Collagen Content in Farmed Atlantic Salmon (*Salmo salar* L.)

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To clarify fish flesh quality problems and softening of fish muscle tissue during chilled storage, the collagen content, types I and V, and its changes in solubility during storage on ice in muscle of farmed Atlantic salmon (*Salmo salar* L.) were analyzed. The contents of acid-soluble, pepsin-soluble, and insoluble collagen in white muscle were determined in fresh fish muscle and after 3 days of storage in ice. The total collagen content was 2.9 g kg^{-1} fresh weight. During storage on ice, a progressive change in solubility of muscle collagen was found. For acid- and pepsin-soluble collagen fractions, significantly higher and lower values, respectively, were detected. The presence and quantification of types I and V collagen in the different collagen fractions was determined also, but no significant difference in solubility during storage was found. The result suggested that collagen fibers of Atlantic salmon have a high solubility in acid solutions and contain few cross-links.

Keywords: *Atlantic salmon; collagen content; type I; type V; storage*

INTRODUCTION

Fish muscle tissue is a highly desirable food due to its moist texture, pleasant mild flavor, and high nutritional value. However, fresh fish is easy perishable and may lose some of its qualities when stored for some time. Further, during the past decade, the feed used in salmon farming has become fatter. During the same time period, quality problems have arisen during processing of farmed Atlantic salmon (*Salmo salar* L.). These problems have become more severe, especially after the introduction of high-energy feeds with fat content exceeding 30% of dry matter. Although the use of such energy-rich diets has increased daily growth, their use has been linked to poor flesh quality (Hillestad and Johnsen, 1994; Sheehan et al., 1996) and problems during processing of the fish, for instance, for smoking (Sheehan et al., 1996). In addition, salmon are often stored for several days after slaughter before reaching the smoking industry, which may result in an increased postmortem softening and occurrence of gaping. Gaping is a phenomenon in which the connective tissues of fish fillets fail to hold the muscle together (Lavéty et al., 1988). Fillets which have gaped cannot be sold from the fishmonger's slab or used in products where appearance is important; therefore, they have to be used in lower priced products and represent an economic loss to the industry (Love, 1973, 1985; Lavéty, 1984). It is a well-known fact that the connective tissues have a great effect on the properties of muscle as food (Sikorski et al., 1990). Collagen, the major component of these connective tissues, has a significant influence on the functional and rheological properties of the flesh and is the main contributor to the tensile strength of the muscles (Sikorski et al., 1990). Studies have focused on the collagen content contribution to the textural properties of fish meat (Sato et al., 1986b; Hatae et al., 1986). Raw fish meat from most fish species softens after only 1 day of chilled storage (Montero and Borderias, 1990; Ando et al., 1991a; Sato et al., 1991). In addition, histological studies have shown that the rapid softening of fish flesh is caused by disintegration of thin collagen fibrils (Hallet and Bremner, 1988; Ando et al., 1991b, 1992). Until now, at least types I and V collagen have been identified in fish intramuscular connective tissue (Sato et al., 1988, 1989a,b, 1991, 1994a,b). It was demonstrated that, in muscle from rainbow trout (*Oncorhynchus mykiss*), the solubility of type V collagen increased during storage on ice, while no change in type I was observed, which may suggest that type V collagen is involved in the rapid softening of fish muscle (Sato et al., 1991).

The aim of the present study was to test if the collagen content in the dorsal muscle of Atlantic salmon changed during storage on ice for 3 days and, further, to investigate changes in the solubility of the different fractions during ice storage. The presence of collagen types I and V was investigated and measured in order to elucidate if any possible effects were seen related with the solubility.

MATERIALS AND METHODS

Atlantic salmon were obtained from Matre Research Station (Institute of Marine Research), Bergen, on the West Coast of Norway. Fish were fed with a commercial diet (Skretting, AS) prior to sampling. Four fish were sampled in January 1998, having an average weight of 4.3 kg and 70 cm fork length. The fish were gutted and transported on ice to the laboratory and immediately prepared upon arrival. The white muscle from the dorsal part of the trunk was separated from skin, bones, and dark muscle and sliced into pieces of 4 cm thickness. Half was used immediately, and the rest was stored in plastic bags on ice at 4 °C until analyses were carried out at day 3.

