

APPLICATION OF BIOLUMINESCENT ENZYMATIC TESTS IN ECOTOXICOLOGY

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Abstract

This paper examines the general principles of bioluminescent enzymatic toxicity bioassays and describes the applications of these methods and the implementation in commercial biosensors. Bioluminescent Enzyme System Technology (BEST) has been proposed in the bacterial coupled enzyme system, wherein NADH:FMN-oxidoreductase-luciferase substitutes for living organisms. BEST was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental, and industrial applications. For wide-spread use of BEST, the multi-component reagent 'Enzymolum' has been developed, which contains the bacterial luciferase, NADH:FMN-oxidoreductase and their substrates, co-immobilized in starch or gelatin gel. 'Enzymolum' can be integrated as a biological module into the portable biodetector-biosensor originally constructed for personal use. Based on the example of 'Enzymolum' and the algorithm of creating new enzyme biotests with tailored characteristics, a new approach was demonstrated in biotechnological design and construction. The examples of biotechnological design of various bioluminescent methods for ecological monitoring were provided. The advantages of enzymatic assays are their rapidity (the period of time required does not exceed 3-5 minutes), high sensitivity, simplicity and safety of procedure, possibility of automation of ecological monitoring; required luminometer is easily available.

Keywords: bioluminescence, luciferase, enzymatic assay, immobilization, integral water toxicity, ecological monitoring

1. INTRODUCTION

Historically, the application of bacterial luminescence in toxicology began with the usage of luminous bacteria for ecological monitoring and they are still widely used (Girotti et al., 2008; Roda et al., 2009; Fernández-Piñas et al., 2014; Xu et al., 2014). These methods made it possible to determine environmental pollution by comparing the light emission intensity of luminous bacteria in control with samples. As opposed to other test objects such as paramecia, algae, crustaceans, and so on, the bioluminescent assay is faster (typically < 30 min). However, as with other living organisms, living luminous bacteria is petulant. The failure to maintain the stable state of bacterial culture during measurements and storage results in low accuracy of measurement, a clear disadvantage of this method caused by the "petulance". The bacteria react to the appearance of toxic substances either by decreasing or by increasing the luminous intensity, often leading to ambiguous interpretation of results. Because of these shortcomings the assay based on luminous bacteria didn't show very good results in ecological laboratories. To overcome those difficulties it was suggested to use enzymes of luminous bacteria NAD(P)H:FMN-oxidoreductase and luciferase in soluble and immobilized forms (Kratasyuk, 1990; Esimbekova et al., 2013). Since 1990, bioluminescent enzymatic toxicity assay has been developed (Kratasyuk, 1990), and is nowadays actively used in ecology, medicine, agriculture, and other areas (Esimbekova et al., 2014; Kratasyuk and Esimbekova, 2015).

The bacterial coupled enzyme system: NAD(P)H:FMN-oxidoreductase + luciferase (Red + Luc) involves two reactions:

Luciferase (Luc)



NAD(P)H:FMN-oxidoreductase (Red)



In Reaction 1, luciferase catalyzes the oxidation of long-chain aliphatic aldehydes involving reduced flavin mononucleotide. One of the products of this reaction is a quantum of light in the blue-green spectrum. To provide luciferase with reduced flavin mononucleotide, the luciferase reaction is coupled with the reaction catalyzed by NAD(P)H:FMN-oxidoreductase (Reaction 2) (Shimomura, 2006).

Application of bioluminescent enzymatic toxicity assays is justified by the fact that Red as a part of enzymatic assays is present in all living organisms, leading to good correlation between the effect of toxic substances on living organisms and that on the coupled enzyme system of luminous bacteria. The main principle of the bioluminescent toxicity enzymatic assays is inhibition of Red and/or Luc activities by the toxic components of analyzed samples.

2. MATERIALS AND METHODS

2.1. Materials

This work was carried out using the lyophilised preparations of highly purified enzymes produced in the laboratory of Nanobiotechnology and Bioluminescence of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Each vial of the lyophilised preparation of enzymes contained 0.5 mg luciferase EC 1.14.14.3 from the recombinant strain *E. coli* and 0.15 units of NAD(P)H:FMN-oxidoreductase EC 1.5.1.29 from the *Vibrio fischeri* culture collection IBSO 836. To prepare the enzyme solutions, 5 mL of potassium-phosphate buffer was added to the vial containing the enzymes.

The immobilised multicomponent reagents 'Enzymolum' were produced by Prikladnye Biosistemy Ltd. (Krasnoyarsk, Russia). The reagents contain enzymes (Red + Luc) co-immobilised with substrates (NADH and myristic aldehyde) into 3% (w/v) starch gel (Enzymolum^{Starch}) or 1% (w/v) gelatine gel (Enzymolum^{Gelatin}) (Russian Federal Service for Intellectual Property, 2011; Bezrukh et al., 2014). The reagent Enzymolum was formed as a disk 6–7 mm in diameter and a dry weight of 1.5 ± 0.2 mg. FMN (Serva); NADH (Gerbu) and tetradecanal (Merck) were used as the substrates of Red and Luc. A 0.0025% (v/v) solution of myristic aldehyde was prepared by mixing 50 μL of 0.25 % (v/v) ethanol solution of aldehyde and 5 mL of 0.05 M potassium-phosphate buffer (pH 6.9). NADH solution was prepared in 0.05 M potassium-phosphate buffer (pH 6.9).

2.2. Methods

The activity of the soluble coupled enzyme system Red + Luc was measured in the reaction mixture containing 300 μL of 0.05 M potassium-phosphate buffer (pH 6.9), 5 μL of enzyme solution, 50 μL of 0.0025% (v/v) aldehyde solution, 50 μL of 0.4 mM NADH solution and 10 μL of 0.5 mM FMN solution. At the beginning, the control luminescence intensity of the enzyme system (I_c) was registered. For I_c registration, all components of the reaction mixture and 50 μL of the control solution were subsequently added to the tube of a Glomax 20/20 luminometer (Promega, USA) and quickly mixed, and the maximum intensity of luminescence was measured. For registration of the luminescence intensity in the presence of the analyzed sample (I_{exp}), 50 μL of the control solution was placed on 50 μL of the analyzed solutions.

