Changes in protein compositions and textural properties of the muscle of skate fermented at 10°C

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ABSTRACT
Chemical properties such as pH, ammonia nitrogen, total volatile base nitrogen, trimethylamine nitrogen, protein composition, and free amino acids composition and textural property were investigated for skate muscle subjected to fermentation at 10°C for 14 days. The values of pH, TVBN, TMAN, and ammonia nitrogen content significantly increased in skate muscle. Principal component analysis indicated a high correlation between changes in textural profiles and ammonia flavor and changes in muscle protein composition. In addition, SDS-PAGE of protein fractions showed that myofibrillar tended to aggregate into large polymers during fermentation. On the other hand, changes in trichloroacetic acid-soluble peptides and free amino acid content during fermentation indicated intensive degradation of muscle proteins. The variations in chemical properties and protein composition of skate muscle during fermentation reveal that decomposition and degradation of muscle proteins are important factors that influence the chemical composition and textural property of skate during fermentation at 10°C.

INTRODUCTION

Skate (Raja kenojei) is a typical elasmobranch fish that is widely consumed in South Korea, especially in the province of Jeollanam-do. Koreans enjoy the wing part of skate, owing to its elastic texture and unique ammonia-like flavor. The traditional processing of skate involves fermentation of fresh skate in a ceramic jar without any additives for 1 week at room temperature. Unlike other fermented fishes such as belachan in Malaysia, kap in Thailand, and yulu in China, skate muscle is known to produce high concentrations of nitrogen components during fermentation. Of these nitrogen components, trimethylamine oxide (TMAO) and urea are degraded into volatile bases of ammonia by the action of microorganisms. As reported by Jang et al. who demonstrated that Gammaproteobacteria plays important role in decomposition of urea during the fermentation of skates. Furthermore, a relatively high concentrations of urea (292–369 mmol/kg wet mass) and urease in skate muscle tissue for counterbalancing osmotic pressure in the deep sea – also responsible for this unique ammonia formation. The elastic texture is possibly attributed to the large polymers of low molecular weight proteins containing disulfide and covalent bonds. In addition, complex biochemical and physical reactions occur owing to the action of endogenous and microbial proteinases that significantly change the initial features during fermentation.

As proteins are the most important constituents of skate muscle, a major change during fermentation involves the conversion of proteins to small peptides, amino acids, and other low molecular weight compounds. Moreover, proteins form networks and structures and interact with...
other ingredients during fermentation. The products formed from protein degradation and protein aggregation play an important role in the physicochemical properties of skates, such as the texture, color, flavor, and nutritional composition. Based on the solubility characteristics, proteins in the skate muscle may be divided into three main groups, including myofibrillar proteins, sarcoplasmic proteins, and alkali-soluble proteins. Sarcoplasmic proteins comprise 18% to 20% of the total muscle proteins and are water-soluble proteins normally found in the cell plasm. The largest proportion (about 65% to 80%) of total muscle proteins includes myofibrillar proteins such as myosin, actin, and tropomyosin, which could be extracted by neutral salt solutions. Myofibrillar proteins have been reported to be associated with the fiber-like structure and muscle activity. The final group of proteins, alkali-soluble proteins, are those involved in the formation of connective tissues surrounding muscle fibers and skin and comprise about 3% to 5% of the total muscle proteins. Several studies have reported the relationship between protein composition and property changes of seafood products during processing. These results demonstrated that the transformation of myofibrillar and sarcoplasmic protein to alkali-soluble protein due to denaturation and aggregation strongly affects texture profiles and microstructure of fish products. Furthermore, proteins in meat product are able to interact with other ingredients and play an important role in the textural, sensory, and nutritional quality of the final product.

