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Hydroxylysyl Pyridinoline Cross-Link Concentration Affects the Textural Properties of Fresh and Smoked Atlantic Salmon (*Salmo salar* L.) Flesh

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A simple HPLC method is presented to quantify the low concentration of hydroxylysyl pyridinoline (PYD) cross-links in Atlantic salmon (*Salmo salar* L.) muscle. The method involved the extraction of tissue with NaOH prior to hydrolysis, which greatly reduced the amount of protein to be hydrolyzed and made downstream operations easier and more reproducible. The concentration of PYD was 426 pmol g^{-1} dry mass muscle in post-*rigor* muscle stored at 0 °C and sampled 3 d after death. Hydroxproline (HYP) concentration was determined following NaOH extraction as a measure of collagen content. In post-*rigor* samples, the alkaline-insoluble HYP fraction comprised 18.3% of the total HYP. Scanning electron microscopy revealed shrinkage of muscle fibers and a retraction of the connective tissue matrix in smoked salmon. PYD concentration was relatively resistant to processing to the smoked product, decreasing by around 11.7%, as compared to a 22.2% decrease in HYP. There was a positive correlation between PYD concentration and the firmness of post-*rigor* muscle samples as measured by an instrumental texture analyzer, explaining 25% of the total variation. A weaker but still significant correlation was found between PYD concentration and firmness in the smoked product. There was no relationship between fillet firmness and total collagen concentration, although the correlation with HYP in the alkaline-insoluble fraction was significant at the 6% level ($P = 0.057$). Our results indicate that only 1−3% of collagen molecules are linked by nonreducible mature cross-links in harvest size farmed Atlantic salmon and that PYD concentration is an important raw material characteristic for flesh quality.

KEYWORDS: Collagen; cross-links; hydroxylysyl pyridinoline; reducible collagen cross-links; fish muscle; smoked salmon; flesh quality; farmed Atlantic salmon

1. INTRODUCTION

Cold smoking of Atlantic salmon (*Salmo salar*) includes salting, drying, and smoking at low temperatures to produce a lightly preserved product with salt content ranging from 2% to 5% and a pH between 5.8 and 6.3 (*1*, *2*). During the selection of fillets for smoking, a proportion are downgraded to lower value products due to soft flesh and gaping, representing a significant economic loss to the industry (*3*). The biological basis of soft flesh and variable processing characteristics are poorly understood (*4*). In Atlantic salmon (*Salmo salar* L.), fillet firmness of the smoked product, as assessed by trained taste panels, was correlated with the density of muscle fibers, explaining around 30−40% of the variation in texture (*5*). Collagen content also influences the texture of raw fish (*6*, *7*). For example, seasonal changes in total collagen in Red Sea bream (*Pagurus* major) were positively correlated with the breaking strength of the muscle (*8*). At least 27 collagen types with a total of 42 distinct α chains, and >20 additional proteins with collagen-like domains, have been identified (*9*). The major collagens present in fish muscle are collagen I with type V collagen as a minor component (*10*, *11*). Raw fish meat can soften after only 1 day of chilled storage (*12*). In rainbow trout, rapid post-mortem softening was associated with the solubilization of collagen V, whereas there was no change in type I collagen (*13*). Ando et al. (*14*, *15*) have suggested that rapid softening of the flesh post-*rigor* is related to the disintegration

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of thin collagen fibrils, perhaps involving collagenase and/or other proteases including cathepsins and calpains.

Collagen synthesis involves multiple posttranslational modifications after the polypeptide chain is secreted into the ER lumin. All collagen molecules comprise three α polypeptide chains and contain at least one domain composed of repeating Gly−X−Y sequences per chain, which are wound together in a tight triple helix. Many of the X and Y positions are occupied by two uncommon residues: proline (X) and hydroxyproline (Y), which fit perfectly inside the helix. Noncollagenous domains are present in addition to the helix (*9*). For fibril-forming collagen such as Collagen I and V, the signal peptide is first removed, followed by hydroxylation on certain proline and lysine, and glycosylation of certain hydroxylysine, and asparagines (*9*). Three C-terminal propeptides associate to form a nucleus for assembly of the triple helix, which propagates from the C-terminus toward the N-terminus. Procollagen molecules are then transported through Golgi stacks, aggregate laterally, and the N and C propeptides are cleaved to facilitate further aggregation, which leads to the self-assembly of fibrils. Cross-linking on collagen occurs between the helical or non-helical ends of the molecule, in an end-to-end fashion, and connect the rigid regions of the molecule. Covalent cross-link is initiated by oxidation of the ϵ-NH_2 group in certain lysine/hydroxylysine, through a series of reducible intermediates, into the final nonreducible products hydroxylysylpyridinoline and lysylpyridinoline (*9*, *16*, *17*). Each pyridinium cross-link is able to connect three collagen molecules.

