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In vitro proteolysis of myofibrillar and sarcoplasmic proteins of white muscle of sea bass (*Dicentrarchus labrax* L.): effects of cathepsins B, D and L

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Abstract

The purpose of this study was to obtain additional information regarding proteolysis mechanisms and disorganization of fish myofibrils resulting in a loss of flesh quality. The ability of cathepsins to degrade in vitro myofibrillar and sarcoplasmic proteins from fish muscle was investigated in order to explain their role in *post mortem* softening. This led to the identification of substrates of the enzymes. Cathepsins degraded myosin heavy chain and α-actinin. Tropomyosin and actin were only susceptible to the action of cathepsin L. Troponin T (assumed 32 kDa component) was resistant only to the action of cathepsin D. Desmin was degraded by cathepsins B and L. Slight changes of some other myofibrillar or cytosolic proteins were also observed (creatine kinase and other unidentified proteins). When compared with protein modifications observed in stored *post mortem* muscle, these results suggest that cathepsin D (if location is in the cytosol and if pH conditions for activity are met in *post mortem* muscle) could be involved in a *post mortem* myofibrillar degradation mechanism.

Keywords: In vitro proteolysis; Cathepsins; Fish muscle proteins; *Post mortem* degradation

1. Introduction

Post mortem tenderization is one of the most unfavourable quality changes in fish muscle and contrasts with muscle of mammalian meats. Therefore, the biochemical processes have been extensively studied in order to identify potential quality indicators or to control *post mortem* degradation. Muscle tissue *post mortem* evolution is characterized by successive biochemical reactions and autolytic modifications, resulting in disorganization of the muscular structure. A proteolytic degradation of myofibrillar and connective tissue components is observed in this tissue. But an understanding of the mechanisms involved in these changes, especially in fish muscle, has not yet been achieved.

There has been considerable debate about the specific protease responsible for *post mortem* changes. The participation of various proteinases in autolytic processes of ice-stored fish depends on the following: location of the enzymes in cytosol and/or factors affecting tissue compartmentization, presence of activators or inhibitors and the susceptibility of the proteins responsible for muscle integrity to *in situ* cleavage by the respective enzymes. Two major intracellular degradative pathways are involved in these degradations: a lysosomal path-way, including cathepsic proteases, and a cytosolic calcium-dependent path-way with calpains. Other proteases, such as metalloproteinases, may also have a role in *post mortem* fish muscle changes, especially with extracellular matrix components, such as collagen (Kubota, Kinoshita, Kubota, Yamashita, Toyohara, & Sakaguchi, 2001). Purified proteasome from lobster (Mykles & Haire, 1995) and rabbit (Matsuishi & Okitani, 1997) hydrolyses myofibrillar proteins but it requires activation by heat or addition of SDS. Thus, its role in *post mortem* degradation could be of less importance, but it also needs to be further clarified. The respective contributions of these systems to myofibrillar protein degradation are still unclear, but it is highly likely that several protease groups could synergistically

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contribute to *post mortem* tenderization. In particular, calpains are believed to initiate the disintegration of the Z line by a titin cleavage (Astier, Labbe, Roustan, & Benyamin, 1991) which weakens the titin/α-actinin interaction and results in the release of intact α-actinin from Z lines (Papa, Alvarez, Verrez-Bagnis, Fleurence, & Benyamin, 1996) before its further proteolysis by proteasome and various cathepsins (Goll, Thompson, Taylor, & Christiansen, 1992; Lamare, Taylor, Farout, Briand, & Briand, 2002), especially in mammalian muscles.

Post mortem changes in sea bass muscle include the weakening and the disorganisation of the Z line structure, the detachment of sarcolemma, and the degradation of dystrophin (Papa et al., 1997) as well as the degradation of titin and nebulin (Astier et al., 1991) and the release and proteolysis of α-actinin from the Z line (Papa, Alvarez et al., 1996). We found that desmin remained unchanged after a 4 day ice storage in cultured sea bass muscle but was highly degraded in wild fish, such as sardine (Verrez-Bagnis, Noël, Sautereau, & Fleurence, 1999). A 16 kDa sarcoplasmic protein was also shown to undergo proteolysis upon storage (Verrez-Bagnis, Ladrat, Morzel, Noël, & Fleurence, 2001).

Three different calpain-like activities have been detected in *post mortem* white muscle from sea bass. They have biochemical properties similar to those of terrestrial vertebrates but are expressed differently throughout the year (Ladrat, Chaplet, Verrez-Bagnis, Noël, & Fleurence, 2000). A previous report found that m-calpain was able to release myofibrillar components in the soluble fraction: tropomyosin, α-actinin and some other unidentified proteins; it degraded myosin heavy chain, α-actinin and desmin but it left actin and tropomyosin intact. A 26.5 kDa sarcoplasmic component was also degraded by calpain (Verrez-Bagnis, Ladrat, Noël, & Fleurence, 2002).

