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Iced storage of Atlantic salmon (*Salmo salar*) – effects on endogenous enzymes and their impact on muscle proteins and texture

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Abstract

Endogenous salmon enzymes and their impact on muscle proteins and texture were studied. To reduce interfering effects of microbial enzymes, the fish were irradiated. Irradiation of salmon fillets resulted in severe inhibition of cathepsin B-like and collagenase-like enzymes. The effect was most pronounced for the cathepsins. The textural properties and amounts of free amino acids and extractable proteins in the irradiated fillets and control group were different after 14 days of iced storage. Even with the increased load of microorganisms seen in the control group, no severe deterioration of fish texture or increase in proteolytic activity was observed during the storage period. It therefore seems that microorganisms are not important for changes in salmon texture.

Keywords: Salmon; Irradiation; Texture; Protein solubility; Cathepsin B-like; Collagenase-like; Proteolytic activity

1. Introduction

The texture of fish is an important quality characteristic, and soft fillets are a problem for the fish industry (Andersen, Thomassen, & Rørå, 1997; Haard, 1992; Hallett & Bremner, 1988; Sigholt, Erikson, Rustad, Johansen, Nortvedt, & Seland, 1997). The texture of fish meat is influenced by several factors, such as fish species, age and size of the fish within the species, fat content and distribution of muscle fat, amount and properties of the proteins, and handling stress before slaughter. Postmortem factors include the rate and extent of pH decline, rigor mortis, rate and extent of proteolysis causing breakdown of myofibrils and connective tissue, and temperature during storage (Andersen et al., 1997; Dunajski, 1979; Haard, 1992; Sigholt et al., 1997; Sigurgisladottir, Torrissen, Lie, Thomassen, & Hafsteinsson, 1997).

The quality of fish muscle will deteriorate during iced storage of raw fish. Endogenous proteases, which are able to hydrolyze different proteins in the muscle, are important early in the deterioration process (Cepeda, Chou, Bracho, & Haard, 1990). Collagenous sheets (myocommata) separate blocks of muscle fibers (Bremner & Hallett, 1985). A fine network of collagen surrounds each muscle fiber and connects it to myocommata. During chilled storage of blue grenadier, it was observed that the attachments between muscle fibers and myocommata, and the whole sarcolemma, were degraded, and muscle fibers were detached from the myocommatal sheets (Bremner & Hallett, 1985; Hallett & Bremner, 1988). A similar degradation is observed with king salmon (Fletcher, Hallett, Jerrett, & Holland, 1997), cod and Atlantic salmon (Ofstad, Egelandsdal, Kidman, Myklebust, Olsen, & Hermansson, 1996). Endogenous collagenases may break down the connective tissue in the fish muscle and thereby lead to undesirable textural changes and gaping (Ando, Yoshimoto, Inabu, Nakagawa, & Makinodan, 1995; Ashie, Smith, & Simpson, 1996b; Bremner & Hallett, 1985; Cepeda et al., 1990). Collagenolytic enzymes have been isolated from the skeletal muscle of fish (Bracho & Haard, 1995;

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Teruel & Simpson, 1995). The enzymatic activities were dependent on fish species, and were most potent at pH values close to neutrality or higher.

Lysosomal catheptic enzymes are involved in deterioration of muscle texture, and different cathepsins may act in concert to autolyze fish muscle (Ashie, Simpson, & Smith, 1996a; Ashie et al., 1996b). The cathepsins are related to protein catabolism of the fish during spawning migration (Yamashita & Konagaya, 1990). Cathepsin B, from chum salmon in spawning migration, hydrolyzed carp myofibrillar proteins in solution, but was not able to hydrolyze carp collagen (Yamashita & Konagaya, 1991). However, when the myofibrils and connective tissue were denatured, the proteins were extremely susceptible to the hydrolytic action of cathepsin B. Lund and Nielsen (2001) investigated changes in the content of free amino acids and low molecular weight peptides during cold storage of salmon. Only small changes were observed, and these were used as an indicator of low activity of exopeptidases in the stored salmon. Most of the proteolysis occurring during cold storage of salmon must therefore be due to endopeptidases cleaving peptide bonds distant to the termini of polypeptide chains.

Hansen, Gill, Røntved, and Huss (1996) studied the relative importance of autolysis and microbial activity on spoilage of cold-smoked salmon. They compared salmon with reduced and normal loads of microorganisms and found that microbiological activity caused production of the characteristic spoilage odors and flavors, while the autolytic enzymes from the fish tissue had a major impact on the textural deterioration (also found by Hansen, Gill, & Huss, 1995). Salmon texture softened before off-odors and off-flavors were observed.

Irradiation of food items is often performed to prevent growth of microorganisms (especially pathogenic microorganisms) and sprouting of vegetables. A lot of attention has been paid to the effect of irradiation on the microbial DNA (Ashie et al., 1996b; Satin, 1996). The fact that radiation treatment influences the alteration and inactivation of other macromolecules, such as proteins and enzymes, has received little attention (Saha, Mandal, & Bhattacharyya, 1995). Among studies concerning irradiation of enzymes, most of the work has concentrated on irradiating enzymes in solution. Only a limited amount of information is available regarding the effect on proteolytic enzymes when intact tissue is irradiated.

In this experiment, different quality characteristics of irradiated salmon fillets and control fillets were determined during storage. The aim of the study was to create a sterile salmon in order to study endogenous enzymes and their impact on salmon texture, without the interfering effects of microbial enzymes. In addition we wanted to discover whether the irradiation process had any effects on quality characteristics such as lipid oxidation and flesh color.

2. Materials and methods

2.1. Salmon sampling and irradiation

Eighteen salmon (*Salmo salar*) were delivered from a fish farm (AKVAFORSK, Averøya) in May 2002. The salmon were live-chilled, bled and gutted, and immediately iced. The day of slaughter was defined as day 0. All fish were filleted on day 4. The fillets were individually vacuum-packed (Nordfilm 213 nylon/polyethylene, 38 cm^3 O_2/m^2/24 h at 23 °C and 85% relative humidity; Nordpak OY, Valkeakoski, Finland) to exclude oxygen during the irradiation and avoid recontamination during the storage period, and divided into two groups (fillets from the same fish were placed in different groups). One group was exposed to irradiation; the other was kept as a control.

The irradiation treatment was carried out at the Institute for Energy Technology (Kjeller, Norway). Radioactive cobalt (^{60}Co) was used as a source of γ radiation. Fillets were exposed to 2.0 kGy at room temperature (3.5 h, 20–22 °C). Fillets in the control group were stored at room temperature (3.5 h; about 21 °C) to ensure that the temperature treatments of the two groups were as equal as possible, and that any differences between the two groups could be attributed to the irradiation. After the irradiation/short storage at room temperature, the fillets were iced and placed in the cold room before analyses. Ice was replenished during storage.

