Kinetic studies of immobilized wheat-based alpha-naphthyl acetate esterase in the detection of organophosphate pesticide

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Abstract

The presence of organophosphate pesticides could inhibit the activity of alpha-naphthyl acetate esterase (ANAE), which could use as the fundamental for biosensor applications. *k*-carrageenan was used for immobilization of ANAE extracted from wheat-based sources. The activity of free and immobilized ANAE was assessed for optimum activity at a range of temperature and pH. Enzyme kinetic studies were performed using Michaelis-Menten kinetic parameters from Lineweaver-Burk, Langmuir, and Eddie-Hofstee linear plots. The optimization studies showed that the relative activity of the enzyme remained high, even above the temperature of 50 °C. The Lineweaver-Burk plot has the best fit with a linear regression coefficient of 94.6%. The kinetic reaction of ANAE showed that the use of the enzyme is promising as the basis of biosensors using enzyme inhibition in the detection of dichlorvos (OP pesticide). From the inhibition kinetics, the value of k_d obtained was very low, which indicates the high sensitivity of ANAE towards OP pesticides.
Keywords: Alpha-naphthyl acetate esterase, Biosensor, Carrageenan, Enzyme immobilization, Wheat flour

1 Introduction

Plants are important to the world's resources for food, fragrances, materials, dyes, fuels, and medicines. A pest can be defined as anything that is considered a threat or disease to crops and plants caused by harmful microorganisms. Organophosphate (OP) pesticides are a standout solution amongst the most widely recognized chemical classes utilized for the protection of crops from pests and pathogens given their high viability and wide range of effectiveness worldwide. OP pesticides consisted of toxic and hazardous chemicals that are harmful to humans [1], especially through food consumption. Therefore, the development of rapid methods to detect pesticides for food safety is significant.

Chromatography is the conventional method to detect OP. However, chromatography is time-consuming, costly, laborious with the requirement of highly skilled operators, and utilizing considerable volumes of solvents [2]. Therefore, the need for a sensitive OP-detecting biosensor based on enzymatic inhibitory effects on OP compounds is significant. Acetylcholine esterase (AChE)

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enzyme has been studied extensively for detecting OP pesticides in biosensor applications [3-6]. However, the extraction and purification of AChE are not feasible and complicated due to its animal origin. Alpha-naphthyl acetate esterase (ANAE) is a promising alternative for pesticide-detection biosensors as it could be extracted from plants. ANAE was reported to be inhibited by OP pesticides, including dichlorvos, dimethoate, malathion, methamidophos, and phoxim [7]. Therefore, ANAE has the potential to be used for the application of biosensors using enzymatic reactions in pesticide detection.

ANAE is a plant esterase enzyme, where wheat is one of the sources for enzyme extraction [2, 8]. The non-immobilized enzyme possesses limited enzyme reusability, complicated recovery and purification, and restricted enzymatic activity. In the development of biosensors, enzyme immobilization on the transducer surface is crucial to enhance sensitivity and selectivity of biosensor, storage stability, and reproducibility with rapid response time [9, 10]. There are several types of enzyme immobilization, which include covalent binding, entrapment, ionic interaction, and physical adsorption [11, 12]. Enzyme immobilization by carrageenan allows enzyme entrapment in the material matrix with excellent affinity to proteins [13]. The use of carrageenan is advantageous due to its excellent biodegradability and biocompatibility [13]. Carrageenan, a biopolymer, is a hydrophilic linear sulfated polysaccharide found in various species of red seaweeds consisting of repeated units of 3,6-anhydro-α-D-galactose and β-D-galactose sulfate [14]. The excellent affinity of carrageenan to proteins assists the immobilized proteins to sustain its high catalytic activities [15]. Carrageenan beads have been proven to be excellent support for enzyme immobilization [2, 15]. The presence of hydroxyl as one of the reactive functional groups facilitates any modification on the molecular surface of carrageenan that will allow direct reaction between the matrix of carrageenan and the enzyme [10]. The application of carrageenan immobilization has the potential to overcome the limitations of a free enzyme, where this strategy could be effective in developing an OP detecting biosensors.

This study includes the extraction of ANAE from wheat sources and immobilization of the enzyme on *k*-carrageenan beads that was amino-functionalized. The goals of this study are to assess the extraction and purification of ANAE from wheat-based sources, the effectiveness and reusability of enzyme immobilization on carrageenan, and the kinetics of inhibition properties of immobilized ANAE on carrageenan via Michaelis-Menten. The kinetic studies were firstly conducted in the absence of the inhibitor using Michaelis-Menten kinetics to calculate the kinetic parameters Michaelis constant (K_m) and the maximum rate of reaction (v_{max}) from the best fitting plot using Lineweaver-Burk, Langmuir and Eddie-Hofstee linear plots. The second part of the kinetics involved the use of dichlorvos, an OP pesticide, to study inhibition kinetics. The application of dichlorvos would inhibit the enzymatic activity of ANAE.

