3:16 Proteolytic action of lysosomal proteinases on the myofibrillar structure. Comparison with the CaANP effects and the post mortem changes

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Introduction

Meat conditioning is a complex process. One mechanism postulated to be implicated in this process is the enzymatic proteolysis of myofibrillar proteins. Although very limited in post mortem muscle, proteolysis seems to be interrelated with post mortem changes and tenderness increase. This is supported by the significant relationship found between some biochemical modifications occurring in the myofibrillar structure and the reduction in toughness[1-3].

At least two intracellular systems of proteinases may be involved in the tenderising process of meat. The first one is composed of two calcium-activated neutral proteinases (CaANP) now called calpain[4]. Their effects on myofibrillar proteins as well as their ability to mimic several structural and biochemical post mortem changes affecting the contractile structure are well documented[5-11]. The second one is the lysosomal complex. Cathepsin D (aspartic proteinase) and cathepsin B (cystéine protéinase) are the first lysosomal proteinases to have been well characterized. Over the last few years, at least six more cysteine proteinases have been described the main ones being cathepsin L and H[12]. Several works have aroused considerable interest in the relationship that the lysosomal enzymes might have to the post mortem alteration of the myofibrillar components and to the tenderising of meat during conditioning[3, 13-17]. This has been strengthened by the proteolytic action of cathepsin D[16, 18-19], B[18, 20] and L[18, 20] either on myofibrils or on isolated myofibrillar proteins.

This work was undertaken in order to study the proteolytic action of cathepsins B, D, H and of new high molecular weight cysteine proteinase (called by mistake cathepsin L in the summary) partially purified from rat liver on myofibrils from both rabbit and bovine muscles. The results are discussed in regards with both the CaANP and the post mortem ageing efects on bovine and rabbit muscle myofibrils.

Materials and methods

- Materials

Samples of about 300 g were cut from bovine Longissimus dorsi (LD) at the level of the 10th rib and Rectus abdominis (RA) muscles 2 hours post mortem and were used immediately to prepare myofibrils according to (21). One rabbit Longissimus dorsi (LD) muscle was excised just after slaughter and myofibrils were also rapidly extracted.

The livers of 20 male Sprague Dawley rats killed by decapitation were quickly removed, washed in 0,02 M Tris-HCl buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and used to prepare the lysosomal enzymes.

- Preparation of the enzymes

Enzymes were purified as described in the following section from an enriched fraction of lysosomes prepared from rat livers according to (22). The lysosomes were homogenized in 0.6 volume of 0.03 M sodium phosphate buffer pH 5.8 containing 0.1 % triton X - 100 and then frozen.

- Measurement of enzyme activities

The assays for N-Benzoyl-DL-Arginine-2-Naphthylamide (BANA), N-Carbobenzoxy-L-Arginine-L-Arginine-2-Naphthylamide (2-Arg-Arg-Nap) and L-arginine-2-Naphthylamide (Arg-Nap) were carried out according to (23) and (24). The activity against N-Carbobenzoxy-L-phenylalanyl-L-Arginine-4 Methyl-7-coumarylamine (Z-Phe-Arg-N-Mec) was performed as decribed in (25). Activity against haemoglobin was followed as previously reported[9].

- Enzymatic treatment of myofibrils

Myofibrils (40 mg of proteins) were incubated with the different enzymes at 30°C in 0.05 M sodium phosphate buffer pH 5.8, 1 mM EDTA, 10 mM dithiotreitol (DTT), 1 mM NaN_3 for 1 hour. For cathepsin D, DTT was omitted and the incubation was carried out for 6 hours.

The reaction was stopped by centrifugation at 5 000 g for 10 min. and the myofibrils extensively washed with a solution containing 0.05 M KCl, 5 mM mercaptoethanol (MCE) and 1 mM NaN_3. The pellet was then dissolved in 0.05 M Tris-HCl buffer pH 8.8, 7.5 % glycerol, 1 % MCE and 3 % sodium dodecyl sulfate (SDS) and heated at 100°C for 5 min.

- Electrophoresis

Electrophoresis of the myofibrillar proteins was performed according to (26) on 12.5 % polyacrylamide slab gels as previously reported[10]. In every case, 15 ug of proteins were loaded into the wells.

- Protein concentration

The concentration of myofibrillar proteins was determined by the biuret method[27] with bovine serum albumin as a standard.

