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In vitro proteolysis of myofibrillar and sarcoplasmic proteins of European sea bass (Dicentrarchus Labrax L) by an endogenous m-calpain

Véronique Verrez-Bagnis*, Christine Ladrat, Joëlle Noëlle, Joël Fleurence

Laboratory of Protein Biochemistry and Quality, IFREMER, Rue de l'Ile d'Yeu, BP 21105, F-44311 Nantes Cedex 3, France

*: Corresponding author : Véronique Verrez-Bagnis (vverrez@ifremer.fr)

Abstract: The effects of m-calpain isolated from the skeletal muscle of sea bass on sarcoplasmic and myofibrillar proteins isolated from the same tissue were examined in vitro. Incubation of sarcoplasmic proteins with m-calpain resulted in only a slight decrease (0.7 kDa) in the molecular weight (MW) of a 26.5 kDa protein. Degradation of myofibrils, monitored by quantification of TCA-soluble peptides generated, resulted in the maximum amount of peptides being generated after 1 h of incubation at 25 °C. Noticeable modifications in the SDS-PAGE profile of digested myofibrils were observed, including partial denaturation of myosin heavy chain and the release of tropomyosin, 69 and 27 kDa doublet bands and a few polypeptides of MW lower than 20 kDa in the soluble fraction. Examination of the degradation patterns of myofibrillar proteins using Western blotting showed that -actinin was partially degraded, with release of native -actinin and its fragments from myofibrils, whereas desmin was highly degraded after 2 h of digestion.

Keywords: myofibrillar protein • calpain • sarcoplasmic protein • proteolysis • Dicentrarchus labrax L

INTRODUCTION

Loss of fish freshness, followed by spoilage, is the result of complex microbiological, physiological, chemical and biochemical processes. The initial steps in deterioration consist of hydrolytic reactions, catalysed by endogenous enzymes, which produce nutrients that allow subsequently bacterial proliferation. Among these hydrolytic reactions, proteolysis should be considered with special attention due to its likely influence on textural changes undergone by fish muscle during cold storage. [1]

The post-mortem protein degradation in muscle cells is probably the result of the synergistic action of calpains and cathepsins on key myofibrillar proteins, [2-3] even if only calpains are sometimes described to mediate the proteolysis [4-5] or the early stage of this process. [6] Calpains are also involved in accelerated post-mortem degradation which results in fish flesh deterioration, such as burnt tuna. [7]

The changes associated with the action of calpains occurring in fish myofibrils and cytoskeletal proteins include a limited proteolysis of titin, [8] a huge degradation of dystrophin [9] and a release of α-actinin from the myofibrillar structure and loss of the Z-line integrity. [6,10] In contrast, little degradation of desmin was observed in different fish species, with an exception to sardine. [11] Very few studies are related to changes in sarcoplasmic proteins during fish post-mortem storage. The main reason was that the composition of this heterogeneous fraction of proteins depended on the type of muscle (white or red), the species and the physiological condition of fish. [12] In a previous study, [13] we have, however, shown the gradual disappearance of a sarcoplasmic protein band of 16 kDa in post-mortem sea bass muscle. But the mechanism of this loss remained unknown.

In our previous paper, [14] we have described the purification and characterization of three calpain-like enzymes, differing from each other at least in net charge as indicated by elution from an anion exchange column, from the skeletal muscle of European sea bass. From the Ca^{2+} concentration required for activity, one was a μ-calpain and the two others were m-calpains. They were differently expressed throughout the year and this could be of great importance to the resulting rate and extent of degradation of fish flesh after death.

Attempts in vitro, to duplicate events in vivo, can never be absolute, [15] and often, in vitro incubation is performed using enzyme:substrate ratios greater than those in muscle. [16] However, keeping in mind these observations, the objectives of the present study were to evaluate the extent of myofibril and sarcoplasmic protein hydrolysis by European sea bass m-calpain and to identify the m-calpain–induced degradation products using post-mortem-like conditions. The m-calpain used was purified and was the major isoform expressed all through the year with a maximum during the spawning period.

MATERIALS AND METHODS

Materials

Sea bass (*Dicentrarchus labrax* L.) (300- 450 g) was obtained from a local sea farm in Vendée (France), brought back alive to the laboratory and killed by decapitation. Sea bass dorsal skeletal muscle was minced with a meat chopper, and calpain purification and myofibrils preparation were performed immediately. In the same time, samples from dorsal muscle were taken immediately after death, frozen in dry ice and kept at -80°C until used for the preparation of sarcoplasmic proteins.

Preparation of myofibrils and sarcoplasmic proteins

Sea bass myofibrils were isolated from the skeletal muscle according to the method of Wang (1982), suspended in 50% (vol/vol) glycerol and stored at – 20°C until used. Extraction of sarcoplasmic proteins was performed as previously described. [17] Dorsal white fish muscle (0.3 g) was homogenised in 3 ml of buffer A (50 mM Tris-HCl (pH 7), 5 mM 2-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA)) in a Potter RGL 100 (Heidolph, Kelheim, Germany) for 1 min. The extracts were centrifuged at 10 500 x *g* for 10 min and the clear supernatant was collected.