Until now, the effect of cathepsins on sea bass muscle proteins has not been investigated. Lysosomes are known to harbour about 13 cathepsins (Kolodziejska & Sikorski, 1995). Among them, cathepsins B, D, L, L-like have been purified from fish muscle. Cathepsins B, D and L are considered to be critical in fish muscle *post mortem* modifications (Aoki & Ueno, 1997; Jiang, Lee & Chen, 1996; Jiang, Wang, & Chen, 1992; Ogata, Aranishi, Hara, Osatomi & Ishihara, 1998; Yamashita & Konagaya, 1990a) or in gel softening during setting of mackerel surimi (Ho, Chen, & Jiang, 2000; Yamashita & Konagaya, 1991). Cathepsins A and C contribute to the hydrolysis of muscle protein in a concerted action with the other cathepsins (Kolodziejska & Sikorski, 1995).

In this paper, the effects of cathepsins on myofibrillar and sarcoplasmic components are investigated to analyse their role in *post mortem* degradation of fish muscle. Especial attention is paid to the lysosomal enzymes cathepsins B, D and L.

2. Material and methods

2.1. Fish

Sea bass (*Dicentrarchus labrax* L.) was purchased alive from a local sea farm in Vendée (France) and killed by decapitation in the laboratory. Dorsal white muscle samples were frozen in dry ice and kept at −80 °C until used for the preparation of sarcoplasmic proteins just before enzymatic digestion.

2.2. Protein preparations

Sarcoplasmic proteins were extracted from muscle frozen at death, by homogenization in 4 volumes (w/v) of water containing 1 mM EDTA. Solutions were centrifuged twice at 10 000 *g*, 4 °C for 15 min. Protein content was determined by Bradford assay (Bradford, 1976) using Bio-Rad reagent (Bio-Rad, Ivry-sur-Seine, France). Myofibril preparation was done on fresh sea bass muscle according to the method of Wang (1982), suspended in 50% (v/v) glycerol and stored at −20 °C until use. Protein content was estimated at 12.5 mg/ml by Bradford assay. Before incubation with proteolytic enzymes, one aliquot of this preparation (800 μl) was taken, centrifuged thrice at 4000 rpm for 15 min at ambient temperature with the pellet being resuspended each time in 1000 μl water. The final pellet was used directly for the reaction mixture.

2.3. Protease preparations

Since no cathepsin from fish is commercially available, cathepsins from other organisms were chosen, making the assumption that they exhibit a similar action toward proteins with respect to the cleavage specificity (Yamashita & Konagaya, 1990b, 1990c). *Paramecium tetraucelia* Cathepsin L was purchased from CALBIOCHEM (Merck Eurolab, Fontenay-sous-bois, France). cathepsin B (C6286) and cathepsin D (C3138) both from bovine spleen were from SIGMA (Saint-Quentin-Fallavier, France).

2.4. Proteolytic digestions

The digestion mixture with cathepsins contained 5 mg/ml sarcoplasmic or myofibrillar proteins, 2 mM EDTA, 0.1 M KCl, enzymes and other additives, as required, in 50 mM acetate buffer, pH 5.5. 7 μg cathepsin B were used for 1 ml of digestion mixture in the presence of 5 mM DTT at 25 °C. 23 μg cathepsin D per millilitre of incubation solution were used at 25 °C. 3 μg/ml cathepsin L were used at 25 °C in the presence of 5 mM DTT. Incubations lasted up to 22 h with aliquots sampled at 0–30 min, 1, 2, 4, 6 and 22 h.

Controls were carried out without enzymes. Each digestion was done and analysed twice.

Two aliquots from the sarcoplasmic protein digestions were removed at specific intervals. Digestion in the first aliquot was stopped by rapid addition of 4× concentrated SDS-containing tracking dye and immediate heating for 3 min at 100 °C while one volume of 5% TCA was added in the second aliquot. For myofibrillar protein breakdown, the first aliquot was added to one volume of 5% TCA; the second aliquot was first centrifuged at 4000 rpm, at room temperature for 15 min in the presence of inhibitors to stop the reaction (0.01 mM E64, 0.01 mM pepstatin A). Supernatant containing proteins released from myofibrils was added to 4× concentrated SDS-containing tracking dye. The pellet was solubilized in the same volume (as initial aliquot) of 40 mM Tris buffer containing 8 M urea; 4× SDS-containing tracking dye was added before boiling for 3 min.

TCA samples were treated as previously described (Verrez-Bagnis et al., 2002) to determine the amount of soluble peptides produced whose increase during digestion was evidence of effective activity of enzymes under the conditions used. Samples with SDS and dye were frozen until submission to SDS–PAGE.

2.5. SDS–PAGE and Western blots

SDS–PAGE and Coomassie Blue staining were performed in order to gain a general insight into modified proteins and blotting with immunodetection of specific proteins was performed. Tropomyosin, α-actinin and desmin were followed by this method because of their key roles in myofibrillar integrity; they have also been reported to undergo changes during *post mortem* storage in sea bass.