Many studies have been published on the classification, physicochemical properties, and nutrition composition of skate. However, the changes in the physicochemical properties in terms of protein composition, protein–protein interaction involved in the formation of gel texture during skate fermentation are yet unknown. In this direction, we investigated the changes in the protein composition (myofibrillar, sarcoplasmic, and alkali-soluble proteins) and physicochemical properties of skate muscle during low-temperature fermentation (10 ± 1°C) for 14 days. The relationships between protein composition and physicochemical properties (pH, ammonia nitrogen, total volatile base nitrogen, and trimethylamine nitrogen content, and textural profiles, et.) in skate muscle could contribute to the advancement in knowledge on alkaline fermented fish products, which is a less known fermented food groups.

Materials and methods

Preparation of fermented skate

Fresh skates (Raja kenoye) (size: 72 × 64 cm; average weight: 4 kg) were purchased from a local market in Gwangju, South Korea. Upon arrival to laboratory, skates were immediately distributed uniformly between the layers of sterilized rice straw without any additive in a ceramic jar. The ceramic jar was incubated at 10 ± 1°C and 70−80% humidity for up to 2 weeks. During fermentation, the edible muscle parts from wing pieces were randomly selected at days 0, 2, 4, 6, 8, 10, 12, and 14, and evaluated for their physicochemical properties and muscle protein composition.

PH and ammonia nitrogen level

The pH of skate muscle was determined according to the method of Xu et al. A total of 5 g of sample was homogenized (T25-S1, Janke & Kunkel GmbH & Co KG, Breisgau, Germany) in 10 volumes of distilled water at 11270 × g for 1 min and the pH was measured using a pH meter (EF-7732, Istek, Seoul, Korea). Ammonia nitrogen content was determined by the distillation method described by Dissaraphong et al. with slight modifications. Skate muscle (4 g) was homogenized with 100 mL of distilled water and placed in a 400-mL Kjeldahl flask with 3 g of magnesium oxide (MgO). The mixture was distilled and the distillate was collected in 50 mL of 4% boric acid containing the mixture of indicators (methyl red, bromocresol green, and methylene blue). The distillate was finally titrated with 0.05 M sulfuric acid (H₂SO₄) until a color change from green to gray was observed. The ammonia nitrogen content was calculated as following:
Ammonia nitrogen (g/kg) = \frac{28 \times M \times V}{W} \quad (1)

where M: the concentration of H$_2$SO$_4$ (M), V: volume of H$_2$SO$_4$ (mL), W: weight of the sample (g)

**Total volatile base nitrogen (TVBN) and trimethylamine nitrogen (TMAN) content**

The contents of TVBN and TMAN of the skate muscle were measured according to the method described by Ng.\[^{13}\] In brief, 2 g skate muscle was homogenized with 8 mL of 4% trichloroacetic acid (TCA) using homogenizer at a speed of 11270 × g for 1 min. After centrifugation, 1 mL of the supernatant and 1% boric acid containing Conway indicator were placed in the outer and inner ring of the Conway apparatus, respectively. For reaction initiation, 1 mL of saturated potassium carbonate (K$_2$CO$_3$) solution was added to the outer ring and the Conway unit was immediately closed and incubated at 37°C for 1 h. The content of TMAN was measured in the same manner as described above, but 1 mL of 10% formaldehyde was added to the sample extract. The inner ring solution was titrated with 0.02 N hydrochloric acid (HCl) until a color change from green to pink was observed.

**TCA-soluble peptides**

The content of TCA-soluble peptides from skate muscle was measured according to the method of Greene and Babbitt.\[^{14}\] Briefly, 3 g of skate muscle was treated with 27 mL of 5% (w/v) TCA solution and homogenized at 11270 × g rpm for 1 min, followed by centrifugation at 1500 × g for 15 min. TCA-soluble peptide content in the supernatant was measured by the method of Lowry et al.\[^{15}\] and expressed as micromole of tyrosine released per gram sample.

