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Research Article

Carboxylesterase from edible legume seeds and their sensitivity to organophosphate and carbamate pesticides

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Abstract

Carboxylesterase from edible legume seeds, including black gram bean (*Vigna mungo* (L.) Hepper), mung bean (*Vigna radiata* (L.) Wilczek) and red bean (*Vigna umbellata* (Thunb.) Ohwi and Ohashi) has been partially purified using the ammonium sulphate salting-out procedure. The specific activity of the partially purified carboxylesterase extracted from black gram bean, mung bean and red bean are 3.96±0.26, 1.51±0.10 and 1.29±0.08 U/mg, respectively ($p<0.05$). The carboxylesterase from black gram bean showed very high sensitivity to 24 types of organophosphate pesticides (OPs) and 7 types of carbamate pesticides (CAs) at the concentrations of 0.001-3.0 μg/ml. The limit of detection (LOD) obtained for OPs and CAs pesticide residues were 0.002 and 0.01 mg/kg, respectively. For performance assessment of methods for pesticide determination in vegetables, all performance parameter values, including sensitivity, accuracy, and specificity of the carboxylesterase enzyme inhibition method, are 100% compared with commercial pesticide test kits and are 70.5%, 90%, and 100%, respectively, compared with chromatographic method analysis.

Keywords: carbamate; carboxylesterase; legume seeds; organophosphate; pesticide residues

Introduction

Organophosphates (OPs) and carbamates (CAs) are the most common pesticides used to protect crops from insects in plants and agricultural production. Although these are useful, they are highly poisonous to human and animal health (Pimsen *et al*., 2014). Long-term accumulated contamination in the environment and residues in agricultural products during the growing stage of plants and post-harvesting were frequently reported (Nieto, Romero and Garrido, 2015). For this reason, the regulatory authorities, including the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO), the European Community, and the World Health Organization (WHO), have set the maximum levels of pesticide residues in original plant products and agricultural products (Ben Oujji *et al*., 2013). Various analysis methods based on chromatographic methods, including gas chromatography (GC) and high-performance liquid chromatography (HPLC), were reported as reference methods for the sensitive detection of pesticide residues

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in food products (Lin *et al*., 2010; Jin *et al*., 2012; Miao *et al*., 2013; Yang *et al*., 2013, Song *et al*., 2014). Although these techniques offer advantages for qualitative and quantitative analysis with low limits of detection (LOD), they are all expensive instruments, time-consuming, complicated techniques that require highly trained persons to operate, use large volumes of solvents, and cause inconvenience for field applications (Chauhan *et al*., 2011; Wang *et al*., 2012). Therefore, the method for detection of pesticide residues based on the biological detection system is a good alternative method because it is more fast, inexpensive, sensitive, simple, and convenient than classical methods (Ben Oujji *et al*., 2013).

Carboxylesterase (EC 3.1.1.1) is classified into the hydrolase group, which catalyzes various types of esters to carboxylic acid and alcohols (Bhavith *et al*., 2014). These enzymes are widely distributed in nature and are found in animals, plants, and microorganisms. The biological functions of these enzymes, including cell expansion, abscission, fruit ripening, and insecticidal resistance against infection, also preferably catalyze the hydrolysis of ester-containing xenobiotic molecules (Hou *et al*., 2012; Shivashankar and Premkumari, 2014). Carboxylesterases in plants belong to the group of hydrolases that can act on ester bonds and involve themselves in biological processes. It is widely distributed in multiple forms in plants, including wheat, rice, soybean seeds, and sorghum (Hou *et al*., 2012). Carboxylesterases extracted from various types of legume seeds show a reaction with OPs and CAs pesticides like animal-origin acetylcholinesterase (AChE). Moreover, these enzymes also showed very high sensitivity to OPs and CAs with the lowest LOD (Li *et al*., 2009; Wang *et al*., 2012).

The present study was performed to determine OPs and CAs residues in vegetables by enzyme inhibition method using -naphthyl acetate as its substrate. These carboxylesterase enzymes were extracted from selected edible legume seeds and partially purified by the ammonium sulphate salting-out method. Since they are cheaper, easier, and more convenient to extract than AChE, the capability of these enzymes for OPs and CAs detection was investigated using commercial pesticide test kits and also evaluated by standard methods with chromatographic analysis.