SDS–PAGE was carried out in a Mini-protean dual slab cell (Bio-Rad) according to the method of Laemmli (1970), using 4–20% polyacrylamide gradient gels. Five microlitres of sample from the digestion of sarcoplasmic proteins, 5 μl of sample prepared from the pellet of digested myofibrillar proteins and 10 μl from the corresponding supernatant were loaded into separated wells. Several sets of molecular weight markers were used: a mixture of SDS-6H and SDS-7 from SIGMA for Coomassie blue stained gels and prestained markers from Bio-Rad for immunoblots, as well as for some blue gels (Broad Range and Broad Precision prestained markers). Average molecular weights were estimated from several analysed gels. After migration, gels were subsequently stained with Coomassie Brilliant Blue R250 or blotted overnight at 15 °C onto a 0.45-μm nitrocellulose membrane at 30 V, using a Mini-Transblot Cell (Bio-Rad). Transfer buffer was as follows: 150 mM glycine, 20 mM Tris, 20% methanol, 0.01% SDS. Membranes were saturated with 8% skimmed milk, reconstituted in 0.9% NaCl, incubated with rabbit antibodies diluted in 0.9% NaCl and left for 2 h at room temperature. They were then washed with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer, pH 7.4) containing 0.05% v/v Tween 20 and subsequently incubated with secondary goat anti-rabbit IgG alkaline phosphatase conjugate, diluted with 0.9% NaCl containing 5% bovine serum albumin. Enzymatic detection was carried out using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as the substrate, resulting in brown-red bands. Primary antibodies included polyclonal anti-α-actinin, produced in the laboratory (1/300 dilution) and used for sarcoplasmic and myofibrillar protein digestions, polyclonal anti-desmin (D8281, SIGMA, 1/200 dilution) and polyclonal anti-tropomyosin (T3651, SIGMA, 1/200 dilution). The two latter antidodies were used on myofibrillar proteins for in vitro proteolysis only.

2.6. Image analysis

The Coomassie Blue-stained gels, as well as the immunoblots, were photographed with a CCD camera system (Image Master VDS-CL, Amersham Biosciences, Orsay, France) and analysed with 1D-Elite program (Amersham Biosciences).

3. Results

3.1. General

The present study made an inventory of the qualitative changes affecting muscular proteins from sea bass when digested by cathepsins, in order to assess their ability to reproduce, in vitro, the *post mortem* changes observed in muscle.

3.2. Sarcoplasmic proteins

Sarcoplasmic proteins are mainly composed of enzymes associated with energy-producing metabolism (e.g. glycolysis, citrate cycle) (Nakagawa, Watabe, & Hashimoto, 1988b). Except for parvalbumins (Gerday, 1982), they have only partially been characterized. Sarcoplasmic extracts resulted in 13 major protein bands (Fig. 1A) and are comparable with the samples described by Nakagawa (Nakagawa, Watabe, & Hashimoto, 1988a). The most abundant protein bands are as follows in average molecular weights: 97 kDa, a doublet band at 60 kDa, 51 kDa, a 41–39 kDa huge band, which is most probably a doublet band [these two bands are assumed to be, respectively, creatine kinase and aldolase by comparison with the patterns described by Nakagawa et al. (1988a)], and a 36 kDa component assumed to be glyceraldehyde-3-phosphate dehydrogenase; supplementary bands were observed at 34, 27, 25, 21.5, and 17 kDa. The last one was shown to disappear upon cold storage (Verrez-Bagnis et al., 2001) and was further

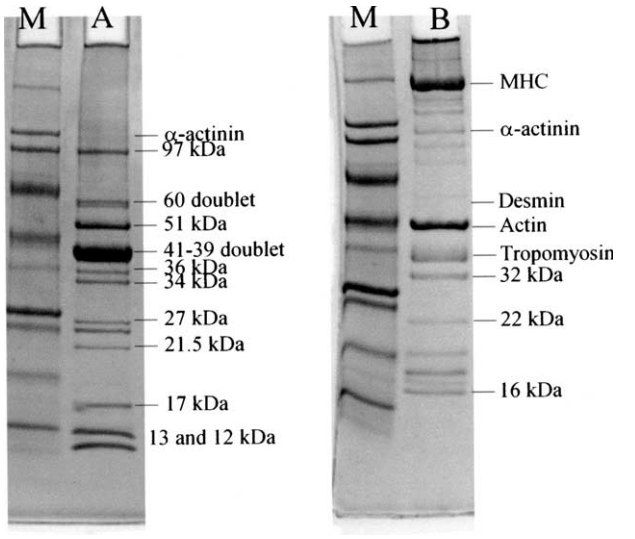

Fig. 1. Annotated major sarcoplasmic and myofibrillar proteins. A: sarcoplasmic extract. B: myofibrillar extract. M: SDS-6H and SDS-7 markers: Myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase *b* 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, glyceraldehyde-3-phosphate dehydrogenase 36 kDa, carbonic anhydrase 29 kDa, trypsinogen 24 kDa, trypsin inhibitor 20.1 kDa, α-lactalbumin 14.2 kDa.

identified as a nucleoside diphosphate kinase by N-terminal sequencing (data not shown). Finally, two protein bands, at about 13 and 12 kDa, could be parvalbumins (Focant, Mélot, Collin, Chikou, Vandewalle, & Huriaux, 1999; Girija & Rehbein, 1988).

