The effect of recombinant caspase 3 on myofibrillar proteins in porcine skeletal muscle

C. M. Kemp and T. Parr†

Division of Nutritional Sciences, School of Biosciences, Sutton Bonington Campus, The University of Nottingham, Leicestershire, LE12 5RD, UK

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The objective of this study was to investigate the potential role of the caspase protease family in meat tenderisation by examining if caspase 3 was capable of causing myofibril protein degradation. Full-length human recombinant caspase 3 (rC3) was expressed in Escherichia coli and purified. The rC3 was active in the presence of myofibrils isolated from porcine longissimus dorsi muscle (LD) and retained activity in a buffer system closely mimicking post mortem conditions. The effect of increasing concentrations of rC3, incubation temperature, as well as incubation time on the degradation of isolated myofibril proteins were all investigated in this study. Myofibril protein degradation was determined by SDS-PAGE and Western blotting. There was a visible increase in myofibril degradation with a decrease in proteins identified as desmin and troponin I and the detection of protein degradation products at approximately 32, 28 and 18 kDa with increasing concentrations of rC3. These degradation products were analysed using MALDI-TOF mass spectrometry and identified to occur from the proteolysis of actin, troponin T and myosin light chain, respectively. The production of these degradation products was not inhibited by 5 mM EDTA or semi-purified calpastatin but was inhibited by the caspase-specific inhibitor Ac-DEVD-CHO. The temperature at which isolated myofibrils were incubated with rC3 was also found to affect degradation, with increasing incubation temperatures causing increased desmin degradation and cleavage of pro-caspase 3 into its active isoform. Incubation of isolated myofibrils at 4°C for 5 days with rC3 resulted in the visible degradation of a number of myofibril proteins including desmin and troponin I. This study has shown that rC3 is capable of causing myofibril degradation, hydrolysing myofibril proteins under conditions that are similar to those found in muscle in the post mortem conditioning period.

Keywords: caspase, myofibrils, proteolysis, tenderization

Introduction

Meat tenderisation results from the weakening of the myofibrillar structures and associated proteins and has been attributed to endogenous proteolytic enzymes. Research into proteases involved in post mortem proteolysis has predominantly focused on calpains and cathepsins, with the calpain system being considered to be a major contributor to meat tenderisation (Sentandreuet al., 2002). However, it has been suggested that meat tenderisation is a multi-enzymatic system and other proteases such as proteasome and caspases could also contribute (Ouali et al., 2006). The cysteine protease family of caspases is primarily associated with apoptosis, functioning in both cell disassembly (effector caspases, such as 3, 6 and 7) and in initiating this disassembly in response to pro-apoptotic signals (initiator caspases, such as 8, 9 and 12) (Thornberry and Lazebnik, 1998). Once activated, caspases target and specifically cleave a number of substrates including cytoskeletal proteins such as vimentin, desmin and spectrin that have an important structural role (Wang, 2000).

In all the meat animals, the process of exsanguination deprives all cells and tissues of nutrients and oxygen. It has therefore been suggested that muscle cells have no alternative but to engage towards cell death (Herrera-Mendez et al., 2006). Caspases are expressed in varying proportions in different skeletal muscles (Kemp et al., 2006a). Their activity changes during the post mortem conditioning period and preliminary data indicate that there is a relationship between caspase activity and meat tenderness as determined by shear force (Kemp et al., 2006b). The objective of this study was to further investigate the potential role of caspases in meat tenderisation by determining if recombinant caspase 3 could degrade myofibrillar proteins.

† E-mail: tim.parr@nottingham.ac.uk
Materials and methods

Myofibril isolation and preparation

A single sample of porcine longissimus dorsi (LD) muscle was dissected from the carcass within 15 min of slaughter and myofibrils were subsequently prepared according to the procedure of Goll et al. (1974). Myofibrils were stored in 50% glycerol and 100 mM NaCl at −20°C. Prior to incubation reactions, myofibrils were prepared by washing three times in a mixed salt solution (MSS: 12 mM NaCl, 12.6 mM MgSO₄ · 7H₂O, 70 mM KH₂PO₄, 3.4 mM NaOH, 64.2 mM KOH, 11.1 mM H₂SO₄, 132 mM lactic acid, 100 mM 2-[(N-Morpholino)ethanesulfonic acid (MES); the pH was adjusted to 5.8 with 1 M MES, 4°C). After the third wash, myofibrils were resuspended in MSS with 1.3 mM CaCO₃. This buffer was designed to simulate the post mortem conditions in the muscle in experiment (Winger and Pope, 1981). The protein concentration of the myofibrils was determined using the Bradford assay (Bio-Rad, Hemel Hempstead, UK).

