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Postmortem Changes in Spinal Cord-damaged Olive Flounder (*Paralichthys olivaceus*)

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Abstract Our study intends to delay the onset of rigor mortis and prolong the freshness by damaging the spinal cord of the olive flounder. The initial pH of nondamaged and damaged meat of the spinal cord was 7.07±0.05 and 7.10±0.12, respectively, but the pH at 20 hours postmortem dropped to 6.11±0.04 and 6.52±0.08, respectively. The initial TVB-N contents of the olive flounder meat with and without spinal cord damage were 2.0±0.1 and 2.5±0.3 mg/100 g, respectively. TVB-N contents at 20 hours postmortem were 10.4±0.6 mg/100 g in nondamaged meat and 8.4±0.8 mg/100 g in damaged meat. In the initial hour postmortem, the contents of APT in the meat were 5.80 and 5.65 mg/100 g in the control and spinal cord-damaged sample, but their contents decreased to 3.02 and 4.12 mg/100 g at 20 hours postmortem, respectively. The K-value of the control of the initial postmortem time was 0.5, but it increased to 16.8 at 20 hours postmortem. However, the K-value of the spinal cord-damaged samples increased from 0.6 to 9.6 during the postmortem time. The rigor mortis of the control started at 6 hours after death, and the rigor index rapidly increased to reach the maximum of 90.0% at 20 hours postmortem. In the spinal cord-damaged samples, the onset of rigor mortis was 12 hours postmortem, and the rigor index reached 74.3% at 20 hours postmortem. The breaking strength in meat without spinal cord damage reached its maximum value at 4 hours postmortem and sharply decreased until the end of the experiment. However, the breaking strength in meat with spinal cord damage recorded a maximum value at 8 hours postmortem, and its value was somewhat lower than that without spinal cord damage. Our results indicated that the freshness of the spinal cord-damaged sample extended approximately 4-8 hours beyond that of the nondamaged control.

Keywords: *spinal cord, ATP-related compound, rigor mortis, K-value*

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1. Introduction

The process of postmortem change in fish is an important factor in evaluating fish quality characteristics. After the fish are slaughtered, the muscles undergo postmortem changes, including rigor mortis, autolysis and spoilage by various biochemical and physical parameters [1,2].

Rigor mortis is one of the most important changes in fish postmortem, and many studies related to this have been carried out [3,4,5]. After the death of the fish, intramuscular oxygen supply is interrupted, resulting in anaerobic glycolysis, rapid degradation of adenosine triphosphate (ATP), and partial ATP synthesis by the action of the reserved creatine kinase in the fish muscle. These reactions correspond to various conditions that induce muscle contraction in the rigor mortis stage. The lactic acid formed by the degradation of glucose depends on the amount of glycogen reserved in the muscle prior to death. The amount of glycogen in muscles is closely related to the stress factors of fish, such as slaughtering methods and handling procedures prior to death, and is one of the important factors in determining the onset of rigor mortis.

One consequence of the accumulation of lactic acid in the muscle is the lowering of pH from near neutrality to the lactic acid range [6]. The decline in pH tends to increase muscle hardness and drip, affecting the quality of the fish tissue [7]. In the muscles of living fish, ATP is converted to adenosine diphosphate (ADP) and phosphoric acid, and ADP is used to synthesize ATP by receiving phosphoric acid from creatine phosphate (Cr-P). After death, the one-way degradation of ATP occurs and the amount of ADP in the muscle increases rapidly. Although Cr-P is hydrolyzed by creatine kinase and a portion of ATP is synthesized, the concentration of creatine in the muscle is not infinite and will soon be exhausted. In the final stage of ATP degradation, the intramuscular ATP content is reduced below extreme levels and the binding between actin and myosin in the

presence of the calcium ion, called actomyosin, lead to contraction, which is irreversible.

Within a few hours after the initiation of rigor mortis, autolysis progresses to make the muscles tender using some tissue enzymes present in the muscle. After that, muscle tenderization is accelerated more quickly by microbial contamination, which eventually leads to spoilage.