Electrophoretic separation of hydrolysis products by cathepsin B (Fig. 2A) revealed that the most susceptible protein to degradation was the huge band around 40 kDa which is then visibly dissociated into two bands. The 41 kDa creatine kinase is likely degraded by cathepsin B. Two fragments (30.5 and 20.5 kDa) were generated at the end of the incubation with cathepsin B. The other protein bands remained unchanged. Cathepsin D (Fig. 2B) was not able to degrade any sarcoplasmic proteins, as shown on the Coomassie blue-stained gel, whereas cathepsin L (Fig. 2C), like cathepsin B, was able to dissociate the 41-39 doublet, probably by degrading both bands. The 21.5 kDa component is also susceptible to proteolysis by cathepsin L and two fragments (24 and 20.5 kDa) appeared upon incubation with cathepsin L.

It was possible to detect some α-actinin by the immunological method in the sarcoplasmic fraction. It was not degraded by cathepsin B, or cathepsin D or cathepsin L (data not shown).

3.3. Myofibrillar proteins

After electrophoresis and Coomassie blue staining of the myofibrillar proteins, 14 bands were observed. Myofibrillar patterns are also similar to those described elsewhere (Ball & Johnston, 1996; Lund & Nielsen,

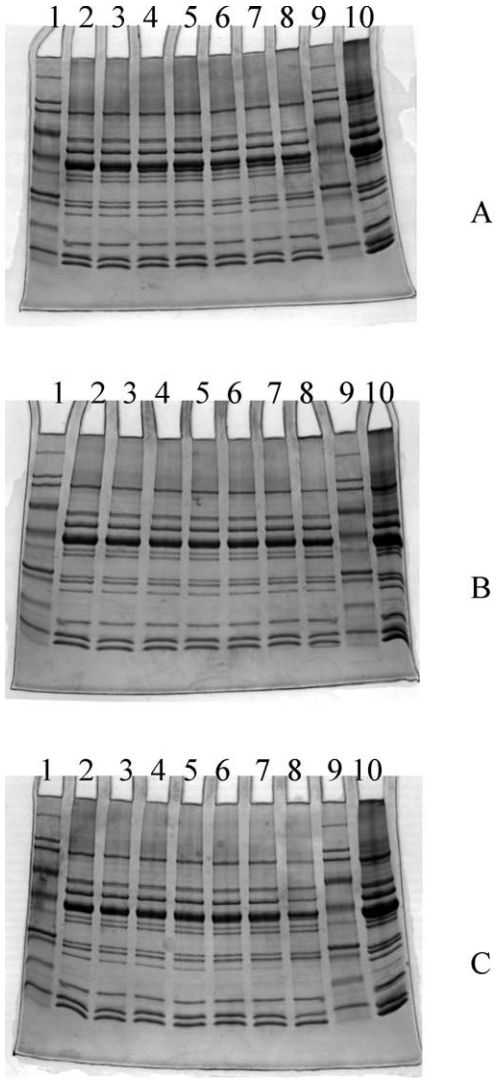

Fig. 2. Effect of cathepsins on sarcoplasmic proteins analysed by SDS–PAGE and Coomassie blue staining. A: cathepsin B; B: cathepsin D, C: cathepsin L. Lanes 1 and 9: SDS6H and SDS-7 markers (see legend to Fig. 1), lane 2: 0 min of incubation, lane 3: 30 min, lane 4: 1 h, lane 5: 2 h, lane 6: 4 h, lane 7: 6 h, lane 8: 22 h, lane 10: sarcoplasmic extract.

2001; Ogata et al., 1998; Swartz, Moss, & Greaser, 1997). By comparison of molecular weights, major structural proteins have been identified. Typical bands are indicated on the left side of Fig. 1B. Much more proteolytic degradation by cathepsins was observed on myofibrillar proteins than on sarcoplasmic proteins.

Myosin heavy chain (MHC, ~200 kDa) seemed to be very sensitive to all three cathepsins and was degraded into two major fragments of about 150 and 140 kDa (Fig. 3). At the end of the incubation (21 h), MHC had been completely degraded by cathepsin B. It is noteworthy that cathepsin L had completely digested myofibrillar proteins within 21 h since only MHC fragments were visible, apart from a smear all over the rest of the lane.

