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Developing an in-house colorimetric method for detecting organophosphate and carbamate residue using cricket cholinesterase

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Abstract

Background Pesticides such as organophosphates and carbamates are used to control pests, leaving residues on fruits and vegetables. The assessment of residues in food materials is required for consumer and agricultural worker health protection. The purpose of this study was to develop an in-house colorimetric method for detecting organophosphate and carbamate residues using cricket cholinesterase that can be used as a low-cost, simple-to-use, and widely used test.

Results The method's parameters, including substrate specificity, kinetics, and incubation time, were optimized. The results indicated that acetylthiocholine iodide was more active than butyrylthiocholine iodide. Using a buffer with a pH of 8.0 resulted in the greatest activity after 5 min. The same enzyme activity was observed at 25 °C and 30 °C, which was greater than at 35 °C and 40 °C. In this study, the Km and Vmax values for cricket cholinesterase were 3.75 mM and 35 μM/min, respectively. The specificity and purification factor of the produced ChE were 1.54 μM/min/mg and 1.43-fold, respectively. The limit of detection for detecting organophosphates and carbamates was in the range of 0.002–0.877 ppm. The validity of the method yielded the following results: relative sensitivity of 95%, relative specificity of 90%, relative accuracy of 93%, positive predictive value of 95%, and negative predictive value of 90%.

Conclusions The developed method is simple and inexpensive, and it can analyze organophosphates and carbamates in a variety of vegetables at an appropriate detection level. Cholinesterase from cricket has the potential to be used for the development of a variety of methods for detecting residues, and the sensitivity could be enhanced using new techniques in the future.

Keywords Colorimetric method, Cricket cholinesterase, Cholinesterase, Organophosphate, Carbamate

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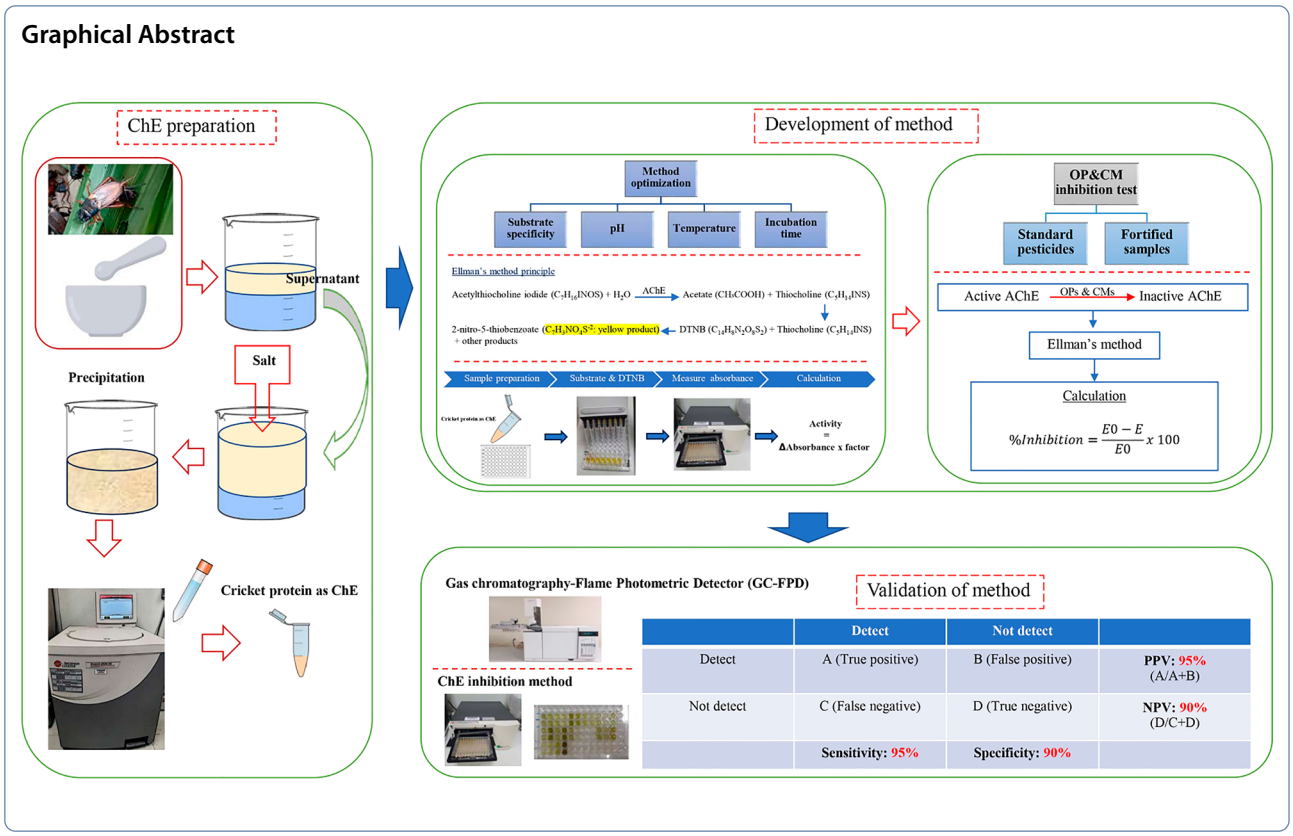

Background

Pesticides such as organophosphates (OP) and carbamates (CM) are used to control pests. However, these chemicals can leave residues on crops after application, which can be harmful to human health if consumed in large quantities. They work by inhibiting acetylcholinesterase, an enzyme that is essential for the proper functioning of the nervous system. Chronic exposure to these pesticides has been linked to a range of health problems, including neurological and developmental disorders, cancer, and reproductive issues [1]. The assessment of residues in food materials is required for consumer and agricultural worker health protection.