Purification of recombinant caspase 3

A pET-23 recombinant protein expression vector containing a cDNA insert encoding full-length human caspase 3 was a kind gift from Henning R. Stennicke (Nova Nordisk, Bargøerd, Denmark). This vector contains a poly-histidine tag located at the C-terminus of caspase 3 cDNA insert, thereby allowing the expressed protein to be tagged. Recombinant caspase 3 (rC3) was expressed in Escherichia coli BL21 (DE3) cells, with expression induced by the addition of 0.2 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The type of recombinant caspase isoform produced is dependent on the incubation conditions. Incubation for 45 min at 30°C with 0.2 mM IPTG will yield the pro-caspase 3 isoform, whilst incubating for 3 h will yield the cleaved active isoform. This enables both types of caspase 3 to be expressed and eliminates any pre-activation step that is required if the active isoform is needed (Stennicke and Salvesen, 1999). Following induction at 30°C for 3 h, rC3 was purified using the ÄKTA Explorer liquid chromatography system (GE Healthcare Life Sciences, Little Chalfont, UK) fitted with a 1 ml HiTrap HP affinity column (GE Healthcare Life Sciences) according to the procedure by Stennicke and Salvesen (1999). The HiTrap HP affinity column purifies the histidine tagged rC3 using immobilized metal ion affinity chromatography. In brief, the column was equilibrated with 5 column volumes of Buffer A (100 mM NaCl, 100 mM Tris, pH 8.0) before the cell lysate was applied to the column at a flow rate of 28 ml/h. Non-specifically bound material was washed off with 50 column volumes of Buffer B (500 mM NaCl, 20 mM imidazole, 100 mM Tris, pH 8.0). rC3 was eluted off the column with a 20 column volume linear imidazole gradient (0 to 200 mM) in Buffer A and 1 ml fractions collected. An aliquot of 100 µl of each fraction was taken and an equal volume of 2 × SDS buffer (125 mM Tris·HCl pH 6.8, 4% (w/v) SDS, 10% glycerol, 10% beta-mercaptoethanol, 0.01% (w/v) bromophenol blue) was added. Fractions were analysed by SDS-PAGE and the gel was Coomassie stained (0.2% Coomassie Blue, 0.2% amido black, 10% isopropanol, 10% acetic acid) for 15 min and then destained with changes of 10% acetic acid until bands could be visualised. Fractions containing rC3 were pooled and stored in 100 µl aliquots at −80°C (Stennicke and Salvesen, 1999).

Purified rC3 activity was determined using Apo-One Homogeneous Caspase 3/7 assay (Promega, Southampton, UK) and a 1:1 volume ratio of caspase reagent to purified rC3 was used for the assay. Fluorescence was measured at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm on a Fluostar galaxy spectrometer (BMG Labtechnologies, Aylesbury, UK) every 5 min for 5 h to determine the rate of the reaction. A range of standards containing 0 to 100 µM of rhodamine 110 reference standard (Invitrogen, Paisley, UK) was used to quantify the fluorescence generated (as recommended by Promega). Protein concentration of purified rC3 was determined using a Bradford assay (Bio-Rad). One unit of rC3 was defined as the amount of enzyme that cleaved 2.94 mM rhodamine 110 substrate/min per µg purified protein.

Effect of increasing concentrations of rC3

Aliquots of 5 mg of isolated myofibrils were incubated with 1, 2, 5, 10 and 20 units of rC3. All myofibril incubation reactions were carried out in a total volume of 500 µl, adjusted to by the addition of MSS buffer. Controls were set up containing no rC3 and 10 units of rC3 with Ac-DEVD-CHO (Sigma, Poole, UK), the caspase 3-specific inhibitor to a final concentration of 0.1 µg/µl. To ensure that any proteolysis observed could not be attributed to endogenous calpain activity, 5 mM ethylenediaminetetraacetic acid (EDTA) was added to specific control reactions with and without 10 units of rC3. In addition 50 µl semi-purified porcine skeletal muscle calpastatin (using the heated calpastatin extraction protocol, as described by Kent et al., 2005) was also added to a myofibril aliquot containing 10 units of rC3. Activity of the isolated calpastatin was 7.87 × 10⁻⁷ units of fluorescence/kg tissue as defined by Sensky et al. (1996). Reactions were incubated with a total of 700 units of semi-purified calpastatin. All reactions were incubated at 37°C for 24 h. After incubation, myofibrils were centrifuged at 6000 × g for 3 min at 4°C. The supernatant was removed and the pellet resuspended in 100 µl MSS with 1.3 mM CaCO₃. Aliquots of supernatant and resuspended pellet samples were taken for determination of protein concentration using the Bradford assay (Bio-Rad). Samples were prepared for SDS-PAGE by the addition of an equal volume of 2 × SDS buffer.

Effect of incubation temperature

Aliquots of 5 mg myofibrils with either 1 unit or 10 units of rC3 were incubated at 25, 15 and 4°C for 24 h, to mimic the change in carcass temperature during the post mortem conditioning period. After incubation, samples were prepared for SDS-PAGE as described above.

Recombinant caspase 3 and myofibrillar protein degradation

Effect of incubation temperature

Aliquots of 5 mg myofibrils with either 1 unit or 10 units of rC3 were incubated at 25, 15 and 4°C for 24 h, to mimic the change in carcass temperature during the post mortem conditioning period. After incubation, samples were prepared for SDS-PAGE as described above.
Effect of incubation time

Aliquots of 5 mg myofibrils with either 1 unit or 10 units of rC3 were incubated at 4°C for 0, 1, 2 and 5 days. Control incubations of 5 mg of myofibrils containing no rC3 were incubated at 4°C for 0, 1, 2 and 5 days. After incubation, samples were prepared as before.