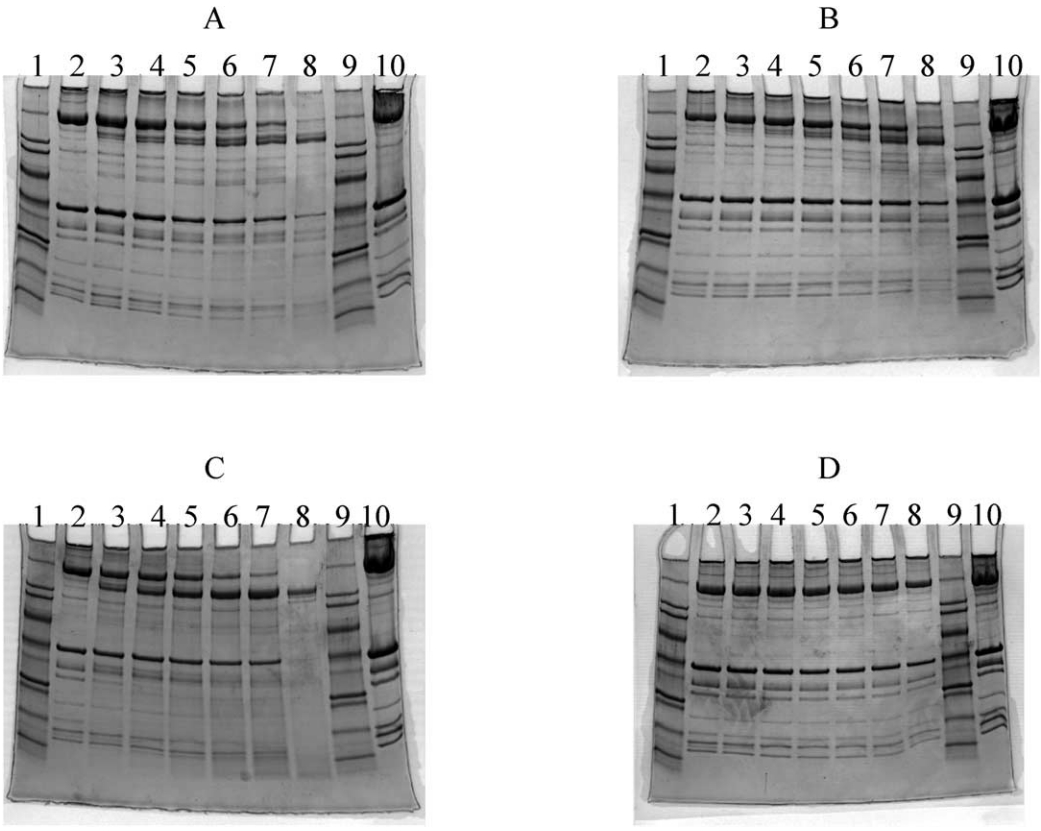

Fig. 3. Effect of cathepsins on structural proteins analysed by SDS–PAGE and Coomassie blue staining. A: cathepsin B, B: cathepsin D, C: cathepsin L, D: control without cathepsin. Lanes 1 and 9: SDS-6H and SDS-7 markers (see legend to Fig. 1), lane 2: 0 min of incubation, lane 3: 30 min, lane 4: 1 h, lane 5: 2 h, lane 6: 4 h, lane 7: 6 h, lane 8: 22 h, lane 10: initial myofibrils extract.

Actin (42 kDa) intensity evolution was not significantly different from the control except with cathepsin L, since actin had completely disappeared at the end of the incubation (Fig. 3).

In the area of tropomyosin (~34 kDa), another slightly higher molecular weight band of 37 kDa appeared and the 32 kDa component was lost when incubation was performed with cathepsin B and L. This latter protein is assumed to be troponin T. Two fragments of 30 and 27 kDa appeared when using cathepsin B, but only the one of 27 kDa with cathepsin D and the one of 30 kDa with cathepsin L (Fig. 3).

Three other lower molecular weight bands (22, 20.1 and 18 kDa) were degraded by cathepsin B, as indicated by the decrease of the band intensity. Three fragments appeared to be more intense soon after the beginning of incubation with cathepsin B (30 min): 90 kDa, 78 and 73 kDa (Fig. 3).

The origin of fragments cannot be determined precisely without using specific techniques, such as partial sequencing or immunochemical tools. Western blots on myofibrillar protein digestions were performed with antibodies to α-actinin, tropomyosin and desmin when working on pellets and only α-actinin on supernatants. α-Actinin (105 kDa) was slightly degraded by cathepsin B (Fig. 4A), as indicated by the decrease of the immunochemical intensity of the band as well as the appearance of immunoreactive fragments of 97, 90, 80, 67 and 55 kDa in 21 h. Cathepsin D was able to degrade α-actinin into fragments of 80 kDa (major one), 72 kDa, 67, 56 and 53 kDa (Fig. 4B). Cathepsin L was able to achieve the degradation of α-actinin (Fig. 4C) in 21 h. Intermediate fragments of 84, 78, 72, 67, 64, 58 can be observed, with the 84 kDa fragment being the major one. The patterns of degradation were then fairly different, depending on the type of cathepsin. The final product of the α-actinin degradation was the same for all the three cathepsins (37 kDa). The desmin (53 kDa) band, probed by the antibody, became fainter upon proteolysis by each cathepsin but this decrease was also observed in the control (Fig. 5). However, a more rapid disappearance seemed to be induced by cathepsins B and L. No degradation fragment was observed. Desmin is not degraded by cathepsin D.

The tropomyosin (34 kDa) hybridised band became fainter upon the action of cathepsin B and cathepsin D but no hydrolytic fragments were visible with the antibody used (Fig. 6A, B). In contrast, cathepsin L degraded tropomyosin from 34 kDa to an about 32 kDa protein which was hybridized as well by the antibody (Fig. 6C).

