## INHIBITION OF PROTEASE ACTIVITY 2. EXAMINATION OF MYOFIBRILS USING LIGHT AND ELECTRON MICROSCOPY

**David L. Hopkins[1], Patrick J. Littlefield[1], Peter R. Garlick[2] and John M. Thompson[1]**

[1]Co-operative Research Centre for the Cattle and Beef Industries, University of New England, NSW, 2351, Australia.
[2]Electron Microscope Unit, University of New England, NSW, 2351, Australia.

**Background.**

*Hopkins et al. (2000b)* showed that the cysteine protease inhibitor E-64 prevented tenderisation when injected into pre-rigour muscle, whilst a specific inhibitor for the cathepsins B and L (Z-Phe-Ala-$CHN_2$) had little effect. *Dransfield (1999)* proposed that E-64 inactivated the calpains by binding to the active site of these enzymes, which was consistent with other reports of E-64 binding to the reactive thiol sites of cathepsin L *(Fujishima et al., 1997)*. However, *Tatsumi et al. (1998)* concluded that a number of protease inhibitors caused deterioration of the sarcomere proteins titin and nebulin by binding to these proteins and as a result, suppressing their ability to bind calcium. This result was advanced as evidence in support of the calcium theory of tenderisation, in which a role for enzymes in tenderisation was dismissed *(Takahashi 1996)*. Some of the evidence advanced by *Tatsumi et al. (1998)* to support the notion that inhibitors cause deterioration of sarcomere proteins included light microscope images of homogenised myofibrils, which had been incubated in specific protease inhibitors. If this was a real effect then electron microscope images should provide the opportunity for closer examination of the localities within the sarcomere which are denatured by inhibitors. To our knowledge such images have not been previously reported.

**Objective.**

This study examined, using both light and electron microscopy the effect of cysteine proteases on the integrity of myofibrils and sarcomeres in muscle that had been injected with specific protease inhibitors during the early post-mortem period.

**Methods.**

*Animals and slaughter procedures:* Muscle samples were taken from the carcasses of 4 lambs of which 3 had been injected with E-64 and 1 with Z-Phe-Ala-$CHN_2$. The lambs were part of a larger experiment, which examined the mechanisms controlling proteolysis using a combination of low voltage stimulation, and specific protease inhibitors, as described by *Hopkins et al. (2000b)*. For preparation of electron microscopic images, samples were selected from 8 portions of the m. *longmissius lumborum* (LL) at 24 hours postmortem. The number of samples used was constrained by the limitations on processing the samples fresh. The rationale in selecting the 8 samples was to match injected samples with a control sample from some of the carcasses, and also to sample more carcasses injected with E-64. Fresh samples of approximately 1-2 grams were first bathed in a primary fixative for 30 mins and then processed as described by *Hwang (1999)*. For each sample, sections from 2 blocks were examined and photographic images were captured (25,000 x). Representative images for 6 samples taken from 4 carcasses are shown here. A description of the samples used for electron microscopy (EM) is as follows;

Sample 1 = E-64, no stimulation (cranial portion)
Sample 2a = saline, no stimulation (cranial portion)
Sample 4a = saline, no stimulation (cranial portion)
Sample 3 = E-64, stimulated (cranial portion)
Sample 2b = Z-Phe-Ala-$CHN_2$, no stimulation (caudal portion)
Sample 4b = E-64, no stimulation (caudal portion)

Samples 2a and 2b came from the same carcass, as did samples 4a and 4b, with the remaining samples coming from different carcasses. Samples of frozen (-20°C) muscle were thawed, homogenised and the myofibrillar fragmentation index determined as described by *Hopkins et al. (2000c)*. After homogenisation a drop of suspension containing myofibrils was placed on a microscope slide and examined under a light microscope (Lecia DMLB, Leica Cambridge Ltd, UK). Representative images were captured using an image processing and analysis system linked to a CCD camera at x250 magnification. Light microscope images are shown below with their matching EM image.

**Results and Discussion.**

For comparison the myofibrillar fragmentation index (MFI) values are given for each sample in brackets. For samples 1 and 3 the corresponding control samples had MFI values of 90 and 70. Thus E-64 reduced MFI values from 10 to 23 units, whereas the Z-Phe-Ala-$CHN_2$ inhibitor had no effect on MFI values.

