PURIFICATION OF THE INTRACELLULAR TRIACYLGLYCEROL LIPASE OF *Oospora lactis*

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A method for the isolation of homogeneous triacylglycerol hydrolase from the mycelium of the fungus *Oospora lactis* is described. The homogeneity of the enzyme has been shown by gel filtration through Sephadex G-100, by ultracentrifugation, and by disc electrophoresis in polyacrylamide gel. Some properties of the enzyme have been studied: molecular weight, 43,000; optimum action temperature, 35-37°C; pH optimum, 7.5.

Lipase (triacylglycerol acyl-hydrolase, E.C. 3.1.1.3) has been detected in many microorganisms [1-3], plants [4], and various animal organs [5].

The aim of the present paper is to describe the selection of conditions for the isolation and purification of the intracellular lipase from the mycelium of a highly active strain of *Oospora lactis*, UzLM-2 [3], the study of its component composition and some characteristics of the homogeneous enzyme.

In the first stages of the work with the aid of disc electrophoresis in polyacrylamide gel and gel chromatography on Sephadex G-100 we studied the component composition of the isopropanol powder. As a result it was established (Figs. 1 and 2) that the products of the vital activity of the fungus *Oospora lactis* form a complex combination containing high- and low-molecular-weight proteins. The lipase activity was exhibited in the form of three factions differing in their charges (disc electrophoresis) and molecular weights (Fig. 1). The second lipase fraction had the highest specific and total activities (∿50% of the overall total activity) and therefore it was necessary to purify it to the homogeneous state and to study some of its properties. The active fractions after separation on Sephadex G-100 (fractions 24-28) was deposited on a column of CM-cellulose. Elution of the proteins from the CM-cellulose with a stepwise gradient gave two fractions containing lipase (Fig. 2). The ratio of the lipase activities of the first and second fractions was approximately 10:1. The maximum of the enzymatic activity was eluted by 0.02 M acetate buffer, pH 5.6 (fraction 1), while fraction 2 was eluted with 1 M acetate buffer.

Fraction 2 of the lipase activity was less stable and on rechromatography it was completely inactivated, and it was therefore not subjected to further purification. Fraction 1, corresponding to the first peak of lipase activity, was desalted by dialysis first against distilled water and then against $5 \cdot 10^{-3}$ M phosphate buffer, pH 7.5, and was deposited on a column of DEAE-cellulose. It was found that with the use of $5 \cdot 10^{-3}$ M phosphate buffer, pH 7.5, the enzyme was scarcely sorbed (Fig. 3) and was eluted with the initial buffer. The following and final stage, leading to the complete purification of the enzyme, was gel filtration on a column of Sephadex G-200 (Fig. 4). The protein was eluted as a single symmetrical peak with a high lipase activity. It must be mentioned that the specific activity here was 5000 units lower than in the previous stage of purification (Table 1). The D_{280}/D_{260} ratio was constant, which shows the chromatographic homogeneity of the lipase. Homogeneity was also shown by analytical disc electrophoresis in 7.5% polyacrylamide gel. The protein moved as one band with a R_f value of 0.45 (Figs. 1 and 4), which exhibited a violet color in the specific reaction for lipases (Abe test) (Fig. 5). The homogeneity of the enzyme was also shown by the sedimentation method in the ultracentrifuge. The sedimentation coefficient of the enzyme was determined by the same method; it was 4.03 S.

Thus, the scheme described above can be used for the purification of one of the three forms of extracellular lipase from the fungus *Oospora lactis*. Table 1 gives the results of

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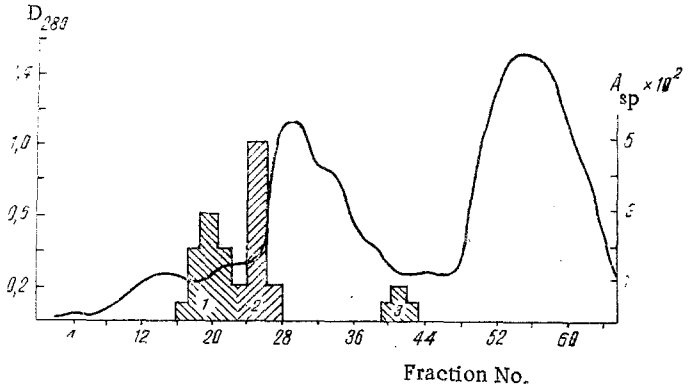

Fig. 1. Gel chromatography of the intracellular protein complex of *Oospora lactis* on Sephadex-G-100 (for conditions, see text; the hatched areas exhibited lipase activity).

