Podcasts

ORIGINAL RESEARCH ARTICLE

Silver-based plasmonic nanoparticles for biosensor organophosphate pesticides using a single film containing acetylcholinesterase/choline oxidase

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ABSTRACT

BACKGROUND AND OBJECTIVES: To address the potential harm caused by the intensive use of pesticides in pest control in agriculture, there is a need for accurate and efficient methods to detect and monitor pesticide residues. Therefore, this study aimed to develop a biosensor that can detect organophosphate pesticides highly toxic to humans and the environment.
METHODS: Biosensor organophosphate pesticides using a single film containing acetylcholinesterase/choline oxidase have been designed using silver-based plasmonic nanoparticles as a colorimetric indicator. In the presence of acetylcholinesterase, acetylcholine is hydrolyzed to choline and acetic acid, then choline oxidase catalyzes the oxidation of choline to hydrogen peroxide and betaine. Hydrogen peroxide reacts with the silver nanoparticles, and the discoloration of the brown solution occurs due to the oxidation of silver+.
FINDINGS: As a biosensor indicator, silver nanoparticles were extremely accurate, sensitive, and stable over a long period of storage. Transmission Electron Microscope images confirmed the reduction in size of nanoparticles from 16.82 ± 4.36 to 9.63 ± 2.29 nanometers. The analyte profenofos, one of the organophosphate pesticides, inhibits the activity of acetylcholinesterase, thereby reducing the concentration decrease of silver nanoparticles by releasing less hydrogen peroxide. Optimum conditions for biosensors were achieved with a potential of Hydrogen of 7, buffer, and acetylcholinesterase concentrations of 7 and 70 millimolar, respectively, with an incubation time of 5 minutes. Biosensor response showed a linear range at profenofos concentrations of 0.05-2.00 milligrams per liter, with limits of detection and quantization of 0.04 and 0.13 milligrams/liter, respectively. Biosensor also has excellent sensitivity, reproducibility, and stability, with a Relative Standard Deviation of 2.5 percent and a stable response of up to 4 months. Subsequently, using a biosensor in the chilli as a sample resulted in a profenofos level of 0.04 milligrams per liter, making it safe for consumption.
CONCLUSION: Biosensor measurement outcome aligned with the gas chromatography-mass spectrometry result, which is the accepted standard method for detecting profenofos. Additionally, the proposed biosensor offers several advantages such as ease of use, fast, low-cost, and on-site analysis. Hence, this method is suitable for monitoring and controlling pesticide residues, particularly organophosphate, in agricultural products and the environment.

