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ISOLATION, PURIFICATION, AND CHARACTERIZATION OF TWO FORMS OF LIPASE FROM THE FUNGUS *Mucor miehei*

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Two forms of lipases (A and B) have been isolated from the fungus Mucor miehei *UzLT-3 and purified to the homogeneous state, and molecular masses of 43 and 40 kDa have been established for them from the results of disk electrophoresis and gel filtration. The isoelectric points of the lipases are 4.7 for form A and 4.9 for form B.*

At the present time, high-purity lipase preparations are being used for the stereospecific analysis of membrane structure, for enzyme-linked immunoassay, for the creation of biological microinstruments with enzymatic amplification, and for the preparative synthesis of lipids with predetermined structures, and also in medicine [1]. However, the production of high-purity lipases presents some difficulty in view of the fact that microbial lipases are characterized by a multiplicity of forms [2-5]. For example, characteristic for the fungus *Rhizopus microsporus* is the presence of five forms of lipase, three of which are secreted into the medium [6]. All five forms of lipase differ in molecular mass, electrophoretic mobility, and catalytic properties [6, 7]. The lipases from *Mucor* fungi are also represented by various forms [8].

The aim of the present work was to develop a method for isolating and purifying the molecular forms of lipases from the fungus *Mucor miehei* UzLT-3.

In the first stage of purification, the proteins were precipitated from the culture liquid of the fungus *Mucor miehei* with ammonium sulfate (0-70%) at 4°C, followed by separation of the precipitate so formed by centrifugation at 3400 *g* for 30 min. At this stage the specific activity of the protein had risen 2.5-fold. After dialysis, a solution of the enzyme was deposited on a column (1.4 × 35 cm) of DEAE-TSK-650 M equilibrated with 0.05 M phosphate buffer, pH 6.0. Proteins were eluted with the initial buffer and then with a linear gradient of NaCl (0-0.6 M) at the rate of 60 ml/h. Fractions with a volume of 4 ml were collected. Protein with lipase activity was eluted as a single peak. Here the specific activity of the enzyme had increased 12-fold in comparison with the initial activity (Fig. 1). Further purification of the lipase was achieved by gel filtration on TSK-55 HW. It must be mentioned that on gel filtration there was a separation of proteins with lipase activity (Fig. 2). At this stage of purification, the overall yield in terms of protein was 3.9%, of which 2.4% was due to the first peak and 1.5% to the proteins of the second peak. The last stage of purification was a second gel filtration of the active fractions, on Sephadex G-100 (Fig. 3). As a result, a 60-fold purification of the first fraction (which has been called form A) and a 30-fold purification of the second fraction (form B) were achieved. The results on the purification of the extracellular lipases are given in Table 1.

The homogeneity of the individual forms was established by gel filtration and electrophoresis. The molecular masses of the individual forms were determined by disk electrophoresis in the presence of Na-DDS and 2-mercaptoethanol and by gel filtration. The disk-electrophoretic method gave molecular masses of 40 and 43 kDa, respectively, for the A and B forms. Gel filtration on Sephadex G-100 using proteins with known molecular masses confirmed these results.

The isoelectric points of the two forms of lipases, determined by isoelectric focusing, were 4.7 and 4.9, respectively, for the lipase forms A and B.

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TABLE 1. Purification of the Extracellular Lipases of *Mucor miehei* UzLT-3

Stages of purification	Total amount of protein, mg	Lipase activity		Yield, %		Degree of purification
		specific, U/mg of protein	total, units	as protein	as activity	
Filtrate of the culture liquid	5000	800	4000000	100	100	1
Precipitation by ammonium sulfate	1620	2050	3321000	32.4	83	2.5
DEAE-TSK 650 M	325	9600	3120000	6.5	78	12
TSK-55 HW 1(A)	120	16000	1920000	2.4	48	20
2(B)	76.6	12000	920000	1.5	23	15
G-100 1(A)	35	48000	1680000	0.7	42	60
2(B)	30	24000	720000	0.6	18	30

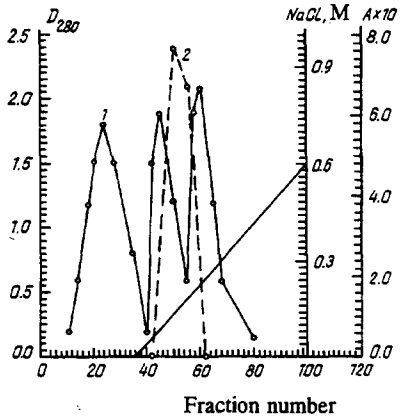

Fig. 1. Ion-exchange chromatography of the lipase fraction on DEAE—TSK 650 M (column 1.4 × 35 cm, elution by 0.05 M phosphate buffer, pH 6.0 with a NaCl gradient from 0 to 0.6 M); here and in Figs. 2 and 3: *1*) optical density of the protein at 280 nm; *2*) lipase activity, U/ml.

