1 ***Post mortem* degradation of myosin heavy chain in intact fish muscle: Effects of pH and**
2 **enzyme inhibitors.**

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Abstract

Fish muscle is rapidly degraded during *post mortem* storage due to proteolytic enzymes acting probably both on muscle cells and connective tissue. In this work we have developed a model system which may be used to study the enzymatic degradation occurring in intact *post mortem* fish muscle. Degradation of myosin heavy chain (MHC) was monitored in muscle with pH adjusted to 6.05, 6.3 and 6.9 and in the presence of the enzyme inhibitors PMSF, EDTA, Phenanthroline, Pepstatin A, Antipain, E-64 and the cysteine proteinase activator Dithiothreitol (DTT). After storage, myofibrillar proteins were isolated and MHC-specific antibodies used to study the degradation in the different samples. MHC from muscle with pH 6.05 and 6.3 was degraded while no severe degradation was observed at pH 6.9. Introduction of enzyme inhibitors into the muscle tissue clearly showed that mainly cysteine and aspartic proteinases are responsible for the *in situ* MHC-degradation. This is supported by the severe breakdown of MHC in the muscle samples containing DTT.

Keywords: Atlantic cod, *post mortem* proteolytic degradation, myosin heavy chain, cathepsins.

1. Introduction

Fish muscle is rapidly degraded during *post mortem* storage due to proteolytic enzymes acting probably both on muscle cells and connective tissue (Cepeda, Chou, Bracho & Haard, 1990; Delbarre-Ladrat, Cheret, Taylor & Verrez-Bagnis, 2006). Unlike in mammalian meats where a certain amount of degradations usually are required for tenderization, the effects of the processes in fish are generally undesirable. These changes which are progressive during storage, may have significant impact on the quality and consumer acceptance of the fish fillets (Foegeding, Lanier & Hultin, 1996).

There are several proteolytic systems present in fish muscle tissue which may be involved in *post mortem* muscle degradation. These include the proteasome, an heat-stable alkaline proteinase, matrix metalloproteinases (MMPs), the calpains, and the lysosomal cathepsins (Delbarre-Ladrat et al., 2006). It has been suggested that cathepsins B, L and/or D could have major roles in degradations of proteins in *post mortem* fish muscle (Ladrat, Verrez-Bagnis, Noël & Fleurence, 2003).

Three main strategies have traditionally been used when studying *post mortem* proteolytic degradation of fish muscle. One is to homogenize fresh muscle tissue and incubate extracted sarcoplasmic and/or myofibrillar proteins with selected purified enzymes and then follow the degradation of muscle proteins by methods such as SDS-PAGE and Western blotting with antibodies directed against specific muscle proteins (Aoki & Ueno, 1997; Delbarre-Ladrat, Verrez-Bagnis, Noel & Fleurence, 2004; Folco, Busconi, Martone, Trucco & Sanchez, 1984; Geesink, Morton, Kent & Bickerstaffe, 2000; Jiang, Lee & Chen, 1996; Ladrat et al., 2003; Ogata, Aranishi, Hara, Osatomi & Ishihara, 1998; Yamashita & Konagaya, 1991). As stated by Godiksen, Morzel, Hyldig, & Jessen, (2009) a major weakness with homogenizing muscle and extracting proteins is that the natural control mechanisms in the intact muscle tissue are lost. Sub-cellular compartments are disrupted, enzyme-inhibitor

complexes may be formed or dissociated and enzyme precursors may be activated. It is well known that myofibrillar proteins (surimi) obtained after homogenization and washing of fish muscle possess proteolytic enzyme activities (An, Weerasinghe, Seymour & Morrissey, 1994; Cao, Jiang, Zhong, Zhang & Su, 2006; Hu, Morioka & Itoh, 2008; Wang, Martinez & Olsen, 2009). It is however an open question if these activities are native to the myofibrillar proteins or if enzyme–myofibrillar complexes are formed during the preparation of the myofibrillar fraction. A second strategy is to correlate the activities of the proteases present with specific properties of the fish or muscle tissue (Bahuaud, Mørkøre, Østbye, Veiseth-Kent, Thomassen & Ofstad, 2010; Cheret, Delbarre-Ladrat, de Lamballerie-Anton & Verrez-Bagnis, 2007; Yamashita & Konagaya, 1992). Both when isolated protein fractions are incubated with a specific protease and when the activity of a protease in a muscle is determined, the conditions used are most often highly optimized and may therefore be quite different from the *in situ* conditions in the *post mortem* muscle. For example when incubating with or analysing the activity of the cysteine protease cathepsin B, both EDTA and a reductant such as dithiothreitol, are usually included (Kirschke, Wood, Roisen & Bird, 1983). A third strategy is to store intact muscle for a certain period under different conditions and then analyze the degradation of proteins by electrophoretic techniques during the storage (Bonnal et al., 2001; Jasra, Jasra & Talesara, 2001; Martinez, Friis & Careche, 2001; Papa, Alvarez, Verrez-Bagnis, Fleurence & Benyamin, 1996; Tsuchiya, Kita & Seki, 1992). With this strategy it is not possible to determine the protease or proteases that are responsible for the degradation.