SDS-PAGE and immunoblotting

The incubated myofibrillar protein samples in 2× SDS buffer were prepared for SDS-PAGE analysis by boiling for 5 min to denature the proteins before centrifuging at 15 000 g for 3 min. Either 20 μg of resuspended pellet protein or 30 μg supernatant was loaded onto 12.5% SDS-PAGE gels. The gels were electrophoresed on a vertical dual-plate unit (Fisher, Loughborough, UK) at a constant of 200 V for 1 h in 1× SDS buffer (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS. The separated proteins were either transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences) by Western blotting (Towbin et al., 1979) using a Trans-blot cell with plate electrodes (Bio-Rad) containing Western blot buffer (0.4 M glycine, 0.025 M Tris, 5% (v/v) isopropanol) at a constant current of 350 mA for 2 h or Coomassie stained. Western blots were immunoprobed with either anti-human caspase 3 polyclonal antibody diluted 1:2000 (Merck Biosciences, Nottingham, UK) or anti-chicken desmin polyclonal antibody diluted 1:500 (Sigma Aldrich, Poole, UK). Protein bands were detected using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences). The chemiluminescence was captured on ECL Hyperfilm (GE Healthcare Life Sciences) and the resulting bands were quantified using a Fluor 5 Max multi-imager (Bio-Rad) enabling densitometry to be performed on the digital image. On the Coomassie SDS-PAGE gels the intensity of the identified bands in each lane were expressed relative to the alpha-actinin band intensity.

Protein identification

Specific myofibrillar proteins were identified based on the description by Porzio and Pearson (1977) of a SDS-PAGE separation of myofibril proteins and also Precision Plus Protein Standard (Bio-Rad) was used to identify proteins’ molecular weights. A number of protein bands that appeared to result from degradation were excised from the Coomassie-stained gels using a scalpel blade and sent for analysis to the Biopolymer Synthesis And Analysis Unit at the Queens Medical Centre, University of Nottingham, UK. Protein bands were analysed using MALDI-TOF (Matrix-Assisted Laser Desorption Ionisation-time of flight) mass spectrometry (Waters Corporation, MA, USA) and identification by peptide mass mapping was performed using the database search program MASCOT PMF (Matrix Science Ltd, London, UK).

Data analysis

Statistical analysis between caspase 3/7 activities of the samples was performed using ANOVA with Genstat for Windows (version 7.2) (Hemel Hempstead, UK).

Results and discussion

Purification and determination of rC3 activity

The trace of the 280 nm absorbance measured on the ÄKTA Explorer liquid chromatography system showed that absorbance peaked between column volumes 35 and 40 (Figure 1a). These fractions were subsequently analysed by SDS-PAGE and Coomassie staining and both the inactive 32 kDa isoform and the active 20 and 11 kDa isoforms of rC3 were identified (Figure 1b). The absorbance values in Figure 1a were converted to fluorescence using the following equation: fluorescence = 550 × absorbance + 30000. This equation was determined by least-squares regression analysis and used for all the samples, including the control. The activity of rC3 was determined by using a synthetic substrate (N-Bz-Val-Asp-Val-Asp-Glu-AMC) in a fluorimeter. The concentration of the rC3 was determined using the absorbance peak value at 280 nm. The time course of the rC3 activity was determined using a synthetic substrate (N-Bz-Val-Asp-Val-Asp-Glu-AMC) in a fluorimeter.

Figure 1 (a) Absorbance (280 nm) of eluted fractions of bacterial lysate containing expressed rC3, purified using ÄKTA Explorer liquid chromatography system fitted with HisTrap HP affinity column and eluted with a linear gradient of 0 to 200 mM imidazole in Buffer A. Flow rate: 28 ml/h. Fraction volume: 1 ml. Pooled fractions are indicated (b) SDS-PAGE (12.5%) loaded with 10 μg of pooled elution fractions containing purified rC3. Molecular weights of inactive and active isoforms of rC3 are indicated. (c) rC3 proteolytic activity, used to determine the initial rate of reaction, the amount of fluorescence generated is directly proportional to the amount of cleavage from its pro-isofrom to its active isoform.
caspase 3 were detected and these fractions pooled together (Figure 1b). Coomassie staining confirmed that the pooled fractions were homogenous with no other proteins detected other than the inactive and active isoforms of rC3. Pro-caspase 3 is a 32-kDa peptide and during the process of activation, peptide fragments of 20 to 17 and 11 kDa are generated (Liu et al., 2005). Coomassie staining indicated that rC3 was predominantly present in the active isoforms. The predominance of the active isoform was expected as protein expression was induced for 3 h with IPTG to yield the active isoform of rC3 (as described by Stennicke and Salvesen, 1999). The specific rC3 activity of the pooled fractions was measured and the initial rate of reaction determined as 479 units of fluorescence generated/min (Figure 1c). The fluorescence generated was calibrated and determined as the amount of enzyme that cleaved 2.94 mM of rhodamine 110 substrate/min per μg purified protein.

