

Characterization of antigenicity of the surimi and kamaboko protein of Japanese threadfin bream (*Nemipterus japonicus*)

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Abstract. Japanese threadfin bream (*Nemipterus japonicus*) contains highmyofibril protein which can be used as raw material in processing of surimi and kamaboko, however both of those products are able to cause allergies in people who have hypersensitivity to allergenic proteins. Parvalbumin is a sarcoplasmic protein which is the main source of allergies in fish. The washing process in surimi processing can remove sarcoplasmic proteins. Therefore, this research was conducted to determine the allergenic protein content in each processing process of surimi and kamaboko Japanese threadfin bream fish. The protein profiles of surimi and washing water were visualized using *sodium dodecyl sulphate polyacrylamide gel electrophoresis* (SDS-PAGE) and total parvalbumin protein was calculated using *enzyme-linked immunosorbent assay* (ELISA). The SDS-PAGE results showed that the washing process removed several allergens as indicated by a decrease in the protein band at a molecular weight of 10-13 kDa and for ELISA the absorbance value decreased with the number of washing processes in surimi processing. The content of parvalbumin and other allergy-causing proteins such as aldolase A and β -enolase decreased after the meat underwent the washing process.

1 Introduction

Indonesia is a country with two-thirds of the country's territory with a water Indonesian fishing commodities, including quite abundant with various types of fish caught for consumption. The rate of fish consumption in Indonesia in 2021 reached the value of 55.37 kg year⁻¹ capita⁻¹ which increased from 2020 which was 54.56 kg year⁻¹ capita⁻¹ [1]. Japanese threadfin bream or kurisi (*Nemipterus japonicus*) is one of the economically important fish in the Nemipteridae family.

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These fish do not migrate naturally and live in waters with mud or sand substrate [2]. Japanese threadfin bream is commonly used by consumers and sold in fresh form, fermentation, basic production of surimi, fish flour, kamaboko and dried salt. The result of recording fisheries catch statistics data by the Ministry of Marine Affairs and Fisheries (KKP) on fisheries production caught is known to have increased from 1813.11 tons in 2017 to 5154.55 tons in 2019.

Japanese threadfin bream has a characteristic myofibril protein which can be used as a raw material that is often used in surimi processing. Surimi or minced meat is a myofibril protein concentrate product from raw fish white meat [3]. Surimi is a fish protein concentrate obtained by washing the fish to remove blood and most of the fat, enzymes and proteins from the sarcoplasm. With its unique gel and emulsification properties, surimi can be processed into various productions of fish balls, fish tofu, cikuwa shrimp sticks or other seafood analogues marketed in the market [4]. The raw material for surimi is generally chosen from marine fish with white flesh because it can produce surimi with good gel quality and color [5]. The type of fish that is often used as raw material for surimi production in Indonesia comes from fish with low economic value, for example catfish, sea bream (*kurisi*), sulfur goatfish (*kuniran*), purple-spotted bigeye (*swanggi*), greater lizardfish (*beloso*) and lefttail croaker (*gulamah*) [6].

Fish is included in foods that have high protein content. Muscle proteins in fish can be categorized into three groups, namely sarcoplasmic, myofibril, and stroma or connective tissue proteins. Myofibril protein is a long fibril protein that is considered the main component of skeletal muscle which accounts for 60-70% of total muscle protein. This protein mainly consists of myosin (thick) and actin (thin) components [7]. Myofibril proteins play an important role in producing meat products with the required functional characteristics namely gel strength [8]. Myosin is the main component of myofibrillar protein which has an effect on the three-dimensional structure of meat protein and gelation of meat protein. Myosin is the most abundant and asymmetric myofibrillar protein molecule, with two heavy polypeptide chains and four light polypeptides. Actin is another major component of myofibrillar proteins that has an important role in strengthening the myosin gel structure [9].

