THE LIPASE OF THE FUNGUS Rhizopus microsporus, UZLT-1

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The presence in the culture liquid of the fungus Rhizopus microsporus, UZLT-1, of an extracellular lipase (E.C. 3.1.13 – glycerol ester hydrolase) has been reported previously [1, 2]. In the present communication we give the results of the purification of the enzyme and the study of some of its properties.

Purification was begun with the gel filtration of the preparation on columns of Sephadex G-75 in 0.005 M phosphate buffer at pH 7.8 (Fig. 1a). After separation the activity of the enzyme preparation had risen five- to sixfold (Table 1). Further purification was performed by ion-exchange chromatography on a column of CM-cellulose. The column was equilibrated with $5 \cdot 10^{-3}$ M phosphate-citrate buffer, pH 6.0. Elution was carried out first with the initial buffer and then with a stepwise gradient formed by increasing the ionic strength of the buffer with sodium chloride (Fig. 1b). The protein was eluted from the column in the form of three peaks. Lipase activity was shown mainly in the second peak (hatched area). The active fraction was concentrated and was passed through a column of Sephadex G-150 equilibrated with the initial buffer (Fig. 1c). The protein was eluted from the column as three fractions, two of which showed lipolytic activity (hatched area). After gel filtration the activity of the enzyme had doubled. Electrophoresis on polyacrylamide gel of the enzyme material in the second active peak showed the presence of two protein bands. The active fraction (33-60) (Fig. 1c) was collected, concentrated, and rechromatographed on a column of Sephadex G-150 under the same conditions. Elution gave a single protein peak with a high specific activity. The results of the investigations are given in Table 1 (all the determinations were performed at +4°C).

The results of the investigation of the dependence of the activity of the lipase on the pH showed that the total enzyme had two pH optimas; in the case of olive oil and triolein as substrates the optima were present in the pH ranges 4.2-4.8 and 7.6-8.0, and in the case of tributyrin they were at pH 3.8-4.8 and 6.0-6.8. In triolein and olive oil the enzyme showed greater activity in the alkaline pH region, and in the case of tributyrin in the acid region. The purified enzyme had a pH optimum of 8.0.

We studied the action of sodium deoxycholate and sodium cholate on the activity of the lipase. Sodium deoxycholate in concentrations of 0.025-0.15% activates the lipase activity, while sodium cholate acts as an inhibitor in these concentrations. Other inhibitors of lipase activity are zinc and copper chlorides and, to a smaller degree, cobalt and iron chlorides. An investigation of the heat stability of the lipase showed that at 0°C the enzyme does not lose activity for 30 h. The half-decomposition of the enzyme in aqueous solution at 20, 37, 45, and 50°C takes 20 h, 6 h, 130 min, and 60 min, respectively.

EXPERIMENTAL

The fungus was grown in 250-ml flasks in a medium of the following composition (%): maize extract – 2; cottonseed oil – 1; $CaCO_3$ – 1.5.

The protein content in the solutions was determined by the biuret reaction and by the Warburg–Christian method [3].

The lipase activity was determined by the method of Ota and Yamada [4]. The activity was expressed in milliliters of 0.1 N alkali necessary for the titration of the fatty acids formed. The reaction mixture for determining lipase activity had the following composition: 1 ml of enzyme solution, 6.5 ml of 0.001 M phosphate-citrate buffer, pH 8.0, and 2.5 ml of an emulsion of olive oil in a 2% solution of poly(vinyl alcohol). Incubation was performed in a Warburg apparatus under standard conditions at 37°C for 60 min. After the end of incubation, 30 ml of ethanol was added to the flask to prevent the hydrolytic dissociation of the oil. The mixture

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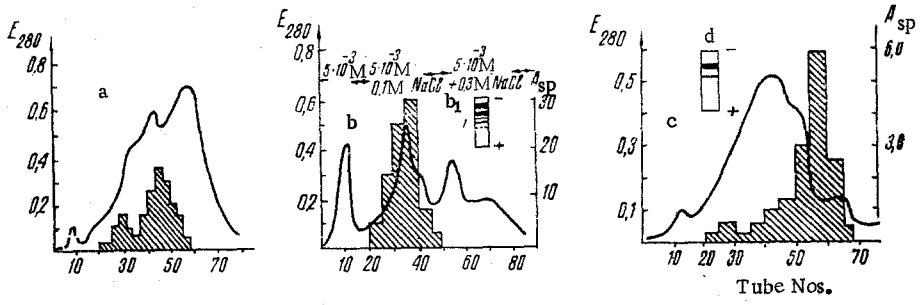

Fig. 1. Gel filtration of the total protein fraction on Sephadex G-75 (the hatched area represents the lipase activity) (a); chromatography of the active fraction after Sephadex G-75 on a column CM-cellulose (b); electrophoretogram (b_1); and gel filtration of the active fraction from the CM-cellulose column on Sephadex G-150 (c).

