Characterization of Trimethylamin-N-Oxide Demethylase (TMAOase) of Lizardfish from Indonesian Waters

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Abstract. Formation of formaldehyde in fish can occur naturally through the breakdown of trimethylamine oxide (TMAO) become formaldehyde and the dimethylamine by-product in the fish during the postmortem phase by trimethylamine-N-oxide demethylase (TMAOase). The objective of this study was to characterize TMAOase which isolated from Saurida tumbil. The study was carried out by homogenizing of fish muscle using Tris-acetate buffer (pH 7.0) and centrifuged 10,000xg for 30 minutes. The supernatant was then added 1 M NaCl up to pH 4.5 and centrifuged at again. The supernatant was neutralized and heated at 80°C for 20 minutes, then centrifuged at 10,000xg for 30 minutes and then characterized by temperature, pH, cofactor effect on enzyme activity, and molecular weight. The same supernatant and then was dialyzed for 2 hours and determined its enzyme activity, protein concentration, and molecular weight. The result showed that TMAOase enzyme had optimum activity at pH 7 and temperature at 50°C and cofactor combination of cysteine, ascorbate, and FeCl2 could increase TMAOase activity. The dialysate had yield 3.71%, 1.78 fold purification, and molecular weight 20 kDa.

Keywords: dimethylamine, formaldehyde, greater lizardfish, TMAOase

1. Introduction

The use of formaldehyde in foods including fish is a serious concern given that formaldehyde is a chemical that is harmful to humans because it is carcinogenic. Currently, there have been many reports about the use of formaldehyde in fresh fish. The government's attention, in this case, the Ministry of Health, the Food and Drug Supervisory Agency, and the Ministry of Maritime Affairs and Fisheries, regarding the use of formalin in fish is a serious concern, this is evidenced by the absence of formaldehyde in fish and its derivative foods, but in fact the use of formaldehyde in fish is still being carried out. Darjamuni reported in the 2016 city administration that as much as 20 tons of moonfish (Mene maculate) had been destroyed by the Department of Marine Agriculture and Food Security (KPKP) because it was indicated that formaldehyde reached 91 ppm and was not suitable for consumption. This can occur due to the addition of formalin which is done intentionally and occurs naturally in the fish's body.

The presence of formaldehyde in fish can naturally occur but based on this study only occurs in certain fish and which have been stored at low temperatures, based on the results of previous studies also obtained information that there is a correlation between TMAOase and formaldehyde enzyme activity. Therefore, it is necessary to extract, purify, and characterize the TMAOase enzyme. The information obtained can be used as a basis for the design of naturally formed formaldehyde detection methods based on TMAOase.
2. Related Works

Lizardfish (Saurida tumbil) is a fish with a high protein content of 19.74% [1], but this fish is less popular because it has a lot of fine spines so that this fish is widely used as raw material for processed products [2]. Lizardfish have the potential as raw material for making high-quality surimi in some countries because of their abundant availability, white flesh color, and high gel strength [3]. Lizardfish can experience a quality decline. One indicator is the formation of formaldehyde during storage. Several studies show the fact that formaldehyde is formed naturally in fish [4-8]. Formaldehyde can form during storage in some fish species such as cod, pollack, and haddock [9]. Formaldehyde is detected with a high amount only in Gadidae fish. Natural formaldehyde and dimethylamine are enzymatic decomposition products of trimethylamine oxide (TMAO) in fish during post-mortem storage [10]. The enzyme Trimethylamine-N-Oxide Demethylase (TMAOase) is an endogenous enzyme that plays a role in the breakdown of TMAO into formaldehyde and dimethylamine. TMAOase is an enzyme found in the muscles and internal organs of various types of fish [11]. TMAOase can accumulate in marine animals because in its work TMAOase requires TMAO substrates. This compound is mostly found in seawater fish because it has a function in osmoregulation [12]. Apparently, TMAO could also be synthesized in tilapia through choline which is associated with intestinal microorganisms and mono-oxygenase tissue in freshwater conditions [13]. TMAOase activity from European hake is influenced by biological and seasonal conditions [14]. The lizardfish kidney had the highest activity compared to other internal organs. Various studies have shown that there is a correlation between the activity of TMAOase enzymes and the formation of formaldehyde and dimethylamine [15]. There is a close correlation between TMAOase activity in situ gadiform white meat with formaldehyde formation [16]. TMAOase extracted from the lizardfish kidneys was stable at 50°C and pH 7.0 with a molecular weight of 128 kDa [13]. TMAOase extracted from jumbo squid was stable at 50°C and stable at pH 7.0-9.0 and had a molecular weight of 17.5 kDa [17]. TMAOase extracted from walleye pollack muscle showed optimum pH 7.0 and optimum temperature of 45°C and had a molecular weight of 25 kDa [18].

