
Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax L.*)

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Abstract:

The effects of a milli(m)-calpain isolated from the white muscle of sea bass (*Dicentrarchus labrax L.*) and commercial cathepsins B, D and L, used in combination on the myofibrillar and sarcoplasmic proteins were examined. Protein digestion was first performed by the endogenous m-calpain, during 2 h before the addition of a mixture of cathepsins B, D and L and a further incubation up to 22 h. Calpain degraded a 27 kDa sarcoplasmic component as well as myosin heavy chain, α -actinin, desmin and a 32 kDa component from the myofibrillar fraction. A 97 kDa component and the assumed creatine kinase–aldolase doublet were degraded during the incubation of sarcoplasmic proteins with the cathepsin mixture while, among the myofibrillar proteins, myosin, actin, tropomyosin, and the 22 kDa component were digested by the same enzymatic preparation. Myofibril-bound α -actinin and tropomyosin were released into the soluble fraction by m-calpain and the cathepsins mixture. Some smaller fragments were also produced. This appears to be the result of the cumulative action of each protease, as already investigated in our previous studies, except for the degradation of the sarcoplasmic 97 kDa component. It was only degraded if the cathepsins were mixed; this indicates that the three cathepsins functioned synergistically on muscle proteins. No difference in the proteolytic effect of cathepsins was observed, whether calpain was acting first or not. This work showed that calpain did not modify any muscle protein prior to later hydrolysis by the catheptic proteinases.

Keywords: Neutral calcium-dependent protease; Cathepsins; Proteolysis; Fish muscle; Postmortem aging

Introduction

Despite awareness that proteolysis plays a critical role in *post mortem* aging of muscle resulting in tenderization, a clear understanding of the mechanisms responsible remains elusive. In contrast to mammalian muscles for which tenderness is beneficial to meat quality, tenderization of fish muscle results in its deterioration. The initial steps in this process include endogenous hydrolytic reactions brought about by enzymes. The role of cathepsins and calpains in the *post mortem* proteolysis of fish muscle has received limited attention compared to the extent of studies on these enzymes in mammalian tissue.

Calpains (neutral calcium-dependent proteases) and cathepsins (lysosomal proteases) are the two major degradative pathways usually described as involved in the *post mortem* aging process. Two ubiquitous forms of calpains exist within the cells: micro(μ)-calpain, and milli(m)-calpain. Fish muscle calpains have received only little attention compared to mammalian counterparts (Geesink, Morton, Kent & Bickerstaffe, 2000 ; Toyohara & Makinodan, 1989 ; Jiang, Wang & Chen, 1991). It has been shown that, in sea bass muscle, they are differently expressed depending on the season of the year (Ladrat, Chaplet, Verrez-Bagnis, Noël & Fleurence, 2000). On the other hand, cathepsins B, D, L and H are the major cathepsins within the fish lysosomes (Aoki, Yamashita & Ueno, 2000) ; some of them have been studied for their enzymatic features (Lee, Chen & Jiang, 1993 ; Jiang, Lee & Chen, 1994 ; Goldman-Levkovitz, Rimon & Rimon, 1995 ; Heu, Kim, Cho, Godber & Pyeun, 1997) and for their ability to degrade muscle proteins (Jiang, Wang & Chen, 1992 ; Aoki & Ueno, 1997 ; Aranishi, Ogata, Hara, Osatomi & Ishihara, 1998).

It would be expected that the respective action of each protease is possibly affected by the other proteases. In particular, endoproteases could degrade proteic substrates to be further proteolysed by other proteases. Moreover, calpains have been believed to initiate the proteolytic degradation of myofibrils by causing a little hydrolysis on proteins making them more susceptible to other proteases (Goll, Thompson, Taylor & Christiansen, 1992 ; Lamare, Taylor, Farout, Briand & Briand, 2002 ; Papa, Alvarez, Verrez-Bagnis, Fleurence & Benyamin, 1996).