Processing can influence and change the structure and chemical properties of a protein. During the processing process, the quality of myofibrillar proteins is considered to be the most important aspect because it influences the intermolecular interactions of proteins [10]. The washing process in surimi production will dissolve water-soluble proteins or sarcoplasm, one example is the sarcoplasmic protein parvalbumin. Parvalbumin is a fish muscle protein that binds calcium with a molecular weight of 10-13 kDa. Protein is the main allergen that contributes to more than 95% of allergy cases caused by fish in the world. Surimi processing, especially in the washing and salting process, is thought to change the structure, gelation, function, physicochemical properties, and fish allergies. Research conducted by Abidin [11] shows that the washing process in surimi production can reduce parvalbumin levels using SDS-PAGE as a detection method, but the detection limit is still high. With the background explained, an attempt was made to compare myofibril protein with meat protein in the surimi production process and an ELISA test was carried out to detect parvalbumin as a substitute for SDS-PAGE because the detection limit was still high.

2 Methods

This research uses several materials and tools. The main ingredient used in this research was a sample of Japanese threadfin bream obtained from Muara Baru, Penjaringan, North Jakarta. Other materials used are ice cubes, CaCl₂, PBS solution (Phosphate Buffer Seline), 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-RadUSA), ammonium persulfate (APS) 15% (Bio-Rad, USA), TEMED (Bio-Rad, USA), acetic acid 5%, isopropanol 20%, SDS 15%, destaining solution, bromophenol blue (BPB), KCl, NaCl, urea, β-mercaptoethanol, AgraQuant Fish Elisa Plate (Romer Labs). The tools used in this research include kitchen equipment (scalpel, basin, cutting board), test tubes (IWAKI), Erlenmeyer (IWAKI), digital scales (OHAUS AX225 New Jersey, America), homogenizer, UV-Vis instrument spectrophotometer (RS spectrophotometer UV-2500), centrifugation (FC5178R1220V), electrophoresis equipment (PEQ Lab, Erlangen, Germany), photocapt software, Image Studio 5.2 version.

2.1 Sample preparation

The samples used in the study included the fresh meat, the first wash, the second wash, and the washing water. The production of surimi begins with the separation of the flesh from the skin, the bones, the viscera and the head. The meat was then minced using a grinder. The meat was then washed with ice water (4 °C) with a water ratio to meat 4:1 and mixed for 15 min. The washing step was carried out twice. The surimi was further processed to make kamaboko by adding calcium chloride and subsequently heated at 40 °C for 30 minutes and 90 °C for 20 minutes.

2.2 Protein extraction

Protein from fish tissues was extracted according to the previous method [12] with minor modifications. Samples of bream fish meat (BM), first washed meat (WM1), second washed meat (WM2) and kamaboko (KC) were homogenized in phosphate buffer solution (0.02 mmol/L PB, pH 7 with 0.6 mol /L KCl) using a homogenizer. The homogenized solution was centrifuged at 8000 rpm for 20 minutes at 4 °C using a refrigerated centrifuge. The supernatant resulting from centrifugation was filtered using Whatmann filter paper No. 1 and the resulting filtrate was designated as myofibril protein. The obtained myofibril proteins were stored at -20 °C. Additionally, protein from washing water was used directly without any extraction and designated as washing water 1 (WW1) and washing water 2 (WW2).

2.3 Chemical composition

The chemical composition of bream fish was analyzed following standardized procedure according to AOAC 2005 [13]. Five components were analyzed including moisture, fat, protein, ash and carbohydrate.

2.4 Turbidity

Determination of turbidity of myofibril protein samples was carried out based on [12]. A concentration of 0.5 mg/mL myofibril protein was prepared in 50 mmol/L

phosphate buffer (0.6 mol/KCl, pH 7.0) and the turbidity of the myofibril protein was measured at 360 nm using UV-VIS spectrophotometer.

