
Calpain and cathepsin activities in post mortem fish and meat muscles

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Abstract:

Post mortem tenderization is one of the most unfavourable quality changes in fish muscle and this contrasts with muscle of mammalian meats. The tenderization can be partly attributed to the acid lysosomal cathepsins and cytosolic neutral calcium-activated calpains. In this study, these proteases from fish and bovine muscles were quantified and compared. The cathepsin B and L activities were in more important amounts in sea bass white muscle than in bovine muscle. On the other hand, cathepsin D activity was 1.4 times higher in meat than in fish muscle, while cathepsin H was negligible in both muscles. Calpain activities were similar in both types of muscle. Moreover, calpastatin (calpain endogenous inhibitor) level is 3.9 times higher in sea bass white muscle. These differential activities are considered in relation to their probable involvement in post mortem degradation of muscle.

Keywords: Calpain; Cathepsin; Protease; Fish; Meat

Introduction

Texture is considered to be one of the most important quality attributes of fish and meat; it determines consumer acceptance and hence the marketability of the final products. Firmness is an important aspect of fish quality as opposed to tenderness which is appreciated in meat consumption. In contrast to fish, weakening of the mammalian muscle structure during *post mortem* storage is desirable since it improves tenderness. During the *post mortem* ageing of muscle under chilled conditions, degradation of muscle proteins contributes to the rapid softening of flesh. The *rigor mortis* is completed between 3 and 18 h after killing in the case of pelagic fish (Fauconneau, 2004 and Sainclivier, 1983). In the case of bovine muscle, rigor mortis occurs 24 h after the death of the animal. In bovine muscle, the acquisition of optimal tenderness requires at least 14 days (Ouali, 1990 and Ouali, 1995).

The main myofibrillar proteolysis can be attributed to endogenous protease activity. Currently, two characterized proteolytic systems are known to hydrolyze myofibrillar proteins during post mortem storage of meat and fish muscle: calpains and cathepsins (Jiang, 2000 and Ouali, 1992). Most of the studies agree that there is a synergistic proteolytic action of calpains and cathepsins on key myofibrillar proteins, whereas the role of proteasome in post mortem tenderization needs to be further clarified (Lamare, Taylor, Farout, Briand, & Briand, 2002).

The calpains (EC 3.4.22.17), intracellular neutral cysteine proteases, are calcium-dependent. These enzymes are further subclassified into μ -calpain and m-calpain, which differ in sensitivity to calcium ions. Both are heterodimers: the large subunit and the small subunit for μ - and m-calpain have molecular weights near 80 kDa and 28 kDa, respectively. The small subunit might play a regulatory role or act as a chaperone (Suzuki et al., 1986). The active site is localized in the large subunit but, for the full activity, the presence of the small subunit is required (Tsuji & Imahori, 1981). Calcium plays a key role in the activation mechanism of calpains, leading to dissociation and/or autoproteolysis, even in the presence of an alternative substrate. Calpastatin is known to be the endogenous specific inhibitor of the calpains.

Cathepsins are acid proteases located in the lysosomes. They may be liberated into both the cytoplasm and the intracellular spaces as a consequence of lysosomal disruption occurring after cell death due to a pH fall (Duston, 1983). Lysosomes are known to harbour about 13 types of cathepsins (Goll et al., 1983). Among these, lysosomal enzymes (four classes) can be distinguished according to the active site: aspartic, cysteine, serine and metallo-proteases. The main cathepsins involved in muscle ageing are cathepsins B (EC 3.4.22.1), L (EC 3.4.22.15), H (EC 3.4.22.16) and D (EC 3.4.22.5). The cathepsins B, H and L are regulated *in vivo* by a protease inhibitor called cystatin (Turk & Bode, 1991).

Several studies indicate that some of the changes occurring in mammalian muscle post mortem are associated with the action of calpains (Goll et al., 1998 and Koohmaraie, 1996). The amounts of calpain activity at death (initial time), as well as the amount of calpastatin, are related to the extent of tenderization in mammalian muscles (Zamora, Debiton, Lepetit, Dransfield, & Ouali, 1996). So, muscle enzymes could be used as final quality indicators (Toldra & Flores, 2000). In fish muscle, the calpain role in flesh tenderization remains controversial (Verrez-Bagnis, Ladrat, Noël, & Fleurence, 2002) and lysosomal cathepsins could be responsible for myofibrillar or connective tissue degradation (Eggen and Ekholt, 1995, Ladrat et al., 2003, Sato et al., 1997 and Yamashita and Konagaya, 1990).

