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Short Communication

Impact of enzyme stabilizers on the characteristics of biomodules for bioluminescent biosensors

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ABSTRACT

The biomodule of bioluminescent biosensor based on a coupled enzyme system NADH:FMN-oxidoreductase and luciferase, co-immobilized with substrates in dried starch or gelatin gels, has been developed. We studied the impact of several stabilizers—dithiothreitol (DTT), bovine serum albumin (BSA) and mercaptoethanol (ME) on the biomodule's activity, storage stability and sensitivity to toxic substances. The inclusion of stabilizers increases the activity of the biological module by more than 150%. To achieve the combination of high activity, prolonged storage time and acute sensitivity to toxic substances within maximum permissible concentration we used starch gel as a carrier adding 100 μM DTT to the immobilized preparation. The gelatin-based biological module had greater storage stability than the starch-based one but demonstrated less sensitivity to toxic substances.

1. Introduction

Enzymes from bioluminescent systems are widely used in analytical studies from the analysis of metabolites and enzyme activity to the integral toxicological analysis of various environments [1–5]. Bioluminescent reactions are known to provide remarkable sensitivity within the entire sample while modern photon counting technologies allow more precise measurements [1,6,7]. However, despite the evident advantages of bioluminescent enzyme-based methods, whole-cell bioluminescent bacterial sensors are most commonly used [8–10]. The application of enzyme-based bioluminescent sensors is still limited. The reason lies in the need to design a biomodule that could resist chemical and physical extremes (pH, temperature and others) and stay consistently active during storage [11,12].

Modification of the biomolecule microenvironment by variety of immobilization techniques is one of the latest strategies aimed at full structural and functional stabilization of biomolecules [13]. To obtain stable (during storage and use) preparations the enzymes of luminous organisms are immobilized in various carriers and different stabilizing additives are used [1,14–16]. A substantial advance was achieved in using starch gel to immobilize the bioluminescent enzyme system of luminescent bacteria [2,17]. Recently we reported that there is a commercially available product (immobilized multicomponent reagent) that can be used to assess integral toxicity of environment [2,18]. However, the problem to save the activity of the reagent during storage has not been definitively solved yet. In general, the characteristics of the reagent could be improved by co-immobilizing the enzymes, substrates and specialized stabilizers [13,19,20]. Those improved reagents can be used as a biological module of the enzyme-based bioluminescent biosensors.

This work examines the impact of stabilizer (type and amount) on the characteristics of the coupled enzyme system NADH:FMN-oxidoreductase and luciferase (R+L) co-immobilized with their substrates in starch or gelatin gel. The result is proposed as a base for biological module creation to be used in bioluminescent sensors to achieve high activity, stability during storage and use, sensitivity to toxic substances within maximum permissible concentrations (MPC).

2. Material and methods

2.1. Substances and solutions

The lyophilized preparations of highly purified enzymes were obtained from the Laboratory of nanobiotechnology and bacterial bioluminescence of the Institute of Biophysics SB RAS (Krasnoyarsk). One vial of the lyophilized preparation of enzymes contained 0.5 mg of luciferase (L) EC 1.14.14.3 from

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the recombinant strain *Escherichia coli* and 0.15 activity units of NADH:FMN-oxidoreductase (R) EC 1.5.1.29 (*Vibrio fischeri*).

The following reagents were used: NADH, FMN, mercaptoethanol, bovine serum albumin (BSA) and dithiothreitol (DTT) (Serva, Germany); myristic aldehyde (Merck, Germany); potato starch, gelatin, toluquinone, thymoquinone, naphtoquinone, benzoquinone (Sigma, Germany); hydroquinone, pyrocatechin, $CuSO_4$, $CrCl_3$, $HgCl_2$ (AR grade). To prepare the solutions of enzymes and substrates, a 0.05 M potassium phosphate buffer with pH 6.9 was used. Samples of the studied compounds (quinones, phenols and heavy metal salts) were dissolved in distilled water. Substances not easily soluble in water were dissolved in alcohol, the amount of which did not exceed 3% in the final reaction mixture.

2.2. Preparing the biological module

Twenty ml of 3.15% starch suspension (or 2% of gelatin) were heated until complete dissolution, and then cooled to a temperature of 25 °C. Then components were added one by one—luciferase, NADH:FMN-oxidoreductase, NADH, myristic aldehyde, and thoroughly mixed. The mixture was measured out (25 μl) on a mylar film using an epMotion 5075 automatic station (Eppendorf, Germany) and then dried at 4 °C for 24 h. This biomodule formed a dried disc with a diameter of 6–7 mm; its weight was 1.5 ± 0.2 mg.

2.3. Measuring the activity of the biological module

One disc and 300 μl of distilled water were added into a cuvet (control). A bioluminescent reaction was initiated by adding 10 μl of 0.5 mM FMN solution. These were immediately mixed, placed in the cuvet section of a Lumat LB 9507 bioluminometer (Berthold Technologies, Germany) and the bioluminescence signal was measured. The impact of the stabilizers on biomodule characteristics was detected through changes in the maximum luminous intensity (I_{max}), the time needed to reach the maximum luminescence (t_{max}) and the luminescence decay coefficient (k_d) in their presence, as compared to the control. The luminescence decay coefficient was calculated according to the following formula: $k_d = (\ln[I_1/I_2])/\Delta t$, where I_1 is the peak of bioluminescence intensity, I_2 is the bioluminescence intensity at the certain moment of time after reaching the bioluminescence maximum, and Δt is the time (minutes) needed for I_1 to reach I_2.