NUMBER OF REFERENCES	NUMBER OF FIGURES	NUMBER OF TABLES
31	6	1

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INTRODUCTION

Pesticide is the most effective solution in controlling pests such as insects, fungi, and weeds due to its effectiveness in killing nuisance organisms (Tudi *et al.*, 2021). The advantages of using pesticides include their ease of use, high success rate, easy to obtain, readily available, and relatively low cost. Their significant role in reducing disease and increasing crop yield has resulted in their widespread use, which, in turn, has led to excessive use of pesticides, including increased dosage, usage frequency, and the use of different types and compositions. Although some pesticides have a low persistence in the environment, they are harmful to humans and the ecosystem due to their high active toxicity (Damalas and Koutroubas, 2016; Mesnage *et al.*, 2014). Consumers are exposed to pesticide residues present in crops, including residues from the soil that are absorbed by the roots and tubers of harvested plants. To ensure food safety, the Indonesian National Standard Agency regulated the maximum allowable limit for pesticide residues in plants, with the concentration of organophosphate residues in vegetables not exceeding 0.05 milligrams per kilogram (mg/kg) (SNI, 2008). Ensuring the safety of agricultural products necessitates the detection of pesticides. Although several analytical methods have been reported in the literature for determining organophosphate pesticides, ultraviolet-visible (UV-Vis) and Fourier Transform Iinfrared (FTIR) spectrophotometry remain the standard analysis methods due to their simplicity (Li *et al.*, 2018; Sahu *et al.*, 2020). However, this method uses reagents that are toxic and not environmentally friendly; this will cause new problems when the measured waste is disposed of into the environment. Other methods such as gas chromatography (GC) (Gaber, 2014), gas chromatography-mass spectrometry (GC-MS) (Yang *et al.*, 2018), high-performance liquid chromatography (HPLC) (Rajput *et al.*, 2018), high-performance thin layer chromatography (HPTLC) (Hussain *et al.*, 2020), and liquid chromatography-mass spectrometry (LC-MS) (Stachniuk *et al.*, 2017) have also been commonly used in determining organophosphate because of their efficiency. However, these methods require relatively long preparation, skilled labor, and sophisticated and expensive equipment. To overcome this problem, enzyme-based biosensors have been developed as an alternative method. For example, various types of cholinesterase biosensing agents were used to measure the inhibition of enzyme activity due to the addition of pesticide analytes in enzymatic reactions (Kuswandi *et al.*, 2008). Acetylcholinesterase (AChE) biosensor has been proposed for detecting organophosphate pesticides using potentiometric (Mashuni *et al.*, 2022), amperometric (Zhang *et al.*, 2019), and optical transducers (spectrometry or fluorometric) (Yan *et al.*, 2019; Shah *et al.*, 2021). The optical sensor, especially colorimetric sensors employ an indicator for detecting the pesticide, such as a zwitterionic polymer (Zhu *et al.*, 2008) and bromothymol blue (Kuswandi *et al.*, 2008) but these indicators are toxic and have a limited range of action due to their dependence on the potential of hydrogen (pH). The weakness can be overcome by using silver nanoparticles (AgNPs) as a colorimetric detector. AgNP has unique properties, such as a high-sensitivity SPR spectral effect, making it a colored solution. (Loisea *et al.*, 2019). The optical biosensor for detecting pesticides is proposed to utilize the SPR of AgNPs. The sensor mechanism is based on AChE inhibition due to the presence of analytes (Kaur and Singh, 2020). AChE enzyme hydrolyzes acetylcholine (ACh) to produce choline (Ch) and acetic acid in the presence of oxygen and water is oxidized to betaine and hydrogen peroxide (H_2O_2) through the catalytic activity of choline oxidase (ChO) (Bodur *et al.*, 2021). Hydrogen peroxide spontaneously undergoes a redox reaction in the presence of colloidal AgNPs in an aqueous system (Sequeira, 2021; Tagad *et al.*, 2013), where the AgNPs serve as an indicator. By combining these two enzymes with a colloidal AgNPs coupled biosensor system, biosensors are being developed. As the mechanism of action of the biosensor developed is based on the activity of the AChE enzyme, pesticides with a mechanism of action to inhibit AChE can be detected using this biosensor, such as organophosphate and carbamate pesticide. In this study, prefonofos (an organophosphate) is used as an analyte that inhibits AChE activity, while the enzymes AChE and ChO were trapped in one film by a simple procedure without further chemical modifications. Subsequently, enzyme immobilization on a suitable matrix can maintain its activity and increase its resistance to changes in reaction condition such as pH and temperature (Hermanto *et al.*, 2020). The film was placed into colloidal AgNPs with added substrate and analytes, and measurements were carried out in a batch system. The validation of biosensor results compared to GC method was described. The application of biosensor

in determining profenofos in the real sample also was conducted. Therefore, this study aimed to develop a biosensor for organophosphate determination and was carried out in Mataram, West Nusa Tenggara, Indonesia in 2023.

MATERIALS AND METHODS

Chemicals

AChE from Electrophorus electricus (EC 3.1.1.7, type Type VI-S, 200 units/milliliter, units/mL), ChO from Alcaligenes sp. (EC 1.1.3.17, the activity of ≥10 units/mL), ACh chloride (≥99 percent: %, TLC), sodium alginate from brown alga (300-400 centipoise, cP), chitosan from crab shell (95% deacetylated), and profenofos (Sigma Pestanal®) were obtained from Sigma Aldrich (St. Louis, Missouri, USA). The 2-pyrimidine aldoxime methiodide (2-PAM) was purchased from Merck. All chemicals were of analytical grade and used as received without further purification, and double distilled water was used to prepare the solutions.

Preparation of film and immobilization procedure

Based on the previous study, a solid support film for the immobilization of AChE and ChO was prepared by mixing both chitosan and alginate hydrosols (Hermanto *et al.*, 2020). The alginate-chitosan hydrosol 3 microliter (µL) was added to 1 µL Tris hydrochloric acid (HCl) buffer (pH 6.5). The buffer mixture was added to 10 µL of the bi-enzyme mixture. The prepared bi-enzyme mixture consisted of AChE (10 microliters: µL, 200.0 units/mL) and ChO (200 µL, 10.0 units/mL) at a 1:1 enzyme ratio in 40 µL of Tris-HCl buffer solution (pH 7.0). Furthermore, the enzyme

activity was maintained using a Tris-HCl buffer in a modified hydrosol process. The mixture was flattened immediately using a magnetic stirrer (300 rotations per minute (rpm) for 10 seconds, then stored for the aging process for 72 hours (h) and 4 degree Celsius (°C). The produced film was stored in a closed container at 4°C until used (Hermanto *et al.*, 2022).

Preparation colloidal AgNPs

The proposed method for preparing colloidal AgNPs is a conventional synthesis method using green electrolysis, as described by Hermanto, *et al.* (2023). The synthesized colloidal AgNPs were further separated and purified by centrifugation (Tomy Centrifuge MDX 310, Japan) at 12,000 rpm, followed by freeze drying (Freeze dryer Alpha 1-2LDplus with RZ 2.5 vacuum pump, Germany) before being used as an indicator biosensor.

