Utilization of Diamine Oxidase Enzyme from Mung Bean Sprouts (Vigna radiata L) for Histamine biosensors

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Abstract. This research is aimed to utilize the diamine oxidase enzyme (DAO) which isolated from mung bean sprouts (Vigna radiata L) to develop histamine biosensors based on electrode enzyme with the amperometric method (cyclic voltammetry). The DAO enzyme is trapped inside the membrane of chitin-cellulose acetate 2:1 and glutaraldehyde which super imposed on a Pt electrode. Histamine will be oxidized by DAO enzyme to produce aldehydes and H₂O₂ that acting as electron transfer mediators. The performance of biosensors will be measured at various concentrations of glutaraldehyde, temperature changes and different range of pH. Recently, it has been found that the optimal conditions obtained from the paramaters as follows; at 25% of glutaraldehyde, temperature of 37°C and pH of 7.4. Eventually, the results provided an expectation for applying histamine biosensors in determining the freshness and safety of fish specifically skombroidae families.

1. Introduction
Histamine is extremely decisive for the quality and freshness of fish specifically the skombroidae family such as tuna and skipjack. Histamine is formed from oxidation process of essential amino acid, histidin, either by bacterial or enzymatic activity that can cause poisoning and allergies. According to Rudi of Research and Development Product Processing and Biotechnology of Marine and Fisheries (BBP4BKp), the content of histamine in fresh fish is below 100 ppm and the levels of 500-1000 ppm is generally considered as indangerous extent and can cause poisoning in people who are sensitive [1]. Therefore, it is required a fast and accurate histamine analyses on fish and its derivatives.
Research has been undergone in order to discover the best method to detect and determine the levels of histamine in fish particularly on skombridae family. Currently histamine analyses can be applied on a variety of ways and methods. According to the Indonesian National Standard, histamine analysis was conducted using spectrofluorometry and HPLC [1]. Both of these methods require specialized personnel trained and skilled because its initial preparation of samples is quite complicated with a long time, besides its equipment is relatively expensive. Thus, to reduce the time required in the analysis and to offer a rapid screening method to test the quality of the food industry products, some enzymatic method and amount of enzyme sensors have been described by several researchers [2]. Aziz [3] using the biosensors constructed of DAO enzyme and immobilized on PVA membrane. Keow, et al [4], DAO enzyme was
immobilized on a poly-HEMA membrane to detect histamine quickly using SPE-carbon paste electrodes. Research Telsnig, et al [5] using the DAO enzyme of pea seeds amine oxidase (PSAO), with MnO2 electrode modified with carbon paste. Biosensor applications generally show various advantages such as enabling faster analysis with preparation/processing samples less even not needed at all [6].

DAO enzyme is widely found in plants Leguminaceae (pulses), such as Cicer arietinum, Lathyrus sativus and Vigna radiata (mung bean) [7]. DAO enzyme was first purified from pea sprouts. This enzyme oxidizes diamine, such as histamine, and also some primary monoamines, with little or no activity against secondary and tertiary amine [8]. Currently DAO is commercially available which obtained from pig kidney. DAO has a low specific activity even when it has been purified, in contrast to the enzyme from plants that possessing a higher activity. Likewise the DAO enzyme which isolated from green beans has a relatively high activity [9].

Analysis of biosensor is expected to replace the procurement of modern chemistry instruments which is relatively much more expensive [10]. An advantageous of biosensors analysis because it is easily taken to the field, relatively low acquisition costs and sensitive enough to identify a particular substance or chemical compound. Biosensors analysis can be performed with the use of transducers electrochemical (amperometric, potentiometric). Transducer design with amperometric or potentiometric method can be done with the development of enzyme electrodes.

Enzyme electrodes that is effective, sensitive, and accurate is highly determined by membrane composition [11]. One component of the membrane that play a role in binding the DAO enzyme is glutaraldehyde. Enzyme electrode which will be developed in this study is the DAO enzyme electrode using glutaraldehyde at various concentrations, temperatures and pH for the analysis of histamine contained in certain materials, for example in the fish from skombroidae family such as tuna and skipjack. Levels of histamine in fish and food derivatives will determine the degree of freshness and safety of food stuffs.

