Application of pepsin enzyme from yellowfin tuna gastrics on the physical and histological characteristics of beef

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Abstract. The increase in tuna production in Indonesia has introduced waste-management challenges. Utilizing pepsin from tuna stomachs for beef tenderization is a potential solution for the food industry. This study aimed to assess the effects of different pepsin concentrations on beef characteristics. This study used a completely randomized factorial design with one factor: beef was treated with 0, 14,000, and 28,000 U/mg pepsin and stored at 4 °C for four days. The results showed that 28,000 U/mg pepsin significantly lowered pH, decreased weight percentage change, increased water-holding capacity, and reduced texture compared to other treatments. Histological examination revealed gradual degradation of meat structure, especially in collagen-deficient areas. Tuna stomach pepsin plays a crucial role in altering the physical and histological characteristics of the beef.

1 Introduction

Tuna is one of Indonesia's most significant export goods. Between 2016 and 2022, there was an average annual increase in tuna output of 4.08% and a 1.24% increase in export value [1]. As a result, the number of by-products that are wasted or used to create low-value items increases. This scenario might be lessened using biotechnological methods to produce biomolecules in high demand in the market, such as enzymes, polyunsaturated fatty acids, and high protein content. Because of the massive volumes of waste produced, fisheries management technologies require greater focus to reduce waste and raise product values, which will enhance the sustainability of marine resources and the global blue economy [2]. The head, skin, breasts, bones, and scales are all found in the tuna [3]. This waste accounts for as much as 70% of the fish's total weight and contains high-quality lipids, proteins, and vitamins, making it a valuable resource for the pharmaceutical, cosmetic, and nutraceutical sectors [4].

Important protease enzymes such as aspartate protease pepsin, serine protease trypsin, chymotrypsin, collagenase, and elastase are found in wastes containing 12–18% protein [5]. Pepsin is an endopeptidase that breaks down peptide bonds that bind proteins, such as tryptophan and tyrosine [6]. One way the fishing industry processes its waste is by applying pepsin enzymes, which also have a positive fermentation effect on protein digestion [7]. Beef

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is one of the foods with high protein content. Beef must be prepared precisely and swiftly because of its high water and nutritional content, which makes it vulnerable to microbial harm.

Meat processed foods that are widely consumed and have a long cooking process one of them is rendang. Usually, silverside parts of beef are used in these recipes. The slice was a caudolateral thigh with dry features and 6.2% body weight. A total of 72.84% of the silverside consists of water [8]. Because of their sensory qualities, silverside cuts are thought to have lower commercial value [9]. Texture, hardness, and histological structure are the factors that affect the quality of fresh meat. Postmortem interventions can be used to increase the amount of meat consumed to soften fresh meat. According to [10], post-mortem interventions employed in the preparation of fresh meat fall into three primary categories: enzymatic, chemical, and physical. These categories depend on the treatment functions.

A study reviewed a system of physical and enzymatic interventions to improve the texture and soften meat [11]. Physical interventions result in structural changes in meat by providing style or physical stimulation such as aging or aging conditions. Enzymatic interventions include infusions, marination, and protease injections. These enzymes function on myofibrillar proteins and bind to tissues to varying degrees to produce more fragmented and destroyed structures. The application of enzymes to beef is a promising approach for obtaining juicy and juicy meat, which can increase consumer acceptance. So far, studies have not explored the use of pepsin derived from fish waste on one of the food commodities, namely beef. Thus, further research is needed to determine the influence of enzyme activity on the physical and histological characteristics of beef.

2 Materials and methods

2.1 Tools and materials

The substances used in this study are pepsin enzymes from the stomach of tuna, silverside meat, aquades, liquid nitrogen, butyl alcohol 10%, HCl (Merck, Darmstadt, Germany), NaOH, tris base (Sigma, Missouri, USA), TCA, hemoglobin (Segma, Misouri, America), Coomassie Briliant Blue (AppliChem, DARMstadt, German), ethanol (Mercker, Darmstadt, Deutschland), NaCl (Merck, Darmstadt, Netherlands), formalin neutral buffer (BNF), formaline, NaH_2PO4 (Merk, Darmnstadt, GERMANY), Na_2HPO_4, alcohol 70%, 80% alcohol, 90% alcohol, 95% absolute alcohol I, II, III, paraffin, xylol, I,II, and III, hemotoxillin, eosin, Entellan®, massively trikomonous.

