ACCELERATION OF PROTEOLYSIS IN BEEF BY ADDITION OF CALCIUM IONS

A. ALARCON-ROJO AND E. DRANSFIELD
Institute of Food Research, Langford, Bristol, BS18 7DY, U.K.

SUMMARY: The scanning trace from SDS electrophoresis of myofibrillar proteins from the inhibitor-treated meat was subtracted from the one of the calcium-treated meat. The most marked changes were the decrease of 175, 145, 98, 44, and 41 kDa proteins which showed 58.4, 45.8, 65.1, 23.2, and 90% reduction in peak area; these proteins would correspond to myosin, protein C, α-actinin, actin, and troponin T respectively. Other important changes were the appearances of new un-identified peaks of 166, 160, 56, 37, 32, and 29 kDa molecular weight proteins. These changes suggest that the calcium ions activated the calpains, most probably calpain II, and accelerated the proteolysis which occurs during ageing of meat.

INTRODUCTION: Chemical and microscopic studies have shown that, during ageing, a number of distinct changes occur within the myofibrillar structure, the most notable being the loss of Z disc integrity and the appearance of gaps at the same structural level leading to disruption of these structures. Cathepsins B and D and the calcium-activated neutral proteinases (Calpains) are the only proteolytic enzymes which degrade myofibrillar proteins and mimic the changes found in conditioned meat (Penny,1980).
Different chemical and physical techniques have been used to follow the structural changes in meat during ageing. The use of SDS-PAGE has become the usual method to study the structural changes in myofibrillar proteins associated with post-mortem autolysis, since it first demonstrated the disappearance of Troponin T (Olson *et al.*, 1977) in association with the appearance of the 30 kDa component. SDS-PAGE studies have also demonstrated that Calpains produce degradation in the myofibrillar component (Parrish, 1977; Slinde and Kryvi 1986), and that desmin and α-actinin are significantly hydrolysed in post-mortem muscle.
Furthermore, it is known that calcium salts produce tenderisation of meat but the mechanism, as well as the effect on the meat structure, have not been studied. Most of the published work on the degradation of meat proteins has been carried out by enzyme treatment of isolated myofibrils. The present work was carried out to study the effect of calcium ions on the protein profile of beef myofibrils prepared from calcium-treated beef and to compare it with the unaged meat.

MATERIALS AND METHODS: *Semitendinosus* muscles from 18 month old bovines were excised at 24 h post-mortem and cut into 30 mm thick slices across the fibre axis. Each slice was cut into 30x20x4mm strips along the fibre axis, and 100 g of strips were soaked in 250 ml of tris-maleate buffer with a final salt concentration of 100 mM and pH 5.5. To inhibit spoilage, 2 ml of 0.1M sodium azide were added per litre of soaking solution. The solutions also contained either 30 mM calcium chloride or 0.1 mM calpain peptide inhibitor (N-acetyl-leu-leu-norleucinal). At 18 μM inhibitor concentration calpain II is partially inhibited and at 40 μM calpain I is also inhibited. The strips were stored at 10°C for 1 day when samples were taken for myofibril preparation and SDS-PAGE analysis were carried out.

Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE): SDS-PAGE of the myofibrillar proteins in the presence of 0.2% SDS was performed using the system of Laemmli (1970) with some modifications. The stacking gel was 4% (w/v) acrylamide and the resolving gel 11% (w/v) acrylamide with a ratio of acrylamide to bisacrylamide of 30:1. The pellets of myofibrillar protein were mixed with equal volume of 0.1 M tris-glycine buffer, pH 8.8 containing 8 M urea, 2% SDS, 5 mM EDTA, and 5 mM DTT (sample buffer) and heated at 60°C for 30 min. These solutions were dialysed overnight against 0.025 M tris-HCl, 0.02% SDS and 2 mM mercaptoethanol, pH 7.4 (dialysing buffer) at 2°C. The dialysates were diluted in sample buffer to a final protein concentration of approximately 1 mg/ml and 20% (v/v) glycerol. 75 μg protein samples were loaded into each gel slot. After loading, 2200 ml of running buffer (0.124 M tris, 0.934 M glycine and 0.5% SDS pH 8.3) were poured into the electrophoresis chamber, and tap water was circulated to keep the gel temperature low during the experiment. The electrophoresis was run at a maximum of 300 V or 50 mA per gel in a Bio-Rad gel electrophoresis apparatus (Bio-Rad Lab. Ltd. U.K.) with a constant current power supply (Pharmacia fine chemicals) attached to it. The gel was run until the front reached 1 cm above the gel edge. The gels were stained overnight in 40% methanol, 10% acetic acid and 0.0125% Coomasie Brilliant Blue R. After clearing the excess stain with a wash of 40% methanol and 10% acetic acid for 2 hours, the gels were photographed and scanned subsequently in an Ultroscan XL Enhanced Laser Densitometer (LKB Produkter AB, Bromma, Sweden). The scanning data collected by the Laser Densitometer was analysed with the GelScan XL™ Software Package (LKB Produkter AB, Bromma, Sweden). Comparison of the data of two gel scans were made and an automatic scan-to-scan subtraction was performed on the calcium and inhibitor gels. The data resulting from the integration calculations was printed out in tabulated and plotted form. To simplify the comparison each scan was scaled and moved in both the 'X' and 'Y' axes as well as altered to eliminate known artefacts.

Molecular weight markers: One or two slots per gel were loaded with 20 µl SDS-protein markers from a pre-stained solution containing either a mixture of bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and a-lactalbumin, which molecular weights are 66, 45, 36, 29, 24, 20.1, and 14.2 kDa respectively; or a mixture of a_2-macroglobulin, b-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, lactic dehydrogenase and triosephosphate isomerase which molecular weights are 180, 116, 84, 58, 48.5, 36.5, and 26.6 kDa respectively. The relative mobilities of the markers were plotted against their molecular weight and the best fit drawn by hand and used for the estimation of the protein molecular weight.

RESULTS: The densitometric profiles of the calcium and the inhibitor treated samples at day 1 storage are illustrated in the Figure 1. To demonstrate the effect of calcium, the trace for inhibitor was subtracted from that of calcium and the results are shown in the lower part of Figure 1. This Figure indicates that some new proteins appeared after soaking in calcium for 1 day, as it can be observed in the lower curve (peaks at 166, 160, 56, 37, 32 and 29 kDa), whereas other myofibrillar protein decreased (myosin, protein C, α-actinin, actin and troponin T) with calcium treatment.

The peak areas given by the gel scanning densitometer were used to quantify the changes in protein content and they are presented in the Table 1. The major changes observed in the protein profile were the decreases in the proteins of myosin (175 kDa), protein C (145 kDa), α-actinin (98 kDa), actin (44 kDa), and troponin T (41 kDa). Peak at 175 kDa was decreased by 58.4% after calcium treatment, the other decreases were 45.8, 65.1, 23.2, and 90.0 % reduction in peak area respectively. The appearances of peaks of 166, 160, 56, 37, 32 and 29 kDa molecular weight proteins occurred only in calcium treatment.

The decrease of α-actinin and troponin T, and the appearance of new peaks of 37, 32, and 29 kDa molecular weight observed in the gel scans were also readily seen in the stained gels (not shown).

DISCUSSION: The changes found in the protein profile of the control as well as the calcium-treated meat, as analysed by SDS-PAGE, were obviously the result of post-mortem proteolysis of the myofibrillar components. In the present experiment, the control did not show major changes in its protein profile at 1 day storage, and was similar to that of the inhibitor-treated meat, thus the myofibrils of the meat soaked in inhibitor were used as a reference of unaged meat. Several major changes in the myofibrillar pattern were detected at subsequent times and the patterns were compared

by subtracting the inhibitor trace from the calcium-treated meat trace. These indicated that the myofibrillar proteins of 175 and 145 kDa, which would correspond to myosin and protein C respectively, were degraded and could have produced, after 1 day soaking in calcium, the new proteins of 166, 160, and 56 kDa as a result of proteolysis. Changes in low molecular weight proteins were also detected. The 98 kDa protein which would be expected to be a-actinin, showed 65.1% degradation, whereas the 44 kDa protein, most probably actin, showed 23% proteolysis. The most marked decrease was found in the 41 kDa protein which could be attributed to proteolysis of troponin T, since that protein is known to be greatly degraded during ageing. The new 37 kDa peptide could be a previously unidentified product of troponin T degradation, or it may be the same as the 30 kDa component observed by many others (MacBride and Parrish, 1977). The other two new products (29 and 32 kDa) might be the degradation products of any myofibrillar protein of higher molecular weight.

