography (TLC) on high-performance TLC Silicagel 60 plates (Merck, Darmstadt, Germany) with hexane/ether/acetic acid (80:20:1.5, by vol.). Unesterified fatty acids, diacylglycerols, monoacylglycerols and unhydrolyzed triacylglycerols were separated. Radioactivity found in different products of hydrolysis was detected on the chromatographic plates by scanning proportional counting with a Berthold LB 2723 Dünnschicht Scanner II apparatus (Germany) and quantified. Appropriate standards, run simultaneously, were visualized by exposure to iodine vapors. Assay mixtures without enzyme preparations were incubated and processed simultaneously as controls. The lipase activity was expressed as μmol triacylglycerols hydrolyzed per mg protein in 1 min. Protein content

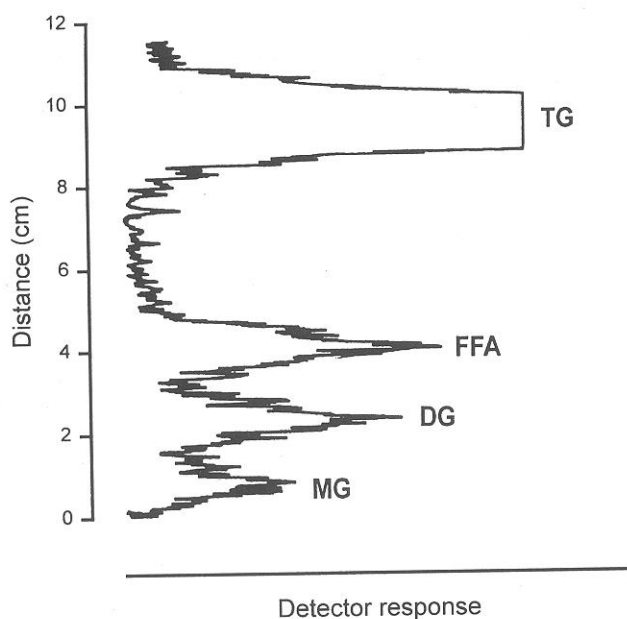


FIG. 1. Typical radioactivity distribution among different products of hydrolysis during the degradation of [ $1\text{-}^{14}\text{C}$ ] triolein by extracts from *G. versiforme* spores. Total lipids were separated by TLC and labeled lipids were detected on the plates by scanning counting. TG: Triacylglycerols, FFA: Free fatty acids, DG: diacylglycerols, MG: monoacylglycerols.

of lipase preparations was determined according to the method of Lowry et al. (1951).

#### RESULTS

Results of the radioactive assays using triolein  $^{14}\text{C}$  as substrate showed the release of labeled free fatty acids, diacylglycerols, and monoacylglycerols, demonstrating lipase activity (FIG. 1) in the total spore homogenate. In order to optimize the lipolytic reaction the following conditions were determined. The pH dependence of the lipase activity was observed over the range pH 7–8.5 in the incubation mixture and the optimal activity was found at pH 8. The protease inhibitors such as Aprotinin and PMSF did not show any effect on lipase activity. The emulsifier Triton X-100 was found to be better than sodium desoxycholate. The presence of  $\text{MgCl}_2$ ,  $\text{CaCl}_2$  and  $\text{NaHCO}_3$  enhanced activity. Triolein hydrolysis by the crude lipase preparations from *G. versiforme* spores increased gradually up to 15 h of incubation (FIG. 2); then it continued increasing but at a slower rate.

Measurement of the triolein enzymatic hydrolysis was also performed in subcellular fractions obtained by differential centrifugation. Lipase activity was more pronounced in the sediment at 13 000 g than in the other fractions when compared to that found in total spore homogenates (TABLE I); the specific