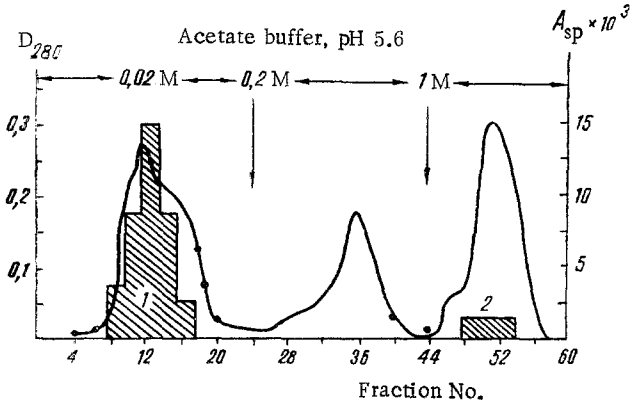

Fig. 2. Chromatographic separation of the 2nd active fraction after Sephadex G-100 on CM-cellulose.

the purification of the enzyme: A 15-fold purification was achieved with retention of 10% of the activity. Such a low yield of the total activity on purification is possibly connected with the quaternary structure of the enzyme, a discussion of which is premature. It must be mentioned that a low yield in purification has also been observed in the case of lipases from other biological materials [6, 7].

The molecular weight of the homogeneous enzyme determined by the sedimentation method was about 43,000, and this was also confirmed by gel filtration on a column of Sephadex G-100.

TABLE 1. Purification of the Lipase from the Fungus *Oospora lactis*

Stage of purification	Protein, mg	Lipase activity		Activity yield, %	Degree of purification
		specific, μmole/min/mg of protein	total, μmole/min		
Extract*	500	1200	600000	100	1
Sephadex G-100	11.2	2500	280000	46.6	2
CM-cellulose	1,8	1500	27000	45,0	12,5
DEAE-cellulose	1,1	18550	20405	17,0	15 45
Sephadex G-200	0 95	13500	12825	10,6	11,2

*From 2 g of isopropanol powder.

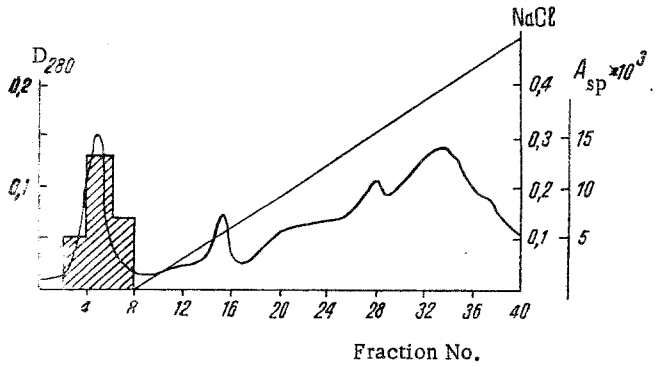

Fig. 3. Chromatography of the active fraction after CM-cellulose on DEAE-cellulose.

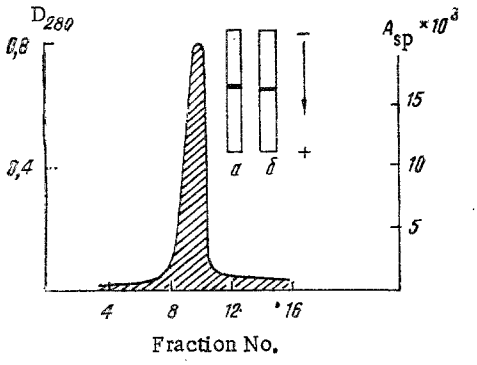

Fig. 4

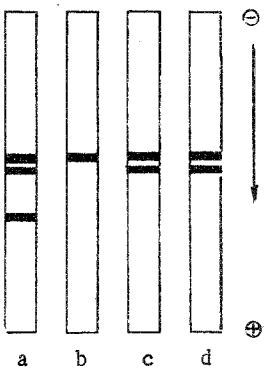

Fig. 5

Fig. 4. Gel chromatography of the active fraction after DEAE-cellulose on Sephadex G-200: a) electrophoretogram on PAAG; b) enzymogram (Abe test [2]).

