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Myosin heavy chain degradation during post mortem storage of Atlantic cod (*Gadus morhua* L.)

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ABSTRACT

Post mortem proteolytic degradation of fish fillets leads to textural changes like muscle softening and gaping. In this study proteolytic degradation of myosin heavy chain (MHC) was monitored during storage of muscle and of isolated myofibrils at different temperatures and pH-values by the use of MHC-specific antibodies. The ability of cathepsin D to associate to myofibrillar proteins was also studied. Muscle stored at 6 °C and isolated myofibrils stored at 0 °C, 6 °C and 20 °C were degraded at pH 6.3 or lower. Cathepsin D could be found associated with extensively washed myofibrils. Inhibition of cathepsin D during storage affected the observed MHC-degradation at pH 5.5, but not at pH 6.3. This indicates that cathepsin D to a less extend than formerly believed, is responsible post mortem degradation of MHC.

1. Introduction

One of the most important quality characteristics for both animal and fish meat is texture. In animal meat post mortem proteolytic degradation of muscle proteins is often desired to obtain a tender product whilst in fish such degradation is usually unwanted due to quality loss (Foegeding, Lanier, & Hultin, 1996). Unwanted textural changes such as muscle softening and fillet gaping are often seen in wild and especially in cultured fish (Kristoffersen et al., 2006). The degree of degradation varies between species and seasons. The process is a result of complex physiological, physical, biochemical and subsequently microbiological processes. The underlying mechanisms causing muscle softening are not fully understood, but endogenous proteolytic enzymes are thought to play an important role in this process. Different proteolytic systems exist within a muscular cell. Amongst these are the multicatalytic proteasome, the lysosomal cathepsins, the cytosolic calpains, cytoplasmic aminopeptidases and some connective tissue hydrolytic enzymes (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). Calpains are reported to be responsible for the observed proteolysis in mammalian meat leading to tenderness (Koohmaraie, 1996). In fish the calpain system and the cathepsins, in synergy, are suggested to be responsible for post mortem muscle protein degradation (Delbarre-Ladrat et al., 2006). Lysosomal cathepsins like B, D and L and calpains have been reported to be able to use myofibrillar proteins as substrates *in vitro* (Delbarre-Ladrat, Verrez-Bagnis, Nöel, & Fleurence, 2004; Ogata, Aranishi, Hara, Osatomi, & Ishihara, 1998). Several fish muscle proteins such as titin (Seki & Watanabe, 1984), nebulin (Astier, Labbe, Roustan, & Benyamin, 1991), dystrophin (Papa et al., 1997), α-actinin (Ogata et al., 1998; Papa, Alvarez, Verrez-Bagnis, Fleurence, & Benyamin, 1996; Tsuchiya & Seki, 1991) and tropomyosin (Astier et al., 1991; Ogata et al., 1998) have been shown to be susceptible to post mortem degradation.

The main protein component of striated fish muscle is myosin. Myosin is a protein of approximately 500 kDa composed of six subunits; the two myosin heavy chains (MHCs) and four myosin light chains (MLCs). It is assembled into thick filaments which form the functional myosin in muscle. Some studies indicate post mortem proteolysis of fish myosin heavy chain (Busconi, Folco, Martone, & Sanchez, 1989), particularly when stored at room temperature (Astier et al., 1991). Others have found that MHCs are fairly stable when fish muscle is stored in ice for prolonged time (Jasra, Jasra, & Talesara, 2001; Tsuchiya & Seki, 1991; Verrez-Bagnis, Ladrat, Morzel, Nöel, & Fleurence, 2001). In these studies proteolytic degradation was studied by protein staining after SDS-polyacrylamide gel electrophoresis (SDS-PAGE), a method which might be relatively insensitive to detect changes in the protein pattern. Kjærsgård and Jessen (2003) and Schiavone, Zilli, Storelli, and Vilella (2008) have used 2-dimensional gel electrophoresis to study changes in the low molecular mass muscle proteins during post mortem storage of fish. Both studies reported increased solubilisation of MLCs during storage and it was indicated that this could be due to

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degradation of MHCs. Martinez, Friis, and Careche (2001) studied muscle protein degradation in Arctic (*Pandalus borealis*) and tropical shrimps (*Penaeus japonicus and Penaeus monodon*) during post mortem storage in ice for 4 days. SDS-PAGE and immunoblotting using polyclonal antibodies directed against fish MHC, clearly showed that MHC became degraded in the Arctic, but not in the tropical shrimps during storage.