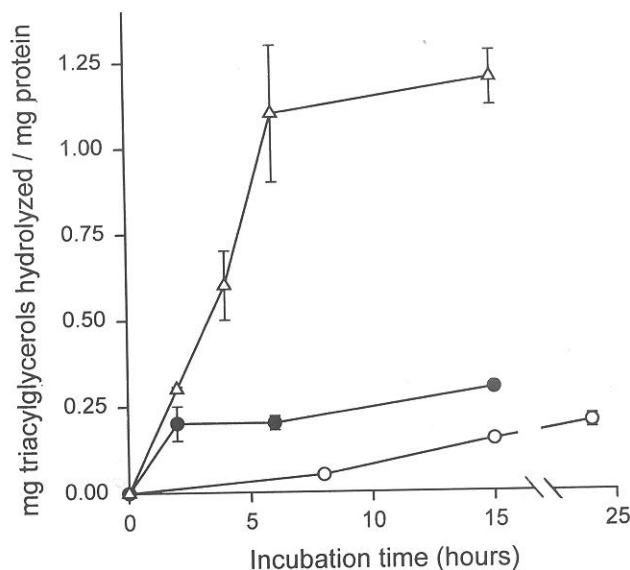


FIG. 2. Time-course of the triolein hydrolysis catalyzed by: O—O total homogenate from spores of *G. versiforme*, ●—● 13 000 g sediment for 30 min,  $\Delta$ — $\Delta$  acetone powder from total homogenate. Error bars indicate SD.

activity in this fraction was 12 times the homogenate values. Product formation proceeded at a linear rate up to 2-h incubation and, thereafter it increased slowly (FIG. 2).

Both cellular preparations, total homogenate and sediment at 13 000 g were used for further lipase purification. Lipolytic activity observed in acetone powder prepared from total homogenates were 20-fold and 2-fold greater than found in total homogenate and sediment at 13 000 g, respectively. Triacylglycerol hydrolysis rises sharply to the maximum at 6 h (FIG. 2) with no further increase. The subcellular fraction pelleted at 13 000 g was further treated with Triton -100 and the extract of soluble proteins was fractionated by gel permeation chromatography. The screening of lipase activity among different subfractions eluted from the column re-

TABLE I. Subcellular coarse localization of lipase activity in *G. versiforme* spores

Cell-fraction	Triacylglycerol hydrolyzed ( $\mu\text{g}/100$ spores) <sup>a</sup>
Total spore homogenate	11.2 $\pm$ 1.1
Sediment 5000 g 10 min	6.1 $\pm$ 0.4
Supernatant 5000 g 10 min	4.6 $\pm$ 0.2
Sediment 13 000 g 30 min	16.3 $\pm$ 0.7
Supernatant 13 000 g 30 min	1.5 $\pm$ 0.1

<sup>a</sup> Incubated at 30 C for 15 h. The values are the mean of three determinations  $\pm$  SD.

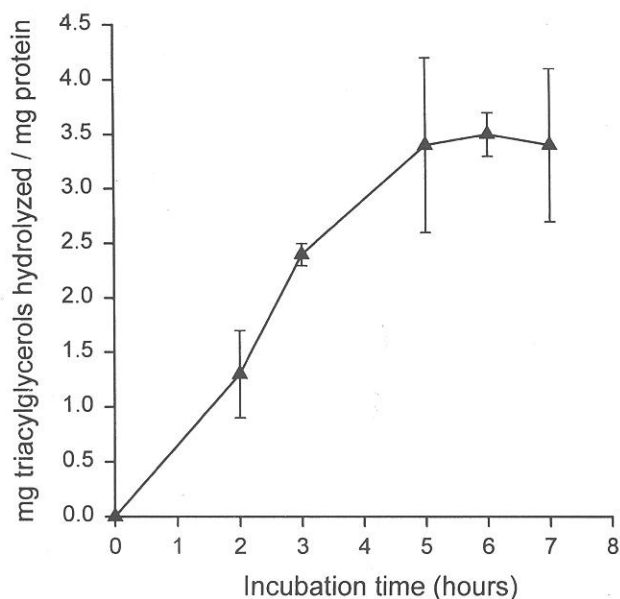


FIG. 3. Time-course of the triolein hydrolysis catalyzed by a lipolytic fraction obtained after gel permeation chromatography. Error bars indicate SD.

vealed a protein with an approximate molecular weight of 30 kDa as the sole active fraction. The time-course of the reaction is shown in FIG. 3. After 2-h incubation, triacylglycerol hydrolysis was 4- and 6-fold greater than found in the acetone powder and sediment at 13 000 g, respectively.

Values of specific activity and other parameters corresponding to different degrees of lipase purification, from total homogenate to chromatographic fraction, are summarized in TABLE II.

