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Design of bioluminescent biosensors for assessing contamination of complex matrices

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ABSTRACT

The presence of potentially toxic xenobiotics in complex matrices has become rather the rule than the exception. Therefore, there is a need for highly sensitive inexpensive techniques for analyzing environmental and food matrices for toxicants. Enzymes are selectively sensitive to various toxic compounds, and, thus, they can be used as the basis for detection of contaminants in complex matrices. There are, however, a number of difficulties associated with the analysis of complex matrices using enzyme assays, including the necessity to take into account properties and effects of the natural components of the test media for accurate interpretation of results. The present study describes the six-stage procedure for designing new enzyme sensors intended for assessing the quality of complex matrices. This procedure should be followed both to achieve the highest possible sensitivity of the biosensor to potentially toxic substances and to minimize the effect of the uncontaminated components of complex mixtures on the activity of the biosensor. The proposed strategy has been tested in designing a bioluminescent biosensor for integrated rapid assessment of the safety of fruits and vegetables. The biosensor is based on the coupled enzyme system NAD(P)H:FMN-oxidoreductase and luciferase as the bioregnition element. The study describes methods and techniques for attaining the desired result in each stage. The proposed six-stage procedure for designing bioluminescent enzyme biosensors can be used to design the enzymatic biosensors based on other enzymes.

1. Introduction

Complex environmental samples such as soil and bottom sediments as well as plant-based foods tend to accumulate potentially hazardous compounds [1,2]. Toxic compounds, which enter the living organisms via various pathways, are able to impede molecular processes and start a cascade of unpredictable effects, resulting in health problems [3–9]. Therefore, one of the priorities of healthy risk assessment is to develop procedures for assessing the level of contamination of complex matrices [10]. Complex matrices are mixtures consisting of many organic and inorganic components. Depending on their origin, they are divided into complex food matrices (for example, fruits and vegetables) and complex environmental matrices (e.g. soil).

Physicochemical analytical methods are very popular and informative for detecting and identifying toxicants in environmental and food matrices. These methods are highly selective and sensitive and, thus, are capable of detecting even trace concentrations of pollutants [11,12]. However, they have a number of limitations: these methods are labor-intensive and costly, sample preparation takes a long time [13], and the actual hazard associated with contamination of, e.g., soil or fruits and vegetables grown on soils often remains underestimated [14,15], especially if samples contain some unknown pollutants.

An alternative approach is to assess toxicity of the environment when it is affecting a living organism using methods of bio-diagnostics such as bio-marking, bio-indication, and bioassay [16,17]. This approach is used to study responses of biological systems with different levels of organization to natural and anthropogenic factors. The state of biota and the quality of its habitat are assessed based on the strength of response [18–21].

Owing to their considerable advantages, enzyme assays, based on effects of activation or inhibition of enzymes by toxic compounds proportional to the amount of toxicants in the sample, have recently become very popular [22–25]. Enzyme assays were developed using the high sensitivity of enzymes to the effects of different classes of toxic

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compounds, which had been proved in numerous experiments. For example, cholinesterases and serine proteases of different fish species [26,27]; alanine transaminase, phosphatase, and amylase [28]; catalase and glutathione reductase [29]; acid and alkaline phosphatases of soil bacteria were found to be highly sensitive to pesticides [30].

Enzymes have been widely used as recognition elements of biosensors to detect toxic compounds in various environmental objects. This has been the subject of numerous research papers and review articles [31–36]. However, the issue of using enzyme biosensors to analyze real natural samples rather than laboratory model mixtures has not been resolved yet [37–39]. The use of biosensors to analyze complex media is an especially serious problem. Despite the advantages of enzyme-based biosensors, before they are used to assess contamination of heterogeneous and complex media, a great number of preliminary experiments should be conducted to estimate the sensitivity of the biosensor to toxicants, depending on the assay conditions, e.g., what solvent is used for a definite toxicant [40]. Moreover, results of enzyme assays are significantly affected by the presence of background interfering natural organic compounds in the samples [41].

The present study considers stages of designing enzyme-based biosensors for toxicological analysis of multicomponent media taking into account the issues and limitations of examination of complex matrices (Fig. 1). The study proposes the procedure consisting of six sequential stages: 1) assessing enzyme sensitivity to the effect of toxicants; 2) selecting conditions of the assay to obtain sensitivity at the maximum allowable concentrations (MACs); 3) selecting sample preparation conditions to reduce the interfering effect of non-toxic components of the test samples; 4) assessing the sensitivity of enzymes to the effect of specially prepared mixtures of model toxic compounds and uncontaminated samples of complex matrices containing interfering background substances; 5) designing the biological module of the sensor; and 6) designing the biosensor including the choice of a proper transducer. Stages 5 and 6 can only be implemented if the first four stages have been successfully fulfilled, confirming the practicability of the idea of developing a new enzyme-based method for assessing complex matrices. This strategy was employed in constructing new bioluminescent biosensors to analyze the safety of fruits and vegetables, in which the coupled enzyme system of luminous bacteria, NAD(P)H:FMN-oxidoreductase-luciferase (Red + Luc), was used as the biorecognition element. Mechanism of reactions of coupled enzyme system Red + Luc is on the scheme below, where RCHO and RCOOH are aliphatic aldehyde and the corresponding fatty acid.