2 Materials and methods

2.1 ANAE extraction from wheat-based sources

Two wheat sources which were wholemeal wheat (Atta) flour and wheat grain (ground), were used for alpha-naphthyl acetate esterase (ANAE) extraction based on the study by Yang et al. [13]. 0.45 µm cellulose acetate membrane and 0.22 µm polyethersulfone (PES) non-protein binding cellulose membranes were used for vacuum-filtered crude ANAE. The filtered ANAE was stored (4 °C**)** for further use within 10 days.

Enzyme purification was conducted using an aqueous two-phase system (ATPS) as described previously [13]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to assess the molecular weight of enzyme using the Laemmli method [16].

2.2 Enzyme immobilization on functionalized *k*-carrageenan

The synthesis, cross-linking and amino-functionalization of the carrageenan beads was based on previous studies [2]. Olive oil (80 mL) (Table 1) was added into the carrageenan solution with 0.3 M potassium chloride (KCl) buffer (300 mL) [2]. The carrageenan gel beads formed were cured for 0.5 h followed by soaking in ANAE enzyme solution for 24 h.

Table 1. Physicochemical properties of olive oil [17].

Properties	Density (kg/m^3)	Moisture content (%)	Boiling point (°C)	Saponification value (mg KOH/g)	Peroxide value ($m_{eq}\,O_2/kg$)	Acid value (mg KOH/g)	Iodine value (g I_2/100 g oil)
Value	906	0.47	160	185.8	12.43	4.2	78.38

2.3 Characterization of ANAE by protein concentration assay and loading efficiency of ANAE

For protein concentration determination, a 100 µL ANAE enzyme was mixed with working reagents from Bicinchoninic acid assay kit (A:B=50:1) (2 mL). Enzyme concentration was determined based on the standard curve of bovine serum albumin (BSA). The mixture was incubated in a water bath at the temperature of 37 °C for 30 min. The absorbance was assessed via UV-vis spectrophotometer (wavelength of 562 nm). Loading efficiency (Equation (1)) was calculated to determine immobilized ANAE loading efficiency on carrageenan.

$$\%\ Loading\ efficiency\ = \frac{(Free\ enzyme\ concentration)}{(Free\ enzyme\ concentration - residual\ enzyme\ concentrations)}\ \mathrm{x}100\% \quad (1)$$

2.4 Enzyme activity assay ANAE

The colorimetric method was used to conduct the activity assay of ANAE in soluble and immobilized states [7, 18, 19]. The enzyme activity was determined based on the concentration of hydrolysis product of substrate (1-naphthyl acetate (NA)), which is 1-naphthol using Equation (2) and the standard curve of 1-naphthol. The relative activity percentage was calculated using the formula shown in Equation (3) [20].

$$Activity\ unit\ = \frac{concentration\ x\ volume\ of\ the\ assay}{enzyme\ volume\ x\ incubation\ time} \quad (2)$$

$$\%\ Relative\ activity\ = \frac{Enyme\ activity}{Maximum\ enzyme\ activity}\ \mathrm{x}100\% \quad (3)$$

2.5 Temperature and pH optimization studies and reusability test of ANAE

The optimization study was conducted at 30-50 °C of temperature and pH 5.0-10.0. Enzyme activity assay and reusability test were conducted afterward. The reusability test was performed by repeating the enzyme activity assay once every day using the optimum pH and temperature of the enzyme obtained for 11 consecutive days.

2.6 Michaelis-Menten kinetics (in the absence of inhibitor) and inhibition kinetics (in the presence of inhibitor)

Michaelis-Menten kinetics was performed through the hydrolytic activity of different substrate concentrations of NA (12, 16, 20, 24, and 28 mM) in phosphate buffer solution (PBS) for a fixed volume of immobilized ANAE beads. Similar to the activity assay, a mixture of 50 µL of NA diluted in acetone, 1.95 mL of 0.04 M PBS (a total mixture of 2 mL), and 0.2 g of immobilized ANAE were mixed

for different incubation time slots (2, 5, 10, 15, 20 and 25 minutes) at 30 °C for every substrate concentration. For the determination of the enzyme activity, 500 µL of 1% fast blue B salt was added after incubation time. Within 10 minutes, the absorbance at 595 nm was recorded using UV-vis spectrophotometer. Finally, the concentration and activity were measured using Equation (*2*) and the 1-naphthol standard curve, respectively. The data were plotted in three plots which were Lineweaver-Burk, Langmuir, and Eadie-Hofstee plots for fitting the kinetics of immobilized ANAE based on Equation (4), Equation (5), and Equation (6) respectively.

$$\frac{1}{v} = \frac{K_m}{v_{max}} \frac{1}{[S]} + \frac{1}{v_{max}} \quad (4)$$

$$\frac{[S]}{v} = \frac{1}{v_{max}} [S] + \frac{K_m}{v_{max}} \quad (5)$$

$$\frac{v}{[S]} = -\frac{1}{K_m} v + \frac{v_{max}}{K_m} \quad (6)$$

where *v*, K_m, v_{max}, and *S* are respectively referring to the rate of reaction, the Michaelis constant, the maximum rate of reaction, and substrate concentration [21].

