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The Modeling of Viscous Microenvironment for the Coupled Enzyme System of Bioluminescence Bacteria

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***Abstract*—**Effect of viscosity of media on kinetic parameters of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase was investigated with addition of organic solvents (glycerol and sucrose), because bioluminescent enzyme systems based on bacterial luciferases offer a unique and general tool for analysis of the many analytes and enzymes in the environment, research and clinical laboratories and other fields. The possibility of stabilization and increase of activity of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase activity in vicious aqueous-organic mixtures have been shown.

***Keywords*—**The coupled enzyme system of bioluminescence bacteria NAD(P)H:FMN-oxidoreductase–luciferase, glycerol, stabilization of enzymes, sucrose.

I. Introduction

BIOLUMINESCENT enzyme systems based on bacterial luciferases offer a unique and general tool for analysis of the many analytes and enzymes in the environment, research and clinical laboratories and other fields. It is a useful tool in environmental risk assessment and monitoring of various aquatic and terrestrial ecosystems. The coupled enzyme system NADH:FMN-oxidoreductase–luciferase emits light at 490 nm in the presence of FMN, NAD(P)H, a long-chain aliphatic aldehyde and molecular oxygen [1]-[5]. The use of bioluminescent toxicity testing based on measurements of the glow of luminous bacteria and their enzymes (luciferase) has grown steadily in recent years.

Development of physico-chemical basis of bioluminescence assay, extension of the scopes of bioluminescence assay, increase of activity, selectivity and stability of enzymes of coupled enzyme system of bioluminescence bacteria are great importance now. An addition, the interaction between proteins and solvents is a general problem concerning the understanding of enzyme catalysis mechanisms. The protein—solvent interaction is a general problem concerning the understanding of enzyme catalysis mechanisms simple alteration of the solvent composition may modulate enzyme activity for biotechnological applications or for reproducing such behaviors observed *in vivo*.

One of approach to solve these problems is reaction media design.

To solve the basic problem on coupling and operating mechanisms of enzyme metabolic chains in the cell we have developed experimental models where the chain linking luciferase with other enzymes of luminous bacteria is realized in gel matrix. The tow prototypes of experimental models were developed: the thermodynamic, kinetic and spectral parameters of bioluminescence reaction in the presence of sucrose and glycerol have been investigated. The models imitate enzymes activity in vicious microenvironment.

The protein–solvent interaction is a general problem concerning the understanding of enzyme catalysis mechanisms too. Over the last few years, many studies have focused on the effects induced by organic solvents, water–organic solvent mixtures and super-critical fluids on the structure and on the catalytic properties of proteins [6]-[10]. A protein that is swelled or dissolved in a non-aqueous medium is modified in its secondary and tertiary structure, since the solvent alters inter- and intra-molecular electrostatic and hydrophobic interactions.

In earlier reports [11]-[13], we described the effects of organic solvents on catalytic activity of bacterial luciferases. Most of the organic solvents tested are effective in stimulating bacterial bioluminescence in vitro with the photoreduced $FMNH_2$ to a greater or lesser degree. If the spectral properties of the emitter of bacterial bioluminescence reaction are influenced by not only its interaction with luciferase, but properties of reaction mixture (dielectric constant, logP, polarity index, ionic strength, viscosity, pH and et. al.), addition of organic solvents might affect the bioluminescence.

A perspective approach in the study of interaction types at the formation of enzyme–substrate complexes is the kinetic analysis of the fermentative act at the addition of organic solvents in a reaction medium.

The effects of viscosity and pH of organic solvents on enzymes are accounted for both as their direct influence on the hydrate shell and/or active centre of the protein, and changes of electrostatic and hydrophobic intra- and intermolecular interactions, which accordingly change the efficiency of contacts between the biocatalytic reaction participants.