The instruments used in this study are UV-Vis spectrophotometers (Rayleigh, Beijing, China), incubators, erlenmeyer tubes, pipe, reaction tube, measuring glass (Pyrex), filter paper, Whatman, 10-100 µL micropipette, 100-1000 µL, and 5000 µL (Thermo Fisher Scientific), pH meters (Hanna Instruments, Rhode Island, America), vortex (DLAB MX-S, US), analytical scales (OHAUS AX224, New Jersey, USA), centrifuges (OHAUS FC5718R, New Jersey, US), Yamato RV-240 microtome, texture analyzer (TAXT2i texture analyser, UK), Olympus CX41 light microscope and Olympus DP21 camera along with Prime Strime Software.

2.2 Methods

2.2.1 Preparation and activity analysis of pepsin enzyme

The enzyme used was the result of tuna stomach extraction, which was performed by Muttaqin [12]. Frozen gastric tuna from Gorontalo were melted and cleaned with flowing water. A clean body was morphometrically measured and cut to a size of 1×1 cm with a knife. The tuna was then given liquid nitrogen to the solid as ice and then smoothed with a blender.

The extraction process was performed using Tris-HCl buffer (pH 7.5), and the mixture was centrifuged for 15 min at 10,000 × g at 4 °C. Pepsin activation was achieved by lowering the pH of the pepsinogen to 2 by adding HCl 3 N and leaving for 10 min, then adding $NaHCO_3$ 2 N to raise the pH to 2.75. The sample was dampened for six hours, filtered to obtain a supernatant pepsin solution. The pepsin enzyme used in this study was extracted in the form of a 1 year old liquid preparation and stored at -23 °C. The frozen liquid pepsin extract was melted, and pepsin activity and protein concentration tests were performed to determine the specific activity of the pepsin produced.

2.2.2 Preparation and analysis of beef

The sample is the silverside portion of the Brahman Cross cattle carcass with an age range of 1.5-4 years from the IPB Farming Faculty's cutting house. The beef used in this study was frozen for ±2 weeks from slaughter. The beef was removed from the fat layer for further analysis. Fresh beef was tested for pH [13], drip loss, and water-holding capacity [14].

2.2.3 Pepsin enzyme application to beef

Beef was cut into a square with dimensions of 2 × 3 cm (thickness 1,5 cm). The meat was macerated using pepsin enzymes after treatment with 0, 14,000, and 28,000 U/mg. The immersion time was four days, and the samples were stored at 4°C. The meat was then analyzed for pH [13], water-holding capacity [14], drip loss, texture profile [15], and histology [16].

2.2.4 Pepsin enzyme activity analysis

Pepsin enzyme activity was analyzed as described by Jurado [17]. The test used a substrate of hemoglobin produced at a concentration of 2%, and then the pH was lowered to 2 by adding HCl. The substrate (1 mL) was removed and inserted into the reaction tube, and an extract of 0.2 mL pepsin was added. The enzyme solution was incubated for 10 min at 50 °C. The next stage of the sample was added to 5% TCA of 2 mL and left to sit. The results were filtered using a cylindrical paper. The obtained filter was analyzed using a spectrophotometer at a wavelength of 280 nm. This measurement was performed to determine the oligopeptide content of the supernatant. Oligopeptide content was defined as enzyme activity. One unit of activity was expressed as an increase of 0.001 at 280 nm per minute, so the enzyme activity can be expressed as follows:

$$U = \frac{A_{280} - A_0}{0.001 \times t \times VE}$$

U/mL = unit of enzyme activity
U = enzyme activity in U per mL
VE = volume of pepsin solution in
activity test A280 = absorption at 280 nm
t = incubation time (minute)
A0 = absorption of samples prepared with the same treatment without the add
pepsin

2.2.5 Pepsin protein concentration analysis

Pepsin protein concentration analysis was performed as described by Bradford [18]. The concentration analysis began with the addition of 5 mL of Bradford reaction into the reaction tube containing the 0.1 mL sample. Incubation was then carried out for 5 min, and the measurement was performed using a spectroscopic photometer with a wavelength of 595 nm. Bradford's pre-action was made by dissolving 10 mg of coomassie brilliant blue into 5 mL of 95% ethanol, then adding 85% phosphate acid as much as 10 mL (b/v) and aquades 250 mL until the solution is mixed. The solution was filtered using Whatman filter paper number 1 before use. The production of a standard solution began by dissolving bovine serum albumin (BSA) at a concentration of 2 mg/mL for use as a stock solution. The standard stock solution was then given a standard concentration ranging of 0.1-1.0 mg/ml. The absorbance of the normal solution was measured using a spectrophotometer at a wavelength of 595 nm.