Results

- **Purification of the lysosomal proteinases**

1 - Gel filtration on Sephadex G 75

The frozen lysosomal homogenate was thawed, centrifuged at 100 000 g for 60 min. and the supernatant was adjusted to 80 % saturation $(NH_4)_2SO_4$. The precipitate was dissolved in a minimum volume of 0.03 M sodium phosphate buffer pH 5.8, 0.05 % triton X - 100 (buffer A) and dialysed against this same buffer. This solution was clarified by centrifugation and applied on a Sephadex G 75 superfine column (90 x 2.5 cm) pre-equilibrated with buffer A. Proteins were eluted at a flow rate of 10 ml. h^{-1} and fractions of 5 ml were collected.

Three main peaks of proteolytic activity were resolved by gel filtration (fig. 1). The P_1 fraction cleaved mainly 2-Phe-Arg-N-Mec and exhibited a molecular weight of 55 Kd (Kilodaltons). The P_2 fraction degraded haemoglobin and had a Mw of 42 Kd. Fraction P_3, which had a Mw of about 25-30 Kd hydrolyzed Z-Phe-Arg-N-Mec, Z-Arg-Arg-Nap, BANA and Arg-Nap.

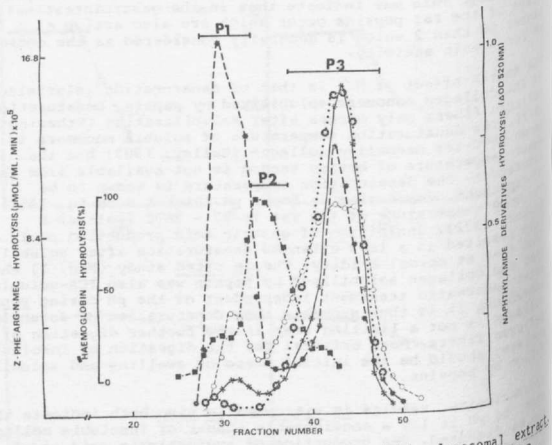


Fig. 1 : Sephadex G 75 gel filtration of a soluble lysosomal extract. Activities againts Z-Phe-Arg-N-Mec (*), Z-Arg-Arg-Nap (○), Arg-Nap (●), BANA (✳) and haemoglobin (■).

2 - Purification of the 55 Kd proteinase

The pooled P_1 fraction was applied on a CM-Sepharose column (10 x 2 cm) pre-equilibrated with buffer A. The 55 Kd proteinase which was retained on the column was eluted in a single peak with 0.6 M NaCl. This proteinase cleaved Z-Phe-Arg-N-Mec and to a lesser extent Z-Arg-Arg-Nap and BANA. It exhibited an absolute requirement for thiol compounds such as DTT or cysteine and was strongly inhibited by leupeptin and $HgCl_2$. A more detailed study of this cysteine proteinase will be published elsewhere. One unit of this enzyme released one umol. of 4-Methyl-7-counmarylamine, ml^{-1} min^{-1} at pH 6.0 and 37°C with Z-Phe-Arg-N-Mec as a substrate.

3 - Purification of cathepsin D

The pooled P_2 fraction was concentrated by ultrafiltration with an immersible CX 10 Millipore membrane and applied on a CM-Sepharose column in the same conditions as for the 55 Kd proteinase. The haemoglobin hydrolysing activity was aluted in the unretarded fraction. This proteinase showed no thiol requirements and was totally inhibited by 5 10^{-7} M pepstatin but not by leupeptin or $HgCl_2$. The partially purified proteinase was therefore identified as cathepsin D. At pH 3.5 and 37°C, one unit of cathepsin D caused an increase in the OD 280 nm of 0.001 absorbance unit per hour.

4 - Purification of cathepsin B and H by chromatofocusing

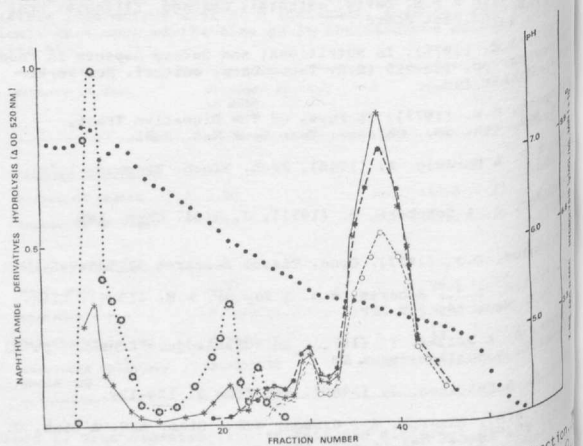


Fig. 2 : F.P.L.C. chromatofocusing on a mono P column of the P_3 fraction. pH (●●) and activities against Z-Phe-Arg-N-Mec (*), Z-Arg-Arg-Nap (○) and BANA (✳) were measured.