2.5 Surface hydrophobicity

Surface hydrophobicity of the myofibrillar protein samples was measured following Wang et. al. [12]. In brief, one milliliter of protein extract was mixed with 200 μ L bromophenol blue (BPB) solution (0.1% (b/v) in distilled water). The control sample was prepared using 50 mmol/L (pH 7) phosphate buffer solution and 200 μ L BPB. The mixture was shaken at 37 °C for 10 minutes in a water bath shaker. Subsequently, the sample was centrifugated for 15 minutes at 2000 rpm. The supernatant absorption was read at 595 nm. The number of BPB bonds as an indication of was calculated using the following formula:

$$\text{Amount of BPB bound } (\mu\text{g}) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 200 \mu\text{g} \quad (1)$$

2.6 Determination of chemical bonds

Chemical bonds in myofibril proteins were determined using methods [14]. Myofibril protein samples were treated with different buffer solutions due to their capacity to disrupt different types of specific bonds. The buffers used are (A) 0.05 mol/NaCl, (B) 0.6 mol/L NaCl, (C) 0.6 mol/L NaCl + 1.5 mol/L urea, (D) 0.6 mol/L NaCl + 8 mol/L urea, and (E) 0.6 mol/L NaCl + 8 mol/L urea + β -mercaptoethanol. A 1 g myofibril protein sample was added to 9 mL of each buffer solution and homogenized for 2 minutes using a homogenizer. The resulting solution was stirred for 2 minutes followed by centrifugation at 4 °C, 8000 rpm for 10 minutes. The protein concentration in the supernatant was estimated by the method of Lowry et. al. (1951). Chemical bonds, namely ionic bonds, hydrogen bonds and sulfide bonds, are calculated by the difference in protein content between buffer solutions A and B (ionic bonds), C and B (hydrogen bonds) E and D (disulfide bonds), respectively.

2.7 Molecular weight determination

Determination of molecular weight using SDS-PAGE based on Laemmli [15]. The sample was dissolved in 5% SDS and then incubated for 1 hour at 85 °C in a temperature-controlled water bath. The mixture was centrifuged at 8500 x g for 5 minutes at room temperature. The supernatant obtained was then mixed with buffer (Tris HCL 60 mM, pH 6.8, 2% SDS and 25% glycerol with a ratio of 1:10 v/v and containing 10 β -mercaptoethanol β -ME). The mixture was then heated in boiling water for 2 minutes. Next, 15 μ L of sample was put into a polyacrylamide gel consisting of 15% running gel and 15% stacking gel. Electrophoresis for 2 hours at a constant current of 50 mA/gel. After that, the gel was marked with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 1 hour, then the sample was marked with a 30% mixture (v/v) methanol and 10% (v/v) acetic acid for 1 hour. Destained is carried out again for 30 minutes.

2.8 Determination of allergen content

Determination of parvalbumin content was determined using the AgraQuant fish ELISA testkit from Romer Labs [16]. The first procedure was to prepare the test sample by dissolving it in extraction buffer with a sample to buffer ratio of 1:20

(buffer solution is diluted 1:10 using distilled water) then vortexing. Samples that has been dissolved in buffer are incubated for 15 minutes at a temperature of 60 °C then centrifuged for 10 minutes at a speed of 2000 g. The supernatant was taken from the centrifugation results and then 100 µL was taken into a microwell that had been coated with antibodies. Samples inserted into the microwell were incubated for 20 minutes at room temperature. The next process is washing 5 times using washing buffer (washing buffer is first diluted using distilled water with a ratio of 1:10), then add 100 µL of conjugate buffer and incubate at room temperature for 20 minutes and after that wash 5 times using buffer washer. The next step, 100 µL of substrate buffer solution was put into a microwell and then incubated in a dark place for 20 minutes. The stop solution was prepared in 100 µL and injected into each sample. Then read the absorbance of each well within 10 minutes after adding the stop solution at 450 nm using an ELISA reader.

