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Extracellular Triacylglycerol Lipases Secreted by New Isolate of Filamentous Fungus

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Abstract Two different types of lipases (lipase I and lipase II) secreted into culture medium by *Rhizopus* sp. L-1 were purified using a hydrophobic chromatography and were partially characterized. Both enzymes were monomeric as revealed by SDS-PAGE and gel filtration. The molecular masses of the enzymes were identified as 45 kDa (lipase I) and 69 kDa (lipase II). The isoelectric points were estimated to be 3.6 and 5.2 for lipase I and lipase II, respectively. pH and temperature activity optima for lipase I were as 7.5 and 50°C, respectively, whereas the corresponding parameters for lipase II were 6.0 and 45°C. The amino terminal sequences of lipase I and lipase II, determined by Edman degradation, were found to be Leu-Val-Met-Ile-Gln-Arg and Leu-Val-Met-Lys-Gln-Arg, respectively. By western blotting analysis, the two lipases were found to have a common antigenic determinant. Immuno-electron cytochemistry conducted with polyclonal anti-lipase I antibody indicated the enzyme located in both the periplasm and the adjacent vesicles of fungal hyphae. Fortunately, the sites on the cell envelope where lipase was exported into the culture medium was also identified.

Key words: Lipase, *Rhizopus*, hydrophobic chromatography, multiple forms of enzyme, immuno-electron cytochemistry

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyze the hydrolysis of ester bonds in triacylglycerol lipids, releasing the constituent diacylglycerols, monoacylglycerols, free fatty acids, and glycerol moieties. One of the characteristics of the lipases is its unique ability to catalyze reactions in the hydrophobic phase of emulsified materials via the water/organic phase interface [10], rendering such systems difficulty to study [14]. This class of enzymes can also catalyze reactions in organic solvents [4]. Since lipases hydrolyze the glycerol esters of long chain fatty acids (water insoluble), they differ from esterases (EC 3.1.1.1) which hydrolyze the water soluble esters of short chain fatty acids.

Lipases are widely distributed and exhibit great diversity in living organisms [23]. Many lipases from microorganisms have been purified and their properties were carefully investigated. The biotechnology industry has a great interest in exploring new lipase-producing microorganisms, which have many potential uses in different industrial areas [6, 15, 24].

A great number of studies have been performed on the subject of isolation and purification of lipases from various filamentous fungi. These enzymes significantly differ from each other in their physico-chemical and catalytic properties. As a rule, a single strain of fungi secretes two or more forms of lipase having different M_r values and other characteristics [8, 9, 12, 17].

The genetic and biochemical properties of fungal lipases have been intensively investigated. The three-dimensional structure and details of the molecular architecture of lipases from several different fungi have been analyzed [5, 11] as well. These investigations are crucial because they provide insights into the nature of the catalytic machinery. However, the elucidation of the mechanisms of lipase secretion is much delayed and, even today, the mechanism of how large protein molecules such as lipases pass through the fungal cell wall and eventually go into the environment is not clear.

In this paper, we report the results on purification and characterization of the extracellular lipases which were produced by the newly isolated filamentous fungus *Rhizopus* sp. L-1 and on their localization during the secretion process. The lipase-containing structures that apparently cross the cell wall are also presented.

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MATERIALS AND METHODS

Microorganism

The filamentous fungus strain exhibiting lipolytic activity was isolated from the soil in a hot spring in the area of Ashkhabad city (Turkmenistan). The fungus was partly identified as *Rhizopus sp.* (strain L1), and the strain was maintained on wort-agar slants and stored at 4°C.

Media and Culture Conditions

Rhizopus sp. L-1 was batch cultured as follows: Erlenmeyer flasks were filled up to 10% of their volume with a medium of the following composition (%): yeast autolysate - 0.7; $(NH_4)_2SO_4$ - 0.3; $CaCl_2$ - 0.1. Conidia inoculum was pregrown on this liquid medium with 0.5% glucose as the carbon source. The cultivation was carried out at 41°C for 18-20 h with shaking (200 rpm). To investigate lipase secretion, mycelia were inoculated to the above media containing 1% of olive oil instead of glucose. Incubations were carried out under the same conditions as above for 30-40 h.

