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Acetylcholinesterase Immobilized on Glass Rod for Organophosphorus Pesticides Detection: Application on Milk Analysis

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***Abstract*— Immense use of an insecticide such as malathion has initiated a hindrance to the environment and has increased possible risk to human health via milk and milk products. The presence of insecticide residues in milk products is an alarming signal to set up a string monitoring system. Since their risk to human health, the determination of organophosphate pesticide levels in dairy products is essential. This study shows biosensors' development for the detection of residues of the pesticides generally used in agriculture to increase productivity, which has high toxicity and selective action, a significant part of which falls into crop and livestock products. The test system described in this paper is based on the immobilization of acetylcholinesterase on glass rods by colorimetry as a detection technique. The application of the biosensor to the analysis of the real sample confirmed its reliability. The developed biosensor test system showed excellent analytical performance with limits to detecting the concentration range of residual amounts of the acetylcholinesterase enzyme inhibitor - malathion insecticide (up to 0.089 mg/kg), along with acceptable reproducibility and stability. Distinct advantages of enzyme-based biosensors are high sensitivity, convenience, quick response, cost-effectiveness, portability, and a possibility to use for environmental detection purposes. The biosensor can be used for the analysis of organophosphorus pesticides with anticholinesterase action. It can be concluded that the benefits of using immobilized enzymes are significant since it will bring advantages for the food, pharmaceutical, and medical device industries.**

***Keywords*— Analytical devices; food safety; glass rods; insecticide; malathion; milk analysis.**

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I. INTRODUCTION

Throughout the last century, pesticides and agrochemicals have become a significant part of global agriculture systems to control pests, enabling boosting agricultural yields and food production. Nevertheless, pesticides' harmful side effects are becoming more apparent in terms of effects on human vitality and the environment. The development of fast, reliable, and economical biosensors to detect these chemicals' trace amounts is vital for consumer protection and a healthy diet. Almost 2 million tons of pesticides are used annually, and it has been predicted that it can increase up to 3.5 million tons [1]. The widespread use of pesticides leads to pollution of soils, groundwater and drinking water sources, and food. Of particular danger is the ability of many pesticides to accumulate in plant and animal organisms with a marked increase in concentration in subsequent food chain links. Growing demand for more food that can be able to produce quickly and inexpensively, pesticide application rates from 2010 to 2017 are doubled in Kazakhstan, too [2]. Toxicity of OPs is based on the inhibition of acetylcholinesterase (AChE) enzyme in the central nervous system, and as a result, the synaptic concentration of acetylcholine will be higher than usual [3]. The results of automated monitoring, which provides information on the levels of various pesticides and organophosphate compounds in food products, show an increase in the total amount of pesticides in dairy products [4-7] due to veterinary practice against insects by applying directly in dairy plants and agriculture.

One of the new devices for analyzing and processing information designed to control the environment using immobilized enzymes is biosensors [8]-[10]. These biosensors showed simplicity and low-cost detection compared to conventional methods, and they are fast, portable, and vastly sensitive devices. Various aspects of the use of AChE in the analysis of pesticides are reflected in several reviews [11], [12]. Several AChE-based biosensors have been developed and recommended on fluorescence [13], amperometry [14], colorimetric [8], [15], and various nanomaterials-based methods [16]. The use of immobilized enzymes for the biochemical determination of various toxicants in biological samples has immense potential to improve pesticide detection effectiveness.

Some of the well-known requirements on the methods used to analyze environmental samples are high sensitivity, accuracy, reproducibility, speed of the analysis, ease of sample preparation, ease of operation with the device, the ability to work in the field, and acceptable cost of the material. Among all methods and requirements mentioned, a colorimetric biosensor is an assuring substitute to conventional methods and techniques and is based on changing the color in the object sample's presence. Malathion is a highly toxic organophosphorus insecticide that penetrates well through the skin and can inhibit the enzyme acetylcholinesterase's action. The objective of the present study was to design a biosensor suitable to detect organophosphorus pesticides. The activity of AChE in the presence of pesticide is inhibited, and we describe the results of the developed biosensor test system based on natural sodium alginate as a gel matrix with an immobilized enzyme, in which a glass rod or a strip of filter paper (option) used as a transducer. This test system was developed to detect the residual amount of malathion in milk by studying the immobilized enzyme acetylcholinesterase's specific activity on a glass rod. The study focused on developing a low-cost, simple, quick, and sensitive device, which will be possible to operate without a highly experienced person. Furthermore, the glass-rod-based sensor can advance the sensitivity of the detection of pesticides at a low level of concentrations.

