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CHARACTERISTICS OF THE MOLECULAR FORMS OF LIPASES SYNTHESIZD BY THE FUNGUS *Rhizopus microsporus*

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The changes in the total extra- and intracellular activities of the lipase and its individual forms during the growth of the fungus Rhizopus microsporus *(white strain) have been studied. The fungus synthesizes five forms of intracellular lipases, three of which are secreted by intact cells into the surrounding medium. The forms of the lipases differ in the charge of the molecules, their molecular masses, the action of inhibitors, and the kinetic parameters of the triolein-cleaving reaction. A similarity of the physicochemical and enzymatic properties of corresponding pairs of extra- and intracellular lipases has been revealed.*

Characteristic for the lipases of microorganisms is their existence as numerous molecular forms [1-5]. We have shown previously that, as a result of repeated subculturing, and also on prolonged storage, the fungus *Rhizopus microsporus*, UzLT-1, splits into two variants (white and gray) differing in a number of characteristics and, particularly, in their capacity for synthesizing extracellular lipases. The productivity of the white variant of the fungus is 1.3-1.5 times greater than that of the initial culture and 1.7-2.0 times greater than that of the gray variant; consequently, in our experiments to elucidate the functional role of the individual forms of the lipases we used the white variant.

We have also reported a considerable activation of the lipases of the fungus *R. microsporus*, UzLM-1, during the purification of these enzymes. The presence of a lipase inhibitor in the culture liquid was also noted [4]. These results impelled us to carry out a purification of the lipases at all stages of the growth of the fungus in order to evaluate the level of change in the total lipase activity and the activities of the individual forms of the enzyme.

The ion-exchange chromatography on DEAE-Sephadex A-50 of the complex preparations of extra- and intracelllar lipases obtained on cultivation of the fungus for 12, 18, and 24 h led to an increase in lipase activity. In the dynamics of growth, a preferential increase in the extracellular over the intracellular activity was observed, particularly on the cultivation of the fungus for 24 h. It is interesting that the degree of activation of a lipase on purification depended on the age of the culture. The greatest activation — a sevenfold increase in the intracellular (Fig. 1, a) and a twofold increase in the extracellular lipase activity (Fig. 1a and b, respectively) — was detected in a 24-hour culture.

The results on the increase in the activity of the lipases on purification have not yet permitted definite conclusions to be drawn concerning the mechanism of inhibition. The nature of the inhibitors also remains unclear. However, it may be assumed that the inhibitors are compounds of high molecular mass, since they were not eliminated on dialysis or on ultrafiltration through a IM-10 membrane filter, which retains polymers with molecular masses greater than 10,000. Regardless of how the lipase activating effect that we observed may be interpreted, in any case lipase inhibitors must be eliminated in the quantitative evaluation of the level of lipase activity.

To determine the qualitative and quantitative changes in the fungal lipases, we obtained electrophoretically homogeneous preparations of all the forms of extra- and intracellular lipases produced by 12-, 18-, and 24-hour cultures. Purification was achieved by a scheme developed previously [3].

Up to the stationary phase of development of the fungus, the lipase activity was due to the presence of five intracellular (L_1—L_5) and two extracellular (L_{1e} and L_{3e}) lipases, and then (24 h) one more protein fraction possessing lipase activity (L_{5e}) appeared.

A comparison of the corresponding individual forms of the lipases isolated in various stages of the growth of the fungus showed that they had the same properties: molecular form, isoelectric point, etc., which gave grounds for assuming the absence of major modifications of the enzyme in the proces of growth, including the secretion period. Of the lipases detected in the

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TABLE 1. Characteristics of the Different Forms of Lipase from the Fungus *R. microsporus* (white variant)*

Index	Form							
	L_1	L_{1c}	L_2	L_3	L_{3e}	L_4	L_5	L_{5e}
Molecular mass, kDa	**28**	**28**	**39**	**43**	**43**	**64**	**67**	**67**
Isoelectric point	**4,25**	**4,35**	**4,0**	**4,35**	**3,55**	—	**4,40**	**4,60**
Temperature optimum, °C	**36**	**36**	**37**	**37**	**37**	**35**	**39**	**39**
pH optimum (substrate triolein)	**8,2**	**8,2**	**4,8**	**7,4**	**7,4**	**6,0**	**7,6**	**7,6**
Concentration required for elution from DEAE-Sephadex A-50	**0**	**0**	**0,04** **0,20**	**0,35**	**0,40**	**0,50** **0,60**	**1,0**	
Specific activity, units $\times 10^{-3}$	**24**	**30**	**21**	**28**	**45**	**15**	**18**	**27**

*The properties of the individual forms of the lipases isolated from 12-, 18-, and 24-hour cultures of the fungus were identical. The results given in the table were obtained in experiments with a 24-hour culture.

