IMMOBILIZED *Oospora lactis* LIPASE PREPARATIONS AND THEIR PROPERTIES

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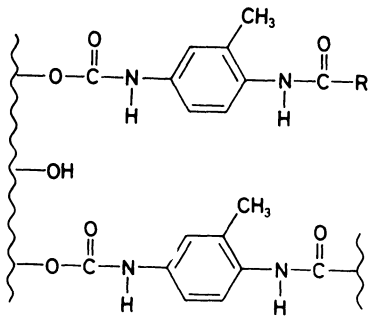
It has been shown that adsorption on microcrystalline and AE-cellulose partially modified by the palmitoyl residue is an effective method of immobilizing the lipases of fungus *Oospora lactis*. The kinetics of the lipases are characterized by substrate inhibition. Immobilization considerably increases the heat stability of the lipases.

The study of the immobilization of lipolytic enzymes (EC 3.1.1.3) is of great interest in connection with broadening the possibilities of their practical utilization[1]. An immobilized lipase, particularly in combination with an esterase, can be used in analytical investigations and also for the preparative cleavage of lipids. There have been several publications on the immobilization of lipases[2-6].

The aim of the present work was to obtain active preparation of immobilized lipases and to evaluate their catalytic properties and stability. We have used partially purified preparations of the lipases of the fungus *Oospora lactis*[7]. The enzymatic activity was determined in a pH-stat with automatic recording of the kinetic curves, using as the substrate an emulsion of olive oil stabilized with polyvinyl alcohol.

The choice of support and the method of immobilizing the lipases has certain characteristic features. In the first place the use of the usual macroporous inorganic supports is excluded since the dimensions of the emulsion considerably exceed the dimensions of the pores. In the second place, the well-known affinity of lipases for a water–lipid surface shows the possibility of using methods of immobilization based on hydrophobic interaction[8,9].

Supports containing hydrophobic residues were obtained by modifying microcrystalline cellulose with toluylene-2,4-isocyanate followed by the addition of compounds with a mobile hydrogen atom. In the first stage of the reaction, the diisocyanate reacts partially as a bifunctional reagent, forming cross linkages, and partially as a monofunctional reagent with the retention of a free isocyanate group[10,11]. Subsequently, to the latter are attached compounds containing aliphatic chains of various lengths: 1-butanol, 1-octanol and palmitic and sebacic acids. The schematic structure of the supports is shown by formula *I*.



I

a, R = C_4H_9O *b*, R = $C_8H_{17}O$ *c*, R = $C_{15}H_{31}$ *d*, R = $C_8H_{16}COOH$
f, R = $C_{15}H_{31}$ *g*, R = $C_{15}H_{31}$ and $C_8H_{16}COOH$ (1 : 1)

In one case (*Ig*) we used an equimolar mixture of palmitic and sebacic acid to obtain a support containing negatively charged groups capable of removing the products of the hydrolysis of lipids. The activities of the lipases immobilized on the supports are given in Table I.

As follows from the results of immobilization adsorption on hydrophobic supports provides the possibility of obtaining active preparations. On additional crossing of the immobilized preparations with glutaraldehyde it was found that glutaraldehyde (concentration 1%, temperature 4°C, time 60 min) completely inactivates the lipase.

Active preparations of immobilized lipases from fungus *Rhizopus* microspores have been obtained previously by adsorption on DEAE-cellulose[11,12]. Consequently, it was of interest to use supports for immobilization that contained positively charged groups and hydrophobic residues simultaneously. These supports were obtained by

TABLE I

The activities of the immobilized lipases (U/g) from fungus *Oospora lactis*. Initial ratio protein to support: A 1 : 20; B 1 : 50; C 1 : 100

Support[a]	A	B	C
Ia	2·700	1·950	1·330
Ib	3·100	2·040	1·450
Ic	3·600	2·230	1·750
Id	3·800	2·470	1·950
If	4·500	3·400	2·200
Ig	4·200	3·150	2·050

[a] See formula.

partial protection of the amino groups in AE-cellulose by the palmitoyl residue. The results show that such a combination is in fact effective. Figures for the activity of lipases immobilized on cellulose derivatives are given in Table II. The activity of the lipases adsorbed on partially modified AE-cellulose was greater than with the use of unprotected or completely acylated AE-cellulose.

TABLE II

Preparation of immobilized lipases of the fungus *Oospora lactis*. Weight ratio of enzyme to support 1 : 30.