The average length and weight (±standard error of mean) of gutted fish with heads were 63.8 ± 0.5 cm and 3272 ± 72 g, respectively.

The muscle between the gills and the dorsal fin was used for analyses of textural properties, water content, solubility properties of proteins, pH, color and proteolytic activity. The part posterior to the dorsal fin was used for analyses of microbiology and properties of fat. Analyses were made on day 5, 10 and 14. Six fillets from each treatment were analyzed at each sampling. Distilled water was used, and all chemicals were of analytical grade.

2.2. Textural properties

Textural properties were measured with a TA.XT2 Texture Analyser (Stable Micro Systems, UK) by a modification of the method described by Einen and Thomassen (1998), as previously described (Hultmann & Rustad, 2002). Four measurements were run on each fillet. Textural parameters were calculated as described by Bourne (1978) (breaking strength (fracturability), hardness, cohesiveness and gumminess). Resilience was calculated as the ratio of the upstroke area to the downstroke area of the first compression.

2.3. Muscle pH, water content and color measurements

Muscle pH was measured directly in the fillets with a Flushtrode P electrode (Hamilton Company, USA) after determining the textural properties. Four measurements were done on each fillet.

Water content was determined by drying samples of 2 g at 105 °C for 24 h (to constant weight). The analyses were run in duplicate.

Fillet color was determined with a Minolta Chroma Meter CR-200 (Minolta, Japan) calibrated with a calibration plate of typical salmon colour. The measurements were taken directly on the fillets. The analyses were run in triplicate.

2.4. Extraction of proteins

Proteins were extracted from white muscle by a modification of the methods of Anderson and Ravesi (1968) and Licciardello, Ravesi, Lundstrom, Wilhelm, Correia, & Allsup, (1982), as previously described (Hultmann & Rustad, 2002). The extraction procedure was carried out once on each fillet.

The amount of proteins in the extracts was determined after centrifugation (7840*g*, +4 °C for 10 min) with BioRad protein assay, using bovine serum albumin as a standard (Bradford, 1976). The analyses were run in triplicate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on salt soluble proteins, according to Laemmli (1970), using PhastGel Gradient 4–15 gels, SDS Buffer Strips, High and Low Molecular Weight Standards, and the gels were stained with Coomassie brilliant blue. All equipment for electrophoresis was delivered from Amersham Pharmacia Biotech (Uppsala, Sweden). The analyses were carried out according to the instructions of the manufacturer.

The extractions of proteolytic enzymes were performed as previously described (Hultmann, Rørå, Steinsland, Skåra, & Rustad, in press). The extraction procedure was carried out once on each fillet. The extracts were frozen and stored at −40 °C until analyzed.

2.5. General proteolytic activity

General proteolytic activity at pH 6.0 and 6.5 was determined by a modification of the method described by Barrett and Heath (1977), with the method adjusted to micro scale as previously described (Hultmann et al., in press).

The proteolytic activity was expressed as mg TCA soluble peptides liberated/g wet weight/hour (using bovine serum albumin as a standard), and given as the arithmetic mean of three individual measurements.

2.6. Activity of collagenase-like enzymes and cathepsin B-like enzymes

After thawing, the amount of proteins in the extracts was determined with BioRad protein assay (Bradford, 1976). The analyses were run in triplicate. Centrifuged samples were diluted with distilled water to get a protein content of about 1.5 mg/ml.

Activities of collagenase-like enzymes were measured against a synthetic fluorogenic substrate, *N*-succinyl-glycine-proline-leucine-glycine-proline-7-amido-4-methylcoumarin (SGP) (Bachem, Bubendorf, Switzerland) (Kinoshita, Toyohara, Shimizu, & Sakaguchi, 1992). Concentration of SGP was 0.0625 mM in 100 mM bis–Tris, 5 mM $CaCl_2$, pH 6.5. Activities of cathepsin B-like enzymes were measured against a synthetic fluorogenic substrate, N_α-carbobenzoxy-arginine-arginine-7-amido-4-methylcoumarin (CAA) (Sigma Chemical Co., St. Louis, MO, USA) (Barrett & Kirschke, 1981). Concentration of CAA was 0.0625 mM in 100 mM bis-Tris, 20 mM EDTA, 4 mM dithiothreitol, pH 6.5.

Enzyme activities were determined as previously described (Hultmann et al., in press). Increase in fluorescence intensity was used to calculate the activity, given as increase in fluorescence/g wet weight/minute during incubation. The analyses were run in triplicate.

2.7. Microbiology

A rectangular piece of fish skin was aseptically cut using a template of 5×2 cm, and homogenized in filter bags (2×30 s, 200 rpm, Stomacher Model 400 Filter Bags, Seward, London, UK) with 9 parts sterile peptone saline (pH 7.2), using a stomacher (Stomacher 400 Labsystem, Seward, London, UK). Homogenates were further diluted with peptone saline. Appropriate dilutions were incubated in iron agar (Lyngby) (Oxoid Ltd., Hampshire, UK) with cysteine (Sigma Chemical Co., St. Louis, MO, USA), with a thin covering layer of iron agar (pH 7.4 ± 0.2), as described by Gram, Trolle, and Huss (1987). Plates were incubated at 15 ± 1 °C for 3 days. Results for individual samples were calculated assuming Poisson distribution. Average values for treatment groups were calculated using values for individual fish and the assumption that the numbers of colony forming units on skin from different fishes were normally distributed.

2.8. Free amino acids

The contents of free amino acids were determined by reversed phase HPLC in enzyme extracts after precipitating the proteins with sulfosalicylic acid, as previously described (Hultmann et al., in press) Glycine and arginine were determined together, as their peaks merged. Amino acid concentrations were determined in duplicate for each sample. Results from individual samples were used to

calculate the average content of specific amino acids within each treatment/storage time group (6 samples in each group). The fraction of each individual amino acid within a group was calculated from the average content of the specific amino acid within the group.

2.9. Analyses of fat

The total amount of fat was determined by the Bligh and Dyer method (Bligh & Dyer, 1959), as modified by Hardy and Keay (1972). Oxidation of lipids was determined as amount of 2-thiobarbituric acid reactive substances (TBARS) and peroxide values (PV). TBARS was determined as described by Ke and Woyewoda (1979) using 1,1,3,3-tetraethoxypropane as standard. PV was analyzed as described by the International Dairy Federation (Anon., 1991), with the modifications of Ueda, Hayashi, and Namiki (1986) and Undeland, Stading, and Lingnert (1998).