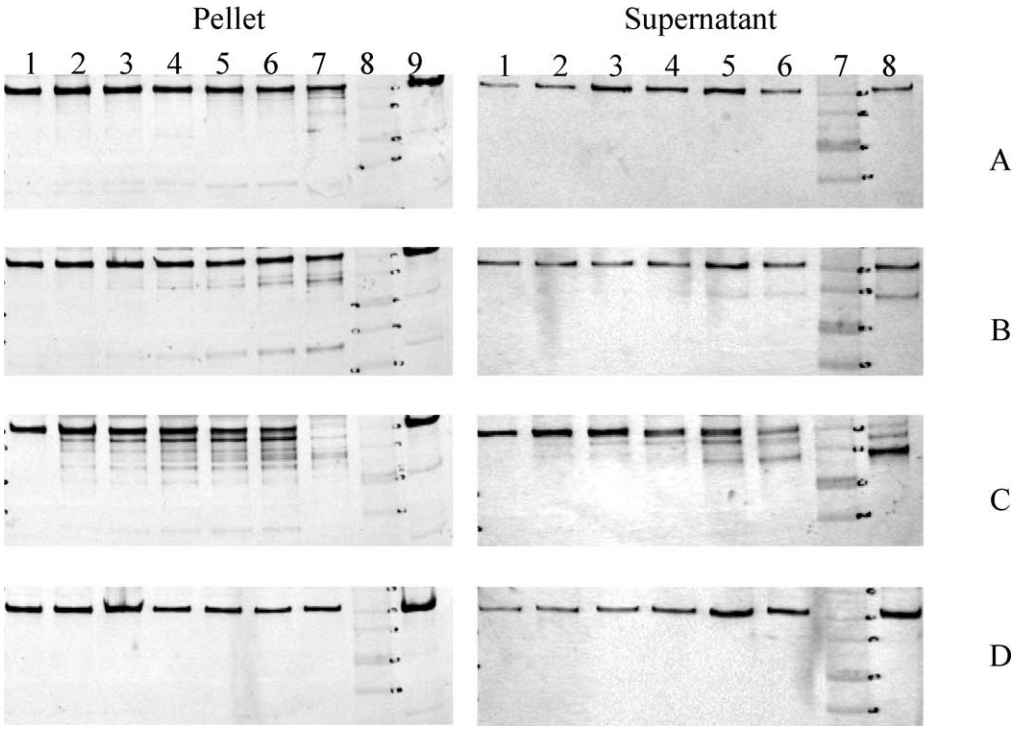

Fig. 4. Effect of cathepsins on α-actinin detected by immunoblots. A: cathepsin B, B: cathepsin D, C: cathepsin L, D: control without cathepsin. Pellet: lane 1: 0 min, lane 2: 30 min, lane 3: 1 h, lane 4: 2 h, lane 5: 4 h, lane 6: 6 h, lane 7: 22 h, lane 8: prestained markers, lane 9: initial myofibrils. Supernatant: lane 1: 0 min, lane 2: 30 min, lane 3: 1 h, lane 4: 2 h, lane 5: 4 h, lane 6: 6 h, lane 7: prestained markers, lane 8: 22 h.

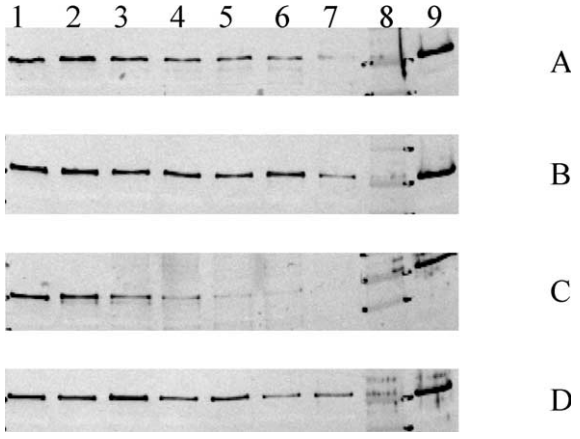

Fig. 5. Effect of cathepsins on desmin detected by immunoblots. A: cathepsin B, B: cathepsin D, C: cathepsin L, D: control without cathepsin. Lane 1: 0 min, lane 2: 30 min, lane 3: 1 h, lane 4: 2 h, lane 5: 4 h, lane 6: 6 h, lane 7: 22 h, lane 8: prestained markers, lane 9: initial myofibrils.

The supernatants of the digestion of the myofibrils were constituted mostly of a fraction of myofibrillar proteins. They also can be considered to include proteins that are dissociated from complexes bound to the particulate fraction of the muscle cells.

As for the pellets, degradations of MHC, actin and 34 kDa tropomyosin components are observed. A slight amount of α-actinin was present in the early stages of incubation in the supernatant (data not shown). The amount of α-actinin did not increase in the supernatants produced by cathepsin B but it rose with cathepsins L and D (Fig. 4). The α-actinin 105 kDa subunit from the supernatant was progressively lost with cathepsins D and L and some degradation fragments appeared at the end of the incubation (69 kDa for D and 98 and 68 kDa for L). Cathepsin B neither released nor degraded α-actinin present in the supernatant.

