Farmed Atlantic cod (*Gadus morhua* L.). Comparative studies on fillets produced pre- and post-rigor and on the drip lost during ice storage.

MASTER THESIS IN FISHERY SCIENCE

SEAFOOD SCIENCE
60 credits

by Birthe Vang

Department of Marine Biotechnology
Norwegian College of Fishery Science
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Acknowledgements

This master thesis was carried out in Institute for Marine Biotechnology (IMAB) at the Norwegian College of Fishery Science and marks the end of 5 long, but amazing years here at NFH. There are several people that have contributed in various ways in this thesis.

First and foremost I would like to express my gratitude to my supervisor professor Ragnar L. Olsen. For you're support, extensive knowledge, patience and good advice I am truly grateful!

To my supervisor Silje Kristoffersen, thank you teaching me everything in the lab, especially Murphy's law. I couldn't have done this without you!

To my friends; Kristine for keeping me sane. Lisa, Alice, Ingvill, Hanne for cheers along the way, and everybody else at C-458 and C-268 you are the best!

And finaly to my family, especially my parents, my brother Eirik and my boyfriend Peter. You're love, support and encouragement has meant the world to me!

Sammendrag

Atlantisk torsk (*Gadus morhua* L.) er en lovende og relativt ny art i norsk fiskeoppdrett. Det er flere fordeler forbundet med oppdrett av torsk, blant annet en jevn tilgang av fersk torsk hele året og at den økende etterspørselen kan møtes uten at det trenger å gå ut over torskebestandene. Forbedrede slakteprosedyrer med blant annet reduksjon av ante mortem stress gir mulighet til å prosessere fisken før den går inn i dødsstivhet (rigor mortis). Det vil si filetering oftest innen 4 timer etter slakting. Slik filetering fører til at filetspaltingen, som ofte er et problem hos oppdrettstorsk, reduseres. Pre-rigor filetering vil føre til reduserte transportkostnader og at den ferske torsken blir tilgjengelig for kunden ved et tidligere tidspunkt. Det fins lite kunnskap om hvordan pre-rigor filetering påvirker drypptapet fra torskefiletene, men den lave vannbindingsevnen som er rapportert i torsk antyder at det er mer alvorlig enn for laks.

Målet med denne masteroppgaven var å undersøke vekt og lengde forandringer under is-lagring av ferske fileter av oppdrettstorsk. I tillegg ble biokjemiske egenskaper som pH, vanninnhold og vannbindingsevne til fileten bestemt. Proteinkonsentrasjonene, gelatin degraderende enzymer og cathepsin D i muskel og drypp tap ble også undersøkt.

I oppgaven ble 24 oppdrettstorsk av god markedsstørrelse (3-3,5 kg) slaktet med slag til hodet og utblødning i vann før sløying. Tolv fisk ble filetert pre-rigor innen 2 timer etter slakting og 12 fisk ble filetert post-rigor (5 dager etter slakting). Den sløyde fisken, og filetene i lukkede plastposer ble lagret i is i til sammen 11 dager etter slakting.

Resultatene viser at den pre-rigor fileterte oppdrettstorsken hadde et vekttap på 10 % etter en lagringsperiode på 11 dager. Dette tapet var dobbelt så stort som hos de like gamle post-rigor produserte filetene. Det store vekttapet kan indikere at pre-rigor produksjon av skinnfrie fileter fra oppdrettstorsk, kanskje ikke er det mest økonomisk lønnsomme foredlingsmåten. Sammentrekningen i de pre-rigor produserte filetene resulterte i en krymping av fileten på 27 % dag 2 etter slakting. På slutten av lagringsperioden var krympingen av fileten redusert til 19 %, mens de post-rigor produserte filetene kun hadde blitt forkortet med 4 %. Formen på de pre- og post-rigor produserte filetene var også ganske forskjellig.

Både myofibril og sarkoplasmatiske proteiner i muskel syntes å være stabile under lagringsperioden. Men mer følsomme metoder enn generell proteinfarging, er nødvendig for å få detaljert kunnskap om post mortem forandringer i disse muskelproteinene. Proteinene i drypp tapet var veldig likt de sarkoplasmatiske proteinene fra muskel ekstraktene, men noe degradering av disse kunne spores i dryppvæsken. Både cathepsin D og de gelatin degraderende enzymene ble funnet i dryppene. På samme måte som ved de sarkoplasmatiske proteinene, virket disse enzym aktivitetene mindre stabile i dryppene enn i muskelvevet under lagringen.

Summary

Atlantic cod (*Gadus morhua* L.) is a promising and relatively new species in Norwegian fish farming. Benefits of cod farming includes stable deliveries of fresh cod all year round, and that the supply of a growing marked can be met without depletion of the cod stocks. Improved slaughter procedures by live chilling and reduction of ante mortem stress, give the opportunity to process the fish pre-rigor, usually within 4 hours pm. Such processing not only reduces fillet gaping which often is a serious problem in well-fed cod fishes filleted post-rigor, but may also lower the transportation costs and make the fresh fillets available to the markets at an earlier stage. Little is known about how pre-rigor filleting affects the drip loss of cod fillets, but the low WHC reported for cod might suggest that it is more severe than in salmon.

The aims of this thesis were to determine weight and length changes during ice storage of farmed Atlantic cod fillets produced pre- and post-rigor. In addition, biochemical characteristic such as pH, water content and water holding capacity (WHC) of the fillets were determined. The concentrations of proteins, gelatine degrading enzymes and cathepsin D in the muscle and drip losses during storage were also investigated.

In this thesis, 24 farmed Atlantic cod of good marked size (3-3,5 kgs) were slaughtered by stunning and bleeding, and then gutted. Twelve individuals were filleted pre-rigor (2 hours post mortem) and 12 post-rigor (day 5 post mortem). The gutted fish, and fillets in closed plastic bags, were then stored in ice for 11 days after slaughter.

The results showed that pre-rigor filleting of farmed Atlantic cod resulted in a 10 % weight loss during an 11 day long storage period after slaughter. This loss was twice the amount found in cod filleted post-rigor (5 days post mortem). Such a large weight loss may indicate that pre-rigor production of skinless fillets from farmed cod may not be an economical feasible practice for the industry. The rigor contraction of the pre-rigor produced fillet resulted in a shortening of 27 % on the second day post mortem. At the end of the storage, the shortening of the fillets were approximately 19 % while the post-rigor produced fillets merely had contracted 4 %. The shapes of the pre- and post-rigor produced fillets were quite different.

Both the myofibrillar and sarcoplasmatic proteins were apparently very stable during the ice storage. However, more sensitive methods than general protein-staining are necessary to obtain detailed knowledge on post mortem changes of these proteins in the muscles. The proteins present in the drip losses were very similar to the sarcoplamatic proteins extracted from the muscles. However, some degradation could be detected to have occurred in the drips. Both cathepsin D and gelatine degrading enzymes were detected in the drip losses. As with the sarcoplasmatic proteins, these enzyme activities appeared to be less stable in the drips than in the muscles

Content

1. Introduction

Atlantic cod (*Gadus morhua* L.) is a promising and relatively new species in Norwegian fish farming. In 2006 Norway exported a total volume of 1.9 million tonnes seafood with a value of 35.6 billon NOK, and the income from fish farming did for the first time exceed the income generated from traditional fisheries (Norwegian Seafood Export Council, 2007). Benefits of cod farming includes stable deliveries of fresh cod all year round, and that the supply of a growing marked can be met without depletion of the cod stocks.

Most often both farmed and wild caught fish have been filleted after the resolution of rigor mortis (rm), approximately 3 to 5 days post mortem (pm). Improved slaughter procedures by live chilling and reduction of ante mortem stress, give the opportunity to process the fish pre-rigor, usually within 4 hours pm. Such processing not only reduces fillet gaping, but may also lower the transportation costs and make the fresh fillets available to the markets at an earlier stage (Sigholt *et al.*, 1997; Robb *et al.*, 2000; Skjervold *et al.*, 2001a; Skjervold *et al.*, 2001b; Kristoffersen *et al.*, 2006b). However, such early filleting leads to length shortening of the fillet, observed both in salmon (*Salmo salar*) and cod. In farmed cod the shortening of the fillets are much more pronounced than in salmon (Sørensen *et al.*, 1997; Einen *et al.*, 2002; Kristoffersen *et al.*, 2006a). In addition, farmed cod show a significantly lower water holding capacity (WHC) than salmon (Ofstad *et al.*, 1996; Kristoffersen *et al.*, 2006a). Little is known about how pre-rigor filleting affects the drip loss of cod fillets (Tobiassen *et al.*, 2006), but the low WHC reported for cod might suggest that it is more severe than in salmon.

A low WHC results in excessive loss of protein containing fluid, called drip, purge or exudate. Large amount of drip is not only unattractive to the consumers because it may affect appearance, juiciness, texture and flavour, but also important commercially since meat is sold by weight (Offer *et al.*, 1984). In the meat industry, this is so important that especially designed methods to determine drip loss have been implemented (Christensen, 2003; Correa *et al.*, 2007).

The mechanisms behind the post mortal changes in fish are not fully understood, but it is assumed that endogenous proteolytic enzymes are of importance. These enzymes from the calpain system and lysosomal cathepsins like cathepsin D (Huff-Lonergan & Lonergan, 2005; Geesink *et al.*, 2006; Toyohara & Makinodan, 1989; Geesink *et al.*, 2000; Verrez-Bagnis *et al.*, 2002; Saito *et al.*, 2007; Yamashita & Konagaya, 1990; Aoki & Ueno, 1997;

Ho *et al.*, 2000; Ladrat *et al.*, 2003; Cheret *et al.*, 2007) as well as connective tissue degrading enzymes like Matrix-metalloproteinases (MMPs) (Bracho & Haard, 1995; Saito *et al.*, 2000; Delbarre-Ladrat *et al.*, 2006; Olsson *et al.*, 2006), have been implicated in the post mortem degradation process of fish muscle.

The overall aim of this thesis was to investigate the drip loss from pre-rigor produced fillets of farmed Atlantic cod.

Specific goals:

1. Determine weight and length changes during ice storage of fillets produced pre- and post-rigor.

2. Analyse the biochemical characteristic such as pH, water content and water holding capacity (WHC) of the fillets.

3. Analyse the content of proteins, gelatine degrading enzymes and cathepsin D in the muscle and drip, and changes occurring in proteins and these enzymes during ice storage.

2. General background

2.1   General muscle structure

The skeletal muscles in teleost fish run on both sides of the body, extending from head to tail, and are divided into muscle segments called myotomes, separated from each other by connective tissue called myocommatas (figure 1).
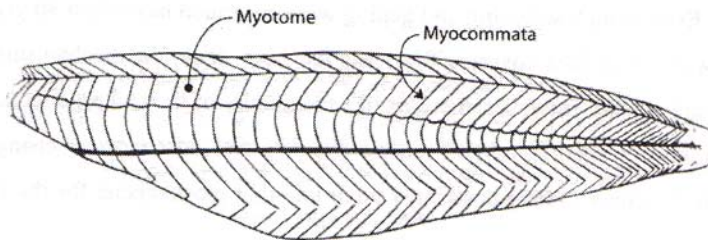
Figure 1. The muscle structure in a fillet from Atlantic cod (*Gadus morhua* L.) after removal from the skeleton (Adapted from Love 1969).

The myotomes essentially consist of parallel running muscle fibres (muscle cells) that are at each end connected to myocommata by fine collagenus fibres. In addition to connecting the myotomes, the myocommata is also connected to the skeleton and the skin.
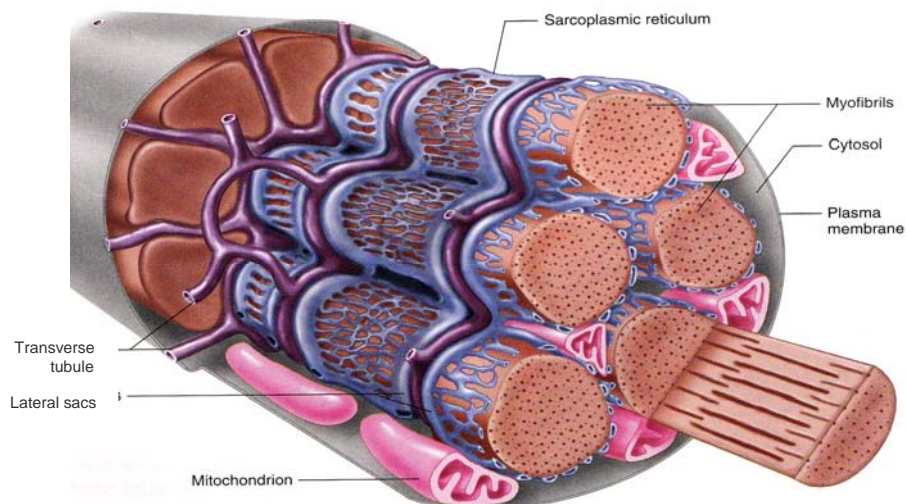
Figure 2. The transverse tubules (T-tubule) and sarcoplasmic reticulum structure surrounding the bundles of parallel running myofibrils in one skeletal muscle fibre (Widmaier *et al.*, 2004).

