Relationship between the hydration degree of poly-*N*-isopropylacrylamide gel and activity of immobilized α-chymotrypsin

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The influence of the pH, temperature, and dimethyl sulfoxide concentration on the hydration degree of the poly-*N*-isopropylacrylamide gel and the activity of α-chymotrypsin immobilized into the polymer was studied. The behavior of more hydrophilic preparations based on polyacrylamide and copolymer of acrylamide and acrylic acid was studied for comparison. An increase in both the temperature and dimethyl sulfoxide content decreases the hydration of the poly-*N*-isopropylacrylamide, which correlates with a decrease in the activity of the immobilized enzyme. The use of substrates with different structures and an irreversible inhibitor proves that the change in the properties of α-chymotrypsin immobilized into the poly-*N*-isopropylacrylamide gel is related to the change in the rate constants of enzymatic reactions. Comparison of all experimental data obtained suggested an opportunity of local interactions between the protein globule and polymeric chains with a change in the hydration degree of poly-*N*-isopropylacrylamide during its phase transition.

Key words: poly-*N*-isopropylacrylamide, phase transition, hydration degree of gel, α-chymotrypsin, enzyme activity.

It is known that the activity of a biocatalyst often depends on the state of its microenvironment: a change in this state changes the enzyme activity. This assertion can be illustrated by examples of enzyme-containing systems both *in vivo*[1—3] and *in vitro*.[4] The main route of regulation of the activity of membrane-immobilized enzymes in living nature is the phase transition "solid—liquid crystal" (melting) of phospholipid membranes (supports of a biocatalyst) with temperature changing.[5] Thus, the lipid membrane (matrix-support) acts as a unique "antenna," *i.e.*, it receives the temperature signal, changes its state, and regulates the enzyme activity of immobilized enzymes through this changed state. It is remarkable that similar phase transitions are reversible. This implies a possibility of reversible regulation of the enzyme activity with changing the temperature around the melting point of the membrane-support. Is it possible to model artificially the natural scheme of regulation of the biocatalyst activity?

For the class of the so-called stimulus-sensitive polymers,[6] the energy parameters of chain interaction change as a response to some physical (temperature, light) or chemical (pH, concentration of an organic solvent) effect (stimuli). As a result, solvent molecules are displaced from the solvate shell of the polymer by the polymer units (this is the phase transition from the viewpoint of physicochemistry[7]). For linear water-soluble polymers, the phase transition, exfoliation of the true solution, occurs usually in a very narrow interval of changing the external signal. For three-dimensional polymeric networks (hydrogels), the phase transition is expressed as a change in the hydration degree (compression), *i.e.*, a decrease in the linear sizes and weight proportionally to the amount of water "extruded" from the sample.[8] This process depends on many factors (nature and concentration of the monomer, concentration of the cross-linking agent, *etc.*) and can occur in a rather wide interval of the external effect.[9] Temperature-sensitive hydrogels are representatives of the described above family of materials. Note that the phase transition in these polymers is reversible, as in the case of natural supports of biocatalysts (lipid membranes).

The first studies devoted to the activity of asparginase immobilized into the thermosensitive poly-*N*-isopropylacrylamide gel showed an interesting result: breaks and even regions with the apparent negative activation energy of the reaction appeared in the plots of the logarithm of the enzymatic reaction rate *vs.* inverse temperature.[10,11] Later similar data were demonstrated for α-chymotrypsin,[12] alkaline phosphatase,[13] urease,[14] *etc.*, and the change in the activity of the immobilized enzyme was manifested in the region of temperatures corresponding to those of the phase transition of the polymeric matrix.[10,11] Pronounced analogies in the temperature behavior of enzymes incorporated into membranes and immobilized into thermoreversible gels allowed the assumption about the resembling character of the influence of the phase transition of the matrix on the activity of the immobilized biocatalyst. However, it remains unclear whether the activity of the immobilized

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enzyme is really regulated by the precisely phase transition of the matrix-support (and the corresponding change in its hydration degree) or this phenomenon is related to an increase in the density of the polymeric network regardless of the mechanism of its compression (for example, due to the compensation of the electrostatic repulsion of the likely charged groups in the polymer composition). The search for the answer to this question is the subject of this study.