In mammals, the relation between the collagen content and flesh firmness is not a simple one, as collagen content remains nearly constant with the age of the animal, whereas the mechanical stability increases (*18*). Cross-links increase the mechanical and thermal stability of collagen fibers as well as their tensile strength, and this is correlated with the toughness of meat (*19*). Recent research suggests that the development of strength of tendons requires the conversion of highly viscous liquidlike assemblies of collagenous macromolecules into solid structures, which involve the sequential stretching of flexible segments within the collagen triple helix and in turn requiring end-to-end cross-linking within a fibril (*16*).

Very few studies have determined collagen cross-link in fish muscle (*20*), and no information is available on the relationship between pyridinoline cross-link concentration and the textural properties of the flesh. In the present study on Atlantic salmon (*Salmo salar*), the first aim was therefore to develop a rapid and sensitive HPLC method to quantify the low concentrations of pyridinoline cross-links in fish muscle. The second aim was to investigate the contribution of pyridinoline cross-link concentration and collagen content to fillet firmness measured post-*rigor* and after smoking.

2. MATERIALS AND METHODS

2.1. Fish Husbandry. Atlantic salmon (*Salmo salar* L.) comprising seven families from the Stofnfiskur breeding program (Stofnfiskur A/S, Iceland) were grown in a dedicated trial unit at Salar, South Uist, Western Isles, Scotland, comprising duplicate 5 m × 5 m × 5 m net pen sea cages. Atlantic salmon smolts from each family were evenly distributed between the cages. The fish were fed a commercial ration of the Ecolife series (BioMar Ltd., Grangemouth, Scotland). The diets consisted of 25/47 (oil/protein) for the 4.5 mm, 30/40 for 6.5 mm, and 33/38 for 9 mm pellet sizes. The total feed input into each cage (energy input) was controlled using a computer-operated demand feeding system (AKVAsmart Uk Ltd.).

Harvest Procedure and Carcass Processing. In October 2002, the fish were starved for 5 days, harvested, killed by percussion stunning, bled in ice slurry, eviscerated, gill-tagged, and packed on ice in polystyrene fish boxes, and then transported to Uniq Prepared Foods (Annan, Scotland) for secondary processing and smoking. Secondary processing commenced on day 3 after slaughter when *rigor* had resolved. The gutted fish were washed, and one fillet was removed for an assessment of texture and gaping before proceeding for smoking under commercial conditions. The other fresh fillets were left on the skeleton, placed in fillet bags, and then packed on ice in polystyrene boxes and dispatched to The University of St. Andrews for texture analysis and muscle tissue sampling. Fillets were robotically salted using dry salt adjusted in proportion to the fillet weight and left to cure for 48 h at <10 °C. Cured fillets were transferred to a horizontal flow kiln and smoked for 14 h using hardwood oak. Kiln temperature and humidity levels were preprogrammed and consistent for each batch. Following smoking, the fillets were chilled to <5 °C and held in maturation for 3 days to allow smoke and salt equilibrium. The fillets were trimmed, vacuum-packed, and dispatched to The University of St. Andrews for texture analysis and further sampling.

Muscle pH Measurements. Muscle pH was measured for each fish at slaughter (pre-*rigor*), on day 3 after slaughter (post-*rigor*), and in the smoked fillet. Duplicate pH measurements were made directly using a calibrated pH electrode with knife (model K/KNIpHE/11, Thermo Russell, Scotland) in the epaxial myotomes below the dorsal fin for the pre-*rigor* sampling, and anterior to the first dorsal fin ray in the post-*rigor* and smoked fillets.

Instrumental Texture Measurements. The flesh quality attribute of texture was measured as fillet firmness, described by Johnston et al. (*21*). Briefly, firmness was evaluated by a shear test using a TA-HDi Texture Analyzer controlled with Texture Expert Exceed 2.52 software from Stable Micro Systems, Surrey, England. To ensure the flesh temperature was kept constant during testing, each fish was kept on ice until the flesh samples were excised, and the temperature of the muscle tissue was measured for individual fish chosen at random. A shear test was performed in duplicate for a post-*rigor* (side A) and a smoked sample (side B) from each fish using two 4 × 4 × 2 cm blocks of fast muscle excised from the epaxial myotomes anterior to the first dorsal fin ray. The probe used was a 60° knife edge blade (not sharpened) with a slotted blade insert located in a heavy duty platform. A 100 kg load cell was used, and the test speed was set at 1 mm s^{-1}. Each texture profile was analyzed using Texture Expert Exceed 2.52 software, and two fillet firmness measurements were determined (*22*). The maximum shear force, measured in newtons, is the highest peak of the curve, representing the maximum resistance of the sample to shearing. The area under the curve during shearing was also calculated as the work done (WD) in millijoules during the shear test.

