Oospora lactis LIPASE: ISOLATION AND PROPERTIES

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Lipase (EC 3.1.1.3) of *Oospora lactis* UzLM-2 was purified by ammonium sulfate (0·40—0·80) fractionation, gel-filtration on Sephadex G-100, column chromatography on DEAE-Sephadex and CM-Sephadex, gel-filtration on Sephadex G-75 and was finally crystallized in concentrated aqueous solution. Crystalline preparation of the enzyme is homogenous at disc-electrophoresis and ultracentrifugation. Sedimentation coefficient ($S_{20}w$) is 3·6 S, isoelectric point (pI) at pH 4·2 and molecular mass of the enzyme is 40—43 kD. Amino acid composition analysis showed that none of sulfur-containing amino acids were detected in the crystalline enzyme, but it contains about 8% of carbohydrates and a small amount of lipids. Lipase from *Oospora lactis* was most active at pH 7·5 and 37°C in olive oil, stable in the range of pH from 5·7 to 8·0 at 30—40°C for 18 h and retained stability at 50°C for 10 min.

As a result of our study on lipase, a strain of *Oospora lactis* UzLM-2 was selected as one potent lipase producer. As previously reported, it was found that this fungi inducibly produces lipase only in the presence of specific substances such as vegetable oil or fatty acid in a cultivation medium[1,2]. Some reports have been published on the production and specificity of *Oospora* lipase[3,4]. Although the application of *Oospora lactis* lipase to the study on glyceride structure has also been described in the above mentioned papers[4], the purification of the enzyme preparation has not been carried out so far.

The present work deals with the purification and crystallization procedures and with some of the enzymatic properties of *Oospora lactis* lipase.

EXPERIMENTAL

Cultivation: The medium used for cultivation of *Oospora lactis* was composed of 0·7% of yeast autolyzate, 1% of cotton oil and 0·3% ammonium sulfate, 0·1% $CaCO_3$ and was adjusted to pH 5·5 to 5·7. Five to ten liters of the medium were put into a 30-liter jar fermentor and were sterilized at 120°C for 30 min. Inoculation was made by addition of 120 ml of seed culture broth obtained after 48 h of reciprocal shaking in the same medium as above in Erlenmeyer flasks at 30°C. The cultivation was carried out by aeration 1·0 l l^{-1} min^{-1} and agitation 200 r.p.m.) at 28°C for 22 h. After cultivation, the cells were filtered off and the filtrate was subjected to the purification of lipase.

Lipase activity was assayed as described previously[4]. The activity which liberated 1 mole equivalent of fatty acid from olive oil per minute was defined as unit.

Protein content in the enzyme preparation was estimated by measurement of the absorbancy at 280 nm with Hammerstein milk casein as a standard. Specific activity of enzyme was expressed as units per mg of protein.

Physical measurements: Ultraviolet absorption spectrum was measured with a spectrophotometer SF-26. Homogeneity of purified enzyme preparation was checked by electrophoresis and ultracentrifugation. Disc-electrophoresis was done by the method proposed by Davis[5], using Reanal's firm model 71 (Hungary). The estimation of the isoelectric point of lipase was accomplished by isoelectric focusing. The carrier ampholytes were selected to give a pH gradient of 3 to 6 and the density gradient was provided by sucrose. Sample was applied to a vertical electrophoresis column of 110 ml capacity (LKB-Products). Analytical centrifugation was carried out on a MOM-3170 analytical ultracentrifuge.

Analysis of amino acid composition: 3·02 mg of the crystallized lipase preparation was hydrolyzed with 6M HCl at 110°C for 24, 48 or 72 h, respectively. Amino acids in the hydrolyzate were analyzed by the amino acid analyzer AAA-881.

Analysis of lipid: 75 mg of the crystallized lipase preparation were suspended in a chlorophorm–ethanol mixture (2 : 1 v/v) and sonicated by ultrasonic device. The resulting solution was evaporated by a rotor vaporizer in vacuum and the solid matter was suspended in ethyl ether, with insoluble residue removed. The soluble fraction was saponified with alcoholic 0·5M KOH and acified with HCl to pH 2·0. After removing the alcohol, ethyl ether was added to the solution. Methylation of the ether soluble fraction was then carried out by the addition of diazomethan. The methylated material was subjected then to gas-liquid chromatography (Column: 5% diethylene glycol succinate, 165°C).

