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Endogenous proteolytic enzymes - Studies of their impact on fish muscle proteins and texture

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PREFACE

The work presented in this thesis was carried out at the Department of Biotechnology, Faculty of Natural Sciences and Technology, Norwegian University of Science and Technology. Financial support was given by the Research Council of Norway.

The scope of the thesis has been to study endogenous proteolytic enzymes in fish muscle, and their impact on muscle proteins and textural properties. The overall aim has been to increase the understanding of the mechanisms of fish muscle softening.

An introduction to the topic is found in Chapter 1, together with general information about the task. In addition, a brief introduction on some proteolytic enzymes and instrumental texture analysis is given. A more extensive and detailed description of the scope of the thesis is found in Chapter 2.

The first part of Chapter 3 deals with some general aspects of fish storage, and choice of methods for determination of textural properties and activities of proteolytic enzymes. The chapter continues with a brief summary of the papers, before a discussion of the importance of proteolytic enzymes based on results from all the papers. It is recommended to read the papers before the summaries, as the summaries are more like extended abstracts. Information concerning the specific results (graphs and discussions) is only included in the papers.

Some concluding remarks are given in Chapter 4, and a few suggestions for further studies in Chapter 5.

SUMMARY

This thesis covers studies on endogenous proteolytic enzymes and their impact on fish muscle proteins and texture. The studies have been performed using Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) subjected to different treatments and storage conditions.

The textural properties were very different in the two species. Salmon fillets were significantly softer and less resilient than cod fillets, and the properties changed somewhat differently during storage experiments. Different proteolytic enzymes have been reported to participate in muscle softening. Some of these enzymes were investigated, and specific proteolytic activities were detected throughout the storage periods. Collagenase-like enzymes seem to be the most important for cod muscle texture. Microorganisms and/or microbial enzymes seem not to be important for changes in salmon muscle texture. Results suggest that the cathepsin B-like enzymes are important for salmon texture. The activities of the proteolytic enzymes may be greatly affected by the muscle pH, and by the treatment(s) the fish are subjected to. In any case, changes caused by differences in proteolytic activities may need some time to be detectable or have significant impact on fish quality.

When cod fillets are stored in ice, it is highly recommended to keep the temperature low. Even a relatively mild temperature abuse was sufficient to result in less favorable textural characteristics, and make the fillets seem older than their days of storage.

Salmon fillets are often subjected to cold-smoking. The smoking temperature was important for the solubility properties of the muscle proteins, and for their composition, but did not affect the proteolytic activity. The effects of the processing parameters were most important early in the product's shelf life, as the differences caused by the different smoking temperatures were reduced by further storage of the smoked samples.

CONTENTS

LIST OF PAPERS

This thesis is based on a series of papers, which are referred to in the text by their Roman numerals.

Paper I. 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.

Paper II. Hultmann, L., & Rustad, T. (2004). Iced storage of Atlantic salmon (*Salmo salar*) – effects on endogenous enzymes and their impact on muscle proteins and texture. *Food chemistry*. 11 p. In press.

Paper III. Hultmann, L., Rørå, A. M. B., Steinsland, I. Skåra, T., & Rustad, T. (2004). Proteolytic activity and properties of proteins in smoked salmon (*Salmo salar*) – effects of smoking temperature. *Food Chemistry*. 85(3), 377-387.

Paper IV. Hultmann, L., & Rustad, T. (2003). Texture, proteins and proteolytic enzymes in farmed Atlantic cod (*Gadus morhua*). *Submitted to Food Chemistry*. 24 p.

1. INTRODUCTION

1.1. General aspects of fish quality

Traditionally, fishing has been very important in Norway, both as food supply and for the economy. Today the stocks of many fish species are decreasing, while there is an increasing demand for fish as a healthy food. A solution to this is fish farming. In Norway, Atlantic salmon (*Salmo salar*) has been farmed for decades, and other species, such as Atlantic cod (*Gadus morhua*), are emerging. It is therefore important to know how the farmed species respond to different treatments and storage conditions they may be exposed to during different types of processing.

By farming fish, the needs for increasing amounts of food for the increasing human population can be met. The consumers' acceptance of fishery products depends on several attributes of food quality (Haard, 1992). Important attributes include safety, freshness, nutrition, flavor, texture, color (especially of pigmented species) and appearance and the suitability of the raw material for processing and preservation. The order of importance of the different characteristics depends on the specific product and its use. Fish farming allows the fish farmer to influence the quality of the fish by means of different rearing conditions, and thereby achieve fish with desired quality characteristics.

During storage after harvest, many fish species exhibit changes in textural properties, long before they are spoilt (Bremner, 1992). This may result in flesh softening and gaping, and trimming losses occur (or increase), resulting in reduced fillet yield. In addition, products may have a poorer appearance and are therefore downgraded and/or returned to the producer, with accompanying loss of reputation and economical losses. In extreme cases, the fillets may fall apart in the skinning operation, being too soft to be usable in mechanical processing. It is therefore important to understand the mechanism(s) of this softening, in order to avoid it or even use it beneficially.

1.2. Fish muscle composition and structure

Fish muscles are divided into myotomes separated from each other by thin sheets or membranes made up of connective tissues (myocommata). Within each myotome, the

muscle fibers run approximately parallel to each other, but at varying angles to the myocommatal sheets to give the necessary moment for swimming during contraction (Figure 1). The muscle cells run roughly parallel to the longitudinal axis of the fish (Dunajski, 1979).

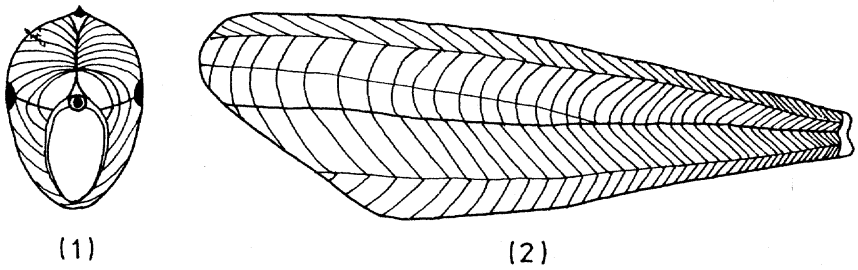

Figure 1. The metameric structure of fish muscles. The pattern of lines on the cross (1) and longitudinal (2) sections represents the arrangement of sheets of connective tissue in the muscles. From Dunajski (1979).

The muscle proteins can be divided into three groups, based on solubility properties (Haard, 1992; Foegeding, Lanier, and Hultin, 1996). These are water-soluble or sarcoplasmic proteins (mainly enzymes), salt soluble or myofibrillar proteins (the contractile network) and the insoluble proteins (primarily collagen). Fish muscle contains a relatively high concentration of myofibrillar proteins and a low concentration of insoluble proteins compared to muscles from land animals (Table 1).

Table 1. Distribution of protein fractions in fish muscle. From Haard (1992).

Source	Sarcoplasmic protein (%)	Myofibril protein (%)	Stroma protein (%)
Fish, general	10-25	70-90	3-10
Atlantic cod	21	76	3
Carp	24	71	5
Flounder	21	76	3
Beef	16-28	39-68	16-28

This implies that the myofibrillar proteins are very important for the textural and water-holding properties of fish muscle. In addition, the large differences in composition and structure of mammalian and fish muscle make a direct transfer of

results on proteins and textural properties from mammalian muscle to fish muscle impossible.

A fine network of collagen surrounds each muscle fiber and proceeds into the myocommata (Bremner, 1992; Bremner and Hallett, 1985). The collagen content of fish muscle varies considerably between species, and is found in increasing proportion in the tail region (Bremner, 1992). Concentrations of 0.3-3% are common in the main edible portion, depending on season and nutritional status. During chilled storage of blue grenadier (*Macruronus novaezelandiae*) it was observed that the attachments between muscle fibers and myocommata, and the whole sarcolemma, was degraded, and muscle fibers were detached from the myocomma (Bremner and Hallett, 1985; Hallett and Bremner, 1988). A similar degradation is observed with king salmon (*Oncorhynchus tshawytscha*) (Fletcher, Hallett, Jerrett, and Holland, 1997), Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) (Ofstad, Egelandsdal, Kidman, Myklebust, Olsen, and Hermansson, 1996).

The intermolecular cross-links in collagen, the main constituent of fish connective tissue, are thought to be responsible for the stability, physical strength and mechanical properties of the connective tissue and other components of the extracellular matrix (Bracho and Haard, 1995). Breakdown of the connective tissue by endogenous collagenases may lead to undesirable textural changes in the fish.

The present thesis focuses on cod and salmon. Both are economically important for Norway, and both are available as wild and farmed fish. The two species are bony fish, and the left and right fillets are considered to be equal. The composition of the fish muscle differs between the fatty salmon and the lean cod. Salmon muscle contains approximately 64% water, 20-22% protein, 13-15% lipid and 1.3% ash, whereas the corresponding values in cod muscle are 81%, 18%, 0.5% and 1% (Foegeding *et al*., 1996). The composition varies considerably even within a species, due to fish size and age, season, nutritional status, and sexual maturation of the fish. In addition, the proximate composition varies between different fillet parts (Katikou, Hughes, and Robb, 2001; Rasmussen, 2001). The lean cod muscle generally has a shorter shelf life than the fattier salmon muscle (reviewed by Sivertsvik, Jeksrud, and Rosnes, 2002).

1.3. Proteolytic enzymes

1.3.1. General

Proteolytic enzymes are found in all tissue, although both the distribution of different enzymes and their activities show considerable variation. The highest activities are found in fractions such as viscera and liver, but there are significant proteolytic activities in muscle tissue as well, where the enzymes play an important role in protein turnover. Endogenous fish muscle proteases are located in intracellular fluids and in the sarcoplasm, or they are associated with various cell organelles. In the live animal, the proteases function in muscle protein turnover. After death, the biological regulation of the enzymes is lost, and the enzymes hydrolyze muscle proteins and resolve the *rigor mortis* contraction (Foegeding *et al*., 1996).

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, and Haard, 1990; Kolodziejska and Sikorski, 1996). Proteolytic enzymes are proteins, and they act by cleaving the peptide bonds of proteins and peptides, as shown schematically (reaction from right to left) in Figure 2.

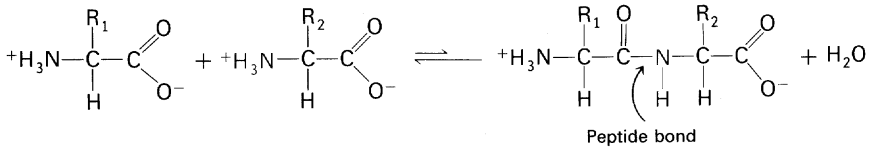

Figure 2. General action of a proteolytic enzyme. From Stryer (1995).

Proteases are generally classified according to the active center as cysteine, serine, metallo, and aspartyl proteases. In addition, proteases may also be divided in two groups according to their mode of action: endopeptidases, which hydrolyze peptide bonds in the middle of polypeptide chains, or exopeptidases, which remove terminal amino acid residues from polypeptide chains (Palmer, 1995).

The proteolytic activity depends on location in the muscle, the life cycle of the fish, pH and temperature. In addition, the activity *in situ* is controlled by the presence of endogenous activators (mostly reducing compounds and metal ions) or inhibitors (both

broad and specific inhibitors may be found in fish muscle). The enzymes are involved in protein catabolism in the muscle, and are important for the creation of gonads during the sexual maturation process and spawning migration (when the fish is not eating, but is using its own proteins for building gonads). For farmed fish, a change in culture conditions may change the enzyme content and proteolytic enzyme activity of fish muscle (Haard 1992).

Hansen, Gill, Røntved, and Huss (1996) studied the relative importance of autolysis and microbial activity on spoilage of cold-smoked salmon. Salmon with reduced and normal loads of microorganisms were compared. The microbiological activity caused formation of the characteristic spoilage odors and flavors, while the autolytic enzymes from the fish tissue had major impact on the textural deterioration (also found by Hansen, Gill, and Huss, 1995). Texture softened before off-odors and off-flavors were observed. Lund and Nielsen (2001) investigated changes in the contents of free amino acids and low molecular weight peptides during cold storage of salmon. Only small changes were observed, and this was 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.

The rate of enzyme-catalyzed reactions can influence quality attributes of the meat (Haard, 1992). The content of free amino acids in the muscle of aquatic animals is normally higher than in muscle of land animals. The free amino acids play an important role as taste and flavor components, and the free amino acid content in muscle from aquatic organisms normally ranges from about 0.5% (w/w) to 2% of muscle weight. Cultured fish tend to contain less free amino acids than wild fish. In addition, both the distribution and total amounts of free amino acids vary with intrinsic (such as nutritional status and sexual maturation) and extrinsic (such as water salinity) factors.

General proteolytic activity is usually measured against hemoglobin. The activity varies with origin of the enzymes, in addition to incubation temperature and pH in the reaction mixture (Stoknes and Rustad, 1995; Stoknes, Rustad, and Mohr, 1993). Generally, the activities are very low (or not even detectable) at temperatures below 20 °C, regardless of species, tissue fraction and assay pH. The content of trichloroacetic acid (TCA) soluble peptides may also be used as a measure of proteolytic activity

(Yamashita and Konagaya, 1990b). However, this will only detect exopeptidases. An increase in TCA soluble components was detected in chum salmon muscle with highest autolytic activity during storage at 4 ºC for 6 days.

In this thesis, we have chosen to investigate a few of the proteolytic enzymes that were believed to be important for the textural properties of fish muscle, namely calpains, cathepsins, and collagenases.

1.3.2. Calpains

The calpains are thought to participate in *post mortem* degradation of fish muscle by cleaving myofibrillar proteins (Geesink, Morton, Kent, and Bickerstaffe, 2000; Kolodziejska and Sikorski, 1996; Ladrat, Chaplet, Verrez-Bagnis, Noel, and Fleurence, 2000). Calpains (EC 3.4.22.17) are neutral cysteine endopeptidases found in the sarcoplasm of muscles. They are composed of two subunits, and are activated by calcium ions and thiol compounds. The enzymes are most active at neutral pH (6.9-7.5) and 30 °C (Kolodziejska and Sikorski, 1996). Many different forms have been identified, with varying calcium requirements (as shown in Figure 3). Calpain I is often called μ-calpain, and calpain II m-calpain (indicating the high and low level of calcium sensitivities, respectively). Forms of intermediate Ca^{2+} sensitivity also exist. An endogenous inhibitor protein, calpastatin, has also been characterized.

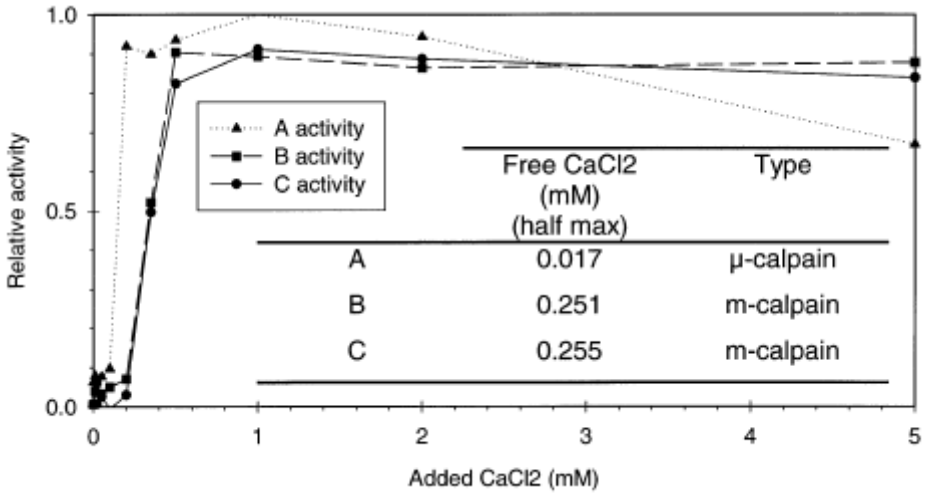

Figure 3. Calpain-like activities as a function of added calcium and free calcium to obtain half of the maximum activity. From Ladrat *et al*. (2000).

