Evaluation of Antioxidant Activity and Amino Acids in the Mucus of Mackerel for Cosmetic Applications

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Abstract: To identify antioxidants for improving rough skin, we aimed to analyze the amino acid composition of fish mucus and antioxidant activity of the mucus component. Specifically, we aimed to examine the antioxidant properties of dialyzed mucus components secreted from mackerel, which can be used as raw materials for producing cosmetics. The amino acid composition of hydrolyzed mucus was examined by ultra-high-performance liquid chromatography after dialyzing mucus. In addition, the antioxidant activity of the mucus was evaluated via oxygen radical absorption capacity and Trolox equivalent antioxidant capacity assays. The amino acid composition differed between the low-molecular-weight and high-molecular-weight fractions. Moreover, the low-molecular-weight fraction of farmed mackerel mucus exhibited antioxidant activity with high specificity. The results suggest that antioxidant peptides or free amino acids are present in the low-molecular-weight fraction of farmed mackerel mucus.

Key words: mucus, mackerel, antioxidant activity, amino acid

1 Introduction

The stratum corneum of the skin is located at the boundary between the inside of the skin and outside environment, functioning as a barrier against stimulation from the outside environment. When barrier function decreases, the water retained in the stratum corneum evaporates, leading to dry skin, which can cause inflammation and itching. This study is based on interview survey results that revealed improvement in symptoms such as atopic dermatitis and rough, itchy hands in people who had been handling mackerel for a long time. Previous studies of fish mucus reported the structure and functions of glycoprotein lectins, mucin, antioxidant, and antimicrobial peptides. Glycoprotein, lectins, and mucin are high-molecular-weight materials, whereas antioxidants and antimicrobial peptides are low-molecular-weight materials. The mucus of fish is predominantly composed of the mucins of glycoprotein, whose amino acid compositions differ depending on the species. Antioxidants and antimicrobial peptides are less susceptible to heat denaturation than proteins such as lectins and mucins.

Stress conditions (e.g., handling stress, confinement, food deprivation, exposure to toxic substances) can also change mucus production and composition (e.g., level of proteins and immune molecules), compromising fish health and increasing the fish susceptibility to bacterial pathogens. Compared to natural fish, farmed fish are constantly under high stress, and it was predicted that the secreted mucus components (e.g., proteins, peptides, amino acids, etc.) were different. However, although the components of fish mucus have been investigated, few studies have examined functions such as their antioxidant and anti-glycation effects for applications in cosmetics and human dermatology.

Specifically, mackerel meat hydrolysates exhibit a fatigue recovery effect; thus, they may contain active ingredients involved in antioxidant and anti-aging effects. Moreover, the mucous components of mackerel may contain active ingredients that can improve rough skin or atopic dermatitis. Therefore, the objective of this study was to evaluate the antioxidant activity of mucous components secreted from mackerel and explore their potential as raw materials.
for cosmetics. The amino acids in the hydrolysate of the mucus component of mackerel were analyzed by UHPLC. A recent 2018 study revealed that dry skin is related to oxidative stress caused by reactive oxygen species\(^8\). Therefore, the antioxidant activity of mucus was evaluated in an oxygen radical absorption capacity (ORAC) assay and Trolox\(^\text{TM}\) equivalent antioxidant capacity (TEAC) assay. Methods for evaluating antioxidant activity are classified into two types according to the reaction mechanism of the reactive oxygen species. The first is the hydrogen atom transfer (HAT) method, in which substrate oxidation is suppressed by donating hydrogen atoms. The second is the single electron transfer (SET) method, in which the substrate is reduced by donating electrons. When compared according to the reaction mechanism, the ORAC evaluation method more closely resembles the \textit{in vivo} environment than TEAC\(^9\). Foods and natural products are mixtures of hydrophilic and lipophilic chemicals; therefore, there may be differences in the radical scavenging ability if different measurement principles and detection methods are used. Thus, within this field, it is common to evaluate the antioxidant properties of a test sample using two or more methods. In this study, two methods were adopted to evaluate the antioxidant activity because the specific antioxidants contained in the mucus have not been purified.

