Multicomponent thermosensitive systems for biocatalysts

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Composite matrices based on macroporous silica modified by *N*-vinylcaprolactam copolymers with diallyldimethylammonium chloride and with 2-hydroxyethyl methacrylate were obtained. Lipase from *Pseudomonas fluorescens* was immobilized on the obtained materials. The temperature dependence of the hydrolytic activity of the immobilized lipase preparations in the triacetin hydrolysis was investigated. The hydrolytic activity of lipase immobilized on the matrix modified by the *N*-vinylcaprolactam copolymer with 2-hydroxyethyl methacrylate can be regulated by varying the temperature of the reaction medium. The temperature dependence of the hydrolytic activity of the immobilized enzyme has a maximum at 40 °C, the activity of the immobilized lipase being ~3.5 times higher compared to that at 20 °C. After immobilization on these composite materials, lipase retained the activity in the acetylation of 1-(*RS*)-phenylethanol with vinyl acetate in Bu^{t}OMe.

Key words: composite materials, lipase, immobilization, copolymers, *N*-vinylcaprolactam, diallyldimethylammonium chloride, 2-hydroxyethyl methacrylate, regulation of enzyme activity.

In recent decades, rapt attention of researchers is attracted by synthetic polymers able to change their conformation and properties in response to minor changes in the environment, in particular, temperature.[1] These smart polymers are used for enzyme and cell immobilization,[2] controlled delivery of drugs,[3] preconcentration of metal ions,[4] affinity precipitation,[5] and so on.

By using thermo- and/or pH-sensitive polymeric materials, one can prepare systems ensuring the retention of functional properties of the biomolecules incorporated into it and control the preparation activity by measuring the temperature and/or pH. A series of biocatalysts obtained by enzyme immobilization into gels based on thermosensitive polymers are currently known. These are, in particular, urease and alkaline phosphatase incorporated into poly(*N*-isopropylacrylamide),[6,7] trypsin in the poly(*N*-isopropylacrylamide)—methacrylic acid copolymer gel,[8] asparaginase and β-galactosidase incorporated into gels of poly(*N*-isopropylacrylamide) copolymer with acrylic acid,[9,10] and trypsin, B carboxypeptidase,[11] α-chymotrypsin[12] incorporated in the poly(*N*-vinylcaprolactam)-Ca-alginate gel. It was shown in the above-mentioned studies that enzymes immobilized in composite gels retain the catalytic activity in both aqueous and mixed water—organic media, as they are stabilized by the smart polymers incorporated in gels. In some cases, the enzymatic activity can also be regulated in these systems by varying the temperature. However, the accessibility of the active sites of the enzymes incorporated in the gel bulk usually decreases due to the diffusion restrictions for the transport of the substrate and the enzymatic reaction products. Apparently, immobilization of the enzymes on solid surfaces modified by thin layers of thermally sensitive polymers would increase the efficiency of the biocatalysts obtained, in particular, by increasing their mechanical strength, high sorption capacity and the lack of diffusion restrictions for substrate transport toward the enzyme. Thus it has been shown[13] that the adsorption of subtilisin on a silica surface results in a thousand-fold increase in the enzymatic activity. However, the use of such systems modified by smart polymers implies a more efficient regulation of the functional activity of immobilized biomolecules, in particular, by changing their hydrophobic-hydrophilic environment as the temperature changes. In this study, we prepared biocatalysts by using macroporous silica modified by thermosensitive copolymers of *N*-vinylcaprolactam with diallyldimethylammonium chloride (copolymer **1**) and with 2-hydroxyethyl methacrylate (copolymer **2**). These polymeric modifying agents were chosen due to the possibility of performing efficient modi-

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fication of the silica surface and some structural features that determine differences between the protein affinities of these compounds.

