Physical and Chemical Properties Measurement of Collagen Isolated from the Skin and Bone of Mackerel by Subcritical Water Hydrolysis

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ABSTRACT

Most commercial collagens are obtained from bovine bone, bovine hide, calf skin and porcine skin. However, the outbreaks of bovine sponge encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) have raised anxiety among some consumer of collagens from land base animals. Furthermore, porcine collagen and other collagens from animals that were not religiously slaughtered are unacceptable to some religious such as Jews and Muslims. Therefore, increasing interest has been paid to the alternative collagen sources, especially fish skin and bone due to their abundance and low cost. Additionally, high molecular weight of collagen mixed with cosmetics cannot penetrate in to the human skin directly. Low molecular weight of collagen peptide (below 3 kDa) production has great importance in the cosmetic industries. In this study, pepsin-solubilized collagen (PSC) was isolated and characterized from mackerel (*Scomber japonicas*) bone and skin to make more effective use of underutilized resources. The yield of PSC (8.10%) from skin was much higher than that of PSC (1.75%) from bone. Based on protein patterns, both PSC were type I, containing two α-chains. Viscosity of PSC from bone and skin was 18.34 centipose (cP) and 20.26 centipose (cP), respectively. Very low molecular weight peptides (lower than 1650 dalton) were generated from both PSC after subcritical water hydrolysis treatment. Therefore, PSC hydrolyzate can be incorporated as a good ingredient in the food, cosmetic and pharmaceutical industries.

INTRODUCTION

Mackerel (*Scomber japonicas*) is an important food stuff that is consumed worldwide. The Korean peoples consume a wide range of mackerel every day. It has been widely eaten as sashimi, fried and boiling food. For making of those foods, requires the removal of fish by-products such as bone, skin, viscera and etc. During the fish processing, a large amount of wastes are generated from original raw materials [1] in fish shops and fish processing factories. Improper disposal of these wastes may cause serious environmental pollutions with offensive odor. Nutritional values of fish wastes are very high. By the proper utilization of these wastes, environment pollution may be protected and commercially benefitted due to

production of useful materials.

Collagen is one of the structural proteins in vertebrates and it is generally found in bone, skin and other connective tissues [2]. Traditionally, collagen has been isolated from the bones and skins of land based animals such as cow, bovine and pig, and it has been widely used in food, cosmetic, biomedical and pharmaceutical industries due to excellent biocompatibility, biodegradability and weak antigenicity [3]. In recent years, some consumers have anxiety on collagen produced from land base animals because of the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza (AI) [4]. Additionally, collagen obtained from pig cannot be used as a component of some foods for religious reasons. Therefore, there is a strong need to develop alternative collagen sources. Since the collagen from fish wastes such as bone and skin is unlikely to be related to BSE, TSE, FMD and AI and will not be forbidden for religious regions, for that reason it has been increasing attention as alternative collagen resources [5].

To get effective functional materials need macromolecules breakdown. Subcritical water hydrolysis (SWH) is a clean and fast macromolecules hydrolysis method which can be used as an alternative acid, basic and enzymatic hydrolysis with the advantages of shorter reaction time, no use of toxic solvents, and lower formation of degradation products [6]. However, acid and alkali hydrolysis needs violent reaction conditions which can create serious pollution of the environment. Enzyme hydrolysis is specific for macromolecule breakdown and it can take long time for completing production cycle.

Now a day, consumers are increasingly aware of the benefits of nutritionally enhanced foods. Collagen hydrolyzates can help to provide the nutraceutical and/or functional properties which are necessary to create new, innovative and healthy food products. The low molecular weight peptides are also easier for the body to absorb and utilize than whole proteins. Additionally, high molecular weight of collagen mixed with cosmetics products cannot deep penetrate into the human skin. Low molecular weight of collagen peptide (below 3 kDa) production has great importance in the cosmetic industries [7].

Recently, collagens from several fish species have been isolated and characterized [3,4,8]. Nevertheless, collagen from different fish species, habitats and tissues were significantly different in terms of properties. To make more effective use of the wastes generated during the fish processing, collagen was isolated from the bone and skin of mackerel and characterized for their potential in commercial applications.

MATERIALS AND METHODS

Materials

The mackerel (*Scomber japonicus*) samples were collected from F & F Co., Busan, Korea and transported to the laboratory on ice. Pepsin, protein marker, standard collagen from calf skin and bovine achilles tendon were purchased from Sigma-Aldrich (St. Louis, Mo., USA). All other reagents and solvents used in this study were of analytical or HPLC grade.