The pooled P_3 fraction was dialyzed against 0.05 % triton X 100 in order to eliminate phosphate ions and concentrated by ultrafiltration with an immersible CX 10 Millipore membrane. Samples of 1.5 ml were applied on the mono P column of a Pharmacia FPLC system pre-equilibrated with 25 mM imidazole - HCl buffer pH 7,0 containing 0.05 % triton X 100. Polybuffer 74 diluted 10 fold and adjusted to pH 4.0 and to 0.05 % triton X 100 was used as an eluent at a rate of 0.9 ml min^{-1} and fractions of 0.8 ml were collected.

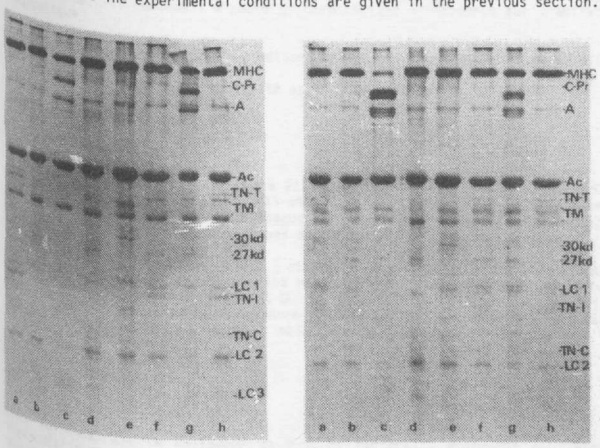
As shown in figure 2, three peaks of activity against Arg-Nap were eluted at pH 7.2, 6.0 and 5.9 respectively, the first one being predominant. All the fractions required a thiol activator and were inhibited by 50 % at concentrations of leupeptin were higher than 10^{-5} M. Moreover, an activity ratio for Arg-Nap to BANA was found. According to (28), these fractions were identified to cathepsin H and pooled together. At pH 6.0 and 37°C, one unit of cathepsin H caused an increase in the OD 520 nm of 0.001 absorbance unit per min. with the Arg-Nap as substrate.

Two peaks of activity towards Z-Arg-Arg-Nap, BANA and Z-Phe-Arg-N-Mec were eluted at pH 5.5 and 5.25 respectively the last one largely prevailing. The two fraction required a thiol activator and were inhibited to 50 % by less than 10^{-6} M leupeptin. According to their substrate specificity, their elution pH and their sensitivity to leupeptin, these fractions were identified to cathepsin B[29]. One unit of cathepsin B degraded one umol. of Z-Phe-Arg-N-Mec ml^{-1} min^{-1} at pH 6.0 and 37°C.

A very small peak of activity towards Z-Phe-Arg-N-Mec without any associated activity against Z-Arg-Arg-Nap was eluted at pH 5.8 - 5.9. This cathepsin-L-like activity was negligeable and was not considered in this work.

- Proteolytic action of these lysosomal proteinases on rabbit and bovine muscle myofibrils

In every case, 40 mg of myofibrillar proteins were incubated with 16 U of cathepsin D or 0.045 U of cathepsin B or 0.18 U of the 55 Kd proteinase or 35 U of cathepsin H. The experimental conditions are given in the previous section.



① ②

Fig. 3 : Action of cathepsins B (b), H (f), D (g) and of the 55 Kd proteinase (c) on rabbit LD (1) and bovine RA (2) muscle myuofibrils. Control myofibrils incubated without enzyme in presence of DTT for one hour (a) or in the absence of DTT for 6 hours (h) as well as myofibrils from rabbit (d) and bovine (e) LD muscles stored for 10 days at + 4°C were also loaded on each gel. The abbreviations used are for myosin heavy chain (MHC), C-protein (C-Pr), α-actinin (A), actine (Ac), troponin (TN), tropomyosin (TM) and myosin light chains (LC).

