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Proteolytic activity and properties of proteins in smoked salmon (*Salmo salar*)—effects of smoking temperature

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Abstract

The effects of smoking temperature and storage period on different properties related to proteins and enzymes in cold-smoked salmon were investigated. The smoking temperature was important for the solubility properties and the composition of myofibrillar proteins in smoked salmon. Increasing the smoking temperature reduced the extractability of myofibrillar proteins, and their composition was greatly affected. SDS-PAGE analysis revealed that the intensity of the myosin heavy chain band was reduced with increasing smoking temperature and with further storage of smoked samples. The content of free amino acids increased with smoking and further storage, but smoking temperature did not affect the total content or the composition of free amino acids. The smoking temperature did not affect cathepsin B-like activity or the general proteolytic activity. Differences caused by different smoking temperatures were reduced by further storage (+7 days). In conclusion, the effect of the processing parameters was most pronounced early in the product's life.

Keywords: Smoked salmon; Smoking temperature; Protein solubility; Proteolytic activity

1. Introduction

Smoking is an old method used to preserve fish, and today large amounts of the farmed salmon produced in Norway are processed to smoked products. The texture of fish is an important quality characteristic, and the texture of smoked salmon is influenced by several factors. These include fish age and size, fat content and distribution of muscle fat, amount and properties of the proteins, and processing conditions. *Post-mortem* factors include the rate and extent of pH decline, *rigor mortis*, rate and extent of proteolysis causing breakdown of myofibrils and connective tissue (Andersen, Thomassen, & Rørå, 1997; Dunajski, 1979; Haard, 1992; Sigholt, Erikson, Rustad, Johansen, Nordtvedt, & Seland, 1997; Sigurgisladottir, Torrissen, Lie, Thomassen, & Hafsteinsson, 1997). When raw fish is stored in ice, the quality of muscle will deteriorate. The initial steps are brought about by endogenous proteases, which hydrolyze different proteins in the muscle (Cepeda, Chou, Bracho, & Haard, 1990).

The effects of different smoking temperatures on the microstructure and texture of Atlantic salmon have been studied by Sigurgisladottir, Sigurdardottir, Torrissen, Vallet, & Hafsteinsson (2000). Higher shear force was required in smoked salmon when the smoking temperature was increased from 20 to 30 °C, but the difference was not significant. The average cross-sectional area of muscle fibers decreased when the salmon was smoked. Changes in microstructure were most pronounced for the samples smoked at 30 °C, and the values obtained for the dry-salted samples depended on raw material characteristics.

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Many studies have been performed to investigate microbial quality and sensory properties of smoked salmon (Cardinal et al., 2001; Hansen, Gill, & Huss, 1995; Hansen, Gill, Røntved, & Huss, 1996; Leroi & Joffraud, 2000; Leroi, Joffraud, & Chevalier, 2000), but little is known about how the properties of the proteins and enzymes in the salmon are affected by different smoking processes.

Hansen et al. (1995, 1996) studied the relative importance of autolysis and microbial activity on the spoilage of cold-smoked salmon. They 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. The texture softened before off-odours and off-flavours were observed.

Lund and Nielsen (2001) investigated changes in free amino acids and in the composition of myofibrillar proteins during cold storage of fresh salmon for up to 23 days and after smoking. Only small changes in the contents of free amino acids and low molecular weight peptides were observed (both due to storage and to smoking), indicating 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. This was supported by studies of the composition of myofibrillar proteins during storage and after smoking. During storage, the myofibrillar proteins were subjected to proteolysis. The pattern that was seen in fresh salmon after 23 days of storage appeared in the smoked salmon prepared from salmon stored for only 4 days, indicating that the smoking process increased the activity of muscle proteases already active in the salmon.

Lysosomal catheptic enzymes are involved in deterioration of muscle texture, and different cathepsins may act in concert to autolyze fish muscle (Ashie, Simpson, & Smith, 1996; Ashie, Smith, & Simpson, 1996). Cathepsins are related to protein catabolism in the fish muscle during spawning migration (Yamashita & Konagaya, 1990). When the myofibrils and connective tissue were denatured, the proteins were extremely susceptible to the hydrolytic action of cathepsin B. Cathepsin B from white muscle of chum salmon in spawning migration has been purified and characterized (Yamashita & Konagaya, 1990). The activity was maximal between pH 5.5 and 6.0, and remained high as the pH increased towards 7.0. When incubated with oligopeptides, the enzyme acted mainly as a dipeptidyl carboxypeptidase (releasing dipeptides from the C-terminus), but it also showed endopeptidase activity. Varying salt concentration may influence the activity and hydrolysis pattern of cathepsins (Jiang, Lee, & Chen, 1996; Reddi, Constantinides, & Dymsza, 1972).

In the present experiment the solubility properties of proteins and proteolytic activities in smoked salmon fillets were investigated. The aim of the study was to investigate the effects of smoking temperature and storage period on different properties related to proteins in smoked salmon, and to relate properties of proteins to textural characteristics of the smoked fillets.

2. Materials and methods

2.1. Salmon and processing

Atlantic salmon (*Salmo salar*) were delivered from a fish research station (AKVAFORSK, Averøy, Norway) in November 2001. The salmon were live-chilled, bled and gutted, and were immediately iced. The salmon were originally part of a feeding trial at AKVAFORSK's research station, where feed based on fish and vegetable oils were tested. No significant effects of dietary treatment were found in this study and therefore the diet groups are pooled. Further information about the feeding trial can be found in Regost, Jakobsen, and Rørå (submitted). The day of slaughter was defined as day 0. Six fillets from separate fish were analyzed on day 2 as fresh samples.