2.2. Collagen Cross-Link Assay. The fish muscle samples used for texture analysis were stored at −80 °C until chemical analysis. Duplicate samples of 1 g of fast muscle were minced by hand before homogenization in 9 mL of cold water (4 °C) for 1 min at 30 000 rpm using a Polytron PT2100 (Kinematica AG, Switzerland). Ten milliliters of ice cold 0.2 M NaOH was added immediately, and the sample was mixed on a wheel roller at 4 °C for 4 h. The homogenate was centrifuged at 10 000*g* for 30 min at 4 °C, and the pellet containing the alkaline-insoluble collagen was resuspended in 2 mL of 6 M HCl and transferred to an 8 mL glass vial with a screw lid. The sample was hydrolyzed at 110 °C for 20 h, after which 2 μL of hydrolysate was removed for hydroxyproline (HYP) assay, and the remaining sample was dried in a vacuum rotary evaporator (Jouan RC1022, Nantes, France), and resuspended in 3 mL of H_2O/acetic acid/*n*-butanol (v/v/v 1:1:4). The hydroxylysyl pyridinium cross-links (PYD) were extracted using cellulose columns (cat. No. 12102095, Varian Ltd., Oxford, England) based on published methods (*23*, *24*). After the sample was loaded, the column was washed with 3 × 3 mL of the same buffer, and PYD was eluted with 3 mL of water, dried, and resuspended in 600 μL of injection sample buffer containing 250 nM pyridoxine (internal standard). After filtration through a 0.2 μm PVDF micro-cartridge (Pall), the sample was loaded onto HPLC autosampler.

The HPLC system (ProStar, Varian) consisted of a 230 SDM Ternary Solvent Delivery System, 410 Autosampler with sample tray cooling, and a 363 Fluorescence Detector. Star LC workstation software version 5.5, run under Microsoft Windows 2000, was used for data acquisition

and processing. The RP-HPLC column (4.6 × 150 mm; Micropak ODS-80TM; 5 μm spherical silica particles with 80 Å pore size; Varian) was thermostated at 40 °C with the Autosampler column oven. The column was eluted using a binary solvent system. Solvent A was 5% (v/v) CH_3CN, 0.1% (v/v) heptaflurobutyric acid (HFBA), and solvent B was 95% (v/v) CH_3CN, 0.1% (v/v) HFBA. The gradient and flow rate were as follows (v/v), 0−15 min, 100% A, 1 mL min^{-1}; 15−30 min, 85% A, 15% B, 1 mL min^{-1}; 30−31 min, 20% A, 80% B, 1.4 mL min^{-1}; 31−40 min, 20% A, 80% B, 1.4 mL min^{-1}; 40−41 min, 100% A, 1.4 mL min^{-1}; 41−49 min, 100% A, 1.4 mL min^{-1}. Fluorescence of the eluted peaks was monitored at $\lambda_{ex} = 295$ nm and $\lambda_{em} = 400$ nm. The Star LC workstation software was used to identify and quantify the peaks for pyridoxine and pyridinoline by comparison with a calibration curve prepared previously with purified pyridinoline (kindly gifted by Simon Robbins, Rowlett Institute, Aberdeen).

2.3. Hydoxyproline Assay. The 2 μL aliquot of hydrolysate obtained from the cross-link assay was mixed with 200 μL of water and dried in an eppendorf tube using a rotary vacuum evaporator (Jouan RC1022, Nantes, France). The pellet was resuspended in 200 μL of 0.1 M boric buffer, pH 11.4 containing 11.2 μM homoarginine as an internal standard. The fluorenylmethoxycarbonyl (FMOC) derivatized amino acids were separated using a ternary gradient described by Bank et al. (*25*). Solvent A was 20 mM citric acid, 5 mM tetramethylammonium chloride, 0.01% (w/v) sodium azide, adjusted to pH 2.85 with 20 mM sodium acetate, 5 mM tetramethylammonium chloride, 0.01% (w/ v) sodium azide. Solvent B was 80% (v/v) of 20 mM sodium acetate, 5 mM tetramethylammonium chloride, 0.01% (w/v) sodium azide, 20% (v/v) MeOH, adjusted to pH 4.5 with concentrated phosphoric acid. Solvent C was CH_3CN. The column was eluted at 1.4 mL min^{-1} with the following gradient (v/v), 0−11.5 min, 75% A, 0% B, 25% C; 11.5−13 min, 60% A, 0% B, 40% C; 13−13.1 min, 0% A, 64% B, 36% C; 13.1−18 min, 0% A, 62% B, 38% C; 18−25 min, 0% A, 30% B, 70% C; 25−30 min, 0% A, 25% B, 75% C; 30−32 min, 0% A, 25% B, 75% C; 32−32.1 min, 75% A, 0% B, 25% C; 32.1−40 min, 75% A, 0% B, 25% C. The eluate was monitored for fluorescence at $\lambda_{ex} = 254$ nm and $\lambda_{em} = 630$ nm. The Star LC workstation software was used to identify and quantify the peaks for homoarginine and hydroxyproline by comparison with a calibration curve prepared previously with collagen hydrolysate (Sigma).