Within each muscle cell there are bundles of parallel running myofibrils (figure 2). The sarcoplasmic reticulum (SR) forms a series of segments around each myofibril. A separate

tubular structure is the transverse tubule (T-tubule) that crosses the myofibrils at the level of the Z-line. T-tubule is continuous of extracellular matrix surrounding the muscle fibres. Major constituents of the extracellular matrix include collagens, non-collagenus glycoproteins and proteoglycans (Lødemel, 2004).
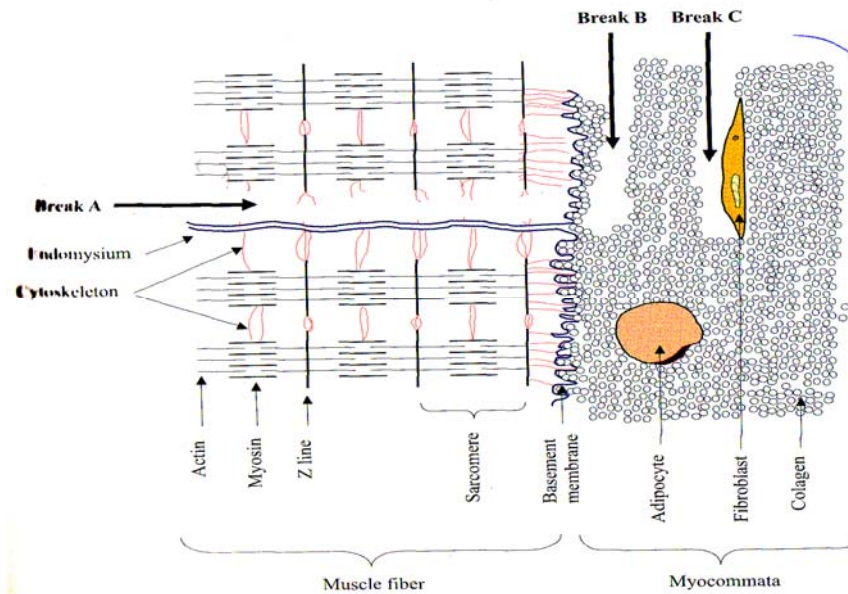

Figure 3. Close-up illustration of a part of myocommata and a myotome. Outside the plasma membrane (sarcolemma) there is a tight mesh called the basement membrane. Outside the basement membrane, there is a thin layer of connective tissue surrounding each muscle cell called endomysium. The endomysium connects to the perimysium, which is contiguous with the myocommata (Adapted from Skjervold, 2002)

Outside the plasma membrane (sarcolemma) there is a tight mesh called the basement membrane (figure 3). The basement membrane predominantly consists of collagen IV. The endomysium consists mainly of collagen I and V and is a thin layer of connective tissue outside the basement membrane, surrounding each muscle cell. The endomysium connects to the perimysium, which is contiguous with the myocommata (Ofstad *et al.*, 2006) Perimysium and endomysium are a part of the intramusclular connective tissue (IMCT) along with the myocommata. Break A, B and C indicates ruptures in the connective tissue (fillet gaping).
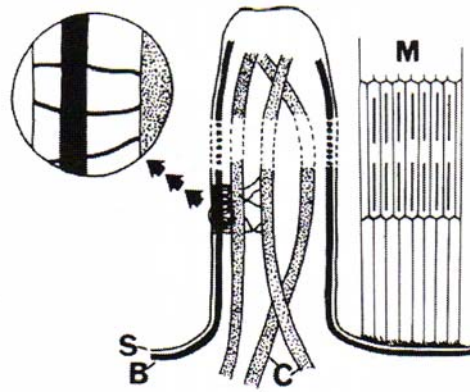
Figure 4. Myofibrills (M) are connected to the sarcolemma (S) of the muscle cell by fine filaments. The basement membrane (B) is tight mesh located outside the sarcolemma (cell membrane). The collagen fibres (C) are connected to the sarcolemma and basement membrane by microfilaments (Adapted from Hallet & Bremner, 1988).

Myofibrills are connected to the sarcolemma (cell membrane) of the muscle cell by fine filaments (figure 4). The basement membrane is tight mesh located outside the sarcolemma. The collagen fibres are connected to the sarcolemma and basement membrane by microfilaments.

2.2 Structure of the contractile apparatus
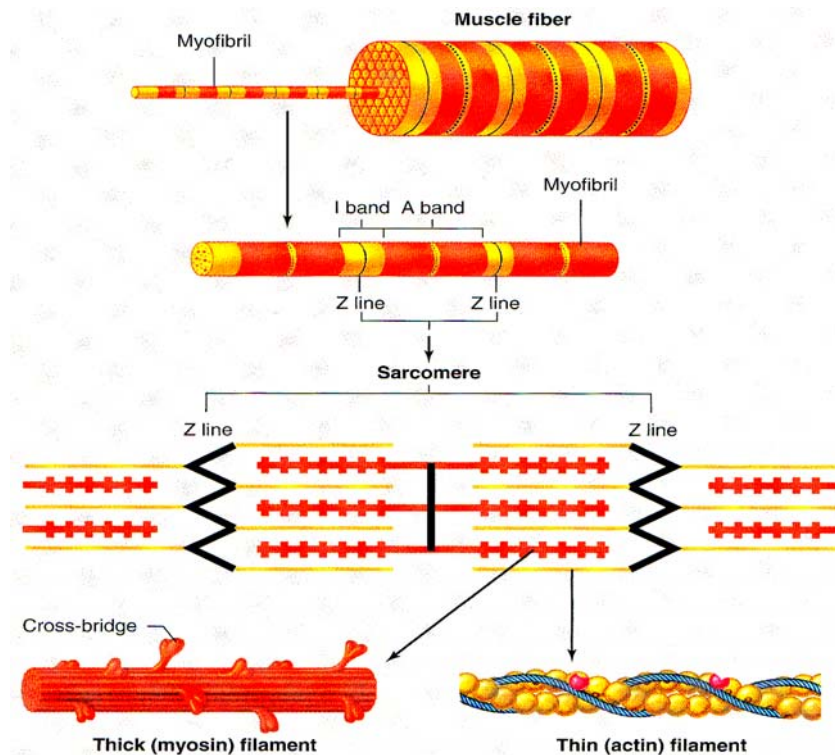
Figure 5. The muscle fibres consist of a bundle of myofibril filaments. Each myofibril is divided into a repeating structural unit called sarcomere. The sarcomere is composed of thick myosin filaments and thin action filaments, witch extends between two adjacent Z lines (Widmaier *et al.*, 2004).

Each myofibril is divided into a repeating structural unit called sarcomere composed of many actin and myosin filaments (figure 5). The myosin filaments are located in the middle of each sarcomere and are known as the A-band. The sarcomere contains two sets of actin filaments which overlap the myosin filaments and are anchored to interconnective proteins called the Z-line. One sarcomere extends between two adjacent Z lines. The I-band extends between to A-bands of two adjacent sarcomere and consists of actin filaments and the Z-line. The H-zone is in the middle of the A-band and corresponds with the distance between the two sets of actin filaments in each sarcomere. In the centre of the H-zone a linkage between the adjacent myosin filaments called the M-line is located.

Figure 6. Contraction of the skeleton muscle. The overlapping thick myosin filament and the thin actin filament in each sarcomere m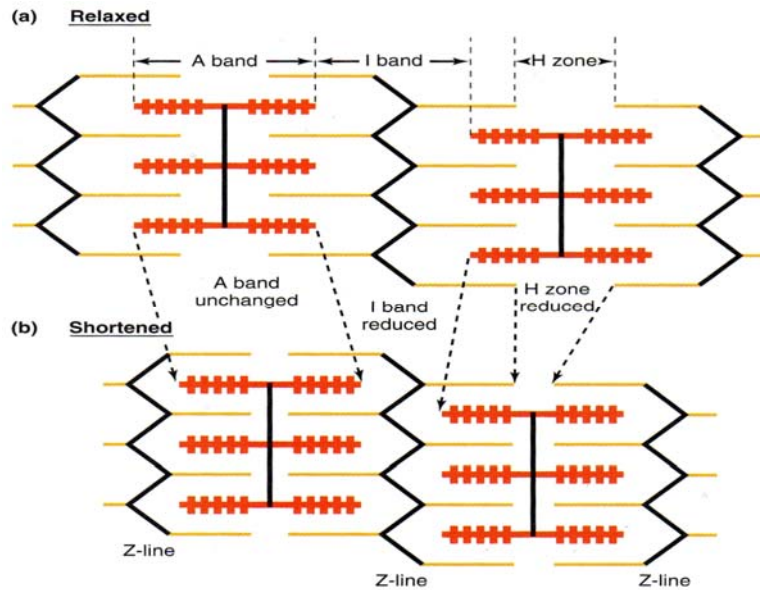ove past each other. During this shortening of the sarcomere there are no change in the myosin or actin filaments, but the I-band and the H-zone are reduced (Widmaier *et al.*, 2004).

When skeleton muscle contracts, the overlapping myosin filament and the actin filament in each sarcomere move past each other in the sliding-filament mechanism forcing the actin filaments towards the centre of the sarcomere (figure 6). During this shortening of the sarcomere there are no changes in the myosin or actin filaments. The A-band and Z-lines are unchanged, but the I-band and the H-zone are reduced.

In the skeletal muscle much of the water can be found in the intra- and extramyofibrillar spaces, namely within and between the myofibrils, between the myofibrils and the cell membrane. In addition, water is also found in the extracellular space between muscle cells and between muscle bundles. A change in the cell structure caused by rigor mortis contraction can therefore influence the ability of muscle cells to retain water (Huff-Lonergan & Lonergan, 2005). The ability of muscles to resist water loss is referred to as liquid- or water holding capacity (WHC) and it is most often determined as liquid loss after a centrifugation procedure (Ofstad *et al.*, 1996).

In addition to proteins (Honikel *et al.*, 1986; Savage *et al.*, 1990; den Hertog-Meischke *et al.*, 1997), other water soluble nutrients are also lost in the drip (Lambert *et al.*, 2001; Meade *et al.*, 2005; Larsen *et al.*, 2007). The liquid holding capacity or drip loss of fish muscle is species dependent, and has also been reported to be influenced by season and is often related to the nutritional status affecting the ultimate muscle pH. Intensive feeding leads to low muscle pH which results in a low WHC (Ang & Haard, 1985; Ingòlfsdòttir *et al.*,

1998). Low muscle pH alone is however not the only reason for a reduced WHC (Rustad, 1992; Olsson *et al.*, 2003b). It has been shown that post mortem rate of muscle degradation is of importance since pronounced degradation resulted in a reduced WHC (Ofstad *et al.*, 1996; Olsson *et al.*, 2003a). Ante mortem stress may also reduce the WHC in fish muscle, but it is not known whether this is due to a lower muscle pH or to a stronger rigor mortis (rm.) contraction (Kiessling *et al.*, 2004).

Limited knowledge is available about the mechanisms behind the development of drip loss in meat, but it has been hypothesized that protein linkages within the muscle cell are of importance and that the calpain system play a major role in the post mortem proteolysis (Huff-Lonergan & Lonergan, 2005; Geesink *et al.*, 2006). Far less is known about the role of the calpain system in fish muscle (Toyohara & Makinodan, 1989; Geesink *et al.*, 2000; Verrez-Bagnis *et al.*, 2002; Saito *et al.*, 2007). Other proteolytic enzymes such as cathepsins B, D and L (Yamashita & Konagaya, 1990; Aoki & Ueno, 1997; Ho *et al.*, 2000; Ladrat *et al.*, 2003; Cheret *et al.*, 2007) and connective tissue degrading enzymes like Matrix-metalloproteinase (MMPs) (Bracho & Haard, 1995; Saito *et al.*, 2000; Delbarre-Ladrat *et al.*, 2006; Olsson *et al.*, 2006), have also been implicated in the post mortem degradation process of fish muscle.