After packaging, the salmon were sent to Norconserv (Stavanger, Norway) and subjected to salting and smoking. The fish were filleted 5 days after slaughter, using a Carnitech filleting machine (Carnitech A/S, Støvring, Denmark), and dry-salted with fine refined salt (Akzo Nobel, Fint Raffinert Salt, minimum 99.8% NaCl, Dansk Salt A/S, Mariager, Denmark) for 18 h at 4 °C. A Bastramat C1500 smoking chamber with MC700 microprocessor and Bastra FR 100 smoke generator (Bayha Strackbein GmbH, Arnsberg, Germany) with Reho Räucher Gold HBK 750/2000 chips (J. Rettenmaier & Söhne GmbH, Rosenberg, Germany) was used for smoking and drying according to the procedure shown in Fig. 1, giving a total drying time of 3 h 10 min and a smoking time of 3 h 20 min. Airspeed was 1 m/s and percentage of filling 7.1 ± 0.2 kg/m^3. The aim was to reach smoking temperatures of 20, 24, 28 and 30 °C, but the temperature log in the chamber showed average temperatures of 21.5, 24.3, 28.2 and 29.9 °C. Relative humidity (%) was logged during the smoking/drying process, and results (average ± standard error of the mean) were 73.2 ± 0.7, 69.8 ± 0.6, 69.2 ± 0.5 and 68.3 ± 0.6, respectively. After smoking the fillets were stored at 14 °C for 20-30 min before weighing, vacuum packing at 99% vacuum (Webomatic C60 D/W/U, Webomatic Machinenfabrik GmbH, Bochum, Germany) and storage at 0–4 °C prior to analyses.

Analyses of smoked samples were done on day 9 and six fillets from each smoking temperature were analyzed. Samples smoked at 21.5 and 29.9 °C were further stored at 0–4 °C and analyzed again on day 16 (referred to as continued storage).

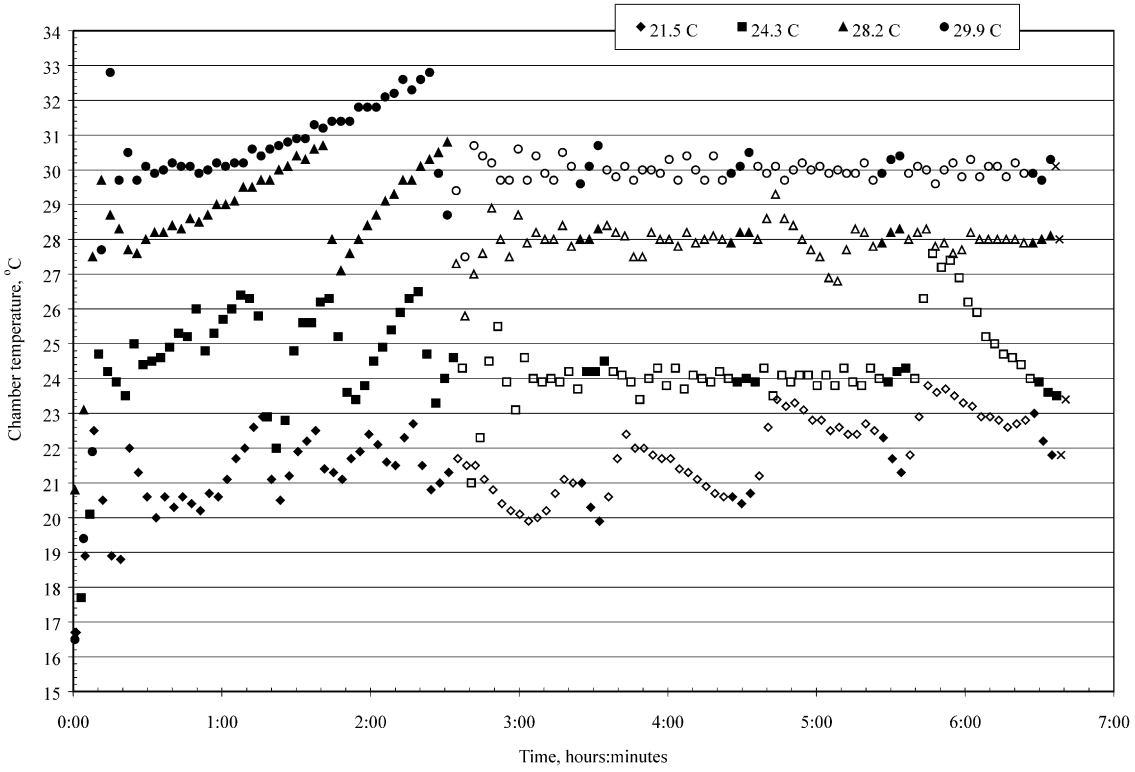

Fig. 1. Chamber temperature during the drying and smoking of salmon fillets. Average smoking temperatures: 21.5 °C (◆◇), 24.3 °C (■□), 28.2 °C (▲△) and 29.9 °C (●○). Filled symbols indicate drying, open symbols indicate smoking, and the Xs indicate evacuation period.

All analyses were performed using the muscle between the gills and the dorsal fin. Distilled water was used, and all chemicals were of analytical grade.

2.2. pH, salt and water content

pH was measured directly in the fresh fillets with a Flushtrode P electrode (Hamilton Company, USA). One measurement was done on each fillet.

The salt content was determined using a Dicromat 11-6 Salt analyser (PCL Control Instrumentation, Leicester, UK).

The 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.

2.3. Textural properties

Fillet texture was measured instrumentally, using a Texture Analyser TA-XT2 (Stable Micro Systems, Surrey, UK) equipped with a load cell of 5 kg and a cylindrical plunger (12.5 mm diameter), performing texture profile analysis (TPA). The measurements were made in front of the dorsal fin, about 1.5 cm above the lateral line. The plunger was pressed into the fillets at a constant speed of 2 mm/s until it reached 60% of the sample height. The maximum force obtained during compression (denoted F_{max}) was recorded, together with the force at 60% of the distance where the maximal force was obtained (denoted F_{60}). The gradient up to F_{60} was calculated. Three measurements were performed on each fillet.