Fabrication optical biosensor

A single alginate-chitosan-immobilized AChE/ChO film was cut into a cuvette size (10 × 45 millimeters (mm) and carefully placed into the cuvette (Fig. 1). Furthermore, 2 mL of colloidal AgNP was added to the cuvette (10 mg of separated AgNP was taken and redispersed using 1 L double distilled water). The measurements were carried out using atomic absorption spectrophotometer (AAS) Thermo scientific iCE3000 USA, obtaining an Ag concentration of 10.38 milligram/liter (mg/L). Next, 1 mL of ACh chloride (substrate) was added to the cuvette. Finally, at the same time, 1 mL of organophosphate pesticide (profenofos pesticide) was also added to the cuvette
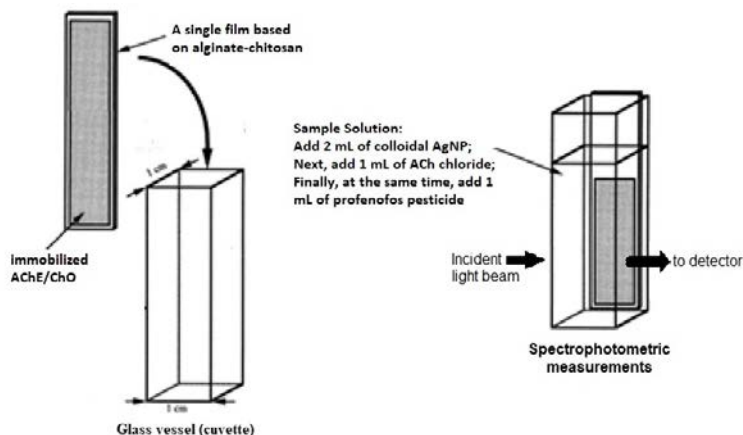

Fig. 1: Biosensor design

with various concentrations to make a standard curve. Incubation for 30 minutes (min) was required to complete the inhibition reaction. A blank solution was prepared using the above procedure without pesticide profenofos.

Measurement procedure

The absorbance of colloidal AgNPs as indicator of biosensor was then measured using UV-Vis Spectrophotometer (Spectrophotometer 7809, Labo-hub, China). The increase in the concentration of profenofos pesticide is proportional to the increase in inhibition. It affected the intensity of the SPR absorbance of the AgNPs colloid due to the redox reaction between hydrogen peroxide (product of the enzyme-substrate reaction) and AgNPs. The response of the optical biosensor is shown as a calibration curve, in which there is a linear correlation between the concentration of profenofos and the SPR intensity of colloidal AgNPs.

Determination with GC-MS methods

For comparison, GC-MS detection method (GC–MS QP210 Ultra, Shimadzu) equipped with an RTX®-5MS fused-silica capillary column (methyl polysiloxane type containing 5% Diphenyl and 95% Dimethyl Polysiloxane, length 30 meters (m) × 0 .25 mm id. × 0.25 micrometer (μm) film thickness in static phase) was also used as a reference method for determining profenofos pesticide in actual samples. High-purity helium carrier gas was used as the mobile phase (1.5 mL/min). The automatic injection process (Auto sampler carousel, AOC, Ahimadzu type) in GC-MS device was performed by injecting 1 μL at an injection temperature of 250 °C. In contrast, the interface temperature and the ion source were set at 300 °C (Alen *et al.*, 2016). Identifying profenofos pesticide based on peak chromatograms was performed, then its concentrations were determined using a calibration curve of peak area versus profenofos concentration.

Determination of profenofos in real sample

A real sample of Chilli (Capsicum frutescens L.) was obtained from the local market (Pagesangan, Mataram-Indonesia). A total of 100 g of crushed chilli was mixed with 50 mL of pH 7 Tris-HCl buffer under stirring conditions. The mixture was filtered and centrifuged at 8000 rpm for 5 min. The supernatant was separated and analyzed for the profenofos content.

