Characterization and Effect of Processing on Parvalbumin Content in Indonesian Shortfin Eel *(Anguilla bicolor bicolor)*

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Abstract

Indonesian shortfin eel (*Anguilla bicolor bicolor*) is a high economic value fish and have a high nutritional content. However, their utilization can be limited because of hypersensitivity cases in individuals allergic to fish. This study aimed to determine the characteristics of the fish major allergenic protein, parvalbumin in different parts of the eel fillet and the effects of boiling technique on the content of this protein. The samples were boiled with 100 mL of water in an Erlenmeyer flask at 95°C for 10 min. The eel fillet was divided into three parts: the front, middle, and rear part of the body. Protein profiles from each part were identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Bradford assays. Parvalbumin was further purified by ammonium sulfate precipitation. The concentration of protein in the different parts of the eel was not significantly different, whereas the processing treatments (meat with water (F1 extract) and meat without water (F2 extract)) affected protein concentration. Eel meat contained proteins with molecular weights ranging from 10 to 186 kDa. Parvalbumins are highly water-soluble, as their content was reduced in the fillet, but were observed at a high concentration in the water after boiling. Parvalbumins of eel were purified by ammonium sulfate 70-90% with high purity. Interestingly, two different bands were observed in SDS-PAGE, suggesting the presence of a protein variant. The molecular weight of parvalbumin obtained from purification ranged from 10 to 11 kDa, similar to that of other fish.

Keywords: allergen, molecular weight, parvalbumin, protein, purification

Introduction

Fish are animal protein sources that are widely consumed by the community and have a high nutritional value. The national fish consumption rate reached 56.48 (kg) per capita in 2022 (Ministry of Marine Affairs and Fisheries, 2022). The average fish consumption in 2022 increased by 2.39% compared to the previous year, which was also the highest recorded in the last decade. One of the fish species that is widely consumed, high in protein and has economic value is Indonesian shortfin eel (*Anguilla bicolor bicolor*).

Indonesian shortfin eel has high nutritional value and is known as an export commodity. The Ministry of Marine Affairs and Fisheries statistics for 2020 state that Indonesia's total exports of eel reached 9,676 tons, which increased from the previous year to approximately 7,763 tons. Eel is very popular in many countries, one of which is Japan. Eel fish have high fat and protein contents. Wijayanti & Susilo (2018) reported that the protein content of eel reached 18.09%. Nafsiyah et al. (2018) stated that eel has a high nutritional content, especially vitamin A, vitamin E, and polyunsaturated fatty acids (EPA and DHA). *A. bicolor pacifica* has a water content of 71.1%, crude protein content of 17.4%, EPA content of 3.28% and 2.93% DHA (Ahn et al., 2015). Eels also have different nutritional contents in each part of their body. This is in line with the statement by Wijayanti & Susilo (2018) that eel body parts, including the head, flesh, and rear, have different nutritional contents. However, several cases of allergy due to eel proteins have been reported. Tamura et al., (2018) reported a case of Japanese woman experiencing an allergy to baked eel. Other studies have demonstrated eel specific IgE in groups of adults and children having food allergy with atopic

dermatitis and/or respiratory symptoms (Sten et. al., 2004; de Martino et. al., 1990).

Allergy is a disease caused by an abnormality in the human immune system which considers fish protein to be a dangerous substance for the body. Fish allergy is caused mainly by a protein called parvalbumin, in addition to other fish proteins such as aldolase, enolase, and collagen (Stephen et al., 2017). Parvalbumin is responsible to over 95% of fish allergy. Parvalbumin belongs to a group of muscle proteins that bind calcium, is acidic and water soluble, has molecular weights ranging from 10 to 13 kDa, and contains six α-helical structures and short β-sheet segments (Stephen et al., 2017). The binding activity towards calcium correlate with muscle relaxation. Thus, migratory fish showed a lower parvalbumin content than sedentary fish (Kobayashi et al., 2016).