**Instrumental textural profile analysis (TPA)**

Textural profile analysis of skate muscle was performed by two-cycle compression tests using a texture analyzer (500 N, Zwick GmbH & Co. KG, Ulm, Germany) equipped with a 50-mm-diameter cylindrical probe, as per the method of Riebroy et al.\[^{16}\] with slight modifications. The skate muscle was cut into the shape of a cylinder (30 mm height × 20 mm diameter). Samples were measured at a test speed of 2.0 mm/s with a 60% compression strain and a holding time between compressions of 3 s. Hardness, springiness, gumminess, chewiness, and adhesiveness were calculated and recorded.

**Protein composition**

The protein composition of skate muscle was evaluated based on the method of Hashimoto et al.\[^{9}\] with little modifications. A total of 20 g of skate muscle was extracted in 200 mL of I= 0.05 phosphate buffer (pH 7.5) using homogenizer at 11270 × g for 1 min. The homogenate was centrifuged at 1500 × g for 15 min, and the residue was extracted in the manner described above. The supernatants were combined and treated with up to 5% TCA. The precipitate was collected and used as the sarcoplasmic protein fraction, whereas the filtrate was used as the non-protein nitrogen fraction. The pellet was homogenized with 200 mL of I = 0.5 potassium chloride (KCl) phosphate buffer (pH 7.5) and centrifuged. The two supernatants were combined and used as the myofibrillar protein fraction. The obtained pellet was extracted with 200 mL of 0.1 M sodium hydroxide (NaOH) with overnight stirring. After centrifugation, the supernatant was used as the alkali-soluble protein fraction. All operations were quantitatively performed at 3–4°C. The nitrogen content of all protein and non-protein fractions was determined by the Kjeldahl method.\[^{17}\]
Free amino acid analysis

A free amino acid composition of skate muscle during fermentation at 0, 5, 10, and 14 days was measured according to the methods of Wu et al.,[18] with a slight modification. In brief, 5 g of skate muscle was homogenized with 30 mL of 16% TCA solution at 11,270 × g for 1 min. The homogenized solution was placed in boiling water for 10 min. The final volume was made up to 50 mL with distilled water and the solution was centrifuged at 3,000 rpm for 10 min. Following centrifugation, 1 mL of the supernatant was mixed with 2 mL of 0.02 N HCl and filtered through 0.2-μm membrane filters. An amino acid autoanalyzer (L-8900, Hitachi, Japan) equipped with an ion exchange column (4.6 × 60 cm) was used to determine the free amino acid composition. About 20 μL of each sample was injected for free amino acid analysis. An ultraviolet detector was used to detect proline at a wavelength of 440 nm and other amino acids at 570 nm. External standard methods were used to calculate the quantity of each free amino acid.

Protein electrophoresis

Changes in protein patterns of sarcoplasmic, myofibrillar, and alkali-soluble proteins from skate muscle during fermentation at 0, 5, 10, and 14 days were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[16] These three protein types were extracted according to the method described by Hashimoto et al.,[9] and the protein content was determined by the Kjeldahl method.[17] Each protein fraction was mixed with SDS-PAGE loading buffer (2×) containing 2 mM β-mercaptoethanol. Each sample was loaded at 10 μg protein/well to obtain high-intensity band. SDS-PAGE was conducted using 4% stacking gel and 12% separating gel at a constant voltage of 120 V. After electrophoresis, protein bands were stained using Coomassie Brilliant Blue R-250 (0.2%) in 25% methanol and 10% acetic acid for 1 h and then destained with 25% ethanol and 10% acetic acid. The equipment for electrophoresis was purchased from Bio-Rad Laboratories (Mini-Protein II, Richmond, CA, USA).