#### DISCUSSION

We have detected lipolytic activity in *in vitro* assays performed with *G. versiforme* spore homogenates at pH 7.5–8.5 similar to lipase activity of several species of filamentous fungi (Stöcklein et al. 1993). The presence of a detergent in the reaction medium was necessary to achieve better contact between the aqueous phase containing the enzyme and the lipid phase containing the substrate. Best results were obtained using Triton X-100 and ultrasonics. Different results were reported for lipases of *Candida rugosa*, *Geotrichum candidum* and *Rhizopus arrhizus* that had greater lipolytic activity when the substrate was emulsified in gum arabic, whereas the activity was less when Triton X-100 is used (Veeraragavan, 1990).

Though many fungi produce extracellular lipases that are easily separated from mycelia by means of either filtration or centrifugation of the culture medium (Iwai et al., 1984), other fungi contain membrane-bound lipases (Cooke and Whipps, 1993). This

TABLE II. Summary of different parameters in the purification of lipase from *G. versiforme* spores (means of three independent experiments)

Purification steps	Protein <sup>a</sup>	Specific activity <sup>b</sup>	Activity <sup>c</sup>	Purification factor <sup>d</sup>
Total spore homogenate	80.0	0.14	0.011	1.20
Sediment 13 000 g	46.7	1.66	0.077	14.23
Acetone powder	17.4	2.79	0.048	23.91
Chromatographic fraction	3.4	10.42	0.035	89.31

<sup>a</sup> µg/100 spores.

<sup>b</sup> µg triacylglycerols hydrolyzed/min × mg of protein.

<sup>c</sup> Activity recovered/100 spores.

<sup>d</sup> Referred to lipolytic action of whole cells determined in *in vivo* assays (Gaspar et al., 1994).

seems to be the case of the *G. versiforme* lipase activity on triolein. Among the different subcellular fractions assayed, a 13 000 g sediment showed the greatest lipase activity. The sedimentation characteristics of this fraction suggest that it may be rich in mitochondria and microbodies such as peroxisomes. These organelles have essential functions in the metabolism of n-alkanes and fatty acids in yeast cells (Tanaka and Ueda, 1993; Kunau et al., 1995), and they could play a similar role in triacylglycerols metabolism in AMF.

The partial purification of the lipase from the total homogenate from *G. versiforme* spores revealed increasing enzyme specific activities in the following order: homogenate < sediment at 13 000 g for 30 min < acetone powder < protein fraction obtained by gel permeation chromatography. The considerably greater amount of activity in the purified fractions compared to the total spore homogenate suggests that we are either activating latent activity, removing an interfering activity, or removing an inhibitor(s). This means that the purification factors could only indicate changes in measured specific activity.

Acetone powder has been a successful procedure for the purification of other microbial lipases (Shimada et al., 1993; Rapp, 1995). In our case, the acetone powder obtained from *G. versiforme* spores increases lipase specific activity 20 times compared to that of total homogenate. As other membrane-bound enzymes, *G. versiforme* lipase was well extracted with detergent; the resulting extract fractionated by HPLC on a gel permeation column yielded a lipolytic protein fraction with a molecular weight similar to the one found in other fungal lipases (Iwai et al., 1975; Macrae, 1983; Yamane, 1987). The marked increase in lipase activity determined in this fraction indicates a good extractability of the enzyme in the presence of detergent.

The activity of the sediment at 13 000 g or the

acetone powder from *G. versiforme* spores was similar to that of the extracellular lipases from *Penicillium cyclopium*, *Rhizopus arrhizus* and *Rhizopus delmar* (Benzonana et al., 1974; Iwai et al., 1984). Other fungal lipases such as extracellular lipases from *Aspergillus niger* and *Geotrichum candidum* had higher lipase activities. It is due to the fact that no significant esterification by these enzymes (inverse reaction) was observed during incubation (Okumura et al., 1981).

The presence of an active lipase in *G. versiforme* spores is consonant with Harley's (1989) suggestion of the importance of lipid accumulation in AMF spores. Triacylglycerol degradation in *G. versiforme* spores was corroborated in in vivo (Gaspar et al., 1994b) as well as in in vitro assays reported here. Such degradation suggests a functional role for these lipids in these fungi. Large amounts of triacylglycerols accumulated in *G. versiforme* spores provide energy-rich material for subsequent consumption during spore germination.

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