The concentration of Ca^{2+} necessary for activation of μ-calpain is similar to that prevailing in the sarcoplasm of the living muscle. *Post mortem* concentration of free Ca^{2+} may increase to amounts sufficient to activate both the low- and high-calcium requiring types of calpain, due to the loss of calcium retaining ability of the sarcoplasmic reticulum. Calpains are active against many myofibrillar proteins, where they cleave proteins at specific sites and therefore cause only a limited proteolysis. The proteins are degraded into large fragments, and this enhances the susceptibility of the proteins to other proteinases (Kolodziejska and Sikorski, 1996; Ladrat *et al*., 2000). Calpains are most active at neutral pH, but are still quite active at pH 5. Optimum temperature for calpain from carp and tilapia was 30 °C and 20% of the activity was retained at 0 °C (tilapia calpain, pH 7.5, Wang and Jiang, 1991). Ladrat *et al*. (2000) studied calpains from sea bass muscle (*Dicentrarchus labrax* L.). Two of their partially purified enzymes had optimum activity between 19 and 25 °C, and the activity decreased with decreasing temperature. The activity against bovine skin gelatin was very low, suggesting that calpains are not able to break down collagen in muscle. Geesink *et al*. (2000) studied calpains from chinook salmon (*Oncorhynchus tshawytscha*) muscle. They found little proteolysis of myofibrillar proteins during storage. Results were reproduced when myofibrils were incubated with calpains.

During *post mortem* storage of ovine muscle, the levels of active calpains decreased rapidly (Ilian, Bickerstaffe, and Greaser, 2003). Active enzymes were degraded by other proteases, and were converted to inactive proteins with a slightly lower molecular weight than the intact calpains. Other studies have shown that calpain activity of chinook salmon (*Oncorhynchus tshawytscha*) was stable the first days *post mortem*, while the activity of calpastatin increased during storage (Geesink *et al*., 2000). Autolysis patterns of salmon calpains differed from those of ovine calpains and the activities were less than those of mammalian calpains. Calpastatin activity was relatively high and comparable to that of bovine muscles. Altogether, this indicates that the calpain system may not be as important to fish muscle tenderization as it is to meat tenderization in mammalian muscles.

1.3.3. Cathepsins

The cathepsins are involved in deterioration of muscle texture, and different cathepsins may act in concert to autolyze fish muscle (Ashie, Simpson, and Smith, 1996; Ashie, Smith, and Simpson, 1996; Kolodziejska and Sikorski, 1996). The cathepsins are related to protein catabolism of the fish during spawning migration, as the activity in white muscle is markedly higher in fish during spawning migration compared to fish in feeding migration (Yamashita and Konagaya, 1990a). Cathepsins are found in the lysosomes of muscle fibers and in phagocyte cells. Lysosomes are intracellular organelles that contain large quantities of hydrolytic enzymes and serve a digestive role in the cell. The lysosomes may also originate from phagocyte cells present in the circulatory system (Foegeding *et al*., 1996). Many types of cathepsins have been identified, with different amino acids at the catalytic site, and cathepsins B (EC 3.4.22.1, a cysteine protease) and L (EC 3.4.22.15, a cysteine protease) are probably the most important for deterioration of muscle texture (Aoki, Yamashita, and Ueno, 2000; Jamdar and Harikumar, 2002; Kolodziejska and Sikorski, 1996). The activities differ between muscle fractions and fish species (Aoki *et al*., 2000; Yamashita and Konagaya, 1990a). The optimum activity is reported to be at about 40-50 °C, and the activity decreases with reducing temperatures. Although they generally perform best at pH 3-4, some of them retain fairly high activity also at pH 6.0-6.5. Cathepsins B and L are activated by reducing compounds (thiol compounds). In addition, results suggest that hormones inducing sexual maturation directly regulate the activities of cathepsins.

The degradation pattern of myofibrillar proteins of softened chum salmon muscle was almost reproduced when myofibrils were treated with purified cathepsin L (Yamashita and Konagaya, 1990b). This indicates that cathepsin L is the most probable enzyme responsible for extensive muscle autolysis and softening observed in mature chum salmon. Cathepsin B from chum salmon in spawning migration hydrolyzed carp myofibrillar proteins in solution, but was not able to hydrolyze carp collagen (Yamashita and Konagaya, 1991). However, when the myofibrils and connective tissue were denatured, the proteins were extremely susceptible to the hydrolytic action of cathepsin B. The relative amounts of these two enzymes in the *post mortem* muscle are not known.

Any treatment that causes disruption of lysosomes will lead to an increased autolytic activity (Yamashita and Konagaya, 1990b). Because of this, the fish muscle will be more vulnerable to deterioration of muscle texture. This may be especially important after thawing of fish, where the lysosomes may have been disrupted by ice crystals during the freezing process or the frozen storage.

1.3.4. Collagenases

Endogenous collagenases may break down the connective tissue in the fish muscle and thereby lead to undesirable textural changes and gaping, in addition to rendering the components of the extracellular matrix more vulnerable to attack by other proteases (Ando, Yoshimoto, Inabu, Nakagawa, and Makinodan, 1995; Ashie *et al*., 1996; Bracho and Haard, 1995; Bremner and Hallett, 1985; Cepeda *et al*., 1990). Collagenases (EC 3.4.24.7) are metalloproteinases found in skeletal muscle (Kolodziejska and Sikorski, 1996). Many different proteases belong to this class. The matrix metalloproteinases are believed to be involved in normal degradation of the matrix. Different catalytic subunits (single polypeptide chains) exist, with varying calcium requirements, and the activity varies between fish species. They are secreted proteins, and thereby placed in a proper location for degradation of the extracellular matrix (Bracho and Haard, 1995). Generally, the initial attack on the collagen triple helix is by specific collagenases. Once the initial cleavage has been achieved, other non-specific proteases can pursue attack (Kristjansson, Gudmundsdottir, Fox, and Bjarnason, 1995). The collagenases are synthesized in latent form as zymogens. After activation (a wide variety of proteolytic enzymes can perform the activation), the collagenases are controlled by Tissue Inhibitors of Metallo Proteinases (TIMP), which are small cationic glycoproteins (Bremner, 1992). Collagenolytic enzymes have been isolated from the skeletal muscle of fish (Bracho and Haard, 1995; Hernandez-Herrero, Duflos, Malle, and Bouquelet, 2003; Teruel and Simpson, 1995). The enzymatic activities were dependent on fish species, and were most potent at pH values close to neutrality or higher. The enzymes are most active at pH values close to neutrality (7-8), and are activated and stabilized by metal ions (Ca^{2+}) and other activators (such as stress, injury, infection or heat) and inhibited by calcium chelators (Bracho and Haard, 1995; Bremner, 1992). Two metalloproteinases from skeletal muscle of Pacific rockfish (*Sebastes* species) were

most active at pH 7.5-8.5 (Bracho and Haard, 1995). The activity against collagen and gelatin was very high, whereas almost no activity was detected against proteins unrelated to the extracellular matrix. This indicates that the metalloproteinases could participate in degradation of collagen and other extracellular matrix proteins and thereby play a role in the loss of integrity of the muscle structure in fish held at abuse temperatures.

1.4. Textural properties

1.4.1. General definition

The term "texture" can be defined in many ways, indicating that it is a complex sensory phenomenon, and a large number of words are used to describe textural properties (Hyldig and Nielsen, 2001). The texture of fish meat is both a valued sensory characteristic for consumers and an important attribute for the mechanical processing of fillets by the food industry (Dunajski, 1979; Haard, 1992). Consumers generally prefer a firm and elastic fish meat (Rasmussen, 2001). According to Szczesniak (2002), there is a general agreement that "texture is the sensory and functional manifestation of the structural, mechanical and surface properties of foods detected through the senses of vision, hearing, touch and kinesthetics". This definition conveys important concepts of texture, such as:

- it is a sensory property, and can only be perceived and described by a human being. Instruments can only detect and quantify certain physical parameters, which needs interpretation in terms of sensory perception;
- it is a multi-parameter attribute, which covers a wide range of characteristics;
- it derives from the structure of the food, both molecular, microscopic and macroscopic structure; and
- it is detected by several senses, where the senses of touch and pressure are the most important.

1.4.2. Factors influencing textural properties of fish muscle

Texture is one of the most important quality characteristics of fish as a food. In most terrestrial animals it is desirable to accelerate and increase meat softening. The situation is opposite in the case of seafood like fish, and soft fillets are a problem for the fish

industry (Andersen, Thomassen, and Rørå, 1997; Haard, 1992; Hallett and Bremner, 1988; Koteng, 1992; Sigholt, Erikson, Rustad, Johansen, Nordtvedt, and Seland, 1997). The "background" texture of fish muscles is softer than that of land animals, because fish muscles have about one-tenth the collagen and less cross-links than muscles from terrestrials (Haard, 1992). 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 connective tissue, and handling stress before slaughter. *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, and temperature during and length of storage period (Andersen *et al.*, 1997; Dunajski, 1979; Haard, 1992; Sigholt *et al*., 1997; Sigurgisladottir, Torrissen, Lie, Thomassen, and Hafsteinsson, 1997). The collagen content and its properties contribute to the texture of raw fish, but is far less important for textural properties of cooked fish. Generally, the *post rigor* fish muscle softens with increasing storage time on ice (Andersen *et al*., 1997; Ando, Toyohara, Shimizu and Sakaguchi, 1993; Einen and Thomassen, 1998; Veland and Torrissen, 1999; Yamashita and Konagaya, 1990b). The amount of muscle glycogen at death influences the meat quality by determining the ultimate pH of the meat (Haard 1992). The acidity of the muscle is probably the most important single factor affecting the textural properties, and fish with a low *post mortem* pH normally has firm texture (Dunajski, 1979; Foegeding *et al*., 1996). The effect of pH is largest below pH 6.7. However, the rate of *post mortem* pH decline in the muscle is also important, as a rapid pH decline may cause soft texture and poor water-holding capacity of the meat even when the ultimate pH is low (Ang and Haard, 1985). The muscle glycogen content may be lowered by *ante mortem* stress (during capture of wild fish and netting/transport of farmed fish) or starvation (Haard, 1992; Rustad, 1992; Sigholt *et al*. 1997). In addition, farmed fish tend to have a softer, less preferable texture than free-living fish (Haard, 1992). It is therefore very important to understand how different processing operations and/or conditions may influence the proteins and proteolytic enzymes and thereby result in changes in the quality of the fish product.

1.4.3. Determination of textural properties

Different methods for instrumental texture measurements have been developed. A wide variety of knives, spheres and cylinders have been used to cut or press into fish fillets, cutlets or other pieces of given geometry, both parallel to and perpendicular to the muscle fibers (Botta, 1991; Bourne, 1978; Einen and Thomassen, 1998; Hyldig and Nielsen, 2001; Jonsson, Sigurgisladottir, Hafsteinsson, and Kristbergsson, 2001; Mørkøre and Einen, 2003; Sigurgisladottir, Hafsteinsson, Jonsson, Lie, Nortvedt, Thomassen, and Torrissen, 1999; Sigurgisladottir *et al*., 1997). The preferred methods have been based on shearing, with recent research focusing on less destructive methods such as compression. However, there is no universally recommended method for determining textural properties of fish muscle. In the industry, the finger method (a person evaluates fish firmness by pressing a finger on the fish or fillet) has been used extensively. Development of instrumental methods has made it possible to get more objective measurements of textural properties, in addition to avoiding the high costs of highly trained personnel for sensory evaluations. Many attempts have been made to correlate instrumental measurements with sensory evaluation of fish texture, with varying results (Hamann and Lanier, 1987; Hyldig and Nielsen, 2001; Mørkøre and Einen, 2003; Sigurgisladottir *et al*., 1997). Lack of correlation between instrumental measurements and sensory evaluation of fish texture may at least partly be explained by the heterogeneous nature of the fish muscle, which makes both sampling and measurements difficult to reproduce. In addition, the instrumental and sensory evaluations are basically very different in the way texture is measured. The sensory evaluation is a combination of several parameters covering all impressions from the first contact between the fish and the sensory evaluator (such as the smell of the sample or the first contact between the fish and a surface in the mouth), until it is completely masticated. As earlier noted, many different methods of instrumental texture measurements are used, with different advantages and drawbacks. This makes it difficult to compare results from different investigations.

1.4.4. Texture profile analysis

To determine the textural properties, a Texture Profile Analysis (TPA) can be carried out. Texture profiling involves compressing the test substance at least twice and

quantifying the mechanical parameters from the recorded force-time curves. TPA is a kind of test that attempts to imitate with an instrument the conditions to which the food is subjected in the mouth or on the plate. More specifically, it imitates the masticatory action of the jaw, except the fact that the speed of travel of the probe is constant and does not follow the sine-wave speed pattern of the human jaw. The force against compression is recorded when a probe is pressed a given distance into a sample. After a specified time, the compression is repeated (at the same position). A force-time curve is generated, as shown in Figure 4.

A constant compression speed is used, and the areas under the force-time curve are therefore directly proportional to the work performed by the probe during the downstroke and by the fillet during the upstroke.

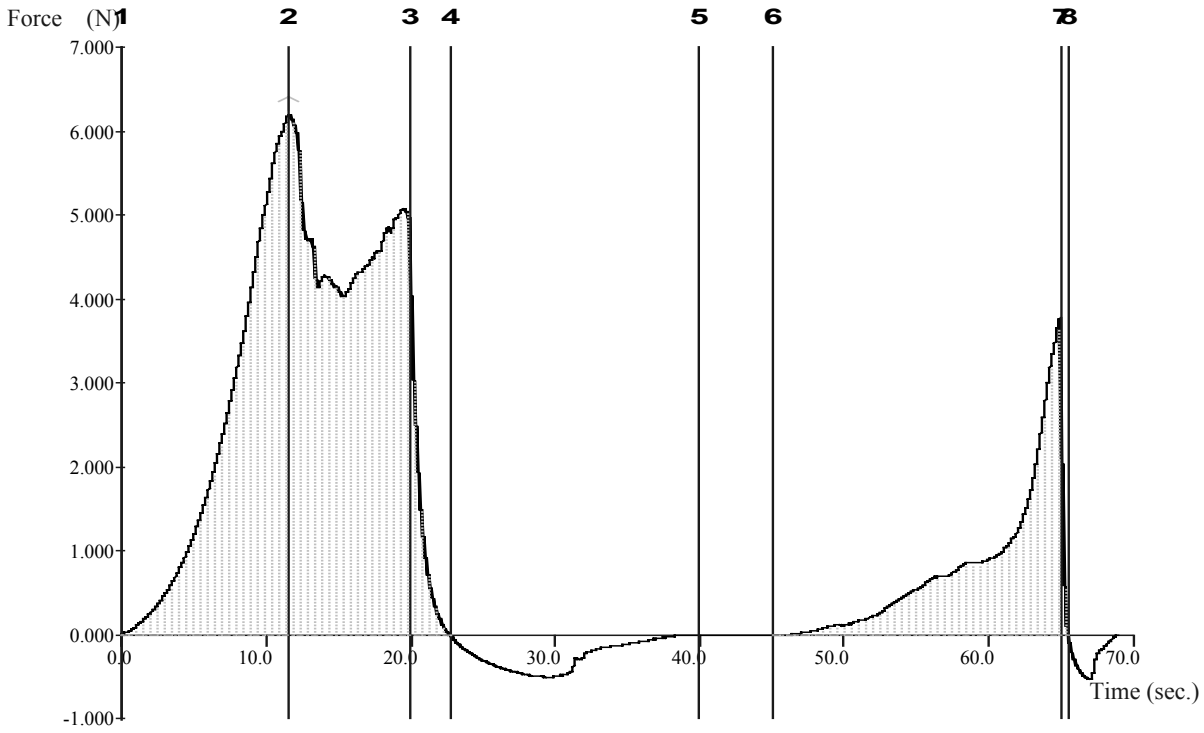

Figure 4. A general instrumental texture profile curve.