$$\mathrm{NAD(P)H + FMN} \xrightarrow{\mathrm{NAD(P)H:FMN-\ oxidoreductase}} \mathrm{FMN^{*}H_2} + \mathrm{NAD(P)^{+}}$$

$$\mathrm{FMNH_2 + RCHO + O_2 \overset{Luciferase}{\rightarrow} FMN + \ RCOOH + H_2O + h\nu}$$

Bioluminescent enzyme inhibition-based assay was used in this study because it is rapid, simple to perform, and highly sensitive to various toxic chemical compounds present in environmental and food matrices. The bioluminescent assay is based on detection of the inhibitory effect of the toxic compounds on the bacterial coupled enzyme system Red + Luc by recording changes in light emission intensity [42–44]. It was developed for the environmental monitoring of aquatic ecosystems and industrial wastewater, that is, samples with a few background interfering substances [44]. Nevertheless, that assay was found to be potentially useful for analyzing complex matrices such as soil, biological fluids, and foods [45–49].

2. Materials and methods

The reagents used in the study were FMN ("Serva", Germany), NADH ("Gerbu", Germany), tetradecanal ("Merck", Germany), and freeze-dried enzyme preparations produced at the Laboratory of Nanobiotechnology and Bioluminescence at the Institute of Biophysics SB RAS (Krasnoyarsk). One vial of enzyme preparation contained a mixture of 0.5 mg luciferase EC 1.14.14.3 from the recombinant strain of *E. coli* and 0.15 U (activity units) of NAD(P)H:FMN-oxidoreductase EC 1.5.1.29 from *Vibrio fischeri*. Enzyme solutions were prepared using 0.05 M potassium-phosphate buffer at pH 6.8. Model toxicant were commercially available state standard reference samples of heavy metal salt solutions produced at the Urals Plant of Chemical Reagents (Russia) and the following pesticides: α- and γ-isomers of hexachlorocyclohexane, 4.4-dichloro-diphenyl-ethylene, 4.4-dichloro-diphenyl-trichloro-methyl-methane (NPATs "Ekolan", Russia), diazinon, malathion, deltamethrin, imidacloprid, glyphosate, cypermethrin, metribuzin (Sigma Aldrich, U.S.), and copper (II) sulfate (Khimreaktivsnab, Russia). Pesticide solutions were prepared using distilled water or acetonitrile 99.9% ("Panreac", Spain) as solvents.

Fruit and vegetable samples for contamination experiments were purchased in grocery stores in Krasnoyarsk, Russia. Samples used as uncontaminated ones (control) were tested for toxicants employing chemical analysis techniques at the accredited laboratory at the State Regional Center for Standardization, Metrology and Testing in Krasnoyarsk. To prepare supernatant from vegetable samples, they were crushed and centrifuged at 10000 g for 5 min at 25 °C.

To make biological module of bioluminescent biosensor solutions of luciferase and NAD(P)H:FMN-oxidoreductase and their substrates, NADH and aliphatic aldehyde, were mixed with 3% starch gel which was prepared by boiling 3% starch suspension for 2 min and cooling to 25 °C. Then, 25-μl drops were placed onto fluoropolymer film and dried at 8 °C. The coupled enzyme system Red + Luc immobilized with the

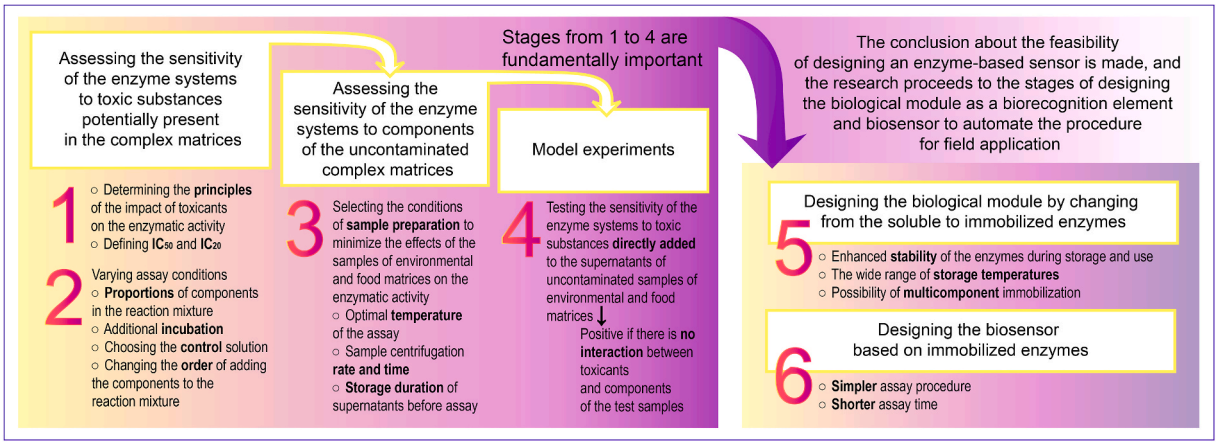

Fig. 1. The six-stage procedure for designing new enzyme sensors.

substrates had the form of disks with 4 mm in diameter, 50 μm thick and dry weight of 9 mg. One disk was used for one measurement.

