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Effect of Thermosensitive Matrix-Phase Transition on Urease-Catalyzed Urea Hydrolysis

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Abstract

Temperature dependencies of kinetic and equilibrium parameters of urea hydrolysis catalyzed by native urease and the urease immobilized in a thermosensitive poly-N-isopropylacrylamide gel have been studied. The swelling ratio of the collapsed urease-containing gel is shown to increase in the presence of urea. Below a lower critical solution temperature (LCST) of the polymer, the immobilized urease actually has the same catalytic properties as the native enzyme. At temperatures above LCST, the observed catalytic activity of the immobilized enzyme depends chiefly not only on the thermoreversible matrix state, but also on gel water content.

Index Entries: Enzyme kinetics; urease; urea hydrolysis; thermoreversible hydrogels; phase transition; enzyme activity thermoregulation.

Abbreviations: E_a, activation energy, kJ/mol; K_m, Michaelis constant, M; V, reaction rate, mM/min; V_{max}, maximum reaction rate, mM/min; $V_{max,\,opt}$, maximum reaction rate in a pH optimum of enzyme activity, mM/min; T, temperature, K; t, temperature, °C; ($[NH_2)_2CO$), urea concentration, M.

Introduction

Recently, the gels that undergo reversible phase transitions during small changes at the medium parameters (pH, ionic strength, solvent concentration) or under certain physical effects (temperature, light, electromagnetic field) have been of great interest for investigators. This interest is the result of numerous possible applications of these gels for both scientific and technological purposes *(1,2)*. The studies of phase transition effects on

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the properties of biocatalysts immobilized in these gels are of especially great importance *(3,4)*.

Thermosensitive hydrogels undergo a phase transition when temperature rises above a certain critical point (so-called lower critical solution temperature, LCST). This phenomenon results in a sharp decrease in gel volume and gel water content *(5,6)*. The Arrhenius correlations for enzymes immobilized in thermosensitive gels reveal the areas with an apparent negative activation energy *(7–12)*. Thus, an increase in temperature above a certain critical point leads to a decreased observed activity of the gel preparations. In some cases (because of the nature of polymer, monomer, and crosslinking agent concentrations, enzyme immobilization methods, and so on), the observed preparation activity decreases to zero *(13,14)*. However, it reaches its initial level with repeatedly decreasing temperature because of reversibility of the phase transition of a polymer matrix.

The reversible shrinking and swelling of a matrix at cyclic changes in temperature can be used in biotechnology to increase the mass exchange in the processes catalyzed with immobilized biocatalyst in a bioreactor *(15)*, and for temperature-controlled "switching on–off" preparation enzyme activity *(7,10)*. The phase transitions in biomembranes during their melting also result in breaks in the Arrhenius dependencies for membrane-bound proteins *(16,17)*. Thus, the studies of enzymes immobilized in a thermoreversible gel (a simple model system) may clarify the mechanisms of enzyme activity regulation in living things.

The temperature dependencies of kinetic and equilibrium constants of the reactions catalyzed by enzymes immobilized in thermoreversible gels show that the matrix-phase transition does not affect the equilibrium reaction constants, and decreasing enzyme activities at the temperature increasing above LCST are caused by a decrease of maximal rates of the reactions *(13)*. The ratio between the decreased and initial enzyme activities correlates well with the swelling ratio (water content) of the matrix *(11)*. However, it is not clear whether the state of the gel matrix (polymer chain conformation, interaction of polymer chains, structure of hydration water, and so on) or of free water content (swelling ratio of a gel) controls the observed phenomena.

Literature data show that water content of the shrunken gel can be controlled by external pumping of ions of the same charge. The repulsion of these ions results on the reversible swelling of the shrunken gel matrix *(18)*. The biospecific processes can also affect the water content of the thermoreversible gel *(19)*. The report concerning the urease-containing copolymer of N-isopropylacrylamide (NIPAA) and acrylic acid *(2,14)* is the most interesting. In the presence of urease substrate, i.e., urea, this preparation undergoes, not the discontinuous-phase transition, but the continuous one. This effect shifts its LCST to a higher temperature region and to the gel swelling after urea addition. The authors think that this phenomenon is caused by the accumulation of charged species in the course of enzymatic urea hydrolysis in the gel and dissociation of carboxyl groups.

