UNIVERSITY OF TROMSØ UIT

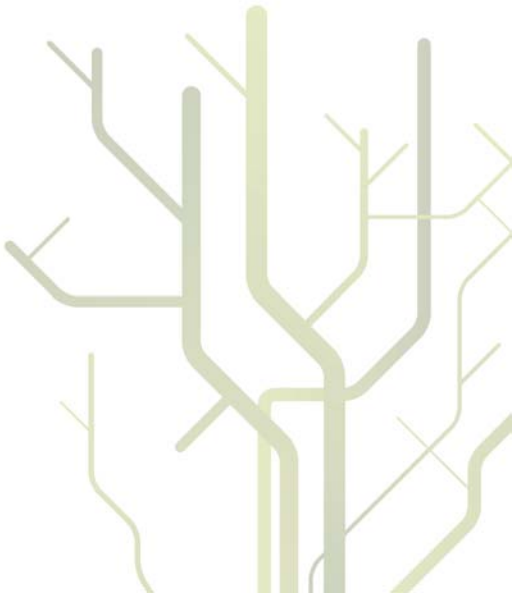
Faculty of Biosciences, Fisheries and Economics
Norwegian College of Fishery Science

Post mortem proteolytic degradation of myosin heavy chain in skeletal muscle of Atlantic cod

Pål Anders Wang

A dissertation for the degree of
Philosophiae Doctor

Spring 2011



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Contents

1 Acknowledgements

This study was carried out during the period 2006-2010 at the Norwegian College of Fishery Science, with prof. Ragnar L. Olsen as supervisor. The work was financed by the University of Tromsø.

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Pål Anders Wang, October 2010

2 List of publications

The thesis is based on the following publications.

Paper 1

Wang, P.A, Steinvik, J., Larsen, R., Mæhre, H & Olsen, R.L. (2007). Cathepsin D from Atlantic cod (*Gadus morhua* L.) liver. Isolation and comparative studies. *Comparative Biochemistry and Physiology, Part B,* 147, 504–511

Paper 2

Wang, P.A., Martinez, I. & Olsen, R.L. (2009). Myosin heavy chain degradation during *post mortem* storage of Atlantic cod (*Gadus morhua* L.). *Food Chemistry* 115, 1228–1233

Paper 3

Wang, P.A., Vang, B., Pedersen, A.M., Martinez, I. & Olsen R.L. (2011). *Post-mortem* degradation of myosin heavy chain in intact fish muscle: Effects of pH and enzyme inhibitors. *Food Chemistry* 124, 1090–1095

3 Sammendrag

En av de viktigste kvalitetsparameterne for kjøtt fra både varmblodige dyr og fisk er tekstur. Den *post mortem* degraderingen av muskel- og bindevevsproteiner som skjer under mørningsprosessen i kjøtt fra varmblodige dyr, er ikke ønskelig hos fisk på grunn av at den fører til redusert kvalitet av produktet. Myk og spaltet filet er et vanlig problem både hos oppdrettstorsk og villtorsk og har stor betydning for kvaliteten og forbrukernes oppfatning av produktene. Hvilke mekanismer som står bak disse forandringene i tekstur er ikke kjent, men endogene proteolytiske enzymer ser ut til å spille en viktig rolle i prosessen.

En muskelcelle inneholder flere ulike proteolytiske systemer. Blant disse er det multikatalytiske proteasomet, lysosomale cathepsiner, cytosoliske calpainer og metalloproteinaser. Det er vist at calpainer er viktig for den degraderingen som gir mørt kjøtt hos varmblodige dyr. Hos fisk er det foreslått at cathepsiner og calpainer, i samarbeid, står bak den observerte *post mortem* degraderingen av muskelproteiner. Det er vist at mange av cathepsinene og calpain er i stand til å degradere isolerte myofibrillproteiner i *in vitro* systemer. Bruk kunstige systemer har tradisjonelt vært hovedstrategien ved undersøkelser av proteindegradering etter slakting i fiskemuskel. En svakhet med slike systemer er at oppmaling av muskelvev og påfølgende isolering av proteiner fra homogenatet fører til at muskelens interne kontrollsystem settes ut av spill. Et annet problem med bruk av slike systemer er at forholdene ofte er veldig optimaliserte og dermed svært forskjellig fra de normale forholdene i muskelen. Ofte er pH og temperatur forskjellig fra normal *post mortem* muskel.

I denne avhandlingen har cathepsin D, et av enzymene som kan stå bak *post mortem* muskeldegradering hos torsk, blitt studert. Enzymet ble isolert fra torskelever, og et spesifikt antistoff mot enzymet ble produsert i mus. Det isolerte enzymet hadde lavere temperaturtoleranse enn tilsvarende kommersielt tilgjengelig enzym isolert fra storfe. Det produserte antistoffet detekterte cathepsin D i ekstrakter fra flere andre fiskearter. Videre ble degraderingen av den tunge kjeden i myosin (MHC) studert. Degraderingen av MHC i isolerte myofibriller skjedde raskest ved høy temperatur (20ºC) og lav pH (5.5), men selv ved lagring på is og ved relevant muskel-pH, foregikk det nedbryting. For å sammenlikne ble intakt muskel lagret før isolering av myofibrillproteiner. Resultatene viste at degraderingen av MHC foregikk i samme mønster, men degraderingen var mindre tydelig. Det ble også påvist at cathepsin D var assosiert med myofibrillene og enzymet var ikke mulig å fjerne fullstendig fra myofibrillene selv etter gjentatte vasketrinn.

Et modellsystem ble etablert for å studere hvordan små forandringer i pH og hemmere av protelytiske enzymer påvirket nedbrytning av MHC i intakt muskelvev etter slakting. En lav muskel-pH (6,05 -6,3) førte til degradering av MHC mens ved pH 6,9 forble proteinet uforandret. Hemmere av cystein- og asparginsyreproteinaser var tydelig mer effektive enn hemmere av serin- og metalloproteinaser i å hindre degradering av MHC. En overraskende observasjon var at EDTA, en hemmer av metalloproteinaser, førte til økt degradering av MHC.

Konklusjonen i avhandlingen er at lysosomale cathepsiner av typene cystein og asparginsyre proteinaser er ansvarlig for nedbrytning av MHC intakt muskel. Det utviklede modellsystemet vil være velegnet for å studere hvordan andre muskelproteiner brytes ned i intakt muskelvev etter slakting og hvilke enzymer som er involvert.

4 Abstract

One of the most important parameters of quality regarding meat both from mammals and fish is texture. The *post mortem* degradation of muscle and connective tissue proteins during tenderization of meat from warm-blooded animals is unwanted in fish due to a loss of product quality. Soft fillet and gaping are common problems both in wild and farmed cod and have huge impact on the quality and the consumers' perception of the products. The underlying mechanisms responsible for the changes in texture are not known, but endogenous proteolytic enzymes seem to play an important role in the process.

A muscle cell contains several proteolytic systems. Among these are the multicatalytic proteasome, lysosomal cathepsins, cytosolic calpains and metalloproteinases. It has been shown that calpains are important in the degradation process resulting in tender meat in warm-blooded animals. In fish, the calpains and cathepsins have been suggested to act in cooperation resulting in the observed *post mortem* degradation of muscle proteins. Many of the cathepsins and calpains have been shown to be able to degrade myofibrillar proteins in *in vitro* systems. Such systems have traditionally been a main strategy when studying protein degradation *post* slaughter in fish muscle. A weakness in such systems is that grinding of muscle tissue and isolation of proteins from the homogenate leads to a loss of the internal control system in the muscle. Another problem by using such systems is that the conditions are often highly optimized and therefore very different from normal *post mortem* muscle. The temperature and pH is often different from normal *post mortem* muscle.

In this thesis cathepsin D, one of the candidate enzymes being responsible for *post mortem* muscle degradation in cod has been studied. The enzyme was purified from cod liver, and an antibody specific against the enzyme was produced in mouse. The isolated enzyme had lower tolerance towards temperature than a similar commercial available enzyme isolated from bovine. The antibody detected cathepsin D in extracts from several other fish species. Further, the degradation of myosin heavy chain (MHC) was studied. The degradation of MHC in isolated myofibrils occurred at the highest rate at high temperature (20ºC) and low pH (5.5), but degradation occurred even when stored on ice and at relevant muscle-pH. For comparison, intact muscle was stored prior to isolation of myofibrils. The results showed that the MHC-degradation occurred in a similar pattern in intact muscle, however less prominent. It was also shown that cathepsin D was associated with the myofibrils, and the enzyme could not be completely removed even after several washing steps.

A model system was established, that made it possible to study how small changes in pH and inhibitors of proteolytic enzymes influence on the MHC-degradation in intact muscle tissue *post* slaughter. A low muscle-pH (6.05-6.3) did lead to degradation of MHC, while the protein stayed unchanged at pH 6.9. Inhibitors of cysteine and aspartic acid proteinases were obviously more efficient in preventing MHC-degradation than inhibitors of serine and metalloproteinases. A surprising observation was that EDTA, an inhibitor of metalloproteinases, increased the degradation of MHC.

The conlusion of this thesis is that lysosomal cathepsins of the cysteine- and aspartic acid-types are responsible for the MHC-degradation in intact muscle. The new model system will be suitable when studying how other muscle proteins are degraded in intact muscle tissue *post* slaughter, and which enzymes that are involved.

5 Introduction

5.1 Fish muscle structure and composition

5.1.1 General structure and composition

There are three different types of muscle in vertebrates: skeletal, cardiac and smooth muscle. In the following it will only be focused on skeletal muscle. The overall structure of fish muscle differs a lot from the structure of skeletal muscle of warm blooded animals. Fish has a segmented fillet, where each segment is a separate muscle (Fig. 1). The muscle segments are termed myotomes and are separated by intramusclular connective tissues (IMCT), classified as myocommata, perimysium and endomysium (Bremner & Hallett, 1985). The myocommata are clearly visible collagenous sheets separating, the fish muscle myotomes. Each myotome consists of several cells called muscle fibres (myomeres) that are running parallel to the long axis of the fish (Fig. 1). The individual muscle cells are surrounded by the sarcolemma cell membrane, with the basement membrane and the endomysial layer of collagen fibres running outside of it. The endomysial layer is running into the perimysium which is contiguous to the myocommata. To increase the tensile strength, the sarcolemma has invaginations containing collagen fibres that run into the base of the muscle fibres. There are also fine connections running between the sarcolemma, the basement membrane and the collagen fibres.