Inhibition kinetics was performed by assessing the enzyme activity for different dichlorvos concentrations (440, 880, 1330, 1770, and 2210 ng/mL) using the protocol described by Silva Filho et al. [19]. The inhibition mechanism of enzyme could be described by the following chemical equation (Equation (7)), where *E* is the enzyme concentration and *I* is the inhibitor (dichlorvos) concentration [19].

$$[E] + [I] \overset{k_d}{\leftrightarrow} [EI] \overset{k_p}{\rightarrow} [E - I] \quad (7)$$

The percent inhibition and enzyme inhibition kinetics were calculated based on Equation (8) and Equation (9) correspondingly, where E_0 is the initial enzyme activity incubated for 1 min, E_1 is the enzyme activity incubated in dichlorvos for 2–5 min (residual enzyme activity), k_p is the rate constant for phosphorylation and k_d is the dissociation constant [19]. Linearization of the kinetic plot was performed with the use of Equation (10), which was by taking the reciprocal of the slope ($1/k_{app}$) from inhibition kinetic plots.

$$\%\ inhibition\ = \frac{E_0 - E_1}{E_0} \text{x } 100 \quad (8)$$

$$ln\ \frac{E_1}{E_0} = -\frac{k_p t}{1 + k_d/[I]} \quad (9)$$

$$\frac{1}{k_{app}} = \frac{1}{k_p} + \frac{k_d}{k_p} \frac{1}{I} \quad (10)$$

3 Results and discussion

3.1 Characterization of immobilized ANAE on carrageenan

One activity unit (U) of ANAE refers to the amount needed to catalyze the production of 1-naphthol (1 µM) per min at 30 °C [22]. ANAE was extracted from two wheat sources in this study, followed by immobilization on *k*-carrageenan beads that were amino-functionalized. The filtered crude flour-derived ANAE weighted at 66.2 kDa based on the result of SDS-PAGE.

During the formation of carrageenan beads, olive oil was used with the potassium chloride buffer to avoid beads sticking with each other, leading to the formation of an oil-phase floating above the buffer due to the density difference. The use of peppermint oil and olive oil was reported to enhance the roughness, morphology, and buoyancy level of the gel beads [23]. Carrageenan beads were amino-functionalized prior to immobilization, where amine (NH_2) functional group was incorporated to improve the stability of enzymes in extreme chemical environment, and to prevent enzyme

leaching [24]. Carrageenan beads treated with polyethyleneimine (PEI) were reported to exhibit high stability and superior resistance to heat and abrasion [25]. Loading efficiency is the measurement of the bound and unbound enzymes to the carrageenan by quantifying their concentrations as shown in Table 2. The beads were incubated in the enzyme solution at a fixed curing time of 1 hour. Further increase in incubation time did not affect enzyme loading. ANAE extracted from wholemeal wheat flour showed better stability and immobilization performance in terms of protein concentrations and the loading efficiency percentage compared to ground wheat grains. The loading efficiency obtained from wholemeal flour-derived ANAE immobilized on *k*-carrageenan was comparable to that of carboxyl esterase immobilized on calcium carbonate [26]. The result from the study indicated 20% of carboxyl esterase enzyme was loaded in the pores of calcium carbonate during the process of adsorption [26].

Table 2. Loading efficiency analysis of esterase enzyme immobilization.

Enzyme	Enzyme source	Immobilization matrix	Immobilized enzyme concentration (µg/mL)	Loading efficiency (%)	Reference
ANAE	Wholemeal wheat flour	*k*-carrageenan	679.44	19.15	This study
ANAE	Wheat grains	*k*-carrageenan	90.92	2.87	This study
Carboxyl esterase	*Rhizopus oryzae* fungi	Calcium carbonate	133.33[a]	20	[26]

[a] Calculated based on 1.5 mL buffer solution

3.2 Optimization study of ANAE

The optimum temperature for immobilized alpha-naphthyl acetate esterase (ANAE) from both wheat sources was observed to be 35 °C, wherein for free-state ANAE, the optimum temperature for the grain-derived enzyme was 40 °C, and 45 °C for the flour-derived enzyme (Figure 1(a)). The results for free-state ANAE are in agreement with the optimum enzyme activity obtained from the free-state wheat-derived ANAE at 40 °C at pH 8 by Abdullah et al. [8]. The relative activity continued to be above 90% even up to 50 °C, demonstrating that the enzyme could work well even at elevated temperatures. Immobilized enzymes exhibited high stability in enzyme activity, even with the temperature change from 35 to 45 °C. The immobilization of enzyme provides rigidity to the structure of the protein molecule of enzyme even upon exposure to high temperature [27]. Another study on the optimum temperature of free-state wheat-derived ANAE showed a wide range of relative enzyme activities from 40-80% for temperature variation from 20 to 60 °C [8]. Regardless of the wheat source, immobilized ANAE catalyst shows greater stability for almost an entire range of temperature, especially at higher temperatures, in comparison to the free-state enzymes. This is in conformity with the trend reported by other studies [2, 28, 29]. The results are also comparable to the effect of temperature on the enzyme activity of carboxyl esterase immobilized on calcium carbonate which appeared to be more stable [26]. The activity of immobilized carboxyl esterase remained above 94% at the temperature of 55 °C [26].

