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The effect of temperature on the activity of μ- and m-calpain and calpastatin during post-mortem storage of porcine *longissimus* muscle

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ABSTRACT

The experiment was conducted to determine the effect of temperature during post-mortem muscle storage on the activity of the calpain system, the myofibril fragmentation and the free calcium concentration. Porcine *longissimus* muscle were incubated from 2 h post-mortem at temperatures of 2, 15, 25 and 30 °C and sampling times were at 2, 6, 24, 48 and 120 h post-mortem. After 120 h at 30 °C the free calcium concentration increased to 530 μM from 440 μM at 2 °C. Incubation at temperatures higher than 2 °C resulted in the appearance of autolyzed m-calpain activity and a decrease of native m-calpain activity. Native m-calpain decreased more slowly than native μ-calpain, and the autolysis process started later. Myofibril fragmentation increased with storage time and incubation temperature, while calpastatin activity decreased. The study showed that high temperature incubation not only rapidly activated μ-calpain but at higher temperatures and later time points also m-calpain.

1. Introduction

The calpain system has been shown to be an important contributor to proteolytic degradation of myofibrillar proteins during storage of meat (Hopkins & Thompson, 2002; Koohmaraie & Geesink, 2006). μ-Calpain and m-calpain are thought to be the primary enzymes involved in the proteolytic tenderization of meat, and their activity is regulated by the inhibitor calpastatin. Both enzymes are heterodimers composed of distinct 80 kDa catalytic subunits and a common 28 kDa regulatory subunit, are calcium dependent and autolyze in the presence of calcium (Cong, Goll, Peterson, & Kapprell, 1989). In addition to calcium concentration, other parameters like temperature and pH influence the activity of the calpain system (Dayton, Reville, Goll, & Stromer, 1976). m-Calpain activity was shown to be significantly faster at 25 °C compared to 0 °C, and the pH optimum was close to 7.5 (Dayton et al., 1976), whereas μ-calpain was reported to be more active at pH 6.5 than at 7.5 (Maddock, Huff-Lonergan, Rowe, & Lonergan, 2005).

In previous studies muscle has been exposed to high temperatures soon after slaughter and the effect on post-mortem proteolysis and on calpain activity has been reported (Geesink, Bekhit, & Bickerstaffe, 2000; Hwang, Park, Cho, & Lee, 2004; Thomson, Gardner, Simmons, & Thompson, 2008; White, O'Sullivan, Troy, & O'Neill, 2006). Western blot analysis of the large subunit of μ-calpain from muscle incubated at elevated temperatures for 24 h showed a faster μ-calpain activation at temperatures of 25 to 35 °C in lamb (Geesink et al., 2000) and at 36 °C in beef (Hwang et al., 2004). Incubation at temperatures above 25 °C during rigor also caused a decrease in the activity of calpastatin at days 1, 3 and 14 (Geesink et al., 2000). Hot boned pieces of beef longissimus incubated at a temperature of 25 °C for 8 h post-mortem showed faster proteolysis (White, O'Sullivan, Troy, et al., 2006). Thomson et al. (2008) also showed faster proteolysis up to day 3 post-mortem for beef that had entered rigor at 15 °C and thereafter incubated at 37 °C for up to 3 h. However, in concomitance with a faster proteolysis early post-mortem, less proteolysis during prolonged ageing was reported (Geesink et al., 2000; Thomson et al., 2008). The lower stability of calpain at higher temperature (Thompson, Goll, & Kleese, 1990) could be the reason of this reduced muscle degradation later post-mortem.

