Small Heat Shock Proteins and Tenderness in Intermediate pHu Beef

Lomiwes, D.[1], Farouk, M. M.[1], Frost, D. A.[1], Dobbie, P. M.[1] and Young, O. A.[2]

[1]AgResearch Ltd, Ruakura Research Centre, East Street, Hamilton, New Zealand
[2]AUT University, 34 Saint Paul Street, Auckland, New Zealand

Abstract – **This study tested the hypothesis that toughness and inconsistent tenderness in intermediate ultimate pH (pH_u) beef is due to the protective function of small heat shock proteins (sHSP) combined with the low proteolytic activities in this pH_u group. *M. longissimus dorsi* from 94 bulls were categorised into high (pH ≥ 6.2, n = 28), intermediate (pH 5.8 – 6.19, n = 14) and low (pH ≤ 5.79, n = 52) pH_u and aged for up to 28 days *post mortem* at -1.5°C. Cathepsin B, μ-calpain, HSP20, HSP27 and αβ-crystallin levels were determined on a subset from each pH_u group at 0, 1, 2, 7, 14 and 28 days *post mortem*. Shear force was measured for all samples from 1 day *post mortem*. Intermediate pH_u samples were generally tougher and more variable in tenderness compared with high and low pH_u samples. sHSP levels decreased with pH_u and time *post mortem*. μ-Calpain activity – as indicated by autolysis – was highest and occurred earlier in high pH_u samples. The greater and less variable tenderness of the high and low pH_u samples could be due to the elevated activities of calpains and cathepsin B, respectively. The low level and high variability in tenderness of intermediate pH_u samples could be attributed to the combination of a larger pool of active sHSP with the sub-optimal cathepsin B and intermediary μ-calpain activities in this pH_u group.**

Keywords – **Small heat shock proteins, tenderness, intermediate pH**

I. INTRODUCTION

Intermediate pH_u meat (pH_u 5.8 – 6.19) is notorious for being inconsistently tender and the biochemical mechanisms causing this are poorly understood [1]. It is widely accepted that μ-calpain and cathepsins hydrolyse key myofibrillar proteins in *post mortem* muscle [2, 3]. This results in the degradation of the highly-organised myofibrillar structure, consequently leading to higher tenderness of the cooked meat.

After slaughter, muscle cells inevitably engage towards apoptosis due to the termination of nutrients and oxygen supply to the muscles [4]. In response to impending cell death, small heat shock proteins are up-regulated to maintain cell homeostasis [5].

The ultimate pH_u of *post mortem* muscle is an indicator of total soluble sHSP levels in the sarcoplasm. As muscle pH falls below 6.2, sHSP precipitate from the soluble protein phase so that at pH 5.4, little sHSP remains in the sarcoplasm [1]. Additionally, the activities of calpain and cathepsins have been shown to be sub-optimal in the intermediate pH_u range, resulting in the delayed degradation of desmin and troponin T [6]. Based on these studies, it was hypothesised that toughness in intermediate pH_u beef is due to the less extensive degradation of myofibrillar proteins during ageing. This is owing to the low activity of endogenous proteases combined with the high bio-available pool of sHSP that bind to proteolytically damaged myofibrillar proteins, preventing further degradation. Thus, maintaining the integrity of the muscle structure, consequently leading to unacceptably tough meat.

The aim of this study was to test this hypothesis by following the trends of sHSP and proteolytic enzymes during ageing and relating this to meat toughness as observed in intermediate pH_u beef.

II. MATERIALS AND METHODS

A. Animals and sampling

94 bulls slaughtered in a commercial abattoir were used in this project. *M. longissimus dorsi* (LD) were hot boned within 1 h *post mortem* and the pH measured. 10 g were dissected from the anterior end of each muscle, frozen in liquid nitrogen, then stored at -80°C until analysed. The remaining LD was vacuum packed and stored at -1.5°C for 24 hours.

After 24 hours, each LD was cut into six equally sized sub-samples and vacuum packed. The sub-samples were randomly allocated to an ageing timepoint (1, 2, 7, 14 and 28 days *post mortem*) and stored at -1.5°C. 10 g were excised from each sub-sample for subsequent biochemical analysis

B. pH and shear force measurements

The pH of each sub-sample was measured with a Testo® 230 pH meter (Lenzkirch, Germany).

Shear force of all sub-samples were determined by cooking in a 100°C water bath until the internal temperature of the loin reached 75°C then measured with a MIRINZ tenderometer as described by Pulford *et al.* [1].