To determine if there was any affect of the MSS buffer and the myofibrillar proteins on rC3 activity, a series of incubation reactions were performed: (1) 10 units of rC3 (2) 1 mg myofibril proteins (3) 10 units of rC3 + 1 mg myofibril proteins (4) 10 units of rC3 + 1 mg myofibril + Ac-DEVD-CHO (final concentration of 0.1 μg/μl) (5) 50 μl MSS buffer (6) 10 units of rC3 + 50 μl MSS buffer, an equal volume of Caspase 3/7 reagent was added to each reaction and the fluorescence measured (Table 1). Results indicated that there was no apparent endogenous caspase 3/7 activity in isolated myofibrils, rC3 was active in the presence of myofibrils and this activity could be inhibited using the caspase-specific inhibitor Ac-DEVD-CHO. Caspase 3/7 activity in reactions 1, 3 and 6 were found to be significantly higher than that in reactions 2, 4 and 5, which contained no rC3 or rC3 activity was inhibited (P < 0.001).

Furthermore the activity of rC3 was not affected by the MSS buffer indicating that there was caspase 3 activity in an incubation which was not at the optimal pH 7.4 for available rC3 (pH of MSS buffer was 5.8) (Table 1). This could therefore suggest that caspases can remain active over a wide range of pH, which would be important and necessary if caspases are to maintain their proteolytic activity in muscle post mortem, where muscle pH decreases from around 7 to 5.3–5.8 (Smulders et al., 1992).

**Effect of increasing concentration of rC3 on myofibril degradation**

Caspases are highly selective in the substrates that they target and cleave. Whilst most proteins contain sites that are susceptible to protease activity, because of the specificity of the position of the amino acid residues required; Asp in the P1 and the P4 position for caspases, the peptides generated by their actions are limited and provide 'fingerprints' of their activity (Wang, 2000). The specificity of the amino acid sequence motif required by caspases is a safety mechanism, enabling controlled caspase activation and cleavage and preventing any inappropriate cellular destruction (Fuentes-Prior and Salvesen, 2004). Differences in the patterns of myofibrillar proteins separated by SDS-PAGE were observed in myofibril samples incubated with rC3 for 24 h at 37°C in comparison to those incubated without rC3 (Figure 2a). These differences were presumably caused by rC3-mediated proteolysis of the myofibril proteins whilst being incubated at pH 5.8. In a study examining the effect of incubating μ-calpain with myofibril proteins and their identification of proteolysis by SDS-PAGE, the degradation of desmin and troponin I bands was described (Geesink and Koohmaraie, 1999). Using similar SDS-PAGE analysis of myofibril protein degradation products, the overall effect of increasing concentrations of rC3 was the decrease in band intensities at 55 kDa and 22 kDa and the appearance of degradation products at approximately 32, 28 and 18 kDa (Figure 2a). The 55 and the 22 kDa bands were initially identified as the cytoskeletal proteins desmin and troponin I, respectively, on the basis of their molecular weight.

### Table 1 Effect of MSS buffer, myofibrils and caspase inhibitor Ac-DEVD-CHO on recombinant caspase 3 activity determined using Apo-One Homogeneous Caspase 3/7 assay

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Incubation conditions</th>
<th>Fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 units rC3</td>
<td>35092.05 ± 1445.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1 mg myofibrils&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.3 ± 2.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>10 units rC3 + 1 mg myofibrils&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39925.67 ± 213.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>10 units rC3 + 1 mg myofibrils&lt;sup&gt;b&lt;/sup&gt; + Ac-DEVD-CHO</td>
<td>32.9 ± 2.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>MSS buffer</td>
<td>28 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>10 units rC3 + MSS buffer</td>
<td>36516.33 ± 291.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The amount of fluorescence (arbitrary units) generated is directly proportional to the quantity of substrate cleaved and therefore the caspase activity.

<sup>a</sup>Myofibrils were resuspended in mixed salt solution (MSS) buffer, pH 5.8.

Values are expressed as mean (n = 3) ± s.e.

Values with different superscript letters are significantly different.
weights and also upon comparison with SDS-PAGE reports by Whipple et al. (1990); Geesink and Koohmaraie (1999); Lametsch et al. (2004) and Hwang et al. (2005), who identified similar patterns. Similar to the approach of Lametsch et al. (2004) and Hwang et al. (2005) MALDI-TOF peptide mapping was subsequently carried out and this analysis confirmed these two bands to be desmin and troponin I. The degradation peptides at 32, 28 and 18 kDa were also identified by MALDI-TOF analysis and were found to result from alpha-actinin, troponin T and myosin light chain 3 proteolysis, respectively. Previously, Ho et al. (1994) also identified troponin T to be degraded during post mortem skeletal muscle studies in bovine LD. The relative changes in the bands’ intensity induced by incubation with rC3 are shown in Table 2. In order to normalise for the protein load on SDS-PAGE within each lane, the values for band intensity are expressed relative to the alpha-actinin band intensity, this assumes that alpha-actinin is not degraded by caspase-mediated proteolysis (Table 2). Alpha-actinin is generally resistant to proteolysis, particularly to those proteases involved in post mortem tenderisation (Taylor et al., 1995). Overall increasing the quantity of rC3 present enhanced the degradation of bands identified by MALDI-TOF analysis as desmin and troponin I and lead to the appearance of degradation products at 32, 28 and 18 kDa, corresponding to result from alpha-actinin, troponin T and myosin light chain 3 proteolysis. The changes in these specific bands were not detected in myofibrils incubated without rC3 or with the caspase 3-specific inhibitor Ac-DEVD-CHO (Figure 2a, lanes 3 and 5). These observations corresponded to the caspase 3/7 activity reactions which showed there was no endogenous caspase activity and rC3 activity could be inhibited by the caspase-specific inhibitor Ac-DEVD-CHO (Table 1).