Processing can change the structure and chemical properties of proteins including parvalbumin. During the processing process, proteins can undergo denaturation and aggregation and bind to fat which affects changes in their allergenicity, especially for certain allergies that are labile to processing (Verhoechx et al., 2015). This type of change occurs because of several factors, such as time, environment, type of food, and type of allergen (Cabanillas & Novak 2017). Kuehn et al. (2010) showed a decrease in parvalbumin content due to the boiling process in fish herring and carp that is higher than in fish trout, salmon, *cod*, mackerel, and tuna. Nugraha et al. (2020) showed a decrease in the parvalbumin content in surimi by up to 95% after the washing process, but the heating process with the addition of enzymes did not affect the decrease in parvalbumin. Sletten et al. (2010) showed a decrease in serum IgE binding capacity in Atlantic cod following immersion and salting. Unfortunately, few studies on eel allergens and processing effect on these allergens have been found. Therefore, this study aimed to determine the characteristics of the fish major allergenic protein, parvalbumin in different parts of the eel fillet and the effects of boiling technique on the content of this protein.

Material and Methods

Materials

The materials and equipment used include samples of eel fillet obtained from an eel farming site in Ciampea, Bogor, ammonium sulfate, phosphate buffered saline (PBS) solution, laemmli sample buffer (Bio-Rad, USA), coomassie brilliant blue (Bio-Rad, USA), tris-HCl 1.5 M pH 8.8 (Bio-Rad, USA), tris-HCl 0.5 M pH 6.8 (Bio-Rad, USA), ammonium persulfate (APS) 10% (Bio-Rad, USA), TEMED (Bio-Rad, USA), 5% acetic acid solution, 20% isopropanol, 10% SDS solution, destaining solution. The required equipment included a homogenizer (Omni, USA), spectrophotometer UV-VIS, SDS-PAGE (Bio-Rad, USA), rocker shaker (BioSan), centrifuge (HERMLE), photocap software, laptop (LENOVO), and various glassware.

Sample Preparation

The eels used in this study were obtained from an eel farming site in Ciampea, Bogor. The size of the eel fish used in this study was about 30 cm. The fish were collected in a fresh filleted state. The fish were separated into three equal parts: front, middle, and rear portions of the flesh (Figure 1).

Extraction of Parvalbumin

Parvalbumin protein is typically water-soluble and requires an extraction process to separate from meat matrices (Nugraha et al., 2020). The extraction was performed using 20× PBS (pH 7.2) stored at the chilling temperature. The eel samples were divided into three parts: the front (1), middle (2), and rear (3). Each sample (20 g) was finely chopped. The meat was then placed in an Erlenmeyer flask and supplemented with 100 mL PBS. The mixture was homogenized using a homogenizer for 10 min, followed by extraction using a rocker shaker at 4°C for 24 h. The mixture was then centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was then filtered using a filtration

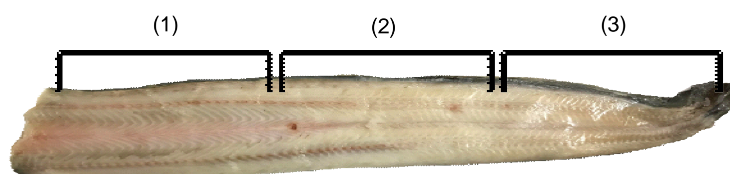

Figure 1. Eel meat section to determine parvalbumin. Notes: (1) front, (2) middle, and (3) rear.

apparatus. The filtered supernatant was then stored in 50 mL tubes at -20°C as raw extract (A). The boiled samples were divided into two groups: meat with water as F1 extract (B) and meat without water as F2 extract (C). Meats were boiled with 100 mL of water in an Erlenmeyer flask, then the treatment of meat without water, the water was excluded, while meat with water and water was still included. The samples were heated at 95°C for 10 min before centrifugation. The samples were then centrifuged at 10,000 × g for 30 min at 4°C. The resulting boiled extract was filtered and stored in 50 mL tubes at -20°C. Samples were analysed for molecular weight using SDS-PAGE (Laemmli, 1970). The final result of the SDS-PAGE was the formation of protein bands on an acrylamide gel. These bands indicate the molecular weight of the proteins and the thickness of the bands reflects the concentration of the proteins. Protein concentration was also analysed using the Bradford method (Bradford, 1976). The total protein concentration was measured calorimetrically in the solution. Coomassie brilliant blue (CBB) dye was used in the Bradford method, which imparts a blue color upon binding to proteins in an acidic solution. The absorbance was measured using a spectrophotometry at a wavelength of 595 nm.