The interrelation between the hydration degree of the poly-*N*-isopropylacrylamide gel that undergoes the phase transition and the activity of immobilized α-chymotrypsin. Unlike methods described in literature, the compression of this matrix was induced by the temperature increase and also in an increase in the concentration of dimethyl sulfoxide in the reaction mixture. In this case, substrates were used for which the native enzyme in water—DMSO mixtures demonstrates different kinetic behavior. Comparative kinetic analysis of the activities was performed using both native α-chymotrypsin and that immobilized in the non-compressible polyacrylamide gel. The influence of the density of the polymeric network on the enzyme activity was studied using the pH-sensitive preparation based in the copolymer of acrylamide and acrylic acid, which does not undergoes the phase transition. In this case, the gel compression occurs due to the compensation of the electrostatic repulsion between the acidic groups in the polymer composition with a decrease in the pH.

Experimental

α-Chymotrypsin (A trade mark, OAO "Samson," Russia), acrylamide (AAm), acrylic acid (AAc), *N*,*N*′-methylenebisacrylamide (MBA), ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (Reanal, Hungary), *N*-acetyl-L-tyrosine ethyl ester (ATEE), and *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA) (BDH, England), phenylmethylsulfonyl fluoride (Sigma, USA), dimethyl sulfoxide (OAO "Khimreaktivkomplekt" plant, Russia), and acryloyl chloride (Merck, Germany) were used. *N*-Isopropylacrylamide (NIPAA) was synthesized by a known procedure.[15] Salts and components of buffer solutions used were not worse than analytical grade (Reakhim, CIS).

Immobilized preparations of α-chymotrypsin. The monomer (AA) (200 g, NIPAA, AAm/AAc 85/15) and cross-linking agent (MBA; [MBA]/[monomer] 1/750 (mol L^{-1})/(mol L^{-1}) were added to a solution (1 mL) of the enzyme (5 mg mL^{-1}) preliminarily modified by acryloyl chloride.[12] The reaction was initiated by the addition of an 0.78 *M* aqueous solution (0.02 mL) of ammonium persulfate and TEMED (0.01 mL), and block copolymerization was carried out at ~20 °C.

Determination of the activity of α-chymotrypsin. In order to measure the activity of preparations, the obtained block-copolymer was crushed in a homogenizing reactor to particles of 20—150 μm in size and washed with a salt solution (0.2 *M* NaCl + 0.02 *M* $CaCl_2$) to the complete absence of activity in washing water. The activity of α-chymotrypsin preparations was determined from the initial rates of hydrolysis of the corresponding substrate at the pH-optimum of effect of the specified sample. In the case of ATEE, the potentiometric method was used: the hydrolysis rate was measured in a thermostatted cell of an RTS-822 pH-stat (Radiometer, Denmark) by the titration of the *N*-acetyl-L-tyrosine that formed with an alkali; the ionic strength being created by a salt solution (0.2 *M* NaCl + 0.02 *M* $CaCl_2$). In the case of BTNA, the activity of preparations in a 0.05 *M* borate buffer was measured on a UV-265FW spectrophotometer (Shimadzu, Germany); the extinction coefficient of the reaction product (*p*-nitroanilide) was determined in an independent experiment for each used concentration of an organic solvent. The concentrations of the substrate and DMSO, temperature, and pH of the solution were varied.

In the first approximation, the mechanism of enzymatic hydrolysis of substrates with α-chymotrypsin is close to that of the effect of a heterogeneous catalyst[16]: the equilibrium stage of enzyme-substrate complex formation followed by the kinetic stage of hydrolysis to form the reaction products and active enzyme regeneration

$$E + S \rightleftharpoons ES \longrightarrow E + \Sigma P,$$

The plot of the observed reaction rate *vs.* concentration of the reactants is described by the Michaelis—Menten equation

$$V = V_{max}[S]_0/(K_m + [S]_0),$$

where $[S]_0$ is the substrate concentration; $V_{max} = k_{cat}[E]_0$ ($[E]_0$ is the enzyme concentration; k_{cat} is the turnover number of the catalyst per unit time, *vz.*, the maximum reaction rate at the complete saturation of the enzyme with the substrate at $[S]_0 \gg K_m$; and K_m is the Michaelis constant numerically equal to the substrate concentration at which a half of the maximum reaction rate is achieved. The V_{max} and K_m parameters for the hydrolysis of the substrates with various preparations of α-chymotrypsin were determined from the plots of the initial hydrolysis rates *vs.* substrate concentration in the double inverse Lineweaver—Burk coordinates ($1/V - 1/([S]_0)$).

