

Comparison of Collagen Characteristics of Sea- and Freshwater-Rainbow Trout Skin

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Abstract Proximate composition, volatile basic nitrogen content, and concentrations of collagen in skin samples from either sea- (S-RT) or freshwater-rainbow trout (F-RT) were characterized and compared, to assess the effect of the sea or freshwater habitat on these parameters. Results of amino acid composition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, thermal denaturation assay and Fourier transform infrared (FT-IR) spectroscopy of acid-soluble collagens were comparable between the two sample sets. Both acid-soluble collagens from sea- and freshwater-rainbow trout skins contained glycine as the major amino acid and high alanine, proline, and hydroxyproline contents, and was found to be predominantly composed of $\alpha 1$ -, $\alpha 2$ -, and β -chains. FT-IR spectra of ASCs from S-RT and F-RT skins were quite similar. These findings suggest that different rainbow trout habitats (seawater and freshwater) do not affect amino acid composition and molecular weight properties of ASCs from S-RT and F-RT skins.

Keywords: sea-rainbow trout, trout, fish skin collagen, acid-soluble collagen, *Oncorhynchus mykiss*

Introduction

Rainbow trout (*Oncorhynchus mykiss*), a Salmonidae, which also includes Pacific trout and salmon, is a human food source rich in omega-3 oils, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); functional free amino acids, such as taurine and anserine; and vitamin A and D (1). An adult rainbow trout weighs between 2 and 3 kg. It is an important commercial fish in Korea, the EU, and USA. These fish live in the upper, cold-water sections of rivers and seas. Similar to other trout, the habitats and food sources of rainbow trout determine their actual colors and shapes (2). Like the Pacific salmon, some Pacific populations of rainbow trout are anadromous—they spend their adult years in the ocean then return to freshwater to spawn. Unlike salmon, however, anadromous rainbow trout do not spawn once then die, but rather, return to spawn several times (3). Although the natural habitat of rainbow trout is in freshwater at approximately 12°C in summer, the species tolerates water temperatures ranging from 0 to 25°C, and the anadromous strain makes runs to the sea (4).

Because it can tolerate a wide range of water temperatures, the rainbow trout may be a suitable species to substitute for warm-water fish species for farming along the south coast of Korea, where

water temperatures frequently hit abnormal lows (lower than 8°C) during the winter (every January–February) (5).

Collagen is a major component of extracellular matrices and improves tissue strength and resistance. Collagens from the skins of various fish species have different properties, particularly denaturation temperatures; these properties are influenced by the environments within which the different fish species thrive (6–8). Recent studies have focused on extraction and characterization of fish collagens (9–11). However, no information on the effect of different environmental conditions on characteristics of collagen in the skin of rainbow trout is available.

The objective of this study was to compare the characteristics of acid-soluble collagen (ASC) from sea-rainbow trout (S-RT) skin to those of ASC from freshwater-rainbow trout (F-RT) skin.

Materials and methods

Materials Sea- and freshwater-rainbow trouts (S- and F-RT) (*Oncorhynchus mykiss*), 38.7–45.3 cm (41.1±2.3 cm) and 36.5–42.8 cm (38.9±2.3 cm), respectively, in body length and 1.0 kg and 0.9 kg, respectively, in body weight, were cultured by feeding (Aller® Silver

EXTM; Aquafeed Co., Christiansfeld, Denmark) in fish farms (13–17°C and 12–16°C, respectively) located in Tongyeong and Geochang, respectively, and obtained in May 2014. The S-RT was also cultured in 3.0% salt concentration. Skins of the RT were separated from fillet with a deskiner, and adhering muscles were removed using a sharp knife. After washing thoroughly with running water, the skins were cut into small pieces (2 to 3 cm) with scissors and placed in polyethylene bags before storing at –20°C for up to 3 months.

Protein markers (wide range: 10–250 kDa) used to estimate the molecular of the collagen were purchased from Bio-Rad Lab., Inc. (Hercules, CA, USA). All of other reagents were of analytical grade.