Pesticide residues are currently identified using gas chromatography (GC) and high-performance liquid chromatography (HPLC). Due to the complexities of analysis and processing, these methods are either expensive or time-consuming. In most cases, sophisticated equipment is required, which is frequently unavailable in many analytical laboratories. Furthermore, these methods are unsuitable for large-scale data analysis [2–5]. Immunoassays are analytical methods that rely on an analyte interacting with an antibody that recognizes it with high affinity and specificity. However, this type of determination necessitates expensive equipment and highly skilled technicians. The majority of them used animals to produce the antibodies [6, 7]. Several enzyme-inhibition-based techniques and devices have been developed, including optical colorimetric assays, fluorometric assays, electrochemical biosensors, rapid test cards, and microfluidic devices [8–12]. Most of them were developed using the cholinesterase enzyme. A bioactive paper-based sensor was developed to detect acetylcholinesterase (AChE) inhibitors, such as organophosphate and carbamate pesticides, in food. Utilizing the Ellman colorimetric assay, this sensor incorporates a chitosan gel that is crosslinked with glutaraldehyde and includes both AChE and 5,5-dithiobis (2-nitrobenzoic) acid (DTNB). Acetylthiocholine iodide (ATCh) is used as an outside reagent. The assay is performed by immersing the paper strip in a pesticide solution and, after incubation, placing it in the ATCh solution. Enzyme catalysis of the substrate initiates a yellow color change, indicating the presence of AChE inhibitors. The sensor can detect certain pesticides with good detection limits and quick response times, demonstrating its potential as a rapid, sensitive, and cost-effective tool for pesticide detection [9]. In addition, a previous study explored the use of a thermostable esterase-2 (EST2) enzyme from *Alicyclobacillus acidocaldarius* as a biosensor for detecting organophosphate

pesticides in food and the environment. Initial characterization of the enzyme involved determining its optimal pH, thermophilicity, and kinetics. The enzyme's activity was tested with several organophosphates, and it was found that paraoxon completely inhibits its activity. Notably, paraoxon and methyl-paraoxon significantly inhibited the fluorescence probe method. The enzyme biosensor was also successfully used to detect pesticides in actual food samples [13].

Acetylcholinesterase is an enzyme that rapidly hydrolyzes the neurotransmitter acetylcholine into choline and acetic acid at cholinergic synapses [14]. It plays an important role in pesticide residue detection as it is inhibited by organophosphate and carbamate pesticides [15]. While Butyrylcholinesterase (BChE) is an enzyme produced in the liver that degrades various compounds, including the neurotransmitter acetylcholine and various drugs. It is less effective than AChE at breaking down acetylcholine. Due to genetics and medical conditions, BChE levels can vary from person to person. In humans, plasma BChE decreases more rapidly than red blood cell AChE and recovers more rapidly following cessation of OP exposure [16].

Insect cholinesterase hydrolyzes acetylthiocholine slightly faster. The majority of the cholinesterase (ChE) activity in insects is found in the central nervous system compared to the peripheral nervous system. A special cholinesterase that hydrolyzes acetylthiocholine and is inhibited by physostigmine was discovered in the motor end plates of muscles in crickets. Insecticide application revealed that a critical site of action in the cricket's brain and nerve cord was involved in a knockdown [17]. Therefore, cholinesterase from cricket is an attractive alternative for developing methods to detect contamination with neurotransmitter-inhibiting pesticides, such as OP and CM.

Since the previous methods have some limitations for use as a screening test for pesticide exposure, such as high cost, high-tech equipment, and complexity, with in-house production of cholinesterase from cricket, which is widely available and inexpensive, combined with the development of a sample preparation method, the cost of the test can be reduced. Using common equipment such as a spectrophotometer in conjunction with an easy-step method can provide widespread use for screening residues, particularly in low-income countries.

The purpose of this study was to develop an in-house colorimetric method for detecting organophosphate and carbamate residues using cricket cholinesterase that can be used as a low-cost and simple-to-use test.

Materials and methods

Insects

Three different species of crickets were bought from a cricket farm in San Kamphaeng, Chiang Mai, Thailand, including *Teleogryllus mitratus* (Oriental ground cricket), *Gryllus bimaculatus de Geer* (African cricket), and *Acheta domestica* (House cricket). All crickets were frozen at − 20 °C prior to extraction.

Chemicals

Standard pesticides were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), including dicrotophos, dichlorvos, mavinphos, carbaryl, carbosulfan, and methomyl. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide (ATCh), and butyrylthiocholine iodide (BTCh) were purchased from Sigma-Aldrich.

Extraction of ChE from crickets and distribution of ChE content in different regions of the body

For comparing the activity of ChE in different regions of the body of three species of cricket, the extraction ratio at 1 mg sample: 8 mL of Phosphate Buffered Saline (PBS) buffer was prepared as 5 mg of the head, thorax, abdomen, and whole body of the cricket were each ground to a paste in a mortar and then resuspended in 40 mL of 1% Triton X-100 in PBS pH 7.2. Following that, 30 mL of the supernatant solution was collected after 30 min of performing a 10,000 g centrifugation at 4 °C. The extracted solution was maintained at − 20 °C for further measurement of ChE activity as described in "Measurement of ChE Activity".