C. Western blots

Proteins from whole muscle and sarcoplasmic extracts were resolved by SDS-PAGE using 7.5% Tris-HCl for µ-calpain and 12% Bis-Tris for sHSP, respectively, on a Bio-Rad Criterion Cell system. 40 µg and 20 µg of protein were loaded onto each well for µ-calpain and sHSP, respectively. Immunoblots for µ-calpain were conducted as described by Kim *et al.* [7] and blots for HSP20, HSP27 and αβ-crystallin were performed as described by Pulford *et al.* [1] on a subset of low (n = 4), intermediate (n = 5) and high (n = 7) pH_u samples.

D. Cathepsin B activity

Cathepsin B activity at all ageing timepoints were determined on a subset of low (n = 8), intermediate (n = 14) and high (n = 7) pH_u samples as described by Caballero *et al.* [8]. Cathepsin B activity was expressed as mU mg $protein^{-1}$.

E. Data analysis

Statistical analysis of was conducted using the REML directive of Genstat [9]. One-way analysis of variance was conducted to determine significant difference between pH_u for shear force.

III. RESULTS AND DISCUSSION

Taking 11 kgF as the upper limit for acceptable cooked tenderness [10], shear force measurements of high pH_u samples (n = 28) showed that they were already acceptably tender at 1 day *post mortem* (Fig. 1).

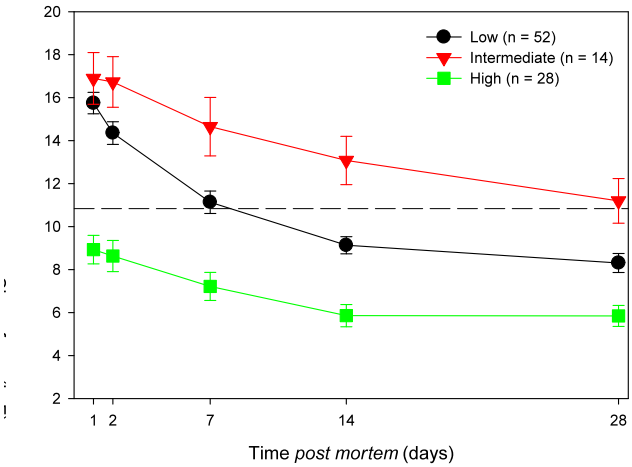

Fig. 1: Shear force of low, intermediate and high pH_u samples. Loins were vacuum packed and aged at -1.5°C for up to 28 days *post mortem*. Error bars are standard error fo the mean

Although the low pH_u (n = 52) samples were initially tough, they eventually reached acceptable tenderness after 14 days ageing. On average, intermediate pH_u (n = 14) samples did not attain acceptable tenderness until 28 days *post mortem* and was more variable in shear force compared with the high and low pH_u meat throughout the ageing period.

Initial µ-calpain autolysis is an indicator of enzyme activation and activity. Further autolysis inactivates µ-calpain, so that once the large subunit had been autolysed to 76 kDa, it becomes proteolytically inactive in meat. The rate of µ-calpain autolysis is apparently pH dependent, with enzyme degradation becoming more extensive as the samples were aged (Fig. 2). Early autolysis of µ-calpain was observed in high pH_u meat with the autolysed 78 kDa large sub-unit of the enzyme detected at 0 day *post mortem*. The autolytic rates of µ-calpain in the high and low pH_u samples were generally equivalent after 1 day *post mortem* with the enzyme in these groups having fully autolysed to 76 kDa after 28 days ageing. However, µ-calpain bands were notably denser in high pH_u samples, suggesting greater enzyme levels present in this group. µ-Calpain autolysis was delayed and occurred at a much slower rate in intermediate pH_u meat with the 78 kDa sub-unit still detectable after 28 days *post mortem*.

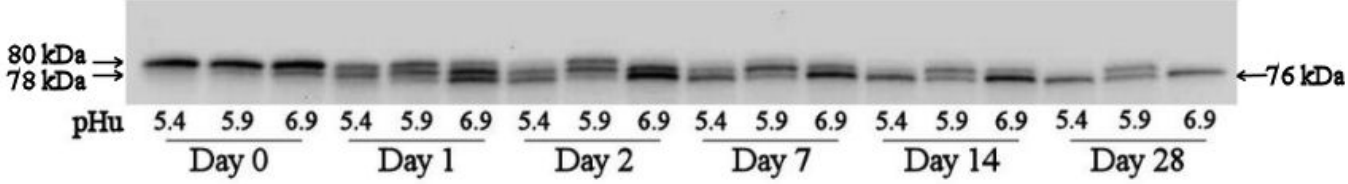

Fig 2: Western blots probed with µ-calpain antibody to determine the autolysis of µ-calpain in representative low, intermediate and high pH_u *M. longissimus dorsi* samples that were vacuum packed and aged at -1.5°C