2.9 Statistical analysis

One-way ANOVA was used to analyze the data. The experimental design used was a completely randomized design with treatment of different types of meat during the making of surimi kamaboko (BM, WM1, WM2, WW1, WW2, and KC) with three replications. The data obtained then analyzed using ANOVA with Duncan's advanced test at the 5% level using Statistical Process for Social Science (SPSS) software.

3 Results and Discussion

The chemical composition of Japanese threadfin bream can be determined using proximate analysis. Proximate analysis carried out on Japanese threadfin bream included levels of protein, fat, ash, water and crude fiber. The results of the analysis of the chemical composition of Japanese threadfin bream can be seen in Table 1.

Table 1. Chemical composition of japanese threadfin bream meat

Chemical composition	Percentage (%)	
	Minced meat	Minced meat [17]
Moisture content	78.93±0.04	79.41±0.88
Ash content	1.46±0.14	2.53±0.24
Fat content	0.76±0.14	1.50±0.38
Protein content	18.73±0.04	16.47±0.38

Japanese threadfin bream meat has a fat percentage of 0.76%, which puts it in the low-fat fish category, according to the proximate analysis results. Japanese threadfin bream was found to have 1.50% fat [17]. The fish's body fat content was less than 5%, placing it in the low-fat fish category [18]. As a result, defatting or fat removal was not necessary before making surimi of Japanese threadfin bream because the fish has a low fat content. The following finding showed that Japanese threadfin bream had a protein content of 18.73%, while Wati and Hafiludin's [17] research found that Japanese threadfin bream had a protein level of 16.47%. Japanese threadfin bream had a comparatively high protein content. Fish with a 15-20% protein percentage were considered high protein fish [19].

Japanese threadfin bream had a moisture content of 78.93%, according to the results of the proximate analysis. The greatest substance found in all living organisms is water. Most fish have a 60-80% moisture content, while certain species have a higher moisture content [20]. The proportion of moisture content was a reliable measure of the proportions of protein, calories, and fats [21]. As an indicator of the mineral composition of the fish, the ash content of the fish used in this study was 1.46%. Fish meat typically contains 0.6% to 1.5% of the fish's total weight in minerals. The findings of this study's ash and moisture contents were similar to those of Wati and Hafiludin's [17], which found that Japanese threadfin bream had an ash content of 2.53% and a water content of 79.41% without crude fiber content.

The Bradford method determined the protein content in Japanese threadfin bream flesh. Colorimetric methods were used to detect protein concentrations in solutions. When proteins in an acidic solution bind to the Coomassie Brilliant Blue (CBB) reagent, a blue color was produced. The amount of protein in the sample will determine how intense the blue hue is, and spectrophotometric methods can be used to estimate the absorbance. 595 nm is the wavelength that was employed for the measurement. Figure 3 shows the protein concentration during the processing of kamaboko and surimi.

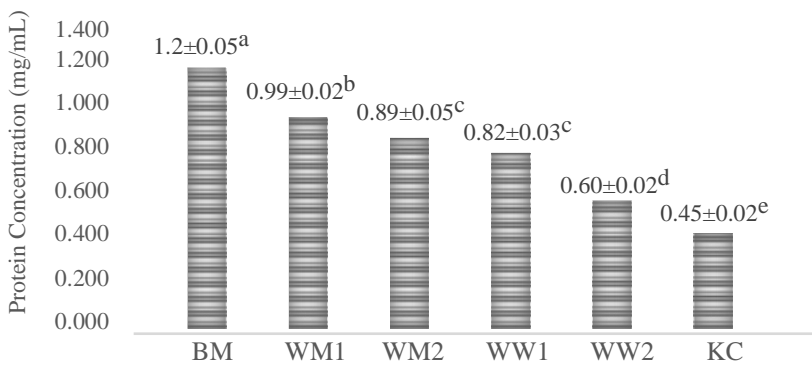


Fig. 1. Protein concentration of surimi, washing water and kamaboko of Japanese threadfin bream. BM = minced meat; WM= washing meat; WW= washing water; KC= kamaboko