Determination of protein

Protein concentration was determined according to the method of Bradford [18] using Bio-Rad Protein Assay kit and bovine serum albumin (BSA) as the standard.

Purification of sea bass m-calpain

The m-calpain used in this study was partially purified according to the method previously described. [14] No non Ca^{2+}-dependent proteolytic activity was detected in calpain preparation. The whole procedure was carried out in ice or at 4°C. Briefly, a portion of 50 g of minced muscle was homogenized in 150 ml of 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol and 5 mM EDTA. After centrifugation, 0.5 M NaCl was added and this supernatant was directly run to the first chromatographic column (Phenyl Sepharose, ∅ 26 mm, L 11 cm). The non-absorbed proteins were washed with the equilibration buffer – composed of 50% buffer B (50 mM Tris-HCl (pH 7), 5 mM 2-mercaptoethanol and 1 mM EDTA, 1 M NaCl) and 50% buffer A (buffer B without NaCl). The calpain activity fraction was then eluted in batch with buffer A, and subsequently applied to the DEAE Sepharose column (∅ 16 mm, L 19 cm). Elution was carried out with a linear 0-400 mM NaCl gradient in 500 ml. The m-calpain corresponding to the third activity peak (eluted at 266 mM NaCl) was dialysed against buffer A, concentrated using ultrafree 20 concentrators (10 000 NMWL – PLGC) and kept in ice in a 4°C fridge. Digestion procedure was performed as soon as possible to minimize loss of activity.

Proteolysis measurement

Calpain activity on myofibrils or sarcoplasmic proteins was routinely determined at 25°C by measuring the release of trichloroacetic acid (TCA)-soluble polypeptides resulting from the digestion by the m-calpain as described previously. [14]

Incubation procedure

Digestion was performed according to the procedure described by Huff-Lonergan *et al.* [19] with some modifications. For each assay, 4 ml of glycerinated myofibrils was centrifuged at 10 000 x *g* for 15 min at room temperature. Pellets were washed with 2 ml of deionised water and then centrifuged at 12 000 x *g* for 15 min at room temperature. Concentrations of washed myofibrils and sarcoplasmic proteins were adjusted with buffer (50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 5 mM 2-mercaptoethanol, 100 mM KCl, 5 mM$CaCl_2$) and m-calpain was added to the ratio around 1:400 (wt/wt; m-calpain:protein substrate), the exact ratio was indicated in the figure captions. Control samples were the same, except that EDTA was added (final concentration 20 mM) to the mixture before the addition of m-calpain

(calpain control), or the mixture was incubated without addition of m-calpain (buffer control). Final reaction volumes were 2.5 ml for myofibrils digestion and 1.1 ml for sarcoplasmic proteins proteolysis. Samples were removed after 0, 15, 30, 60, and 120 min of digestion by m-calpain at 25°C and EDTA was added to a final concentration of 20 mM to stop the reaction. All digestions were done in duplicate. Samples were centrifuged at 10 000 x *g* for 15 min at 20°C, and supernatants and pellets were added to 0.5 volume of sodium dodecyl sulfate (SDS) sample buffer as described by Wang [20] and stored at –20°C until used. In the same time, 125 µl of samples were stopped by 125 µl of 5% TCA and concentration of TCA-soluble peptides released by digestion was measured using Bradford assay.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were heated to 100°C for 2 min before loaded. SDS-PAGE was performed in a Mini-protein dual slab cell (Bio-Rad, Richmond, CA) according to the method of Laemmli [21] using 4–20% (for myofibrils digestion)- and 8-20% (for sarcoplasmic proteins digestion)-polyacrylamide gradient 1 mm thick gels. Electrophoresis was carried out at 7.5 mA per gel for 12 min followed by 20 mA per gel until the dye front reached the end of the gel. Gels were subsequently stained with Coomassie Brillant Blue R-250.
To estimate the molecular weight (MW) of proteins, each band was integrated by means of an Epson GT-12000 scanner (Seiko Epson, Nagano, Japan) coupled with ImageMaster® 1D Elite software (Amersham Pharmacia Biotech Europe GmbH, Orsay, France). Molecular weight markers (FMC BioProducts, Rockland, ME and Bio-Rad) were also run with protein samples.

Western immunoblotting

After electrophoresis, proteins were transferred overnight at 15°C onto a 0.45 µm nitrocellulose membrane with buffer consisting of 0.4 M Tris base, 150 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS. Transfer was performed at 20 V in a Trans-Blot cell (Bio-Rad). Membranes were saturated in 8% skimmed milk powder reconstituted in 0.9% NaCl and incubated with rabbit antibodies diluted in 0.9% NaCl, for 2 hours at room temperature. Primary antibodies used in the Western blotting procedures included anti-α-actinin in-house produced antibodies, polyclonal anti-desmin (D8281, Sigma Immuno Chemicals,St. Louis, MO) and polyclonal anti-tropomyosin (T3651, Sigma Immuno Chemicals). After washing with phosphate buffered saline (PBS) (150 mM NaCl in 10 mM Na_2HPO_4 / NaH_2PO_4 (pH 7.2)) containing 0.05% Tween 20 (v/v) solution, membranes were incubated in anti-rabbit IgG antibodies labelled with phosphatase, diluted in 5% BSA in 0.9% NaCl. The proteins immunologically reacted with the antibodies were subsequently revealed using nitro blue tetrazolium/5-bromo –4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate.