The freshness of fish used in sashimi is very important, and even though the use of fish in sashimi during postmortem changes varies depending on the difference in food culture, sashimi is usually used within the autolysis stage after death. In Korea, olive flounder is mainly consumed in sashimi during the pre-rigor stage, it is called "live fish sashimi". The sashimi of Japan is somewhat different from that of Korea. The sashimi in Japan, which is called sushi, uses fish meat in the autolysis stage. Thus, it is necessary to extend the onset of rigor mortis as long as possible and to consider the time until the fish is consumed after being slaughtered. In this paper, we investigated the postmortem changes in olive flounder by storing the fillets at a low temperature and preparing the fillets that had spinal cord-damage and/or did not have damage after being slaughtered by cranial spiking.

2. Materials and Methods

2.1. Materials

The olive flounder (*Paralichthys olivaceus*, body weight (1,120±36 g)) used in this experiment was purchased from a flounder farm in Gijang - gun, Busan, and transferred to the laboratory by placing it in a water tank controlled by a seawater temperature of 16-17°C. After transporting the fish to the laboratory, it was held for 2 hours while oxygen was supplied to the tank to avoid stress.

The controls were slaughtered by cranial spiking of olive flounders. Slaughtered fish were sealed in a polyethylene bag and stored in a 10°C refrigerator. The spinal cord-damaged samples were passed through the neural canal three to five times with wires after deing slaughtered by cranial spiking, and they were stored in the same manner as above.

2.2. pH

Ten grams of the fish meat was homogenized with 20 mL of distilled water at 12,000 rpm for 1 min under 5°C. The pH of the homogenized sample was measured using a pH meter.

2.3. Total Volatile Basic Nitrogen (TVB-N)

Two grams of the fish meat was homogenized with 8 mL of 4 % trichloroacetic acid (TCA). The mixture was kept at ambient temperature for 30 min and then centrifuged at 3,000 rpm for 10 min. The supernatant was made up to 10 mL with 4 % TCA and determined to be TVB-N, according to the method of Hasegawa [8].

2.4. ATP-related Compound Analysis

Analysis of nucleic acid-based substances was carried out according to the method of Ryu et al [9]. Ten milliliters of 10% perchloric acid (PCA) was added to 0.5 g of the fish meat, homogenized, centrifuged at 4,000 rpm, and the supernatant was separated. Ten milliliters 10% PCA was added to the precipitate, and the supernatant of the mixture was collected by repeating this aforementioned process twice. The supernatant was filtered, and the mixture was adjusted with 5 N KOH to a pH of 6.5 and then the volume was increased up to 100 mL with 10% PCA solution. The mixture was allowed to stand at 0°C for 30 minutes, then filtered through a 0.45-μm membrane filter and stored at -60 °C for use as a sample for HPLC analysis. The standard materials ('5-ATP, '5-ADP, '5-AMP, '5-IMP, inosine, hypoxanthine) used in the experiments were purchased from Sigma-Aldrich (Sigma-Aldrich, Co, St Louis, USA). The analysis conditions of HPLC (UV-Vis 200 Series, Perkin Elmer, USA) for analyzing nucleic acid-related substances are as follows. Columns were analyzed with an Eclipse XDB-C18 column at a temperature of 40°C, a mobile phase with 1% triethylamine-phosphoric acid (pH of 6.5), and a flow rate of 1.0 mL / min, as well as detected with a UV-Vis detector (254 nm).

2.5. K-values

Freshness of fish meat was expressed by the K-value using the following formula [10].

$$K-value(\%) = \frac{[H\times R]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[H\times R]+[Hx]}\times 100.$$

where, each compound is expressed as a molar concentration.

2.6. Rigor Index

The rigor index was determined by the method of Bito et al. [11]. The rigor index of the fish was measured at 1 hour intervals after placing the upper half of the whole fish on the horizontal table surface and the other half of the tail portion facing the outside of the table edge. The rigor index was calculated as:

$$Rigor\ index\ (\%) = [(L_0 - L)/L_0]x100.$$

where, L_0 is the vertical distance between the tail fin and the horizontal extension line of the table surface immediately after the slaughtering of the fish, and L is measured in the same manner as above for each measurement time.