2.2.6 pH testing

The pH of 10 g of beef was measured and mixed with 100 mL of aquades using a blender [12]. The solution was then measured using a pH meter that was calibrated with pH buffers 4 and 7. The measurements were performed twice.

2.2.7 Drip loss

Drip loss was calculated from the difference in weight before and after the meat sample was stored at 4 °C, and the result was expressed as an average proportion. The percentage of weight change during the next four days of storage was drip loss.

$$Drip\ loss\ (\%) = \frac{(\text{sample weight before cooling} - \text{sample weight after cooling})}{(\text{sample weight before cooling}) \times 100}$$

2.2.8 Water holding capacity

Water-holding capacity (WHC), commonly referred to as water retention capacity, is determined using centrifugation techniques. A total of 15 g of meat samples were placed in a 2.8×11 cm centrifugal tube, 22.5 mL of 0.6 M NaCl solution was added, and the contents were mixed for 1 min with vortex. After holding the mixture for 15 min at 4 °C, the pulverized meat was remixed for 1 min and centrifuged at 12, 000×g for 15 min. Supernatant layers were then poured and recorded in terms of volume. The amount of additional solution retained by the meat was recorded as the water retention capacity (mL per 100 g of meat).

$$\text{WHC}\ (\%) = \frac{(\text{Volume of NaCl after centrifuge} - \text{Volume of NaCl before centrifuge})}{(\text{Volume of NaCl before centrifuge}) \times 100}$$

2.2.9 Texture profile

Texture profile analysis was performed according to Maqsood [15], with modifications. The meat texture properties were measured using a texture analyzer (CT3-4500, Brookfield Engineering Laboratories, Middleboro, USA) with a cylindrical probe (diameter 50 mm). The samples were cut into cylinders (length 30 mm × width 15 mm × thickness 10 mm) and placed at the base of the instrument. The test was performed using two compression cycles. TPA texture parameters were measured at room temperature under the following test conditions: crosshead speed 5.0 mm/s, tension 50%; surface sensing style, 99 g; threshold,

30 g; time interval between the first and second compressions, 1 sec. Texture Expert software (version 1.0; Stable Micro Systems, Surrey, UK) was used to collect and process the data. The time-style curves generated for each sample contained hardness, springiness, and cohesiveness data.

2.2.10 Meat histology

Meat histology was performed as described by Angka [16]. Analysis of beef tissue begins with the preparation and observation of the preparation under a light microscope. Beef (1×1×0.5 cm2) was scrubbed with a fixative solution of BNF, then dehydrated using a standard alcohol ranging from 70% for 24 h, 80% for 2 h, 90% for two hours, 95% for 2 h, and 100% for 12 h. The sample was then cleaned by immersing the sample in boiling liquid alcohol: xylol (1:1) for 30 min, followed by the impregnation phase (embedding). The embedding phase removed the clearing agent from the tissue and replaced it with paraffin. The samples were immersed in a solution of xylene: paraffin (1:1) in a glass cup for 45 min in an oven at 60 °C. The samples were then printed with liquid paraffin to 1/8 parts of the mold size (2×2×2) cm^3 until slightly frozen. Liquid paraffin was subsequently administered until soaking and left to freeze at room temperature for 24 h.

The paraffin block-shaped sample was then trimmed using a silette and cut to a thickness of 4 μm using a rotating microtome to obtain the prepared slide. Paraffin tape containing the tissue was then attached to the glass of the object. The staining process on the glass of the object is performed such that the tissue elements are contrasted and can be observed through a microscope. The coloring begins with deprafinification of the glass, submerging the object into xylol I and xylole II for 2 min, followed by submergence in 100%, 95%, 90%, 80%, 70%, and 50%, respectively, for 2 min. The object was rinsed with aquades for 2 min, then inserted into the hematoxylin and tricom massom dye for 7 min and washed with running water to remove excess dye that was not absorbed. The tissue preparations were then incubated in alcohol with concentrations increasing slowly by 50%, 70%, 85%, 90%, 100%, xylol I, Xylol II for 2 min. The next process was the closure of the glass of the object with the administration of a mounting agent or Canada Balsam on the glass, followed by drying for 24 h. The preparation was observed with an Olympus CX41 light microscope at a magnification of up to 400× and then photographed with an Olympus DP21 camera.