2.4. Scanning Electron Microscopy. Small bundles of fast muscle fibers were dissected from pre-*rigor*, post-*rigor*, and smoked samples and pinned at their resting length to Sylgard (RS Ltd., Corby, Northamptonshire, UK) strips and fixed overnight in 2.5% (v/v) gluteraldehyde, 2.5% (v/v) paraformaldehyde. The fixed samples were washed three times in distilled water, frozen, and lyophilized. The dried samples were placed onto metal stubs to be gold sputter coated and viewed under a JEOL JSM 3SCF scanning electron microscope at 10 kV and photographed using Ilford FP4 Pro black and white film.

3. RESULTS

3.1. The HPLC Assay of Hydroxylysyl Pyridinoline (PYD) Cross-Links in Salmon Muscle. Figure 1A shows a typical chromatogram of a muscle hydrolysate from a post-*rigor* sample. The peak corresponding to the pyridinoline cross-links was well separated. We measured PYD in the same muscle samples without the NaOH wash (compare **Figure 1A** and **B**). This was difficult because of the interference from other substance such as lipids, and the results were more variable. Most, if not all, of the PYD in the samples was retained in the pellet, for example, for three smoked samples 99%, 100%, and 92%. The alkaline-wash also decreased the variation between duplicates; for example, for the same three samples we obtained a 3.6%, 2.7%, and 4.6% difference between duplicates with the NaOH wash, as compared to a 54%, 2%, and 43% difference without the alkaline-wash. The concentration of PYD was 426 pmol g^{-1} dry mass muscle in post-*rigor* muscle stored at 0 °C and sampled 3 d after death (**Table 1**). On average, the concentration of PYD decreased by 11.7% after smoking relative to the post-*rigor* sample ($P < 0.001$) (**Table 1**).

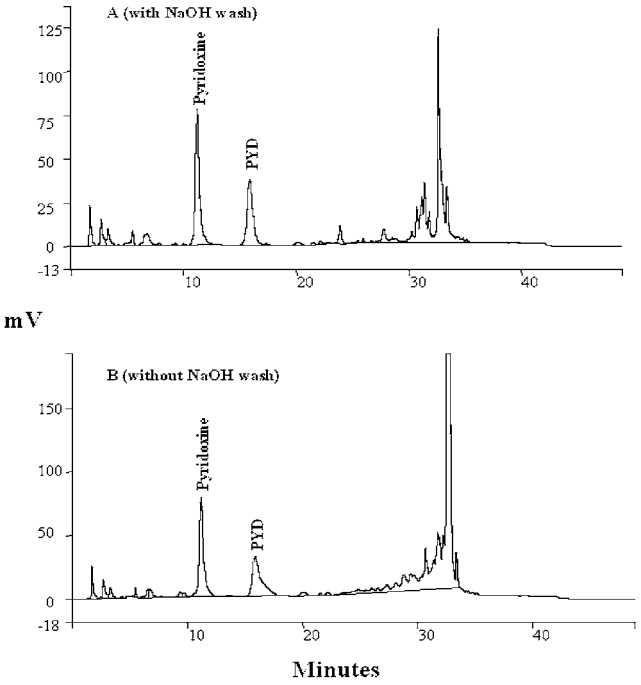

Figure 1. HPLC analysis of PYD (hydroxylysylpyridinoline) from salmon muscle: (**A**) with NaOH wash before hydrolysis; (**B**) without NaOH wash.

Table 1. Hydroxyproline and Pyridinoline Cross-Link Concentration and Measurements of Texture in Fast Muscle Samples of Atlantic Salmon (*Salmo salar* L.)[a]

	units	post-*rigor*	smoked product	% decrease with smoking
hydroxyproline (pellet)	μmol g^{-1} dry mass	8.41 ± 0.37	6.55 ± 0.23*	22.2
hydroxyproline (supernatant)	μmol g^{-1} dry mass	43.81 ± 2.63	35.37 ± 2.00**	19.3
pyridinoline cross-links	pmol g^{1} dry mass	426 ± 21	376 ± 21**	11.7
maximum shear force	N	39.72 ± 1.06	37.46 ± 1.08	5.7
shear work (WD)	mJ	604 ± 15	515 ± 17**	14.8

[a] The results represent mean ± SE, 33 fish. The asterisks represent that they are significantly different at the $P < 0.01$ (*) and $P < 0.001$ (**) levels.