2.7. Breaking Strength

Texture analysis was measured using a texture analyzer (Brookfield, CT3, USA). The meat of the dorsal muscle was taken from the same area of each fish and cut into 25 × 25 × 10 mm cubes. Samples were measured using a cylinder (Ta-DSJ), with pre-test and posttest speeds of 5 $mm.s^{-1}$ at a 10kg maximum weight. Hardness (g) was evaluated for 7 samples from each storage period using TexturePro CT software (1.8.31) and the results are expressed as an average value.

2.8. Statistical Analysis

The results from three replications of two trials were subjected to analysis of variance (ANOVA) and Duncan's multiple range test with significant differences at $p < 0.05$ [12].

3. Results and Discussion

3.1. Postmortem pH Changes

The pH changes during the postmortem time for the spinal cord-damaged sample and nondamaged control are shown in Figure 1. The initial pH of the control, which did not have a damaged spinal cord, was 7.07±0.05, and it gradually decreased until 4 hours postmortem and decreased rapidly after 6 hours of storage. The pH at the end of storage, 20 hours postmortem, dropped to 6.11±0.04. The initial pH of the samples with spinal cord damage was 7.10±0.12, and no significant change was observed until 6 hours postmortem. At 8 hours postmortem, the pH value dropped sharply and was recorded as 6.52±0.08 at 20 hours postmortem.

The decrease in the muscle pH after death is due to the generation of H^+ ions associated with the production of lactic acid by glycogen as well as the degradation of ATP in muscle [13,14]. These two factors are closely related to stress during the slaughtering process, including the handling conditions of the fish before death [15]. After death, the initial pH value in the mammal was approximately 7.2, dropping to 5.6 in the postmortem period, while the fish pH decreased from 7.4 to 6.0 and sometimes below [6].

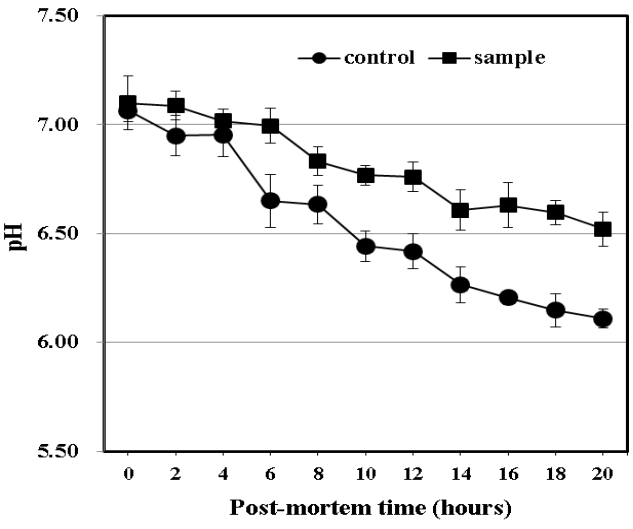

Figure 1. Changes in pH in olive flounder meats without (control) or with (sample) spinal cord damage. Different letters indicate significant differences between postmortem times according to Duncan's test ($p < 0.05$)

In our results, the pH of the control showed a tendency to decrease rapidly 4 hours postmortem, but the spinal cord-damaged sample showed little change until 6 hours postmortem. The pH value at 20 hours postmortem was also higher in spinal cord-damaged samples than that of the control. These results suggest that the production of lactic acid in spinal cord-damaged muscle is slower than in muscle without spinal cord damage after the fish's slaughter by cranial spiking.

