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# Effects of temperature abuse on textural properties and proteolytic activities during *post mortem* iced storage of farmed Atlantic cod (*Gadus morhua*)

Lisbeth Hultmann *, Turid Rustad

*Department of Biotechnology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway*

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**Abstract**

Farmed cod (*Gadus morhua*) fillets were subjected to a temperature abuse, which caused changes in muscle pH, textural properties and proteolytic pattern early in the *post mortem* 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 cohesive, and had a higher content of small peptides than the iced fillets. From the proteolytic profiles, it is suggested that 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

## 1. 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, & Rørå, 1997; Haard, 1992; Hallett & Bremner, 1988; Sigholt et al., 1997). Several 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, & 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 this deterioration process (Cepeda, Chou, Bracho, & Haard, 1990). Key structural proteins in both myofibrils and extracellular matrix are degraded, together with proteins involved in linkages between myofibrils and the sarcolemma (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006).

Blocks of muscle fibers are separated by collagenous sheets (myocommata), and a fine network of collagen surrounds each muscle fiber and connects it to myocommata (Bremner & Hallett, 1985). During chilled storage of blue grenadier it was observed that the sarcolemma was degraded, and muscle fibers were detached from the myocommatal sheets (Bremner & Hallett, 1985; Hallett & Bremner, 1988). A similar degradation has been observed in king salmon (Fletcher, Hallett, Jerrett, & Holland, 1997), Atlantic cod and salmon (Ofstad, Egelandsdal, et al., 1996). 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

* Corresponding author. Tel.: +47 73 59 33 20; fax: +47 73 59 12 83.
*E-mail address:* lisbeth.hultmann@biotech.ntnu.no (L. Hultmann).

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pursue attack (Kristjansson, Gudmundsdottir, Fox, & Bjarnason, 1995). 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, 1996; Bremner & Hallett, 1985; Cepeda et al., 1990). The collagenolytic activities from skeletal muscle of fish were dependent on fish species, and were most potent at pH values close to neutrality or higher (Bracho & Haard, 1995; Hernandez-Herrero, Duflos, Malle, & Bouquelet, 2003; Teruel & Simpson, 1995).

Calcium-activated neutral proteases (calpains) participate in *post mortem* degradation of mammalian and avian muscle, and possibly also fish muscle (Delbarre-Ladrat et al., 2006; Geesink, Morton, Kent, & Bickerstaffe, 2000; Kolodziejska & 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 may enhance the susceptibility of the proteins to other proteinases (Delbarre-Ladrat et al., 2006; Kolodziejska & Sikorski, 1996; Ladrat, Chaplet, Verrez-Bagnis, Noël, & Fleurence, 2000).

Cathepsins (endogenous lysosomal cysteine proteases) are related to protein catabolism of the fish during spawning migration (Yamashita & 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 has been associated with soft textured fillets exhibiting high drip loss (Ang & 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.

The effect of storage temperature on quality characteristics of farmed salmon during storage for up to 10 days have been studied (Sigholt et al., 1997). Even a minor increase of storage temperature (from 0.4 to 3.3 °C) significantly increased the K-value (degradation products of ATP) of raw salmon fillets, affected the sensory properties of cooked fillets, and seemed to mask some effects of handling stress. However, the storage temperature did not affect quality parameters such as muscle pH, protein solubility characteristics, or textural properties (fillet firmness and breaking strength). The effects of storage temperature (4 or 20 °C) and handling stress (3 min air exposure) on color and texture of *pre rigor* filleted farmed cod has been investigated (Stien et al., 2005). Fillets stored at the high temperature were softer than the others (after ~4 days). The high storage temperature also masked the majority of effects caused by the handling stress.

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 specified quality 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. The abuse itself was also applied to possibly alter the proteolytic profile, and thereby investigate relationships between specific proteolytic activities and given quality characteristics.