2 Experimental

2.1 Samples

The samples used were natural mackerel produced in Nagasaki prefecture, Japan and farmed mackerel produced in Karatsu, Japan. General reagents were purchased from FUJIFILM Wako Pure Chemical Co., (Osaka, Japan). Trifluoroacetic acid was purchased from Watanabe Chemical Industries, Ltd. (Hirosima, Japan). The labeling reagents 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), fluorescein sodium salt, Trolox\(^\text{®}\), 2,2’-azobis(2-methylpropanamidine) dihydrochloride (AAPH), and ABTSTM were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Amino acid analysis of proteins in mucus by UHPLC

For hydrolysis treatment of proteins in mucus, 6 M HCl 200 µL was added to 10 mg of lyophilized powder obtained from natural and farmed mackerel. A mixed solution of hydrochloric acid and mucus was hydrolyzed at 110°C for 24 h under a reduced pressure atmosphere. This sample solution was derivatized with NBD-F. The derivatization sample was prepared by mixing 0.1 M borate buffer (pH 9.96), a reaction solution comprising 1 mL of ethanol and 5 mg of NBD-F, and the sample solution. The sample was heated at 60°C for 5 min. Subsequently, 200 µL of 0.1% trifluoroacetic acid was added to stop the reaction, which was passed over a 0.45-µm filter before amino acid analysis by UHPLC.

2.3 Evaluation of antioxidant activity via ORAC assay

The mucus of natural and farmed mackerel were diazoyzed using Thermo Fisher Scientific (Waltham, MA, USA) DIALYSIS TUBING (12,000–14,000) and then freeze-dried. The residual material in the dialysis tube was named fraction H, which had molecular weight higher than 12,000–14,000. The external dialysate was collected completely and lyophilized and was named fraction L. The sample solutions were prepared by adding distilled water to the lyophilized powder from natural and farmed mackerel. Fluorescein solution (4.2 mM) was prepared in 10 mL of 75 mM phosphate buffer (pH 7.4) with 16 mg of fluorescein sodium salt. Trolox\(^\text{®}\) solution (20 mM) was prepared in 50 mL of 75 mM phosphate buffer (pH 7.4) with 0.25 g of Trolox\(^\text{®}\). AAPH solution was prepared in 10 mL of 75 mM phosphate buffer (pH 7.4) with 2.6 g of AAPH. These solutions were suitably diluted with phosphate buffer before the measurements. Twenty-five microliters each of phosphate buffer solution and Trolox\(^\text{®}\) solution with the sample solution were placed into a 96-well plate (black type), and 50 µL fluorescein solution was added. Next, 25 µL of AAPH solution was added to the plate, and the plate was maintained at a temperature of 37°C for 40 min, with measurements performed every 2 min. A multi-mode plate reader DTX800 (Beckman Coulter, Brea, CA, USA) was used for the measurements. Fluorescence intensities were measured at wavelengths of λex = 485 nm and λem = 535 nm. The ORAC value (µmol TE/g) was calculated from the area of the graph obtained from the value of fluorescence intensity.

2.4 Evaluation of antioxidant activity via TEAC assay

The sample solution was prepared as described for the ORAC assay. ABTSTM working solution was prepared by mixing 5 mL of 7 mM ABTS\(^\text{TM}\) solution and 88 µL of 140 mM potassium persulfate solution. This sample was incubated in the dark at room temperature for 12–16 h and then diluted to an absorbance of 0.70 ± 0.02 at 620 nm with phosphate buffer. In the following procedure, 100 µL of ABTS\(^\text{TM}\) working solution and 10 µL of the sample solution were added to the 96-well plate and stirred. These solutions showed an absorbance of 620 nm after being incubated at 30°C for 4 min. The TEAC value (µmol TE/g) was calculated from the graph obtained from their absorbance values.