Pomponio et al. (2008) demonstrated that in porcine muscle not only μ-calpain, but later also m-calpain has potential to autolyze during post-mortem storage at refrigerated temperature, suggesting that the sarcoplasmic calcium level in porcine muscle under certain time–temperature combinations rises to the level required for activation of μ-calpain and later also m-calpain. The effect of temperature incubation on the free calcium level in porcine muscle is not known. In the study on beef by Hwang et al. (2004) an increase in extractable free calcium was measured during the onset of rigor following incubation at a temperature of 36 °C. Based on this we hypothesized that increasing temperature will activate first μ-calpain and later m-calpain in porcine muscle. To test this hypothesis, we incubated prerigor *longissimus* muscle at different temperatures (2, 15, 25 and 30 °C) for up to 5 days and followed the activity of μ- and m-calpain,

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calpastatin and myofibrillar fragmentation. Long incubation time at elevated temperatures was included in the study to obtain a more complete understanding of the activation of m-calpain.

2. Materials and methods

2.1. Sample descriptions

Longissimus muscles were obtained 1 h post-mortem from the right carcass side of 5 Danish pigs slaughtered at a local abattoir. The *longissimus* muscles were transported to the lab and the section from the 6th to the 13th rib was used in the experiment. At 2 h post-mortem temperature and pH were measured. pH was measured at the 6th rib with a PHM 201 pH meter (Radiometer, Copenhagen, Denmark) equipped with a Metrohm probe-type glass electrode WOC (Metrohm Ag, Herisau, Switzerland). The mean pH and temperatures values +/− SD at 2 h were 6.24 ± 0.29 and 34 °C ± 1.97. From each loin one 2 h reference sample was frozen in liquid nitrogen immediately and 16 slices of approximately 30 g each were sampled, vacuum packed and stored at four different temperatures: 2, 15, 25 and 30 °C. Samples were collected at different time points: 6, 24, 48 and 120 h post-mortem, frozen in liquid nitrogen and stored at −80 °C until enzyme extraction (2, 6, 24, 48 and 120 h), measurements of sarcomere length (48 h), free calcium (24 and 120 h) and myofibril fragmentation (24, 48 and 120 h).

2.2. Extraction procedure for calpain and calpastatin determination

From each sample, frozen at −80 °C, duplicates of 1.5 g of meat were homogenized at 13,500 rpm using an Ultra-Turrax T25 Mixer (Ika Labortechnik, Staufen, Germany) in 9 mL of an extraction buffer (100 mM Tris; 5 mM EDTA; 10 mM Monothioglycerol; pH 8.0) and centrifuged for 30 min at 4 °C with a speed of 15,000 × *g*. An aliquot of the supernatant was mixed with glycerol to a final concentration of 30% and was stored at −80 °C until analysed for calpain activity. This procedure of freezing samples both before and after extraction was done to be able to perform extraction and activity measurements in parallel for all samples. After addition of glycerol calpain retained activity upon freezing and thawing and we observed that the activities of the calpain system were stable during frozen storage of the meat at −80 °C (Kristensen, Christensen, & Ertbjerg, 2006). For calpastatin determination another aliquot (1 mL) of supernatant was heat treated in a water bath at 100 °C for 5 min and centrifuged for 5 min with a speed of 20,000 × *g*. The heat treated supernatant was stored at −20 °C until calpastatin activity measurements.

