PREPARATION OF IMMOBILIZED LIPASES OF THE FUNGUS *Rhizopus microsporus* UzLT-1 AND THEIR PROPERTIES

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The study of the immobilization of enzymes is of great interest in connection with a broadening of 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. In this connection, it must be observed that the hydrolysis of emulsified lipids by an immobilized lipase is a special case, since both the enzyme and the substrate are present in a water-insuluble state. The aim of the present work was to obtain active preparations of immobilized lipase and to evaluate their catalytic properties and stability.

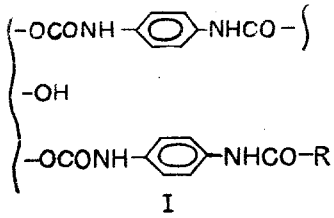
There have been several publications on the immobilization of lipases (E.C. 3.1.1.3.) [2-7]. When emulsified substrates are used, the activity of the immobilized enzymes is usually low.

We have used partially purified preparations of the lipases of the fungus *Rhizopus microsporus* UzLT-1: intracellular (IC-lipase) [8, 9] and extracellular (EC-lipase) [10]. 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 poly(vinyl 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 [11]. At the same time, the interaction of the support must not prevent the functioning of the "anchor section" of the enzyme [12, 13] which brings about reversible binding with the substrate.

Supports containing hydrophobic residues were obtained by modifying microcrystalline cellulose with 2,4-toluylene diisocyanate followed by the addition of compounds with mobile hydrogen atoms. 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 [14]. Subsequently, to the latter are attached compounds containing aliphatic chains of various lengths: n-butanol, n-octanol, and palmitic acid.

The schematic structure of the supports is shown by formula I. In one case we used an equimolar mixture of palmitic and sebacic acids to obtain a support containing negatively charged groups capable of removing the products of the hydrolysis of lipids.



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a. R=C_4H_9,
b. R=C_8H_{17},
c. R=$C_{15}H_{31}$,
d. R=$C_{15}H_{31}$ and $C_8H_{16}COOH$ (1:1).

The activities of the lipases immobilized on the supports I were as follows (initial ratio of protein to support A — 1:20; to B — 1:100):

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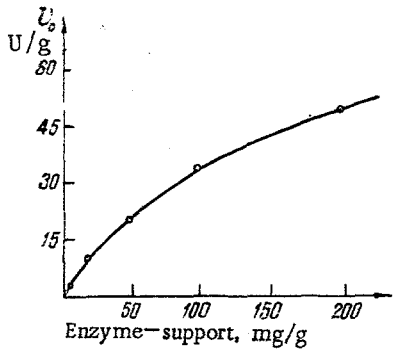

Fig. 1

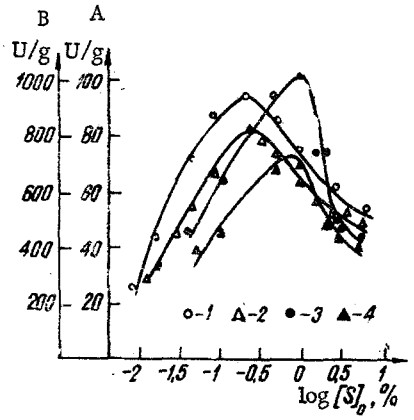

Fig. 2

Fig. 1. Dependence of the activity of immobilized EC-lipase on the initial weight ratio of enzyme to support.

Fig. 2. Dependence of the activity of the lipases on the concentration of the substrate: A — immobilized preparations; B — soluble preparations; 1) soluble IC-lipase; 2) soluble EC-lipase; 3) immobilized IC-lipase; 4) immobilized EC-lipase.

R	EC-lipase, U/g		IC-lipase, U/g
	A	B	A
C_4H_9	7.4	1.8	4.1
C_8H_{17}	5.3	1.7	10.5
$C_{15}H_{31}$	3.6	2.2	-
$C_{15}H_{31}$ and $C_8H_{16}COOH$ (11)	4.1	2.7	6.1

As follows from the results of immobilization, adsorption on hydrophobic supports provides the possibility of obtaining active preparations. The optimum degree of hydrophobicity is different for the two lipases. On additional cross-linking of the immobilized preparations with glutaraldehyde it was found that the glutaraldehyde (concentration 1%, temperature 4°C, time 2 h) completely inactivates both the EC- and the IC-lipases.

Active preparations of immobilized EC-lipases have been obtained previously by adsorption on DEAE-cellulose [15]. Consequently, it was of interest to use supports for immobilization that contained positively charged groups and hydrophobic residues simultaneously. These supports were obtained by the partial protection of the amino groups in AE-cellulose by the palmitoyl residue. The results show that such a combination is in fact effective.