Materials and Methods

Plant materials

Legume seeds (black gram bean (*Vigna mungo* (L.) Hepper), mung bean (*Vigna radiata* (L.) Wilczek), and red bean (*Vigna umbellata* (Thunb.) Ohwi and Ohashi) and fresh vegetables (Chinese cabbage, lettuce, cabbage, coriander, cilantro, chilli, sweet basil, yard long bean, pennywort, and cucumber) were purchased from a local market in Chonburi Province, Thailand, during January- August 2020.

Chemicals and pesticide standards

Sodium dihydrogen phosphate, sodium hydroxide, disodium hydrogen phosphate, sodium carbonate, copper sulphate, sodium potassium tartrate and ammonium sulphate anhydrous were purchased from Merck (Germany) and Honeywell. Bovine serum albumin (BSA), α-naphthyl acetate, α-naphthol, fast blue B salt and Folin Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Chemical Co. Sodium phosphate buffer (0.1 M, pH 7.0) was used in this experiment. All chemicals used were analytical or HPLC grade. OPs pesticide standards including acephate, azinphos-methyl, chlorpyrifos, diazinon, dichlorvos, dicrotophos, dimethoate, EPN, ethion, fenitrothion, malathion, methamidophos, methidathion, mevinphos, monocrotophos, omethoate, parathion, parathion-methyl, phosalone, pirimiphos-methyl, profenofos, propargite, prothiofos and triazophos and CAs pesticides standards including aldicarb, carbaryl, 3-OH-carbofuran, carbofuran, methomyl, methiocarb and oxamyl were purchased from Dr. Ehrenstorfer Co., Augsburg, Germany. All standards are >99.0% purity.

Carboxylesterase extraction and partially purification

Carboxylesterase of legume seeds (black gram bean, mung bean and red bean) was extracted by the modified method of Li *et al.* (2009) and Wang *et al*. (2012). A total of 12 g of legume seeds powder was added to 30 ml of phosphate buffer (0.1 M, pH 7.0), shaken for 30 min, then allowed to stand overnight at 4 °C. The homogenates were centrifuged (6660 Series, Centurion, Scientific Ltd, UK.) at 5,000 rpm for 10 min at 4 °C. The supernatant of the crude extracts as carboxylesterase were collected. Crude carboxylesterase extracts were partially purified by dialysis as follows: 3.90 g of ammonium sulphate was added slowly to the 10 ml of crude extracts to achieve 60% saturation. The mixtures were incubated for 2 hours at 4 °C and then centrifuged at 5,000 rpm for 15 min at 4 °C. The sediment containing enzyme was dissolved with phosphate buffer (0.1 M, pH 7.0) and dialysed in the same buffer, placed overnight at room temperature. The dialysed enzyme was stored at 4 °C prior to use.

Carboxylesterase activity assay

Carboxylesterase activity was measured by the modified method of Ali *et al*. (2013) Briefly, the mixture of 1.0 ml of crude or dialysed carboxylesterase extracts and 5.0 ml α-naphthyl acetate (0.3 mM) as a substrate were added into a test tube and incubated for 15 minutes at room temperature. The reaction was stopped by adding 1.0 ml of DBLS reagent (the mixture of 2 parts of 1% fast blue B salt and 5 parts of 5% sodium lauryl sulphate) and was incubated for 15 min. Finally, the absorbance of developed colour was monitored by spectrophotometer at 600 nm (Perkin Elmer Instruments, USA). In the control, phosphate buffer (0.1M, pH 7.0) was used for the reactions. Carboxylesterase activity was calculated from the standard curve of α-naphthol (3.0-27.0 μM). All reactions were performed in triplicate. One unit of carboxylesterase activity was defined as the amount of carboxylesterase required to produce 1 μmol of α-naphthol per min at 25 °C.

The protein content of legume seed extracts was measured by the modified method of Lowry *et al*. (1951) using the spectrophotometer at 700 nm with BSA as the standard.

Sensitivity test to standard organophosphate (OPs) and carbamate (CAs) pesticides by enzyme inhibition assay

The 1.0 ml of partially purified carboxylesterase extracts was added into 1.0 ml of the various concentrations of standard pesticide solution (0.001-3.0 μg/ml) including OPs group (24 types) and CAs group (7 types). The mixtures of samples were incubated for 15 min at room temperature and then 5 ml of α-naphthyl acetate (0.3 mM) was added. Again, the mixture was incubated for 15 min at room temperature. The procedure below was the same as the carboxylesterase activity assay described above. The non-pesticide tube was used as the control.