Figure 1(b) shows that the activities of the free-state enzymes from both wheat sources were not greatly impacted by the change in pH from pH 5 to 10. The results demonstrated that the optimum pH, based on the maximum activity of the immobilized flour-derived enzyme, was pH 6. The free-state enzyme extracted from both wheat sources has the same optimum pH at pH 7. The pH profile of free-state wheat-derived ANAE reported by Abdullah et al. was broad at 20 to 80% of relative enzyme activity corresponding to variation from pH 5 to 10, with optimum enzyme activity at pH 8 [8].

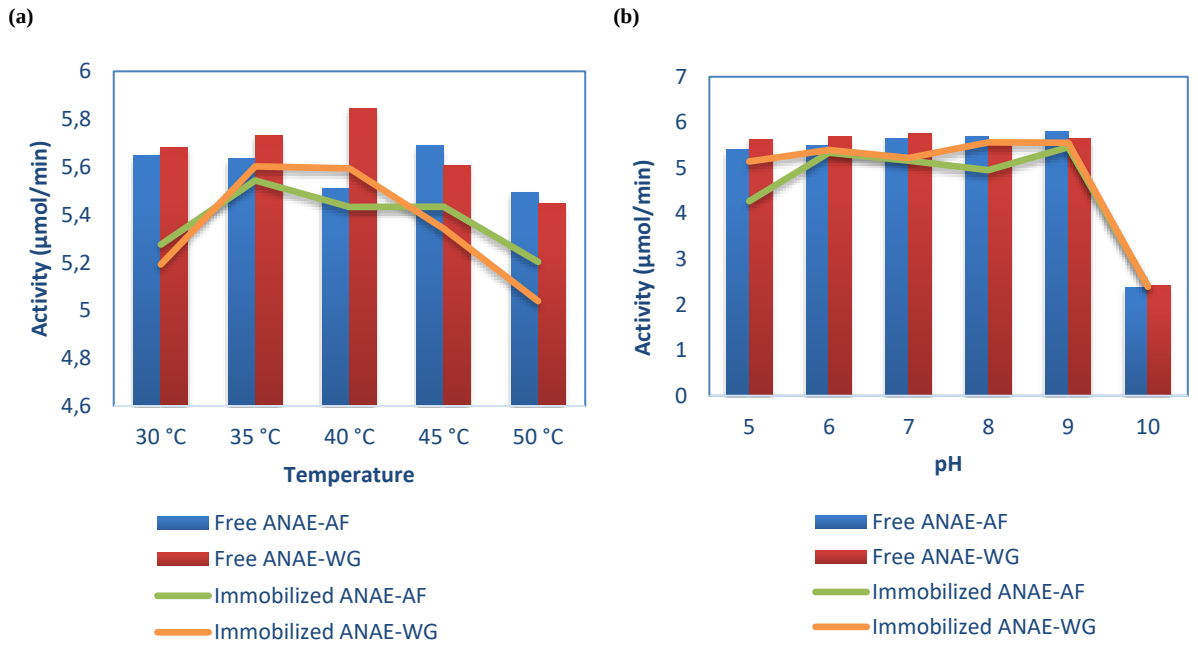

Figure 1. (a) The optimum temperature and (b) optimum pH for ANAE enzyme that was extracted from wholemeal wheat flour (AF) and wheat grains (WG).

The immobilization of ANAE enzyme showed excellent re-usability of the enzyme (Figure 2). The immobilized grain-derived ANAE retained higher activity throughout more than 10 re-usages, compared to ANAE extracted from commercial wheat flour. The immobilization of carboxyl esterase on calcium carbonate resulted in similar enzyme re-usability with stable catalytic activity even after 10 cycles [26].

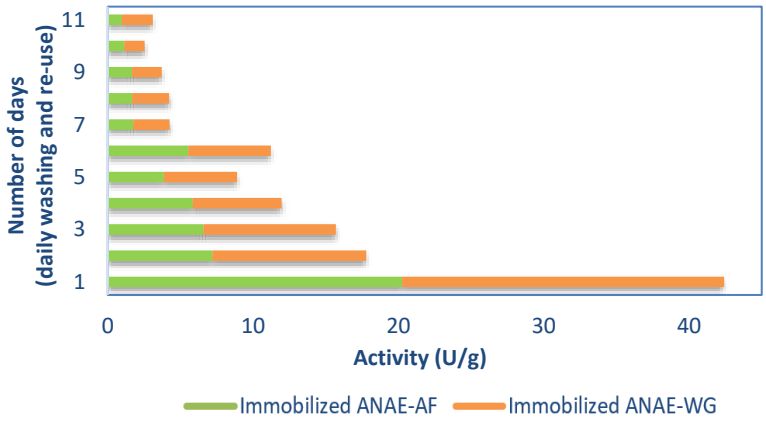

Figure 2. The reusability of immobilized ANAE enzyme extracted from wholemeal wheat flour (AF) and wheat grains (WG) through repeated washing and re-use per day.