3.2. Total Volatile Basic Nitrogen (TVB-N)

The TVB-N resulting from the degradation of protein and nonprotein nitrogen compounds has been used as an indicator of fish freshness. The TVB-N, including trimethylamine (TMA), dimethylamine (DMA) and ammonia, is generated extensively in fish meat during the storage and distribution of fish. In particular, TMA produced by reducing trimethylamine oxide (TMAO), one of the fish's taste components, forms DMA and formaldehyde by the endogenous enzyme present in the muscle, which causes unpleasant smells of fish and indicates that fish meat has spoiled [16,17].

Fish meat that has a TVB-N content of 10 mg/100 g or less is extremely fresh, and fish meat with a TVB-N content of 10-20 mg/100 g is fresh. When the TVB-N content is 25-30 mg/100 g, the initial spoilage of fish meat has started. When the TVB-N content is above 30 mg/100 g, the spoilage in fish meat has progressed considerably, although it depends on the species of fish [18]. In general, the fish meat used as sashimi has a TVB-N content of 20 mg/100 g or less.

The difference in the TVB-N content during the postmortem time according to the pretreatment of olive flounder is shown in Figure 2. The initial TVB-N contents of the olive flounder meat with and without spinal cord damage were 2.0±0.1 and 2.50.3 mg/100 g, respectively. The control, which did not have damage to the spinal cord, gradually increased up to 8 hours postmortem and rapidly increased after 10 hours postmortem.

On the other hand, the spinal cord-damaged sample gradually increased until 12 hours and increased rapidly after 14 hours postmortem time. The TVB-N contents at 20 hours postmortem were 10.4±0.6 in nondamaged meat and 8.4±0.8 mg/100 g in damaged meat. These results show that the freshness of meat with spinal cord damage is prolonged by approximately 4 hours compared to meat without spinal cord damage.

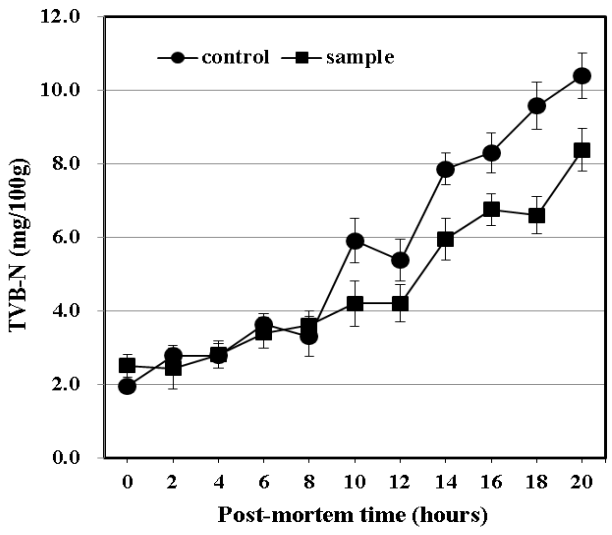

Figure 2. Changes of TVB-N in olive flounder meats without (control) or with (sample) spinal cord damage. Different letters indicate significant differences between postmortem times according to Duncan's test ($p < 0.05$)

Table 1. ATP-related compounds of olive flounder meat without (control) or with (sample) spinal cord-damage

Items		Postmortem time (hours)					
		0	4	8	12	16	20
ATP	control	5.80±0.12	5.43±0.06	4.86±0.07	4.31±0.09	3.54±0.51	3.02±0.14
	sample	5.65±0.12	5.31±0.01	5.08±0.10	4.67±0.06	4.39±0.07	4.12±0.10
ADT	control	0.10±0.07	0.29±0.04	0.27±0.04	0.15±0.02	0.23±0.02	0.28±0.03
	sample	0.30±0.04	0.25±0.06	0.27±0.02	0.28±0.02	0.28±0.03	0.27±0.03
AMP	control	0.12±0.03	0.39±0.03	0.35±0.04	0.28±0.02	0.26±0.03	0.34±0.02
	sample	0.37±0.03	0.29±0.02	0.32±0.02	0.35±0.04	0.36±0.02	0.47±0.04
IMP	control	0.12±0.00	0.24±0.02	0.39±0.06	0.64±0.04	0.93±0.02	1.61±0.07
	sample	0.13±0.03	0.19±0.02	0.31±0.02	0.58±0.05	0.91±0.09	1.33±0.07
HxR	control	0.02±0.01	0.21±0.08	0.18±0.00	0.32±0.02	0.38±0.06	0.59±0.07
	sample	0.02±0.02	0.08±0.07	0.15±0.04	0.28±0.05	0.29±0.02	0.35±0.04
Hx	control	0.01±0.01	0.06±0.02	0.20±0.04	0.36±0.03	0.44±0.03	0.49±0.08
	sample	0.02±0.02	0.04±0.03	0.09±0.04	0.20±0.02	0.27±0.02	0.31±0.02