RESULTS AND DISCUSSION

Action of m-calpain towards sarcoplasmic proteins

Fig. 1 represents the evolution in TCA-soluble peptides generated during incubation of sarcoplasmic proteins with m-calpain. The enzyme concentration employed for the treatment is similar to that currently used for digestion studies. Results show a slight proteolysis appearing during the first stage of incubation. The peptides generated by incubation reach a maximum in the first 15 min and proteolysis seems nearly stopped after that time. In contrast, the control samples (calpain control: sarcoplasmic proteins incubated with m-calpain in

presence of 20 mM EDTA and buffer control: sarcoplasmic proteins incubated without m-calpain) show no significant proteolysis indicating that the changes in peptides are due to m-calpain activity and not to the incubation conditions. The presence of endogenous inhibitor of the calpains (including calpastatin) in the sarcoplasmic fraction could have been an explanation, but the ratio enzyme:substrate used for the hydrolysis is such that m-calpain is in large excess compared to the inhibitors.

SDS-PAGE of sarcoplasmic proteins after incubation with m-calpain shows that m-calpain has a very limited and specific effect on sarcoplasmic proteins (Fig. 2). Only a slight decrease in the molecular weight of a 26.5 kDa protein is detectable using Coomassie blue staining. This change corresponds to the generation of a 700 Da peptide (or generation of several peptides corresponding to the loss of 700 Da from the 26.5 kda protein) and occurs in the first 15 min of incubation. This is in accordance with results obtained from the quantification of TCA-soluble peptides generated by incubation where a slight proteolysis appeared during the first stage of incubation.

Figure 1. Kinetic of TCA-soluble peptides generated during incubation of sarcoplasmic proteins with m-calpain (ratio enzyme:proteins 1:364). ● Sarcoplasmic proteins incubated with m-calpain and sampled after 0, 15, 30, 60 and 120 min of incubation . ◆ Calpain control sample (sarcoplasmic proteins incubated with m-calpain in presence of 20 mM EDTA). ■ Buffer control sample (sarcoplasmic proteins incubated without m-calpain).

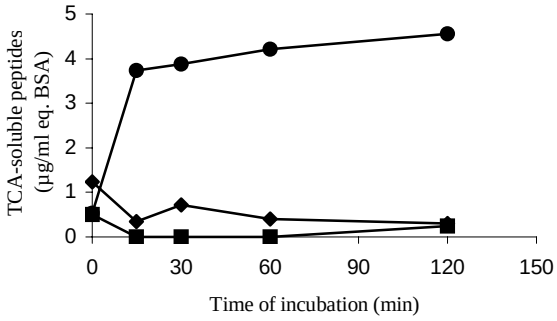
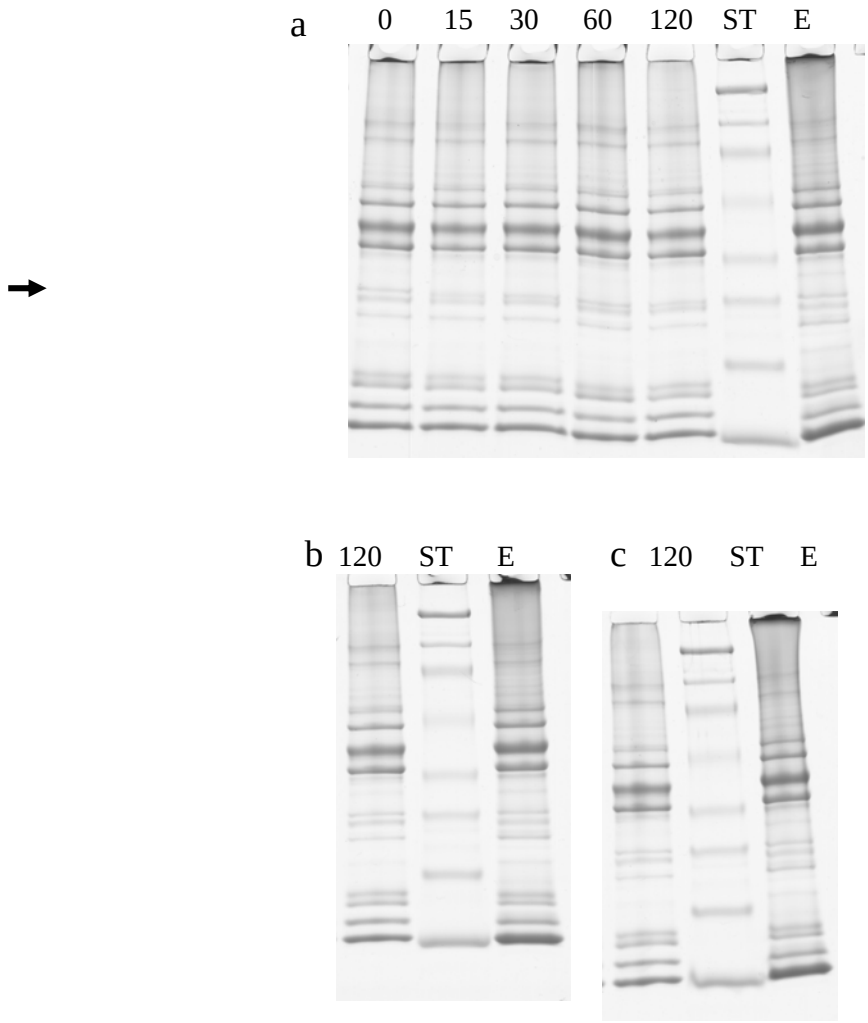