Sample preparation

The bone and skin were manually separated from raw mackerel and the residues of adhering tissues were also removed manually. After washing thoroughly with running cold water, the samples were dried in a freeze-drier for about 72 h. The dried samples were crushed in a

mechanical blender and stored at -20 ^{o}C until used.

Proximate analysis of mackerel bone and skin

Moisture, ash, crude lipid and crude protein contents of mackerel bone and skin were determined according to the AOAC method [9]. Non protein content was calculated by subtracting the sum of weight of moisture, ash, lipid and protein from total weight.

Isolation of collagen from mackerel bone and skin

The isolation of collagen from mackerel bone and skin was done according to the method of Liu et al. [3] with slight modifications. All experimental procedures were carried out at 4 ^{o}C.

To remove non-collagenous substances, the crushed bone was treated with 0.1 M NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was continuously stirred for 24 h using a magnetic stirrer at a speed of 250 rpm and every 6 h alkaline solution was changed. Next, sample was washed with cold distilled water until a neutral pH of washing water was obtained and lyophilized by freeze drier (EYELA FDV-2100, Rikakikai Co. Ltd., Tokyo, Japan). Subsequently, the insoluble bone was decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 7.5) at a sample/EDTA solution ratio of 1:10 (w/v) for 4 days by changing the EDTA solution once a day. After washing the residue with cold distilled water, fat was removed with 10% (v/v) butyl alcohol at a sample/solvent ratio of 1:10 (w/v) for 24 h. The residue was again washed with cold distilled water. After that, the remaining residue was extracted with 0.57 M acetic acid containing 0.1% (w/v) pepsin for 3 days and the extract was centrifuged at 12,000 rpm for 50 min. The residue was re-extracted with the same solution for 3 days and the extract was centrifuged using the same conditions. Each viscous solution was mixed and salted out by adding NaCl to a final concentration of 2.0 M. The solution was kept 24 h for checking the precipitation. The resultant precipitate was collected by centrifugation at 12,000 rpm for 30 min and it was dissolved in 0.57 M acetic acid. The solution was dialyzed against 0.1 M acetic acid and distilled water using a dialysis bag for 2 days and then lyophilized.

The crushed mackerel skin was treated with 0.1 M NaOH for 24 h where sample/alkaline solution ratio of 1:35 (w/v) was used to ensure the mixture by a magnetic stirrer. The solution was changed 4 times in a day and lyophilized after washing with the cold distilled water. Next, the lyophilized sample was defatted by 10% (w/v) butyl alcohol for 24 h with the above ratio. The defatted residues were washed with cold distilled water and suspended with 0.57 M acetic acid containing 0.1% (w/v) pepsin for 3 days where sample/solution ratio was 1:35 (w/v). Further procedures were carried out according to the collagen isolation from mackerel bone.

Characterization of PSC

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein pattern was analyzed by SDS-PAGE according to the method of Laemmli [10] using a 3.0% stacking gel and a 5.0% resolving gel made in the laboratory. PSC sample (2 mg) was dissolved in 1.0 mL of 0.02 M sodium phosphate buffer (pH 7.2). Solubilized samples were mixed with the sample buffer (1 M Tris-HCl, pH 6.8 containing 10% SDS, 25% glycerol, 2% bromophenol blue and 5% 2-mercaptoethanol) at 1:1 ratio (v/v). Then 20 µL of the mixtures sample (20 µg protein) were loaded onto polyacrylamide gel and subjected to electrophoresis at a constant current flow of 30 mA. Collagens from calf skin and bovine achilles tendon were used as standard sample according to the above procedure to compare with the protein pattern of collagen from mackerel bone and skin. After electrophoresis, the gel was stained using a

0.1% (w/v) Coomassie Brilliant Blue R-250 for 1 h and destained using 7.5% (v/v) methanol and 10% (v/v) glacial acetic acid until clear protein band. The high molecular weight (MW) markers were used to estimate the MW of proteins.

Measurement of viscosity

A Brookfield DVII + Pro viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA 02346 USA) was used to measure the viscosity of PSC from mackerel bone and skin according to the method of Ogawa et al. [11] with slight modification. Exactly, 8 mL of 0.1% (w/v) PSC in 0.1 M acetic acid incubated at 10 ^{o}C for 20 min was put into the vessel. Spindle SC4-18 and agitation 150 rpm was used for viscosity measurement. Viscosity unit was expressed as centipose (cP).