The muscle fibres are large multinucleated cells formed by fusion of many myoblasts. Each cell contains many parallel myofibrils which contain contractile units called sarcomeres. Due to the regularly repeating sarcomeres, vertebrate myofibrils show a striated appearance (Fig. 2). Two types of protein myofilaments comprise the sarcomeres, actin monomers and myosin molecules. The myosin filaments and part of the actin filaments are forming the A-

band, while the rest of the actin filaments are forming the I-band. All the myosin filaments in the A-band are connected to each other through a structure forming the M-line. The actin filaments are connected through the Z-disc (Z-line) forming the I-band of adjacent sarcomeres. The Z-line structure is running perpendicular to the myofibril and connects neighbouring sarcomeres.

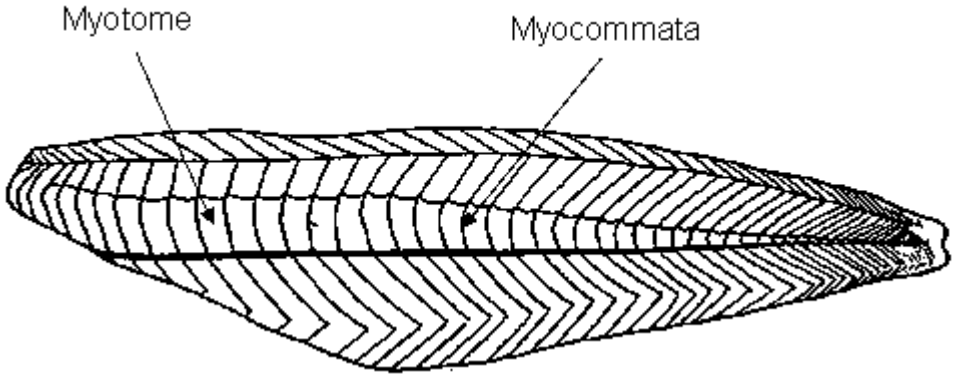

Figure 1. Fillet structure of Atlantic cod (*Gadus morhua* L.) after removal from the skeleton. The myocommata is indicated by black lines and separate each muscle segment (Myotome). Adapted from Love (1970).

The myosin aggregates are also connected to the Z-line through elastic titin filaments, which span from the Z-line to the M-line. In addition to the Z-disc interconnection, sarcomeres are attached through intermediate filaments composed of desmin. There are also cytoskeletal structures present that connect the Z- and M-lines to the sarcolemma. These structures are called costameres, and are composed of actin binding proteins bound to actin and membrane associated integrins. The integrins are further bound to extracellular matrix proteins like collagens and fibronectin (Du et al., 2010a; Kim, Nakamura, Lee, Hong, Pérez-Sala & McCulloch, 2010). The actin binding proteins of the costamere includes spectrin, vinculin, α-actinin, dystrophin, talin and filamin.

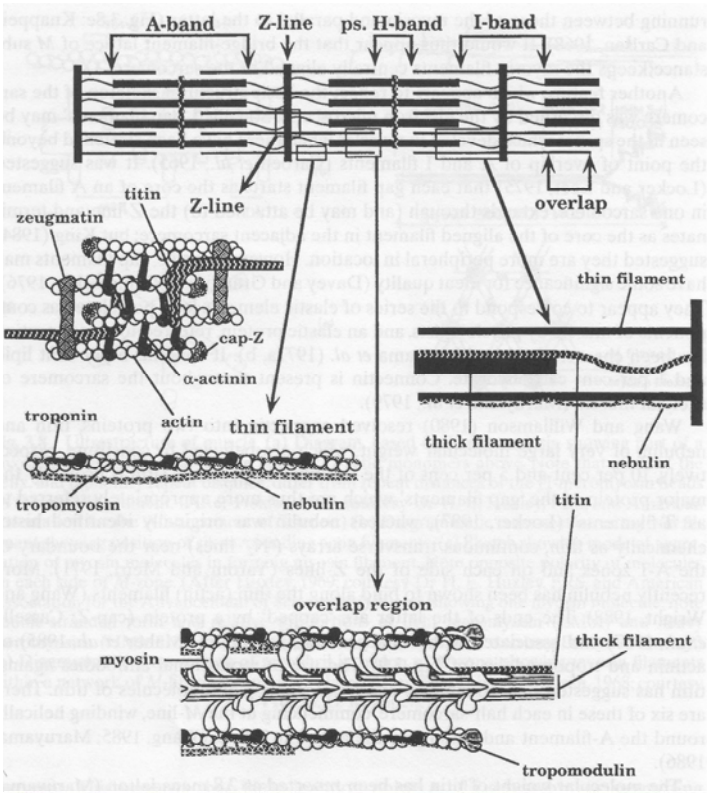

Figure 2. Upper panel: Simplified model of the relationship between the different proteins in the sarcomere and the interaction between actin, titin and other proteins at the Z-line. From Lawrie & Ledward (2006).
Lower panel: Electron microscopic photograph of the ultrastructural organization of a sarcomere. Adapted from Ottenheijm, Heunks & Dekhuijzen (2008).

5.1.2 Myosin

Myosins constitute a large superfamily of proteins that share a common domain which has been shown to interact with actin, hydrolyze ATP and produce movement. Myosin is the motor protein in the thick filaments of striated muscle and is the most abundant protein in skeletal muscle. The protein is a hexamer, with a molecular mass of approximately 520 kDa, consisting of one pair of 220 kDa heavy chains (MHC) and two pairs of 20 kDa light chains

(MyLC-1 and MyLC-2). The carboxyl-terminal half of the heavy chain consists of coiled-coil forming sequence which homodimerizes to form the long rod which usually terminated in a short non-helical segment (Hodge, Cross & Kendrickjones, 1992) and has two globular heads attached at one end, formed by the amino-terminals of the MHCs and the MyLCs (Fig. 3).

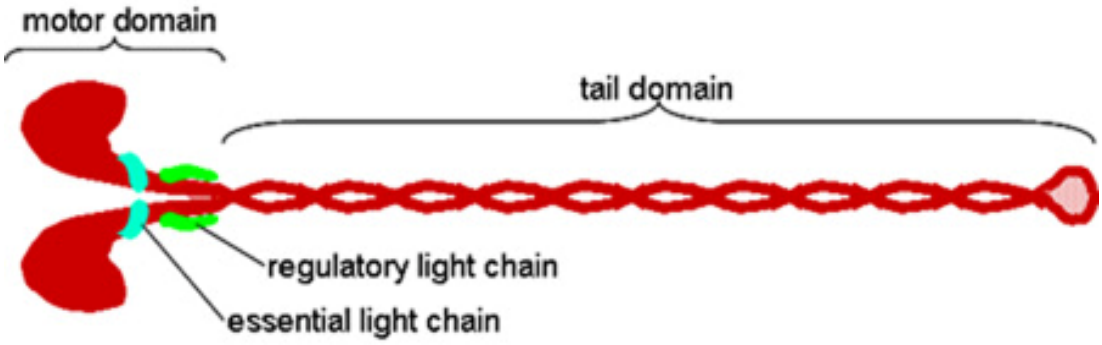

Figure 3. Myosin molecule with heavy chains divided into motor and tail domains and the regulatory and essential light chains (http://www.duke.edu/~jcs27/research.html).

The protein has three functional subdomains: (1) the amino-terminal motor domain which binds ATP and interacts with actin, (2) the neck domain which binds light chains or calmodulin, and (3) the tail domain which serves to anchor and position the motor domains so that it can interact with actin. Myosin motor domains are essentially built of four subdomains connected by flexible linkers (Houdusse, Kalbokis, Himmel, Szent-Gyorgyi & Cohen, 1999) (Fig. 4). The amino-terminal subdomain forms an SH_3-like motif (Dominguez, Freyzon, Trybus & Cohen, 1998) and is connected to the upper subdomain called 50 kDa, which is further connected to the lower 50 kDa subdomain. The name 50 kDa relates to the fact that there are two proteolytically sensitive surface loops that connects them with the rest of the myosin molecule. These loops give rise to bands on SDS-PAGE gels of 50 kDa (Mornet, Bertrand, Pantel, Audemard & Kassab, 1981). These two domains comprise most of the actin binding interface of myosin. The fourth subdomain of the motor domain is termed the

converter region. This domain bind to both light MyLCs through a long helix and the binding sites are highly specific for their respective light chains.

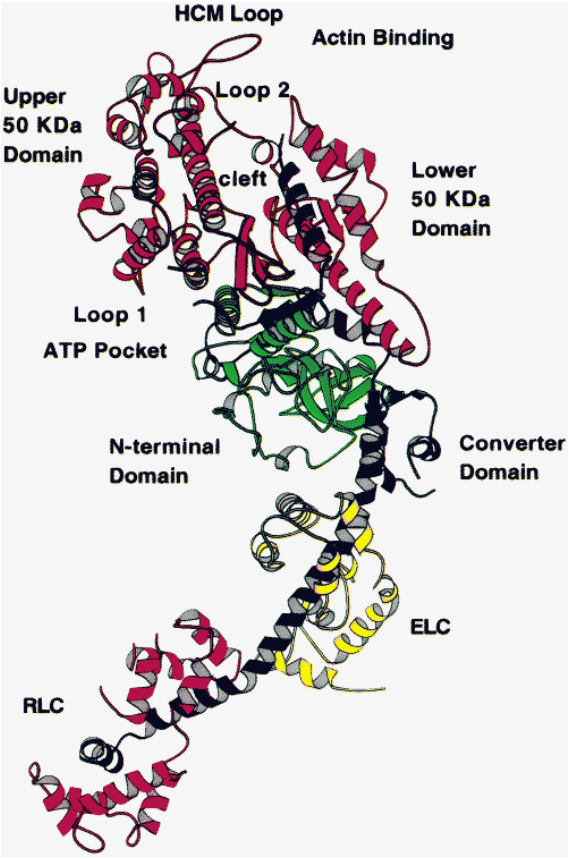

Figure 4. The four subdomains of the myosin motor domain, the N-terminal domain, the two 50 kDa domains and the converter domain. ELC = essential light chain; RLC = regulatory light chain; HCM = hypertrophic cardiomyopathy. From Sellers (2000).