Statistical analysis

All experiments were performed twice, and all measurements were performed in triplicates. Values are expressed as mean ± standard deviation and analyzed by one-way analysis of variance. The mean comparison was performed by Duncan’s multiple range test and significant differences were defined at p < 0.05. Statistical analyses were performed using the SPSS 17.0 for windows software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Analysis of pH and ammonia nitrogen content

Figure 1a shows the pH profile of skate muscle during fermentation. The pH of skate muscle significantly increased from 8.24 to 9.13 during the first 10 days of fermentation, resulting in the rapid increase in ammonia nitrogen content (p < 0.05). This observation may be attributed to the accumulation of alkaline ammonia components, amines, and other basic substances produced from the degradation of proteins by endogenous and microbial enzymes.[19] Unlike other fish species, skate retains high urea and trimethylamine N-oxide (TMAO) in their muscle tissue as organic osmolytes at relatively high concentrations urea at 292–369 mmol/kg wet mass and TMAO at 85–168 mmol/kg wet mass, which leads to a significant increase in pH values and formation of unique ammonia flavors by the action of microorganisms during the fermentation.[7] A bacterial succession during the curing process of a skate has been investigated by Reynisson et al.[20] that the key players in skate samples during fermentation are organisms belonging to Oceanisphaera, Psychrobacter, Pseudoalteromonas genera and some species within the Pseudomonas genus. Both Oceanisphaera and Pseudoalteromonas had a strong urease activity and were present in high relative
quantity in the beginning of the fermentation. In addition, the increase in pH value was related to
the liberation of inorganic phosphate and ammonia from the degradation of ATP.[21] In the studies
of Lee et al.,[22] a significant increase in the pH of skate muscle was observed during low-temperature
fermentation. A similar increase in pH value has been reported for other fish species such as sardine
and hake during processing.[23]

A significant increase in the ammonia nitrogen content of skate muscle was observed during
fermentation and the value ranged from 1.99 g/kg at day 0 to 11.96 g/kg at day 10, followed by a slow
increase (Figure 1b). These results suggest that rapid degradation of proteins and deamination of
nitrogenous compounds occur during the first 6 days of fermentation. The increase in ammonia nitrogen
content may be associated with the higher concentration of nitrogen components produced by the
degradation of muscle proteins.[5] It has been reported that urea can be decomposed by microorganism
producing urease during fermentation, in which ammonia nitrogen is the major products.[24] In the

Figure 1. Changes in pH (a), ammonia nitrogen (b), TVBN (c), TMAN (d) and TCA-soluble peptides (e) content of skate muscle
during fermentation. Bars represent standard deviation from three determinations. \(^{a-b}\) Means followed by different letters are
significantly different (\(p < 0.05\)).
study of Jang et al. (6), it has been reported that the urea concentrations in the skate samples were 9-fold higher than those of fermented skates. While a much higher ammonia nitrogen content in fermented skate samples was observed than that of fresh skates. According to Reynisson et al (20) and Cho et al. (25), Genus Pseudomonas and Thiopseudomonas were a predominant bacterium in fermented skate sample. And those two genera have been reported that predominantly produces alkaline proteolytic enzymes, which catalyze the cleavage of peptide bonds in meat proteins. These nitrogen components are degraded into volatile bases of ammonia, owing to the action of microorganisms. The increase in the content of ammonia nitrogen may be related to the activity of microbial and endogenous enzymes. (27) Similar results have been reported during the fermentation of traditionally fermented shrimp paste for 12 months by Faithong and Benjakul. (3) The increase in ammonia nitrogen content over the entire fermentation period resulted in an increase in pH through the degradation of small peptides and amino acids, as well as urea.