The activity of the immobilised reagent 'Enzymolum' was measured in the reaction mixture containing the following: 1 disc of the reagent Enzymolum, 300 µL of distilled water, 50 µL of the analyzed solution (or control solution) and 10 µL of 0.5 mM FMN solution.

3. RESULTS AND DISCUSSION

3.1. Principles of the bioassays based on the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase

Application of enzymatic bioassays based on the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase for ecological monitoring instead of living organisms such as intact luminescent bacteria, daphnia, algae and other organisms that can be legally used in Russia is justified by the fact that NADH:FMN-oxidoreductase as part of enzymatic bioassays is present in all living organisms. That is why good correlation is expected between the effect of toxic substances on living organisms and on the coupled enzyme system of luminous bacteria. Integrated luciferase-based biotesting methods are mainly used for continuous rapid environmental monitoring in industrial and agricultural regions, for control over pollutant emissions of enterprises, for estimation of the efficiency of waste water detoxification and of the control of water and wastewater treatment facilities, as well as for ecological risk assessment.

Bioluminescent integrated assays demonstrate changes in the bioluminescent signal as a reaction to the appearance of large-scale classes of polluting substances in the analyzed media. But the development of such assays depends on the influence of pollutants and xenobiotics on bioluminescence (Kudryasheva et al. 1998, 2002; Vetrova et al. 2007b).

Enzymatic bioluminescent bioassays are based on the classification of bioluminescence inhibitors and activators according to the mechanism of their influence on fundamental physico-chemical processes. The patterns of exogenous compound influence on bioluminescent systems are classified (Kudryasheva 2006). According to this classification, there are four possible ways in which exogenous compounds act on a bioluminescent system: 1) influence of molecules on energy transport processes in a bioluminescent system; 2) influence of molecules on hydrogen transport processes in a bioluminescent system; 3) influence of molecules on electron transfer processes in a bioluminescent system; 4) interaction of molecules with the enzymes of a bioluminescent system. Knowing the physico-chemical basics of luminescent biotesting, it is possible to predict the analysis results and change the sensitivity of bioassays to certain pollutant groups (Kratasyuk et al. 1991; Kudryasheva et al. 2000, 2002; Vetrova et al. 2007b).

When enzymatic biotesting is carried out, several analysis schemes can be used (Fig. 1). The first variant of bioluminescent enzymatic bioassay is the quickest and demonstrates good repeatability of results. The following sequence of operations is performed: a cuvette with all the necessary components of a coupled enzyme system of luminous bacteria, their substrates and buffer solution are placed into a bioluminometer, and after the maximum light emission intensity I_c (control) is registered, 5 to 50 µL of the test water sample or toxic substance solution are added, and the maximum light emission intensity I_{exp} is registered again (Fig. 1a).

The intensity of the residual luminescence $(I_{exp}/I_c) \cdot 100\%$ and the decay constant k_d are estimated. The decay constant is calculated according to the following formula: $k_d = (\ln(I_2/I_1))/\Delta t$, where I_1 is the peak of bioluminescence intensity, I_2 is the bioluminescence intensity at the moment of certain time after reaching the bioluminescence maximum, and Δt is the time (minutes) needed for I_1 to reach I_2 .

When analyzing toxicity of the water samples, the luciferase index (LI) or toxicity coefficient (TC) are calculated according to the formulas:

$$LI = (I_{exp} / I_c) \times 100 \%$$

$$TC = [(I_c - I_{exp}) / I_c] \times 100 \%$$

LI and TC are the residual luminescence and the degree of inhibition of the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase in the presence of analyzed sample correspondingly and $TC = 100 - LI$.

The criterion of toxicity is a 50 % decrease in the maximum luminescence level of the bacterial coupled enzyme system after the analyzed sample is added, as compared to the luminescence level in the control sample.

In the second case it is possible to achieve higher sensitivity of assays to the toxic substances. In this variant, testing of the control sample (usually, distilled water or buffer solution) and analysis sample is performed in different cuvettes (Kratasyuk and Esimbekova, 2011a). The reaction of the bioassay is also determined by the values of TC and k_d . But in that case, it is possible to use one more parameter for toxicity analysis – the time when the coupled enzymatic system reached the luminescence maximum (T_{max}) (Fig. 1b).