3.3. ATP-related Compounds and K-values

The changes in ATP-related compounds during the postmortem time of the olive flounder meat with or without spinal cord-damage are shown in Table 1.

Fish are characterized by rapid degradation of ATP after death compared to cattle, pigs and poultry. When a fish is alive, ADP degraded from ATP binds to a single molecule of Cr-P and is then synthesized into ATP. However, after the fish is dead, 1 ADP from 2 ADP is used for the synthesis of 1 ATP until the Cr-P in muscle is completely lost, and 1 ADP is degraded to 1 AMP (adenosine monophosphate) through phosphorylation by myokinase. Subsequently, AMP is released from ammonia by the action of AMP deaminase and is converted to IMP (inosine 5'-monophosphate), which is accumulated in the muscle [19]. IMP, the major nucleotide of fish muscle, is degraded into inosine (HxR), which is related to the freshness of postmortem muscles in fish [10,20]. HxR is formed by nucleoside phosphorylase as hypoxanthine (Hx), and then Hx is finally converted to xanthine and uric acid by xanthine oxidase [16,21,22]. In the initial hour postmortem, the contents of ATP in meat were 5.80 and 5.65 mg/100 g in the control and spinal cord-damaged sample, respectively, but their contents decreased to 3.02 and 4.12 mg/100 g at 20 hours postmortem, respectively.

ADP and AMP remained at a low concentration during the postmortem time. IMP was very low, with concentrations of 0.12 and 0.13 mg/100 g at the beginning of the postmortem period, but increased to 1.61 and 1.33 mg/100 g after 20 hours postmortem.

Accumulation of IMP in the fish meat is one of the most important postmortem changes in the sashimi, since IMP, umami taste, is an important component of the taste in fish and shellfish along with IMP, guanosine monophosphate (GMP), AMP, TMAO and glutamic acid.

The initial HxR levels during the storage were all 0.02 mg/100g, but gradually increased with postmortem time and increased to 0.59 (control) and 0.35 mg/100 g (sample) at 20 hours postmortem. Increased concentrations of HxR indicate loss of freshness in fish meat. The lower concentration of HxR in the spinal cord-damaged sample indicates that the spinal cord damage of the fish can maintain freshness for several hours after death.

Fish meat quality is closely related to ATP-related compounds during the postmortem time. The freshness indicator, that is, the K-value, is expressed as the ratio of the concentration of ATP, and its degradation products, ADP, AMP, IMP, HxR and Hx to that of HxR and Hx. Although many chemical methods for evaluating freshness, including moisture, volatile basic nitrogen, protein and lipid changes, can be applied to the quality of fish meat [23], the concentration ratios of ATP and its degradation products are useful for indicating the quality of fish meat before microbial spoilage [10].

The results of the K-value are shown in Figure 3. The K-value of the control of the initial postmortem time was 0.5, but increased to 16.8 after 20 hours postmortem. However, spinal cord-damaged samples increased from 0.6 to 9.6 during the postmortem time, and their ratio of increase was significantly lower than that of the control.

The K-value of fish meat with very good freshness is less than 20; those with K-value between 20 and 50 have moderate freshness, and those above 70 have been evaluated as not fresh anymore [10]. It is known that fish meat quality can be judged based on the K-value of sashimi [24].