Below we give figures for the activity of lipases immobilized on cellulose derivatives (initial weight ratio of enzyme to support 1:20):

Support	Ratio (molar) of AE-cellulose to palmitic acid	IC-lipase, U/g	EC-lipase, U/g
AE-cellulose	-	18	-
"	1:0.5	67	23
"	1:5	55	-
DEAE-cellulose	-	-	12.4

The activity of lipases adsorbed on the partially protected AE-cellulose was greater than with the use of unprotected or completely acylated AE-cellulose. Preliminary determinations of heat stability have shown that the preparations obtained, unlike those immobilized by adsorption on DEAE-cellulose [15], are fairly stable. When the palmitoyl residue was replaced by an n-butyl residue, the activity of the immobilized preparation was considerably less.

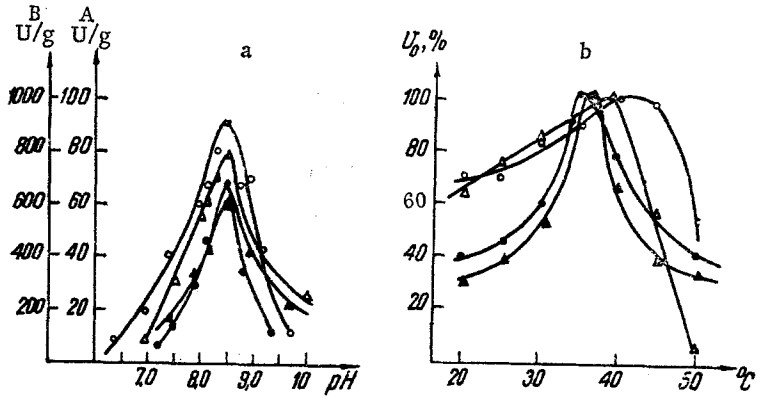

Fig. 3. Dependence of the activity of the lipases on the pH (a) and on the temperature (b). For an explanation of the symbols, see Fig. 2.

We determined in more detail the properties of the EC- and IC-lipases immobilized by adsorption on partially protected AE-cellulose. The dependence of the activity of the immobilized preparation of EC-lipase on the initial weight ratio of enzyme to support is illustrated in Fig. 1. The activity yield was 6-13%.

In the majority of cases, the kinetics of the lipolysis of emulsified substrates is described formally by a Michaelis—Menten-type equation in which, in place of the concentration of the substrate, the concentration of interphase surface is used [16, 17]. 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 in the case of the immobilized lipases inhibition by the substrate took place (Fig. 2). As a result of the treatment of the experimental results by graphical methods according to the kinetic scheme of inhibition by the substrate (II) [18] we obtained the kinetic parameters for the soluble lipases:

$$E + S \underset{K_S}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k} E + P$$
$$\Updownarrow$$
$$ES_2 \xrightarrow{2k} ES + P$$

$$V = \frac{\left(k = \frac{2k[S_0]}{K_S}\right)[E]_0 \cdot [S]_0}{K_m + [S]_0 + \frac{[S]_0^2}{K_S}} \quad \text{II}$$

Preparation	K_M, %	K_S, %	k, $M \cdot min^{-1} \cdot g^{-1} \cdot 10^{-6}$	β
IC-lipase	0.036	0.37	1220	0.33
EC-lipase	0.039	0.46	1010	0.44

The values of the 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 unsatisfactorily. The peak of enzymatic activity in the case of the immobilized preparations is found at higher concentrations of emulsion than in the case of the soluble preparations (see Fig. 2). 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 EC- and IC-lipases have maxima at pH 8.2-8.6 (Fig. 3a). No appreciable shift of the pH optimum appears on immobilization. The dependence of the initial rate on the temperature is shown in Fig. 3b. The optimum temperature both for the soluble and for the immobilized lipases is 35-40°C. The immobilized lipases differ from the soluble enzymes by their considerably greater heat stability (Fig. 4). The time of half-inactivation at 50°C increases more than 10-fold for the immobilized preparations.

Under the optimum conditions (concentration of emulsion 1%, temperature 35°C, pH 8.5), the immobilized lipase preparations obtained possess the following activities: IC-lipase — 122 U/g; EC-lipase — 93.5 U/g. The activity yields are, respectively, 32 and 13%. The observed difference in the activities of the two enzymes shows that the method of immobilization used corresponds more to the structural features of the intracellular enzymes, which are in fact membrane enzymes.