The activity of the soluble coupled enzyme system Red + Luc co-immobilized with the substrates was determined as the maximum light emission intensity, I_{max}, expressed in relative units. In experiment with soluble enzymes, reaction mixture contained the following components: 250 μl 0.05 M potassium-phosphate buffer pH 6.8, 50 μl 0.0025% tetradecanal solution, 5 μl solution of enzyme preparation Red + Luc, 100 μl 0.4 mM NADH solution, 50 μl control solution or solution of the sample analyzed, and 50 μl 0.5 mM FMN solution. Distilled water or acetonitrile, no more than 2% of the reaction mixture, was used as the control solution.

To measure the activity of immobilized enzymes, 1 disk with co-immobilized enzymes and substrates, 350 μl distilled water or solution of the test substance at a preset concentration, and 10 μl 0.5 mM FMN solution were incubated in 10 s and added into the cuvette of the bioluminometer. One disk with co-immobilized enzymes and substrates is used for one assay.

The effect of the test samples on the Red + Luc bioluminescent enzyme-based system was estimated from the residual luminescence intensity, I_{exp}/I_c, %, where I_{exp} and I_c are the maximum light emission intensity of the coupled enzyme system Red + Luc in the presence of the test sample and of the control solution, respectively. The degree to which the contaminants affected the activity of the coupled enzyme system Red + Luc was quantified using the values of IC_{20} and IC_{50} – concentrations of the test compounds that caused a 20% and a 50% reduction in the activity of the Red + Luc system, respectively.

Bioluminescence was measured using a Lumat LB 9507 bioluminometer ("Berthold Technology", Germany). Five replicate measurements were made for each experimental point. Statistical processing of the results was done using Student's *t*-test. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Stage 1. Assessing the effect of toxicants on the activity of the coupled enzyme system Red + Luc

The purpose of Stage 1 in designing enzyme-based sensors was to determine the range of toxicants 'concentration capable of affecting the activity of the enzymes used as recognition elements. In the present study, model experiments were performed to assess the inhibitory effects of pesticides and heavy metals, as the most commonly occurring contaminants of soil and vegetables, on the bioluminescence parameters of the soluble coupled enzyme system Red + Luc.

Pesticides such as α- and γ-isomers of hexachlorocyclohexane (HCH), 4.4-dichloro-diphenyl-ethylene (DDE), 4.4-dichloro-diphenyl-trichloro-methyl-methane (DDT), diazinon, malathion, deltamethrin, imidacloprid, cypermethrin, copper (II) sulfate, glyphosate, metribuzin, and fenvalerate inhibited the activity of the coupled enzyme system Red + Luc to different extents. The IC_{50} and IC_{20} obtained for α- and γ-isomers of HCH, DDE, DDT, diazinon, deltamethrin, cypermethrin, and copper (II) sulfate were equal to or lower than their MACs in foods. The inhibitory effect of malathion, imidacloprid, and fenvalerate was less pronounced, and the IC_{50} and IC_{20} values of these pesticides were several orders of magnitude higher than their MACs. The glyphosate and metribuzin herbicides used within the range of concentrations from 4 g/L to 0.1 g/L tested in this study did not produce any effect on the coupled enzyme system Red + Luc.

The effects of zinc, copper, mercury, and chromium ions on the coupled enzyme system Red + Luc were tested in similar experiments, and their concentrations were below their MACs in foods. Copper ions had the greatest inhibitory effect on the activity of the coupled enzyme system, and IC_{50} was 0.03 mg/L. Lead and aluminum ions inhibited the activity of the Red + Luc system insignificantly: for lead, only the value of IC_{20} was determined, and it was higher than the MAC – 0.6 mg/L. For aluminum ions, no IC_{20} and IC_{50} values could be determined within the concentration range 0.6 mg/L to 6 μg/L tested in this study.

Thus, Stage 1 showed that enzymes of the bacterial bioluminescent system were more sensitive to the inhibitory effects of insecticides compared to herbicides and fungicides. Limitations that prevented the assessment of the sensitivity of the coupled enzyme system Red + Luc to a number of pesticides included precipitation of the compounds or turbidity of the solution. Those processes were associated with the physicochemical properties of the pesticides: poor solubility or interaction with the reaction medium components.

3.2. Stage 2. Selecting conditions of the assay to increase the sensitivity of the Red + Luc system to the toxicants

An important advantage of enzyme assays is that their sensitivity to the effects of toxicants can be varied to enable testing at MAC levels.

This study showed that sensitivity of the coupled enzyme system Red + Luc to the effects of a number of potentially hazardous compounds increased as the amounts of the enzymes in the reaction mixture were decreased. The greatest inhibitory effect of deltamethrin at a concentration of 0.5 mg/L on the activity of the coupled enzyme system Red + Luc was observed with 0.2 μg Luc and 0.06 mU Red in to the reaction mixture (Fig. 2). Thus, by decreasing the amounts of the enzymes in the reaction mixture, the initial IC_{50} value for deltamethrin was reduced by a factor of 7.5. The decrease in the amounts of the Red and Luc enzymes in the reaction mixture resulted in the higher sensitivity of the coupled enzyme system Red + Luc to lead and aluminum ions as well. The value of IC_{20} for lead dropped by a factor of 2 compared to the previously obtained value – to 0.3 mg/L, and IC_{50} was 0.6 mg/L. In addition, the lower amounts of the enzymes in the reaction mixture made it possible to determine IC_{20} for aluminum ions: 0.6 mg/L. Yet, we did not manage to achieve sensitivity at MAC levels to heavy metals such as cadmium, nickel, tin, cobalt, bromide, manganese, and arsenic.

