

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DOMOIC ACID DETECTION IN SELECTED SHELLFISH SEAFOOD USING α -AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONIC ACID (AMPA) RECEPTOR

(Pembangunan Imunojerapan Berpaut Enzim Berpesaingan Tak Langsung (ELISA) bagi Pengesanan Domoik Asid dalam Makanan Laut Bercengkerang Terpilih menggunakan Reseptor α -Amino-3-Hidroksi-5-Metil-4-Isoksazolepropionik Asid (AMPA))

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Abstract

In this study, a selective and sensitive enzyme-linked immunosorbent assay (ELISA) method using ionotropic glutamate receptor of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was successfully developed in order to determine the presence of domoic acid (DA) in shellfish seafood. The optimum concentration of AMPA receptor and goat anti-mouse IgG alkaline phosphate (AP) were obtained at 1.3 ppm and 1.7 ppm, respectively. Absorbance response of the optimised assay towards DA recorded limit of detection (LOD) and limit of quantification (LOQ) at 0.72 ppm and 1.2 ppm, respectively. Cross-reaction with glutamic acid (GA) showed that the assay was less sensitive than DA, with cross-reactivity values of 12.96%. The competency of the immunoassay towards real samples analysis was studied using mussel and cockle. Detection of non-spiked samples recorded the presence of DA at 0.96 ppm for mussel and 0.56 ppm for cockle. Meanwhile, recovery of the spike samples with 3 ppm DA was found at 2.69 ppm for mussel and 2.60 ppm for cockle, with recovery percentages of 86.7% and 89.7%, respectively.

Keywords: enzyme-linked immunosorbent assay, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, domoic acid, shellfish seafood

Abstrak

Dalam kajian ini, satu kaedah imunojerapan berpaut enzim berpesaingan tak langsung (ELISA) yang selektif dan sensitif dengan menggunakan reseptor glutamat ionotropik asid α -amino-3-hidroksi-5-metil-4-isoksazolepropionik (AMPA) telah berjaya dibangunkan untuk menentukan kehadiran asid domoik (DA) dalam makanan laut bercengkerang. Kepekatan optimum reseptor AMPA dan anti-tikus kambing IgG alkali fosfat (AP) masing-masing adalah didapati pada 1.3 ppm dan 1.7 ppm. Tindak balas penyerapan terhadap ujian optimum DA mencatatkan had pengesanan (LOD) dan had kuantifikasi (LOQ) masing-masing diperolehi sebanyak 0.72 ppm dan 1.2 ppm. Tindak balas silang dengan asid glutamat (GA) menunjukkan bahawa ujian GA adalah kurang sensitif daripada DA, dengan nilai tindak balas-silang 12.96%. Kecekapan imunoasai bagi analisis sampel sebenar telah dikaji menggunakan kupang dan kerang. Pengesanan sampel yang tidak dipaku mencatatkan kehadiran DA pada 0.96 ppm untuk kupang dan 0.56 ppm untuk kerang. Sementara itu, perolehan semula sampel yang dipaku dengan 3 ppm DA diperolehi pada 2.69 ppm bagi kupang dan 2.6 ppm bagi kerang, dengan peratusan pemulihan masing-masing sebanyak 86.7% dan 89.7%.

Kata kunci: Ujian imunojerapan berpaut enzim berpesaingan tak langsung, asid α -mino-3-hidroksi-5-metil-4-isoksazolepropionik, asid domoik, makanan laut bercengkerang

Introduction

Domoic acid (DA) is frequently associated with Red Tide phenomenon, which occurs when certain phytoplankton species with reddish pigments bloom in the sea. Red tide usually occurs in marine, estuarine or fresh waters, where algae accumulate rapidly in the water columns. These algae, which are in the form of phytoplankton, can form visible and dense patches near the water surface [1]. DA is a toxin produced by marine diatoms that belongs to the members of the genus *Pseudo-nitzschia*, a tiny plant, in the form of microscopic algae, which lives in the sea and obtains energy from sunlight in the daytime [2]. A total of 22 species of the genus *Pseudo-nitzschia* have been identified, including 14 new records in Malaysia. Among these, nine species have been identified as the cause of amnesic shellfish poisoning (ASP) outbreak that has been documented worldwide [3].