2.2.11 Statistical analysis

The experimental design used in this study was a Complete Random Design (CRD) with one factor, namely pepsin enzyme activity, used at 0, 14,000, and 28,000 U/mg. Statistical analysis was performed for pH, DL, WHC, texture profile, and meat histology. Parametric data from enzyme activity testing, protein concentration, and specific activity were further processed using data normalization analysis. Research data can be categorized as normally distributed when they have a significance value greater than α (α=0,05). The test data were obtained twice for each sample. If the results of the analysis differed significantly ($P<0.5$), a further Duncan test was performed. The confidence interval was set at 95%.

3 Results and discussion

3.1 Pepsin enzyme raw extract activity

The specific activity of pepsin was tested using 2% hemoglobin in 0.2 M HCl as its substrate. The pepsin enzyme used was a liquid preparation that had previously undergone the process of pepsinogen extraction and activation using Tris-HCl pH 7.5. The concentration of protein in the raw pepsin extract of tuna stomach was 0.0437 mg/mL, whereas the enzyme activity

was only 1,226.67 U/ml so thus, the enzymatic activity produced was 28,011.50 U/mg. The specific activity of pepsin has been studied with similar species of fish and obtained a value of 22,173.36 U/mg [19]. Specific activity of the same species fish obtaining value of 10,879.99 U/mg [20]. The values of pepsin enzyme activity vary due to several factors, such as fish type, fish habitat, and fish stomach size. The weight ratio of yellow shrimp tuna was 8.71% [21]. Aspartate proteases from the stomachs of fish have been studied, for example, in tuna and salmon of different origins. Albacora tuna, shellfish tuna and yellow shrimp tuna produced from Thailand have pepsin activity of 0.1129±0.17, 0.0983±0.61 and 0.1021±0.74 U/mg [22]. Black Sea salmon gastric produced from Turkey has pepsin activity of 144.57±9.64 U/mg [23]. Other factors that can affect pepsin enzyme activity include fish size, season, and origin [24].

3.2 pH of meat

The pH was measured by dissolving the meat in aquades to homogeneity. The solution was then tested using a pH meter. The pH test results for meat are presented in Figure 1.

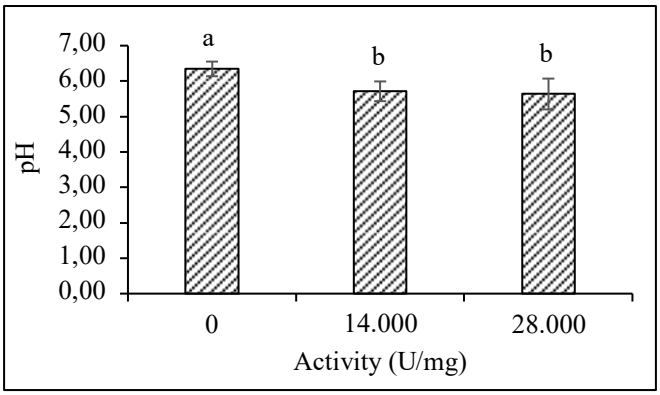

Fig. 1. Effect of pepsin activity on the pH of meat. The same letter indicates no significant difference (P >0.05). Description: 0 = beef soaked with Tris-HCl buffer 7.5, 14,000 = meat soaked with pepsin 14,000 U/mg, 28,000 = flesh soaked with pepsin 28,000 U/mg.