3.2. The Alkaline-Soluble and Insoluble Collagens. Collagen content is usually determined by the assay of the hydroxyproline concentration. To monitor the effect of the NaOH wash, we also compared the HYP ratio in samples with and without NaOH wash (**Figure 2A** and **B**). **Figure 2C** shows the HPLC profile of the supernatant after NaOH wash. It is clear that the collagen was enriched by the NaOH wash step, because the ratio of HYP to other amino acids was higher in the pellet than in the total lysate, while the latter was higher than that in the supernatant. The retention of HYP (mean ± SE) was 18.3 ± 1.8% ($n = 33$) for post-*rigor* samples and 17.6 ± 1.6% ($n = 33$) for smoked salmon. We designated the HYP left in the pellet as alkaline-insoluble collagen, and the HYP in the supernatant as alkaline-soluble collagen. The mean values of HYP are summarized in **Table 1**. The total hydroxyproline concentration in the post-*rigor* sample was 52.21 μmol g^{-1} dry mass, of which 16.1% was alkaline-insoluble (**Table 1**). The concentration of alkaline-resistant HYP decreased by 22.2% after smoking ($P < 0.01$) as compared to 19.3% for the soluble HYP ($P < 0.001$) (**Table 1**).

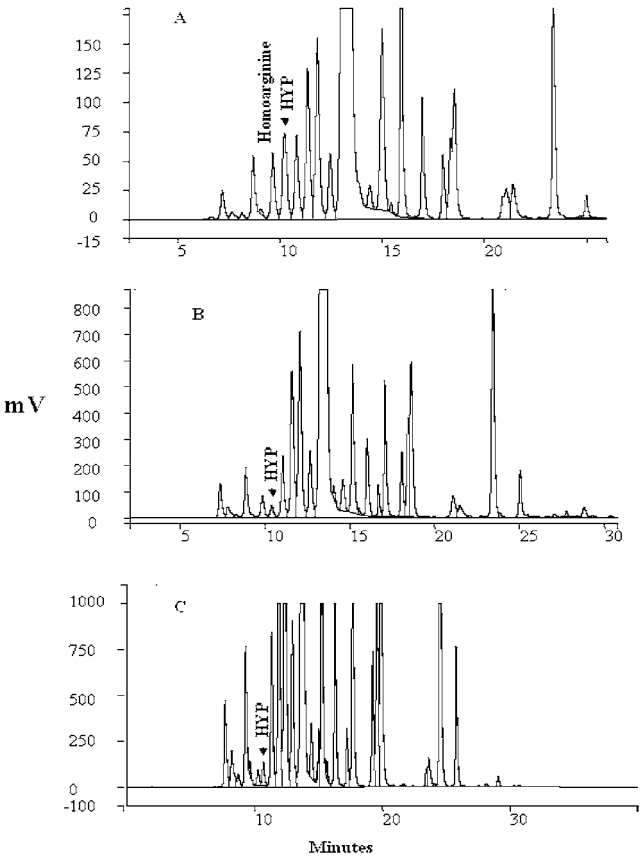

Figure 2. HPLC analysis of HYP (hydroxyproline) from smoked salmon muscle. Homoarginine was the internal standard. (**A**) Pellet (alkaline-resistant) prepared after the NaOH wash; (**B**) hydrolysate of the whole muscle sample without the NaOH wash; and (**C**) supernatant (alkaline soluble) prepared after the NaOH wash.

3.3. The Relationship between Fillet Firmness and Collagen. The relationship between muscle texture and collagen content was investigated in fast muscle sampled post-*rigor* (3 d after death) and in the smoked product. There was a significant positive correlation between the work done (mJ) to shear a standardized slab of muscle (firmness) and PYD concentration in the post-*rigor* samples ($P = 0.003$), explaining 26% of the total variation (**Figure 3**, solid symbols). On average, the firmness of the muscle decreased by 14.8% after smoking (**Table 1**). There was a much weaker correlation between PYD and firmness in the smoked product ($P = 0.05$), explaining only 12% of the total variation (**Figure 3**, open symbols). There was no significant correlation between total HYP and firmness (not shown). However, the relationship between alkaline-insoluble collagen and firmness was significant at the $P = 0.057$ level in the post-*rigor* sample (**Figure 4**), but was not significant for smoked salmon (not shown). Values for the maximum shear force and fillet firmness (WD) of post-*rigor* and smoked samples are summarized in **Table 1**. A highly significant correlation was also found between WD post-*rigor* and WD in the smoked fillets ($P = <0.001$) (not shown).

3.4. Muscle pH. At the time of slaughter, the average muscle pH was 7.23 ± 0.02 S.E.; following resolution of *rigor* the pH had dropped to 6.33 ± 0.01 and was 6.35 ± 0.01 in the smoked product. No significant correlation was found for muscle pH (whether measured in pre- or post-*rigor*, in smoked fillets or calculated pH drop) with any other parameter measured.

3.5. Muscle Structure before and after Processing. Representative SEM pictures of the muscle at slaughter, post-*rigor*, and post-smoking are shown in **Figure 5A**, **B**, and **C**, respectively. At slaughter, the muscle fibers are covered with continuous sheets of connective tissue and lipid droplets are common, particularly in the region of the myosepts (arrows in **Figure 5A**). **Figure 5B** shows a post-*rigor* tissue sample fractured to show the connective tissue sockets (arrows) into which the tendons of muscle fibers insert at the myosepta. Following smoking, the connective tissue sheet covering the fibers retracted and the fibers shrunk in diameter (arrows) relative to the post-*rigor* sample. The smoked product still contained appreciable amounts of connective tissue matrix and lipid inclusions at the myosepta (**Figure 5C**).