The inhibition percentage (*%I*) of carboxylesterase activity was determined by the equation:

$$\%I = \frac{(Abs_{control} - Abs_{sample}) \times 100}{Abs_{control}}$$

The degree of sensitivity to the pesticides was evaluated by using a linear slope obtained from a calibration graph of common logarithm of pesticide concentration vs. inhibition percentage *(%I)*.

Sensitivity test to pesticide residues (OPs and CAs) and limit of detection (LOD)

The sensitivity to pesticide residues was determined by enzyme inhibition assay according to the modified methods of Li *et al*. (2009) and Hou *et al*. (2012). Lettuce and apples, as the representative fruits and vegetables according to the Codex Alimentarius Commission (1993) were used as the test samples. The various concentrations of pesticides (0.001-3.0 μg/ml) were added to homogeneous samples and extracted by the modified QuEChERS method. Briefly, the pesticides were added to the mixture of 10 g of samples and 10 ml of 1% acetic acid in acetonitrile and transferred into a 50 ml centrifuge tube. After shaking for 1 min, the homogeneous sample was mixed with 4 g of magnesium sulphate and 1 g of sodium acetate, shaken for 1 min,

and was centrifuged for 5 min at 3,500 rpm. Then, the 5 ml aliquots of the acetonitrile layer were transferred into a 15 ml centrifuge tube. The d-SPE (mixture of 150 mg of magnesium sulphate, 50 mg of primary, secondary amine and 2.50 mg of graphitized carbon) was added. After centrifugation for 15 min at 3,500 rpm, a total of 2 ml of the aliquot was transferred into vials, then the sample was evaporated by drying in a stream of nitrogen. Finally, the 2 ml of extract sample was adjusted by phosphate buffer (0.1 M, pH 7.0). For enzyme inhibition assay, the procedure was the same as described above. Sensitivity test to individual standard (OPs and CAs) pesticides in lettuce and apple, the concentration of 0.02 μg/ml of each standard pesticide and 0.02 μg/ml of partially purified carboxylesterase extract of black gram bean was used.

Determination of pesticide residues in vegetables by commercial pesticide test kit method

The determination of pesticide residues in vegetables using commercial pesticide test kits was investigated. A portion of 5 g of homogenized fresh vegetable samples from the local market was weighed into the sample bottles, and 5.0 ml of solvent extract was added, then allowed to stand for 5 min. The supernatant sample (1.0 ml) was transferred to an aluminum cup that included the reaction paper. The reaction occurred at 48±2 °C in a water bath until the residue sample was completely absorbed. The reaction paper was moved into another test tube and 0.5 ml of partially purified carboxylesterase extracts from black gram bean was added in place of the enzyme from commercial pesticide test kit. After mixing and standing for 15 min, 1 ml of α-naphthyl acetate (0.3 mM) was added, mixed, and allowed to stand for 10 min. Then, 0.5 ml of DBLS reagent were added and mixed well. The determination of pesticide residues was evaluated by comparing the colour of samples with a standard indicator that indicated three levels of pesticide contamination: no pesticide detection or pesticide detection at the safety level, pesticide detection at the not-safety level, and pesticide detection at the toxic level. All of steps were performed in water bath at 32-36 °C.

The sensitivity to pesticide residues was investigated by a carboxylesterase enzyme inhibition assay. The 10 species of fresh vegetables were extracted by the modified QuEChERS method and followed by an enzyme inhibition assay. For the enzyme inhibition assay, the procedure was the same as described above.

Validation of the method

Ten species (50 samples) of fresh vegetables from the local market were used to validate the carboxylesterase enzyme inhibition method. The accuracy of the method was confirmed by GC with a flame photometric detector (GC-FPD) for OP pesticide residues and by HPLC with a fluorescence detector (HPLC-FLD) for CA pesticide residues. For the determination of OPs pesticide residue, 10 g of fresh vegetables were extracted by the modified QuEChERS method described above. In the final steps, the extracted samples were adjusted by the mixture of n-hexane and ethyl acetate (3:1). Then, OPs pesticide residue was determined by capillary gas chromatography with a flame photometric detector (GC-FPD). The condition of GC was performed using Agilent Technology 7895 with DB-1701 capillary column (30 m, 0.25 mm, i.d. 0.25 μm film thickness, Agilent Technologies Inc., Santa Clara, USA). The oven temperature program had an initial temperature of 80 °C, held for 1 min, then rising by 15 °C/min to 180 °C and by 3 °C/min to 205 °C, held for 7 min and then rising by 40 °C to final temperature of 260 °C, held for 18 min. Nitrogen gas was used for making up at a flow rate 60 ml/min. FPD detector temperature was 220 °C.

