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Small heat shock proteins and toughness in intermediate pH_u beef

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ABSTRACT

Bull *M. longissimus dorsi* (n = 94) categorised into high (n = 28), intermediate (n = 14) and low (n = 52) ultimate pH (pH_u) were aged at −1.5 °C for 28 days. Shear force was higher and more variable ($p < 0.05$) in intermediate pH_u samples during ageing. Titin, filamin and desmin degradation was also less extensive in intermediate pH_u samples compared to the other two pH categories. The extent of the decline of HSP20, HSP27 and αβ-crystallin concentrations during *post mortem* ageing was pH_u related such that high pH_u meat maintained the highest concentration of small heat shock proteins followed by intermediate and low pH_u meat. μ-Calpain autolysis was slowest in intermediate pH_u and cathepsin B activities remained consistently low during ageing in this group ($p < 0.05$). Meat toughness in the intermediate pH_u group may be attributed to the combination of a larger pool of sHSP with a sub-optimal cathepsin B activity and intermediary μ-calpain activities.

1. Introduction

There is a curvilinear relationship between meat ultimate pH (pH_u) and tenderness in beef and lamb (Bouton, Harris, & Shorthose, 1971; Devine, 1994; Jeremiah, Tong, & Gibson, 1991) and on average, meat with the lowest tenderness has intermediate pH_u — that is, meat with a pH_u between 5.8 and 6.19. A recent survey of New Zealand beef found that 18% of meat from bulls aged between 24 and 36 months old attained intermediate pH_u (Wiklund et al., 2009) accounting for much of the variation and suboptimal meat quality of bull beef.

Meat tenderisation is caused by the hydrolysis of cytoskeletal and myofibrillar proteins by proteases resulting in the structural degradation of muscle fibres. Three proteolytic systems have been recognised as being potentially involved in *post mortem* proteolysis leading to meat tenderness. These are lysosomal cathepsins (Ouali, 1992), calpains — particularly μ-calpain (Huff-Lonergan et al., 1996; Koohmaraie & Geesink, 2006) and the multicatalytic proteinase complex (Sentandreu, Coulis, & Ouali, 2002). There are several views as to which of these protease systems are variably involved in meat tenderisation, and the matter still remains contentious among meat scientists.

The marked biochemical changes taking place in muscle *post mortem* purportedly triggers apoptosis of muscle cells (Ouali et al., 2006). Apoptosis is a process of programmed cell death and is induced when a cell is damaged, infected or exposed to adverse environmental conditions. In this process, the metabolic insult triggers a cascade of biochemical reactions leading the cell to self destruct without damaging surrounding healthy cells. Ouali et al. (2006) suggested that after slaughter, muscle cells inevitably tend towards apoptosis due to the loss of nutrients and oxygen supply to the muscles. In response to impending cell death, small heat shock proteins (sHSPs) are synthesised to prevent unnecessary apoptosis and thus attempt to maintain cell homeostasis (Beere, 2004).

sHSPs belong to a large group of chaperone proteins that typically have a molecular weight ranging from 15 to 43 kDa (Arrigo, 2005). Studies have found that sHSPs have a protective role in cells, and their expression is particularly prominent where cells are exposed to harmful conditions such as hyperthermia (hence the name heat shock), hypoxia and harmful oxidants. They are also known to be involved in protein repair and maintain cell survival by keeping proteins from fatally aggregating in stressed cells (Soti, Sreedhar, & Csermely, 2003).

Small heat shock proteins are abundant in skeletal muscle (Kato et al., 1994) and are believed to be implicated in the apoptotic processes in *post mortem* muscle that consequently affect meat quality (Ouali et al., 2006). However, very little work has been conducted to investigate the contribution of sHSP with regard to meat quality, and their role in the conversion of muscle to meat is still an enigma to meat science.

