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Comparative Study of Immobilized and Soluble NADH:FMN-Oxidoreductase–Luciferase Coupled Enzyme System

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Abstract—The properties of a coupled enzyme system (NAD(P)H:FMN-oxidoreductase and luciferase) from luminous bacteria were studied. The enzymes and their substrates were immobilized in polymer gels of different types: starch (polysaccharide) and gelatin (polypeptide). Maximum activity yield (100%) was achieved with the enzymes immobilized in starch gel. An increase in $K_{m\ app}$ was observed in both immobilized systems as compared with the soluble coupled enzyme system. Immobilization in starch and gelatin gels increased the resistance of the NAD(P)H:FMN-oxidoreductase and luciferase coupled enzyme system to the effects of external physical and chemical factors. The optimum pH range expanded both to the acidic and alkaline regions. The resistance to concentrated salt solutions and high temperature also increased. The coupled enzyme system immobilized in starch gel (with activation energy 30 kJ/mol) was characterized by the best thermostability. The immobilized coupled enzyme system can be used to produce a stable and highly active reagent for bioluminescent analysis.

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Luminescent bacteria and the enzymes isolated from them, including the bacterial NADH:FMN-oxidoreductase–luciferase (L+R) coupled enzyme system, are extensively used in luminescent analysis for solution of analytical problems [1-4]. The coupled enzyme system of luminescent bacteria is a reaction catalyzed by two enzymes (NADH:FMN-oxidoreductase and luciferase), where light is one of the reaction products [5]. However, application of the enzymes of luminescent bacteria is complicated by their instability under various conditions: high temperatures, extreme pH values, etc. [6, 7]. Immobilization of the enzymes makes it possible to obtain stable enzyme preparations suitable for bioluminescent analysis [8, 9]. Successful application of immobilized enzyme preparations is determined largely by the choice of a suitable carrier and immobilization method and by the knowledge of kinetic and thermodynamic peculiarities of catalysis by immobilized enzymes. More than ten methods of immobilization of the enzymes of luminescent bacteria on different carriers have been proposed [10-12]. One of the most promising immobilization methods is entrapment of enzymes in polymer gels. This method facilitates the formation of the optimal microenvironment for enzymes and yields high catalytic activity, which is maintained during the long-term storage of immobilized preparations. The microenvironment is optimized by selection of an appropriate gel-forming system.

In the present work, gelatin and starch gels have been chosen as carriers for immobilization of the coupled enzyme system of luminescent bacteria. The selection of polysaccharide (starch) and gelatin (polypeptide) gels is determined by their nontoxicity and neutrality [11-14].

Abbreviations: C_{14}, myristic aldehyde; L+R, NADH:FMN-oxidoreductase–luciferase coupled enzyme system; L+R+C_{14}+NADH, multicomponent immobilized reagent including the NADH:FMN-oxidoreductase–luciferase coupled enzyme system, myristic aldehyde, and NADH.

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Such a gel environment is supposed to provide conditions for the functioning of these enzymes that are close to the conditions *in vivo*, e.g. inside the bacterial cell [15].

The goal of the work was to compare the kinetic and thermodynamic characteristics of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system, soluble and immobilized in polymer gels of different nature, to select the method for obtaining a stable and highly active reagent for bioluminescent analysis.

MATERIALS AND METHODS

Lyophilized preparations of high-purity enzymes produced at the Laboratory of Bacterial Bioluminescence (Institute of Biophysics, Siberian Branch of the Russian Academy of Sciences (SB RAS), Krasnoyarsk) were used in the work. One vial of lyophilized preparation contained 0.5 mg/ml of luciferase (EC 1.14.14.3) from recombinant strain of *Escherichia coli* and 0.15 U NADH:FMN-oxidoreductase (EC 1.5.1.29) from *Photobacterium leiognathi*. Before the measurement, the lyophilized enzymes were dissolved in 0.05 M potassium phosphate buffer (pH 6.8).