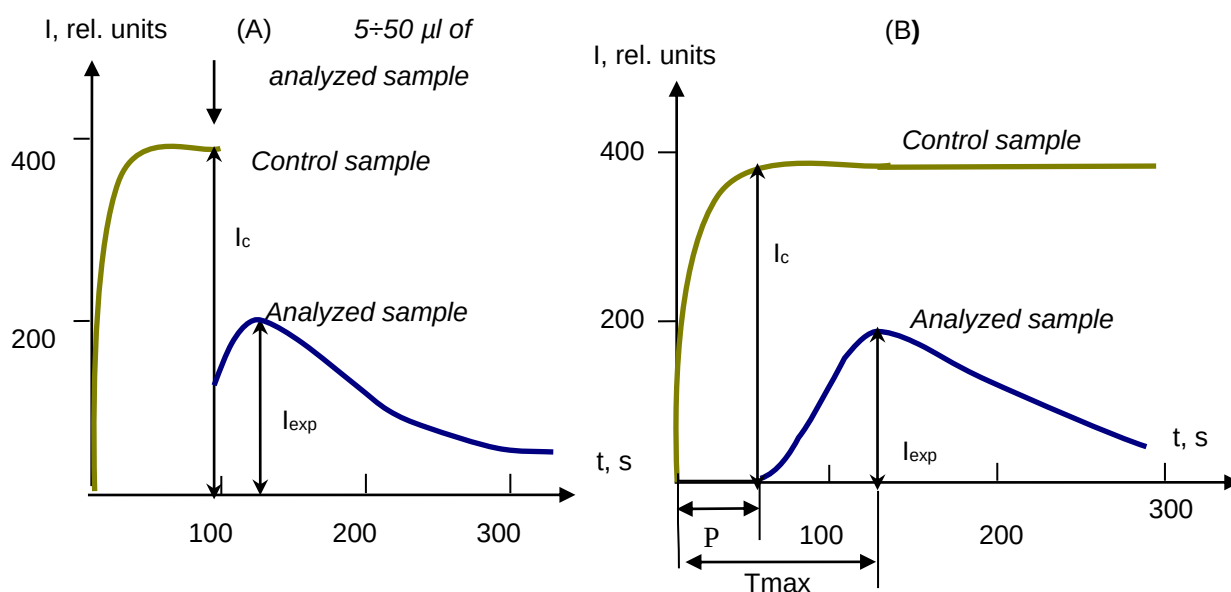


Fig. 1. Bioluminescent testing diagram (A). Modified diagram of bioluminescent testing (B).

Application of the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase for ecological monitoring is justified in quite a large number of works (Kratasyuk et al. 1996, 2001; Vetrova et al. 2007b). Bioluminescent bioassays based on the coupled enzyme system: NADH:FMN-oxidoreductase-luciferase were successfully used for the analysis of water in the Yenisei river and its tributaries, drinking water in various districts of Krasnoyarsk and Altai Territories, salt lake Shira, Lake Baikal (Kratasyuk et al. 1999; Vetrova et al. 2002) as well as air and soil pollutions (Rimatskaya et al., 2012, 2014).

A new trend in using bioluminescent enzymatic toxicity assay is the assessment of detoxification of pollutant solutions by water-soluble humic substances (HSs). This method is based on the quantitative determination of the antioxidant activity of HSs. There were a few studies that promote application of the assay to monitor toxicity of pollutants of oxidative nature in environmental and waste waters during remediation procedures (Tarasova et al., 2015). The bioluminescent enzymatic toxicity assays were applied to monitor changes in the toxicity of homologous quinones with different redox characteristics under exposure to HSs. Toxicities of general and oxidative types were evaluated using bioluminescent kinetic parameters—bioluminescence intensity and T_{max} , respectively. Antioxidant activity of HSs was attributed to their ability to decrease both general and oxidative toxicities. The HSs antioxidant efficiency was characterized with detoxification coefficients D_{GT} and D_{OxT} ,

respectively. Dependency of D_{GT} and D_{OxT} on HSs concentration and time of preliminary incubation of the oxidizers with HSs were demonstrated. The optimal conditions for detoxification of the oxidizers were > 20 -min incubation time and from $50 \mu\text{M}$ to 0.2 mM of HSs concentration.

3.2. The set of bioluminescent enzymatic toxicity assays

Bioluminescent enzymatic toxicity assay provides an instrument to solve a problem of complex evaluation of environmental toxicity. It is well-known that to estimate environmental toxicity it is necessary to use the battery of bioassays. Usually they represent different levels of organisms such as cells, organs, organisms and ecosystems. Due to the coupling with bacterial luciferase, it is possible to design new enzymatic bioassays in toxicology and combine them into a set to provide the toxicity control at the enzymatic level (Kratasyuk and Gitelson, 1987). The set includes enzymes of different classes, or key enzymes of metabolic processes in living organisms. The bacterial luciferase may be the terminal enzyme in coupling chains for more than 100 enzymes including such as lactate dehydrogenase, trypsin, glucose-6-phosphate dehydrogenase, and others, making it possible to measure the enzyme activities according to the light emission intensity.

To develop the set of bioluminescent enzymatic toxicity bioassays different enzyme interaction mechanisms were suggested (Fig. 2). For example, in research by Kratasyuk et al. (2001) to estimate toxicity of water samples two enzymes were chosen: alcohol dehydrogenase (ADH) and trypsin, because they belong to different classes (oxidoreductases and hydrolases), and secondly, because they interact differently with bacterial luciferase, providing sensitivity to the different toxic substances (Kudryasheva et al., 1999, 2003).