2.10. Statistics

Mean values for the different quality characteristics (Y_i) were calculated for the individual samples, and were used for statistical analyses of the data. Three different analyses were performed, with significance level $p = 0.05$.

The effect of storage time (t_j, $j = 1, 2, 3$) on a given variable (Y_i) was investigated by linear regression, using the model $Y_i = \beta_{0i} + \beta_{1i}t + \varepsilon$, where $\varepsilon \sim N(0, \sigma_\varepsilon^2)$. The null hypothesis ($H_0$) tested was $\beta_{1i} = 0$.

The effect of treatment (r_k, $k = 1, 2$) was investigated using paired test of the differences (d) between quality characteristics of irradiated (r_1) and control (r_2) samples, $d_{i\,tj} = Y_{i\,tj\,r1} - Y_{i\,tj\,r2}$. The model used was $d_{i\,tj} \sim N(\mu_d, \sigma_d^2)$. The null hypothesis ($H_0$) tested was $\mu_d = 0$.

In addition, the t-test assuming equal variances was applied to investigate differences in a given variable between the first and last sampling day, using the model $Y_{i\,tj\,rk} \sim N(\mu_{i\,tj\,rk}, \sigma^2)$. The null hypothesis ($H_0$) tested was $\mu_{i\,t1\,rk} = \mu_{i\,t3\,rk}$.

3. Results and discussion

3.1. Microbiology, color, fat, Muscle pH and water content

To confirm that the irradiated fillets had lower bacterial loads than the control group, samples of fish skin were subjected to microbiological analyses. By pour plating into iron agar, H_2S-producing bacteria (reported to be specific spoilage organisms of fish, Gram et al., 1987) give rise to black colonies, while black and white colonies together constitute a measure of total viable count. The irradiated fillets had low numbers of microorganisms during the whole storage period (Table 1), while the number of colony forming units in the control group increased during storage. H_2S-producing bacteria were only detected in the control group, after 14 days of iced storage.

In addition to reducing the number of microorganisms, the irradiation also caused color changes in the fillets (Table 2). Irradiated fillets were generally decolorized, appearing pale throughout the thickness of the fillet. Measured as Hunter color values, the irradiated fillets were significantly lighter than the control fillets (Table 6). The control fillets were significantly more red and yellow (a^*- and b^*-values, respectively) than the irradiated fillets.

Due to practical reasons (problems with getting ice), the irradiation was performed at room temperature. Freezing the samples prior to irradiation could have curtailed undesired processes such as decolorization, but would have affected the solubility characteristics of the proteins (Mackie, 1993; Rodger & Wilding, 1990). It was therefore concluded that performing the irradiation at room temperature was both feasible and acceptable.

When the vacuum packages were opened, there was a smell of old fish from the irradiated samples, even on day 5. No attempts were made to collect or analyze the volatile components. Possible oxidation products were measured as TBARS and PV. No significant linear dependence was found between TBARS and storage time (Tables 3 and 5). The irradiated fillets showed a decrease in TBARS during the storage period, but the changes were only significant at 90% level. The PV increased for both groups during storage, but the changes were not significant (Tables 3 and 5). The effect of irradiation was investigated for each sampling day (Table 6). The irra-

Table 2
Hunter color values at day 14

	Control	Irradiated
L^*	45.4 ± 0.7	48.5 ± 0.6
a^*	9.9 ± 0.2	2.3 ± 0.4
b^*	10.3 ± 0.2	7.9 ± 0.5

Average ± standard error of the mean ($n = 6$).

Table 1
Microbial counts on Iron Agar, log cfu/cm^2 (min and max values in brackets)

Storage days	White colonies		H_2S-producing bacteria	
	Control	Irradiated	Control	Irradiated
10	3.66 (2.99–4.25)	1.93 (1.52–2.29)	<2.81	<0.85
14	4.39 (3.46–5.47)	1.81 (1.41–2.39)	2.22 (<0.79–3.59)	<0.83

Table 3
Muscle pH, water content, solubility properties of proteins, proteolytic activity and oxidation of lipids during iced storage of vacuum-packed fillets

Storage day	Muscle pH	Water content (%)	WSP (%)	SSP (%)	ExP (%)	GPA 6.0 (mg/(g*h))	GPA 6.5 (mg/(g*h))	TBARS (μmol/g)	PV (meq/kg)
Control									
5	6.18 ± 0.02	66.1 ± 0.8	8.15 ± 0.10	8.3 ± 0.3	16.5 ± 0.3	0.05 ± 0.04	0.08 ± 0.03	0.98 ± 0.18	7.7 ± 1.5
10	6.25 ± 0.02	64.7 ± 0.7	7.97 ± 0.12	8.4 ± 0.4	16.4 ± 0.3	0.06 ± 0.03	0.08 ± 0.03	0.91 ± 0.10	8.1 ± 0.6
14	6.30 ± 0.02	66.5 ± 0.5	7.02 ± 0.29	8.3 ± 0.1	15.3 ± 0.3	0.11 ± 0.04	0.08 ± 0.02	1.19 ± 0.04	9.4 ± 0.4
Irradiated									
5	6.20 ± 0.02	66.8 ± 1.2	8.47 ± 0.18	8.6 ± 0.2	17.1 ± 0.4	0.08 ± 0.07	0.05 ± 0.03	1.26 ± 0.14	6.9 ± 1.4
10	6.24 ± 0.02	64.2 ± 0.5	7.91 ± 0.18	7.7 ± 0.3	15.6 ± 0.3	0.13 ± 0.05	0.11 ± 0.05	0.91 ± 0.10	7.4 ± 1.0
14	6.32 ± 0.01	67.6 ± 0.6	7.11 ± 0.13	7.4 ± 0.3	14.5 ± 0.4	0.01 ± 0.04	0.01 ± 0.02	0.97 ± 0.07	9.4 ± 0.8

WSP, water-soluble proteins; SSP, salt soluble proteins; ExP, extractable proteins (sum of WSP and SSP); GPA, general proteolytic activity at indicated pH value; TBARS, 2-thiobarituric acid reactive compounds; PV, peroxide value. Water content and protein solubility characteristics are given in % of wet weight, general proteolytic activity as mg TCA soluble peptides liberated/g wet weight/hour, TBARS in μmol malonaldehyde/g fat and PV in milliequivalents peroxide/kg fat. Average ± standard error of the mean ($n = 6$).

diated fillets had significantly lower TBARS values than those in the control group after 14 days of iced storage. No significant effect of irradiation on TBARS was evident earlier in the storage period or for PV during the whole storage period. The difference in TBARS could be due to damage of enzymes in the irradiated group, as endogenous enzymes may be important for the lipid oxidation in fish muscle (Ashie et al., 1996b). Nam, Du, Jo, and Ahn (2002) studied irradiated pork, and found that TBARS values were not affected by irradiation dose or storage time. They concluded that lipid oxidation in vacuum-packed pork did not increase during storage because no oxygen was available for hydroperoxide formation. The same conclusion may be valid in our experiment. In addition, lipids may have been oxidized, and the products reacted further with proteins and/or carotenoids, without resulting in components detectable with the PV and TBARS methods.