The outcomes of each treatment's protein concentration yield varying values, as seen in Figure 1. Protein level, specifically sarcoplasmic protein content, can be decreased during the surimi-making process by washing the fish. This outcome is consistent with previous research [11], which found that the protein content of surimi dropped following the washing process. The concentration of dissolved protein will drop after boiling surimitill it turns into kamaboko [22]. This finding is consistent with the boiling process of surimi. When producing surimi, washing can lower the number of water-soluble proteins, blood (pigments), odor-causing lipids, and allergenic proteins, particularly parvalbumin [23]. Sarcoplasmic proteins can be dissolved during the washing process, increasing the amount of myofibril protein in surimi [24]. According to research findings, sarcoplasmic protein has been found in surimi washing wastewater.

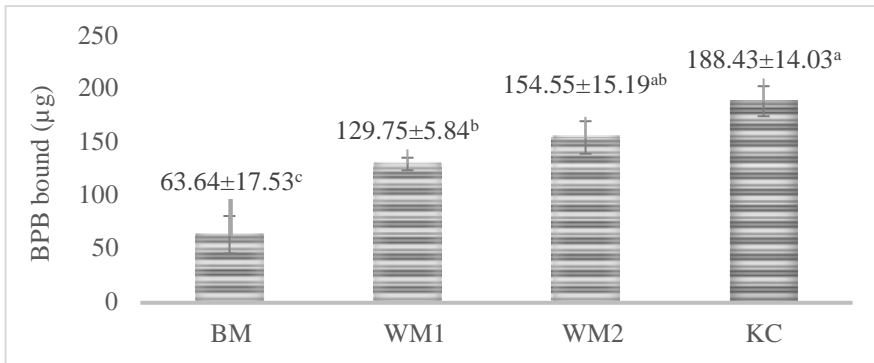


Fig. 2. Surface hydrophobicity of meat, surimi, and kamaboko of Japanese threadfin bream
BM = minced meat; WM=washing meat; KC= kamaboko

The hydrophobicity of the protein surface was measured using BPB (bromophenol blue) dye solution. Calculation of surface hydrophobicity based on the amount of bound BPB. The surface hydrophobicity of Japanese threadfin bream meat proteins during the manufacture of surimi and kamaboko can be seen in Figure 2. For every treatment, the data shows varying values of surface hydrophobicity. A sample's emulsifying qualities are reflected in its surface hydrophobicity, which also correlates with protein solubility. There are more hydrophobic regions in the protein structure the higher the BPB value linked to the myosin molecule [25]. The original structure of proteins has the propensity to both disperse hydrophilic groups across the surface and conceal their hydrophobic core. More non-polar amino acid residues will be exposed as the myofibril protein structure opens, as indicated by a higher bound BPB value [26]

Surface hydrophobicity and hydrophobic interactions are correlated; protein aggregation is typically thought to be caused by greater hydrophobic interactions. When a protein transforms into insoluble cross-fibers, it loses its typical solubility, a condition known as protein aggregation occurs physically [27]. Hydrophobic interactions between proteins also reveal a protein's tertiary structure, where the strength of these interactions influences the stability and functional characteristics of the protein by preserving its structure [28]. Variations in hydrophobicity during the surimi-making process may be a sign that the hydrogen bonds between the hydrophilic and globular protein molecules are breaking. Hydrophobicity can be used as a metric to gauge protein denaturation [10]