Preparation of Collagen. All operations were performed in a cold room at 4 ± 1 °C. Acid-soluble (ASC), pepsin-soluble (PSC), and insoluble collagen fractions (ISC) were prepared as described by Sato et al. (1988), which involved a preliminary extraction with cold 0.1 N NaOH solution to remove non-

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collagenous proteins. The alkali extraction included homogenization of muscle with 10 volumes (v/w) of 0.1 N NaOH, followed by centrifugation at $10000g_{av}$ for 20 min. The residue was added to 20 volumes of NaOH solution, stirred overnight, and centrifuged ($10000g_{av}$). The NaOH addition followed by centrifugation was repeated four times. The final precipitate was washed with distilled water before centrifugation. To the residue was added 10 volumes (v/w) of 0.5 M acetic acid. This was stirred for 2 days and centrifuged at $10000g_{av}$ for 20 min, and the supernatant was collected and used as ASC fraction. PSC was rendered soluble by limited digestion with porcine pepsin (Sigma, 2× crystallized) at an enzyme:substrate ratio of 1:20 (w/w, wet weight) in 0.5 M acetic acid. The digestion was performed at 4 °C for 2 days before centrifugation at $10000g_{av}$ for 20 min. The final supernatant was the PSC, and the insoluble matter was the ISC.

Fractionation of Collagen into Types I and V. Preparation of types I and V collagen from ASC, PSC, and ISC fractions was performed according to the method of Sato et al. (1991). The collagen preparation was fractionated by different salt precipitation at neutral and acidic pH. Solid NaCl was added to the preparation in 0.5 M acetic acid to make a final concentration of 2.0 M NaCl. The resultant precipitate was harvested by centrifugation at $10000g_{av}$ for 20 min and then washed with 2.0 M NaCl solution. The residue was washed with 4.4 M NaCl−50mM Tris-HCl buffer, pH 7.5. The residue was mixed with 2.4 M NaCl−50mM Tris-HCl buffer, pH 7.5. The suspension was stirred overnight and centrifuged at $10000g_{av}$ for 20 min. This procedure was repeated twice. Solid NaCl was added to the supernatant to give a final concentration of 4.4 M. The resultant precipitate was harvested by centrifugation at $10000g_{av}$ for 20 min and redissolved with 11.5% (w/v) ammonium sulfate−0.5 M acetic acid. The resultant precipitate was removed by centrifugation at $10000g_{av}$ for 20 min. Ammonium sulfate was further added to the supernatant to give a final concentration of 20% (w/v). The resultant precipitate was harvested by centrifugation at $10000g_{av}$ for 20 min and used as the type V collagen fraction. The residue after extraction with 2.4 M NaCl−50 mM Tris-HCl buffer, pH 7.5, was mixed with 1.7 M NaCl−50mM Tris-HCl buffer, pH 7.5. The suspension was stirred overnight and centrifuged at $10000g_{av}$ for 20 min. This procedure was repeated twice. Solid NaCl was added to the supernatant to give a final concentration of 2.4 M. The resultant precipitate was harvested by centrifugation at $10000g_{av}$ for 20 min and used as the type I collagen fraction. The collagen fractions were suspended in water, dialyzed successively against 20 mM Na_2HPO_4 and distilled water, and then freeze-dried.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Laemmli (1970) using ready gels (Bio-Rad) with 7.5% and 10% Tris HCl. Gels were stained in Coomassie Brilliant Blue R-250 solution (Bio-Rad) and destained in a solution of acetic acid, methanol, and water (10:40:50 v/v/v). Two replications of the samples were performed in order to be sure that the fractions salted out of type I and V collagen were pure. Human type I and V collagen isolated from acid-soluble placenta was purchased from Sigma (St. Louis, MO) and was used as standards.

Chemical Analysis. All the chemical analyses were run in duplicate. Crude protein in salmon muscle was determined by Kjeldahl digests reaction as described by Crook and Simpson (1971). The solubility of the muscle protein was determined as trinitrobenzosulfonic acid (TNBS)-positive material as described by Adler-Nissen (1979). L-Leucine was used as a standard. Total fat was determined gravimetrically after extraction with ethyl acetate (Losnegard et al., 1979).

The isolated type I and V collagen samples were hydrolyzed in 6 N HCl for 22 h at 110°C. Amino acids were then analyzed by using the Waters HPLC analyzer system (PicoTag) after prederivatization with phenyl isothiocyanate (PITC) according to Cohen et al. (1989) using 0.25 mM norleucine as internal standard.