The aim of this study was to characterize and compare fish flesh and meat initial amount of proteolytic activity in order to evaluate the possible differential role of proteases in both types of muscles. The methods used for quantification of the proteolytic activity (calpains; cathepsins B, D, H and L) within the muscles are described. This better knowledge would help us to understand the ageing mechanisms in fish and meat.

1. Materials and methods

1.1. Materials

Unless specified, chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The chromatographic gels were from Amersham Biosciences (Uppsala, Sweden).

1.2. Animal samples

Dairy cows (62 months old, 300–400 kg) were slaughtered in a local abattoir (SOVIBA, Le Lion d'Angers, France). At one day *post mortem*, the *biceps femoris* muscle was excised, minced and vacuum packed in 50 g portions.

Sea bass (*Dicentrarchus labrax* L., 4 years old, 250–350 g) were obtained from a local sea farm in Vendée (France) and brought back alive to the laboratory. Fishes were killed by decapitation; white muscle was excised, minced and vacuum-packed in 30 g portions.

Both types of muscles were frozen at $-80\text{ }^{\circ}\text{C}$ prior to use.

1.3. Preparation of sarcoplasmic proteins from sea bass muscle

A 30 g portion of minced fish muscle was homogenized twice for 30 s with an Ultra Turrax macerator (T25, IKA, Labor Technik, Staufen, Germany) equipped with a 18 mm diameter head (S 25–18 G) in 90 ml of buffer A containing 50 mM Tris-HCl (pH 7.5), 10 mM β -mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 10,000g (GR 20.22, Jouan, France) for 40 min at $10\text{ }^{\circ}\text{C}$, the supernatant was recovered and referred to as crude extract (Verrez-Bagnis et al., 2002). Six different crude extracts were prepared.

1.4. Preparation of sarcoplasmic proteins from bovine muscle

A 50 g portion of minced bovine muscle was homogenized twice for 30 s with the Ultra Turrax macerator in 150 ml of buffer A. After centrifugation at 25,000g for 20 min at $4\text{ }^{\circ}\text{C}$, the supernatant was collected and filtered through glass wool and referred to as crude extract (Koochmariaie, 1990). Six crude extracts were obtained.

1.5. Purification of calpastatin and calpains from muscle

The whole procedure was carried out at $4\text{ }^{\circ}\text{C}$. The chromatographic column (Phenyl Sepharose CL-4B, \varnothing 26 mm, L 10.5 cm) was balanced with equilibration buffer – composed of 50% buffer A and 50% buffer B (buffer A with 1 M NaCl).

Fifty millilitres of crude extract, supplemented with 0.5 M NaCl, were directly applied to the chromatographic column. The non-absorbed proteins, including calpastatin, the endogenous inhibitor of calpains, were washed with the equilibration buffer. The calpain-active fraction was then eluted in a batch with 50% buffer A and 50% glycol ethylene. The different fractions were collected in ice.

1.6. Determination of protein

The amount of proteins was determined by measurement of the optical density at 280 nm (Abs.) and expressed as grammes per litre, using bovine serum albumin as the standard. The values were the means of three measurements for each sample.

1.7. Activity measurement of calpains

Calpain activity was determined in duplicate at 30 °C in a 303 µl reaction mixture containing 3 µl of 0.5 M CaCl₂, 6 µl of 5% CHAPS {3-[3(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} and 5 µl of synthetic fluorogenic substrate SucLT (*N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin) prepared in methanol at 20 mM. The reaction was initiated by adding 255 µl of enzymatic sample. During two hours, fluorescence was recorded in microplate wells, with an excitation wavelength set at 355 nm and emission wavelength set at 460 nm, using the spectro-photo-fluorometer FLUOstar OPTIMA POLARstar OPTIMA reader (BMG LABTECH, Champigny sur Marne, France). A control, in which 3 µl of 0.5 M CaCl₂ was replaced by 3 µl of 0.5 M EDTA, was also performed. In this assay, the specific activity was expressed in FU (units of fluorescence) increase per minute per gramme of muscle. The values were the means of three measurements for each sample.

1.8. Quantification of calpastatin inhibitory activity

Calpastatin inhibitory activity was measured with a calpain-active sample produced separately from a whole fish white muscle or meat muscle, as described above. The activities of the calpain samples from the two different muscles were adjusted to the same level with buffer A. 55 µl of calpastatin sample (or buffer for the control) were mixed with 200 µl of calpain sample and the residual calpain activity was measured on SucLT fluorogenic substrate, as previously described. One unit of calpastatin activity was defined as the amount which inhibited one unit of calpain activity.