The consumption of shellfish mollusks contaminated with DA can lead to ASP. Humans affected by ASP could experience nausea, vomiting, abdominal cramps, diarrhea, temporary memory loss as well as death [4]. DA poisoning was first detected in 1987 in Canada, with four casualties [5]. Since then, numerous DA-related incidents have been reported around the globe [6]. In Malaysia, DA screening in seafood has been carried out at two sites of cage cultures, in Tebrau Straits and in Johor in 2015, to evaluate the potential occurrence of DA [7]. Enforcement in the European Union (EU) of the regulatory limit for DA is 20 $\mu\text{g/kg}$ (Regulation EC, 2074/2005), this implies that samples with DA levels below this value show no ill effects on human (tolerable daily intake (TDI) of DA for humans is 0.075 ppm) and would be released on the market [8].

α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), is known as an ionotropic glutamate receptor, that was named after the activated agonists of α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate. AMPA is a nonselective cation channel that allows the passage of Na^+ and K^+ , and in some cases small amounts of Ca^{2+} [9]. Figure 1 shows the ionotropic glutamate receptor of AMPA adapted from Chelsea & Shawn, 2017 [10].

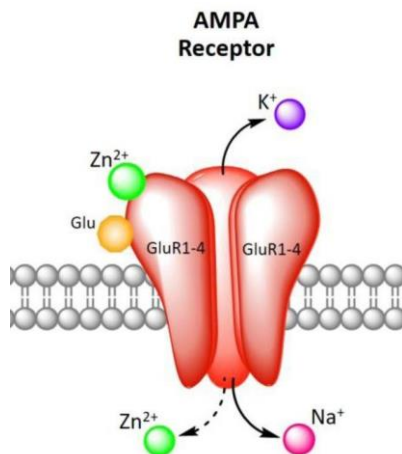


Figure 1. Ionotropic glutamate receptors AMPA

DA is an analogue to the neurotransmitter L-glutamate and excitatory amino acid. Occurrence of DA in the neurons subsequently activates AMPA. The activation causes an uncontrollable increasing level of intracellular Ca^{2+} , which then induces glutamate release [11]. Complementing the glutamic acid that is needed by the human body, DA will bind predominantly to the AMPA receptors in the central nervous system after the consumption of contaminated shellfish. This cause the depolarization of the neurons and the increase of intercellular Ca^{2+} concentration, resulting in sustained activation of calcium-sensitive enzymes, eventually leading to neuronal swelling, depletion of energy and cell death [12].

High performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) are commonly used in the detection of DA [13]. Although these techniques have the advantages of high speed, high

resolution, high sensitivity and accuracy, they also possess some disadvantages such as bulky instrument, time consuming, large amounts of sample extract is needed which can lead to shortened column life, high maintenance and clean-up cost, and also require trained personnel to operate [14]. In order to overcome the limitations of these instruments, researchers have shifted to the development of a suitable bio-chemical sensors for DA detection.

An attempt of developing an AMPA as an immunoassay for the detection of DA in selected shellfish seafood was made using the enzyme-linked immunosorbent assay (ELISA) method. ELISA is a plate-based assay technique designed for quantifying and detecting substances such as proteins, hormones, peptides and antibodies. The main advantages of ELISA are that, it is sensitive, the equipment needed is relatively cheap and it has been formally validated in an interlaboratory study [15]. ELISA has the ability to selectively detect DA in shellfish samples with a detection limit in parts per trillion (ppt) [16]. Although the application of the ELISA method in determining DA is not new, researchers agree that ELISA is the best method for the regular monitoring of DA in shellfish samples due to its accurate quantification, high recovery and tolerable precision [17]. Therefore, in this study, the implementation of AMPA receptor as a sensing reagent that replace the use of antibodies in ELISA for DA detection was utilized, and the total performance of the assay towards DA was evaluated for the future development of the AMPA-ELISA immunosensor.

