HIGH PRESSURE PROCESSING EFFECTS ON TENDERNESS, MYOFIBRILLAR FRAGMENTATION AND CALPAINS OF PRE-RIGOR LONGISSIMUS DORSI FROM PRIME AND BULL STOCK

Roy Bickerstaffe[1], Grant Pearson[2], Hannah Lee[1], Stephanie J.Smithson[2] and James D. Morton[1].

[1] Department of Wine, Food and Molecular Biosciences, Lincoln University, Lincoln,7647, Canterbury, New Zealand

[2]Silver Fern Farms Limited, P.O. Box 283, Christchurch, 8140, New Zealand

Abstract -The commercial potential of HPP hot boned pre-rigor LD muscles from prime and bull stock was investigated. Application of HPP improved the tenderness of the meat from tough to tender. MFI was reduced with increased tenderness indicating the method of meat tenderisation by HPP is different to that occurring in traditional cold chain chilled meat. Casein zymography showed HPP promoted the autolysis of calpain 1 and Western Blotting showed that HPP stimulated an increased appearance of sarcoplasmic 80 kDa calpain 1 in the LD muscles. There was evidence of nebulin degradation. The results indicate that HPP promotes proteolytic changes that are detrimental to the structural integrity and strength of the sarcomeres - hence the improvement in meat tenderness. HPP may cause structural sarcomere changes independently of the enzymatic effects. There are commercial HPP benefits according to the meat cut, type of stock, processing capacity and intended markets

Key words: HPP, pre-rigor hot boned meat, tenderness improvements, MFI reduction, autolysed calpain 1, 80 kDa calpain changes, commercial benefits.

I. INTRODUCTION

The review on high pressure processing (HPP) of fresh meat by Ma *et al* [1] concluded there were potential benefits by applying pressure treatments to hot boned pre-rigor meat but that commercial exploitation of the process had not been realised because of the cost and complexities of hot boning.

The aim of this research was to investigate the benefits of commercial high pressure processing of hot boned pre-rigor Longissimus dorsi muscles from prime and bull animals. The impact of the process on meat tenderness (an important consumer eating factor), myofibrillar fragmentation index (an indicator of proteolytic breakdown of myofibrillar proteins) and autolysis of calpain 1 and 2 were also determined. The objective of these measurements was to gain an insight into the mechanism(s) by which high pressure processing impacts on tenderness.

II. MATERIALS AND METHODS

A 55 L Hiperbaric HPP machine was installed by Silver Fern Farms Limited in the boning room of a commercial hot boning beef plant to collect meat samples for objective, biochemical and sensory measurements.

Four prime animals and four bull animals were randomly selected and slaughtered by standard procedures. Longissismus dorsi (LD) muscles (strip loins) were removed by hot boning from the carcasses within 45 to 60 minutes of slaughter. Each strip loin was divided into 30cm lengths and each portion was placed in vacuum pack bags. The first portion (A) from each animal was the control LD. The second portion (B) was placed in the Hiperbaric chamber and exposed to pressures between 100 to 300 MPa for 1 to 5 minutes. After the HPP treatment, all the LD samples were vacuum packed, chilled for 24 hours at -1ºC and then frozen at -20°C. Therefore, there were four prime control LD matched with four prime HPP LD from the same carcasses and four bull control LD matched with four bull HPP LD from the same carcasses.

From the frozen LD strip loins, 25 mm thick steaks were removed for tenderness measurements using the method described by Bickerstaffe *et al*.[2]. A 25 mm steak was also removed and stored at -40ºC for biochemical measurements when 15 mm cores were removed from the frozen steaks using an electric drill with a keyhole bit. The myofibrillar fragmentation index (MFI) was determined using the method described by Culler *et. al.* [3] with minor modifications. In summary, 2g of LD was homogenised in a 50 ml centrifuge tube containing 20 ml of extraction buffer (25mM potassium phosphate buffer (pH 7), 1mM EDTA, 0.1M KCl, 1mM sodium azide and protease inhibitors) using a Polytron homogeniser set at 10,000 rpm. Two 30 second bursts were applied. After centrifugation at 1000g for 15 minutes the supernatant (sarcoplasmic) and precipitate (myofibrillar) fractions were collected. The precipitate was washed three times, and the final precipitate suspended in 12 ml of phosphate buffer. The protein content of the suspension was determined using the biuret reagent. Aliquots of the suspended myofibrils were diluted with buffer to a final protein concentration of 0.5mg/ml and the turbidity of the suspension read at 540 nm using a spectrometer. The mean of duplicates multiplied by 200 gives the MFI value