1.9. Activity measurement of cathepsins

1.9.1. Cathepsin D

Cathepsin D activity was determined with haemoglobin as the substrate according to Anson's method ([Anson, 1938](#)). Activity was determined at 37 °C in a 1000 µl reaction mixture consisting of 250 µl of 2% (w/v) denatured haemoglobin and 250 µl of 0.2 M sodium acetate/ acid acetic buffer, pH 4, containing 10 mM β-mercaptoethanol and 1 mM EDTA. The reaction was initiated by adding 500 µl of sarcoplasmic protein extract. After a determined time interval, up to 100 min of incubation, the reaction was stopped by adding 125 µl of 10% trichloroacetic acid (TCA) to 125 µl of mixture reaction. After overnight incubation at 4 °C, the sample was centrifuged at 18,000g for 15 min at 10 °C. 150 µl of supernatant were reacted with 150 µl of Bio-Rad Protein assay (BIO-RAD Laboratories GmbH, München, Germany) for the quantification of TCA-soluble peptides released by digestion. The absorbance was measured spectrophotometrically at 595 nm. In this assay, the specific activity was expressed as absorbance (at 595 nm) increase per minute per gramme of muscle. The values were the means of three measurements for each sample.

1.9.2. Cathepsins B, H and L

B, H and L cathepsin activities were determined at 30 °C in a 298 µl reaction mixture consisting of 6 µl 5% CHAPS, 1 µl of 1.40 M 2-mercaptoethanol, 16 µl of 5% (w/v) Brij[®] 35, 5 µl of synthetic fluorogenic substrate prepared in methanol at 20 mM and 70 µl of 0.4 mM acetate/acid acetic (pH 4) buffer containing 10 mM β-mercaptoethanol and 1 mM EDTA; Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride, L-Arginine-7-amido-4-methylcoumarin hydrochloride were used as the substrates for cathepsin B, cathepsin B and L, and cathepsin H, respectively. The reaction was initiated by adding 200 µl of protein extract. A control with buffer A instead of enzymes was run in parallel. In this assay, the specific

activity was expressed in FU (units of fluorescence) increase per minute per gramme of muscle. The values were the means of three measurements for each sample.

2. Results

2.1. Purification profiles: Phenyl Sepharose CL-4B chromatography

Cathepsins were directly measured in the sarcoplasmic extract but the presence of calpastatin, in amount sufficient to totally inhibit calpain activity in this extract, made purification steps necessary before measuring calpain activity.

The elution profiles on the Phenyl Sepharose CL-4B column obtained on the bovine muscle and on the sea bass white muscle extracts are shown in [Fig. 1](#) and [Fig. 2](#), respectively. Calpastatin does not bind to the chromatographic gel and is eluted during the washing with 0.5 M NaCl. The fraction eluted at 0 M NaCl, containing calcium-dependent enzymatic activity, is shown with an arrow. The only notable difference on the profiles of both chromatograms is a shoulder in the peak of non-absorbed proteins of fish including calpastatin.

2.2. Enzyme assays

The cathepsin, calpain and the calpastatin activities in sea bass white muscle and in bovine muscle are shown in Table 1 to allow the comparison between fish and meat.

Our results show that there are some differences in protease amounts in muscle, depending on the family of proteases considered. The cysteine endopeptidase cathepsin B and L activities were detected in more important amounts in sea bass white muscle than in bovine muscle. There are 29.7 and 4.7 times more cathepsins B and B + L, respectively, in fish muscle than in meat. Cathepsin H can be considered negligible in both types of muscle as well as cathepsin B in bovine muscle. Cathepsin L activity in fish muscle is four-fold higher than that in bovine muscle. Cathepsin D (aspartic protease) activity is 1.4 times higher in meat than in fish muscle. Activity units are specific to this enzyme and comparison with other enzyme levels is not justified.

On the other hand, the calpain amounts are similar in both muscles but the calpastatin level is 3.9 times higher in sea bass white muscle, showing the high inhibition potential of calpain in this muscle.

3. Discussion

Zamora et al. (1996) reported that a beef muscle initially rich in enzymes becomes more tenderized. Thus, in this study, we determined the total proteolytic activity present in muscle. The measured levels of cathepsin H suggest that this enzyme has only a negligible role in *post mortem* proteolysis of fish and bovine muscle. Cathepsin B might have a role in postmortem deterioration of fish muscle, as suggested by its high activity level but it is in too low amount in bovine muscle to have a role in *post mortem* proteolysis of this muscle. Thus cathepsin L could have a role in the tenderization process in both muscles, but cathepsin B in fish muscle only.