Materials and Methods

Chemicals and Reagents

Domoic acid, glutamic acid, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, bovine serum albumin (BSA) and *para*-nitrophenylphosphate (*p*-NPP) were purchased from Sigma, USA. Sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate, sodium carbonate buffer and sodium bicarbonate buffer were purchased from Thermo Fisher, USA. Goat anti-mouse IgG alkaline phosphate (AP) was purchased from Santa Cruz Biotechnology, USA. Diethanolamine (DEA) was purchased from Merck, Germany. All reagents were of analytical grade.

Instrumentation

Mini incubator (VWR, China) was used to incubate samples at 37 °C. An Ascent Multiscan ELISA reader (Thermo Scientific) was used for optical characterization.

Preparation of buffer solutions and substrate

Buffer solutions and substrate were freshly prepared prior to assay. 0.1 M of sodium bicarbonate, sodium carbonate-bicarbonate and sodium carbonate buffer solutions were used as coating buffers. The washing buffer, phosphate buffer saline (PBS, pH 7.4) was prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium phosphate and 0.24 g of monopotassium phosphate in deionized water. Mixture of 0.1 g (1%) BSA and PBS were used as blocking buffer (pH 7.4). AMPA was prepared in the coating buffer. DA and AP were diluted with PBS. 1 ml *para*-nitrophenylphosphate (*p*-NPP) substrate was diluted in 9 ml 1 M DEA buffer (pH 9.5).

Optimization of reagents and biomolecules:

Types of coating buffer

Three different types of coating buffer, which are carbonate buffer, bicarbonate buffer and carbonate-bicarbonate buffer, were applied in indirect ELISA. The performance of the assays using 50 μ l of 0.1 M bicarbonate buffer, 50 μ l of 0.1 M carbonate buffer and 50 μ l of 0.1 M carbonate-bicarbonate buffer were compared.

Influence of blocking step

The effect of blocking step in ELISA was evaluated by comparing the performance of assays with and without a blocking buffer.

AMPA and AP concentration

50 μ L of AMPA and 50 μ L of AP in varying concentrations were used to determine the optimum condition and the optimum performance of the assays.

ELISA for DA Detection

50 μ L of carbonate-bicarbonate buffer was added to each well. The wells were incubated for 1 hour at 37 °C. 200 μ L PBS was used to wash the plate by inverting the plate frame over a sink and tapping against a pile of paper towels to remove the remaining liquid completely before proceeding to the next step. 50 μ L AMPA was added to each well. The wells were incubated for 1 hour at 37 °C, and 200 μ L PBS was used to remove the unbound receptors. 50 μ L of serial dilutions of DA with different concentrations were added to each well. The wells were incubated for 1 hour at 37 °C, and 200 μ L PBS was used to remove the unbound DA molecule. 50 μ L AP was then added to the wells and the mixture was incubated for 1 hour at 37 °C. 200 μ L PBS was used to remove the unbound AP. The reaction was initiated by the addition of 200 μ L *p*-NPP to each well. A change of color was observed, and the plate was measured at 405 nm after 1 hour.

Control assays

Control assays were performed to evaluate the performance of optimized biomolecules and reagents. The procedures for the assays were similar to the procedures described in ELISA for DA detection, but with the exception of certain components. There were five types of control assays tested, namely, substrate (*p*-NPP) only, control 1 (no AMPA), control 2 (no DA), control 3 (no AP) and normal assay.

Cross-reactivity with glutamic acid

The ELISA procedure was repeated by replacing DA with GA for measuring a cross-reactivity of DA with GA.

Measurement with real samples

The ELISA procedure was repeated for DA measurement in real shellfish samples of cockle and mussel.

