BIOPHYSICS AND BIOCHEMISTRY

Immobilization of Lipase on Poly(N-Vinyl Pyrrolidone)

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 4, pp. 395-397, April, 2011
Original article submitted April 4, 2010

Immobilization of lipase from *Rhizopus niveus* on poly(N-vinyl pyrrolidone) was carried out and optimal conditions for manifestation of catalytic activity of this enzyme were determined. Kinetic aspects of substrate hydrolysis by free and immobilized lipase were studied.

Key Words: *lipase, poly(N-vinyl pyrrolidone), immobilization*

Lipase (EC 3.1.1.3), an enzyme hydrolyzing triglycerides, is used for enzyme replacement therapy and as the agent specifically destroying harmful metabolites in human body [1]. Since the use of immobilized enzyme preparation reduces their allergenicity and prolongs their action, the search for new carriers, which are also drugs, is of special importance. Poly(N-vinyl-pyrrolidone, PVP), an amorphous linear polymer obtained by radical polymerization and a component of blood substitutes, is applied to modify the properties of liposomes. Creation of PVP nanoparticles both with anti-angiogenic peptide (angiostatin and endostatin) and non-peptide (silymarin and silibinin) drugs considerably improves their antitumor effectiveness [3,5].

The purpose of the work was to evaluate the possibility of lipase immobilization on PVP.

MATERIALS AND METHODS

The object of research was the enzyme lipase from *Rhizopus niveus* (Sigma). Medical PVP with molecular weight of 10 kDa was used as the carrier molecule.

In order to immobilize lipases by adsorption, 10 mg carrier was added to 2 ml enzyme solution (10^{-5} mol/liter) and left for 2 h (25°C) with constant stirring.

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Molecular weight of free and immobilized lipase was determined by gel chromatography on Sephadex G-100 equilibrated with phosphate buffer (0.1 mol/liter, pH 7.0) using a 500-mm chromatographic column (20-mm diameter, 400 mm height of the gel layer). The volume of the sample applied to a column was 1 ml at a flow rate of 9 ml/h; 3-ml fractions were collected. Absorbance was measured on an SF-46 spectrophotometer at 280 nm. The chromatographic column was calibrated using catalase (230 kDa), BSA monomer (67 kDa), BSA dimer (134 kDa), and horseradish peroxidase (48 kDa).

Infrared absorption spectra of lipase were recorded on a Specord M-80 infrared spectrophotometer in the range of 4000-400 cm^{-1}.

During the analysis of absorption spectra of immobilized lipase, the method of comparison of IR spectra of the immobilized enzyme and IR spectra of the carrier and free lipase was used.

Protein content of the free enzyme preparation was determined by the method Lowry, protein estimation in the immobilized enzyme preparation was done by modified Lowry method. Catalytic activity of free and immobilized lipase was detected spectrophotometrically by the method of Anderson—McCarthy.

Hydrolysis of triglycerides was studied using UTU-4 thermostat connected to two fermentors and placed on a magnetic stirrer. In one fermentor, the enzyme and the substrate were incubated for 20 min

0007-4888/11/1514-0415

at pH 7.0 and 37°C. In the other fermenter, the same amount of phosphate buffer (pH 7.0) was placed instead of lipase solution. During the analysis of catalytic activity of immobilized enzyme preparation, the time of substrate hydrolysis was 40 min at 40°C.

Statistical analysis was performed using Student's *t* test.

RESULTS

We carried out adsorption immobilization of lipase on PVP. It was shown that the immobilized lipase retained 79% native enzyme activity.

The decrease in catalytic activity of immobilized lipase could be due to reduced mobility of the tertiary structure responsible for the formation of the enzyme-substrate complex.

To prove binding of the enzyme to the carrier, we performed gel chromatography of free and PVP-immobilized lipase. Chromatography showed that the enzyme was eluted in a single volume (molecular weight 96±3 kDa). Binding of lipase to the carrier (5×10^{-4} mol/liter) increased the molecular weight of the resulting complex to 110±3 kDa. After reducing PVP concentration to 5×10^{-6} mol /liter, we recorded two peaks with molecular weights of 96±3 and 110±3 kDa.