Our objective in the current study, therefore, was to improve our understanding of the mechanisms involved in *post mortem* deterioration of fish muscle and to acquire an insight into the concept of the synergistic effect of calpains and cathepsins. In our previous studies, the involvement of calpain and cathepsins in the proteolysis of sea bass white muscle during refrigerated storage was investigated using enzymes one by one (Verrez-Bagnis, Ladrat, Noelle & Fleurence, 2002 ; Ladrat, Verrez-Bagnis, Noël & Fleurence, 2003). In this work, these enzymes are used in combination.

Material and methods

Fish

Live sea bass (*Dicentrarchus labrax L.*) were purchased from a local sea farm in Vendée (France) and killed by beheading. In a cold room, viscera and skin were removed and white muscle was recovered.

Protein preparations for enzymatic digestions

Sarcoplasmic proteins were extracted by homogenising the sea bass muscle frozen at death at -80°C in 4 volumes (w/v) of water containing 1 mM EDTA. The supernatant recovered after two centrifugations at 10000 g, 4°C for 15 minutes was used as the sarcoplasmic preparation. The protein content (9.3 mg/ml) was determined by Bradford assay (Bradford, 1976) using Bio-Rad reagent (Bio-Rad, Ivry-sur-Seine, France), and bovine serum albumin as the standard.

Myofibrils were isolated from fresh sea bass muscle according to the procedure of Wang (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. When needed, 800 μl of this preparation were taken and centrifuged three times at 4000 rpm for 15 minutes at ambient temperature. Each time, the pellet was washed in 1 ml water. The final pellet was used directly for myofibrillar protein digestion.

Proteases preparation

Cathepsin L from *Paramecium tetraucleia* was purchased from CALBIOCHEM (Merck Eurolab, Fontenay-sous-bois, France). Cathepsin B (Ref C6286) and cathepsin D (Ref C3138), both from bovine spleen, were from SIGMA (Saint-Quentin-Fallavier, France). No fish cathepsins were commercially available, but most data on the catalytic activity and molecular properties of cathepsins are consistent regardless of the source (Gildberg, 1988 ; Yamashita & Konagaya, 1990a ; Yamashita & Konagaya, 1990b). Milli-calpain was purified from sea bass muscle as previously described (Ladrat, Verrez-Bagnis, Noel & Fleurence, 2002).

Proteolytic digestions

Digestions were performed at 25°C during two hours with m-calpain and then a mixture of the three above cathepsins was added. Highly purified m-calpain (18.6 $\mu\text{g}/\text{ml}$ enzyme) was added to sarcoplasmic or myofibrillar proteins to a final ratio of 1/500 (w/w) in the presence of 5 mM CaCl_2 and 5 mM β -mercaptoethanol in 50 mM Tris buffer pH 7. After two hours of incubation, 1 volume of 50 mM citrate phosphate buffer pH 5.5 was added in order to decrease the pH to 5.5 and the cathepsins mixture containing 35 μg cathepsin D (final 19 $\mu\text{g}/\text{ml}$), 10 μg cathepsin B (final 5.4 $\mu\text{g}/\text{ml}$) and 2 μg cathepsin L (final 1.1 $\mu\text{g}/\text{ml}$) in 50 mM citrate phosphate buffer was added. Incubations lasted up to 22 h. Aliquots were sampled at 0-30 min and 1.5 hour (corresponding to the action of calpain) and at 2 h-3 h-5 h and 22 h total incubation duration (corresponding to the digestion by the mixture of cathepsins).

Three different treatments were performed in this combined experiment with calpain and cathepsins: the first one (treatment A) with both calpain and cathepsins mixture as indicated above, the second one (treatment B) with calpain and addition of 50 mM citrate phosphate buffer pH 5.5 instead of cathepsins, and the last one (treatment C) with 50 mM Tris buffer pH 7 instead of calpain with addition of the mixture of the cathepsins. A negative control (D) was carried out without any enzyme but with the appropriate buffer instead. Each digestion was done and analysed twice.