## 2. 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, Norway) in May 2003. Experiments from the earlier life stages have been published by others (Hemre et al., 2004; Karlsen, Hemre, Tveit, & Rosenlund, 2006; Rosenlund, Karlsen, Tveit, Mangor-Jensen, & Hemre, 2004). After this, the cod was reared in cages, fed standard commercial feed (Skretting, Norway) and exposed to natural light (Karlsen, personal communication). 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, *post rigor*) 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 h, to simulate inadequate storage conditions (later referred to as abused fillets). 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 Fig. 1. Ice was replenished when needed during storage.

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

The two fillets from each fish (with and without temperature abuse) were analyzed on the same day. Six fish 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.

### 2.1. 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 (Einen & Thomassen, 1998) as previously described (Hultmann & 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 (Bourne, 1978) (breaking

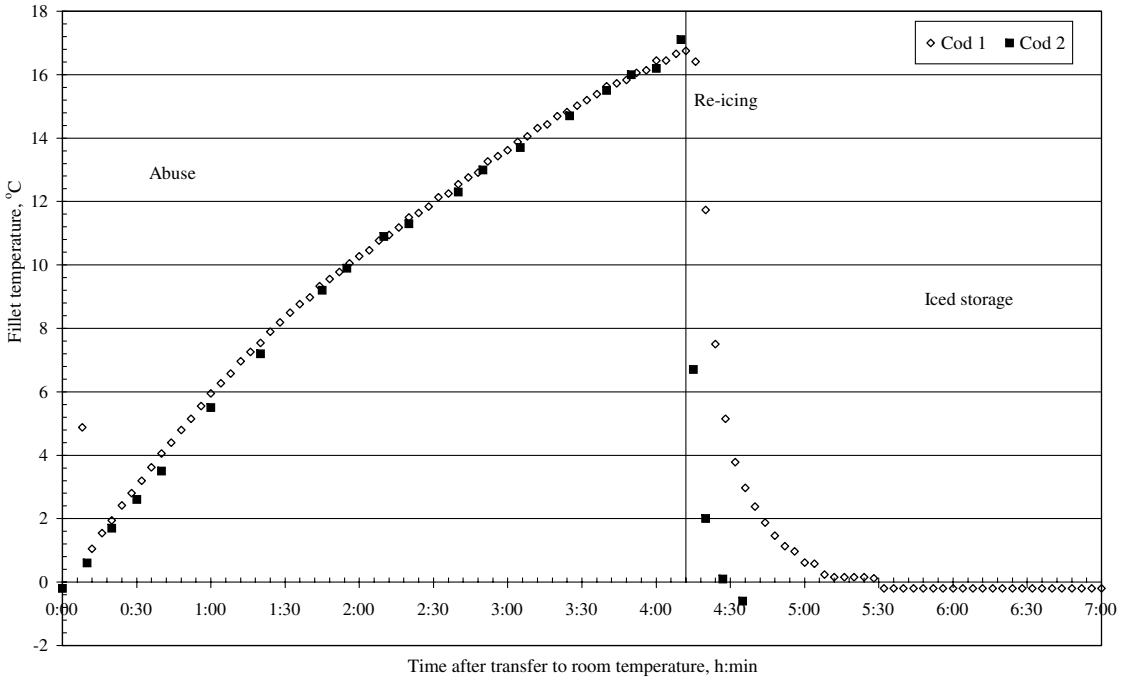


Fig. 1. Fillet temperature during temperature abuse and re-icing of cod fillets, recorded in two fillets.

strength (fracturability), hardness, cohesiveness and gumminess). Resilience was calculated as the ratio of the upstroke area to the downstroke area of the first compression.

### 2.2. 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 h. The analyses were run in duplicate.

### 2.3. Solubility properties of proteins

Proteins were extracted from white muscle by a modification of previously described methods (Anderson & Ravesi, 1968; Licciardello et al., 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.

The composition of the salt soluble protein fractions was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (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.

### 2.4. Activity of proteolytic enzymes

The extractions of proteolytic enzymes were performed as previously described (Hultmann, Rørå, Steinsland, Skåra, & 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 (Barrett & 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 (TCA), 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 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 by replacing protein extract with buffer in the assay, and was found to be small. This was, however, subtracted from the results.