2.4. Liquid holding capacity

Liquid-holding capacity (LHC) was analyzed according to a modification of the method described by Gomez-Guillen, Montero, Hurtado, and Borderias (2000). One slice of muscle was cut about 3–4 cm in front of the dorsal fin, from the lateral line toward the belly. The skin and the belly flap fat were removed. The samples (15 g) were weighed and placed in a tube with a weighed filter paper (V_1) (Schleicher & Schuell GmbH, Dassel, Germany). The tubes were centrifuged at 500 × g for 10 min at 10 °C, and the wet paper was weighed (V_2) before drying at 50 °C to constant weight (V_3). The percentage liquid loss was calculated on a wet weight basis as $100\times(V_2-V_1)\times S^{-1}$, with S = weight of muscle sample, water loss as $100\times(V_2-V_3)\times S^{-1}$ and fat loss as $100\times(V_3-V_1)\times S^{-1}$, respectively. The analyses were run in duplicate.

2.5. Protein solubility

Proteins were extracted from white muscle by a modification of the methods of Anderson and Ravesi (1968) and Licciardello, Ravesi, Lundstrom, Wilhelm,

Correia, and Allsup (1982). The extractions were done in a cold room (+4 °C). White muscle (4 g) was homogenized for 20 s in 80 ml phosphate buffer (0.05 M phosphate, 0.5% triton X-405, pH 7.0) using an Ultra Turrax homogenizer. After centrifugation (20 min at 8700 × *g*, 4 °C), the volume was made up to 100 ml with phosphate buffer. Neglecting the initial salt content of the sample, this was regarded as the water-soluble fraction. The precipitate was homogenized for 10 s in phosphate buffer with KCl (0.05 M phosphate, 0.6 M KCl, 0.5% triton X-405, pH 7.0), and centrifuged as above. The supernatant was adjusted to 100 ml with KCl-phosphate buffer. This was the salt soluble fraction. The extraction procedure was carried out once on each fillet.

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.

2.6. Preparation of enzyme extracts

The extractions of proteolytic enzymes were done in a cold room (+4 °C). White muscle (45 g fresh or 20 g smoked) was homogenized for 20 s in 90 ml cold distilled water using an Ultra Turrax homogenizer. The homogenates were kept on ice for 30 min and occasionally stirred. After centrifugation (20 min at 14,600 × *g*, 4 °C), the volume was made up to 100 ml with distilled water. The extraction procedure was carried out once on each fillet. The extracts were frozen and stored at −80 °C until analyzed.

2.7. General proteolytic activity

General proteolytic activity was determined according to Barrett and Heath (1977), with the method adjusted to micro scale. Incubation was performed in Silent Screen plates with Loprodyne membrane bottom, 3.0 μm pore size (Nalge Nunc International, Roskilde, Denmark). The incubation mixture consisted of 100 μl phosphate-citric acid buffer (McIlvaine, 1921) pH 6.0 or 6.5, 100 μl enzyme solution (filtered through 0.45 μm filter) and 100 μl 1% hemoglobin (filtered through 0.20 μm filter) in one well. The filter plates were incubated for 2 h at 20–25 °C. The reaction was arrested by the addition of 50 μl 17.5% (w/v) trichloroacetic acid, and the mixture filtered (Event 4160 microplate percolator, Eppendorf AG, Hamburg, Germany). A blank was prepared, as described above, except that trichloroacetic acid was added before the enzyme solution, and the blank was filtered without incubation.

The Folin-positive material in the filtrate was determined according to Lowry, Rosebrough, Farr and Randall (1951), with the method adjusted to micro scale. Filtrate/standard (45 μl) was mixed with 230 μl alkaline copper reagent, and the mixture was shaken for at least 30 min (IKA-Schüttler MTS 2, 700 rpm). Folin-Ciocalteu's phenol reagent (25 μl) was added, and the mixture was incubated for 30 minutes with shaking. Within this period, the plates were centrifuged (1801×*g* for 5 min, Centrifuge 5810R, Eppendorf AG, Hamburg, Germany) to remove gas bubbles in the wells. Optical density at 750 nm was read in a well plate reader (SpectraMax Plus, Molecular Devices Ltd., Wokingham, UK) using the software SoftMax Pro Version 3.1.2 (Molecular Devices). Bovine serum albumin was used as a standard.

The proteolytic activity was expressed as mg TCA soluble peptides liberated/g wet weight/hour, and given as the arithmetic mean of three individual measurements.

2.8. Activity of cathepsin B-like enzymes

After thawing the extracts were centrifuged (7840 × *g*, 4 °C for 10 min). The amount of proteins in the extracts was determined by BioRad protein assay, using bovine serum albumin as a standard (Bradford, 1976). The analyses were run in triplicate. Samples were diluted with distilled water to get a protein content of about 1.5 mg/ml.

The activity of cathepsin B-like enzymes was measured against a synthetic fluorogenic substrate, N_{α}-carbobenzoxy-arginine-arginine-7-amido-4-methylcoumarin (Sigma Chemical Co., St. Louis, MO, USA) (Barrett & Kirschke, 1981). Enzyme extract (0.15 ml, suitably diluted) was incubated with 0.15 ml substrate (0.0625 mM in 100 mM bis–Tris, 20 mM EDTA, 4 mM dithiothreitol, pH 6.5) at 4 °C. The reaction was arrested after 10 min by adding 3 ml 1% SDS in 50 mM bis–Tris, pH 7.0. A blank was prepared by adding distilled water instead of enzyme extract to the reaction mixture.

When the enzymes cut the synthetic substrate, 7-amino-4-methylcoumarin (AMC) is liberated. Fluorescence of AMC was measured at 460 nm (5 nm slits) after excitation at 360 nm (10 nm slits) (Perkin Elmer 3000 Fluorescence Spectrometer, PerkinElmer Inc., Buckinghamshire, UK). 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.

2.9. Free amino acids

The content of free amino acids was determined in enzyme extracts after precipitating the proteins with sulfosalicylic acid (Osnes & Mohr, 1985) and diluting the supernatant with deionized water. Reversed phase HPLC, by precolumn fluorescence derivatization with *o*-phthaldialdehyde (SIL-9A Auto Injector, LC-9A Liquid Chromatograph, RF-530 Fluorescence HPLC Monitor, all parts from Shimadzu Corporation, Japan), was performed using a NovaPak C18 cartridge (Waters, Milford, MA, USA), using the method of Lindroth and

Mopper (1979), as modified by Flynn (1988). Amino acid concentrations were determined once in each extract. Glycine/arginine and methionine/tryptophan were determined together, as their peaks merged. Results from individual samples were used to calculate average contents of specific amino acids within each smoking temperature/storage time group (six 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.