3.3 Michaelis-Menten kinetics for ANAE for potential biosensor application

The response of a biosensor is depicted by the concentration of the hydrolysis product formed, upon the addition of a substrate, due to the reaction of enzyme on the surface of the sensor [30]. The fundamental principle of ANAE-based biosensor for the detection of pesticides could be accomplished through amperometric biosensors (Figure 3(a)), where the presence of enzyme inhibitor (i.e. OPs) inhibits substrate (NA) hydrolysis by ANAE into 1-naphthol that gives rise to

change in electric current that is generated at the electrode's surface of biosensor (Figure 3(b)) [31, 32]. The study of enzyme kinetics is important to evaluate the enzyme's maximum reaction velocity and its binding affinities to substrates and inhibitors (i.e. OP) [33]. From the Michaelis-Menten kinetics, v_{max} is the rate as the substrate concentration goes infinite and K_m is the value of substrate concentration at halfway to v_{max} ($v = v_{max}/2$) [21].

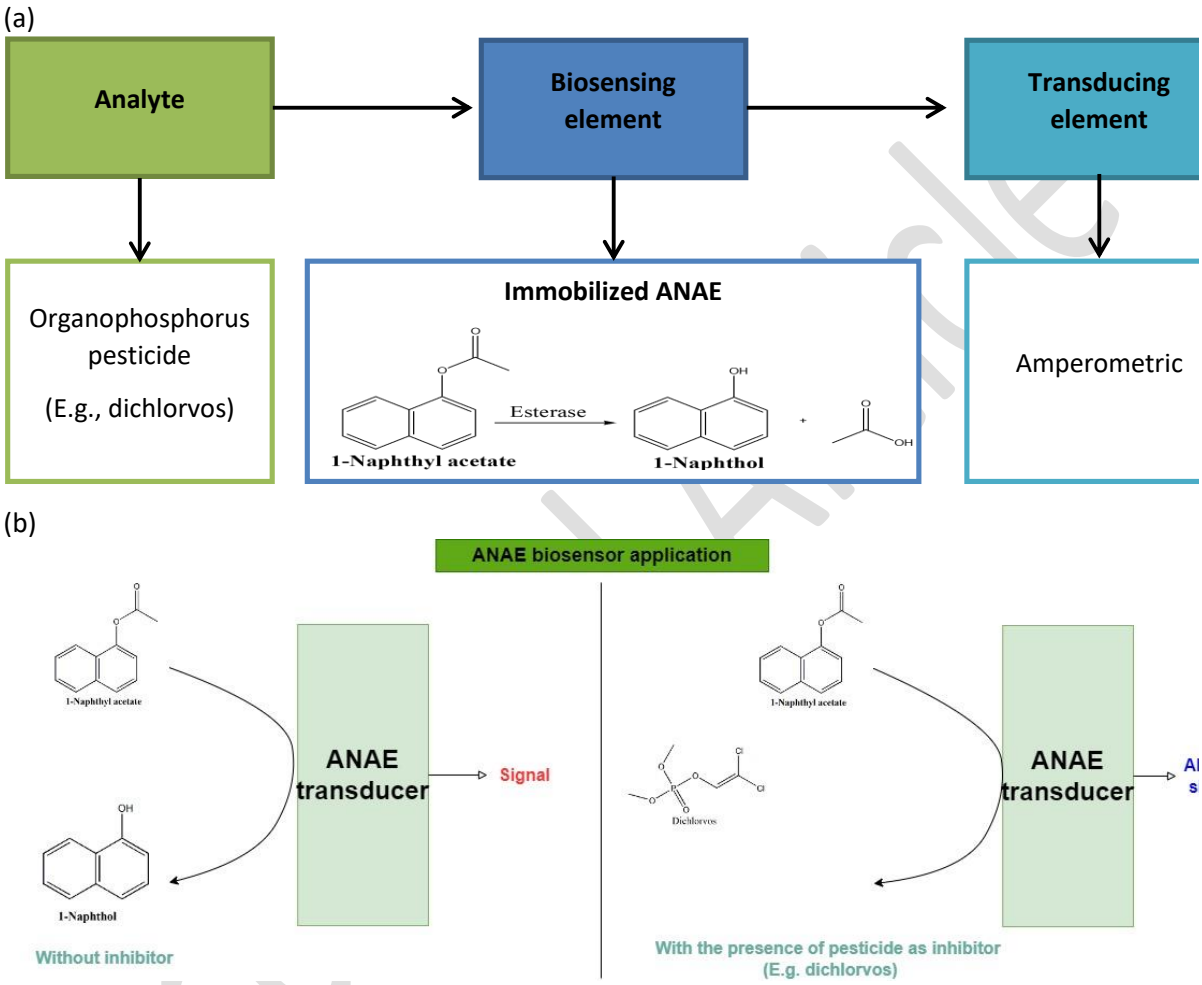

Figure 3. (a) The use of ANAE in enzyme inhibition-based biosensor in pesticide detection [4, 34]. (b) The mechanism of the detection of pesticide, where the presence of pesticide inhibits hydrolysis of NA by ANAE into 1-naphthol that gives rise to change in electric current that is generated at the surface of the biosensor's electrode.