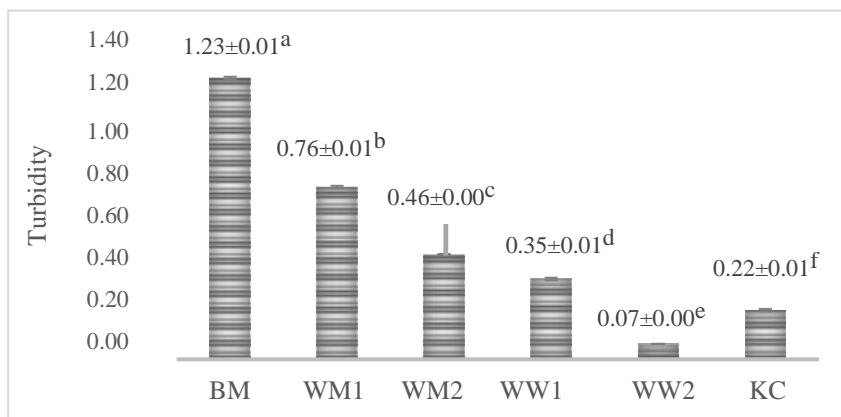


Fig. 3. Turbidity of meat, surimi, surimi washing water and kamaboko of Japanese threadfin bream. BM = minced meat; WM= washing meat; WW= washing water; KC= kamaboko

Turbidity can be measured using a dual beam spectrophotometer. The wavelength of the spectrophotometer used is 360 nm. Samples of meat, surimi, kamaboko and washing water were prepared using a phosphate buffer solution. The results of turbidity measurements can be seen in Figure 3. The turbidity values for each treatment vary from one another, according to the results. The minced meat (BM) sample had the highest turbidity value, measuring 1.234 ± 0.006 , whereas the first and second washed surimi (DP1) samples had lower values, measuring 0.755 ± 0.006 and 0.459 ± 0.000 , respectively. In kamaboko, the turbidity was 0.215 ± 0.006 , less than that of the surimi sample that was washed twice. The analysis of variance results indicates that there was a substantial ($p < 0.05$) effect on the turbidity or turbidity value during the surimi-making process.

Higher turbidity values could be the result of large protein aggregates that were constantly forming and causing light scattering and inhibition of light transmission [29]. Variations in particle size and protein aggregation rate lead to variations in turbidity values [26]. Variations in the quantity of hydrophobic and disulfide bonds were suggested as one of the potential reasons for variations in aggregation and will result in turbidity alterations [30]. A decrease in ionic and hydrogen bonding was probably what's causing the turbidity levels to drop. After the heating process was completed to make kamaboko, the turbidity value of the second washing surimi (DP2) fell. Turbidity reduces when the temperature hits 90 °C, mostly due to the suspension's rapidly aggregating proteins [31].



Fig. 4. Chemical bonds, surimi, surimi washing water and kamaboko japanese threadfin bream. BM = minced meat; WM= washing meat; KC= kamaboko

The disruption of hydrogen bonds between water and protein molecules leads to the unfolding of protein molecules, and this results in a gradual decrease in apparent viscosity. The presence of more hydrogen bonds is possibly to be the reason for more bound surface hydrophobicity in kamaboko. Ionic and hydrogen bonds play important roles in gel formation and stabilizing protein conformation. It is stated that the disulfide bonds involve cross- linking of denatured protein molecules as well as aggregation [28]. It is usually accepted that inherent disulfide bonds stabilize the properly folded conformation of proteins and/or destabilize denatured conformations by decreasing conformational entropy.

The electrophoresis method can be used to qualitatively visualize protein content or surimi protein molecular weight. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was the electrophoresis technique employed, and Coomassie brilliant blue (CBB) staining was used. Figure 5 illustrates the surimi protein's molecular weight.