CAs pesticide residue was determined by HPLC-FLD (Water-Alliance system e2695, USA). The solid phase extraction (SPE) method was used for sample preparation following published procedures (Payanan *et al*., 2015). Briefly, 20 g of homogeneous vegetable samples and 100 ml of methanol:phosphate buffer [1:1] were added into a beaker, and the mixture was homogenized with an Ultra-Turrax homogenizer for 2 min. After filtration, the filtrate was evaporated by rotary evaporator. The 100 ml of sample was extracted with SPE (EXtrelut™ NT packed column), and then 20 ml of eluent (hexane: dichloromethane; 1:1) was passed through the column. After concentration with a rotary evaporator, the extract was made up to 2 ml of methanol before determination. The extracted sample was filtered by syringe filter PTFE (13 ml 0.20 μm). Injection volume was

20 µL. HPLC column was Zorbax SB-C8 (25 cm, 4.6 cm) with the oven temperature at 30 °C. The fluorescence detector (Ex 345 nm, Em 455 nm.) was used. The gradient elution was used at flow rates of 1.0 ml/min with gradient elution of acetonitrile and water. The post-column used reagent flow 0.3 ml/min, reactor temperature was 100 °C

Performance assessment of the method

The 10 species (50 sample) of vegetables were selected to determine pesticide residues (OPs and CAs) by difference methods. Performance assessment of the method by commercial test kit (AChE), carboxylesterase from black gram bean extract inhibition assay and chromatographic method was investigated according to the method of the National Association of Testing Authorities (NATA: 2018; General Accreditation Guidance-Validation and verification of quantitative and qualitative test methods) that shown in Table 1. The acceptance criteria of performance parameters including sensitivity, specificity and accuracy are about 80% -100% (Jamras, 2017; National Association of Testing Authorities Australia (NATA), 2018; Tongbor, Puidacha and Lerprasert, 2018).

Table 1. Performance testing method

Performance testing method	Calculation
% Sensitivity	(TP/TP+FN) x 100
% Specificity	(TN/FP+TN) x 100
% Accuracy	(TP+TN/ Total sample) x 100

TP; True Positive, FP; False Positive, FN; False Negative, TN, True Negative

Results and Discussion

Carboxylesterase extraction and partially purification and Carboxylesterase activity

Carboxylesterases were extracted and purified from various sources, including plants, animals, and microorganisms. They have been purified from various plant sources by employing different purification processes, including ammonium sulphate fractionation, ion exchange chromatography, and gel filtration chromatography (Subramani *et al*., 2012). Purification of the enzyme generally improved the activity of the enzyme. In this study, the ammonium sulphate salting-out procedure was used for the partially purification of carboxylesterase enzymes from the selected legume seeds since is a common, simple, and widely-used method. Carboxylesterase extracted from legume seeds can catalytically hydrolyse specific substrate α-naphthyl acetate into α-naphthol, which can react with fast blue B salt to form a purple-colored diazonium dye (Subramani *et al*., 2012; Bhavith *et al*., 2014; Shivashankar and Premkumari, 2014). The reaction was shown in Figure 1. The results of legume seed extraction and partially purified carboxylesterase enzymes are summarized in Tables 2-3. Partially purified carboxylesterase enzyme preparations had specific activity ranging from 1.29 to 3.97 U/mg with about 2-fold of crude extracts, which is similar to previous reports by Subramani *et al*. (2012) and Chandre *et al*. (2014) and the highest specific activity was found in black gram bean purified extracts (3.97±0.26 U/mg), whereas the lowest value was found in red bean purified extracts (1.29±0.08 U/mg). However, specific activity was also affected by temperature and pH on hydrolysis of -naphthyl acetate (Subramani *et al*., 2012; Chandre *et al*., 2014).