Calpains in bovine muscle and in fish muscle, are in similar amounts but this enzyme is highly regulated and, in particular, the amount of calpastatin should be taken into consideration. Table 1 shows that calpastatin is present at concentrations high enough to effectively inhibit all measurable calpain activity. But the calpastatin levels are different in the studied muscles; in particular, the sea bass muscle calpastatin/calpain ratio is 3.6 times higher than in bovine muscle showing the high inhibition potential of calpain in fish muscle. Ouali and Talmant (1990) have already reported that muscle calpastatin level is not the same among mammalian families. Goll, Thompson, Li, Wei, and Cong (2003) showed that calpastatin activity exceeds activity of the calpains in most, but not all cell and tissues. In bovine meat, the relatively low calpastatin/calpain ratio may support calpain involvement in *post mortem* proteolysis. In fish muscle, this ratio is very

high, so calpain may be less active in *post mortem* muscle. However, the regulation of calpain activity by calpastatin may also depend on protein localization and on the calpastatin to calpain ratio in the considered place. Indeed, the rate of meat tenderization has been shown to be related to the enzyme/inhibitor ratio more than to the calpain content (Koochmaraie, 1996). Geesink, Morton, Kent, and Bickerstaffe (2000) partially purified calpains from salmon and compared their activities to sheep and beef calpain. Salmon had about as much calpastatin as sheep but 100-fold lower *post mortem* calpain activity. In our study, sea bass had as much calpain as beef but 4-fold higher calpastatin activity. This does not support a role of calpains in fish *post-mortem* tenderisation.

On the other hand, environmental *in situ* conditions have to be taken into account to better understand the activity of the enzymes in the muscle after death. The level of activities was determined at the optimal pH of each enzyme activity, using synthetic or normal (in excess) substrates and, in the case of calpain, in absence of inhibitor (calpastatin) and in the presence of calcium, allowing complete activation. Therefore, the results could not totally reflect what happens *in situ*. Calpains require an *in vitro* calcium concentration much higher than usual physiological concentrations, even if calcium increases in sarcoplasm after death; this would not support a role of calpains in *post mortem* tenderization. But other activators, such as membrane phospholipids, other cations, protein activators and the phosphorylation process, could participate in calpain activation at low calcium concentration (Baki et al., 1996, Johnson, 1990 and Suzuki and Ohno, 1990).

In fish muscle, after the death of fish, and during the *rigor mortis* period, the pH drops from 7.0 to 6.5 and later rises to a value close to 7 (data not shown and Sainclivier, 1983). In meat, the pH is close to 6.5 and shows a rapid decrease after death, reaching 5.7–5.4 after 24 h of storage (Ouali, 1990). Thus, cathepsins B, L and calpains may be more implicated in fish muscle *post mortem* changes than cathepsin D, since their optimal pH is closer to *post mortem* pH (between 6.5 and 7.0) while the cathepsin D optimal pH is below 5.0 (Jiang, 2000 and Makinodan et al., 1982). In the case of meat, the pH is more appropriate for activity of lysosomal proteases (precisely the cathepsins D, B and L). In addition, during the early *post mortem* period, the pH does not drop quickly and it may allow the calpains to be active in meat and fish flesh.

Cathepsins are usually located in lysosomes and thus are inactive in living tissue because they are not in contact with their substrates. However, they may become released in the cytosol due to lysosome disruption after death.

Conclusion

In this study, cathepsins and calpain as well as calpastatin have been quantified in fish and bovine muscles. In relation to enzyme environment features in both muscles, the possible roles of these enzymes in *post mortem* degradation of muscles are discussed.

Many questions remain fully not elucidated. These results suggest that the principal cause of *post mortem* degradation of sea bass white muscle is partly the action of cathepsins B and L. The calpain system is implicated in a secondary role. In bovine muscle, the low calpastatin/calpain ratio and the high amount of cathepsin L indicate that these two systems can act in a synergistic way in the promotion of *post mortem* tenderization. Moreover, after a few *post mortem* hours, the physiological conditions of pH in the meat may become more favourable to the action of cathepsin D.

A better identification of the role of each protease requires a demonstration that the enzymes are active in the *post mortem* muscle environment and also requires the identification of their muscular substrates.