Different textural parameters are read and calculated from the force-time curve as described by Bourne (1978) and Stable Micro Systems (1993) (letters and numbers refer

to Figure 4, and Ax:y refers to the area under the force-time curve between anchors x and y):

Breaking strength:	The force at the first significant break in the curve during the first compression, anchor 2. The force required to bite through the surface of the fillet.
Hardness:	The resistance at maximum compression during the first compression, anchor 3. The hardness of the sample at the first bite.
Resilience:	The upstroke area divided by downstroke area (both of first compression), A3:4/(A1:2 + A2:3).
Cohesiveness:	The ratio of the positive force area during the second compression to that during the first compression, (A6:7 + A7:8)/(A1:2 + A2:3 + A3:4).
Gumminess:	The product of hardness and cohesiveness

Resilience gives a relative and dimensionless measure of the elasticity of the muscle, and considers not only the distance, but also the force and speed with which the fillet bounces back after the initial deformation (Veland and Torrissen, 1999). If the value of resilience is 1, all the work performed by the probe during the first downstroke is returned by the fillet during the upstroke. If the value of resilience is less than 1, the fillet has not recovered completely to its original thickness, or has recovered with less force or speed than it was compressed with.

Cohesiveness gives a relative and dimensionless measure of how much of the muscle's strength is retained after the deformation of the first compression (Veland and Torrissen, 1999). If the value of cohesiveness is 1, the muscle has maintained its strength and regained its structure completely during the pause between the compressions, and offers the same resistance to the second compression as to the first. If the value of cohesiveness is less than 1, the deformation of the first compression has been partly irrecoverable.

1.4.5. Mechanisms of post mortem *softening*

Fish skeletal muscle contains many different proteases, and the softening rate of the fish flesh after death varies considerably among species (Ando *et al*., 1993, Ando, Nishiyabu, Tsukamasa, and Makinodan, 1999; Bremner, 1992; Yamashita and Konagaya, 1990b).

There are three possible explanatory mechanisms for *post mortem* softening: some major components within either the myofibrils or in the extracellular connective tissue degrade; or links, bonds and connections that organize and stabilize the structure between the muscle components degrade; or both of these mechanisms occur (Bremner, 1992).

The *post mortem* textural changes have been attributed to processes that cause changes in the physico-chemical state of the myofibrillar proteins (Dunajski, 1979). However, protein hydrolysis may not be important, at least during the first period *post mortem*. Myofibrillar proteins of softened chum salmon muscle was extensively degraded, and the degradation pattern was almost reproduced when myofibrils were treated with purified cathepsin L (Yamashita and Konagaya, 1990b). *Post mortem* changes in muscle include several modifications in the myofibrillar proteins (Foegeding *et al*., 1996; Ladrat, Verrez-Bagnis, Noel, and Fleurence, 2003). *In vitro* proteolysis of sea bass (*Dicentrarchus labrax* L.) myofibrillar proteins have been investigated (Ladrat *et al*., 2003). Different types of cathepsins were able to hydrolyze some of the myofibrillar proteins, and myosin heavy chain seemed to be very sensitive. Some of the results agreed with protein modifications observed in stored *post mortem* muscle. However, the changes in the myofibrils are not large, especially when compared to mammalian muscle. In addition, it must be emphasized that even if the proteases are able to degrade myofibrillar proteins *in vitro*, there may be other mechanisms dominating *in situ*.

Several studies indicate that the myofibrillar proteins are largely unaffected during *post mortem* storage. The softening of muscle is due not to the breakdown of myofibrils but probably to proteolytic digestion of minor cell components that link the major structural units together (Ando *et al*., 1995; Bremner and Hallett, 1985; Hernandez-Herrero *et al*., 2003). Proportionately small changes in these minor components may have disproportionately large effects on the flesh structure, and thereby the flesh texture, before any changes in the electrophoretic pattern are detected (Bremner, 1992).

Studies with different microscopy techniques have revealed that the fine collagen fibrils are progressively deteriorated, resulting in detachment of the muscle fiber from the myocommata (Bremner, 1992). In addition, there is large deterioration within the muscle fiber ends. Bremner and Hallett (1985) suggested that endogenous collagenases

and proteases might be responsible for the destruction of fine collagenous fibrils in the skeletal muscle of blue grenadier (*Macruronus novaezelandiae*). Pelagic fish meat softening and degradation of collagen fibrils are accelerated when fish are killed without bleeding, suggesting influence of some proteases in the fish blood (Ando *et al*., 1999). However, bleeding had no effect on the muscle firmness of several demersal fish, indicating that the mechanism of softening may vary considerably between species.

In general, the published mechanisms of *post mortem* fish muscle softening are often contradictory. In addition, the different research groups often consider only a limited number of proteins, enzymes or species, making the knowledge fragmented.

2. AIMS OF THE WORK

The aim of the work was to investigate enzymatic properties of fish fillets, and increase the understanding of the mechanisms leading to fish softening and excessive soft fish. Biochemical properties such as solubility and composition of muscle proteins were studied, together with activities of different proteolytic enzymes. These properties were then related to textural changes in the fish muscle. To achieve a better understanding of the mechanisms of textural changes in fish muscle, both salmon and cod were investigated. In addition, the fish were subjected to different treatments to obtain a broader range of raw material characteristics within the species.

More specifically, the following questions have been addressed in separate papers:

- Do textural properties of cod and salmon develop differently when the fish are stored in ice? Are there any differences in the solubility properties of proteins and activities of proteolytic enzymes in the two species?
- How do specific endogenous proteolytic enzymes contribute to changes in textural properties and protein solubility of salmon?
- What is the importance of smoking temperature when salmon is cold-smoked? How are the protein solubility characteristics and proteolytic activities influenced? And what happens when the smoked salmon is further stored (like at the consumer's)?
- How important is it to maintain low temperature during storage of fresh fish? How are textural properties of cod fillets influenced by a controlled temperature abuse?

3. RESULTS AND DISCUSSION

3.1. Choice of system

In order to study properties of fish proteins, various systems and treatments can be used. These include whole fish, fish fillets, smaller pieces, fish minces, direct icing, plastic bags, vacuum packaging, iced storage, freezing, super-chilling, temperature abuse, irradiation and smoking. The various systems are not expected to behave equally during storage, and it may therefore be impossible to compare the results directly. In general, it is very important to choose the most relevant storage system when performing experiments, to be sure that the results can be related to real situations.

When using treatments to reduce the load of microorganisms, it is important to avoid recontamination after the treatment. When irradiating samples, it is also important to exclude air. The most relevant system is therefore vacuum-packed fillets. This system is very different from iced storage of whole fish.

In the experiments, we have aimed at investigating fish of similar size (within each experiment) to ensure that the observed effects are due to different treatments investigated and not only resulting from differences in fish size. With the number of biochemical analyses (and thereby number of weighing and extractions) performed, it was not practically possible to investigate a large number of individuals. Therefore, to be able to accomplish the experiments with a relatively low number of fish individuals and with low interference from fish size, we have tried to keep the size and prehistory of the fish within each experiment as equal as possible.

3.2. Textural properties

There is no agreement about which methods are the best for measuring textural properties of fish, and there is no method universally recommended (Hyldig and Nielsen, 2001). Veland and Torrissen (1999) investigated textural properties of salmon fillets, and compared a spherical probe (25 mm diameter) with the Warner-Bratzler shear test. The shear test was slightly more sensitive than the compression test for differentiating between recently killed salmon and salmon stored on ice for up to 24 days. In addition, it was demonstrated that the fillet temperature during textural

measurements is important (the fish softened when the temperature was increased), and the fillets should have as equal temperature as possible. Mørkøre and Einen (2003) compared different systems for instrumental determination of textural properties of raw and cold-smoked Atlantic salmon (*Salmo salar*) cutlets. Significant differences were detected with the two cylinders and the sphere, but not with the Warner-Bratzler blade. There are advantages and drawbacks with all methods. It is possible that the chosen cylindrical probe is not the best method for detecting small changes in textural properties. It has, however, been found to differentiate between raw and smoked salmon cutlets (Mørkøre and Einen, 2003), and raw salmon fillets after different periods of storage (Einen and Thomassen, 1998).

The method for measuring textural properties was chosen based on available equipment at the department. It was also desirable to use an established and published method that does not require extensive sample preparation and handling. The Warner-Bratzler shear method requires a pre-cut muscle piece of a defined geometry, which is then cut by a blade. The sharpness of this blade should be stable, and every muscle piece should be cut in the same manner. Some of the other published methods also require extensive sample preparation. This may influence the textural properties of the sample, and the handling may even destroy the original characteristics of the sample. In addition, extensive sample preparation may not lead to the desired geometry in every sample, and the preparation procedure can be very time-consuming. According to Sigurgisladottir *et al*. (1999), the area under the dorsal fin is suitable for measuring textural properties in parallel in an individual. It is quite difficult to place the probe on the fillet without hitting connective tissue or some part of the bone structure (if the fillets are not boned). Together with expectance of large individual variations, we chose to measure each fillet more than once. The fillets were kept in ice until measured.

The textural properties were determined using Texture Profile Analysis (TPA). A compression of 60% of the sample height was chosen to ensure a breaking point in almost all samples, and the holding time between the two compressions was 5 s. A flat-ended cylindrical plunger with diameter 12 mm was pressed into the fillet, perpendicular to the muscle fibers. The area tested was between the gills and the (second for cod) dorsal fin, above the lateral line. Three to five measurements were run on each fillet.

A high degree of compression fractures the sample after the first compression, leading to a change in sample dimension and structure before the second compression is performed. It may be discussed whether it is possible to calculate any properties including the second compression (Hyldig and Nielsen, 2001). On the other hand, the destructive test may be preferred, as it corresponds better to the mastication of food in the mouth (Bourne, 1978; Dunajski, 1979; Mørkøre and Einen, 2003). In this thesis, this only affects the discussion of cohesiveness and gumminess, the only two mentioned textural properties calculated from the second compression.

3.3. Proteolytic activities

Most of the published work about proteolytic enzymes in fish muscle has been on characterization of different enzymes. That is, the research is directed towards finding ways of purifying enzymes, finding natural and synthetic substrates and inhibitors for the enzymes, finding optimum conditions for enzyme activities, and learning about how the enzymes are influenced by other components (such as salts, detergents, and activators). Most papers do not report how the activities of different enzymes change during the commercial storage life of fish muscle, or how the composition of enzyme activities may change during storage. Only a limited amount of information is available from the early *post mortem* period (Geesink *et al*., 2000; Ladrat, Verrez-Bagnis, Noel, and Fleurence, 2004).

The general proteolytic activity was measured against hemoglobin, and the method was adapted to micro wells. The pH of the buffer was adjusted to give the desired pH in the reaction mixture. A blank was prepared for each sample by adding the trichloroacetic acid (TCA) before the enzyme extract. The blank samples were not incubated, as it was found that the content of TCA soluble components increased during incubation, possibly due to acid hydrolysis. Optical density of the filtered solutions (after precipitation with TCA) at 280 nm was not suitable as a measure of the proteolytic activity. The content of TCA soluble components was determined by Lowry's method, with the volumes adjusted to get a steeper slope of the standard curve. In addition, a centrifugation step was included to remove gas bubbles in the wells.

The methods for determination of the specific proteolytic activities were based on the description of Stoknes and Rustad (1995). As the different substrates employed had

similar excitation and emission properties to their common product, preliminary scans of the substrates and the product were performed in order to choose excitation and emission conditions that would measure the product without extensive influence from the substrates. The concentration of fluorogenic substrate was chosen after economical and practical considerations, and the enzyme concentrations were adjusted to get activity measures practically independent of the protein content in the enzyme extracts. Some preliminary experiments were performed to determine appropriate incubation times, and concentrations of activators and inhibitors (such as Ca^{2+}, EDTA, and dithiothreitol). Blank samples were first prepared for each sample by adding substrate after inactivating the enzymes by sodium dodecyl sulfate (SDS) in solution (stopping solution). However, as it became clear that the fluorescence of the blanks were equal, it was decided that blanks should be prepared for each substrate, by replacing the enzyme extract with distilled water.

Accurate determination of the specific activity of an enzyme requires a practically pure enzyme. In addition, the enzyme must retain its activity throughout the (often complex) purification process. In the present thesis, no attempts were made to fully characterize the different proteolytic enzymes present in the fish muscles. Instead, the available activities were measured, implying that most of the different enzyme activities were measured in plain water extracts. No attempts were made to remove (the effect of) endogenous inhibitors (calpastatin, TIMP, and others), or activate a given activity by applying special extraction procedures.

Different specific proteolytic activities were measured against synthetic substrates. These may not give an accurate picture of the proteolytic potential in the fish muscle. *In vivo*, the substrates are most often large proteins, which may cause sterical effects. These effects may be both beneficial, as binding of the substrate protein at locations distant to the active site may improve the catalytic activity. It is, however, more likely that the large proteins cause sterical inhibition. In addition, the specific activities were measured in solution, after addition of activators (to example Ca^{2+} or thiol compounds). By this, we alter the activity compared to the physiological situation. Increase in fluorescence was used as a measure of specific proteolytic activities. It is not possible to determine whether this increase is caused by a high number of enzyme molecules with low to moderate activity, or a low number of enzyme molecules with high activity. The

obtained activities are therefore not a direct measure of the amounts of proteolytic enzymes *in vivo*, but an indication of the potential proteolytic activity. No attempts were made to study myofibril fragmentation by incubating partially purified myofibrillar proteins with enzyme extracts, and subsequently detect changes in protein composition by SDS-PAGE (or other techniques).

Many different fluorogenic substrates based on 7-amino-4-methylcoumarin have been described (see for example Barrett and Kirschke, 1981; Kinoshita, Toyohara, Shimizu, and Sakaguchi, 1992; Sasaki, Kikuchi, Yumoto, Yoshimura, and Murachi, 1984). The only difference between them is the peptide sequences attached. Some of the substrates may be cleaved by more than one protease, and a given protease may cut more than one substrate. The efficiency of a substrate may also depend on the source of the enzyme. Extensive testing of different proteases against different substrates has led to the assumption that the specificity is sufficient to accurately quantify activity levels for individual protease types without significant cross assay interference, without preparation of subcellular fractions to distinguish cytoplasmic and lysosomal proteases.

In conclusion, it is assumed the total activity against physiological substrates *in vivo* is lower than the activity against synthetic substrates *in vitro*, and that the applied methods are sufficient.

3.4. Brief summary of results from the papers

3.4.1. Iced storage of farmed salmon and wild cod

Quality characteristics of farmed Atlantis salmon (*Salmo salar*) and wild Atlantic cod (*Gadus morhua*) were determined during iced storage, as described in Paper I. The aim of this study was to relate textural properties to biochemical and enzymatic changes in the fish muscles during iced storage, and to compare the two species. In addition, the experiment was performed in order to compare later investigations of different processing with the changes observed with only iced storage.