The goal of this work was to study temperature dependencies of kinetic and equilibrium parameters of urea hydrolysis catalyzed by urease immobilized in thermoreversible nonionic poly-NIPAA gel. The authors intended to clarify the effect of the matrix collapse on temperature dependencies of the reaction parameters and the relation between the matrix water content and the enzyme activity of the preparations.

Materials and Methods

Jack bean urease (328 U/mg) was obtained from Serva (Germany). N,N'-methylene-*bis*-acrylamide (MBA), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, and dithiotreitol (DTT) were from Reanal (Hungary). NIPAA was synthesized by the method given in Plaut and Ritter *(20)*. Bovine serum albumin (BSA), urea, and components of buffer solutions of pure-for-analysis grade were from Reakhim (Moscow, Russia).

Production of Immobilized Preparations of Urease

To 0.9 mL solution containing 0.2 g NIPAA and 12 mg BSA (protein protector against enzyme inactivation during immobilization), 0.1 mL urease solution (2–6 mg/mL), 0.02 mL MBA solution (18 mg/mL) and initiators (0.02 mL aqueous 0.78 *M* ammonium persulfate solution and 0.02 mL TEMED) were added at 0°C, and with continuous stirring; the stirring was stopped, and block copolymerization was carried out for 1 h. The resulting block copolymer was ground in a homogenizer (particle size, 20–100 μm). Then 10 mL 5×10^{-6} *M* DTT solution was added. The gel particles were separated by centrifuging at 3500 *g* for 15 min. The supernatant was removed, and the washing and centrifugation steps were repeated (usually four times) to the complete absence of the enzyme activity in the washing solutions. After the last washing and decanting of a pellet, 15–20 mL of 5×10^{-6} *M* DTT solution was added, and thus 20–25 mL gel suspension with the immobilized enzyme was produced.

Assay of Activities of Native and Immobilized Urease

Activities of native and immobilized urease were determined by recording the initial rates of urea hydrolysis with a RTS-622 pH-stat (Radiometer, Denmark) with a 5-mL thermostatted vessel. The ionic strength was established with 0.2 *M* NaCl. All measurements were made at [DTT] = 5×10^{-6} *M*. The substrate concentration in the vessel was varied from 7.0×10^{-4} to 9.0×10^{-2} *M*, temperature from 20 to 50°C, and pH from 4.5 to 8.0 for the native enzyme, and from 3.5 to 7.5 for the immobilized enzyme. The concentration of native enzyme, in the vessel was 5×10^{-3} mg/mL. For the immobilized enzyme, 0.25 mL of the suspension was added to the vessel.

Determination of Kinetic and Equilibrium Constants of Enzymatic Reaction

Parameters of the enzymatic reaction were evaluated from the dependencies of the initial rates of urea hydrolysis on the substrate concentration in Hanes-Woolf [S/V–S] coordinates.

Determination of Swelling Ratio of Poly-NIPAA Preparations

The block copolymer was thoroughly washed to remove unreacted reagents, and then was cut into slices with $5 \times 5 \times 2$ mm vol. Water droplets were gently removed from the slice surfaces by filter paper. The slices were weighed and equilibrated for 3 h at the selected temperature, either in 0.2 *M* NH_4Cl–HCl buffer solution containing 5×10^{-6} DTT or in the same solution, but in the presence of 1.0 *M* urea. At the end of 3 h, the samples were withdrawn, water droplets were gently removed by filter paper, and the samples were reweighed. Water content (swelling ratio) was expressed as percent ratio between the sample weight at the selected temperature and the sample weight at 20°C. For each temperature, the mean value of the swelling ratio was calculated using the results of three independent experiments. The temperature was varied from 20 to 50°C, and pH from 4.5 to 5.25.

Results

Native Urease

Temperature Dependence of pH Profile of Native Urease Activity

When studying the temperature dependencies for enzymes with rather narrow pH profiles of activities, one should take into account a possible shift of pH optima to the region of more acidic pH with increasing temperature *(21)*. Figure 1 shows experimental results concerning the pH dependence of the native enzyme activity. The figure demonstrates the increase in temperature from 20 to 50°C to shift the pH optimum from 6.25 to 5.5 (*see also* Fig. 4).