Calpain proteolytic activity was determined in the sarcoplasmic fractions by casein zymography. Samples were loaded onto a non-denaturing casein gel according to Raser [4] with minor modifications [5,6] and calpains separated into four bands corresponding to native calpain 1, autolysed calpain 1, native calpain 2 and autolysed calpain 2. The location and extent of calpain activity in each band was determined by incubating the gel in a calcium based buffer (20mM Tris-HCl, pH 7.4, 10mM DTT and 20mM $CaCl_2$). The gels were stained with Coomassie Blue to locate and quantify the calpains.

Protein levels of calpain 1, calpain 2 and nebulin in the sarcoplasmic fractions were determined by separating the proteins on 4 - 12% gradient PAGE gels transferring the proteins onto PVDF membranes and Western Blotting the membranes with specific antibodies [6,8]. The antibody response was visualised with an alkaline phosphatase conjugate substrate kit, and the optical density measured by Doc EQ and Quantity One software (BioRad).

III. RESULTS AND DISCUSSION

(i) The tenderness of LD are in Table 1.

Table 1.Shear Force (KgF) of control and HPP treated LD from bull animals

Stock	N	Mean KgF (± s.d.)	T. Test (paired)
Bull control	4	18.13±2.64	
Bull HPP	4	5.91±1.34	*p*=0.0005

The shear force of cooked steaks from control bull LD of 18.1±2.6 KgF would be perceived tough by consumers [2]. In contrast, after HPP, the shear force of the same LD from the bull animals were significantly reduced to 5.91±1.3 KgF which would be considered tender by consumers. A similar improvement in tenderness was obtained with the prime LD. Thus, HPP has improved meat tenderness by 63 to 67%. Preliminary sensory testing confirmed that consumers prefer the HPP treated steaks to the control steaks.

The improvement in tenderness could be due to physical sarcomere structural changes in the sarcomeres and/or proteolytic breakdown of the myofibrillar proteins that contribute to the structural integrity of the sarcomeres by calpains. Whether proteolysis was changed by HPP is investigated in the next sections.

(ii) MFI has been promoted as an indicator of myofibril breakup and monitors the rate of proteolytic breakdown of myofibrillar proteins. The MFI results are in Table 2.

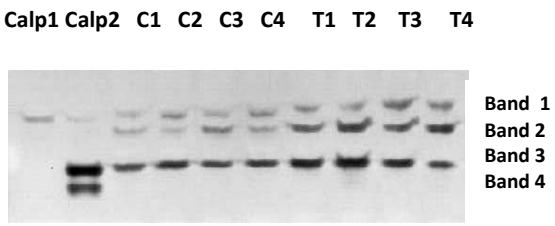
Table 2. MFI of control and HPP treated LD from bull animals.

Stock	N	Mean MFI (± s.d.)	T. Test (paired)
Bull control Bull HPP	4 4	71.8±5.1 39.1±11.5	 p = 0.024.

HPP reduced the MFI of the bull LD by 46% as the muscles underwent tenderisation. A similar result was obtained with the prime LD. The reduction in MFI associated with the HPP tenderisation of pre-rigor muscles is opposite to that which occurs in cold chain chilling of post-rigor muscles when MFI increases as tenderisation proceeds. This indicates the mechanism of meat tenderisation by HPP is different to the mechanism of tenderisation that occurs during the cold chain chilling of post-rigor muscles.

(iii) Calpain activity in the LD sarcoplasmic fractions of the prime and bull animals was determined by casein zymography. The results for the bull LD are in Figure 1.

Fig 1. Casein zymography of calpains in 40.6 µg of sarcoplasmic fractions from control (C1-C4) and HPP (T1 -T4) bull LD



Band 1, calpain 1; Band 2, autolysed calpain 1; Band 3, calpain 2; Band 4; autolysed calpain 2.