Preparation of collagen fractions, concentration and yield of collagen

All analyses were done in a cold room (5°C). Native collagen from RT skins was prepared as described by Park *et al.* (12). Frozen S- and F-RT skins were thawed for 20 h in cold tap-water. Cleaned fish skin pieces were squeezed by hand to remove excess water. To the residues, 10 volumes (v/w) of 0.1 M NaOH were added to remove non-collagenous protein and to inhibit the endogenous proteases on collagen (13). The homogenate was continuously stirred overnight using a stirrer (Standard Jar Tester; Phipps & Bird Co., Richmond, VA, USA) before centrifuging at 10,000×g (Supra 22K; Hanil Co. Ltd., Daejeon, Korea) for 20 min. The alkali-extraction was repeated 3 additional times. The skins were washed thoroughly with cold distilled water until a neutral pH (6.91; Metrohm Co., Herisau, Switzerland). Then, 10 volumes (v/w) of 0.5 M acetic acid were added. Suspensions were stirred for 3 days and then centrifuged at 10,000×g for 20 min. The acid-extraction was repeated once more. The skins were then washed with cold distilled water at a 1:2 (w/v) ratio. The supernatant from the acid-extraction and the filtrate from washing were combined and salted out by adding NaCl to 2.0 M before centrifuging at 20,000×g for 20 min. The precipitate was re-dissolved in 0.5 M acetic acid and then dialyzed [molecular weight cut-off of 10,000 kDa according to the manufacturer (Dialysis tubing cellulose membrane; Sigma-Aldrich, St. Louis, MO, USA)] against cold distilled water to remove salt. The salting-out followed by dialysis was repeated twice more for further purification of the collagen. The final dialyzed solution was lyophilized using a freezer-dryer (EYELA freeze dryer; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and this ASC was packed and stored at –25°C until.

Insoluble collagen was prepared as described by Kim and Park (10) with slight modifications. Separately from the preparation of ASC, the centrifuged residue obtained after the last acid-extraction was heated with 5 volumes (v/w) of distilled water in an autoclave at 121°C for 1 h and then centrifuged at 10,000×g for 20 min. The precipitate was rinsed using hot distilled water (95°C) at a 1:2 (w/v) ratio. The supernatant from centrifugation and the vacuum-filtrate from rinsing were combined, and lyophilized. The freeze dried matter was used as a sample of acid-insoluble collagen (AISC) for calculating total collagen (ASC+AISC)-N concentration described in the lower parts.

Concentration and yield of collagen were calculated, respectively, as [total collagen-N concentration, %/total-N concentration of skin, %]×100 and (ASC-N concentration, %/total collagen-N concentration, %) ×100, respectively.

Proximate composition and volatile basic nitrogen (VBN) ASC and PSC were subjected to proximate analyses, moisture, total protein and crude ash contents, according to the AOAC (14) method Nos. 950.46, 920.153 and 928.08, respectively. In addition, total lipid was extracted into a methanol-chloroform mixture according to the method of Bligh and Dyer (15). The content of VBN was determined by the method of Conway described by Kapute *et al.* (16).

Amino acid composition and hydroxylation of proline and lysine

Amino acid composition was determined using an amino acid analyzer (Biochrom 30; Pharmacia Biotech, Cambridge, UK) according to the method described by Kimura *et al.* (17). The sample was hydrolyzed in 6 N HCl in evacuated/sealed tubes at 110°C for 24 h. The hydrolysates was evaporated to dryness in a vacuum evaporator at 40°C and then diluted up to 25 mL with Li⁺-citrate buffer (pH 2.2, Sigma-Aldrich) before injecting into an amino acid analyzer for analysis of amino acids containing hydroxyproline (Hyp) and hydroxylysine (Hyl). The composition of each amino acid was calculated based on the area of the corresponding peak on elution curves of the sample and standard (Sigma-Aldrich).