3. Materials and Methods

3.1. Materials

The main material used in this study is lizardfish (Saurida tumbil) obtained from TPI Cituis Tangerang Regency, Banten. The fish was brought to the laboratory using coolbox. The ingredients used for the extraction and purification of the TMAOase enzymes consist of tris base (Merck, Germany), HCl (Merck, Germany), acetic acid (Merck, Germany), NaCl (Merck, Germany), trichloroacetic acid (TCA) (Merck, Germany), FeCl₂ (Merck, Germany), TMAO substrate (Sigma-Aldrich, UK), cysteine (Merck, Germany), ascorbic acid (Merck, Germany), dimethylamine oxide (DMA) (Merck, Germany), bovine serum albumin (BSA) (Sigma-Aldrich, UK), coomassive brilliant blue (Applichem), ethanol (Merck, Germany), phosphoric acid (Merck, Germany), Whatman filter paper, 15 g ammonium acetate (Merck, Germany), 0.3 mL acetic acid (Merck, Germany) and 0.2 mL acetyl acetone (Merck, Germany), 25 g ammonium acetate (Merck, Germany), 0.2 cooper sulfate (Merck, Germany), 25 mL 40% NaOH (Technical), 20 mL ammonia (Merck, Germany), EDTA (Titriplex III PA) (Merck, Germany), acrylamide (Merck), sodium dedocyl sulphates (SDS) (Merck), ammonium persulphates (APS) (Sigma), tetramethylethylenediamine (TEMED) (Merck) dialysis bags cut off size 12 kDa (Sigma, USA), and distilled water.

3.2. Methods

3.2.1. Extraction and characterization of TMAOase enzymes [17]. Muscle of lizardfish homogenized with four times the volume of 20 mM Tris-acetate (pH 7.0) containing 1 M NaCl, centrifuged 10 000 g for 30 minutes, followed by the addition of 1 M HCl to pH 4.5. The homogenate is centrifuged 10 000xg for 30 minutes. The supernatant was neutralized and heated to an optimum temperature of 50, 60, 70, 80°C for 20 minutes, then centrifuged again at a speed of 10 000xg for 30 minutes. Supernatants obtained are then characterized. Characterization of the TMAOase enzyme includes determining the optimum pH, optimum temperature, and the influence of cofactors on enzyme activity and molecular weight profiles. The supernatant was then dialyzed in a buffer (20 mM Tris-acetate pH 7.0) using a dialysis bag measuring 12 kDa for two hours at 4°C, after which enzyme activity and protein
concentration were tested.

3.2.2. Analysis.

3.2.2.1. **TMAOase enzyme activity** [15]. A total of 2.5 mL of the reaction mixture (120 mM Tris-acetate; 24 mM TMAO; 2.4 mM cysteine; 2.4 mM ascorbate; and 0.24 mM FeCl₂, pH 7), with the addition of 0.5 mL of added enzyme solution. Incubation was carried out at 25°C for 20 minutes and added 1 mL of 5% TCA. The reaction mixture is centrifuged at 8 000xg for 15 minutes. The supernatant is used to determine dimethylamine levels. The blank is tested with the same steps and ingredients, but no enzymes are added. One unit (U) of TMAOase activity was defined as the production of 1 µmol dimethylamine per minute and testing of dimethylamine was carried out by the copper-ammonia method [19].

3.2.2.2. **Protein concentration** [20]. Bovine serum albumin (BSA) as standard. Bradford preparations were carried out by dissolving 10 mg of Coomassie brilliant blue in 5 mL of 95% ethanol, then adding 10 mL of phosphoric acid 85% (v/v). The distilled water is added to 250 mL and filtered with Whatman number 1 filter paper just before use. A total of 0.1 mL of the sample was put into a test tube and then 5 mL of Bradford reagent was added. The mixture was incubated for 5 minutes and measured with a spectrophotometer at a wavelength of 595 nm. Standard solutions are measured in the same way as sample solutions. The standard solution used is 0.02 - 0.30 mg/mL. The next stage is to create a standard curve with absorbance as an ordinate (y-axis) and protein concentration as abscissa (x-axis). The absorbance value obtained is then put into the Bradford standard curve to determine the protein concentration contained in the sample.