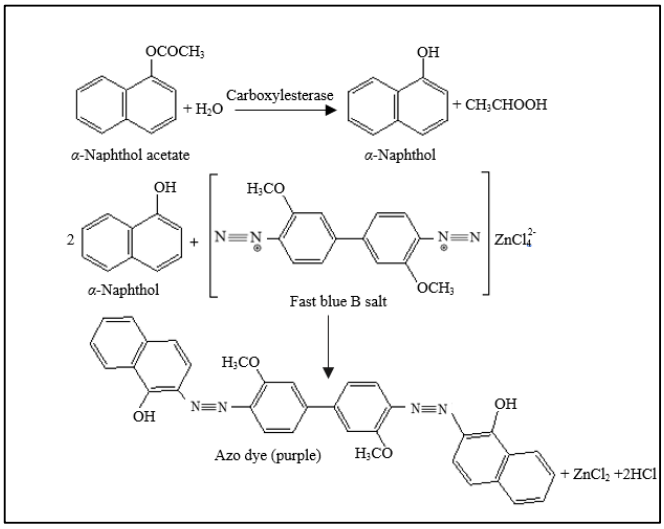

Figure 1. The reaction of carboxylesterase enzyme with α-naphthyl acetate

Table 2. Carboxylesterase activity of crude extracts from selected legume seeds

Legume seeds	Unit enzyme (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Black gram bean	1306.2±117.1[b]	861.9±56.8[a]	1.53±0.22[b]
Mung bean	666.9±32.0[a]	851.7±101.4[a]	0.79±0.09[a]
Red bean	607.4±41.4[a]	834.7±18.5[a]	0.73±0.05[a]

Means ± standard error with different letters (a, b) within the same column are significant differences at the $p < 0.05$ level according to Duncan test.

Table 3. Carboxylesterase activity of partially purified extracts from selected legume seeds

Legume seeds	Unit enzyme (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Black gram bean	1960.1±109.0[b]	493.6±12.6[a]	3.97±0.26[c]
Mung bean	777.6±67.8[a]	512.9±51.3[a]	1.51±0.10[b]
Red bean	826.4±44.5[a]	639.9±31.0[b]	1.29±0.08[a]

Means ± standard error with different letters (a, b, c) within the same column are significant differences at the $p < 0.05$ level according to Duncan test.

Sensitivity to standard OPs and CAs pesticides

Carboxylesterases (EC 3.1.1.1) extracted from plants could react with OPs and CAs and play an important role in the rapid determination of OP and CA residues (Gershater and Edwards, 2007; Li *et al*., 2009; Hou *et al*., 2012; Kantharaju and Murthy, 2014) because of their low cost, easy extraction, convenient preservation, sensitivity, and accuracy (Li *et al*., 2009). In this study, the sensitivity to various concentrations of standard pesticides, both OPs and CAs (0.001-3.0 μg/ml), was investigated by carboxylesterase enzyme inhibition assay. The inhibition percentage of enzyme activity in selected legume seeds at various concentrations of standard pesticides was calculated using Equation 1. The relationships between the inhibition percentage and the concentrations of OPs and CAs are shown in Figure 2 (A-B). The inhibitory percentage of enzyme activity was enhanced by the increase in pesticide concentration. The results indicated that partially purified carboxylesterase extracted from black gram beans was more sensitive than those from mung beans and red beans based on the percentage of inhibition. Furthermore, black gram bean carboxylesterase has shown very high sensitivity to both OPs and CAs standard pesticides. Therefore, the

partially purified carboxylesterase extracted from black gram beans was used for the study of the sensitivity to pesticide residues (OPs and CAs) and LOD.

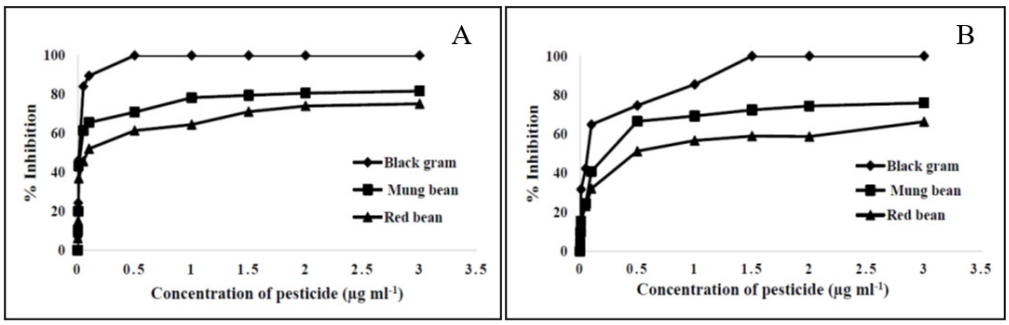

Figure 2. Inhibition percentage of carboxylesterase enzyme (*%I*) from legume seeds extracts for OPs (A) and CAs pesticides (B)