For protein precipitation, the extracted samples with the highest enzymatic activity were chosen. The protein was prepared using the salting out technique. To compress the solvation layer and increase protein–protein interactions, ammonium sulfate $(NH_4)_2SO_4$ was used. Through aggregation and precipitation activity, increasing salt concentration caused the protein to separate and fall to the bottom of the sample solution [18]. To achieve the desired salt levels, 10 mL of the extract was mixed with an appropriate volume of saturated ammonium sulfate salt and then stirred at room temperature for 1 h using a magnetic stirrer. The centrifugation was carried out at 12,000 g for 30 min at 4 °C. After removing the supernatant, the protein pellet was resuspended in 2 mL of PBS, pH 7.2. The protein solution was kept at − 20 °C before being tested for protein concentration and ChE activity.

Preparation of the differently precipitated protein; The extracted sample was serially precipitated by preparing a

0–30% salt mixture before collecting the protein (Sample No. 1), as previously described. The supernatant was then prepared as a 30–50% salt mixture, and the second-step protein was collected (Sample No. 2). In the third step, the supernatant was collected and prepared to be a 50–75% salt mixture before collecting the protein (Sample No. 3). Samples No. 4, No. 5, and No. 6 were prepared to be 0–40%, 0–50%, and 0–75% salt mixtures, respectively. The protein was then collected in the manner described previously.

The Bradford's Protein Assay [19] and the microwell plate technique were performed briefly, and protein standards ranging from 0.1 to 0.5 mg/mL were prepared in distilled water using a BSA standard. Ten µl of each protein standard was added to the wells of the 96 well plates, and 10 µl of distilled water (DW) served as a control. The samples were prepared in DW at three different dilutions of 1:10, 1:50, and 1:100 and added to the tested wells. The incubation time was set at 5 min at room temperature (25 °C) after preparing 1:5 Bradford reagent in DW and adding 300 µl of it to the tested wells, respectively. The absorbance at 595 nm was then measured, and the protein concentration of the samples was calculated using a standard curve obtained from five concentrations of standard BSA.

Measurement of ChE activity

The method was carried out using a modified version of the methods described in the Ellman assay, as well as some modifications based on our previous research. The Ellman method was used to detect this thiocholine using Ellman's reagent, also known as DTNB. Thiocholine can break the disulfide bond in DTNB to produce 2-nitro-5-thiobenzoate ions, which have a yellow color and can be detected by their absorbance at 405 nm. As a result, the acetylcholinesterase enzyme activity can be measured by measuring the increase in yellow color over time [20, 21].

The overall reaction is

$$\text{Acetylthiocholine iodide } (C_7H_{16}INOS) + H_2O \xrightarrow{AChE} \text{Acetate } (CH_3COOH) + \text{Thiocholine } (C_5H_{14}INS)$$

$$\text{Thiocholine } (C_5H_{14}INS) + \text{DTNB } (C_{14}H_8N_2O_8S_2)$$
$$\downarrow\rightarrow 2-\text{nitro}-5-\text{thiobenzoate}\left(C_7H_3NO_4S^{-2}:\ \text{yellow product}\right) + \text{other products}$$

The cholinesterase activity was measured in the sample using 7.5 mmol/l ATCh as a substrate and 0.25 mmol/l DTNB as chromogen for measuring the ChE activity. Briefly, 10 µl of the sample was diluted in 240 µl PBS (pH 8.0) in a microplate, and then 70 µl of a mixture solution of 5:2 DTNB and substrate was added. The absorbance change at 405 nm was detected every 1 min for 5 min. The activity was calculated and reported in units per milliliter (units/mL), where one unit of ChE activity was defined as the amount of enzyme needed to hydrolyze 1 µmol of ATCh per min at 25 °C under the assay condition. Then, the calculations of specific activity (µM/min/mg), purification (fold), and yield (%) were performed [22].

Calculation of ChE activity

ChE activity (A) change/min as follows:

$$ChE\ activity\ (units/mL) = \Delta Absorbance\ x\ factor$$

$$\text{Factor} = \left[1000/\left(1.36\ x10^4 x\ 1\right)\right]\ x\ \text{dilution factor}$$

where 1.36×10^4/M/cm = extinction coefficient of yellow anion; 1000 = conversion from mM/mL to /µM/mL. [20]

Optimization of the method for ChE activity test

As described in "Measuring ChE Activity," 0–60 mM/mL substrates were used to determine the substrate type and rate of reaction for ATCh and BTCh substrates. The Michaelis–Menten graph was constructed. The Michaelis constant (Km) and the maximum velocity (Vmax) were read from the graph. The Michaelis–Menten model is one of the fundamental concepts in the field of enzymology, which is the study of enzymes. It describes the rate of enzymatic reactions by relating reaction rate (v) to substrate concentration ([S]).

The equation is represented as

$$v = Vmax[S] / (Km + [S])$$

where v is the rate of the reaction. Vmax is the maximum rate achieved by the system, at maximum (saturating) substrate concentrations. [S] is the substrate concentration.

Km is the Michaelis constant, which is the substrate concentration at which the reaction rate is half of Vmax.

It's an important measure of the enzyme's affinity for the substrate; a smaller Km means a higher affinity, because less substrate is needed to achieve the maximum rate. The substrate that showed a lower Km and a higher Vmax was chosen for the next step [23].

The pH levels for the method were optimized. Six pH levels of PBS (pH 6.5–8.0) were used as the diluent, and ChE activity was measured using 7.5 mmol/mL ATCh as the substrate, as mentioned in the previous section. The results were compared, and the pH level of PBS with the highest activity was chosen for the next steps.