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

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. The high molecular weight standard contained the following proteins: rabbit muscle myosin heavy chain (220,000), bovine plasma α_2-macroglobulin (170,000), *E. coli* β-galactosidase (116,000), human transferrin (76,000), an unidentified protein, and bovine liver glutamic dehydrogenase (53,000). The low molecular weight standard contained the following proteins: rabbit muscle phophorylase b (97,000), bovine serum albumin (66,000), chicken egg white ovalbumin (45,000), bovine erythrocyte carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and bovine milk α-lactalbumin (14,400). All equipment for electrophoresis was delivered from Amersham Pharmacia Biotech (Uppsala, Sweden). The analyses were carried out according to the instructions of the manufacturer.

2.11. 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 $P = 0.05$.

Effect of smoking temperature (T_j, $j = 1, 2, 3, 4$) on a given variable (Y_i) was investigated by linear regression, using the model $Y_i = \beta_{0i} + \beta_{1i}\ T + \varepsilon$, where $\varepsilon \sim N(0, \sigma^2_\varepsilon)$. The null hypothesis ($H_0$) tested was $\beta_{1i} = 0$.

Effect of further storage (storage time t_k) of samples smoked at 21.5 and 29.9 °C was investigated using paired tests of the differences (d) between quality characteristics before (storage time t_1) and after (storage time t_2) additional cold storage of the smoked samples, $d_{i\ Tj} = Y_{i\ Tj\ t2} - Y_{i\ Tj\ t1}$. The model used was $d_{i\ Tj} \sim N(\mu_d, \sigma^2_d)$. 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 highest and lowest smoking temperatures, using the model $Y_{i\ Tj\ tk} \sim N(\mu_{i\ Tj\ tk}, \sigma^2)$, $k = 1, 2$. The null hypothesis (H_0) tested was $\mu_{i\ T1\ tk} = \mu_{i\ T4\ tk}$.

3. Results and discussion

Salmon fillets were dry salted (18 h) and subsequently cold-smoked (6.5 h) at four different temperatures between 20 and 30 °C. Although the aim was to reach smoking temperatures of 20, 24, 28 and 30 °C during the drying and smoking of fillets, chamber temperature recordings showed that actual average temperatures were 21.5, 24.3, 28.2 and 29.9 °C, respectively. The temperature curves for the smoking chamber (temperature as a function of process time) differed between the smoking temperatures investigated (Fig. 1). However, it may be assumed that the fish responded more slowly to fluctuations in the smoking temperature. Because of the problems in keeping the temperature and relative humidity in the smoking chamber constant, it is impossible to determine what parts of the observed differences are true effects of the smoking temperature alone.

The fresh fillets had pH values of 6.41 ± 0.03 (average ± standard error of the mean) when analyzed on day 2, indicating a raw material with low variation among individuals. As expected, the water content was reduced after smoking salmon fillets (Table 1). The results indicate that salmon smoked at 29.9 °C had a lower water content than salmon smoked at lower temperatures, but the difference was not significant (Table 2 and 3). After continued storage, the water content increased slightly, but was still lower than in fresh salmon. Salmon smoked

Table 1
Water (% of wet weight) and salt content (g/100 g water) in processed salmon. Average ± standard error of the mean ($n = 6$)

Processing	Water	Salt
F	72.4 ± 0.6	ND[a]
21.5 °C	65.0 ± 1.8	5.3 ± 0.4
24.3 °C	65.5 ± 2.3	5.3 ± 0.3
28.2 °C	65.7 ± 1.8	5.8 ± 0.2
29.9 °C	60.2 ± 2.9	6.1 ± 0.5
21.5 °C, S	65.7 ± 0.8	ND
29.9 °C, S	64.2 ± 0.3	ND

[a] ND: not determined. F: fresh fish; "number" C: average smoking temperature (°C); S: continued storage of smoked samples.

Table 2
Results from linear regression against smoking temperature

Variable (Y_i)	n	β_0	β_1	P
Water	24	74.874	−0.415	0.246
Salt	24	2.937	0.104	*0.059*
Water-soluble proteins	23	7.492	−0.037	**0.044**
Salt soluble proteins	23	14.464	−0.384	**0.000**
General proteolytic activity (pH 6.0)	22	0.535	−0.009	0.669
General proteolytic activity (pH 6.5)	22	0.116	0.001	0.914

Model $Y_i = \beta_{0i} + \beta_{1i}\ T + \varepsilon$, where $\varepsilon \sim N(0, \sigma^2_\varepsilon)$. The null hypothesis ($H_0$) tested was $\beta_{1i} = 0$. P values in bold are significant at 95% level, those in italic at 90% level.

Table 3
Effect of further storage (storage time t_k) of smoked samples investigated using paired test of the differences (d) between quality characteristics before and after additional cold storage

Variable (Y_i)	Storage, $\mu_d = 0$				Upper and lower temperature, $\mu_{i\ T1\ tk} = \mu_{i\ T4\ tk}$	
	21.5 °C		29.9 °C		Day 9	Day 16
	μ_d	P	μ_d	P	P	P
Water	0.77	0.693	3.97	0.212	0.194	*0.077*
Salt	–	–	–	–	0.198	–
Water-soluble proteins	−1.02	**0.008**	−0.30	0.280	*0.072*	0.139
Salt soluble proteins	−2.61	**0.002**	−0.38	*0.053*	**0.000**	**0.003**
Free amino acids	2.79	**0.001**	2.71	**0.009**	0.595	0.636
General proteolytic activity (pH 6.0)	−0.13	0.599	0.16	0.409	0.750	0.667
General proteolytic activity (pH 6.5)	−0.08	0.575	0.02	0.913	0.791	0.996
Cathepsin B-like activity	−407	**0.000**	−369	**0.000**	0.537	0.799

Differences in a given variable between the highest and lowest smoking temperatures investigated, using t-test assuming equal variances. P values in bold are significant at 95% level, those in italic at 90% level.

at 29.9 °C still had a lower water content than those smoked at 21.5 °C (significant at 90% level).