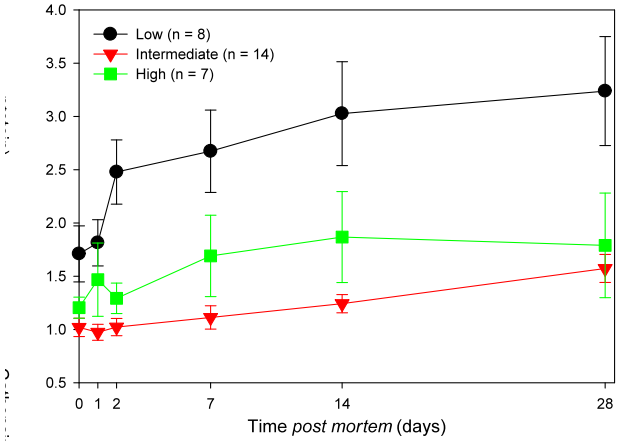

Fig. 3: Cathepsin B activity in low intermediate and high pH_u *M. longissimus dorsi* samples that were aged for up to 28 days *post mortem* at -1.5°C. Error bars are standard errors of the mean.

Cathepsin B activity in low the pH_u samples dramatically increased within the first 2 days *post mortem*, and remained at its peak thereafter. Although cathepsin B activity was dynamic in high pH_u samples early *post mortem*, mean enzyme activity remained relatively constant during ageing. With the exception of 0 and 28 days *post mortem*, the intermediate pH_u samples had lower activity compared with low and high pH_u meat during ageing.

Toughness in intermediate pH_u beef may be partly explained by the low proteolytic activity in this group during ageing. This has been proposed to be due to the limiting action of pH on μ-calpain and cathepsin B which are optimal at pH 7.5 [11] and 5.0-6.0 [3], respectively. Thus, the early activation of μ-calpain in high pH_u beef may explain the rapid tenderisation in this group, and the synergistic action of μ-calpain and cathepsin B *pre* and *post rigor*, respectively, may explain the tenderness in low pH_u beef.

At present, the reasons to why a portion of intermediate pH_u is tender while others remain unacceptably tough are still largely unknown.

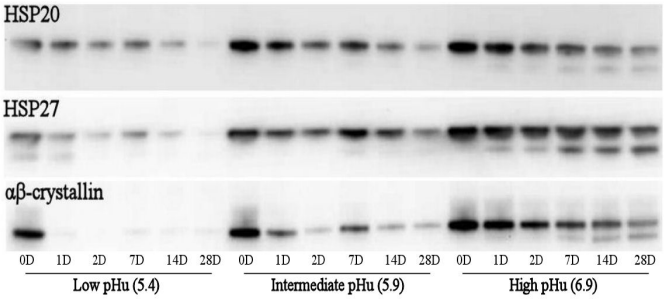

Fig. 4: Representative Western blots of sarcoplasmic HSP20, HSP27 and aB-crystallin from *M. longissimus dorsi* of low, intermediate and high pH_u samples aged at -1.5°C.

Previous studies have demonstrated the up-regulation of small heat shock proteins in tough meat [12, 13]. Due to the anti-apoptotic function of sHSP, it was speculated that the up-regulation of these sHSP has some role in preventing the irreversible damage of myofibrillar proteins, consequently leading to meat toughness. The present study determined the expression of the sHSP HSP20, HSP27 and αβ-crystallin in the sarcoplasm of representative low, intermediate and high pH_u samples during ageing.

The Western blots showed the more rapid disappearance of all three sHSP from the sarcoplasm in the low pH_u sample (Fig. 4). This has been attributed to the precipitation of proteins from the soluble phase of muscle as the pH decline in low pH_u and drops to levels far below the isoelectric point (pI) of each of the sHSP (pH 6.49, 5.98 and 6.76 for HSP20, HSP27 and αβ-crystallin, respectively [1]). Given that the pI of HSP20 and αβ-crystallin are within the high pH_u range, sHSP may be more available to protect from myofibrillar degradation in high pH_u meat. However, Western blots revealed the progressive degradation of all three sHSP during ageing in the high pH_u sample. This may be due to proteolytic activity of endogenous enzymes, however this requires further investigation. Nonetheless, any protective effects of sHSP may be nullified by their susceptibility to degradation in high pH_u meat.