In experiments examining the sensitivity of the biomodule to toxic substances, 300 μl of the analyzed substance were added instead of water. The impact of toxic substances on the activity of biomodule was detected through the ratio of the maximum luminous intensity in their presence to the maximum luminous intensity of the control (I_i/I_0, %). Each experimental point was measured in at least three parallel measurements.

3. Results and discussion

3.1. Impact of stabilizers on biological module activity

To enhance the activity of the immobilized coupled enzyme system, stabilizing additives of different mechanisms were introduced. DTT and ME were used as stabilizers for enzyme SH-groups [2], and BSA was used as a component to increase the solution viscosity due to a higher protein concentration. Biomodules with different quantities of BSA, DTT or ME above the main components were obtained. The inclusion of stabilizers into biomodule increased the I_{max} by 150% and more (Fig. 1).

For the biomodule based on starch gel, the highest luminous intensity in the result sample was induced with 50 μM BSA. Here the luminous intensity was almost 10 times brighter than that

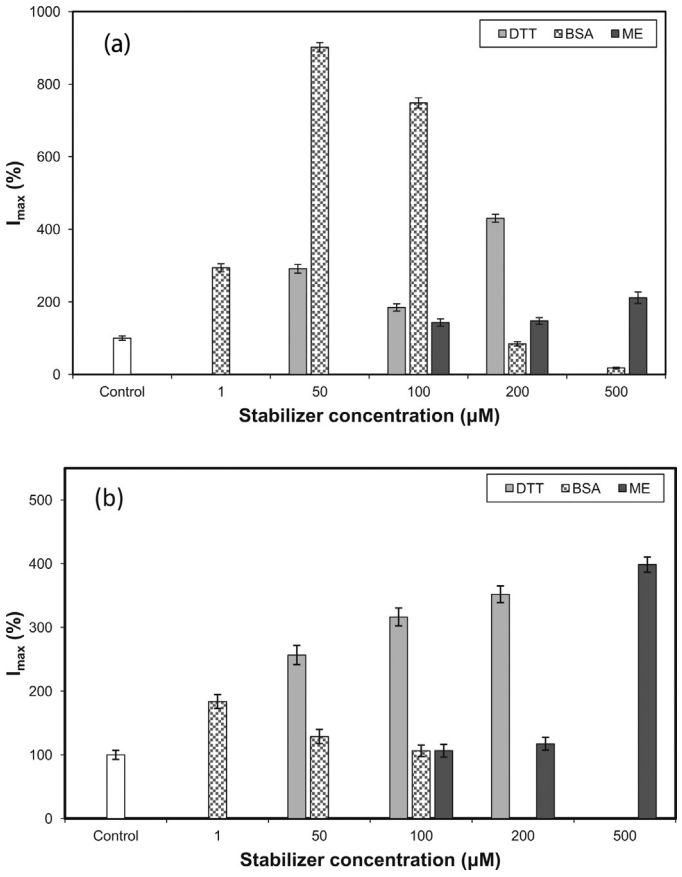

Fig. 1. Maximum luminous intensity of the biological module based on starch (a) and gelatin (b) gels containing enzyme stabilizers.

in the control sample without stabilizer. When smaller BSA concentrations were added, the activating effect did not appear, or was much less evident. If the BSA content was increased, the biomodule stabilization tended to reduce and could even inhibit the activity of the coupled enzyme system. Higher BSA concentrations cause "protein-protein" type interactions, which lead to changes in the functional activity of luciferase and/or oxidoreductase [21]. Moreover, BSA affects the amount of light gained from bacterial luciferase via pulling out one of the luciferase substrates—the aldehyde [22]. As for inclusion of DTT or ME, the highest biomodule activity was observed with 200 μM (Fig. 1a).

Adding stabilizers to gelatin gel-based biomodules increases the activity of the coupled enzyme system by approximately 4 times as compared to control biomodule (Fig. 1b). The highest activity is induced by of 500 μM of ME.

3.2. Impact of stabilizers on bioluminescence parameters and biological module characteristics during storage

The changes in biomodule activity containing 100 μM of DTT within 6 months storage at 4 °C are presented in Fig. 2. In control sample the activity decreased 5 times after 6 months. Stabilizers allow to reduce the drop of activity during storage. I_{max} in the sample containing 100 μM DTT after 6 months remained at 80% of the initial one. When 200 and 500 μM of ME were added, the biomodule retained up to 70% of its activity. The activity drop in biomodule with BSA as a stabilizer was also not prominent: 50 μM BSA retained about 90% of its activity after 6-months storage. The 100 μM DTT biomodule was characterized not only by the great activity and adequate stability during storage, but also by