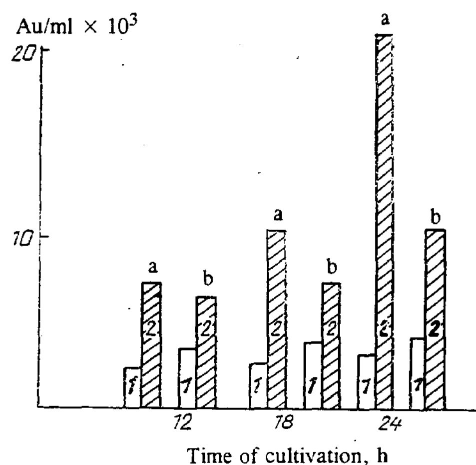

Fig. 1. Change in lipase activity during the growth of the fungus *Rhizopus microsporus*. Total activities of the intracellular (a) and the extracellular (b) lipases: 1) activity in the initial samples; 2) activity after chromatography on DEAE-Sephadex A-50.

cells, L_{1e}, L_{3e}, and L_{5e} were transported into the medium. A similarity of the properties of L_1 and L_{1e}, of L_3 and L_{3e}, and of L_5 and L_{5e} permitted the assumption that the regulation of the secretion of these forms takes place at the stage of the issuance of the enzymes from the cell, which was confirmed by the lower rate of accumulation of of the extracellular forms of the enzymes in comparison with the corresponding intracellular forms. The other two forms of lipases (L_2 and L_4) are true intracellular types and were not secreted into the surrounding medium by intact cells.

The main physicochemical properties of corresponding pairs of extra- and intracellular lipases (L_1 and L_{1e}; L_3 and L_{3e}) were identical, but some differences were observed in their catalytic properties. Lipases L_1 and L_{1e} differed in the degrees of their inhibitions by Tweens. The lipase forms L_3 and L_{3e} differed in their rates of cleavage of triolein, which was 1.6 times

higher for the extracellular enzyme. The specific activity (for the substrate triolein) of the extracellular form 5 (L_{5e}) was likewise 1.5-1.6 times higher than for the corresponding intracellular form. It is not excluded that on leaving the cell the lipase molecules (L_{1e}, L_{3e}, and L_{5e}) undergo a slight modification, like other enzymes secreted by fungi, which leads to a change in their catalytic properties.

Regardless of the stage of growth of the fungus, the forms L_3 and L_{3e} were the main ones, and the two others were minor forms. A comparison of the physicochemical properties of the different forms showed that they differed not only by the charge on the molecule but also by their catalytic properties: they behaved differently under the action of bile acid salts and of metal ions, and also differed in specific activity (Table 1). Thus, the main contribution to the splitting of triolein is made by lipases L_3 and L_{3e}, and the functions of the other two forms are possibly connected with the transesterification and synthesis of new triglycerides in the cell of the microorganism. It will be possible to make a decision on the functions of the two truly intracellular forms of the lipases (L_2 and L_4) after additional experiments have been performed, especially a study of the substrate specificities of these forms of the enzyme.

The question of the genesis of the molecules of the extra- and intracellular lipases still remans unclear.

The molecular masses of the purified lipases practically confirmed facts described previously [3, 5]. In addition, the molecular masses coincided (see Table 1). On the basis of the results obtained in the determination of the molecular masses of the native proteins (gel filtration through Sephadex G-100) and the denatured proteins (electrophoresis in PAAG in the presence of NaDS) it may be assumed that the lipases of the fungus *R. microsporus* are not subunit proteins and apparently consist of a single polypeptide chain. An investigation of the interrelationship of the individual forms of the lipases synthesized by the fungus *R. microsporus* (white variant) is the object of our further investigations.

EXPERIMENTAL

The source of lipases was the fungus *Rhizopus microsporus* (white variant). The conditions for the growth of the fungus and for obtaining a complex lipase preparation were identical with those described previously [2].

Lipase activity was determined by the method of [3]. As the unit of lipase activity we took that amount of enzyme which splits out 1 μmole of fatty acid from a 40% emulsion of triolein in a 2% solution of poly(vinyl alcohol) in 1 h at 37°C

The extracellular lipases were isolated from a filtrate of the fungal culture liquid.

The intracellular lipases were isolated after the successive washing of the mycelium with distilled water and 0.2 M NaCl solution, disruption of the mycelium in a mortar with glass beads, extraction of the proteins with 0.1 M phosphate buffer, pH 7.4, containing 5×10^{-3} M 2-mercaptoethanol, and 0.2 M NaCl, and centrifugation at 6000 rpm for 20 min.

The purification of the extra- and intracellular lipases was carried out by a scheme developed previously [3, 5].

The homogeneity of the preparations was determined with the aid of electrophoresis in 7.5% polyacrylamide gel in Tris-glycine buffer, pH 8.6, in the presence of NaDS [6].

The molecular masses of the lipases were determined by gel filtration through Sephadex G-100 using Serva (FRG) proteins as standards: aldolase (M — 147 kDa); phosphatase (100 kDa); albumin (67 kDa); DNase(31 kDa); and myoglobin (17 kDa); and by electrophoresis in the presence of NaDS [6].

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