RESULTS AND DISCUSSION

Biosensor scheme

In this study, an optical biosensor was developed for detecting organophosphate pesticides based on a single film of alginate-chitosan modified with silver-based plasmonic nanoparticles. For this purpose, a simple alginate-chitosan film was prepared by immobilizing the double enzymes (AChE and ChO). The scheme of the enzymatic reaction of AChE and ChO enzymes immobilized on film with ACh is shown in Fig. 2. First, the hydrolysis reaction (reaction with water, H_2O) of ACh to Ch and acetic acid occurs with AChE as a biocatalyst (Kuswandi *et al.*, 2008; 2021). Under the role of ChO, Ch released is oxidized to betaine. The ChO prosthetic group, namely Flavin Adenine Dinucleotide (FAD), is reduced to 1,5-dihydro-FAD ($FADH_2$) by accepting electrons. This reaction is reversible. In the presence of oxygen (O_2) as the electron acceptor, $FADH_2$ is oxidized to FAD. Therefore, the enzyme returns to its original form, while the oxygen as the electron
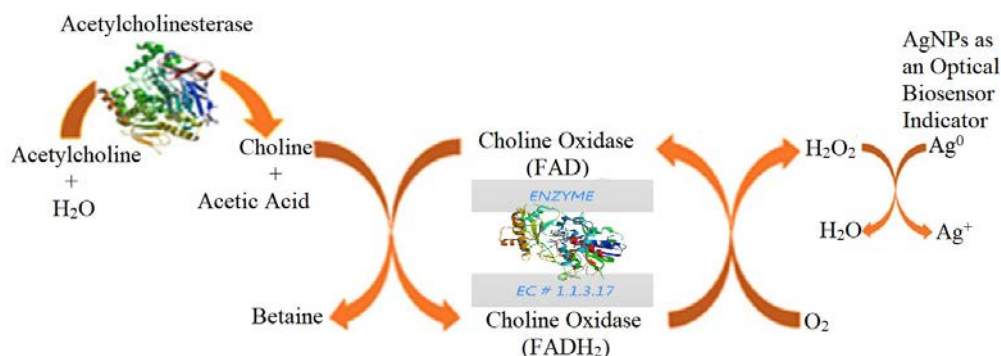

Fig. 2: Scheme of enzymatic reaction (AChE/ChO)

acceptor is reduced to hydrogen peroxide (H_2O_2) (Bodur *et al.*, 2021). The hydrogen peroxide produced by this reaction enters the aqueous system with colloidal AgNPs. Hydrogen peroxide is oxidized into water molecules, while AgNPs as a biosensor indicator will decrease in concentration because silver (Ag^0) is reduced to ionic silver (Ag^+ ion) (Sequeira, 2021; Tagad *et al.*, 2013). The determination of ACh can be carried out by measuring changes in the intensity of the SPR absorbance of the AgNPs. The presence of profenofos (an organophosphate pesticide) as an inhibitor of ACh hydrolysis of the enzymatic reaction in Fig. 2 caused a reduction in the amount of H_2O_2 released, and hence the discolouration of AgNP was reduced compared to the absence of profenofos.

In this study, a color change from brown to pale yellow causes a decrease in the intensity of the SPR AgNPs signal. This aspect is important because the operating range of AgNPs as an optical biosensor indicator closely matches the changes in hydrogen peroxide released in enzymatic reactions. On the other hand, the colloidal AgNPs are evenly distributed in the aqueous system to allow easy access to the enzymatic reaction products, resulting in the high sensitivity of the optical biosensor indicator, which is also responsible for reaction inhibition (the presence of organophosphate pesticide analytes). Besides reducing the SPR-AgNP signal intensity, the presence of hydrogen peroxide associated with the enzyme-substrate reaction leads to the erosion of the AgNP. This erosion results from the redox reaction, and hydrogen peroxide is reduced to a water molecule while AgNP is oxidized to Ag^+. It is demonstrated in Transmission Electron Microscope

(TEM) images of AgNPs before and after reacting with hydrogen peroxide associated with the enzyme-substrate reaction (Fig. 3).

In this study, Fig. 3a shows the TEM images of the AgNPs prepared. As shown above, nanoparticles are spherical with a quasi-uniform size, and the estimated mean diameter is 16.82 ± 4.36 nanometer (nm) (Hermanto *et al.*, 2023). Fig. 3b shows a TEM image of an AgNP undergoing oxidation due to interaction with hydrogen peroxide. Nanoparticles have a quasi-uniform sphere size but decreasing size; In the mean distribution histogram, its diameter is 9.63 ± 2.29 nm. Subsequently, TEM images confirm that the AgNPs before and after the reaction with hydrogen peroxide are spherical. This spherical AgNP allows all sides to interact well and spontaneously in the presence of hydrogen peroxide, and it can be used as a biosensing agent.

Optimization of experimental parameters

Before the analytical properties of biosensors can be determined, various parameters must be optimized through a series of preliminary investigations. Table 1 shows some of the experimental parameter ranges investigated and their optimum values.

The investigated experimental parameters influenced the enzymatic reaction, namely pH, buffer and substrate concentration (ACh), and incubation time. Tris-HCl buffer was utilized for this system, while phosphate buffer was avoided because of its interaction with AChE because it has the potential to compete with profenofos pesticide, which has a phosphate group. It was found that the buffer