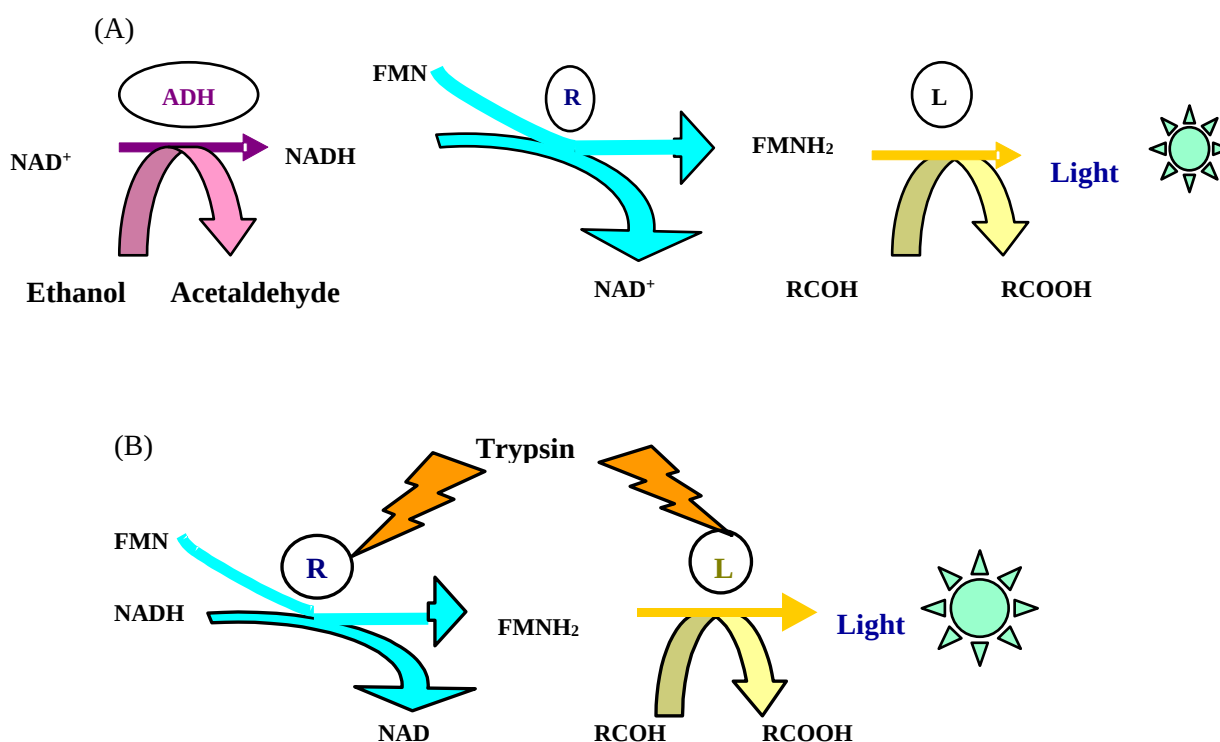


Fig. 2. Examples of coupling of the enzymatic reactions. (A) The sequence of enzymes in the triple enzyme system: ADH + Red + Luc (Petushkov et al., 1987); (B) interaction of enzymes in the triple enzyme system: trypsin + Red + Luc (Njus et al., 1974).

The effect of toxic substances on the activities of the triple enzyme system with ADH and trypsin were measured using the bioluminescence decay constant (Fig. 3).

Moreover, it became possible to regulate the sensitivity of enzymatic toxicity assays. For example, it was shown that the sensitivity of enzymatic assays to the toxic substances may be increased by extending the coupling chain of enzymatic reactions (Fig. 4).

The set of bioluminescent enzymatic toxicity assays was used for monitoring natural and laboratory aquatic ecosystems (Kratasyuk et al., 2001) and for studying the seasonal dynamics of zooplankton non consumptive mortality (Dubovskaya et al., 2002), as well as for toxicity analysis of pesticides (Vetrova et al., 2007a), nanomaterials (Esimbekova et al., 2017a,b) and sanitary assessment of natural polymers polyhydroxyalkanoates (Shishatskaya et al., 2002).

3.3. Other fields of application of bioluminescent enzymatic toxicity assays

Bioluminescent enzymatic toxicity assays are used not only in ecology but also in other sectors, such as agriculture, food industry and medicine.

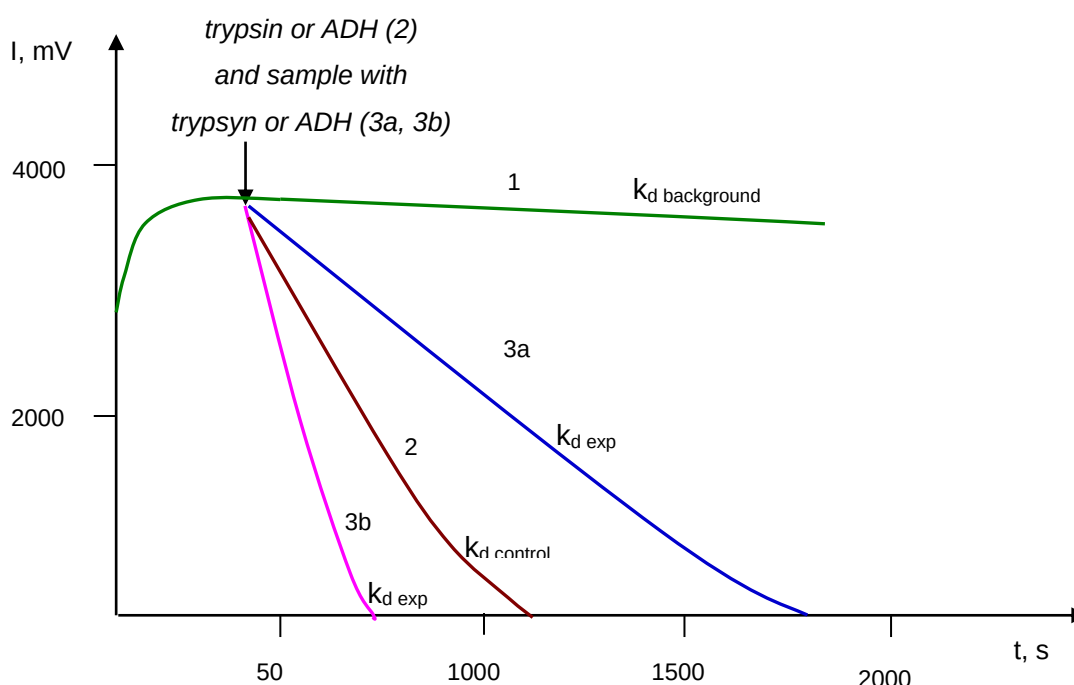


Fig. 3. Scheme of ADH (or trypsin) activity measurement using the bioluminescence decay constant: $k_d \text{ background}$ is the decay constant for the coupled enzyme reaction Red + Luc (1); $k_d \text{ control}$ is the decay constant for the triple enzyme reaction with ADH or trypsin in the presence of control solution (2); $k_d \text{ exp}$ is the decay constant for the triple enzyme reaction with ADH or trypsin with toxic substances: 3a, the pollutant inhibits trypsin or ADH activity; 3b, the pollutant activates trypsin or ADH.