3 Results and Discussion

3.1 Analysis of amino acids by UHPLC

Figure 1 shows a UHPLC chromatogram of an amino acid obtained by hydrolyzing mackerel mucus with an acid. High- and low-molecular fractions and natural and farmed fractions were analyzed, respectively. Peaks were assigned from a chromatogram of the standard amino acid solution.
In addition, the area of each peak was calculated and displayed as a percentage of each amino acid, as shown in Figs. 2 and 3. Natural mackerel had a clearly lower y-axis intensity and peak area than farmed mackerel. This indicates that natural and farmed mackerel vary in the total numbers of amino acids. Figure 2 shows the percentages of each amino acid calculated from the UHPLC peak area value in fraction L. The number of each amino acid per 1000 residues and according to the ratio of farmed to natural mackerel is shown in Table 1. Considering the ratio of natural and farmed mackerel, L-Methionine exhibited the highest UHPLC peak area. L-Histidine also exhibited a high value. The sulfur atom of L-Methionine is easily substituted in vivo by the selenium atom, including in mackerel. Selenium substitutions of L-Methionine are known as L-Selenomethionine or L-Selenoneine. These amino acids have high antioxidant activities, and L-Selenomethionine can suppress allergic dermatitis. Thus, the high value for L-Methionine was likely due to the existence of L-Selenomethionine and L-Selenoneine. If the mucus of farmed mackerel contains L-Selenomethionine and L-Selenoneine, it should be rich in selenium, which is an essential trace element and component of glutathione peroxidase, an in vivo antioxidant. Selenium is also involved in the development of malignant tumors. Elemental selenium in the mucus of farmed and natural mackerel was quantified by...
M. Tagami and J. Kuwahara

*J. Oleo Sci.*

inductively coupled plasma mass spectrometry; the selenium concentration of natural mackerel was below the minimum determination limit (<0.1 ppm), whereas the selenium concentration of farmed mackerel was 0.3 ppm.

Furthermore, the ratio of L-Histidine was higher than that of the other amino acids. L-Histidine shows more fatigue-alleviation effects than other amino acids. For example, imidazole dipeptides such as L-Carnosine or L-Arginine also exhibit L-Histidine residue and antioxidant activities. These activities may be conferred by the imidazole group of L-Histidine. We found that L-Histidine and L-Methionine levels were high in fraction L. However, as the amino acids were only analyzed qualitatively, further quantitative analysis should be performed.

The results obtained for fraction H are shown in Fig. 3 and Table 2. The ratio of L-Proline was highest among all amino acids assigned by UHPLC measurements. The main component of mucus is mucin; moreover, it has been reported that squid-derived mucin contains the highest amount of L-Proline. Therefore, high L-Proline level is reasonable because the high-molecular-weight fraction likely contains mucin components.

### 3.2 Evaluation of antioxidant activity by ORAC assay and TEAC assay

Figure 4 shows the ORAC values calculated from the area of the graph obtained from the ORAC measurements. Figure 5 shows the TEAC values. The data on the y-axis are described in μmol Trolox equivalents per 1 g of lyophilized powder. As shown in Fig. 4, the ORAC values of natural and farmed mackerel in fraction L were 60 and 333 μmol TE/g, respectively. In contrast, those of fraction H were 164 and 178 μmol TE/g, respectively. In fraction L of natural and farmed mackerel, the TEAC values were 2.9 and 7.0 μmol TE/g, respectively. In fraction H, the values were 4.3 and 3.6 μmol TE/g, respectively. Both samples showed significant differences in fraction L (p < 0.05). These results indicate that fraction L of farmed mackerel contained many active antioxidant ingredients. In preli-

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**Table 1** Number of different amino acids per 1000 residues in the low-molecular-weight fraction.