II. Materials and Methods

A. Materials, Instrumentation, and Solution Preparation

The enzymes acetylcholinesterase (AChE) (type VI-S from electric eel, lyophilized powder, and 1.000 units/mg protein), acetylcholine chloride 99% (AChCl), sodium alginate (98%), and malathion (purity of 99.5%) were obtained from Sigma-Aldrich Co (USA). All commercially available solvents and reagents were of analytical grade and used without further purification. As a transducer - a glass rod with a diameter of 5 mm and a length of 220 mm was purchased from Sigma-Aldrich Co. (USA). Whole cow's milk samples were obtained from a local market. Fifteen samples of 1 L were collected in glass vials with plastic tops and kept at the temperature of 4-6 ^{0}C. Studies were performed in a period of 1 to 3 d after collection. pH indicator phenol red was used as a 0.02% aqueous solution. To avoid the absorption of CO_2 from the air, the phenol red solution was kept in a bottle protected by a tube. The water used to prepare all the solutions was first distilled in a glass distillatory, deionized, and degassed before use. Spectrophotometric measurements were performed using a KFK - 5 photocolorimeter (Russia) at a wavelength of 540 nm with a cell layer thickness of 10 mm. The AChE enzyme solutions were set freshly on the day of examinations in tetraborate buffer (pH 8.4). The specific activity of the enzyme was calculated as mmoles AChCl hydrolyzed/mg protein/min. The AChCl was prepared at a concentration of 2% in distilled water. A buffer solution (pH 8.4) was prepared from 6.2 mL of a sodium tetraborate solution with a concentration of 0.05 mol/L mixed with 3.8 mL of a hydrochloric acid solution with a concentration of 0.1 mol/L. The sodium alginate was used as a gel matrix to entrap an enzyme. Alginate solutions (0.5, 1, 2, and 4%, w/v) were prepared in distilled water. The pesticide stock solutions were prepared daily and were not used for more than three hours after preparation to reduce the degradation. A stock solution of pesticide malathion (0.1 mol/L) was prepared in water by dissolving suitable quantities in 5.0 mL of water in a volumetric flask. To promote dissolution, an ultrasound bath was used. Continual reductions with distilled water achieved further diluted solutions. For malathion, 0.1652 g of malathion was dissolved with water in a 5.0 mL volumetric flask to get a concentration of 0.1 mol/L. From this solution, 500 μL was transferred to another 5.0 mL flask, and diluted with distilled water to a concentration of 0.01 mol/L. Pesticide solution was prepared in a fume hood due to its high toxicity. Chemical name for malathion is 2-(dimethoxyphosphinothioylthio) butanedioc acid diethyl ester. In Kazakhstan, malathion is also known as carbaphos.

B. Determination of AChE Activity

AChE dividing acetylcholine into choline and acetic acid leading to a decreasing pH of medium (Scheme 1). Our work focuses on immobilization of acetylcholinesterase (AChE) onto glass rod with stabilization in alginate and buffer solution (pH 8.4) and based on the color change of the glass rod from bright pink to yellow. The determination of the specific activity of the immobilized enzyme acetylcholinesterase, a technique was developed based on the research and the approach established by Filippova et al. [17]. The organophosphorus pesticide malathion was used to amend the AChE activity inhibition. To 0.1 mL of an aqueous solution of the enzyme acetylcholinesterase, 2 mL of a buffer solution with a pH of 8.4 was added and incubated for 30 min at a 37^{0}C. At the same time, the prepared 2% aqueous solution of acetylcholine chloride used as a substrate was incubated. To set up an enzymatic reaction, 0.5 mL of a 2% solution of acetylcholine chloride was added to the studied solution of acetylcholinesterase, and then the mixture was incubated for 30 min at a temperature of 37^{0}C. Then 0.2 mL of a 1% aqueous solution of malathion was added and incubated for 10 min at 37^{0}C. As a test sample, a mixture was used in which the inhibitor was added before incubating. After 10 min of incubation, 2.1 mL of distilled water and 0.3 mL of red phenol indicator (0.02% aqueous solution) were added to each tube. The secreted amount of acetic acid was evaluated by a bright pink color on FEC-KFK-5 at a wavelength of 540 nm in a cell with a layer thickness of 10 mm. The amount of acid released was determined by the difference between the control and experimental samples' values, taking into account the calibration curve in the optical density's coordinates and the

amount of acetic acid in mg. The specific activity of AChE was calculated according to the Eq. (1).