Sensitivity test to pesticide residues (OPs and CAs) and limit of detection (LOD)

Since QuEChERS (Quick, Easy Cheap, Effective. Rugged and Safe method) is currently of great significance to ensuring accurate, reliable results and widely used for pesticide determination (Regina *et al*., 2011; Xuejin *et al*., 2020; Fatemeh *et al*., 2022; Divya *et al*., 2023; Xiaoli and Zimian, 2023), modified QuEChERS extraction method for preparing the sample was used before sensitivity testing by enzyme inhibition method. In this study, the concentration of OPs and CAs standard pesticides that produced 10% inhibition of enzyme activity (*IC*10) from the linear equations was taken as the LOD. (Jeanty and Marty, 1998; Wang *et al*., 2012; Amine *et al*., 2016). The LOD values for OPs and CAs pesticides were 0.002 and 0.01 mg/kg, respectively. Moreover, the LOD values of this method did not exceed the default limit as described in Notification of the Ministry of Public Health, Thailand No. 387 (AD 2017) Re: Food Containing Pesticide Residues (Pesticide Residues in Food). It shows the LOD value for OPs was less than the default limit, while the LOD value for CAs was the same as the default limit (Table 4).

The sensitivity of the black gram bean carboxylesterase enzyme to single standard pesticides (24 types of OPs and 7 types of CAs) in matrix samples (lettuce and apple) and phosphate buffer (pH 7.0) is shown in Figure 3. Since the oxon bond (P=O or P=S bond) of OPs (No. 1-24) displayed stronger toxicity than the thio bond (N=R) of CAs (No. 25-31), and their bond is providing free radicals or electrons to the specific nucleophile of the active site of the carboxylesterase enzyme (Hou *et al*., 2012), the carboxylesterase enzyme is more sensitive to organophosphate pesticides than carbamates pesticides. The inhibition reaction of the carboxylesterase enzyme in both lettuce and apple samples was similar in phosphate buffer. The average percentages of enzyme inhibition (*%I*) for OPs and CAs are 28 and 13, respectively. Furthermore, the carboxylesterase enzyme from black gram beans showed the highest sensitivity to Dichlorvos, with 44% inhibition of enzyme activity. Similar results for carboxylesterase enzyme extracted from plant seeds have been reported by Hou *et al.* (2012) and Kantharaju and Murthy (2014).

At present, the animal-origin acetylcholinesterase (AChE) is the main enzyme used in the enzyme inhibition method for detecting organophosphate and carbamate pesticides (Forget and Leboulenger, 2002; Frasco *et al*., 2006; Sehrish *et al*., 2022) and is a commercial product for sale as a pesticide test kit. There have been some reports on the development of class-specific determination of organophosphate and carbamate pesticides based on inhibition of phytoesterase (Fahmy *et al*., 2008; Li *et al*., 2009; Chandrashekharaiah *et al*., 2011; Subramani *et al*., 2012) which were extracted from wheat and plant seeds. From the results above, the determination of pesticide residues in local vegetables by the commercial pesticide test kit method using carboxylesterase enzyme extracted from black gram bean was investigated in this study.

Table 4. LOD values of carboxylesterase inhibition assay for OPs and CAs pesticides and the default limit

Pesticide	Linear equations	R^2	LOD from IC_{10} (mg/kg)	Default Limit (mg/kg)
OPs	y = 4309.5x-0.503	0.99	0.002	0.01
CAs	y = 1084.1x-1.189	0.99	0.01	0.01