In the present study we have investigated post mortem degradation of myosin in muscle of Atlantic cod by the use of SDS-PAGE and immunoblotting with antibodies directed against myosin heavy chain. Isolated myofibrils were used to examine myosin degradation under various pH and temperature conditions and for detection of a possible association between proteolytic enzymes and myosin.

2. Material and methods

2.1. Fish

The fish used in this study were raised at the Tromsø Aquaculture research station at Skulgamsbukt. Six farmed Atlantic cod (*Gadus morhua* L.) with weight of 4–6 kg, were carefully netted from the cage and stunned by a cranial blow, followed by cutting of the isthmus and bleeding in fresh water before gutting. The fish were filleted *pre rigor* and samples for myofibril and muscle storage experiments were removed. The remaining fish muscle was immediately frozen at −50 °C.

2.2. Isolation and storage of myofibrils

Isolation of myofibrils was done according to Cao, Jiang, Zhong, Zhang, and Su (2006) with some modifications. Fish muscle (200 mg) was homogenised by an T25 Ultra Turrax (Ika Laboratory and Analytical Equipment, Staufen, Germany) using an S25N-10 G dispersing tool (Ika) for 30 s in 800 μl of 50 mM sodium phosphate buffer, pH 7.5, containing 0.02% NaN_3. The homogenate was then centrifuged for 15 min at 4 °C and 4000 *g* in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany). Following this the supernatant which contained sarcoplasmic proteins was removed and the pellet resuspended in 800 μl of the same buffer. This washing step was repeated twice. The third wash was performed with the same type of buffer with the desired storage pH. After the last wash the pellet was dissolved in 50 mM sodium phosphate buffer with the desired pH, containing 0.02% NaN_3 and 500 mM NaCl. The buffers contained NaN_3 to avoid any microbial growth during storage of myofibrils and NaCl to keep the myofibrils soluble. Isolated myofibrils were incubated at 20 °C, 6 °C and on ice at 0 °C. After the desired incubation time the myofibril samples were dissolved 1:1 in 10% SDS and heated to 100 °C to dissolve all myofibrillar proteins and then frozen at −50 °C prior to simultaneous analysis of all samples. In one experiment a fresh muscle sample was homogenised, washed and centrifuged 6 times, instead of three times as described above, to remove as much as possible of soluble proteolytic enzymes prior to extraction of the myofibrils.

Myofibrils were also incubated with the aspartyl protease inhibitor pepstatin A (Calbiochem, San Diego, CA, USA). Pepstatin A was added to myofibrils isolated from frozen muscle samples at pH 5.5 and 6.3 in a final concentration of 0.01 mM. The myofibrils were then incubated at 6 °C and 20 °C for 120 h prior to heating and freezing as described earlier.

In order to compare the deterioration of MHC in intact muscle and in myofibrils, samples of fish muscle and isolated myofibrils from the same fish were stored at 6 °C. Prior to storage of the muscle, it was submerged in MilliQ water containing 0.02% NaN_3 for 30 min at 20 °C to remove microbial surface activity and to inhibit growth during storage (Olsson, Ofstad, Lødemel, & Olsen, 2003). The myofibrils were extracted from the muscle samples after 6, 12, 24, 48 and 120 h of storage and analysed together with isolated myofibrils stored for equally long periods.