Aliquots from the sarcoplasmic protein digestions were removed at specific intervals, mixed with 4x concentrated SDS-containing tracking dye and immediately heated for 3 min at 100°C . To follow the breakdown of myofibrillar proteins, a sampled aliquot was 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) and insoluble material precipitated. The pellet was dissolved in the same volume as initial aliquot of 40 mM Tris buffer containing 8 M urea and 4x SDS-containing tracking dye was added before boiling for 3 min. The remaining supernatant containing proteins released from myofibrils was added to 4x concentrated SDS-containing tracking dye and boiled.

SDS-PAGE

SDS-PAGE was carried out in a Mini-protean dual slab cell (Bio-Rad) according to the method of Laemmli (Laemmli, 1970) using 4-20 % polyacrylamide gradient gels. 5 μl 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.

Molecular weight markers were from Bio-Rad (Broad Precision recombinant standards) or from SIGMA (SDS-6H and SDS-7 mixture: 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). Average molecular weights were estimated from several analysed gels.

After migration, gels were subsequently stained with Coomassie Brilliant Blue R250 or blotted overnight onto a membrane.

Western blots of structural proteins

Electrophoresis gels were blotted overnight at 15°C onto a 0.45 μ m nitrocellulose membrane (Bio-Rad) at 30 V using a Mini-Transblot Cell (Bio-Rad) with transfer buffer containing 150 mM glycine, 20 mM Tris, 20 % methanol and 0.01 % SDS. Membranes were saturated with 8 % skimmed milk prepared in 0.9 % NaCl, washed with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer pH 7.4) containing 0.05 % v/v Tween 20 and further incubated for 2 hours at room temperature with primary rabbit antibodies in 0.9 % NaCl. After being washed in PBS with 0.05 % v/v Tween 20, blots were 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-coloured bands on the membrane. Polyclonal anti- α -actinin produced in the laboratory (1/300 dilution) was used as primary antibody on sarcoplasmic and myofibrillar proteins digestions while polyclonal anti-desmin (SIGMA, Ref D8281, 1/200 dilution) and polyclonal anti-tropomyosin (SIGMA, Ref T3651, 1/200 dilution) were used as primary antibodies for the *in vitro* proteolysis of myofibrillar proteins only.

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) and analysed with 1D-Elite software (Amersham Biosciences).

Results

Sarcoplasmic proteins combined digestion

In this experiment in which both types of proteases were used in combination, the only modification on sarcoplasmic proteins caused by calpain was observed for the 27 kDa band which was fragmented into a band of very close molecular weight as previously described (Figure 1A) (Verrez-Bagnis et al., 2002). But cathepsins were able to degrade the 97 kDa protein and the creatine kinase-aldolase assumed doublet (41-39 doublet) as it was identified by comparisons of fish proteins pattern in a previous article (Ladrat et al., 2003). In this area, the real protein affected is not clear because of the existence of huge bands. A fragment around 34 kDa was increasing. Two fragments (25 and 20.5 kDa) were produced during the incubation with cathepsins. A 21.5 kDa component was degraded. A 20.5 kDa band was appearing in each experiment performed even when no enzyme was added giving evidence for an unidentified and uncontrolled occurring mechanism (Figure 1D).

Comparing results from Figure 1C and 1A possibly indicated the synergistic effect of calpain and cathepsins. Treatment without the calpain but with the mixture of cathepsins (Figure 1C) showed that the 97 kDa component, the creatine kinase-aldolase doublet and the 21.5 kDa proteins were degraded while the 34 kDa and 24 kDa band were also increasing. Treatment B together with negative control on Figure 1 indicated that the cathepsins are really responsible for these changes in protein patterns.

α -actinin present in the soluble fraction of muscular proteins was affected neither by calpain nor by cathepsins (Figure 2) confirming the already observed stability in relation to each cathepsin action (Ladrat et al., 2003).