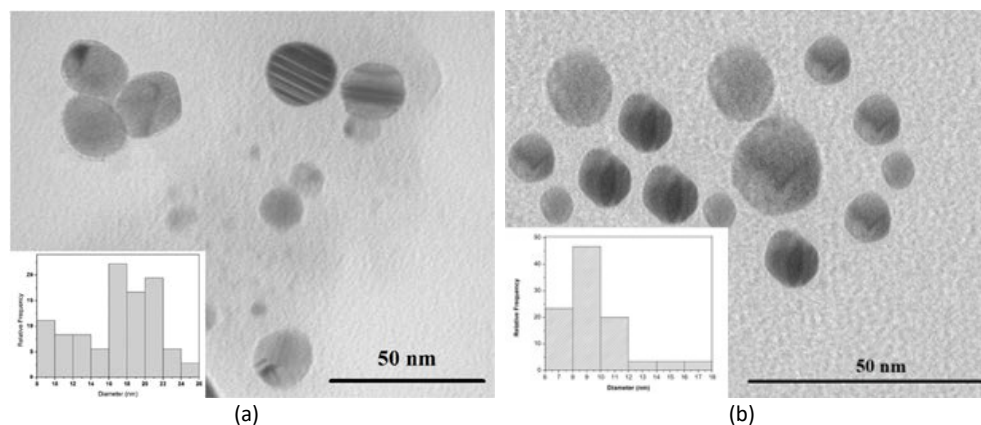(a)   (b)

Fig. 3: TEM images of AgNPs (a) before (b) after reacting with hydrogen peroxide released in enzymatic reactions (Hermanto *et al.*, 2023)

concentration was 7 millimolar (mM), and pH 7 was the optimum buffer solution; this was used for further measurement. The enzymatic reaction proceeds efficiently when the concentration of ACh as a substrate is proportional to the activity of AChE enzyme. In order to achieve good reproducibility, the substrate concentration was optimized in the range of 10 to 100 mM ACh. The concentration of 70 mM is the optimum ACh concentration and is used for further measurements. The chemical environment around the enzyme influences biosensor response. In this case, the alginate-chitosan film act as the enzyme immobilization matrix. The substrate must diffuse and penetrate the film to interact with the enzyme, and a measurable response change was obtained. Incubation time to ensure the optimal enzymatic reaction was achieved and induced AgNPs color change. In this study, the reaction incubation time was 5 min.

The response of optical biosensor

The dynamic response of the profenofos biosensor was determined by changes in the SPR absorbance signal before and after inhibition at 425 nm under optimal conditions (Fig. 4a). The response of plasmonic-based biosensor to AChE and ChO activity

due to the presence of the profenofos pesticide is plotted as a calibration curve. The biosensor system detected a series of profenofos pesticide solutions at concentration intervals of 0.05–2.00 mg/L with three replicates (Fig. 4b). Samples containing profenofos interacted with AChE resulted in an inhibitory response to ACh production. Regeneration of inhibited enzyme activity was carried out by adding the 2-PAM solution to restore immobilized enzyme activity (Kuswandi and Suwandari, 2007). However, inhibited AChE regeneration cannot restore 100% of its activity due to the strong interaction between AChE and profenofos pesticide (as a competitive inhibitor). Therefore, it is considered an irreversible reaction, and regeneration can only be carried out on film. On the other hand, it is difficult to regenerate AgNPs as an indicator of optical biosensors due to the impossibility of obtaining their original shape and size. From Fig. 4a, it is known that the increase in the concentration of profenofos pesticide is proportional to the increase in SPR absorbance. Fig. 4b shows the relationship between the concentration of profenofos pesticide and SPR absorbance in a standard curve (linear plot).

A calibration curve was constructed by evaluating the level of inhibition against the profenofos pesticide

Table 1: Optimization of experimental parameters

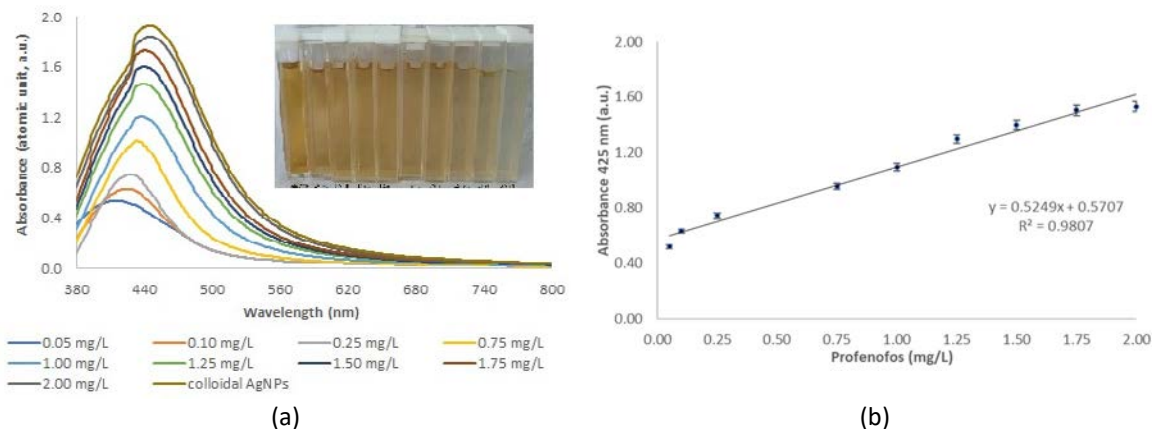| Experimental parameters	Value range	Optimal value
The concentration of buffer (mM)	1 – 10	7
pH	6 – 8	7
ACh concentration (mM)	10 – 100	70
Incubation time (min)	1 – 10	5