In this study, the kinetic study for ANAE was performed at different substrate concentrations ranging from 12-28 mM. From the three kinetic plots based on the enzyme activity assay represented in Figure 4, it can be inferred that the Lineweaver-Burk plot fits the data with a high linear regression coefficient of 94.62%. On this basis, the kinetic constant values from the Lineweaver-Burk plot based on the slope and the intercepts are -37.56 mM and -0.897 U/g respectively for K_m and v_{max}. K_m indicates how rapid the rate of reaction increases with substrate concentration. K_m could also be used as an indicator of the enzyme's affinity for its substrate. A higher K_m corresponds to a lower affinity for the substrate and vice versa. Abdullah et al. performed kinetic studies on free-state ANAE

enzyme with the ratio of v_{max}/K_m obtained from the Langmuir plot was 8.60 ×10^{-3} min^{-1} [8]. However, in that study, the fitting of kinetic data was not tested with different kinetic models, only Langmuir plot was used for the determination of the kinetic constants [8]. The ratio of v_{max}/K_m obtained in this study from the best fit plot of Lineweaver-Burk was 23.9 ×10^{-3} min^{-1}, which showed that immobilized ANAE has superior enzymatic rate than free-state ANAE. The enzyme showed the highest activity when incubated for 10 min under standard conditions (temperature of 30 °C and pH 8).

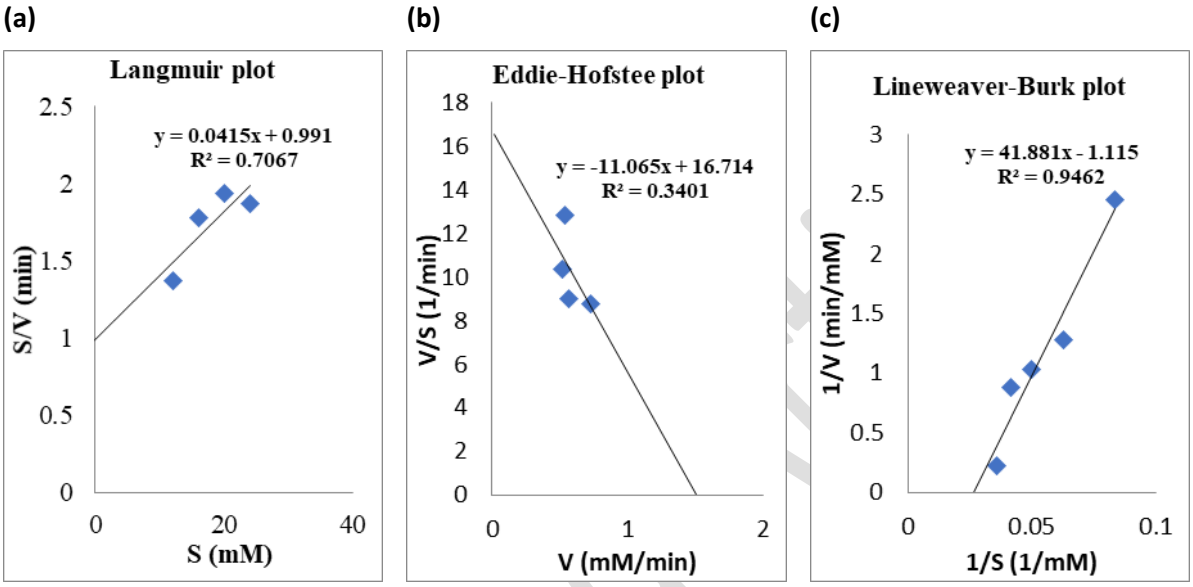

Figure 4. Michaelis-Menten kinetics plotted via (a) Langmuir, (b) Eddie-Hofstee, and (c) Lineweaver-Burk plots.

For the development of a biosensor based on enzyme inhibition, the investigation of the inhibition kinetics of free-state and immobilized ANAE is critical. The study on inhibition kinetics is crucial as the mechanism of detection of pesticides ANAE biosensor is centered on the activity of ANAE that is inhibited by OP pesticide. Dichlorvos (OP pesticide) was used to examine the inhibitory effects, in which the presence of dichlorvos causes the inhibition of the production of 1-naphthol catalyzed by ANAE from NA. k_d value is an important indicator for assessing the lowest detection limit [4]. The inhibition percentage was calculated and plotted at a constant concentration (16 mM) of the hydrolysis product (1-naphthol) (Figure 5(a)). The highest inhibition percentage was obtained at 2210x10^{-6} mg/mL of dichlorvos. It was expected to exhibit a higher inhibitory effect at higher concentrations of dichlorvos. The plots of inhibition kinetic are shown in Figure 5(b-c). The similar slope for each concentration of dichlorvos indicated that ANAE concentration selected was sensitive to detect the concentration of dichlorvos as low as 10 µM (Figure 5(c)), which is comparable to AChE biosensor in the detection of methyl-paraoxon from 0.1-3.3 µM [19]. The reciprocal of the slope ($1/k_{app}$) for each dichlorvos concentration trend from Figure 5(c) was plotted against the reciprocal of the dichlorvos concentrations for plotting the standard linear inhibition kinetic plot (Figure 5(b)). Figure 5(b) shows that the inhibition kinetic model fits the kinetic study well with the linear regression coefficient of 88.6%. From the slope of the plot, k_d was determined to be -0.36 nM. The low value of k_d shows the high sensitivity of ANAE towards dichlorvos (OP pesticide). This is because k_d and k_p that have high values could indicate rapid breakup of complex enzyme-inhibitor compounds [19].