EXPERIMENTAL

As the source of the enzyme we used the fungus *Mucor miehei*, strain UzLT-3, which was isolated by workers in the laboratory of microorganism enzymes.

The enzyme was isolated from the culture liquid obtained in the deep cultivation of the fungus *Mucor miehei* UzLT-3 on a circular shaking machine at 40°C for 96 h.

Determination of Lipase Activity. Lipase activity (LA) was determined by a modification of Ota and Yamada's method [9], which is based on the titration of the free fatty acids formed in the hydrolysis of the substrate under the action of the enzyme. As the unit of lipase activity we took that amount of enzyme which liberates 1 μmole of oleic acid from a 40% emulsion of olive oil in a 2% solution of poly(vinyl alcohol) in 1 h under selected conditions (pH 8.7, temperature 55°C).

Proteins were determined by Lowry's method and spectrophotometrically at wavelengths of 260 and 280 nm. Bovine serum albumin (Reanal) was used as standard.

The molecular masses of the enzymes were determined by gel filtration on Sephadex G-100. A column of Sephadex G-100 was calibrated with proteins of known molecular mass, and the free volume of the column was found with the aid of Dextran Blue. The standards used were chymotrypsinogen (25 kDa), β-N-acetylglucosaminidase (20 kDa), aldolase (subunit, 40 kDa), bovine serum albumin (67 kDa), and ferritin (subunit, 220 kDa).

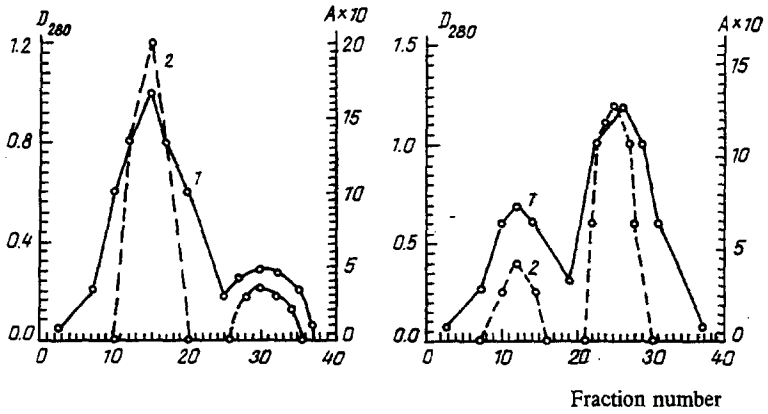

Fig. 2. Gel chromatography of the active fractions on TSK-HW 55.

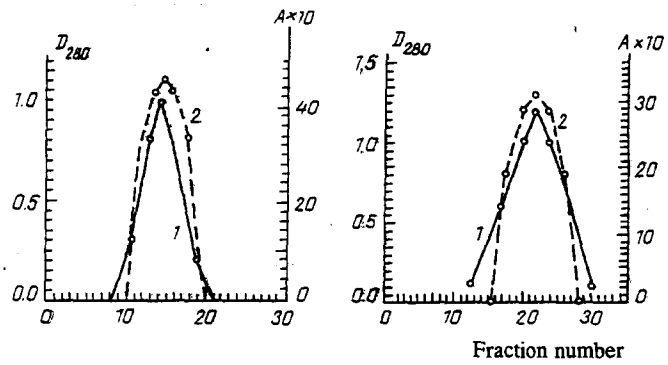

Fig. 3. Repeat gel chromatography of the active fractions on Sephadex G-100.

Disk electrophoresis was conducted in 7% polyacrylamide gel (PAAG) at pH 8.3 in the presence of Na-DDS and 2-mercaptoethanol. The gel was stained with a solution of Coomassie R-250 after it had been fixed with 7% TCA solution (5 min). The molecular masses of the proteins detected were calculated from a plotted calibration graph of the dependence of the relative electrophoretic mobility on the molecular masses of standard proteins. As the standard proteins we used the combined proteins of Pharmacia Electrophoresis Calibration Kits (Sweden).

The isoelectric focusing of the enzymes was carried out by Vesterberg's method [10] at 4°C in a glycerol density gradient from 0 to 60%, using ampholines with pH values from 3 to 10 in a column with a volume of 110 ml. The electrofocusing of the enzymes was conducted at 100 V for 72 h. After the volume of the column, excluding the anode and cathode solutions, had been divided into fractions each containing 2 ml of solution, protein contents, LAs, and pH values were determined.

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