As many as 300 myosin molecules exists in each thick filament (Miroshnichenko, Balanuk & Nozdrenko, 2000). They have the ability to form filaments via the self-association of the rod-like coiled-coil α-helical tail. Because of this, bipolar filaments such as those found in sarcomeric muscles can be formed by a combination of antiparallel tail interactions. These filaments have a bare central zone, not populated by motor domains, designed to pull actin filaments toward the center. Myosin molecules can be proteolytically cleaved into discrete functional domains. The junction between the head and the tail is one site of cleavage, in which a soluble fragment termed subfragment one (S1) is produced together with a rod fragment that retains the solubility properties of the parent molecule. S1 binds to actin and

nucleotides and contains the two light chains. Another site of cleavage in myosin is approximately 40 kDa into the rod. Cleavage at this site produces two fragments called heavy meromyosin (HMM) and light meromyosin (LMM). HMM contains the head region and a portion of the coiled-coil sequence (subfragment 2 or S2) which dimerize into a two-headed fragment.

5.2 Post mortem muscle degradation

The popularity and consumption of seafood has increased markedly during the recent years. One main reason is that seafood and its compounds are increasingly recognized as an important factor for improved human health (Brunner, Jones, Friel & Bartley, 2009; Børresen, 2008; Hansen, Dahl, Bakke, Frøyland & Thayer, 2010; Li & Hu, 2009; Shahidi & Cadwallader, 1997; Wergedahl et al., 2004). The most important product from the seafood industry is fish muscle. In the period from the fish is caught or slaughtered several biochemical processes occur inside the fish muscle that influence the product bought and consumed by consumers. To be able to offer a product that satisfies the customers, an understanding of these processes is necessary. The term fish quality is difficult to define and consists of many different attributes and has been used to describe a wide range of fish characteristics. Generally, a product has a good, or correct, quality when it meets the demands and expectations of the consumers. Among the quality attributes are food safety, nutritional quality, organoleptic features and aptitude to industrial transformation. These factors influence consumption and acceptability of fish as food. Stress around time of slaughter has been shown to affect the attributes of the fish both physically and biochemically (Kiessling, Helge Stien, Torslett, Suontama & Slinde, 2006; Misimi, Erikson, Digre, Skavhaug & Mathiassen, 2008; Robb, Kestin & Warriss, 2000). Changes in texture and sensorial properties are closely linked to the freshness of fish, and degradation *post mortem* is one of the most important quality attributes of fish muscle (Delbarre-Ladrat, Cheret, Taylor &

Verrez-Bagnis, 2006). It is however important to remember that quality implies different things to different people. Taking this into consideration, some might regard that a fish is at its best quality a few hours post catch, while others find that a slightly older fish has a better quality because it is easier to process. Most consumers buy fish that has undergone *rigor mortis* and this implies that the fish has been stored for some time before purchase. The time of onset of *rigor mortis* is affected by biological condition, *ante mortem* stress, species differences, individual differences and storage temperature (Haard, 1992; Kiessling et al., 2006; Love, 1988; Skjervold, Fjæra, Østby & Einen, 2001). The level of exercise, activity and stress prior to slaughter effect the amount of tension developed in muscle and may have an adverse effect on fillet texture (Kiessling, Espe, Ruohonen & Mørkøre, 2004; Robb et al., 2000; Roth, Slinde & Arildsen, 2006; Sigholt, Erikson, Rustad, Johansen, Nordtvedt & Seland, 1997).

In meat from warm-blooded animals, *post mortem* proteolytic degradation by endogenous proteolytic enzymes is associated with meat tenderness (Kemp, Sensky, Bardsley, Buttery & Parr, 2010; Koohmaraie, 1996; Lonergan, Zhang & Lonergan, 2010). The proteolysis contribution to meat tenderness is predominantly regulated by the amount of proteases present in the muscle at slaughter, duration of *post-rigor* aging and protease activities during aging (Koohmaraie & Geesink, 2006). During the *post mortem* period, both regulatory and cytoskeletal proteins of myofibrillar proteins are degraded into fragments (Bond & Warner, 2007; Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson & Robson, 1996). Many studies have shown that weakening of the myofibers is the key event in tenderization and that the most consistently reported structural change associated with tenderization is breaks at the the I-band and Z-disk (Abbott, Pearson, Price & Hooper, 1977; Davey & Dickson, 1970; Dutaud, Aubry, Guignot, Vignon, Monin & Ouali, 2006; Dutson, Pearson, Merkel & Spink, 1974; Ho, Stromer & Robson, 1996; Koohmaraie et al., 2006;

Taylor, Geesink, Thompson, Koohmaraie & Goll, 1995a). The proteins that are degraded during myofiber degradation are myofibrillar and cytoskeletal proteins like troponin-I, troponin-T, desmin, vinculin, *meta*-vinculin, dystrophin, nebulin and titin (Fig. 5).

Several studies show that myofibrillar proteins are hydrolyzed when treated with purified proteasome (Dutaud et al., 2006; Koohmaraie, 1992; Robert, Briand, Taylor & Briand, 1999; Taylor et al., 1995a; Taylor, Tassy, Briand, Robert, Briand & Ouali, 1995b). The proteasome has also been suggested to be one of the major endogenous proteolytic systems contributing to *post mortem* meat texture (Sentandreu, Coulis & Ouali, 2002).

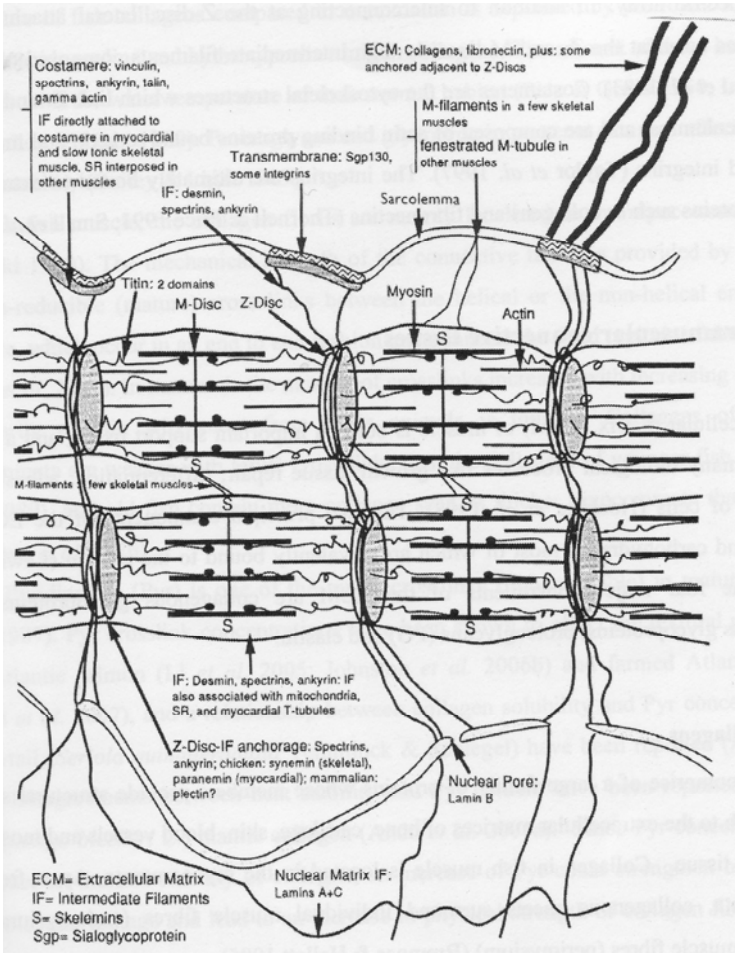

Figure 5. A model of the intermediate filament cytoskeleton in relation to the myofibrillar lattice of the thick and thin filaments of the sarcomeric structures. From Price (1991).

However, Koohmaraie (1992) and Solomon & Goldberg (1996) found that the intact structure of myofibrils was not changed when incubated with purified proteasome. This can be explained by differences in conditions between incubation in an artificial system and *post*

mortem condition of muscle. In addition to this, the proteasome can not degrade myofibrils directly because the myofibril filaments are not able to enter the core of the proteasome (Goll, Neti, Mares & Thompson, 2008). This suggests that other proteolytic systems are involved at an earlier stage in the degradation process of meat.