3. Materials and methods

3.1   Cod used in the experiments

Farmed Atlantic cod (*Gadus morhua* L.) with an average weight of 1.7 kg were delivered in November 2003 to Aquaculture Research Station (Kårvika, Tromsø) and fed Marine Feed (Skretting, Norway), containing 18% fat and 52% protein until slaughter. At slaughter in March 2006, the fish were carefully netted and stunned by a blow to the head followed by cutting of the isthmus and bled in freshwater (4ºC) for 30 min before gutting. The biological data were obtained at the time of gutting and are presented in Table 1. The fork length, total body weight, the weight of liver and the gonads were used to calculate Fulton's condition factor (K-factor) [(total body weight (g) / fork length (cm) ^{3}) x 100], the hepatosomatic index (HSI) [(liver weight (g) / total body weight (g) x 100)] and the gonadosomatic index (GSI)) [(gonad weight (g) / total body weight (g) x 100)].

Twelve cod (8 ♀, 4 ♂) were hand filleted and skinned pre-rigor (2 hours post-mortem) and the fillets were stored separately in pre-weighed sealed plastic bags in ice. In addition, 12 cod (6 ♀, 6 ♂) were stored as gutted fish in ice with the belly down to avoid accumulation of ice-water in the abdominal cavity. After 5 days storage, the gutted post-rigor fish were filleted and skinned and the fillets were stored separately as described above. The average fillet weight of pre- and post-rigor fillets were 436 ± 85 grams (n = 24) and 412 ± 93 grams (n = 24) respectively. During the storage period, all fillets and the gutted fish were stored in ice in a cold room (4ºC). At filleting (day 0 and 5 pm), the fillets were trimmed (approximately 1-2 cm at each end), and the cut offs were used for determining the water content of the fillets. The length and weight of each fillet were then measured and subsequently measured each day throughout the storage period. The volume of the drip loss from each fillet was measured daily during the storage period and the combined drips stored at -20ºC for later analysis. For each pre-rigor produced fillet, the drips from the first 5 days (day 1-5 pm) and from day 6-11 pm, were combined separately. For each post-rigor produced fillet all drips (day 6-11 pm) were combined.

At the end of the storage period (11 days pm) the fillets were minced in a food processor before determining the water holding capacity. The minces were frozen at -20ºC and used for later analysis. The water content of each fillet was determined before mincing.

3.2   General methods

The pH of the muscle was determined using a WTW pH-meter (pH 330, Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany) equipped with a Hamilton double pore glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) which were inserted into the cut side of the fillet. The pH in the pooled drip losses were measured by using the same electrode. The water content was determined after drying triplicates of each fillet sample at 105 °C for 24 hours. The water holding capacity was determined on each fish (6 replicates) at day 11 pm. by the centrifugation method described by Rørå (2003) and expressed as liquid loss on wet weight basis.

In most cases, the proteins concentrations were determined using the Bio-Rad Detergent Compatible Protein Assay (Bio-Rad, Herkules, CA, USA) using bovine serum albumine as standard protein. The protein concentrations in gelatine bound fraction from the affinity chromatogrphy were determined at OD 280 nm using Nanodrop-ND 1000 (Nanodrop Technologies Inc. Wilmington DE, USA). An absorbance of 1.0 was defined as a protein content of 1.0 mg/ml.

3.3   SDS-PAGE and Western blotting

Muscle and drip loss were homogenized by ultra sonication (30 sec. 4°C) in four volumes of sodium dodecyl sulphate (SDS) sample buffer (0.1 M Tris-HCl pH 6.8, 4 % sodium dodecyl sulphate, 20 % glycerol) lacking dithiotreitol (DTT) and bromphenolblue. Isolation of myofibril and sarcoplasmic proteins from muscle were performed as described by Cao (2006) with minor modifications. Frozen, minced muscle was homogenized in 4 volumes of ice-cold 20 mM phosphate buffer, pH 7.5 and clarified by centrifugation for 10 min. at 4000xg (4ºC). The supernatant was collected as the sarcoplasmic protein fraction and stored at -80ºC. The pellet was then washed 4 times with 4 vol. of the same buffer and centrifugation conditions as above. The myofibrillar proteins were obtained by re-suspending the pellet in 4 vol. of 20 mM phosphate buffer with 0.5 M NaCl, pH 8.0. Prior to protein determinations, the samples were mixed with equal volume of SDS sample buffer, heated at 95-100°C for 2 min and centrifuged for 10 min at 14 000 rpm.

SDS-PAGE was performed according to Laemmli (1970). Protein adjusted aliquots (125 µl) of the muscle and drip loss samples, were added 4 µl 0.1M dithiotreitol (DTT) and 1 µl 0.1% bromphenolblue (BFB) and boiled for 5 min. For the SDS-PAGE analysis, samples

(15 µl/12 µg protein) were run on 12 % SDS-polyacrylamide gel along with the pre-stained electrophoresis standards (Bio-Rad) in a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). The gels were stained with 0.1 % Coomassie Brilliant Blue in a solution of 45 % methanol and 10 % acetic acid and destained in a solution of 10 % methanol and 10 % acetic acid.

For the Western blot, samples (15 µl per well) were prepared as described by Wang *et al.* (2007) and run on 10 % SDS-polyacrylamide gel along with the MagicMark XP Western Protein Standard (Invitrogen, Berkeley, CA, USA) in a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA) as described by Towbin *et al.* (1979) by electrophoretic blotting (90 min. at 400 mA) in a Bio-Rad Mini Transblot Electrophoretic Transfer Cell. The incubation conditions and detection were performed as described by Wang *et al.* (2007). Membranes were then incubated in blocking solution (5 % skimmed milk powder in TTBS (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.3 % Tween 20) for 1h at room temperature. The primary antibody used (3300 x dilution) had been raised against purified Atlantic cod liver cathepsin D (Wang *et al.*, 2007). The membrane was incubated with primary antibody diluted in blocking solution for 20h at 4°C. The membrane was washed three times in TTBS and incubated 1h at room temperature with Horseradish peroxidise-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:10 000 in TTBS as the secondary antibody. The membrane was washed three times in TTBS and once in distilled water before detection was performed using the Super Signal West Pico Chemiluminiscent Substrate (Pierce, Rockford, USA) and the membrane was exposed to autoradiography film, as described by Wang *et al.* (2007)

3.4   *Gelatine degrading enzymes*

3.4.1 Fractionating of gelatine degrading enzymes

The gelatinolytic activities in muscle and drip loss were fractionated by gelatine Sepharose affinity chromatography as described by Lødemel and Olsen (2003). Minced fillets (2 g) were homogenised in 10 ml extraction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.05 % Brij-35 and 0.02 % NaN_3) and agitated for 30 min at 4 °C prior to centrifugation (4000 x g, 30 min, 4 °C). The supernatants (muscle extracts) were stored at – 50 °C prior to use.

Muscle extract and drip loss were added to 1 ml of gelatine Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) and slowly agitated for 30 min prior to centrifugation (2000 x g, 1 min, 4°C). The supernatants containing the unbound proteins were stored at -50°C until analysis. The gelatine Sepharose pellet was re-suspended in 7 ml of extraction buffer and centrifuged (2000 x g, 1 min, and 4°C). This washing step was repeated twice. The gelatine bound proteins were released by adding 4 ml of extraction buffer containing 10 % dimethyl sulphoxide (DMSO), followed by mixing for 30 min and subsequent centrifugation (2000 x g, 2 min,4°C). The supernatant were concentrated 30-fold by centrifugation (4000 x g, 20 min, 4°C) using an Ultrafree-4 unit with a Biomax-10 membrane (Millipore, Billeria, MA, USA).

3.4.2 Gelatine zymography

The concentrated samples were diluted with an equal amount of modified Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 4 % SDS, 25 % glycerol and 1 % bromophenol blue). Non heated, non reduced samples (10 μl) were loaded into the wells of the SDS polyacrylamide gel (5 % polyacrylamide in the stacking gel and 9 % polyacrylamide in the separating gel). The protein contents of all samples were adjusted with sample buffer so that 25 μg protein was applied to each lane. The separating gel contained 0.1 % gelatine (Bio-Rad). The gels were run in a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad) placed in an ice bath. The gels were washed in 2.5 % Triton X-100 for 2 x 15 min prior to incubation in 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$ and 0.02 % NaN_3 for 20 h at 38°C. The gels were stained as the SDS-PAGE gels. The gelatinolytic activities were identified as clear zones estimated by visual inspection. The MMP standards used were a mix of serum free conditioned medium from PMA (phorbol 12-myristate 13-acetate) stimulated THP-1 macrophage cells and serum free conditioned medium from human skin fibroblasts. The former shows two bands in zymography, a monomer of 92 kDa and a homodimer of approximately 225 kD, identified as MMP-9 (92 kDa gelatinase). The latter medium is rich in MMP-2 (72 kDa gelatinase) with a proform at 72 kDa and an active form at 62 kD.

3.5   *Statistical analysis of results*

The t-test was used to determine the significance of differences between the samples.

4 Results

4.1 *Biological properties of the farmed Atlantic cod*

The round weights of the farmed Atlantic cod used in this thesis were 3477 ± 590 grams, and 3320 ± 587 grams in the pre- and post-rigor groups' respectively (Table 1). The gutted weights in the two groups were 55 % and 53 % of the round weights, respectively. The results showed that the gutted fish (the post-rigor group) did not experience any weight changes during ice-storage before filleting. The fillet yields based on round weights were about 25 %. The K-factor was 1.4 ± 0.1 and 1.3 ± 0.1 in the two groups' respectively. Hepatosomatic index (HSI) was 11.4 ± 2.4 and 9.5 ± 1.8. The gonadosomatic index (GSI) was 16.7 ± 2.1 and 20.3 ± 4.2. The combined liver and gonads accounted for 28-30 % of the round weights.

Table 1 Biological characteristic of the farmed Atlantic cod filleted pre-rigor (n = 12) and post-rigor (n = 12). Weight and length were determined at slaughter and fillet weight at time of filleting.

		Pre-rigor ♀= *8,* ♂= *4*	*Post-rigor* ♀= *6,* ♂= *6*
Weight (g)		3477 ± 590	3320 ± 587
Gutted weight (g)	Day 0	1915 ± 314	1747 ± 342
	Day 5	-	1755 ± 341
Fillet weight (g)		436 ± 85	412 ± 93
Fork length (cm)		62.7 ± 2.0	63.1 ± 4.0
K-factor		1.4 ± 0.1	1.3 ± 0.1
HSI		11.4 ± 2.4	9.5 ± 1.8
GSI		16.7 ± 2.1	20.3 ± 4.2

There was not the same number of male and female fish in the pre- and post-rigor groups of the fillets. Therefore, the biological properties were also calculated on basis of sex, independent of when the cod were filleted (table 2). The round weight in the female group of farmed Atlantic cod was 3491 ± 628 grams, and in the male group 3269 ± 513 grams. No differences were found in fillet yields, K-factors and GSI between the two sexes. However, the HSI was significantly higher (p < 0.01) in the female group. The liver and gonads accounted for 31 % of the round weights of the female and 27 % of the male round weights.

Table 2 Biological characteristic of the female (♀= **14)** and male **(**♂**= 10**) farmed Atlantic cod.

	♀= *10*	♂= *14*
Weight (g)	3491 ± 628	3269 ± 513
Gutted weight (g) Day 0	1843 ± 355	1815 ± 316
Fillet weight (g)	429 ± 93	417 ± 85
Fork length (cm)	62.7 ± 2.9	63.1 ± 3.2
K-factor	1.4 ± 0.1	1.3 ± 0.1
HSI	11.5 ± 2.1**	9.0 ± 0.1
GSI	19 ± 5	18 ± 2

** Significant difference between the female- and male farmed Atlantic cod ($p < 0.01$)

4.2 Drip loss from pre- and post-rigor produced fillets of farmed Atlantic cod

The amount of drip lost from pre- and post-rigor produced fillets during ice-storage for 11 days are shown in figure 7. The drip loss was estimated by measuring the weight reduction of fillets. Eleven days after slaughter, the pre-rigor fillets had lost drip equivalent to 10 % weight reduction, while the post-rigor produced fillets had lost approximately 5 %.
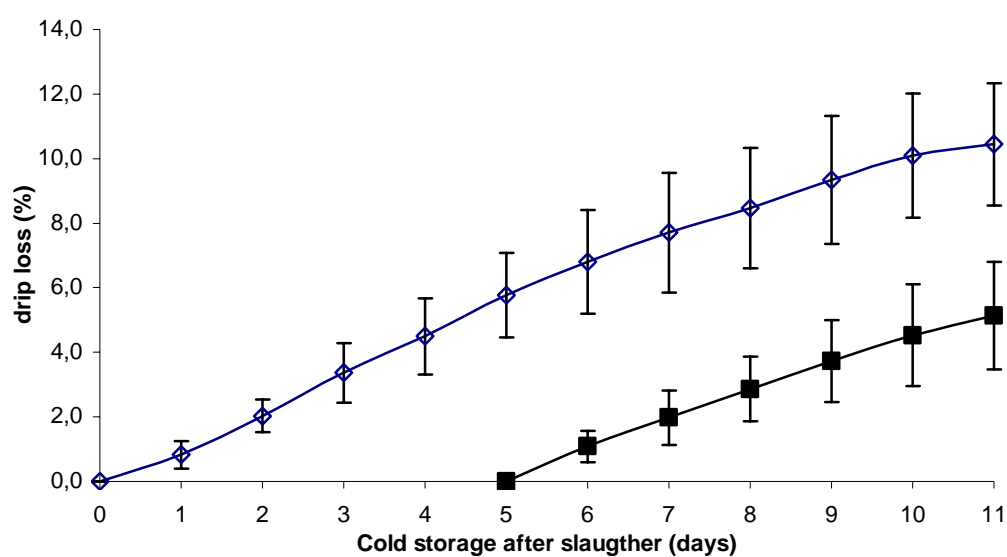

Figure 7 Drip loss (% weight loss) from fillets of pre- (◊) and post-rigor (■) filleted farmed Atlantic cod during cold storage. n = 24 in each group.

