The action of Cathepsin L and Ca activated neutral proteases on myofibrillar proteins

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The increase in tenderness in beef which has been conditioned by post-mortem storage has been known for a long time but the underlying mechanism of the changes which produce the increase in tenderness is still far from clear. Earlier results indicated that there was an increase in the fragility of the fibres resulting from a weakening of the Z-line of myofibrils during conditioning (1,2). Examination of myofibrils from conditioned beef by SDS polyacrylamide electrophoresis revealed that a polypeptide of molecular weight of 30,000 was produced (3) and that troponin T was degraded (3,4), the loss of the latter being shown to parallel the decrease in toughness (5). More recently it has been found that the cytoskeletal protein, desmin, which surrounds myofibrils at the Z-line and links adjacent myofibrils (6) was degraded during conditioning (7). It has also been suggested that the protein titin (mol. wt ~1 x 10^6) (8), which has been postulated to form filaments linking thick filaments in adjacent sarcomeres through the Z-line, and another high molecular weight protein, nebulin (mol. wt ~5 x 10^5) (9), which is a major component of the transverse M_2-line of myofibrils, could be broken down during conditioning (10). The loss of these proteins, which form major structural elements within the myofibril, could make a considerable contribution to the increased tenderness of conditioned meat.

It is generally agreed that proteolytic enzymes are responsible for the loss of these proteins during conditioning (11,12) but it is not known which enzymes are involved. Since the discovery of a Ca-activated neutral protease (CANP), which degrades Z-lines in myofibrils (13), this enzyme has been considered to be an important agent in conditioning (11,12). There are two forms of the enzyme, one optimally activated by 1.5 mM Ca^{2+} (mM CANP) and the other optimally activated by 100 uM Ca^{2+} (uM CANP) (14,15). It is expected that the concentration of free Ca^{2+} ions in post-mortem muscle would rise sufficiently to activate the latter (16). The optimum pH for these enzymes is pH 7.0 to pH 7.5 (13) and although they are still active at the ultimate pH of meat, pH 5.5 to pH 5.8, their activity is considerably reduced. CANP has been shown to remove the Z-lines of myofibrils by causing the release of α-actinin (17). The enzyme also degrades troponin T, troponin I (3,11,12) and desmin (18).

The catheptic enzymes of the lysosomes may also take part in conditioning. Cathepsins B and D can degrade myosin, actin and troponin (19). These enzymes have optimum activity between pH 3.5 and 4.5 (19) and, therefore, as with CANP, their activity would be greatly diminished in meat. Cathepsin L (20,21) however, has an optimum pH of pH 5.5 to pH 6.0 and therefore could be active in meat. This enzyme has been shown to degrade myosin, actin, actinin and troponin (20).

In this paper we have re-assessed the possible role of CANPs and cathepsin L in conditioning. Myofibrils from 1-dorsi muscle of a beef carcass have been digested with mM CANP, uM CANP and cathepsin L and the changes analysed by SDS polyacrylamide gel electrophoresis. The results have been compared with those obtained from the myofibrils from conditioned beef and from homogenates incubated with and without Ca^{2+} and with an endogenous inhibitor to CANP.

Methods

Preparation of mM CANP and uM CANP The muscles from a rabbit were removed immediately after death and homogenised in 2 1/2 volumes of 0.025 M NaCl, 10 mM Tris acetate, 4 mM EDTA, 2 mM mercaptoethanol, 0.5 mM PMSF pH 7.0. The extract was centrifuged at 20,000 g for 30 min. The supernatant was adjusted to pH 7.5 by addition of 1 M Tris and recentrifuged. The clarified extract was applied to a column of DEAE-Sephacel and after washing with 0.025 M NaCl the uM CANP and mM CANP were recovered separately in a gradient of .025 M NaCl to 0.5 M NaCl. All these solutions and others subsequently used in the purification were made in 10 mM Tris acetate, 0.2 mM EDTA, 2 mM mercaptoethanol, pH 7.5. Most of the fractions containing uM CANP also contained an inhibitor to CANP. The uM CANP and mM CANP were separately applied to columns of Phenyl Sepharose in 0.5 M NaCl and eluted in 1% ethylene glycol. The inhibitor in the uM CANP fractions was not absorbed in 0.5 M NaCl on this column. The fractions containing enzyme activity were concentrated and passed through a column of Sephacryl S-200 in 0.5 M NaCl and the enzymes were finally applied to columns of Phenyl Sepharose and eluted in a gradient from 0.2 M NaCl to 1% ethylene glycol.