II. Materials and Methods

The coupled enzyme system NADH:FMN-oxidoreductase–luciferase (the luciferase from *Photobacterium leiognathi*, strain 208) used in the work has undergone high purification by ion-exchange chromatography [8].

In this work we used the recombinant luciferase from *Escherichia coli* SL-60 strain with cloned genes lux A and lux

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B of the luciferase from *Photobacterium leiognathi* luminescent bacteria from collection of Institute of biophysics of SD RAS [14].

To measure the control a reaction was carried out in the mixture of the following composition: 10μL of $(0.07–0.13)\cdot10^{-7}$ M luciferase, 50μL of aqueous solution of aldehyde, 440μL of $2\cdot10^{-2}$ M phosphate buffer, of 0.5mL of $7.6\cdot10^{-5}$ M aqueous solution of FMNH2 (Sigma, USA) with 10mM EDTA (Serva, USA), 200μL of 0.4 mM NADH, pH=7. Initiated by reduced $FMNH_2$, the bioluminescent reaction is a long flash of light with pronounced maximum (I_{max}) and decay of bioluminescence. The long-chain aldehyde – tetradecanal (C_{14}, Merck, Germany) was used as substrate. Measurements were carried out with a bioluminometer of «Terner BioSystems» (USA).

In experiments, the phosphate buffer was substituted for a water-organic mixture. Glycerol and sucrose (Sigma, USA) were used as organic solvents. They were used at the highest purity grade. Concentrations of solvents were expressed in volume per cent. pH in the reaction mixture were varied 5.8; 6.4; 6.9; 7.3; 7.8 for each chosen concentration of organic solvent.

The experimental results obtained have been statistically processed by Excel for Windows-98.

III. Results and Discussion

Effect of viscosity of media on kinetic parameters of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase was investigated with addition of organic solvents (glycerol and sucrose). Addition of organic solvents into the reaction medium of the coupled bioluminescent reaction changes the kinetic parameters of the light flash: maximum reaction rate (I_0), quantum yield (Q) and light emission decay constant (k_d).

Addition of organic solvents into the reaction medium of bioluminescent reaction changes kinetic parameters of the light flash. Addition of glycerol and sucrose increase the viscosity of the reaction medium decrease activity of the coupled enzyme bioluminescent system. Glycerol and sucrose inhibited the enzymes activity by about 50% with compared with initial light intensity, when concentrations reached different values - threshold concentrations - C_{50}.

Organic solvent concentrations at which half of the maximum activity is obtained (C_{50} – the threshold concentration of organic solvent) were determined for both water-miscible organic solvents. It should be noted that the loss of luciferase activity resulting from the action of the organic solvent is completely reversible at solvent concentration which is smaller than C_{50} value. Above threshold concentrations the catalytic activity of the coupled enzyme system studied in organic solvents decreased dramatically. Threshold concentrations of organic solvents (C_{50}) for glycerol exceed these C_{50} values for sucrose.

Among the used organic solvents, sucrose has the strongest activation on the coupled enzyme system NADH:FMN-oxidoreductase–luciferase.

With addition of solvents when pH ranges from 5.8 to 7.8 increases of the Imax and the Q depend on the balance between the capacity of the solvent to form hydrogen bonds and moderate hydrophobic contacts. The pH-optimum for the coupled enzyme system NADH:FMN-oxidoreductase–luciferase was 6.9.

Addition of glycerol and sucrose that increase the viscosity of the reaction medium at all used pH decrease activity of the coupled enzyme bioluminescent system (see Table I). The effects of organic solvents on the activity of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase were pH dependent.

TABLE I
Threshold Concentration (C_{50}) of Water-Miscible Organic Solvents at Different pH

C_{50}, M / pH	Sucrose	Glycerol
5.8	*0.30*	*7.29*
6.4	*1.93*	*6.53*
6.9	*2.70*	*8.10*
7.3	*1.53*	*7.57*
7.8	*1.21*	*7.80*

The results show that in the presence of sucrose at pH=5.8 the inhibition affect of viscosity was more affective, while glycerol inhibit strongly the coupled enzyme system NADH:FMN-oxidoreductase–luciferase activity at pH= 7.84.