Our results in the accompanying paper, *Hopkins et al. (2000b)* clearly implicate cysteine enzymes in tenderisation and also proteolysis, as indicated by MFI values. The light microscope images showed that the injection of E-64 in our study did not cause denaturation and aggregation of the myofibril fragments, unlike the images of *Tatsumi et al. (1998)*. In our images normal striations can be readily seen (see sample 2b), suggesting that the integrity of the myofibrils was retained. *Hattori and Takahashi (1982)* found that when myofibrils were incubated for 24 hours with 0.2 mM E-64 some aggregation of the myofibrils occurred, although normal striations could still be seen. The aggregation was also minimal compared to that shown by *Tatsumi et al. (1998)* for myofibrils incubated for 18 hours in 0.1 mM calpastatin domain I. In this latter study the effect of the protease inhibitor leupeptin (1 mM) was minimal compared to that of calpastatin domain I indicating that the reported effects could not be ascribed to all protease inhibitors. We did not observe these effects after muscle had been injected with E-64 and held for 24 hours. Furthermore even samples held for 48 hours post-mortem did not show denaturation *(Hopkins et al., 2000a)*.

The electron microscope images provided a more detailed view of the structure of the sarcomere. Scrutiny of these images did not reveal any modification to the sarcomere that could be ascribed to treatment with either of the inhibitors. There was no

visual evidence of denaturation and treated samples showed no structural differences compared with control samples. For example the Z-disk and A-band of the sarcomeres were clear and intact as indicated for Sample 3. *Tatsumi et al. (1998)* claimed that the inhibitors used in his study bound to titin and nebulin, and therefore prevented the binding of calcium ions, although this wasn't the case when 1-mM leupeptin was used. For inhibitors to bind to proteins such as titin and nebulin they must have the ability to chemically interact with these proteins. *Tatsumi et al. (1998)* did not provide an explanation of how inhibitors such as E-64 bind to sarcomere proteins, yet they suggested that the inhibitor calpastatin domain 1 also bound to other proteins including myosin heavy chain, α-actinin, actin and troponin C. Since calpains bind calcium ions for activation and titin has been shown a good substrate for these enzymes *Robson et al. (1997)* it is reasonable to suggest that wherever calpains bind to proteins there will be evidence of calcium ions. These facts could explain some of the reported effects of calcium ions on sarcomere proteins. Study of the level of free calcium in treated muscle may provide more insight into this issue.

**Acknowledgments.**

Support for DLH through a Junior Research Fellowship by Meat and Livestock, Australia is acknowledged. The assistance of Andrew Blakely, Diana Perry, Samuel Bruley, Benoit Delord, David Edmonds and In Ho Hwang is also noted with much appreciation.

**References.**

Dransfield, E. (1999). Proc. 45th ICoMST, Yokohama, 220-228.
Fujishima, A., Imai, Y., Nomura, T., Fujisawa, Y., Yamamoto, Y., and Sugawara, T. (1997). FEBS Letters. **407,** 47-50.
Hattori, A. and Takahashi, K. (1982). J. of Biochemistry. **92,** 381-390.
Hopkins, D. L., Littlefield, P. J., and Thompson, J. M. (2000a). Asian-Australasian Journal of Animal Science. (in press).
Hopkins, D. L., Littlefield, P. J., and Thompson, J. M. (2000b). Proc. 46th ICoMST, Buenos Aires, Argentina. (in press).
Hopkins, D. L., Littlefield, P. J., and Thompson, J. M (2000c). Meat Science. (in press).
Hwang, I.H. (1999). Mechanisms for optimizing meat quality using electrical stimulation. PhD. Thesis, University of New England.
Robson, R. M., Huff-Lonergan, E., Parrish, Jr. F. C., Ho, C. H., Stromer, M. H., Huiatt, T. W., Bellin, R. M., and Sernett, S. W. (1997). Proc. Reciporal Meat Conference, Iowa, USA; **50,** 43-52.
Takahashi, K. (1996). Meat Science. **43,** s67-s80.
Tatsumi, R., Hattori, A., and Takahashi, K (1998). Bio. Biotechnology and Biochemistry. **62,** 927-934.