TABLE 1. Purification of the Extracellular Lipase from the Fungus Rhizopus microsporus, UZLT-1

Stage of purification	Protein, mg	Activity		Yield, %		Degree of purification
		specific	total	in protein	in activity	
Culture liquid	8680	565,14	4905068,0	100	100	1
Isopropanol extract	3741	1106,8	4140538,8	43,8	84,5	2
Separation on a column of Sephadex G-75						
fractions 4—12	1158	—	—	13,46	—	—
13—30	944,1	1000 0	944100	10,8	19,2	2
31—37	592,2	500,0	296100	6,9	6,04	1
38—42	601,2	2500	1503100	6,9	30,6	5
43—58	184,05	7590	1396939,5	2,1	28,5	13,5
Separation of the active (43-58) fraction on CM-cellulose						
Peak I	55,6	—	—	—	—	—
Peak II	58,1	24000	1394400	0,67	28,3	42,3
Peak III	35,6	—	—	—	—	—
Separation of peak II on Sephadex G-150						
fractions 20—32	24,0	22000	528000	0,03	10,8	35,2
33—60	16,5	50000	825000	0,02	16,9	88,5
61—72	12,3	—	—	—	—	—
Rechromatography of peak II on Sephadex G-150	11,2	55000	616000	0,015	12,6	100

obtained was titrated with 0.1 N KOH to phenolphthalein. Control samples were treated in the same way but the culture liquid was added after the ethanol. The results of the titration of the control were deducted from the results of the experiment. The specific activity is the number of units referred to 1 mg of protein in the enzyme preparation.

Precipitation by Isopropanol. The culture liquid was separated from the fungal mycelium by filtration and was cooled in a refrigerator to 0°C. Then, with constant stirring, cooled isopropanol was added dropwise. At a ratio of culture liquid to isopropanol of 1:6, a voluminous precipitate deposited, which was collected by centrifuging at 6000g for 15 min. The precipitate was washed three times with cooled isopropanol and then with acetone, and was dried in a vacuum desiccator over anhydrous calcium chloride.

Disk Electrophoresis in Polyacrylamide Gel. Vertical electrophoresis was performed in polyacrylamide gel. The concentration of the concentrating gel was 2.5% in 0.025 M tris-glycine buffer, pH 6.6, and the concentration of the separating gel was 7.5%, pH 8.6. The size of the glass tubes for filling with the gels was 0.6 × 10 cm. Before deposition on the gels, the samples were saturated with sucrose in an amount of 250 mg/ml. The current strength during electrophoresis was 3.5 mA on each tube.

Separation on a Column of Sephadex G-75. After saturation with isopropanol, the precipitate obtained was deposited on a column (3.0 × 50 cm) of Sephadex G-75 previously equilibrated with $5 \cdot 10^{-3}$ M phosphate buffer, pH 8.0. The rate of elution was 24 ml/h. Fractions of 6 ml each were collected in an automatic collecotr. In each fraction the activity was measured by the method described above and the extinction was determined on an SF-4 spectrophotometer at 280 and 260 nm (see Fig. 1a).

Separation on CM-Cellulose. The active fraction (184 mg) after gel filtration (Sephadex G-75) was concentrated and deposited on a column containing CM-cellulose (1.0 × 17 cm) equilibrated with the initial buffer. The rate of elution was 12 ml/h. Elution was performed first with the initial buffer and then with a stepwise gradient with an increasing ionic strength of the buffer. Fractions with a volume of 3.0 ml were collected in an automatic collector. The activity of each fraction was determined by the method described above and the extinction was measured on the SF-4 spectrophotometer at 280 and 260 nm. A graph was plotted from the spectrophotometric results (see Fig. 1b). Three peaks were obtained. The activity was determined in each fraction and was found to be shown by one peak (see Fig. 1).

Gel Filtration on Sephadex G-150. The active fraction (58 mg) after separation on CM-cellulose was concentrated and deposited on a column of Sephadex G-150 (3.0 × 50 cm) equilibrated with $5 \cdot 10^{-3}$ M phosphate-citrate buffer, pH 8.0. The rate of elution was 12 ml/h, and the fraction volume 3 ml. The graph of the elution of the protein has three peaks (see Fig. 1c). The lipase activity was checked in each test tube; it appeared in two peaks.

Repeat Gel Filtration on Sephadex G-150. The active protein from the second active peak (16.5 mg) was concentrated and deposited on a column of Sephadex G-150 (2.2 × 25 cm) equilibrated with the initial buffer. The rate of elution was 12 ml/h. Fractions with a volume of 3 ml were collected. The protein was eluted in one symmetrical peak with a high lipase activity.

All the operations in the purification of the enzyme were performed at +4°C. The influence of various ions was studied after incubation of the enzyme for 15 min in a solution of the active substance at room temperature followed by a determination of activity.

The heat stability of the enzyme was studied by incubating solutions of the enzyme for a definite time at the given temperatures in a water bath, bringing the temperature to that of the room, and determining the activity.

SUMMARY

1. An active lipase enzyme has been isolated from the culture liquid of the fungus Rhizopus microsporus, UZLT-1, by precipitation with isopropanol, gel filtration on Sephadexes G-75 and G-150, and chromatography on CM-cellulose. Some properties of the purified enzyme (optimum pH, heat stability, influence of various ions) have been studied.

LITERATURE CITED

1. M. Z. Zakirov, L. G. Sultanova, and K. Davranov, Prikl. Biokhim. Mikrobiol., 11, No. 4, 546 (1975).
2. K. Davranov and M. Rizaeva, Khim. Prirodn. Soedin., 279 (1975).
3. O. Warburg and W. Christian, Biochem. J., 310, 384 (1941).
4. Y. Ota and K. Yamada, Agric. Biol. Chem., 30, 351 (1966).