(a)

(b)

Fig. 4: SPR spectra of AgNPs as a biosensor detection system for profenofos at various concentrations (a); calibration curve (b)

concentration. A linear correlation (r) of 0.99 was obtained for the working range of profenofos pesticide concentrations from 0.05 to 2.00 mg/L, as shown in Fig. 4b. A flat biosensor response was obtained for higher concentrations of pesticide profenofos, indicating that the response is approaching the optimal maximum SPR uptake of AgNP. Since the maximum concentration of analyte in the sample that falls within the linear range is 2.00 mg/L profenofos, for samples that provide absorbance above the linear range, dilution is required until the concentration is within the linear range. The limits of detection (LOD, 3σ) and limits of quantization (LOQ, 10 σ) of the calculated results were 0.04 mg/L and 0.13 mg/L of profenofos pesticide, respectively. Determination of the pesticide chlorpyrifos by a previous study (Kuswandi *et al.*, 2008) through a fiber-optic biosensor based on AChE and bromothymol blue on a single sol-gel film was able to detect 0.04 mg/L of chlorpyrifos with a concentration range of 0.05-2.00 mg/L. This value represents the same LOD and linear range as this proposed biosensor. Another visible sensor that uses poly(sulfobetaine methacrylate)-coated paper for detecting profenofos has a higher detection limit of 4.891 mg/L (Zhu *et al.*, 2023). The proposed biosensor is better and allows the detection of profenofos at the maximum residue level (0.05 mg/kg) approved by the Indonesian government by Menkes and Mentan (1996) and SNI (2008). As a comparison for determining profenofos using HPLC in previous studies (Mahajan and Chatterjee, 2018), it showed a detection limit of 0.104 mg/L. The developed biosensor provides better analytical features with a lower measurement limit of 0.04 mg/L. AgNP as an indicator offered better sensitivity than the previous indicator, which used poly(sulfobetaine methacrylate) (Zhu *et al.*, 2023) and bromothymol blue (Kuswandi *et al.*, 2008) in visual/optical sensors for pesticide determination. It is indicated by the higher slope value of the change in pesticide absorbance per unit concentration, and the higher the slope value, the more sensitive the sensor. In this study, the slope was 0.5249 a.u L/mg, while the slope in the previous study was 0.0085 and 0.0331 a.u L/mg, respectively. In addition, AgNPs are highly accurate and stable over a long storage period (Hermanto *et al.*, 2023). In fabricating enzyme-based optical biosensors, recovery of inhibited enzymes is an advantage, and it is one of the purposes of immobilizing enzymes, repeatedly used, enabling biosensors to be more economical, effective, and practical in handling. The phosphate group of profenofos has a strong interaction with AChE, and AChE exposed to profenofos has an ester bond between the phosphate and serine groups on AChE, making it difficult to restore its activity (competitive inhibitor) and repeated reactivation using Tris-HCl buffer solution and ACh was not achieved. However, reactivation using 1 mM 2-PAM can restore AChE activity effectively due to its strong nucleophilic character towards the electrophilic phosphorus atom of phosphorylated AChE, and phosphorus attached to AChE is released, producing a free and active form of AChE (Kuswandi and Suwandari, 2007). Then, washing with Tris-HCl buffer solution spontaneously restored ChO activity effectively. Biosensor reproducibility is another important analytical performance. The reproducibility of silver-based plasmonic nanoparticles proposed for biosensors is expressed as Relative Standard Deviation (RSD) or the Coefficient of Variation (CV) of determining the response of biosensor at a concentration of 0.05 to 2.00 mg/L with 3 repetitions on a same single film containing AChE/ChO. The reproducibility of biosensors (RSD) was an average of 2.5% (Fig. 4b), meaning the reproducibility was good. The repeated use of film from this biosensor produces a good response after being reactivated, which is expressed as the stability of the biosensor. The stability of this biosensor is good, and even after being used repeatedly for a week for the detection of profenofos pesticide, this biosensor can still provide a sensor response of about 90% of the initial response, as long as a film containing AChE/ChO is stored at 4 °C. Fig. 5 shows that sensor response can last up to 4 months, after which it gradually decreases, and the acceptable biosensor response ≥ 60% of the initial response. At four months of the storage period, namely the seventh measurement, biosensor response was reduced to 60%, probably due to the leaching of AChE/ChO from the film during film washing, which was carried out during the enzyme reactivation process in the film using a solution of 2-PAM, in this case, reactivation was carried out six times. In these conditions, film on biosensors can no longer be used to determine profenofos, and this result is better than the previous study (Kuswandi *et al.*, 2008). Considering the use of low-cost material for synthesizing film and AgNP, the production cost per biosensor unit is 5 USD. A technique of immobilizing the enzyme on film facilitates the reuse of the enzyme, offering a biosensor production
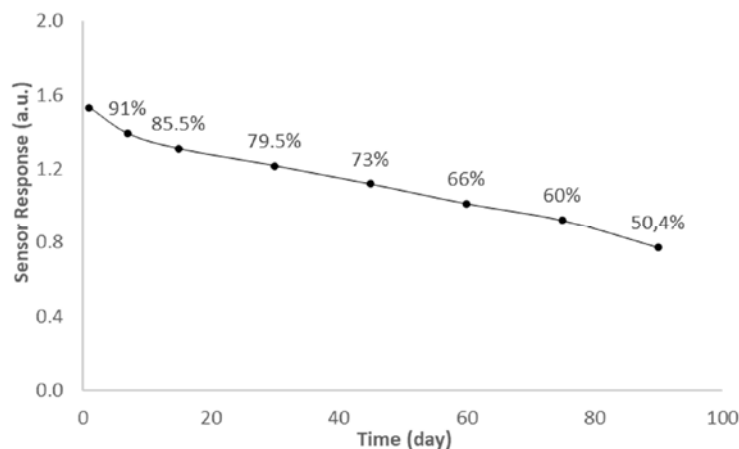
Fig. 5: Decrease in sensor response as a function of time, measurement using 2 mg/L profenofos
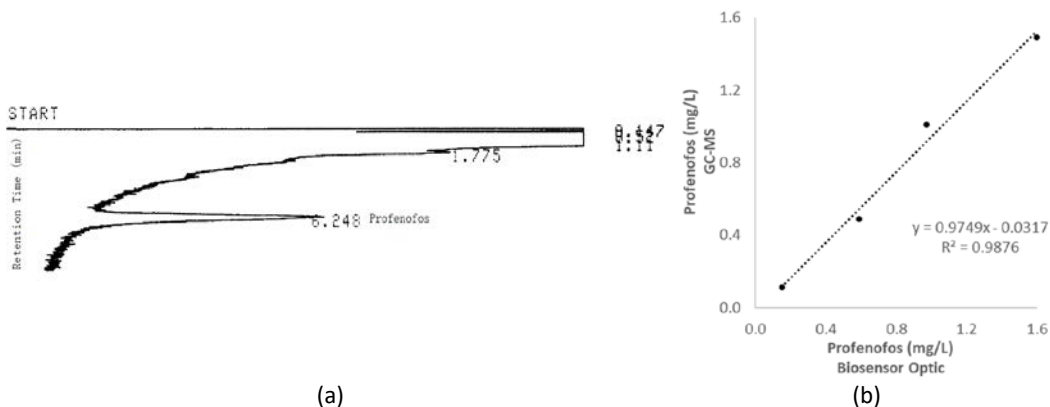