The sensitivity of the coupled enzyme system Red + Luc to toxicants was tested in experiments with aliphatic aldehydes with different lengths of hydrocarbon chain, namely, decanal (C_{10}) and tetradecanal (C_{14}), used as the substrate of luciferase. First, we determined concentrations of the C_{10} and C_{14} aldehydes that enabled the maximum intensity of light emission by the soluble coupled enzyme system Red + Luc: 50 μM and 11 μM of the C_{10} and C_{14} aldehydes, respectively. In the experiment with C_{14} used as luciferase substrate, the maximum intensity of light emission by the coupled enzyme system Red + Luc was twice

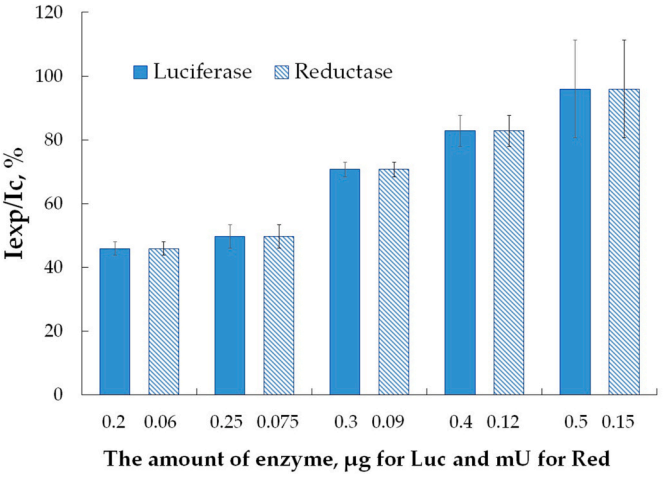

Fig. 2. The residual luminescence intensity of the coupled enzyme system Red + Luc in the presence of 0.5 mg/L of deltamethrin as dependent on the amount of luciferase and reductase in the reaction mixture. Deltamethrin MAC in vegetables is 0.5 mg/L. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

higher than in the experiment with C_{10}. Moreover, in experiments with copper (II) sulfate, sensitivity of the coupled enzyme system to toxicants was higher when C_{14} was used, and IC_{50} was 1 mg/L; in the presence of C_{10}, the value of IC_{50} for copper (II) sulfate was one order of magnitude higher.

Table 1 lists IC_{20} and IC_{50} values for pesticides and heavy metals, showing that the sensitivity of the coupled enzyme system Red + Luc was high enough to detect some of the compounds at environmentally relevant levels.

3.3. Stage 3. Model experiments to assess the effects of uncontaminated food matrices on the activity of the Red + Luc system

Stage 3 experiments showed that the addition of supernatants of various vegetable and fruit samples to the reaction mixture affected the intensity of residual luminescence of the coupled enzyme system Red + Luc, resulting in considerable stimulation or inhibition of the activity of the system. Steps were taken to optimize the sample preparation procedure in order to decrease the effect of the uncontaminated samples of food matrices on the activity of the Red + Luc system.

To achieve the desired effect, we investigated the degree to which uncontaminated tomato and cucumber samples affected the activity of the Red + Luc system by varying the rate and duration of centrifugation of the samples, which had been preliminarily crushed and filtered through two layers of gauze, and by varying the amounts of the samples added to the reaction mixture. The minimal effect on the activity of the Red + Luc system was observed in the experiment with the samples centrifuged at 10 000 g for 5 min (Fig. 3, a) and with no more than 50 μL of the supernatant added to the reaction mixture.

In addition, the effects of the supernatants on the activity of the Red + Luc system were studied as dependent on the storage duration and temperature (Fig. 3, b). The results showed that the samples prepared for analysis could be stored for 24 h at 4 °C and for no more than 1 h at room temperature. The increase in the sample storage time would cause a change in the strength of the effect on the activity of the Red + Luc system and, hence, distort results of assay.

3.4. Stage 4. Assessing the effect of the mixture of toxicants with food samples on the activity of the Red + Luc system

The problem with estimating the degree of contamination or safety of environmental and food matrices is that it is impossible to eliminate interactions of toxicants with the natural components of these matrices. For example, in soil, toxicants interact with humic and fulvic acids. Therefore, a necessary stage in developing new enzyme-based methods is to test the effects of the mixture of complex matrix components and toxicants on enzyme activity.

In the present study, we investigated changes in the sensitivity of the coupled enzyme system Red + Luc to pesticides and heavy metals in experiments where the toxicants were added to the vegetable supernatants (test samples). Results were compared to the previously obtained data on the effects of those contaminants dissolved in distilled water (control samples) on the activity of the coupled enzyme system Red + Luc. Experiment with copper added to the vegetable sample showed that the sensitivity of the coupled enzyme system Red + Luc to that metal remained high enough to detect copper MAC for food but was inferior to the sensitivity of the system to copper ion solutions in the control sample (Fig. 4). At the same time, experiment with mercury ions added to the cucumber sample demonstrated a considerably weaker effect on the coupled enzyme system Red + Luc (Fig. 4).

Similar experiments were conducted to assess the effects of the fenvalerate and diazinon insecticides, representatives of the pyrethroids and organophosphates, respectively, on the coupled enzyme system Red + Luc. The sensitivity of the Red + Luc to fenvalerate was 2.5 times lower in the presence of cucumber extract (test sample) compared to the control sample (Fig. 5). The IC_{50} values for fenvalerate in acetonitrile (control sample) and for the solution of fenvalerate in acetonitrile in the presence of cucumber extract were 6 mg/L and 16 mg/L, respectively.