The fillet water content was not significantly dependent on storage time (Tables 3 and 5) or treatment (Table 6). The muscle pH values increased significantly during the storage period for both treatments (Tables 3 and 5). However, no significant changes in muscle pH were observed due to irradiation (Table 6). Changes in other quality characteristics observed during the storage period may be related to the changes in pH, but neither water content nor pH values can explain observed differences between the treatment groups (see below).

3.2. Textural properties and protein solubility

Different textural properties of the fillets are shown in Fig. 1. No significant linear dependences were found between the textural properties investigated and storage time (Table 5). In the control group, only resilience showed a weak linear dependence on storage time (negative, significant at 90% level). In addition, the values at day 5 were not significantly different from the values at day 14. The results are quite different from those obtained earlier with larger fresh salmon, where both breaking strength and hardness decreased significantly during iced storage (11 days) while resilience and cohesiveness increased (Hultmann & Rustad, 2002). However, the values were of the same order of magnitude, stressing the importance of fish size and storage system when comparing textural properties of fish. The effect of irradiation was investigated during the storage period (Table 6). A significant effect of irradiation was seen at the end of the storage period, as the control fillets were softer and showed lower breaking strength than the irradiated fillets. This may be due to reduced release of Ca^{2+} ions from the sarcoplasmic reticulum in the irradiated samples, and thereby decreased activity of calcium-activated proteolytic enzymes (as shown in grass shrimp by Yang & Perng, 1995). Compared to the control group the irradiated fillets were harder after 5 days of iced storage, and displayed increased gumminess after 14 days (both significant at 90% level).

The muscle proteins are important for quality characteristics such as textural properties. A significant reduction in the amount of water-soluble proteins was observed for both treatments during the storage period (Tables 3 and 5). No significant changes in the content of salt soluble proteins were observed in the control group. During storage, the irradiated fillets showed a significant decrease in extractability of salt soluble proteins. Altogether, there was a significant reduction of total protein extractability in both groups. The irradiation process caused a decrease of the extractability of salt soluble proteins, and thereby of the total protein extractability, evident after 14 days iced storage (significant at 90% level, Table 6). This difference in protein extractability supports the observed differences in textural properties between the two groups. Even if significant, the changes in protein solubility were not great.

Given the decreased extractability of salt soluble proteins in the irradiated fillets, the composition of salt soluble proteins was investigated by SDS–PAGE.

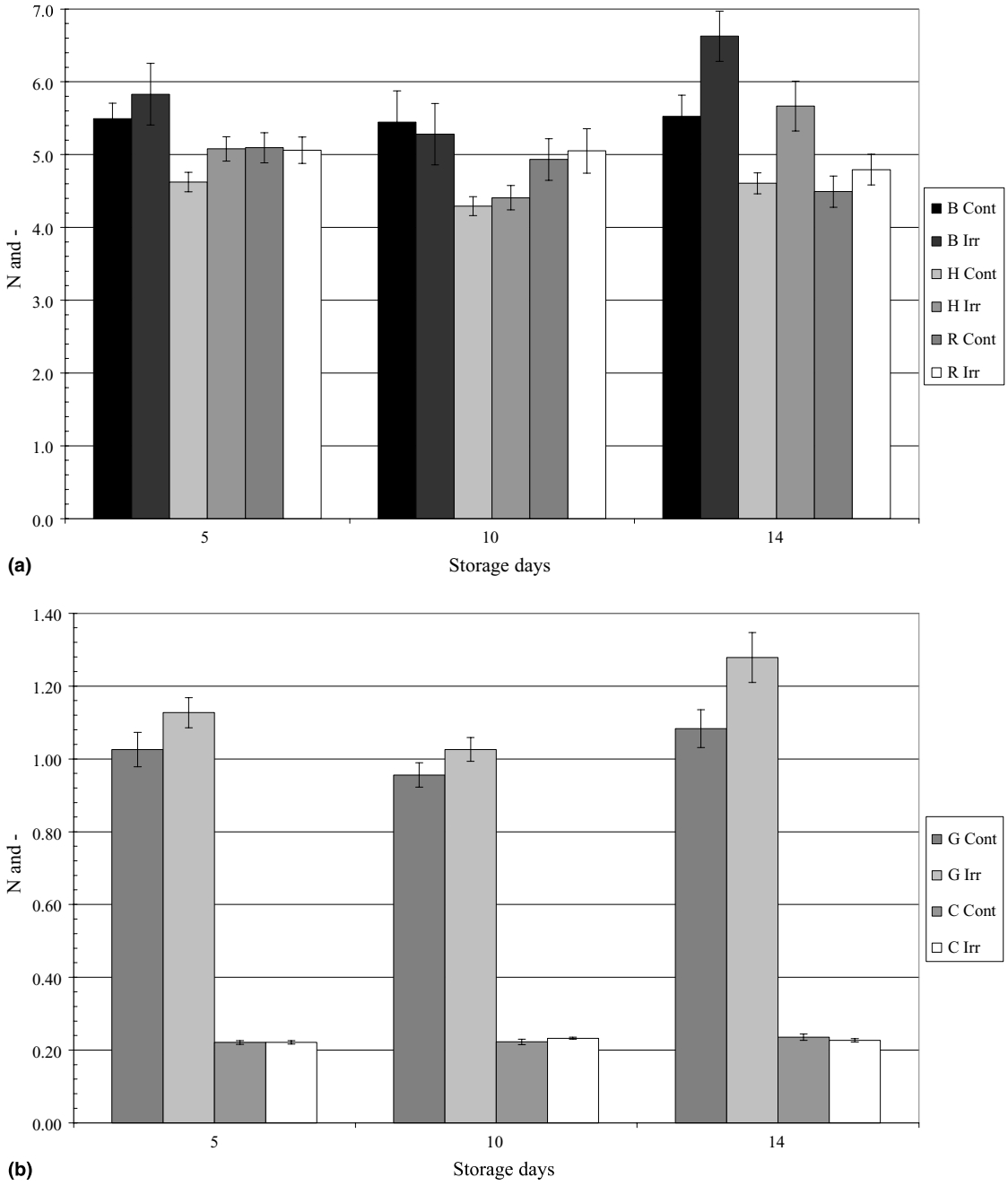

Fig. 1. Textural properties of salmon fillets during iced storage. (a) B, breaking strength (N); H, hardness (N); R, 100 * resilience (–). (b) G, gumminess (N); C, cohesiveness (–). Cont, control fillets; Irr, irradiated fillets. Bars indicate standard errors of the means ($n = 6$).