Myofibrillar protein digestion

During *in vitro* proteolysis of myofibrillar proteins by calpain followed by cathepsins (Figure 3), partial degradation of myosin heavy chain (MHC) was observed all over the incubation. Two fragments (140 and 78 kDa) were generated by the action of calpain and subsequently degraded during the incubation with the mixed cathepsins. Calpain left actin intact before its degradation by cathepsins. Tropomyosin (34 kDa) was degraded only after the addition of the cathepsins. Calpain caused the loss of the 32 kDa component (assumed to be troponin T). Cathepsins cleaved the 22 kDa band. Three other fragments appeared during the incubation with cathepsins: 68, 33 and 30 kDa. At the end of the incubation, all the proteins were completely hydrolysed and only a smear was visible on the lane. The comparison of the different treatment groups and control (Figure 3) showed that the above mentioned changes are due to calpain and/or to cathepsins as indicated and not to an unidentified and uncontrolled factor as it was the case for the sarcoplasmic proteins. Moreover, no difference was observed in the changes induced by cathepsins between the experiments in which calpain was acting first (treatment A, Figure 3A) or not (treatment C, Figure 3C), thus suggesting m-calpain did not modify myofibrillar proteins in a manner that rendered them more susceptible to the action of cathepsins.

As we can see in Figure 4II, α -actinin was degraded into a set of fragments by calpain (90 kDa, 80, 71, and 37 kDa). They were all degraded subsequently by the cathepsins but these latter proteases produced also a 99 kDa and a 104 kDa fragment from the remaining undegraded α -actinin subunit. Tropomyosin remained unchanged upon action of calpain as we can see on immunoblots on pellets (Figure 4III). It was afterwards degraded into a 32 kDa fragment by cathepsins (Figure 4III). Control showed only a slight decrease in the probed band intensity without any degradation (data not shown). Desmin was rapidly and highly degraded by calpain (Figure 4IV) with degradation fragments visible as early as the beginning of incubation. No desmin was visible in the second sampling. This result confirmed the previous observation of Verrez-Bagnis et al. (2002).

Some proteins were released from myofibrils into the supernatant before being completely degraded during treatment A ; in particular, two fragments of 62 and 54 kDa were released into the soluble fraction (Figure 5I) by cathepsins. No visible difference in the action of the cathepsins was observed between treatments involving first action of calpain and the treatment without calpain (data not shown). Intact α -actinin and some of its breakdown fragments were released in the soluble fraction (Figure 5II). Calpain and cathepsins were able to liberate tropomyosin from myofibrils into the soluble fraction but only cathepsins could degrade it (Figure 5III).

The observed action of the mixture of the all three cathepsins at pH 5.5 appears to be the result of the cumulative action of each cathepsin studied in a previous work (Ladrat et al., 2003) except for the 97 kDa sarcoplasmic component which was broken down only by the mixture of the three cathepsins all together.

Conclusion

Our previous results showed that MHC, α -actinin, desmin, actin, troponin T, tropomyosin and other non identified protein bands can be degraded by cathepsins D, B, or L (Ladrat et al., 2003). This work showed that when these three cathepsins are mixed, each of the above proteins is also degraded. In addition, a 97 kDa component was degraded by the mixture but not by each cathepsin taken apart from the others. In this case, the three cathepsins act in

synergy. When the cathepsin B, D and L mixture acted after the action of calpain, no difference was observed with the treatment without calpain suggesting calpain did not modify proteins and did not make them more susceptible to the further degradation by cathepsins as it was hypothesized by (Goll et al., 1992 ; Lamare et al., 2002 ; Papa et al., 1996). A combined incubation in which the myofibrils were first hydrolyzed by m-calpain then later subjected to a lysosomal extract has already been studied by Whipple and Koohmaraie (Whipple & Koohmaraie, 1991). It also led the authors to the conclusion that m-calpain did not alter any myofibrillar protein to be later hydrolyzed by the acidic proteinases.

In vitro degradation of muscle proteins by isolated or purified proteases is far more extensive than the degradation occurring with normal postmortem storage. Attempts *in vitro* to reproduce events *in vivo* can never be absolute because of reaction conditions (temperature, pH, enzyme:substrate ratio). In particular, an accurate enzyme:substrate ratio selection close to the physiological ratio is complicated by the presence of naturally occurring inhibitors. Nevertheless, our analysis substantiates the idea that calpain and cathepsins do not function synergistically in *post mortem* muscles but does not rule out the action of calpain in the early phases of *post mortem* aging followed by the cathepsins action.