Purification of Parvalbumin

The extracted samples were purified using ammonium sulfate precipitation (Ruethers et al., 2018). The amount of ammonium sulfate was determined using the website http://www.encorbio.com/protocols/AM-SO4.htm. The crystalline form of ammonium sulfate was first crushed and then transferred into 2 mL microtubes. Ammonium sulfate was weighed in the ranges of 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, 80–90%, and 90–100%, which corresponded to the predetermined saturation ranges. The extraction samples, totalling eight microtubes, were added to 1 ml each. The samples were sequentially mixed with ammonium sulfate, homogenized, and incubated at 4°C for 1 h on a rocker-shaker. The mixture was then centrifuged at 10,000 × g at 4°C for 15 min. The supernatant was carefully poured into 2 mL microtubes. Next, ammonium sulfate was poured again for a second time, followed by centrifugation at the same speed, temperature, and duration. The supernatant from the sample was separated from the precipitate and the precipitate was stored at -20°C until further use. Samples were analysed for molecular weight using SDS-PAGE (Laemmli, 1970), and protein concentration was analysed using the Bradford method (Bradford, 1976).

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with a 95% confidence interval ($\alpha = 0.05$). Significant results were further tested using Duncan's test. The data were processed using the Excel 2007 application.

Results and Discussion

Protein Concentration in Different Parts of Eel (*A. bicolor bicolor*)

Protein concentration analysis of different parts of the eel is presented in Figure 2.

Protein concentrations in the front, middle, and rear parts of the raw extract were 0.486 ± 0.01, 0.510 ± 0.02, and 0.516 ± 0.03, respectively. The protein concentrations in the front, middle, and rear parts of the F1 extract were 0.258±0.06, 0.231±0.01, and 0.249±0.03, respectively. The protein concentrations in the front, middle, and rear parts of the F2 extract were 0.237±0.04, 0.270±0.03, and 0.292±0.02, respectively. The ANOVA results for protein concentration in eels with treatments of the front, middle, and rear parts showed no significant effect on the soluble protein values ($p>0.05$). The result showed no significant because from the same sample (meat eel). The protein concentrations in the body of the eel (*A. bicolor bicolor*) reported by Nafsiyah et al. (2018) reached 16.78%. High protein content in eels is essential for meeting the nutritional requirements of the human body. The protein concentrations in fish generally increases with fish size. Suwandi et al., (2014) state that the protein concentrations in fish meat can be influenced by several factors, such as fish species, diet, habitat, and food availability.

Protein Concentration in Processed Eel (*A. bicolor bicolor*)

The results of protein concentration analysis are shown in Figure 2. Fresh eel had the highest protein concentrations, but the protein concentrations decreased after boiling. The ANOVA results for fresh and boiled eels showed a significantly different protein concentration. Based on Duncan's post-hoc test, there was no significant difference between F1 extract and F2 extract. This is consistent with Utami et al. (2016), who stated that the protein concentrations in seluang fish after several cooking processes were lower compared to that in fresh seluang fish. A decrease in protein concentrations may occur because boiling involves a high temperature. Boiling at high temperatures causes loss of free water and coagulation, resulting in a denser meat texture. Simultaneously, the proteins undergo denaturation to form simpler structures. This process leads to a decrease in the protein content of a substance. This is supported by Erkan & Ozden (2011),