The results of this experiment are in line with the findings of others. The appearance of new bands of 27 and 30 kDa was observed by Troy *et al.* (1986) after storing bovine *M. Longissimus dorsi* at 37°C for 24 h, they also reported that these bands appear at earlier time post-mortem in electrical stimulated meat (Troy and Tarrant, 1987).

Although there is no direct evidence of the effects of calpain II on the muscle structure, the findings of the present study suggest that the proteolytic effect of calpains at high calcium levels, most probably of calpain II, produced the changes observed in the myofibrils since they were not present in the control or inhibitor-treated meat. Calcium effects on myofibrils have been observed by Slinde and Kryvi (1986) who found disintegration of myofibrils by purified calpains in presence of 10 mM calcium chloride, and their results may suggest that calpain II could be the enzyme activated under the conditions of that experiment.

Changes in the large proteins of the muscle have previously been reported. Locker and Wild (1984) found disappearance of nebulin (500-600 kDa) after 2 days storage of ovine *Longissimus dorsi* at 15°C, whereas new bands of high molecular weight (114, 76, 85 kDa) were also observed in beef by Troy *et al.* (1986) after 6 h storage at 37°C. Although it has been reported that myosin proteolysis does not take place during ageing of meat at chilling temperatures (Koohmaraie, 1988), some reduction in myosin content by calcium was observed in the present experiment. The explanation could be an enhancement of calpain II activity, which would not be active in normal ageing, or the higher temperature of storage (10°C) which had enhanced myosin proteolysis. Similar results were found by Bechtel and Parrish (1983) who observed degradation of myosin when storing ground beef for 7 days at 37°C. They also found that 10 mM calcium chloride produced more intense degradation of myosin than the control at the same storage time. Degradation of myosin has also been attributed to proteolysis by cathepsin

D which has been found to be very effective in hydrolising that protein (Okitani *et al.*, 1981), but this does not account for the degradation of proteins, including myosin, in the presence of calcium ions and inhibition of the degradation in the presence of calpain inhibitor.

In conclusion, calcium chloride accelerated myofibrillar degradation and it produced, in 1 day at 10°C, changes which are comparable to those of fully aged meat.

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Table 1. Changes in protein profile due to calcium addition

SDS gels of bovine *Semitendinosus* myofibrils from meat treated with 0.1 mM calpain peptide inhibitor or 30 mM calcium chloride for 1 day at 10°C were scanned and subtracted. The molecular weights were estimated from the protein relative mobility. The change (calcium-inhibitor treatment) of each protein is expressed as the percentage of the total peak area from the gel scanning trace.

Relative Mobility (R_f)	Estimated Molecular Weight	Protein		Change (%)
0.09	175	Myosin	-	58.4
0.10	166	New	+	∞
0.11	160	New	+	∞
0.13	145	Protein C	-	45.8
0.21	98	α-actinin	-	65.1
0.35	56	New	+	∞
0.54	44	Actin	-	23.2
0.58	41	Troponin T	-	90.0
0.62	37	New	+	∞
0.70	32	New	+	∞
0.74	29	New	+	∞