(a) (b)

Fig. 6: Chromatogram of profenofos (a); and the linearity relationship of profenofos pesticide concentrations were measured using an optical biosensor and GC-MS as a reference method (b).

cost reduction of about 80%, and per unit film can be measured up to 7 times (6 times enzyme reactivation).

Validation of analytical methods is the evaluation of specific parameters using laboratory experiments to demonstrate that they meet the requirements for their use. Optical biosensor validation uses GC-MS as a standard method to determine profenofos pesticide in real samples. Subsequently, the samples were analyzed using both methods, in which samples spiked with profenofos were made in four different concentrations, 0.1, 0.5, 1.0, and 1.5 mg/L, with three times repetition. Fig. 6 shows the linear relationship between the measurements of profenofos pesticide using both methods.

Fig. 6a is the chromatogram of the profenofos pesticide with a retention time as a qualitative parameter of about 6.248 min. Meanwhile, the peak area parameter of the chromatogram was used quantitatively by plotting it in the linear regression equation of the profenofos calibration curve to obtain the profenofos residue level in the sample. Based on Fig. 6b, the linear regression analysis shows a linear relationship (y=1.1637x–0.1795), with a good agreement between the two methods (R^2 = 0.9876). The regression coefficient is ≈1, meaning there is no significant difference when comparing the optical biosensor method and GC-MS in determining profenofos pesticide. It is supported by the analysis of the slope of the linear regression curve of 0.9749 (close to the ideal value of 1.0). The measurement

results of the proposed optical biosensor, therefore, agree with the reference method (GC-MS), and the determination of the profenofos content in chilli as a real sample was determined using the proposed biosensor. The profonefos level in chilli was 0.04 mg/L; this value is below the maximum residue threshold permitted by the Government of Indonesia, making chilli safe for consumption. Compared to conventional method such as GC-MS and HPLC, the proposed biosensor is cheaper for fabricating and maintaining the apparatus and sample preparation, more accessible, faster, and allows on-site analysis. The reusability of biosensors can be achieved by using film as an immobilizing matrix for AChE/ChO enzyme and reactivation process. In addition, this method minimizes expensive solvents such as those used in HPLC analysis. Therefore, the developed biosensor is suitable for monitoring and controlling pesticide levels in agricultural products and the environment to ensure the safety of its consumption and minimize the negative impact of pesticides on non-target living things and the environment.