The temperature levels for the method were optimized. As mentioned in the previous section, ChE activity was measured in the water bath at 4 different temperatures (25–40 °C). The results were compared, and the temperature with the highest activity level was chosen for the following steps.

The effect of methyl alcohol on ChE activity was investigated. Six concentrations of methyl alcohol (MeOH) in PBS (0–5% *v*/*v*) were used as buffer solutions in ChE activity measurements. The results of ChE activity were compared, and the concentration of MeOH, which has no effect on ChE activity, was used in the step of pesticide extraction from vegetable samples.

Optimization of the method for ChE inhibition test

Acetylcholinesterase is a critical enzyme in the nervous system that degrades the neurotransmitter acetylcholine. Its function is critical to maintaining proper nerve cell communication. The chemical equation below represents the typical cholinesterase-catalyzed reaction:

$$\text{Acetylcholine } (C_7H_{16}NO_2+) \xrightarrow{\text{AChE}} \text{Acetic acid } (CH_3COOH) + \text{Choline } (C_5H_{14}NO+)$$

Organophosphates and carbamates, on the other hand, can bind to the active site of the cholinesterase enzyme, inhibiting its activity. When these pesticides bind to the active site of cholinesterase, they prevent it from breaking down acetylcholine. This results in an excess of acetylcholine, which causes overstimulation of certain bodily systems. A colorimetric assay can detect this change in cholinesterase activity. These assays typically use a substrate that changes color when cholinesterase acts on it. Under normal conditions, cholinesterase will catalyze the breakdown of this substrate, causing the solution's color to change. This color change is reduced or absent when organophosphates or carbamates inhibit cholinesterase. It is possible to estimate the extent of cholinesterase inhibition and, thus, the presence of organophosphate or carbamate pesticides [24].

ChE activity was measured in the manner described in "Measurement of ChE Activity". Four dilutions of extracted ChE (1:1–1:8) were used as the sample, and two concentrations of ATCh (7.50 and 3.75 mmol/mL) were used as the substrate. The absorbance of each reaction was measured. To further develop the ChE inhibition test, a combination of diluted ChE and concentrated ATCh with an absorbance difference of about 1 was chosen.

The optimal time for an inhibition test was performed briefly: 10 µl of cricket ChE was added into the well, followed by 100 µl of 1 part per million (ppm) mevinphos in 5% MeOH PBS pH 8.0 as an inhibitor, and 140 µl of PBS pH 8.0, and then incubated at 6 different times (10–40 min). The activity of ChE was measured, as mentioned in the previous section, at room temperature (25 °C) and pH 8.0 conditions. The inhibition percentages were calculated, and the appropriate incubation time was used in the method for ChE inhibition by OP and CM pesticides. The inhibition percentage was calculated by

$$\%\,Inhibition = \frac{E0 - E}{E0} \times 100$$

where [E0] is the initial enzyme activity in the absence of an inhibitor, and [E] is the enzyme activity in the presence of an inhibitor.

ChE inhibition by organophosphate and carbamate pesticides

Ten µl of cricket ChE was added into the well, followed by 100 µl of the inhibitors OPs and CMs at different concentrations (0.005–5.000 ppm) in 5% MeOH PBS pH 8.0 and 140 µl of PBS pH 8.0, and incubated for 30 min. The activity of ChE was measured, as mentioned in the previous section, at room temperature (25 °C) and pH 8.0 conditions. The calibration curve was obtained, and the half maximal inhibitory concentration (IC_{50}) and 25% inhibition concentration (IC_{25}) were calculated using the Prism program version 4.01. The IC_{25} was used as the limit of detection (LOD) of this method.

Extraction of pesticides from vegetable samples

Five grams of each vegetable sample were chopped finely and shaken for 5 min with 10 mL of dichloromethane. Five milliliters of the extract were transferred to a glass tube and dried in hot water to completely remove the solvent. The pellet was resuspended with 200 µl of dichloromethane, then 200 µl of 5% MeOH PBS, pH 8.0, was added and vigorously mixed. Dichloromethane was then completely removed using hot water. The extracted

solution was used in a further step of ChE activity measurement.

ChE inhibition by organophosphate and carbamate pesticides in vegetables

The pesticide-free vegetables (cabbage), confirmed to have no OP residues by gas-chromatography coupled with flam photodetector (GC–FPD) as described in the previous study [25], were spiked with pesticides at several concentrations (0.005–5.000 ppm) of dicrotophos, dichlorvos, mevinphos, carbarly, carbosolfan, and methomyl. All samples were extracted as mentioned in the previous section, and the developed method was performed to determine an inhibition. Briefly, 10 µl of the twice diluted ChE and 100 µl of the sample were added into a microplate, then 140 µl PBS (pH 8.0) was added and incubated at room temperature (25 °C) for 30 min. In a further step, 70 µl of a mixture solution of 5:2 DTNB and 3.75 mmol/mL ATCh substrate was added. The absorbance changes at 405 nm were detected every 1 min for 5 min at 405 nm. The inhibition was calculated as described previously. The calibration curve was obtained, and the IC_{50} and IC_{25} were calculated using the Prism program version 4.01. The IC_{25} was used as the LOD of this method.