Experiments with diazinon showed that the sensitivity of the coupled enzyme system Red + Luc to that pesticide remained the same regardless of whether the control sample or the test sample was added to the reaction mixture.

3.5. Stage 5. Designing the biological module for bioluminescent sensors based on the coupled enzyme system Red + Luc immobilized in the starch gel

For the bioluminescent sensor to be effective, its biological module should meet the following requirements: a) to have high activity; b) to be sufficiently sensitive to toxicants to be able to detect them at maximum allowable concentrations or below; c) to exhibit long-term storage stability. In the present study, these requirements were fulfilled by co-immobilizing the coupled enzyme system Red + Luc and substrates in starch gel.

A study was performed to estimate the activity of the coupled enzyme system Red + Luc co-immobilized with NADH and aldehydes with different lengths of the hydrocarbon chain, C_{10} and C_{14}, in starch gel and the sensitivity of the system to toxicants. The immobilized preparations in the form of single-use starch disks contained 50 μM C_{10} or 11 μM C_{14}, and the amounts of other components did not differ: 0.5 μg Luc, 0.15 mU Red, and 1.6 μg NADH. The release of the activity of the coupled enzyme system Red + Luc co-immobilized with the substrates in the disks containing C_{10} and C_{14} was 11% and 18%, respectively. The sensitivity of the immobilized preparation containing C_{14} to copper (II) sulfate was one order of magnitude higher than the sensitivity of the preparations containing C_{10}; IC_{50} values were 5 and 45 mg/L, respectively. Moreover, the immobilized preparations containing C_{14} retained their high activity for long periods: over the three-month storage at 4 °C, their activity decreased by a factor of 1.4, while the activity of the disks containing C_{10} dropped by a factor of 3.

The sensitivity of the immobilized preparations to toxicants was enhanced by decreasing the amounts of enzymes in the starch disks (Fig. 6). The highest sensitivity to copper (II) sulfate was achieved in the experiment with the starch disk containing 0.2 μg Luc and 0.06 mU Red. In that experiment, the IC_{50} was 1.25 mg/L, and that was close to the IC_{50} value obtained in the experiment with enzyme solutions.

The experiments in which the amounts of other components of the

Table 1
IC_{20} и IC_{50} values for the effect of heavy metals and pesticides on the activity of the coupled enzyme system Red + Luc.

Toxicant	IC_{20}, mg/L	IC_{50}, mg/L	Permissible level, mg/L	
			RF [50,51]	EC [52–54]
Pesticides				
α- HCH	0.02	0.03	0.5	0.01
γ- HCH	0.04	0.1	0.5	0.01
DDE	0.002	0.01	0.1	0.05
DDT	0.002	0.01	0.1	0.05
Diazinon	0.06	6	0.5	0.01
Malathion	3.8	19.4	0.2	2.0
Deltamethrin	0.2	0.5	0.2	0.2
Imidacloprid	110	880	1.0	1.0
Cypermethrin	0.9	1.8	2.0	2.0
Copper (II) sulfate	0.4	1	5.0	5.0
Fenvalerate	2	6	3	0.3
Heavy metal ions				
Lead, Pb	0.3	0.6	0.5	0.1
Zinc, Zn	2	6	10.0	–
Copper, Cu	0.002	0.03	5.0	5.0
Mercury, Hg	0.03	0.06	0.02	0.01
Aluminum, Al	0.6	–[a]	–	–
Chromium, Cr	0.06	0.3	0.5	–

«[a]» - at the metal concentrations used in the test, the value of the parameter was not determined. «- » - allowable concentrations in food are not established.

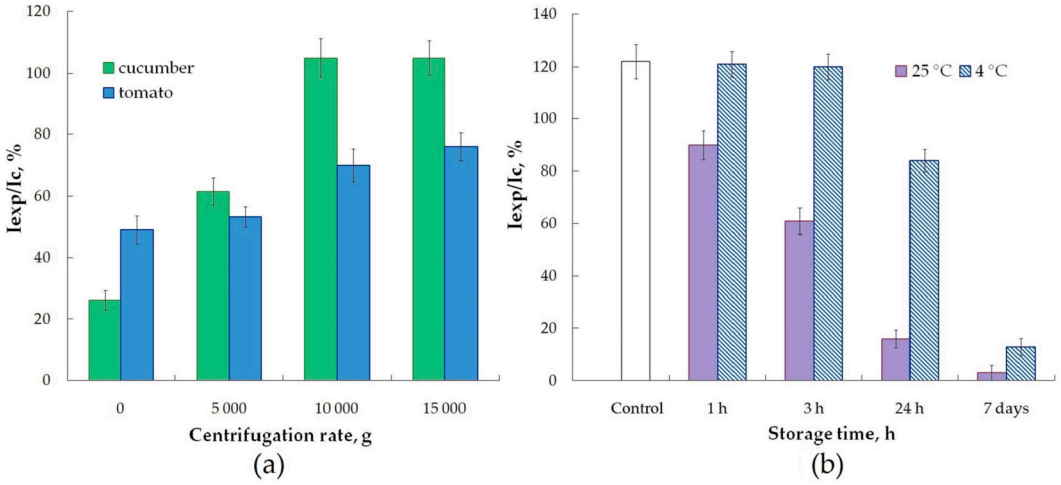

Fig. 3. The residual luminescence intensity of the coupled enzyme system Red + Luc in the presence of the vegetable sample supernatant (**a**) as dependent on the rate of centrifugation of the samples at room temperature for 5 min, (**b**) as dependent on the time and temperature of the storage of the cucumber sample supernatant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