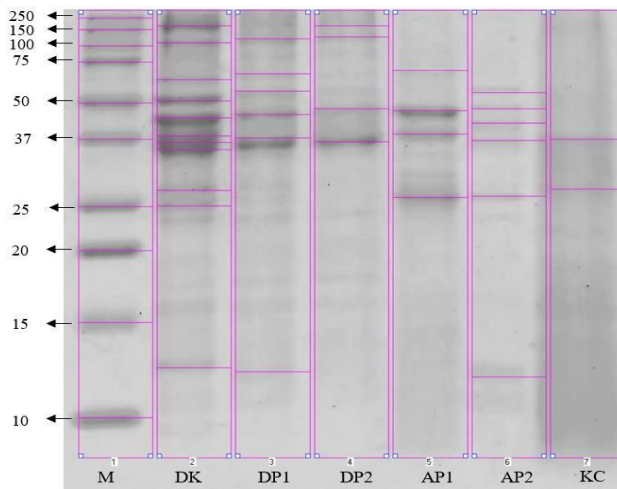


Fig. 5. Surimi protein profile on SDS-PAGE, M= marker 250 kDa; BM = minced meat; WM= washingmeat; WW= washing water; KC= kamaboko

After the surimi protein was separated by SDS-PAGE, photocap software was used to determine its molecular weight. The findings of assessing the protein molecular weight in each sample indicate varying values, which were consistent with the SDS-PAGE results previously mentioned. The 10-250 kDa marker is employed. Table 2 displays the findings of the protein molecular weight study. The SDS-PAGE method was used to visualize the protein profile observations made in the meat and washing water of Japanese threadfin bream throughout the preparation of kamaboko and surimi. The results indicated that the protein bands formed had a molecular weight ranging from 12.58 to 178.36 kDa. The findings of the electrophoresis demonstrated that the protein bands varied in thickness. Figure 5 illustrates that a thicker band signifies a larger concentration of protein [32]. According to Table 2, there are ten bands in Japanese threadfin bream meat (DK) with a molecular weight range of 12-180 kDa, six bands in first washing meat (DP1) with a molecular weight range of 12-120 kDa, four bands in second washing meat (DP2) with a molecular weight range of 36-120 kDa, and two bands in kamaboko (KC) with a molecular weight range of 27-37 kDa. Four bands with molecular weights between 26 and 69 kDa are present in the first washing water (AP1), and six bands with molecular weights between 12 and 55 kDa are present in the second washing water (AP2).

Table 2. Molecular weight of surimi (SDS-PAGE)

Number	Marker (kDa)	BM (kDa)	WM1 (kDa)	WM2 (kDa)	WW1 (kDa)	WW2 (kDa)	KC (kDa)
1	250	178.36	119.72	178.36	69.35	55.52	37
2	150	108.30	67.20	125.90	46.98	47.73	27.72
3	100	50.97	56.26	47.73	38.60	42.37	
4	75	44.28	45.50	36.49	26.36	36.75	
5	50	37.98	37.39			26.56	
6	37	36.37	12.38			12.11	
7	25	34.98					
8	20	27.50					
9	15	25.09					
10	10	12.58					

Three protein components were present in the process of creating kamaboko and surimi: β -enolase (55 kDa), aldolase-A (35 kDa), and beta-parvalbumin (10–13 kDa) [33]. Sarcoplasmic proteins, lipids, and other nitrogen-containing substances from crushed fish flesh can be dissolved during the washing process, affecting the protein composition of surimi [34]. The three proteins just discussed belong to the sarcoplasmic proteins family, which also includes proteins that trigger allergies in fish. The similarity in protein profiles between surimi and washing water in this study indicated that the washing process could eliminate most of the major proteins. According to Abidin's research [11], the profile of the probable proteins parvalbumin (10–13 kDa), aldolase-A (35 kDa), and β -enolase (55), found in the first and second washing water, indicated that the washing water conveyed the allergen. According to the SDS-PAGE results, the meat that had been washed had less intensity in the protein band in the 10–13 kDa region that was thought to contain parvalbumin than the meat that had been ground up before washing. This

indicates that the allergen content, more specifically parvalbumin can be decreased during the meat- washing step of the surimi production process.