To be able to identify the enzyme mechanisms responsible for the *post mortem* proteolytic degradation in intact fish muscle we have adapted the system developed by Huang, Huang, Xi, & Zhou (2009) for studies of poultry muscle degradation. The aims of our study were to investigate how small changes in muscle pH and how the introduction of enzyme

76 inhibitors and activators into the intact muscle, affected the post mortem degradation of
77 myosin heavy chain (MHC) during storage of the muscle.

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2. Material and Methods

2.1. Fish

The results presented are from an Atlantic cod (*Gadus morhua* L.) of approximately 5 kgs purchased from a local fish supplier in Tromsø. After slaughter the gutted fish had been stored in ice for 4 days before hand filleted (*post rigor*). The fillets were then frozen at -50°C prior to further use. Similar results were found using a cod of similar size obtained from the Kårvika Aquaculture Station, Tromsø, but these are not shown.

2.2. Adjusting the pH in intact fish muscle samples

Cubes, approximately 2 grams, of fish muscle were incubated at room temperature (RT) in 8 ml 200 mM phosphate buffers, pH 5.7, 6.3 and 7.0 with 0.02% NaN₃ included. After 0, 1, 5, 15, 30 and 60 min and after 1, 2, 3 and 5 days of incubation, muscle cubes were homogenised 1:1 (w/v) in 150 mM KCl for 30 seconds using a T25 Ultra Turrax (Ika Laboratory and Analytical Equipment, Staufen, Germany) with a S25N-10 G dispersing tool (Ika). The pH was measured in the solution immediately after homogenisation. To ensure that the pH measured in the samples was not due to buffer left on the surface of the muscle pieces, the cubes were carefully wiped with a tissue paper prior to homogenisation and pH measurement.

2.3. Storage of muscle in the presence of protease inhibitors and an activator

Storage of muscle was performed as described under section 2.2 at pH 5.7, 6.3 and 7.0. The inhibitors used were Phenylmethylsulfonyl Fluoride (PMSF) (Calbiochem, San Diego, CA, USA), EDTA (Calbiochem), Phenanthroline (Sigma-Aldrich Inc., St. Louis MO, USA), Pepstatin A (Calbiochem), Antipain (Sigma) and E-64 (Sigma). The concentration of the different inhibitors is indicated in the figure legends. Fish muscle cubes, 0.5 g, were

individually incubated with 2 mls of buffers containing the different inhibitors or activator in glass containers with a plastic lid at room temperature. After the desired incubation times indicated in the figures, myofibrils were isolated from the samples as described below. One sample was incubated in the presence of the cysteine proteinase activator Dithiothreitol (DTT) (Calbiochem).

2.4 Isolation of myofibrils

Isolation of myofibrils was done according to Cao et al. (2006) with some modifications. 0.5 g of fish muscle were homogenized by Ultra Turrax (Ika) for 30 seconds in 2 mls of 50 mM phosphate buffer, pH 7.5, containing 0.02% NaN₃. The homogenate was then centrifuged for 15 minutes at 4°C and 4000g. The supernatant which contained sarcoplasmic proteins was removed and the pellet resuspended in 0.8 ml of the same buffer. This washing step was repeated three times. The third wash was performed with the same type of buffer with pH of the of the incubation solution. After the last wash the pellet was dissolved in 50 mM phosphate buffer with pH of the incubation solution, containing 0.02% NaN₃ and 500 mM NaCl. Then the sample volume was doubled using 10% SDS to totally dissolve all proteins.

