SUMMARY

From a chymotryptic hydrolyzate of the CM-7S-globulin of cotton seeds we have isolated and characterized in relation to amino-acid composition and N-terminal amino acids 65 homogeneous peptides, 38 of which contain arginine or lysine residues and are overlapping.

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ISOLATION OF AN INTRACELLULAR LIPASE FROM THE HEAT-TOLERANT FUNGUS Rhizopus microsporus UzLT-1 AND ITS PROPERTIES

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The comprehensive study of the intracellular and extracellular enzymes secreted by microorganisms is important for explaining the physiological roles of particular enzymes and the routes of their biosynthesis and secretion by the cells. We have previously reported that the fungus Rhizopus microsporus UzLT-1 discharges into the surrounding medium two extracellular lipases [1] and have described methods for their purification [2]. In the present paper we give a method for the isolation and purification of an intracellular lipase from the mycelium from the fungus Rhizopus microsporus, UzLT-1.

The fungus was grown by a known method [1]. The fungal mycelium was separated from the culture liquid by filtration and was washed with distilled water until the filtrate was clear. The washed mycelium was broken down in 0.005 M phosphate buffer, pH 7.5, with glass beads in a mortar and was then separated by vacuum filtration on a Buchner funnel. The enzyme was concentrated with the aid of dry washed Molselekt G-25 (from the firm "Reanal"). All the subsequent purification was carried out at 2-4°C. The concentrated enzyme solution was dialyzed first against distilled water and then against 0.005 M phosphate buffer, pH 7.5. The precipitate that deposited after dialysis was separated by centrifuging. The supernatant liquid, containing 39.84 units of lipase activity, was deposited on a column of DEAE-Sephadex A-50 equilibrated with the initial buffer. The protein was eluted with phosphate buffer, using a stepwise concentration gradient from 0.005 to 0.2 M followed by the use of the buffer containing 0.3 M NaCl.

Three protein fractions were obtained (Fig. 1). The first fraction, eluted by the initial buffer, and the third, eluted by 0.2 M phosphate buffer containing 0.3 M NaCl, exhibited lipase activity.

The fraction corresponding to the first active peak (Fig. 2) was concentrated and dialyzed against 0.005 M acetate buffer, pH 5.6 overnight. The dialyzed enzyme solution was filtered and deposited on a column of SE-Sephadex C-50 previously equilibrated with 0.005 M acetate buffer, pH 5.6.

Under these conditions, the bulk of the inactive protein was washed out with the free volume. The column was washed with the initial buffer at the rate of 30 ml/h until the absorption of the eluate at 280 nm reached 0.01. The lipase was eluted with acetate buffer with stepwise increasing concentration to 0.05 M, pH 5.6. The results of purification are given in Table 1. After purification an electrophoretically homogeneous fraction of lipase the specific activity of which was 16 times greater than the specific activity of the first extract was obtained.

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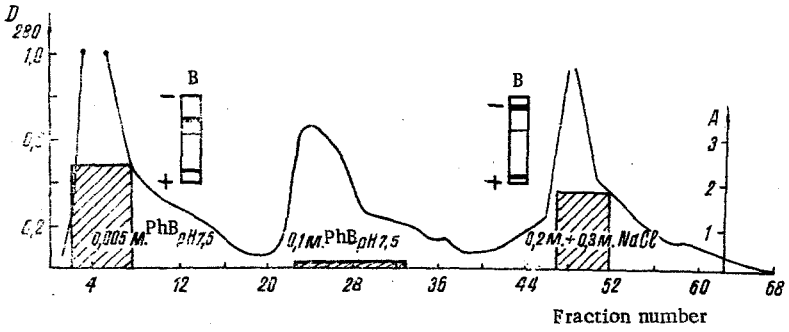

Fig. 1

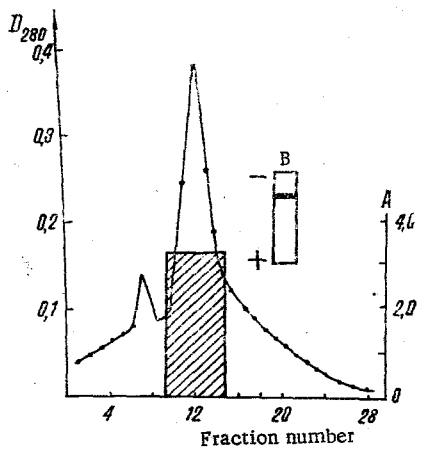

Fig. 2

Fig. 1. Chromatogram of the intracellular lipase on DEAE-Sephadex A-50: A) activity, ml of 0.1 M KOH; B) electrophoretogram of the individual fraction.

Fig. 2. Chromatogram on SE-Sephadex C-50 of the 1st active fraction after DEAE-Sephadex A-50 (arbitrary symbols the same as in Fig. 1).

TABLE 1. Purification of the Intracellular Lipase of UzLT-1

Stage of purification	Total lipase activity, ml of 0.1 N KOH	Total amount of protein, mg	Specific activity, units/mg of protein	Yield (%) in		Degree of purification
				activity	protein	
Filtrate from the disintegrated cells	48,0	200,0	0,215	100	100	1
Concentration of filtrate	40.50	90,0	0,45	93,0	45,0	2,7
Dialysis	39,84	75,0	0,525	92,6	37,2	2,4
Chromatographic separation on DEAE-Sephadex A-50 with 0.005 M phosphate buffer, pH 7.5	23,5	10,8	2,35	54,7	5,4	10,9
With 0.1 M phosphate buffer, pH 7.5	1,87	27,2	0,07	4,3	13,7	0,3
With 0.2 M phosphate buffer, pH 7.5, + 0.3 M NaCl	14,4	12,6	1,80	33,3	6,3	8,4
Chromatography of the 1st active fraction on SE-Sephadex C-50*	17,3	5,4	3,2	40,3	2,7	15,9

*8.4 mg of the enzyme with a total activity of 19.7 units was deposited on the comumn of SE-Sephadex C-50.