2.3. SDS-PAGE and Western blotting

SDS-PAGE was performed basically according to Laemmli (1970) using 7.5% polyacrylamide gels in a Mini-PROTEAN III Electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). To get sharper protein bands, 20% glycerol was included in the gels. 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 min prior to electrophoresis. The staining of the gels was performed using Coomassie brilliant blue. If not otherwise stated in the figure legends, a total amount of 1 μg of protein was loaded to each well on gels destined for Coomassie staining and an amount of 0.5 μg of total protein was loaded in each well on gels determined for Western blotting. Measurement of sample protein content was performed with the Bio-Rad DC Protein Assay kit (Bio-Rad) according to the manufacturer's protocol. The molecular mass standard used was Seeblue Plus2 Pre-Stained Standard (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). 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 400 mV for 90 min at room temperature. Immediately after the blotting, the membrane was washed 2 × 5 min 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 min (blocking buffer). The membrane was then incubated with primary antibody in blocking buffer at 4 °C overnight. The antisera dilutions used in the immunoblotting experiments are 1:40,000 for the anti-MHC antibody (anti-MHC) (Martinez & Pettersen, 1992) and 1:5000 for the anti-cathepsin D antibody (αClcD) (Wang, Stenvik, Larsen, Mæhre, & Olsen, 2007). The next day the membrane was washed 3 × 5 min in TBS containing 0.05% Tween (TTBS), followed by incubation for 1 h 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 × 5 min in TTBS followed by 5 min in MilliQ water. Subsequently, the membrane was incubated with 750 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. MHC-degradation in stored myofibrils

Myofibrils were isolated from 6 freshly slaughtered cod and stored at 6 °C pH 6.3 for up to 10 days. Results from SDS-PAGE and Western blotting analysis of samples from two cods are shown in Fig. 1. Coomassie staining might indicate a slight decrease in the intensity of the intact MHC (220 KDa) after 5–7 days of storage (Fig. 1A and C). Simultaneously, an increase in bands with molecular weights of approximately 125 KDa could possibly be distinguished. No other changes could be observed. Western blotting using antibodies against MHC confirms the results indicated by the protein staining (Fig. 1B and D). The 220 KDa protein band was reduced after 5 days of storage (lane 6) and at least for myofibrils from one of the fishes the bands in the area of 125 KDa showed a distinct increase (Fig. 1D). In this fish, bands with