Fig. 5. Enzymogram of the lipases (Abe test [2]: a) complex preparation of the intracellular lipase; b) 2nd active fraction of the intracellular lipase on a column of Sephadex 100; c) the same after precipitation with acetone at pH 4.0; d) the same after a second gel filtration on Sephadex G-100.

EXPERIMENTAL

Enzyme. The source of enzyme was the *Oospora lactis*, strain Uz LM-2 [3]. The initial material for the isolation of the lipase was the filtered-off and washed mycelium after the producing agent had been grown in 0.25-liter flasks and in 10-liter fermenters.

The fungal mycelium was ground in a mortar with glass beads, using liquid nitrogen for freezing. The freezing-thawing process was repeated three times. The enzyme–protein complex from the broken down mycelium was extracted with 0.1 M phosphate buffer, pH 7.5, with centrifugation at 10,000 rpm for 15 min. The enzyme was precipitated from the supernatant liquid with six volumes of cold isopropanol.

After being kept for +2°C for four hours, the precipitate that had formed was separated off by filtration through a Buchner funnel and was dried by washing with cold isopropanol in the air (isopropanol powder). The isopropanol powder (5.0 g) was suspended in 50 ml of 0.005 M acetate buffer, pH 5.6, and the suspension was centrifuged at 18,000 rpm for 10 min. The supernatant liquid (45 ml) was deposited on a column of Sephadex G-100 (6.0 × 130 cm) previously equilibrated with the same buffer. The proteins were eluted from the column with the same buffer. The rate of elution was 40 ml/h and the volume of the fractions from the column was 10 ml.

Fractions 24-28 were combined (50 ml), dialyzed against double-distilled water, and freeze-dried. The product was dissolved in the minimum amount of 0.02 M acetate buffer, pH 5.6, and chromatographed on a column (1.0 × 20 cm) of CM-cellulose previously equilibrated

with the same buffer. The proteins were eluted from the column in a stepwise gradient, the ionic strength of the buffer being increased while the pH was kept constant. Fractions with a volume of 3.5 ml were collected every 15 min.

Fractions 8-18 (66.5 ml) were combined and lyophilized, and the residue was dissolved in 3 ml of double-distilled water and deposited on a column of DEAE-cellulose (0.8 × 10 cm) previously equilibrated with $5 \cdot 10^3$ M phosphate buffer, pH 7.5. Elution was carried out with 3 ml of buffer solution having a linear concentration gradient of NaCl from 0 to 0.5 M.

Fractions 4-7, containing the bulk of the lipase (12 ml) were dialyzed against double-distilled water and lyophilized. The lyophilized enzyme was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.8, and was deposited on a column of Sephadex G-200 (2.1 × 75 cm). The protein in fractions 41-43 (9 ml) consisted of a homogeneous lipase, which was freeze-dried and used for the further characterization of the enzyme.

The determination of lipase activity has been described by us previously [2]. Protein was determined by Lowry's method and also, in the stages of purifying the enzyme, spectrophotometrically by measuring the optical densities at 260 and 280 nm.

Disc Electrophoresis in Polyacrylamide Gel. The concentration of the separating gel was 7.5% and that of the concentrating gel 2.5%, pH 8.9; the current strength during the passage through the concentrating gel was 3 mA and through the separating gel 5 mA, temperature +4°C. The proteins were stained with a 0.25% solution of Coomassie in the methanol–acetic acid–water (5:1:5) system after the fixation of the gel in a 7% solution of trichloroacetic acid (5 min).

The lipase activity in the gel was determined by a method described previously [2] and also on cut-out discs 1.5 mm thick after their extraction with 0.1 M phosphate buffer, pH 7.5, and incubation in a substrate mixture [2].

Molecular Weight Determination. The molecular weight of the enzyme was determined by two methods: sedimentation in an MOM-3170 analytical ultracentrifuge, and in a calibrated column of Sephadex G-100 (2.0 × 120 cm). The marker proteins used were bovine serum albumin (mol. wt. 68,000), aldolase (40,000), pepsin (35,000), and trypsin (23,000).

CONCLUSION

A method has been described for the isolation and purification of a triacylglycerol lipase from the fungus *Oospora lactis*. The homogeneity of the enzyme has been shown by disc electrophoresis, ultracentrifugation, and gel filtration in a column of Sephadex G-100. The molecular weight of the enzyme has been determined (43,000).

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