The methods for modification of porous silica matrices by these polymers were chosen from the following considerations. Previously,[14] it has been shown that poly(diallyldimethylammonium chloride) (polymer **3**) can be sorbed from solutions in aqueous alcohols on a bulk-porous silica to give a stable coating due to the charge difference between the support surface and the nitrogen-containing groups of the polymer. Procedures for the preparation of polymer-containing composite sorbents based on bulk-porous silicas by immobilization of polymers from solutions on the silica surface with solvent evaporation have been proposed.[15] We immobilized copolymer **1** on the silica surface by adsorption from a solution, while coating by copolymer **2** was performed by distilling off the solvent. The introduction of monomer units differing in the protein affinity into *N*-vinylcaprolactam-based copolymers may induce structural differences in the microenvironment of enzymes immobilized on polymer surfaces to produce biocatalysts. Previously, it has been shown[16] that silica sorbents modified by polymer **3** are suitable for the isolation of the total protein fraction from complex mixtures containing polysaccharides, nucleic acids, and monomeric compounds, together with proteins, because this polymer has an enhanced protein and peptide affinities. Conversely, the 2-hydroxyethyl methacrylate polymer is known to have low protein and peptide affinities; hence, it can be used, in particular, as a capillary modifier in the capillary electrophoresis, and nonspecific sorption in both organic[17] and aqueous[18] media is reduced.

As the model protein for the preparation of the thermosensitive biocatalyst, we chose the thermally stable lipase from the microorganisms *Pseudomonas fluorescens* (CF 3.1.1.3), whose activity does not change much over a broad temperature range.[19] In addition, lipases occupy a special place in the series of hydrolytic enzymes used as catalysts of organochemical transformations, as they can be used both in the hydrolysis of lipids and esters and in the enantioselective esterification and transesterification in organic solvents or in water-organic media.

Experimental

The study was performed using the Trisopor-500 macroporous silica with an average pore diameter of 50 nm, a specific pore volume of 1.2 $cm^3\ g^{-1}$, and a specific surface area of 108 $m^2\ g^{-1}$ (Shuller GmbH, Germany); *N*-vinylcaprolactam, diallyldimethylammonium chloride, 2-hydroxyethyl methacrylate (Sigma—Aldrich GmbH, Germany); 2,2′-azobis(isobutyronitrile) (AIBN), Bu^{t}OMe, vinyl acetate, 1-(*RS*)-phenylethanol (Fluka Chemie AG, Switzerland); triacetylglycerol (triacetin) (Merck, Germany); chemically pure grade methanol, acetone, inorganic salts, and sodium hydroxide (Reachim, Russia). The commercial preparation of lipase from *Pseudomonas fluorescens* was provided by the RÖHM Pharma Polymers company (Germany); the specific activity of the initial sample with respect to triacetin was 120 (mmol of the product) h^{-1} (g of the preparation)$^{-1}$.

Radical copolymerization of *N*-vinylcaprolactam with diallyldimethylammonium chloride and 2-hydroxyethyl methacrylate. The reaction was carried out in anhydrous MeOH in the presence of AIBN as an initiator under oxygen-free conditions for 16 h at 60 °C with gentle stirring. The *N*-vinylcaprolactam-enriched fractions of the copolymers were precipitated with acetone. The unreacted *N*-vinylcaprolactam and acetone-soluble fractions were removed by the thermal precipitation of copolymers at 75 °C. The total yields of the acetone-insoluble fractions of copolymers **1** and **2** determined by gravimetry were 76 and 83% of the initial content of comonomers in the mixture, respectively.

To estimate the weight-average molecular masses of copolymers **1** and **2**, their diffusion coefficients (*D*) were determined in solutions (in water for copolymer **1** and in methanol for copolymer **2**) at 20 °C. The resulting *D* values were substituted into the Polson equation

$$D = a\mathrm{M}^{-1/3},$$

where $a = 2.74 \cdot 10^{-5}$,[20] and the weight average molecular masses were calculated.