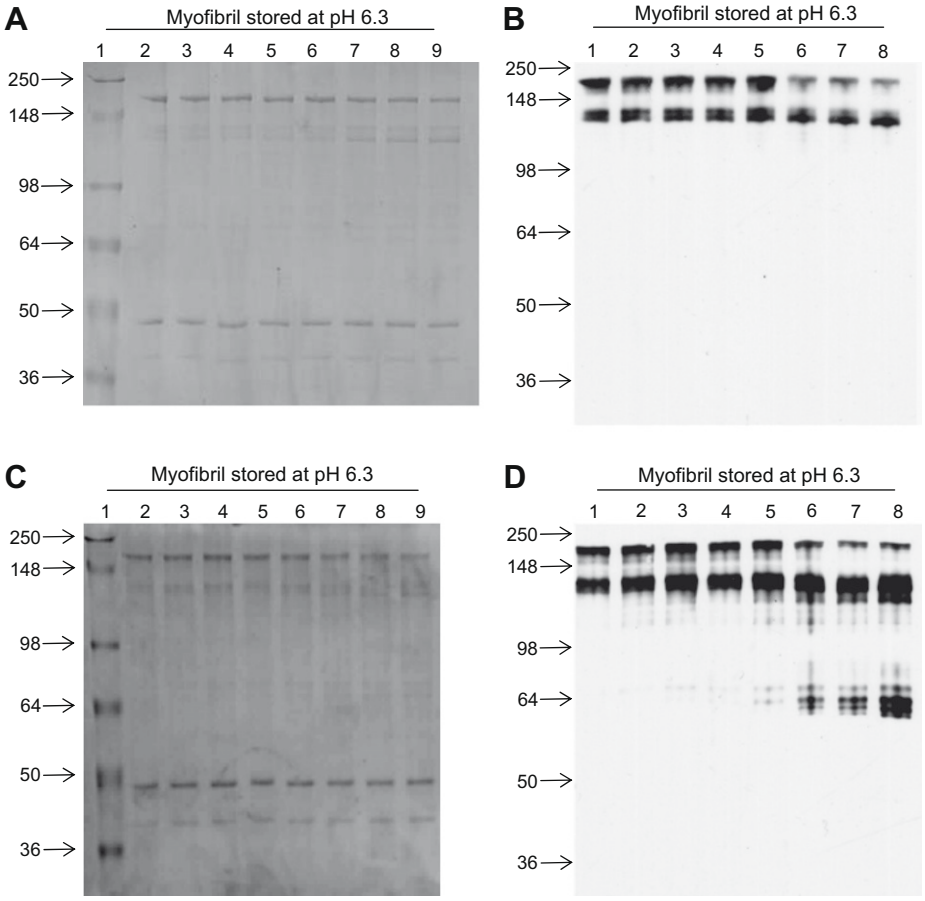

Fig. 1. SDS-PAGE (A and C) and Western blot (B and D) with anti-MHC as primary antibody of isolated myofibrils stored at 6 °C and pH 6.3. The samples in A and B were from fish #1 and in C and D from fish #2. A and C; Lane 1: MW marker, lane 2: 0 h storage, lane 3: 6 h storage, lane 4: 12 h storage, lane 5: 24 h storage, lane 6: 2 days storage, lane 7: 5 days storage, lane 8: 7 days storage and lane 9: 10 days storage. B and D; Lane 1: 0 h storage, lane 2: 6 h storage, lane 3: 12 h storage, lane 4: 24 h storage, lane 5: 2 days storage, lane 6: 5 days storage, lane 7: 7 days storage and lane 8: 10 days storage. Similar results were obtained with the other cods examined.

molecular weights of around 65–75 kDa became increasingly visible after 2–5 days of storage of the isolated myofibrils. The myofibrils isolated from the other four fishes showed intermediate degradation patterns indicating individual differences.

Myofibrils isolated from the same fish muscle were stored at 6 °C for 7 days at different pH-values (Fig. 2). Western blotting analysis showed that the MHC degradation was pH-dependent. Most profound degradation of the 220 KDa band took place during storage at pH 5.5. Some degradation could be observed at pH 6.3 whilst no apparent changes were observed at pH 7.0 and 8.0 but, more importantly, there were differences in the molecular mass of the proteolytic fragments produced depending on the pH. Myosin heavy chain degradation in isolated myofibrils during 5 days of incubation at different temperatures was also studied (Fig. 3). Very little degradation was detected when incubating at 0 °C (lanes 1–3). However, a slight increase was seen in the 125 kDa band during the storage period. As previously observed, some degradation appeared to have occurred at 6 °C (lanes 4–6) whilst a large proportion of the 220 KDa band had been lost after 5 days at 20 °C (lanes 7–9). Unlike in the case of the degradation produced under different pH conditions, there did not seem to be a difference in the molecular mass of the proteolytic fragments that could be attributed to either the length of the storage period or the temperature.

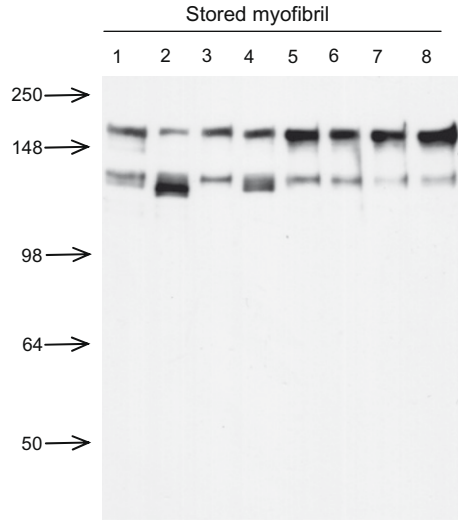

Fig. 2. Western blot with anti-MHC as primary antibody of isolated myofibrils stored at 6 °C at different pH. Lanes 1 and 2 stored in pH 5.5 for 0 h and 7 days, respectively. Lanes 3 and 4 stored in pH 6.3 for 0 h and 7 days, respectively. Lanes 5 and 6 stored in pH 7.0 for 0 h and 7 days, respectively. Lanes 7 and 8 stored in pH 8.0 for 0 h and 7 days, respectively.