The accumulated drip loss was also related to time of filleting (figure 8). Both groups of fillets appeared to lose similar amount of drip during the first few days after filleting. However, on days 5 and 6 after filleting, the pre-rigor produced fillets lost significantly (* $p < 0.05$, ** $p < 0.01$) more drip.
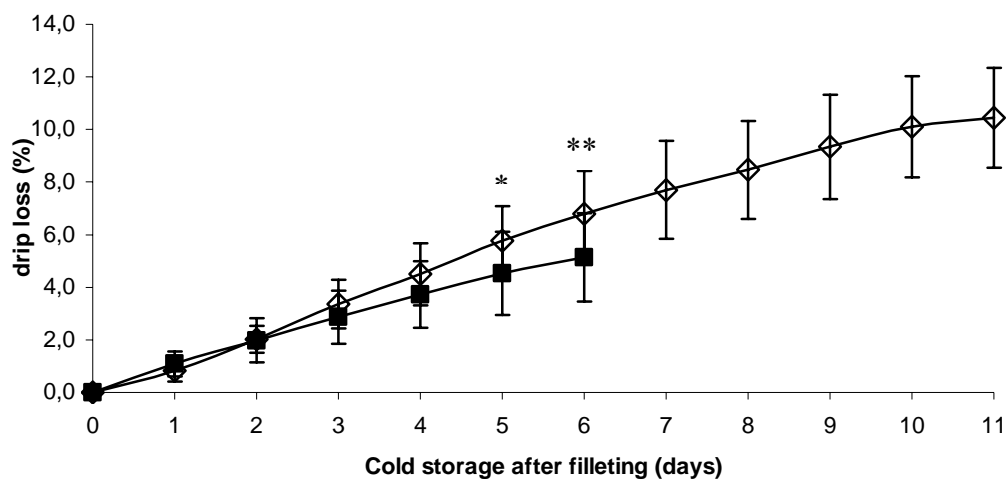

Figure 8 Drip loss (% weight loss) from fillets of pre- (◊) and post-rigor (■) filleted farmed Atlantic cod during cold storage. n = 24 in each group. * ($p < 0.05$), ** ($p < 0.01$) significant differences in drip loss between corresponding days after filleting for pre- and post-rigor produced fillets.

The daily weights losses from the fillets are expressed in table 3 as grams weight lost pr kilo fillet. On day 2 there were an increase in the drip lost from the pre-rigor produced fillets. This corresponds with the onset of rigor mortis, and the drip loss remained high in the following 4 days. In the post-rigor produced fillets the highest drip loss was experienced at day 1 after filleting, after this there was a reduction in the drip lost. On day 5 and 6 after filleting there were a significantly ($p < 0.01$) higher weight lost from the pre-rigor produced fillets, than from the post-rigor produced fillets.

Table 3 Daily weight loss (g/kg) from fillets of pre- and post-rigor filleted farmed Atlantic cod stored in ice.

Storage time as fillets (days)	Pre-rigor	Post-rigor
	n = 24	n = 24
1	8.3 ± 4.3	10.4 ± 4.8
2	12.0 ± 2.0	8.8 ± 5.1
3	13.6 ± 5.1*	8.8 ± 3.5
4	11.7 ± 3.8	8.4 ± 4.7
5	13.4 ± 3.9**	8.1 ± 4.0
6	11.0 ± 4.1**	6.0 ± 2.3
7	9.7 ± 5.0	
8	8.3 ± 2.6	
9	9.6 ± 4.2	
10	8.3 ± 2.8	
11	3.8 ± 3.5	

Significant difference between fillets from pre- and post-rigor groups * (p < 0.05) and ** (p < 0.01)

4.3 Length reductions of pre- and post-rigor produced fillets

Length reductions in fillets from farmed Atlantic cod after slaughter are shown in figure 9. The pre-rigor produced fillets had a high rigor contraction on the first day after slaughter as shown in figure 9. On day 2 pm the mean shortening of the fillets were 27 %. After this there was a reduction in the contraction that corresponded with the resolution of rigor mortis. On day 11 pm the reduction of the pre-rigor fillets had a mean value of 19 %. The post-rigor fillets showed a small shortening after filleting, and the length decreased steadily throughout the storage period. At day 6 pm (day 1 after filleting) the shortening of the post-rigor fillets had a mean value of 1 % and on day 11 pm the mean reduction value was 3 %.
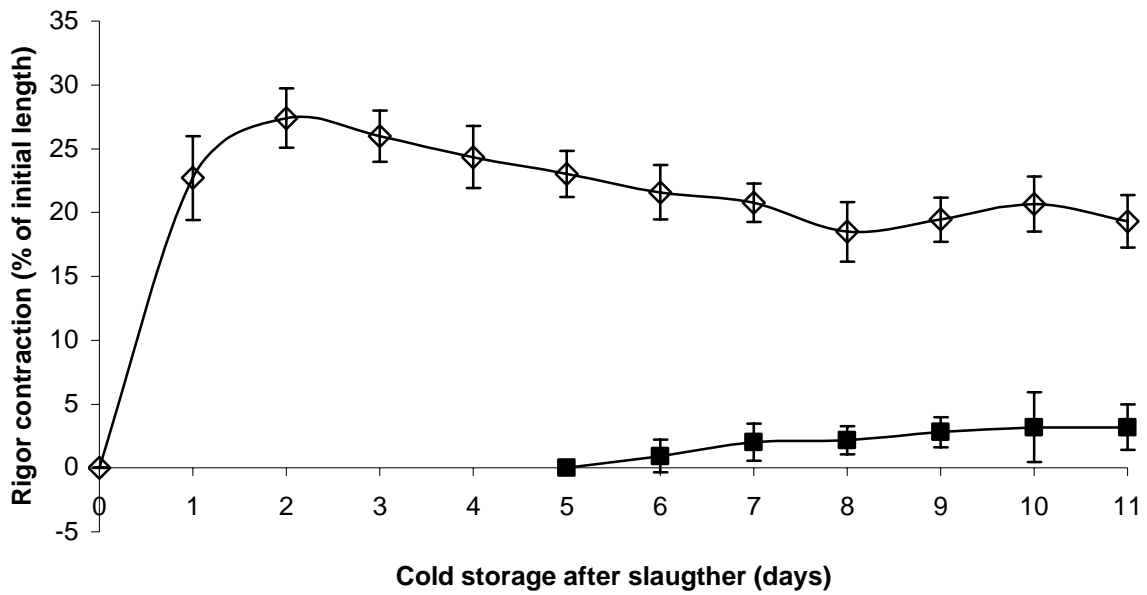
Figure 9 Development of rigor contraction (percentage of initial length) during cold storage of fillets from pre- (◊) and post-rigor (■) filleted farmed Atlantic cod, n = 24 in each group. The points and error bars denote means and standard errors, respectively.

The length reductions of the pre- and post-rigor produced fillets were also related to time of filleting, as shown in figure 10. As mentioned previously, the maximum rigor contraction of 27 % in the pre-rigor fillets was observed on the second day after filleting. At the same time after filleting the post-rigor produced fillets had a length reduction of only 2 %.
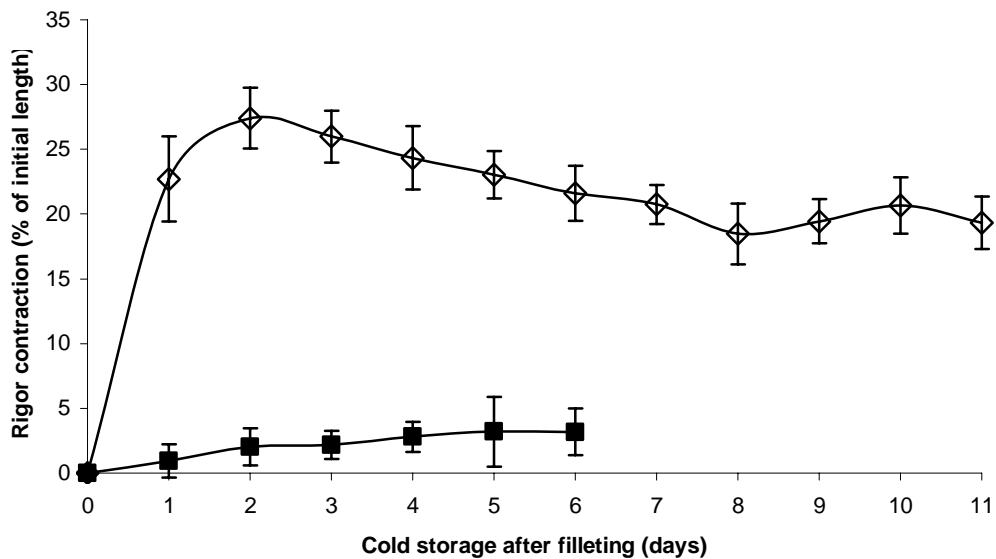

Figure 10 Development of rigor contraction (percentage of initial length) during cold storage of fillets from pre- (◊) and post-rigor (■) filleted farmed Atlantic cod, n = 24 in each group. The points and error bars denote means and standard errors, respectively.

The visual length reduction of pre-and post rigor produced fillets from the same fish on day 6 (144 hours) after slaughter is also documented (figure 11). The left fillet was filleted pre-rigor within two hours pm. The right fillet with skin on, was left on the vertebrate for 5 days before filleting (post-rigor filleting). The pre-rigor fillet was approximately 19 % shorter than the post-rigor fillet in this specific fish. This finding was similar to the results presented in figure 9 and 10. Figure 11 also illustrates the difference in shape between skin-free fillets produced pre- and post-rigor. It is possible that the pre-rigor produced fillets also are thicker, but this was not measured.

Figure 11 Illustration of the rigor contraction on fillets from the same fish day 6 after slaughter. The right (lower) fillet was filleted post-rigor while the left (upper) fillet was filleted pre-rigor.

4.4 Biochemical characteristics of pre- and post-rigor produced fillets

Biochemical characteristics of fillets from farmed Atlantic cod produced pre- and post-rigor were also examined (table 4). On day eleven, the water holding capacity (WHC) expressed as liquid loss was significantly ($p < 0.01$) lower (6.8 ± 1.8 %) in the pre-rigor produced fillets than in the post-rigor produced fillets (5.4 ± 1.2 %). There was also a significant difference ($p < 0.05$) in the muscle pH between the two groups. The pH-muscle values were 6.34 ± 0.05 and 6.40 ± 0.09 in the pre- and post-rigor groups respectively. The water content on day 11 pm were 78.7 ± 0.9 % and 79.6 ± 1.1 % in the pre- and post-rigor produced fillets respectively. The difference was not significant. The pH in the drip lost from

the pre-rigor produced fillets day 1-5 and day 6-11 were 6.59 ± 0.07 and 6.47 ± 0.38 respectively. The pH in the drip from the post-rigor produced fillets was 6.56 ± 0.13. The pH in the drips were higher compared to the muscle pH of the corresponding fillets. There was a slight increase in the amount of protein lost from pre-rigor fillets day 1-5 (0.42 ± 0.21 %) to pre-rigor fillets day 6-11 (0.47 ± 0.12 %). The post-rigor drip contained 0.46 ± 0.15 % protein.

Table 4 WHC (%) expressed as liquid loss (LL), water content (%) and pH in the pre- and post-rigor produced fillets. As well as pH and protein lost (g/100 g fillet) from the pooled drips from pre- and post-rigor produced fillets.