The structure of copolymer **1** was determined by ^{13}C NMR spectroscopy. ^{13}C NMR (D_2O), δ: 123 (C=O); 35.4 (NMe); 34.4 (α-CH_2); 32.5 (δ-CH_2); 29.7 (γ-CH_2); 27.4 (β-CH_2); 23.1 (ε-CH_2); 16.2 (Me); 15.9 (CH_2); 6.8 (CH). The structure of the water-insoluble copolymer **2** was determined by IR spectroscopy (film from MeOH). IR, ν/cm^{-1}: 1400 (CH_2OH); 1650 ($CONR_2$); 1700 (CO); 2900 (CH_2). The composition of copolymers was confirmed by elemental analysis. Copolymer **1**. Found (%): C, 64.86; H, 9.59; Cl, 9.59; N, 10.00; O, 6.49. Copolymer **2**. Found (%): C, 67.42; H, 9.18; N, 8.89; O, 14.39.

The lower critical solubility temperatures (LCST) of the copolymers were determined based on light scattering using a Coulter N4 submicron particle analyzer (France).

The temperature dependence of hydrophobic-hydrophilic properties of the copolymers was studied by the capillary method using an improved procedure[21] in which the capillaries with an inner diameter of 2 mm were filled with solutions of copolymers (0.5 g ml^{-1}) in MeOH and then the solvent was evaporated *in vacuo*. Then the capillaries were immersed into a dish with water at different temperatures and the height of the liquid column was rapidly measured.

Preparation of the composite sorbents. The specified weight portions of the Trisopor-500 macroporous glass were placed into an evacuated reactor. The samples (10 g) were evacuated to remove air and the vapor that condensed during the storage of silica. The layer of the support particles was wetted with 60 mL of a solution of a thermosensitive copolymer with a concentration of 0.033 g mL^{-1} (in the case of copolymer **1**, an aqueous solution was used and for copolymer **2**, a solution in MeOH). The resulting suspension was treated in an ultrasonic bath for 15 min to attain a more uniform distribution of macromolecules over the sorbent pore bulk. When immobilizing copolymer **1** containing diallyldimethylammonium chloride residues on the

support surface, the suspension was kept for 3 h with gentle stirring on a magnetic stirrer and the sorbent thus produced was washed with water (a 10-fold volume) and acetone (a 10-fold volume) on a Büchner funnel. For immobilization of copolymer **2** containing 2-hydroxyethyl methacrylate residues, the solvent was evaporated using a rotary evaporator. The resulting sorbent was also washed with water (a 10-fold volume) and acetone (a 10-fold volume). The products were dried *in vacuo* at 30 °C to a constant weight.

To estimate the strength of retention of the polymer modifiers by the support surface, the weight portions of the sorbents (300 mg) were incubated in water for 1 h at 20, 30, and 50 °C, the sorbent particles were precipitated by centrifuging for 10 min at 3000 rpm, supernatants were withdrawn, and UV spectra were recorded on a Beckman DU-70 spectrophotometer (USA).

The morphology of the resulting material was studied by mercury porosimetry on a PoreSizer 9300 instrument (Micromeritics, USA).

Determination of the hydrolytic stability of the sorbents. The hydrolytic stability of the obtained materials was estimated by incubating samples of the initial silica and the composite sorbents (1 g) in 10 mL of a buffer solution (pH 9.5) containing sodium borate (0.025 mol L^{-1}) and NaOH (0.1 mol L^{-1}) for 8 h at ~20 °C. The aliquot portions (0.25 mL) taken every hour were mixed with equal volumes of a 0.05 *M* solution of sodium molybdate acidified with conc. H_2SO_4 (1/200 v/v with respect to the sodium molybdate solution), and then the absorbance was measured on a Beckman DU-70 spectrophotometer (USA) at $\lambda = 320$ nm.

Lipase immobilization on a composite sorbent. To prepare the biocatalyst, a sample (1 g) of the obtained composite material was re-suspended in a 3 mL of a lipase solution (50 mg in a 0.2 *M* solution of KH_2PO_4, pH 7.5), and the mixture was incubated for 24 h at 4 °C with gentle stirring. The resulting suspension was centrifuged for 10 min at 3000 rpm and the supernatant was withdrawn. The biocatalyst was washed on a Schott filter with a 20-fold volume of the phosphate buffer and subjected to freeze-drying. The enzyme content incorporated in the composite material was calculated from the residual hydrolytic activity of the enzyme in the supernatant and in the filtrates.