In this research, enzyme biosensors based histamine amperometric will be developed, namely the biosensors to detect and determine the content of histamine in certain materials. The use of histamine biosensors with DAO enzyme electrodes which immobilized in the membrane of chitin-cellulose acetate and glutaraldehyde can be done. Research objective is to develop histamine biosensors based on electrode enzyme with the amperometric method (cyclic voltammetry).

2. Material and Methods

2.1 Materials
Ammonium sulfate, wire Pt, wire Cu, wire Ag, distilled water, HCl, NaOH, tin, glutaraldehyde, acetone, histamine, mung beans, cellophane, Na2HPO4.2H2O, NaH2PO4.H2O, sephadex G-75.

2.2 Equipment
Cyclic voltammetry–eDAQ ED410-159, pH meter/potentiometer Orion 710 A, an enzyme electrode, magnetic stirrer, spectrophotometer IR Shimadzu Prestige-21, spectrophotometer UV/Vis, oven, analytical balance, solder, and glassware used in the laboratory.

2.3 Procedure
2.3.1 Preparation of phosphate buffer solution
a. Preparation of NaH2PO4.H2O solution 0.2 M.
A number of 2.7598 gram NaH2PO4.H2O was diluted with distilled water and put in a 100 mL volumetric flask. After wards it was diluted until reach the boundary mark (solution A).
b. **Preparation of Na$_2$HPO$_4$.2H$_2$O solution 0.2 M**

A number of 1.7799 gram Na$_2$HPO$_4$.H$_2$O was diluted with distilled water and put in a 50 mL. Then it was diluted until the boundary mark (solution B). The creation of phosphate buffer solution was conducted by mixing both solution A and solution B in accordance with a ratio of each to desired pH buffer.

2.3.2 **Isolation of DAO Enzyme of Mung Beans (Vigna radiata L.)**

a. **Crude extract of DAO Enzyme.**

A total of 100 grams of mung beans were washed and soaked in distilled water overnight. Furthermore, drained and leveled above porous containers are lined with filter paper, stored at room temperature humid. To help the process of growing sprouts in order to more quickly and evenly, the container can be placed in the pot (lid slightly open) or covered with a napkin and moisture is maintained by sprinkling water on a regular basis. For the isolation and purification of the DAO enzyme of mung bean sprouts, sprouts aged 3 days were collected and washed with distilled water and the outer skin removed. Mung bean sprouts that have clean homogenized with an equal amount (w / v) of 0.05 M phosphate buffer pH 7.0 cold by using a blender. Crude extract obtained by squeezing homogenates through four layers of gauze and centrifuged at 10,000 rpm for 20 min at 4°C. The obtained result (solution) is called DAO enzyme crude extract [9].

b. **Ammonium Sulfate Fractionation**

DAO enzyme crude extract was fractionated with ammonium sulfate at saturation degree of 0-80 % with an interval of 20 units which added gradually while stirring with a magnetic stirrer until completely dissolved and stored over night at 4°C. After that, it was centrifuged at 4°C for 20 minutes. The attained supernatant was proceeded for further fractionation, whereas the precipitate was dissolved in a small volume of 0.2 M phosphate buffer pH 7.0 and its activity was measured. Fractions that have the highest activity continued to a process which called dialysis [9].

c. **Dialysis.**

Ammonium sulphate precipitate fraction with high activity was dissolved in 5 mL of 0.2 M phosphate bufferpH 7.0. The solution was put in a cellophane bag and then dialyzed with 0.05 M phosphate buffer pH 7.0 and stirred with a magnetic stirrer for 12 hours at 4°C. The buffer is changed every 3 hours [9].

d. **Gel Filtration.**

The active fraction which has undergone dialysis process in incorporated into the matrix column with sephadex G-75 (column 35 cm long and 1 cm in diameter), previously equilibrated with 0.05 M phosphate buffer pH 7.0 for one night. Subsequently it was eluted with the same buffer. The volume of each fraction as much as 3.0 mL and each fraction is measured for its activity and protein content. Fractions that have high activity will be saved and used as the DAO enzyme for further research [9].