Analysis of carbohydrate: Total sugar content in the crystallized lipase preparation was determined by phenol-sulfuric acid method. The sugar composition was analysed by the following procedure. One mg of the crystallized lipase preparation was hydrolyzed with 0·5M H_2SO_4 at 100°C for 3 h. The hydrolyzate was adjusted with $BaCO_3$ to pH 5·3. After removal of the precipitate by filtration, the filtrate was concentrated to 0·1 ml under reduced pressure. The sugar components of the hydrolyzate were analyzed by paper chromatography using a solvent system of 1-butanol–pyridine–water (6 : 4 : 3) with double ascending development. Reducing sugars were detected by spraying with aniline hydrogen phthalate.

Reagents: Sephadex G-100, Sephadex G-75, DEAE-Sephadex A-50, CM-Sephadex C-50 were the products of Pharmacia Co, Ltd. The Carrier ampholytes for electrofocusing were products of the LKB-Products. All other materials used were special grade reagents purchased from Reanal.

RESULTS AND DISCUSSION

Purification Procedures of Lipase

Ammonium sulfate fractionation: The culture filtrate derived from a cultivation of *Oospora lactis* was subjected to purification of lipase. Solid ammonium sulfate was added to 25 liters of the culture filtrate of *Oospora lactis* up to 0·40 saturation and the precipitate formed was filtered of. To the filtrate, solid ammonium sulfate

was further added to give an 0·80 saturation. The resulting precipitate was collected by filtration and dissolved in a minimum amount of deionized water.

Gel filtration on Sephadex G-100: The concentrated solution was chromatographed on a column of Sephadex G-100 (6·0 × 130 cm), equilibrated with 0·1M phosphate buffer, pH 7·5. Elution was performed with the same buffer at a flow rate of 30 ml per h and the effluent was collected in 7·5 ml fractions. A typical chromatographic profile obtained in gel filtration is represented in Fig. 1*a*. The active fractions (175 to 240) were combined and dialyzed against deionized water for 2 days and lyophilized.

DEAE-Sephadex column chromatography: The lyophilized powder (1 306·0 mg) was dissolved in 30 ml 0·01M acetate buffer, pH 5·0. After removal of the precipitate, the filtrate (185 ml) was applied to a (6 × 60 cm) column of DEAE-Sephadex A-50 which was equilibrated with the 0·01M acetate buffer, pH 5·0. The column was washed thoroughly with the same buffer, and the adsorbed protein was eluted with a linear concentration gradient of NaCl at a flow rate of 20 ml per h. The effluent was collected in 5 ml portions. The main fractions were combined and concentrated to 20 ml by dialysis against polyethylene glycol (Fig. 1*b*).

CM-Sephadex column chromatography: The concentrated solution was chromatographed on a (3·5 × 45 cm) column of CM-Sephadex, equilibrated with 0·02M acetate buffer (pH 5·6). The column was washed thoroughly with the same buffer,

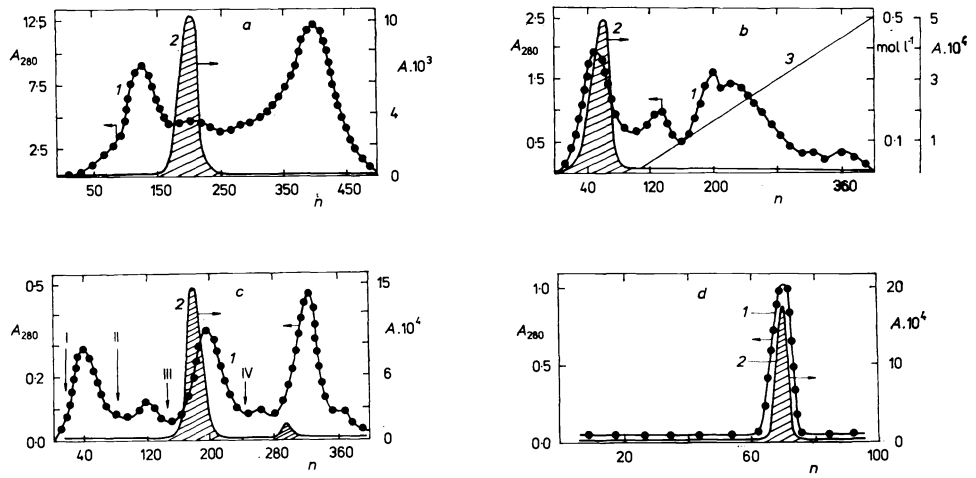

FIG. 1

Column chromatography of *Oospora lactis*: *a* on Sephadex G-100: *1* A_{280} protein, *2* lipase activity; *b* on DEAE-Sephadex A-50: *3* NaCl; *c* on CM-Sephadex C-50 (elution: I 0·03M acetate buffer, II 0·1M-NaCl, III 0·4M-NaCl, IV 1M-NaCl); *d* on Sephadex G-75

and the adsorbed protein was eluted with a linear concentration gradient of NaCl at a flow rate of 20 ml per h. The effluent was collected in 5 ml fractions. The main fractions (70–80) were combined and concentrated to 16 ml by dialysis against polyethylene glycol (Fig. 1*c*).