The degradation of myofibrillar proteins was also observed in myofibril samples incubated with rC3 and either 5 mM EDTA or crude calpastatin extract (Figure 2a lanes 2 and 4, respectively). The endogenous calpain inhibitor calpastatin (50 μl of semi-purified crude extract) was included in the myofibril incubations to try to ensure any degradation found could not be attributed to the actions of any residual calpain in the samples. Calpastatin is also a known caspase substrate and has been reported to be degraded to 75 and 30 kDa fragments by both caspases 3 and 7 (Wang et al., 1998). Caspase activity and the degradation of calpastatin in early apoptosis are thought to compromise plasma membrane and cytoskeletal integrity, resulting in increase of intracellular Ca²⁺ levels and calpain activation (Wang, 2000). The interaction between calpain and caspase protease system is multifaceted; Neumar et al. (2003) reported that human neuroblastoma cells over-expressing calpastatin not only inhibited calpain activity but also up-regulated caspase activity and accelerated apoptosis. The exact mechanism behind this up-regulation remains unclear. In the current study, it appears that co-incubation of calpastatin and myofibrils with rC3 induced an increase in band intensities of the 32, 28 and 18 kDa cleavage products (Figure 2a, lane 4). Similarly, in myofibril samples co-incubated with rC3 and 5 mM EDTA band intensities of the degradation products also appeared to be more intense, in particular the 32- and 28-kDa peptides (Figure 2a lane 2) in comparison to myofibrils incubated without EDTA. Assessment of band intensities using densitometry showed a fourfold increase in the band intensity of the 28-kDa peptide and a twofold increase in the band intensity of the 32-kDa peptide in myofibrils incubated with either

### Table 2: Protein levels of bands identified by SDS-PAGE in myofibrils subjected to incubation with different levels of rC3 at 37°C for 24h

<table>
<thead>
<tr>
<th>rC3 units</th>
<th>Desmin 32 kDa</th>
<th>28 kDa</th>
<th>Troponin I 18 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>62.1</td>
<td>15.9</td>
</tr>
<tr>
<td>1</td>
<td>101.9</td>
<td>60</td>
<td>13.8</td>
</tr>
<tr>
<td>2</td>
<td>91.2</td>
<td>67.8</td>
<td>30.6</td>
</tr>
<tr>
<td>5</td>
<td>86.1</td>
<td>127.2</td>
<td>50.2</td>
</tr>
<tr>
<td>10</td>
<td>67.5</td>
<td>109.9</td>
<td>88.7</td>
</tr>
<tr>
<td>20</td>
<td>43.3</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Figures are expressed as percentage of densitometry units of bands from Coomassie-stained SDS-PAGE relative to 0 units for Desmin, and Troponin I and 20 units for 32, 28 and 18 kDa degradation products.
Recombinant caspase 3 and myofibrillar protein degradation

calpastatin or EDTA and rC3 in comparison to those incubated with just rC3. In a study by McGinnis et al. (1999), incubation of human neuroblastoma cells with the Ca\(^{2+}\) chelator EGTA exhibited increased caspase activity and apoptosis, whilst calpain activity was inhibited. It is therefore plausible to speculate that EDTA may have a similar effect in myofibers, increasing the ability of caspase 3 to mediate protein degradation. Inhibiting the calpains by including EDTA and calpastatin in the myofibril incubation reactions suggests that the 32-, 28- and 18-kDa peptides are not products of calpain-mediated proteolysis. Although both the studies by McGinnis et al. (1999) and Neumar et al. (2003) were performed in human neuroblastoma cells, in which apoptotic events are well characterised, it is becoming widely accepted that caspase-mediated apoptosis occurs under a number of conditions in skeletal muscle (for review see Tews, 2005). The apparent increased proteolysis observed in the myofibrils incubated with calpain inhibitors accentuates the complexity of post mortem proteolysis and suggests that meat tenderness could be multi-enzymatic and further investigation is therefore needed to understand the mechanisms behind it.

Western blots probed with the anti-caspase 3 antibody detected immunopositive bands at 32 and 20 kDa that could correspond to the inactive and active isoform (Figure 2b). The 11 kDa active isoform is not recognised by this antibody (Merck Biosciences). Caspase 3 was detected in all the myofibril samples incubated with rC3, as expected the higher the concentration of rC3 in the incubation samples, the greater the protein levels of caspase 3 detected by Western blotting. More importantly, the inclusion of EDTA, calpastatin and Ac-DEVD-CHO inhibitor did not appear to have any effect on caspase 3 protein levels. Purified rC3 (10 units) was used as a positive control.