Determination of the hydrolytic activity of the enzyme. Triacetin (2 mL) was dissolved in 100 mL of a solution of NaCl (0.05 mol L^{-1}) and $CaCl_2$ (0.05 mol L^{-1}) in water (pH 7.0). The resulting solution of the substrate (5 mL) was placed in the cell of a Radiometer Copenhagen TTT80 titrator and 50 μL of a solution containing 5 μL of the enzyme or 120 mg of the immobilized lipase (containing 5.88 μg of the enzyme in the biocatalyst modified by copolymer **1**, or 4.44 μg of the enzyme in the biocatalyst modified by copolymer **2**). The acetic acid formed in the enzymatic reaction was titrated with a 0.01 *M* solution of NaOH at pH 7.0 for 10—15 min. The results of titration were used to calculate the hydrolytic activity of the enzyme in (μmol AcOH) h^{-1}.[22] The scatter of the values did not exceed 2.6%.

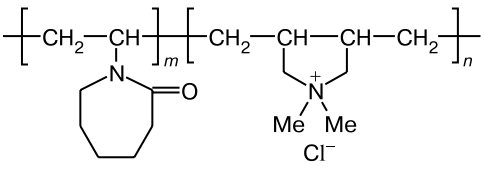
Determination of the esterification activity of the enzyme. The lipase activity in esterification was determined from the initial rate of 1-(*RS*)-phenylethanol acetylation with vinyl acetate to give 1-(*R*)-phenylethyl acetate. The initial lipase preparation (14 mg; protein content, 35 μg) or the solid support (100 mg; protein contents of 4.9 and 3.7 mg for the biocatalysts based on silicas modified by copolymers **1** or **2**, respectively) was placed into a conical flask containing 10 mL of Bu^tOMe containing 5 mmol of the racemic substrate, 1-(*RS*)-phenylethanol, and 15 mmol of vinyl acetate. The suspension was shaken with a velocity of 200 vibrations per min at 20—40 °C. Aliquots (0.5 mL each) were taken from the reaction mixture. The solid catalyst particles were separated on a centrifuge and the supernatant was analyzed by HPLC on a Beckman liquid chromatograph (System Gold, USA) with a LiChroCART RP 18 column (5 μm) (240×4 mm), Merck (Germany). The absorbance was recorded at $\lambda = 260$ nm at a flow rate of 0.8 mL min^{-1} using a MeCN—water mixture (1 : 1) for elution. The retention times of 1-(*RS*)-phenylethanol and 1-(*R*)-phenylethyl acetate were 5.9 and 13.5 min, respectively. The results were used to calculate the esterification activity of the enzyme in (μmol 1-(*R*)-phenylethyl acetate) h^{-1}. The scatter did not exceed 2%.

Results and Discussion

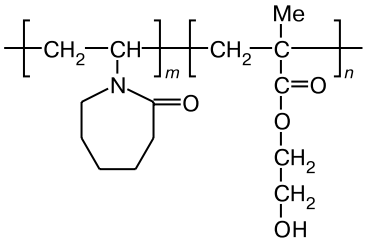
We synthesized polymeric modifiers **1** and **2** by radical copolymerization. The model composite biocatalysts were prepared using random copolymers of *N*-vinylcaprolactam containing, according to elemental analysis, 40% diallyldimethylammonium chloride or 12% 2-hydroxyethyl methacrylate. Copolymer **2** swells in water and dissolves upon the addition of 40% (v/v) MeOH.

The weight-average molecular masses calculated from the Polson equation[20] using the diffusion coefficient found experimentally by correlation laser spectroscopy were 20000 and 25000 for copolymers **1** and **2**, respectively.