Hydrolyzate production of collagen by subcritical water hydrolysis (SWH)

A small-scale of SWH apparatus was used for PSC hydrolyzate production. The SWH was carried out in 200 mL of a batch reactor made of 276 Hastelloy with temperature controller. Accurately, 0.5 g of PSC and water was put in the reactor and closed. Liquid CO_2 was applied into the reactor for getting initial pressure 2 bar where material to water ratio was 1:200 (w/v). The reactor was heated by an electric heater which was previously heated to the desired temperature (200-250 ^{o}C) and pressure (30-70 bar). The temperature and pressure in reactor of each experiment was measured by temperature controller and pressure gauge, respectively. The sample was stirred by magnetic stirrer at 150 rpm. The reaction time for each sample was taken 3 min. After rapidly cooling to room temperature, the hydrolyzate samples from the reactor were collected and filtered using a filter paper.

Peptide molecular weight analysis

Exactly, 1 µL of the PSC hydrolyzate sample solutions from mackerel bone and skin were spotted onto a polished steel 384 target plate, mixed together with 1 µL of 2,5-dihydroxybenzoic acid (DHB) matrix solution containing 1% THF and dried. Each of the sample was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrum (MALDI-TOF MS). MALDI-TOF mass spectra were obtained on an Ultraflex III mass spectrometer (Bruker Daltonics, Germany) equipped with a 337 nm pulse nitrogen laser. Measurement in the m/z range 700-6000 was acquired in the positive ionization and reflection mode by accumulating data from 200 laser shots.

RESULTS AND DISCUSSION

Proximate composition of mackerel bone, skin and PSC

The protein content of freeze dried mackerel bone and skin was 47.25 ± 1.25% and 49.49 ± 1.52%, respectively as their major component and also contained moisture (6.68 ± 0.07% and 4.60 ± 0.06%), ash (6.89 ± 0.06% and 4.50 ± 0.05%), lipid (17.59 ± 1.10% and 29.47 ± 1.26%) and non-protein (21.59 ± 1.28% and 11.94 ± 0.76%) shown in Table 1. Compared to mackerel bone and skin, the protein and lipid content was highest in skin. However, the moisture, ash and non-protein contents were higher in bone than skin. The yield of PSC from mackerel bone and skin was 1.75 ± 0.07% and 8.10 ± 0.12%, respectively shown in Table 2. Generally, pepsin can be cleaved intermolecular cross-links at the telopeptide region, leading to increased solubilization [2]. Therefore, we had used pepsin for collagen isolation. Some

researchers have been reported the yield of collagen (wet weight basis) from fish bone was 1.6% of bigeye snapper and 1.3% of bighead carp, respectively [1,3] and the yield of collagen (wet weight basis) from fish skin was 7.1% of bigeye snapper (*Priacanthus macracanthus*) and 7.7% of striped catfish, respectively [12,13]. The yield of PSC differences can be attributed to the differences in fish species, biological conditions and preparative methods. These observations were agreement with Liu et al. [3]. Protein content of PSC from mackerel bone and skin was little different and the value was 90.05 ± 2.34% and 86.89 ± 2.48%, respectively shown in Table 2. Additionaly, PSC from mackerel bone and skin contained moisture (6.28 ± 0.12% and 7.48 ± 0.10%) and ash (3.48 ± 0.09% and 5.37 ± 0.06%) with a lower fat content (0.19 ± 0.02% and 0.26 ± 0.03%). Similar results have been reported of collagen from blacktip shark [14].

Table 1: Proximate composition of bone and skin of mackerel

Sample name	Moisture (%)	Ash (%)	Crude lipid (%)	Crude protein (%)	Non-protein (%)
Freeze dried mackerel bone	6.68 ± 0.07	6.89 ± 0.06	17.59 ± 1.10	47.25 ± 1.25	21.59 ± 1.28
Freeze dried mackerel skin	4.6 ± 0.06	4.5 ± 0.05	29.47 ± 1.26	49.49 ± 1.52	11.94 ± 0.76

Means ± SD (n = 3).

Viscosity of PSC and its thermal behaviors

High viscosity is one of the characteristics of collagen. Viscosity of PSC from mackerel bone and skin was 18.34 ± 0.25 and 20.26 ± 0.21 centipose (cP) shown in Table 2. However, viscosity of PSC from mackerel skin was little higher than bone, which might be on account of the high proportion of polymers of collagens, resulting in a higher average molecular weight.