It is noteworthy that, when myofibrils were digested by cathepsin B and cathepsin D, the intensity of the 34 kDa band corresponding to tropomyosin increased in the supernatant (data not shown) as confirmed by immunoblotting (Fig. 6B). Thus, tropomyosin was not degraded by either cathepsin B or cathepsin D but was actually released from myofibrils in the soluble fraction. In contrast, the degradation of tropomyosin by cathepsin L in a 32 kDa fragment was visible on immunoblots (Fig. 6B). A third fragment (28 kDa) was produced during the degradation of tropomyosin by cathepsin L.

No release of desmin in the soluble fraction of the digests was observed (data not shown).

4. Discussion

The breakdown of fish muscle proteins is probably caused by several categories of proteases. Much of the focus on proteases and their role in *post mortem* processus

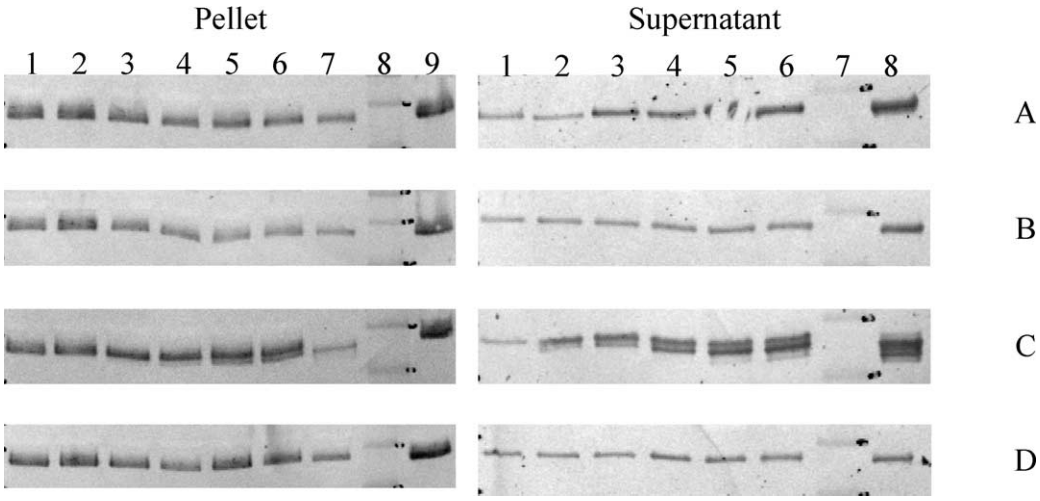

Fig. 6. Effect of cathepsins on tropomyosin detected by immunoblots. A: cathepsin B, B: cathepsin D, C: cathepsin L, D: control without cathepsin. Pellet: lane 1: 0 min, lane 2: 30 min, lane 3: 1 h, lane 4: 2 h, lane 5: 4 h, lane 6: 6 h, lane 7: 22 h, lane 8: prestained markers, lane 9: initial myofibrils. Supernatant: lane 1: 0 min, lane 2: 30 min, lane 3: 1 h, lane 4: 2 h, lane 5: 4 h, lane 6: 6 h, lane 7: prestained markers, lane 8: 22 h.

has been directed toward calpains, cathepsins B, L and D (Hopkins & Thompson, 2002). Some studies dealt with cathepsin H as well. Cathepsin L has been shown to play an important role in proteolysis in migrating chum salmon (Yamashita & Konagaya, 1990a), in mackerel (Aoki & Ueno, 1997) and carp muscle (Ogata et al., 1998). It has also been involved in the degradation of myofibrillar and sarcoplasmic proteins in salmon (Lund & Nielsen, 2001). Jiang et al. (1992) also showed the ability of cathepsin D to degrade tilapia myofibrils.

A number of in vitro studies have clearly demonstrated the susceptibility of numerous myofibrillar proteins by proteolysis calpains and lysosomal proteinases. Our results reveal that MHC, α-actinin, desmin, actin, troponin T, tropomyosin and other unidentified protein bands can be degraded by cathepsins B, D or L and are in accordance with those reported by several investigators. Aoki and Ueno (1997) found that cathepsin L, in mackerel, degraded myosin, troponin T, troponin I and tropomyosin in contrast with cathepsin B, which was unable to hydrolyse any of the myofibrillar proteins from mackerel white muscle. The effect of cathepsin L on myofibrillar proteins is similar to that reported by Aranishi, Ogata, Hara, Osatomi, and Ishihara (1998) and Ogata et al. (1998) for carp cathepsin L. Yamashita and Konagaya (1991) also showed that cathepsin B produced less hydrolysis than cathepsin L on salmon muscle structural proteins and substrate specificity suggested that cathepsin L was the most probable enzyme participating in the muscle softening process. Jiang et al. (1996) extensively studied the proteolysis of actomyosin by cathepsins B, L, L-like and X from mackerel and found that MHC was degraded by cathepsin B, cathepsin L while actin was only degraded by cathepsin L. Sarcoplasmic creatine kinase (41 kDa band) was already shown to be proteolysed by m-calpain (Purintrapiban, Wang, & Forsberg, 2001). It was also degraded by cathepsin B and L.