Figure 2. Coomassie-stained 8 to 20% gradient SDS-PAGE gels of sarcoplasmic proteins incubated with m-calpain (ratio enzyme:proteins 1:364). (a) Sarcoplasmic proteins incubated with m-calpain and sampled after 0, 15, 30, 60 and 120 min of incubation . (b) Calpain control sample (sarcoplasmic proteins incubated with m-calpain in presence of 20 mM EDTA) after 120 min of incubation. (c) Buffer control sample (sarcoplasmic proteins incubated without m-calpain) after 120 min of incubation. 0 to 120 at the top of the gels refer to min of incubation. Abbreviations are as follows : E = sarcoplasmic extract, ST = molecular weight standard (from top to the bottom of the gel : 203, 118, 82, 50.4, 33.4, 26.7, 19.6, 7.4 kDa). Arrow designates the position of the protein undergoing a slight decrease in its molecular weight.

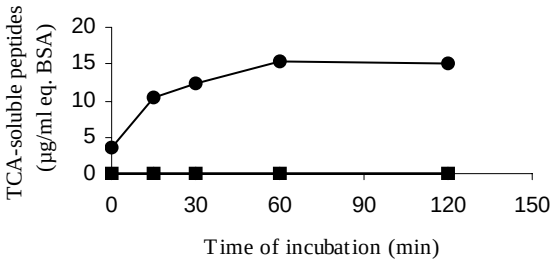


The identity of the protein involved in this change is, up to now, unknown. Our result is, however, in accordance with those of Waxman [22] who observed that the calpains rapidly cleaved a few specific sarcoplasmic proteins such as phosphorylase kinase and a variety of other kinases and phosphatases. On the other hand, Goll *et al.* [23] found that they do not cause general degradation of sarcoplasmic proteins to fragments soluble in 2.5 % TCA. The limited action of calpains on sarcoplasmic proteins is explained by the fact that, if the calpains are involved in muscle protein turnover, their action is directed exclusively to the myofibrillar or cytoskeletal proteins on which their effects result in disassembly of the myofibril and in release of large polypeptides. [23] Moreover, calpains are able to induce only limited proteolysis of substrates.

Action of m-calpain towards myofibrils

Fig. 3 represents the evolution in TCA-soluble peptides generated during incubation of myofibrils with m-calpain. This experiment indicates that m-calpain cleaves myofibrillar proteins with production of free amino acids and/or peptides. In contrast, the two control samples (calpain control and buffer control) show no significant proteolysis indicating that the changes in peptides are due to the activity of m-calpain and not to the incubation conditions. The amount of peptides generated by hydrolysis of myofibrils by m-calpain reaches a maximum after one hour of incubation at 25°C.