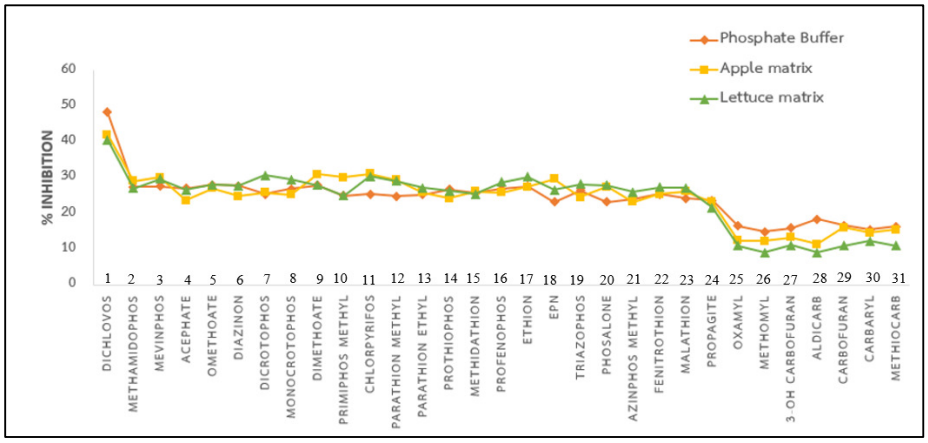

Figure 3. Inhibition of partially purified carboxylesterase enzyme (*%I*) from black gram bean on standard pesticides (OPs and CAs)

Determination of pesticide residues in vegetables by commercial pesticide test kit method

The 10 species of fresh vegetables were selected to determine the pesticide residues by the commercial pesticide test kit method, using carboxylesterase enzyme extracted from black gram beans in place of the enzyme (AChE) from the commercial pesticide test kit. The reaction shown developed a variety of colours, including a dark blue colour that indicated no pesticide detection or pesticide detection at the safety level, a light blue colour for pesticide detection at the safety level, and a light blue colour for detection at the toxic level (Figure 4). For the determination of pesticide residues, a commercial pesticide test kit was used to compare with black gram bean carboxylesterase. It was found that the quantitative and qualitative results of the two methods were the same (Table 5). This result indicated that the carboxylesterase enzyme extracted from black gram bean was sufficiently accurate to evaluate pesticide residues in vegetables the same as AChE from commercial test kit. The sensitivity of carboxylesterase enzyme extracted from black gram beans to pesticide residues by enzyme inhibition assay was compared with AChE from a commercial test kit. The percentage of enzyme inhibition (*%I*) is shown in Table 6. The carboxylesterase enzyme extracted from black gram beans was more sensitive to pesticide residues at toxic levels that showed more than 80% inhibition.

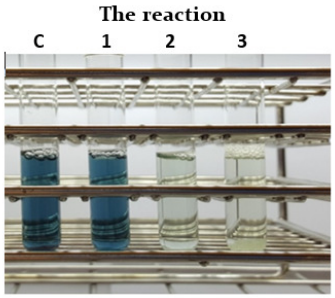

Figure 4. The reaction involved in pesticide residues detection in vegetables by enzyme inhibition assay using the commercial pesticide test kit method

Table 5. Results of pesticide residue determination in vegetables by commercial pesticide test kit and carboxylesterase from black gram bean extract inhibition method

Fresh vegetable	Commercial pesticide test kit	Black gram bean enzyme extract
Chinese cabbage	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Lettuce	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Cabbage	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples))
Cucumber	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Coriander	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Chili	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Sweet basil	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Yard long bean	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)
Cilantro	1) Not detected / Detected in safety level (2 samples) 2) Detected in not safety level (3 samples)	1) Not detected / Detected in safety level (2 samples) 2) Detected in not safety level (3 samples)
Pennywort	Not detected / Detected in safety level (5 samples)	Not detected / Detected in safety level (5 samples)

Table 6. Results of enzyme inhibition (*%I*) from reaction involved in pesticide residues detection in vegetables

Tube no.	Evaluation	% Inhibition	
		Commercial pesticide test kit	Black gram bean enzyme extract
1	Not detected /Detected in safety level	<50%	<40%
2	Detected in not safety level	50%-70%	40% - 80%
3	Toxic level	>70%	>80%

Validation of the method

The results of pesticide residues detection by the carboxylesterase enzyme inhibition method and the chromatographic methods in selected fresh vegetable samples are shown in Table 7. The 13 samples contaminated with OPs and the 9 samples contaminated with CAs were detected by the traditional

chromatographic methods of GC-FPD and HPLC-FLD, respectively, while 12 samples were detected by the carboxylesterase enzyme inhibition method. However, the quantitative results of the two methods were the same. This result indicated that the carboxylesterase enzyme inhibition method was sufficiently accurate to evaluate pesticide residues in vegetables. Thus, the method can be used as a convenient test kit to assure the safety of vegetables that are being marketed.