The purified preparation showed no esterase activity (substrates: ethyl acetate and methyl butyrate). On electrophoresis in polyacrylamide gel at pH 8.3, the purified lipase migrated towards the anode faster than the extracellular lipase, in the form of a sharp band.

Some properties of the purified enzyme have been studied: the optimum pH is 6.0, and the maximum activity is shown at 40°C.

The results of an investigation of the influence of various ions on the intracellular lipase of Rhizopus microsporus, UzLT-1, have shown that the cleavage by the enzyme of olive oil does not require the presence of metal ions. Alkali-metal salts in phosphate buffer have a slight activating effect which is apparently connected with the nonspecific influence of the ionic strength. The ions Cd^{2+}, Mg^{2+}, Mn^{2+} and Ni^{3+} are inhibitors of the intracellular lipase. It is possible that the inhibiting action of Mg^{2+} ions is due to metal-enzyme interaction. In a concentration of $1 \cdot 10^{-4}$ M, Co^{2+} ions did not suppress the activity of the intracellular lipase, while the extracellular enzyme is inhibited under similar conditions [3]. The inhibition of the lipase by Cd^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{3+} ions was shown more distinctly in Tris-HCl buffer than in phosphate buffer.

Inhibition was also observed at high concentrations of Ca^{2+} ions. This effect can apparently be explained by the removal of metal ions from the sphere of the enzyme reaction because of their interaction with phosphate ions.

On comparing the changes in lipase activity in aqueous solution, in buffer, and in buffer containing sodium chloride at pH 6.0 it can be seen that the activity at 60°C falls sharply. Incubation at 45°C in phosphate buffer and in phosphate buffer containing 0.06 M NaCl in the course of an hour (5, 10, 30, 45, and 60 min) showed that an increase in the ionic strength of the buffer by the addition of NaCl raises the heat-stability of the lipase. Thus, for example, at 45°C the enzyme incubated in phosphate buffer for 30 min loses 50% of its activity, while in the presence of sodium chloride it loses only 20%.

EXPERIMENTAL

Source of the Enzyme. The fungus Rhizopus microsporus, UzLT-1, was grown as described previously [1, 2]. The lipase was obtained from the mycelium of a 24-hour culture of the fungus by grinding with glass beads that had been washed with concentrated sulfuric acid. The enzyme was extracted with 0.005 M phosphate buffer, pH 7.5. The cell fragments were separated by centrifuging or by vacuum filtration on a Buchner funnel. The enzyme solution was concentrated with the aid of Molselekt G-25 in an amount of 1.0 g/4.0 ml at 2-4°C.

Chromatography on DEAE-Sephadex A-50. An amount of enzyme solution containing 39.84 mg of protein (4 ml) was deposited on a column of DEAE-Sephadex A-50 (3.0 × 60 cm) equilibrated with 0.005 M phosphate buffer, pH 7.5. The protein was eluted first with the initial buffer and then with 0.1 M phosphate buffer containing 0.3 M NaCl. Lipase activity was shown in the fractions of the first and third peaks. The fractions corresponding to the first peak were collected, concentrated, and dialyzed first against distilled water and then against 0.001 M acetate buffer, pH 5.6, and were deposited on a column of SE-Sephadex C-50.

Chromatography on SE-Sephadex C-50. The dialyzed solution of the enzyme after separation on DEAE-Sephadex A-50 (8.4 mg) was deposited on a column (3.0 × 50 cm) of SE-Sephadex C-50. Elution was performed with 0.005 M acetate buffer, and then the concentration of the buffer was raised to 0.05 M at constant pH. The lipase activity was eluted by the 0.05 acetate buffer. The fractions were collected, dialyzed against double-distilled water overnight, and freeze-dried. The lipase preparation so obtained contained 40.3% of the initial activity.

Disk electrophoresis in polyacrylamide gel was performed as described previously [2].

Purity of the Enzyme. The lipase preparation was homogeneous according to disk electrophoresis in polyacrylamide gel. After the staining of the gel with Amido Black 10B and Coomassie Blue, and also on a zymogram obtained by Abe's method [4], only one band was revealed.

Optimum pH. The lipase activity was determined in 0.1 M Tris-HCl buffer and 0.1 M phosphate-citrate buffer at pH 4.2-9.5. It was found that in the buffer solution studied there was one pH optimum located in the pH range of 5.8-6.2.

Optimum Temperature. In studying the influence of the temperature on the activity of the enzyme, the reaction mixture used in the standard activity determination was incubated for an hour at every 5°C in the temperature range from 20 to 70°C. The maximum activity of the intracellular lipase was observed at 40°C.

Heat Stability. Solutions of the enzyme in water, in 0.1 M phosphate buffer, pH 6.0, and in the same buffer containing 0.06 M NaCl were heated at 30, 40, 45, 50, and 60°C for from 5 to 60 min. After rapid cooling, the lipase activities were measured in the usual way.

The influence of salts was studied after incubation of the enzyme in salt solutions for 15 min at room temperature followed by activity determinations.

SUMMARY

1. A method of isolating and purifying an intracellular lipase of the fungus Rhizopus microsporus, UzLT-1, has been described.

2. Some properties of the purified enzyme have been studied and it has been shown that the intracellular lipase differs from the extracellular lipase in its pH optimum and electrophoretic mobility.

3. The presence of sodium chloride (0.06 M) in the reaction mixture raises the heat stability of the purified lipase.

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