The salt content of smoked salmon fillets was 5.3–6.1 g/100 g water (Table 1). There was a slight increase in the amount of salt in the water phase with increasing smoking temperature (significant at 90% level) (Table 2), but the t-test, assuming equal variances, did not reveal any differences in salt content in samples smoked at 21.5 and 29.9 °C (Table 3). The difference in salt content was possibly due to the reduced water content in samples smoked at increasing temperatures. Salt contents of individual samples ranged from 4.0 to 7.2 g/100 g water.

The solubility properties of proteins are shown in Fig. 2. The amount of salt soluble proteins was reduced as a result of smoking, and this is in accordance with the results of Gomez-Guillen et al. (2000). A significant negative linear dependence between extractability of water-soluble proteins and smoking temperature was found (Table 2). The value of this regression parameter was low, and the t-test, assuming equal variances, did not reveal any differences in amounts of water-soluble proteins extracted from samples smoked at 21.5 and 29.9 °C, regardless of storage period (Table 3). When samples were further stored, the extractability of water-soluble proteins was significantly reduced for samples smoked at 21.5 °C, while no significant changes were observed for the samples smoked at 29.9 °C. A significant negative linear dependence was also found between extractability of salt soluble proteins and smoking temperature (Table 2). Further cold storage of

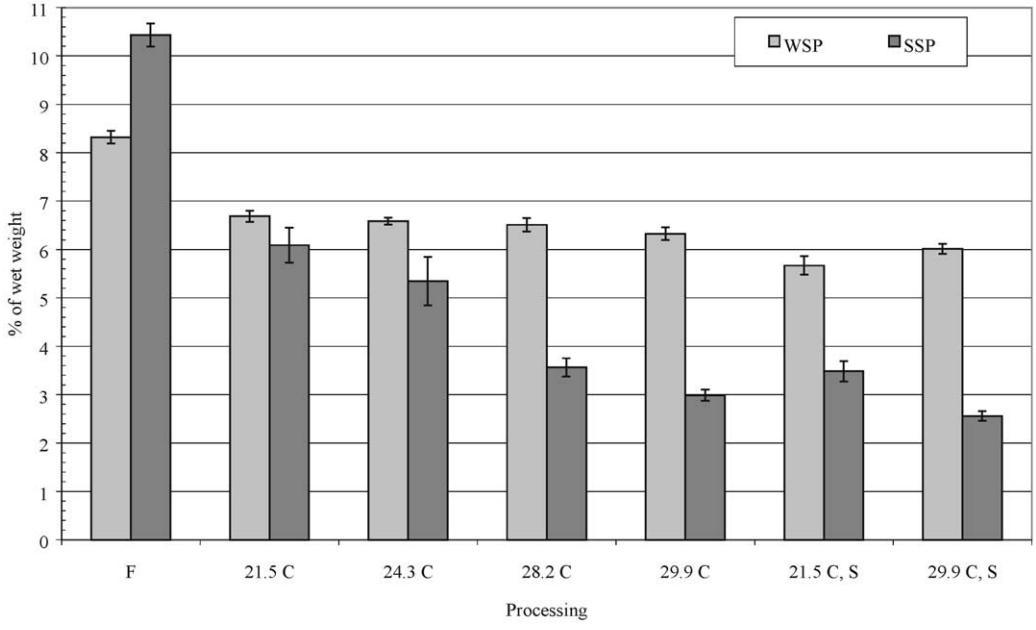

Fig. 2. Solubility properties of proteins in processed salmon. WSP: water-soluble proteins; SSP: salt soluble proteins. Bars indicate standard error of the mean ($n = 6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

smoked salmon caused a significant reduction in the extractability of salt soluble proteins (for salmon smoked at 29.9 °C at 90% level) (Table 3), and the values for samples smoked at 29.9 °C were significantly lower than those of samples smoked at 21.5 °C.

The content of free amino acids in smoked salmon was higher than in the fresh salmon, but was not significantly different for the two smoking temperatures tested (Table 3 and 4). This result, together with the results obtained for extractability of proteins as a function of smoking temperature, indicates that the extractability of salt soluble proteins was reduced due to denaturation/aggregation of the myofibrillar proteins. Total amounts of free amino acids were independent of smoking temperature, but increased significantly with further storage for both smoking temperatures. This indicates activity of exopeptidases in the smoked samples.

Lund and Nielsen (2001) investigated changes in free amino acids (using ethanol extraction) during cold storage of fresh salmon for up to 23 days and after smoking (at 28 °C). The dominating amino acid in all samples was histidine, constituting about 48% (weight %) of total free amino acid content in salmon and about 43% in smoked salmon. Alanine, glutamic acid and glycine were also important, constituting about 22% of total free amino acid content. Only small changes in the concentrations of individual amino acids were observed due to storage time or smoking. However, the study indicates a clear decrease in histidine concentration in smoked salmon prepared from salmon after more than 20 days of storage, possibly due to formation of histamine in the salmon. Only small amounts of free histidine were found in our study (Table 4), and this is in accordance with the results reported by Rustad, Sigholt, Aursand, Seland, and Berg (2000). Glycine/arginine (coeluting) and alanine dominated the fresh samples, constituting about 55% of the total amount of free amino acids (mol%), followed by lysine, threonine and glutamic acid. After smoking, glycine/arginine and alanine constituted only about 40% of the total amounts of free amino acids (values of gly/arg were most reduced). Methionine was only detected in smoked samples. Only small changes were observed in the relative amounts of different amino acids when the smoked samples were further stored for one week, suggesting that the change in free amino acid composition was due to the smoking process. Within the smoked samples, the amino acid profiles were independent of smoking temperature, indicating that the same proteolytic enzymes were active, irrespective of smoking temperature.