Table 7. Results of validation of method for pesticide residues determination in vegetables

Fresh vegetable	Chromatography method (mg/kg)		Carboxylesterase inhibition assay
	OPs	CAs	OPs and CAs
Chinese cabbage	Not detected (5 samples)	Not detected (5 samples)	Not detected (5 samples)
Lettuce	1) Not detected (3 samples) 2) Chlorpyrifos <0.05 (2 samples)	1) Not detected (3 samples) 2) Carbofuran 0.01-0.02 (2 samples)	1) Not detected (3 samples) 2) Detected (2 samples)
Cabbage	Not detected (5 samples)	Not detected (5 samples)	Not detected (5 samples)
Cucumber	Not detected (5 samples)	1) Not detected (4 samples) 2) Carbofuran <0.01 (1 samples)	Not detected (5 samples)
Coriander	1) Not detected (3 samples) 2) Chlorpyrifos <0.05-1.73 (2 samples)	Not detected (5 samples)	1) Not detected (4 samples) 2) Detected (1 samples)
Chili	1) Not detected (4 samples) 2) Chlorpyrifos 0.13 (1 samples)	1) Not detected (4 samples) 2) Carbofuran 0.05 (1 samples)	1) Not detected (4 samples) 2) Detected (1 samples)
Sweet basil	1) Not detected (2 samples) 2) Chlorpyrifos ≤0.05 (2 samples)	1) Not detected (2 samples) 2) Carbofuran 1.8, aldicarb 0.02 (1 samples)	1) Not detected (4 samples) 2) Detected (1 samples)
		3) Carbaryl 0.04 (1 samples) 4)Carbofuran 0.08 (1 samples)	
Yard long bean	Not detected (5 samples)	1) Not detected (3 samples) 2) Methomyl 0.04-0.21 (2 samples)	1) Not detected (3 samples) 2) Detected (2 samples)
Cilantro	Chlorpyrifos 0.05-1.76 (5 samples)	Not detected (5 samples)	Detected (5 samples)
Pennywort	Not detected (5 samples)	Not detected (5 samples)	Not detected (5 samples)

LOD of chromatographic method; GC-FPD = 0.02 mg/kg, HPLC-FLD=0.003 mg/kg: LOD of carboxylesterase inhibition assay = IC10

Performance assessment

The percentages of performance parameters, including sensitivity, specificity, and accuracy, are shown in Table 8.

Table 8. The performance parameters of the methods

Enzyme inhibition assay	Performance parameters		
	% Sensitivity	% Accuracy	%Specificity
Commercial pesticide test-kit	100	100	100
Chromatographic method	70.5	90	100

Chromatographic method; GC-FPD, HPLC-FLD

All of the performance parameters of the enzyme inhibition method are 100% compared with commercial pesticides test kits. This result indicated that the partially purified carboxylesterase from black gram bean was sufficiently accurate to evaluate the pesticide residues (OPs and CAs) in vegetables by enzyme inhibition assay, the same as using a commercial pesticides test kit. However, performance parameters, including sensitivity, accuracy, and specificity, by the enzyme inhibition method are 70.5%, 90%, and 100%, respectively, compared with the chromatographic method. The percentage of sensitivity (70.5) was below the criteria acceptance value (80-100%) due to the fact that the LOD of the chromatographic method was lower (0.003 mg/kg) than the LOD for CAs (IC10) of the enzyme inhibition method (0.01 mg/kg).

Conclusions

In this study, the specific activity of partially purified enzymes by the ammonium sulphate salting-out procedure of selected legume seeds including black gram bean, mung bean and red bean was investigated. The sensitivity of carboxylesterase extracted from black gram bean to pesticides (OPs and CAs) by enzyme inhibition method and the LODs were determined. The results showed that partially purified carboxylesterase enzyme extracted from black gram bean indicated the highest sensitivity to pesticides with LODs that do not exceed the default limit of Notification of the Ministry of Public Health, Thailand No. 387 (AD 2017) Re: Food Containing Pesticide Residues (Pesticide Residues in Food). The results of the validation study indicated that the partially purified carboxylesterase from black gram bean by inhibition method could be used to evaluate pesticides residues in vegetables based on commercial pesticide test kit to ensure the safety of vegetables for human health. But it was not sufficiently accurate for pesticides detecting in term of sensitivity performance comparing the traditional chromatographic method. Future studies should focus on the method of enzyme purification to improve better sensitivity for pesticide residues detection in foods.

Authors' Contributions

The study was conceptualized and designed by CN and SC. CN: designed research study and/or evaluation. CN, MC and SC drafted the manuscript and reviewed it before submitting it to the journal.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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