Among the used organic solvents, sucrose has the strongest inhibition on the coupled enzyme system NADH:FMN-oxidoreductase–luciferase in almost cases (different pH).

Figs. 1, 2 are example of this behave for the coupled enzyme system NADH:FMN-oxidoreductase–luciferase in water-glycerol and water-sucrose mixes.

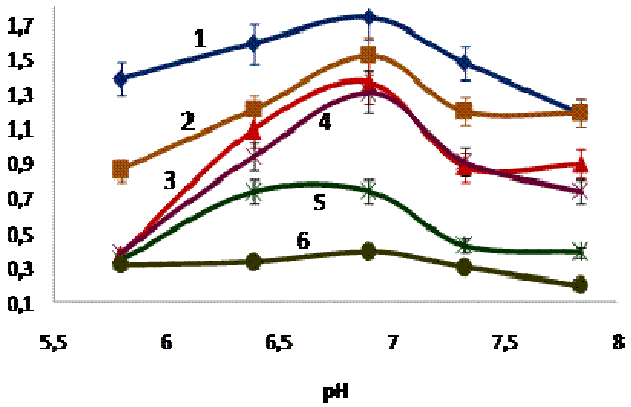

Fig. 1 Effect of pH on the luminescence intensity (%) of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase at sucrose concentrations : 1- control; 2- 0.06 M; 3- 0.51 M; 4-1.01 M; 5- 2.03 M; 6-4.06 M

Addition of glycerol and sucrose decreases the decay rate of excited emitter (light decay rate constant). Value of pH of reaction media attenuates electrostatic interactions. Glycerol and sucrose helps stabilize the excited intermediate of the reaction at all used pH. Sucrose caused the smaller decrease in the decay rate of the long-lived intermediate of the coupled

enzyme system NADH:FMN-oxidoreductase–luciferase.

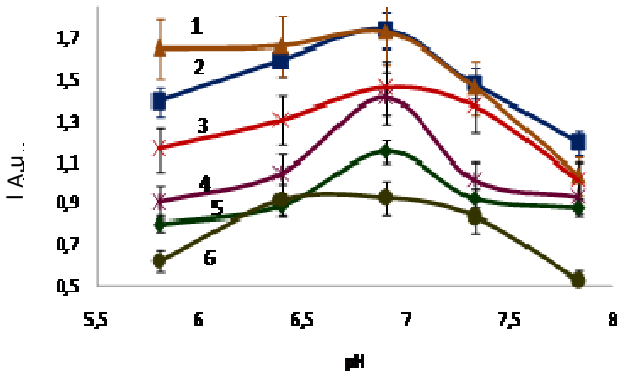

Fig. 2 Effect of pH on the luminescence intensity (%) of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase at glycerol concentrations : 1- 0.91 M-; 2- control ; 3- 1.09 M; 4- 2.72 M; 5- 5.44 M; 6-8.15 M

The quantum yield (Q) - total number of photons released in the course of reaction calculated from intensity and the rate constant is affected by the variation of both these reaction parameters. The value of Q does not essentially change in the presence of sucrose, but increases with addition of glycerol.

We found out that bioluminescent activity of luciferases was observed with not a wide range of organic solvent concentrations. Concentrations at which the luciferases lost half of its activity were 2-3 times less than the respective concentrations for several other enzymes [8], [11], [15]. At the same time, however, it is known that activity of some enzymes maintained or increased at higher solvent concentrations (after 50% v/v). Some enzymes may be used in 100 % (v/v) organic solvents with great efficiency. In the present study, possibility of stabilization and increase of activity of the coupled enzyme system NADH:FMN-oxidoreductase–luciferase activity in vicious aqueous-organic mixtures have been shown.

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