Enzyme Production and Batch Cultures

Time course of fungal growth and lipase production rates were investigated as follows: *Rhizopus sp.* L-1 was cultivated under batch conditions for 40 h. Samples were taken every 2 h for analysis. Supernatants from the batch culture suspensions (triplicates) were separated from the residual fungal biomass by vacuum filtration through a Buchner funnel with a membrane filter. Pellets were washed with 50 ml of distilled water and dried to a constant weight (80°C dry-heat-vacuum oven). The lipase activities in both culture filtrates and ground cell preparations were obtained. For detection of the latter, the mycelium was harvested on a membrane filter, washed with a 0.05 M Tris-HCl buffer (pH 7.5) and then disintegrated in a French pressure cell. The fluid obtained was used without further treatment for assaying the lipase activity.

Lipase Activity Assay and Protein Determination

Lipase activity was measured by titrating free fatty acids which were released by hydrolysis of triacylglycerides using a modified method [9]. A lipid emulsion was prepared by emulsifying 100 ml of olive oil in a 150 ml of 2% (w/v) polyvinyl alcohol solution in water for 10 min at 10,000 rpm in a Waring blender. The emulsion was then stored at 4°C for 1 h. The reaction mixture containing 2.5 ml of olive oil emulsion, 6.5 ml of 0.02 M phosphate-citrate buffer, pH 6.0 or 8.0, and 1 ml of the enzyme solution, was incubated at 40°C for 60 min by shaking. Enzyme action was terminated by the addition of 30 ml of acetone-ethanol (1:1 v/v). Fatty acid release was quantified by titration at pH 10.2 using 0.05 N NaOH. One lipase unit (U) is defined as the amount of enzyme which liberates 1 μmole of free fatty acids/min under the experimental conditions. Protein concentrations were determined by the Bradford method [2].

Preparation of Hydrophobic Matrix

The hydrophobic matrix was prepared as follows: 3.65 ml of toluene 2,4-diisocyanate was added to a suspension of 15 g of dry microcrystalline cellulose (MCC) in 150 ml of 1,4-dioxane, and the mixture was slowly stirred at 20°C for 6 h. Then, the MCC was removed by filtration, rinsed with dioxane and *n*-butanol on the filter, resuspended in n-butanol and again stirred slowly for 5 h at 95-100°C. The matrix was rinsed successively with 250 ml of *n*-butanol, 300 ml of acetone, and 500 ml of 10 mM phosphate buffer (pH 8.0).

Purification of Extracellular Lipase

All purification steps were carried out at 4°C. After removal of the mycelium of *Rhizopus* sp. L1, the lipase containing culture supernatant was precipitated with four volumes of cold (-6°C) isopropyl alcohol, and the precipitate was dissolved in 10 mM phosphate buffer (pH 8.0) to a protein concentration of 30 mg/ml. The protein solution (50 ml) was applied to a column (15×350 mm) packed with 10 g of hydrophobic matrix (modified cellulose) and equilibrated with 10 mM of phosphate buffer (pH 8.0). During the hydrophobic chromatography, other proteins were separated by elution with 10 mM phosphate buffer (pH 8.0). The lipases were then eluted with a 0-20% (v/v) 1,4-dioxane gradient. The flow rate used was 25 ml/h. Fractions containing lipase activity were combined, concentrated in an Amicon cell (UM-10 ultrafilter, Millipore, Bedford, U.S.A.) and used for the next purification step. Each fraction (8 ml) was applied to a Sephadex G-100 column equilibrated with 10 mM of phosphate buffer (pH 8.0) containing 0.1 M NaCl. FPLC gel filtration was performed at a flow rate of 30 ml/h and fractions of 4 ml were pooled.

Estimation of Molecular Masses

The molecular masses of the purified lipases were determined by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) [12] and gel filtration, and protein bands after electrophoresis were visualized by Coomassie Blue and silver staining [7]. In order to estimate molecular masses under non-denaturing conditions, the Sephadex G-100 gel filtration column was calibrated with standards of ribonuclease (13 kDa), chymotrypsin (22.5 kDa), pepsin (35.5 kDa), peroxidase (40 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 kDa).

N-terminal Sequencing

The purified lipase preparations were prepared for analysis in a Perkin Elmer Applied Biosystems Model 140C PTH

amino acid analyzer (Nowark, U.S.A) according to the manufacturer's instructions. The proteins were then subjected to Edman degradation as reported by Tarr [25].