Days post mortem	Pre - rigor	Post - rigor
	n = 12	n = 12
WHC (LL)		
11	6.8 ± 1.8**	5.4 ± 1.2
Water content		
0	79.6 ± 0.9	-
5	-	80.6 ± 1.1
11	78.7 ± 0.9	79.6 ± 1.1
Muscle pH		
5	6.34 ± 0.05*	6.40 ± 0.09
pH in drips		
1 to 5	6.59 ± 0.07	-
6 to11	6.47 ± 0.38	6.56 ± 0.13
Protein lost in drip		
1 to 5	0.42 ± 0.21	-
6 to 11	0.47 ± 0.12	0.46 ± 0.15

Significant difference between pre- and post-rigor groups * ($p < 0.05$), ** ($p < 0.01$).

The relationship between protein concentration in drip and amount of drip lost from pre-rigor (day 1-5 pm), pre-rigor (day 6-11 pm) and post-rigor (day 6-11 pm) produced fillets expressed as weight loss (%) are shown in figure 12. The mean values of the drip lost from both fillets of each fish were used when determining the protein concentration in the drip loss. In the pre-rigor produced fillets day 1-5 drip there was a higher loss of drip compared to pre-rigor day 6-11. There are however no distinctive differences in the protein concentrations between the three groups.
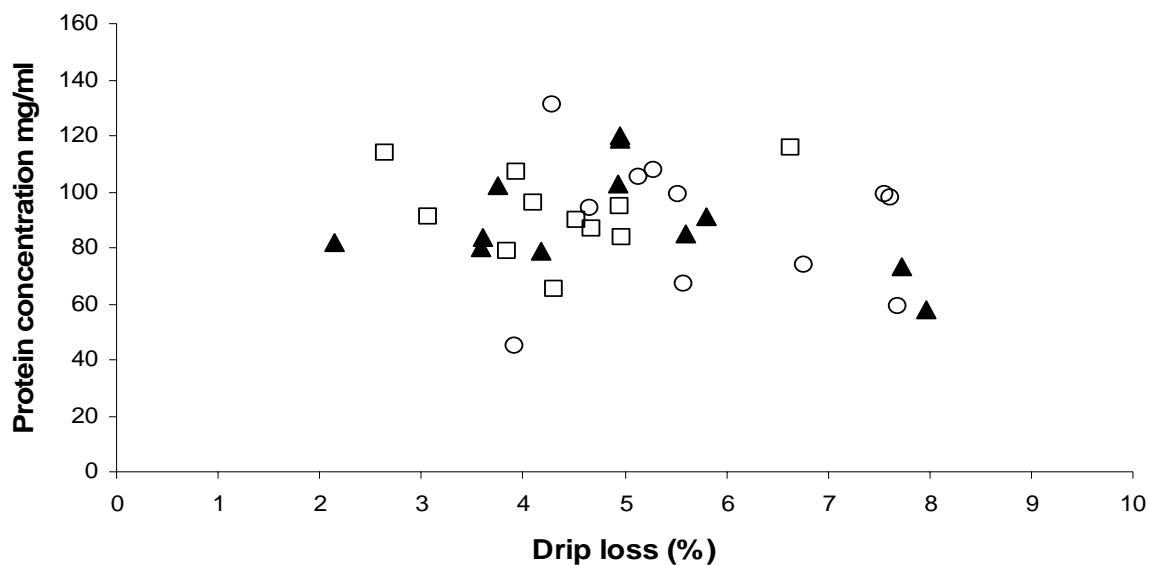
Figure 12 Relationship between protein concentration in drip and amount of drip loss (%) from (o) pre-rigor- (pooled from day 1-5 pm), (□) pre-rigor- (pooled from day 6-11 pm) and (▲) post-rigor (pooled from day 6-11 pm) fillets of farmed Atlantic cod. Each point is the mean values of the two fillets from each fish.

4.4.2 SDS-PAGE

SDS-PAGE analysis of proteins in the drip losses and of the myofibrillar and sarcoplasmatic proteins of the muscle from fillets are shown in figure 13. During these experiments fillets and drip losses from several cods were analysed. However, the results shown are the muscle protein patterns from one and the same fillet. From pre-rigor produced fillets drip losses from 3 fillets were investigated (days 1-5; lanes 5, 6 & 7 and days 6-11; lane 8, 9 & 10). From post-rigor produced fillets drip losses from 2 fillets were investigated (days 6-11; lanes 13 & 14). No detectable differences were observed between the sarcoplasmatic proteins extracted on day 0 and 11 pm from a pre-rigor produced fillet (lanes 3 & 4). Similar observations were made on sarcoplasmatic proteins extracted from a post-rigor produced fillet on day 5 and 11 pm (lanes 11 & 12). The SDS-PAGE analysis of the drip losses also showed very similar protein patterns regardless of which period during the storage they were collected (lanes 5, 6 & 7; 8, 9 & 10 and 13 & 14).

The SDS-PAGE analysis showed that there were only minor differences between the sarcoplasmatic muscle proteins and the proteins in the drips. The sarcoplasmic proteins (lanes 3, 4, 11 & 12) showed a weak band (a) at approximately 106 kDa that is not visible in the drip samples. In all drip samples weakly discernable bands at approximately 98 kDa (b) and 37 kDa (c) are apparent. These could not be detected in the sarcoplasmatic proteins. High molecular weight proteins (approximately 220 kDa) appear to be more visible in the drip losses than in the sarcoplasmatic protein samples. The myofibrillar muscle proteins extracted on the day of slaughter were analysed in lane 2. The myosin heavy-chain (M), actin (A) and tropomyosin (T) are indicated. The myofibrillar proteins were also extracted at different timed during storage. No changes could however be detected even after 11 days of storage pm (results not shown).
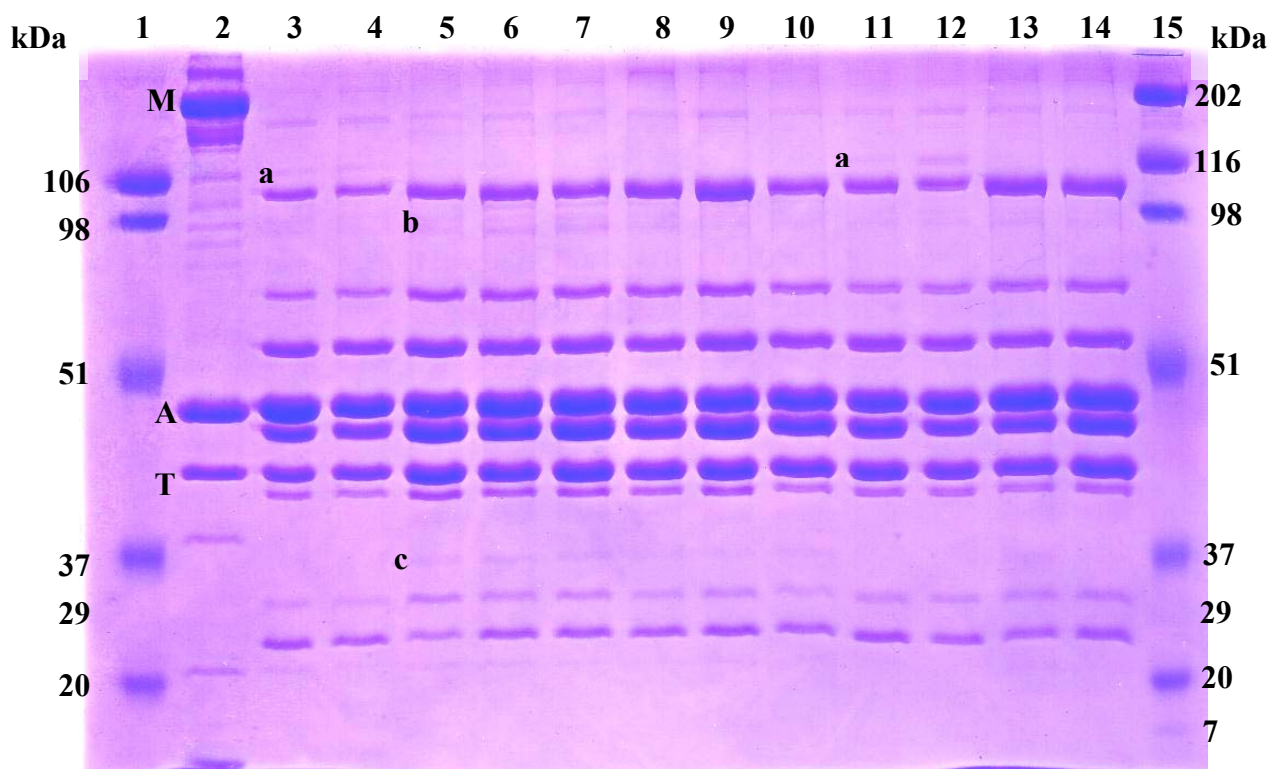
Figure 13 SDS-PAGE analysis of myofibrillar-, sarcoplasmatic- and drip loss proteins. Lane 2: Myofibrillar proteins from pre-rigor muscle day 0 pm. Lane 3-4: Sarcoplasmic proteins from day 0 and 11 pm of a pre-rigor filleted muscle respectively. Lane 5-7: Drip losses from 3 pre-rigor produced fillets (1-5 days pm). Lane 8-10: Drip losses from 3 pre-rigor produced fillets (6-11 days pm). Lane 11-12: Sarcoplasmic proteins from a post-rigor produced fillet day 5 and 11 pm of post-rigor filleted muscle respectively. Lane 13-14**:** Drip losses from 2 post-rigor produced fillets (6-11 days pm). The samples in lanes 3, 4, 6 and 8**,** and in lanes 11, 12 and 14 were from the same individuals**.** The protein concentrations were adjusted so that 12 μg were loaded in each well. Lane 1 and 15**:** Prestained SDS-PAGE standards; low- (19-106 kDa) and broad-range (6-202 kDa).

4.4.3 Gelatine zymography

The gelatinolytic activities in crude drip loss samples from pre- and post-rigor produced fillets are shown in figure 14. Drip losses from 3 fillets sampled on days 1-5 pm are shown in lanes 1- 3. The drip losses from day 6-11 pm of same fillets are shown in lanes 4-6. Lanes 7, 8 and 9 show the drip losses from 3 fillets produced post-rigor. The drips from the 3 fillets analysed in lanes 1-6 showed a specific pattern. The gelatinolytic activities were very similar regardless of the period they were collected. However, individual differences were apparent. The drip losses from the fillet analysed in lanes 1 and 4 were different from the drips of the other two fillets (lanes 2 & 5 and 3 & 6). In these 4 lanes, a high molecular weight activity (band a) was present. The drips from the 3 post-rigor produced fillets were also similar. As a control experiment, fillets were soaked in 0.02 % NaN_3 to prevent microbial growth. No differences in gelatinolytic activities were observed compared with the drips from ordinary stored fillets (results not shown).
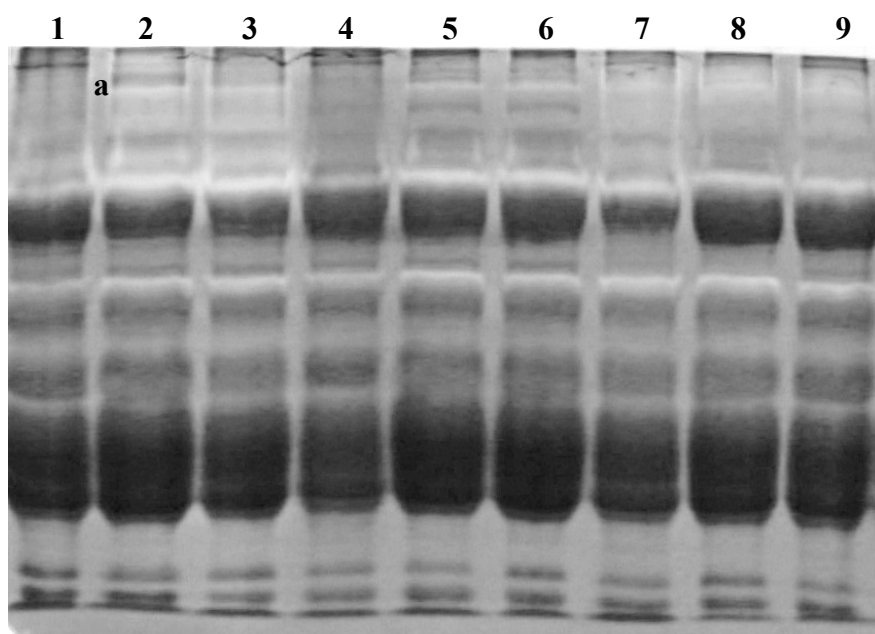

Figure 14 Zymography of gelatine degrading enzymes in crude drip samples from pre- and post-rigor filleted farmed Atlantic cod. Lane 1, 2 and 3 contains pre-rigor pooled samples (day 1-5 pm) from 3 fillets. Lane 4, 5 and 6 contains pre-rigor pooled samples (day 6-11 pm) of the same fillets as in lanes 1-3. Lane 7, 8, 9 and 10 contains post-rigor pooled samples.