The activities of specific proteolytic enzymes were also determined in the water extracts. After thawing, the extracts were centrifuged (7840*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, USA) (Sasaki, Kikuchi, Yumoto, Yoshimura, & 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, $N_\alpha$-carbobenzoxy-arginine-arginine-7-amido-4-methylcoumarin (CAA) (Sigma Chemical Co., St. Louis, MO, USA) (Barrett & 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, & 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 at 4 °C 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.

### 2.5. Statistics

Mean values for the different quality characteristics 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 effect of treatment on a given variable within each sampling day was investigated using paired test of the difference ($d$) between quality characteristics of the abused and iced fillets from each fish.

The effect of storage time on a given variable within each treatment group was investigated, applying one-way $t$-test assuming equal variances.

## 3. Results and discussion

During distribution from producer to consumer, fish may occasionally be subjected to inadequate storage conditions 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 Fig. 1. The recorded temperature curves from the two fillets follow each other closely during the abuse period, and they are taken to represent 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 it stabilized at −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 both 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.

The temperature abuse affected muscle pH, as the ice stored fillets had significantly lower pH values than the abused ones during the whole storage period (Tables 1 and 2). Muscle pH increased for both groups during storage, significantly for the iced group (significant at 90% level for the abused group). pH values were lower than those reported for wild cod (Hultmann & Rustad, 2002; Olsson, Seppola, & Olsen, 2007), but in agreement with results from farmed cod (Mørkøre, 2006; Olsson et al., 2007; Rustad, 1992; Stien et al., 2005). Neither temperature treatment nor storage period affected the fillet water content. The water content was somewhat high (~83%), indicating that the fish were not fully recovered from the spawning/starvation (Love, 1980).

### 3.1. Textural properties and protein solubility

Soft texture and high drip loss has been reported for intensely feeding post spawning Atlantic cod, due to the low ultimate pH of this fish (Ang & 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, & Hermansson, 1996). 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 Fig. 2. The temperature abused fillets were significantly less resilient and had lower values of gumminess during the storage period than those in the control group. Neither resilience nor gumminess changed significantly within each

Table 1
Size parameters, muscle pH, water content and solubility properties of proteins in farmed cod fillets during storage

| Variable | Iced | | Abused | |
|---|---|---|---|---|
| | 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 |

Water content and protein solubility are given in % of wet weight. Average ± standard error of the mean ($n = 6$).

treatment group during the storage period. Further, the abused fillets were somewhat less cohesive, and had somewhat higher breaking strength than the control group. Values for hardness were almost equal. Neither treatment nor storage time caused significant differences in these quality characteristics (Table 2). The results indicate that the temperature abuse caused a decrease in the fillets' ability to regain shape and structure when compressed. These fillets showed textural characteristics observed with iced fillets after a longer storage period, in accordance with the softening observed after elevated storage temperature early in the *post mortem* period (Stien et al., 2005). Textural changes during iced storage have been investigated in wild cod (Hultmann & 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).

Only minor differences in protein solubility between the two treatment groups were observed (Tables 1 and 2). 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. During the storage period the amount of water- and salt soluble proteins decreased slightly in both groups (while the water content increased). In total, the protein extractability was somewhat reduced during the storage period, but the changes due to storage period or treatment were not significant. This is in agreement with results in salmon (Sigholt et al., 1997). The