2.5. SDS-PAGE and Western Blotting

SDS-PAGE was performed basically according to Laemmli (1970) using 4-10 % NuPAGE® Novex® Bis-Tris polyacrylamide gels in an XCell SureLock™ Mini-Cell (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Samples mixed with sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) were heated at 95–100 °C for 10 minutes prior to electrophoresis. The staining of the gels was performed using SimplyBlue™ SafeStain (Invitrogen). The molecular

mass standard used was Seeblue Plus2 Pre-Stained Standard (Invitrogen). Approximately 1 µg of total protein was applied to each well on gels destined for Simplyblue staining and 0.5 µg was loaded on gels destined for western blotting (Fig. 2). Protein concentration in the samples was determined using a BCA Protein Assay - Reducing Agent Compatible kit (Thermo scientific, Rockford, IL, USA). In the western blots of isolated myofibrils from samples incubated with enzyme inhibitors or activator an anti-actin antibody (A2066, Sigma) was used in a 1:1000 dilution as a loading control (Fig. 3). Proteins separated by SDS-PAGE were transferred to a PVDF-membrane, as described by Towbin, Staehelin, and Gordon (1979) by using Power PAC 1000 electroblot apparatus (Bio-Rad). The transfer was performed using 30 V for 90 minutes at room temperature. Immediately after the blotting, the membrane was washed 2x5 minutes at room temperature in TBS buffer (20 mM Tris-HCl, pH 7.5, containing 500 mM NaCl) and blocked with 25 ml TBS containing 0.05% Tween 20 and 5% fat-free milk for 30 minutes (blocking buffer). The membrane was then incubated with primary antibody in blocking buffer at 4 C° over night. The antisera dilutions used in the immunoblotting experiments are 1:80000 for the anti-MHC antibody (anti-MHC) (Martinez & Pettersen, 1992). The next day the membrane was washed 3 x 5 minutes in TBS containing 0.05% Tween (TTBS), followed by incubation for 1 hour at room temperature with a secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:10,000 in TTBS. The membrane was then washed 3 x 5 minutes in TTBS followed by 5 minutes in MilliQ water. Subsequently, the membrane was incubated with 0.75 ml Supersignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure to film (Lumi-Film Chemiluminescent Detection Film, Roche Diagnostics, Indianapolis, IN, USA) and developing.

3. Results

3.1 Adjustment of muscle pH

Pieces of fish muscle were incubated in phosphate buffers with pH 5.7, 6.3 and 7.0 at RT (20-22°C) for up to 5 days, to adjust pH conditions in the muscle tissue during the storage. Initial pH of the samples was 6.3. The results from the incubations for 1 hour are shown in Fig. 1. Incubation for 1 minute did not alter the tissue pH for any buffer used, while after 5 minutes the pH was markedly changed in the samples with high- and low-pH buffer. Samples kept in pH 7.0 had reached a tissue pH close to 6.6, while samples kept at 5.7 had a pH of 6.1. The relatively fast change in pH of the muscle tissue continued during the first 15 minutes and was then stabilized in the high-pH buffer at pH 6.9. The pH of the muscle samples incubated in pH 5.7 obtained a stable pH of approximately 6.05 after 15 minutes. The samples kept in a buffer with pH 6.3 showed no change in pH during the incubation period. Further incubation of muscle samples for up to 5 days did not change the tissue pHs obtained after 1 hour (results not shown).

3.2. MHC-degradation in stored muscle

Myofibrils were isolated from pieces of fish muscle stored under the three different pH-conditions to determine if these adjustments had effect on the MHC-degradation. The results in figure 2 clearly show a pH-dependent degradation of MHC in intact fish muscle.

Simplyblue staining of SDS-PAGE gels showed a decrease in intensity of the protein band corresponding to MHC (220 kDa) for samples kept at pH 5.7 (Fig. 2A) for 5 days. In addition protein bands at approximately 125 kDa appeared with increasing intensity when analyzed after, 1, 3 and 5 days of incubation. The relative amount of the 220 kDa band and the 125 kDa changed during the storage at pH 6.3 indicating similar, but less prominent

degradation. Little or no reduction in the 220 kDa band was seen in the samples kept at pH 7.0 and no appearances of bands at 125 kDa were detected.

The same samples were subjected to Western blotting with primary antibody directed against MHC and corresponding results were found as in Fig. 2A. A marked decrease of intact MHC (220 kDa) was detected in the samples kept at pH 5.7, together with an increase of the 125 kDa band (Fig. 2B). The same relative changes in intensities of the 220 kDa and 125 kDa bands were also seen at pH 6.3. No degradation was observed during the incubation period in samples kept at pH. 7.0.