Table 4
Free amino acids in fresh and processed salmon fillets, fraction (%) of total amount (from μmol/g wet weight)

Amino acid	Fresh	Smoked at 21.5 °C		Smoked at 29.9 °C	
	Day 2	Day 9	Day 16	Day 9	Day 16
Aspartic acid	0.0	6.8	6.7	5.9	5.3
Glutamic acid	9.2	7.5	6.7	8.1	7.3
Asparagine	0.0	0.0	0.0	0.0	0.0
Histidine	1.6	1.8	1.9	2.5	2.6
Serine	4.0	5.1	5.6	5.1	5.0
Glutamine	1.7	3.1	3.6	3.8	4.0
Glycine/Arginine	29.5	18.3	17.5	18.8	18.3
Threonine	10.1	7.7	7.1	7.7	7.2
Alanine	24.2	21.2	20.0	20.1	20.0
Tyrosine	1.9	2.6	2.6	2.4	2.4
Methionine	0.0	1.3	1.8	1.3	1.6
Valine	2.9	4.3	4.6	4.4	4.5
Phenylalanine	0.9	2.7	2.8	2.5	2.7
Isoleucine	1.1	2.1	2.3	2.0	2.3
Leucine	2.2	4.7	5.3	5.1	5.8
Lysine	10.6	10.8	11.3	10.3	11.0
Total amount	11.2±0.5	20.6±0.8	23.4±1.0	19.9±0.9	22.6±1.2

Total amounts (μmol/g wet weight) are given as average±standard error of the mean ($n=6$).

Changes in the composition of the salt soluble proteins were investigated by SDS-PAGE. The results are shown in Fig. 3. No differences were observed between samples within a treatment. When salmon were smoked at 21.5 °C, two bands with molecular weights of about 150 and 170 kDa appeared. The intensity of a 97 kDa band and two bands with molecular weights slightly less than 45 kDa increased compared to the fresh samples. The two latter bands were not so intense after further storage. Increasing the smoking temperature to 29.9 °C resulted in a great reduction of the intensity of the myosin heavy chain (MHC) band and a band slightly heavier than 20.1 kDa. The two bands with molecular weights of about 150 and 170 kDa were not seen in samples smoked at 29.9 °C. The relative intensity of the MHC band decreased when the smoked salmon were further stored, for both smoking temperatures. In total, the intensity of the MHC band was reduced with increasing smoking temperature and with further storage of smoked samples. This could be due to the activity of cathepsins, which are known to be able to degrade myosin and actin (Ashie & Simpson, 1997; Hara, Suzumatsu, & Ishihara, 1988; Koohmaraie, 1994; Makinodan, Nakagawa, & Hujita, 1991). Smoking or storage did not seem to cause a great increase in large fragments due to breakdown of proteins too large to be seen on the gels or aggregation of proteins, or small fragments resulting from proteolytic breakdown of proteins. New proteins may be present at too low concentrations to be detected by SDS–PAGE, and small changes in molecular weight of proteins will not be detected by SDS–PAGE.

Lund and Nielsen (2001) studied changes in myofibrillar proteins from salmon smoked (at 28 °C) after different storage periods, using SDS–PAGE. Several bands appeared or increased in intensity in the 43–150 kDa range. The intensity of the MHC band decreased while no change in the actin band was observed. This indicates proteolysis of large cytoskeletal proteins, with

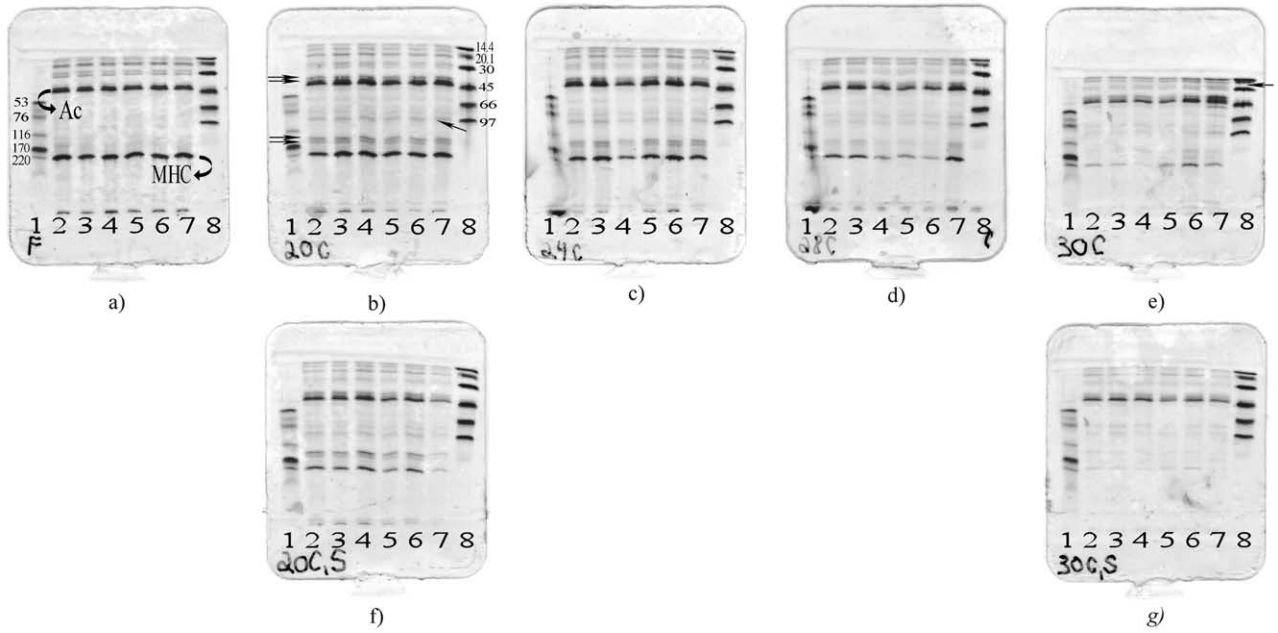

Fig. 3. Changes in salt soluble proteins in salmon caused by smoking and subsequent cold storage. High and low molecular weight standards are in well 1 (left side) and 8 (right side), respectively, on all gels. Molecular weights of proteins in the standards are given in kDa in (a) and (b). Protein concentrations are given in brackets. (a) Fresh salmon (~2 mg/ml). Salmon smoked at (average temperatures given) (b) 21.5 °C (~1.2 mg/ml), (c) 24.3 °C (~1.1 mg/ml), (d) 28.2 °C (~0.7 mg/ml), (e) 29.9 °C (~0.6 mg/ml). Salmon smoked at (f) 21.5 °C (~0.7 mg/ml) and (g) 29.9 °C (~0.5 mg/ml) and analyzed after 7 days' further storage. Ac indicates actin, and MHC indicates myosin heavy chain. Arrows indicate other bands whose intensity increase/decrease during smoking and storage.