Hydroxylations (%) of proline (Pro) and lysine (Lys) were calculated on the basis of the amino acid composition according to the following equations:

$$\text{Hydroxylation of Pro (\%)} = [\text{Hyp}/(\text{Pro} + \text{Hyp})] \times 100$$

$$\text{Hydroxylation of Lys (\%)} = [\text{Hyl}/(\text{Pro} + \text{Hyl})] \times 100$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done using the method of Laemmli (18). For SDS-PAGE analysis, the samples (50 mg) were dissolved in 5 mL of 8 M urea containing 2% β-mercaptoethanol and 2% SDS solution. The collagen solution was mixed at 4:1 (v/v) ratio with the sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS (w/v, pH 8.3), 10% glycerol, 2% β-mercaptoethanol and 0.002% bromophenol blue] and then heated at 95°C for 3 min. The samples (20–40 μg collagen) were loaded onto the 10% Mini-PROTEAN TGXTM Precast gel (Bio-Rad Lab. Inc.) and subjected to electrophoresis for about 1.5 h at a constant current of 2 mA per well using a Mini-PROTEAN Tetra cell (Bio-Rad Lab. Inc.). After running the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 for 15 min. Destaining was carried out for 3 day in a solution containing acetic acid, methanol, and water (1:2:7, v/v/v). Molecular weight of protein bands was estimated using a protein marker.

Thermal denaturation temperature (TDT) TDT was performed according to the method of Zhu and Kimura (19). The Ostwald

viscometer (Sigma-Aldrich) was filled with 5 mL of ASC solution (30 mg ASC dissolved in 100 mL of 0.1 M acetic acid solution) or 0.1 M acetic acid as a control sample. The viscometer was immersed in a 15°C water bath for 30 min to allow the collagen solution to equilibrate. The temperature was incrementally increased up to 45°C and checked data at each temperature (15 to 31°C at every 2°C, and at 45°C) for 30 min.

Efflux time of the solution was measured and the specific viscosity (η_{sp}) was calculated according to the following equation:

$$\text{Specific viscosity } (\eta_{sp}) = (t - t_0) / t_0$$

t = efflux time of the collagen solution

t_0 = efflux time of the 0.1 M acetic acid

The above described efflux time was checked with a digital timer (4-channel memory timer, JM Science, Seoul, Korea).

It was assumed that the collagen helical conformation was undenaturated at 15°C, whereas breakdown was completed at 45°C (20).

The TDT of the collagen solution was defined as the temperature at which the change in viscosity was 50% of the total change. Fraction change was calculated according to the following equation:

$$\text{Fraction change} = [(a_2/C) - (a_3/C)] / [(a_1/C) - (a_3/C)]$$

C = collagen concentration (mg/mL);

a_1 = specific viscosity at 8°C;

a_2 = specific viscosity at a measured temperature (°C)

a_3 = specific viscosity at 45°C

Fourier transform infrared spectroscopy (FT-IR) FT-IR was determined as described by Muyonga *et al.* (21). For FT-IR spectra analysis, lyophilized samples placed onto the single reflection germanium crystal cell and the cell was clamped into the mount of the FTIR spectrophotometer (Bruker IFS 88; Bruker, Billerica, MA, USA). All spectra were obtained from 4,000 to 1,000 cm^{-1} at a data acquisition rate of 2 cm^{-1} and compared to a background spectrum collected from the clean empty cell. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker).

Statistical analysis Statistical analysis was done using SAS (version 8.0; SAS Institute Inc., Cary, NC, USA). Analysis of variance using the General Linear Model procedure and the difference between means using the Duncan test were determined at an α level of $p < 0.05$.