**Analysis of TVBN and TMAN content**

The contents of TVBN and TMAN were considered as the products of nitrogenous materials produced by microbial and endogenous enzymes. Thus, both values are widely used as quality indices in the evaluation of different fresh and fermented fish products. (28) The changes in TVBN and TMAN contents of the skate muscle during fermentation are shown in Figure 1c–d. The initial TVBN and TMAN values were 142.50 and 12.88 mgN/100 g, respectively, and a progressive increase in these values was observed during fermentation, especially during the first 8 days of fermentation. The high values of initial skate samples might be attributed to the ammonia present in the raw material after capture. After 14 days, TVBN and TMAN contents reached to 520.80 and 90.72 mgN/100 g, respectively. The TVBN and TMAN content of fermented skate in our study were consistent with the results of Lee et al. (22) who evaluated TVBN content of ready-to-eat skate products brought from the market and observed that TVBN content of fermented skates was higher than that of other fermented fish products such as sardines, tuna viscera, and Yu-lu. (4,12,23) Higher concentrations of nitrogen components in skate were produced during fermentation, resulting in higher level of TVBN. (5) In addition, a slight decrease in TVBN content was observed after 12 days of fermentation. Our results are in line with those reported by Jiang et al., (4) wherein the TVBN value of fermented fish sauce showed a little decrease after 4 months of fermentation. In general, TVBN, including nitrogen from ammonia, TMA, and amine, reflects the extent of degradation of proteins and non-protein nitrogen compounds. The decrease in TVBN content during long-term fermentation may be related to the endogenous enzyme activity. (29) TMA was produced from the decomposition of TMAO by bacteria such as Pseudomonas spp, Photobacterium phosphoreum and Shewanella putrefaciens, which are the primary ingredients responsible for the spoilage of fish and has a typical fishy odor. (30,31) In the present study, TMA showed a similar trend with TVBN content in skate muscle during fermentation.

**Analysis of TCA-soluble peptides**

A significant increase in the level of TCA-soluble peptides in skate muscle was observed during fermentation (Figure 1e), which was consistent with the increase in the amino type nitrogen and non-protein nitrogen content. This observation may be attributed to the degradation of myofibrillar and sarcoplasmic proteins to yield peptides and amino acids, as evident from the decrease in the band intensity of myofibrillar and sarcoplasmic proteins during fermentation. Similar results have been reported by Xu et al. (8) and Visessanguan et al. (32) in silver carp sausage and Nham sausage, respectively. No significant difference was reported in TCA-soluble peptides after 8 days of fermentation. During the extension of the fermentation process, small peptides may serve as nutrients to facilitate microbial growth. In addition, the degradation of peptides into amino acids contributed to the decrease in TCA-soluble peptide content. (33) The peptides produced from endogenous and microbial enzyme activities are
thought to be responsible for the development of flavor and aroma of fish products. The higher TCA-soluble peptide content indicated greater hydrolysis of skate muscle during fermentation.

Textural profile analysis

The results of textural profile evaluation, including hardness, springiness, gumminess, chewiness, and cohesiveness of skate muscle during fermentation are shown in Table 1. A slight decrease in the hardness and cohesiveness was observed at the end of the fermentation than during the initial fermentation stages, whereas similar values were observed for springiness, chewiness, and gumminess (p > 0.05). This observation contradicts the general knowledge about raw fish fermentation that softening occurs after 1 day of fermentation or cold storage. In general, the decrease in textural parameters is related to the degradation, deterioration, as well as oxidation of muscle tissue. However, the elastic texture of the skate muscle may be related to the denaturation and gelation of muscle proteins, leading to the formation of a protein network. It has been reported that the conformation changes in myofibrillar proteins induced by alkaline processing would expose more functional groups for trans-glutaminase, which resulted in protein-protein crosslinking and protein–water interactions. In addition, the loss of water from skate muscle during fermentation may have contributed to the characteristic hardness. The decrease in springiness at later stages of fermentation was probably owing to the extensive formation of covalent cross-linking among proteins through disulfide bonds and non-disulfide covalent bonds. Our results differ slightly from those of Riebroy et al. studies, wherein the hardness of fermented Thai fish mince increased during fermentation. This observation could be explained by the gradual decrease in pH from the aggregation of proteins that is thought to be associated with firmness. Riebroy et al. reported that the decrease in pH might result in an increase in the firmness and mouthfeel because of the acid denaturation of muscle proteins. In our study, the gel formation of myofibrillar protein produced by higher pH of skate muscle during fermentation may have been responsible for the elastic textural properties of skate muscle, which confirmed the results of increment of alkali-soluble proteins content in protein composition analysis.