$$A = \frac{1000 * m_{CH3COOH}}{m_{enzyme}} \quad (1)$$

where $m_{CH3COOH}$ - the mass of acetic acid, mg; m_{enzyme} - the enzyme's mass, mg; 1000 - mg into g conversion unit.

C. Enzyme Immobilization and Gel Matrix Preparation

The alginate biopolymer was adapted as a polymer matrix for enzyme stabilization. Gel mixture of the biopolymer, buffer sodium tetraborate (pH=8.4), AChE enzyme, and red phenol indicator were prepared in every optimization development testing. The combined mixture was stirred for 5 min and was used to fabricate the biosensor.

D. Fabrication of Glass-rod based Biosensor with AChE

The fabrication of biosensor is shown in a flow chart (Fig. 1). A biosensor that did not include AChE was made similarly and used as a control test.

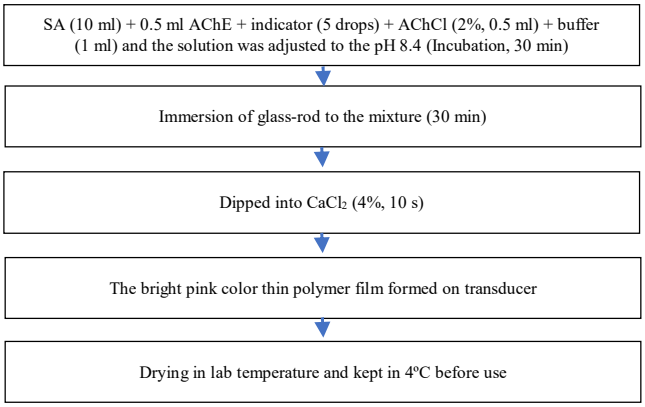

Fig. 1 A flow-chart of the fabrication of the biosensor.

E. Optimization of Glass-rod based biosensor.

To build a biosensor it is necessary to take into account all the factors affecting the enzyme activity. For an objective analysis, the effect of different parameters such as the pH, concentration of AChE, alginate biopolymer, $CaCl_2$, incubation time, temperature, and color intensity were studied to improve the bioactive assay. The gel's appropriate viscosity for the immobilization method was selected after investigating different concentrations (0.25, 0.5, 0.75, 1, 3, and 4%) in the tetraborate buffer solution (pH=8.4). To improve the crosslinking process, different volumes (1-50 µL) of 4% $CaCl_2$ stock solution were added to the final amount of the gel 10 mL. For the optimization of the AChE activity, different amounts from the enzyme extract were mixed with 10 mL of alginate gel solution, incubated for 5 min, added five drops of red phenol indicator and applied on glass rod by immersing later into the $CaCl_2$ for 5, 10, 20, 30 s, 1, 5, 10 min. Then the transducer was dried at 25^0C for 20 min. Results showed that alginate at the concentration of 0.5%, 4% $CaCl_2$, and 10 s time to the developed thin layer of the polymer is the best for our work. The glass-rod sensor's performance under enhanced conditions was evaluated directly by immersing in the different concentrations of pesticides in milk.

F. Detection of Organophosphate Pesticides using Glass-rod based Biosensor

Malathion inhibitory effects were evaluated on glass-rod-based biosensors by determining the lessening in the pink color intensity and changing into yellow color. The change of color development was converted to the concentration of an inhibitor by visual detection. The bioactive glass rod's detecting area was dipped in a prepared milk sample with various concentrations of malathion (0.03; 0.05; 0.07; 0.09, and 0.12 mg/kg). A positive model resulted in reduced or withdrawn pink color. In the absence of malathion, the fuchsia color turns yellow; if it is present, it remains. For visual detection, the minimum detection limit was defined as the lowest concentration level of the pesticide that was scored "positive" in five tests by six test persons. Assays in the absence of inhibitor and the absence of enzyme-inhibitor (blank) worked as control.