3.2.2.3. **Characterization of TMAOase Enzymes** [17]. Determination of optimum temperature was carried out with variations in incubation temperature when testing the activity of enzymes at temperatures of 30, 40, 50, 60, 70, 80 and 90°C. Determination of optimum pH was carried out with variations in buffer pH used pH 5-10. The effect of cofactor on TMAOase enzyme activity is carried out with added cysteine and FeCl₂, ascorbate and FeCl₂, also cysteine and ascorbate and FeCl₂ into the enzyme which will be determined the activity. The measurement of enzyme activity in each TMAOase enzyme characterization was carried out in accordance with the procedure of [15].

3.2.2.4. **Measurement of molecular weight** [21]. Molecular weight measurements were carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The concentration of acrylamide used in this analysis is 15% separating gel and 3% stacking gel. This is presented in table 1.

| Materials   | Separating gel (15%) | Stacking gel (3%) |
|-------------|----------------------|-------------------|
| Distilled water | 1.75 mL              | 3.70 mL           |
| 30% acrylamide     | 3.75 mL              | 0.50 mL           |
| 1.5 M Tris-HCl pH 8.8 | 1.85 mL              | -                 |
| 0.5 M Tris-HCl pH 6.8 | -                  | 0.65 mL           |
| 10% SDS             | 75.00 µL              | 50.00 µL          |
| 10% APS             | 75.00 µL              | 50.00 µL          |
| TEMED                | 7.50 µL               | 5.00 µL           |

Samples of 20 µL were mixed with 5 µL of sample buffer then put into the well. The included marker is 10 µL. The gel is installed in an electrophoresis device by pouring electrophoresis buffers into the chamber. The electrophoresis process lasts for 65 minutes at a voltage of 180 volts and 50 mA in an electrophoresis device, after which the gel is removed from the glass plate. Gel soaked in coomassie gel stain solution for two hours followed by destaining until protein bands were obtained. The protein composition of SDS-PAGE markers can be seen in table 2.
Table 2. Composition of SDS-PAGE protein markers.

| Molecular weight (Da) | Types of protein               |
|-----------------------|--------------------------------|
| 250,000               | Myosin                         |
| 150,000               | B-galactosidase                |
| 100,000               | Bovine serum albumin           |
| 75,000                | Glutamat dehydrogenase         |
| 50,000                | Ovalbumin                      |
| 37,000                | Carbonic anhydrase             |
| 25,000                | Myoglobin                      |

4. Result and Discussion

4.1. Optimization of TMAOase extraction temperature
Optimization of extraction temperature was carried out to determine the optimum extraction temperature in producing enzymes with high activity. The optimization results of the TMAOase enzyme extraction temperature are presented in figure 1. Figure 1 shows that the use of extraction temperature of 80ºC has the highest TMAOase enzyme specific activity of 5.94 U/mg. It is suspected that the higher the extraction temperature, the purer TMAOase enzyme is produced so that it can increase enzyme-specific activity. The results of this study are in accordance with the research of [17] which is an increase in the specific activity of the TMAOase enzyme extracted from squid meat at a heat treatment stage of 80ºC which is from 2.39 U/mg to 6.78 U/mg.

![Figure 1](image1.png)

Figure 1. Specific activity of TMAOase from lizardfish on various extraction temperature.

4.2. Characterization of TMAOase
4.2.1. Optimum pH TMAOase: Determination of the optimum pH is carried out by adding buffers in the pH range 5-10. Buffers are an important component of enzymatic reactions because of their ability to maintain pH. The results of the measurement of TMAOase enzyme activity from lizardfish at several pH are presented in figure 2.