Results and Discussion

Food component composition of rainbow trout skin The proximate composition of S-RT and F-RT skins was as follows: 74.2 and 75.3% moisture; 22.4 and 21.2% crude protein; 3.3 and 3.6% crude lipid; and 0.3 and 1.0% ash, respectively (Table 1). Skin from S-RT showed slightly higher in crude protein content than that from F-RT. The sum

Table 1. Proximate composition, collagen concentration and volatile basic nitrogen (VBN) content of sea- and freshwater-rainbow trout (S-RT and F-RT) skins

Food component		Fish skin	
		S-RT	F-RT
Proximate composition (g/100 g)	Moisture	74.2±0.2 ^{b2)}	75.3±0.1 ^a
	Crude protein	22.4±0.1 ^a	21.2±0.1 ^b
	Crude lipid	3.3±0.3 ^a	3.6±0.2 ^a
	Ash	0.3±0.1 ^b	1.0±0.1 ^a
Collagen (%) ¹⁾		61.6±0.2 ^a	60.9±0.1 ^b
VBN (mg/100g)		2.7±0.3 ^a	2.7±0.2 ^a

¹⁾Collagen concentration was calculated as (total collagen-N concentration, %/total-N concentration of skin, %)×100.

²⁾All values are mean±standard deviation ($n=3$). Means in a row followed by different letters differ significantly ($p < 0.05$).

of ash and lipid contents of S-RT skin was 3.6% and was slightly lower than that of F-RT skin (4.6%).

Concentration of collagen in S-RT skin was 61.6%; this was slightly higher than that in F-RT skin (60.9%).

VBN content in S-RT skin was 2.7 mg/100 g, which is within the acceptable limit (<20 mg/100 g) for processed seafood products for human consumption (22). No difference in the VBN content in S-RT and F-RT skins was observed. Kim *et al.* (20) reported that the VBN content in rockfish skin was 4.2 mg/100 g. The relatively low VBN contents of fish skin in our study were probably due to the samples being immediately processed for analysis during preparation of sliced raw fish.

SDS-PAGE patterns Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) patterns of ASCs from S-RT and F-RT skins are shown in Fig. 1. Three distinctive chains were detected in both ASCs from S-RT and F-RT skins: 2 α bands (α_1 , upper; α_2 , lower) with molecular weights of approximately 100 kDa, and their β -cross linked components, with a molecular weight of 200 kDa. Overall, electrophoretic patterns of ASCs from S-RT and F-RT skins were similar to those for collagens from the skin and muscle of other species like hake, including *M. merluccius* L. (23), and *M. productus* (10) trout (23) and Alaska pollock (24). No difference was observed in the relative mobility of α_1 and α_2 chains between ASCs from S-RT and F-RT skins. High molecular weight cross-links were also formed, as seen on the top of each lane in Fig. 1. These large molecules are yet to be identified, and their significance is not known.

Amino acid composition Amino acid composition, expressed as amino acid residues per 1,000 total amino acid residues, and hydroxylation ratios of proline (HDP) and lysine (HDL) in ASC from S-RT and F-RT skins are shown in Table 2. Glycine was the most abundant amino acid present in both S-RT and F-RT. In general, glycine occurs uniformly at every third residue, except for the 14 amino acids from N-terminus and the first 10 from the C-terminus, for most collagen molecules. Alanine and proline compositions were

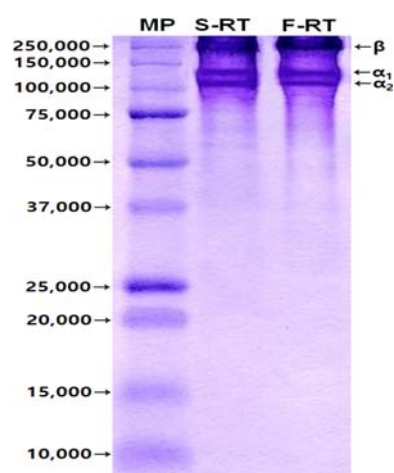


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) patterns of acid-soluble collagens (ASCs) from sea- and freshwater-rainbow trout (S- and F-RT) skins. Compared with MP (Protein marker, 4038; Sigma-Aldrich Co, St. Louis, MO, USA).

also rich in ASCs from both S-RT and F-RT. Threonine, methionine, isoleucine, tyrosine, phenylalanine, hydroxylysine and histidine, however, showed significantly lower concentrations, and cysteine was not detected at all.