2.3.3 **Design and characterisation of histamine biosensors**

Plastic-coated copper wire with a size of 5 cm, 1 mm diameter which connected to a Pt wire of1.5 cm with 0.4 mm diameter by soldering used lead wire. Furthermore, the tip of the Pt wire is curved back into the solder point (Pt electrode, Ep). Ep Pt electrode wire section was immersed in a homogeneous solution of chitin-cellulose acetate 2:1 in acetone [10]. As soon as the membrane layer is formed, the electrode is dipped into the water repeatedly. Then, on the wire that has been coated with chitin-cellulose acetate membrane is soaked in a solution of glutaraldehyde (E1=15, E2=20, E3=25%) for 6 hours. After the membrane layer containing gluteraldehyde formed, the electrode was washed with phosphate buffer pH 7.0 (Em). DAO enzyme electrode was made by soaking Em in phosphate buffer pH 7.0 containing DAO enzyme for 16 hours. Further DAO enzyme electrode design results were characterized by cyclic voltammetry at various temperatures and pH. Characterization is conducted with a standard solution of histamine: 1, 10, 100, 1000, 10000 ppm. Each of these enzymes is done by scanning
electrode potential from -1.5 to +2.0 V at a rate of 100 mV/ sec from a low concentration to a higher concentration. The formed cyclic voltammogram results are analyzed to obtain a peak flow changes caused by alteration in the concentration of the solution used.

3. Results and Discussion

3.1 DAO Enzyme Isolation

The yield of isolation and fractionation DAO enzyme protein with (NH₄)₂SO₄ can be seen in Table 1. It is shown that 40-60% of saturated fraction was the fraction with the highest DAO enzyme activity (3326.00 mU/mL). Therefore 40-60% of saturated fraction was followed by dialysis and gel filtration process in order to obtain more pure DAO enzyme quantity.

After having done the dialysis and gel filtration processes on sephadex G-75 column, it was gained the fraction 14 which had protein level at 0.63 mg/mL, 116.00 mU/mL of activity and its specific activity was 185.01 mU/mg protein. The specific activity of DAO enzyme from results isolation of mung bean sprouts was 185.01 mU/mg protein which was much larger than DAO enzyme from pig kidney in the market (50 mU/mg protein). So the DAO enzyme isolated from mung bean sprouts can be further used in the manufacture of electrodes for the DAO enzyme for histamine biosensor.

| No | Protein Fraction | [Protein] (mg/mL) | Activity (mU/mL) | Specific Activity (mU/mg protein) |
|----|------------------|------------------|-----------------|----------------------------------|
| 1  | Crude extract    | 25.20            | 1226.00         | 48.65                            |
| 2  | 0 - 20 %         | 20.49            | 1336.00         | 65.20                            |
| 3  | 20 - 40 %        | 24.34            | 2042.67         | 83.92                            |
| 4  | 40 - 60 %        | 29.03            | 3326.00         | 114.57                           |
| 5  | 60 - 80 %        | 24.65            | 1329.33         | 53.93                            |
| 6  | Fraction 14      | 0.63             | 116.00          | 185.01                           |

3.2 DAO enzyme electrode characterization with cyclic voltammetry

3.2.1 The influence of the concentration of glutaraldehyde

Results of measurement (cyclic voltammogram) various concentrations of histamine in various glutaraldehyde concentrations are shown in figure 1.
Figure 1. Cyclic voltammograms of various concentrations of histamine at a glutaraldehyde concentration of; 15% (a), 20% (b) and 25% (c).

At Figure 1 it was appeared that the type of electrode E3, glutaraldehyde concentration of 25% (1c) showed a clearer peak current and occurred to the histamine concentration of 1 ppm up to 10,000 ppm compared with the type of electrode E2 and E1 namely glutaraldehyde concentration of 20% (1b) and a 15% glutaraldehyde (1a). On the membrane with glutaraldehyde concentration of 20%, the peak current is clearly visible on the histamine concentration of 1 ppm to 100 ppm. At a concentration of 15% glutaraldehyde, peak currents only occur at a concentration of 100 ppm histamine. Based on these results it can be said that the type of electrode with glutaraldehyde concentration of 25% is better than 20% glutaraldehyde and 15% on the membrane of chitin-cellulose acetate 2:1. It means that the higher the concentration of glutaraldehyde, the better the DAO enzyme electrode is produced [12]. This happens because the higher the concentration of glutaraldehyde, the easier the glutaraldehyde under going the polymerization process inter molecules and binding to DAO enzyme. Thus the membrane electrode will contain more DAO enzymes that will eventually oxidize more histamine.