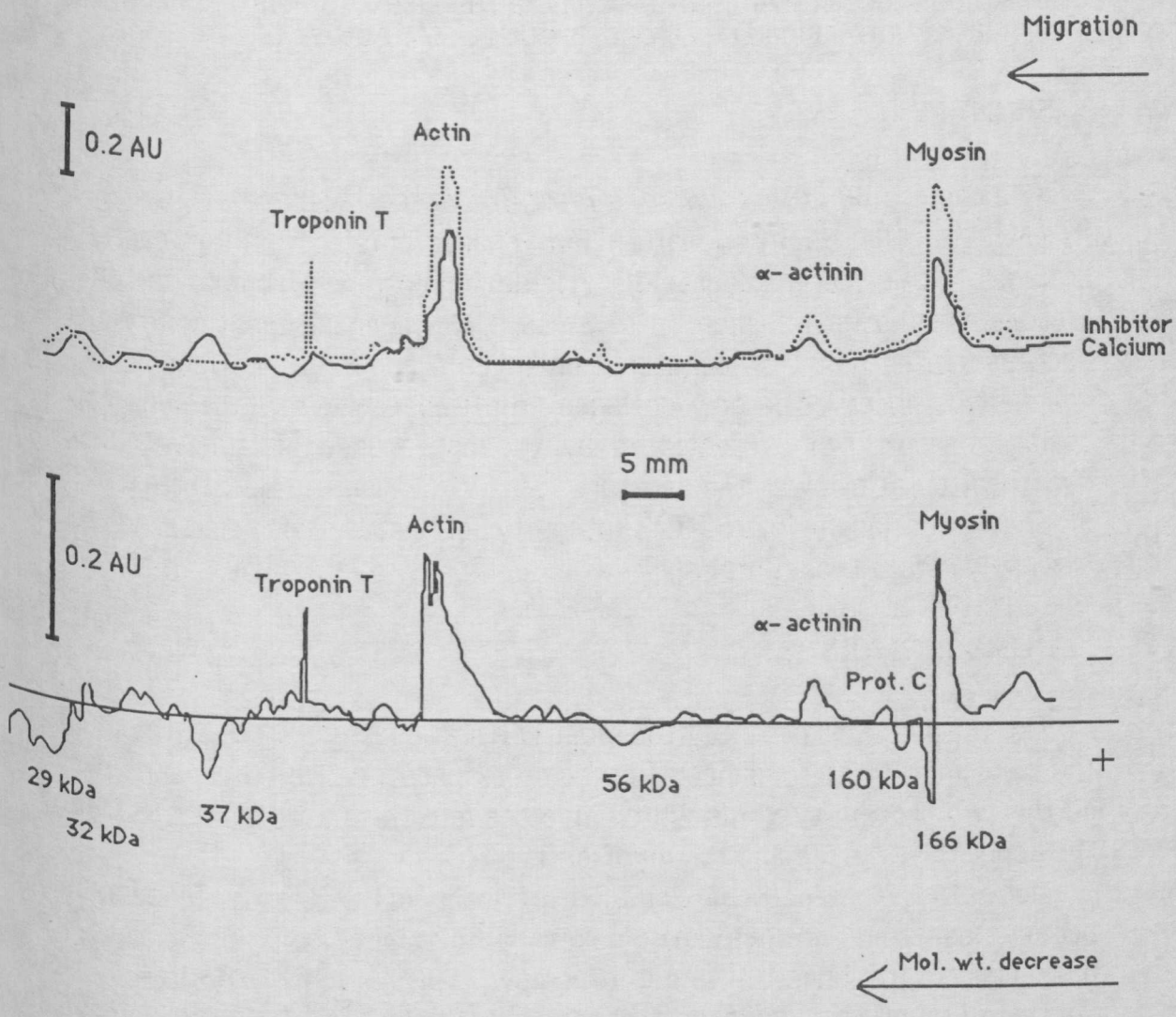

Figure 1 Densitometric scan of the SDS gel

SDS gels of bovine *Semitendinosus* myofibrils from meat treated with 0.1 mM calpain inhibitor or 30 mM calcium chloride were scanned and subtracted. The result after the subtraction is presented in the lower part of the graph. The horizontal line (lower curve) indicates the 'y' zero position, the peaks above that line represent decreases or disappearances (-) and the ones below indicate appearances (+) of new protein bands. X axis refers to gel migration distance in mm and Y axis represents Absorbance Units Full Scale (A.U.F.S.) which range is selected between 0-4 A.U.F.S. (these units are arbitrary and the selected scale is equivalent to an analogue signal of 0 to 1 volt in the UltraScan XL).