Figure 15 shows the gelatinolytic activities in drip losses from pre- and post-rigor produced fillets after Gelatine Sepharose affinity chromatography. Lanes 1-3 contains fractionated activities from drip loss (day 1-5 pm) of a pre-rigor produced fillet while lane 4-6 shows the fractionated activities of drip loss (day 6-11 pm) from the same fillet. Lanes 7-10 show fractionated activities from the drip of a post-rigor produced fillet. Crude drip loss samples were run in lane 1, 4 and 7 while unbound activities are shown in lanes 2, 5 and 8. Activities eluded from the gelatine gel are shown in lanes 3, 6 and 10. Lane 9 shows the results after extensive washing of the affinity gel. The results showed that at least 2 gelatinolytic activities, one very high molecular weight (band a) and one high molecular weight (band b), were not bound to the affinity ligand. Two activity bands (bands c and d) bound specifically to the gelatine ligand. It was noted that the gelatinolytic patterns are similar regardless of period of drip collection or time of filleting. An exception is seen in lane 4 where the high molecular band (a) was relatively weak.
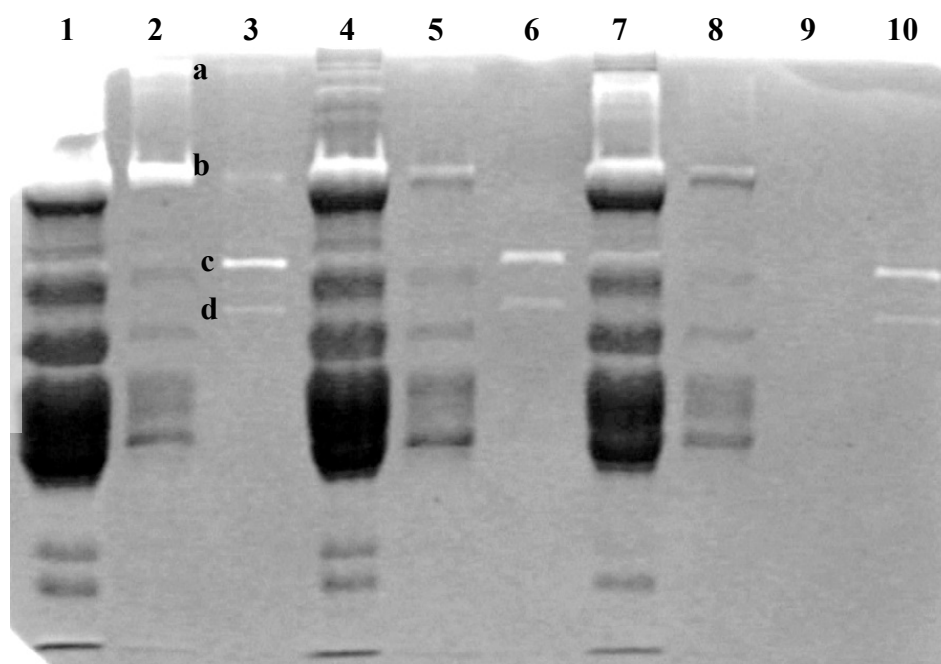

Figure 15 Zymography of gelatine degrading enzymes in drip samples from pre- and post-rigor filleted farmed Atlantic cod after fractionating by Gelatine Sepharose affinity chromatography. Lanes 1, 2 & 3 respectively contain crude, unbound and eluted activities from drip (days 1-5 pm) of a pre-rigor produced fillet. The fractionated samples from drip (days 6-11 pm) of the same pre-rigor produced fillet are shown in lane 4, 5 & 6 respectively. Lanes 7, 8 & 10 respectively contain crude, unbound and eluted activities from drip loss of a post-rigor produced fillet. Lane 9 indicates results after extensive washing of the affinity gel used for the sample in lane 7.

Gelatinolytic activities were also analysed in extracts of fillets stored for different periods of time and compared with the activities in the drip losses (Figure 16). All muscle and drip samples were collected from the same fillets, one pre- and one post-rigor produced fillet. Lane 1 contains the MMP standards. The activities in muscle extracts obtained on day 0 (lane 2) and day 11 (lane 3) were from a pre-rigor produced fillet and from a post-rigor produced fillet on day 5 (lane 4) and day 11 pm (lane 5). The activities of the corresponding muscle extract samples eluted from the gelatine affinity gel are shown in lanes 6, 7, 8 and 9, respectively. Drip loss from the pre-rigor produced fillet are shown in lane 10 (day 1-5 pm) and lane 11 (day 6-11 pm). There were obvious differences in the muscle extract samples between the pre- and post-rigor produced fillets, both in the crude and in the gelatine affinity gel fractionated samples. The pre-rigor produced fillet showed distinctly weaker gelatinolytic activities than the post-rigor produced fillet. There were small differences between the samples regarding extraction time. The drip loss samples showed that there were no major differences between the two pre-rigor filleted groups and the post-rigor filleted group. However, the eluted drip samples (day 1-5 pm) from the pre-rigor produced fillet showed a relatively high activity of band a.
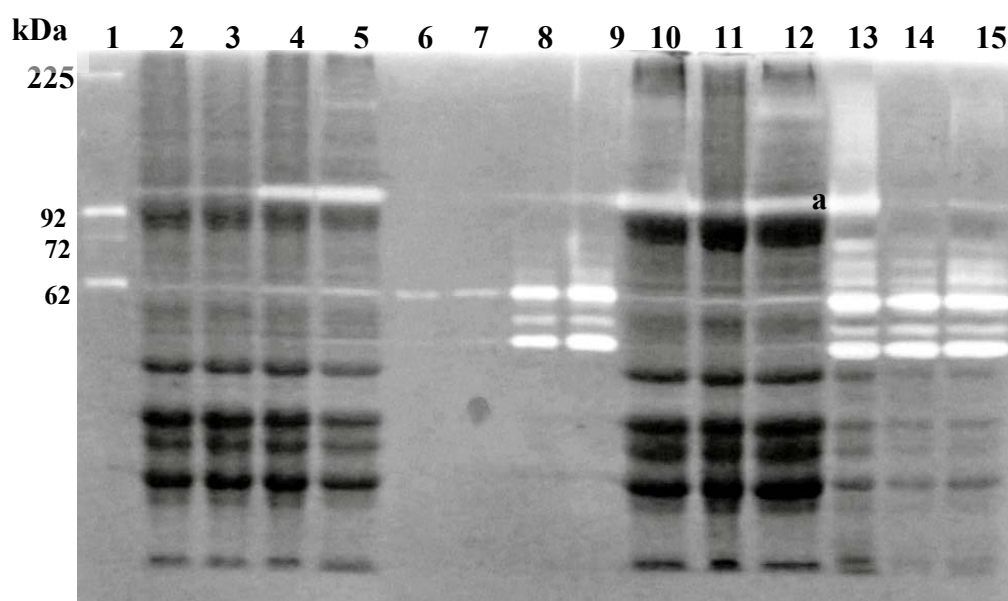

Figure 16 Zymography of gelatine degrading enzymes from crude samples and after isolation by Gelatine Sepharose affinity chromatography from muscle and drip lost from pre- and post-rigor produced fillets. Lane 1 contains MMP standards. Lanes 2-5 show crude muscle samples from the pre-rigor produced fillet (days 0 & 11 pm) and the post-rigor produced fillet (days 5 & 11 pm), respectively. Lanes 6-9 show the Gelatine Sepharose affinity chromatography eluted samples from the same fillets in the same order. Lanes 10-12 contain crude drip samples collected from the pre-rigor (days 1-5 & days 6-11) and post-rigor produced fillets, respectively. Lanes 13-15 show eluted drip samples from the Gelatine Sepharose affinity chromatography. The drips are from the fillets analysed in lanes 10-12 (same order).

Samples eluted from the affinity gel, from several drip losses and fillets were also analysed by gelatine zymography. Lane 1 contains the MMP standards. Lanes 2-4 show eluted activities from drip losses collected from 3 fillets on days 1-5 pm. The drip losses from day 6-11 pm of same fillets are shown in lanes 5-7. Lanes 8, 9 and 10 show the drip losses from 3 fillets produced post-rigor. Muscle samples from a pre-rigor produced fillet day 11 pm (lane 11) and day 0 pm (lane 12) and a post-rigor produced fillet day 11 pm (lane 13) and day 5 pm (lane 14) are also shown. The results indicate that activities are lost during the storage. The drip losses from the late phase of pre-rigor produced fillets (lanes 5-7) have clearly reduced activities of bands a, b and c. The drip losses (day 6-11 pm) from the post-rigor produced fillets (lanes 8-10) showed reduced activities of bands a and b in particular. In all affinity purified muscle samples the bands a, b and c were absent or strongly reduced. It was also observed that the low molecular weight bands (d and e) appear to weaken during prolonged storage.
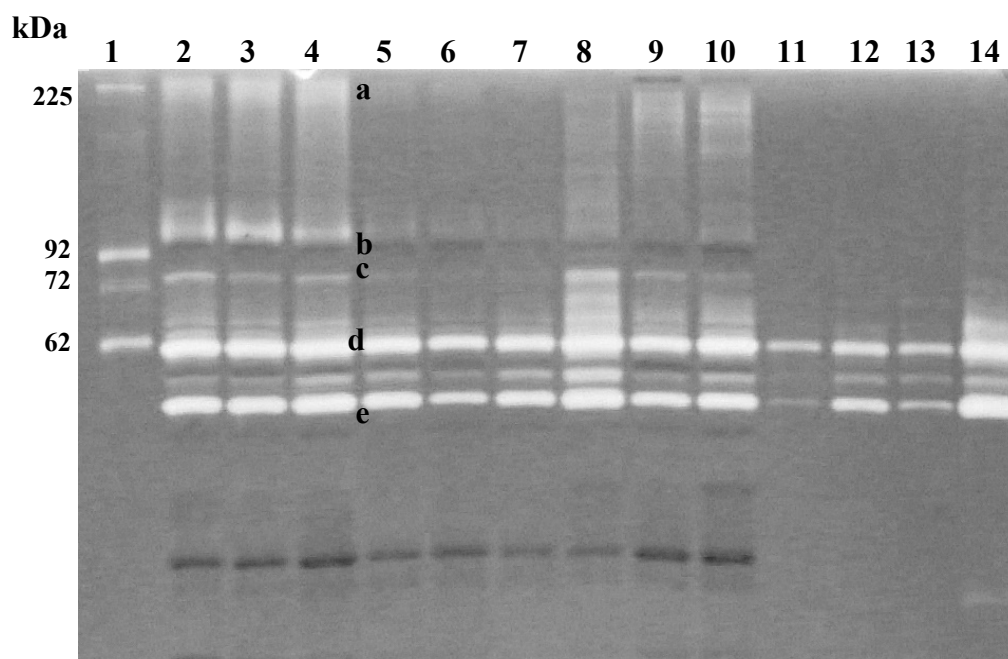
Figure 17 Zymography of gelatine degrading enzymes in drip and muscle samples from pre and post rigor filleted farmed Atlantic cod, after isolation by Gelatine Sepharose affinity chromatography. Lane 1 contains the MMP standards. Lanes 2, 3 and 4 contains pre-rigor drip samples (days 1-5). Lanes 5, 6 and 7 contains pre-rigor drip samples pooled from day 6-11 pm. Lanes 8, 9 and 10 contains post-rigor drip samples. Lanes 11 and 12 contain muscle samples from a pre-rigor produced fillet on day 11 and day 0, respectively. Lanes 13 and 14 contains muscle samples from a post-rigor produced fillet day 11 and day 5, respectively.