3.2. MHC-degradation in stored muscle

Incubation of isolated myofibrils and pieces of muscle from the same fish were done at 6 °C to determine whether the MHC-degradation observed in isolated myofibrils also happened in whole muscle. Fig. 4A shows the degradation of MHC in muscle from

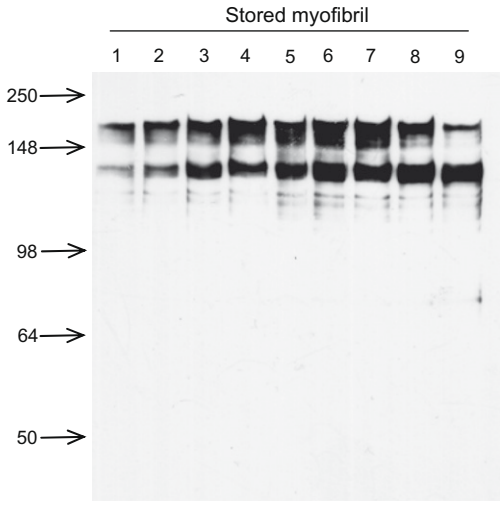

Fig. 3. Western blot with anti-MHC as primary antibody of isolated myofibrils stored at 0 °C, 6 °C and 20 °C at pH 6.3. Lanes 1, 2 and 3 stored at 0 °C for 0 h, 2 days and 7 days, respectively. Lanes 4, 5 and 6 stored at 6 °C for 0 h, 2 days and 7 days, respectively. Lanes 7, 8 and 9 stored at 20 °C for 0 h, 2 days and 7 days, respectively.

one cod as determined by Western blotting during storage for 5 days at 6 °C. Similar degradation patterns were observed in stored muscle from the other 5 cods (results not shown). The results showed that degradation of MHC occurred both in stored muscle and in the isolated myofibrils (Fig. 4). The reduction in intensity of the 220 kDa MHC protein band was more apparent in the stored myofibril (Fig. 4B) than in stored muscle (Fig. 4A). Bands of 65–75 KDa also became visible in the samples from isolated myofibrils, but could not be detected in samples from stored muscle. However, the increase of protein bands of molecular weights around 125 KDa were observed both in muscle and in isolated myofibrils. As could be expected due to the different conditions and possible presence of different proteases, MHC proteolytic fragments of different size were produced in stored muscle than in stored myofibrils.

3.3. Cathepsin D in isolated myofibrils

Western blotting with antibodies to cod cathepsin D was used to determine if this enzyme could be found associated with myofibrillar proteins after extensive washing. The results showed that a substantial amount of cathepsin D was found in the initial homogenate and in the 2 first wash fractions (Fig. 5, lanes 1–3). More extensive washing resulted in a strongly reduced amount of immunoreactive cathepsin D and in the 6th wash fraction it was barely visible (lanes 4–7). The myofibrillar proteins extracted by using high sodium chloride concentration (lane 8) indicate a higher concentration of cathepsin D present in the myofibrillar fraction than in the last wash fractions.

Isolated myofibrils were incubated for 5 days at pH 5.5 and at pH 6.3 in the absence or presence of 0.01 mM pepstatin A, an inhibitor, amongst others of cathepsin D. The incubations were carried out at 6 °C and at 20 °C. Fig. 6 show the degradation of MHC when myofibrils were incubated with and without 0.01 mM pepstatin A. At pH 5.5 (Fig. 6A) the sample treated with pepstatin A (lane 2) had an identical MHC-signal (220 kDa) to the control (lane 1) and a stronger signal than the non-treated sample (lane 3) at 6 °C. The untreated sample had much stronger immunoreactive bands around 125 kDa. The samples incubated at 20 °C had no or very weak immunoreactive protein bands at 220 kDa, whilst the bands around 125 kDa were stronger than the ones of the same size at 6 °C. The sample not treated with pepstatin A had immunoreactive bands at 64 and 70 kDa which were not detected in any other samples at pH 5.5.