CONCLUSION

A successful optical biosensor has been developed for detecting organophosphate pesticides based on an alginate-chitosan film containing AChE and ChO. In this system, the alginate-chitosan single film was used as a matrix of double enzymes (AChE and ChO), and silver-based plasmonic nanoparticles serve as a colorimetric indicator. As an indicator, the excellences of AgNP are highly accurate, sensitive, and stable over a long storage period. Silver nanoparticles are oxidized to silver ions in the presence of hydrogen peroxide produced by enzyme activity. A decrease in the concentration of nanoparticles is indicated by the fading of the solution color from brown to pale yellow in the system. Profenofos inhibits the ACh hydrolysis, reducing the amount of H_2O_2 released. Therefore, the discoloration of AgNP was reduced compared to the absence of profenofos, and the increase in the concentration of profenofos pesticide was proportional to the increase in SPR absorbance of AgNP. The size reduction of AgNPs was confirmed by TEM images from 16.82 ± 4.36 to 9.63 ± 2.29 nm due to its reaction with H_2O_2. The optimum biosensor performance was at 7 mM buffer concentration, pH 7, 70 mM ACh concentration, and 5 min incubation time. Biosensor response due to inhibition of profenofos showed a linear relationship with the concentration of profenofos in the range of 0.05 to 2.00 mg/L, with LOD 0.04 mg/L and LOQ 0.13 mg/L. The proposed organophosphate biosensor also has excellent sensitivity, reproducibility, and stability, with RSD of 2.5% and a stable response of about 4 months. The method validation through GC-MS analysis as a standard method in profenofos concentration of 0.1 to 1.5 mg/L shows good agreement between the results of the two methods. Applying biosensor in chilli as a sample gives profonefos level of 0.04 mg/L, and therefore it is safe for consumption. The advantages of biosensor include its simplicity, ease, cheapness, short time, and on-site analysis, making it is suitable for routine analysis to ensure the safety of consumption of agricultural products and reduce the negative impact of pesticides on the environment. The film containing AChE/ChO enzymes must be stored at 4 °C when not in use to ensure the performance of the biosensor remains good for on-site detection of organophosphate pesticide.

AUTHOR CONTRIBUTION

D. Hermanto performed the study conceptualization, methodology and writing-original draft. N. Ismillayli, perfomed the validation, data curation and the study supervision. R. Wirawan worked on the study software, resources and methodology. S.R. Kamali has done the study investigation, methodology and visualization. H. Muliasari investigated the formal analysis and project administration. All authors contributed to the study writing, review and editing.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

ABBREVIATIONS

%	Percent
^{o}C	Degree Celsius
μ	Micro
μg	Microgram
μL	Microliter
AAS	Atomic Absorption Spectrophotometer
ACh	Acetylcholine
AChE	Acetylcholinesterase
Ag^{+}	Ionic silver
Ag^{0}	Silver
AgNP	Silver nanoparticle
AOC	Auto sampler carousel
a.u.	Atomic unit
C	Celsius
Ch	Choline
ChO	Choline oxidase
cP	Centipoise
CV	Coefficient of variation
EC	The Enzyme Commission number
FAD	Flavin adenine dinucleotide
$FADH_2$	1,5-dihydro-FAD
FTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography combined with mass spectrometry
h	Hour
H_2O	Dihydrogen monoxide or water
H_2O_2	Dihydrogen dioxide or hydrogen peroxide
HCl	hydrochloric acid
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
kg	Kilogram
L	Liter
LC-MS	Liquid chromatography combined with mass spectrometry
LOD	Limits of detection
LOQ	Limits of quantization
MDX	Magnetically-driven sealless circulator
m	Meter
mm	Millimeter
mg	Milligram
mg/kg	milligram per kilogram
mL	Milliliter
mg/L	Milligram per liter
min	Minute
mM	Millimolar
Menkes	Menteri Kesehatan
Mentan	Menteri Pertanian
nm	Nanometer
O_2	Oxygen
PAM	2-pyrimidine aldoxime methiodide
pH	Potential of hydrogen
QP	Quadrupole
R	Linier Regression
RSD	Relative standard deviation
RTX	Column GC, Fused silica nonpolar phase
RZ	Rotary vane pump

Rpm	Rotations per minute
SNI	Standar Nasional Indonesia
SPR	Surface plasmon resonance
TEM	Transmission electron microscopes
TLC	Thin layer chromatography
USA	United States of America
UV	Ultraviolet
Vis.	Visible

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