The normality test of the beef pH value showed normal spread data with a significance value > α (α=0.05). The results of the polymorphic analysis (α = 0.05) show that different pepsin activities have a real influence on the meat pH value. Furthermore, Duncan's results showed that the treatment without pepsin (0 U/mg) had the highest pH value and a significant difference from the pH value after treatment with pepsin 14,000 U/mg and 28,000 U/mg activity. The highest meat pH was obtained with an enzyme treatment of 0 U/mg, which had a value of 6.34. The decreased pH value of meat is presumed to be due to the influence of pH conditions of acid pepsin enzymes (pH 2). Meat softens faster at a high pH of ≥6.2, with the degradation of titin protein and filamine nebulin, like the process of tenderization at the time of early post-mortem of beef [25]. Meat consumption at a low pH of ≤5.79 is associated with protein fragmentation with smaller molecular weights, such as desmin, and delayed processes due to larger molecular weight degradation. This corresponds to the effectiveness of the enzymes that are affected by pH.

3.3 Drip loss

Drip loss (DL) was determined by measuring the mass of meat before and after immersion in the enzyme. The results of the meat drip loss tests are shown in Figure 2.

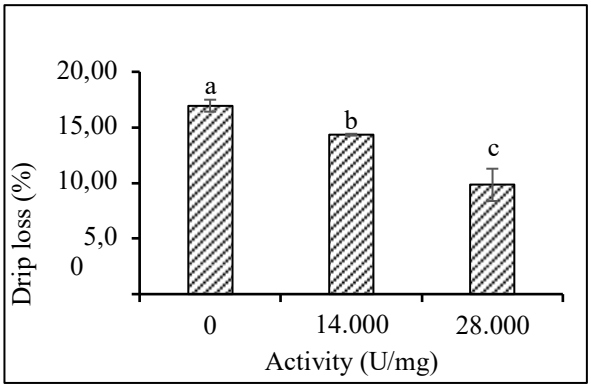

Fig. 2. Effects of pepsin activity on meat DL. Description: 0 = beef soaked with Tris-HCl buffer 7.5, 14,000 = meat soaked with pepsin 14,000 U/mg, 28,000 = beef soaked with pepsin 28,000 U/mg.

The normality test of beef DL showed normal spread data with a significance value > α (α=0.05). The results of the analysis (α = 0.05) shows that different pepsin activities have a real effect on meat DL. Further testing by Duncan's test showed that any treatment had a significant effect on the meat DL value. Figure 2 shows that the meat DL obtained ranges from 9.83-16.95%). The higher the pepsin activity, the higher the DL of the meal. The highest meat DL was received from meat with an enzyme treatment of 0 U/mg, which had a value of 16.95±0.54%. The addition of pepsin influences DL in meat. It is believed that the interfiber septum protects the entrance of enzymes into the meat fibers, especially in strong epimysium tissue. Drip Loss can be affected by the loss of fluid from meat during storage. Fresh meat stored untreated tends to lose weight because of the presence of WHC [26]. This weight loss is believed to be due to storage at 4 °C for 4 days. High DL values in meat indicate that the meat is dehydrated or decreases in quality during storage [27].

3.4 Water holding capacity

The WHC is measured by dissolving meat in aquadest to be homogeneous and then centrifuging and measuring the volume of the superfood. The results of the meat WHC tests are shown in Figure 3.

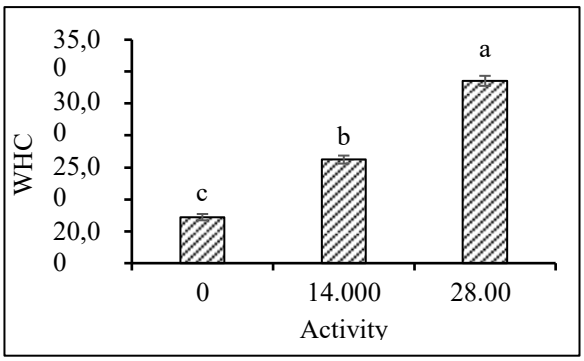

Fig. 3. Effect of pepsin activity on WHC. Description: 0 = beef, s = meat soaked with pepsin 14,000 U/mg; 28,000 = beef soaked with peptin 28,000 U/mg.