Samples incubated at pH 6.3 (Fig. 6B) behaved differently than the ones incubated at pH 5.5. At 6 °C it does not appear to have been much degradation of MHC and the protein bands corresponding to MHC in the treated samples were both similar in intensity to the control (compare lanes 2 and 3 with lane 1). The immunoreactive bands at 125 kDa were slightly stronger in the treated samples than in the control, whilst a small band around 110 kDa had disappeared in both of the stored samples.

The samples incubated with and without pepstatin A at pH 6.3 and 20 °C (Fig. 6B, lanes 4 and 5) were also similar to each other,

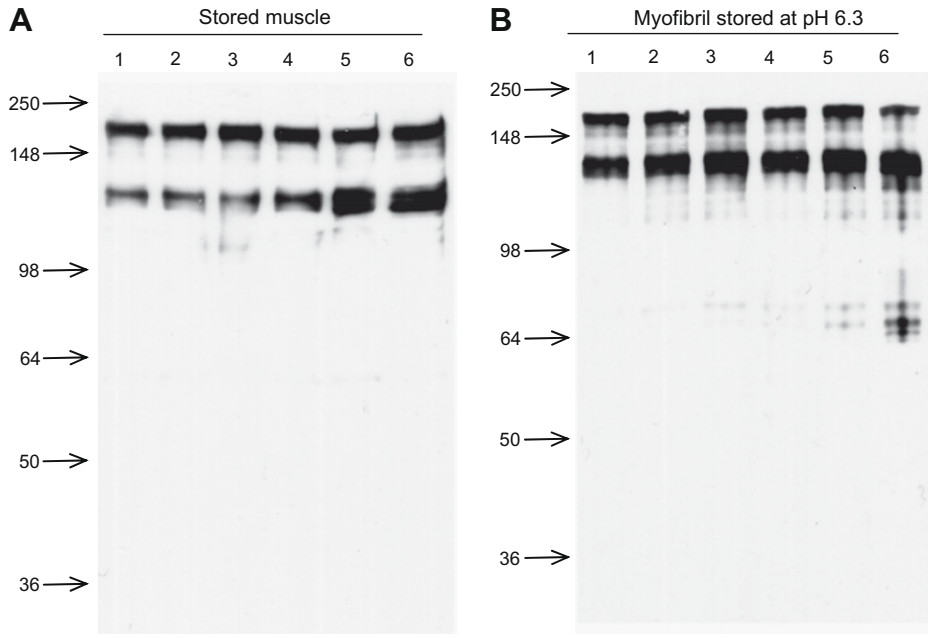

Fig. 4. Western blot with anti-MHC as primary antibody of myofibrils isolated from muscle stored at 6 °C (A) and of isolated myofibrils stored at 6 °C (B). Myofibrils were isolated at pH 6.3. Lane 1: 0 h storage, lane 2: 6 h storage, lane 3: 12 h storage, lane 4: 24 h storage, lane 5: 2 days storage and lane 6: 5 days storage.

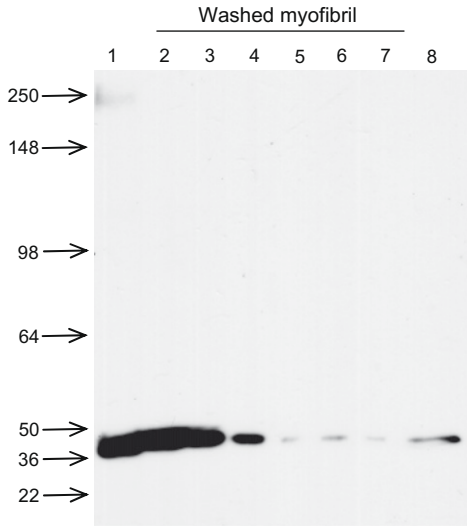

Fig. 5. Western blot with αClcD as primary antibody of myofibrils washed six times during the isolation procedure. Protein content in each sample was not determined, but the same volume of sample was applied to each well (10 μl). Lane 1: initial homogenate, lane 2: wash fraction 1, lane 3: wash fraction 2, lane 4: wash fraction 3, lane 5: wash fraction 4, lane 6: wash fraction 5, lane 7: wash fraction 6 and lane 8: isolated myofibrils.