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

| | Treatment | | | | Storage | |
|---|---|---|---|---|---|---|
| | Day 4 | | Day 10 | | Ice $p_s$ | Abuse $p_s$ |
| | $d$ | $p$ | $d$ | $p$ | | |
| Muscle pH | 0.04 | **0.018** | 0.04 | **0.005** | **0.028** | *0.058* |
| Water content (%) | −0.09 | 0.198 | 0.06 | 0.225 | 0.317 | 0.261 |
| Water-soluble proteins (%) | −0.09 | 0.279 | −0.05 | 0.227 | 0.212 | 0.243 |
| Salt soluble proteins (%) | 0.03 | 0.421 | 0.17 | 0.340 | 0.140 | 0.266 |
| Extractable proteins (%) | −0.06 | 0.426 | 0.12 | 0.390 | 0.146 | 0.250 |
| TCA soluble peptides | −0.02 | **0.006** | 0.14 | **0.034** | **0.000** | **0.000** |
| Breaking strength (N) | 0.21 | 0.424 | 0.67 | 0.362 | 0.298 | 0.120 |
| Hardness (N) | −0.11 | 0.470 | 0.30 | 0.406 | 0.484 | 0.406 |
| Resilience (–) | −0.030 | **0.035** | −0.008 | **0.045** | 0.128 | 0.340 |
| Cohesiveness (–) | −0.028 | 0.110 | −0.012 | *0.058* | 0.277 | 0.300 |
| Gumminess (N) | −0.49 | **0.028** | −0.18 | 0.155 | 0.461 | 0.312 |
| General activity pH 6.0 | −0.03 | **0.009** | 0.02 | 0.289 | 0.323 | **0.020** |
| General activity pH 7.0 | 0.00 | 0.420 | 0.04 | 0.109 | 0.380 | *0.080* |
| Calpain-like activity pH 6.0 | −4.1 | **0.014** | 2.0 | 0.321 | **0.028** | 0.117 |
| Calpain-like activity pH 7.0 | 2.6 | 0.249 | −6.7 | 0.199 | 0.134 | *0.081* |
| Collagenase-like activity pH 6.0 | 21.4 | 0.237 | 9.3 | 0.295 | **0.002** | **0.001** |
| Collagenase-like activity pH 7.0 | 50.2 | 0.264 | 18.4 | 0.344 | **0.006** | **0.001** |
| Cathepsin B-like activity pH 6.0 | −277.3 | **0.019** | −64.2 | 0.198 | 0.455 | 0.256 |
| Cathepsin B-like activity pH 7.0 | −303.5 | **0.042** | −71.8 | 0.185 | 0.496 | 0.191 |

Effects of further storage of samples within each treatment, investigated using $t$-test assuming equal variances (reported as $p_s$). 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.