Table 1. Total Collagen (g kg^{-1}) and Relative Distribution of Collagen Content (%) of Acid-Soluble (ASC), Pepsin-Soluble (PSC), and Insoluble (ISC) Collagen Fractions of Farmed Atlantic Salmon ($n = 4$) during 3 Days of Storage on Ice

fraction	day 0	day 3	SEM pooled	correlation % against time
total (g kg^{-1})	2.9	3.0	0.27	
ASC (% of total[a,b])	23.7	33.8	2.86	$r^2 = 0.7096$, $P = 0.049$
PSC (% of total[a,b]	70.5	59.2	3.10	$r^2 = -0.7231$, $P = 0.043$
ISC (% of total)	5.78	6.96	0.69	$r^2 = 0.4235$, $P = 0.296$

[a] Significant difference between day 0 and day 3 ($P < 0.05$) with use of ANOVA. [b] Significant difference of the development of % ASC and % PSC against time ($P < 0.05$).

Protein contents in collagen types I and V were determined by the Lowry method (Bio-Rad Protein Assay Kit II) using BSA as a standard.

Hydroxyproline content was determined according to a colorimetric method described by the International Organization for Standardization (ISO, 1978), which basically is a method for determination of hydroxyproline content in meat and meat products. The first step in this method, hydrolysis of the crude sample, was slightly modified to adjust for the determination of hydroxyproline in the prepared sample solutions of ASC, PSC, and ISC from fish. One milliliter of sample from the prepared collagen fractions was added to 1 mL of 60% sulfuric acid. After being hydrolyzed overnight (12 h) at 105 °C, the samples were diluted to 50 mL to make the final concentration of hydroxyproline between 0.5 and 2.4 μg mL^{-1}. The later steps in the method were performed as described in the ISO method, by oxidation of hydroxyproline with chloramin-T, followed by addition of 4-dimethylaminobenzaldehyde, giving a colored complex which was measured photometrically at 560 nm. To convert the amount of hydroxyproline to collagen in salmon muscle, a factor of 11.42 was used (Sato et al., 1991).

Statistical Methods. The results are given with standard error of mean (SEM) in Tables 1 and 2. Analyses of variance (ANOVA), followed by Tukey's HDS test, were used to calculate significant differences in the amounts of ASC, PSC, and ISC versus storage time. Significant differences were assessed with a given level of $P < 0.05$. The development of ASC, PSC, and ISC over time was tested by Pearson product−moment correlation analyses. All statistics were performed using CSS Statistica (Statsoft Inc., 1991, Tulsa, OK).

RESULTS AND DISCUSSION

No significant differences were observed in the composition of salmon muscle when fresh and after storage for 3 days related to the crude protein, fat content, and solubility. For day 0, a value of 232 g kg^{-1} was found for the crude protein, 210 g kg^{-1} for fat content, and a value of 0.118 was achieved for the solubility of protein, given as milliequivalents of L-leucine α-NH_2 per gram of protein.

According to the results given in Table 1, the total collagen content in fresh Atlantic salmon muscle was 2.9 g kg^{-1} of wet weight. This value is lower than values reported for dorsal white muscle in other fish species (Sikorski et al., 1984; Sato et al., 1986a,b) and also for the same fish species (Eckhoff et al., 1998). A variation of total collagen from 3.4 (sardine, *Sardinops melanostictus*) to 21.9 g kg^{-1} (conger eel, *Conger myriaster*) was found by Sato et al. (1986b) between 24 different fish species. Eckhoff et al. (1998) found a value of 6.6 g kg^{-1} for the Atlantic salmon. The higher values reported by Eckhoff and co-workers might be due to the different sampling time used, which might be related with a different biological stage (January vs May). Another

Table 2. Changes in Collagen Types, Given as Relative Percent Distribution in Atlantic Salmon Muscle when Fresh and after Storage for 3 Days at 4 °C[a]

storage time, days	collagen content, mg % of dry muscle		solubility of collagen,[b] %					
			type I			type V		
	type I	type V	ASC	PSC	ISC	ASC	PSC	ISC
0	1208 ± 25	25 ± 2.8	37.1 ± 13.5 (448.2)	46.6 ± 6.97 (562.9)	16.4 ± 6.6 (198.1)	14.7 ± 2.4 (3.7)	37.7 ± 3.4 (9.4)	47.5 ± 6.1 (11.9)
3	1259 ± 32	28 ± 3.5	32.2 ± 11.0 (405.4)	40.5 ± 10.6 (509.9)	28.1 ± 3.8 (353.8)	33.4 ± 13.5 (9.4)	42.2 ± 17.5 (11.8)	24.5 ± 10.9 (6.9)