Results and Discussion

Optimization of Reagents and Biomolecules:

Types of coating buffer

In immunoassays, a coating step is basically conducted to allow the binding of biological elements (antibody or antigen) onto the solid support by passive adsorption [18]. This first step of immunoassay is very crucial, and thus a few considerations must be taken into account before the coating process, especially the selection of the coating buffer. Carbonate-type buffers are commonly used buffers in immunoassays. Figure 2 shows that, a carbonate-bicarbonate buffer had the most significant absorbance reading of 0.12 a.u. compared to bicarbonate buffer and carbonate buffer, which the absorbance obtained were 0.09 a.u. and 0.10 a.u. respectively. Carbonate-bicarbonate buffer provided better absorbance as it has a pH value of 9.6 which was proven efficient in coating step compared to bicarbonate and carbonate buffer with pH value of 8.3 and 11.0 respectively. Carbonate-bicarbonate buffer with pH 9.6 aids solubility of a variety of proteins, and makes sure that most proteins are unprotonated with an overall negative charge, which helps when binding to positively charged plate [19].

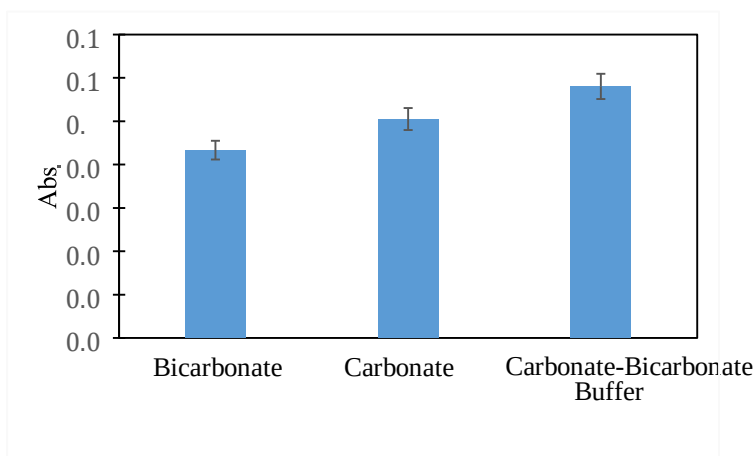


Figure 2. Absorbance reading of different coating buffers

Influence of blocking step

Blocking step is as important as coating step because it can improve the sensitivity of the assay by increasing the signal-to-noise ratio and reducing background interference. Blocking agents such as BSA, non-fat dry milk, and whole serum are commonly used to eliminate the residual binding capacity of the wells. Besides that, blocking agents can help in reducing non-specific interactions, and stabilize the biomolecules bound to the well surface [20]. Figure 3 shows that the assay with a blocking step is likely to have higher absorbance compared to the one without a blocking step. This study has proven that a blocking step was able to improve the sensitivity of the assay, as the remaining binding surface had been blocked to prevent the nonspecific binding of the antibodies. The blocker used in this study was 1% BSA in PBS buffer.