Gel filtration on Sephadex G-55: The concentrated solution was chromatographed on a (3·5 × 120 cm) column of Sephadex G-75, equilibrated with 0·01M acetate buffer, pH 5·6. Elution was made with the same buffer at a flow rate of 16 ml per h and the effluent was collected in 4 ml portions. As shown in Fig. 1*d*, a symmetric peak of protein with a constant specific activity of lipase was obtained. The fractions having more than 1 200 units per ml were combined and dialyzed against deionized water for 2 days.

Crystallization: The dialyzed solution was concentrated in a collodion bag under reduced pressure. The crystals appeared when the concentration of protein solution reached about 12%. After standing the concentrated solution in a cold room (4°C) for 48–60 h under reduced pressure, the crystallization was almost completed. The microphotograph of crystals of lipase is shown in Fig. 2. The crystals formed were washed three times with cold deionized water and lyophilized. The overrall procedure of purification and the results are summarized in Table I. The purifications results in a 218-fold increase in activity with a 25% recovery of the original activity.

Some properties of crystalline lipase of *Oospora lactis*: The homogenity of the crystallized preparation of lipase from *Oospora lactis* was examined by analytical

FIG. 2
Crystal of lipase from *Oospora lactis* (17 v)

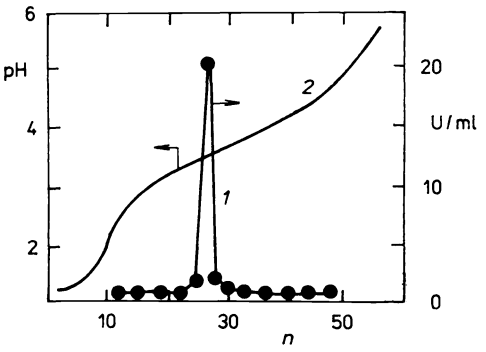

FIG. 3
Electrofocusing of *Oospora lactis* lipase. The experimental details are described in the text. *1* Lipase activity, *2* pH, *n* fraction number

disc-electrophoresis on polyacrylamide gels and sedimentation. Resulting data showed that the preparation is homogeneous.

The sedimentation constant, $S_{20}w$ was found to be 3·6 S, when the experiment was performed with a protein concentration of 0·385 to 0·927%. The molecular weight of lipase was estimated to be 39 000–40 000 by gel filtration using Sephadex G-100 (1·5 × 110 cm) column. Chymotrypsinogen (M.w. 25 000), ovalbumin (M.w. 45 000), bovine serum albumin (M.w. 67 000) were used as standard proteins for molecular weight determination. The isoelectric point of the lipase was determined by the isoelectric-focusing method. It was estimated to be pH 4·2 from the effluent profile shown in Fig. 3.

Chemical Composition of the Crystalline Lipase of Oospora lactis

Carbohydrate content of the crystalline lipase preparation was estimated to be 8% mannose, xylose, arabinose and galactose. The paper chromatogram suggests mannose as the main component among the sugars detected.

Table II summarizes the results of amino acid analysis of the crystallized lipase preparation. Sulfur-containing amino acid such as cystein and methionine were not detected in the hydrolyzate. The lipase is characterized as an enzyme without sulfur-containing amino acid.

By extraction with a chloroform–methanol (1 : 1 v/v) mixture about 0·7 mg of oily substance was obtained from 70 mg of the crystallized lipase preparation. This suggests a lipoprotein nature of homogeneous lipase.

Enzyme Property

Effect of temperature upon activity: The effect of temperature on the enzyme activity using olive oil as a substrate was examined under conditions described in Experimental and the optimal temperature was found to be 35–37°C (Fig. 4*a*).

Effect of pH upon stability: One ml aliquots of the crystalline lipase solution were adjusted to different pH values by adding 1 ml each of 0·1M McIlvain buffer and were kept at 37°C for 2 h. The residual activities of the enzyme were assayed after diluting the samples with 0·1M phosphate buffer (pH 7·5). The lipase was stable in the pH range between 7·0 and 9·0 (Fig. 5*a*).