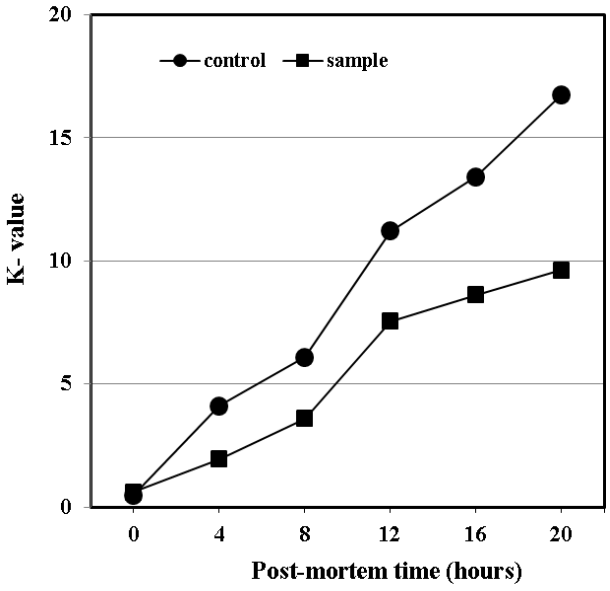

Figure 3. Changes in the K- value in olive flounder without (control) or with (sample) spinal cord damage

In the market, it is generally understood that a K-value of less than 20 is valuable as sashimi. Therefore, various

meat methods including slaughtering, storage temperatures, high-pressure treatment, and bleeding have been attempted to extend the postmortem onset in fish.

Our results indicated that the spinal cord-damaged sample extended by approximately 4-8 hours the K-value compared to the control that did not have a damaged spinal cord.

3.4. The Rigor Index and Breaking Strength

The rigor index of the fish and the breaking strength of the fish meat are shown in Figure 4 and Figure 5, respectively. We stored the olive flounder at 10°C after slaughtering. That is because the onset of rigor mortis is more prolonged when stored at 10°C rather than at 0 °C and 5°C [25].

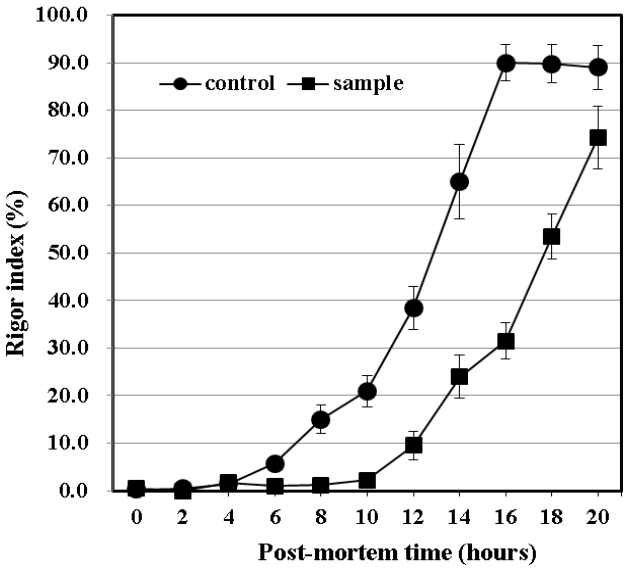

Figure 4. Changes in the rigor index in olive flounder bodies without (control) or with (sample) spinal cord damage. Different letters indicate significant differences between postmortem times according to Duncan's test ($p < 0.05$)