The following reagents were used: potato starch, NADH, and FMN (Serva, Germany); tetradecanal (Merck, Germany); gelatin (State Standard 11293-89; Russia). Solutions were prepared with 0.05 M potassium phosphate buffer (pH 6.8).

The effect of temperature on the activity of the coupled enzyme system was studied using a VT-8 liquid circulation ultrathermostat (Thermex-2, Russia). The measurements were taken with a BLM 8801 bioluminometer manufactured at the Nauka Special Engineering and Design Bureau (Krasnoyarsk Research Center, SB RAS, Krasnoyarsk) connected with a 2210 recorder (LKB, Sweden).

The activity of the soluble and the immobilized coupled enzyme system was determined by the value of maximal luminescence intensity I_{max} expressed in millivolts (mV) in reaction mixture containing: 5 µl of enzyme solution, 50 µl of 0.002% tetradecanal, 50 µl of 0.5 mM FMN, 200 µl of 0.05 M potassium phosphate buffer (pH 6.8), 200 µl of 0.4 mM NADH. All components were introduced into a bioluminometer cuvette in series and quickly mixed; the cuvette was put into the bioluminometer, and the maximal luminescence intensity I_{max} was recorded. Immobilized enzymes prepared in the form of a disc were introduced instead of the enzyme solution for measurement of their activity.

L+R was immobilized in gelatin gel as follows: 5 ml of 5% gelatin suspension was heated to complete dissolution of the gelatin, filtered through two layers of filter paper, and cooled to 30°C followed by introduction of 0.25 ml of the enzyme solution into the gel. The resulting mixture was dosed with a micropipette by 50-µl portions onto lavsan substrate and dried for 12 h at room temperature.

L+R immobilization into starch gel was performed in much the same way, using 3.5% starch suspension and sample drying at 4°C. The activity yield of immobilized enzymes was calculated by the ratio of maximal luminescence intensities of immobilized and soluble enzyme preparations. At multicomponent immobilization (L+R+C_{14}+NADH), substrate solutions were introduced into the gel in addition to the enzymes: 125 µl of 0.02% tetradecanal and 125 µl of 1.6 µM NADH. The reaction was initiated by adding 50 µl of 0.5 mM FMN and 450 µl of 0.05 M phosphate buffer (pH 6.8).

The immobilized reagent was a disc, 7-8 mm in diameter, 50-60 µm thick, dry weight 9 ± 0.5 mg. The quantities of luciferase and NADH:FMN-oxidoreductase in a particular membrane corresponded to their quantities in the reaction mixture for a single measurement.

Apparent Michaelis constants ($K_{m\ app}$) for the soluble and immobilized coupled enzyme systems were determined from the analysis of reaction rate dependencies on substrate concentrations in Eadie–Hofstee coordinates. The rate of coupled enzyme reaction was determined by luminescence intensity.

Thermostability of the L+R coupled enzyme system, soluble and immobilized in starch and gelatin gels, was determined by variation of the maximal luminescence intensity after incubation of the enzyme preparations at different temperatures (5 to 70°C) for 5 min. Thermoinactivation of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system in the soluble and immobilized states was carried out by incubation of enzyme preparations in a thermostat for different periods of time at 30-70°C. The inactivation constant was determined by the slope of the dependence of residual activity logarithm on time; then the activation energy for the reaction of the coupled enzyme system was calculated graphically in Arrhenius coordinates.

Experimental data were obtained from three series of experiments; the measurements of each experimental point were taken in no less than five parallel measurements.

RESULTS AND DISCUSSION

The activity yield of the immobilized NADH:FMN-oxidoreductase–luciferase coupled enzyme system depends on the nature of the polymer gels (Fig. 1). The activity yields of immobilized L+R and the enzymes immobilized jointly with substrates (L+R+C_{14}+NADH) were 100% on immobilization in starch gel and less than 20% on immobilization in gelatin gel.