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Figures

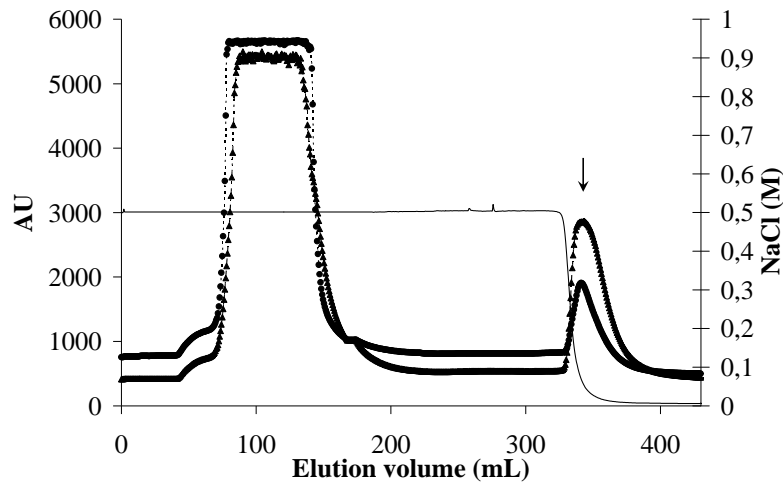


Fig. 1. Phenyl Sepharose CL-4B elution profile of meat extract. Arrow indicates position of calcium-dependent active peak. Absorbance units (AU) at 280 nm (▲), 254 nm (•) and concentration of NaCl (M) (—) are monitored and plotted against the elution volume.

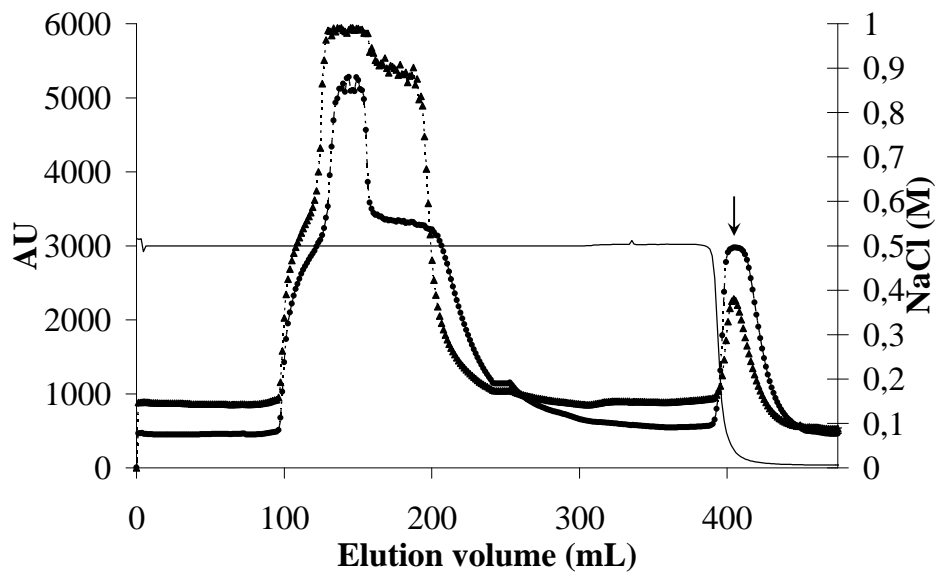


Fig. 2. Phenyl Sepharose CL-4B elution profile of fish extract. Arrow indicates position of calcium-dependent active fractions. Absorbance units (AU) at 280 nm (▲), 254 nm (•) and concentration of NaCl (M) (—) are monitored and plotted against the elution volume.

Proteolytic activities	Total activities	
	Sea bass white muscle	Bovine muscle
Cathepsin D	1.8 ± 0.21	2.4 ± 0.03
Cathepsin H	170 ± 39	212 ± 26
Cathepsin B	2168 ± 79	73 ± 13
Cathepsins (B+L)	11823 ± 681	2507 ± 620
Cathepsin L*	9655	2434
Calpains	1308 ± 261	1224 ± 76
Calpastatin	22444 ± 4021	5705 ± 1492
Calpastatin / calpain ratio	17.2	4.7

Table 1.

Activities of proteolytic enzymes (cathepsin D, cathepsin H, cathepsin B, cathepsins (B + L), cathepsin L and calpains) and of calpastatin in sea bass white muscle and bovine muscle

^a The units for the different activities are: increase in absorbance at 295 nm per min and per g of muscle for cathepsin D and increase in FU per min and per g of muscle for the other cathepsin activities and calpain activities. One unit of calpastatin inhibits one unit of calpain. *Cathepsin L was deduced by subtracting cathepsin B activity amount from the cathepsins B + L level. Sea bass calpastatin activity was measured using purified calpain (130 FU/min) from sea bass muscle while bovine calpastatin was measured using purified calpain (130 FU/min) from bovine muscle.