The calpains are believed to play a major role in the tenderization process of meat and have been and still are extensively investigated (Lonergan et al., 2010). The first evidence for their role in this process was the rapid loss of Z-disks in skeletal muscle myofibrils treated with calpains (Busch, Suzuki, Stromer & Goll, 1972). Later, a large set of evidence strengthens the role of calpains in *post mortem* proteolysis. Huff-Lonergan et al. (1996) showed that μ-calpain degraded titin, nebulin, filamin, desmin and troponin-T in bovine muscle in an *in vitro* system with low pH and temperature. Some authors state that calpains are responsible for 95% of all proteolytically induced tenderization (Delgado, Geesink, Marchello, Goll & Koohmaraie, 2001a). The concentrations and ratio of calpain/calpastatin is also stressed as to be predictors for the ultimate tenderness in beef (Ouali & Talmant, 1990; Shackelford et al., 1991; Thomson, Dobbie, Cox & Simmons, 1999; Warner, Greenwood, Pethick & Ferguson, 2010). Koohmaraie (1996) states that a protease must meet three specific criteria to be a candidate for involvement in *post mortem* tenderization. These criteria are; (1) the protease must be endogenous to skeletal muscle cell, (2) the protease must have the ability to reproduce *post mortem* changes in myofibrils in an *in vitro* setting and (3) have access to myofibrils in tissue. According to Koohmaraie (1996) are calpains the only proteases that meet all of these requirements. Chéret, Delbarre-Ladrat, de Lamballerie-Anton & Verrez-Bagniz (2007a) suggest that calpains and cathepsin L act in synergy to promote tenderization in mammalian meat, while the conditions are more favourable for cathepsin D a few hours *post mortem*. This involvement of cathepsins in meat tenderization has been a controversial question during the last decades. Cathepsin activities have been shown to fail to explain

differences in tenderness of meat samples (Koohmaraie, Seideman, Schollmeyer, Dutson & Babiker, 1988; Whipple, Koohmaraie, Dikeman, Crouse, Hunt & Klemm, 1990). Studies have also shown that some cathepsin inhibitors were not able to suppress *post mortem* proteolysis (Sentandreu et al., 2002), while a general cysteine peptidase inhibitor prevented degradation in rabbit meat (Prates, Ribeiro & Correia, 2001). When myofibrillar proteins were incubated with cathepsins, the degradation patterns were different from those occurring during *post mortem* storage of muscle and it is then doubtful that cathepsins are released from lysosomes in early *post mortem* muscle (Koohmaraie, 1988).

Less is known about the *post mortem* degradation processes in fish muscle compared to muscles of mammalian species. Fish muscle is rapidly degraded during *post mortem* storage due to proteolytic enzymes acting probably both on muscle cells and connective tissue (Caballero et al., 2009; Cepeda, Chou, Bracho & Haard, 1990; Delbarre-Ladrat et al., 2006). In fish muscle, proteolysis of cytoskeletal components results in myofilament degradation (Ayala et al., 2010; Busconi, Folco, Martone & Sanchez, 1989; Ofstad, Egelandsdal, Kidman, Myklebust, Olsen & Hermansson, 1996). Degradation of myofibrillar proteins like titin (Du et al., 2010b; Seki & Watanabe, 1984), nebulin (Astier, Labbe, Roustan & Benyamin, 1991; Du et al., 2010b), dystrophin (Caballero et al., 2009; Papa et al., 1997), α-actinin (Jasra, Jasra & Talesara, 2001; Papa, Alvarez, Verrez-Bagnis, Fleurence & Benyamin, 1996; Tsuchiya & Seki, 1991), myosin (Wu et al., 2010) and tropomyosin (Astier et al., 1991; Delbarre-Ladrat, Verrez-Bagnis, Noel & Fleurence, 2004b) has been observed in muscle of different fish species. The degradation of myosin heavy chain (MHC) leads to destructuration of the myofibril, which will influence the integrity of the fillet (Astier et al., 1991; Busconi et al., 1989). The stability of MHC during *post mortem* storage of fish muscle is debated as Busconi et al. (1989) and Astier et al. (1991) found that myosin was degraded, while others have reported that it is stable when the muscle is stored on ice (Jasra et al., 2001; Tsuchiya et al.,

1991; Verrez-Bagnis, Ladrat, Morzel, Nöel & Fleurence, 2001). Degradation of MHC has also been found in two species of shrimp stored on ice (Martinez, Friis & Careche, 2001).

There are apparently differences in the *post mortem* degradation between species as desmin is degraded in turbot and sardine, but not in sea bass (Verrez-Bagnis, Nöel, Sautereau & Fleurence, 1999) and croaker (Busconi et al., 1989). This is different from *post mortem* bovine muscle were desmin is largely degraded during aging (Taylor et al., 1995a). In salmon and sea bass the costameres which link sarcomeres to the sarcolemma are degraded within the first 24 h after slaughter (Papa et al., 1997; Taylor, Fjaera & Skjervold, 2002). The myofibrils of fish, in contrast to myofibrils in beef and sheep, are believed to undergo few structural changes *post mortem* and the I-bands are thought to almost never be broken (Busconi et al., 1989; Ofstad et al., 1996; Taylor et al., 2002). These observations based on electron microscopy are contradicted when myofibers are purified, since breaks then are quantified to be extensive (Tsuchiya, Kita & Seki, 1992). Also the connective tissue collagen is degraded in fish after death and this process correlates with the *post mortem* tenderization (Ando, Yoshimoto, Inabu, Nakagawa & Makinodan, 1995). A decrease of type V collagen content in fish meat has been shown to correlate with softening during chilled storage (Ando, Nishiyabu, Tsukamasa & Makinodan, 1999; Sato et al., 1997; Shigemura, Ando, Tsukamasa, Makinodan & Kawai, 2003).

The responsible factors for *post mortem* autolytic changes in fish muscle are far from understood. In contrast to in mammals where most of the tenderness is associated with calpain activities (Koohmaraie, 1996), Delbarre-Ladrat et al. (2006) have suggested that the calpains and the cathepsins probably act in a complementary way and in synergy at different levels of myofibrillar protein breakdown in fish. The pH-fall inside fish muscle after death gives a favourable environment for lysosomal acidic proteinases and these can therefore be suspected to be active in the degradation process. However, this demands that they are

liberated from their lysosomes and reaching the substrate. Three possible candidates are cathepsins B, D and L (Delbarre-Ladrat et al., 2004b; Ladrat, Verrez-Bagnis, Noel & Fleurence, 2003). A low ultimate pH in fish muscle is earlier reported to have negative effects on fish muscle quality, such as poor liquid-holding capacity (Ang & Haard, 1985; Love, 1979; Ofstad et al., 1996; Rustad, 1992), increased fillet gaping (Love, 1980) and rapid degradation of muscle tissue (Ofstad et al., 1996). The rapid degradation of connective tissue in fish also supports the role of cathepsins (Sato et al., 1997), as connective tissue proteins are susceptible to cathepsins but not to most other proteases.

Siebert (1958) was probably one of the first to describe activity of cathepsins in fish muscle, when finding that the cathepsin activity in fish was ten times higher than that of mammalian tissue. Since 1950 a number of cathepsins have been purified and characterized. Groninger (1964) purified a cathepsin D-like proteinase from fish. The response to activators and inhibitors and substrate specificity was similar to cathepsin D. This enzyme has probably been studied earlier without being specified. Several other cathepsins have also been found in fish muscle (Delbarre-Ladrat et al., 2006). Yamashita & Konagaya (1990a) found activity of cathepsins B, D, H and L in chum salmon muscle and Aoki, Yamashita & Ueno (2000) have published an overview over the distribution of cathepsins B, B-like, L and D in red and white muscles of 24 different marine and freshwater species. The cathepsins B, D and L are considered by several groups to be critical in modifications of fish muscle *post mortem* (Aoki & Ueno, 1997; Jiang, Lee & Chen, 1996; Ogata, Aranishi, Hara, Osatomi & Ishihara, 1998; Yamashita & Konagaya, 1990b). Several structural muscle proteins in fish have been shown to be susceptible to cathepsins. Exogenous cathepsins B, D and L are shown to degrade and release α-actinin and degrade MHC (Ladrat et al., 2003). The same study showed that tropomyosin and actin only were susceptible to the action of cathepsin L, while troponin-T and desmin were degraded by cathepsin B and cathepsin L. Degradation of myofibrillar

proteins has severe consequences for the muscle tissue. α-actinin degradation leads to weakening of the Z-disc, while proteolysis of myosin, tropomyosin, troponin T and desmin destructurates the myofibril (Delbarre-Ladrat et al., 2006). Bahuaud et al. (2010) showed that pre-slaughter stress significantly increased muscle cathepsin L gene expression and also tended to increase cathepsin B gene expression and activity in farmed Atlantic salmon. The increased cathepsin B activity was correlated to increased muscle degradation and increased cathepsin L gene expression to muscle degradation and texture, so that stress-induced cathepsin activity accelerates muscle degradation. The same link between cathepsins and fish muscle quality has also been found by others. Godiksen, Morzel, Hyldig & Jessen (2009) correlated muscle protein profiles of rainbow trout (*Oncorhynchus mykiss*) with firmness and found that exogenous cathepsins B, D and L affected 10, 9 and 4 out of the 17 obtained protein bands respectively. Most changes induced by cathepsin D was unfavourable to firmness, showing that this enzyme is involved in textural changes in trout.

5.3 Cathepsins

5.3.1 General

The name cathepsin was introduced by Willstätter and Bamann (1929) to describe acid proteinases distinct from pepsin in mammalian gastric mucosa. Historically, all intracellular enzymes were named cathepsins from the Greek word for "to digest". The enzymes now known as the cathepsins were discovered in the first half of the 20th century. The first one to be characterized was cathepsin C, initially known as dipeptidyl peptidase I or DPPI. Decades later, in the early 1970s, cathepsins B, H and L were identified (reviewed by Guha & Padh, 2008). Little was known about the physiological and pathological role of these enzymes until the 1990s when the crystal structure of cathepsin B was determined. This discovery gave a rapid progress in cathepsin research and introduced a golden era of lysosomal cysteine protease research. In this period several cathepsin knockout mice demonstrated that the role of

cathepsins is not simply that of scavengers (Chapman, Riese & Shi, 1997; Turk, Turk & Turk, 2000). In addition the genetic disorder, *pycnodysostosis*, was found to be linked with cathepsin K (Gelb, Shi, Chapman & Desnick, 1996).

The cathepsins are a series of proteases grouped into cysteine, serine and aspartic proteinases, based on differences in their active sites. The knowledge of cysteine cathepsins have been reviewed in several articles by Turk and co-workers (Stoka, Turk & Turk, 2007; Turk et al., 2000; Turk & Guncar, 2003; Turk, Turk & Turk, 2003; Turk, Turk & Turk, 2001). All known lysosomal cysteine proteinases are cathepsins, but there are cathepsins that are neither lysosomal nor cysteine proteinases. Cathepsins D and E are aspartic proteinases, while cathepsins A and G are serine proteinases; cathepsins E and G are non-lysosomal proteinases. Thirteen different human cathepsins have so far been identified (Guha et al., 2008). It is interesting to notice that although most of these enzymes are stored in lysosomes, the endosomes are the major functional centers of *in vivo* proteolysis (Pillay, Elliott & Dennison, 2002; Tjelle, Brech, Juvet, Griffiths & Berg, 1996).