III. Results and Discussion

A. AChE Immobilization and Biosensor Preparation

Foodstuff and environmental samples with pesticide residue and detection are essential safety issues due to inherent toxicity, and they can lead to long-term hazards for the environment. Although the conventional methods of detecting pesticides have prominent sensitivity and specificity, necessities for sophisticated sample treatment in the research facility, skillful personnel, precise time operation, and analyzing the data are not beneficial to real-time and productive monitoring of samples on the field. Therefore, it is crucial to build up adequate mobile and rumbustious analytical tools correctly and quickly track the pesticide residue and find contamination sources. Besides, biosensors nowadays have taken place as a rising technique due to their uncomplicatedness and much cheaper than conventional methods. The development of biosensors based on the inhibition of AChE has been studied exceedingly to discover and detect organophosphorus pesticide [9], [13], [18]. The method described in our work is based on the quantitative determination of acetic acid formed by acetylcholinesterase enzymatic reaction immobilized on glass rod with the substrate acetylcholine with inhibition of malathion. Fig. 1 shows the enzymatic reaction of AChE. For the duration of the enzyme immobilization procedure, the enzyme's resistance rises against environmental changes like temperature and pH and makes possible longer shelf life for the immobilized enzyme [19]. Using biopolymer structures for immobilization of AChE is a significant part of biosensing purposes. Biosensors with a high amount of active and stable AChE are essential. In this work, we proposed an immobilization method that can design cheaper biosensors for the detection of organophosphorus pesticides. The enzymatic reaction mixture (AChE, ACh, alginate, and phenol red) after an incubation time of 30 min was immobilized on the glass rod by immersing glass-rod into the mixture for 10 min, following the immersion into $CaCl_2$ to crosslink the alginate and entrap enzyme mixture in polymer matrix colored in bright pink. The AChE hydrolyzes its substrate acetylcholine into choline during enzyme immobilization. This enzyme-immobilized glass rod transducer, when immersed in OP solution, the produced acetic acid shifts the pH of the incubation mixture to the acidic region, which is detected

using an indicator and quantified spectrophotometrically at 540 nm.

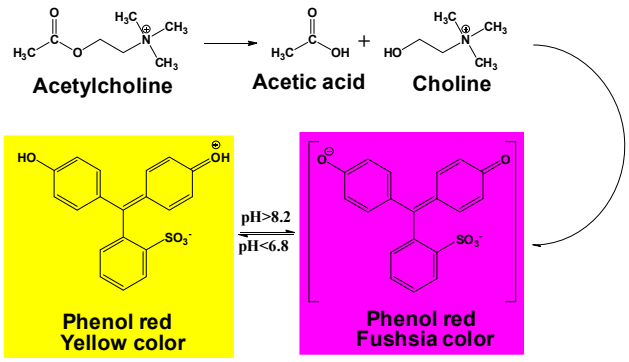

Fig. 2 The enzymatic process of acetylcholine.

AChE hydrolyzes acetylcholine into choline and acetic acid leading to a decreasing pH of the medium and can be determined by changing of color of an indicator. Several spectrophotometric indicators like cresol red, phenol red, bromocresol purple, and others have been used for pesticide detection [20]. The authors [21] used as an optical biosensor based on a lipophilic chromoionophore doped on sol-gel films for determination of AChE inhibition caused by dichlorvos; fluorescent pH-sensitive dye in PVC gel matrix was used in the fabrication of biosensor [13], and authors [22] design a biosensor with AChE and bromothymol blue in gel film. Our work focuses on the immobilization of acetylcholinesterase (AChE) onto a glass rod with stabilization in alginate gel and phosphate buffer solution (pH 8.4) and based on the color change from bright pink to yellow (Fig. 1). This immobilization type shows an easy technique for performing and allows a simple design in which enzyme, substrate, and other additives can be applied in the same transducer layer. Compared to other methods of immobilization techniques, it allows the anchorage of a sufficient amount of enzyme and increased working and storage stability. The long-term stability of AChE was also investigated. Our findings showed that the immobilized enzyme retained >75% of its initial activity over three months when kept at 4^0C, whereas free enzyme activity decreased in 4 d. However, after three months, the sensitivity of the color change for the detection of the residual amount of malathion was almost absent.