Hydration degree of gels. In order to measure the hydration degree of gels, the block-copolymer samples were cut to plates of 10×2×5 mm. The samples were equilibrated under specified conditions for 3 h. The temperature, concentration of DMSO in the mixture, and pH of the solution (0.05 *M* acetate-ammonia, 0.05 *M* Na-phosphate, and 0.05 *M* borate buffers for pH intervals of 4—6, 5.5—8, and 7.5—10.5, respectively) were varied. The hydration degree of gels was determined as a ratio of the gel weight under certain conditions (m) to the gel weight under the conditions of maximum swelling of the sample (m_0) and expressed in %.

Results and Discussion

Influence of the pH on the hydration degree of gels and activity of α-chymotrypsin

The plots of the hydration degree of the obtained gels *vs.* pH of the medium are presented in Fig. 1. It is seen that the nonionized polyacrylamide (**1**) and poly-*N*-isopropylacrylamide (**2**) preparations are insensitive to this parameter. Only the charged gel of poly(acrylamide/acrylic acid) (**3**) changes its hydration degree when the pH decreases from 8 to 5. Recall that, in this case, the collapse mechanism is determined by blocking of the Coulomb repulsion of the carboxyl groups in the gel rather than the phase transition of the polymer.

The pH plots of the maximum reaction rate of ATEE hydrolysis by native α-chymotrypsin and its immobilized preparations are presented in Fig. 2. As can be seen, the immobilization of the enzyme shifts the

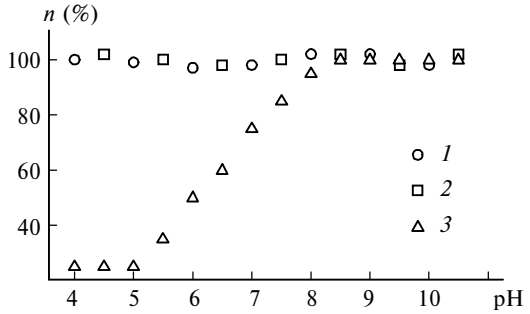

Fig. 1. Plots of the hydration degree (*n*) of hydrogels **1** (*1*), **2** (*2*), and **3** (*3*) *vs.* pH of the solution.

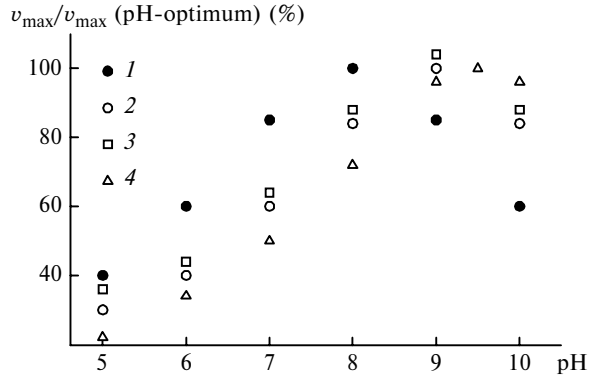

Fig. 2. pH-Plots of the relative maximum reaction rates of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester with native α-chymotrypsin (*1*) and its immobilized preparations (*2—4*): **1** (*2*), **2** (*3*), and **3** (*4*). The activity of the preparation at the pH-optimum of its effect was taken as 100%.