Effect of temperature on myofibril degradation

During the post mortem conditioning period the carcass temperature decreases from 37°C to 4°C (Savell et al., 2005). Incubation reactions with commercially available rC3 are recommended to occur at 37°C pH 7.4, as this is presumably when enzyme activity is at an optimum. It was therefore of interest to determine if rC3 would be active both at pH 5.8 and lower temperatures (analogous to those in a cooling carcass) and whether the degradation patterns observed in the increasing concentration of rC3 experiment also occurred at lower temperatures. Myofibrils incubated with 10 units of rC3 at 4°C, 15°C and 25°C (Figure 3a, lanes 6 to 8) showed visibly high levels of protein bands produced as a result of protein degradation in comparison the samples incubated with 1 unit of rC3 (Figure 3a, lanes 3 to 5). With changes in the desmin band and the appearance of troponin T and myosin light chain 3 degradation products at approximately 28 and 18 kDa detected, when compared relative to the alpha-actinin band (Table 3).

Caspases are expressed as proenzymes and are activated by the removal of the prodomain (Earnshaw et al., 1999). The isoform of the recombinant caspase is dependent on the length of incubation with IPTG. In this experiment, expression of rC3 was induced for 3 h to yield the active caspase 3 isoform. However, SDS-PAGE and Western blot analysis showed that the inactive isoform of caspase 3 was also present, although to a much lesser extent (Figure 1b). Western blots were probed for caspase 3 to determine whether temperature had any effect on the activation of rC3 as indicated by changes in the caspase 3 immunopositive bands at 32 and 20 kDa. In the myofibril samples incubated with 10 units of rC3, protein levels of the inactive 32 kDa isoform were found to decrease as temperature increased, suggesting that there was more activation from its pro-isoform to its active isoform at higher temperatures (Figure 3b, lanes 7 to 9). This pattern was also observed in the myofibrils incubated with lower concentrations of rC3 but was only visible when the quantity of total protein loaded onto the gel was increased from 20 to 50 µg (Figure 3b, lanes 1 to 3). Western blots loaded with 20 µg of myofibril samples incubated with 1 unit of rC3, showed protein levels of the active 20 kDa isoform of caspase 3 were lower at 4°C than at 25°C (Figure 3b, lanes 4 to 9). This may indicate that there was less cleavage of caspase 3 from its pro-form to its active form as the temperature decreased, although this was not apparent in myofibrils with 10 units of rC3 (Table 2). This was not noticeable in Western blots loaded with 50 µg of myofibril sample as the increased protein quantity required for detection of the inactive 32 kDa caspase 3 isoform in the samples resulted in saturation of the signal from the band representing the active 20 kDa isoform (Table 4). In a similar study by Koohmaraei et al. (1986) µ-calpain activity at 4°C was found to be 24% to 28% of that at 25°C and was also capable of causing myofibrillar degradation and does not need to be at its optimum activity level to cause proteolysis.

An increase in desmin degradation was observed in myofibrils incubated with increasing concentrations of rC3 and also increasing temperatures, as detected by Coomassie staining. The intermediate filament protein desmin is part of the cytoskeletal structure of skeletal muscle, cross-linking myofilaments at the Z-disc as well as linking myofibrils to the sarcolemma via costameres and is susceptible to post mortem proteolysis (Taylor et al., 1995). Western blot analysis was therefore performed to determine if desmin was cleaved by rC3 under the experimental incubation conditions. The myofibril supernatant was used for desmin analysis due the detection of non-specific products and high background levels found with the pellet samples. Western blot analysis detected a band at 55 kDa that corresponds to desmin. The rate of desmin degradation varies between muscles and also between the same muscle in different animals, with faster degradation occurring in tender muscle in comparison to muscle classified as tough (Hopkins and Thompson, 2002). Western blot analysis showed that there was an increased disappearance of the desmin band with increasing temperatures for myofibril samples incubated with either 1 unit or 10 units of rC3. The isolated myofibrils incubated at 25°C had approximately twice as much...
desmin degradation in comparison to those incubated at 4°C (Figure 3c, Table 4). Although few differences were observed by Coomassie staining on overall myofibril degradation, Western blot analysis showed that temperature did have an effect on both the cleavage of pro-caspase 3 into its active isoform and also on the extent of desmin degradation, with increased caspase activation and desmin proteolysis detected in myofibrils incubated at higher temperatures. However, an important observation was that whilst rC3 was apparently more active at higher temperatures it was still active and capable of causing myofibrillar degradation at 4°C.

Effect of incubation time on myofibril protein degradation

The recommended conditioning period for pork is between 5 and 10 days (Warriss, 2000). Myofibrils were incubated with either 1 unit or 10 units of rC3 at 4°C, pH 5.8 for a period of up to 5 days to try and replicate the in situ post mortem muscle conditions in vitro. Reactions were terminated after 1, 2 and 5 days, with one sample stopped as soon as the incubation was set up (0 days). Myofibril incubation reactions with time were also set up without rC3 to ensure any degradation that was observed could not be attributed to incubation alone. Incubation of myofibrils with rC3 over 5 days at 4°C resulted in visible differences in the bands detected by SDS-PAGE. In the myofibrils incubated

Table 3  Protein levels of bands identified by SDS-PAGE of myofibrils subjected to incubation with 10 units of rC3 at different incubation temperatures for 24 h

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Desmin 28 kDa</th>
<th>18 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>98.3</td>
<td>57.3</td>
</tr>
<tr>
<td>15</td>
<td>88.9</td>
<td>85.3</td>
</tr>
<tr>
<td>25</td>
<td>85.8</td>
<td>100</td>
</tr>
</tbody>
</table>

Figures are expressed as percentage of densitometry units obtained from bands on Coomassie-stained SDS-PAGE relative to the negative control for Desmin, and 25°C for 28 and 18 kDa degradation products.