Application of developed method to vegetable samples

The 30 vegetable samples examined using GC–FPD came from our previous study [26], such as kale (*Brassica alboglabra L.H. Bailey*), cabbage (*Brassica oleracea var. capitata L.*), bok choy (*Brassica rapa. L.*), long bean (*Vigna unguiculata ssp. Sesquipedalis*), and morning glory (*Ipomoea aquatica Forssk.*). All samples were extracted, as mentioned in the previous section, to determine their inhibition using the developed method. The inhibition was calculated in the manner mentioned previously. Samples showing an inhibition test result greater than 25% were interpreted as having the presence of an insecticide. For relative sensitivity, relative specificity, relative accuracy, positive predictive value, and negative predictive value, the results are calculated and compared with GC–FPD results [27].

Statistical analysis

ChE activity results were reported as the mean and standard deviation. The inhibition curve was conducted, and the IC_{50} was calculated using GraphPad Prism 4.01. A one-way ANOVA was used to compare ChE activity and inhibition percentage across the study. The statistical tests were two-sided, with a significance level of 0.05. The SPSS statistical program, version 17, was used for the analyses.

Table 1 ChE activity in different body regions in three cricket species

Body region	ChE activities (Mean ± SD)		
	Oriental ground cricket (units/mL)	African cricket (units/mL)	House cricket (units/mL)
Head	3.40 ± 0.37[a]	3.17 ± 0.17[a]	7.76 ± 0.35[a]
Thorax	4.87 ± 0.22[b]	2.88 ± 0.22[a,b]	5.31 ± 0.27[b]
Abdomen	5.27 ± 0.33[b]	2.23 ± 0.33[b]	6.08 ± 0.49[a,b]
Whole body	2.97 ± 0.17[a]	2.98 ± 0.64[a]	11.97 ± 0.53[c]

Mean within columns followed by the same letter are not significantly different ($P > 0.05$; One-Way ANOVA; post Hoc Benfferoni Multiple Comparisons)

Note: In the table, it was indicated that there were not significantly different cholinesterase levels in several samples, including the head and the whole body of oriental ground cricket, the head, the thorax, and the whole body of African cricket, and the head and abdomen of the house cricket, as shown in the same letter (a). Following the letter (b), the thorax and abdomen of all crickets were not significantly different. However, the whole body of the house cricket showed a higher cholinesterase level than the others, as shown in the letter (c)

ChE Cholinesterase

[a] Indicated that there were not significantly different cholinesterase levels in several samples, including the head andthe whole body of oriental ground cricket, the head, the thorax, and the whole body of African cricket, and the head and abdomen of thehouse cricket

[b] The thorax and abdomen of all crickets were not significantlydifferent

[c] The whole body of the house cricket showed a higher cholinesterase level than the others

Results

House cricket cholinesterase activities

According to the results in Table 1, ChE activity in the house cricket was found to be superior to others in every body region, with the whole body of cricket showing the best at 11.97 ± 0.53 units/mL. In this study, the whole body of the house cricket was used for enzyme production.

Table 2 displays the protein concentration and enzyme activity of PBS-extracted samples and precipitated proteins. PBS-extracted total protein had the highest concentration (95.50 mg), whereas 0–50% precipitated protein exhibited the highest enzyme activity (27.75 units/mL), followed by 0–40% precipitated protein (27.70 units/mL). Specific activity and purification were calculated, and the 0–40% salt-precipitated protein had the highest specific activity (1.54 µM/min/mg) and purification factor (1.43-fold). The yield percentage of the 0–50% salt-precipitated protein yield was the highest at 53.91%, followed by the 0–40% and 0–75% salt-precipitated protein yield percentages at 53.81% and 53.07%, respectively. According to the highest specific activity and purification factor, the 0–40% salt-precipitated protein of the house cricket was used to determine substrate specificity and kinetics in subsequent steps.

Table 2 Summary of protein concentration and ChE activity results from crude purification at different salt levels

Specification	PBS extracted	Ammonium sulfate salt precipitation level (%)					
		0–30	30–50	50–75	0–40	0–50	0–75
Volume (mL)	10.00	2.00	2.00	2.00	2.00	2.00	2.00
Protein (mg/mL)	9.55	14.11	13.17	5.73	17.95	21.47	21.31
Total protein (mg)	95.50	28.22	26.34	11.46	35.90	42.94	42.62
Total activity (μM/min)	102.95	41.05	38.05	1.83	55.40	55.50	54.64
Specific activity (μM/min/mg)	1.08	1.45	1.44	0.16	1.54	1.29	1.28
Purification factor (fold)	1.00	1.35	1.34	0.15	1.43	1.20	1.19
Yield (%)	100.00	39.88	36.96	1.77	53.81	53.91	53.07