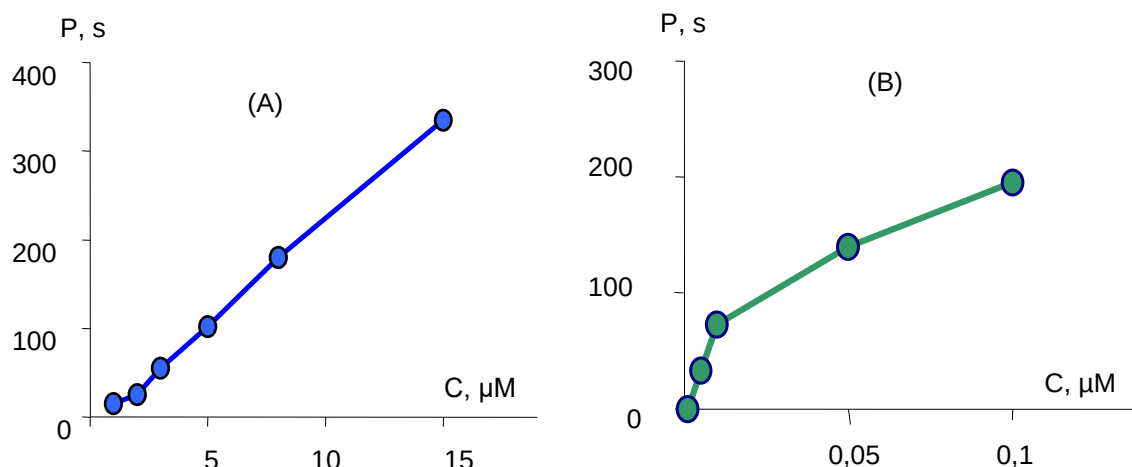


Fig. 4. Dependence of induction period on the concentration of 1,4-benzoquinone in the reaction mixture, (A) - for the coupled enzyme system R + L, and (B) - for the triple enzyme system ADH + R + L. Increasing the number of links in the coupling chain of enzymatic reactions results in a tenfold increase of sensitivity to benzoquinone

Source: N.S. Kudryasheva et al, Chemosphere, vol. 38 (February 1999), pp. 751–758.

Mostly method of assessing bacterial contamination, based on firefly ATP-dependent luciferase is applied. Methods based on the use of enzymatic bioluminescent bacterial systems are also applied for food product quality. For example, methods for detecting living bacterial cells were developed using luminous bacteria enzymes (Mei et al., 2009; Peng et al., 2014). The bioluminescent assay for determination of L- and D-lactate in beer has been designed (Girotti et al., 2000). The effects of the mycotoxins produced by *Fusarium* fungi on the coupled enzyme system: NADH:FMN-oxidoreductase and luciferase were studied (Kratasyuk et al., 1998a) and method for evaluation of wheat grain infection with *Fusarium* has been developed (Kratasyuk et al., 1998b). A possibility was demonstrated to use bioluminescent methods for safety evaluation of food preservatives (Esimbekova et al., 2017c). Nevertheless methods based on the use of enzymatic bioluminescent bacterial systems have been applied for food product quality rarely yet.

Bioluminescent enzymatic toxicity assays are also very promising for medical research, for example, for evaluating the gravity of endotoxemia during treatment in surgery and therapy. The bioluminescent assay for evaluating the gravity of endotoxemia is based on the fact that the effect of the blood serum of donors on enzymatic activity differed markedly from that of the patients. It has been shown that blood serum of a patient inhibits bioluminescence less than that of donor.

Comparative analysis of application efficiency for luciferase index LI and other laboratory parameters to assess the severity of patients with peritonitis have been made (Sovtsov and Kratasyuk, 1991). LI index was useful in case of doubts on the abdominal inflammation location and severity of disease. Bioluminescent assays allow to estimate the severity of a patient's condition as satisfactory, of middle seriousness, severe and the critical one. They can be used also for disease course prediction, estimation of the efficiency of the used detoxification methods, and control of drainage procedure with semipermeable membranes. Most important is applying LI in prognostic plan as far as the long low-positive LI dynamics could indicate the need for change of treatment plan.

It was reported that bioluminescent enzymatic toxicity assay can be used as a reliable criterion to monitor the course of disease for therapeutic patients with bronchitis, ulcerous disease or chronic colecystitis (Esimbekova et al., 1999). The most important advantages of the proposed approach are

the very short time interval between sample collection and results, high sensitivity, low traumatism, and simplicity of the method.

A very interesting and promising trend in the development of bioluminescent enzymatic toxicity assay is the design of rapid analysis for the assessment of human organism reaction to physical and mental stress. Analysis is made by comparing the light emission intensity of the coupled enzyme system Red + Luc in the presence of a person's saliva taken before and after a physical stress. The main advantages of this method are not only its rapidity, high precision, and sensitivity, but also noninvasiveness, because human saliva is analyzed, which reflects the functional state of a person just as blood does.

It is possible to use bioluminescent assay *in vitro* for selective analysis also, for example to measure stress in plants. Pyridine nucleotides are key redox carriers in the soluble phase of all living cells and play crucial roles in pro- and antioxidant metabolism and non-redox mechanisms in particular in plant stress responses. Therefore, the determination of pyridine nucleotide content and the ratio of their oxidized and reduced forms can be the basis for monitoring of plant response to stress caused by environmental changes. Bioluminescent method to determine pyridine nucleotide NAD(P)H, NAD(P)⁺ in plant tissues grown in closed hypobaric growth chambers have been developed. The experiments model conditions that would be used for plant chambers on the Moon or Mars as indicators of oxidative stress caused by a low pressure environment (Kratasyuk et al., 2011) (Fig. 5).