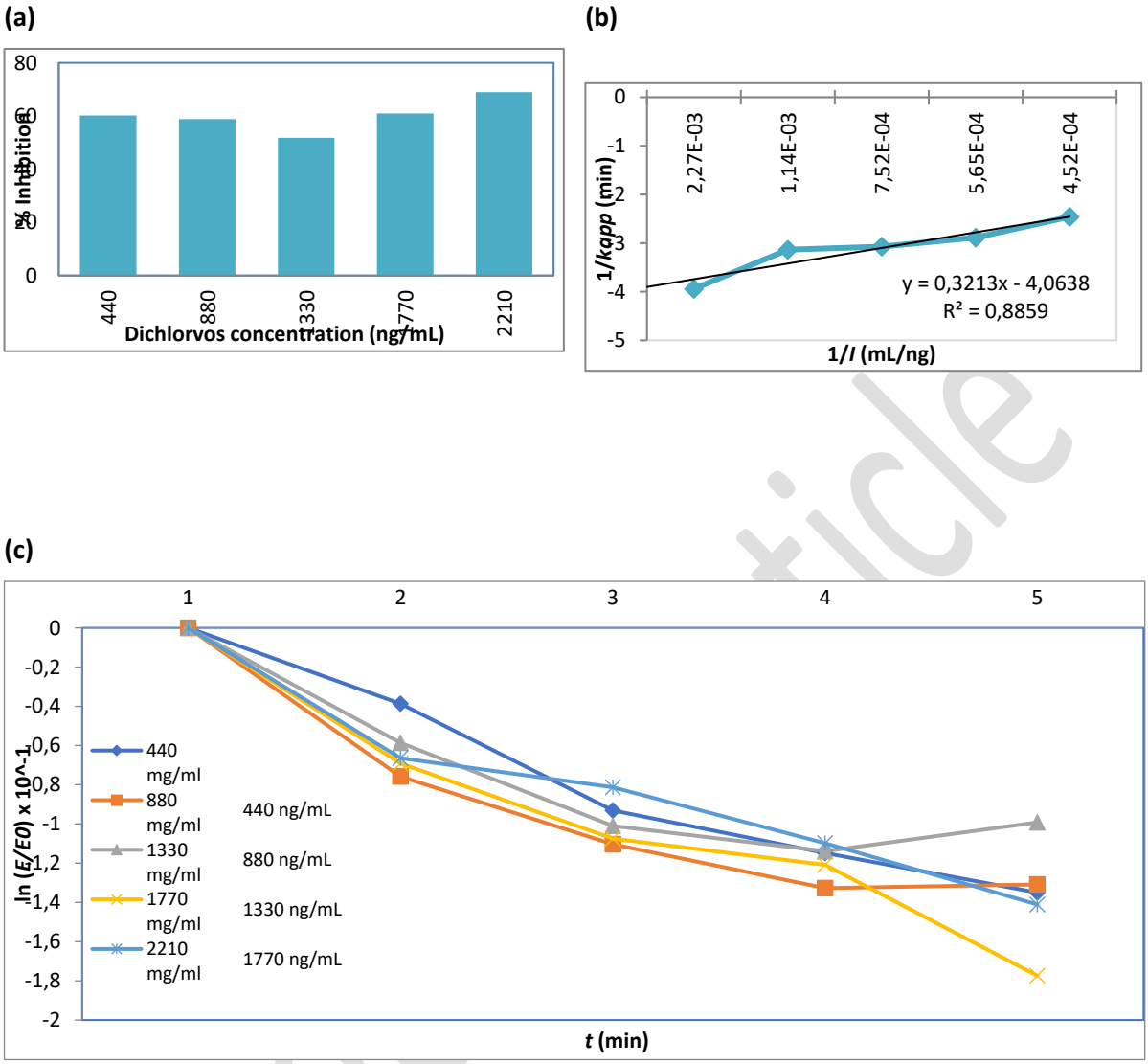

Figure 5. (a) Inhibition of ANAE activity by dichlorvos. (b) Linearization of enzyme inhibition kinetics plot. (c) Inhibition kinetics of the residual enzyme activity.

4 Conclusion

The extraction of alpha-naphthyl acetate esterase (ANAE) from two wheat sources (wholemeal wheat flour and wheat grain) was performed and further immobilized on *k*-carrageenan. The optimum temperature for the free-state enzyme from both sources of wheat was 40-45 ℃. The free-state enzyme, regardless of the sources, has the same optimum pH at pH 7. The kinetic study showed that the Lineweaver-Burk plot has the best fit with a linear regression coefficient of 94.62%. The kinetic reaction of ANAE in the presence or absence of dichlorvos showed that ANAE is promising as the basis biosensor in the detection of OP pesticide. From the inhibition kinetics, the value of k_d obtained was very low, which indicates high sensitivity of ANAE towards dichlorvos (OP pesticide). The enzyme immobilization using carrageenan may enhance the reusability of ANAE as it could be recycled for more than 10 times with effective enzyme activity.