[a] Values are given as average of four measurements ± SEM. No significant difference was detected in each column ($p > 0.05$). [b] ASC, acid-soluble collagen; PSC, pepsin-soluble collagen; ISC, insoluble collagen. Values in parentheses are mg (100 g)$^{-1}$ white muscle (dry wt).

study done by Yoshinaka et al. (1990) showed that the total collagen content ranged between 3.7 g kg^{-1} for red sea bream (*Pagrus major*) to 12.8 g kg^{-1} for Japanese eel (*Anguilla japonicus*). In addition, some previous studies have demonstrated a certain degree of correlation between the collagen content and the meat toughness. In the study of Sato et al. (1986b), it was found that all the fish species with a low total muscle collagen content (3.4−5.1 g kg^{-1}) had a tender meat vulnerable to gaping. The relationship between collagen content and texture was further confirmed by Hatae et al. (1986), who showed that a high collagen content resulted in firm meat. Connell (1995) also reported that, in some fish species, when the protein collagen content of the flesh increases, the fat content falls and an effect of strengthening of the tissue is observed, making the fish hold together better during processing. Based on this, we conclude that the low collagen content found in farmed salmon muscle results in a tender meat vulnerable to gaping.

The relative distributions of concentrations of collagen fractions given in Table 1 were 23.7% for ASC, 70.5% for PSC, and 5.8% for ISC; corresponding to 0.74, 1.96, and 0.17 g kg^{-1}, respectively. Previous data had reported 6%, 93%, and 1% for ASC, PSC, and ISC, respectively (Eckhoff et al., 1998). This shows that almost all of the collagen found in white muscle of salmon (approximately 94.2%), before ice storage, was soluble in acetic acid solution. This fact indicates that salmon, like other fish species, in opposition to collagen present in mammalian meat, have a unique collagen with a high solubility in dilute acid (Sato, 1993). The addition of pepsin, used to cleave nonhelical domains of collagen (Sato, 1993), left only 5.8% of the ISC fraction, indicating that there are few cross-links of collagen in salmon muscle.

During storage, there was a progressive change in the solubility of muscle collagen. After 3 days of ice storage, the ASC fraction increased to more than one-third of the value achieved at day 0, from 0.74 to 1.02 g kg^{-1}. The relative collagen distribution in this fraction, by the Tukey HDS test, exhibited a significant difference ($P = 0.048$). A positive correlation was also found for the development of ASC with storage time ($r^2 = 0.7096$), and the change was significant ($P = 0.049$). The PSC fraction decreased significantly ($P = 0.042$) from an initial value of 1.96 to 1.74 g kg^{-1} in white muscle. A negative correlation was found for the development of pepsin-solubilized collagen over time ($r^2 = -0.7231$), and the change was significant ($P = 0.043$). Although no significant changes were seen for the insoluble collagen fraction, an increase in this fraction from 0.17 to 0.20 g kg^{-1} was measured during storage (Table 1). These results are contradictory to values previously reported for Atlantic salmon (Eckhoff et al., 1998). The explanation may be that we slightly modified and improved the method of preparation of the different collagen fractions. Actually, we performed the digestion of porcine pepsin at an enzyme:substrate ratio of 1:20 (w/w) at 4 °C instead of 37 °C. In doing so, the digestion was limited, and no significant difference for the ISC was found, in opposition to the results for the ASC and PSC fractions. Further, by mistake, an excess of pepsin was added in the previous work, also contributing to the amount found by the method used for analyzing collagen. The variation of the content of collagen fractions during storage was probably a result of environmental changes in the salmon muscle postmortem, where enzymes such as collagenases, neutral proteinases, and acid proteinases have cleaved parts of the triple helix (Pearson and Young, 1989).