3.2.2 The Influence of pH. Cyclic voltammograms of various concentrations of histamine at various pH measurements can be seen in figure 2.

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**Figure 1.** Cyclic voltammograms of various concentrations of histamine at a glutaraldehyde concentration of; 15% (a), 20% (b) and 25% (c).
Figure 2. Voltammogram cyclic various concentrations of histamine at pH; 6.2 (a), 6.6 (b), 7.0 (c), 7.4 (d) and 7.8 (e).

It was illustrated that at pH 6.2 the peak current (though not too sharp) occurs in histamine concentration of 1 ppm to 100 ppm. Whereas at pH 6.4 to 7.8 peak flow occurs at all concentrations of histamine. This indicates that the DAO enzyme electrode works at pH ranges are quite wide and of course this is a profitable thing [13]. However, to see the most optimum pH, it can be noted regarding the linearity of the relationship between the peak current (anodic or cathodic) with concentrations of histamine. The Linearity can be seen via coefficient linearity (R) as shown in Table 2. Table 2 shows that the linearity flow of the highest peak of histamine concentrations of histamine shown in cathodic current at pH 7.4 (R = 0.9935), whereas the highest linearity on the anodic current at pH 7.8 (R = 0.9884). R value is calculated without involving concentration of 10000 ppm because at this concentration peak currents generated is far deviating and it is also shown that the measurement does not reach the maximum limit of 10000 ppm.
**Table 2.** The relationship between the concentration of histamine with anodic and cathodic peak currents at various pH.

| pH  | [Histamine] (ppm) | I anodic (mA)  | R anodic | I cathodic (mA) | R cathodic |
|-----|-------------------|----------------|----------|-----------------|------------|
| 6.2 | 1 - 10000         | 0.063 - 0.090  | 0.9072   | -0.010 - -0.059 | 0.9089     |
| 6.6 | 1 - 10000         | 0.083 - 0.101  | 0.9518   | -0.052 - -0.084 | 0.9576     |
| 7.0 | 1 - 10000         | 0.065 - 0.100  | 0.9560   | -0.022 - -0.086 | 0.9760     |
| 7.4 | 1 - 10000         | 0.079 - 0.100  | 0.9685   | -0.041 - -0.079 | 0.9935     |
| 7.8 | 1 - 10000         | 0.080 - 0.097  | 0.9884   | -0.051 - -0.088 | 0.9824     |

3.2.3 *The Influence of temperature.* Cyclic voltammograms on various concentrations of histamine at diverse temperatures can be seen in Figure 3.

![Figure 3](image)

**Figure 3.** Voltammogram in a variety of histamine concentrations at a temperature of; 31°C (a), 34°C (b), 37°C (c) and 40°C (d).

It was described that the temperature of 31°C (3a) does not give a peak current for the entire concentration of histamine, it means that the DAO enzyme electrode can not work and detect histamine at that temperature. Both temperature of 34°C and 40°C (3b and 3d) provided anodic peak currents were quite good but the peak cathodic was unclear. At 37°C (3c) seen that for the whole concentration of histamine giving anodic and cathodic peak currents were clear and orderly. It can be concluded that the DAO enzyme electrode can work optimally at 37°C. This temperature was the same as the optimum temperature of the DAO enzyme isolated from mung bean sprouts [14].

**4 Conclusions**

Some principal findings are: DAO enzyme isolation of mung bean sprouts obtained specific activity of 185.01 mU/mg of protein and can be used in the make of DAO enzyme electrodes for histamine biosensors. Comparison of chitin-cellulose acetate 2:1 with 25% of glutaraldehyde levels which is the best membrane composition on making the DAO enzyme electrode for histamine biosensors. The DAO enzyme electrode obtained will work optimally at a temperature of 37°C and pH 7.4.
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