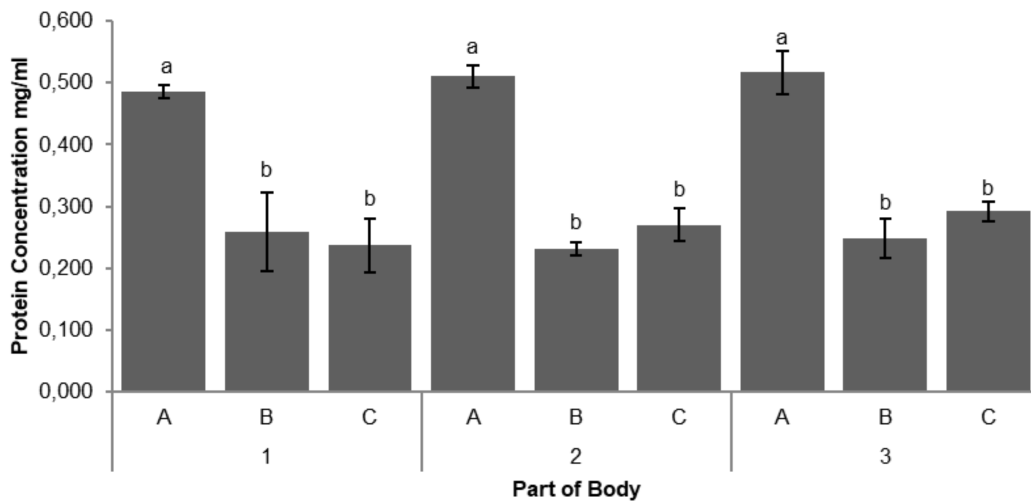

Figure 2. Soluble protein concentration in various parts of the eel body with different processing methods. Notes A= raw extract; B= F1 extract; C= F2 extract; 1,2,3= front, middle, and rear; a and b: significantly different; b and b: not significantly different.

who stated that heat causes some proteins in meat to be lost along with the water that comes out of the meat. The most influential factors affecting the level of damage during the heat treatment were the heating temperature and duration (60-90°C at 1 hour).

Protein Molecular Weight in Different Parts of Eel (*A. bicolor bicolor*)

The specific molecular weight of the protein was measured using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The SDS-PAGE results for different treatments of eel (*A. bicolor bicolor*) are shown in Figure 3.

Analysis of the protein profiles in different parts of the eel showed that the molecular weight of the proteins ranged from 10 to 186 kDa, there are parvalbumin (10-13 kDa), tropomyosin (29.7-36.3 kDa), actin (37.8-46.2), and myosin (180-220 kDa). The number of protein bands observed in the front, middle, and rear parts of the raw extract was 22, 19, and 21, respectively. In the F1 extract, the number of protein bands in the front, middle, and rear parts was 8, 6, and 7, respectively, whereas in the F2 extract, the number of bands was 14, 11, and 13, respectively. Based on these results, it can be concluded that the division of the eel into front, middle, and rear parts does not significantly affect its protein concentration. Visualisation of protein bands indicated the concentration of proteins present. Thicker bands represent higher protein concentrations, whereas thinner bands indicate lower protein concentrations. The thickness or thinness of the bands was determined by the number of migrated protein molecules, with thicker bands representing the fusion of multiple bands. The F1 extract showed thicker parvalbumin bands (10-13 kDa) than the F2 extract, suggesting higher parvalbumin cocentration in the former. This is likely because parvalbumin is soluble in water, and therefore, boiling water retains a higher concentration of parvalbumin. The F2 extract showed thinner parvalbumin bands because the water used for boiling was not included in the analysis. In summary, the analysis revealed that the different parts of the eel did not have a significant influence on protein content. Visualisation of protein bands provides insights into the concentration of proteins, with thicker bands

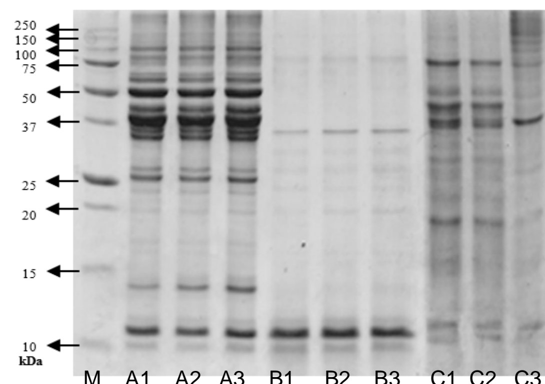

Figure 3. Eel protein profile by SDS-PAGE. M= 10-250 kDa marker; A= raw extract; B= F1 extract; C= F2 extract; 1,2,3= front, middle, and rear.

indicating higher protein concentrations. The presence of thicker parvalbumin bands in the F1 extract suggests a higher parvalbumin content owing to its solubility in water.