pH-optimum of the reaction, which depends on the nature of the matrix. The effect of the pH-optimum shift for immobilized hydrolases is always observed and imaginary because it is related to a change in the local pH value inside the polymeric network.[17] At the same time, for different immobilized preparations, the maximum reaction rates at the pH-optima are the same and independent of the nature of the matrix. The values of the Michaelis constants during immobilization increase compared to that of the native enzyme, however, at the pH-optima they are also equal for all matrices (the K_m values for native α-chymotrypsin and its immobilized preparations at the pH-optimum of their effect are $6 \cdot 10^{-4}$ and $4 \cdot 10^{-3}$ mol L^{-1}, respectively). In addition, it is evident that the pH profiles of the maximum reaction rates have virtually identical shapes. The shift of the pH-optimum by 1 or 1.5 pH units depends on the hydrogel composition rather than the hydration degree of the matrix (*cf.*, Figs. 1 and 2). Thus, we may conclude that the similar procedure of enzyme immobilization into polymeric gels with similar spatial three-dimensional structures gives preparations with almost identical kinetic properties of α-chymotrypsin at the pH-optimum of its effect. The second important conclusion: the pH-induced compression of the matrix of **3** (an increase in the density of the polymeric network without changing its phase state) has no effect on the kinetic characteristics of the immobilized enzyme.

Temperature influence of the hydration degree of gels and activity of α-chymotrypsin

The temperature plots of the hydration degrees of the gel preparations are presented in Fig. 3. It is seen that both polyacrylamide and polyacrylamide/acrylic acid are not sensitive to a change in this parameter. Only the thermosensitive gel of poly-*N*-isopropylacrylamide undergoes the phase transition accompanied by the compression of the polymeric network in a temperature interval of 30—50 °C. What does occur with the activity of immobilized α-chymotrypsin in this case?

As can be seen in Fig. 4, enzyme immobilization increases the temperature coefficient of the enzymatic reaction of *N*-acetyl-L-tyrosine ethyl ester (**4**) hydrolysis. This value is the same for all immobilized preparations, including **2**, in the 18—30 °C interval (*i.e.*, under the conditions where the hydration degrees of the gels are equal). However, in the case of preparation **2**, with the further temperature increase the region (35—45 °C) with the observed "negative activation energy" appears. This demonstrates clearly that this is precisely the phase transition in the polymeric matrix (and the phase transi-

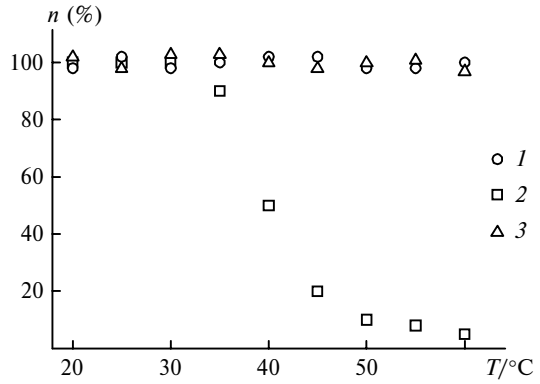

Fig. 3. Temperature plots of the hydration degree (*n*) of hydrogels **1** (*1*), **2** (*2*), and **3** (*3*).

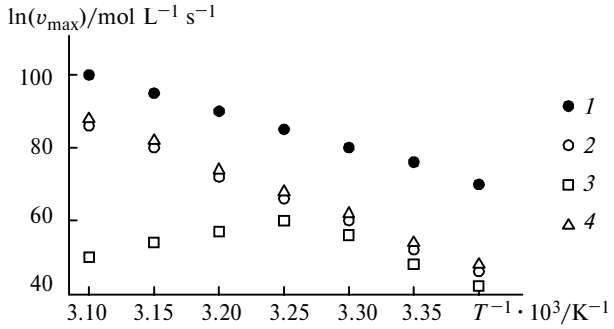

Fig. 4. Temperature plots of the maximum reaction rates of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester at the pH-optimum of the effect of native α-chymotrypsin (*1*) and its immobilized preparations **1** (*2*), **2** (*3*), and **3** (*4*) in the Arrhenius coordinates.