Textural properties, protein solubility, water-holding capacity and activity of calpain- and collagenase-like enzymes of salmon (*Salmo salar*) and cod (*Gadus morhua*) fillets were measured during iced storage. Large differences were observed between the two species, which behaved differently when stored on ice. The differences between the two

species were greater than the changes observed during the storage period within each species. Both breaking strength and hardness of fillets were reduced during iced storage (significantly in salmon). Properties related to fillet elasticity and its ability to resist repeated deformation (resilience and cohesiveness, respectively) showed decreasing values in cod, and increasing values in salmon during the storage period. The salmon fillets were significantly softer and had lower values of resilience than the cod fillets. Salmon fillets had a significantly higher content of extractable proteins than the cod fillets, and a lower fraction of salt soluble proteins. As expected, the salmon muscle pH and water content was lower than the values in cod, but the water-holding capacity was better. Compared to salmon, the changes in textural properties were larger in cod, and cod also had more active proteolytic enzymes during the entire storage period. The activity of collagenase-like enzymes was virtually constant during the storage period. Compared with the collagenase-like activity, calpain-like enzyme activity was very low, and it was low to not detectable in salmon.

3.4.2. Iced storage of irradiated salmon fillets

When salmon is stored in ice, it is assumed that the endogenous muscle enzymes are responsible for quality changes early in the storage period, while microbial enzymes play a greater role during the last part of the storage period. The aim of the study described in Paper II was to investigate endogenous enzymes and their impact on salmon texture, without interfering effects of microbial enzymes. To accomplish this, half the fillets were irradiated. In addition we wanted to discover whether the irradiation process had any effects on quality characteristics such as lipid oxidation and flesh color.

The irradiation process was successful in that it reduced the number of microorganisms present on the fish surface. The higher number of microorganisms found in the control group was not accompanied with severe deterioration of fish texture or increased proteolytic activity during the storage period. This indicates that leakage of microbial enzymes into the fish flesh is not important for changes in salmon texture during storage.

Both the cathepsin B- and collagenase-like activities were severely inhibited by the irradiation. The activity of cathepsin B-like enzymes was about twice as high as that of collagenase-like enzymes in the control group. After irradiation, the activities were

approximately equal. The difference in inhibition by irradiation treatment reflects the different structures of the enzyme molecules, with the thiol group in the cysteine residue in the active site of cathepsin B being susceptible to damage by the irradiation.

The textural properties did not change significantly during the storage period in either group. However, a significant effect of the 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. Similar results were obtained with protein extractability and content of free amino acids. This indicates that structural and biochemical differences caused by differences in proteolytic activities require time to become evident.

The irradiation was detrimental for the salmon color, as the irradiated fillets appeared pale orange throughout the thickness of the fillets. In addition, the fish smelled somewhat old already at the first sampling, but no differences in lipid oxidation (measured as TBARS and PV) were detected until late in the storage period.

3.4.3. Cold-smoked salmon - effects of smoking temperature and storage time

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. With an increased focus on lightly processed food products, it is common to subject the salmon to cold smoking. In this process the smoking temperature is below 30 °C. The experiment in Paper III was performed in cooperation with AKVAFORSK and Norconserv. The aim of the study was to investigate the effects of smoking temperature and storage period on different properties related to proteins and enzymes in cold-smoked salmon, and to relate these properties to textural characteristics of the smoked fillets.

Increasing the smoking temperature from 20 to 30 °C had a large impact on the solubility properties of the muscle proteins, as the extractability of myofibrillar proteins was significantly reduced. The extractability of water-soluble proteins was almost unaffected by smoking temperature. Protein extractability was further reduced during continued storage (+7 days). The composition of myofibrillar proteins was also greatly affected by the smoking temperature. The intensity of the myosin heavy chain band was reduced both with increasing smoking temperature and further storage of the smoked samples. The general proteolytic activity was low to not detectable, and was not

significantly affected by smoking temperature or further storage. Generally, the activity was higher at pH 6.0 than at pH 6.5, but the variation between individuals was large. The activity of cathepsin B-like enzymes was not significantly dependent on smoking temperature, but was significantly reduced during the storage period. However, the activity was still high after the cold storage, and may contribute to the observed increase in free amino acids during the storage period. The content of free amino acids increased with both smoking and further storage. Nevertheless, the smoking temperature affected neither the total content nor the composition of free amino acids, in good agreement with the proteolytic activities. In total, the differences caused by the different smoking temperatures were reduced by further storage in the cold room, indicating that the effects of processing conditions are most pronounced early in the product's life.

3.4.4. Quality of farmed cod after different storage conditions

Freshness is one of the most important quality characteristics of raw, fresh fish. In order to preserve the high quality of fresh fish, the normal procedure is to store the fish in ice. However, the fish may occasionally be subjected to inadequate storage conditions, such as temperature abuse, for a limited period during the distribution from slaughter to consumer. The effects of storage conditions on proteolytic enzymes, muscle proteins and textural properties of Atlantic cod (*Gadus morhua*) fillets have been investigated, as described in Paper IV. To simulate inadequate storage conditions of fresh fish; ice-stored cod fillets were subjected to a temperature abuse (4.25 hours at room temperature) before the iced storage continued.

The temperature abuse was relatively short and gentle, as the muscle temperature (as measured in the thick part of the fillets) never exceeded 18 °C. This was, however, sufficient to cause undesirable changes in the fillet quality. The textural properties within each treatment group did not change significantly during the storage period. However, the temperature abused fillets had reduced ability to regain their shape and structure when compressed, compared with the control group. Generally, the temperature abused fillets exhibited characteristics observed with iced fillets after a longer storage period, that is, the fillets seemed older than their iced counterparts. The extractability of proteins was not significantly affected by the storage temperature or sampling day. The temperature abuse seemed to cause an increase in a 30 kDa fragment

of the salt soluble proteins early in the storage period, but no large changes in the composition of the salt soluble proteins were observed. The content of small peptides (TCA soluble peptides) increased significantly in both groups during the storage period. At the last sampling day, the content of small peptides was highest in the temperature abused fillets. This indicates that endogenous enzymes might have been activated during the temperature abuse, thereby leading to and increased breakdown of proteins in the abused fillets compared with the iced group.

The cathepsin B-like enzymes dominated throughout the storage period, followed by collagenase-like enzymes. Calpain-like activity was low or not detectable, and decreased even further during the storage period. Generally, the temperature abuse caused decreased activity of cathepsin B-like enzymes, and increased activity of collagenase-like enzymes throughout the storage period. In addition, the enzyme activities were highly dependent on assay pH. The general proteolytic and the cathepsin B-like activities were always significantly higher at pH 6.0 than at pH 7.0, regardless of treatment and storage period. The opposite result was obtained with the calpain- and the collagenase-like activities, with the greatest effect obtained with the collagenase-like enzymes. These results are in agreement with other studies, indicating that the proteolytic enzyme activity profile in a given sample is highly dependent on the sample pH. The muscle pH was significantly higher in the temperature abused fillets than in the control group. Altogether, it is assumed that the collagenase-like activity *in situ* is higher in the abused fillets than in the control fillets. Together with a general increase in activity with increasing temperature, this indicates that the collagen in the muscle of temperature abused fillets is more degraded than in the control group. In conclusion, the present results indicate that collagenase-like enzymes may have caused the textural changes observed after temperature abuse.

3.5. Importance of proteolytic enzymes

When performing the different experiments referred to in this thesis, the fish were always of high quality. We did not have access to fish with extremely soft texture during the experimental period. This may indicate that a lot of the fish is of good quality. However, it was a bit unsatisfactory for the work in this thesis, as a higher variability in raw material characteristics would have been appreciated. Instead, the fish

were subjected to different treatments (iced storage, irradiation, smoking and temperature abuse) to affect the muscle proteins and the activity of endogenous proteolytic enzymes, and thereby study the impact of enzymes on muscle proteins and texture.

During storage, the textural properties have been shown to change, although the changes were not significant. Cod fillets had generally higher values of breaking strength, hardness, resilience and gumminess than the salmon fillets. The values of cohesiveness have been approximately equal. The wild cod seemed to have larger changes in textural properties than farmed cod, which changed more than farmed salmon. This is in contrast to the ultrastructural changes observed by Ofstad *et al*. (1996). Both muscle softening and gaps in the fillets may be a problem during chilled storage of fish. This gaping may occur after filleting, and the severity depends on fish species, muscle pH, and the nutritional status of the fish (Dunajski, 1979; Einen and Thomassen, 1998; Love, Lavety, and Garcia, 1972). The higher frequency of gaping and larger changes in textural properties observed in cod compared to salmon could be explained by the higher activity of collagenolytic enzymes in cod (see below).

Cod fillets contained more collagen than the salmon fillets, and the collagen solubility properties were very different. This may contribute to the observed differences in textural properties between the two species. A positive correlation between muscle collagen content and toughness of raw sliced fish has been reported (Sato, Yoshinaka, Sato, and Shimizu, 1986). Both the total collagen content and its degree of cross-linking may affect the textural properties. Weakening of the pericellular tissue has been related to muscle softening (Ando *et al*., 1993, 1995). Montero and Mackie (1992) reported that collagen in cod (*Gadus morhua*) muscle degraded during iced storage of fillets. The collagen might be more important for the textural properties in cod than in salmon, due to the higher water content of cod. In addition, cod lack the stabilizing amorphous material filling the intra- and extracellular spaces in salmon muscle (Ofstad *et al*., 1996).

No severe degradation of myofibrillar proteins was observed, indicating that changes in the myofibrillar proteins may be of less importance for the textural changes of cod fillets. However, SDS-PAGE is not a very sensitive technique. Minor changes may not be discovered, and there are important proteins that are too large to be suitable for

separation on SDS-PAGE. In addition, the total protein conformation and functional properties of the proteins may change without being seen as changes in solubility properties. Therefore, this may not be taken as complete evidence against the influence of myofibrillar protein fragmentation on fillet textural properties. Besides, the changes in textural properties may be due to proteolytic digestion of minor components that link the major structural units together (Bremner, 1992; Bremner and Hallett, 1985; Yamashita and Konagaya, 1991).

Specific proteolytic activities were detected during the entire storage periods in all experiments. Of the specific proteolytic activities investigated, cathepsin B-like activity resulted in the highest increase in fluorescence, followed by collagenase-like activity, both in salmon and cod. Neither cathepsin B- nor collagenase-like activities in fresh fish were significantly dependent on storage time. In general, the activities were higher in cod than in salmon. Both cathepsin B- and collagenase-like activity was severely inhibited by irradiation of salmon fillets. In addition, cathepsin B-like activity decreased during storage of cold-smoked salmon. Calpain-like activity has been found to be low or not detectable in both salmon and cod, and decreases even further during storage of the fish. This is in agreement with results obtained by Geesink *et al*. (2000) and Ladrat *et al*. (2004).

There has been a debate about the importance of calpains and cathepsins on meat textural properties (Blanchard and Mantle, 1996). Some workers claim that calpains are the most important. Other groups argue that the calpains need Ca^{2+} for activation, at concentrations obtained only after the muscle pH has fallen below the range of activity for the calpains, and that catheptic enzymes therefore are responsible for the tenderization. It has also been suggested that the tenderization results from the synergistic action of calpains and cathepsins. Lysosomes in fish muscle are commonly associated with the connective tissue. *Post mortem* disruption of the lysosomes leads to release of cathepsin B, which could activate the collagenases (Bremner, 1992). The different proteolytic enzymes may therefore act synergistically at two levels, both by activating other proteolytic enzymes and by performing subsequent attacks on the same protein substrates. An investigation of proteolytic enzyme levels in chicken, pig, lamb and rabbit muscle revealed that there was no general relationship between muscle proteolytic capacity and meat tenderization rates (Blanchard and Mantle, 1996).

According to our results, the cathepsin B-like enzymes may be more important for changes in textural properties of salmon fillets than the collagenase-like enzymes, provided they are released in sufficient amounts in the *post mortem* muscle. On the other hand, the collagenase-like enzymes seem to be more important in cod than in salmon. This is supported by the pH dependence of the enzymes, and the activity profiles in the temperature abused and ice-stored fillets. Gelatinolytic enzymes from Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), and spotted wolffish (*Anarhichas minor*) muscle showed both quantitative and qualitative differences (Lødemel and Olsen, 2003). This indicates that both the composition and the enzyme activity differ between species, and that the importance of the collagenase-like enzymes may vary with fish species.

4. CONCLUDING REMARKS

The textural properties of fish fillets did not change significantly during storage in our experiments. Activities of specific proteolytic enzymes were detected throughout the storage period of salmon and cod. It seems that cathepsin B-like and collagenase-like activities are the most important in salmon and cod, respectively.

Proteolytic activities depend on muscle pH , and this may have a great impact on the stability of processed products (salted, marinated, …). In addition, the proteolytic enzymes may be affected by treatments such as smoking or irradiation, and the effect may depend on the type of enzyme considered. The irradiation treatment we performed was successful in reducing the microbial load on the surface of the fillets. However, the impact on the enzymes varied considerably.

It has been documented that it is important to keep the temperature low during iced storage of cod fillets. A relatively mild temperature abuse was sufficient to make the fillets appear older, with less favorable textural characteristics than the iced fillets. Collagenase-like enzymes may cause the textural changes.

During production of cold-smoked salmon, the smoking temperature influenced the solubility and composition of proteins. The smoking temperature did not affect the proteolytic activity. The differences caused by different smoking temperatures were reduced by further cold storage. Hence, the effect of processing parameters was most pronounced early in the product's life. No sensory evaluation was performed, and it is therefore not possible to conclude about sensory changes due to different protein solubility. There are no suggestions that smoking temperature in the investigated area is important for the storage stability of the smoked salmon.

5. SUGGESTIONS FOR FURTHER STUDIES

In order to be able to compare results on fish texture from different groups, more research on different methods for determining textural properties is needed. An agreement between international researchers on recommended method(s) for determining textural properties of different species or products would be appreciated. A method has been used extensively on salmon and trout, both by researchers and in the industry (Mørkøre and Einen, 2003). This method is often called "The Norwegian method" in the industry (Dr. Turid Mørkøre, personal communication), and involves pressing a 12 mm diameter flat-ended cylinder into fish cutlets. The method gives good correlation between instrumental and sensory analyses of fish texture, but it is not generally recommended as a standard procedure (at least not yet).

It would also be interesting to follow salmon fillets more closely during the smoking process, to see if there are enzymatic differences due to smoking temperature. In addition, other treatments of the raw material, such as super-chilling, could be included.

There is a need for further characterization of proteolytic activities during spawning/life cycle of different fish species. This may generate knowledge about when the activities of different enzymes increase. In addition, there may be important differences between the fish that mature and spawn only once before dying, and those spawning every year. The first group may have higher autolytic activity, as the fish are predetermined to die and have no need to maintain the muscle structure for later recovery.

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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

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

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 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.