1 - Proteolytic action on rabbit LD muscle myofibrils

Cathepsin B seems to alter the high Mw myofibrillar proteins only slighly (fig. 3.1b). Nevertheless, proteolytic breakdown products can be detected just below C-protein and α-actinin on SDS-polyacrylamide gel electrophoresis. They have a Mw of about 130 Kd for the first one and in the range of 80 to 95 Kd for the others. At least some of these components might orginate from myosin heavy chain (MHC) since, with a higher cathepsin B to myofibrillar proteins ratio, similar fragments appear concomitantly with the decrease in the MHC content. The main ones have a Mw of 130 Kd and 95 Kd. Cathepsin B degrades the lower Mw myofibrillar proteins more extensively. Thus, in cathepsin-B-treated myofibrils, troponin (TN) T, ß-tropomyosin (TM), TN-I and TN-C have almost completely disappeared. At the same time, the appearance of two proteolytic components with a Mw of 27 and 26 Kd and an increase in the intensity of the myosin light chain 3 (LC 3) can be noted. These changes are very similar to those observed in stored rabbit meat (fig. 3.1d).

The 55 Kd proteinase affects the high as well as the low Mw myofibrillar proteins more extensively than cathepsin B (fig. 3.1c). Decreasing in MHC is concomitant with the increase in the content of two main proteolytic fragements with a Mw of about 130 Kd and 95 Kd respectively. TN-T, ß-TM, LC 1, TN-I, TN-C and to a lower extent, LC 2 are degraded. As for cathepsin B, for shorter incubation periods or lower proteinase to myofibrillar proteins ratio, a 27 Kd and to a lesser extent a 26 Kd components have been observed as in conditioned meat.

Similarly to the 55 Kd proteinase, cathepsin D digests MHC and causes the release of the same hydrolytic products of Mw 130 and 95 Kd respectively (fig. 3.1g). As regards to the lower Mw myofibrillar proteins, cathepsin D seems to be less efficient than both the 55 Kd proteinase and cathepsin B since only TN-I and, to a lesser extent, TN-T and LC 2 decrase in intensity. In cathepsin D treated myofibrils, only the presence of the 27 Kd component was noted. For each of these proteinases, when the conditions (incubation time and/or proteinase to proteins ratio) are not well adapted, the 26 and/or the 27 Kd fragments which appear transiently can be totally absent.

Cathepsin H hardly hydrolyses myofibrillar proteins since, none of these proteins seem to be altered. Only a 27 Kd breakdown component can be seen.

2 - Proteolytic action on bovine RA muscle myofibrils

Because the different lysosomal proteinases studied have similar effects on both bovine LD and RA muscle myofibrils, only the results obtained with RA muscle myofibrils will be considered.

The 55 Kd proteinase causes a large decrease in the MHC content of these myofibrils (fig. 3.2c). As MHC is degraded the appearance of two proteolytic breakdown components with Mw of about 120 and 130 Kd is observed. Five other components with Mw ranging from about 70 to 95 Kd can be seen below α-actinin. In comparison with the hydrolytic products generated by this enzyme from rabbit muscle MHC, boine muscles MHC is split into a larger number of fragments. The 55 Kd proteinase also digests the lower Mw proteins very extensively. TN-T, ß-TM, LC 1, TN-I, TN-C and to a lesser extent LC 2 have almost completely disappeared. As for rabbit muscle myofibrils, a 25 Kd component is transiently observed in well-adapted experimental conditions.

Cathepsin B had only a slight action on MHC and at least three hydrolytic components with Mw of about 130, 120 and 95 Kd could be detected (fig. 3.2b). This enzyme was much more efficient on the lower Mw porteins where TN-T, ß-TM, TN-I and LC 1 were digested. Concomitantly the appearance of a 27 Kd component alone was noted.

Cathepsin D hydrolyses preferentially MHC and as the MHC content decreases, an increase in the concentration of two components with a Mw of 130 and 120 Kd as well as four other ones with Mw in the range of 80 to 95 Kd was observed (fig. 3.2g). The action of cathepsin D on MHC was very similar to that reported for the 55 Kd proteinase. By contrast, only a little effect was noted on the lower Mw proteins in spite of the presence of the 27 Kd component.

In cathepsin H treated myofibrils, the main change observed was the apperance of a 27 Kd breakdown product.