4.4.4 Western blot analysis of cathepsin D in muscle and drip losses from pre- and post-rigor produced fillets

The muscle extracts and drip losses were also investigated for the presence of Cathepsin D immunoreactivity by using Western blot analysis. In figure 18, extracts from 5 pre-rigor produced fillets at day 0 pm (lanes 2-6) were analysed. The polyclonal antibody reacted with two bands with molecular weights of approximately 40 and 85 kDa. Both bands had about the same intensity in all 5 samples. In one of the fillets, the reactivities were apparently weaker than in the other 4 fillets although the same amounts of muscle proteins were applied to each lane.
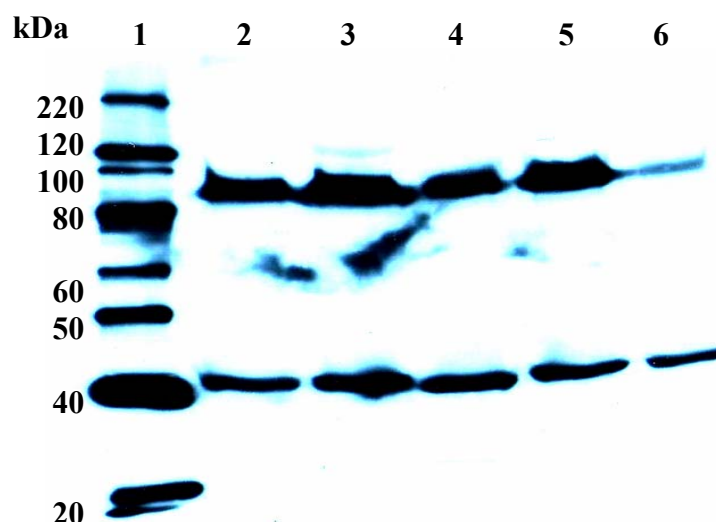
Figure 18. Western blot analysis of Cathepsin D extracted from 5 pre-rigor filleted muscles of farmed Atlantic cod at day 0 pm (lanes 2-6). 5 µg of proteins were applied in each lane. Lane 1 contains the MagicMark XP Western Protein Standard.

The pre-rigor produced fillets were also analysed for Cathepsin D immunoreactivity after 11 days of storage (figure 19). The results showed that the Cathepsin D antibody also in these samples cross reacted with the 2 protein bands (40 and 85 kDa). However, cross reactivity with the low molecular weight band was much weaker now.
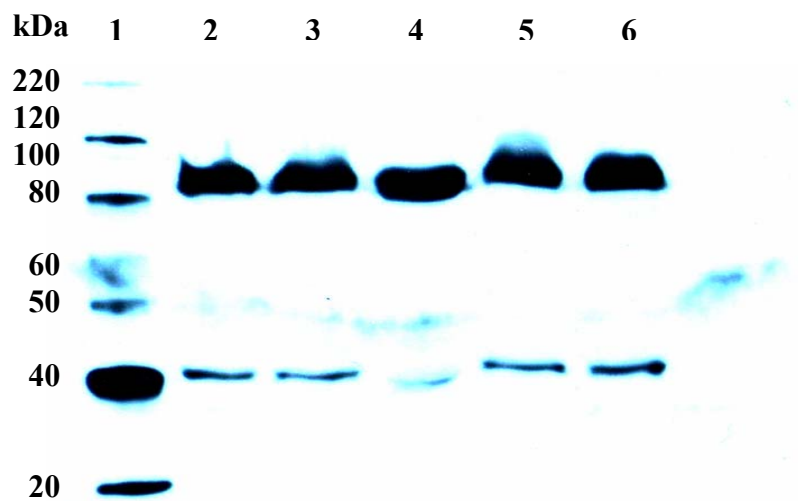
Figure 19. Western blot analysis of Cathepsin D in muscle extracts from 5 pre-rigor produced fillets at day 11 pm (lanes 2-6). 5 μg of proteins were applied in each lane. Lane 1 contains the MagicMark XP Western Protein Standard.

Drip losses collected from 8 pre-rigor produced fillets days 1-5 pm were also subjected to Western blot analysis (figure 20). The results showed that mainly the 40 kDa band was detected. In all samples, the band seemed to be split in two bands. Some lower molecular weight bands (40 < 20 kDa) were weakly detected in some of the samples. Faint bands with molecular weights of approximately 80 kDa and 220 kDa could be detected in some of the samples.

Figure 20 Western blot analysis of Cathepsin D in drip-loss collected from 8 pre-rigor produced fillets days 1-5 pm (lanes 2-9). 10 μg of proteins were applied in each lane. Lane 1 contains the MagicMark XP Western Protein Standard.

Drip losses collected from 6 pre-rigor produced fillets days 6-11 pm were subjected to Western blot analysis (figure 21). The results showed that both the 40 and the 85 kDa bands were strongly stained in all 6 drip losses. The 40 kDa band appeared split in two and some weak reativities with molecular weights between 40 and 85 kDa were also faintly present.
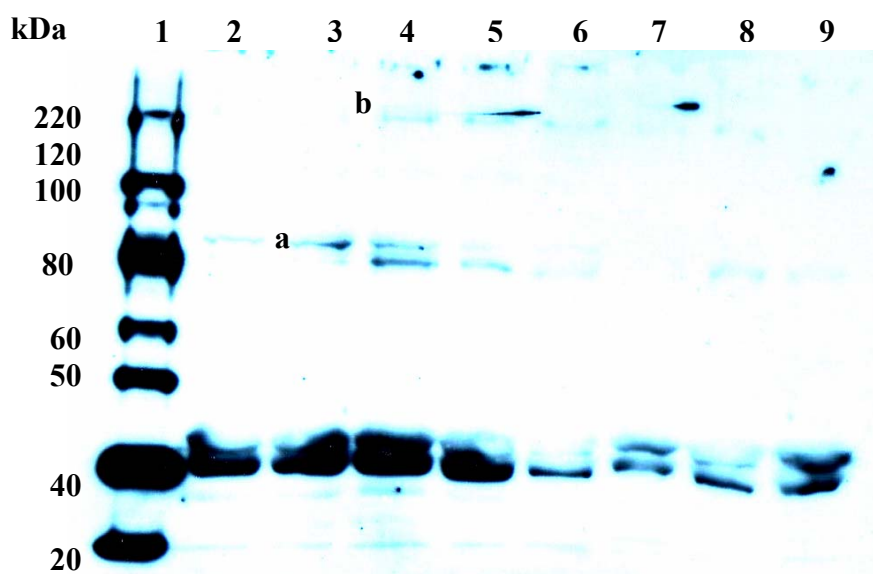

Figure 21 Western blot analysis of Cathepsin D in drip-loss collected from 6 pre-rigor produced fillets from days 6-11 pm (lanes 2-7).). 10 μg of proteins were applied in each lane. Lane 1 contains the MagicMark XP Western Protein Standard.

Drip losses collected from 7 post-rigor produced fillets days 6-11 pm were subjected to Western blot analysis (figure 22). The results showed that the 40 kDa band was present as a double band in probably all 7 drip loss samples. The 85 kDa band could be weakly detected in 3 of the fillets.
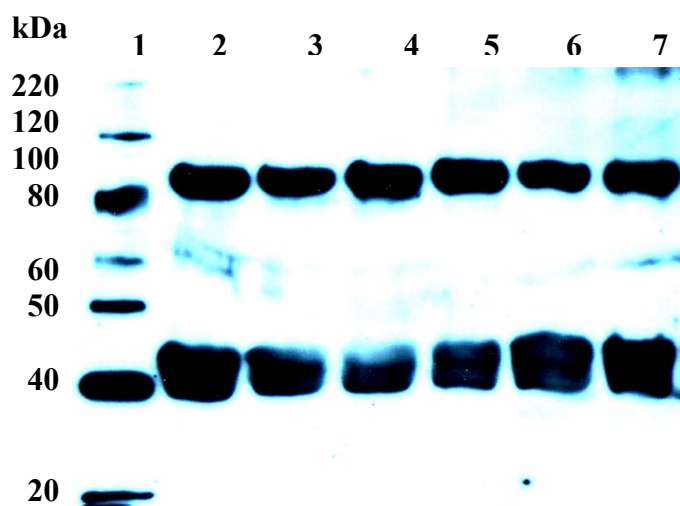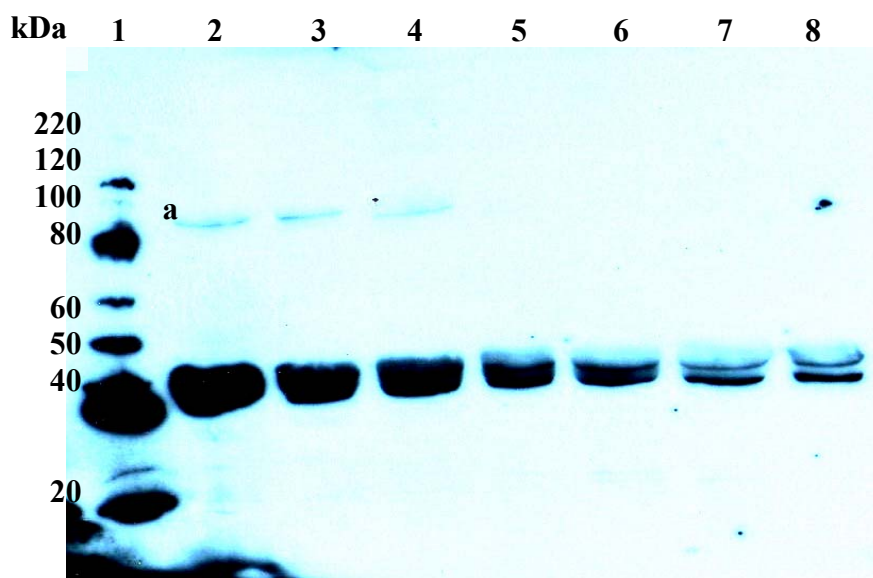
Figure 22 Western blot analysis of Cathepsin D in drip loss collected from 7 post-rigor produced fillets (lanes 2-8). 10 µg of proteins were applied in each lane. Lane 1 contains the MagicMark XP Western Protein Standard.

Finally, the muscle extracts and drip losses were subjected to Western blot analysis on the same gel (figure 23). Lanes 2 & 3 contain muscle extract samples from a pre-rigor produced fillet after storage for 11 days and at time of filleting (day 0 pm), respectively. Lanes 4 & 5 contain muscle extracts samples from a post-rigor produced fillet at the end of storage (day 11) and at time of filleting (day 5 pm), respectively. Drip losses from the fillet analysed in lanes 2 & 3 are run in lanes 6 (day 1-5 pm) and lane 7 (day 6-11 pm). Lane 8 contains drip loss (day 6-11 pm) from the post-rigor produced fillet analysed in lanes 4 & 5. The results showed that the 40 and 85 kDa bands were present both on the day of slaughter and after 11 days of storage of the pre-rigor produced fillets. However, the low molecular weight band stained much more strongly. In the post-rigor produced fillets basically only the 40 kDa band could be detected 5 and 11 days pm. In the drip losses, the 40 kDa band was reduced with the appearance of smaller bands of 35 kDa and equivalent or less than 20 kDa. In addition, different high molecular weight bands from 50-220 kDa were observed. Some of these bands were very faint.
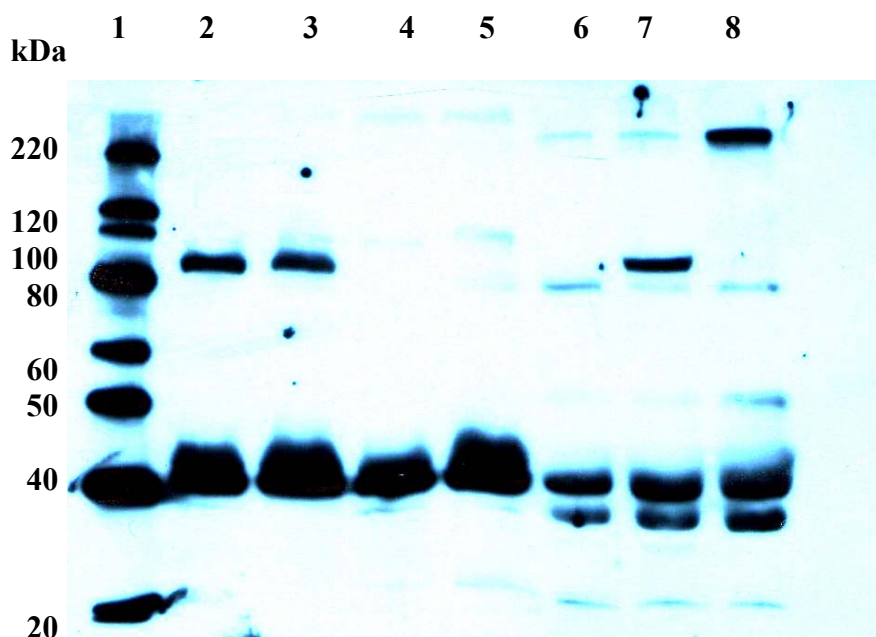
Figure 23 Western blot analysis of cathepsin D in muscles extracts and drip losses from fillets of pre- and post-rigor produced fillets. Lane 1 contains the MagicMark XP Western Protein Standard. Lanes 2 & 3 contains muscle extract samples from a pre-rigor produced fillet day 11 and day 0 pm, respectively. Lanes 4 & 5: muscle extracts samples from a post-rigor produced fillet at day 11 and day 5 pm, respectively. Drip losses from the fillet analysed in lanes 2 & 3 are run in lanes 6 (day 1-5 pm) and lane 7 (day 6-11 pm). Lane 8 contains drip loss (day 6-11 pm) from the post-rigor produced fillet analysed in lanes 4 & 5. The protein concentrations of applied muscle extract and drip loss samples to each well were 7.5 and 15 μg, respectively.