Measurement of CANP activity The enzyme was added to 1.5 ml of 0.5 % casein in 0.1 M Tris-acetate, 15 mM mercaptoethanol, 5 mM Ca^{2+} pH 7.0 and incubated for 15 min at 30°C. 1.5 ml of 5% TCA were added and the solution filtered. The absorption of the filtrate was measured at 280 nm. A unit of CANP activity was defined as that amount which gave an increase in absorption of 0.001 in 1 min.

Preparation of inhibitor to CANP The fractions which were not absorbed in 0.5 M NaCl on Phenyl Sepharose in the preparation of uM CANP were heated to 70°C and held at that temperature for 15 min. The resulting precipitate was removed by centrifuging and the inhibitor recovered by precipitation in 30% to 75% saturated $(NH_4)_2SO_4$. The inhibitor was further purified by elution on Sephacryl S-200. The fractions containing inhibitor were pooled and concentrated. 20 ul of inhibitor solution completely inhibited 20 units of CANP activity.

Preparation of Cathepsin L Cathepsin L was prepared from rabbit livers by the method of Mason et al (21). 1 unit of enzyme digests 1 u mol of Z-Phe-Arg-NMec/min at 37°C.

Conditioning of beef Slices of 1. dorsi muscle from a beef carcass were removed 24 h after slaughter. The slices were dipped in a solution of 5 mM NaN_3 and blotted. They were packed in polythene pouches and stored at either 10°C for 7 days or 30°C for 24 h.

Homogenates of beef Chopped pieces of beef were coarsely homogenised at half-speed in a Sorval Omnimixer for 20 secs with an equal volume of 0.1 m NaCl containing 10 mM NaN_3. 10g portions of the homogenate were weighed out and adjustments made as follows.

(a) Two samples were adjusted to pH 7.0 by the addition of 1 M Tris. To one $CaCl_2$ solution was added to 2.5 mM. To the other 500 ul of CANP inhibitor was added (this was calculated to be more than enough to inhibit the amount of CANP likely to be present in the sample) together with $CaCl_2$ to 2.5 mM. These samples were incubated at 20°C for 24 h.

(b) Three samples were held at pH 5.7. One of these incubated at 20°C for 24 h, another was incubated at 30°C for 24 h and to the third, 500 ul of inhibitor solution and $CaCl_2$ to 2.5 mM were added and incubated at 30°C for 24 h. Myofibrils were prepared from these samples.

Preparation of myofibrils Myofibrils were prepared by homogenising in 80 ml of 0.1 M NaCl, 10 mM Tris-HCl, 4 mM EDTA pH 7.0 at full speed for 1 min, 5g of beef or 10g of homogenates and filtered through "stockinette" to remove connective tissue. The homogenates were centrifuged at 3,000 g for 10 min. The myofibrils were resuspended in 0.1 M NaCl, 10 mM Tris-HCl pH 7.0 and centrifuged. This was repeated 5 times.

Preparation of KI residues Myofibrils (300 mg) were extracted 3 times for at least 2 h with 0.6 M KI, 10 mM Tris HCl pH 7.0. The precipitates were recovered by centrifuging at 30,000 g for 15 min. The precipitates were washed twice with 0.1 M NaCl. In these residues the desmin which is insoluble in KI is more evident than in myofibrils after SDS electrophoresis.