Figure 2 shows the dependence of luminescence intensity of the NADH:FMN-oxidoreductase–luciferase

coupled enzyme system on substrate concentration in Eadie–Hofstee coordinates. The calculated apparent Michaelis constants for the soluble and immobilized coupled enzyme systems are given in Table 1. As a result of immobilization, $K_{m\ app}$ increases for all three substrates as compared with soluble enzymes. However, for the enzymes immobilized in starch gel the $K_{m\ app}$ values increase 2- and 2.5-fold for FMN and NADH, respectively. For the enzymes immobilized in gelatin gel, the changes of $K_{m\ app}$ as compared with the soluble coupled enzyme system are more significant: $K_{m\ app}$ increases 4- and 5-fold for FMN and NADH, respectively. Tetradecanal showed an increase of $K_{m\ app}$ of 4- and 6-fold for the enzymes immobilized in gelatin and starch gels, respectively. The increase in $K_{m\ app}$ values for immobilized enzymes can be explained by the fact that the process of enzyme immobilization is accompanied by limitation of the conformational lability of protein molecules reflected in the rate of the catalytic act [16]. This limitation was aggravated by the fact of joint immobilization of the two enzymes. The substrate for luciferase is a reaction product catalyzed by oxidoreductase, $FMNH_2$, which is an extremely unstable compound quickly oxidized by air oxygen to FMN.

Another reason for the lowering affinity of substrates to the enzymes is the fact that not only the luciferase and oxidoreductase complexes, but also these enzymes separately, are immobilized in the matrix structure. In this case, Michaelis constants increase due to diffusion limitations appearing in the course of $FMNH_2$ delivery between the enzymes molecules spatially dispersed in the gel.

It has been shown that immobilization changes enzyme properties, including the dependence of catalytic activity on pH, ionic composition and other parameters of the medium, and affects the stability of enzymes with respect to denaturing effects of different kinds. Figure 3

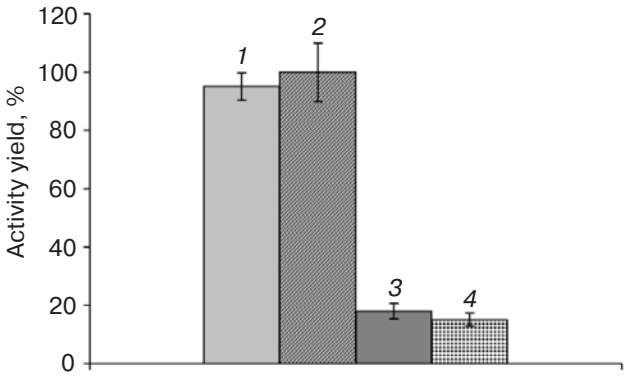

Fig. 1. Activity yield of immobilized NADH:FMN-oxidoreductase–luciferase coupled enzyme system: *1*) L+R immobilized in starch gel; *2*) L+R+C_{14}+NADH immobilized in starch gel; *3*) L+R immobilized in gelatin gel; *4*) L+R+C_{14}+NADH immobilized in gelatin gel.