Negative control is myofibril samples incubated without rC3 at 37°C.
with 1 unit of rC3, there was a clear increase in intensity, relative to alpha-actinin, in a band at approximately 18 kDa from 2 days, identified to occur from degradation of myosin light chain 3 (Figure 4a and Table 5), which were presumably generated by rC3-mediated proteolysis. In comparison, in the myofibrils incubated with 10 units of rC3 the
degradation patterns were much more pronounced and there were distinct changes in a number of bands relative to alpha-actinin (Figure 4 and Table 5). This is not unexpected, if rC3 is responsible for the proteolysis of myofibrillar proteins observed more degradation would be detected in the reactions containing increased concentrations of rC3, providing the substrate did not become limiting. The 18 and 28 kDa degradation products were detectable from the start of the incubation, suggesting that the action of high concentrations of caspases on myofibril degradation is relatively rapid (Figure 4 and Table 5). Caspase activation is known to occur quickly with the period from apoptotic stimuli to caspase activation happening in approximately 10 min and once committed cell death is inevitable. Although initiation of activity may be rapid, the time between the initial apoptotic trigger to complete cellular destruction can take hours or even days (Green, 2005). In these studies, myofibrils were incubated with rC3 predominantly in its active isoform and therefore its effects on myofibrillar proteins can occur immediately as it does not require activation. Apoptosis is translationally regulated process permitting immediate and selective changes in protein levels and therefore no new transcription synthesis is required for complete cellular destruction (Holčík and Sonenberg, 2005). Consequently, it can be assumed that the proteolysis observed in vitro is also happening in vivo. The band identified as troponin-I, by its molecular weight and MALDI-TOF peptide mapping, appeared almost completely degraded after 2 days of incubation (Figure 4a) and when compared, relative to alpha-actinin, it was not detectable by densitometry after 1 day when myofibrils were incubated with 10 units of rC3 (Figure 4 and Table 5). The caspase-mediated myofibril degradation patterns observed with time are similar to those shown by Geesink and Koohmaraie (1999) in bovine myofibrils incubated with μ-calpain, who found the appearance of protein degradation products in the 28 to 32 kDa region after 1 day of incubation and the degradation of troponin-I after 2 days. A major qualitative difference between our observations and those of Geesink and Koohmaraie (1999) was the presence of a peptide at approximately 18 kDa, which was identified to arise from myosin light chain 3 degradation. However, the degradation of myosin light chains has been has been related to pig meat quality (Lametch et al., 2004; Hwang et al., 2005). The detection of a myosin light chain degradation product in myofibrils incubated with caspasases and not in reactions incubated with calpains is not unsurprising as myosin light chain has previously been identified as a caspase substrate (Fischer et al., 2003). In the myofibril samples incubated with 10 units of rC3, there appeared to be an increase in the 28 kDa troponin T degradation product and the 18 kDa myosin light chain degradation peptide with time (Figure 4a lanes 9 to 12), suggesting increased rC3-mediated proteolysis. In comparison, there was no apparent degradation in the myofibril samples incubated for 5 days at 4°C with no rC3 added (Figure 4a), indicating that rC3 was responsible for the degradation observed and that the process of incubation alone had no visible detrimental affects on the myofibrils.

The effect of incubation time on caspase 3 is shown in Figure 4b. There was little difference in protein levels of the active 20 kDa caspase 3 isoform detected by Western blotting across the incubation period for myofibrils incubated with either high or low concentrations of rC3. However, in the myofibrils incubated with high concentrations of rC3, protein levels of the inactive 32 kDa isoform of caspase 3 were found to decrease across the incubation period. This would suggest that the inactive 32 kDa isoform of caspase 3 is being cleaved and activated into the proteolytically active isoform with time (Table 6). Caspase 3 protein levels were shown to be affected by incubation temperature (Figure 3b and Table 4), with more inactive isoform present in myofibrils incubated at lower temperatures (Figure 3b, lanes 7 to 8). However, increasing the incubation temperature appears to compensate for the decreased activity at 4°C allowing more caspase cleavage from the inactive isoform and therefore presumably enabling more proteolysis to occur.