In a study to determine the kinetics of sHSP early *post mortem* in bull beef, it was demonstrated that the concentration of total sHSP in the soluble phase of meat *post rigor* was determined by the muscle's pH_u (Pulford et al., 2008) due to the precipitation of sHSP from the soluble phase at pH_u values 6.2 and lower. In addition, the proteolytic activity of calpain and cathepsin enzyme systems were found to be pH-related with the reduction of μ-calpain activity concomitant with the rise of cathepsin B activity as pH_u decreased (Pulford et al., 2009). Authors from these studies hypothesised that the protective effect of total soluble sHSP and sub optimal proteolytic activities of calpains and cathepsins observed at intermediate pH_u levels may explain higher toughness observed in intermediate pH_u beef.

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This study sought to test the hypothesis that a combination of the protective function of sHSP and low enzyme activity in intermediate pH_u meat maintains the integrity of the muscle structure resulting in meat toughness or delayed ageing as observed in intermediate pH_u beef.

2. Materials and methods

2.1. Animals and muscle sample collection

Bulls (n = 94) slaughtered at a commercial abattoir were used in this study. Animals were head-only electrically stunned as is routine for the Halal slaughter of beef in New Zealand. Upon reaching the hot boning floor (<1 hour *post mortem*), the *M. longissimus dorsi* (LD) from the left side of each carcass was excised and 10 g muscle samples were cut from the anterior end of each muscle, frozen in liquid nitrogen then stored at −80 °C. The remaining LD was packaged in vacuum bags then stored at −1.5 °C for 24 h.

After 24 h, each LD was cut into six equally sized sub-samples and vacuum packed. The six sub samples from each LD were randomly allocated an ageing timepoint and were stored −1.5 °C for 1, 2, 7, 14 and 28 days *post mortem*. At each timepoint, 10 g was excised from the muscle sample, frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. The rest of the sub sample was used for pH and shear force measurements.

2.2. pH and shear force measurements

The pH of each loin at all ageing timepoints was measured with a Testo® 230 metre (Lenzkirch, Germany). The pH metre was calibrated at pH 7.0 and 4.0 with buffers (Mallinckrodt Chemicals, USA) stored at room temperature (20 °C).

Meat tenderness was determined by cooking the loins in weighted plastic bags in a water bath at 100 °C until the internal temperature of the loin reached 75 °C as measured by a thermocouple. The cooked loins were immediately chilled in an ice water bath and cooled to below 10 °C. The shear force from 10 mm × 10 mm cross sections (n = 10) was determined for each loin using a MIRINZ tenderometer. Shear force values for each loin were expressed as kgF.

2.3. Meat sample preparation and protein determination

Sarcoplasmic fractions were prepared from the frozen muscle samples for all timepoints as described by Pulford et al. (2008).

Whole muscle protein (WMP) fractions were prepared for all timepoints from frozen muscle samples. Muscle, 0.4 g, was powdered with a mortar and pestle then homogenised in WMP extraction buffer (10 mM sodium phosphate buffer, 0.2% SDS; pH 7.0). The homogenate was centrifuged at 1500 *g* for 15 min at 25 °C and the resulting supernatant was recovered.

Protein concentration of sarcoplasmic and WMP fractions at all timepoints were determined as described by Lowry, Rosenbrough, Farr, and Randall (1951) using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

2.4. Coomassie blue sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 0.5 g frozen muscles from all timepoints were crushed and homogenised in 5 mL of extraction buffer (50 mM Tris–HCl (pH 5.8), 10% glycerol, 2% SDS and 2% 2-mercaptoethanol) followed by centrifugation at 10,000 *g* for 5 min at 4 °C. An aliquot of the supernatant was collected for protein determination by RC-DC protein kit (BioRad).

For SDS-PAGE, an aliquot of the supernatant was mixed with an equivalent volume of reduced sample buffer (62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.02% bromophenol blue), then heated for 20 min at 50 °C. After centrifugation at 10,000 for 10 min, a total of 100 μg of protein from each sample was loaded onto wells of 5% Tris–HCl gels (BioRad Laboratories) and separated in a BioRad Criterion Cell system at room temperature at 10 mA for 17 h. Gels were subsequently stained with colloidal Coomassie blue (17% ammonium sulphate, 2% phosphoric acid, 30% methanol, 0.04% Coomassie G-250) and scanned with a GS700 calibrated densitometer scanner (BioRad Laboratories).