Isoelectric Focusing

Experiments were performed according to the recommendations of the manufacturer (Pharmacia, Piscataway, U.S.A). The separation was carried out on a LKB-8100 (Bromma, Sweden) isoelectric focusing apparatus with 1% ampholine solution (pH 3.0–10.0) and sucrose gradient. One-tenth N H_3PO_4 and 0.1 N NaOH were used as anode and cathode solutions. Isoelectricfocusing was performed at 400 V and 4°C for 48 h. Two ml fractions were collected and the pH was measured at 4°C.

Raising and Purification of Anti-Lipase Antibodies

Two rabbits were immunized by intramuscular injection with purified lipase I and lipase II preparations dissolved in 0.85% (w/v) NaCl plus an equal volume of Freund's complete adjuvant. Each rabbit was given another three injections subcutaneously over 2 week period, using Freund's incomplete adjuvant. Blood tests were initiated 15 days after the last injection by performing double diffusion on an agar gel. The crude antisera were successively subjected to double fractionation with ammonium sulphate, dialyzed against 10 mM phosphate buffer (pH 7.5) and separated by anion-exchange chromatography on a column with DE-52 cellulose (Whatman, England). The IgG-containing fractions were combined and concentrated on an UM-10 ultrafilter.

Western Blotting

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes (Amersham, Arlington Height, U.S.A) by a Semi-dry blotter (Pharmacia, Piscataway, U.S.A) in a transfer buffer (Tris-glycin-methanol) for 2 h, according to the manufacturer's instructions. Blocking of unspecific binding was performed with Tris-saline-tween 80 buffer (TBST) containing 1% BSA for 1 h or overnight at 4°C. Nitrocellulose filters were then incubated successively with TBST-diluted rabbit anti-lipase primary antibody, anti-rabbit (swine) peroxidase-conjugated secondary antibodies, and stained with diaminobenzidine hydrochloride-hydrogen peroxide for peroxidase until a suitable brown color was obtained without an excessive background.

Immuno-Electron Cytochemistry

The mycelium of *Rhizopus* sp. L-1 was grown in an olive oil containing liquid medium for 20 h. It was then immobilized in Ca-alginate gel and incubated in the same medium for an additional 20 h. Granules of the immobilized fungus were fixed in a 50 mM sodium cacodylate buffer (pH 7.2) containing 2.5% formaldehyde and 0.5% glutaraldehyde at 4°C for 2 h. The samples were then dehydrated in an ethanol series. Embedding was performed in Lowicryl K4M of the low-temperature resin [3]. Ultrathin sections were cut with a diamond knife, and mounted on formvar film-covered nickle grids. Immunocytochemical labeling were performed using a protein A-gold technique [18]. The sections were then stained with 1% uranyl acetate and lead citrate.

RESULTS

Examination of Lipase Activities

Growth rates of *Rhizopus* sp. L-1 on olive oil during batch cultivation and extracellular and cell-bound lipase production are presented in Fig. 1. Lipase activity in the supernatant of the culture medium and in the ground cell preparations showed that the maximum intracellular activity appeared at 16 h of growth, but maximal extracellular activity was detected in the early stationary phase (20 h). Nevertheless, the proportion of the cell-bound activity during the early phase of growth was higher than that observed extracellularly, and the latter represented 75% of the total activity during the stationary phase of growth.

Extracellular Lipase Purification

Lipase activities measured during various steps of purification for *Rhizopus* sp. L1 culture filtrates are shown in Table 1. After hydrophobic chromatography two separate groups of active fractions were observed, and they were designated as lipase I and lipase II (Fig. 2). The specific activities of these fractions were found to be much higher than that of the enzyme extract obtained by the isopropyl alcohol precipitation. Further fractionations of the active lipase I and lipase II preparations were performed using a Sephadex G-100 gel filtration, which enhanced about 1.5-fold of the specific activity for both lipases. The purification was monitored by SDS-PAGE.

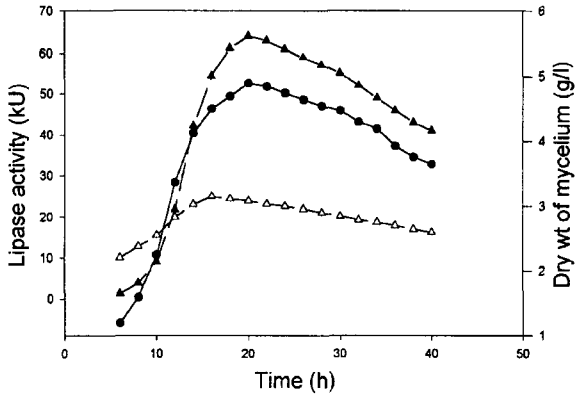

Fig. 1. Growth rates and lipase activity of *Rhizopus* sp. L1. ●, dry weight of mycelium; ▲, extracellular lipase activity; △, cell-bound activity.