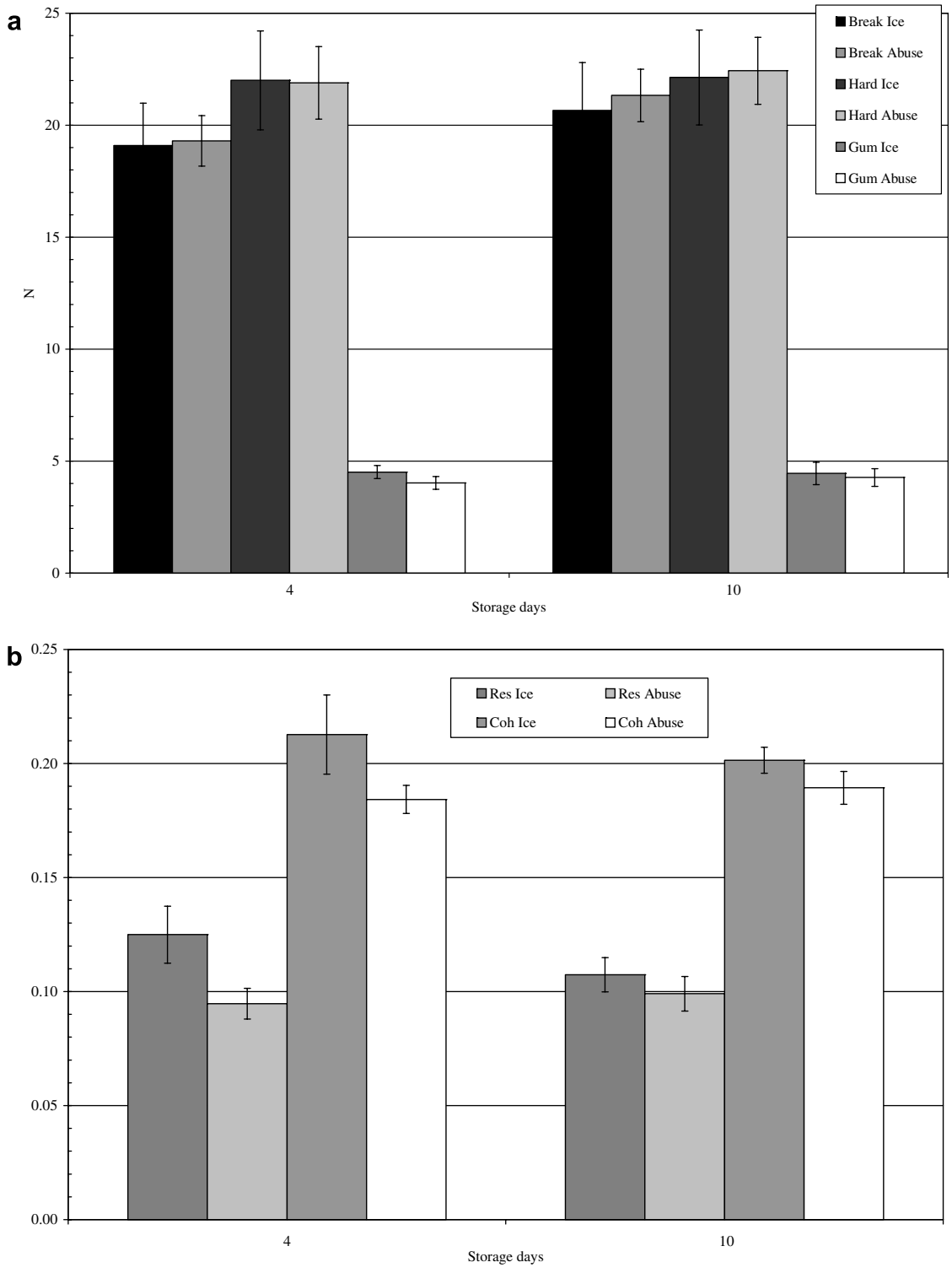


Fig. 2. Textural properties of farmed cod fillets during storage. (a) Break: breaking strength (N), Hard: hardness (N), Gum: gumminess (N). (b) Res: resilience (–), Coh: cohesiveness (–). Ice: iced storage throughout the storage period. Abuse: temperature abuse day 3, with iced storage during the rest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

textural changes discussed above could therefore not be explained by changes in protein solubility characteristics. The observed solubility properties of protein are different from those reported for wild cod, where protein extractability was significantly reduced during iced storage (Hultmann & 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. The low extractability of proteins could be explained by the

long period without feeding, when the fish mobilize proteins from white muscle to cover energy requirements (Beaulieu & Guderley, 1998). No attempts were made to measure total protein content of the fish muscle.

Possible changes in the composition of the salt soluble proteins were investigated using SDS-PAGE (Fig. 3). As expected, the myosin heavy chain 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 (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 (Fig. 4 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, and the abused fillets contained significantly higher levels of soluble peptides than the control group. This indicates that endogenous enzymes may have been activated during the temperature abuse, leading to an increased breakdown of proteins in the abused fillets.

### 3.2. Activity of proteolytic enzymes

The general proteolytic activity (against hemoglobin) was investigated at pH 6.0 and 7.0 (Fig. 4). 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). During the storage period, the proteolytic activity increased significantly in the abused fillets. At day 10 the temperature abused fillets had somewhat higher activities than the control group at both pH values. The activity was relatively low in all samples, as has also been found in smoked salmon (Hultmann et al., 2004).