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Figure 1: SDS-PAGE followed proteolysis of sarcoplasmic proteins by m-calpain for 2 h (lanes 2 to 4) followed by cathepsins mixture (lanes 5 to 8). A: combined experiment with m-calpain and cathepsins, B: treatment without cathepsins, C: treatment without m-calpain, D: control without any enzyme. Lanes 1 and 9: SDS-6H and SDS-7 molecular weight markers. Lane 2: initial reaction mixture at time 0 after the addition of the calpain, lane 3: reaction mixture after 30 min incubation, lane 4: 1.5 h incubation, cathepsins were added and sample was withdrawn at 2 h of total incubation (lane 5), lane 6: 3 h total incubation, lane 7: 5 h, lane 8: 20 h.

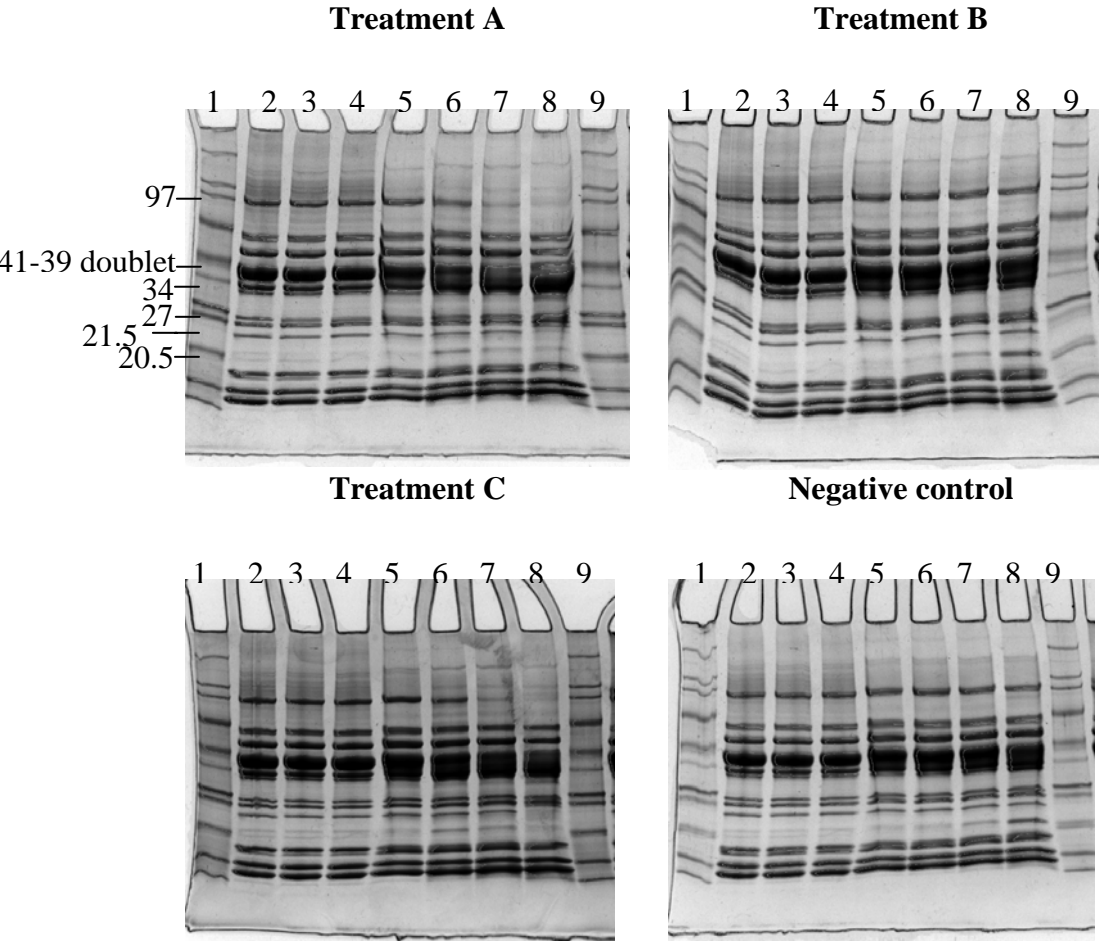


Figure 2: α -actinin on Western blots of sarcoplasmic proteins digested during the combined experiment in the presence of m-calpain and cathepsin mixture. Lane 1: initial reaction mixture at time 0 after the addition of the calpain, lane 2: reaction