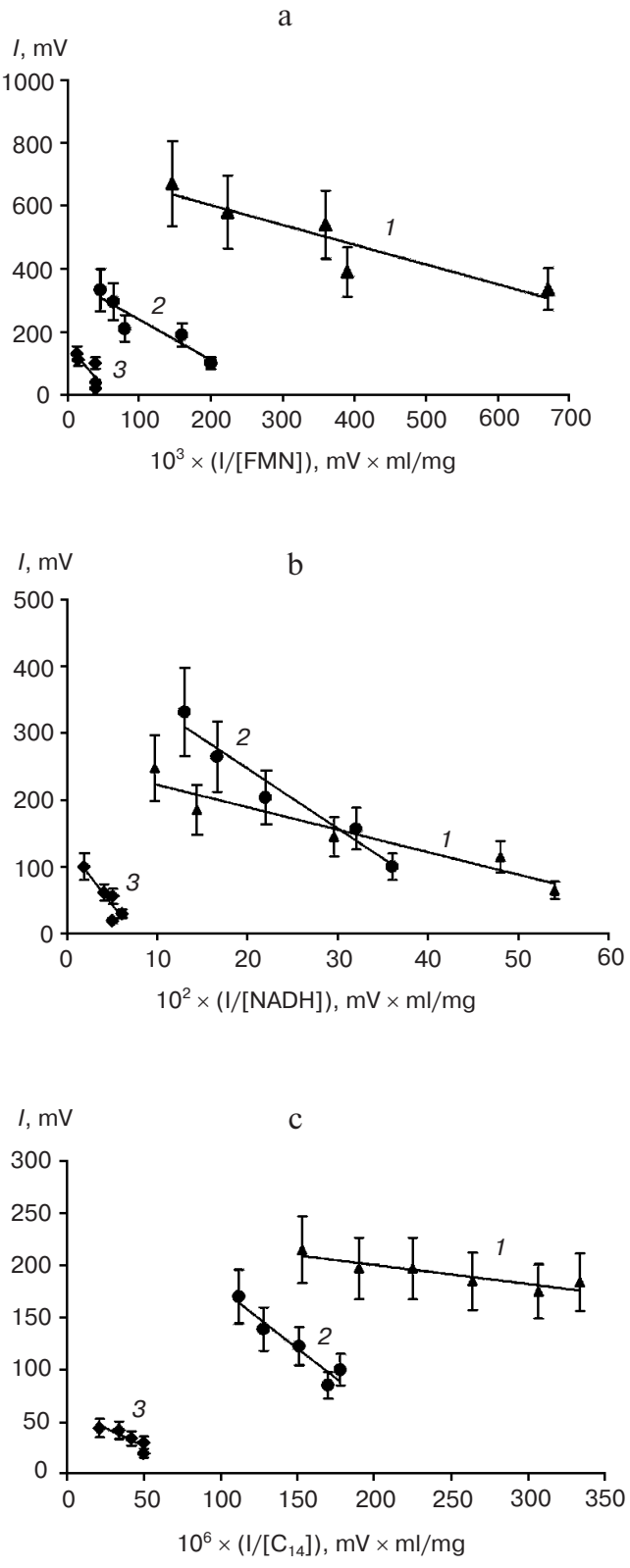

Fig. 2. Dependence of the luminescence intensity of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system on substrate concentrations in Eadie–Hofstee coordinates: a) FMN; b) NADH; c) myristic aldehyde. *1*) Soluble L+R; *2*) L+R immobilized in starch gel; *3*) L+R immobilized in gelatin gel.

Table 1. Values of apparent Michaelis constants for the NADH:FMN-oxidoreductase–luciferase coupled enzyme system

Coupled enzyme system \ Substrates	$K_{m\ app}$, mg/ml		
	FMN	NADH	tetradecanal
Soluble L+R	$(0.6 \pm 0.1) \times 10^{-3}$	$(3.4 \pm 0.7) \times 10^{-2}$	$(0.18 \pm 0.04) \times 10^{-6}$
L+R immobilized in starch gel	$(1.3 \pm 0.3) \times 10^{-3}$	$(9.0 \pm 1.8) \times 10^{-2}$	$(1.2 \pm 0.2) \times 10^{-6}$
L+R immobilized in gelatin gel	$(2.8 \pm 0.6) \times 10^{-3}$	$(17.9 \pm 3.5) \times 10^{-2}$	$(0.7 \pm 0.1) \times 10^{-6}$

presents the pH dependence of the luminescence intensity of the coupled enzyme system. One can see that the pH optimum of soluble enzymes is 6.8, and the immobilized enzymes demonstrate a shift of the pH optimum to the alkaline region. At the same time, the luminescence intensity of the coupled enzyme system immobilized in starch gel was no less than 80% relative to the maximum activity over the studied range of pH values, with the pH optimum in the range of 5.8 to 7.8. For the multicomponent reagent immobilized in gelatin gel, the pH optimum was narrower (pH 6.6-7.3).