Temperature Dependencies of K_m and V_{max}

Figure 6 indicates that, in pH optimum area, K_m equals $(2.6 \pm 0.5) \times 10^{-3}$ *M*, and is virtually temperature-independent. Literature data show that the K_m values for urea hydrolysis catalyzed by jack bean urease depend on enzyme preparation, buffer, ionic strength, and pH, and usually lie in the range $(1–10) \times 10^{-3}$ *M (22,23,25)*. Note that the determined K_m value is one order lower than the substrate concentration used for estimation of pH profiles of the enzymatic reaction. Thus, the enzyme activity values should be close to V_{max} values of the reaction in the area of pH optimum. Indeed, the V_{max} values that were determined in independent experiments corresponded well to pH profiles of the enzyme activity (Fig. 1). The Arrhenius dependence for the maximal reaction rate in the pH optimum (Fig. 7) gives a reaction activation energy equal to 32 ± 2.0 kJ/mol. This value also agrees well with literature data *(22,24–26)*.

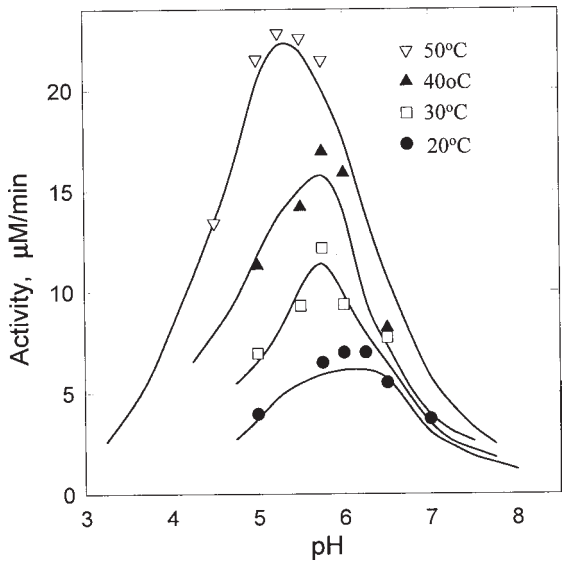

Fig. 1. Dependence of the observed activity of native urease on pH at different temperatures (solid line) and the calculated V_{max} values (symbols) in the region of pH optimum of urease activity. The substrate concentration is 4×10^{-2} *M*.

Immobilized Urease

Diffusion Effects in Preparations of Immobilized Urease

To verify the possible occurrence of diffusion limitations in the preparations with immobilized urease, and to correct the obtained K_m and V_{max} for urea hydrolysis catalyzed by immobilized urease, the concentration of enzyme solution taken for the immobilization was varied. Figure 2 shows the dependencies of the preparation activity on initial enzyme activities taken for the immobilization. These dependencies are linear, and a decrease in the initial enzyme concentration results in the preparation activity tending to zero, both at 20 and 50°C. This fact suggests the absence of diffusion limitations during catalytic hydrolysis of urea by immobilized urease in the interval of concentrations taken for the immobilization.

Temperature Dependence of a pH Profile of Immobilized Urease Activity

Figure 3 shows experimental data concerning pH dependence of the activity of the immobilized enzyme. The pH optimum of immobilized urease, compared with that of the native enzyme, is shifted by approx 1 pH unit to a more acidic area (Fig. 4). This is because of the formation of positively charged ammonium ions during the reaction. This process results in a local pH shift in the gel matrix, which is responsible for the necessity of maintenance of more acid pH in surrounding solution. As in the case of the native enzyme, a shift of the pH optimum to a more acidic area with increasing temperature occurs. However, the position of the pH optimum abruptly changes in the interval from 30 to 40°C. Moreover, the approximately sym-

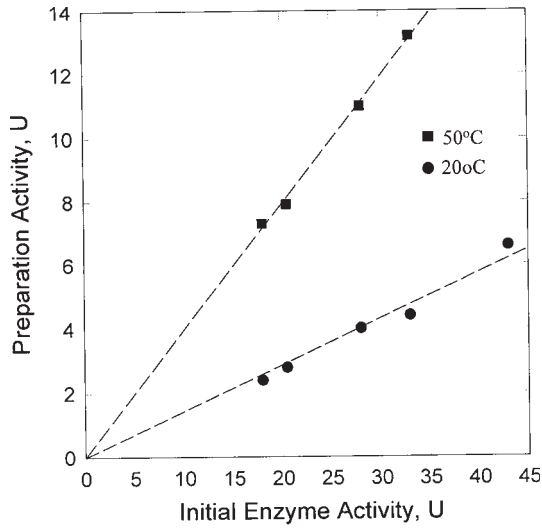

Fig. 2. Dependence of the observed activity of urease-containing preparations on urease activity taken for immobilization. The substrate concentration is 4×10^{-2} *M*.