5.3.2 Aspartic cathepsins

The name aspartic originates from the fact that Aspartic acid residues are ligands of the activated water molecule in all examples for which the catalytic residues have been identified. All the so far described members of this group are endopeptidases. The members of the aspartic acid proteinase group, like the well-characterized cathepsin D, yeast proteinase A and pepsin, all have a high degree of sequence similarity and common motifs. The conserved features in this group include, among others, two aspartic residues in the active site surrounded by Asp-Thr-Gly triads, long conserved stretches around the triads and a minimum of three disulfide linkages. They also have significant similarities in their primary, secondary and tertiary structures. Peptidases in the A1 family are formed by a bi-lobal structure and both

lobes are similar (Fig. 6). Another common structure in aspartic proteinases is a α-helix that covers the active-site cleft and obstructs self-activation.

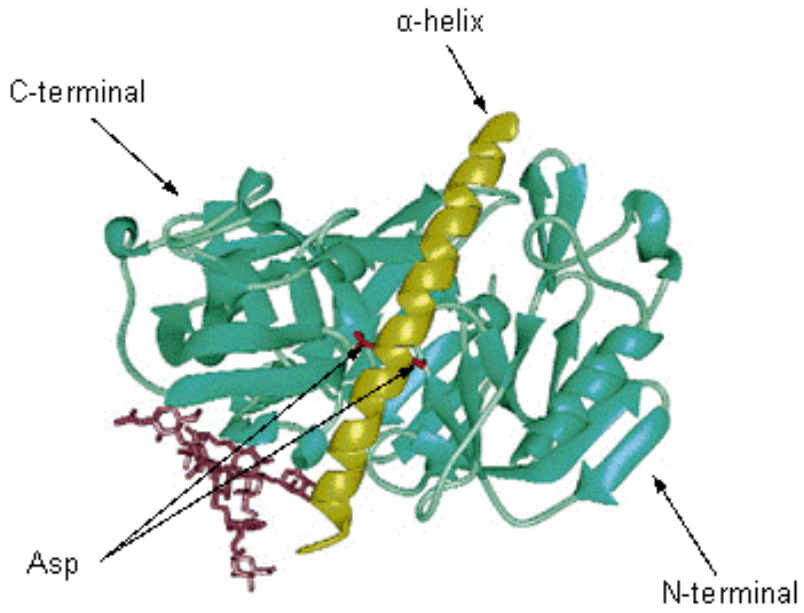

Figure 6. Structure of Yeast Proteinase A (YprA), a typical aspartic proteinase. The N-terminal domain is shown on the right handside of the figure, and the C-terminal domain is on the left handside. Each domain contributes with one aspartic acid (Asp) to the catalytic apparatus. The alpha-helix aligns within the active site of YprA to prevent cleavage of a substrate. Modified from Li et al. (2000).

Cathepsin D is a lysosomal proteinase, which is found in nearly all cells and tissue types in mammals (Sakai, Saku, Kato & Yamamoto, 1989). The concentration of cathepsin D inside rat liver lysosomes can be as high as 0.7 mM (Dean & Barrett, 1976) and comprise approximately 10% of the total soluble lysosomal protein. Cathepsin D is an endopeptidase with a pH optimum between pH 3.5 and 5.0, dependent on type of substrate and assay conditions. The enzyme will of course show activity, albeit lower, outside of the pH optimum. Early isolations showed that cathepsin D had as many as 12 different isoforms with different size, number of polypeptides and isoelectric point, but similar enzymatic properties (Sapolsky & Woessner, 1972). The active site of this enzyme has been shown to be similar to the active center of pepsin (Cunningham & Tang, 1976; Keilova & Lapresle, 1970), confirming its identity as an aspartic proteinase. The relationship with other aspartic proteinases has been confirmed by N-terminal sequencing (Huang, Huang & Tang, 1979). The same method has

also been used to predict that the cathepsin D precursor, found by studies of cathepsin D biosynthesis (Erickson & Blobel, 1979; Hasilik & Neufeld, 1980), is inactive (Erickson, Conner & Blobel, 1981). The precursor is, dependent on species, undergoing two to as many as five proteolytic cleavages to generate the forms isolated from tissues. The mature N-terminus as isolated from cells and tissue, is not always generated by autoproteolysis. This difference probably reflect aminopeptidase activity either in the lysosome or during purification (Conner, 1998).

Cathepsin D functions in general protein degradation inside lysosomes, but is involved in other processes as well. Animals deficient in cathepsin D have been shown to suffer from several disorders; Mice deficient in cathepsin D gene die in a state of anorexia 4 weeks after birth (Saftig et al., 1995) and show signs of neurodegeneration (Yamasaki et al., 2007), while cathepsin D deficient *Drosophila* suffer from neurodegeneration in the brain and retina (Kuronen, Talvitie, Lehesjoki & Myllykangas, 2009). It is also involved in the antigenic peptide-presentation as it is co-localized with immune system components in the endosomes (Guagliardi, Koppelman, Blum, Marks, Cresswell & Brodsky, 1990) and the specificity and activity suggest that it processes antigens for presentation (Vannoort, Boon, Vanderdrift, Wagenaar, Boots & Boog, 1991).

Signs of another aspartic proteinase in addition to cathepsin D, were early reported in vertebrate cells and tissues (Lapresle, Puizdar, Porchonbertolotto, Joukoff & Turk, 1986). Cathepsin E is present in lungs, kidneys, bladder, spleen, thymus and other lymphoid-associated tissues in mammals (Arbustini et al., 1994) and lower vertebrates including fish and bullfrog (Inokuchi, Kobayashi & Horiuchi, 1994). It has however a more limited distribution than cathepsin D and is as mentioned a non-lysosomal enzyme. It has a pH optimum of 3.1 towards haemoglobin (Yamamoto, Katsuda & Kato, 1978). The structure of cathepsin E is distinct from other known vertebrate aspartic proteinases in that it exists as a

dimer with molecular mass of 84 kDa, instead of as a single polypeptide chain with a ~40 kDa mass. Autoactivation at acidic pH generates the mature dimeric enzyme. Cathepsin E has been shown to be able to process neuropeptides and act on serum albumin (Kageyama, Ichinose & Yonezawa, 1995). The physiological role of cathepsin E has been predicted to be in the biogenesis of the vasoconstrictor peptide endothelin (Bird et al., 1992; Lees, Kalinka, Meech, Capper, Cook & Kay, 1990), antigen processing in human primary B-cells (Burster, Reich, Zaidi, Voelter, Boehm & Kalbacher, 2008) and neurodegeneration (Nakanishi, Tominaga, Amano, Hirotsu, Inoue & Yamamoto, 1994). It also seems to have anti-tumour activity (Kawakubo et al., 2007).

5.3.3 Inhibitors of aspartic proteinases

Inhibitors of aspartic proteinases can be produced by several species. Such inhibitors have been found in microorganisms, plants and some lower animals. Microorgansims of the genus *Streptomyces* can produce inhibitors called pepstatins. The pepstatins inhibit the activities of cathepsin D, cathepsin E, renin, pseudorenin, pepsin and aspartic proteinases (Gacko, Minarowska, Karwowska & Minarowski, 2007). Also potato can contain several inhibitors of cathepsin D and trypsin both in their bulbs and leaves (Baudys et al., 1991; Dash, Kulkarni, Dunn & Rao, 2003; Goulet, Benchabane, Anguenot, Brunelle, Khalf & Michaud, 2010; Guevara, Daleo & Oliva, 2001; Majer, Collins, Gulnik & Erickson, 1997). The potato inhibitors do not inhibit the action of pepsin. Inhibitors of aspartic acid proteinases have also been found in many other plant types. Among these are Lupins (Molina, Zamora & Blanco-Labra, 2010), beans (Kulkarni & Rao, 2007) and tomato (Lison, Rodrigo & Conejero, 2006). In lower animals, a cathepsin D inhibitor has been found in a sea anemone named *Actinia equine* (Galesa, Pain, Jongsma, Turk & Lenarcic, 2003; Lenarcic & Turk, 1999) and an inhibitor of cathepsin E, rennin and pepsin in two *Ascaris* species (Martzen, McMullen, Smith, Fujikawa & Peanasky, 1990; Ng et al., 2000; Valler, Kay, Aoyagi & Dunn, 1985).

Synthetic inhibitors of aspartic proteinases also exist. These are low molecular mass organic compounds that estrifies carboxyl groups in the catalytic site. Examples of such compounds are diazoacetyl norleucine methyl ester, diazoacetyl-glycine ethyl ester, diazoacetyl-phenyloalanin methyl ester, diazoacetyl-2,4-dinitrophenyl-ethylenediamine and other diazol compounds. Some of these compounds are reviewed by Gacko et al. (2007).

5.3.4 Cysteine cathepsins

There are 11 known lysosomal cysteine cathepsins which all belong to the papain subfamily of cysteine proteinases (Rawlings, Morton & Barrett, 2006). The cysteine cathepsins are mainly endopeptidases located intracellularly in endolysosomal vesicles. Cysteine cathepsins such as cathepsins B and L are thought to participate in general protein turnover. Surprisingly, mice deficient of the constitutively expressed cathepsins L and B have shown that these enzymes also have specific functions in certain tissues (Dennemarker et al., 2010; Hsing et al., 2010).