The structures of copolymers **1** and **2** were established by ^{13}C NMR and IR spectroscopy, respectively. The data thus obtained were compared with the spectra of the poly(*N*-vinylcaprolactam) homopolymer (homopolymer **4**). As the analytical ^{13}C NMR signals of copolymer **1**, we used the signal at δ ~29 from the methylene bridge, which links the ring units of the macromolecule of polymer **3**, and the multiplet signal at δ 47—50, corresponding to the α-C atom in macromolecule **4**. The presence of diallyldimethylammonium chloride residues in the copolymer results in a lower intensity of the signal at δ 47. In addition, the broad line at δ 180.3 corresponding to the lactam carbonyl in homopolymer **4** decreases and a weak signal at δ 181—182 simultaneously increases. The spectra of the obtained copolymers exhibit additional signals at δ 27 and 32.5; the group of signals at δ 30—31.5 becomes more intense, which, in our opinion, is also indicative of the appearance of the methylene group adjacent to the β-C atom of the *N*-vinylcaprolactam unit and the methylene group near the α-C atom of this unit. Analysis of the IR spectra of copolymer **2** showed well-resolved peaks at 1400 cm^{-1} corresponding to the absorption of hydroxyethyl groups of 2-hydroxyethyl methacrylate, in addition to the absorption peaks at 1650, 1700, and 2900 cm^{-1}, typical of homopolymer **4**. Thus, the IR-spectroscopy data confirmed the formation of copolymers under these conditions. The structural formulas of the copolymers are presented below.



Copolymer **1** ($m : n = 4 : 1$)



Copolymer **2** ($m : n = 9 : 1$)

The LCST for a solution of copolymer **1** was 38—39 °C. For a solution of copolymer **2**, a sharp change in the turbidity with an increase in the temperature was observed at 32—33 °C. For comparison, the LCST for a solution of homopolymer **4** (M = 20000) was determined by this method; this was equal to 37 °C. The influence of the temperature around the LCST on the hyrophobic-hydrophilic properties of copolymers **1** and **2** was also studied. This was done using an improved procedure[21] based on the measurement of the height of the liquid meniscus in capillaries with inner walls modified by the layer of the thermosensitive polymer. The height of the liquid column in the capillaries treated with copolymers **1** and **2** proved to be lower than the value found for the untreated capillary (32 mm), being equal to 31 and 28 mm in cold water and 25 and 20 mm in hot water (70 °C), respectively. These data indicate the ability of the thermosensitive copolymers to change the hydrophilic-hydrophobic balance on passing the LCST. A more pronounced decrease in the height of the liquid column in the capillary treated with copolymer **2** attests to its higher hydrophobicity with respect to copolymer **1**. Thus, on phase transition due to temperature rise above the LCST, copolymer **2** loses the hydration shell to a greater extent.

Copolymer **1** was immobilized by incubating the support in an aqueous solution of the copolymer. This was accompanied by spontaneous sorption of the copolymer on the support surface due to the charge difference between the silica surface and the diallyldimethylammonium chloride units incorporated in the copolymer. Water-insoluble copolymer **2** was deposited onto the support surface from a methanol solution followed by solvent evaporation. The contents of the polymer modifiers **1** and **2** in the obtained composite materials were 12 and 15%, respectively.

The morphology of the polymer-containing composite sorbents was studied by mercury porosimetry. For samples of both sorbents, similar porosigrams were obtained (Fig. 1). It follows from their analysis that not only the external surface of particles but also the internal surface of the pores is modified by the polymer, which is accompanied by a decrease in the average pore diameter from 48 to 43 nm. Thus, the average effective thickness of the polymer coating was ~25 Å. In addition to the decrease in the average diameter, their specific volume also decreases (from 1.2 to 0.87 and 0.78 $cm^3\ g^{-1}$ for copolymers **1** and **2**, respectively), which is also indicative of immobilization of the polymer layer on the inner surface of the pores. The porosity of the support is largely retained. The presence of a continuous polymer film on the pore surface is also confirmed by testing the stability of the resulting materials against alkaline hydrolysis. The incubation of the nonmodified support samples and the materials obtained in an alkaline buffer (pH 9.5) for 4 h resulted in 7 and 9 times greater amounts of silicic acid in the case of nonmodified silica than in the case of samples containing copolymers **1** and **2**, respectively.