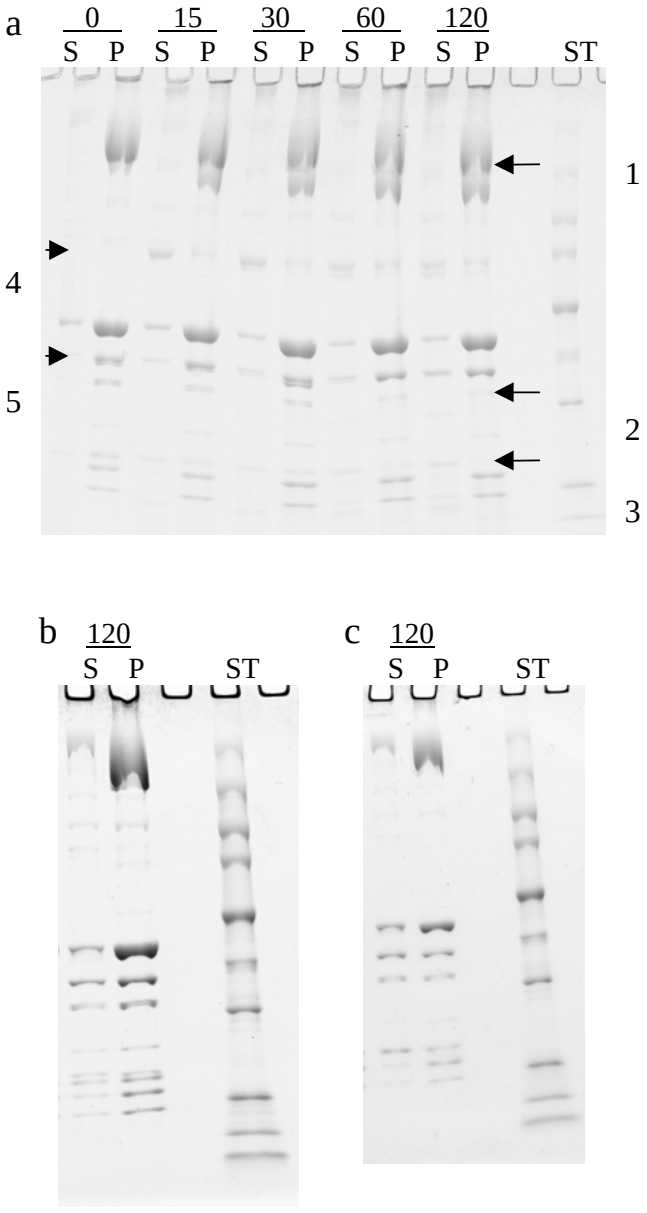
Figure 3. Kinetic of TCA-soluble peptides generated during incubation of myofibrillar proteins with m-calpain (ratio enzyme:proteins 1:420). ● Myofibrillar proteins incubated with m-calpain and sampled after 0, 15, 30, 60 and 120 min of incubation . ■ Calpain control sample (myofibrillar proteins incubated with m-calpain in presence of 20 mM EDTA) and buffer control sample (myofibrillar proteins incubated without m-calpain) (these two control



General changes in myofibrils due to action of m-calpain can be seen in the 4 to 20% gradient gels and compared to the two controls in figure 4. Changes in myofibrillar proteins due to m-calpain incubation are significant compared to protein profiles of the two controls where no detectable change is noted even after 2 hours of incubation. Noticeable modifications can be observed from the myofibril profile as the partial degradation of myosin heavy chain, the release of tropomyosin in the supernatant (as seen also from Western blot on fig 5c) accompanied by the loss of the lower band of the doublet as well as the loss of a double band of around 27 kDa in the remaining myofibrils after incubation, and the solubilization of a double band of MW around 69 kDa and the release in the supernatant of few polypeptides with MW< 20 kDa. These remarkable changes confirm the previous result obtained on TCA-soluble peptides which has indicated that proteolysis occurred in myofibrillar proteins due to action of m-calpain. Our results corroborate those of Geesink *et al.* [24] who studied, in particular, the effect of salmon m-calpain on salmon myofibrils. They also observed the release of several degradation products from the myofibrils into the soluble fraction during the incubation. Nevertheless, they noticed the appearance at a slow rate of a 31-kDa band as well as during storage of salmon muscle or during incubation of salmon myofibrils with salmon m-calpain. The authors could not check if the 31-kDa band found in salmon myofibrils is a degradation product of troponin-T. Degradation of troponin-T resulting in protein fragments with a molecular weight of approximately 30 kDa is the most reported change in myofibrillar

proteins during storage of mammalian muscles (for a review, see Robson *et al*. [25]) ; until now, no study reported the appearance of a troponin-T degradation fragment in post-mortem fish muscle. Regarding our results, no band with a molecular weight of about 31 kDa is detected in sea bass myofibrils even after 2 hours of incubation. Ertbjerg *et al*. [26] reported also the proteolytic degradation of a 26 kDa band composed of poorly resolved myosin light chain 1 and troponin-I in porcine muscle. They identified a 24 kDa band as a troponin-I degradation product. We only can speculate that the loss of a double band in remaining myofibrils with a molecular weight of 27 kDa, in our study, may be those of troponin-I, but, this hypothesis have to be corroborated by further study using anti-troponin-I antibodies.

Figure 4. Coomassie-stained 4 to 20% gradient SDS-PAGE gels of myofibrillar proteins incubated with m-calpain (ratio enzyme:proteins 1:420). (a) Pellets and supernatants from purified at death myofibrils incubated with m-calpain and sampled after 0, 15, 30, 60 and 120 min of incubation . (b) Calpain control sample (myofibrillar proteins incubated with m-calpain in presence of 20 mM EDTA) after 120 min of incubation. (c) Buffer control sample (myofibrillar proteins incubated without m-calpain) after 120 min of incubation. 0 to 120 at the top of the gels refer to min of incubation. Abbreviations are as follows : S = supernatant, P = pellet, ST = molecular weight standard (from top to the bottom of the gel: 225, 150, 100, 75, 50 (twice the amount of others), 35, 25, 15, 10 and 5). Numbered arrows designate the position of the proteins undergoing changes: (1) corresponds to myosin, (2) to 27 and 26 kda doublet, (3) to 17 kDa protein, (4) to 69 and 64 kDa bands and (5) to tropomyosin.