Preparation of Desmin Desmin was purified from chicken gizzard muscle by the method of Small & Sobieszek (22).

SDS Polyacrylamide gel electrophoresis (PAGE) SDS PAGE was carried out on myofibrils or KI residues by the method of Laemmli (23) on gels of 11%, 7% and 5% acrylamide on an LKB 2001 electrophoresis unit. 40 to 50 ug of protein were applied to the sample wells. 3% tube gels were carried out by the method of Wang et al. (8).

Digestion of myofibrils 100 ul of myofibrils (7 mg/ml) were incubated with 5 units of mMCANP in 2.5 mM Ca^{2+} or with 5 units uM CANP in 0.25 Mm Ca^{2+} in 0.1 MNaCl, 10 mM Tris-HCl, 2 mM mercaptoethanol pH 7.0 for 30 min at 30°C or with cathepsin L in 0.1 M NaCl, 30 mM Na glycerophosphate-HCl, 20 mM Cysteine pH 6.0 for 30 min at 30°C.

Results

Effect of proteolysis on myofibrils When myofibrils were treated with mM or uM CANP, SDS PAGE on 11% gels (Fig. 1a) shows that troponin T and troponin I were digested . Two polypeptides appeared, one with a molecular weight of 30,000 and the other 32,000. The myofibrils digested with 0.0005 units of Cathepsin L were also altered. Troponin T was not degraded but there was a strong band of a component of molecular weight of 30,000 although not at 32,000. This 30,000 band must be derived from a source other than troponin T - perhaps from actin the intensity of which was less than in the control myofibrils. However, with 0.002 units cathepsin L there was extensive degradation of most myofibrillar proteins. Myosin, α-actinin, actin troponins T and I and tropomyosin were all digested. The least affectd proteins were the myosin light chains and troponin C. Numerous bands of components resulting from the digestion of these proteins appeared including the 30,000 but not the 32,000 component.

Changes due to conditioning and treatment of homogenates

Conditioning of meat at 10°C and 20°C caused the loss of troponin T and the production of two polypeptides of molecular weight 30,000 and 32,000, the latter giving the more intense band (Fig 1a). Myofibrils which had been prepared from homogenates which had been incubated with 2.5 mM Ca^{2+} at pH 7.0 at 20°C for 24 h, gave results shown in Fig 1b very similar to the conditioned samples. The myofibrils from the homogenate incubated in the presence of CANP inhibitor showed a strong troponin T band and a slight production of the 32,000 polypeptide. The myofibrils from the homogenate incubated at 20°C at pH 5.7 had a 32,000 polypeptide. When the homogenates were incubated at 30°C, the troponin T and I were digested even when CANP inhibitor was present. In both cases the 32,000 polypeptide and 3 faint bands in the region of the 30,000 components were present.

All these samples were also run on 5% acrylamide gels in order to examine the proteins of molecular weight higher than α-actinin. In Fig. 2a when myofibrils were treated with 0.002 units of cathepsin L the loss of myosin was apparent with a multiplicity of bands appearing below the myosin band. However, in none of the other samples shown in Figs 2a, 2b, was there any evidence that myosin had been degraded. There were two proteins which were digested by CANP but were unaffected by conditioning, one below myosin and one with a molecular weight of ~85,000. There was also a protein of a molecular weight of 95,000 which was present in the lanes of the control samples, cathepsin L (0.0005 units), conditioned at 30°C and homogenates held at 30°C but was absent from the others.

The effect of treatment on titin and nebulin

Of great interest were the protein bands of very high molecular weights. The principal band is believed to be nebulin. Fig 2a shows that CANP caused some breakdown of this protein in myofibrils and cathepsin L (0.002 units) destroyed it completely. This protein appeared to be partly degraded in homogenates treated with Ca^{2+} and also with CANP inhibitor and in homogenates incubated at 30°C (Fig. 2b) but it is not clear in the gel in Fig. 2a whether there was any change in the conditioned samples.