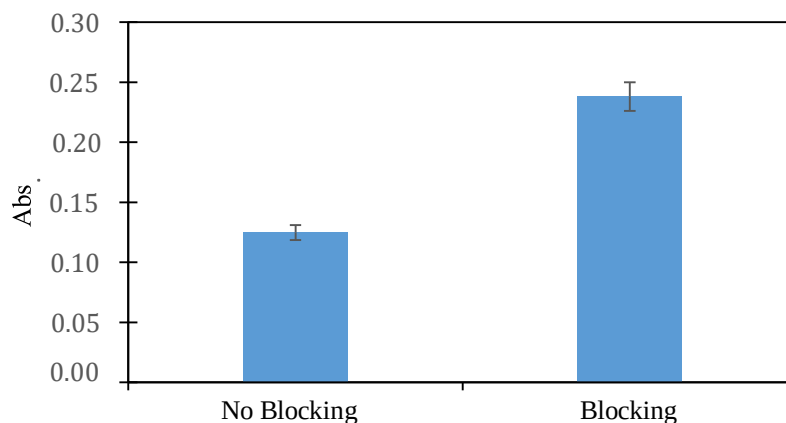


Figure 3. Influence of blocking step during assay

AMPA optimum concentration

The assay was conducted using different concentrations (0.2-2 ppm) of the AMPA receptor in order to find out the optimum condition for AMPA that will react with 1 ppm of DA in the assay. From Figure 4, it can be seen that the absorbance reading started to plateau at 1.3 ppm. As the concentration of AMPA increased, the absorbance reading did not differ much, and the absorbance was maintained at almost a similar value. Therefore at a concentration of 1.3 ppm of AMPA, the reaction between AMPA and DA was proven to be the most active.

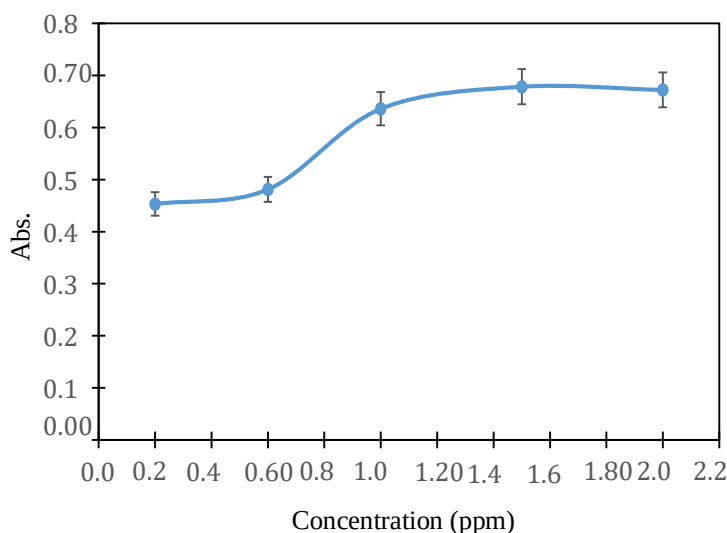


Figure 4. Absorbance reading for different concentrations of AMPA at 405 nm

AP optimum concentration

AP label antibody was used in ELISA for optical performance. The concentration of AP ranged from 1 ppm to 3 ppm. Color change was observed after one hour of *p*-NPP substrate addition, where it changed from colorless to yellow. The change in color implies that there are active formations of bio element complexes in the well. The plate was read at 405 nm and the absorbance reading observed is shown in Figure 5. From the graph, the absorbance started to plateau at 1.7 ppm. After that, the absorbance reading did not differ much and maintained almost similar value even when the concentration of AP had increased. Therefore, AP had been successfully optimized at 1.7 ppm.

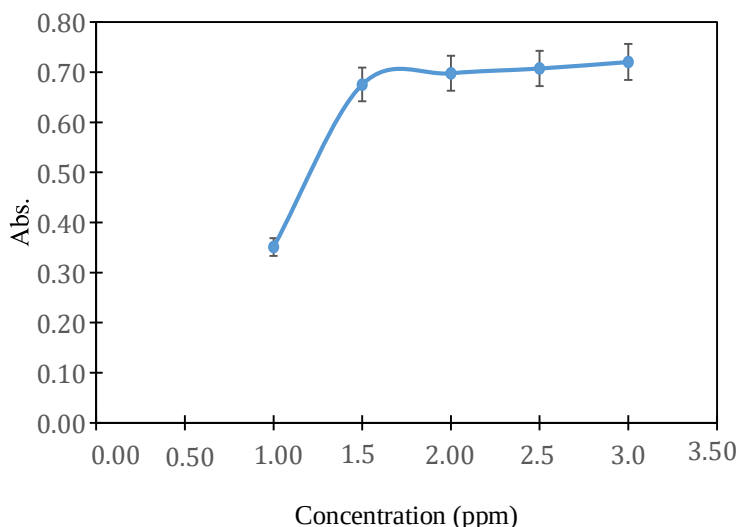


Figure 5. Absorbance for AP label antibody read at 405 nm

ELISA for DA detection

Responses of different concentrations of DA (0.2-2 ppm) in ELISA were examined and the result is shown in Figure 6. The developed ELISA method was verified by a series of experimental parameters including linear range, correlation coefficients (R^2), LOD and LOQ. Good linearity was recorded with R^2 of 0.9156. The calculated LOD and LOQ values for optical response of DA were 0.72 ppm and 1.20 ppm, respectively.