molecular weights too large to be detected on the gels. The smoking process enhanced the formation of degradation products, but did not change the cleavage pattern (compared to iced storage of raw salmon). This strongly suggests that the smoking process increased the activity of muscle proteases already active in the salmon. Some of the changes seen in the smoked salmon may also be due to salt denaturation. The conformation of the myofibrillar proteins may have changed as a result of increased salt content in the muscle, making the proteins more susceptible to attack by endogenous proteases.

Muscle proteins are important for quality characteristics, such as texture and liquid-holding capacity. Given the changes in solubility characteristics due to smoking temperature, possible relationships between protein solubility and textural properties or liquid-holding capacity were investigated. Linear regression analyses were performed, using mean values for individual smoked salmon (without additional storage). Both maximum resistance force (F_{max}) and the force at 60% of the distance where F_{max} was obtained (F_{60}), were somewhat higher in the samples smoked at 29.9 °C than in samples smoked at 21.5 °C (significant at 90% level, Table 5). No linear dependence was found between F_{max} and content of salt soluble proteins ($P = 0.138$). Both F_{60} and gradient$_{60}$ decreased with increasing amount of salt soluble proteins ($F_{60} = 22.619 - 1.173 \times$ salt soluble proteins, $P = 0.010$; and gradient$_{60} = 11.173 - 0.426 \times$ salt soluble proteins, $P = 0.047$). In total, the extractability of salt soluble proteins, in samples smoked at the highest temperatures, was low, and this may contribute to the increased firmness of those fillets. The myofibrillar network retains most of the liquid in muscle, and the properties of the salt soluble proteins are therefore important for the liquid-holding capacity in muscle. A positive relationship between the amount of salt soluble proteins and liquid-holding capacity in the smoked salmon was expected, but not found ($P = 0.866$, 0.582 and 0.580 for water, fat and total liquid loss, respectively). This may be explained by the large variation in textural and liquid-holding properties between individual samples within each smoking temperature (Table 5). The *t*-test, assuming equal variances, did not reveal any significant differences in liquid-holding properties between the samples smoked at 21.5 and 29.9 °C.

General proteolytic activity (GPA) was low or not detectable in all samples, regardless of assay pH (Fig. 4). Smoking temperature or further storage of smoked samples did not have any significant impact on the general proteolytic activity (Tables 2 and 3). Applying *t*-tests on the results, for samples smoked at the same temperature (assuming equal variances), did not reveal any significant differences between the general proteolytic activity at the different pH values tested ($P = 0.291$, 0.186, 0.508 and 0.272 for samples smoked at 21.5, 24.3, 28.2 and 29.9 °C, respectively). The average activity was higher at pH 6.0 than at pH 6.5, for all smoking temperatures, but the variation between individual samples

Table 5
Textural properties and liquid loss in processed salmon

Processing	F_{max}	F_{60}	Gradient$_{60}$	Liquid loss	Water loss	Fat loss
21.5 °C	24.7±0.9	14.6±0.8	8.5±0.5	2.6±0.2	0.72±0.03	1.9±0.2
24.3 °C	26.6±1.4	16.0±0.6	8.3±0.3	3.5±0.4	0.64±0.04	2.9±0.4
28.2 °C	29.2±0.9	19.0±0.7	10.2±0.4	3.5±0.3	0.61±0.03	2.9±0.3
29.9 °C	28.7±1.7	18.9±2.1	9.8±0.9	3.2±0.6	0.76±0.06	2.5±0.5

F_{max}: maximum force obtained during compression (hardness, N); F_{60}: resistance force (N) at 60% of the distance where the maximal force was obtained; Gradient$_{60}$: gradient up to F_{60} (N/s). Losses are given as % of wet weight. Average±standard error of the mean ($n=6$). "Number" C: average smoking temperature (°C).

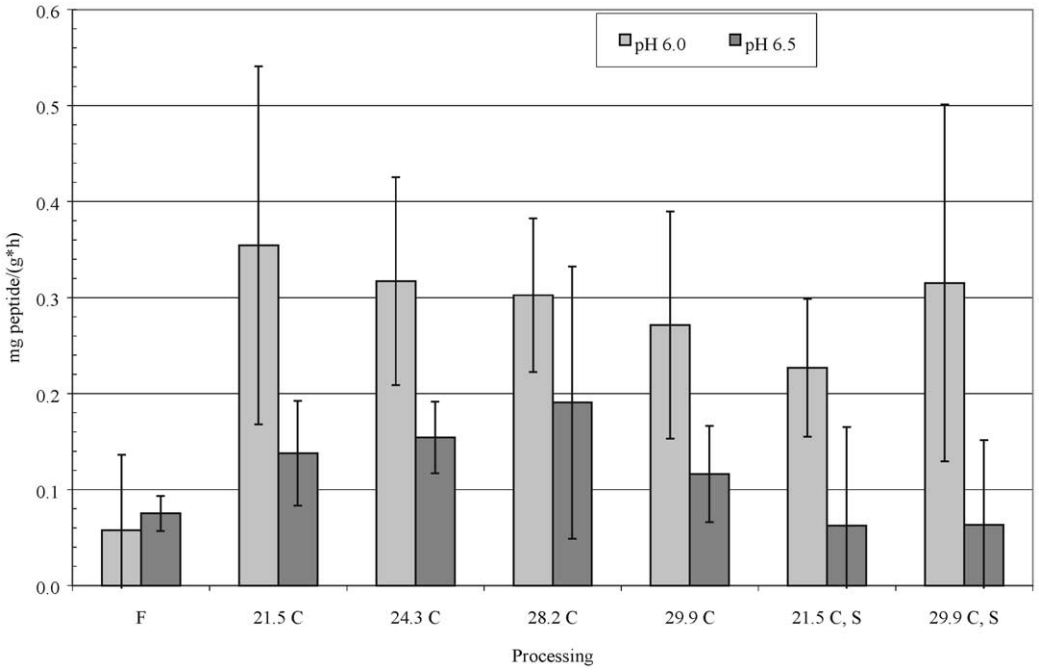

Fig. 4. Proteolytic activity in processed salmon, given as increase in TCA soluble peptides/g wet weight/h, investigated at different pH values. Bars indicate standard error of the mean ($n=6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