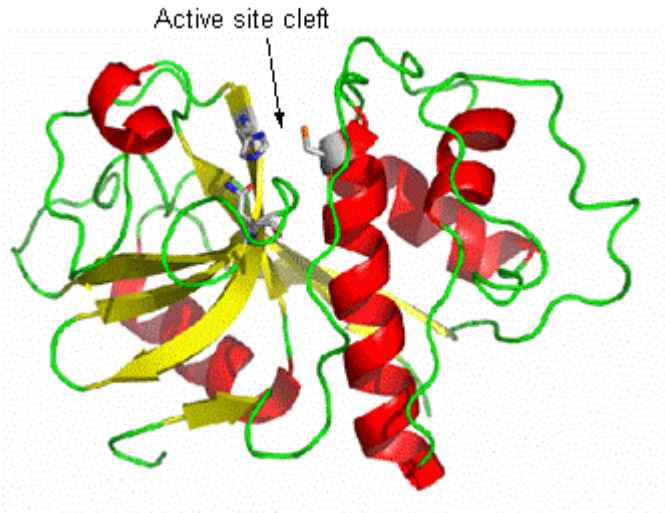

Figure 7. The structure of Papain, showing the general features of cysteine proteinases. A V-shaped active site cleft which separates the right- and left domain.
Modified from http://chemistry.umeche.maine.edu/CHY431/Peptidase9.html.

The cysteine cathepsins mode of action has been well characterized. Most of them require acidic pH for optimal activity. They are generally monomeric, have a molecular mass of

approximately 30 kDa and a two-domain structure with a V-shaped active site cleft extending along the two-domain interface (Fig. 7); the left domain is dominated by three α-helices and the right domain is based on a β-barrel motif with five to six strands (McGrath, 1999). The two domains are highly conserved and all cathepsins show common secondary structures in their respective domains.

Cathepsin B is the first described member of the lysosomal cysteine peptidase family and is probably the most extensively studied mammalian cysteine proteinase. It has been the subject of many protein engineering experiments and crystallographic studies (Musil et al., 1991; Watanabe et al., 2006). However, cathepsin B is not a typical representative for the lysosomal enzymes and appears to have diverged early from the other papain group enzymes (Berti & Storer, 1995). The structural and functional features of the enzyme distinguish it from the cathepsin L branch of the family. Cathepsin B has a role in intracellular protein turnover and has been implicated in diseases, among others, such as cystic fibrosis (Martin et al., 2010), pelvic inflammatory disease (Tsai, Wang, Tee, Lin, Hsieh & Yang, 2009) and Alzheimers disease (Sun et al., 2008). Cathepsin B has been shown to degrade the myofibrillar proteins α-actinin (Godiksen et al., 2009), myosin (Liu, Yin, Li, Zhang & Ma, 2008) and desmin (Baron, Jacobsen & Purslow, 2004). It possesses both endopeptidase and exopeptidase activity. The enzyme has a pH optimum in the range of 7.5-8.0 using different synthetic substrates (Khouri, Plouffe, Hasnain, Hirama, Storer & Menard, 1991), but it is very unstable under these conditions and denatures spontaneously (Mort, Recklies & Poole, 1980; Turk, Dolenc, Zerovnik, Turk, Gubensek & Turk, 1994). Because of this, an assay pH of 6.0 is optimal (Mort, 1998).

Cathepsin L is ubiquitous in eukaryotic cells (Kirschke, 1998), and very active in the degradation of the muscle proteins myosin, α-actinin, troponin-T and –I in the acidic to neutral pH range and also attacks the α-chains of type I collagen (Ogata et al., 1998). It has

also been implicated in a number of disease processes such as bone resorption and tumour metastases (Kakegawa et al., 1993; Yagel, Warner, Nellans, Lala, Waghorne & Denhardt, 1989). Cathepsin L activity is similar to other proteinases such as cathepsins K and S, but is most comparable to the activity of the 78%-identical cathepsin L2, which is found in testis and thymus and is highly expressed in certain cancers (Santamaria, Velasco, Cazorla, Fueyo, Campo & Lopez-Otin, 1998). Cathepsin L2 has also been found in fish muscle (Li, Zhou, Zhang, Liu & Ma, 2008). The activation of procathepsin L can occur autocatalytically at pH 3.0-3.5 (McDonald & Kadkhodayan, 1988; Salminen & Gottesman, 1990), at negatively charged surfaces at pH 5.0-6.0 (Mason & Massey, 1992) or by the action of cathepsin D or metalloendopeptidases (Hara, Kominami & Katunuma, 1988; Nishimura, Kawabata, Furuno & Kato, 1989). The first determined structure of cathepsin L was that of the proenzyme (Coulombe, Grochulski, Sivaraman, Menard, Mort & Cygler, 1996). Cathepsin L-deficient mice exhibit abnormal phenotypes in skin and cardiac muscle (Roth et al., 2000; Stypmann et al., 2002), while cathepsin B-deficient mice exhibit reduced tumour necrosis factor (TNF) - induced apoptosis in certain blood cells (Reinheckel, Deussing, Roth & Peters, 2001).

5.3.5 Inhibitors of lysosomal cysteine cathepsins

Activated cathepsins have enormous disruptive potential since their total concentration inside lysosomes might exceed 1 mM (Turk et al., 2001). Their inappropriate action is controlled by cystatins, which are endogenous protein inhibitors of lysosomal cysteine proteinases, and apparently not by pH (Turk, Dolenc, Turk & Bieth, 1993). Cystatins are divided into stefins, cystatins and kininogens based on their sequence homology. Stefins are intracellular inhibitors, while cystatins and kininogens are extracellular (Turk & Bode, 1991). Two conserved hairpin loops and a cystatin N-terminal trunk are involved in interaction with the conserved features of the active site of the target enzymes, as they surround the catalytic site (Stubbs et al., 1990). The cystatins inhibit endopeptidases and exopeptidases in the

picomolar and nanomolar range, respectively, and they are not very selective (Turk, Turk & Turk, 1997). In addition to the cystatins, several other cysteine proteinase inhibitors have been found. The most important are the thyropins, which are inhibitors homologous to the thyroglobulin type-I domains (Lenarcic & Bevec, 1998). The only known mammalian representative among the thyropins is the major histocompatibility class II-associated p41 invariant chain fragment, which is a selective cathepsin L inhibitor (Bevec, Stoka, Pungercic, Dolenc & Turk, 1996; Guncar, Pungercic, Klemencic, Turk & Turk, 1999). Also the proregions are inhibitors of the enzymes as they occupy the active site cleft in a linear, but backwards orientation. Serpin SCCA1 is also a cathepsin inhibitor. It was initially expected to be a serine proteinase inhibitor, but is actually an inhibitor of cathepsins S, L and K in the micromolar range (Schick et al., 1998).

5.3.6 Serine cathepsins

The serine proteinases are among the best characterized and physiologically most versatile class of enzymes described (Neurath, 1999). Serine proteinases share a common feature in the intermediate transfer of the acyl portion of a substrate to form a covalent bond with a functional group of the enzyme. The cathepsins that belong to this group of enzymes are cathepsin A and cathepsin G.

Cathepsin A is also called lysosomal carboxypeptidase A. It was originally described by the name cathepsin I. It is a multifunctional enzyme that expresses deaminase and esterase activities at neutral pH and carboxypeptidase activity at acidic pH (Jackman et al., 1990). The enzyme has a preference for substrates with hydrophobic amino acid residues in the cleavage piont (Pshezhetsky, Vinogradova, Elsliger & Elzein, 1995). In addition to this the enzyme also shows high affinity towards amino acid residues that are positively charged. Cathepsin A is widely distributed in mammalian tissues with the highest expression in kidney, liver, lung and placenta. It is inhibited by phenylmethanesulfonylfluoride (PMSF), iodoacetamide,

diisopropylfluorophosphate (DFP), thiol reagents in high concentrations and heavy metals such as Hg^{2+}, Ag^{2+} and Cu^{2+} (Chikuma et al., 1996; Itoh et al., 1993; Tranchemontagne, Michaud & Potier, 1990).

Cathepsin G is a single-chain serine proteinase which was first purified from spleen (Baugh & Travis, 1976; Starkey & Barrett, 1976a; Starkey & Barrett, 1976b). Its molecular mass is approximately 28.5 kDa and the optimal pH on most substrates is 8.0. The biological activity of cathepsin G is assumed to be participation in proteolytic degradation of engulfed particles during normal phagocytosis and effects on platelets (Selak, Chignard & Smith, 1988) and proteolysis of certain blood coagulation factors (Schmidt, Egbring & Havemann, 1975; Turkington, 1991).

5.4 Metallopeptidases

Metallopeptidases are together with the aspartic peptidases among the enzymes in which the nucleophilic attack on a peptide bond is mediated by a water molecule. In the metallopeptidases the water molecule is activated by a divalent cation, usually zinc or sometimes cobalt or manganese. The metallopeptidases can be divided into two broad groups depending on the number of metal ion required for catalysis. Usually one zinc-ion is required, but in some families two metal ions act co-catalytically. The peptidases that utilize cobalt or manganese all require two metal-ions. The known metal ligands in metallopeptidases are histidine, glutamic acid, aspartic acid or lysine residues. In addition to the metal ligands, at least one other residue is necessary for catalysis. This ligand is often glutamic acid, but can also be lysine or arginine. Metallopeptidases with cocatalytic metal ions are exopeptidases, while metallopeptidases with one catalytic metal ion may be exopeptidases or endopeptidases.

The matrix metalloproteinases (MMPs) is a family of secreted enzymes that play a significant role in the proteolysis of the extracellular matrix (ECM). They are calsium dependent neutral/alkaline zinc metalloendopeptidases and can degrade several component of

the ECM (Massova, Kotra, Fridman & Mobashery, 1998). The MMPs are divided into six subgroups; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs. Much knowledge has accumulated since the first ECM degrading MMP was discovered by Gross & Lapiere (1962) in resorbing tadpole tails. Until now, 24 human MMP genes have been discovered. MMPs can in addition to being ECM modulators, act as activators or regulators of many aspects of cell biology (Fanjul-Fernandez, Folgueras, Cabrera & Lopez-Otin, 2010; Morrison, Butler, Rodriguez & Overall, 2009; Murphy, 2008). Such functions are potentially regulation of chemokines, cytokines, growth factors, cell adhesion molecules and other membrane associated proteins.