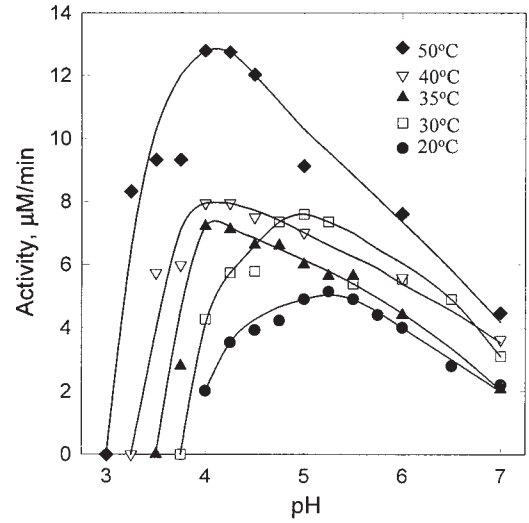

Fig. 3. pH Dependence of the observed activity of immobilized urease at various temperatures. The substrate concentration is 4×10^{-2} *M*.

metric bell-shaped pH dependence of the native enzyme activity becomes clearly asymmetric after the immobilization of urease: The left branch (more acid pH) is more steep than the right branch.

Temperature Dependence of Immobilized Urease Activity

Figure 5 shows the temperature dependence of the preparation activity in pH optimum of the reaction at different urea concentrations. At temperatures above LCST of the gel (35–50°C) and at low substrate concentrations

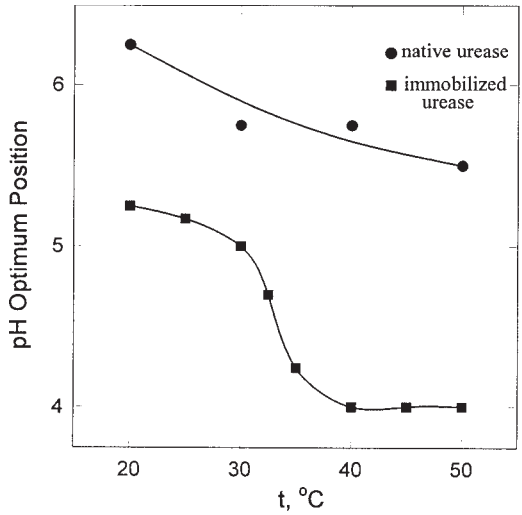

Fig. 4. Temperature dependence of positions of pH optima of enzyme activity for urease-catalyzed hydrolysis of urea for native and immobilized urease.

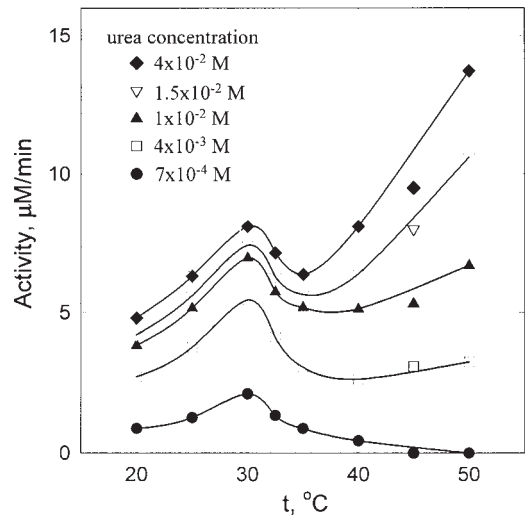

Fig. 5. Temperature dependence of activity of immobilized urease in pH optima of activity of the immobilized enzyme at various urea concentrations.

(lower than $4 \times 10^{-3} M$), the enzyme activity decreases to zero. However, this decrease becomes progressively less sharp with increasing substrate concentration, and the activity increases in the range of 40–50°C. These processes result in the appearance of a minimum at 35°C.