The cathepsin B-like activity was the dominating specific proteolytic activity investigated, followed by collagenase-like activity (Fig. 5). Calpain-like activity was low or not detectable, and decreased even further during storage for both treatments. The temperature abuse caused a significant decrease in the calpain-like activity (measured at pH 6.0) and in the cathepsin B-like activity (at pH 6.0 and

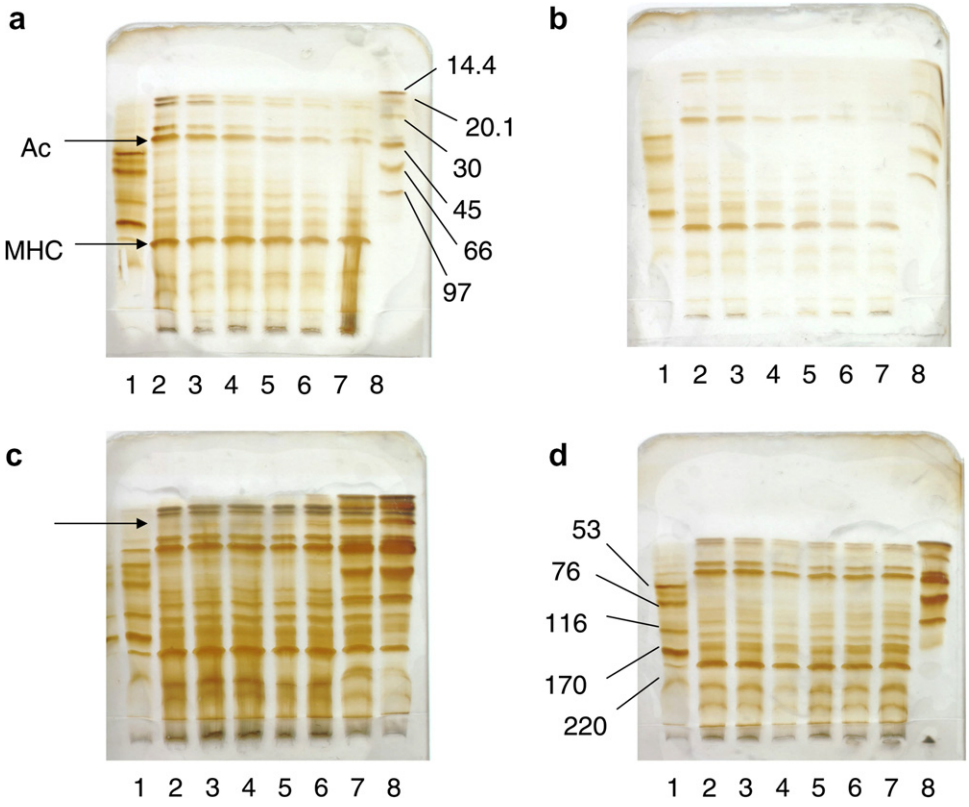


Fig. 3. Composition of salt soluble proteins in farmed cod fillets during storage. High and low molecular weight standards are in well 1 (left side) and 8 (right side), respectively, on all gels. Molecular weights of proteins in the standards are given in kDa in (a) (LMW) and (d) (HMW). Samples from individual fish are in well 2–7, and 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.