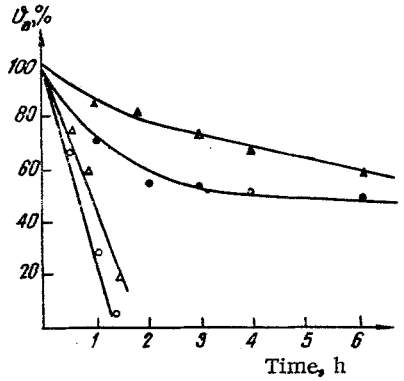

Fig. 4. Heat stability of the lipases: soluble preparations at 50°C, immobilized preparations at 60°C. For an explanation of the symbols, see Fig. 2.

EXPERIMENTAL

The extracellular lipase was obtained by precipitating the culture liquid with isopropanol. The preparation contained 23.2% of protein and its activity was 3.6 U/mg of protein (U — $\mu M \cdot min^{-1}$). The intracellular lipase was obtained by the acetone precipitation of an extract of the mycelium of the fungus. This preparation contained 24.0% of protein and had an activity of 3.9 U/mg of protein. The substrate was a 42.3% emulsion of olive oil containing 1.15% of poly(vinyl alcohol). The particle size of the emulsion, determined by observation under the microscope, was 5-10 μ. The supports — microcrystalline cellulose (Chemapol) and aminoethylcellulose (Olaine factory for chemical reagents), 0.05-0.25 mm fraction — and the 2,4-toluylene diisocyanate (Merck), palmitoyl chloride (Merck), and other reagents were used without additional purification.

Determination of the Lipase Activity. The pH-stat apparatus consisted of a pH-121 pH-meter, a BAT-15 automatic titration unit, a B701 automatic buret, 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 40°C was charged with 7.5 ml of water, 2.0 ml of 0.5 M 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 20 mg/ml) or 20-200 mg (depending on the activity of the preparation) of immobilized enzyme was added. Titration was carried out with 0.01 N KOH solution at pH 8.0. 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 given on the dry weight of the preparation.

Modification of the Microcrystalline Cellulose. A suspension of 5.0 g of microcrystalline cellulose in 50 ml of anhydrous dioxane was treated with 2.0 ml of 2,4-toluylene 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 it was washed on the filter with dioxane and was boiled in 50 ml of n-butanol for 4 h. In other experiments, in place of n-butanol we used n-octanol, a solution of palmitic acid (3.0 g/50 ml) or a solution of a mixture of palmitic acid (3.0 g) and sebacic acid (2.0 g) in dioxane. The support was washed with dioxane, ethanol, and water.

Modification of AE-Cellulose. A suspensions of 5.0 g of AE-cellulose in 50 ml of anhydrous acetone was treated with 0.14 ml of palmitoyl chloride, 0.08 ml of triethylamine, and 2-3 drops of DMF. The mixture was boiled for 4-5 h and filtered, and the residue was washed with acetone and water.

Immobilization. To a solution of a lipase preparation (50 mg of protein) in 10 ml of 0.5 M KCl was added 1 g of support, and the mixture was stirred on a shaking-machine at 4°C for 18-20 h. The immobilized preparation was washed on the filter with a 0.1 M solution of KCl and with water. The preparations were stored in the form of aqueous suspensions containing a few drops of toluene at 4°C.

SUMMARY

It has been shown that adsorption on AE-cellulose partially protected by the palmitoyl residue is an effective method of immobilizing the lipases of *Rhizopus microsporus*. The kinetics of the lipases are characterized by substrate inhibition. Immobilization considerably increases the heat stability of the lipases.

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PREPARATION AND PROPERTIES OF ENZYMES IMMOBILIZED ON SUPPORTS ACTIVATED BY METAL IONS

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The adsorption of enzymes forms the simplest method of immobilizing them [1, 2]. Recently, to improve the parameters of supports they have been modified by coating the surface with hydrophobic compounds [3], by the "grafting on" of hydrophobic radicals [4], or by treating the surface with transition-metal ions [4-15]. Such treatment of the sorbents leads to an increase in the amount of bound enzyme and to an improvement in the stability of the enzyme. The mechanism of the activation of supports by hydrophobic compounds has been studied in detail by Poltorak et al. [3], and the hydrophobic nature of the fixation of the enzymes has been shown. The nature of the activation of supports by metal ions has been little investigated, in spite of the fact that biocatalysts obtained in this way are of great practical interest [6, 11]. Moreover, this method of immobilizing enzyems is used mainly in the region of low pH values. The immobilization and properties of enzymes obtained at neutral pH values have not been studied.

Our aim was to investigate the immobilization of enzymes at neutral pH values on supports activated by transition-metal ions, and also to study the properties of the heterogeneous biocatalysts obtained.

On considering the immobilizaiton of enzymes at neutral pH values on supports activated by metal ions, as enzymes we selected the well-studied proteolytic enzymes α-chymotrypsin

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