However, no differences due to storage or treatment were seen (results not shown). This is in agreement with the results from irradiated beef (Lee, Yook, Lee, Kim, Kim, & Byun, 2000), indicating that muscle proteins may be structurally changed but not degraded by the irradiation treatment.

3.3. Proteolytic enzymes

Proteolytic enzymes are known to degrade muscle proteins and thereby lead to textural changes in the muscle. The activities of different enzymes were studied during the storage period. The general proteolytic activity was determined at pH 6.0 and 6.5, and was low (or not detectable) in all samples (Table 3). No significant effects of storage time or irradiation were seen (Tables 5 and 6). *t*-tests (assuming equal variances) were used in order to compare the proteolytic activities at the two pH values tested, for each sampling day. No significant differences were found (irradiated fillets: $p = 0.658$, 0.768 and 0.902; control fillets: $p = 0.627$, 0.617 and 0.496 after 5, 10 and 14 days of iced storage,

respectively). Low general proteolytic activity at pH 6.0 and 6.5 (25 °C) is in agreement with the results reported by Stoknes and Rustad (1995), where the maximum activities for salmon proteases were found to be at higher temperatures and pH values.

Activities of the specific proteases investigated, cathepsin B- and collagenase-like enzymes, were not significantly dependent on storage time (Fig. 2 and Table 5). In the irradiated fillets, the cathepsin B-like activity was higher after 14 days of iced storage (compared to the first sampling day), but the difference was only significant at 90% level. No significant differences were seen for collagenase-like enzymes or in the control fillets. Both cathepsin B-like and collagenase-like enzyme activities were severely inhibited by the irradiation treatment (Fig. 2 and Table 6), even if the amount of proteins in the enzyme extract was unaffected by treatment. This effect was most pronounced for the cathepsin B-like enzymes. In the irradiated fillets, both peptidase activities showed an increasing trend during the storage period. This may be due to a slight reactivation of enzymes during iced storage after irradiation, or destruction of endogenous inhibitors. Comparison of fillets from the same individual revealed that the cathepsin B-like enzymes in about 90% of the fillets were 75–90% inhibited by the irradiation process. Cathepsin B is a cysteine endopeptidase, and it is reasonable to assume that the cysteine residue in the active site was damaged during the irradiation. Radiation of cysteine residues may lead to irreparable damage if oxygen or water is present (Ashie et al., 1996a; Saha et al., 1995). To our knowledge, only one other study of cathepsin B-like activity in irradiated intact muscle has been published. Jamdar and Harikumar (2002) studied cathepsins in γ-irradiated chicken meat. In intact tissue, the cysteine protease cathepsin B was far more sensitive to irradiation than cathepsins D (aspartyl protease) and H (cysteine protease). At 2.5 kGy, chicken cathepsin B-like enzymes were 25% inactivated. The degree of inactivation of collagenase-like enzymes varied considerably between the fillets. About 70% of the samples showed 45–80% inactivation, but the values varied from a slight activation to 94% inactivation. Collagenolytic enzymes are Zn^{2+}-containing metalloendopeptidases stimulated by Ca^{2+} ions and thiol reagents (Ashie et al., 1996b), and are not as susceptible as sulfhydryl enzymes to damage by irradiation (Saha et al., 1995). Different proteolytic enzymes will respond differently to a given irradiation dose, some being fully inactivated while others are almost unaffected or even activated. The stability of the irradiated fish product may therefore be difficult to predict.

The differences in proteolytic activities due to treatment were largest early in the storage period. However, significant differences in fillet texture were detected only after 14 days of storage (Table 6). The effects of enzymatic processes are only discovered after some time, possibly due to the relatively stable myosin and/or actomyosin complex, in addition to an amorphous material filling the intra- and extracellular spaces in salmon muscle and thereby stabilizing the muscle (Ofstad et al., 1996). Proteolytic degradation of salmon proteins may therefore not lead to severe textural changes early in the storage period.

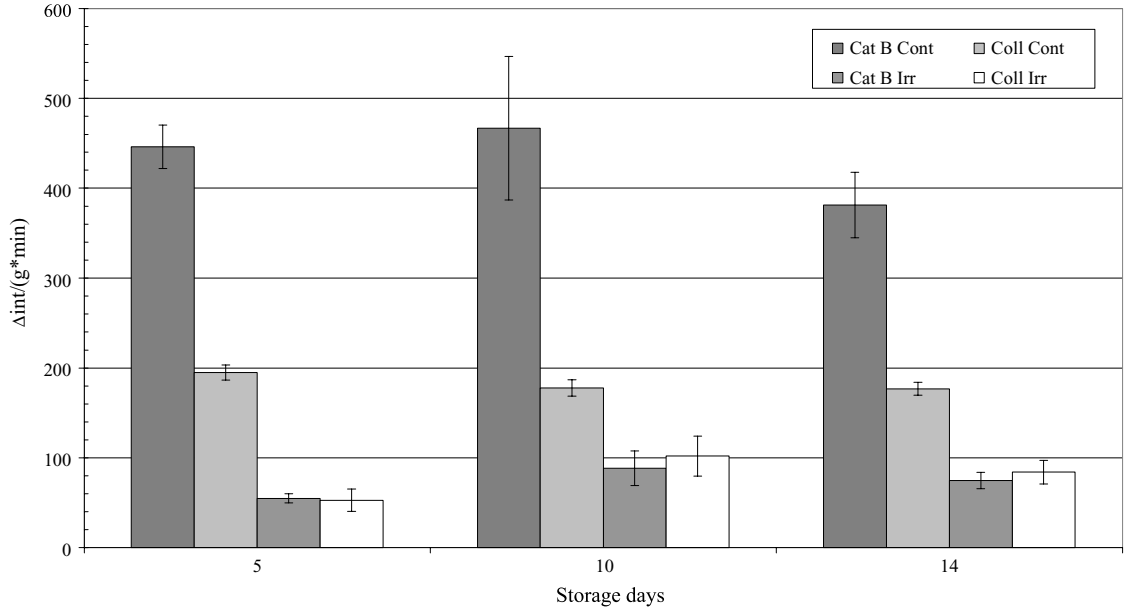

Fig. 2. Activity of cathepsin B-like (Cat B) and collagenase-like (Coll) enzymes during iced storage of salmon fillets, pH 6.5 at 4 °C. Enzyme activities are reported as increase in fluorescence intensity/g wet weight/minute. Cont, control fillets; Irr, irradiated fillets. Bars indicate standard errors of the means ($n = 6$).