tion-induced change in the hydration degree of the gel) which results the anomalous temperature behavior of the matrix-immobilized biocatalyst (*cf.* Figs. 3 and 4). It is also of interest that the temperature run of the Michaelis constant is the same for all immobilized preparations, including α-chymotrypsin immobilized in gel **2**: the plot of the logarithm of the Michaelis constant *vs.* inverse temperature is linear and have no breaks (the data are not presented). This implies that the Michaelis constant does not "feel" the phase transition of the matrix (nonsensitivity to the phase transition of the support for constants of equilibrium stages of reactions of enzymes immobilized into thermosensitive gels have also been observed previously[13,18]). An important moment is the reversibility of the enzyme behavior: when the temperature is multiply increased—decreased, the activity of the preparation is not determined by its thermal prehistory (*i.e.*, the number of cycles of temperature change) but it is determined only by the temperature at which a certain measurement of the activity was performed.[13]

Thus, the observed effect of decreasing the enzyme activity of preparation **2** with temperature is related to precisely the change in the kinetic parameters, *i.e.*, the maximum reaction rate. This fact finds two possible explanations: the compression of the matrix either results in lowering of the catalytic rate constant of substrate hydrolysis or creates steric hindrances for the enzymatic reaction, *i.e.*, "shields" some active sites of the enzyme. To answer this question, we performed the experiment on the inactivation of α-chymotrypsin immobilized into the poly-*N*-isopropylacrylamide gel with benzylsulfonyl fluoride at 50 °C. It has been shown that the all enzyme was bound to the inactivator within at most 10 min. It is substantial that the enzyme activity did not increase with the temperature decrease contradicting the assumption about shielding of some active sites of the enzyme.[18] Thus, even under the conditions of complete compression of the matrix where the rates of the enzymatic reaction are close to zero (*cf.*, Figs. 3 and 4) all active sites of immobilized α-chymotrypsin are accessible for the interaction with molecules comparable in size with the inactivator molecule (including with the substrate used by us). It follows from this that the specific influence of poly-*N*-isopropylacrylamide on immobilized α-chymotrypsin during the phase transition of the polymeric matrix results in the precisely decrease (!) in the catalytic constant of the enzyme with an increase (!) in the temperature. Since this fact contradicts the laws of thermodynamics, it should be assumed that some interactions between the biocatalyst and polymer occur in this system and result in the experimentally observed decrease in the catalytic constant.

Influence of dimethyl sulfoxide on the hydration degree of gels and activity of α-chymotrypsin

The plots of the hydration degrees of preparations **1** and **2** *vs.* DMSO concentration in the reaction medium

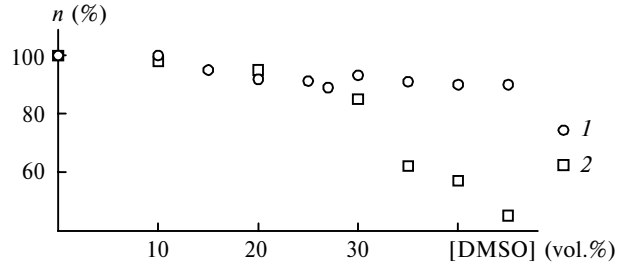

Fig. 5. Plots of the hydration degree (*n*) of hydrogels **1** (*1*) and **2** (*2*) *vs.* dimethyl sulfoxide concentration in the medium.

are presented in Fig. 5. In this case, the phase transition of gel **2** (and the corresponding change in its hydration degree) is observed in an interval of DMSO concentrations of 25—50 vol.%. Since below 20 vol.% DMSO in the mixture the hydration degrees of both preparations are virtually the same, it was reasonable to expect the resemblance in the kinetic behavior of α-chymotrypsin immobilized into **1** and **2** in this range of DMSO concentrations, which, however, they are not.

The different kinetic behaviors of native α-chymotrypsin during the hydrolysis of various substrates in water—DMSO mixtures have previously been described[19—21] (*cf.* data on the hydrolysis of **4** and *p*-nitroanilide-*N*-benzoyl-L-tyrosine (**5**) with the native enzyme, Fig. 6). This phenomenon was explained by local conformational changes in the vicinity of the active site of the enzyme due to the specific interaction of DMSO with the protein globule, presumably, in the substrate-binding protein pocket. This conclusion was drawn from comparison of the data for native α-chymotrypsin and that immobilized into gel **2**. The immobilization into gel **2** protects the enzyme, from the one hand, from the denaturating effect of DMSO and, on the other hand, removes the influence of the substrate structure on the kinetic behavior of immobilized prepa-