For S-RT skin, the ratio was 37.3% for HDP and 23.1% for HDL, these values were similar to those for S-RT skin which were 36.2% (HDP) and 22.0% (HDL). In general, HDP in collagen plays a role in stabilizing the triple helix, whereas HDL contributes to the formation and stabilization of cross-links of nonhydrolyzable bonds (23). The HDPs of ASCs from S-RT and F-RT in this study are lower than those reported for yellow sea bream (40%) and tiger puffer (39%), shark, carp (25) as well as those of land animals and other higher vertebrates (25), but are similar to that reported for Pacific whiting (36.9%) (10).

Thermal denaturation temperature (TDT) TDTs of ASCs from S-RT and F-RT skins are presented in Fig. 2. Within the range of 19–31°C,

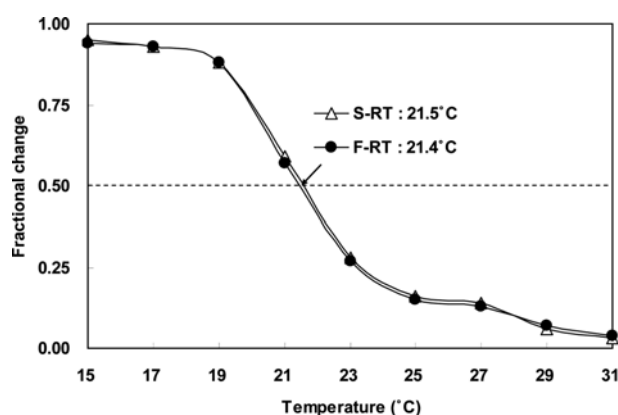


Fig. 2. Thermal denaturation temperature of acid-soluble collagens (ASC) from sea- and freshwater-rainbow trout (S- and F-RT) skins. The denaturation temperature was measured by viscosity in 0.1 M acetic acid of 0.03% collagen solution.

the fractional change of ASC from S-RT skin decreased continuously as the temperature increased; this trend is similar to that of ASC from S-RT skin. Increasing temperature breaks hydrogen bonds between adjacent polypeptide chains of the collagen molecules, and transforms intact trimers into individual chains or dimers this ultimately causes the denaturation of the collagen structure (9). The TDT of ASC from S-RT skin was 21.5°C and was comparable to that of F-RT skin (21.4°C). These results might be due to similar hydroxyproline ratios observed in the ASCs from S-RT and F-RT skins as the hydroxyproline ratio was highly correlated to thermal stability. The TDTs of the ASCs from S-RT and F-RT skins were considerably lower than those for collagens obtained from shark (26) and land animal skins (27).

Fourier transform infrared (FT-IR) spectra FT-IR spectroscopy can provide unique information on molecular structure, which may be useful for determining the secondary structure of proteins (28). FT-IR spectroscopy was used in this study to monitor the functional groups and secondary structure of ASCs from S-RT and F-RT skins. Results

Table 2. Amino acid composition (residues/1,000 residues) and hydroxylation ratios (%) of proline (HDP) and lysine (HDL) of acid-soluble collagens (ASCs) from sea- and freshwater-rainbow trout (S- and F-RT) skins

Amino acid	Rainbow trout ¹⁾		Amino acid	Rainbow trout		Amino acid	Rainbow trout	
	Sea	Fresh water		Sea	Fresh water		Sea	Fresh water
Asp	44	37	Cys	tr	tr	Lys	50	46
Hyp	63	63	Val	25	24	His	8	8
Thr	19	19	Met	9	10	Arg	51	52
Ser	42	41	Ile	10	10	Total	1,000	1,000
Glu	70	72	Leu	21	21	HDP ¹⁾	37.3	36.2
Pro	106	111	Tyr	4	4	HDL ²⁾	23.1	22.0
Gly	335	337	Phe	18	17			
Ala	110	115	Hyl	15	13			

¹⁾HDP (%)=Hydroxyproline/(Hydroxyproline+Proline)×100

²⁾HDL (%)=Hydroxylysine/(Hydroxylysine+Lysine)×100

³⁾tr: trace

All values are mean of duplication.