Table 1. Purification profile of extracellular lipases produced by *Rhizopus* sp. L1.

Purification steps	Total protein (mg)	Total activity (kU)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Culture supernatant*	3712	63	17	1	100
Isopropyl alcohol precipitate	437	58	133	8	92
Hydrophobic chromatography					
Lipase I	27	30	1125	66	48
Lipase II	19	28	1478	87	44
Sephadex G-100 gel filtration					
Lipase I	14	28	1992	117	43
Lipase II	9	19	2133	125	30

*Was taken after 20 h of batch cultivation.

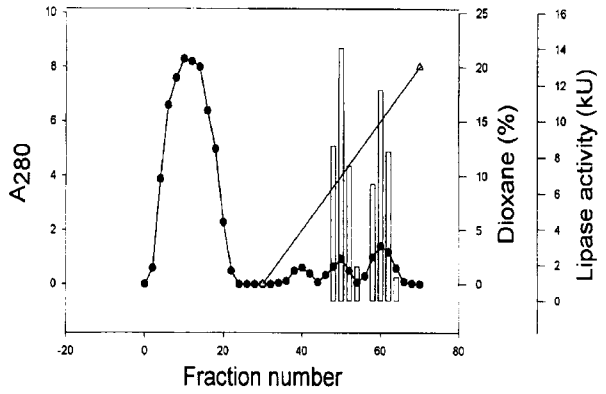

Fig. 2. Purification using hydrophobic chromatography of *Rhizopus* sp. L1 extracellular lipase. Modified cellulose was used as a matrix.

○, protein content was measured by its absorbence at 280 nm; □, lipase activity; ∧, dioxane gradient.

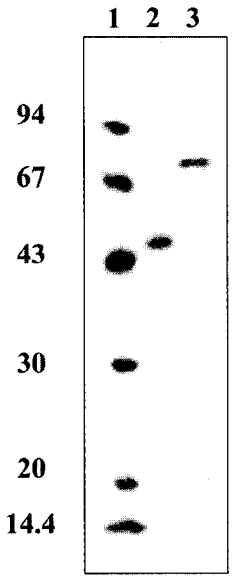

Fig. 3. SDS-PAGE (10% polyacrylamide) of the purified lipase preparations.

Lane 1, molecular weight standards: phosphorylase b (97.0 kDa), bovine serum albumin (66 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.0 kDa), and α-lactalbumin (14.2 kDa); lane 2, lipase I; lane 3, lipase II.

The electrophoretic pattern obtained revealed a single band of each lipase prepared (Fig. 3).

Characterization of Purified Lipases

Figure 3 indicated the SDS-PAGE gave protein bands of 45 kDa and 69 kDa for lipase I and lipase II, respectively, based on the M_r of standard proteins. Also, the samples obtained from Sephadex G-100 gel filtration column, under non-denaturing conditions, showed the presence of two single bands (data not shown). The different values of M_r for lipase I and lipase II were confirmed by both gel filtration in the native form and SDS-PAGE analysis. Lipase I and lipase II showed a single peak of enzyme activity after isoelectric focusing. The N-terminal amino acid sequence for lipase I was Leu-Val-Met-Ile-Gln-Arg and, for lipase II, Leu-Val-Met-Lys-Gln-Arg, thus showing that lipase II had the same first three N-terminal residues as lipase I. However, the forth amino acid was different in that Lys was for lipase II but Ile was for lipase I. The isoelectric points were determined to be 3.6 for lipase I and 5.2 for lipase II. The effect of temperature was measured at various temperatures ranging from 20 to 80°C. The optimal temperatures for enzyme activities were 50°C for lipase I and 45°C for lipase II. pH optima for lipase activities were determined in acetate, glycolic, phosphate, borate, veronal and phosphate-citrate buffers (0.1 M) in the pH range of 3.0–11.0 at a pre-determined optimum temperature of each lipase, and were found to be 7.5 for lipase I and 6.0 for lipase II.