Hatzizisis *et al.* [27] noticed also a clear degradation of myosin heavy chain during treatment of myofibrillar proteins of the arm muscle of *Octopus vulgaris* by a calpain-like proteinase from the same tissue.

In previous studies, we have monitored Z-line disintegration during post-mortem storage of sea bass and sea trout [6] and we have shown the release of α-actinin from the Z-line and its partial degradation according to the fish species. In another previous study on desmin, [11] we have noted that the extent and the rate of degradation of this intermediate filament protein vary considerably among species. No noticeable change in sea bass fillet has been noted, while desmin post-mortem degradation is reported in many terrestrial meat as in beef muscle, [28-30] in pork muscle [31-32] and in chicken. [33] Consequently, Western blots using anti α-actinin, anti-desmin and also anti-tropomyosin antibodies have been done to learn whether these proteins are degraded *in vitro* by m-calpain.

Western blot probed with polyclonal anti α-actinin antibodies shows that myofibrils exhibit a release and a partial degradation of α-actinin (Fig. 5a). α-actinin is found in greater quantity into supernatant after 2 hours of incubation of myofibrils with m-calpain and polypeptide fragments (MW ≅ 87 and 37 kDa in both pellet and supernatant, and 80 in pellet only) are detected as well in remaining myofibrils than in supernatant.

When the myofibril sample which is digested with m-calpain is examined by Western blotting, the polyclonal anti-desmin antibodies recognized the presence of an approximately 45 kDa degradation product since the preparation of myofibrils as this fragment is present in both myofibril sample and control samples at the beginning of incubation (Fig. 5b). This observation is in disagreement with the results of our previous study [11] where no desmin fragmentation in sea bass muscle was detected during post-mortem storage. After 2 hours of digestion of myofibrillar proteins with m-calpain, desmin seems to be highly degraded, the non-degraded desmin band at 57 kDa has nearly completely disappeared while 45 kDa fragment is always detected in pellet and an approximately 75 kDa polypeptide is detected into supernatant. We could hypothesize that this polypeptide is an aggregate of different degradation fragments.

Immunoblot of incubated myofibrils that are probed with polyclonal antibodies to tropomyosin shows that tropomyosin, which migrates generally as a doublet in sea bass, is partially released from the myofibrils after 2 hours of incubation (Fig. 5c). This result confirms the previous observations in muscle tissue obtained from SDS-PAGE profiles.

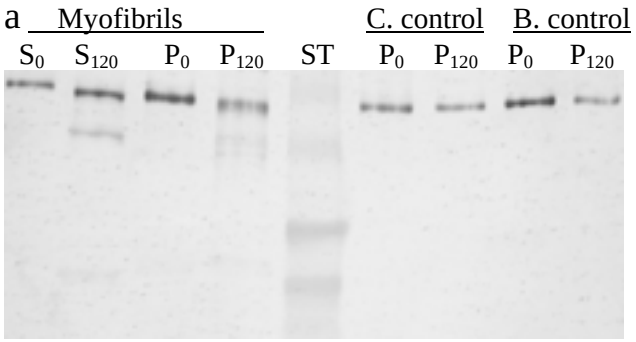
The sea bass m-calpain activities on myofibrillar protein fraction from the same tissue are summarized in table 1 and compared with results obtained in other animals from literature. As can be seen, m-calpain from mammalian sources does not to have the same effect on some myofibrillar proteins as sea bass m-calpain.

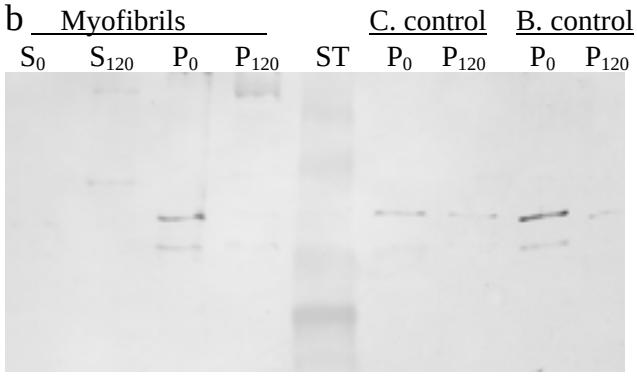
Table 1. Comparaison of the *in vitro* effect of m-calpain on myofibrillar protein fraction from mammalian[a], octopus[b] /salmon[c]/amphibian[d] and sea bass.