PBS Phosphate Buffered Saline

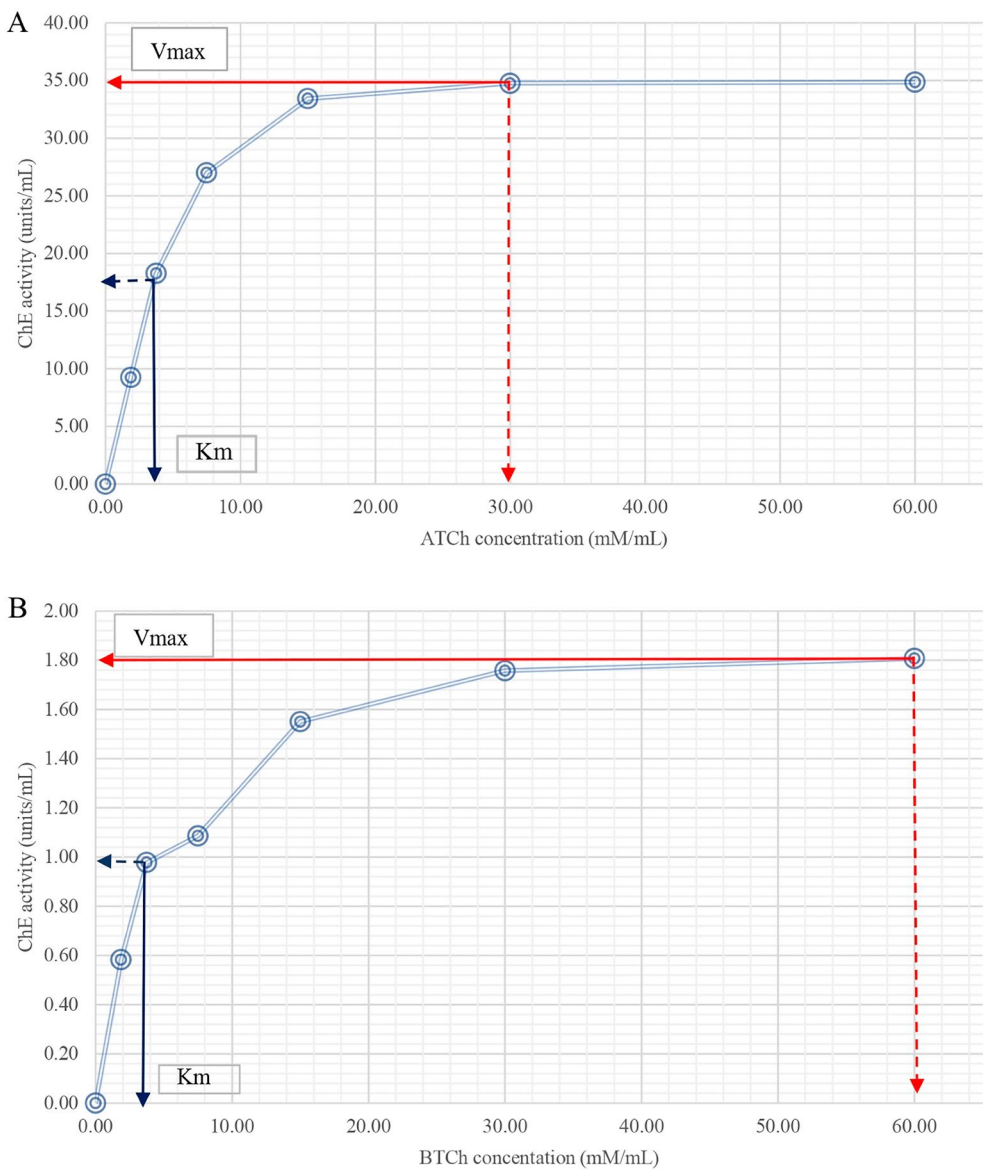

Fig. 1 Michaelis–Menten graph: the result of ChE activities on (**A**) ATCh substrates and (**B**) BTCh substrates

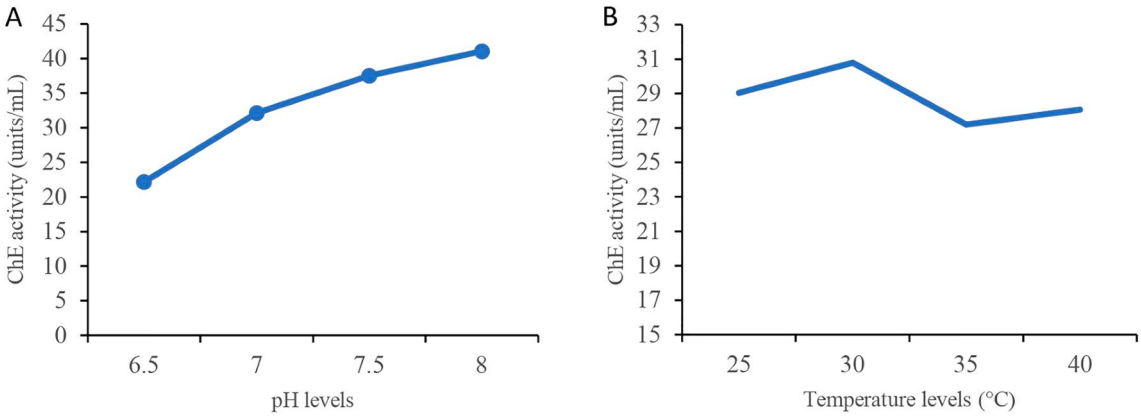

Fig. 2 Optimization results of the method: (**A**) pH levels and (**B**) temperature levels

Table 3 Result of ChE activity in the 6 levels (%*V/V*) of MeOH in PBS pH 8.0

	MeOH in PBS (%)					
	5	**4**	**3**	**2**	**1**	**0**
ChE activity (mean ± SD)	41.80 ± 1.45[a]	42.04 ± 0.66[a]	42.44 ± 0.59[a]	42.15 ± 1.15[a]	40.40 ± 1.20[a]	40.05 ± 1.36[a]

Means followed by the same letter are not significantly different ($P > 0.05$; One-Way ANOVA; post Hoc Bonferroni Multiple Comparisons)

Note: According to the table, there were no significant differences in cholinesterase levels within 0% to 5% of the methanol percentage in PBS, as indicated by the same letter (a), indicating that the methanol percentage in PBS can be increased to 5% without affecting the test

ChE Cholinesterase, *MeOH* Methanol, *PBS* Phosphate Buffered Saline

[a] There were no significant differences in cholinesterase levels within 0% to 5% of the methanol percentage inPBS, (a) indicating that the methanol percentage in PBS can be increased to 5% without affecting the test.