B. Determination of Acetylcholinesterase Activity

The rate of hydrolysis of acetylcholine is a direct measure of the purity and/or the amount of AChE present. The activity of AChE was determined by determining the acid produced from the hydrolysis of choline esters. This method has been most extensively used since this is, as a whole, relatively simple, precise, and accurate. The change in pH is directly related to the amount of acetic acid produced. In this method, developed back in 1949 by Michel [23], ACh's enzymatic hydrolysis occurs in a standard buffer. The decrease in the pH of the buffer caused by the acetic acid formation works as a measure of enzymatic activity. The determination of acetic acid concentration in solution was done by analyzing of the calibration graph, which showed that the optical density of the substrate solution with the enzyme is 1.62, and the mass of acetic acid in the solution is 0.035 mg (Fig. 3).

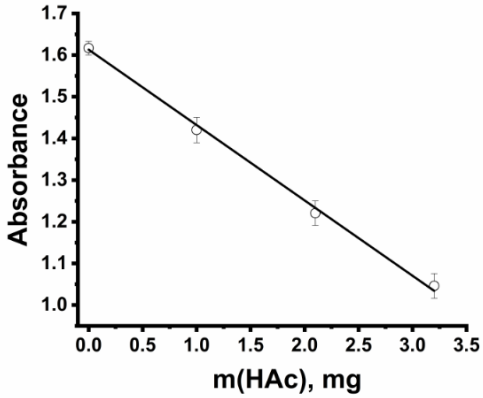

Fig. 3 The calibration curve for determining the concentration of acetic acid in solution.

The specific activity of the enzyme was 5.9 mmol/mL. The study results show that the specific activity of acetylcholinesterase (5.9 mmol/mL) sufficiently catalyzes the hydrolysis reaction with the formation of acetic acid and choline. As a result, the pH of the buffer solution changes.

To prove the presence in the solution of the acetylcholine substrate's decomposition products, a method was proposed to determine acetyl anions with ferric ions ($FeCl_3$), which was used as an indicator (Fig. 4a).

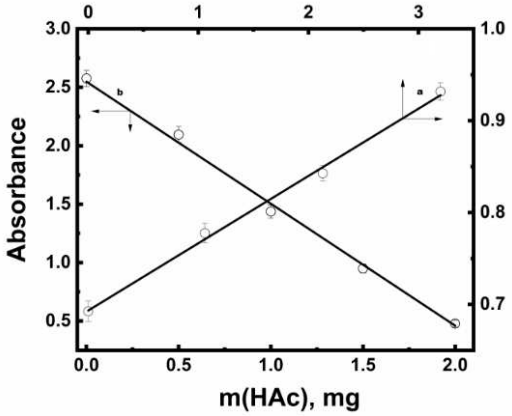

Fig. 4 (a) The calibration curve of the determination of the decomposition products of the acetylcholine substrate; (b) The calibration curve for determining the concentration of acetic acid in milk.

Analysis of the calibration graph shows that the optical density of the substrate solution with the enzyme is 0.703, and the mass of acetic acid in the solution is 0.036 mg. The determination of acetyl ions with ferric ions is shown as Eq. (2):

$$Fe3+ + CH3COO- \rightarrow H3O + Fe\,(CH3COO)3 \qquad (2)$$

The determination of the amount of acetic acid in milk was done as follow: 0.1 mL of the enzyme was mixed with 2 mL of buffer solution (pH 8.4) and kept in an incubator at 37^0C. In another tube, 0.5 mL (0.4; 0.3; 0.2; 0.1) mL of acetylcholine was mixed with 2.0 (2.1; 2.2; 2.3; 2.4) mL of milk and 0.3 mL of a solution of the indicator of phenol red and kept in a thermostat at 37^0C. After 30 min, the solutions were mixed, and the resulting solution's optical density was determined. For this, 1 mL of the solution was mixed in a cuvette with 1 mL of distilled water, and the optical density was determined spectrophotometrically. As a comparison solution, we used a solution of the tested milk in water in a

ratio of 1:1. According to the calibration graph (Fig. 4b), we found the amount of acetic acid in milk. The specific activity of the enzyme is 11 mmol/mL.