![Figure 2](image2.png)

Figure 2. Specific activity of TMAOase from lizardfish at several pH.
The results of this study are consistent with the TMAOase enzyme extracted from meat walleye pollack, which also has optimum pH at pH 7 [22]. The TMAOase enzyme extracted from the lizardfish kidney has an optimum pH of 7 [15], while the TMAOase enzyme derived from squid has an optimum pH at 8 [17]. The difference in optimum pH value is thought to be caused by species differences, differences in body defense conditions, namely the environment in which it lives.

4.2.2. Optimum temperature of TMAOase. Determination of the optimum temperature for TMAOase activity is carried out at a temperature of 30-90°C. The results of the measurement of TMAOase enzyme activity from lizardfish at several temperatures are presented in figure 3. Figure 3 shows that an increase in temperature will cause an increase in TMAOase enzyme activity to a certain point. While a further increase in temperature will make TMAOase enzyme activity decrease. TMAOase enzyme activity from lizardfish in this study increased from incubation temperature of 30 °C to 50 °C and decreased activity from 60°C to 90°C. The optimum temperature range of the TMAOase enzyme produced was 50-60°C with enzyme activity values respectively 0.650 U/mL and 0.611 U/mL.

The optimum temperature of coarse and pure extracts of TMAOase enzymes from squid is 55°C and 70°C [17]. The TMAOase enzyme extracted from the lizardfish kidney had optimal conditions at a temperature of 50°C and decreased at 60°C and no activity was found at 70°C, at high temperatures it was suspected that the enzyme was denatured and lost its activity [15]. The pure TMAOase enzyme extract from meat walleye pollack was a thermostable protein, which continued to be active even after 80°C heating for 30 minutes [22]. The optimum temperature difference for TMAOase enzyme activity from several fish species differ depending on habitat temperature and the purity level of the enzyme [17].
4.2.3. Effect of TMAOase cofactor. The results of testing the relative activity of the TMAOase enzyme in various types of cofactors are presented in table 1. The results of testing various types of cofactors in the TMAOase enzyme showed that the combination of cysteine added with ascorbate and FeCl₂ obtained the highest activity value of 100%. These results indicate that a mixture of cysteine, ascorbate, and FeCl₂ is the best mixture to increase TMAOase enzyme activity compared to other types or mixtures of cofactors. These results also prove that Fe metal ions can increase the activity of TMAOase enzymes so that they can be classified as activators. Cofactor compounds in the form of metal ions that have the potential to increase enzyme activity can be called activators, while metal ions that inhibit or weaken enzyme activity are called inhibitors [23].

| Cofactor | Relatively of enzyme activity TMAOase (%) |
|----------|------------------------------------------|
| Cysteine (2 mM) and FeCl₂ (0.2 mM) | 64.2 |
| Ascorbate (2 mM) and FeCl₂ (0.2 mM) | 89.5 |
| Cysteine (2 mM) and Ascorbate (2 mM) and FeCl₂ (0.2 mM) | 100 |

These results are consistent with research the TMAOase enzyme from the lizardfish kidney, which produced a relative activity value of 100% when adding cysteine, ascorbate and FeCl₂ [15]. The enzyme TMAOase which extracted from meat walleye pollack then reacted with a mixture of cysteine, ascorbate, and FeCl₂ to produce a relative activity of 100% [24]. The metal ions act as a reaction stabilizing agent between enzymes and substrates. Fe metal ions can increase the activity of the TMAOase enzyme because it helps stabilize the transition state in the reaction between enzymes and the substrate so that the enzyme complex of the substrate that is formed cannot be decomposed and tends to react to form the product [25]. The cysteine and ascorbate function to maintain Fe to react optimally to TMAOase enzyme. Cysteine and ascorbate are Fe reducing agents, so they can keep Fe maximum [24].

4.3. Purification of TMAOase
TMAOase enzyme purification consists of several stages: crude enzyme extracts, acid treatment, heat treatment and dialysis (table 2). The greater the value of specific activities shows the higher level of purity. The enzyme specific activity of crude extract TMAOase gets the lowest value of 3.60 U/mg because there are still many impurities contained in it. The presence of several inhibitors in coarse enzymes can reduce the specific activity of TMAOase enzymes [22]. The results of this study are consistent with the result of research showed that the value of the enzyme specific activity of crude TMAOase from the lizardfish kidney had the lowest value of 4.07 U/mg [15].