Table 4 Results of the absorbance difference of the combinations of ChE dilution and ATCh concentration

ATCh (mM/mL)	Difference Absorbance (min5–min1)			
	Dilution (1:x)			
	1	**2**	**4**	**8**
7.50	2.01 ± 0.19	1.30 ± 0.06	0.52 ± 0.11	0.30 ± 0.15
3.75	1.44 ± 0.03	1.00 ± 0.01	0.50 ± 0.16	0.34 ± 0.01

ATCh Acetylthiocholine iodide

Substrate specificity and kinetics

The activity of ChE on ATCh and BTCh substrates was determined using the selected sample (0–40% salt-precipitated protein). The result from the Michaelis–Menten graph showed the Vmax and Km on ATCh substrate at 35 units/mL and 3.75 mM/mL, respectively. While the Vmax and Km on BTCh substrate showed 1.80 units/mL and 3.75 mM/mL, respectively, as shown in Fig. 1, the outcome indicated that the ChE of house cricket is more specific to ATCh than BTCh substrate.

Table 5 Result of inhibition percentage of 1 ppm Mevinphos pesticide in difference incubation time

	Incubation time (min)					
	15	**20**	**25**	**30**	**35**	**40**
Inhibition (Mean ± SD)%	76.21 ± 7.89[a]	77.85 ± 1.74[a,b]	86.55 ± 1.47[b]	91.71 ± 1.03[b,c]	94.81 ± 0.52[b,c]	96.86 ± 0.99[c]

Mean followed by the same letter are not significantly different ($P > 0.05$; One-Way ANOVA; post Hoc Benfferoni Multiple Comparisons)

Note: In the table, it was indicated that there were not significantly different cholinesterase levels at 15 and 20 min of incubation, as shown in the letter (a). Following the letter (b), the incubation times at 20 to 35 were not significantly different. Even though 40 min of incubation showed the highest level, there was no significant difference in the levels at 30 to 40 min of incubation, as shown in the letter (c)

[a] Indicated that there were not significantly different cholinesterase levels at 15 and 20 min of incubation

[b] The incubation times at 20 to 35 were not significantly different. Even though 40 min of incubationshowed the highest level

[c] There was no significant difference in the levels at 30 to 40 min of incubation

Table 6 Detection limits of the standard pesticides and fortified samples using the ChE inhibition method

Pesticides	Detection limits (ppm)	
	Standard pesticides	Fortified samples
Dicrotophos	0.192	0.174
Dichlorvos	0.246	0.426
Mevinphos	0.004	0.009
Carbarly	0.497	0.508
Carbosulfan	0.001	0.002
Methomyl	0.022	0.008

ppm part per million

Table 7 Efficiency of the developed ChE inhibition method for the detection of insecticides in vegetable samples compared with GC–FPD

Methods	Number of positive sample (n)		Number of negative sample (n)	
	True	False	True	False
GC–FPD	20	0	10	0
ChE inhibition method	19	1	9	1

According to Fig. 2, pH conditions of 6.5 showed low activity of ChE, whereas 7.0, 7.5, and 8.0 showed high activity of ChE. In addition, the ChE activity showed no significant difference in pH 7.0–8.0. The pH of 8.0 was chosen for further steps according to its highest activity. The comparison of temperature levels was performed, and the result showed the same activity of enzymes at 25 °C and 30 °C, which was higher than at 35 °C and 40 °C, as shown in Fig. 3. According to the convenience of the method that can be performed without the incubator, a temperature of 25 °C was used for the method in the next step.

Optimization of the method for ChE inhibition

The effects of several concentrations of MeOH (0–5% *V*/*V*) in phosphate buffer solution pH 8.0 were tested. The result showed no difference in ChE activity between the concentrations of 0–5% V/V MeOH, as shown in Table 3. That means we can use 5% MeOH in PBS pH 8.0 as the resuspension solution for the extracted samples.

To select the appropriate combinations of dilution of the produced ChE and substrate concentration for the inhibition method, the optimized ChE activity test method was performed in a variety of combinations. The combination of 1:2 diluted ChE and 3.75 mM/mL ATCh exhibited an absorbance difference of approximately 1.0 at 5 min, as shown in Table 4. This combination was used in the subsequent step of the inhibition test procedure. To optimize the inhibition method, the time required for pesticides and ChE to react completely was determined. At 30 min, the inhibition of ChE by 1 ppm mevinphos was complete, and there was no significant difference, thereafter, as shown in Table 5.

Development and application of ChE inhibition method for vegetable samples

After utilizing the optimized ChE inhibition method, the IC_{50} and IC_{25} of OP and CM pesticides were determined. In this study, the IC_{25} was used as the LOD for the inhibition method, and the LOD values for standard OPs and fortified samples ranged between 0.004–0.246 ppm and 0.009–0.426 ppm, respectively. LODs for standard CMs and fortified samples range between 0.001–0.497 ppm and 0.002–0.508 ppm, respectively. As shown in Table 6, mevinphos was the most potent inhibitor among OP pesticides, while carbosulfan was the most potent inhibitor among CM pesticides.

According to Table 7, the true positive, true negative, false positive, and false negative values for the evaluation of the validity of pesticide detection methods using the developed ChE inhibition method versus the GC–FPD method in 30 vegetable samples were 19, 9, 1, and 1, respectively. Consequently, the relative sensitivity was 95%, the relative specificity was 90%, the relative accuracy was 93%, the positive predictive value was 95%, and the negative predictive value was 90%.