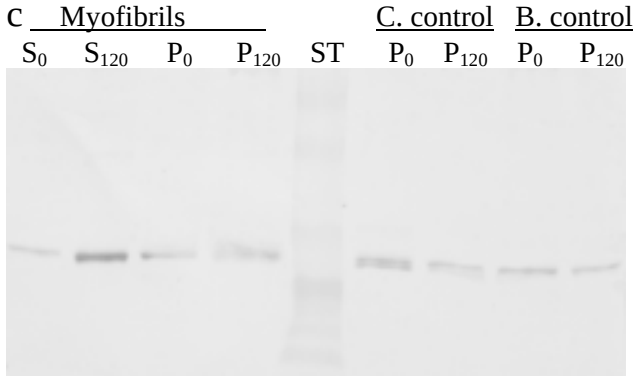
	Mammalian[a]	Octopus, salmon and amphibian	Sea bass
Titin degradation	+		n.d.
Nebulin degradation	+		n.d.
Myosin degradation	-	+[b, d]	+
Dystrophin degradation	+		n.d.
α-actinin solubilization	+		+
α-actinin degradation	-		+
69-kDa doublet solubilization			+
Desmin degradation	+		+
Actin degradation	-	+[b] , + G-actin[d]	-
Troponin-T degradation	+		
Tropomyosin solubilization			+
Appearance of 30-kDa fragment	+	+[c]	-
27-kDa protein degradation			+
Release of < 20-kDa fragments			+

[a]Data taken from Koohmaraie [34]; [b]Data taken from Hatzizisis *et al.* [27] on *Octopus vulgaris*; [c]Data taken from Geesink et al. [24] on salmon (*Oncorhynchus tshawytscha*); [d]Data from Sargianos *et al.* [35] on the amphibian *Rana ridibunda*.

Figure 5. Western blots of pellets and supernatants from purified at death myofibrils incubated with m-calpain (ratio enzyme:proteins 1:488) (lanes 1 to 4) and from calpain control sample (C. control, lanes 6 and 7) (sarcoplasmic proteins incubated with m-calpain in presence of 20 mM EDTA) and buffer control sample (B. control, lanes 8 and 9) (sarcoplasmic proteins incubated without m-calpain). Pellets and supernatants were run on 4 to 20 % gradient gels, transferred to nitrocellulose membranes and immunoblotted with polyclonal anti- (a) α-actinin, (b) desmin and (c) tropomyosin antibodies. Abbreviations are as follows : S_0 and S_{120} = supernatants at 0 and 120 min of incubation, P_0 and P_{120} = pellets at 0 and 120 min of incubation, ST = prestained molecular weight standard (from top to the bottom of the gel: 201, 115, 81, 43.4, 32.9 and 17.9 kDa).







CONCLUSION

Our results obtained *in vitro* showed that one cytosolic protein and some myofibrillar proteins (such as myosin, tropomyosin, α-actinin and desmin) could be a substrate for sea bass m-calpain. Nevertheless, this study does not allow direct correlation with degradation of proteins in post-mortem fish muscle. Effectively, the *in vitro* activities of calpain did not explained all changes in proteins occurring during post-mortem degradation of fish muscle. If calpains are know to be active just after death, other proteases like cathepsins are also likely to be implicated in post-mortem changes.

REFERENCES

[1] Busconi L, Folco EJ, Martone CB and Sanchez JJ, *Post mortem* changes in skeletal elements in fish muscle. *J Food Biochem* **13**:443-451 (1989).

[2] Ouali A, Demeyer D and Raichon C, An introduction to the workshop. *Biochimie* **74**:213-215 (1992).

[3] Ogata H, Aranishi F, Hara K, Osatomi K and Ishihara T, Proteolytic degradation of myofibrillar components by carp cathepsin L. *J Sci Food Agric* **76**:499-504 (1998).

[4] Lowell BB, Ruderman NB and Goodman MN, Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. *Biochem J* **234**:237-240 (1986).

[5] Koohmaraie M, Biochemical factors regulating the toughening and tenderization process of meat. *Meat Sci* **43**:193-201 (1996).

[6] Papa I, Alvarez C, Verrez-Bagnis V, Fleurence J and Benyamin, Y, *Post mortem* release of fish white muscle α-actinin as a marker of disorganisation. *J Sci Food Agric* **72**:63-70 (1996).

[7] Watson C, Burnt tuna: a problem of heat inside or out? In *Environmental and Ecological Biochemistry*, Ed by Hochachka PW and Momsen TP, Elsevier, Amsterdam and New York, **5**:126-145 (1995).

[8] Astier C, Labbé JP, Roustan C and Benyamin, Y, Sarcomeric disorganization in post-mortem fish muscles. *Comp Biochem Physio* **100B**:459-465 (1991).

[9] Papa I, Taylor R, Astier C, Ventre F, Lebart MC, Roustan C, Ouali A and Benyamin Y, Dystrophin cleavage and sarcolemma detachment are early post mortem changes on bass (*Dicentrarchus labrax*) white muscle. *J Food Sci* **62**:917-921 (1997).

[10] Tsuchiya H, Kita S and Seki N, Postmortem changes in α-actinin and connectin in carp and rainbow trout muscles. *Nippon Suisan Gakkaishi* **58**:793-798 (1992).

[11] Verrez-Bagnis V, Noël J, Sautereau C and Fleurence J, Desmin degradation in postmortem fish muscle. *J Food Sci* **64:**240-242 (1999).