Protein composition analysis

Myofibrillar, sarcoplasmic, and alkali-soluble proteins are important components of skate muscle and play important functional and structural roles. As shown in Figure 2, the myofibrillar protein fraction was the dominant protein component in skate muscle, followed by sarcoplasmic and alkali-soluble proteins, consistent with the observation reported in other fish species such as sardine and mackerel. As the fermentation progresses, the changes in protein compositions of skate muscle were evident from the significant decrease in myofibrillar and sarcoplasmic protein contents accompanied with a significant increase in the content of alkali-soluble proteins and non-protein nitrogen. Similar results have been reported by Xu et al., wherein a continuous decrease in myofibrillar and sarcoplasmic protein fractions was observed during fermentation with a concomitant increase in the alkali-soluble protein fraction.

Table 1. Textural profiles analysis in skate muscle during fermentation.

| Fermentation time (days) | Hardness (N) | Springiness | Gumminess (N) | Chewiness (N) | Cohesiveness |
|-------------------------|-------------|-------------|---------------|--------------|-------------|
| 0                       | 0.65 ± 0.01<sup>a</sup> | 0.26 ± 0.04<sup>ab</sup> | 0.76 ± 0.08<sup>ab</sup> | 0.24 ± 0.01<sup>a</sup> | 1.42 ± 0.01<sup>a</sup> |
| 2                       | 0.48 ± 0.05<sup>b</sup> | 0.28 ± 0.03<sup>ab</sup> | 0.70 ± 0.28<sup>ab</sup> | 0.18 ± 0.02<sup>ab</sup> | 1.28 ± 0.06<sup>ab</sup> |
| 4                       | 0.53 ± 0.03<sup>ab</sup> | 0.24 ± 0.06<sup>ab</sup> | 0.62 ± 0.08<sup>ab</sup> | 0.13 ± 0.02<sup>b</sup> | 1.19 ± 0.03<sup>bc</sup> |
| 6                       | 0.59 ± 0.02<sup>ab</sup> | 0.21 ± 0.01<sup>ab</sup> | 0.59 ± 0.11<sup>b</sup> | 0.14 ± 0.06<sup>b</sup> | 1.02 ± 0.01<sup>c</sup> |
| 8                       | 0.52 ± 0.05<sup>b</sup> | 0.20 ± 0.03<sup>ab</sup> | 0.62 ± 0.01<sup>b</sup> | 0.12 ± 0.03<sup>b</sup> | 0.65 ± 0.14<sup>d</sup> |
| 10                      | 0.48 ± 0.07<sup>b</sup> | 0.17 ± 0.01<sup>b</sup> | 0.82 ± 0.07<sup>a</sup> | 0.14 ± 0.02<sup>b</sup> | 0.53 ± 0.13<sup>d</sup> |
| 12                      | 0.47 ± 0.05<sup>b</sup> | 0.18 ± 0.04<sup>b</sup> | 0.68 ± 0.11<sup>ab</sup> | 0.16 ± 0.05<sup>ab</sup> | 0.63 ± 0.10<sup>d</sup> |
| 14                      | 0.54 ± 0.07<sup>ab</sup> | 0.20 ± 0.04<sup>ab</sup> | 0.76 ± 0.06<sup>ab</sup> | 0.16 ± 0.06<sup>ab</sup> | 0.57 ± 0.03<sup>d</sup> |