Loss of sHSP by precipitation may also occur in intermediate pH_u meat but not as extensively as low pH_u meat as the pH does not decline to the same levels in intermediate pH_u meat. In addition, there was no evidence of sHSP protein degradation in the intermediate pH_u sample. Based on these results, any potential activity for sHSP to provide protection against the fragmentation of myofibrillar proteins is in the intermediate pH_u range. Thus, results from this study provides support to the hypothesis that low enzyme activity combined with the available pool of sHSP in intermediate pH_u meat may result in the protection of myofibrillar proteins from degradation resulting in the delayed tenderisation or toughness of intermediate pHu beef. Moreover, the inconsistent tenderness in intermediate pH_u beef may be due to the variability of sHSP in this group so that intermediate pH_u beef with inherently high sHSP levels will be unacceptably tough.

IV. CONCLUSIONS

This study provides strong support to the hypothesis that toughness in intermediate pH_u beef is due to the combination of low proteolytic activity and the protective effects of sHSP from myofibrillar protein degradation. The low proteolytic activity in intermediate pH_u beef was confirmed by the delayed activation and sub-optimal activity of μ-calpain and cathepsin B, respectively. As soluble sHSP were seemingly more stable in the intermediate pH_u beef, we speculate that it is in the intermediate pH_u range where sHSP are at their greatest potential to bind to proteolytically damaged myofibrillar proteins, thereby maintaining the structural integrity of the muscle structure, consequently leading to toughness.

ACKNOWLEDGEMENTS

Funding for this project was provided by New Zealand Foundation of Research, Science and Technology (contract number C10X0708). Acknowledgements to Adam Stuart, Guojie Wu and Kevin Taukiri for their assistance during sample collection and shear force measurements and Dr. Eva Wiklund for her supervisory support.

REFERENCES

1. Pulford, D.J., S. Fraga Vazquez, D.F. Frost, E. Fraser-Smith, P. Dobbie, and K. Rosenvold, *The intracellular distribution of small heat shock proteins in post-mortem beef is determined by ultimate pH.* Meat Science, 2008. **79**(4): p. 623-630.
2. Koohmaraie, M. and G.H. Geesink, *Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system.* Meat Science, 2006. **74**(1): p. 34-43.
3. Ouali, A., *Proteolytic and physicochemical mechanisms involved in meat texture development.* Biochimie, 1992. **74**(3): p. 251-65.
4. Ouali, A., C.H. Herrera-Mendez, G. Coulis, S. Becila, A. Boudjellal, L. Aubry, and M.A. Sentandreu, *Revisiting the conversion of muscle into meat and the underlying mechanisms.* Meat Science, 2006. **74**(1): p. 44-58.
5. Beere, H.M., *`The stress of dying': the role of heat shock proteins in the regulation of apoptosis.* J Cell Sci, 2004. **117**(13): p. 2641-2651.
6. Pulford, D.J., P. Dobbie, S. Fraga Vazquez, E. Fraser-Smith, D.F. Frost, and C.A. Morris, *Variation in bull beef quality due to ultimate muscle pH is correlated to endopeptidase and small heat shock protein levels.* Meat Science, 2009. **83**(3): p. 1-9.
7. Kim, Y.H., S.M. Lonergan, and E. Huff-Lonergan, *Protein denaturing conditions in beef deep semimembranosus muscle results in limited mu-calpain activation and protein degradation.* Meat Sci, 2010. **86**(3): p. 883-7.
8. Caballero, B., V. Sierra, M. Oliván, I. Vega-Naredo, C. Tomás-Zapico, Ó. Alvarez-García, D. Tolivia, R. Hardeland, M.J. Rodríguez-Colunga, and A. Coto-Montes, *Activity of cathepsins during beef aging related to mutations in the myostatin gene.* Journal of the Science of Food and Agriculture, 2007. **87**(2): p. 192-199.
9. Windows, *Genstat*. 2008, VSN International: Oxford.
10. Bickerstaffe, R., A.E.D. Bekhit, L.J. Robertson, N. Roberts, and G.H. Geesink, *Impact of introducing specifications on the tenderness of retail meat.* Meat Science, 2001. **59**(3): p. 303-315.
11. Koohmaraie, M., J.E. Schollmeyer, and T.R. Dutson, *Effect of Low-Calcium-Requiring Calcium Activated Factor on Myofibrils under Varying pH and Temperature Conditions.* Journal of Food Science, 1986. **51**(1): p. 28-32.
12. Bernard, C., I. Cassar-Malek, M. Le Cunff, H. Dubroeucq, G. Renand, and J.F. Hocquette, *New indicators of beef sensory quality revealed by expression of specific genes.* J Agric Food Chem, 2007. **55**(13): p. 5229-37.
13. Hwang, I.H., B.Y. Park, J.H. Kim, S.H. Cho, and J.M. Lee, *Assessment of postmortem proteolysis by gel-based proteome analysis and its relationship to meat quality traits in pig longissimus.* Meat Science, 2005. **69**(1): p. 79-91.