To acquire quantitative data, the allergen content particularly parvalbumin was also assessed using the high-sensitivity, quick-to-process enzyme-linked immunosorbent assay (ELISA) technique. The AgraQuant Fish ELISA Plate ELISA kit is utilized, and it uses photometric measurements to detect and quantify parvalbumin based on the interaction of antigen and specific antibodies. 450 and 630 nm wavelengths were used in the ELISA Reader to measure parvalbumin. Figure 6 shows the outcomes of measuring parvalbumin using the ELISA method during the processing of surimi and kamaboko Japanese threadfin bream.

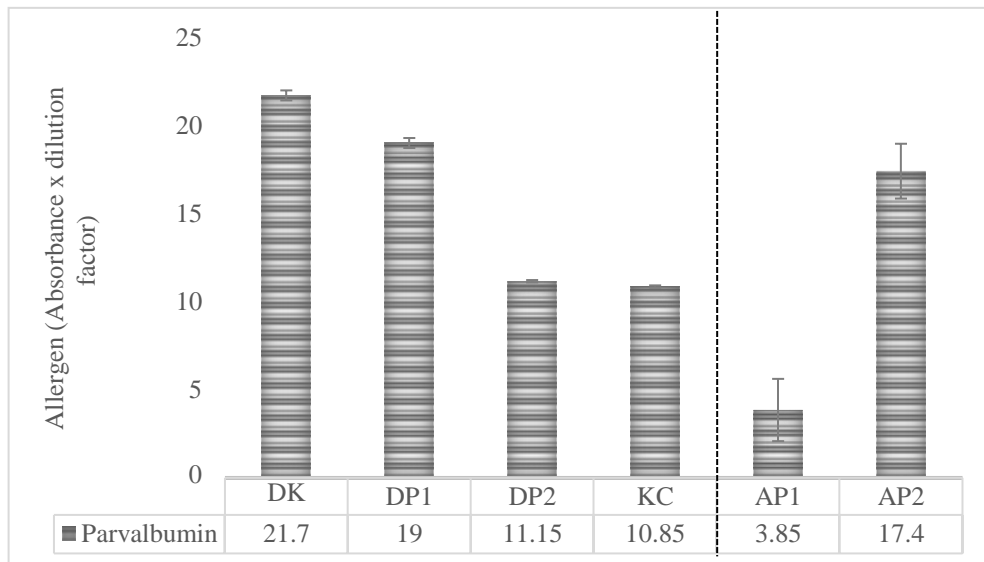


Fig. 6. Allergen content (parvalbumin), surimi, surimi washing water and kamaboko Japanese threadfin bream. BM =minced meat; WM= washing meat; WW= washing water; KC= kamaboko

According to Figure 6, there are variations in the outcomes for the parvalbumin content in each step of the processing of surimi and kamaboko from Japanese threadfin bream. Before the washing process, the control treatment's chopped Japanese threadfin bream flesh had the highest parvalbumin content, while kamaboko had the lowest. In the washing water, the second wash had the highest parvalbumin content and the first wash had the lowest. While the parvalbumin content dropped throughout the washing procedure, it did not significantly reduce during the boiling or kamaboko-making processes. The significant parvalbumin level in AP1 and AP2 indicates that this parvalbumin is removed by washing water. In a different investigation, Alaskan pollock surimi samples were used for allergy testing using ELISA. The samples were reduced by 87% after two washings and by 99% after five washings [23]. Since parvalbumin is a heat-resistant protein, the heating process has no discernible effect on its content [35].

4 Conclusion

Surimi processing results in the presence of allergenic protein content, specifically parvalbumin, aldolase A, and β -enolase. When using the ELISA Kit, results regarding a decrease in allergen content are more accurate. Following the washing procedure, the allergenic protein in the Japanese threadfin bream meat decreased. As the protein dissolves sarcoplasmic reticulum, the quantity of allergenic proteins decreases. Protein properties, including surface hydrophobicity, turbidity, and chemical bonding, alter throughout the processing of surimi.

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