3.3. MHC-degradation in muscle samples stored in the presence of protease inhibitors

Figure 3A shows a Western blot of extracted myofibrillar proteins from intact muscle samples incubated for 120 hours in the pH 5.7 buffer (muscle pH 6.05) together with different enzyme inhibitors and a cysteine proteinase activator (DTT). The results show that EDTA, Pepstatin A, Antipain, E64 and DTT present in the buffer, all influenced the degradation of MHC at pH 6.05. Pepstatin A, Antipain and E64 inhibited while EDTA and DTT accelerated the MHC-degradation compared to the control sample (lane 2). Degradation products of MHC (approximately 100-60 kDa) were present in high amounts in the control muscle sample (no inhibitor) incubated for 120 hours (lane 2) and in the muscle sample incubated with PMSF (lane 3). Less of these degradation products were observed in the muscles incubated with Pepstatin A (lane 6), Antipain (lane 7) and E-64 (lane 8). The sample incubated with Pepstatin A for 120 hours had about the same amount of these degradation products as the control at 0 hours (lane 1). The presence of Phenanthroline in the incubation buffer (lane 5) appeared to slightly reduce the amount of intact MHC and the smaller degradation products (approximately 55-60 kDa) compared to the control and PMSF-containing samples (lanes 2 and 3). The MHC in the muscle sample incubated with DDT appeared to be completely

203 degraded into two major bands of approximately 60 and 50 kDa (lane 9). The actin band
204 (loading control) was also degraded in this muscle sample. A similar 50 kDa band can also be
205 seen in the EDTA-containing sample (lane 4). In this sample, the high MW bands were just
206 faintly visible while the bands in the 60-100 kDa region are clearly visible.

207 Samples incubated at pH 6.3 (Fig. 3B) showed the same general pattern as samples in
208 pH 5.7 buffer (muscle pH 6.05), but the degradation appeared less pronounced. The band
209 corresponding to intact MHC was present with equal intensity in all samples except those
210 containing EDTA and DTT. In these two samples no intact MHC were discernable. The
211 samples with muscle pH 6.9 (Fig. 3C) showed no or very little degradation of MHC. A
212 possible exception is the muscle sample incubated with DTT (lane 9).

4. Discussion

Several reports have shown that a low ultimate muscle pH *post mortem* can lead to negative effects on fish muscle quality. Among these are poor liquid holding capacity (Ang & Haard, 1985; Love, 1979; Ofstad, Egelanddal, Kidman, Myklebust, Olsen & Hermansson, 1996; Rustad, 1992), increased gaping (Love, 1980) and a more rapid degradation of the muscle tissue (Ofstad et al., 1996). The reason for a low ultimate muscle pH is intensive feeding leading to a high glycogen level in the muscle (Ang et al., 1985; Ofstad et al., 1996). The ultimate muscle pH of farmed cod and wild cod is often around 6.3 and 6.8, respectively and it is known that farmed cod suffers more from gaping and undergo more severe structural alterations *post mortem* than wild cod (Ofstad et al., 1996). In this work we have developed a simple system, which enables us to determine the effects of pH and enzyme inhibitors on *post mortem* protein degradation in intact fish muscle. This makes it possible to identify the class of enzymes responsible for the degradation. We have studied MHC breakdown *post rigor*, but the technique could also be used in studies of other muscle proteins and probably also in the *pre-* and *in rigor* phase *post mortem*.

The results show that it is possible to quickly adjust the pH-conditions inside the fish muscle *post rigor*. The muscle pH of 6.3 at the start of the experiment suggests that the cod used was of farmed origin. The pH of the muscles was stabilized at the required level after only 15 minutes incubation. The samples kept in a low-pH buffer (pH 5.7) could not be adjusted lower than approximately pH 6.05. This was probably due to limited buffering capacity of the phosphate buffer in this pH range. A different buffer system could most likely have solved this problem, however the values obtained are relevant for a *post rigor* fish muscle. By altering the pH in the pieces of muscle, different types of enzymes can have conditions suitable for their action, and their ability to degrade muscle structures can be monitored. The increased degradation of MHC during the storage of the intact muscle at the

two lowest pHs indicates that lysosomal enzymes are responsible. Lysosomal cathepsins have acidic pH-optimum while most other proteolytic enzymes in muscle tissue have optimum in the more pH neutral range. Very little degradation of MHC appeared to have occurred during the 120 hours of *post rigor* storage at the near neutral pH, indicating that enzymes with such a pH optimum are not of major importance for this breakdown. We have previously shown that degradation of MHC in isolated myofibrils were similarly dependent on pH (Wang et al., 2009). Myosin has isoelectric point at pH 5.5 (Foegeding et al., 1996) and it is known that proteins may be more susceptible to degradation at pH close to the proteins isoelectric point (Dice & Goldberg, 1975). Consequently this may also contribute in the observed increased degradation at the low pH.