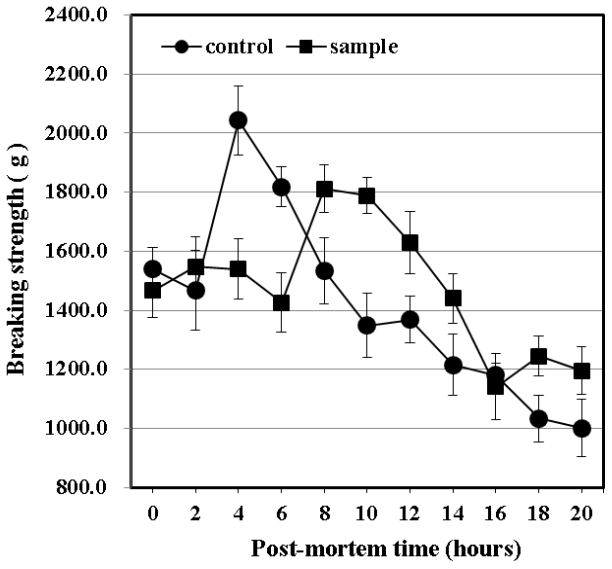

Figure 5. Changes in the breaking strength in olive flounder meats without (control) or with (sample) spinal cord damage. Different letters indicate significant differences between postmortem times according to Duncan's test ($p < 0.05$)

The rigor mortis of the control started at 6 hours after death, and the rigor index rapidly increased to reach the maximum of 90.0% at 20 hours postmortem. The onset of rigor mortis is influenced by factors such as the fish species, stress by the breeding environment and handling methods before death, slaughter methods, and postmortem temperature [22,26,27,28].

In the spinal cord-damaged samples, the onset of rigor mortis was 12 hours postmortem, and the rigor index reached 74.3% at 20 hours postmortem. The maximum value of the rigor index in the control was maintained since 16 hours postmortem but the sample did not seem to reach the maximum of the rigor index until 20 hours postmortem.

In the postmortem period, the point at which the rigor index of the fish's body is maximized and the point at which the breaking strength of the fish meat is maximized are generally inconsistent, and the point of maximum breaking strength appears several hours earlier than that of the rigor index [29,30]. The rigor tension, expressed as the maximum rigor index, is maintained for several hours or more depending on the conditions of the fish, while the breaking strength of the fish meat rapidly drops after reaching the maximum value, which is called tenderization of fish meat. The tenderization of the fish meat is the action of endogenous enzymes that promote muscle protein and connective tissue proteolysis [31].

These enzymes are distinguished from digestive enzymes and are present in muscular cells. The main intracellular proteolytic system in fish is a system involving cathepsins and calpains, as well as alkaline protease and connective tissue hydrolytic enzymes such as elastase and collagenase [32,33,34].

The breaking strength of the control reached its maximum value (2,042 g) at 4 hours postmortem and sharply decreased until the end of the experiment. However, the spinal cord-damaged sample recorded a maximum value (1,811 g) at 8 hours postmortem and its value was somewhat lower than that of the control. Therefore, the results of the rigor index and breaking strength indicate that fish can be further freshened by damaging the spinal cord after slaughtering.

4. Conclusions

The freshness of the fish rapidly decreases after rigor mortis postmortem. Especially in sashimi, various methods for delaying the onset of rigor mortis have been applied, including a slaughter method and changing the storage temperature after slaughtering.

We sought to extend the onset of rigor mortis by slaughtering olive flounder with cranial spiking and then damaging their spinal cord. The initial pH of fish meat postmortem was 7.07 and 7.10 in the meat without and with spinal cord damage, respectively. However, during the 20 hours postmortem, their pH decreased to 6.11 and 6.52, respectively. The TVB-N values, wherein 20 mg/100 g is recommended for sashimi, were 10.4 and 8.4 mg/100 g in meats without and with spinal cord damage at 20 hours, respectively. ATP degradation and the K-values in spinal

cord-damaged meat were delayed by approximately 4 hours compared to those of nondamaged meat.

The rigor index in meat without spinal cord-damage increased to reach the maximum of 90.0% at 14 hours postmortem, but the rigor index in spinal cord-damaged meat reached 74.3% at 20 hours postmortem. The breaking strength in meat without spinal cord damage reached its maximum value at 4 hours postmortem and sharply decreased until the end of the experiment. However, the breaking strength in meat with spinal cord-damage recorded a maximum value at 8 hours postmortem, and its value was somewhat lower than that without spinal cord damage. Our results indicated that the freshness in the spinal cord-damaged sample was extended approximately 4-8 hours compared to the control that did not have damaged spinal cord.

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