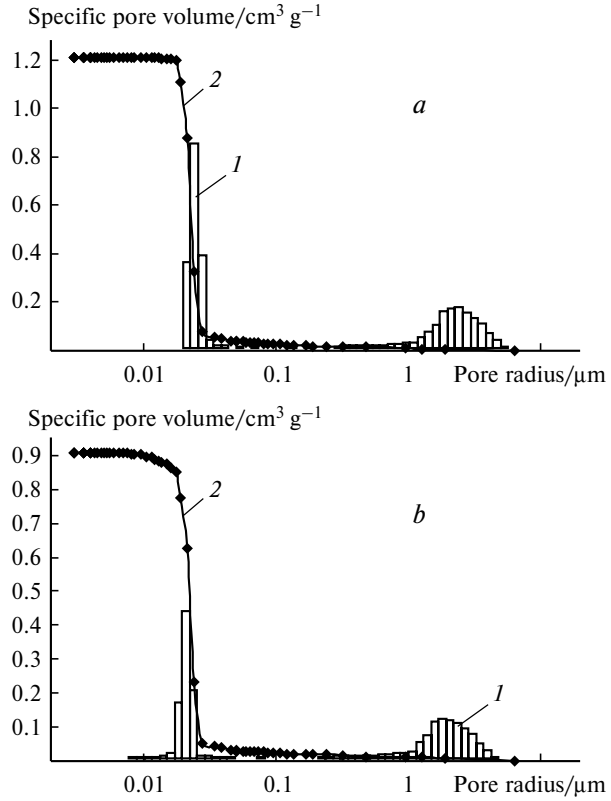


Fig. 1. Differential (*1*) and integral (*2*) pore distribution over effective radii (mercury porosigrams) for nonmodified silica (*a*) and silica modified by copolymer **1** (*b*).

The composite sorbents obtained are able to efficiently bind lipase with retention of its enzymatic activity. The degree of inclusion of the enzyme into silica-based sorbents modified by copolymers **1** and **2**, determined from the residual activity of the supernatants and the filtrates obtained by washing of the biocatalysts after lipase immo-

Table 1. Change in the lipase enzymatic activity in the obtained biocatalysts depending on temperature

T/°C	Activity[a]		
	hydrolytic[b]		esterification[c]
	copolymer **1**	copolymer **2**	copolymer **2**
20	37000	7300	1340
25	37100	11300	1350
30	36600	17800	1380
35	36200	20600	1400
40	36600	27000	1400
45	35500	21400	1400

[a] Expressed in (μmol of the product) h^{-1} (mg of the enzyme)$^{-1}$.
[b] The activity of the initial lipase was 52000 μmol h^{-1} (mg of enzyme)$^{-1}$ at 20 °C.
[c] The activity of the initial lipase was 1500 μmol h^{-1} (mg of enzyme)$^{-1}$ at 20 °C.

bilization, was 98 and 74%, respectively. The greater inclusion of lipase into the sorbent based on silica modified by copolymer **1** is, apparently, due to the electrostatic interaction of the lipase macromolecule, which is negatively charged at neutral pH (p*I* 4.46),[23] with the positively charged diallyldimethylammonium chloride on the matrix surface.

The variation of the hydrolytic activity of lipase in the biocatalysts obtained as a function of temperature is depicted in Table 1. These data indicate, in particular, that the hydrolytic activity of lipase incorporated in the sorbent modified by copolymer **1** varies insignificantly (the scatter does not exceed 2.6%) over the temperature range under study (20—45 °C). The use of the silica-based sorbent modified by copolymer **1** does not seem to allow regulation of the enzymatic activity by varying the temperature under these conditions. This can be due to the fact that the lipase molecules are accumulated in the surface sections composed of poly(diallyldimethylammonium chloride) (Fig. 2, *a*), which are not involved in the phase transition on temperature change, unlike the sections containing mainly *N*-vinylcaprolactam residues. Therefore, the activity of the lipase within the biocatalyst does not increase substantially on temperature rise.