Similar results were obtained with the prime LD samples. Thus, in both the prime and bull LD, HPP activated calpain 1 as shown by the appearance of autolysed calpain 1 (band 2). HPP had no effect on calpain 2 (band 3).The intensity of band 2 from the LD samples were quantified and the results shown in Table 3.

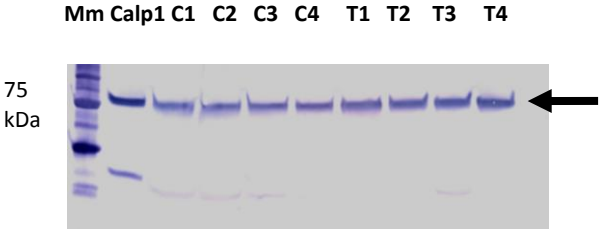
Table 3. Intensity of casein zymograph Band 2 from control and HPP LD from bull animals.

Stock	N	Mean Intensity (± s.d.)	T. Test (paired)
Bull control Bull HPP	4 4	101±41.6 261±69.9	 p = 0.045

Table 3 shows that HPP stimulated the autolysis of calpain 1 (band 2) by at least 250% in the bull LD. Higher rates of autolysis were observed with the prime LD.

To determine whether there were any immuno-reactive calpain proteins, the sarcoplasmic fractions were Western blotted using a monoclonal antibody to calpain 1. Figure 2 illustrates the Western Blot of sarcoplasmic fractions from control and HPP bull LD. Western Blots of the control HPP and prime LD gave similar results.

Fig 2.Western immuno-detection of calpain 1 in 37.5µg of sarcoplasmic fractions from control (C1-C4) and HPP (T1 -T4) bull LD using mouse monoclonal antibody ab49652



The arrow indicates a large sub unit of calpain 1 with a molecular weight of approximately 80 kDa which immuno responded with the monoclonal antibody. The intensity of the bands at 80 kDa were quantified using Quality One software. The results for the bull LD are in Table 4.

Table 4. Intensity of calpain 80 kDa band from control and HPP LD from bull animals.

Stock	N	Mean Intensity (± s.d.)	T-Test (paired)
Bull control Bull HPP	4 4	198.8±7.8 279.8±12.8	 p = 0.003

Thus, HPP increased the appearance of immuno-reactive 80 kDa large sub unit of

calpain1 in the LD from the bull animals by 41%. A similar result was obtained with the prime LD. The results are indicative of HPP stimulating muscle proteolytic activity which has been reported to occur during meat tenderisation.

Nebulin, a protein, which is part of the thin filament complex of myofibrils, is a substrate of calpains, and has been shown to be broken down during meat tenderisation. Proteins from the sarcoplasmic fractions of the prime and bull LD muscles were separated on a 3 - 5 % tris-acetate gel and the separated proteins transferred to a membrane which was Western Blotted using a mouse monoclonal anti-nebulin antibody. Nebulin degradation products were observed with the HPP LD samples (results not shown) verifying that HPP stimulated calpain activity which proteolysed the nebulin; one of its key substrates.

IV. CONCLUSION

There are commercial benefits from HPP pre-rigor LD muscles from hot boned prime and bull carcasses due to the significant improvements in meat tenderness from tough to tender. Shear force (KgF) of the meat was improved by 63 to 67 %. The improvements in tenderness could be due to structural or enzymatic changes in the myofibrillar proteins that contribute to the structure of sarcomeres in muscle myofibrils. An increase in MFI has been correlated with the breakdown of myofibrillar proteins in traditional cold chain meat tenderisation. In this work, HPP of hot boned LD, reduced MFI by 46 to 57% compared to control LD illustrating the mechanism of tenderisation in HPP muscles is different to that occurring in post-rigor cold chain aged muscles. Casein zymography showed autolysed calpain 1 was increased by more than 250%, and Western Blotting that the intensity of the 80 kDa calpain 1 sub-unit was increased by 41 to 58 % in the HPP hot boned LD muscles.

Thus, HPP promotes calpain activity which is recognised as a key proteolytic process regulating the rate of meat tenderisation. Preliminary data indicates that nebulin, a key myofibrillar protein, is degraded by calpain and this would compromise the structural integrity and strength of the sarcomeres. Direct physical disruption of the sarcomeres by HPP is another possible factor contributing to the tenderisation but requires electron microscopic investigations. This is under investigation.

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