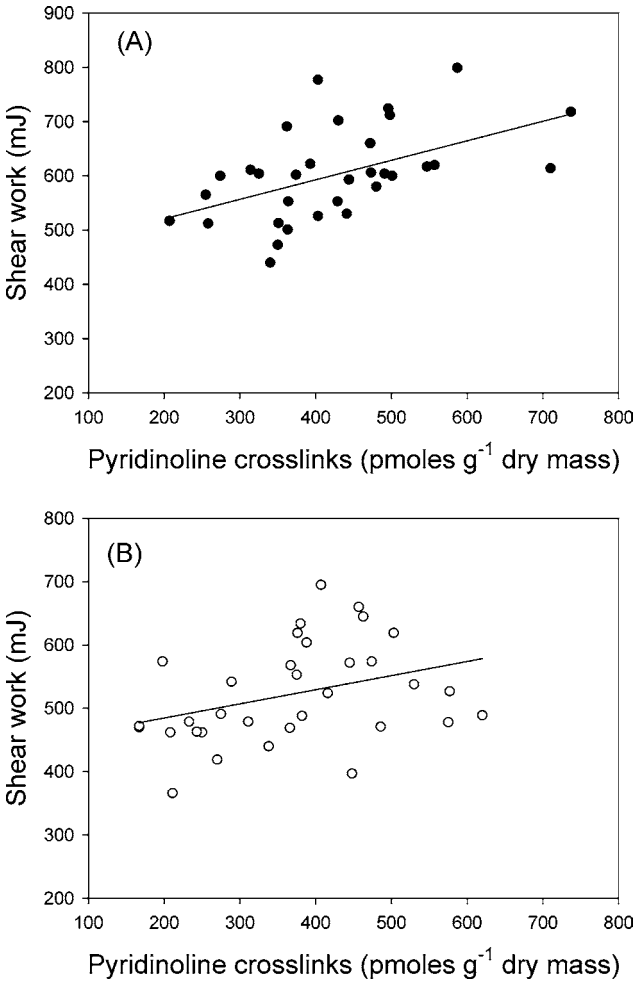

Figure 3. The relationship between pyridinoline cross-link (PYD) concentration and the work done to shear a standardized fillet sample for (**A**) the post-*rigor* (●) and (**B**) the smoked product (○). First-order linear regressions (solid line, post-*rigor*, dashed line, smoked product) were fitted to the data with the following equations: (**A**) post-*rigor* samples, shear work = 449 + 0.36 (PYD); $R_{sqr} = 0.25$; $n = 32$; $P = 0.003$; (**B**) smoked samples, shear work = 440 + 0.22 (PYD); $R_{sqr} = 0.12$; $n = 32$; $P < 0.05$.

4. DISCUSSION

Here, we describe a method to assay the low PYD content in fish skeletal muscle. Our data show that farmed salmon have a mature PYD cross-link concentration of 200−800 pmol/g dry weight. The ratio of PYD/HYP (in alkaline-insoluble collagen) is about 1:20 000, which is equivalent to one cross-link per 100 collagen molecules, assuming each collagen molecule has 3 × 1000 residues (~200 HYP residues). Every PYD potentially connects 3 molecules, so less than 3% collagen molecules (in