The total amount of free amino acids in the control group increased slightly during storage (Table 4). Due to large variation between individuals, the change was not significant (Table 5). During storage, no significant change was observed for the irradiated samples. Irradiation caused a decrease in total content of free amino acids in the fillets (Table 6). These results agree with the radiation-induced inactivation of proteolytic enzymes. The difference in content of free amino acids was significant after 14 days of iced storage. The time lag to discover effects of irradiation is in accordance with results obtained for textural properties and solubility properties of proteins. Lund and Nielsen (2001) investigated changes in free amino acids during cold storage of salmon. Although the total amount of free amino acids seemed to increase slightly during the storage period, only small changes in the content of individual amino acids were observed between salmon samples stored for 3 and 23 days. Ahn, Jo, and Olson (2000) studied irradiation of raw pork, and found that

Table 4
Free amino acids in salmon muscle during iced storage of vacuum-packed fillets, fraction (%) of total amount (from μmol/g wet weight)

Amino acid	Control		Irradiated	
	Day 5	Day 14	Day 5	Day 14
Aspartic acid	1	3	0	1
Glutamic acid	6	6	7	7
Asparagine	0	0	0	0
Histidine	4	4	4	5
Serine	4	7	4	5
Glutamine	3	2	3	2
Glycine/Arginine	23	17	24	19
Threonine	7	6	7	7
Alanine	28	27	29	28
Tyrosine	2	2	1	1
α-butyric acid	2	1	2	2
Methionine	0	0	0	0
Valine	2	3	2	2
Phenylalanine	2	2	1	1
Isoleucine	1	1	1	1
Leucine	3	3	2	2
Lysine	13	16	14	17
Total amount	15.2 ± 1.0	17.9 ± 1.7	14.7 ± 0.6	13.9 ± 1.1

Total amounts (μmol/g wet weight) are given as averages ± standard errors of the means ($n = 6$).

Table 5
Results from linear regression against storage time ($t_j, j = 1, 2, 3$), and differences between the first and last sampling days

Variable (Y_i)	Control				Irradiated			
	β_0	β_1	p	p_{tj}	β_0	β_1	p	p_{tj}
Water content	65.40	0.04	0.751	0.639	65.55	0.07	0.683	0.551
Muscle pH	6.12	0.01	**0.001**	**0.002**	6.12	0.01	**0.000**	**0.001**
Water-soluble proteins	8.89	−0.12	**0.002**	**0.004**	9.28	−0.15	**0.000**	**0.000**
Salt soluble proteins	8.40	−0.01	0.860	0.796	9.22	−0.14	**0.007**	**0.016**
Extractable proteins	17.28	−0.13	**0.029**	**0.031**	18.50	−0.29	**0.000**	**0.001**
Breaking strength	5.46	0.00	0.951	0.930	5.14	0.08	0.256	0.176
Hardness	4.55	−0.00	0.845	0.931	4.50	0.06	0.256	0.155
Resilience	0.055	−0.001	*0.092*	*0.070*	0.052	−0.000	0.445	0.362
Cohesiveness	0.211	0.002	0.178	0.167	0.220	0.001	0.332	0.421
Gumminess	0.97	0.01	0.474	0.434	1.00	0.02	0.131	*0.088*
Fat content	–	–	–	0.767	–	–	–	0.356
TBARS	0.82	0.02	0.281	0.285	1.37	−0.03	*0.067*	*0.086*
PV	6.58	0.18	0.220	0.286	5.29	0.27	0.130	0.150
Cathepsin B-like activity	496.58	−6.75	0.422	0.170	49.60	2.39	0.260	*0.084*
Collagenase-like activity	203.16	−2.07	0.124	0.131	43.44	3.74	0.187	0.112
General proteolytic activity (pH 6.0)	0.01	0.01	0.261	0.289	0.14	−0.01	0.380	0.345
General proteolytic activity (pH 6.5)	0.07	0.00	0.905	0.912	0.09	−0.00	0.604	0.397
Free amino acids	–	–	–	0.190	–	–	–	0.530

Model $Y_i = \beta_{0i} + \beta_{1i}t + \varepsilon$, where $\varepsilon \sim N(0, \sigma_\varepsilon^2)$. The null hypothesis ($H_0$) tested was $\beta_{1i} = 0$, $n = 18$ (reported as β_0, β_1 and p). Differences in a given variable between the first and last sampling day were investigated using t-test assuming equal variances (reported as p_{tj}). Units of measurements are as given in Figs. 1 and 2 and Tables 3 and 4. p values in bold are significant at 95% level, those in italic at 90% level.

Table 6
Effect of treatment investigated using paired test of the differences (d) between quality characteristics of irradiated and control samples

Variable (Y_i)	Day 5		Day 10		Day 14	
	μ_d	p	μ_d	p	μ_d	p
Water content	0.73	0.643	−0.55	0.437	1.10	0.196
Muscle pH	0.01	0.317	−0.02	0.219	0.01	0.502
Water-soluble proteins	0.32	0.201	−0.06	0.841	0.08	0.681
Salt soluble proteins	0.30	0.977	−0.73	0.120	−0.84	*0.067*
Extractable proteins	0.62	0.512	−0.79	0.157	−0.75	*0.095*
Breaking strength	0.34	0.594	−0.16	0.791	1.10	**0.047**
Hardness	0.46	*0.064*	0.12	0.373	1.06	**0.019**
Resilience	0.000	0.810	0.001	0.788	0.003	0.399
Cohesiveness	0.000	0.915	0.010	0.271	−0.009	0.180
Gumminess	0.10	0.215	0.07	0.104	0.20	*0.057*
Fat content	1.78	*0.088*	−0.32	0.788	0.76	0.233
TBARS	0.28	0.113	0.00	0.957	−0.22	**0.020**
PV	−0.78	0.504	−0.68	0.497	−0.01	0.993
Cathepsin B-like activity	−391.18	**0.000**	−378.30	**0.002**	−306.39	**0.000**
Collagenase-like activity	−142.13	**0.000**	−75.80	**0.027**	−92.69	**0.000**
General proteolytic activity (pH 6.0)	0.03	0.729	0.08	*0.053*	−0.11	0.143
General proteolytic activity (pH 6.5)	−0.03	0.696	0.03	0.459	−0.07	0.150
Free amino acids	−0.49	0.272	–	–	−4.04	**0.002**
L^*	–	–	–	–	3.17	**0.014**
a^*	–	–	–	–	−7.62	**0.000**
b^*	–	–	–	–	−2.38	**0.004**