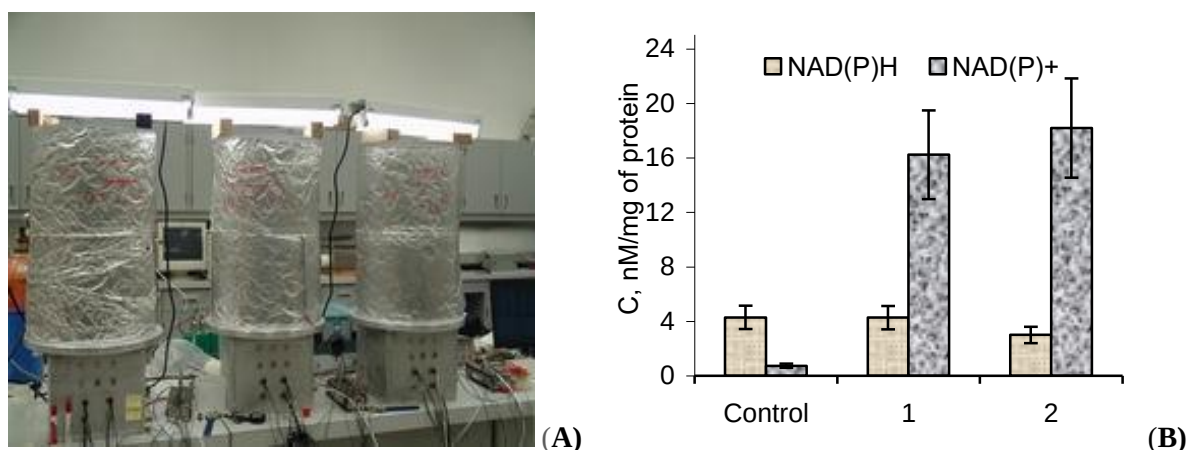


Fig. 5. (A) – Growing of the plant samples in climatic chambers; (B) – Concentrations of NAD(P)⁺ and NAD(P)H in the extracts of radish roots under conditions of modeled stress: control – sample plants grown at 101.5 kPa; (1) experimental plants grown at 32 kPa and oxygen partial pressure 4.7 kPa for 24 h; (2) experimental plants grown at 32 kPa and oxygen partial pressure 6.5 kPa for 24 h and then additionally at the 9 kPa pressure and oxygen partial pressure 3.3 kPa for the next 24 h.

Source: V.A. Kratasyuk et al, Luminescence, vol. 26 (January 2011), pp. 543-546.

3.4. Enzymatic Reagents for Bioluminescent Analysis

Widespread use of the available bioluminescent enzymatic toxicity bioassays is limited by the instability of the enzymes during use, limited shelf-life of enzymes–reagents, the need to control ambient conditions (i.e., pH, temperature, etc.), high manufacturing cost, and other factors. These problems can be solved by using immobilized enzymes that possess high catalytic activity and stability for long-term storage and successfully serve as biological modules of biosensors (Turner, 2000).

For the last 30 years immobilization has been widely used for production of stable reagents for bioluminescent analysis based on various bioluminescent systems: luminous bacteria, and bacterial

and firefly luciferases. Many of the available immobilized reagents are successfully used in analytic measurements and in biosensors, because they simplify the analysis procedure, sometimes enabling full automation. At present, there are more than 40 different methods of immobilizing luminous organisms and enzymes (Kratasyuk and Esimbekova, 2003). An important advantage of immobilized enzymes is the possibility to control the enzyme stability to physical and chemical factors by way of choosing a suitable microenvironment. The optimal microenvironment for bacterial luciferase is natural polymer gels such as gelatin or starches (potato, rice, or corn). By varying gel concentration, time, and mode of drying of immobilized enzymes it is possible to make reagents with different enzymatic activity (Esimbekova et al., 2007, 2015).

It was shown that coupled enzyme system Red + Luc immobilized in starch or gelatin gel, saves its activity for 2 years (Lonshakova-Mukina et al., 2015). Moreover, immobilization in these gels leads to a considerable stabilization of the coupled enzyme system with regard to denaturation treatment: pH optimum of the enzymes expands both to the acid and alkaline areas; high enzyme activity is maintained at increased salt concentration; thermal stability increases essentially, especially in case of starch gel immobilization (Esimbekova et al., 2009; Bezrukikh et al., 2014).

Several substrates of bacterial bioluminescent reaction can be co-immobilized together with the coupled enzyme system to make the final test much simpler. For example, homogeneous multicomponent reagent named 'Enzymolum' contains the enzymes Red and Luc, their substrates (myristic aldehyde and NADH) and buffer salts, co-immobilized in starch or gelatin gel (Kratasyuk and Esimbekova, 2011b). The reagent is currently produced in flake form and can be used in the tube bioluminometer (Fig. 6).



Fig. 6. The multicomponent reagent 'Enzymolum' is a flake of dried gel, diameter 6–7 mm; dry weight 1.5 ± 0.2 mg.

The advantages of enzymatic assays using 'Enzymolum' are their rapidity (the time of analysis does not exceed 3–5 min), high sensitivity, one-step measuring procedure and possibility of automation of analysis.

4. CONCLUSIONS

The new approach to develop the bioluminescent enzymatic biosensors, toxicity bioassays and reagents has been described. To solve the problem of how to detect, identify, and measure the contents of the numerous chemical compounds in environmental monitoring, food product monitoring, and medical diagnostics, the bioluminescent enzymatic toxicity assays were proposed, wherein the bacterial coupled enzyme system NAD(P)H:FMN-oxidoreductase-luciferase substitutes for living organisms. The immobilized reagent Enzymolum was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for toxicological assays. The reagent is easy to use and convenient to be applied not only in toxicology studies but also in course of education, mainly in ecological and enzymological practical courses (Gitelson and Kratasyuk, 2002, Kratasyuk and Kudinova, 1999). Prototype biosensors offer cost advantages, versatility, high sensitivity, rapid response, extended shelf and flexible storage conditions.