mixture after 30 min incubation, lane 3: 1.5 h incubation, cathepsins were added and sample was withdrawn at 2 h of total incubation (lane 4), lane 5: 3 h total incubation, lane 6: 5 h, lane 7: 20 h, lane 8: initial sarcoplasmic extract.

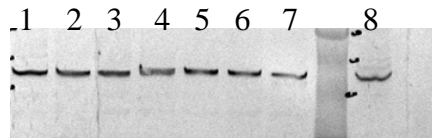
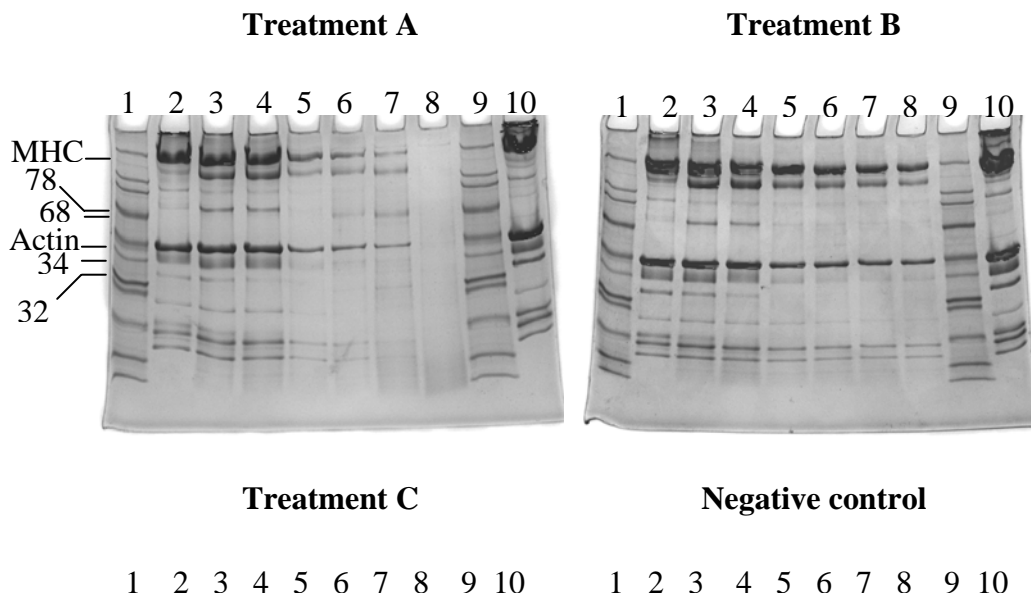


Figure 3: SDS-PAGE analysis of pellet from proteolysis of myofibrillar proteins by m-calpain for 2 h (lanes 2 to 4) followed by cathepsins mixture (lanes 5 to 8). A: combined experiment with m-calpain and cathepsins, B: treatment without cathepsins, C: treatment without m-calpain, D: control without any enzyme. Lanes 1 and 9: SDS-6H and SDS-7 molecular weight markers. Lane 2: initial reaction mixture at time 0 after the addition of the calpain, lane 3: reaction mixture after 30 min incubation, lane 4: 1.5 h incubation, cathepsins were added and sample was withdrawn at 2 h of total incubation (lane 5), lane 6: 3 h total incubation, lane 7: 5 h, lane 8: 20 h.