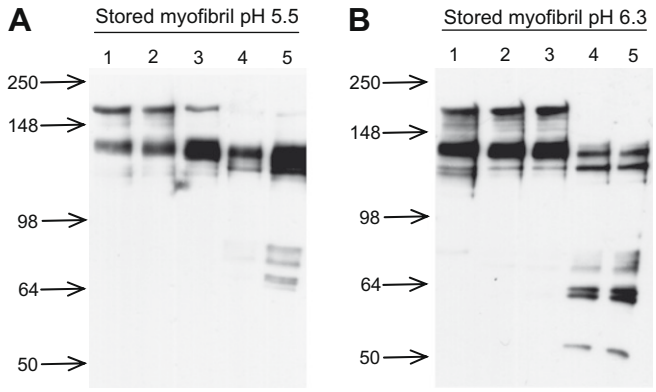

Fig. 6. Western blot with anti-MHC as primary antibody of isolated myofibrils stored with pepstatin A at 6 °C and 20 °C at pH 5.5 (A) and pH 6.3 (B). Lane 1: control (0 h), lane 2: 5 days storage at 6 °C with 0.01 mM pepstatin A, lane 3: 5 days storage at 6 °C without pepstatin A, lane 4: 5 days storage at 20 °C with 0.01 mM pepstatin A and lane 5: 5 days storage at 20 °C without pepstatin A.

but different from the 6 °C samples. At 20 °C the MHC was completely degraded. There were however distinct immunoreactive bands at approximately 125, 110, 70, 65 and 50 kDa. Differences in the molecular mass and intensity of MHC proteolytic fragments originated could be clearly attributed to the pH of storage.

4. Discussion

Post mortem degradation of fish muscle proteins is regarded a major cause for quality loss such as muscle softening and gaping in fish. Traditionally, key proteins subjected to degradation have been reported to be structural myofibrillar proteins such as titin, α-actinin and nebulin and proteins linking the sarcomere to the sarcolemma (reviewed by Delbarre-Ladrat et al., 2006) as well as MHC and α-actinin in Arctic species (Martinez et al., 2001). Our work clearly presents immunologically detected alterations of MHC during post mortem storage of cod muscle and of isolated myofibrillar proteins in a temperature and pH-dependent manner. The use of MHC-specific antibodies makes it possible to monitor the patterns of degradation during the storage period. The observed pattern of proteolysis is similar to the myosin degradation observed in pressurised chicken muscle (Ikeuchi et al., 2001).

When examining samples of stored myofibrillar proteins it is clear that although traditional SDS-PAGE may give a rough estimation of the extent of protein degradation, the use of immunological methods with specific antibodies directed against relevant proteins, such as MHC, provide additional and valuable information such as the degradation pattern and size of the proteolytic fragments. The present approach for example also permits a rough estimation of whether the same or different proteolytic enzymes are active on the MHC. Since different proteases cut the MHC at different locations, the peptide map produced, i.e. the distribution of fragments of MHC (their molecular masses and relative intensities) will be dependant on the proteases active.

In this study we have also showed that there are variations between individuals in the degree of MHC-degradation in cod myofibrils. Individual variations, sometimes large, are often registered when dealing with biological material, such as fish (Olsson, Cooper, Friis, & Olsen, 2006).