Relative Contents of Lipases I and II in Culture Liquid of *Rhizopus* sp. L1

Lipases in culture supernatants of *Rhizopus* sp. L1, sampled at different stages of the batch cycle, were purified using hydrophobic chromatography (Table 2). The relative amounts of the two lipases dramatically changed during the course of fungal growth. Lipase II was the major form that comprised of 71% in the early exponential phase, but then its relative content gradually decreased to as low as 19% during the late stationary phase within 40 h. Lipase I, on the other hand, was shown as a minor

component during the early stages and a major component during the late stationary phase.

Immuno-Blotting Analysis

Polyclonal antibodies were raised against isolated lipase I and lipase II. The object of this study was to investigate the possibility of the enzymes having common antigenic determinants. Figure 4 shows anti-lipase I antibody that recognizes both lipase I and lipase II in a culture liquid obtained from a periodically-grown *Rhizopus* sp. L1. Anti-lipase II antibody also cross-reacted with both enzymes and, interestingly enough, anti-lipase I antibody appeared to cross-react more strongly with lipase I compared to lipase II.

Immuno-Electron Cytochemistry

During the process of secretion and extracellular export, ultrastructural localization of lipase in the fungal hyphae was examined using a polyclonal anti-lipase I antibody. Electron microscopy of ultrathin sections of *Rhizopus* sp. L1 hyphae, batch-cultivated in a liquid medium with olive oil for 20 h and subsequently immobilized in Ca-alginate gel, revealed the presence of lipase bound mostly to the cell envelope area. Lipases concentrated primarily in the periplasm and in vesicles of 0.3 - 1.0 μm diameter were located near the plasmalemma (Figs. 5a, 5b, 5c). In some sections, these vesicles appear to be fused with the periplasmic space (Fig. 5c). Intensive labeling of the growing tip of the hyphae was observed on lateral sections (Fig. 5d). Some sections appeared to show not only that fungal hyphae had structures to pierce through the cell wall, but also that they accumulated a high level of immuno-gold anti-lipase label (Figs. 5e, 5f).

Table 2. A comparison of the relative contents of the two lipases in the culture filtrate of *Rhizopus* sp. L-1 during the course of batch cultivation.

Time of growth (h)	Total activity[a] (kU)	Relative content (%)	
		Lipase I	Lipase II
15	44.2	29	71
20	58.5	45	55
24	53.5	52	48
30	51.5	63	37
40	38.7	81	19

[a]Total activity was calculated from the pooled lipase peaks of both active fractions eluted from the hydrophobic chromatography column.

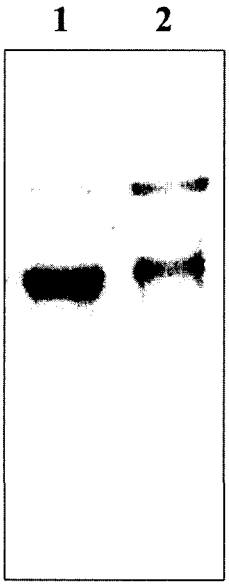

Fig. 4. Western blotting of culture filtrate obtained from the fermentation of *Rhizopus* sp. L1 (20 h batch cultivation).
Anti-lipase I (lane 1) and anti-lipase II (lane 2) antibodies, respectively. SDS-PAGE was performed on 10% polyacrylamide gel.