| Step of purification | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Fold | Yield (%) |
|----------------------|-------------------|-------------------|--------------------------|------|-----------|
| Crude extract        | 222.97            | 61.92             | 3.60                     | 1.00 | 100.00    |
| Acid treatment       | 187.26            | 45.10             | 4.15                     | 1.15 | 83.98     |
| Heat treatment       | 9.24              | 1.83              | 5.11                     | 1.42 | 4.14      |
| Dialysis             | 6.94              | 1.47              | 7.38                     | 1.78 | 3.71      |

TMAOase enzyme purification then proceeds to the acid treatment stage. The acid treatment stage in the study serves to reduce proteins that are not cooled in the crude enzyme extract. This stage is proven to be able to increase the specific activity of the enzyme TMAOase from 3.60 U/mg to 4.15 U/mg. The acid treatment can remove impurity proteins so that it can increase the purity of TMAOase enzymes [15].

The specific activity of TMAOase extracted from walleye Pollack increase from 0.049 U/mg to 3.35
U/mg [24]. The acid treatment in enzyme purification can be used in removing unwanted proteins thus increasing the purity of the TMAOase enzyme [17].

The purification stage then continues to the heat treatment stage. The heat treatment in this study was proven to increase the specific activity of the TMAOase enzyme, from 4.15 U/mg to 5.11 U/mg. The heat treatment can also increase purification by 1.42 times. The heating at high temperatures proved to denature various proteins, but the TMAOase enzyme would not be damaged by this warming. TMAOase is not damaged by heating due to its thermostable nature, which is resistant and stays active in hot temperatures [18].

The results of TMAOase from squid also increase the activity after extraction using heat treatment stage. The specific activity increase from 2.39 U/mg to 6.78 U/mg. Heat treatment in this study uses a temperature of 80°C which aims to remove impurities from coarse enzymes [17]. The dialysis phase is the last stage of purifying the TMAOase enzyme in this study. Dialysis was performed using a prepared 12 kDa dialysis bag. Dialysis is the transfer of molecules from dissolved substances into other substances through a semipermeable membrane. This displacement is influenced by molecular concentration, membrane area, and solution volume. The purification results of TMAOase enzyme by dialysis method in this study can increase specific activity, i.e from 5.11 U/mg to 7.38 U/mg. Dialysis can also increase purity up to 1.78 times. This is because impurities which can affect the purity of the enzyme have been removed in the previous stage.

4.4. Molecular weight of TMAOase
The molecular weight of crude extract, acid treatment, heat treatment and dialysis of lizardfish can be seen in figure 4. SDS-PAGE test results in Figure 4 showed that the TMAOase enzyme still has many protein bands, which means that there are still many protein molecules derived from other impurity cells and proteins. Acid treatment and heat treatment can eliminate impurity proteins. This is indicated by the reduced amount of protein at each stage of purification which indicates that the protein becomes purer. TMAOase that has been dialyzed show one band with a molecular weight of 20 kDa (figure 4). This means that the enzyme is pure.

The TMAOase enzyme extracted from meat walleye pollack has a molecular weight of 25 kDa [22]. The molecular weight of the enzyme TMAOase lizardfish is smaller than the TMAOase enzyme from other tissues, for example, the lizardfish kidney with a molecular weight of 128 kDa [15], and European hake kidney with a molecular weight of 30-100 kDa [25]. The TMAOase enzyme extracted from squid has a molecular weight of 17.5 kDa [17].

![Figure 4. Electrophoresis results of molecular weight of the Beoso fish TMAOase enzyme, M = Marker, C = rough extract, A = acid treatment, H = Heat treatment, and D = Dialysis.](image-url)
5. Conclusion

TMAOase enzymes have been successfully extracted, purified and characterized from the muscle of lizardfish. The TMAOase enzyme of lizardfish has the highest activity value at optimum conditions pH 7, optimum temperature 50ºC, and cofactor combination of cysteine, ascorbate, and FeCl2 can increase TMAOase enzyme activity. The TMAOase enzyme can be purified by several stages, namely, acid treatment with the addition of 1 M HCl to pH 4.5, heat treatment using a temperature of 80ºC, and dialysis for 2 hours with a purity level of 1.78 times. TMAOase enzyme muscle of lizardfish has a molecular weight of 20 kDa.

Acknowledgement

A part of this research was funded by RISTEKDIKTI through Division research grants at 2016.

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