Such effect of pH on the activity of immobilized enzymes can be explained is several ways. First, the charged groups of immobilization carrier can influence proton distribution in the microenvironment of immobilized enzymes. This effect must be marked for enzymes immobilized in gelatin gel containing charged groups. In the case of the enzyme immobilization in starch gel, the weak dependence of activity of the coupled enzyme system on pH may be due to electric neutrality of the carrier. Second, the polymer matrix, increasing viscosity of the medium, prevents free diffusion of protons inside the immobilized enzyme and thus causes a significant change in pH dependence of the activity, which is particularly noticeable with the increase in pH, i.e. decrease in proton concentration in solution. Besides, a combination of the effects of distribution of protons and limitation of their diffusion is possible. Extension of the pH optimum for the multicomponent immobilized reagent is probably due to additional stabilization of enzyme molecules due to entrapment of substrates into active centers of the enzymes during immobilization.

We also studied the effect of temperature on the activity of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system. Immobilized enzyme preparations maintained high catalytic activity under the impact of temperatures in the whole range considered. The luminescence intensity was no less than 60% of the maximal value for the enzyme preparations immobilized in starch gel and no less than 50% for the enzyme preparations

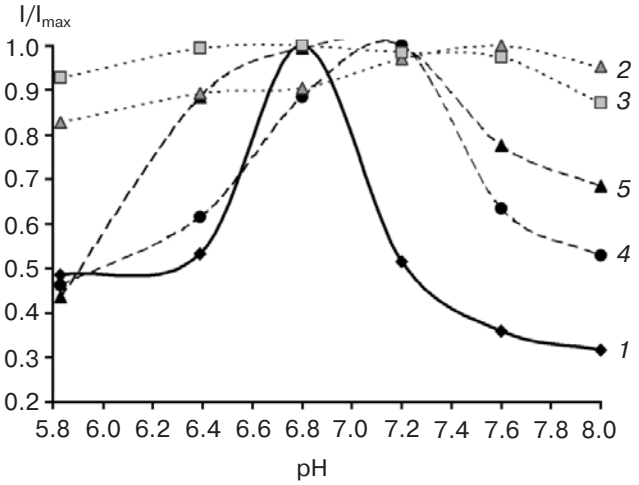

Fig. 3. pH dependence of normalized luminescence intensity of the coupled enzyme system of luminescent bacteria: *1*) soluble L+R; *2*) L+R immobilized in starch gel; *3*) L+R+C_{14}+NADH immobilized in starch gel; *4*) L+R immobilized in gelatin gel; *5*) L+R+C_{14}+NADH immobilized in gelatin gel.

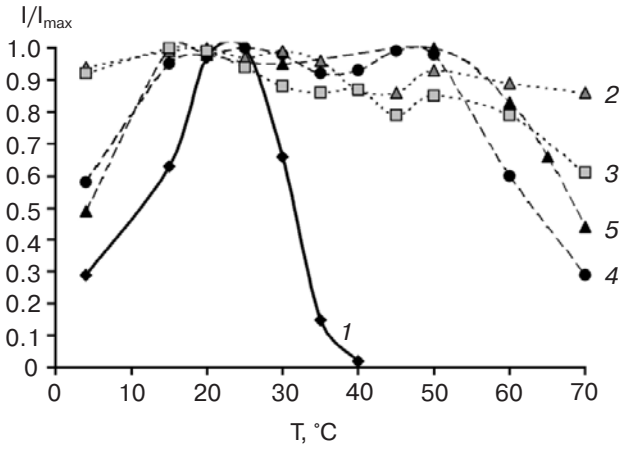

Fig. 4. Dependence of normalized luminescence intensity of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system on incubation temperature: *1*) soluble L+R; *2*) L+R immobilized in starch gel; *3*) L+R+C_{14}+NADH immobilized in starch gel; *4*) L+R immobilized in gelatin gel; *5*) L+R+C_{14}+NADH immobilized in gelatin gel.