Temperature Dependencies of K_m and V_{max}

Figure 6 shows the determined K_m values for the immobilized enzyme at different temperatures and pH. At temperatures lower than 35°C and in

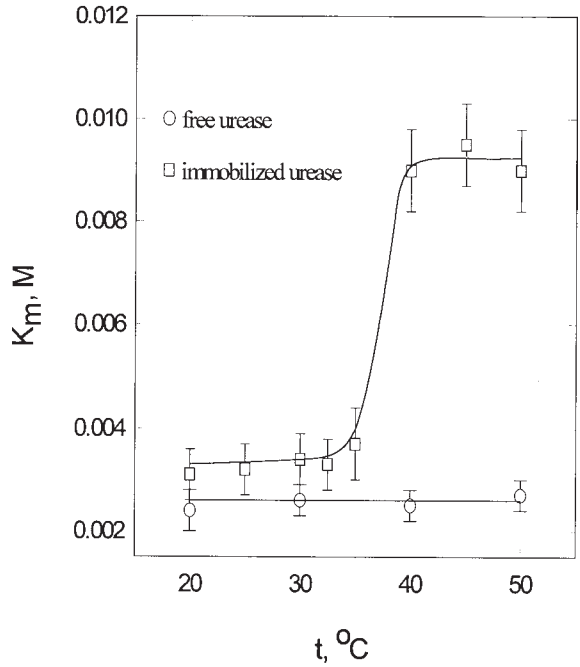

Fig. 6. Temperature dependence of the calculated K_m for urea hydrolysis catalyzed by native and immobilized urease in the region of pH optimum of their activity.

the region of pH optimum of immobilized urease, K_m values are virtually the same as those for the native enzyme. At more elevated temperatures, K_m in the region of pH optimum rises approximately threefold.

Figure 7 shows the temperature dependence of V_{max} in Arrhenius coordinates for pH optimum of the enzyme activity. At the interval 20–30°C, the temperature dependence is similar to that of the native enzyme, and close to E_a for the native enzyme ($E_a = 32 \pm 2$ kJ/mol). However, the enzyme activity decreases with increasing temperature in the range 30–35°C. This effect results in a break in the Arrhenius dependence and is characteristic for a number of enzymes *(7–12)*. However, with a further increase in temperature, the enzyme is reactivated, and nearly complete restoration of the enzyme activity at 50°C occurs.

Swelling Ratio of Matrix

Figure 8 shows the swelling ratios of poly-*N*-isopropylacrylamide gel preparations, which contain and do not contain urease, in 0.2 *M* NH_4Cl–HCl buffer, in the presence and in the absence of 1 *M* urea. The swelling curves of all preparations except the urease-containing gel in the presence of urea are the same. In the latter case, the usual shrinking of the gel at increasing temperature changes to repeated swelling. This swelling correlates with an increase in V_{max} for urea hydrolysis catalyzed with the immobilized enzyme (compare Figs. 7 and 8).

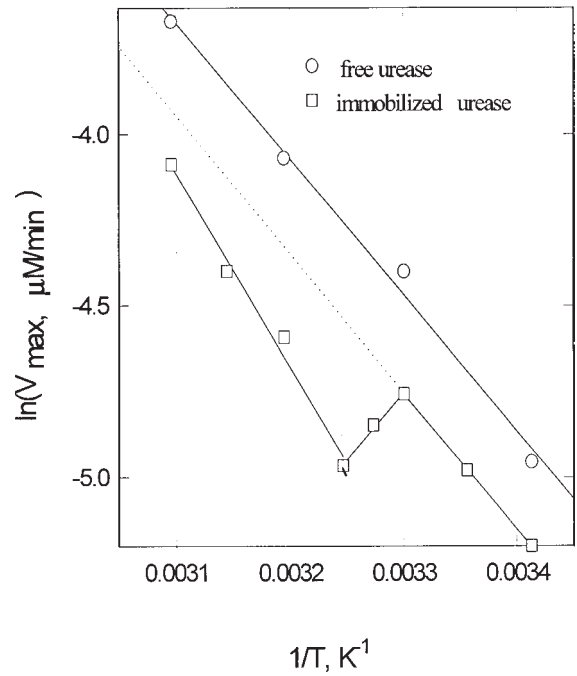

Fig. 7. Temperature dependence of the calculated $V_{max,opt}$ for urea hydrolysis catalyzed by native and immobilized urease in the Arrhenius coordinates. Dotted line, extrapolation of the low-temperature branch to elevated temperature.