Discussion - conclusion

The results of this study show that the 55 Kd proteinase, cathepsin D and cathepsin B degrade both rabbit and bovine muscles myofibrils under post mortem pH condition (5.8) causing a breakdown of MHC and of other lower molecular weight proteins. It also appears that whereas cathepsin D hydrolyses mainly the high Mw myofibrillar proteins, cathepsin B degraded preferentially the lower Mw prteins. The 55 Kd proteinase digests almost all the proteins whatever their Mw. By contrast, cathepsin H hardly affects myofibrilalr proteins, but it is possible that the experimental conditions used here are not well adapted. Therefore, further studies are needed. However, these results conforts the findings of (20) which show that, as opposed to cathepsin B, cathepsin H was unable to degrade actin suggesting that proteins are not good substrate for this last proteinase.

In the high Mw region, cathepsins D, B and the 55 Kd proteinase digested MHC from both bovine and rabbit muscle myofibrils. Regarding, the proteolytic fragments Mw obtained within animal species, these proteinases have quite similar specificities. However, differences between species could be noted. These differences might arise either from the variation in the myosin isoenzymes content of these muscles or from the animal species. MHC proteolytic components appearing in either bovine RA or LD treated myofibrils are identical in spite of their different myosin isoenzymes content and metabolic type. So, the species is likely to be the main factor of variation in the specificities of these enzymes against these two myofibrillar substrates as demonstrated earlier for CaANP[10]. In comparison with cathepsin D and as reported by (18), cathepsin B has a lower effect on MHC.

During post mortem storage of meat, the appearance of similar high Mw proteolytic components have been reported mainly in high temperature conditioned bovine[15, 30-31] and rabbit muscles[32-33]. In meat stored at low temperature, these components were scarcely seen (fig. 3.1 and 3.2d,e). As shown by (15), the concentration of these components increases with the temperature of storage and consequently with the rate of pH fall suggesting that this proteolysis is essentially due to the action of the lysosomal proteinases. Nevertheless, CaANP was able to generate some of these breakdown products[8]. Furthermore, the appearance of a 95 Kd component either in conditioned beef or in CaANP treated bovine muscle myofibrils has been reported by (31). In post mortem beef muscle, a component of similar Mw (95 Kd) has been identified to the myosin subfragement 1 (S1)[15] suggesting that some of these high Mw hydrolytic fragments might arise from MHC.

Whereas α-actinin is unaffected in cathepsins D, B and H treated myofibrils, this proteins seems to be hydrolyzed by the 55 Kd proteinase (fig. 3.1 and 3.2c). For a long time, the disappearance of α-actinin in post mortem muscle has been very questionable[34]. Recently, a quantitative decrase in α-actinin has been reported in beef after prolonged incubations at 37°C but nearly not at 4°C. In these conditions, the degradation of this protein is likely due to the action of the lysosomal system since, it has been shown that CaANP caused a release of α-actinin from the Z-line without any hydrolysis[34].

In the lower Mw region (below actin), the 55 Kd proteinase and cathepsin B degrade most of the proteins and more specially TN-T and TN-I which are known to disappear in post mortem muscle[6, 10, 17]. The degradation of TN-T has been commonly attributed to the action of CaANP. These results show that this protein can be hydrolyzed by at least two acidic proteinases in both rabbit and bovine muscle myofibrils. In contrast, cathepsin D affects only slighly these low Mw proteins whereas cathepsin H has nearly no action.

In rabbit and bovine muscle myofibrils treated with any of the lysosomal enzymes studied the appearance of a 27 Kd component was noted although only transiently in myofibrils incubated with cathepsin B, D and the 55 Kd proteinases. In rabbit myofibrils, cathepsin B and the 55 Kd proteinase also generated a 26 Kd hydrolytic product similar to that observed in conditioned meat (fig. 3.1 and 3.2d). As previously reported[10], CaANP released the 27 Kd component alone in rabbit myofibrils whereas a 30 Kd and a 27 Kd products are observed in both bovine RA and LD CaANP treated myofibrils. It appears therefore that the two proteolytic systems have quite similar effects at least on rabbit myofibrils.

In this range of Mw the appearance of two predominant hydrolytic components with Mw of 27 and 26 Kd has been shown to occur in post mortem rabbit muscle whereas in bovine LD muscle the main proteolytic fragments have a Mw of 30 and 27 Kd respectively[10] (fig. 3.1 and 3.2e). In bovine RA muscle stored for ten days at + 4/+ 5°C, the presence of a 27 Kd component was observed whereas the 30 Kd was nearly totally absent. Thus, these results support our earlier findings[9] and show that if we want to explain all the changes occurring in the myofibrillar structure during meat conditioning a cooperative action of the two proteolytic systems will have to be considered.

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