MMPs have also been detected in fish. The first to propose a presence of MMPs in fish muscle were Bracho & Haard (1996). They described two proteinases with properties similar to MMPs in Pacific rockfish (*Sebastes* sp.) muscle. Later gelatinolytic activities ranging from 25 to 200 kDa were described in muscle of yellowtail (Kubota, Toyohara & Sakaguchi, 1998). Proteolytic breakdown of extracellular macromolecular proteins, such as collagens, by metalloproteinases is supposedly a major cause for fish meat tenderization (Kubota, Kinoshita, Takeuchi, Kubota, Toyohara & Sakaguchi, 2003). Gelatinolytic proteinases with properties similar to MMPs have been proposed to participate in the metabolism of collagens and in the *post mortem* degradation of fish muscle during cold storage in species like red sea bream (Wu et al., 2010; Yoshida et al., 2009), squid (*Loligo opalescens*) (Tamori, Kanzawa, Tajima, Tamiya & Tsuchiya, 1999), Pacific rockfish (Bracho et al., 1996), yellowtail and ayu (Kubota, Kinoshita, Yokoyama, Toyohara & Sakaguchi, 2000; Kubota et al., 1998), common carp (Wu et al., 2008), Atlantic cod, spotted wolfish, and Atlantic salmon (Lødemel & Olsen, 2003).

The MMPs can be inhibited by different chelating agents (Ganea, Trifan, Laslo, Putina & Cristescu, 2007; Visse & Nagase, 2003), but their activity is also regulated by proteins

called tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have been identified in humans with molecular masses ranging from 21 to 30 kDa (Shiomi, Lemaitre, D'Armiento & Okada, 2010). The TIMPs also have other important biological functions such as influencing cell proliferation, apoptosis and angiogenesis and regulating the activation of proMMP-2 and proMMP-9 (Nagase, Visse & Murphy, 2006; Okada, 2009). TIMP-2a, TIMP-2b, TIMP-3 and TIMP-4 have so far been described in fish (Lødemel, Egge-Jacobsen & Olsen, 2004; Tsukamoto, Yokoyama, Suzuki, Mizuta & Yoshinaka, 2006). To my knowledge TIMP-1 has not been described in any fish species and Tsukamoto et al. (2007) stated that the TIMP-1 gene has been lost in fugu (*Takifugu rubripes*).

5.5 Calpains

Calpain-1 (μ-calpain) and calpain-2 (m-calpain) are intracellular cysteine endopeptidases that are ubiquitously distributed and show activity at neutral pH. The name calpain originates from **cal**cium ion dependent pa**pain**-like cysteine proteinase. Both calpains require calcum (Ca^{2+}) at micro- and millimolar concentrations, respectively, for activity. The occurrence of Ca^{2+}-dependent neutral proteinase activity in rat brain was first described by Guroff (1964). Both calpains are dimers in their active form and the large subunit (80 kDa) contains the peptidase activity. The small subunit (30 kDa) is similar in both types. Calpains have been identified as a calcium-activated sarcoplasmic factor (CaSF) hydrolyzing Z-lines (Busch et al., 1972) and as a protein kinase C-activating factor (Takai, Yamamoto, Inoue, Kishimoto & Nishizuka, 1977). Purification of calpain, at that time called CANP (calcium-activated neutral proteinase) to homogeneity was first performed by Ishiura, Murofushi, Suzuki & Imahori (1978).

Three different calpain-like enzymes have been characterized from European sea bass (*Dicentrarchus labrax* L.) (Ladrat, Chaplet, Verrez-Bagnis, Nöel & Fleurence, 2000), all active at neutral pH. Based on their calcium-requirements, two were designated m-calpains

and one μ-calpain. Their expression was found to fluctuate throughout the year, with an elevated activity during the spawning period. Verrez-Bagnis, Ladrat, Noelle & Fleurence (2002) found that m-calpain from sea bass muscle mainly degrades myofibrillar proteins and hardly any sarcoplasmic proteins. m-calpain and μ-calpain have also been described in Chinook salmon (*Oncorhynchus tshawytscha*) (Geesink, Morton, Kent & Bickerstaffe, 2000) and carp (*Cyprinus carpio*) (Toyohara & Makinodan, 1989) muscles.

Calpain has a very specific *in vivo* protein inhibitor, called calpastatin. Structurally, calpastatin contains four repeats of the inhibitory unit and all of them can inhibit calpain independent of each other. In fish, calpastatin has among others been described in Chinook salmon (Geesink et al., 2000), rainbow trout (Saito, Li, Thompson, Kunisaki & Goll, 2007) carp (Toyohara, Makinodan, Tanaka & Ikeda, 1983) and sea bass (Chéret, Delbarre-Ladrat, Verrez-Bagnis & De Lamballerie, 2007b). Unlike in mammals, the activity of calpastatin in fish muscle remains constant during *post mortem* storage (Delbarre-Ladrat, Verrez-Bagnis, Noel & Fleurence, 2004a; Geesink et al., 2000). The level of inhibitor activity did not fluctuate significantly throughout the year in carp muscle (Toyohara, Makinodan, Tanaka & Ikeda, 1985). These findings indicate that high levels of calpastatin in fish muscle normally will suppress calpain-activity, but in periods of high activity the inhibitor-capacity might be exceeded.

6 Aim of study

The main objective of this PhD thesis has been to increase the understanding of the biochemical mechanisms underlying the observed *post mortem* muscle degradation in fish species important in aquaculture and fisheries. This thesis has in particular focused on investigating the presence of the lysosomal cathepsin D in Atlantic cod and the effects of different proteinases on the myosin heavy chain *post mortem*.

The more specific goals of the thesis have been to:

- Isolate cathepsin D from Atlantic cod and produce polyclonal antibodies directed against this proteinase
- Study some characteristics of the isolated proteinase
- Detect the presence of cathepsin D in muscle tissue of different fish species
- Establish a system for studies of the degradation of fish muscle proteins both in isolated myofibrils and intact muscle
- Study the *post mortem* degradation of myosin heavy chain in Atlantic cod and determine responsible candidate proteinases

7 Main results and general discussion

Cathepsin D activity is high in fish muscle (Mommsen, French & Hochachka, 1980) and has also been reported to comprise as much as 10% of the lysosomal proteins in mammalian tissues (Wittlin, Rösel & Stover, 1998). The enzyme has been suggested to be a major factor in *post mortem* fish muscle degradation (Gildberg, 1988).

In this thesis cathepsin D was purified from cod liver and some characteristics of the purified enzyme were described (paper I). Cod liver was chosen as tissue because of the high specific enzyme activity compared to skeletal muscle. The enzyme was purified using ion-exchange and affinity chromatography. A total recovery of 10% enzyme activity was obtained, which is high compared to other purifications of the enzyme (Gildberg, 1987; Goldman-Levkovitz, Rimon & Rimon, 1995; Jiang, Wang & Chen, 1991; Nielsen & Nielsen, 2001). After purification, the identity of the isolated protein was confirmed by SDS-PAGE, western blot with peptide antibodies specific to an amino acid (AA) -sequence in cod cathepsin D and inhibition by pepstatin A. The isolated protein had a MW of approximately 40 kDa. Cathepsin D isolated from cod liver had a pH optimum, with haemoglobin as substrate, of 3.0. This is typical for cathepsin D from fish (Gildberg, 1987; Nielsen et al., 2001). The pH optimum can however vary depending on protein substrate (Doke, Ninjoor & Nadkarni, 1980; Stoknes & Rustad, 1995). Cod liver cathepsin D started to lose activity at 30ºC, a lower temperature than for cathepsin D from bovine spleen. The lower temperature stability can be explained with that cod is ectothermic and adapted to arctic temperatures. The same pattern is also seen for other enzymes from cold-adapted species (Capasso et al., 1999; Gildberg & Øverbø, 1990; Olsen, Øverbø & Myrnes, 1991).

Based on the isolated cathepsin D, a polyclonal antibody was produced in rabbit (Paper I). This antibody showed the same cross reaction pattern as the antibody directed

against the AA-sequence. The antibody based on the isolated enzyme was also shown to detect the native cathepsin D in cod liver. In some comparative studies on other fish species, the antibody produced from the isolated enzyme detected cathepsin D in liver extracts from cod, saithe, wolffish, halibut, Atlantic herring and Atlantic salmon, while cathepsin D was only detected in muscle extracts from cod, saithe, Atlantic herring and Atlantic salmon.

In paper II the degradation of MHC during storage was studied in samples of isolated myofibrils and in pieces of intact muscle of Atlantic cod. In order to detect the MHC-degradation with higher sensitivity than by traditional SDS-PAGE and Coomassie staining, western blotting with a specific anti-MHC antibody (Martinez & Pettersen, 1992) was used. Myofibrils were isolated from freshly slaughtered cod and stored under different pH-conditions and temperatures for up to 10 days. The results showed that degradation products of MHC were detectable already from day two of storage while more severe degradation of MHC was detected after 5 days of storage at pH 6.3 and 6ºC. At pH 5.5 a similar degradation pattern as seen at pH 6.3 was observed. At pHs 7.0 and 8.0 there were no detectable degradation of MHC. When comparing different storage temperatures (0, 6 and 20ºC) at pH 6.3 immunoreactive degradation products of MHC was observed during the incubation period (7 days) even at 0ºC. The same degradation pattern as earlier described was seen at 6 ºC, while a large proportion of the MHC had been degraded at 20 ºC.

To determine whether the *in vitro* system was comparable to the conditions in whole intact muscle during storage, cubes cut from cod muscle was stored at 6ºC for up to 5 days and examined for MHC-degradation. The samples stored as intact pieces of muscle also showed degradation of MHC. However, the reduction of the 220 kDa MHC protein band was more apparent in the stored myofibril than in stored muscle. The observed difference can be explained by that proteinases might associate with myofibrillar proteins during the isolation procedure and then be able to degrade them more heavily during incubation. By using the

antibody produced against the isolated enzyme, it was shown that cathepsin D associated with the myofibrillar proteins and could not be completely removed by extensive washing during the isolation of the myofibrillar proteins. This ability to associate to myofibrillar proteins has also been observed for other cathepsins like B and L (Liu et al., 2008) and μ-calpain (Delgado, Geesink, Marchello, Goll & Koohmaraie, 2001b). Adding an inhibitor of aspartic proteinases to samples of myofibrillar proteins prior to incubation did inhibit the degradation of MHC at pH 5.5. This shows that cathepsin D has a role in the observed degradation of isolated MHC. Another explanation can be the NaCl used in the storage buffer. Salt is added to the buffer to help stabilize MHC in solution and the soluble MHC will be more easily subject to denaturation than if it was in a "*rigor*-like" state associated to actin. The study performed in paper II shows that the *post mortem* degradation of MHC in Atlantic cod occurs even at very low storage temperatures and that it is pH-dependent. The observed effect of pH indicates that acid proteinases play an important role.