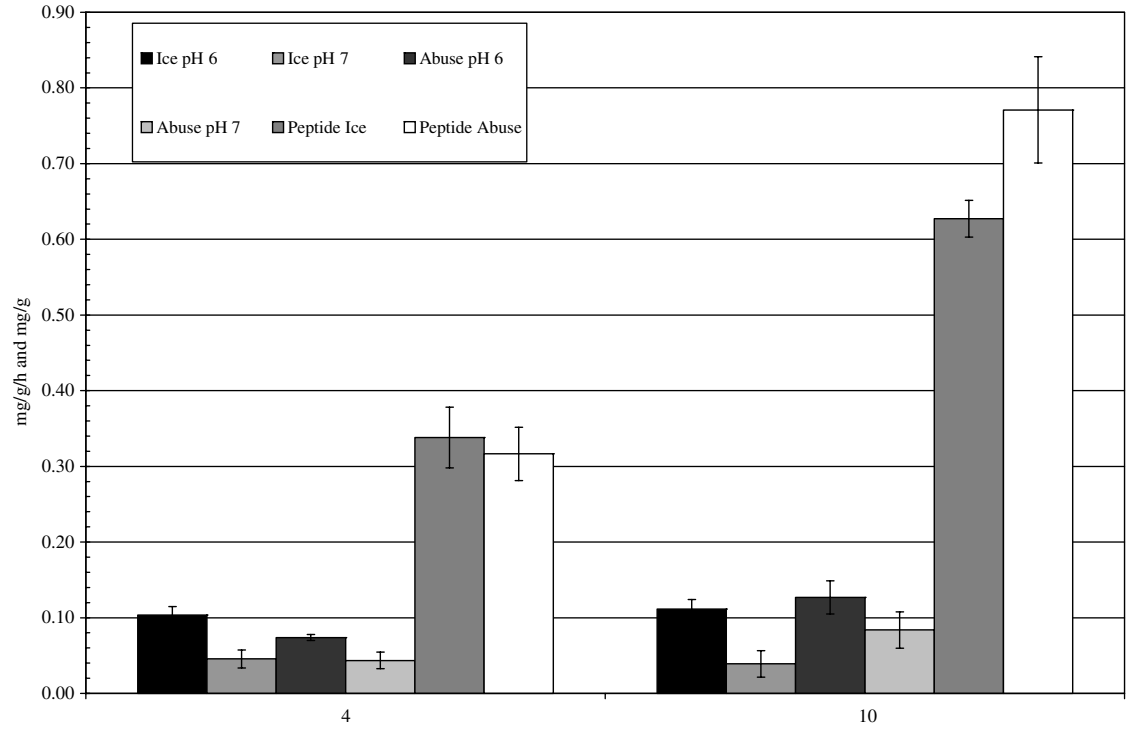


Fig. 4. General proteolytic activity and content of TCA soluble peptides in farmed cod fillets during storage, given as mg TCA soluble peptides liberated/g wet weight/hour and mg TCA soluble peptides/g wet weight, respectively. Bars indicate standard error of the mean ($n = 6$). Ice: iced storage throughout 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.