The possibility of regulating the enzymatic activity by changing the temperature is demonstrated in relation to the biocatalyst based on silica modified by copolymer **2**. It follows from the data presented in Table 1 that the dependence of the enzymatic activity of immobilized lipase toward hydrolysis passes through a maximum at 40 °C (the activity increases more than 3.5-fold with respect to the activity at 20 °C), then the activity decreases somewhat still remaining ~3 times higher at 45 °C than at 20 °C (Fig. 3). This pattern of dependence of the lipase activity on the temperature is apparently due to enzyme immobilization on the fragments of copolymer macromolecules containing poly(*N*-vinylcaprolactam), because the sections containing 2-hydroxyethyl methacrylate residues have a low protein affinity (see Fig. 2, *b*). The lack of a narrow temperature range in which the catalyst activity would sharply change is probably related to the random nature of copolymer **2**. Apparently, a sharp activity change can be expected upon modification of the supports by block or graft thermosensitive copolymers.

The phase transition that takes place as the temperature increases above the LCST of the thermosensitive copolymer and determines the change in the activity of the biocatalyst is caused by the presence of *N*-vinylcaprolactam residue in the copolymers and is accompanied by changes in the structure of the hydration shell of the copolymer macromolecule. One should expect that in nonpolar media without a hydration shell, no activation of immobilized lipase will be observed. Indeed, the esterification activity of lipase incorporated in the biocatalyst based on copolymer **2** in an organic solvent (Bu^{t}OMe) in the 20—45 °C range changes insignificantly (from 89.3 to 93.9%) with respect to the activity of native lipase (see Table 1).

Thus, no temperature sensitivity of the systems studied is observed in an anhydrous medium.

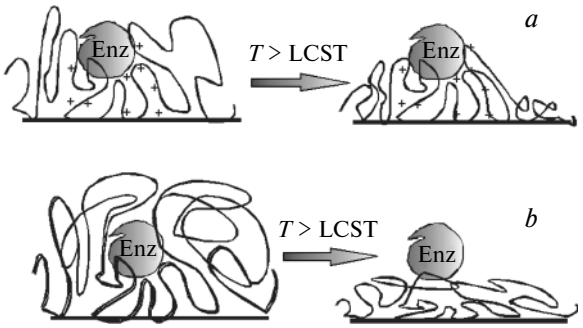


Fig. 2. Schematic diagram illustrating the degree of accessibility of the lipase immobilized on the surface of sorbents modified by copolymers **1** (*a*) and **2** (*b*) depending on temperature.

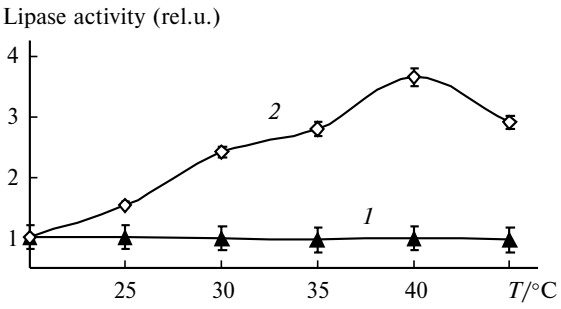


Fig. 3. Variation of the enzymatic activity of lipase in the triacetin hydrolysis: initial lipase (*1*) and lipase immobilized on silica modified by copolymer **2** (*2*) (lipase activity at 20 °C is taken to be unity).

The relatively low activity of lipase incorporated in the sorbent modified by copolymer **2** attests to the need to optimize the ratio of the *N*-vinylcaprolactam to 2-hydroxyethyl methacrylate residues in the macromolecules of the modifying agent.

Thus, these studies resulted in the preparation of biocatalysts based on polymer-modified silicas with immobilized lipase from the microbes *Pseudomonas fluorescens*. The possibility of regulating the catalytic activity of the resulting material in aqueous media on changing the temperature was demonstrated.

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