| Amino acid residue | Natural mackerel | Farmed mackerel | Farmed / Natural |
|--------------------|------------------|-----------------|------------------|
| His                | 11               | 33              | 3.0              |
| Ser/Arg            | 175              | 261             | 1.5              |
| Asp                | 44               | 28              | 0.6              |
| Gly                | 154              | 173             | 1.1              |
| Glu                | 101              | 51              | 0.5              |
| Thr                | 30               | 29              | 1.0              |
| Ala                | 90               | 53              | 0.6              |
| Pro                | 57               | 87              | 1.5              |
| Met                | 1                | 23              | 16.4             |
| Val                | 64               | 56              | 0.9              |
| Cys                | 9                | 0               | 0.0              |
| Ile                | 41               | 36              | 0.9              |
| Leu/Lys            | 179              | 95              | 0.5              |
| Phe                | 21               | 39              | 1.8              |
| Tyr                | 22               | 36              | 1.6              |
| Total              | 1000             | 1000            | 1.0              |
Table 2 Number of different amino acids per 1000 residues in the high-molecular-weight fraction.

| Amino acid residue | Natural mackerel | Farmed mackerel | Farmed / Natural |
|--------------------|------------------|-----------------|-----------------|
| His                | 20               | 25              | 1.2             |
| Ser/Arg            | 54               | 97              | 1.8             |
| Asp                | 89               | 32              | 0.4             |
| Gly                | 121              | 112             | 0.9             |
| Glu                | 120              | 55              | 0.5             |
| Thr                | 67               | 75              | 1.1             |
| Ala                | 80               | 37              | 0.5             |
| Pro                | 59               | 254             | 4.3             |
| Met                | 17               | 26              | 1.5             |
| Val                | 68               | 58              | 0.9             |
| Cys                | 4                | 4               | 1.0             |
| Ile                | 50               | 47              | 0.9             |
| Leu/Lys            | 191              | 69              | 0.4             |
| Phe                | 36               | 63              | 1.8             |
| Tyr                | 23               | 45              | 2.0             |
| Total              | 1000             | 1000            | 1.0             |

In amino acid analysis by UHPLC measurements, large amounts of L-Histidine were found in the mucus of farmed mackerel. Imidazole dipeptides consisting of L-Histidine are known as antioxidant peptides and have important bioregulatory functions such as antioxidant activity, anti-glycation effects, and anti-fatigue action. The ORAC and TEAC values differed significantly between natural and farmed mackerel. The ORAC and TEAC values of the extracted powder of blackcurrant fruit are 17,880 and 6440 µmol TE/g, respectively, which are higher than those of dried mackerel mucus powder. The antioxidants in fruits are predominantly polyphenols derived from pigments, suggesting that the antioxidant mechanism of mackerel mucus involves compounds other than polyphenols. Moreover, most antioxidants react to specific reactive oxygen species to suppress oxidation. According to previous studies by other researchers, polyphenols such as quercetin showed strong antioxidant activity against hydroxyl radicals. The antioxidant activity of hypochlorite and peroxynitrite was weaker than that of the hydroxyl radical. However, imidazole dipeptides such as L-Carnosine and L-Anserine have inhibitory effects on the in vivo production of hypochlorite and peroxynitrite. The following reasons were considered to explain why the mucus of farmed fish was superior to that of natural fish in terms of antioxidant activities. The mucous surface of fish has a biological defense function against stress such as diseases in fish. Particularly, farmed fish are kept in cages as compared to natural fish, and thus farmed fish are always exposed to stress from density effects and human handling.

**Fig. 5** TEAC values of the high-molecular-weight fraction (H) and low-molecular-weight fraction (L). Natural and farmed mackerel are denoted by N and F, respectively.
Therefore, many anti-stress components such as L-Histidine may be present.

Future studies are needed to confirm the presence of other antioxidants in the mucus and conduct evaluations using cells such as Hacat and B16M cells. The evaluation of antioxidant and anti-inflammatory effects using cells may contribute to the development of cosmetic ingredients based on natural resource recycling.

4 Conclusion

UHPLC analysis of amino acids in mackerel mucus revealed high L-Methionine and L-Histidine values. Moreover, the potential presence of L-Selenomethionine, L-Selenomethionine, L-Carnosine, and L-Anserine in the mucus of farmed mackerel should be further investigated. The ORAC and TEAC assay results revealed that the low-molecular-weight fraction of farmed mackerel exhibited high antioxidant activity.

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