<sup>a-d</sup> Means with different superscripts in the same column indicate significant differences (p < 0.05).
Non-protein nitrogen could be derived from free amino acids, small peptides, nucleic acids, urea, and ammonium ions. The increase in non-protein nitrogen content could be explained from the higher concentration of nitrogen components produced during fermentation. Moreover, several measurements in our study have shown that proteolysis during fermentation was reflected by an increase in the non-protein nitrogen content. On the other hand, the decrease in myofibrillar and sarcoplasmic protein fractions was probably associated with the protein degradation by endogenous and microbial proteases. In addition, the gel formation including denaturation and irreversible aggregation of proteins to form large aggregates play an important role in the decrease in the myofibrillar protein fractions. The increase in the content of alkali-soluble protein could be related to the extensive covalent cross-linking of myofibrillar proteins through disulfide and non-disulfide covalent bonds. Several researchers have demonstrated that the conformational changes in the myosin (the main composition of myofibrillar protein) head under alkaline conditions is the main mechanism of myofibrillar protein to form aggregation and gel matrix. Whiles, the role of a disulfide bond in myosin head-to-head aggregation formation has been proved associated with the reactive thiols which showed a high content after alkaline treatment. The characteristics of these protein fractions were indicated by SDS-PAGE.

**Analysis by SDS-PAGE**

The electrophoretograms of SDS-PAGE obtained for sarcoplasmic, myofibrillar, and alkali-soluble proteins at various fermentation stages (0, 5, 10, and 14 days) are shown in Figure 3. Myofibrillar proteins with molecular weights of >130 kDa disappeared within 5 days of fermentation, owing to their proteolysis by endogenous and microbial enzymes. Furthermore, the band intensity of proteins with molecular weight of about 40 kDa continuously decreased while that of proteins with molecular weight of 15–35 kDa increased. The degradation of protein was also evidenced by the increase in the content of amino nitrogen and TCA-soluble peptides. Similar results were reported by Riebroy et al., wherein the dominant protein molecular weight in Thai traditional fermented minced fish was about 40 kDa that underwent degradation during fermentation to give the product its characteristics. In the study by Spaziani et al., high molecular weight proteins showed a similar decrease during ripening in different sausage types. In addition, the aggregation of polypeptides into large polymers through disulfide and covalent bonds during fermentation plays an important role in decreasing the intensity of low molecular weight proteins (40 kDa) and increasing the amount of insoluble proteins.

Unlike myofibrillar proteins, sarcoplasmic protein fraction showed several new molecular weight protein bands. Thus, the peptides produced from the proteolytic degradation of sarcoplasmic proteins.
may aggregate into low molecular weight proteins, as evident from the increase in the band intensity of alkali-soluble proteins with 25–35 kDa weight. These new bands of low molecular weight proteins (15–20 and 30 kDa) were produced after 5 days of fermentation and the band intensity progressively increased with the prolongation of fermentation. These results suggest that peptides produced from the proteolytic degradation of myofibrillar and sarcoplasmic proteins may aggregate into low molecular weight proteins, resulting in the corresponding increase in the band intensity of alkali-soluble proteins.

Interestingly, the lightened protein bands (molecular weights>90 kDa) were recovered in the myofibrillar protein suggested that certain aggregation of myofibrillar protein was induced by alkaline condition during fermentation. While in sarcoplasmic protein (Figure 3b), protein components with high molecular weights showed similar band intensity at different fermentation time. This may be due to different cleavage sites. It has been reported that some endopeptidase could cleave hydrophilic residues such as glutamate, histidine, and lysine, and keep most hydrophobic residues remained. Therefore, hydrophobic interaction could be enhanced, and aggregation could be formed in skate muscle, which resulted in a significant increase in alkali-soluble protein content. The intensity of myofibrillar protein bands markedly decreased during 5 days of fermentation, followed by a small change in the band intensity of high molecular weight proteins, suggesting that myofibrillar proteins were hydrolyzed by endogenous and microbial proteases such as collagenases, calpains, and cathepsins. On the other hand, the increase in polypeptide bands for sarcoplasmic proteins after 5 days of fermentation indicated that the low molecular weight proteins were degraded into smaller peptides and amino acids by bacterial proteases. The proteolysis pattern of skate muscle observed in our study is in line with the results of previous studies with silver carp and dry fermented sausages.