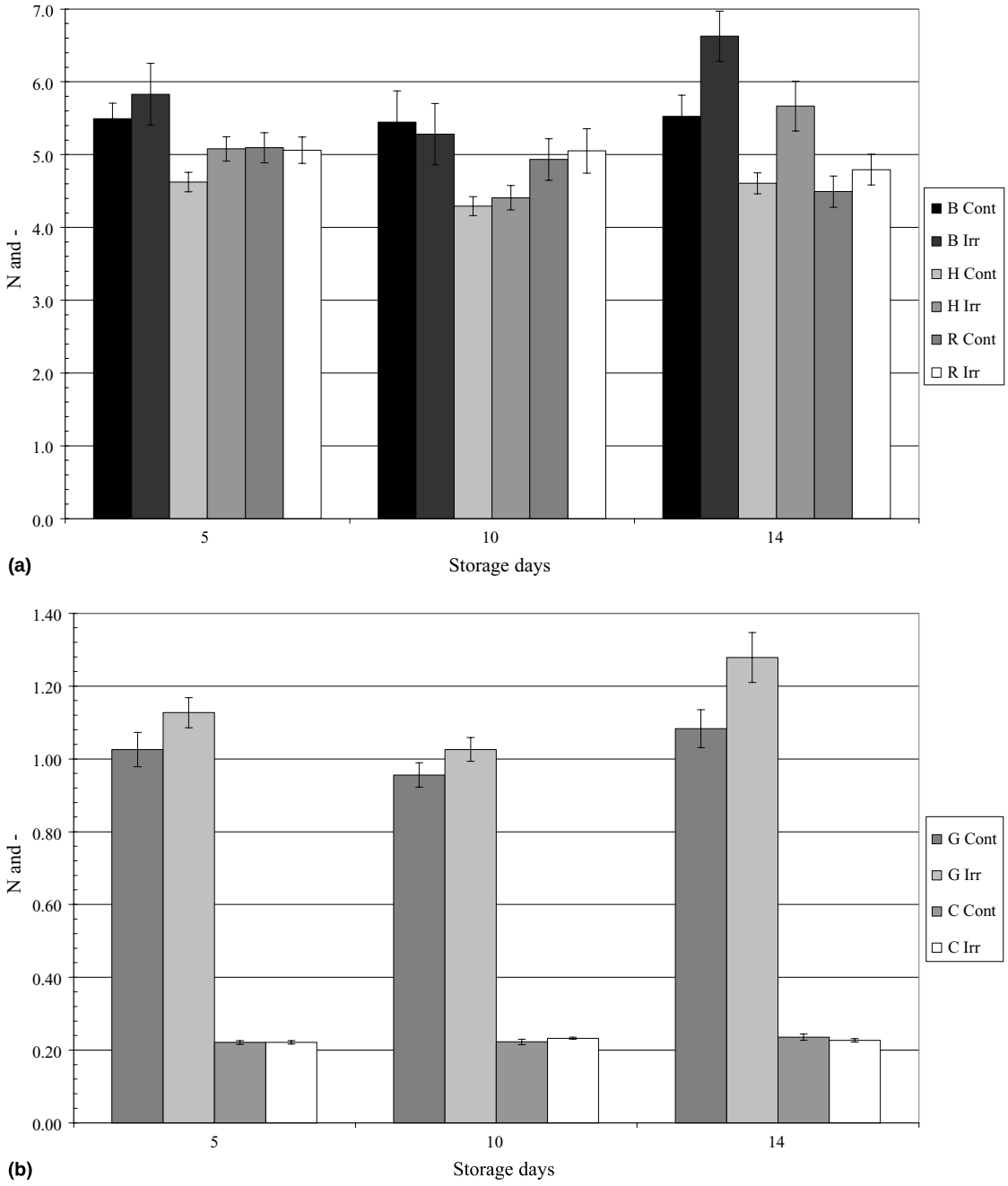

Fig. 1. Textural properties of salmon fillets during iced storage. (a) B, breaking strength (N); H, hardness (N); R, 100 * resilience (–). (b) G, gumminess (N); C, cohesiveness (–). Cont, control fillets; Irr, irradiated fillets. Bars indicate standard errors of the means ($n = 6$).

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.

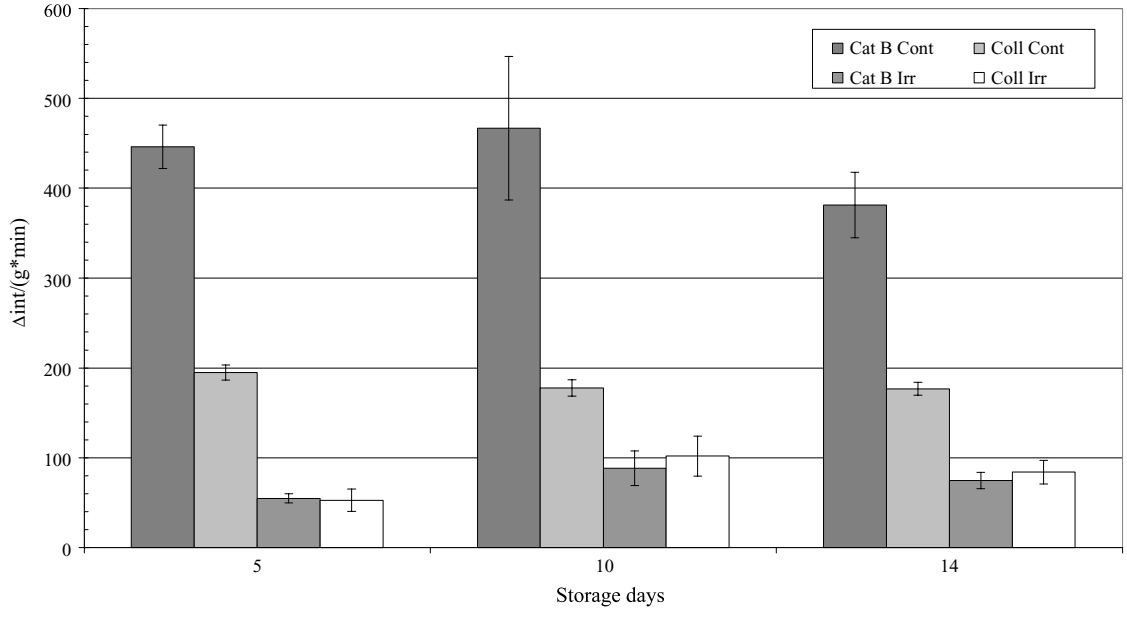

Fig. 2. Activity of cathepsin B-like (Cat B) and collagenase-like (Coll) enzymes during iced storage of salmon fillets, pH 6.5 at 4 °C. Enzyme activities are reported as increase in fluorescence intensity/g wet weight/minute. Cont, control fillets; Irr, irradiated fillets. Bars indicate standard errors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

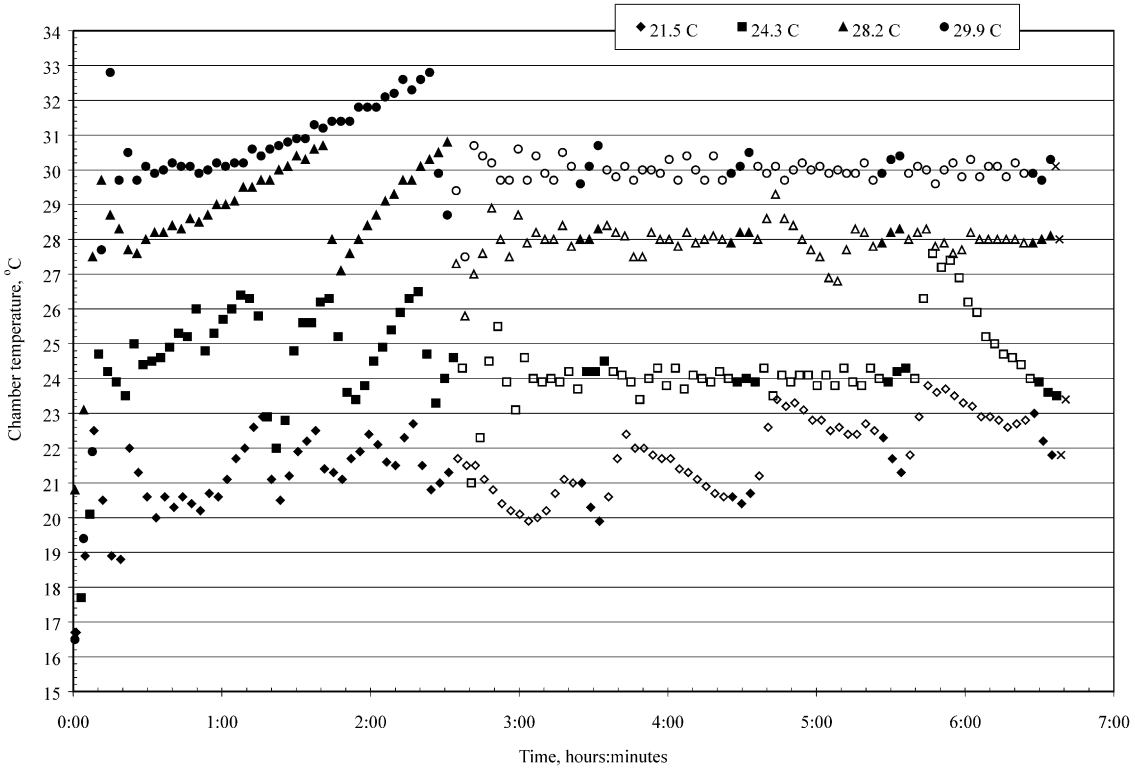

Fig. 1. Chamber temperature during the drying and smoking of salmon fillets. Average smoking temperatures: 21.5 °C (◆◇), 24.3 °C (■□), 28.2 °C (▲△) and 29.9 °C (●○). Filled symbols indicate drying, open symbols indicate smoking, and the Xs indicate evacuation period.

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

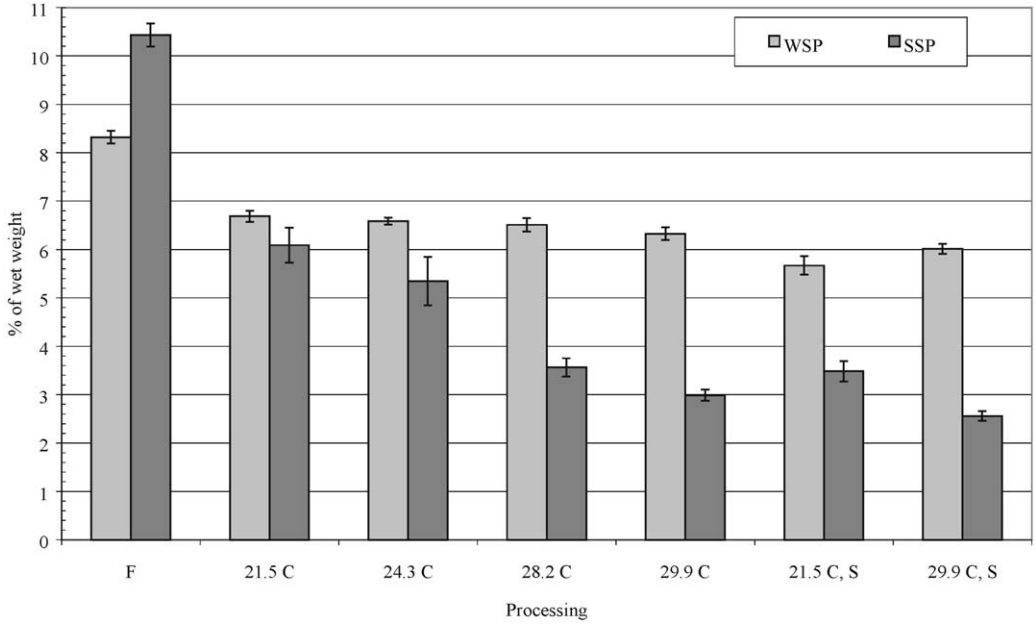

Fig. 2. Solubility properties of proteins in processed salmon. WSP: water-soluble proteins; SSP: salt soluble proteins. Bars indicate standard error of the mean ($n = 6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

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

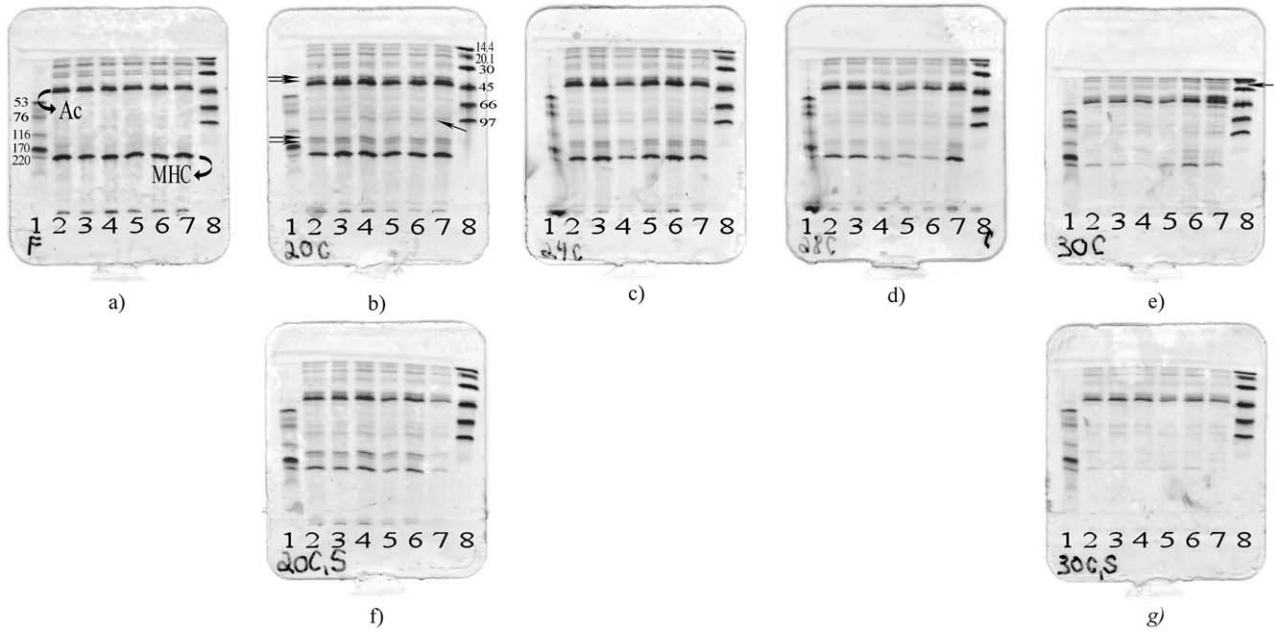

Fig. 3. Changes in salt soluble proteins in salmon caused by smoking and subsequent cold storage. High and low molecular weight standards are in well 1 (left side) and 8 (right side), respectively, on all gels. Molecular weights of proteins in the standards are given in kDa in (a) and (b). Protein concentrations 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).