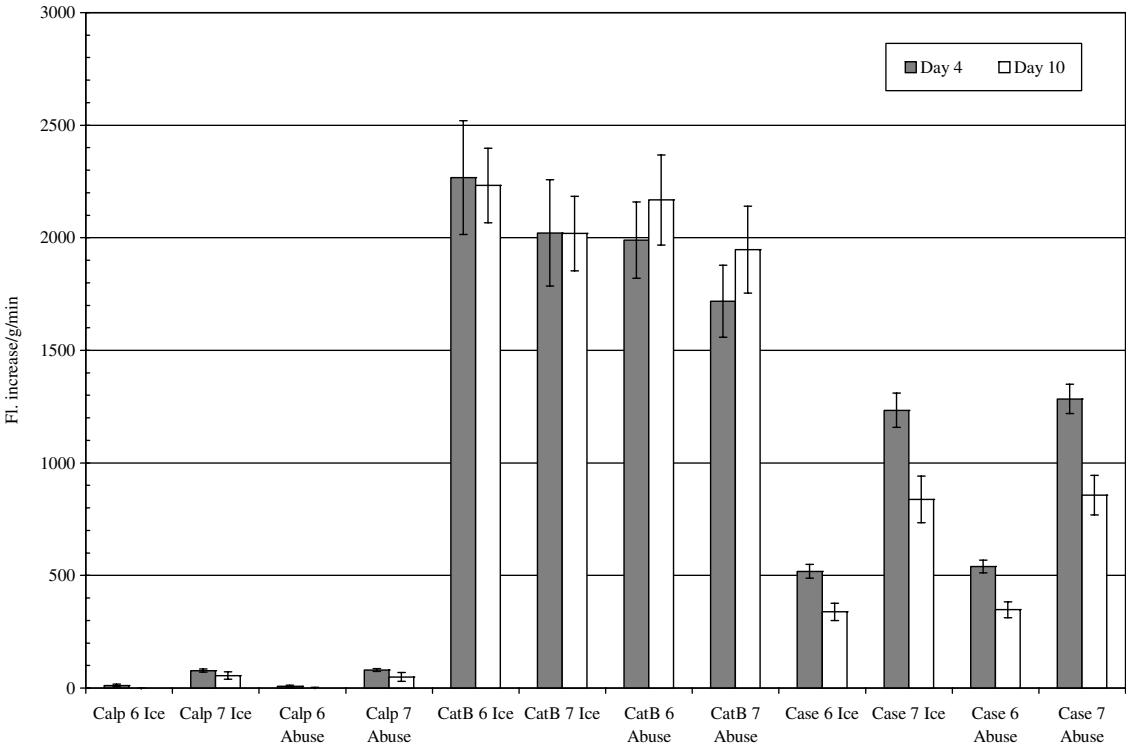


Fig. 5. Activity of specific proteolytic enzymes in farmed cod fillets during storage, given as increase in fluorescence intensity/g wet weight/minute. Bars indicate standard error of the mean ($n = 6$). Calp: calpain-like enzymes; CatB: cathepsin B-like enzymes; Case: collagenase-like enzymes. Numbers 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.

7.0) early in the storage period (day 4, Table 2). Collagenase-like activity increased slightly due to temperature abuse, but this was not significant. The cathepsin B-like activity was not significantly altered during storage, 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 & Rustad, 2002).

Calpains from Chinook salmon muscle has been studied (Geesink et al., 2000). Little proteolysis of myofibrillar proteins during storage were found, 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. In sea bass, m-calpain activity decreased during storage, while calpastatin activity remained constant (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004a). This is in agreement with the low and decreasing (available) calpain activity found in the present experiment, which was also found in wild cod (Hultmann & Rustad, 2002). In a study of *in vitro* proteolysis of myofibrillar proteins from sea bass, it was found that calpains did not modify any muscle protein prior to later hydrolysis by cathepsins (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004b). Other studies suggest that the softening of cod muscle observed during iced storage is caused more by collagenase activity than by proteolysis of myofibrils (Hernandez-Herrero et al., 2003; Montero & Mackie, 1992; Shigemura, Ando, Tsukamasa, Makinodan, & Kawai, 2003). Changes in intramuscular collagen of cod during iced storage has also been investigated (Montero & Mackie, 1992). 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 & 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 significantly higher at pH 6.0 than at pH 7.0. This pattern is in agreement with results obtained with 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 & Konagaya, 1990), for calpains from sea bass muscle (Ladrat et al., 2000), and for collagenolytic enzymes from the muscle of winter flounder (Teruel & Simpson, 1995) and Pacific rockfish (Bracho & Haard, 1995). Altogether, this indicates that the proteolytic profile in a given sample will be highly dependent on the sample pH.

The strength of the myocommata also depends on the surrounding pH and temperature (Love, Lavety, & Garcia, 1972). 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 & 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

Table 3
Effects of pH on 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

| | Ice | | | | Abuse | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 4 | | Day 10 | | Day 4 | | Day 10 | |
| | *d* | *p* | *d* | *p* | *d* | *p* | *d* | *p* |
| General proteolytic activity | −0.06 | **0.003** | −0.07 | **0.006** | −0.03 | **0.022** | −0.04 | *0.097* |
| Calpain-like activity | 66.0 | **0.000** | 60.5 | **0.004** | 72.7 | **0.000** | 51.9 | **0.010** |
| Cathepsin B-like activity | −245.4 | **0.004** | −213.2 | **0.001** | −271.6 | **0.012** | −220.8 | **0.002** |
| Collagenase-like activity | 715.4 | **0.000** | 499.9 | **0.000** | 744.2 | **0.000** | 509.1 | **0.000** |

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.

in the muscle. The observed textural changes may therefore be a result of changes in the connective tissue.

## 4. Conclusions

The temperature abuse caused a decrease in the fillets' ability to regain shape and structure when compressed, and these fillets showed characteristics observed with iced fillets after a longer storage period. This difference was not reflected in the protein extractability properties. 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 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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