### Free amino acids composition analysis

The changes in the concentration of free amino acids in skate muscle during fermentation are shown in Table 2. Total free amino acid concentration was 741.01 mg/100 g at the beginning of fermentation, while a small peak of 802.21 mg/100 g was observed after 5 days of fermentation. In the later stages of fermentation, the total free amino acid concentration significantly decreased to 574.54 mg/100 g, owing to the degradation of these amino acids into volatile compounds. Similar results of changes in total free amino acid concentration were reported in suan-cai, a traditional Chinese fermented vegetable, and semi-dry fermented sausages by Wu et al. and Hughes et al. respectively. Thirty-three different free amino acids were detected in skate muscle during fermentation. Of these, β-alanine, ammonia,
sarcosine, and taurine were the most prominent amino acids that formed up to 60.17% of the total amino acid content at the end of fermentation and primarily respond to the changes in the total amino acids except for taurine, which showed a constant decrease during skate fermentation. In contrast, the concentrations of phosphoserine, 3-methylhistidine, glycine, ethanolamine, and arginine constantly increased. The changes in the concentration of each amino acid may be attributed to the differences in the balance between amino acids produced by protein breakdown and microbial actions. The increase in amino acids may suggest that amino acid production by degradation of protein was more intense than amino acid metabolism by bacteria during later stages of fermentation. Some amino acids such as 3-methylhistidine may serve as indicators of changes in meat proteins. The amino acid 3-methylhistidine was located mainly in myofibrillar proteins; thus, the increase in 3-methylhistidine level was related to the degradation of myofibrillar protein fraction during fermentation.

### Relationships between physicochemical parameters and protein composition changes

The principal component analysis was applied to investigate the relationship between chemical parameters and protein composition (non-protein nitrogen and myofibrillar, sarcoplasmic, and alkali-soluble proteins) changes in skate muscle during fermentation. As shown in Figure 4, the first two principal components showed 89.27% of the total variance. Myofibrillar and sarcoplasmic proteins were negatively related with PC1, whereas other indicators except hardness, springiness, chewiness, and cohesiveness showed a positive correlation with PC1, suggestive of the inverse proportional relationship between these
physicochemical parameters and myofibrillar/sarcoplasmic proteins during fermentation. In addition, these parameters showed a similar trend with non-protein nitrogen and alkali-soluble protein fraction, as evident from the close distribution in the component plot in rotated space. Myofibrillar and sarcoplasmic proteins were known to be degraded into small peptides and amino acids by endogenous and microbial proteases, resulting in a significant increase in TCA-soluble peptide and free amino nitrogen content. The increase in ammonia nitrogen, TVBN, and TMAN may be related to the degradation of small peptides and amino acids. On the other hand, the transformation of myofibrillar and sarcoplasmic proteins into alkali-soluble proteins strongly affects textural profiles of skate muscle. The results of principal component analysis allowed us to obtain a better overall idea of the changes in the protein composition and protein–protein interactions during skate fermentation.

**Conclusion**

In this study, pH value, TVBN, TMAN, and ammonia nitrogen content in skate muscle significantly increased during fermentation, which were highly correlated with the degradation of the myofibrillar and sarcoplasmic protein. The increasing content of TCA-soluble peptides during fermentation indicated intensive degradation of muscle proteins. In the present study, the transformation of myofibrillar and sarcoplasmic protein to alkali-soluble protein strongly affects textural quality properties of skate muscle. In addition, the aggregation of small myofibrillar protein fragments under alkaline condition, as well as decrease of hydrophilic residues such as glutamate, histidine, and lysine during fermentation also played a critical role in the formation of superior textural properties of fermented skate muscle. In this study, the changes in the physicochemical properties in terms of protein composition under alkali conditions during skate fermentation was first time reported, which could contribute to the advancement in knowledge on alkaline fermented foods.
**Funding**

This work was supported by a National Research Foundation of Korea [NRF 2017R1D1A1B03035782].

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