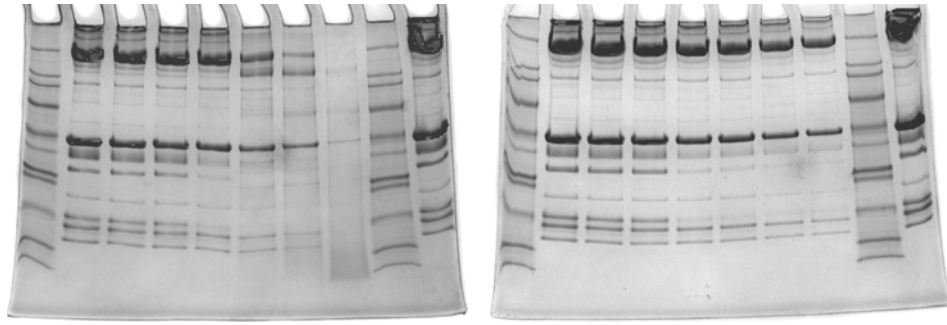


Figure 4: SDS-PAGE and immunoblots of pellet during myofibrillar protein digestion in the combined experiment i.e. treatment A in the presence of m-calpain (lanes 2 to 4 at 0, 30 min and 1.5 h incubation) followed by cathepsins mixture (lanes 5 to 8 at 2, 3, 5 and 20 h incubation). I: SDS-PAGE stained with Coomassie Blue (identical to Figure 3A). II: α -actinin immunoblot. III: tropomyosin immunoblot. IV: desmin immunoblot. Lanes 1 and 9: SDS-6H and SDS-7 markers (see legend to figure 1) for SDS-PAGE or Broad Precision standards for immunoblots. Lane 10: initial myofibrillar extract.

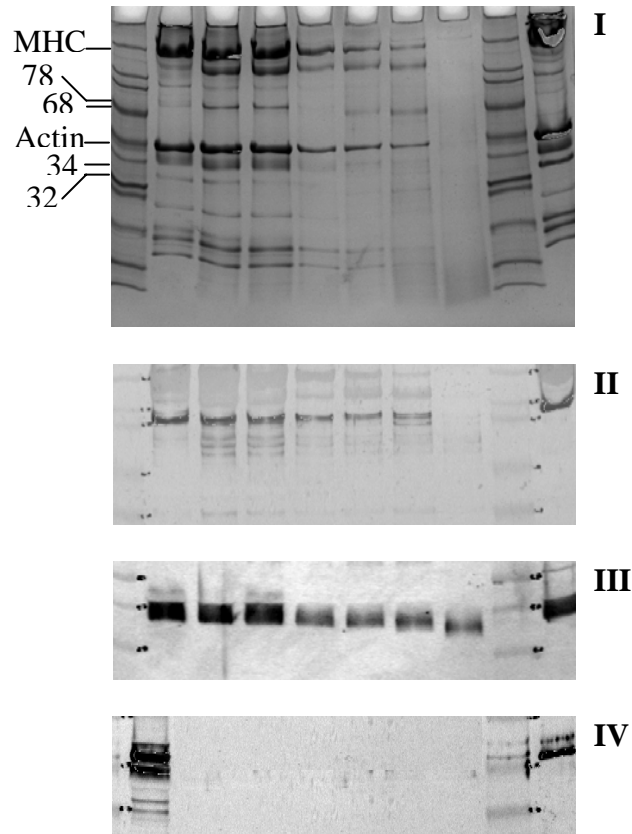


Figure 5: SDS-PAGE and immunoblots of supernatant during myofibrillar protein digestion in the combined experiment i.e. treatment A in the presence of m-calpain (lanes 2 to 4 at 0, 30 min and 1.5 h incubation) followed by cathepsins mixture (lanes 5 to 8 at 2, 3, 5 and 20 h incubation). I: SDS-PAGE stained with Coomassie Blue. II: α -actinin immunoblot. III: tropomyosin immunoblot. Lanes 1 and 9: SDS-6H and SDS-7 markers for SDS-PAGE and Broad Precision standards for blots..