5   Discussion

Over the years numerous reviews concerning the factors influencing WHC (liquid loss) in mammalian and avian meat have been published (Hamn, 1986; Offer & Knight, 1988; Solomon *et al.*, 1998; Honikel, 2004; Huff-Lonergan & Lonergan, 2005). Few studies have however been carried out on WHC or drip loss from fish meat. Results obtained from studies on animal meat may not always apply to fish meat since fish are ectothermic organisms with a structural organisation of the muscles quite different from warm blooded animals. As mentioned in the introduction, heavily feeding of fish may result in inferior quality of the fillet and gadoid species such as Atlantic cod may be especially vulnerable (Love *et al.*, 1969; Ofstad *et al.*, 1996; Kristoffersen *et al.*, 2006a). Pre-rigor filleting while the muscle pH is still high, has been suggested as one way of reducing these problems (Skjervold *et al.*, 2001a; Skjervold *et al.*, 2001b; Kristoffersen *et al.*, 2006a; Tobiassen *et al.*, 2006).

The biological characteristics showed that the farmed cod in our study were of market size and had similar condition factor and hepatosomatic index (HSI) as reported previously (Kristoffersen *et al.*, 2006a; Mørkøre, 2006). Early sexual maturation of farmed cod indicated by the high gonadosomatic index (GSI), is also commonly observed (Svåsand *et al.*, 2004). The slaughter yields of the cod were relatively low (52-55 %) where the gonads accounted for more than half of this weight loss. This indicates that a substantial amount of the feed intake is lost in low value biomass. For example, the livers can not normally be used for cod liver oil production due to the high content of fatty acid linolic acid (18:2n-6) from plant ingredients in the feed. The low fillet yield found is also a reflection of the low slaughter yield. When the experimental cod were grouped on basis of sex, it was found that the female group had a larger liver. This has also been observed by others (Dahle *et al.*, 2003).

One of the main aspects of this thesis was to study how much liquid fillets from farmed cod lost during ice storage. As mentioned in the introduction cod have a low WHC compared to for example salmon. It is assumed that fish starts to loose liquid from the fillets after removal of the protective skin and membranes in the gut cavity. This was confirmed in thesis since no weight loss was detected during ice storage of the gutted cod in the recommended way. After filleting, the muscles lost weight due to drip loss and 11 days post mortem, the total weight loss was about 10% and 5% for the fillets produced pre- and post-rigor respectively. The difference in weight loss seemed reasonable since the fillets in the former group had been stored for 11 days and in the latter only 6 days. Einen *et al.* (2002) have

studied the effects of pre- and post-rigor filleting on the drip loss of Atlantic salmon and reported that pre-rigor fillets had lower drip loss than post-rigor produced fillets. Recently, a study on wild-captured Atlantic cod has reported similar results (Tobiassen *et al.*, 2006). In both these works, the authors related the drip loss to the time of filleting, not time of slaughter. However, it is more reasonable to evaluate quality of products of the same age pm. It is generally accepted that time after slaughter limits the saleability due to microbial growth. When filleting pre-rigor the muscle tissue is exposed to invading microbes which will then start the microbial degradation earlier than in fish filleted post-rigor (4-5 days pm). The lower drip loss observed in salmon (Einen *et al.,* 2002) may be due to the high fat content in the muscle compared with muscle of cod (Ofstad *et al.*, 1996).

When comparing the daily liquid loss during the first few days after filleting, higher losses were found in the pre-rigor produced fillets. This can probably be explained by the extensive shortening of the pre-rigor produced fillets because the fillets are not connected to the vertebrate during the rigor contraction. This shortening may push water from the myofibrils and the intracellular spaces to extracellular areas from where it is more easily lost. This increased daily drip loss results in a significantly higher total drip loss after 4 to 6 days of storage compared to post-rigor produced fillets stored for the same number of days.

The rigor contraction in the pre-rigor produced fillets resulted in a maximum shortening of 27 % on the second day post mortem. Thereafter the contraction decreased to about 19% at the end of the storage period. The post-rigor produced fillets showed a small reduction in length (4 %) at the end of the storage period. Both this decrease in length and the increase observed in the pre-rigor produced fillets after resolution of rigor mortis are probably caused by enzymatic degradation of structural molecules in the muscle cells. The contraction of the pre-rigor cod fillets in this experiment was slightly more than reported in previous works (Kristoffersen *et al.*, 2006a; Mørkøre *et al.*, 2006). Shrinkage of pre-rigor cod fillets appears to be more extensive than pre-rigor produced fillets of Atlantic salmon (Sørensen *et al.*, 1997; Einen *et al.*, 2002). The reason for this species difference is not known. It is however likely that structural features of the muscles are contributing factors (Ofstad *et al.*, 1996). The shortened pre-rigor produced cod fillets looked broader and thicker, and appeared to have a firmer texture. This may affect the eating quality of the product which needs to be tested.

The water content was non-significantly higher in the post-rigor produced fillets at the end of the storage period. This is consistent with the higher liquid loss of the pre-rigor produced fillets. Still, the WHC was significantly lower in the pre-rigor than in the post-rigor

produced fillets on day 11 pm. This may be due to higher water content in extracellular spaces due to the extensive shortening. These findings are in agreement with recently published results (Kristoffersen *et al.*, 2006a).

The pre-rigor produced fillets had a significantly lower muscle pH than the post-rigor produced fillets on day 5 pm. This could have contributed to the higher liquid loss in the pre-rigor fillets due to more protein denaturation at the lower pH. However, in earlier work on farmed cod and halibut, individual differences apparently affected LL more than variations in muscle pH in the low range area (Olsson *et al.*, 2003b; Kristoffersen *et al.*, 2006a). The pH in the drips was approximately 0.2-0.3 units higher than in the muscles. The drips collected from day 1-5 pm and day 6-11 pm had no differences in pH indicating low bacterial growth. The slightly higher pH in the drips than in the muscles is difficult to explain, but differences in buffering capacity or soluble amines, may contribute.

No differences in protein concentrations were found in the drips from the first 5 days and the 6 last days of storage of the pre-rigor produced fillets. In addition, no inverse relationship was found between the amounts of drip and protein concentration in the drips. Similar results have been published from studies on Atlantic halibut (Olsson *et al.*, 2003a). This indicates that proteins are steadily lost during storage. Savage *et al.* (1990) studied the drip loss in pig meat and found a weak inverse relationship between the amount of drip and its protein concentration. In another study on the chemical composition of the exudates from pale, soft, exudative (PSE) and normal pork loins, no correlation between drip volumes and protein concentration were discovered (Ewan *et al.*, 1979). In addition to proteins, other water soluble nutrients such as amino acids and vitamins are also lost in the drip (Larsen & Elvevoll, 2007).

The sarcoplasmatic proteins in the muscle during storage and the proteins present in the collected drips were analyzed by SDS-PAGE. No discernable differences were detected in sarcoplasmatic proteins extracted from fillets stored for 0 and 11 days pm. Myofibrillar proteins were tentatively identified based on previously published results (Ogata *et al.*, 1998; Verrez-Bagnis *et al.*, 2001; Ladrat *et al.*, 2003) and no changes were detected in these or in other bands in the myofibrillar proteins during storage. More specific detection methods such as the use of antibodies or zymography may be necessary to detect changes in both these protein fractions during storage of a fish muscle. Few studies have been carried out on changes in sarcoplasmatic proteins during storage pm and some proteins have been shown to be slowly degraded in fish muscle (Verrez-Bagnis *et al.*, 2001; Hernandez-Herrero *et al.*, 2003). Equivalent to the findings on pig meat (Savage *et al.*, 1990), SDS-PAGE analysis of the drip losses from the cod fillets gave a very similar protein pattern as for the sarcoplasmatic

proteins extracted from the muscles. However, we found some small differences indicating that sarcoplasmatic proteins are changed when lost in the drips. This may suggest that the proteins are more susceptible or exposed to protolytic degradation during the leakage process.

It has been suggested that degradation of extracellular matrix contribute to fish muscle deterioration post mortem (Bremner & Hallett, 1985; Ando *et al.*, 1991; Lødemel & Olsen, 2003). Both matrix metalloproteinase (MMPs) and matrix serine proteinases may be involved in degradation of extracellular components (Woessner, 1991; Koshikawa *et al.*, 1992). Zymography have been shown to be a useful technique to study the presence of proteolytic enzyme activities during storage of muscle tissue (Veiseth *et al.*, 2001). Recently it has been shown by using gelatine zymography that matrix degrading proteinases are present in fish muscles with both species and individual variations (Lødemel & Olsen, 2003; Olsson *et al.*, 2006). Our results show that the gelatinolytic activities are also present in crude drip loss from cod fillets and that these activities are not changed in drips collected early or late during the storage. As reported previously, individual differences are also noticed in this thesis. However, when fractioning the activities by gelatine affinity chromatography it was apparent that a high molecular weight activity, probably MMP-9 homodimer (225 kDa) was reduced in drips collected in the second half of the storage period. Similar was observed for two other activities with molecular weights of approximately 100 kDa and 72 kDa (MMP-2). As with the activities extracted from muscle, the drip losses also contained gelatinolytic activities which did not bind to the gelatine ligand indicating that activities other than MMP-9 and MMP-2 are present. The presence of both serine proteinases and metalloproteinases with gelatinolytic activity were also reported in cod muscle by Lødemel and Olsen (2003).

It has been suggested that deterioration of muscle is not due to breakdown of myofibrils, but to proteolytic degradation of minor components linking the structural units together (Òlafsdottir *et al.*, 1997). With the exception of μ-calpain, the activities of proteolytic enzymes including lysosomal cathepsins, have been suggested to be stable during post mortem storage of muscle tissue (Etherington *et al.*, 1990; Dransfield *et al.*, 1992; Boehm *et al.*, 1998; Gil *et al.*, 1998; Lamare *et al.*, 2002; Hernandez-Herrero *et al.*, 2003; Hultmann & Rustad, 2004). In most of these studies, the enzymes were extracted from the stored muscle prior to activity determinations. In addition to the presence of inhibitors, cellular compartmentalisation may impede the ability of proteolytic enzymes such as the lysosomal proteases, to degrade cellular proteins (Whiting *et al.*, 1975; Ertbjerg *et al.*, 1999; Kubo *et al.*, 2002). By the use of a polyclonal antibody raised against cod liver cathepsin D, we investigated the presence of cathepsin D in the cod fillets during storage and in the collected

drip. Consistent with the reported molecular weight of cod cathepsin D (Wang *et al.*, 2007), the antibody recognized a major band of approximately 40 kDa in all muscle samples. In addition, a band of approximately 85 kDa was also detected. This may be the non-lysosomal cathepsin E which also is aspartic acid proteinase (Tang, 1998).   In the muscle samples, the results indicated no degradation products of cathepsin D during the 11 days of storage. In the drip loss samples however, the cathepsin D appeared to be partly degraded to forms with molecular weights of about 35 and 20 kDa. This indicates that the stability of a protein in expelled liquid might be different than in the flesh during storage. Similar was observed for the 85 kDa immunoreactivity. In most of the samples analyzed, weaker bands with molecular weights of about 220, 90 and 80 kDa are seen. Since such high molecular weights forms are unlikely to be cathepsin D, we suggest that these bands are due to non-specific cross-reactivity because of the relatively low dilution of the antiserum used.

The present study have shown that pre-rigor filleting of farmed Atlantic cod resulted in a 10 % weight loss during a reasonable long storage period. This loss was twice the amount found in cod filleted post-rigor. Such a large weight loss may indicate that pre-rigor production of skinless fillets from farmed cod is not a feasible practice for the industry. It was also found that both myofibrillar and sarcoplasmatic proteins apparently are very stable during ice storage. However, more sensitive methods than general proteinstaining are necessary to determine this. The results showed that proteins and enzymes in the drip losses from the muscles are degraded more easily than when extracted from the muscle tissue.

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