The normality test of the beef WHC test had normal spread data with a significance value of > α (α=0.05). The results of the analysis showed that different pepsin activities had a real effect on the WHC of meat. Further Duncan tests showed that any treatment had a significant impact on the WHC value of meat. Figure 3 shows that the meat WHC ranged between 7.21-28.56%. The higher the pepsin activity, the higher the WHC. The highest meat WHC was obtained from meat with an enzyme treatment of 28,000 U/mg, which had a value of 28.56±0.78%. The WHC value indicates the ability of the meat to hold water. This result is consistent with statement about aging treatment, that in aged meat, WHC is increasing, because a decrease in pH after a mortis rigidity process causes ATP hydrolysis and myosine actin protein binding [28]. Bivalent ions, such as Ca++ and Mg++, can be replaced by Na+ so that emptiness occurs and is filled with water to increase WHC. The WHC value is influenced by the number of hydrogen bonds between the polypeptide groups of proteins, which causes the number of hydrogen bonds to increase. Protease enzymes can accelerate the rate of the protein hydrolysis reaction in meat, which causes muscle fibers to open, giving room for water molecules to increase WHC [26]. One quality of meat is determined by its water binding: the higher its water bonding, the better the meat quality is than the low water bending because the molecules of water in meat have more space that makes meat fluffier [29].

3.5 Meat texture profile

The texture profile was measured by applying pressure on the meat using a pressure tool. The resulting large pressure was recorded and processed with the device, and then the results for each meat immersion with the lowest and highest activities were compared. The observed texture profiles included hardness, springiness, and cohesiveness. The texture profiles of the fresh meat and soaking enzymes with different activities are presented in Table 1.

Table 1. The texture profiles of fresh meat and soaking enzymes with different activities

Parameter	Meat sample		
	Fresh	0 U/mg	28.000 U/mg
Hardness	6426.10^{b}	8119.9^{a}	5359.5^{b}
Springness	48.24^{b}	50.84^{a}	45.34^{c}
Cohesiveness	0.65^{b}	0.75^{a}	0.56^{c}

Note: values with different superscripts show significant differences

Table 1 shows that treatment without pepsin (0 U/mg) had the highest hardness and a real difference with hardness values in fresh meat and meat with pepsin activity of 28,000 U/mg. The treatment without pepsin (0 U/mg) resulted in the highest springiness and differed significantly from that of the other treatments. Additional pepsin (0 U/mg) increased springiness, while pepsin at 28,000 U/mg reduced springiness. The test results showed that every treatment had a significant influence on the cohesiveness of meat. Additional pepsin (28, 000 U/mg) decreased the cohesiveness of beef. Treatment with 0 U/mg improved the texture profile of beef for each parameter. A 7.5 tris-HCl buffer was suspected to be involved in the meat dive. The solution of tris-HCl 7.5 is a mixture of tris(hydroxymethyl) aminomethane salts and HCl [31]. The presence of these tris salts is believed to increase the strength of muscle tissue ions and water retention in muscular tissue. Salt affects the solubility of myogenic fibrin (a protein that is soluble in salt) and increases water retention,

thereby increasing meat hardness [30]. Protein binding in meat is directly related to the compactness/cohesiveness of meat particles and flexibility of the gel matrix formed [32]. This behavior explains the higher cohesiveness value. Salt levels can affect the water retention capacity and binding capacity of the microfibril protein gel. Cross- linking of proteins is carried out through ionic forces, hydrogen bonds, disulfide bonds, and hydrophobic bonds [33]. Pepsin enzymes are thought to play a role in the breakdown of proteins in addition to aging factors from storage at a temperature of 4°C for 4 days. Meats with lower hardness and springiness are attractive to older consumers [34]. The following results prove that pepsin has equivalent effectiveness in beef and is effectively used for fattening. The breakdown of myofibrillar proteins is associated with an increase in their functional characteristics. Overall, the resulting texture profile suggested that the addition of pepsin to beef is a promising option for the development of softer meat products.

3.6 Meat histology

Histological analysis of the meat using a light microscope is shown in Figure 4. Figure 4 shows the structure of beef damaged by the addition of 28, 000 U/mg of pepsin.