was large. Performing the same test on all samples revealed that the activity was significantly higher at pH 6.0 than at pH 6.5 (0.314 and 0.152 mg peptides/g wet weight/h, $P=0.035$). Low general proteolytic activity at 25 °C, pH 6.0 and 6.5, is in agreement with the results reported by Stoknes and Rustad (1995), who found the optimal conditions for the proteases studied in their work to be at higher temperatures and pH values. In samples with high salt content, the general proteolytic activity at pH 6.0 was lower than in samples with low salt content [GPA (pH 6.0) = 1.079−0.138×salt (g/100 g water), $P=0.056$]. No such relationship was found for the activity at pH 6.5 ($P=0.886$).

Activity of cathepsin B-like enzymes was not significantly dependent on smoking temperature, regardless of storage period (Fig. 5 and Table 3). After storage, the activity of cathepsin B-like enzymes was significantly lower for both smoking temperatures. Remaining activity was still high, and this may contribute to the increased amount of free amino acids during storage of smoked salmon. The temperature in the smoking process never exceeded 33 °C, and there is, therefore, no heat inactivation of the proteolytic enzymes in the salmon tissue. Hara et al. (1988) studied carp cathepsin B. The enzyme was purified and characterized, and was found to degrade carp muscle proteins, such as myosin (fragments of molecular weight 150–170 kDa appeared) and actin (a protein band of molecular weight 40 kDa appeared). Cathepsin B activity was affected by salt concentration; activation was observed at NaCl concentrations above 0.58% and the maximum activation occurred at about 1.75–2.34% (activity was measured at pH 6.0 and 37 °C). Based on these results, activation of salmon cathepsins during the salting process may be expected. No significant linear dependence was found between cathepsin B-like activity and salt content in the water phase or in wet weight ($P=0.315$ and $P=0.188$, respectively). Compared to the investigation by Hara et

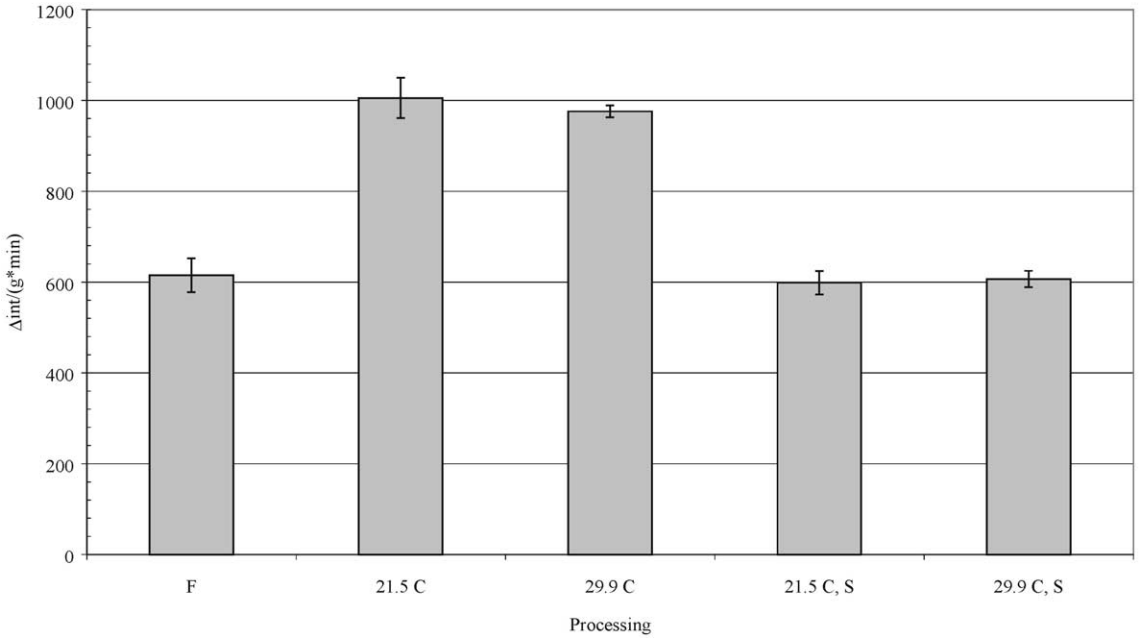

Fig. 5. Cathepsin B-like activity in processed salmon, given as increase in fluorescence intensity/g wet weight/min. Bars indicate standard error of the mean ($n = 6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

al. (1988), the salt content in the water phase of the present samples was higher than the salt content found to be optimal for cathepsin B activity. Nevertheless, the salt content was not high enough to fully inactivate the cathepsin B-like enzymes and thereby contribute to increased stability of the smoked salmon.

4. Conclusions

Increasing the smoking temperature reduces the extractability of myofibrillar proteins, and their composition is greatly affected. SDS–PAGE analysis revealed that the intensity of the myosin heavy chain band was reduced with increasing smoking temperature and with further storage of smoked samples. The amounts of free amino acids increased during storage, indicating active proteases in the smoked salmon. The smoking temperature did not affect cathepsin B-like activity or the general proteolytic activity.

The observed differences in the proteins, caused by different smoking temperatures, were reduced with storage time. Hence the effects of processing conditions seem to be most important early in the product's life.

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