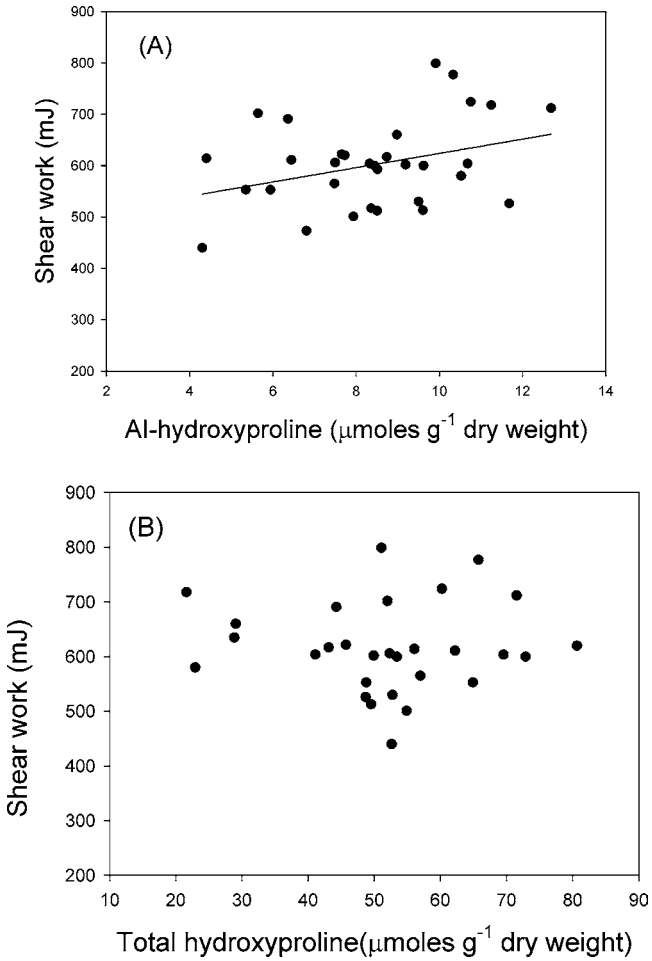

Figure 4. The relationship between hydroxyproline (HYP) concentration in the alkaline-insoluble (AI) hydroxyproline fraction (**A**) and total hydroxyproline (**B**) and the work done to shear a standardized post-*rigor* fillet sample. First-order linear regressions (solid line) were fitted to the data. The regression for the relationship between Shear work and the AI-HYP fraction was statistically significant at the 6% level (shear work = 484 + 13.9 (HYP); $R_{sqr} = 0.11$; $n = 32$; $P = 0.057$).

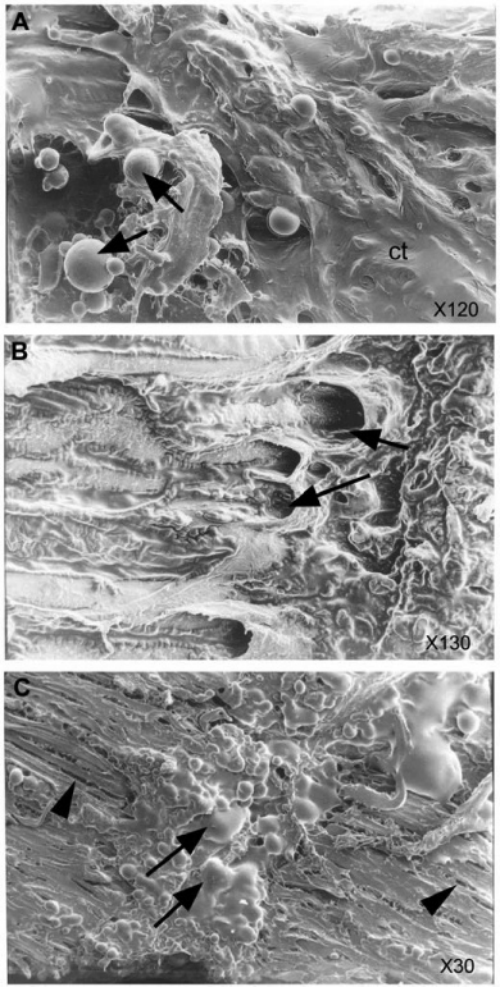

Figure 5. Scanning electron micrographs (SEM) of representative samples from (**A**) muscle at slaughter, (**B**) muscle post-*rigor*, and (**C**) the smoked product. (**A**) The arrows show lipid droplets at the position of the myosepta and a continuous sheet of connective tissue (ct) covering the muscle fibers. (**B**) The arrows show two examples of the connective tissue sockets into which the muscle fiber tendons insert at the myosepta. Note the rougher appearance of the connective tissue sheet (ct) relative to the muscle at slaughter (**A**). (**C**) The arrowheads show spaces between the muscle fibers due to shrinkage during the salting and smoking process. Note the extensive lipid droplets at the position of the myosepta (arrows).

the insoluble collagen fraction) are linked by PYD. For total collagen, the PYD/HYP is about 120 000; therefore, less than 1% are linked by mature cross-links. In contrast, the skeletal muscle of domestic animals (cattle, sheep, pigs) of market age generally contains 0.2−0.35 mol PYD/mol collagen. Because each PYD potentially is able to connect 3 collagen molecules, most of the collagen molecules in these animals are crossed linked by PYD (*26*).

Most published HPLC methods for measuring PYD have a cellulose extraction step after hydrolysis, which extract PYD from the bulk of amino acids. However, due to the low content of PYD in fish muscle, a large amount of muscle sample is required for hydrolysis (equivalent to 10−20 mL of 6 M HCL g^{-1}), which is impractical. Because connective tissue is not distributed evenly in muscle, reducing the weight of each sample will significantly increase the sampling variation. We therefore incorporated an extra washing step with cold 0.1 M NaOH before acid hydrolysis. This treatment has already been used to inhibit proteases and remove most noncollagen proteins from fish muscle (*27*). The prehydrolysis enrichment by the NaOH wash greatly reduces the amount of protein to be hydrolyzed and makes downstream operations much easier and more reproducible. The mature cross-links were contained in the alkaline-insoluble fraction. Sato (*27*) suggested that a NaOH wash would remove most noncollagen proteins, while leaving collagen in the pellet. However, our data showed that when HYP was calculated on the basis of muscle dry weight, only around 18% HYP remained in the pellet after the NaOH wash. The missing fraction was found to be present in the supernatant (**Figure 2C**). Because HYP is a specific product of posttranslational modification of collagen molecules, all of the HYP should come from collagen. Our data show that farmed salmon muscle contains about 6.7% (3−10%) total collagen and 0.5−1.5% alkaline-insoluble collagen. Sato (*7*) found that the collagen content among 24 different fishes was 0.34−2.19%, which corresponds to the alkaline-insoluble fraction, according to the method they used to determine HYP content.