Units of measurements are as given in Figs. 1 and 2 and Tables 2–4. p values in bold are significant at 95% level, those in italic at 90% level.

radiolytic breakdown of amino acids containing sulfur was the major contributor to off-odor in irradiated meat. The amino acids histidine, tyrosine, tryptophan, cysteine and methionine are particularly prone to attack by radicals because of the presence of easily oxidizable functional groups (Saha et al., 1995). Differences in amino acid composition were investigated using paired tests of the differences between contents of individual amino acids in the irradiated and control samples. The irradiation process significantly reduced the content of free aspartic acid, tyrosine, valine, phenylalanine and leucine, while free histidine and methionine contents seemed unaffected. Tryptophan and cysteine were not detected with the selected method, and it is therefore not possible to determine the effect of irradiation on these amino acids in the present study.

Several researchers have reported that microbial enzymes leak through the skin or fillet surface and into the fish muscle, causing changes to fish texture and other properties (Ashie et al., 1996b). Under temperature abuse conditions, or cuts in the fish skin, as a result of improper handling, microorganisms invade the relatively sterile muscle tissue, resulting in more rapid spoilage. Filleted fish is even more susceptible. Peptidase activities did not increase significantly during storage of vacuum-packed fillets (Table 5). In the control group, both cathepsin B-like and collagenase-like enzyme activities showed a decrease during storage, but the changes were not significant. This indicates that the higher numbers of microbes present on the skin (and probably on the fillet surface) of control fillets did not lead to leakage of peptidases through the surface and into the fillets. A raw material of high quality may be more robust towards different treatments than a raw material of inferior quality. The salmon used in this experiment showed good textural properties during the entire storage period, indicating a good quality. Greater differences might have appeared between the two treatment groups if the raw material had been of lower quality.

4. Conclusions

Irradiation of salmon fillets resulted in severe inhibition of cathepsin B-like and collagenolytic enzymes. The effect was most pronounced for the cathepsins. Differences in quality characteristics such as textural properties and amounts of free amino acids and extractable proteins between the irradiated and control fillets, were evident only late in the storage period (after 14 days of iced storage). Even with the increased numbers of microorganisms seen in the control group, no severe deterioration of fish texture or increase in proteolytic activity was observed during the storage period. It therefore seems that microorganisms are not important for changes in salmon texture during storage.

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References

Anon. (1991). *Anhydrous milk fat. Determination of peroxide value*. International IDF Standard 74A. Belgium: International Dairy Federation.

Ahn, D. U., Jo, C., & Olson, D. G. (2000). Analysis of volatile components and the sensory characteristics of irradiated raw pork. *Meat Science, 54*(3), 209–215.

Anderson, M. L., & Ravesi, E. M. (1968). Relation between protein extractability and free fatty acid production in cod muscle aged in ice. *Journal of the Fisheries Research Board of Canada, 25*(10), 2059–2069.

Andersen, U. B., Thomassen, M. S., & Rørå, A. M. B. (1997). Texture properties of farmed rainbow trout (*Oncorhynchus mykiss*): Effects of diet, muscle fat content and time of storage on ice. *Journal of the Science of Food and Agriculture, 74*(3), 347–353.

Ando, M., Yoshimoto, Y., Inabu, K., Nakagawa, T., & Makinodan, Y. (1995). Post-mortem change of three-dimensional structure of collagen fibrillar network in fish muscle pericellular connective tissues corresponding to post-mortem tenderization. *Fisheries Science, 61*(2), 327–330.

Ashie, I. N. A., Simpson, B. K., & Smith, J. P. (1996a). Mechanisms for controlling enzymatic reactions in foods. *Critical Reviews in Food Science and Nutrition, 36*(1&2), 1–30.

Ashie, I. N. A., Smith, J. P., & Simpson, B. K. (1996b). Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science and Nutrition, 36*(1&2), 87–121.

Barrett, A. J., & Heath, M. F. (1977). Lysosomal enzymes. In J. T. Dingle (Ed.), *Lysosomes – a laboratory handbook* (2nd ed.) (pp. 19–145). Amsterdam, The Netherlands: North-Holland.

Barrett, A. J., & Kirschke, H. (1981). Cathepsin B, cathepsin H, and cathepsin L. In L. Lorand (Ed.), *Methods in enzymology. Proteolytic enzymes, Part C*, vol. 80 (pp. 535–561). New York: Academic Press.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method to total lipid extraction and purification. *Canadian Journal of Biochemistry, 37*, 911–917.

Bourne, M. C. (1978). Texture profile analysis. *Food Technology*, 62–66 +72.

Bracho, G. E., & Haard, N. F. (1995). Identification of two matrix metalloproteinases in the skeletal muscle of Pacific rockfish (*Sebastes* sp.). *Journal of Food Biochemistry, 19*, 299–319.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Bremner, H. A., & Hallett, I. C. (1985). Muscle fiber-connective tissue junctions in the fish blue grenadier (*Macruronus novaezelandiae*). A scanning electron microscope study. *Journal of Food Science, 50*, 975–980.

Cepeda, R., Chou, E., Bracho, G., & Haard, N. F. (1990). An immunological method for measuring collagen degradation in the muscle of fish. In M. N. Voigt, & J. R. Botta (Eds.), *Advances in fishery technology and biotechnology for increased profitability: Papers from the 34th Atlantic Fisheries Technological Conference and Biotechnological Workshop, Aug 27–Sept 1, 1989, St. Johns, NF, Canada* (pp. 487–506). Lancaster, Pennsylvania, USA: Technomic Publishing Company.

Dunajski, E. (1979). Texture of fish muscle. *Journal of Texture Studies, 10*, 301–318.

Einen, O., & Thomassen, M. S. (1998). Starvation prior to slaughter in Atlantic salmon (*Salmo salar*) II. White muscle composition and evaluation of freshness, texture and colour characteristics in raw and cooked fillets. *Aquaculture, 169*, 37–53.

Fletcher, G. C., Hallett, I. C., Jerrett, A. R., & Holland, A. J. (1997). Changes in the fine structure of the myocommata-muscle fibre junction related to gaping in rested and exercised muscle from king salmon (*Oncorhynchus tshawytscha*). *Food Science and Technology, 30*(3), 246–252.