Discussion

Several studies have demonstrated that ChE can be extracted from a variety of animals and insects, and their substrate specificity and kinetics have been investigated. Most of them were used in the development of pesticide detection methods. Several purification techniques, including ammonium sulfate precipitation, Sephadex chromatography, DEAE–cellulose chromatography, gel filtration, affinity chromatography, procainamide affinity chromatography, etc., were combined to prepare ChE for use in their studies [22, 28–34]. In these previous studies, the specificity and purification fold ranged from 9.0–1336.7 μM/min/mg to 4.8–3897 fold, respectively. Higher specificity and purification are associated with a more complex and expensive technique. Since the objective of this study was to develop a low-cost in-house method, one step of crude purification (ammonium sulfate precipitation) was used to extract ChE from crickets. The specificity and purification of the produced ChE were 1.08 μM/min/mg, and 1.54-fold, respectively. The lower specificity of this study is due to the ineffectiveness of

the ammonium sulfate precipitation method in isolating different protein sizes compared to the other previously mentioned methods. Nevertheless, one step of crude purification can be used as an easy and low-cost technique for the ChE production method in this study.

The optimal pH and temperature of 8.0 and 25 °C were confirmed. Previous studies that reported identical findings for insect cholinesterase supported the findings [17, 28]. This study revealed that, under optimal conditions, the LOD of standard pesticides was lower than that of pesticide-spiked samples for both OPs and CMs, which may be caused by the vegetable matrix (i.e., color and acidity) and the extraction process (i.e., solvent). Since this study demonstrated that the LOD for OPs and CMs were between 0.002 and 0.508 ppm, it is comparable to the LOD of a paper-based acetylcholinesterase inhibition assay using commercial acetylcholinesterase for the detection of OPs, which was between 0.003 and 0.600 ppm [35]. The results were superior to the qualitative acetylcholinesterase detection kit based on a modification of the Ellman and ELISA methods, using honeybee (*Apis mellifera L.*) heads as the source of ChE with a LOD range of 0.50–4.80 ppm [36]. Furthermore, the newly developed smartphone-based colorimetric sensor technique demonstrated that the low LOD of a method for all pesticides was less than 1.5 × 10(-7) M, indicating greater sensitivity than the U.S. Environmental Protection Agency regulations specify [37]. Indicating that the cricket ChE can be used in method development and is a potential source for the future.

A method of ChE inhibition for monitoring OP and CM pesticides in vegetable extracts was presented. Importantly, the ChE inhibition method was validated in vegetable matrices, and its performance was confirmed by an accredited GC–FPD. The short analysis time (20 min for sample preparation for 10 samples and 30 min for the ChE inhibition method) and low cost of the 96-microwell plate demonstrated that the ChE inhibition method can be used by laboratories with limited resources as a preliminary screening tool. In addition, 48 samples (2 wells/sample in a 96-microwell plate) were able to be analyzed using the developed high-throughput method. In the test, samples of vegetables with an intense dark color showed a false positive, which the extraction method was unable to clear. Indicating that color is one of the matrices in this method, along with other chemicals that can influence ChE, such as heavy metals [38]. Low levels of pesticide residues in the samples led to false negative results. Since it yielded greater than 90% of all efficiency indicators, especially 95% of the positive predictive value, our developed method can be used as a screening test for detecting OP and CM residues in vegetable samples.

Multifaceted approaches can improve cholinesterase (ChE) assay sensitivity and recovery. Concentrating target pesticides and eliminating interfering substances requires improving sample preparation methods. Optimizing assay conditions such as temperature, pH, and incubation time increases enzyme activity. Enzyme enhancers may benefit, while nanotechnology-based methods may improve sensitivity and stability. Microfluidics or lab-on-a-chip technologies may enable efficient, miniaturized testing. Finally, advanced instrumentation such as spectrometry or chromatography could improve detection.

Conclusion

The results of this study demonstrate conclusively that the developed method can analyze OP and CM pesticides at appropriate detection levels in many types of vegetables. The method requires minimal equipment and is simple enough for use in a primary laboratory equipped with a spectrophotometer. The developed method can be easily integrated into pesticide control programs as a screening method. Future method development could utilize cricket as a source of ChE.

Abbreviations

OP	Organophosphates
CM	Carbamates
GC	Gas chromatography
HPLC	High-performance liquid chromatography
GC–FPD	Gas chromatography coupled with flam photodetector
AChE	Acetylcholinesterase
ChE	Cholinesterase
DTNB	5,5′-Dithiobis-2-nitrobenzoic acid
ATCh	Acetylthiocholine iodide
BTCh	Butyrylthiocholine iodide
BSA	Bovine serum albumin
IC_{50}	The half maximal inhibitory concentration
IC_{25}	The 25% inhibition concentration
LOD	The limit of detection
ppm	Part per million

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Author contributions

Conceptualization, AW and SH; methodology, AW and PA; validation, AW, PA and UJ; formal analysis, AW and NS; resources, AW and SH; data curation, AW and PS; writing—original draft preparation, AW; writing—review and editing, AW, SH, NS, WW and PD; visualization, AW, SH, WW and PD; supervision, SH, WW and PD; All authors read and approved the final manuscript.

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All data generated and analyzed during this study are included in this manuscript.

Declarations

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Not applicable.

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Competing interests
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