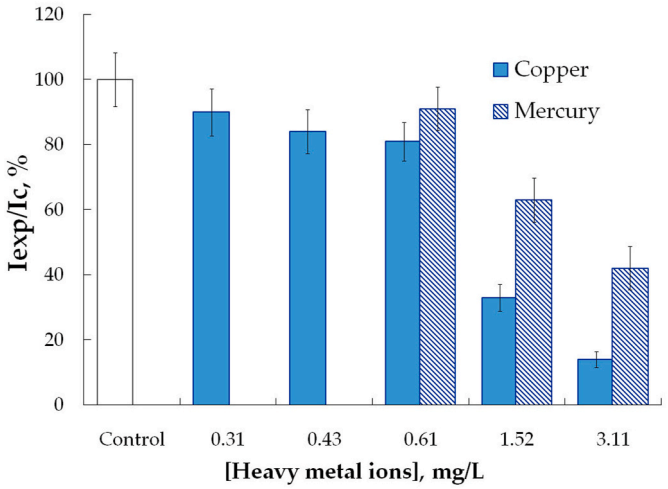

Fig. 4. The residual luminescence intensity of the coupled enzyme system Red + Luc in the presence of (a) copper ions and (b) mercury ions preliminarily diluted with the vegetable sample supernatant. Copper MAC for vegetables is 5.0 mg/L, mercury MAC is 0.02 mg/L. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

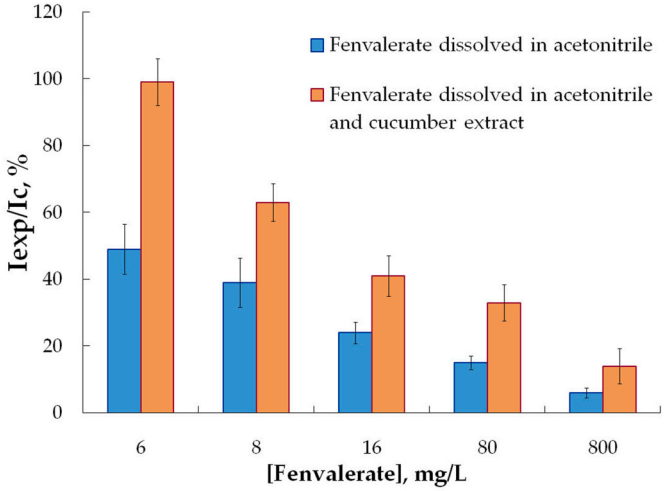

Fig. 5. The residual luminescence intensity of the coupled enzyme system Red + Luc as dependent on concentration of fenvalerate dissolved in acetonitrile (control) and in the mixture of acetonitrile and cucumber extract. The maximum allowable concentration of fenvalerate in vegetables is 2 mg/L. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

immobilized preparation in the disk were varied – NADH was varied between 0.8 and 3.2 μg, and molarity of the buffer used to prepare solutions for immobilization between 0.025 and 0.1 M – did not result in changes of the sensitivity of the preparation to the toxicant.

Thus, to achieve high activity and sensitivity to toxic compounds, the biological module of the bioluminescent sensor based on the coupled enzyme system Red + Luc co-immobilized with the substrates, NADH and aliphatic aldehyde, in 3% starch gel should contain 0.2 μg Luc, 0.06 mU Red, 11 μM C_{14}, and 0.8-3.2 μg NADH. The biological module based on the coupled enzyme system Red + Luc co-immobilized with the substrates retained its 90% activity and sensitivity for two months of storage at 4 °C.

3.6. Stage 6. Designing the biosensor based on the coupled enzyme system Red + Luc co-immobilized with substrates

The biological module developed during Stage 5 can be used as a reagent in enzymatic assays. This is a dry starch disk of small size, which can be placed in a cuvette or well of any bioluminometer; upon addition of FMN, it emits light of intensity that is determined by the test sample (Fig. 7, a).

Nevertheless, based on modern microfluidic technologies and the knowledge about the functioning of the immobilized coupled enzyme system Red + Luc, a more convenient microfluidic chip, in which the coupled enzyme system Red + Luc co-immobilized with the substrates, serves as the biorecognition element (Fig. 7, b). The disposable enzymatic microfluidic chip consisted of a poly(methyl methacrylate) body and gelatin scaffold was used to prevent loss of enzymatic activity. To obtain high luminescence intensity and reproducibility results, uniform distribution of FMN throughout the reaction chamber was achieved by using a sampler as an active mixer [55]. The bioluminescent system retained its activity and sensitivity to toxins for 6 months without significant reduction when storage at −18 °C. Standard variation was nearly to 30%.