Table 3. Main locations of Fourier-transform infrared (FTIR) spectrum peak and their assignments for collagens from sea and freshwater-rainbow trouts (S- and F-RT) skins

Region	Normal wavelength range (cm ⁻¹)	Peak wavenumber (cm ⁻¹) obtained		Assignment ¹⁾
		S-RT	F-RT	
Amide A	3,400-3,440	3,328	3,332	N-H stretch, coupled with hydrogen bond
Amide B	2,850-2,950	2,890	2,898	CH ₂ asymmetrical stretch
Amide I	1,600-1,700	1,674	1,696	C=O stretch/hydrogen bond coupled with COO ⁻
Amide II	1,550-1,600	1,546	1,554	N-H bend coupled with C-N stretch
Amide III	1,320-1,220	1,210	1,214	N-H bend coupled with C-N stretch

¹⁾Jeevithan *et al.* (9) and Wang *et al.* (30)

are shown in Table 3. The amide A band (3,400-3,440 cm⁻¹) is generally associated with N-H stretching frequency. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies; usually 3,300 cm⁻¹ (29,30). The amide A band of ASC from S-RT skin appeared at 3,328 cm⁻¹ and was similar to that from F-RT skin (3,332 cm⁻¹). Liu *et al.* (31) and Wang *et al.* (30) reported that the amide A bands of ASC from the skins of four carp species and from Amur sturgeon occurred in the range of 3,313-3,323 cm⁻¹. The amide B band represents the asymmetric stretching vibration of alkenyl CH, as well as NH₃⁺ and has been observed at 2,850-2,950 cm⁻¹ (31,32). In our study, the amide B bands for ASCs from S-RT and F-RT skins were observed at 2,889 and 2,898 cm⁻¹, respectively.

The regions of amides I, II, and III are known to be directly related with the shape of a polypeptide. Amide I band, located at 1,600-1,700 cm⁻¹, which is mainly associated with stretching vibrations of carbonyl groups in polypeptides, is considered the most important factor in determining the secondary structure of a protein (31,32). The amide I band of ASCs from S-RT and F-RT skins appeared at 1674 and 1,696 cm⁻¹, respectively. Liu *et al.* (31) observed the amide I peak at around 1,650 cm⁻¹ for skin collagens of carp. The amide II band, with a normal absorption in the range of 1,550-1,600 cm⁻¹, is mainly associated with N-H bending coupled with C-N stretching vibrations, and the shift to lower wavelengths suggests the formation of hydrogen bond. The amide II band of ASCs from S-RT and F-RT skins appeared at, respectively, 1,546 and 1,554 cm⁻¹. Muyonga *et al.* (33) observed the amide II peak at 1,540-1,558 cm⁻¹ for Nile perch skin collagen. The amide III band is related to CN stretching and NH, and is involved with the triple helical structure of collagen (34). The amide III band of collagens from ASCs in S-RT and F-RT skins appeared at 1,210 and 1,214 cm⁻¹. This was reconfirmed by the ratio of approximately 1 between the amide III band and the 1,450 cm⁻¹ band for skin collagen of both rainbow trouts. The ratio of collagens from ASCs of S-RT and F-RT skins was 0.99 and 0.96. A ratio of approximately 1 suggests a triple-helical structure of collagen (6). Thus, both collagens from ASCs from S- and F-RT skins generally showed a similar secondary structure. FT-IR spectra of ASCs from S-RT and F-RT skins were quite similar. Therefore, the ASCs seem to have similar secondary structures. This suggests that different rainbow trout habitats (seawater and freshwater) do not affect the

secondary structure of collagen, particularly the triple-helical structure.

According to the results of amino acid composition, SDS-PAGE, thermal denaturation temperature and FTIR spectra of acid-soluble collagens, and different rainbow trout habitats (seawater and freshwater) do not affect amino acid composition and molecular weight properties of ASCs from S-RT and F-RT skins.

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