For analyzing the nature of enzyme-carrier interaction, IR spectra of lipase, carrier, and immobilized enzyme were recorded.

When interpreting spectrograms of free enzyme, the following bands were shown: amide I (1630-1690 cm^{-1}), amide II (1520-1560 cm^{-1}), stretching vibrations of NH_2-groups (3200-3400 cm^{-1}).

Amide I band resulting from stretching of carbonyl group bond indicated the presence of structures with hydrogen bonds in the native lipase molecule. Amide II band indicated the same position of α-helices and β-layers in the secondary structure of the enzyme. Fluctuations that generate it are related to CN-bond stretching and NH-bonds deformation.

In IR spectrum of PVP, the following most intense absorption bands were observed: vibration transitions

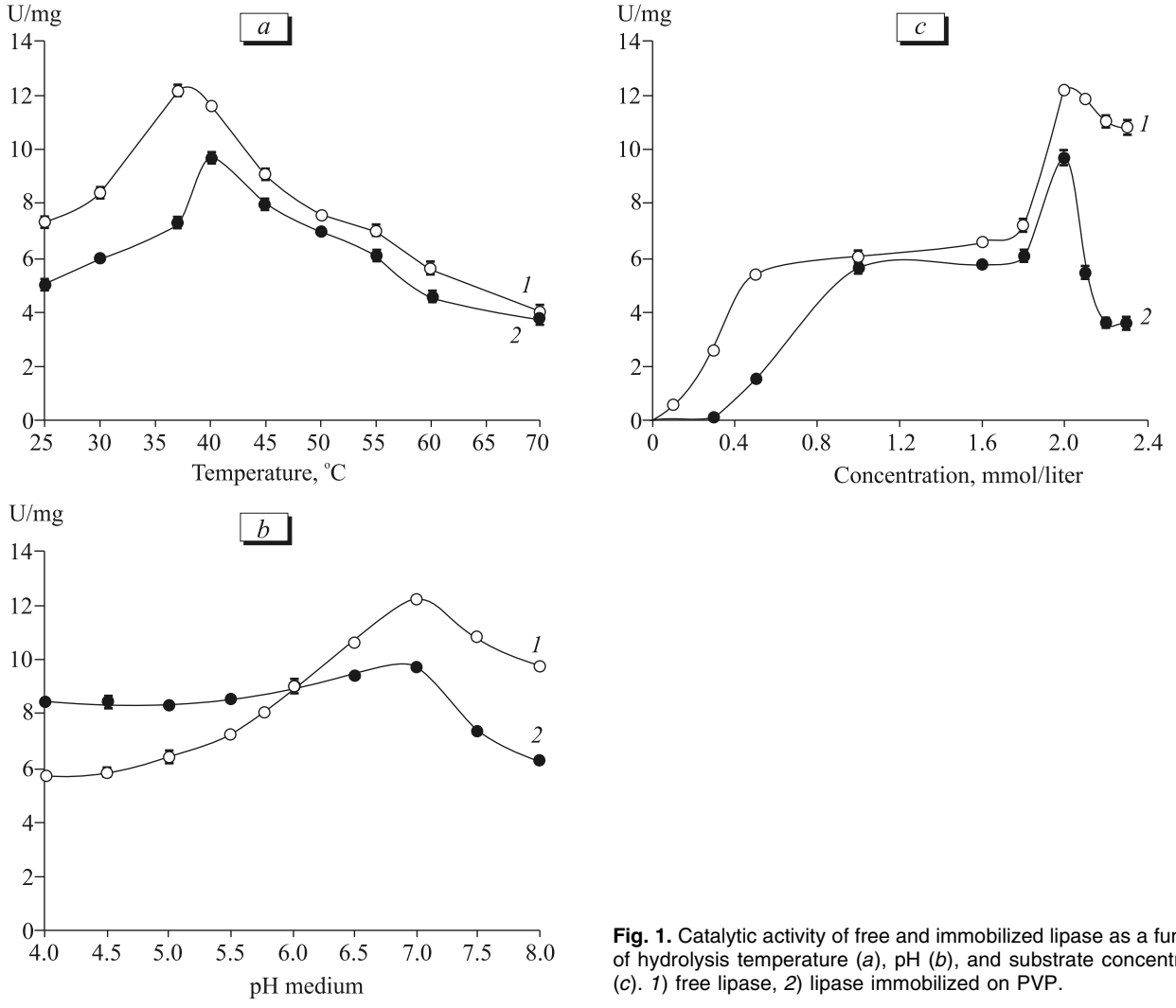

Fig. 1. Catalytic activity of free and immobilized lipase as a function of hydrolysis temperature (*a*), pH (*b*), and substrate concentration (*c*). *1*) free lipase, *2*) lipase immobilized on PVP.

TABLE 1. Kinetic Parameters of Enzymatic Triglycerides Hydrolysis by Free and Immobilized Lipase

Lipase	K_m ($K_{m'}$), mmol/liter	V_{max} ($V_{max'}$), μmol mg/min
Native	0.8	14
Immobilized on PVP	1.0	9.7

as a result of stretching of NH-bond (3200-3450 cm^{-1}), carbon—hydrogen bond stretching vibration (2800-3000 cm^{-1}), and C=O stretching vibrations (1641 cm^{-1}); planar vibrations of nitrogen molecules consisting of the pyrrole ring at a range of 900-1000 cm^{-1} were also recorded.

After absorption immobilization, the following absorption bands caused by the presence of lipase were detected in the spectrum of PVP: stretching vibrations of aliphatic primary alcohols (1045 cm^{-1}), absorption band of two substituted carbon atoms (835 cm^{-1}), vibrations of carbonyl groups at the end-associated COOH groups (1739-1970^{-1}), and amide II band (1578 cm^{-1}). Our findings suggest that binding of lipase to PVP is determined by both electrostatic interactions and hydrogen binding by positively charged nitrogen pyrrolidone loop carrier and negatively charged COO^- groups of lipase.

When studying immobilized enzymes, a key point is the analysis of physical and chemical characteristics of the obtained enzyme—carrier complexes.