When storing muscle pieces in the presence of specific protease inhibitors the results support that mainly cathepsins are responsible for the MHC breakdown confirming more directly the involvement of these enzymes in muscle degradation (Aoki et al., 1997; Bahuaud et al., 2010; Cheret et al., 2007; Jiang et al., 1996; Ladrat et al., 2003; Ogata et al., 1998; Yamashita et al., 1991). The results show that cysteine- and aspartic acid proteinase inhibitors are more effective than serine- and metalloproteinase inhibitors in preventing breakdown of MHC at the slightly acidic pHs of 6.05 and 6.3. No effects of the cysteine- and aspartic acid inhibitors or the other inhibitors could be seen at the near neutral pH of 6.9. The results therefore strongly indicate that cathepsin D and acid cysteine proteinases, possibly B and L, are involved in MHC degradation in intact muscle *post mortem*. Schwartz & Bird (1977) reported that cathepsins B and D isolated from rat liver could degrade myofibrillar proteins at pH below 6. More recently, Ladrat et al. (2003) showed that isolated fish myofibrillar proteins are degraded by cathepsins B, D and L *in vitro*.

The severe breakdown of MHC in muscle samples with Dithiothreitol (DTT) included during the incubation confirms that cysteine proteinases are involved. DTT or other reducing

agents such as free cysteine, reduces oxidized cysteine or cystine in the active site thereby activating such proteinases. This result suggests that the oxidation state of the muscle tissue may be of importance for protein degradation and that the use of reducing agents when determining cysteine proteinase activities may not reflect the true activities in the muscle. The added DTT may also reduce cystine residues within the myofibrillar complex in the muscle sample and thereby making the proteins more accessible to proteolytic attack. Also the accelerated degradation of MHC when EDTA is used as inhibitor is an interesting observation, suggesting that metal-dependent or activated proteinases such as calpains and matrix metalloproteinases may not play a major role in this process. The mechanism behind the increased degradation of MHC in the presence of EDTA is difficult to explain, but activation of non-proteolytic enzymes by EDTA have previously been described (Kirkeby, 1976). Erdös (1960) also saw an activating effect of EDTA and interpreted this effect as binding of an other inhibitor present. Mort (1998) stated that in preparations of cathepsin B, EDTA together with DTT in millimolar concentrations should be used to avoid active site cysteine oxidation and formation of the dimeric amino acid cystine. The usage of metal chelators has been shown to inhibit cysteine oxidation in cell cultures in vitro (Ishii & Bannai, 1985). EDTA may also chelate divalent cations involved in stabilizing the myofibrillar protein structure and consequently making the proteins more susceptible to proteolytic degradation. More work is clearly required to elucidate the mechanisms behind the increased breakdown of MHC in muscle when it is incubated with DTT and in particular EDTA. EDTA, and also DTT, is commonly used in buffers for enzyme activity assays. Our findings indicate that addition of such compounds might speed up the rates of enzymatic reactions. This implicate that the measurements of enzymatic activity show total potential activity and not the amount of active enzyme.

In this paper we have focused on degradation of myosin heavy chain in intact fish muscle after the ultimate pH had been obtained (*post rigor*). The results showed increased breakdown at pH values similar to what found in the flesh of intensive farmed fish and that cathepsin D and cathepsin cysteine proteases probably are the main enzymes involved. However, this increased breakdown of MHC may not be the direct cause of the more rapid quality changes often seen in farmed fish. With the use of antibodies directed against other contractile or structural proteins, the technique could probably be used to clarify which enzymes are responsible for the quality related changes in muscle proteins occurring *post mortem*.

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Figure 1. pH adjustment in muscle samples incubated at room temperature (20-22°C) in 200 mM phosphate buffers. The buffer pHs are shown in the inserted box.

Figure 2. Coomassie stained SDS-PAGE gel (A) and Western blot (B) of myofibrils isolated from Atlantic cod muscle cubes incubated at three different pH-values without enzyme-inhibitors present. The samples were kept at room temperature (20-22°C). Incubation time and pH-conditions are indicated in the top panel of the figure. Primary antibody in the western blot was directed against myosin heavy chain (1:80000).

Figure 3. Western blot of myofibrillar proteins isolated from Atlantic cod muscle cubes incubated at pH 5.7 (A), pH 6.3 (B) and 7.0 (C) in the presence of different enzyme inhibitors. The samples were kept at room temperature (20-22°C) for 120 hours before isolation of the myofibrils. Primary antibodies were directed against actin (1:1000) and myosin heavy chain (1:80000). Type of inhibitor used and concentration is: Lane 1: No inhibitor 0h, lane 2: No inhibitor 120h, lane 3: 1 mM PMSF, lane 4: 5 mM EDTA, lane 5: 1 mM Phenanthroline, lane 6: 0.1 mM Pepstatin A, lane 7: 0.1 mM Antipain, lane 8: 0.1 mM E64 and lane 9: 5 mM DTT

Figure 1

Figure 2

Figure 3