2.5. Immunoblots

Immunoblots for sHSP were conducted on sarcoplasmic fractions that were adjusted to a concentration of 4 mg mL^{-1} with sarcoplasmic extraction buffer. A total of 20 μg protein were loaded onto 12% Bis–Tris gels (BioRad Laboratories, 345-0118) then separated at 120 V at room temperature. For μ-calpain, desmin and myosin immunoblots, WMP fractions were adjusted to a concentration of 4 mg mL^{-1} with WMP extraction buffer and a total of 40 μg protein were loaded onto 7.5% Tris–HCl gels (Bio-Rad Laboratories, 345-0006) and resolved in a Bio-Rad Criterion Cell system at 120 V at room temperature.

Following SDS-PAGE, proteins were transferred onto Immobilon-P PVDF membranes (Millipore, IPVH00010) and blocked by incubating the membrane with 5% non-fat dry milk powder diluted with PBS-Tween (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween) overnight at 4 °C. Membranes were then washed three times with PBS-Tween then incubated with the chosen primary antibody for 1 h at room temperature. Primary (monoclonal) antibodies from mouse included αβ-crystallin (Abcam, ab74441) HSP20 (HyTest, 4HSP20) and HSP27 (HyTest, 4HSP27) and were diluted to 1:10,000 in PBS-Tween. Desmin (Sigma, D1033) and μ-calpain (Thermo Scientific, MA3-940) monoclonals were diluted to 1:5000 in PBS-Tween. After washing, membranes were subsequently incubated with goat anti-mouse IgG (H + L) HRP conjugate (BioRad, #172-1011) diluted to 1:5000 in PBS-Tween at room temperature for 1 h. The membranes were then washed as previously described and the bound antibody was detected using ECL Western blot substrate kit (Pierce, 32106). Western blot images were captured with a G:Box Chemi HR16 image capture instrument (Syngene, Cambridge, UK).

2.6. Cathepsin B and μ-calpain activities

Isolation and measurement of cathepsin B activity were determined as described by Caballero et al. (2007) with minor modifications. Crushed frozen muscle fragments (2 g) were suspended in ice cold homogenisation buffer (10 mM K_2HPO_4, 50 mM NaCl, 250 mM sucrose, 1 mM EDTA; 7.4). Standard curves were prepared from purified cathepsin B (Sigma, C6286) and diluted with incubation buffer (100 mM $C_2H_3NaO_2$, 1 mM EDTA, 5 mM DTT, 0.1% Brij; pH 5.5). Sample lysates and standards were dispensed into 96 well fluorescent plates (Nunc, #265301) and mixed with 70 μL of incubation buffer then warmed to 37 °C for 10 min. An aliquot (5 μL) of 40 μM cathepsin B Fluorogenic Substrate III (Calbiochem, 219392) was then dispensed into each well. Fluorescent measurements were collected every minute for 1 h at 37 °C with a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) fitted with 360 nm excitation and 460 nm emission filters.

Calpain extraction and activities were determined as described by Wiklund, Dobbie, Stuart, and Littlejohn (2010).

2.7. Quantitative determination of small heat shock proteins

For the quantitative measurement of small heat shock proteins in muscle sarcoplasm, sarcoplasmic extracts were adjusted to a concentration of 4 μg mL^{-1} protein with coating buffer (10 mM Na_2HPO_4, 15 mM NaCl; pH 7.4). Aliquots (100 μL) of each sample were dispensed into 96 well Costar® High Binding EIA/RIA polystyrene plates (Corning Inc., 3590) in duplicate. Individual sHSP was measured by indirect enzyme-linked immunosorbent assay (ELISA) according to the