Table 2. Values of E_d (kJ/mol) for the NADH:FMN-oxidoreductase–luciferase coupled enzyme system

Soluble L+R	L+R	L+R+C_{14}+NADH	L+R	L+R+C_{14}+NADH
	immobilized in gelatin gel		immobilized in starch gel	
201 ± 39	79 ± 18	162 ± 33	30 ± 7	129 ± 28

immobilized in gelatin gel (Fig. 4). The soluble coupled enzyme system after incubation within a temperature range of 15 to 30°C maintained no less than 60% of the maximal activity, whereas the enzymes were completely inactivated at 40°C. For the soluble coupled enzyme system, the temperature optimum of activity, when the luminescence intensity of enzyme preparations was maximal, was at 20-25°C. The immobilized enzymes and the enzymes immobilized jointly with tetradecanal and NADH showed an extension of the temperature optimum to 5-50°C for the preparations immobilized in starch gel and to 15-50°C for the preparations immobilized in gelatin gel. The increase in thermostability of the enzymes on their immobilization is due to the lower mobility of the enzymes and their subunits in the gel matrix.

Thus, immobilization in polymer gels stabilizes the coupled enzyme system; however, the effect of stabilization is lower at higher temperatures. The effective values of activation energy (E_d) for the soluble and immobilized coupled enzyme system are given in Table 2. The table shows that the activation energies for immobilized enzymes are lower compared with the activation energy of the soluble enzymes. It seems that in case of immobilized enzyme preparations there are no considerable changes in free energy due to the strengthening of the conformation of the proteins and limitation of their mobility in the immobilized state. Thus, the protein–matrix complex formed in the course of immobilization becomes more advantageous thermodynamically. The values of activation energy for multicomponent systems prove to be higher than for the enzymes immobilized without the substrates, both for starch and gelatin gels. This means that the joint immobilization of enzymes and substrates does not lead to additional stabilization of the enzymes, which is revealed in the experiments on thermal inactivation. The coupled enzyme system immobilized in starch gel is the most stable thermodynamically among the considered reagents, because it is characterized by the lowest activation energy.

Comparison of the properties of enzymes immobilized in gels of different nature showed that on application of gelatin gel the activity yield was much lower, while the $K_{m\ app}$ values for FMN and NADH and the activation energy were higher than for starch gel. Since the only difference under conditions of immobilization of the coupled enzyme system is the nature of the gel used, we believe that it is associated first with the differences in the physicochemical characteristics and the nature of gel-forming polymers. Starch gel is chemically neutral, and maintenance of high enzyme activity in this gel is probably due to the absence of covalent bonds with the active groups of entrapped enzymes. In contrast to starch, gelatin is a polypeptide containing a considerable quantity of polar amino acid residues (e.g. glycine and hydroxyproline constituting 30-35 and 10% of the total content of amino acid residues) and hydrophobic amino acid residues (e.g. proline and alanine, each constituting more than 10% of the total content of amino acid residues) [17], which can form hydrogen, hydrophobic, and other bonds with oxidoreductase and luciferase molecules. Besides, in the course of jelly formation, numerous secondary cross-links (mainly hydrogen bonds) are formed between the α-chains forming gelatin molecules, and collagen-like helical conglomerates are formed between several polypeptide chains [17]. Since enzymes are included in gelatin gel exactly at the moment of jelly formation, it can apparently result in partial deformation of protein conformation and, consequently, decrease in their activity.

Thus, comparison of the kinetic and thermodynamic characteristics of the NADH:FMN-oxidoreductase–luciferase coupled enzyme system, soluble and immobilized in polymer gels, has shown that immobilization in starch and gelatin gels results in substantial stabilization of the coupled enzyme system with respect to denaturing effects and maintenance of high enzyme activity. This demonstrates the possibility of using these methods for obtaining stable and highly active reagents for bioluminescent analysis.

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