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Abbreviations

AChE	Acetylcholine esterase
ANAE	Alpha-naphthyl acetate esterase
E_0	Initial enzyme activity
E_1	Enzyme activity
I	Inhibitor concentration
K_d	Dissociation constant
k_p	Rate constant for phosphorylation
K_m	Michaelis constant
OP	Organophosphate
S	Substrate concentration
v	Rate of reaction
v_{max}	Maximum rate of reaction

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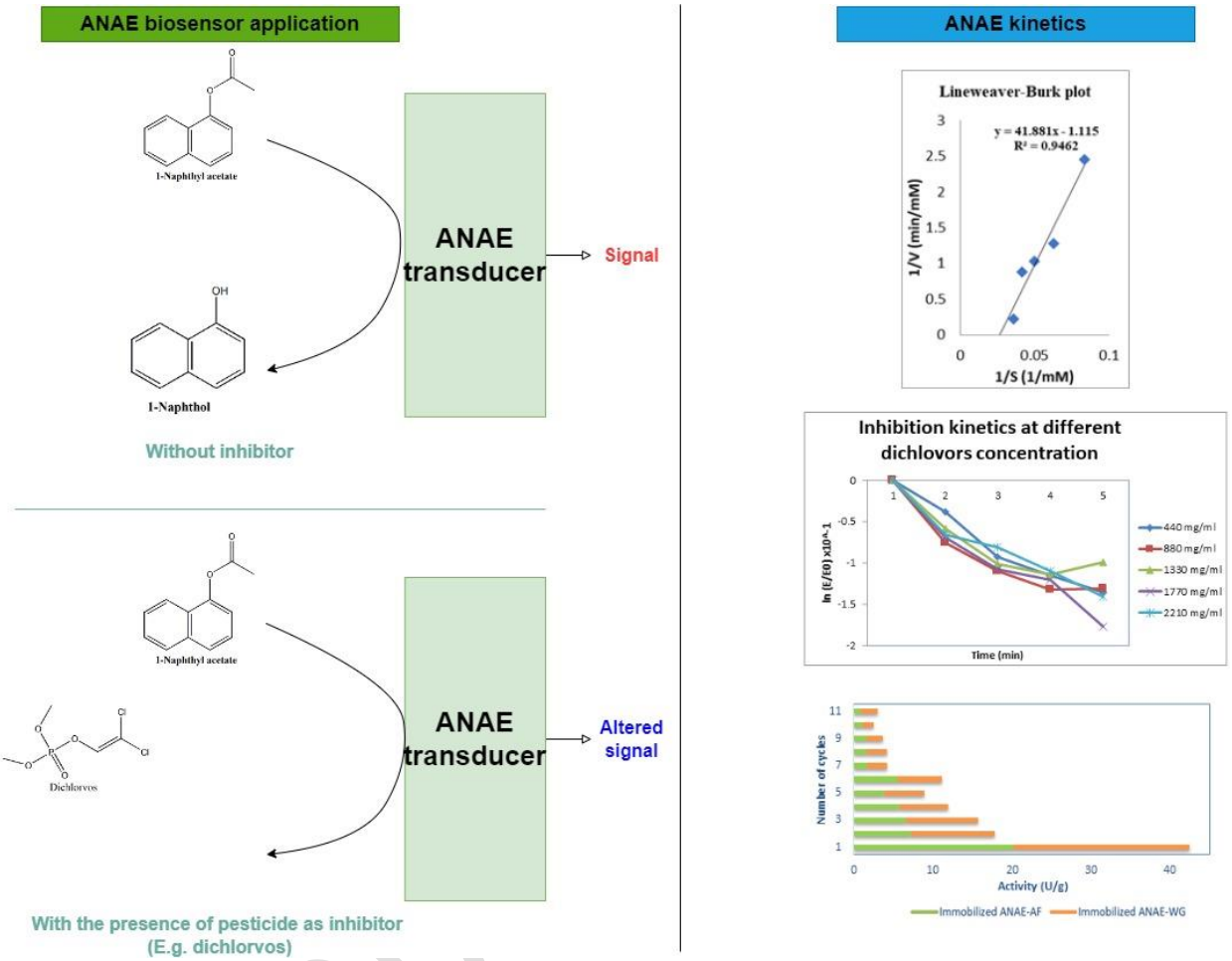
Graphical Abstract



Entry for the Table of Contents

The activity of alpha-naphthyl acetate esterase (ANAE) can be inhibited by organophosphate pesticide for biosensor applications. ANAE extracted from wheat flour and grain, was immobilized on carrageenan and assessed for its protein concentration, loading efficiency, activity, reusability, optimum temperature and pH. Enzyme kinetics was performed using Michaelis-Menten kinetics and inhibition kinetics using dichlorvos pesticide as inhibitor.