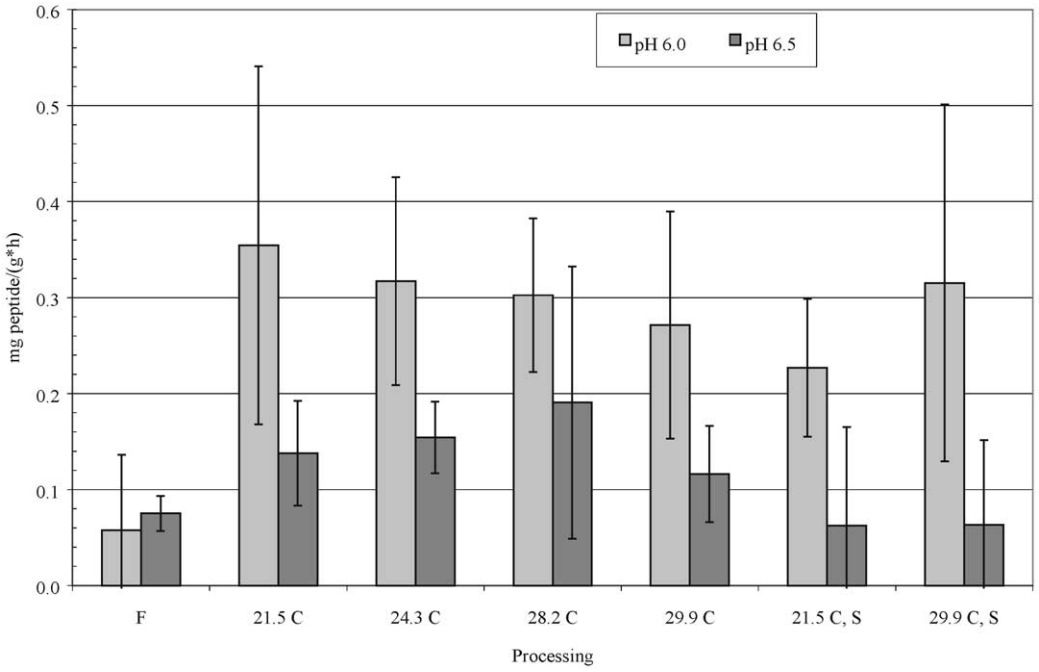

Fig. 4. Proteolytic activity in processed salmon, given as increase in TCA soluble peptides/g wet weight/h, investigated at different pH values. Bars indicate standard error of the mean ($n=6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

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

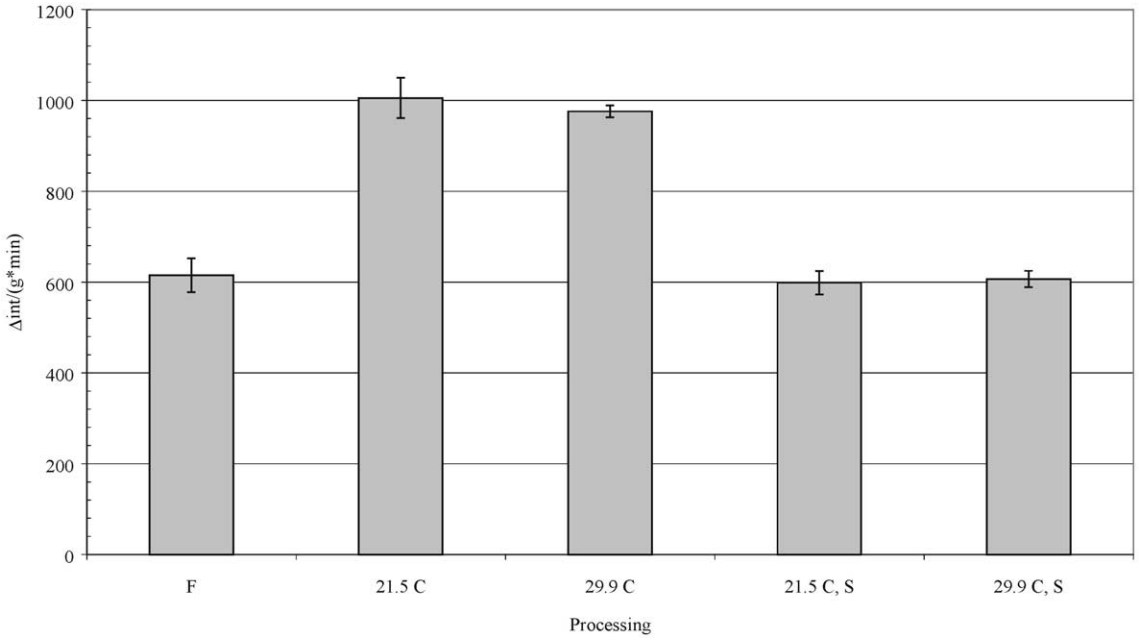

Fig. 5. Cathepsin B-like activity in processed salmon, given as increase in fluorescence intensity/g wet weight/min. Bars indicate standard error of the mean ($n = 6$). F: fresh fish; "number" C: average smoking temperature (°C); S: 7 days' further storage of smoked samples.

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.

Acknowledgements

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Texture, proteins and proteolytic enzymes in farmed Atlantic cod (*Gadus morhua*)

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ABSTRACT

The effects of storage conditions on quality characteristics of farmed cod fillets were investigated. Subjecting cod fillets to a temperature abuse caused undesirable changes in fillet quality. Neither textural properties nor protein solubility characteristics changed significantly during the storage period, but the temperature abused fillets were less resilient and had lower values of gumminess than those subjected to iced storage throughout the storage period. In addition, the abused fillets were somewhat less hard and cohesive, and had a higher content of small peptides than the iced fillets. The textural changes observed after temperature abuse may have been caused by collagenase-like enzymes.

KEYWORDS. Cod, texture, iced storage, temperature abuse, protein solubility, calpain-like, cathepsin B-like, collagenase-like, proteolytic activity.

INTRODUCTION

One of the most important quality characteristics of fresh fish is muscle texture, and excessively soft fillets may cause problems for the industry (Andersen, Thomassen and Rørå, 1997; Haard, 1992; Hallett and Bremner, 1988; Sigholt, Erikson, Rustad, Johansen, Nordtvedt and Seland, 1997). Several different parameters influence fish muscle texture, including *ante mortem* 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 muscle proteins, and handling stress before slaughter. *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, and conditions during storage (i.e.

temperature and duration of storage period) (Andersen *et al*., 1997; Dunajski, 1979; Haard, 1992; Sigholt *et al*., 1997; Sigurgisladottir, Torrissen, Lie, Thomassen and Hafsteinsson, 1997).

During iced storage of raw fish the quality of the fish muscle will deteriorate. Endogenous proteolytic enzymes, able to hydrolyze different muscle proteins, are important early in the deterioration process (Cepeda, Chou, Bracho and Haard, 1990). Collagenous sheets (myocommata) separate blocks of muscle fibers, and a fine network of collagen surrounds each muscle fiber and connects it to myocommata (Bremner and Hallett, 1985). During chilled storage of blue grenadier it was observed that the attachments between muscle fibers and myocommata, and the whole sarcolemma, was degraded, and muscle fibers were detached from the myocommatal sheets (Bremner and Hallett, 1985; Hallett and Bremner, 1988). A similar degradation has been observed in king salmon (Fletcher, Hallett, Jerrett and Holland, 1997), Atlantic cod and salmon (Ofstad, Egelandsdal, Kidman, Myklebust, Olsen and Hermansson, 1996a). 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 and Makinodan, 1995; Ashie, Smith and Simpson, 1996; Bremner and Hallett, 1985; Cepeda *et al*., 1990). In general, the initial attack on the collagen triple helix is by specific collagenases. Once the initial cleavage has been achieved, other non-specific proteases can pursue attack (Kristjansson, Gudmundsdottir, Fox and Bjarnason, 1995). Collagenolytic enzymes have been isolated from the skeletal muscle of fish (Bracho and Haard, 1995; Hernandez-Herrero, Duflos, Malle and Bouquelet, 2003; Teruel and Simpson, 1995). The enzymatic activities were dependent on fish species, and were most potent at pH values close to neutrality or higher.

Calcium activated neutral proteases (calpains) participate in *post mortem* degradation of mammalian and avian muscle, and possibly also fish muscle, by cleaving myofibrillar proteins (Geesink, Morton, Kent and Bickerstaffe, 2000; Kolodziejska and Sikorski, 1996). *Post mortem* concentration of free Ca^{2+} may increase to amounts able to activate both the low- and high-calcium requiring types of calpain. Calpains are active against many myofibrillar proteins, where they cleave proteins at specific sites and therefore cause only a limited proteolysis. The proteins are degraded into large fragments, and

this enhances the susceptibility of the proteins to other proteinases (Kolodziejska and Sikorski, 1996; Ladrat, Chaplet, Verrez-Bagnis, Noel and Fleurence, 2000).

The cathepsins (endogenous lysosomal cysteine proteases) are related to protein catabolism of the fish during spawning migration (Yamashita and Konagaya, 1990). During maturation, endogenous enzyme activity increases, as the fish stop eating and use its own muscle proteins for building gonads. After spawning, the fish is feeding again. Intensely feeding post spawning Atlantic cod (*Gadus morhua*) has been associated with soft textured fillets exhibiting high drip loss (Ang and Haard, 1985). Because of the high proteolytic activity of maturing fish, increased proteolytic activity and thereby reduced stability in post spawning fish may also be expected.

Sigholt *et al*. (1997) studied the effect of storage temperature (0.4 and 3.3 °C) on quality characteristics in farmed salmon during storage for up to 10 days. The storage temperature did not affect quality parameters such as muscle pH, protein solubility characteristics and textural properties (fillet firmness and breaking strength). However, the storage temperature significantly affected the K-value of raw fillets and the sensory properties of cooked fillets.

Textural and biochemical changes during iced storage of wild cod have been reported (Hultmann and Rustad, 2002). Commercial cod farming is growing, and little is published on the textural properties of farmed cod. Wild and farmed or fed cod differ in muscle pH, muscle structure and water-holding capacity (Ofstad *et al*., 1996a; Rustad, 1992). This may influence the textural properties, and it is therefore of interest to investigate textural and biochemical properties of farmed cod.

In order to preserve the high quality of fresh fish, the normal procedure is to store the fish in ice. However, the fish may occasionally be subjected to inadequate storage conditions (temperature abuse) for a limited period during the distribution from slaughter to consumer. In this experiment, the effects of storage conditions on proteolytic enzymes, muscle proteins and textural properties of cod fillets were investigated. To simulate inadequate storage conditions of fresh fish, ice-stored cod fillets were subjected to a temperature abuse before the iced storage continued.

MATERIALS AND METHODS

Twelve female post spawning farmed cod (*Gadus morhua*) were delivered from a fish farm (Institute of Marine Research, Austevoll Aquaculture Research Station, Hordaland) in May 2003. The cod were slaughtered (stunned by a blow to the head, bled and gutted) and was immediately iced. The day of slaughter was defined as day 0. After transport to Trondheim, the fish were filleted (day 3) and placed in plastic bags (one fillet per bag). The fillets were divided into two groups, with the two fillets from the same fish placed in different groups. Twelve fillets were kept in ice in a cold room. The other 12 fillets were placed in room temperature (about 20 °C) for 4.25 hours, to simulate a temperature abuse. The fillets were then iced and placed in the cold room until analyzed. Two additional fillets were used for temperature logging during the abuse period and subsequent re-icing, as shown in Figure 1. Ice was replenished when needed during storage.

The average length and weight (± SEM) of gutted fish with heads were 59.5 ± 0.4 cm and 2.14 ± 0.05 kg, respectively.

The two fillets from each fish (with and without temperature abuse) were analyzed on the same day. Six fillets were analyzed on day 4; the remaining six were analyzed on day 10. The muscle between the gills and the dorsal fin was used for all analyses. Distilled water was used, and all chemicals were of analytical grade.

Textural properties

Textural properties were measured with a TA.XT2 Texture Analyser (Stable Micro Systems, UK), equipped with a load cell of 5 kg, by a modification of the method described by Einen and Thomassen (1998) as previously described (Hultmann and Rustad, 2002). Four (occasionally five) measurements were run on each fillet. The area tested was between the gills and the dorsal fin, above the lateral line. 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.

Muscle pH and water content

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

Water content was determined by heating samples of 2 g at 105 °C for 24 hours. The analyses were run in duplicate.

Solubility properties 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 and Allsup (1982) as previously described (Hultmann and Rustad, 2002). The extraction procedure was carried out once on each fillet.

The amount of proteins in the extracts was determined after centrifugation (7,840 × 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.

The composition of the salt soluble protein fractions was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using PhastGel Gradient 4-15 gels, SDS buffer strips, high and low molecular weight standards, and the gels were silver stained. All equipment for electrophoresis was delivered from Amersham Biosciences (Uppsala, Sweden). The analyses were carried out according to the instructions of the manufacturer.

Activity of proteolytic enzymes

The extractions of proteolytic enzymes were performed as previously described (Hultmann, Rørå, Steinsland, Skåra and Rustad, 2004). The extraction procedure was carried out once on each fillet, and the extracts were frozen and stored at –80 °C till analyzed.

The general proteolytic activity at pH 6.0 and 7.0 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*., 2004), using halved volumes in the incubation mixture. The filter plates were incubated at 25 °C for 2 h. The reaction was arrested by addition of 25 µl 17.5% (w/v) trichloroacetic acid, and the mixture was

shaken before filtering. The general proteolytic activity was expressed as mg TCA soluble peptides liberated/g wet weight/h (using bovine serum albumin as a standard), and given as the arithmetic mean of five individual measurements. The content of TCA soluble peptides in the zero samples was used as a measure of the endogenous amount of small peptides in the samples. The contribution from hemoglobin to the amount of TCA soluble protein was measured and found to be small. This was; however, subtracted from the result.

The activities of specific proteolytic enzymes were also determined in the water extracts. After thawing, the extracts were centrifuged (7,840 × g, 4 °C for 10 min). The amount of protein in the extracts was determined as described above. Samples were diluted with distilled water to a protein concentration of about 1.5 mg/ml.

The activities of calpain-like enzymes were measured against a synthetic fluorogenic substrate, N-succinyl-leucine-tyrosine-7-amido-4-methylcoumarin (SLT) (Sigma Chemical Co., St. Louis, MO) (Sasaki, Kikuchi, Yumoto, Yoshimura and Murachi, 1984). Concentration of SLT (dissolved in dimethyl sulfoxide) was 0.0625 mM in 100 mM bis-Tris, 10 mM $CaCl_2$, 2 mM dithiothreitol, pH 6.0 or pH 7.0. The calcium dependent activity was determined by subtracting the fluorescence values obtained when incubating with 20 mM EDTA (the non-calcium dependent activity) from those obtained when incubating with 10 mM $CaCl_2$ (total activity against SLT).

The 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 and Kirschke, 1981). Concentration of CAA (dissolved in dimethyl sulfoxide) was 0.0625 mM in 100 mM bis-Tris, 20 mM EDTA, 4 mM dithiothreitol, pH 6.0 or pH 7.0.

The 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 and Sakaguchi, 1992). Concentration of SGP (dissolved in dimethyl sulfoxide) was 0.0625 mM in 100 mM bis-Tris, 5 mM $CaCl_2$, pH 6.0 or pH 7.0.

The specific proteolytic activities were determined as previously described (Hultmann *et al*., 2004). A blank was prepared for each type of activity by adding

distilled water instead of enzyme extract to the reaction mixture. Increase in fluorescence intensity was used to calculate the activity, given as increase in fluorescence/g wet weight/min during incubation. The analyses were run in triplicate.

Statistics

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

The effects of treatment (r_k, $k = 1, 2$) on a given variable (Y_i) within each sampling day (t_j) were investigated using paired test of the difference (d) between quality characteristics of the abused (r_2) and iced (r_1) fillets from each fish, $d_{i\,tj} = Y_{i\,tj\,r2} - Y_{i\,tj\,r1}$. It was assumed that the differences were normally distributed with mean μ_d and variance σ_d^2. The null hypothesis (H_0) tested was $\mu_d = 0$.

The effects of storage time (t_j, $j = 1, 2$) on a given variable (Y_i) within each treatment group (r_k) were investigated, assuming that the variables were normally distributed with mean $\mu_{i\,tj\,rk}$ and variance σ^2. Null hypothesis (H_0) tested: $\mu_{i\,t1\,rk} = \mu_{i\,t2\,rk}$, applying t-test assuming equal variances.

RESULTS AND DISCUSSION

During distribution from producer to consumer, fish may occasionally be subjected to inadequate storage conditions (temperature abuse) for a limited period. To simulate inadequate storage conditions of fresh fish, ice-stored cod fillets were subjected to a temperature abuse before the iced storage continued. The muscle temperature was recorded during the temperature abuse and subsequent iced storage (using the thick part of the fillet), and the temperature profiles of two independent fillets are shown in Figure 1. The recorded temperature curves from the two fillets follow each other closely during the abuse period, and they are taken to be representative of all the fillets. The room temperature was around 20 °C during the temperature abuse period, and the thin part of the fillets probably approached this temperature faster than the thick parts. After re-icing the temperature decreased rapidly, and was stable after reaching –0.20 °C.

When working with fish, large variations between individuals within a species have been observed. In order to study the effect of storage conditions, fillets from one

individual was subjected to the two different treatments. This allows a direct comparison between the temperature abused fillets and the control fillets within each sampling day using paired tests, thereby eliminating the variability between individuals. The standard error of the mean (indicated in figures and tables) is therefore not used when comparing results from different treatments within a sampling day. Changes due to storage time (within each treatment group) were compared using t-tests assuming equal variances.