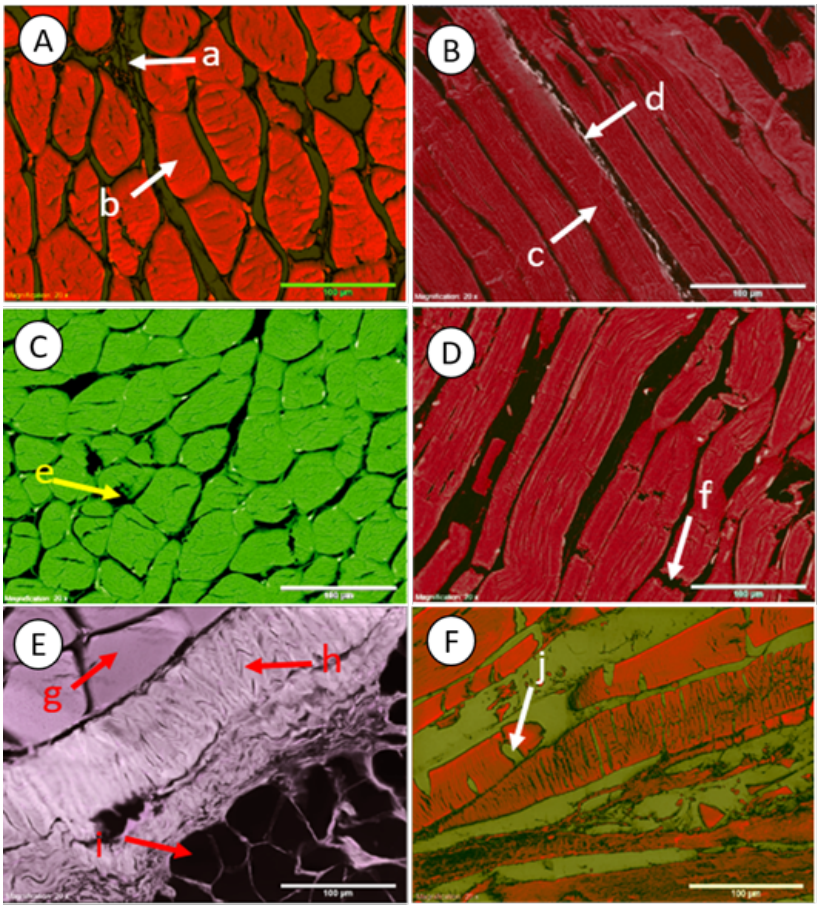

Fig. 4. Histological analysis of fresh meat (A and B), meat immersed in pepsin 0 U/mg (C and D), and meat immersed in peptin 28,000 U/mg (E and F). Notes: (a) and (i) fatty tissue, (b) transversal overlapping of whole meat fibers, (c) longitudinal overlay of whole flesh fiber, (d) myoseptum, (e) and (f) erosion at the edges of the meat fibers; (g) intact meat fibers; (h) epimysium; (j) damaged meat fibers.

Figure A shows the transverse intersection of the meat. The muscle fiber is still intact and shows that the fiber is still compact, and the fiber edge is still smooth and visible on b. Arrow b shows the remainder of the interfibrillar septum, which is seen as filling the interfibrillar space. Figure B shows the longitudinal portion of the flesh. The d arrow indicates the remainder of the septum that is visible due to adjacent meat fibers that have suffered fractures. Figure C shows the cross-section of meat flooded with pepsin 0 U/mg. Meat fibers begin to suffer damage and cracking but are not yet broken. The edge of the meat fiber was eroded in some places, as indicated by arrow e. Figure D shows a longitudinal piece of meat soaked in pepsin 0 U/mg. Some fibers start to break, as shown by the arrows f. Figure E shows a transverse cross-section of pepsin-soaked meat (28, 000 U/mg). The meat fibers (g) were still intact, presumably protected by the epimysium (h), which is composed of several layers of collagen fibers, which are waves. Figure F shows the longitudinal penetration of meat submerged in 28, 000 U/mg of pepsin. The meat fiber starts to break, showing an arrow and crawling.

Initially, intact muscle tissue is broken down by pepsin to form capillary microspaces in the meat fiber. This condition triggers adhesion to the tissue, allowing water to be absorbed into meat. The connective tissue consists of the epimysium located around the muscle and the perimitium located between the fasciculus and endomysium between the muscular fibers. The gap between the fascicles or the arrangement of the connective tissue can be regarded as an indication of the tightness of the meat because the larger the distance between the fascicles, the more compact the consistency of the resulting meat [35].

4 Conclusion

Treatment with pepsin enzymes of tuna gastric pepsin affects the physical and histological characteristics of beef. Treatment with pepsine enzyme reduces the pH, drip loss, and meat texture profile, and increases the WHC of meat. The addition of pepsin with an activity of 28,000 U/mg resulted in damage to beef muscle tissue, with the gradual emergence of points of damage to the histology of meat.

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