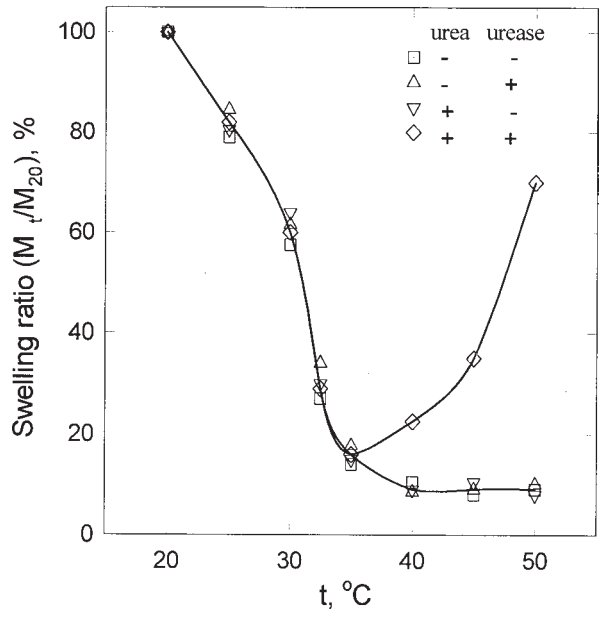

Fig. 8. Temperature dependencies of the swelling ratios of poly-NIPAA preparations with and without urease, in 0.2 *M* NH_4Cl–HCl buffer solution containing 5×10^{-6} *M* DTT in the absence and in the presence of 1 *M* urea.

Discussion

The experimental results suggest the urease immobilized in thermoreversible poly-*N*-isopropylacryamide gel to have the same properties below 30°C as those of the native enzyme. The K_m for urease-catalyzed hydrolysis of urea virtually does not change, the temperature dependence of pH optimum of the enzymatic reaction with regard to the shift caused by immobilization has an analogous pattern, and the activation energy is virtually the same as that for the native enzyme. However, at further increasing temperature, the significant distinctions in kinetics of native and immobilized urease appear. The abrupt shift of the pH optimum occurs (Fig. 4), the K_m changes (Fig. 6), and the break in the Arrhenius dependence for V_{max} of the enzyme reaction occurs (Fig. 7). What is the reason for the observed effects? This difference in kinetic properties of the native and immobilized enzymes is explicable only by a specific effect of products of the enzymatic hydrolysis on the gel. All of the authors' results show the swelling ratio to depend on enzyme activity (the rate of enzyme reaction). The dependencies of the observed activity of the urease-containing preparation on substrate concentration (Fig. 5) serve as an additional confirmation of this pattern. If the matrix swelling degree depends on the enzyme activity, the effect of substrate concentration on the observed activity of the preparation should have a cooperative character. At elevated temperatures (40–50°C), the dependence of the reaction rate on substrate concentration is actually S-shaped. Such dependencies can be considered, from a formal kinetic viewpoint, as quasicooperative. Kinetic and equilibrium parameters of the reaction catalyzed by immobilized urease at elevated temperatures can be estimated by using a Hill-type equation. In this case, the calculated parameters well agree with those obtained in the Hanes-Woolf coordinates with high substrate concentrations (data not shown).

Kokufuta et al. *(14)* suggest that the swelling of urease-containing copolymer of NIPAA and acrylic acid occurs because of the accumulation of hydrolysis products of the same charge and the dissociation of carboxyl groups, i.e., because of electrostatic forces. It is difficult to suppose that the processes associated with a significant change in the ionic strength or in ammonium ion concentration can occur in the gel in the case of 0.2 *M* ammonium buffer. The authors think that the above effects were observed probably because of an inner osmotic pressure induced by the appearance of ammonium ions during enzymatic urea hydrolysis in gel particles. Several studies indicate that osmotic water flows can be higher than diffusion flows in some systems *(27)*. Thus, an increase in molar concentrations of substances in collapsed gel particles caused by hydrolysis of one urea molecule to three product molecules may result in the emergence of the osmotic water flow and increasing swelling ratio.

Conclusion

To summarize the authors' results, the following conclusions can be drawn:

1. In the temperature range below LCST of poly-*N*-isopropylacrylamide gel (<35°C), the immobilized urease has the same catalytic properties as those of the native enzyme.
2. The swelling ratio of the collapsed thermoreversible urease-containing gel increases after addition of urease substrate, i.e., urea.
3. The observed phenomena are caused by a change in concentration of the products of urease-catalyzed hydrolysis of urea in the gel.
4. The observed activity of the immobilized enzyme at temperatures above LCST is chiefly dependent, not on the thermoreversible polymer state, but on water content of the gel matrix.

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