The 3 1/2% acrylamide tube gels in Fig. 3 show more clearly the changes in titin. The untreated control shows mainly the titin 1 band. When myofibrils were treated with mM CANP there was a greater proportion of titin 2 band, which is considered to be a product of proteolysis of titin 1 (8). Cathepsin L (0.002 units) destroyed titin completely. The titin was much less in the myofibrils from the two conditioned samples and mainly in the form of titin 2. However, in the myofibrils from homogenates stored at 20°C the titin was present in all three as the titin 1 band and there was no change even when Ca^{2+} was added.

There was a difference in the intensities of the possible nebulin bands in Fig 3 compared to those in Figs 2a and 2b. The reason for this is not known but it may arise from the different systems of electrophoresis. For the gels shown in Figs 2a and 2b a discontinuous buffer system was used and it is possible that a number of proteins could be concentrated at this position giving sharp dense bands. A continuous buffer system was used for the gels shown in Fig. 3 which may have caused the proteins to spread out and hence give more diffuse and weaker bands. Clearly more work is required to resolve this problem.

The effect of treatment on desmin The changes in desmin were difficult to assess. When KI residues, prepared from unconditioned meat, were incubated with mM CANP, uM CANP or cathepsin L (0.002 units) it was apparent that the desmin had been degraded by CANP but not by cathepsin L as shown in Fig 4a.

Of interest also was the effect of CANP on the residual actin and -actinin in the KI residues. Both these proteins were degraded by mM CANP and uM CANP. Presumably the structures of these have been altered by the treatment with KI in such a way that they have become accessable to the enzymes. The results from the KI residues of conditioned beef were more equivocal (Fig. 4b). It would appear that there was less desmin in the samples which had been conditioned at either 10°C or 30°C and also in the sample from the homogenate which had been incubated with Ca^{2+}. The KI residue from the homogenate incubated with CANP inhibitor contained more desmin. It was not possible to obtain KI residues from the homogenates incubated at 30°C because the actomyosin had denatured and was insoluble in KI.

Discussion

The results from these experiments indicate that when beef was conditioned at 30°C for 24 h or 10°C for 7 days, during which the meat would become tender, there was a loss of troponin T, the production of two polypeptides with molecular weights 30,000 and 32,000, a loss of some desmin and a breakdown of titin and possibly nebulin. There was no evidence of the breakdown of myosin even at 30°C but it has been widely reported that myosin is degraded during storage at temperatures of 30°C to 40°C usually after a period longer than 24 h (3,24,25,26).

The degradation of troponin T, and desmin and the production of the 30,000 and 32,000 molecular weight components were reproduced by the action of mM CANP and uM CANP on myofibrils or in a homogenate containing Ca^{2+} but not when a CANP inhibitor was added. Cathepsin L did not reproduce these changes. Therefore, these changes are most likely to be caused by the action of CANP during conditioning.

CANP had a slight effect on titin in myofibrils, but in homogenates in the presence of Ca^{2+} there was no evident change. Therefore it is far less likely that the degradation of titin during conditioning was caused by CANP. Titin was far more susceptible to the action of cathepsin L.

CANP and cathepsin L degraded nebulin, the latter enzyme being far more effective but the breakdown of nebulin during conditioning was not clear from the gels and more work needs to be done.

Therefore it can be concluded from these results that a role during conditioning can be ascribed to both CANP and cathepsin L. The CANP is responsible for the breakdown of desmin which would result in a loss of adhesion between myofibrils and also for a weakening of the Z-line (3,11,12,13) thus causing a loosening of the general structure within the fibre. The cathepsin L on the other hand would degrade the larger molecules titin and nebulin - and myosin at high temperature - which would considerably weaken the integral structure of the fibre.