Table 2: Yield characteristics of collagen from bone and skin of mackerel

	Collagen	
	Bone	Skin
Yield (% dry weight)	1.75 ± 0.07	8.10 ± 0.12
Protein (% dry weight)	90.05 ± 2.34	86.89 ± 2.48
Viscosity (cP)	18.34 ± 0.25	20.26 ± 0.21

Means ± SD (n = 3).

SDS-PAGE of PSC

The subunit compositions of PSC from mackerel bone and skin are shown in Figure 1. Both of PSC mainly contained two different α-chains (α_1 and α_2) which were similar to the pattern observed from many other fish species [3,8,15]. Approximately, the MW of both PSC was identical and it was 116 kDa for α_1 and 126 kDa for α_2 which was also similar to standard collagen from calf skin and bovine achilles tendon. Based on electrophoresis mobility and subunit composition, it was suggested that the PSC from mackerel bone and skin were mainly composed of type-I collagen, a heterotrimer containing two identical α_1-chains and one α_2-chain in the molecular form of $[\alpha_1(I)]_2\alpha_2(I)$. This observation was agreement with the previous

reports for skin collagen from deep-sea redfish [15] and fins, scales, skins, bones and swim bladders collagen from bighead carp [3]. Zhang et al. [8] reported that collagen from jellyfish mainly consisted of homotrimer α-chains, $[\alpha_1(I)]_3$. The differences in collagen molecular form can be attributed due to different species. Moreover, PSC from mackerel bone and skin contained one β-chain and the MW of β-chain was approximately 205 kDa.

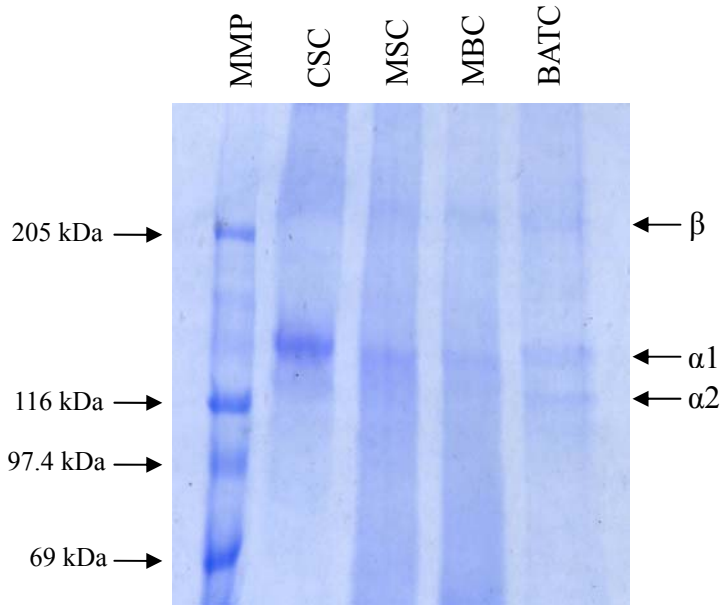

Figure 1: SDS-PAGE pattern of collagens. MMP-molecular weight marker protein, CSC-calf skin collagen, MSC-mackerel skin collagen, MBC-mackerel bone collagen and BATC-bovine achilles tendon collagen.

Hydrolysis yield of PSC

PSC from mackerel bone and skin was completely hydrolyzed at temperature ranging from 200-250 ^{o}C and pressure ranging from 30-70 bar.

Peptide MW identification of PSC hydrolyzates

Molecular weight and peptide was identified of PSC hydrolyzate at temperature (200-250 ^{o}C) and pressure (30-70 bar) from the bone and skin of mackerel by MALDI-TOF mass spectra data shown in Table 1. MW range from 759.12-988.20 dalton obtained of PSC hydrolyzate at temperature 200 ^{o}C and pressure 30 bar from mackerel bone. Among the obtained MW, peptide intensity (3335.74) was very high at MW 984.21 dalton. Peptide peak was not found of PSC hydrolyzate at temperature 250 ^{o}C and pressure 70 bar from mackerel bone within the selected MALDI-TOF MS m/z data range (700-6,000 dalton). Due to high temperature and pressure, lower MW peptide below than 700 dalton and higher organic compound might be produced of PSC hydrolyzate at temperature 250 ^{o}C and pressure 70 bar. MW range from 789.36-1632.92 dalton and 952.30-1368.13 dalton was obtained of PSC hydrolyzate at temperatue (200-250 ^{o}C) and pressure (30-70 bar) from mackerel skin. The highest peptide intensity was 2173.88 in 1216.11 dalton at 200 ^{o}C and 30 bar and 5328.47 in 952.30 dalton at 250 ^{o}C and 70 bar. Smaller MW with higher intensity produced due to apply the high