In paper III the effect of pH on *post mortem* degradation of MHC in intact fish muscle was studied further. Traditionally, studies on *post mortem* proteolytic degradation of muscle have been performed in three different ways. One method used is isolation of muscle proteins or fractions of proteins and then incubation in the presence of added proteinases (Delbarre-Ladrat et al., 2004a; Geesink et al., 2000; Jiang et al., 1996; Ladrat et al., 2003; Ogata et al., 1998). Another method used is correlating the activities of proteinases present in muscle tissue with certain properties of the tissue (Bahuaud et al., 2010; Chéret et al., 2007a). The third method is storage of intact muscle tissue under different conditions and then analysis of the degradation by electrophoretic techniques during the storage (Bonnal et al., 2001; Jasra et al., 2001; Martinez et al., 2001; Papa et al., 1996; Tsuchiya et al., 1992). To be able to identify the enzyme mechanisms responsible for the *post mortem* proteolytic degradation and to avoid that the internal control mechanisms existing in intact muscle were lost because of disruption

of tissue during homogenization (Godiksen et al., 2009), a system that allows studies of the *post mortem* degradation mechanisms in intact fish muscle was developed.

Pieces of fish muscle were incubated in phosphate buffers with pH 5.7, 6.3 and 7.0 at room temperature (RT) for up to 5 days to adjust pH conditions in the muscle tissue during storage (Paper III). The pH in the *post rigor* muscle samples kept in high- and low-pH buffers were markedly changed already after 5 minutes. Samples kept at pH 7.0 had changed from 6.3 to 6.6, while samples kept at 5.7 had changed from 6.3 to 6.1. The fast change in pH inside the pieces of muscle continued during the first 15 minutes of incubation. Then the samples kept in high pH buffer had a pH of 6.9 and the samples kept in low pH buffer had obtained a pH of 6.05. Samples kept in pH 6.3 buffer showed no pH-changes during incubation. Further incubation of samples for up to 5 days did not change the muscle pH obtained after 1 hour. This shows that it is possible to quickly adjust the internal pH of muscle tissue samples. The low pH buffer seemed to have a too low buffering capacity, since the samples only could be adjusted to 6.05. A different buffer system would most likely solve this problem.

This system was used to adjust pH in muscle samples which were stored for up to 5 days. After storage, a specific antibody was used to study the degradation of MHC. The results showed that the degradation of MHC in *post mortem* fish muscle clearly is pH-dependent. Samples kept in pH 5.7-buffer showed a decrease in intensity of the 220 kDa protein band corresponding to MHC. In addition several degradation products with smaller molecular mass were observed. At pH 6.3 similar results were obtained, but the degradation appeared less prominent. At pH 7.0 little or no degradation was detectable. The ultimate pH of farmed cod and wild cod is often around 6.3 and 6.8 respectively. Farmed cod is known to suffer more from gaping than wild cod and undergo more severe structural alterations *post mortem* (Ofstad et al., 1996). The findings in this work show that MHC is degraded more at low pH and this indicates that lysosomal enzymes are responsible. Obvious candidate

enzymes to be responsible for the degradation are the lysosomal cathepsins, which have acidic pH-optimums. Other proteolytic enzymes present in fish muscle tissue have pH-optimums in the more neutral range. This is also supported by the results obtained when cod muscle was stored in the presence of different enzyme inhibitors and a cysteine proteinase activator. Inhibitors of aspartic and cysteine proteinases inhibited degradation of MHC when muscle was stored at pH 6.05. Surprisingly, addition of EDTA to the incubation buffer accelerated the MHC-degradation in a similar way as DTT. DTT reduces oxidised cysteine- or cystine-residues in the active site and will therefore function as an activator of cysteine proteinases. Inhibitors of metallo- and serine proteinases did not affect the degradation of MHC at low pH. Samples incubated at 6.3 showed the same general pattern as samples at low pH (6.05). Samples stored in pH 7.0-buffer showed no or very little MHC-degradation except when stored in the presence of DTT. The observed effect of different inhibitors provide evidence of cathepsin involvement in muscle degradation as suggested by others (Aoki et al., 1997; Bahuaud et al., 2010; Chéret et al., 2007a; Jiang et al., 1996; Ladrat et al., 2003; Ogata et al., 1998; Yamashita & Konagaya, 1991). Since no effect could be detected of any of the inhibitors at near-neutral pH, the results strongly indicate that cathepsin D and acid cysteine proteinases like cathepsin B and L are involved in the MHC-degradation in intact cod muscle. Schwarz & Bird (1977) reported that cathepsins D and L were able to degrade myofibrillar proteins at low pH (pH 6) *in vitro*. An interesting finding was that addition of EDTA to the incubation buffer seemed to speed up the rate of MHC-degradation at acidic pH. This suggests that metal-dependent enzymes, like calpains and matrix metalloproteinases, do not play a major role in this process. This activation-effect of EDTA has previously been described (Erdös, Debay & Westerman, 1960; Kirkeby, 1976). Possible explanations for this effect can be binding of divalent cations that inhibit proteinases, cysteine oxidation and

thereby activation of cysteine proteinases or myofibrillar protein destabilization as a result of metal chelation.

In this thesis the focus has been on cathepsins, in particular cathepsin D, and the degradation of myosin heavy chain in isolated myofibrils and intact muscle from cod *post rigor*. In the future, more work should be performed to study the degradation of other proteins in fish muscle, such as titin, nebulin, dystrophin and α-actinin. By using the system developed in this work to make changes in muscle pH and adding inhibitors, it will be possible to clarify which enzymes that are responsible for the quality-related changes in fish muscle proteins *post mortem*.

8 Conclusion

In this thesis the lysosomal cathepsin D was successfully isolated from liver of Atlantic cod. The pH- and temperature optimum were determined for the proteinase and found to be typical for lysosomal enzymes found in cold adapted fish. Further, a polyclonal antibody against cod cathepsin D was produced. The antibody detected the target protein in several fish species by western blotting. It also detected the native cathepsin D in extract from cod liver by immunoprecipitation.

The degradation of myosin heavy chain (MHC) from skeletal muscle of cod was also studied, both when stored as isolated myofibrils and as intact muscle. Even at low storage temperatures, a degradation of MHC was detectable by using western blotting with a MHC-specific antibody. It was also found that the degradation was more pronounced at low pH. This indicates that acid proteinases play an important role in the process. Cathepsin D seems to associate to isolated myofibrils even after several washing steps. This may be of importance when studying degradation of isolated myofibrils *in vitro*.

To more precisely elucidate which proteinases are involved in the *post mortem* MHC-degradation, a system for adjustment of the pH inside intact fish muscle was developed. By using buffers with different pH for incubation of pieces of intact fish muscle *post rigor*, the pH inside the pieces was raised, kept constant or lowered from the initial value of 6.3, which is a typical ultimate muscle pH in cod of farmed origin. After incubation at different pH-values and together with different enzyme inhibitors the degradation of MHC was studied using the MHC-specific antibody. The results showed increased breakdown at pH-values often found in intensively farmed fish. Cathepsin D and cysteine proteinases are probably the main enzymes involved.

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Paper 1

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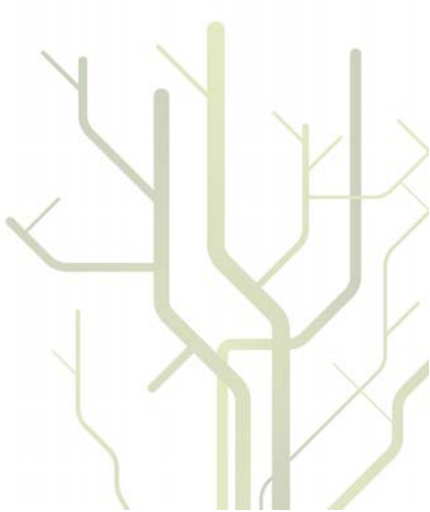

Paper 2

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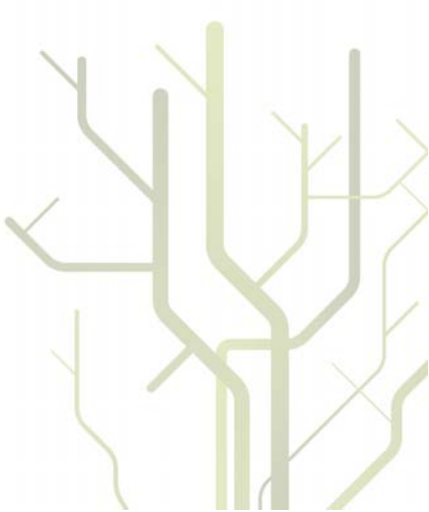

Paper 3

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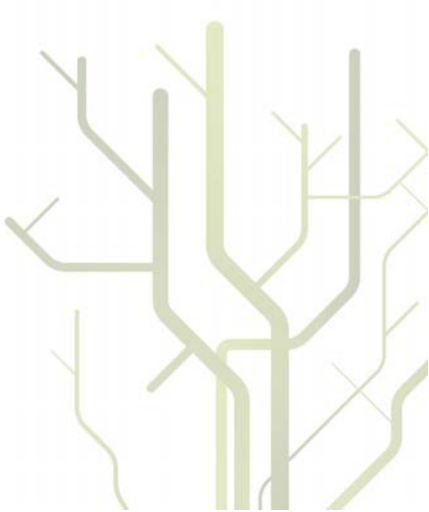

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