In the present study, molecular species of collagen in the white muscle of farmed Atlantic salmon were examined, and the results are listed in Table 2. As might be expected, the major component of the intramuscular connective tissue was found to be the type I collagen, present at 1208 mg % of dry muscle, while the type V collagen was present at 25 mg % of dry muscle. The values found in the literature were 1318 and 35 mg % of dry muscle, respectively, for types I and V collagen in rainbow trout muscle (Sato et al., 1991). In the present study, the type I collagen had a high content of glycine, proline, and alanine and a low content of histidine. Further, in this collagen type, glutamic acid content was also higher compared to the other amino acids present. On the other hand, the type V collagen showed a high content of glycine, isoleucine, proline, and alanine as major amino acids, and the minor quantity of amino acid found was lysine and also some vestiges of taurine. This fact might indicate that this type was not completely pure. However, from the SDS−PAGE patterns of types I and V collagen fractions from Atlantic salmon muscle, they appear to be pure when compared with the standards, as shown in Figure 1, demonstrating an example of the pattern found for the ISC fraction in muscle stored for 3 days on ice.

In fish, it has been suggested that the fine collagen fibrils that arise from myocommata progressively deteriorate during storage on ice (Bremner and Hallett, 1985, 1986; Hallett and Bremner, 1988). Sato et al. (1991, 1994) reported that the solubility of type V collagen in the muscle of rainbow trout increased significantly during chilled storage, while that of type I collagen did not change. This fact suggests that degradation of nonhelical regions or intermolecular cross-links occurs preferentially in type V collagen. Ando and co-workers (1991b, 1992) have demonstrated by light and electron microscopy that postmortem tender-

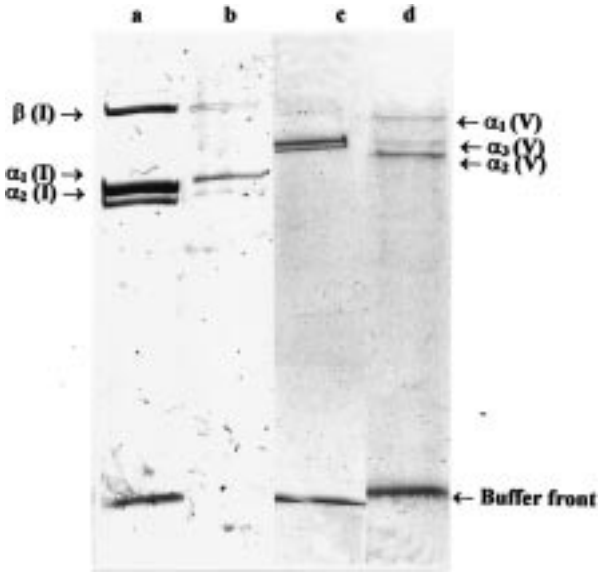

Figure 1. SDS−PAGE patterns of types I (b) and V (d) insoluble collagen fraction from Atlantic salmon stored for 3 days on ice compared with those of human types I (a) and V (c). The polyacrylamide gels used were 7.5% and 10% for the type I and type V, respectively.

ization of rainbow trout muscle is mainly due to the disintegration of collagen fibers in the pericellular connective tissues.

The results given in Table 2 show that the solubility of types I and V collagen did not change significantly during storage on ice ($P > 0.05$). However, after 3 days of storage, some modifications were seen, especially related with collagen type V. The ASC fraction increased, more than doubling, and the ISC decreased by approximately 50%. The type V collagen, in opposition to the type I, was more readily solubilized with acid extraction and pepsin digestion after chilled storage. On the other hand, the solubility of type I ISC fraction increased during storage, while the opposite was observed at the same fraction regarding the collagen type V.

A possible explanation for the divergent results between the solubility of collagen types found in the present study and in the literature might be the difference in the size of the fish used in this experiment, which were about 4 times bigger than those in experiments with rainbow trout (Sato et al., 1991). It has been reported that cathepsin L and serine proteases are capable of hydrolyzing major muscle structural proteins such as collagen (Sato et al., 1994b; Yamashita and Konagaya, 1991). While the extracellular matrix collagenases are active against collagen of types I, IV, and V and are regarded as initiators of breakdown (Bremner, 1992), maybe they will need more time than 3 days of storage at 4 °C to start acting.

Further studies should be performed in order to examine the effects of such factors as fish size, growth rate in fish (fast and slow growing types), storage parameters, and slaughter techniques. The development of techniques for isolation and characterization of fish muscle molecular species would be useful to elucidate the cause of textural changes in fish meat on a molecular level. However, currently there are no rapid methods for determining changes in muscle collagens during postmortem storage. Changes in the characteristics of proteins can be determined only after isolation involving time-consuming and specialized techniques. An attempt to improve these methods seems necessary.

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