It is generally believed that post-mortem changes of connective tissue constituents rather than degradation of myofibrillar protein, such as myosin, connectin, and actin, might be responsible for the rapid softening of raw fish flesh during chilled storage (*13*). Some studies suggest that high collagen content resulted in a firm flesh (*6*, *7*). On the other hand, McCormick found that both mature cross-links and collagen

concentration were related to the toughening of bovine muscle, but the relationship was complex (*26*). In the present study, PYD concentration was positively correlated with fillet firmness in the post-*rigor* samples, explaining 25% of the total variation ($P = 0.003$) (**Figure 3A**). Thus, PYD is one of the explanatory variables contributing to fillet firmness in addition to the density of muscle fibers (*5*). The work done (WD) in shearing the samples was significantly (14.7%) less after smoking ($F_{1,61} = 12.35$; $P = 0.001$) (ANOVA with PYD as covariate ($F_{1,61} = 13.7$; $P < 0.001$)). After smoking, PYD concentration only explained 12% of the variation in WD, although the regression analysis between WD and PYD remained significant ($P < 0.05$) (**Figure 3B**). In contrast, there was no significant relationship between total HYP and fillet firmness (**Figure 4B**), suggesting collagen itself contributed a negligible amount to the texture. We found that the relationship between the concentration of alkaline-insoluble collagen and firmness was significant at the $P = 0.057$ level, explaining 11% of the variation (**Figure 4A**). Because most (if not all) of the PYD cross-links remained in the pellet, our explanation is that the alkaline-insoluble collagen comprises intact, mature, and cross-linked collagen molecules, forming a resilient network, which makes a significant contribution to the tensile strength of the muscle. In contrast, the alkaline soluble-collagen includes degraded collagen, and newly synthesized collagen molecules, which are not cross-linked and make no contribute to muscle firmness. If our calculation of one mature cross-link per 100 collagen molecules is correct, then it may be that many more immature reducible cross-links are present, which help to stabilize the collagen network. Reducible cross-links include dihydroxylysinonorleucine, hydroxylysinorleucine, and lysinonorleucine (*28*). PYD is derived from the adol condensation of two ketoamine cross-links, reducible by borohydride. In rat aorta (*29*, *30*) and human diaphysial femurs (*28*), during early growth and development reducible cross-links (HLNL and DHLNL) decrease and PYD and other mature collagen cross-links increase. A stoichiometric relationship between the disappearance of the reducible cross-link and the accumulation of the trivalent cross-link with aging has been observed (*26*). It would be very informative if the concentration of both reducible and nonreducible cross-links could be assayed. In collagen-rich tissue, this has been achieved using a single HPLC method (*28*). However, in fish, due to the low concentration of cross-links, reducible cross-links have only been assayed with the conventional method using tritium-labeled sodium borohydride, along with a similar enrichment step with 0.1 M cold NaOH (*20*). At present, there is no simple and convenient method to assay both reducible and nonreducible cross-links in fish muscle.

Smoking was found to shrink the muscle fibers and result in some dispersion of the surrounding connective tissue matrix (**Figure 5**). Sigurgisladottir (*2*, *31*, *32*) found that fish muscle fibers shrank during the salting and smoking process, but sarcomere length did not change. After smoking, a considerable number of fat globules remain dispersed among the muscle fibers, particularly at the myosepts (**Figure 5**; Sigurgisladottir, 2001). The water content of fillets was 75−80%, and 5% salt was added prior to smoking, accounting for some of the shrinkage of the protein component. In a previous study, smoking was shown to result in some proteolytic breakdown of myosin and other proteins, although there was no change in the concentration of α-actinin and actin (*33*). The degradation/release of PYD during curing and smoking was only 12% ($P = 0.013$; paired *t*-test) and closely matched the observed decrease in fillet firmness (∼15%) (**Table 1**). In contrast, Sigurgisladottir et al. (*31*) found a higher absolute shear force was required for smoked than fresh salmon fillets. However, in this study, fillets were not vacuum packed after smoking, and we have found similar results when fillets were treated in the same way, which leads to some dehydration and toughening relative to the vacuum packed product.

In conclusion, the results of the present study indicate that, despite their low concentration, the amount of PYD cross-links present in the muscle is an important determinant of the textural properties of the fresh and smoked products. PYD concentration is therefore one raw material characteristic that has a significant impact on flesh quality and is likely to influence downgrading losses during secondary processing.

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