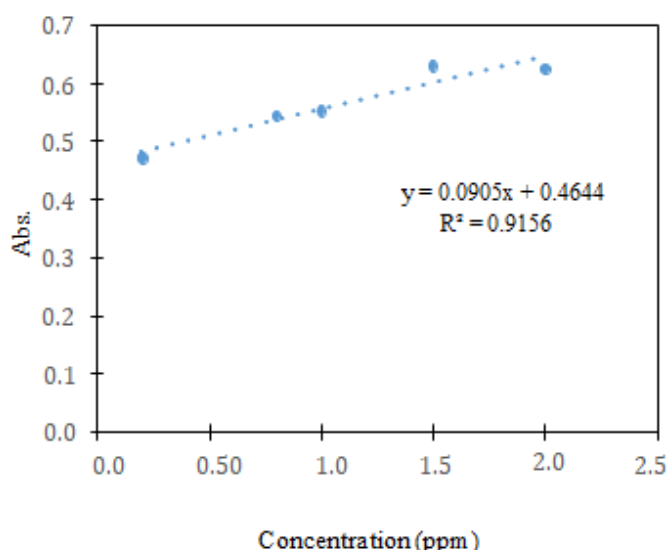


Figure 6. Calibration curve of absorbance against concentration of DA

Control assay

Control assays were conducted in order to assess the performance of each optimized bio element. The absorbance for all assays is shown in Figure 7. From the graph, it can be seen that the normal assay had the highest absorbance reading while the rest had almost similar absorbance readings. *p*-NPP acted as a blank assay. Control 1 had been performed without the addition of AMPA, while Control 2 was conducted without DA. Control 3 was performed without AP. In all the control assays, the absorbance reading was low, because there was no active interaction between the biomolecules. In ELISA, the performance of assay will be affected by any substance that adsorbs on the surface. By comparing control assays and normal assay, it was proven that all biomolecules must be present in order to obtain a better absorbance reading, in which the absorbance signals the formation of complex biomolecules in the assay.

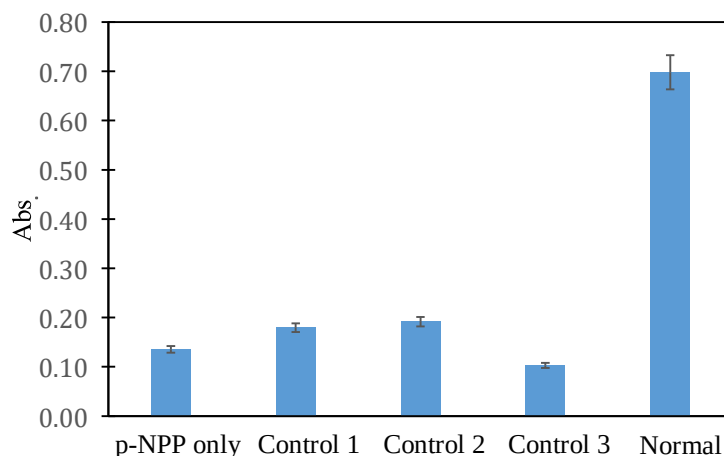


Figure 7. Control assays of biomolecules with indirect ELISA. All assays were read at 405 nm. (Control 1 without AMPA, Control 2 without DA and Control 3 without AP)