Support	Molar ratio of modif. cellulose to palmitic acid	Activities of the lipases U/g
Microcrystalline cellulose	—	150
AE-cellulose	—	280
	1 : 0·5	885
	1 : 1	835
	1 : 2	640
	1 : 5	580
DEAE-cellulose	—	310
	1 : 0·5	1 050
	1 : 1	1 010
	1 : 2	995
	1 : 5	895

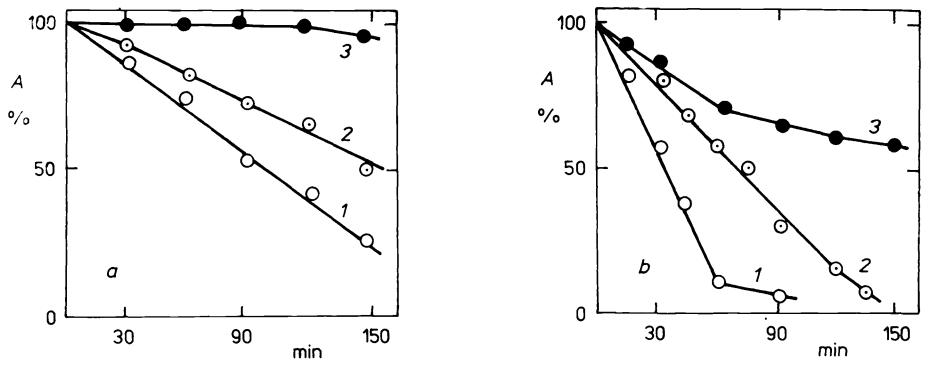


FIG. 1

Thermal stability of *Oospora lactis* lipase: *a* at 40°C; *b* at 50°C. *1* Native lipase; *2* lipase adsorbed on AE-cellulose; *3* lipase adsorbed on AE-cellulose with palmitoyl residue (1 : 0·5)

Preliminary determinations of heat stability have shown that the preparations obtained, unlike those immobilized by adsorption on DEAE-cellulose, are fairly stable (Fig. 1). When the palmitoyl residue was replaced by an 1-butyl or 1-octyl residue, the activity of the immobilized preparation was considerably less.

We determined in more detail the properties of the lipase from fungus *Oospora lactis* immobilized by adsorption on partially modified AE-cellulose. The dependence of the activity of the immobilized preparation of lipase on the initial weight ratio of enzyme to support is illustrated in Fig. 2. The activity yield was 25–30%.

In the majority of cases, the kinetics of the lipolysis of emulsified substrates is described by a Michaelis–Menten-type equation with slight modifications[13,14]. In a study of the dependence of the rate of hydrolysis on the volume concentration of the substrate, we found that both in the case of the soluble and the immobilized lipases inhibition by the substrate took place (Fig. 3). As a result of the treatment of the experimental results by graphical methods according to the kinetis scheme of inhibition by the substrate[15], we obtained kinetic parameters for soluble lipases.

The values of kinetic parameters depend on such experimental conditions as, for example, the size of the emulsified particles and, therefore, the kinetic scheme given is applicable only to an "engineering" description of the behavior of a system under the given conditions. On the analogous treatment of the results for immobilized lipases, linearization takes place insatisfactorily. The peak of enzymatic activity is similar to the peak of soluble preparations (Fig. 3). This corresponds to the increase in the value of diffusion factors for the rate of the reaction that is usually observed on immobilization. The profiles of the pH activities of the lipases from fungus *Oospora lactis* have maxima at pH 7·4–7·8 (Fig. 4). No appreciable shift of the pH optimum appears on immobilization. The dependence of the initial rate on temperature is shown in Fig. 5*a*. The optimum temperature both for the soluble and the immobilized lipases is 37–39°C. The immobilized lipases differ from the soluble enzymes by their considerably greater heat stability (Fig. 5*b*). The time of half inactivation at 50°C increases more than 3 times for the immobilized preparations. Under the optimum conditions (concentration of emulsion 2·5%, temperature 37°C, pH 7·5), the immobilized lipase preparations obtained possess the activity 1 000 U/g.

EXPERIMENTAL

Chemicals: The extracellular lipase was obtained by precipitating the culture liquid with isopropylalcohol. The preparation contained 27·5% of protein and its activity was 2 800–3 100 U/g of protein (U = μmol min^{-1}). The substrate was a 40·0% emulsion of olive oil containing 2·0% of polyvinylalcohol. The particle size of the emulsion determined by observation under the microscope was 5–7 μm. The support-microcrystalline cellulose (Chemapol) and aminoethylcellulose (Olaine factory for chemical reagents), 0·05–0·25 mm fraction- and toluylene-2,4-diisocyanate (Merck), palmitoyl chloride (Merck) and other reagents were used without additional purification.