Desmin is involved in linking Z-disk to Z-disk in the myofibrillar lattice. The degradation of desmin is thought to significantly contribute to tenderisation (Hopkins and Taylor, 2004; Geesink et al., 2006). Research by Koohmaraie et al. (1995) found that in lambs carrying the callipyge phenotype, which is associated with increased levels of

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**Table 5 Protein levels of bands identified by SDS-PAGE of myofibrils subjected to incubation with 1 or 10 units of rC3 over time at 4°C**

<table>
<thead>
<tr>
<th>Protein band : alpha-actinin absorbance ratio</th>
<th>Desmin 32 kDa</th>
<th>28 kDa</th>
<th>Troponin I</th>
<th>18 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (days)</td>
<td>1 unit 10 units</td>
<td>1 unit 10 units</td>
<td>1 unit 10 units</td>
<td>1 unit 10 units</td>
</tr>
<tr>
<td>0</td>
<td>100 100 ND</td>
<td>21.2 ND 29.3</td>
<td>100 100 0</td>
<td>31.1 59.8</td>
</tr>
<tr>
<td>1</td>
<td>112.0 65.5 ND</td>
<td>60.7 ND 56.6</td>
<td>124.7 6.0 83.5</td>
<td>59.8 62.8</td>
</tr>
<tr>
<td>2</td>
<td>96.1 69.7 ND</td>
<td>56.0 ND 83.5</td>
<td>99.0 0 62.8</td>
<td>69.0</td>
</tr>
<tr>
<td>5</td>
<td>88.0 149.3 ND</td>
<td>100 ND 100</td>
<td>77.0 0 100 100</td>
<td>100</td>
</tr>
</tbody>
</table>

ND = not detectable.

*Figures are expressed as percentage of densitometry units obtained from bands on Coomassie-stained SDS-PAGE relative to 0 days for Desmin, and Troponin I and 5 days for 32, 28 and 18 kDa.
Recombinant caspase 3 and myofibrillar protein degradation

Table 6 Protein levels of recombinant caspase 3 and desmin in myofibrils incubated with 1 or 10 units of rC3 over time at 4°C

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Caspase 3 32 kDa isoform</th>
<th>Caspase 3 20 kDa isoform</th>
<th>Desmin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 unit 10 units</td>
<td>1 unit 10 units</td>
<td>Negative 1 unit 10 units</td>
</tr>
<tr>
<td>0</td>
<td>ND 100</td>
<td>100 100</td>
<td>100 100 100</td>
</tr>
<tr>
<td>1</td>
<td>ND 116.8</td>
<td>116.2 99.8</td>
<td>104.3 77.6 84.6</td>
</tr>
<tr>
<td>2</td>
<td>ND 86.5</td>
<td>89.8 117.6</td>
<td>102.8 70.4 53.7</td>
</tr>
<tr>
<td>5</td>
<td>ND 34.5</td>
<td>122.1 114.2</td>
<td>101.8 49.5 19.5</td>
</tr>
</tbody>
</table>

ND = not detectable.
*Figures are expressed as percentage of densitometry units obtained from Western blots relative to 0 days.
Negative = negative control myofibrils incubated without rC3 at 4°C.

calpastatin and increased meat toughness, there was a reduction in the degradation of a number of myofibrillar proteins including desmin in comparison to normal lambs, suggesting its importance with regard to meat tenderness. In this study, Western blot analysis for desmin showed that protein levels of desmin decreased in myofibrillar proteins incubated with rC3 across the time course (Figure 4c, Table 6). Approximately half the total desmin detected in the supernatant of the myofibrillar incubations was degraded by 2 and 5 days in myofibrils incubated with high and low levels of rC3, respectively (Table 6). Desmin degradation by calpain has been shown to occur within 1 day in ovine LD (Veiseth et al., 2004) and in bovine semimembranosus (Taylor et al., 1995) at pH 8.3 and 7.0, respectively, with both groups showing significant loss of desmin at the end of the incubation periods. Although desmin degradation was not as rapid in myofibrils incubated with rC3 in comparison to those studies with calpain, the rC3 incubations were performed at a lower pH than those of calpain, which may affect rC3 activity. To date, there is no data on the effect of pH on the stability and proteolytic activity of caspases; however, it has been speculated that as caspases are neutral peptidases their proteolytic activity of caspases; however, it has been speculated that as caspases are neutral peptidases their activity will be affected by muscle acidification in a similar way to calpains (Sentandreau et al., 2002).

Conclusions

This study has demonstrated evidence that human rC3 is capable of causing degradation of porcine myofibrillar proteins. Recombinant caspase 3 is still active and causes proteolysis of myofibrils in post mortem conditions of 4°C and pH 5.8. Upon incubating for up to 5 days, there was increased degradation of desmin and troponin I and also the appearance of a number of peptides that could result from proteolysis of myosin light chain 3, alpha-actin and troponin T, as detected by SDS-PAGE and MALDI-TOF analysis. Proteolysis of desmin by rC3 over time was also detected by Western blot analysis. The identification that the 28 kDa degradation product was from troponin T proteolysis as detected by Coomassie staining, is an important observation as troponin T is extensively hydrolysed in the post mortem conditioning period (Ho et al., 1994) and its degradation has been shown to positively correlate to tenderness (Huff-Lonergan et al., 1996). Additionally, the myofibrillar protein degradation patterns observed and the degradation of desmin detected by Western blot analysis are similar to those observed in myofibrils incubated with μ-calpain (Taylor et al., 1995; Geesink and Koohmaraie, 1999). These findings therefore strengthen the hypothesis that caspases could contribute to post mortem proteolysis and meat tenderisation is a multi-enzymatic process.

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References