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REFERENCES

1. Bezrukikh, AE, Esimbekova, EN, Nemtseva, EV, Kratasyuk, VA, Shimomura, O 2014, "Gelatin and starch as stabilizers for the coupled enzyme system of luminous bacteria NADH:FMN-oxidoreductase-luciferase", *Analytical and Bioanalytical Chemistry*, vol. 406, pp. 5743-5747.
2. Dubovskaya, OP, Gladyshev, MI, Esimbekova, EN et al 2002, "Study of possible relation between seasonal dynamics of zooplankton nonconsumptive mortality and water toxicity in a pond", *Inland Water Biology*, vol. 3, pp. 39-43.
3. Esimbekova, EN, Asanova, AA, Deeva, AA, Kratasyuk, VA 2017, "Inhibition effect of food preservatives on endoproteinases", *Food Chemistry*, vol. 235, pp. 294-297.
4. Esimbekova, EN, Kondik, AM, Kratasyuk, VA 2013, "Bioluminescent enzymatic rapid assay of water integral toxicity", *Environmental Monitoring and Assessment*, vol. 185, pp. 5909-5916.
5. Esimbekova, EN, Kratasyuk, VA, Abakumova, VV 1999, "Bioluminescent method nonspecific endotoxigenesis in therapy", *Luminescence*, vol. 14, pp. 197-198.
6. Esimbekova, EN, Kratasyuk, VA, Shimomura, O 2014, "Application of enzyme bioluminescence in ecology", *Advances in Biochemical Engineering/Biotechnology*, vol. 144, pp. 67-109.
7. Esimbekova, EN, Kratasyuk, VA, Torgashina, IG 2007, "Disk-shaped immobilized multicomponent reagent for bioluminescent analyses: correlation between activity and composition", *Enzyme and Microbial Technology*, vol. 40, pp. 343-346.
8. Esimbekova, EN, Lonshakova-Mukina, VI, Bezrukikh, AE, Kratasyuk, VA 2015, "Design of multicomponent reagents for enzymatic assays", *Doklady Biochemistry and Biophysics*, vol. 461, no. 4, pp. 102-105.
9. Esimbekova, EN, Nemtseva, EV, Bezrukikh, AE, Jukova, GV, Lisitsa, AE, Lonshakova-Mukina, VI, Rimatskaya, NV, Sutormin, OS, Kratasyuk, VA 2017a, "Bioluminescent enzyme inhibition-based assay to predict the potential toxicity of carbon nanomaterials", *Toxicology in Vitro*, vol. 45, part 1, pp. 128-133.
10. Esimbekova, EN, Nemtseva, EV, Kirillova, MA, Asanova, AA, Kratasyuk, VA 2017b, "Bioluminescent assay for toxicological assessment of nanomaterials", *Doklady Biochemistry and Biophysics*, vol. 472, no. 1, pp. 60-63.
11. Esimbekova, EN, Torgashina, IG, Kratasyuk, VA 2009, "Comparative study of immobilized and soluble NADH:FMN-oxidoreductase-luciferase coupled enzyme system", *Biochemistry (Moscow)*, vol. 74, pp. 695-700.
12. Fernández-Piñas, F, Rodea-Palomares, I, Leganés, F et al 2014, "Evaluation of the ecotoxicity of pollutants with bioluminescent microorganisms", *Advances in Biochemical Engineering/Biotechnology*, vol. 145, pp. 65-135.
13. Gitelson, JI, Kratasyuk, VA 2002, "Bioluminescence as an educational tool", In: Kricka LJ, Stanley PE (eds), "Bioluminescence and chemiluminescence: progress and current applications", World Scientific Publishing Co Pte Ltd, Singapore, pp. 175-182.
14. Girotti, S, Ferri, EN, Fumo, MG et al 2008, "Monitoring of environmental pollutants by bioluminescent bacteria", *Analytica Chimica Acta*, vol. 608, pp. 2-29.
15. Girotti, S, Muratori, M, Fini, F et al 2000, "Luminescent enzymatic flow sensor for D- and L-lactate assay in beer", *European Food Research and Technology*, vol. 210, pp. 216-21.

16. Kratasyuk, VA 1990, "Principle of luciferase biotesting", In: "Biological luminescence, Proceedings of the first international school", Wroclaw, Poland, June 20-23, 1989, World Scientific Publishing Co Pte Ltd, Singapore, pp. 550-558.
17. Kratasyuk, VA, Gitelson, JI 1987, "Application of luminous bacteria in bioluminescent analysis", *Uspekhi microbiologii*, vol. 21, pp. 3–30.
18. Kratasyuk, VA, Egorova, OI, Esimbekova, EN et al 1998b, "A biological luciferase test for the bioluminescent assay of wheat grain infection with *Fusarium*", *Applied Biochemistry and Microbiology*, vol. 34, pp. 622–624.
19. Kratasyuk, VA, Esimbekova, EN 2003, "Polymer immobilized bioluminescent systems for biosensors and bioinvestigations", In: Arshady R (ed) "Polymeric biomaterials", The PBM Series (Introduction to Polymeric Biomaterials), Citus Books, London, vol. 1, pp. 301–343.
20. Kratasyuk, VA, Esimbekova, EN 2011a, *Express method for biotesting of natural, manufacturing waters and water solutions*, RU Patent 2,413,771.
21. Kratasyuk, VA, Esimbekova, EN 2011b, *Bioluminescent biomodule for analyses of various media toxicity and method of its preparation*, RU Patent 2,413,772.
22. Kratasyuk, VA, Esimbekova, EN 2015, "Applications of luminous bacteria enzymes in toxicology", *Combinatorial Chemistry & High Throughput Screening*, vol. 18, I. 10, pp. 952-959.
23. Kratasyuk, VA, Esimbekova, EN, Correll, M, Bucklin, R 2011, "Bioluminescent enzyme assay for the indication of plant stress in enclosed life support systems", *Luminescence*, vol. 26, pp. 543-546.
24. Kratasyuk, VA, Esimbekova, EN, Gladyshev, MI et al 2001, "The use of bioluminescent biotests for study of natural and laboratory aquatic ecosystems", *Chemosphere*, vol. 42, pp. 909–915.
25. Kratasyuk, VA, Kudinova, IY 1999, "Practical enzymology course based on bioluminescence", *Luminescence*, vol. 14, pp. 189-192.
26. Kratasyuk, VA, Kuznetsov, AM, Rodicheva, EK et al 1996, "Problems and prospects of bioluminescence assays in ecological monitoring", *Siberian Journal of Ecology*, vol. 5, pp. 397-403.
27. Kratasyuk, VA, L'vova, LS, Egorova, OI et al 1998a, Effect of *Fusarium* mycotoxins on a bacterial bioluminescence system in vitro, *Applied Biochemistry and Microbiology*, vol. 34, pp. 190–192.
28. Kratasyuk, VA, Makurina, VI, Kuznetsov, AM et al 1991, "Study of effect of sulfo-substituted succinic acid on bacterial luminescence", *Applied Biochemistry and Microbiology*, vol. 27, pp. 127-133.
29. Kratasyuk, VA, Vetrova, EV, Kudryasheva, NS 1999, "Bioluminescent water quality monitoring of salt Lake Shira", *Luminescence*, vol. 14, pp. 193–195.
30. Kudryasheva, NS 2006, "Bioluminescence and exogenous compounds. Physico-chemical basis for bioluminescent assay", *Journal of Photochemistry and Photobiology B: Biology*, vol. 83, pp. 77–86.
31. Kudryasheva, NS, Esimbekova, EN, Rimmel, NN et al 2003, "Effect of quinones and phenols on the triple—enzyme bioluminescent system with protease", *Luminescence*, vol. 18, pp. 224–228.
32. Kudryasheva, NS, Esimbekova, EN, Kudinova, IY et al 2000, "Effects of Quinones on NADH-Dependent Enzymatic Bioluminescent Systems" *Applied Biochemistry and Microbiology*, vol. 36, pp. 409-413.
33. Kudryasheva, NS, Kratasyuk, VA, Esimbekova, EN et al 1998, "Development of the bioluminescent bioindicators for analyses of environmental pollutions", *Field Analytical Chemistry & Technology*, vol. 2, pp. 277-280.