C. Application to Real Samples

The passage of pesticides to milk from pasture depends on many factors, such as the quantity of the ingestion, absorption, metabolism of pesticides, and excretion by animals in production. Several reports about milk are contaminated by pesticides [6], [7], even though most chemicals can be found in animals' urine and feces. Therefore, real-time detection of pesticides is crucial as sample conditions can vary depending on the day and time of collection and shipping to the lab. Hence, there is a real need for sensors that can make it possible to monitor and field-check detection. Researchers have developed portable biosensors that can be implemented in water, tea, juice, and milk with simple operation techniques, which are more desirable [4], [6]-[9], [24]. To determine the presence or absence of an enzyme inhibitor (malathion) in milk transducer with the polymer film formed on it containing the immobilized enzyme is immersed in a prepared milk sample. In the absence of an enzyme inhibitor (malathion), the pink color turns to yellow; if it is present, the color remains pink. The test-system's performance was carried out according to the following scheme: malathion was added to the milk sample in the following amounts: 0.03; 0.05; 0.07; 0.09, and 0.12 mg/kg. Then the activity of cholinesterase in this milk was investigated. The dependence of the enzyme activity on the concentration of pesticide in milk is presented in Fig. 5. As can be seen from Fig. 4b, the activity of the enzyme decreases sharply when reaches the pesticide maximum residual level content in milk. The detection limit of the biosensor showed to be 0.089 mg/kg for malathion. Detection of an organophosphorus pesticide such as malathion in fresh milk collected from 15 different markets showed no contamination in all analyzed samples.

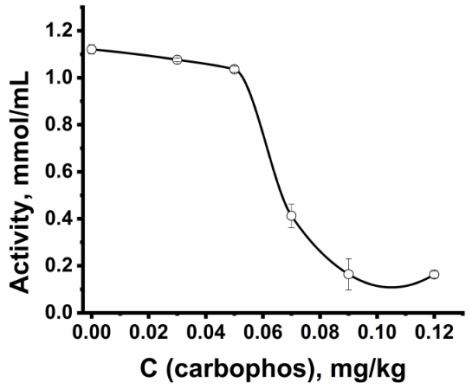

Fig. 5 The dependence of the activity of the AChE enzyme on the concentration of malathion in milk.

D. The Limit of Detection

Our work's purpose was not exact determination (by objective methods) of the finding limit of the whole range of malathion inhibitor. The plan of the study was only to validate whether the newly developed test system could provide results as good as known biosensors while using visual observation of the color change. Organophosphorus pesticide malathion was used for this purpose due to this pesticide in the agricultural treatment of crops. The specified detection limit corresponded to the concentration of malathion. The control sample was yellow. The value of the detection limit was 0.089 mg/kg.

IV. Conclusion

In this work, we have developed and optimized the immobilization of acetylcholinesterase on a glass rod. The dipstick-type AChE inhibitor test-system showed great performance for detecting pesticide malathion in milk with a detection limit of 0.089 mg/kg, in 5 min incubation time. The naked eye can detect the pesticide residue because of the color change due to an indicator's pH drop. A new biosensor is an alternative tool for convenient and straightforward screening of pesticide residue in biological samples with continuous monitoring, low cost, reliable and not required highly trained personnel. Thus, the prepared test systems with the immobilized acetylcholinesterase enzyme subsequently used to formulate the enzymatic reaction in the analyzed solution (milk) to determine organophosphorus pesticides and demonstrated the potential application of the glass stick device for the screening of OP residues in actual samples. The resulting test system is promising for the creation of express tests to determine various toxicants in biological objects. Membranes and screen-printed electrodes immobilized by enzymes are conducive to cheap material used and indulge with fast and reliable measurements. Enzyme entrapment in alginate polymer crosslinked by $CaCl_2$ allowed stable sensors for three months at 4^0C with the enzyme activity >75%. Focusing on developing the color change-based diagnostic platforms is useful in locations where resources are scarce.

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