The temperature abuse affected muscle pH, as abused fillets had significantly higher pH values than those stored in ice during the whole storage period (Table 1 and 2). Muscle pH increased for both groups during storage, but the changes were not significant (significant at 90% level for the iced group). pH values were lower than those earlier reported for wild cod (Hultmann and Rustad, 2002), in agreement with results reported by Rustad (1992). Neither temperature treatment nor storage period affected the fillet water content, which was in the same range as earlier observed in wild cod.

Textural properties and protein solubility

Soft texture and high drip has been reported for intensely feeding post spawning Atlantic cod, due to the low ultimate pH of this fish (Ang and Haard, 1985). Large structural differences have been observed between maturing and post-spawned cod, with the latter having large gaps between the myofibrillar units (Ofstad, Kidman, Myklebust, Olsen and Hermansson, 1996b). In addition, the endogenous proteolytic activity is increased during the maturation, and the muscle proteins may be depleted after the spawning period. Altogether, it may be presumed that post spawning fish is especially vulnerable to temperature abuse during the storage of fresh fillets.

Different textural properties of the cod fillets are shown in Figure 2. The abused fillets showed somewhat lower values for hardness, were less cohesive and had somewhat higher breaking strength than the control group, but neither treatment nor storage time caused significant differences (Table 2). The temperature abused cod fillets were less resilient (significant at 90% level during the storage period) and had lower values of gumminess (significant at 90% level only on the first sampling day) than those in the control group. Neither resilience nor gumminess changed significantly within

each treatment group during the storage period. The results indicate that the temperature abuse caused a decrease in the fillets' ability to regain its shape and structure when compressed. These fillets showed characteristics observed with iced fillets after a longer storage period. Textural changes during iced storage have been investigated in wild cod (Hultmann and Rustad, 2002). In the wild cod, both breaking strength and hardness were reduced during storage, although not significantly. Both cohesiveness and resilience of wild cod was significantly reduced during the storage period. Farmed cod were generally harder than the wild ones, and exhibited higher values for breaking strength and resilience. The differences in properties may be related to the different physiological status (muscle pH, farmed post spawning cod compared with wild cod of unknown age, feeding and degree of maturation), and the fish size (the wild were about twice as heavy as and had 50-75% thicker fillets than the farmed cod).

Minor differences in protein solubility between the two treatment groups were observed. The iced fillets contained higher amounts of water-soluble proteins and lower amounts of salt soluble proteins than the abused fillets during the whole storage period, but the differences were not significant (Table 1 and 2). During the storage period the amount of water- and salt soluble proteins decreased in both groups, but again there were no significant changes. In total, the protein extractability was somewhat reduced during the storage period, but the changes due to storage period or treatment were not significant. The textural changes discussed above could not be explained by changes in protein solubility. The observed solubility properties of protein are different from those reported for wild cod, where protein extractability was significantly reduced during iced storage (Hultmann and Rustad, 2002). In addition, the amount of extractable proteins in the farmed cod was lower than that obtained with the wild cod, but the distribution of proteins (fraction of salt soluble proteins) was about the same.

Possible changes in the composition of the salt soluble proteins were investigated using SDS-PAGE (Figure 4). The difference in solubility of salt soluble proteins between the two treatment groups was also evident after SDS-PAGE, as the abused samples showed more intense bands on the gels (Fig. 4c and d). As expected, the myosin heavy chain (MHC) and actin bands were the most intense in all samples. Neither temperature treatment nor storage time caused large changes in the composition of the salt soluble proteins. The temperature abuse seemed to cause an increase in a 30

kDa fragment, at least early in the storage period. This is in agreement with the results obtained with chinook salmon (*Oncorhynchus tshawytscha*) (Geesink *et al*., 2000).

To further characterize the muscle proteins, the content of TCA soluble peptides was determined. The temperature abused fillets had a significantly lower content of TCA soluble peptides early in the storage period (Figure 3 and Table 2). However, the difference was very small. At the end of the storage period, the content of TCA soluble peptides had increased significantly in both groups. The abused fillets had the highest content, but the difference was not significant. This indicates that endogenous enzymes may have been activated during the temperature abuse, leading to an increased breakdown of proteins in the abused fillets compared with the control fillets.

Activity of proteolytic enzymes

The general proteolytic activity against hemoglobin was investigated at pH 6.0 and 7.0 (Figure 3). The temperature abuse caused a significant reduction in the proteolytic activity at pH 6.0 measured early in the storage period (day 4), whereas the activity at pH 7.0 was unaffected (Table 2). At day 10 the temperature abused fillets had higher activities at both pH values, but the differences were not significant. During the storage period, the proteolytic activity increased (significant for activity measured at pH 6.0 in abused samples). The activity was relatively low in all samples, in agreement with results obtained with smoked salmon (Hultmann *et al*., 2004).

The cathepsin B-like activity was the dominating specific proteolytic activity investigated, followed by collagenase-like activity (Figure 5). Calpain-like activity was low or not detectable, and decreased even further during storage for both treatments, in accordance with results obtained with wild cod (Hultmann and Rustad, 2002). The temperature abuse caused a significant decrease in the calpain-like activity (measured at pH 6.0) and in the cathepsin B-like activity (measured at pH 6.0 and at 90% level measured at pH 7.0) early in the storage period (day 4, Table 2). No significant differences due to temperature abuse were detectable on day 10, or in the other activities on day 4. Generally, the temperature abuse caused decreased cathepsin B-like activity and increased collagenase-like activity throughout the storage period. The cathepsin B-like activity in the ice-stored fillets was only slightly reduced during storage, whereas it increased somewhat for the abused fillets. The changes were not significant, regardless

of pH in reaction mixture. The collagenase-like activity was significantly reduced during the storage period, for both treatments and pH values investigated. The collagenase-like activity was in the same order of magnitude as the activity detected in wild cod at pH 6.5, where it was only slightly reduced during the storage period (Hultmann and Rustad, 2002).

Geesink *et al*. (2000) studied calpains from chinook salmon (*Oncorhynchus tshawytscha*) muscle. They found little proteolysis of myofibrillar proteins during storage, and results were reproduced when calpain was incubated with myofibrils. Calpain activity also seemed to be stable the first days *post mortem*, but the activity of the endogenous inhibitor calpastatin increased during storage. This could explain the very low calpain activity and degradation of myofibrillar proteins found in our experiment. Results obtained by Hernandez-Herrero *et al*. (2003) suggested that the softening of cod muscle observed during iced storage is caused more by collagenase activity than by proteolysis of myofibrils. Montero and Mackie (1992) investigated changes in intramuscular collagen of cod during iced storage. Their results indicate that proteolytic activity may be responsible for the increased acid and heat solubility of cod collagen during the storage period. Only a limited proteolysis of myofibrillar proteins was detected (as earlier noted), indicating that the observed textural changes in the abused fillets were not related to extensive breakdown of myofibrillar proteins. The collagenolytic enzymes could be partly responsible for the degradation of collagen and other extracellular matrix proteins in fish muscle and for the texture softening of seafood products (Ando *et al*., 1995; Bracho and Haard, 1995).

To further characterize the proteolytic activities, the effects of pH in the reaction mixture when measuring different proteolytic activities were investigated (Table 3). The general proteolytic activity against hemoglobin was highly dependent on pH in the reaction mixture, always being higher at pH 6.0 than at pH 7.0, and this pattern is in agreement with results obtained for smoked salmon (Hultmann *et al*., 2004). Both the calpain- and the collagenase-like activities were significantly higher at pH 7.0 than at pH 6.0 regardless of treatment and storage period, whereas the opposite result was obtained for the cathepsin B-like activity. The influence of assay pH on the proteolytic activities was in agreement with results reported for cathepsin B from chum salmon muscle (Yamashita and Konagaya, 1990), calpains from sea bass muscle (Ladrat *et al*.,

2000), and collagenolytic enzymes from the muscles of winter flounder (Teruel and Simpson, 1995) and Pacific rockfish (Bracho and Haard, 1995). Altogether, this indicates that the proteolytic enzyme activity profile in a given sample will be highly dependent on the sample pH. Love, Lavety and Garcia (1972) showed that the strength of the myocommata depends on the surrounding pH and temperature. Both reduced pH and increased temperature weakened the myocommata. The effects were reversible, but this indicates that the myocommata in the temperature abused fillets may be more susceptible to attack by proteolytic enzymes during the temperature abuse period. A change in pH may both change the properties of the collagen molecules and thereby make them more susceptible to attack by endogenous proteases, and activate the proteases directly. Collagenase-like enzymes may cause the textural changes observed due to temperature abuse. As earlier described, the muscle pH was significantly higher in the abused fillets than in the control fillets throughout the storage period. In addition, the endogenous proteolytic enzymes will generally be more active at temperature abuse conditions than at temperatures experienced during iced storage (Ashie *et al*., 1996; Cepeda *et al*., 1990; Teruel and Simpson, 1995). It is therefore expected that the activity of collagenase-like enzymes *in situ* will be higher in the abused fillets, leading to increased breakdown of collagen in the muscle. The observed textural changes may therefore be a result of changes in the connective tissue.

CONCLUSIONS

Neither textural properties nor protein solubility characteristics changed significantly during the storage period. The temperature abused fillets were less resilient and had lower values of gumminess than those subjected to iced storage throughout the storage period. In addition, the abused fillets were somewhat less hard and cohesive. The textural changes observed due to temperature abuse may be caused by activity of collagenase-like enzymes. The activity of the collagenase-like enzymes was highly dependent on pH, the activity being more than doubled when pH was increased from 6.0 to 7.0. The muscle pH in the abused fillets was significantly higher in the abused fillets than in the control fillets throughout the storage period. Altogether, it is assumed that the activity of collagenase-like enzymes *in situ* is higher in the abused fillets than in the control fillets, leading to increased breakdown of collagen in the muscle.

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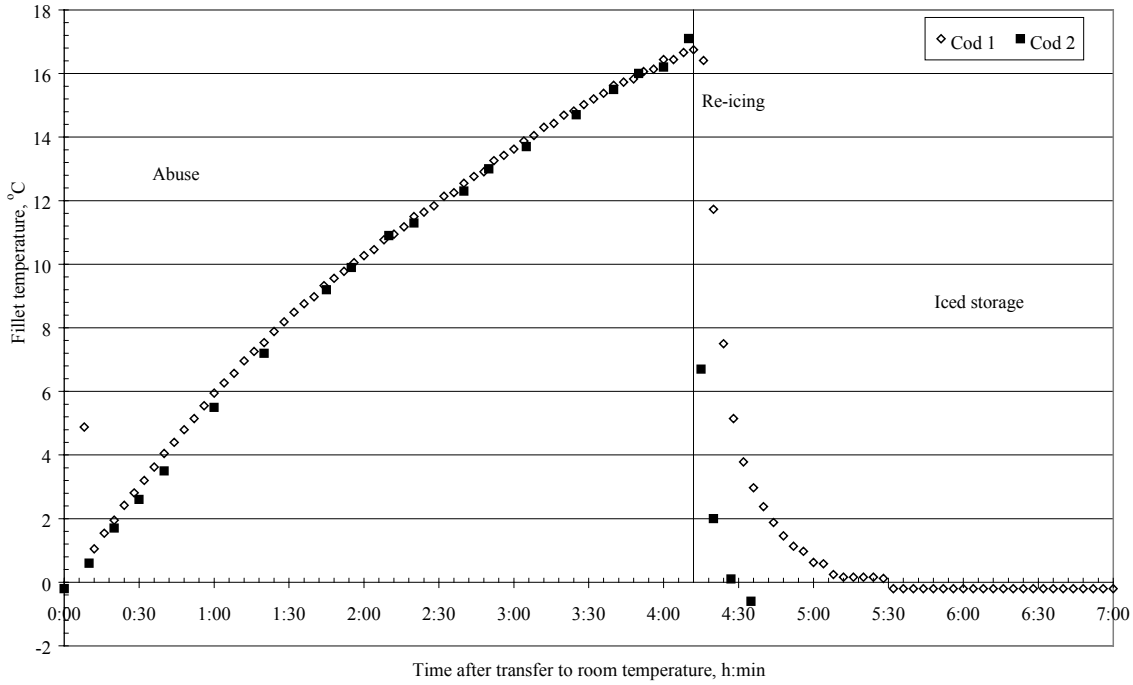
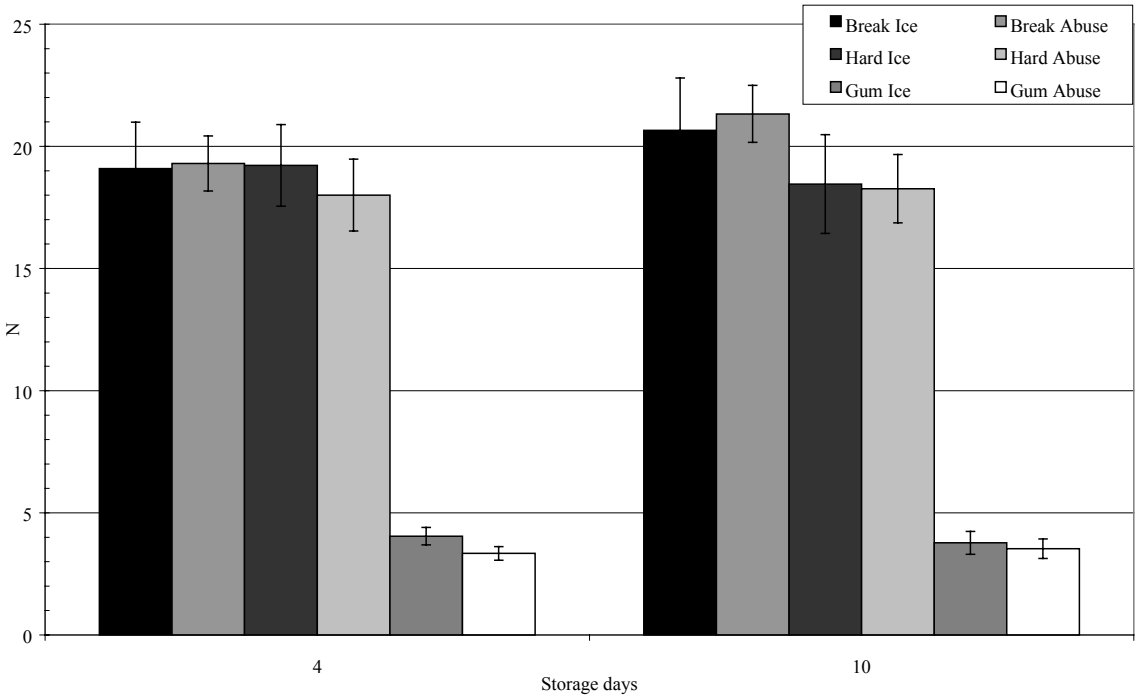
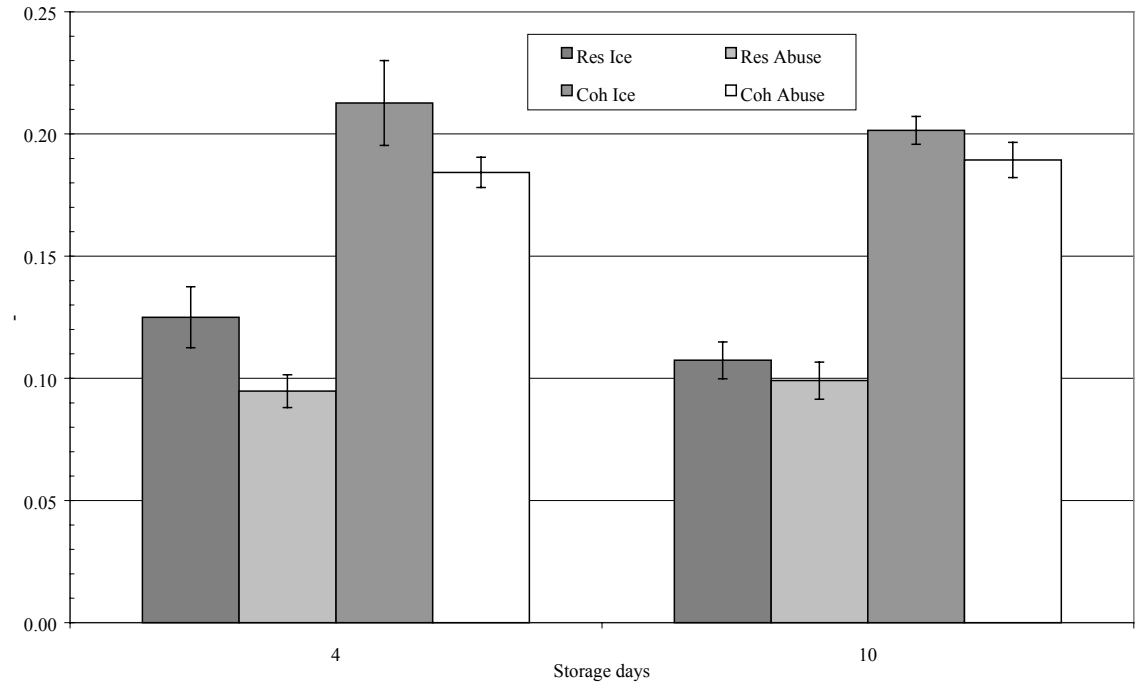


Figure 1. Fillet temperature during temperature abuse and re-icing of cod fillets, recorded in two fillets.



a)



b)

Figure 2. Textural properties of farmed cod fillets during storage. a) Break: breaking strength (N), Hard: hardness (N), Gum: gumminess (N). b) Res: resilience (-), Coh: cohesiveness (-). Ice: iced storage throughout the storage period. Abuse: temperature abuse day 3, with iced storage during the rest of the storage period. Bars indicate standard error of the mean (n = 6).