Protein Molecular Weight in Processed Eel (*A. bicolor bicolor*)

The protein content in the SDS-PAGE bands was quantified by measuring the intensity using a Photocapt application. The intensity of the bands was visualised as peaks, with thicker bands corresponding to higher peaks. The peaks in the analysis represent the separated polypeptide bands obtained by SDS-PAGE, based on their molecular weight. The height of the peak represents the intensity of polypeptide bands. The broader the area under the curve, the wider the band formation. Higher peaks and larger areas under the curve indicated the dominance of specific polypeptide bands in the sample. Based on our research findings (Figure 3), the dominant molecular weights in the fresh eel extract were approximately 50, 30, and 10 kDa. The studies conducted by Kuehn et al. (2013), Fernandes et al. (2015), Mohammadi et al. (2016) and Ruethers et al. (2020) identified bands in the range of 50 kDa, 40 kDa, and 12 kDa as major allergens in several fish species (parvalbumin and tropomyosin allergen). After eel was cooked, some proteins might dissolve in the buffer, resulting in the disappearance of certain bands.

The protein profile results of the fresh eel treatment showed a higher number of formed bands compared to the boiled treatments, whereas the F2 extract had a higher number of bands than the F1 extract. This indicates that different processing methods affect the separation of protein bands, resulting in specific molecular weights. Nugraha et al. (2021) stated that the heating process can reduce the dominance of certain protein bands while also increase the intensity of new protein bands. The fresh eel extract exhibited a molecular weight range of 10-186 kDa, indicating the presence of high-molecular-weight molecules (>150 kDa) in the fresh extract. The molecular weight of the F1 extract from 10 to 79 kDa, whereas of that the F2 extract has a molecular weight from 10 to 133 kDa. The decrease in the number of protein bands was attributed to the boiling process.

Parvalbumin Content

Parvalbumin is the main protein responsible for fish allergies. It is a sarcoplasmic protein with a molecular weight of 10-13 kDa and consists of six á-helical structures and short â-sheet segments. Parvalbumin functions by binding calcium during muscle contraction. The parvalbumin protein content in the eel (*Anguilla bicolor bicolor*) is shown in Figure 4.

The results showed that there were increases and decreases in the intensity of eel meat with different processing methods. The parvalbumin content decreased in the raw extract and F2 extract. This is consistent with the findings of Kuehn et al. (2010), who reported a decrease in parvalbumin content due to boiling in herring and carp. However, parvalbumin content increased in the F1 extract. This is likely because boiling can remove heat-labile proteins, thus increasing the proportion of heat-labile proteins, including parvalbumin. Kuehn et al. (2010) stated that fish of the same species but with different processing methods can yield different changes in parvalbumin content. Basler et al. (2001) suggested that the denaturation process can expose linear epitopes within the secondary structure of proteins, potentially increasing their allergenicity. Mostashari et al. (2023) stated that certain heat processing methods, such as boiling, frying, and grilling, can even lead to the formation of new epitopes. Boye & Godefroy (2010) mentioned that allergens from animals (including fish and shellfish) generally have high thermostability, meaning that their allergenic properties may not be eliminated and can even increase after heating. Therefore, modifications that occur during processing can not only damage epitopes but also mask, alter, or expose them, resulting in a reduction or increase in allergenicity (Rao & Jiang, 2021).

Profile of Eel Parvalbumin after Ammonium Sulphate Precipitation

The results of the parvalbumin protein content stated that the F1 extract had a higher parvalbumin protein content than the F2 extract, so the purification process used a sample of the F1 extract. In this study, purification was performed using the ammonium sulfate precipitation technique with the raw and F1 extracts. This technique is commonly used to isolate water-soluble proteins from plants and animals. The results of purification of the raw and F1 extracts are shown in Figure 5.