[12] Rehbein H, Post-mortem changes of sarcoplasmic proteins of fresh fish, in *Methods to Determine the Freshness of Fish in Research and Industry*, Ed by Olafsdóttir G, Luten J, Dalgaard P, Careche M, Verrez-Bagnis V, Martinsdóttir E and Heia K. International Institute of Refrigeration: Paris, France, pp 238-240 (1997).

[13] Verrez-Bagnis V, Ladrat C, Morzel M, Noël J and Fleurence J, Protein changes in *post mortem* sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. *Electrophoresis* **22**:1539-1544 (2001).

[14] Ladrat C, Chaplet M, Verrez-Bagnis V, Noël J and Fleurence J, Neutral calcium-activated proteases from European sea bass (*Dicentrarchus labrax* L.) muscle: polymorphism and biochemical studies. *Comp Biochem Physiol Part B* **125**:83-95 (2000).

[15] Whipple G and Koohmaraie M, Degradation of myofibrillar proteins by extractable lysosomal enzymes and m-calpain, and the effects of zinc chloride. *J Anim Sci* **69**:4449-4460 (1991).

[16] Ouali A, Garrel N, Obled A, Deval C and Valin C, Comparative action of cathepsins D, B, H, L and a new lysosomal cysteine proteinase on rabbit myofibrils. *Meat Sci* **19**:83 (1987).
[17] Morzel M, Verrez-Bagnis V, Arendt E.K and Fleurence J, Use of two-dimensional electrophoresis to evaluate proteolysis in salmon (*Salmo salar*) muscle as affected by a lactic fermentation. *J Agri Food Chem* **48**:239-244 (2000).
[18] Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254 (1976).
[19] Huff-Lonergan E, Mitsuhashi T, Beekman DD, Parrish FC, Olson DG and Robson RM, Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *J Anim Sci* **74**:993-1008 (1996).
[20] Wang K, Purification of titin and nebulin. *Meth Enzymol* **85**:264-274 (1982).
[21] Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **224**:680-685 (1970).
[22] Waxman L, Calcium-activated proteases in mammalian tissues. *Meth Enzymol* **80**:664-680 (1981).
[23] Goll DE, Thompson VF, Taylor RG and Christiansen JA, Role of the calpain system in muscle growth. *Biochimie* **74**:225-237 (1992).
[24] Geesink GH, Morton JD, Kent MP and Bickerstaffe R, Partial purification and characterization of Chinook salmon (*Oncorhynchus tshawytscha*) calpains and an evaluation of their role in postmortem proteolysis. *J Food Sci* **77**:2685-2692 (2000).
[25] Robson R.M, Huff-Lonergan E, Parrish EC, Ho CY, Stromer MH, Huiatt TW, Bellin, RM and Sernett SW, Postmortem changes in the myofibrillar and other cytoskeletal proteins in muscle. *Proc Recip Meat Conf* **50**:43-52 (1997).
[26] Ertbjerg P, Henckel P, Karlsson A, Larsen L and Møller A, Combined effect of epinephrine and exercise on calpain/calpastatin and cathepsin B and L activity in porcine longissimus muscle. *J Anim Sci* **77**:2428-2436 (1999).
[27] Hatzizisis D, Gaitanaki C and Beis I, Degradation of myofibrillar proteins by a calpain-like proteinase in the arm muscle of *Octopus vulgaris. J Comp Physiol B* **170**:447-456 (2000).
[28] Hwan SF and Bandman E, Studies of desmin and α-actinin degradation in bovine semitendinosus muscle. *J Food Sci* **54**:1426-1430 (1989).
[29] Taylor RG, Geesink GH, Thompson VF, Koohmaraie M and Goll DE, Is Z-disk degradation responsible for postmortem tenderization*? J Anim Sci* **73**:1351-1367 (1995).
[30] Ho CY, Stromer MH and Robson RM, Effect of electrical stimulation on *postmortem* titin, nebulin, desmin and troponin T degradation and ultrastructural changes in bovine longissimus muscle. *J Anim Sci* **74***:*1563-1575 (1996).
[31] Hortos M, Gil M and Sarraga C, Effect of calpain and cathepsin activities on myofibrils from porcine longissimus muscle after conditioning of normal and exudative meat. *Sci Aliments* **14**:503-515 (1994).
[32] Morrison E, Mielche M and Purslow P, Immunolocalisation of intermediate filament proteins in porcine meat. Fibre type and muscle-specific variations during conditioning. *Meat Sci* **50**:91-104 (1998).
[33] Takahashi K, Structural weakening of skeletal muscle tissue during postmortem ageing of meat; the non-enzymatic mechanism of meat tenderization. *Meat Sci* **43S**:S67-S80 (1996).
[34] Koohmaraie M, The role of Ca2+ dependant proteases (calpains) in *post mortem* proteolysis and meat tenderness. *Biochimie* **74**:239-245 (1992).
[35] Sargianos N, Gaitanaki C, Dimitriadis B and Beis I, Proteolytic degradation of isolated myofibrils and myofibrillar proteins by m-calpain from the skeletal muscle of the amphibian *Rana ridibunda*. *J Exp Zool* **276:**30-42 (1996).