It was revealed that the optimum temperature for hydrolysis of olive oil by free enzyme was 37°C. After binding the enzyme to the carrier, it shifted toward higher values and attained 40°C (Fig. 1, *a*).

After immobilization of the enzyme, its catalytically active conformation was fixed and the optimum temperature for enzymatic reaction increases with increasing the number of bonds. Analysis of the dependence of catalytic activity of free and immobilized lipase on pH of the substrate showed that free and PVP-immobilized lipase exhibits optimal catalytic activity at pH 7.0 (Fig. 1, *b*).

Catalytic activity of immobilized lipase was significantly higher than that of the native enzyme in the pH range of 4.0-6.0. This may be due to protonation of >C=O group of PVP pyrrolidone cycle [6] leading to changes in the interactions in lipase—carrier complex and promoting the formation of the enzyme—substrate complex at pH 4.0-6.0.

Kinetics of substrate splitting (Fig. 1, *c*) does not correspond to the Michaelis equation, which is consistent with published data [2]. Kinetic curves has several plateaus, which confirms preservation of the quaternary structure of the enzyme in the interaction with carriers during adsorption. Changes in the kinetics of enzymatic catalysis after immobilization may be indicative of minor conformational changes of individual subunits involved in triglyceride cleavage and increasing the degree of substrate inhibition [1].

Michaelis constant (K_m) and maximum reaction rate (V_{max}) of triglyceride hydrolysis by free enzyme and apparent V_{max} and K_m for immobilized samples were determined using Lineweaver–Burk, Hanes, and Eadie–Hofstee presentations of V–S curves (Table 1). It was shown that immobilization increases Michaelis constant and decreases the maximum reaction rate compared to those of native enzyme.

Analysis of the results of lipase immobilization on PVP allows us to consider it as a promising carrier for enzyme immobilization, in order to obtain soluble, stable against low pH conditions, and highly active drugs with prolonged action.

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