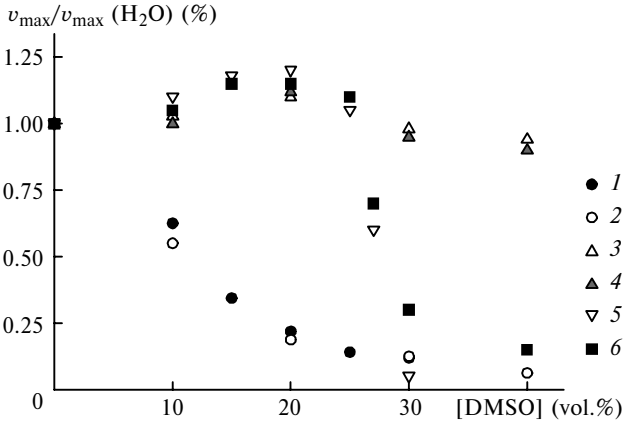

Fig. 6. Plots of the relative maximum reaction rates of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (*1—3*) and *p*-nitroanilide-*N*-benzoyl-L-tyrosine (*4—6*) at the pH-optimum of the effect of native α-chymotrypsin (*1*, *5*) and its immobilized preparations **1** (*3*, *4*) and **2** (*2*, *6*) gels *vs.* dimethyl sulfoxide concentration in the medium.

rations (Fig. 6). In this study, we showed that the kinetic behavior of α-chymotrypsin immobilized into gel **2** does not virtually differ from that of the native enzyme for which the detected activity is determined by the substrate nature. The values of the Michaelis constants for preparations **1** and **2** (the data are not presented) coincide in the whole interval of the used concentrations of an organic solvent, *i.e.*, neither the type of the matrix, nor its state affect the enzyme—substrate equilibrium. Similar facts suggest that specific interactions of the protein globule of α-chymotrypsin with both DMSO and the matrix of **2**, which undergoes the phase transition, are similar and result in local conformation changes near the active site of the enzyme (*cf.* Figs. 5 and 6).

In fact, the driving force of gel **2** compression is an enhancement of the hydrophobic interactions in the polymer with both the temperature increase and an increase in the concentration of an organic solvent in the mixture. If the interactions of α-chymotrypsin with **2** become more energetically favorable than those with water with an increase in the polymer hydrophobicity, we can assume that the polymer (similarly to DMSO) results in local conformational changes in the active site of the enzyme by the interaction with the substrate-binding protein pocket. The same mechanism (the interaction of **2** with the protein with an increase in the polymer hydrophobicity) takes place, most likely, in the case of the temperature compression of the poly-*N*-isopropylacrylamide matrix.

It is substantial that the kinetic effects observed in this case are completely reversible. The multiple transfer of the immobilized preparation from the purely aqueous medium into an aqueous-organic medium and *vice versa* have no effect on the observed activity of the preparation determined at a specified concentration of DMSO in the mixture.

* * *

Thus, the compression of the gel due to blocking of the electrostatic repulsion between the likely charged groups of the support, *i.e.*, a simple increase in the density of the polymeric network, has no effect on the observed activity of immobilized α-chymotrypsin. At the same time, the specificity of the interaction of the protein globule with the matrix is observed for the enzyme immobilized in the poly-*N*-isopropylacrylamide gel in which the compression of the matrix (and the corresponding change in its hydration degree) is induced by the phase transition of the polymer. Analysis of the data on the behavior of α-chymotrypsin immobilized into the poly-*N*-isopropylacrylamide gel during the compression of the matrix in water/dimethyl sulfoxide mixtures suggested the local interactions of polymeric chains of the stimulus-sensitive support with the protein globule. The data presented indicate that the stimulus-sensitive hydrogels (as well as natural supports of biocatalysts — phospholipid membranes) during the phase transition act as unique "antennas," which perceive the external stimulus and, specifically interacting with the protein globule, regulates the activity of the biocatalyst. Especially note the complete reversibility of the observed kinetic effects, which provides a possibility to create catalyst preparations purposefully regulated by various external effects.

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