Cross-reactivity with GA

A cross-reactivity study was selected because in a real human body, AMPA normally reacts with GA to maintain the release of CA^{2+} ions. It was found that only with the presence of DA, the normal flow of CA^{2+} ions was distracted. Figure 8 shows the response carried out for GA in an indirect assay, in order to test the selectivity of the assay towards GA. GA was of GA towards ELISA. Good linearity was observed for the response between GA and the assay. The half maximal inhibitory concentration (IC_{50}) values for DA and GA calculated from Figure 6 and Figure 8 (equation 1) were 0.39 and 3.01, respectively. The low IC_{50} value indicates the high sensitivity of the assay towards the target analyte. Thus, this proves that the assay was evidently more sensitive towards DA than GA.

$$IC_{50} = \frac{(0.5-c)}{m} \quad (1)$$

where c is y-intercept and m is slope of the calibration plot.

Meanwhile, cross-reactivity value of DA with GA calculated using equation 2 was 12.96 %. The low cross-reactivity value obtained was due to the tendency of AMPA to also react with GA, even though it was more sensitive towards DA.

$$Cross - reactivity (\%) = \frac{IC_{50 \text{ DA}}}{IC_{50 \text{ GA}}} \times 100 \quad (2)$$

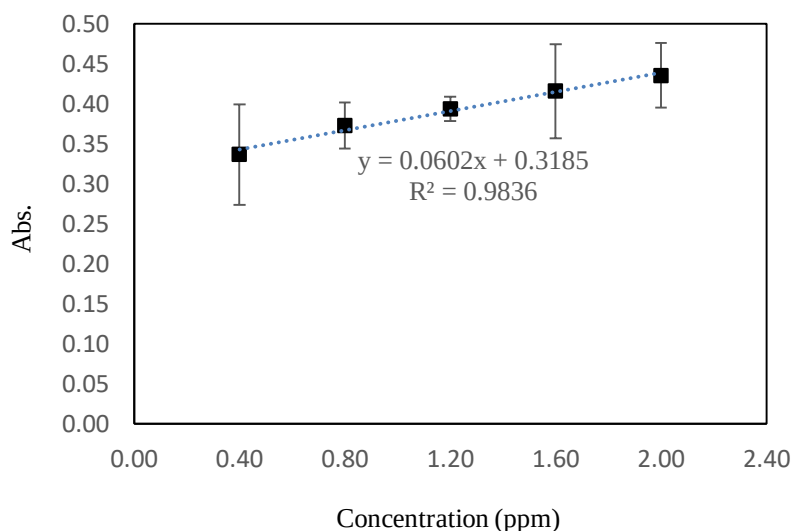


Figure 8. Absorbance reading of GA in indirect ELISA

Measurement with real samples

The sensitivity and selectivity of the developed assays were tested with spiked and non-spiked shellfish samples (mussel and cockle). The optical evaluation was recorded and is tabulated in Table 1. In non-spiked samples, 0.96 ppm and 0.56 ppm DA were found in mussel and cockle, respectively. Meanwhile, in spiked samples, 3 ppm of DA standard was injected into the samples. 2.69 ppm of DA in mussel and 2.60 ppm of DA in cockle samples were recovered with a recovery percentage of 89.7% and 86.7% for mussel and cockle samples, respectively. Low recovery from the spiked samples might be due to the interference of other substances in the samples. The interference might have caused the absorbance reading of DA to be hindered, causing low reading and recovery.

Table 1. Recovery of DA in cockle and mussel samples using optical measurement

Sample	Non-spiked	Spiked (3 ppm)	
	Found (ppm)	Found (ppm)	Recovery (%)
Mussel	0.96	2.69	89.7
Cockle	0.56	2.60	86.7

Conclusion

In this study, an approach of developing an ELISA immunoassay using an AMPA receptor as the recognition reagent for DA determination was successfully conducted. Since the glutamate receptors readily interact with DA in a normal human body, the concept of replacing antibodies with receptors in ELISA was derived out and experimented in the detection of DA in shellfish seafood. The immunoassay shows highly selective and sensitive responses toward DA, even in the case of real sample analysis. The achievements offer a great deal of potential for the future development of receptor-ELISA immunosensors for DA detection.

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