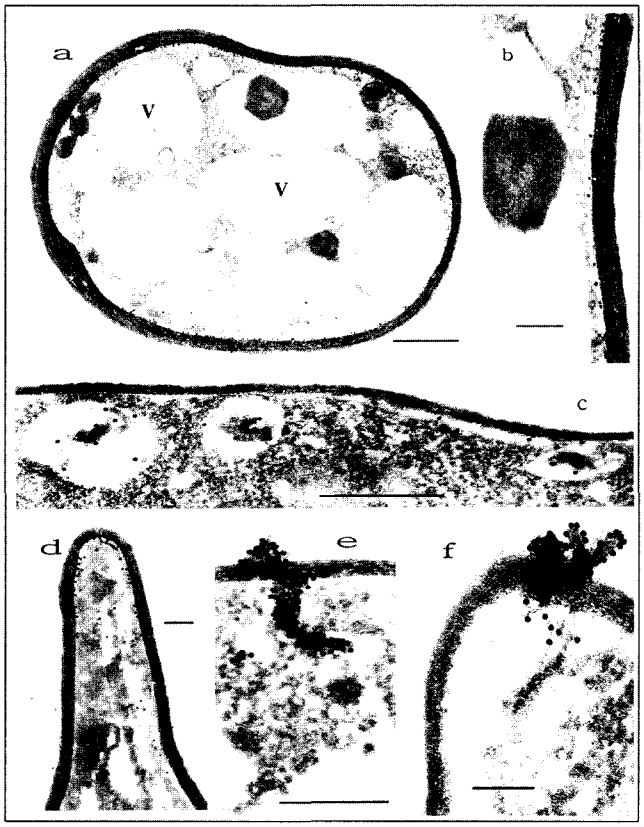

Fig. 5. Transmission electron micrographs of the olive-oil grown mycelium of *Rhizopus* sp. L1.
All the samples were immunocytochemically labeled with specific polyclonal anti-(lipase I) antibody. (a) Survey of transverse section of fungal hyphae. Note the uniform distribution of gold label in periplasm. Bar = 1 μm. (b) Enlarged fragment of the same section. Bar = 0.2 μm. (c) Lateral section of fungal cell showing the secretion vesicles near the plasma membrane, which contain immuno-gold label. Bar = 0.5 μm. (d) Intensive gold labeling occurring within the growing tip of the hyphae. (e and f) Some sections, such as these, featured the structures in the cell envelope, which exhibit intensive immuno-gold anti-lipase labeling. Bar (d - f) represents 0.2 μm. Abbreviations: V, vacuole; M, mitochondrion.

DISCUSSION

The extracellular lipolytic enzymes derived from the culture filtrate of the newly isolated filamentous fungus were successfully purified. The strain seemed to belong to the *Rhizopus* genus and was designated as *Rhizopus* sp. L1. The enzyme preparation was separated into two molecular forms. Two distinct lipases, one with a lower molecular mass (lipase I) and the other with a higher molecular mass (lipase II), were purified by hydrophobic chromatography and gel filtration. Based on SDS-PAGE, gel filtration, and isoelectric focusing studies, both enzymes were found to be homogeneous and composed of a single polypeptide chain.

Most of the studies on extracellular fungal lipases have been performed on different strains of *Geotrichum candidum*, which produced two different types of extracellular lipases. The two forms of the enzyme were shown to be monomeric and differed slightly in their molecular masses, pI and amino acids composition [21, 22, 26]. These two lipases were suggested to be produced by two distinct genes. cDNA clones of lipases I and II were isolated by colony hybridization and the nucleotide sequences were determined [19, 20]. The nucleotide and amino acid sequences of the two forms of lipases (I and II) were very similar except for having different N-termini [22]. However, it was revealed that the two forms of lipases from *Rhizomucor miehei* are different by only one degree of glycosylation [1]. Similar results were obtained in regard to the lipase that was derived from *Penicillium camamberti* [9].

We have demonstrated that the two extracellular lipases secreted by *Rhizopus* sp. L1 have much more profound differences compared to the respective enzymes in *G. candidum, Rh. miehei,* and *P. camamberti.* The heterogeneity of the two forms of the enzymes were based on both charge properties and molecular composition. This was possibly caused by a multiplicity of genes encoding lipase I and lipase II or by post-translational modifications, strain diversity, or a combination of these influences. However, cross-immunoreactivity of the two lipases indicated a partial immunological identity of the two types of the enzymes, i.e., some of their epitopes were shared.

Immuno-electron cytochemical studies provide us with an extraordinary ability to elucidate details of protein secretion and export processes. In the case of filamentous fungi, a growing apical part of the hyphae is known as the major zone in which proteins are exported [27]. In this study, we confirmed the fact that the preferential protein secretion area is recognized as the growing hyphal tip that was revealed by immunolabeling of lipase in the *Rhizopus* sp. L1 strain. Immuno-electron microscopy also showed that the hyphae had immuno-gold anti-lipase labeled peripheral vesicles, which were closely associated with the periplasma and could possibly be the secretory vesicles.

A fungi can excrete through their cell wall protein molecules that are much larger than 20 kDa of free penetration limit [16]. Taking this into account, we initially suggested that the existence of specialized sites in a cell wall of fungi with an unusual permeability for high molecular mass proteins through which there is a regulated exchange of macromolecules between the intracellular and extracellular media was regulated. In the present study, the structures filled with anti-lipase label and located in the cell envelope area were demonstrated in the hyphae of *Rhizopus* sp. L1. These structures are probably the specialized sites for lipase export into the culture liquid.

Acknowledgments

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