The addition of ammonium sulfate reduced the amount of solvent interacting with protein molecules in the solution. Proteins with lower molecular weights have higher solubilities. Sha et al. (2014) reported that low concentrations of ammonium sulfate precipitate proteins with higher molecular weights. The results showed the presence of high-molecular-weight protein bands in the purification results with low saturation levels (Figure 5). Proteins with higher molecular weights showed decreasing concentrations with increasing ammonium sulfate concentrations. This can

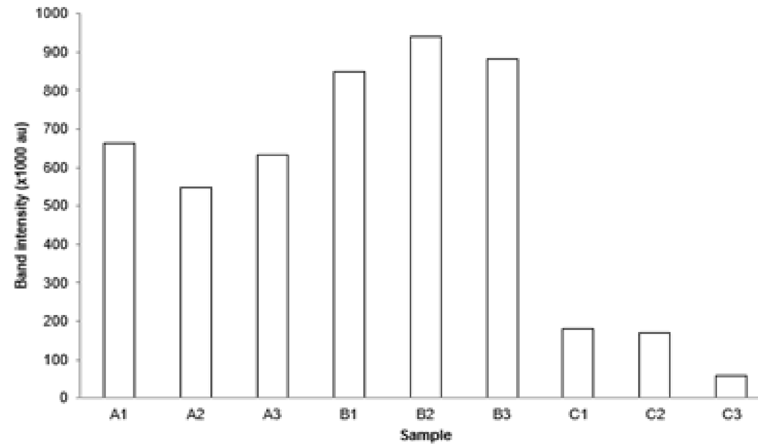

Figure 4. Parvalbumin band intensity in the histogram. A= raw extract; B= F1 extract; C= F2 extract; 1,2,3= front, middle, and rear.

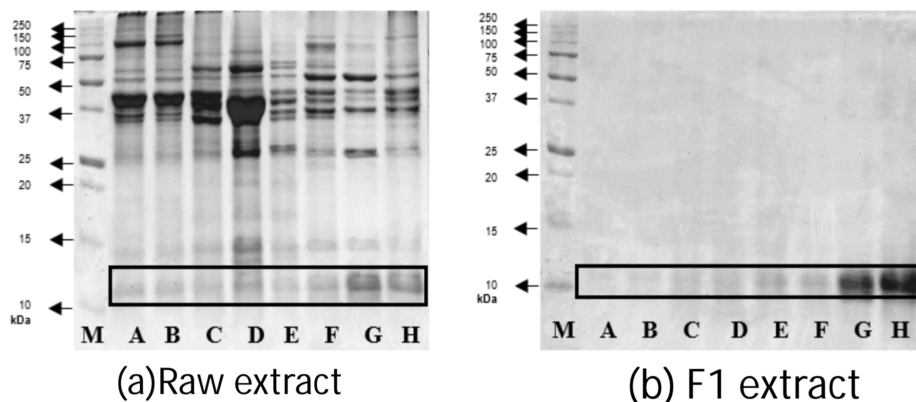

Figure 5. Parvalbumin protein profile after purification with ammonium sulfate. Notes: M= 10-250 kDa marker; saturation; A= 20–30% saturation; B= 30–40% saturation; C= 40–50% saturation; D= 50–60% saturation; E= 60–70% saturation; F= 70–80% saturation; G= 80–90% saturation; H= 90–100% saturation.

be observed by a decrease in the number of protein bands with molecular weights >15 kDa from the saturation range of 60–70% to 90–100%.

The selected ammonium sulfate saturation for the raw and F1 extract was 70-90%. This result is consistent with the study conducted by Sun et al. (2019), who used ammonium sulfate saturation of 60–90% for Japanese Flounder (*Paralichthys olivaceus*) protein samples. The parvalbumin band obtained in the purification results was approximately 10–11 kDa. Sun et al. (2019) identified three parvalbumin isoforms with molecular weights of 10-14 kDa. The purification results indicated that the boiling treatment did not eliminate parvalbumin. According to Yu et al. (2015), heating treatment aims to obtain high-purity parvalbumin by removing fat and other substances from the sarcoplasmic protein.

Conclusion

The different parts of the eel did not contain a significantly different dissolved protein concentration, whereas the processing method had an effect on the protein concentration. Parvalbumin is widely found in cooking water because of its water solubility. The obtained parvalbumin molecular weight ranges from 10 to 11 kDa similar to the content of other fish. Boiling fish without water can reduce the risk of allergies, although this requires further research.

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Supplementary Materials

Supplementary materials is not available for this article.

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