Geesink, Morton, Kent, and Bickerstaffe (2000) identified a 31 kDa degradation product in the washed pellet of myofibrils during refrigerated storage of salmon muscle, as well as during the in vitro digestion of myofibrils by calpain. It could be related to the 30 kDa degradation fragment from troponin T found in tenderized mammalian muscles (Ho, Stromer, & Robson, 1994). In our study, the assumed troponin T was degraded by the cathepsins B and L with a concomitant appearance of a 30 kDa band. Whether it is related to troponin T breakdown should, however, be assessed with immunological tools.

There is an extensive literature on *post mortem* changes in fish muscle, including microscopy and biochemistry. *Post mortem* tenderization of fish muscle may be closely related to gradual degradation of the extracellular matrix (Ando, Toyohara, Shimizu, & Sakaguchi, 1991). In general, fish muscle shows little *post mortem* change in myofibrils, especially in comparison to mammals. The changes, which have been documented, include the weakening and the disorganization of the Z line structure and detachment of sarcolemma (Papa et al., 1997), the degradation of titin, a giant protein anchored in the Z line and nebulin (Astier et al., 1991; Busconi, Folco, Martone, & Sanchez, 1989), as well as the release of α-actinin from the Z line and its degradation (Astier et al., 1991; Papa, Alvarez, et al., 1996).

Titin degradation and α-actinin release may serve as predictors of *post mortem* changes for bass, trout and carp (Papa, Alvarez et al., 1996). α-Actinin degradation yielded 80 and 40 kDa fragments as major bands of autolysis in *post mortem* sea bass muscle. The release of this protein, occurring before the degradation of released

molecules in muscles, was correlated with the calpain activity (Papa, Alvarez et al., 1996). In the present study, the effect of the proteases on α-actinin was complex. All the cathepsins degraded it in the pellet but only cathepsin D and L released it in the supernatant. No degradation of the supernatant form was observed with cathepsin B.

No degradation of MHC or troponin T occurred during *post mortem* storage of sea bass (Verrez-Bagnis et al., 2001). Lund and Nielsen (2001) observed slight change of myosin and no change of α-actinin or actin during cold storage of salmon muscle. Martinez (1992) also showed that fish myosin degradation may take place during chilled storage in a species and tissue-dependent way. The three cathepsins degraded MHC in our experiment and troponin T was degraded by cathepsin B and L.

Desmin remains unchanged after refrigerated storage of cultured fish muscle (Verrez-Bagnis et al., 1999) whereas desmin is degraded during *post mortem* storage of bovine muscle (Olson, Parrish, Dayton, & Goll, 1977). But desmin is highly degraded in wild fish, such as sardine (Verrez-Bagnis et al., 1999). Calpain was able to highly degrade sea bass desmin in vitro (Verrez-Bagnis et al., 2002); cathepsin L seemed to degrade it also, but slightly. Since desmin is stable in *post mortem* sea bass muscle, it is unlikely that calpain is responsible for the initiation of the *post mortem* degradation, as previously proposed.

5. Conclusion

In conclusion, MHC, desmin, actin, tropomyosin and the 32 kDa band were shown to be stable in autolytic *post mortem* sea bass muscle but MHC was degraded in vitro by all three proteases (cathepsins B, D, L); desmin was broken down by cathepsins B and L; tropomyosin and actin were split by cathepsin L, while the 32 kDa troponin T was degraded by cathepsins B and L. Cathepsin L caused extensive degradation of the proteins which was not observed in the autolytic muscle. Taken together, these results suggest that cathepsin D could participate in *postmortem* proteolysis by the release of α-actinin and some other changes. The lysosomal location of the cathepsins could preclude their role in the initiation of the softening process. However, O'Halloran, Troy, Buckley, and Reville (1997) reported that a high proportion of cathepsins B, D and L were present in the soluble fraction early *post mortem*.

To improve the analysis, the evolution of the other huge proteins, such as dystrophin, titin and nebulin, should be taken into account. Due to their high molecular weights, they were not visible on the gels. Dystrophin degradation was shown to be a predictor of freeze-thaw effect in bass and of the duration of chilled storage (Papa, Ventre, Lebart, Roustan, & Benyamin, 1996). Total degradation occurred within 48 h (Papa et al., 1997).

This study led to the identification of in vitro substrates for cathepsins B, D and L but the elucidation of their exact roles among the variety of lysosomal and non-lysosomal proteases should be further clarified, especially by the use in situ of specific inhibitors and by appropriate understanding of proteolytic activity regulation. It is clear that knowledge of the mechanisms underlying *post mortem* evolution of fish flesh is fragmented and often contradictory.

These types of studies could improve our understanding of the mechanisms of sarcomeric disintegration and the loss of flesh texture.

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