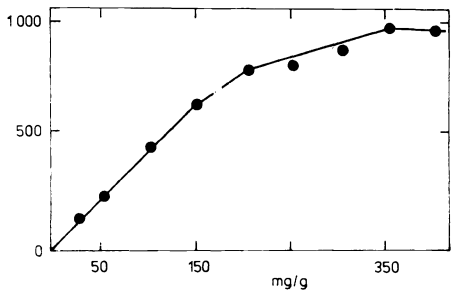


FIG. 2

Dependence of immobilized lipase activity (U/g) on the weight ratio (mg/g) of enzyme to carrier (AE-cellulose with palmitic acid)

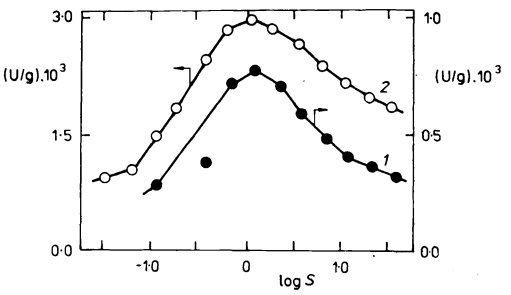


FIG. 3

Dependence of lipase activity upon concentration of the substrate (in %): *1* immobilized lipase (AE-cellulose with palmitic acid), right scale; *2* soluble lipase, left scale

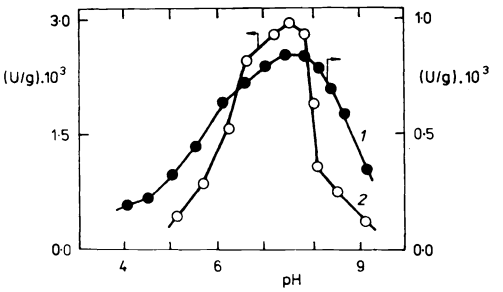


FIG. 4

Dependence of lipase activity on the pH: *1* immobilized lipase (AE-cellulose with palmitic acid), right scale; *2* soluble lipase, left scale

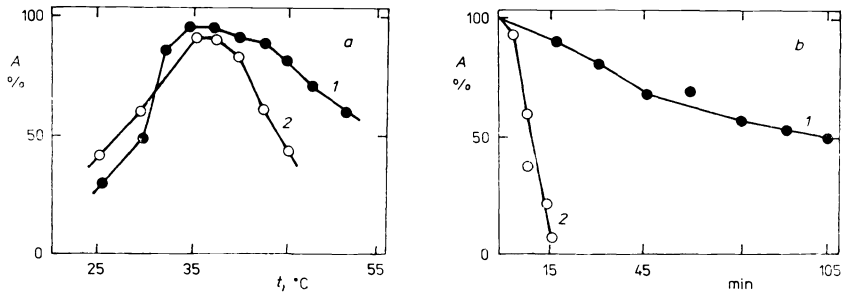


FIG. 5

Dependence of lipase activity on the temperature (*a*) and thermal stability of lipase (*b*) at 60°C. *1* Immobilized lipase (AE-cellulose wwith palmitic acid); 2 soluble lipase

Determination of the lipase activity: The pH-stat apparatus consisted of a pH-121 pH-meter, a BAT-15 automatic titraton unit, a B701 automatic burette, a KSP-4 electronic recording potentiometer and a digital voltmeter. A cell with a volume of 12 ml mounted on a magnetic stirrer and thermostated at 37°C was charged with 7·5 ml of water, 2·0 ml of 0·5M-KCl solution, and 0·2 ml of the substrate, and after the necessary thermostating and correction of the pH, 0·45 ml of enzyme solution (concentration of the preparation 10—20 mg/ml) or 20—200 mg (depending on the activity of the preparation) of the immobilized enzyme was added. Titration was carried out with 0·01M-KOH solution at pH 7·8. The activity of the soluble lipases was calculated from the slope of the steady-state section of the kinetic curve and that of the immobilized preparations by the Newton–Gregory extrapolation method. The activity of the immobilized enzymes is related to the dry weight of the preparation.

Modification of microcrystalline cellulose: A suspension of 10·0 g of microcrystalline cellulose in 10 ml of anhydrous dioxane was treated with 4·0 ml of toluylene-2,4-diisocyanate and five drops of triethylamine, and the mixture was stirred slowly at 80—90°C for 3—4 h. Then the cellulose was filtered off and washed on the filter with dioxane, suspended in 50 ml of 1-butanol and boiled for 4 h. In other experiments, there was 1-octanol instead of 1-butanol, a solution of palmitic acid (6·0 g/100 ml) or a solution of a mixture of palmitic acid (6·0 g) and sebacic acid (4·0 g) in dioxane. The support was washed with dioxane, ethanol and water.

Modification of AE-cellulose: A suspensions of 10·0 g of DEAE or AE-cellulose in 100 ml of anhydrous acetone was treated with 0·1 mmol of palmitoyl chloride, 0·03 mmol of triethyl amine and 5—6 drops of dimethyl formamide. The mixture was boiled for 4—5 h and filtered and the residue was washed by acetone and water.

Immobilization: To a solution of a lipase preparation (100 mg of protein) in 20 ml of 0·5M-KCl were added 2 g of support, and mixture was stirred on a shaking machine at 4°C for 18—20 h. The immobilized preparation was washed on the filter with a 0·1M-solution of KCl and by water. The preparations were stored in the form of aqueous suspensions containing a few drops of toluene at 4°C.

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