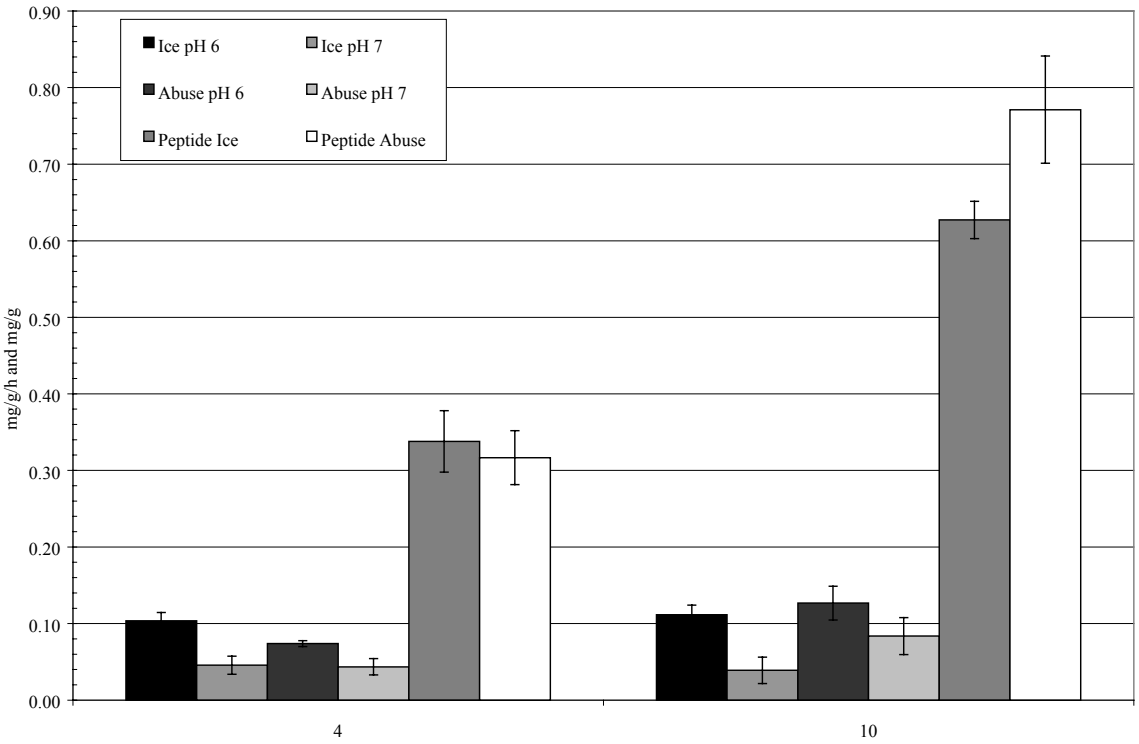


Figure 3. General proteolytic activity and content of TCA soluble peptides in farmed cod fillets during storage, given as mg TCA soluble peptides liberated/g wet weight/hour and mg TCA soluble peptides/g wet weight, respectively. Bars indicate standard error of the mean (n = 6). Ice: iced storage throughout the storage period. Abuse: temperature abuse day 3, with iced storage during the rest of the storage period. Activities were measured at the pH values given. Peptide: content of TCA soluble peptides in the samples.

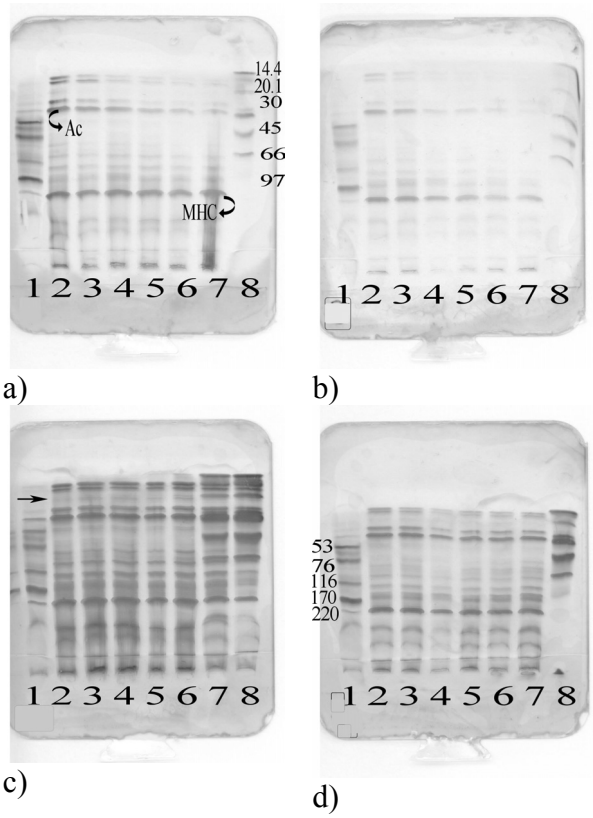


Figure 4. Composition of salt soluble proteins in farmed cod fillets during storage. High and low molecular weight standards are in well 1 (left side) and 8 (right side), respectively, on all gels. Molecular weights of proteins in the standards are given in kDa in a) (LMW) and d) (HMW). Protein concentrations are given in brackets. Fillets subjected to iced storage and sampled at a) day 4 (~1.5 mg/ml) and b) day 10 (~0.7 mg/ml). Fillets subjected to temperature abuse followed by iced storage and sampled at c) day 4 (~1.5 mg/ml) and d) day 10 (~0.7 mg/ml). Ac indicates actin, and MHC indicates myosin heavy chain. Arrows indicate other bands which intensity change due to treatment or during storage.

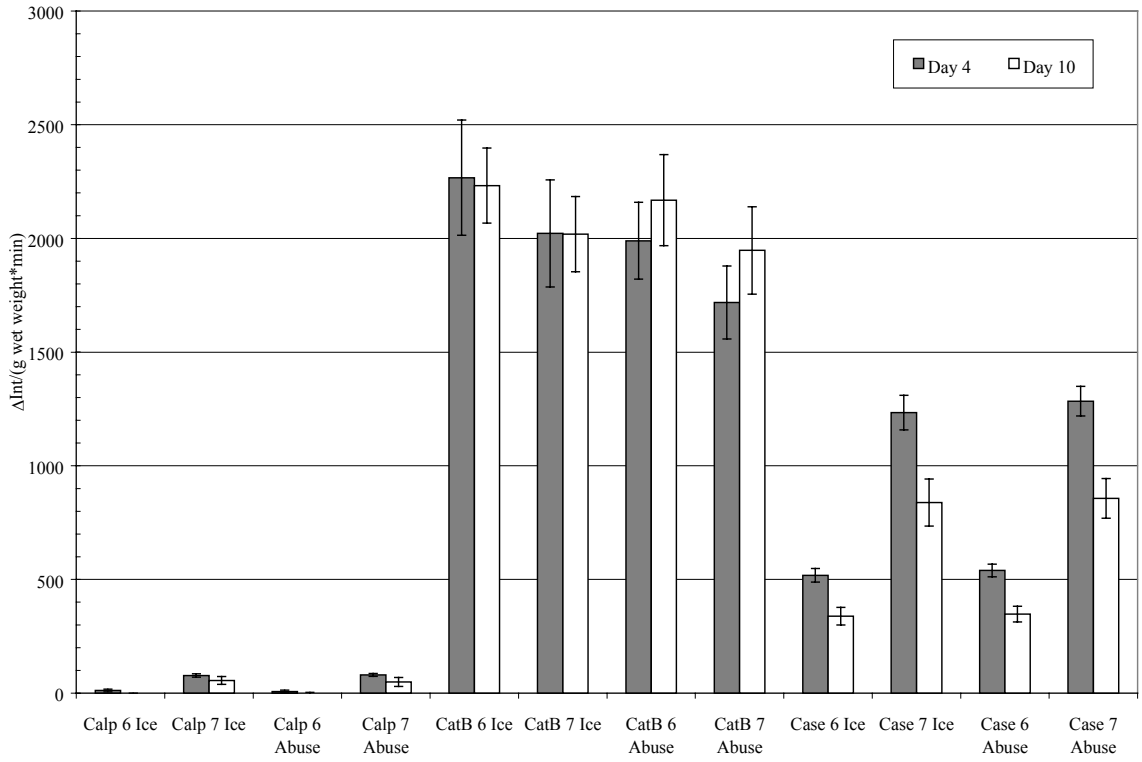


Figure 5. Activity of specific proteolytic enzymes in farmed cod fillets during storage, given as increase in fluorescence intensity/g wet weight/minute. Bars indicate standard error of the mean (n = 6). Calp: calpain-like enzymes; CatB: cathepsin B-like enzymes; Case: collagenase-like enzymes. Numbers indicate pH in reaction mixture. Ice: iced storage throughout the storage period. Abuse: temperature abuse day 3, with iced storage during the rest of the storage period.

Table 1. Size parameters, muscle pH, water content and solubility properties of proteins in farmed cod fillets during storage. Water content and protein solubility are given in % of wet weight. Average ± standard error of the mean (n = 6).

Variable	Iced		Abused	
(Y_i)	Day 4	Day 10	Day 4	Day 10
Gutted weight (kg)	2.18 ± 0.07	2.10 ± 0.07	-	-
Length (cm)	60.2 ± 0.5	58.8 ± 0.6	-	-
Condition factor (g/cm^3)	1.00 ± 0.02	1.04 ± 0.05	-	-
Fillet thickness (mm)	19.5 ± 1.3	20.2 ± 0.8	20.3 ± 0.7	19.1 ± 0.5
Muscle pH	6.30 ± 0.03	6.37 ± 0.02	6.34 ± 0.03	6.41 ± 0.02
Water content (%)	82.8 ± 0.6	83.2 ± 0.7	82.7 ± 0.5	83.3 ± 0.7
Water-soluble proteins (%)	2.43 ± 0.21	2.22 ± 0.14	2.34 ± 0.17	2.18 ± 0.15
Salt soluble proteins (%)	7.22 ± 0.27	6.63 ± 0.43	7.25 ± 0.20	6.80 ± 0.68
Extractable proteins (%)	9.65 ± 0.47	8.85 ± 0.54	9.59 ± 0.36	8.97 ± 0.81

Table 2. Effects of treatment within each sampling day, investigated using paired test of the differences (d) between quality characteristics of fillets from the same individual with and without temperature abuse. Effects of further storage of samples within each treatment, investigated using t-test assuming equal variances (reported as p_{tj}). Water content and protein solubility are given in % of wet weight, specific proteolytic activities are reported as increase in fluorescence intensity/g wet weight/minute (at 4 °C), general proteolytic activities as mg TCA soluble peptides liberated/g wet weight/hour (at 25 °C) and TCA soluble peptides in mg peptides/g wet weight. p values in bold are significant at 95% level, those in italic at 90% level.

Variable	Treatment				Storage	
(Y_i)	Day 4		Day 10		Ice	Abuse
	d	p	d	p	p_{tj}	p_{tj}
Muscle pH	0.04	**0.037**	0.04	**0.011**	*0.056*	0.117
Water content (%)	-0.09	0.397	0.06	0.449	0.635	0.523
Water-soluble proteins (%)	-0.09	0.558	-0.05	0.453	0.424	0.486
Salt soluble proteins (%)	0.03	0.841	0.17	0.680	0.279	0.533
Extractable proteins (%)	-0.06	0.852	0.12	0.780	0.292	0.500
TCA soluble peptides	-0.02	**0.012**	0.14	*0.067*	**0.000**	**0.000**
Breaking strength (N)	0.21	0.848	0.67	0.724	0.595	0.239
Hardness (N)	-1.22	0.239	-0.19	0.812	0.776	0.899
Resilience (-)	-0.030	*0.070*	-0.008	*0.089*	0.256	0.680
Cohesiveness (-)	-0.028	0.220	-0.012	0.115	0.553	0.601
Gumminess (N)	-0.71	*0.098*	-0.24	0.105	0.655	0.699
General activity pH 6.0	-0.03	**0.019**	0.02	0.578	0.646	**0.039**
General activity pH 7.0	0.00	0.840	0.04	0.219	0.759	0.159
Calpain-like activity pH 6.0	-4.1	**0.028**	2.0	0.643	*0.056*	0.234
Calpain-like activity pH 7.0	2.6	0.498	-6.7	0.398	0.267	0.161
Collagenase-like activity pH 6.0	21.4	0.474	9.3	0.591	**0.004**	**0.002**
Collagenase-like activity pH 7.0	50.2	0.527	18.4	0.688	**0.012**	**0.003**
Cathepsin B-like activity pH 6.0	-277.3	**0.038**	-64.2	0.395	0.910	0.512
Cathepsin B-like activity pH 7.0	-303.5	*0.084*	-71.8	0.369	0.993	0.382

Table 3. Effects of pH in proteolytic activity measurements within each sampling day investigated using paired test of the differences (d) between proteolytic activity of cod extracts from the same individual at pH 7.0 and pH 6.0. GPA: general proteolytic activity; Calp: calpain-like activity; CatB: cathepsin B-like activity; Case: collagenase-like activity. Specific proteolytic activities are reported as increase in fluorescence intensity/g wet weight/minute (at 4 °C), and general proteolytic activities as mg TCA soluble peptides liberated/g wet weight/hour (at 25 °C). p values in bold are significant at 95% level, those in italic at 90% level.

Variable	Ice				Abuse			
(Y_i)	Day 4		Day 10		Day 4		Day 10	
	d	p	d	p	d	p	d	p
GPA	-0.06	**0.007**	-0.07	**0.011**	-0.03	**0.045**	-0.04	0.193
Calp	66.0	**0.000**	60.5	**0.008**	72.7	**0.000**	51.9	**0.020**
CatB	-245.4	**0.008**	-213.2	**0.002**	-271.6	**0.024**	-220.8	**0.003**
Case	715.4	**0.000**	499.9	**0.001**	744.2	**0.000**	509.1	**0.000**