Gram, L., Trolle, G., & Huss, H. H. (1987). Detection of specific spoilage bacteria from fish stored at low (0 °C) and high (20 °C) temperatures. *International Journal of Food Microbiology, 4*, 65–72.

Haard, N. F. (1992). Control of chemical composition and food quality attributes of cultured fish. *Food Research International, 25*(4), 289–307.

Hallett, I. C., & Bremner, H. A. (1988). Fine structure of the myocommata-muscle fibre junction in hoki (*Macruronus novaezelandiae*). *Journal of the Science of Food and Agriculture, 44*, 245–261.

Hansen, L. T., Gill, T., & Huss, H. H. (1995). Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. *Food Research International, 28*(2), 123–130.

Hansen, L. T., Gill, T., Røntved, S. D., & Huss, H. H. (1996). Importance of autolysis and microbiological activity on quality of cold-smoked salmon. *Food Research International, 29*(2), 181–188.

Hardy, R., & Keay, J. N. (1972). Seasonal variations in the chemical composition of Cornish mackerel *Scomber scombrus* (L.) with detailed reference to the lipids. *Journal of Food Technology, 7*, 125–137.

Hultmann, L., & Rustad, T. (2002). Textural changes during iced storage of salmon (*Salmo salar*) and cod (*Gadus morhua*). *Journal of Aquatic Food Product Technology, 11*(3-4), 105–123.

Hultmann, L., Rørå, A. M. B., Steinsland, I., Skåra, T. & Rustad, T. Proteolytic activity and properties of proteins in smoked salmon (*Salmo salar*) – effects of smoking temperature. *Food Chemistry*, in press.

Jamdar, S. N., & Harikumar, P. (2002). Sensitivity of catheptic enzymes in radurized chicken meat. *Journal of Food Science and Technology, 39*(1), 72–73.

Ke, P. J., & Woyewoda, A. D. (1979). Microdetermination of thiobarbituric acid values in marine lipids by a direct spectrophotometric method with a monophasic reaction system. *Analytica Chimica Acta, 106*, 279–284.

Kinoshita, M., Toyohara, H., Shimizu, Y., & Sakaguchi, M. (1992). *Modori*-inducing proteinase active at 50 °C in threadfin bream muscle. *Nippon Suisan Gakkaishi, 58*(4), 715–720.

Laemmli, U. K. (1970). Cleavage and structural proteins during assembly of the head of bacteriophage T_4. *Nature, 227*, 680–685.

Lee, J. W., Yook, H. S., Lee, K. H., Kim, J. H., Kim, W. J., & Byun, M. W. (2000). Conformational changes of myosin by gamma irradiation. *Radiation Physics and Chemistry, 58*(3), 271–277.

Licciardello, J. J., Ravesi, E. M., Lundstrom, R. C., Wilhelm, K. A., Correia, F. F., & Allsup, M. G. (1982). Time-temperature tolerance and physical-chemical quality tests for frozen red hake. *Journal of Food Quality, 5*, 215–234.

Lund, K. E., & Nielsen, H. H. (2001). Proteolysis in salmon (*Salmo salar*) during cold storage; Effects of storage time and smoking process. *Journal of Food Biochemistry, 25*(5), 379–395.

Mackie, I. M. (1993). The effects of freezing on flesh proteins. *Food Reviews International, 9*(4), 575–610.

Nam, K. C., Du, M., Jo, C., & Ahn, D. U. (2002). Effect of ionizing radiation on quality characteristics of vacuum-packaged normal, pale-soft-exudative, and dark-firm-dry pork. *Innovative Food Science & Emerging Technologies, 3*(1), 73–79.

Ofstad, R., Egelandsdal, B., Kidman, S., Myklebust, R., Olsen, R. L., & Hermansson, A. M. (1996). Liquid loss as effected by *post mortem* ultrastructural changes in fish muscle: Cod (*Gadus morhua* L.) and salmon (*Salmo salar*). *Journal of the Science of Food and Agriculture, 71*(3), 301–312.

Rodger, G. W., & Wilding, P. (1990). Muscle proteins. In P. Harris (Ed.), *Food gels* (pp. 361–400). London: Elsevier Applied Science.

Saha, A., Mandal, P. C., & Bhattacharyya, S. N. (1995). Radiation-induced inactivation of enzymes – a review. *Radiation Physics and Chemistry, 46*(1), 123–145.

Satin, M. (1996). *Food irradiation: A guidebook* (2nd ed.). Lancaster, Pennsylvania, USA: Technomic Publishing Company.

Sigholt, T., Erikson, U., Rustad, T., Johansen, S., Nordtvedt, T. S., & Seland, A. (1997). Handling stress and storage temperature affect meat quality of farmed-raised Atlantic salmon (*Salmo salar*). *Journal of Food Science, 62*(4), 898–905.

Sigurgisladottir, S., Torrissen, O., Lie, Ø., Thomassen, M., & Hafsteinsson, H. (1997). Salmon quality: Methods to determine the quality parameters. *Reviews in Fisheries Science, 5*(3), 223–252.

Stoknes, I., & Rustad, T. (1995). Proteolytic activity in muscle from Atlantic salmon (*Salmo salar*). *Journal of Food Science, 60*(4), 711–714.

Teruel, S. R. L., & Simpson, B. K. (1995). Characterization of the collagenolytic enzyme fraction from winter flounder (*Pseudopleuronectes americanus*). *Comparative Biochemistry and Physiology, 112B*(1), 131–136.

Ueda, S., Hayashi, T., & Namiki, M. (1986). Effect of ascorbic acid on lipid autoxidation in a food model system. *Agricultural and Biological Chemistry, 50*(1), 1–7.

Undeland, I., Stading, M., & Lingnert, H. (1998). Influence of skinning on lipid oxidation in different horizontal layers of herring (*Clupea harengus*) during frozen storage. *Journal of the Science of Food and Agriculture, 78*(3), 441–450.

Yamashita, M., & Konagaya, S. (1990). High activities of cathepsins B, D, H and L in the white muscle of chum salmon in spawning migration. *Comparative Biochemistry and Physiology, 95B*(1), 149–152.

Yamashita, M., & Konagaya, S. (1991). Hydrolytic action of salmon cathepsins B and L to muscle structural proteins in respect of muscle softening. *Nippon Suisan Gakkaishi, 57*(10), 1917–1922.

Yang, J. S., & Perng, F. S. (1995). Effects of gamma irradiation on the distribution of calcium ions in grass shrimp (*Phenaeus monodon* F.) muscle. *Meat Science, 39*(1), 1–7.