2.3. Casein zymography method

Calpain activity was determined using a casein zymography method as described by Pomponio et al. (2008) with some modification. Each duplicate of the samples was run in triplicate using 12.5% casein precast gels (Bio-Rad Laboratories, Hercules, CA). One volume of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT, pH 6.8) was mixed with 3 volumes of supernatant and 15 μL of the sample was loaded onto the gel. The distribution of samples on the gel was randomized. The electrophoresis was carried out at 80 V for 3 h at 4 °C (running buffer: 25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.3). The gels were incubated with shaking at room temperature in 100 mL incubation buffer (50 mM Tris, 4 mM $CaCl_2$, 10 mM monothioglycerol, pH 7.5) for 1 h, changing the buffer after 30 min. The calpain activity was stopped by washing the gel for 30 min with shaking, using 20 mM Tris, 10 mM EDTA, pH 7.0. The gels were stained overnight with colloidal Coomassie Brilliant Blue G and destained 5 h the next day with change of deionised H_2O every hour. Size of the clear area denoting calpain activity was measured with LabScan (Amersham Bioscience) (UMAX Power Look 1120) analyzing images with the Phoretix 1D version 2003.02; Nonlinear Dynamics Ltd. Quantification of calpain activity was accomplished by expressing the density of the bands from samples relative to the mean density of reference standards within each gel. This standard was obtained by mixing the supernatant of samples belonging to the five different time points (2, 6, 24, 48 and 120 h post-mortem) and to the four different temperatures (2, 15, 25 and 30 °C). Then glycerol was added to a final concentration of 30% and the standard stored at −80 °C until use. Fifteen microliters of four reference standards were run within each gel. Native and autolyzed forms of μ-calpain were quantified relative to the native band of μ-calpain in the reference standard while native and autolyzed forms of m-calpain were quantified relative to the native band of m-calpain.

2.4. Calpastatin activity determination

Calpastatin activity in the supernatant was measured using a casein assay containing 0.2 unit of m-calpain as described (Ertbjerg, Henckel, Karlsson, Larsen, & Moller, 1999). Various amounts (0, 20 and 40 μL) of the heated supernatant from the extraction procedure were incubated with m-calpain in 300 μL of an incubation medium (100 mM Tris, 10 mM monothioglycerol, 5 mg/mL casein and 5 mM $CaCl_2$, pH adjusted to 7.5 with 1 M acetic acid) with a total volume of 400 μL. After 40 min of incubation at 20 °C the reaction was stopped adding 400 μL of 10% trichloroacetic acid, and the solution was centrifuged for 3 min with a speed of 20,000 × *g*. From the decrease in the absorbance at 278 nm an inhibition curve was generated. Calpastatin activity was calculated using the linear part of this curve. One unit of calpastatin was defined as the amount that inhibited one unit of m-calpain activity. The m-calpain used in the above described casein assay was partially purified using pork *longissimus* 3 h post-mortem following the procedure described in Kristensen et al. (2002). Briefly, calpains were precipitated from a homogenate by ammonium sulfate. The pellet was dissolved and separated from calpastatin using a butyl sepharose column and finally μ-calpain and m-calpain were separated into two fractions using ion-exchange chromatography with a NaCl gradient.

2.5. Myofibril particle size

The myofibril fragmentation was measured by particle size analysis as described by (Lametsch, Knudsen, Ertbjerg, Oksbjerg, & Therkildsen, 2007). Briefly, samples of 2.5 g were homogenized in 30 mL cold buffer (100 mM KCl, 20 mM Potassium Phosphate, 1 mM EGTA, pH 7.00) at 20,500 rpm using an Ultra-Turrax T25 equipped with an S25N-18G dispersing element (Ika Labortechnik, Staufen, Germany). Samples were analysed in duplicates and sizes of myofibrils were measured using a Malvern Mastersizer Micro Plus (Malvern Instruments Ltd, Worcestershire, UK). The distribution in the size of the myofibrillar fragments was determined as surface mean diameter D (3,2). D (3,2) is an average particle size calculated from the size distribution as the diameter of a sphere that has the same volume/surface ratio as the measured particles ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$ where n_i is the number of particles with diameter d_i).

2.6. Free calcium concentration

Determination of free calcium concentrations was done by a modification of the procedure described by Hopkins and Thompson (2001). Muscle samples (20 g) held at −80 °C were removed from frozen storage and held at room temperature for 10 min after which they were finely diced, held on ice for 20 min and then centrifuged at 20,000 × *g* for 20 min. An aliquot of the supernatant (4 mL) was mixed with 80 μL of 4 M KCl. The calcium concentration was determined using a calcium selective electrode (ISE25Ca) with a reference calomel electrode, both from Radiometer, Copenhagen, Denmark and