Effect of pH upon activity. The pH dependence of the enzyme activity was examined using 0·1M McIlvain buffers of various pH and the maximal activity was observed at 7·0 to 7·5 (Fig. 5*b*).

Stability against heat treatment: The enzyme solution was adjusted to pH 7·5 and kept at various temperatures. After 15 min the samples were quickly cooled and the residual lipase activity was measured. The enzyme was found to be stable

TABLE I

Purification procedure of lipase of *Oospora lactis*

Fraction and step	Protein mg	Specific activity U/mg	Total activity U	Protein mg	Activity %	Purif. degree
Culture filtrate	250 000	850	$2\,125 . 10^4$	100	100	1
Ammonium sulphate precipitate	5 250	3 400	$1\,785 . 10^4$	2·1	84	4
Sephadex G-100 chromatography	1 306	10 250	13 387 500	0·52	63	12
DEAE-Sephadex chromatography	200	51 020	10 200 000	0·080	48	60
CM-Sephadex chromatography	46·2	147 100	6 800 000	0·018	32	173
Sephadex G-75 chromatography	29·0	184 450	5 350 000	0·012	25	217
Crystals	28·6	185 300	5 312 500	0·012	25	218

TABLE II

Amino acid composition of the lipase from *Oospora lactis*

Amino acid	Numbers of residue per mole of the enzyme[a]	Amino acid	Numbers of residue per mole of the enzyme[a]
Lysine	12	Alanine	24
Histidine	8	Half-cystine	0
NH_3	32	Valine	18
Arginine	14	Methionine	0
Aspartic acid	38	Isoleucine	12
Threonine	16	Leucine	28
Serine	20	Tyrosine	12
Glutamic acid	22	Phenylalanine	20
Proline	18	Tryptophan[b]	6
Glycin	28		

[a] Calculated on the basis of the value obtained by subtracting the carbohydrate constant (8%); [b] by the method of Goodwin and Mowton[6].

at temperatures below 45°C but was rapidly inactivated when the temperature was raised above 50°C (Fig. 4*b*).

Action upon olive oil and methyl butyrate: Whereas olive oil, triglycerides of fatty acids were almost completely hydrolyzed by the enzyme, methyl butyrate, a short-chain ester and typical substrate for esterases, was not attacked at all by the enzyme.

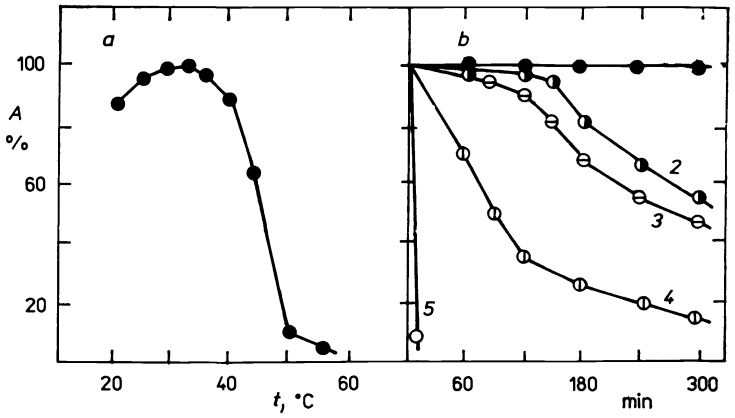

FIG. 4

Temperature influence on lipase activity of *Oospora lactis*: *a* activity dependence of temperature, *b* thermostability (*1* 30°C; *2* 40°C; *3* 45°C; *4* 50°C; *5* 60°C)

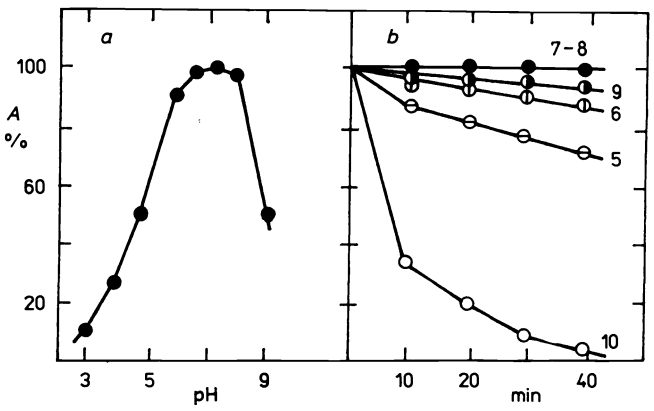

FIG. 5

pH Influence on lipase activity of *Oospora lactis*: *a* activity dependence of pH reactive mixture, *b* pH-stability. pH Values are given in figure

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