temperature and pressure. Based on peptide MW, PSC hydrolyzate at different temperature and pressure from mackerel bone and skin were totally different in terms of amino acid sequence and composition. Kittiphattanabawon et al. [14] and Liu et al. [3] reported that MW of collagen maximize reduced 48.8 kDa and 20 kDa using V8-protease from blactip shark and bighead carp which was higher than PSC hydrolyzate of mackerel bone and skin. Therefore, it may be suggested that subcritical water hydrolysis was a good treatment for smaller MW production.

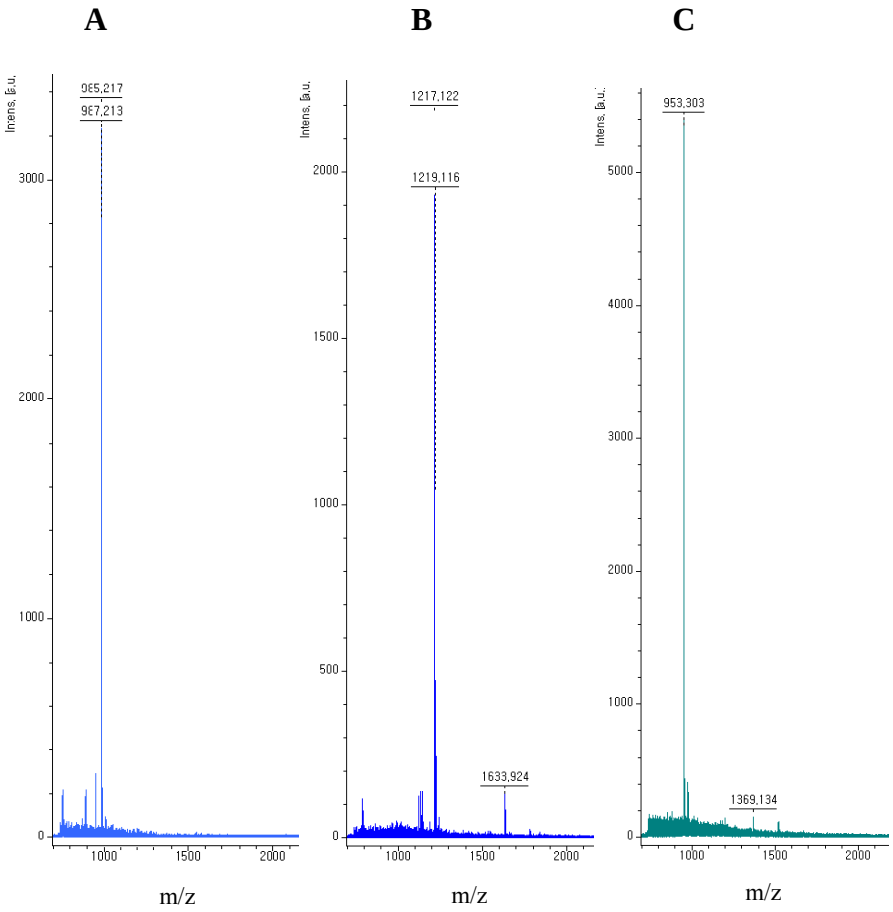

Figure 2: Mass spectra chromatogram of (A) bone collagen hydrolyzate (200 ^{o}C, 30 bar), (B) skin collagen hydrolyzate (200 ^{o}C, 30 bar) and (C) skin collagen hydrolyzate (250 ^{o}C, 70 bar).

CONCLUSIONS

PSC was successfully isolated from bone and skin of mackerel and much higher yield was obtained from skin than bone. Both PSC were most likely type I collagen and maintained their triple helical structure. Different smaller size of MW containing peptide was produced of both PSC after subcritical water hydrolysis treatment. Therefore, PSC hydrolyzate from mackerel bone and skin has the potential to be an alternative source of isolated collagen in various fields.

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