The pH-dependence of MHC-breakdown during storage of myofibrils is supporting the thesis that endogenous cathepsins may be the main enzymes responsible for the observed MHC-degradation. Cathepsins B and D are, in contrast to calpains (Ladrat, Chaplet, Verrez-Bagnis, Nöel, & Fleurence, 2000), known to have acidic pH-optimum. The fact that fillets from farmed cod suffer more gaping and undergo more severe structural alterations than wild cod, is often explained by the lower muscle pH (Kristoffersen et al., 2006; Ofstad et al., 1996). It is tempting to suggest that this lower pH-value provides more favourable working conditions for the cathepsins. Schwartz and Bird (1977) have reported that cathepsin D from rat liver and skeletal muscle were able to degrade purified myosin up to pH 6.0. The near neutral muscle pH of wild cod will, unlike the more acidic muscle pH of farmed cod, provide more unsuitable working conditions for the cathepsins.

The observed MHC-breakdown in stored myofibrillar proteins at high temperatures (20 °C) is similar to what others have reported. In addition, we have shown that MHC in cod muscle is also degraded at temperatures between 0 and 6 °C. This is probably due to the fact that cod is a cold water species adapted to Arctic temperatures, as the Arctic shrimp examined by Martinez et al. (2001), and therefore its endogenous enzymes such as proteases are likely to be active at very low temperatures.

It is well known that proteolytic enzymes can be found associated with isolated myofibrils from fish muscle. Much focus has been on so called myofibril bound serine proteases (Cao, Weng, Liu, Hara, & Su, 2007; Jiang et al., 2006; Kinoshita, Toyohara, & Shimizu, 1990). When storing pieces of muscle and comparing the pattern of MHC-breakdown with stored myofibrils both types of samples show degradation, but they are different from each other. The proteases present in the muscle might be able to associate with myofibrillar proteins during the isolation procedure and then degrade them more heavily during the incubation. In the present work the observed extent of the degradation might be affected by the 500 mM NaCl used in the storage buffer. On one hand, the high amount of salt used to help stabilizing MHC will probably make it more resistant to proteolytic attack, than if it was denatured. On the other hand the soluble protein will be more easily subject to proteolytic degradation than if it was in a compact "rigor-like" actomyosin state. Finally, the activity of the proteases themselves may be either activated, inhibited or remain unaffected by the salt in the buffer. For example, it is known that cathepsin D-like activity from fish muscle is inhibited by high NaCl concentrations (Gómez-Guillén & Batista, 1997). In any case, association of protease and myofibril is less likely to occur during the storage

of muscle pieces, due to the cellular compartmentalisation. We observed that cathepsin D associated strongly with the myofibrillar proteins and could not be removed totally by extensive washing. This can also be the case with other proteases. Liu, Yin, Li, Zhang, and Ma (2008) have reported that activities of cathepsins B and L remain after washing of silver carp mince. Whether the association happens during the myofibril protein isolation procedure or has occurred prior to isolation, is not known. There are however reports that μ-calpain associates to myofibrils during post mortem storage of bovine muscle (Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001).

Adding an inhibitor of aspartyl proteases to samples of myofibrillar proteins prior to incubation has an inhibiting effect on the degradation of MHC at pH 5.5. This shows that cathepsin D has a role in the MHC-degradation observed in this study. Other authors have reported that cathepsin D is capable of degrading MHC at temperatures above 20 °C and pH below 6.3 (Ladrat, Verrez-Bagnis, Nöel, & Fleurence, 2003; Weng, Hamaguchi, Osako, & Tanaka, 2007) and that it is detrimental to the firmness of fish fillets (Godiksen, Morzel, Hyldig, & Jessen, 2009). Moreover, the different degradation pattern of MHC at pH 5.5 and 6.3 seems to indicate that there are differences in the proteases active at these two pH-values.

In summary, this study shows that degradation of cod MHC occurs even at very low storage temperatures and that it is pH-dependant. The effect of pH on the degree of degradation indicates that acid proteases play an important role in the process. Since the inhibiting effect of pepstatin A was negligible at relevant muscle pH, the role of cathepsin D might be less important than other cathepsins.

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