Finally, a portable luminometer, which has been designed earlier [56], can be used to analyze contamination of environmental matrices in

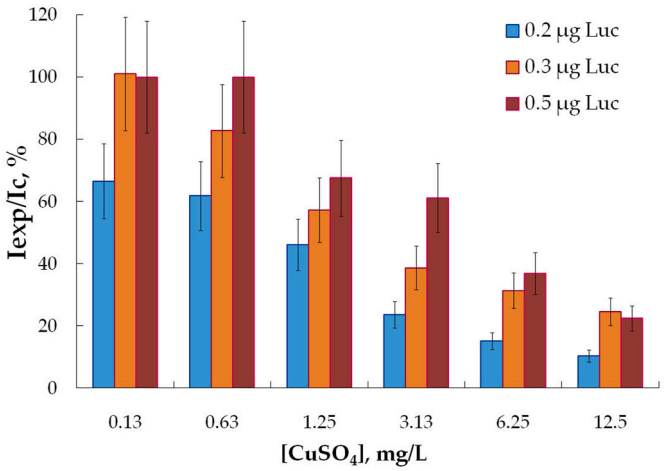

Fig. 6. The residual luminescence intensity of the coupled enzyme system Red + Luc co-immobilized with NADH and C_{14} and containing different amounts of Luc in the presence of copper (II) sulfate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

laboratory and in the field (Fig. 7, c). The compact luminometer has a size of 80 × 140 × 41 mm^3 and weight less than 300 g. It consists of a sample compartment, a silicon photomultiplier with a thermal stabilization system, a battery and a microcontroller electronics. Portability and possibility of autonomous operation allow the proposed biosensor to be applied as an early warning system to assess the safety of fruits and vegetables. As all materials and reagents used to fabricate the chips are commercially available and relatively inexpensive, the compact bioluminometer complete with the chips is a competitive device.

4. Discussion

This study described the step-by-step activities aimed at designing new enzyme-based sensors to analyze complex matrices, using bioluminescent biosensors for testing the safety of vegetables and fruits.

Most enzymes exhibit specificity, although not absolute specificity, to different types of toxicants [57–59]. A number of studies show high specificity of cholinesterases to the carbamate and organophosphate pesticides [24]. However, this specificity is not absolute, as cholinesterases are inhibited by other toxic compounds as well [60,61]. Biosensors based on the principle of activation of the enzymes by the test compounds (toxicants) are more sensitive, as toxicants are substrates of these enzymes [35,62]. One example is organophosphorus hydrolase (OPH): sensors based on the free or immobilized enzyme are highly sensitive to organophosphates and are capable of detecting parathion and paraoxon at a detection limit of 1 nM and 3 nM, respectively [35, 63]. Therefore, the purpose of the first stage in the development of a new enzyme-based sensor is to define the range of the toxicants to which the enzyme used in the sensor is sufficiently sensitive and, thus, decide whether the biosensor will be sufficiently effective. In the present study, the sensors exhibited sensitivity at levels of maximum allowable concentrations to such food contaminants as heavy metals – zinc, copper, mercury, and chromium ions – and pesticides – α- and γ-isomers of HCH, DDE, DDT, diazinon, deltamethrin, cypermethrin, and copper (II) sulfate.

If the toxicant is capable of producing an effect on the enzyme but it is present at concentrations higher than MAC, the sensitivity of the enzyme to this toxicant can be increased using various techniques: by changing the composition of the reaction mixture (amounts of enzymes and substrates, the amount of the toxic mixture); by changing the order in which reaction components are added to the mixture; by incubating the enzymes in the test sample; by using different enzyme preparations (e.g., enzymes purified to different degrees, mutant forms), etc. [43,64]. Studies in which the sensitivity and the selectivity of enzyme assays were enhanced by using new mutant forms of the enzymes and nanomaterials were cited in the review [24] and other studies [59,65].

However, sometimes, whatever efforts are made, the enzyme assays do not exhibit sensitivity to the test toxicant. In our study, the coupled enzyme system Red + Luc did not show sensitivity to a number of heavy metals and pesticides, such as cadmium, nickel, tin, cobalt, bromide, manganese, arsenic, glyphosate, and metribuzin, which again supported selective sensitivity of the enzymes. In addition, these results suggest that effective comprehensive analysis of environmental matrices for various potentially toxic substances can only be performed using a battery of tests. This may be a set of enzyme-based biosensors, which rapidly and accurately perform the initial screening for toxicants in various media.

Unfortunately, even if the sensitivity of the enzyme to the toxicant is high enough, it may be ineffective for analyzing complex environmental and food matrices. Analysis of complex matrices should take into account properties and effects of the natural components of the test media, or else no accurate interpretation of results can be made. Fruits and vegetables are complex matrices, as they contain various biomolecules – so-called natural products. These chemical substances are components of whole organisms and their parts, extracts from organisms and pure compounds from them [66], represented by phytochemicals, vitamins, fibers, minerals, etc. The presence of natural products in food matrices hinders detection and identification of toxicants by both physicochemical analytical methods and enzyme assays [67,68]. Natural products present in food matrices are able to interact with the components of the enzyme system and distort the results. Similar difficulties, associated with the substantial effect of natural components of such environmental