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Fig 1a

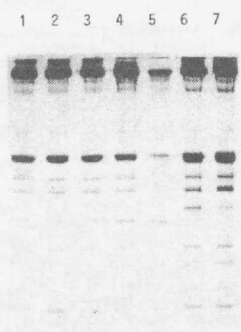

Fig. 1b

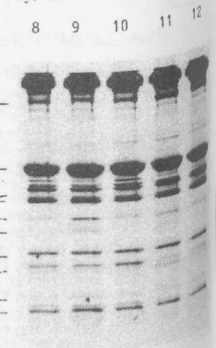

Fig. 2a

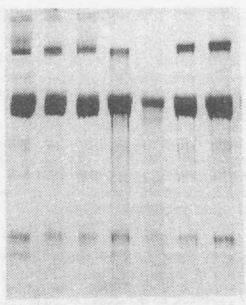

Fig. 2b

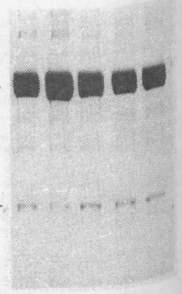

Fig. 1a, 1b, 2a, 2b

SDS PAGE of myofibrils. (1a, 1b) in 11% acrylamide; (2a, 2b) in 5% acrylamide. Fig. 1a, 2a: Lanes (1) control, (2) with mM CANP, (3) with μM CANP, (4) with 0.0005 units cathepsin L, (5) with 0.002 units cathepsin L, (6) from meat conditioned 7 days at 10°C, (7) from meat conditioned 24 h at 30°C. Fig 1b, 2b: myofibrils from meat homogenates incubated 24h at 20°C, (8) at pH 5.7, (9) at pH 7.0 with 2.5 mM Ca^{2+}, (10) at pH 7.0 with CANP inhibitor: myofibrils from meat homogenates incubated 24 h at pH 5.7 at 30°C, (11) no addition, (12) with CANP inhibitor.

Fig. 3

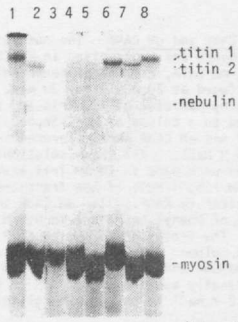

Fig. 3

SDS PAGE of myofibrils in 3½% acrylamide. Lanes (1) control, (2) with mM CANP, (3) with cathepsin L, (4) from meat conditioned 7 days at 10°C, (5) from meat conditioned 24 h at 30°C; from meat homogenates incubated for 24 h at 20°C, (6) at pH 5.7, (7) at pH 7.0 with 2.5 mM Ca^{2+}, (8) at pH 7.0 with CANP inhibitor.

Fig 4a

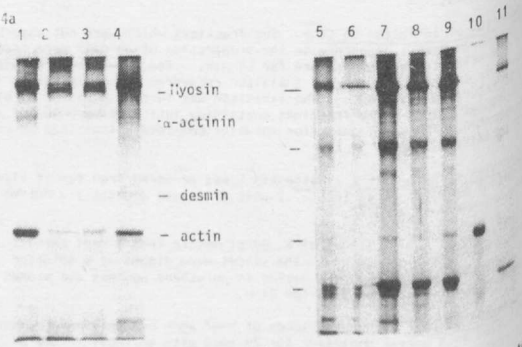

Fig. 4a, 4b

SDS PAGE of KI residues in 7% acrylamide. Fig. 4a: KI residues treated with, lane (1) control, (2) mM CANP, (3) μM CANP, (4) 0.002 units cathepsin L. Fig. 4b: KI residues from (5) meat conditioned for 7 days at 10°C, (6) meat conditioned for 24 h at 30°C: from homogenates incubated for 24 h at 20°C, (7) at pH 5.7, (8) at pH 7.0 with 2.5 mM Ca^{2+}, (9) at pH 7.0 with CANP inhibitor: (10) purified desmin, (11) control KI residue.