34. Kudryasheva, NS, Kudinova, IY, Esimbekova, EN et al 1999, "The influence of quinones and phenols on the triple NAD(H)-dependent enzyme systems", *Chemosphere*, vol. 38, pp. 751–758.
35. Kudryasheva, NS, Vetrova, EV, Kuznetsov, AM et al 2002, "Bioluminescent assays: effects of quinones and phenols", *Ecotoxicology and Environmental Safety*, vol. 53, pp. 221-225.
36. Lonshakova-Mukina, VI, Esimbekova, EN, Kratasyuk, VA 2015, "Impact of enzyme stabilizers on the characteristics of biomodules for bioluminescent biosensors", *Sensors and Actuators B: Chemical*, vol. 213, pp.244–247.
37. Mei, C, Wang, J, Lin, H et al 2009, "Quantitative detection of NADH by in vitro bacterial luciferase bioluminescent", *Wei Sheng Wu Xue Bao*, vol. 49, pp. 1223–1228.
38. Njus, D, Baldwin, TO, Hastings, JW 1974, "A sensitive assay for proteolytic enzymes using bacterial luciferase as a substrate", *Analytical Biochemistry*, vol. 61, pp. 280–287.
39. Peng, Y, Jin, Y, Lin, H et al 2014, "Application of the VPp1 bacteriophage combined with a coupled enzyme system in the rapid detection of *Vibrio parahaemolyticus*", *Journal of Microbiological Methods*, vol. 98, pp. 99–104.
40. Petushkov, V, Shefer, L, Rodionova, N et al 1987, "Bioluminescent method of determination of NAD(P)H dehydrogenase activity", *Applied Biochemistry and Biotechnology*, vol. 23, pp. 270–274.
41. Rimatskaya, NV, Baigina, EM, Kazanceva, MA et al 2014, "Application of bioluminescent enzymatic method for assessment of the state of the soil", *Luminescence*, vol. 29, pp. 66-67.
42. Rimatskaya, NV, Nemtseva, EV, Kratasyuk, VA 2012, "Bioluminescent assays for monitoring of air pollution", *Luminescence*, vol. 27, pp. 154.
43. Roda, A, Guardigli, M, Michelini, E et al 2009, "Bioluminescence in analytical chemistry and in vivo imaging", *Trends in Analytical Chemistry*, vol. 28, pp. 307–322.
44. Shimomura, O 2006, "Bioluminescence: chemical principles and methods", World Scientific Publishing Co Pte Ltd, Singapore.
45. Shishatskaya, EI, Esimbekova, EN, Volova, TG et al 2002, "Hygienic assessment of polyhydroxyalkanoates—natural polyethers of new generation", *Gigiena Sanitaria*, vol. 4, pp. 59–63.
46. Sovtsov, SA, Kratasyuk, VA 1991, *Determination of endotoxemia under surgery*, USSR Patent 1,714,512.
47. Tarasova, AS, Stom, DI, Kudryasheva, NS 2015, "Antioxidant activity of humic substances via bioluminescent monitoring in vitro", *Environmental Monitoring and Assessment*, vol. 187, p. 89.
48. Turner, APF 2000, Biosensors—sense and sensitivity, *Science*, vol. 290, pp. 1315–1317.
49. Xu, T, Close, D, Smartt, A et al 2014, "Detection of organic compounds with whole-cell bioluminescent bioassays", *Advances in Biochemical Engineering / Biotechnology*, vol. 144, pp. 111-151.
50. Vetrova, EV, Esimbekova, EN, Rimmel, NN et al 2007a, "A bioluminescent signal system: detection of chemical toxicants in water", *Luminescence*, vol. 22, pp. 206–214.
51. Vetrova, EV, Kudryasheva, NS, Kratasyuk, VA 2007b, "Redox compounds influence on the NAD(P)H:FMN-oxidoreductase – luciferase bioluminescent system", *Photochemical & Photobiological Sciences*, vol. 6, pp. 35-40.
52. Vetrova, EV, Kratasyuk, VA, Kudryasheva, NS 2002, "Bioluminescent characteristics of Shira Lake water", *Aquatic Ecology*, vol. 36, pp. 309–315.