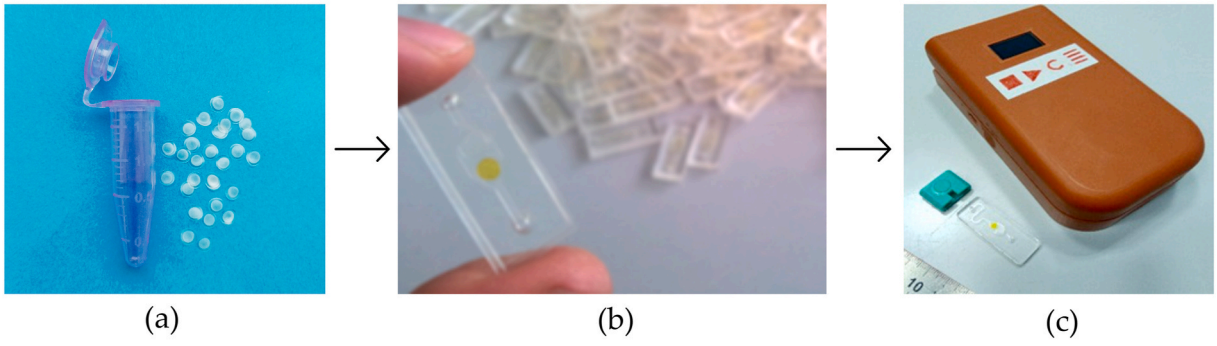

Fig. 7. (**a**) The external form of immobilized preparations based on the Red and Luc enzymes co-immobilized with their substrates, NADH and C_{14}, in starch gel; (**b**) single-use microfluidic chips. The reaction chamber contains two dried drops of starch gel, one encapsulating the Red and Luc enzymes and the NADH and C_{14} substrates and the other – FMN. Reproduced from Ref. [55] with permission from Wiley; (**c**) A portable bioluminometer. Reproduced from Ref. [56] with permission from MDPI. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

matrices as soil extracts on the activity of the coupled enzyme system Red + Luc, affected the development of the enzyme assay for testing soil contamination [45].

Researchers designing biosensors usually try to resolve this problem by modifying the sensing device or optimizing sample preparation procedure [69–74]. For example, the sensitivity of the optical sensor based on methyl parathion hydrolase to methyl parathion was 4 μM [75], while the sensor based on the same enzyme but modified by nanomaterials showed the sensitivity that was one order of magnitude higher – 0.338 μM [76]. Drechsel et al. [73] proposed the unique automated lab-on-a chip platform, which in turn dipped a biosensor into the reagents and sample, thus incorporating sample preparation into the biosensor. Zheng et al. [74], in order to enhance the sensitivity of the biosensor to organophosphates in complex food matrices, additionally incubated the biosensor with the extracted sample solution prior to measurement. Additional sample preparation procedures, however, considerably complicate and lengthen the duration of the analysis, making it less suitable for in situ testing [77,78]. Having optimized sample preparation, we avoided coloring of the sample solutions, and we removed large cellular components by centrifugation. In addition, we managed to take into account optical parameters of the solutions of the test substances to achieve accurate interpretation of results of bioluminescent enzyme assays.

After all the objectives of the stages confirming the appropriateness of the definite enzyme or enzyme system for the analysis of complex environmental and food matrices have been accomplished, a new challenge is to keep the enzyme stable during storage and use, as most enzymes, which are proteins, are very labile under variations in temperature, pH, and other factors. The most commonly used approach to enzyme stabilization is chemical and physical immobilization [59, 79–83]. The researcher should be able to choose the best technique for the enzyme both to remain stable during storage and use and to retain its high sensitivity to toxicants. Fennouh et al. reported [84] that sensitivity of acetylcholinesterase to paraoxon increased by a factor of 250 when 5% cyclohexane was used as the solvent for this pesticide, but no such effect was found in experiments with the immobilized enzyme. In the present study, the coupled enzyme system was stabilized by co-immobilizing the enzymes and their substrates in starch gel, as previous studies showed that the enzymes immobilized in the gel and then dried remained highly active, sensitive, and stable during storage and use [85–87].

Finally, limits of detection of toxicants using enzyme biosensors are largely determined by the types of the transducer and electrode. For example, the optical biosensor based on acetylcholinesterase for detection of paraoxon had detection limit as low as 10.5 pM, whereas the sensitivity of the photoelectrochemical biosensor was considerably higher: 10 fM [74,88]. The review by Pundir et al. (2019) [22] discussed in detail changes in the sensitivity of biosensors to organophosphorus pesticides as dependent not only on the enzyme used but also on the type of sensor for detecting compounds.

The procedure of designing a bioluminescent enzyme biosensor for integrated rapid assessment of the safety of complex matrices was successfully tested using an expanded range of toxicants.

5. Conclusions

The present study, for the first time, proposed the six-stage procedure for designing bioluminescent enzyme biosensors intended for toxicological analysis of complex matrices. Researchers designing biosensors for analysis of complex environmental and food matrices face certain problems, including the probable effect of natural components on the activity of the enzymes. Therefore, the main principles underpinning development of such biosensors should be achievement of the highest possible sensitivity of the biosensor to potentially toxic substances and selection of the conditions minimizing the effect of the uncontaminated complex matrices on the enzymes used as the recognition element. A considerable advantage of the procedure of designing enzyme biosensors proposed in the present study is that it is not specific to the bioluminescent enzyme biosensors described here and can be used to design biosensors based on other enzymes or enzyme systems.

Author statement

Elena N. Esimbekova: Conceptualization, Project administration, Funding acquisition, Writing – review & editing. Valeriya P. Kalyabina: Investigation, Formal analysis, Writing – original draft, Visualization. Kseniya V. Kopylova: Investigation, Formal analysis, Writing – original draft, Visualization. Irina G. Torgashina: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Valentina A. Kratasyuk: Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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