BIOCHEMISTRY OF MEAT TENDERIZATION DURING POSTMORTEM AGING: ASSOCIATION WITH CALPAIN 3 (p94)

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Background.

Meat tenderisation is a biochemical process which involves fracturing proteins contributing to the inter and intra-linkages or proteins linking myofibrils to the sarcolemma by the calpain system (Taylor et al., 1995). Calpains are a family of intracellular Ca^{2+}-dependent neutr proteinases (Sorimachi et al., 1997). In mammalian skeletal muscle, calpains comprise the ubiquitous calpains 1 and 2 and the tissue-specific calpain 3 (p94). The role of these calpains in meat tenderisation is not known.

Current literature on the role calpains in meat tenderization refers almost exclusively to the ubiquitous calpains as it is these that can routinely assayed. Until recently, it was not possible to study the potential role of calpain 3 in postmortem meat tenderisation because of a la of the necessary probes to quantify the expression of calpain 3 in livestock animals. However, with the development of S1 nuclease protectio assay (Ilian and Bickerstaffe, 1999) and antibodies against calpain 3 as described in this study for the ovine and by Parr et al. (1999) for the porcine some information about the role of calpain 3 in meat tenderization can be obtained. Two published studies examined the role of calp 3 in tenderisation of meat. Ilian and Bickerstaffe (1999) reported a strong correlation between the intermuscular variation in tenderness and t expression of calpain 3 mRNA in sheep. On the contrary, Parr et al. (1999) reported no correlation between calpain 3 level at slaughter and t porcine longissimus (LD) tenderness.

Objective.

To further investigate the role of calpain 3 at the protein level in postmortem tenderisation using lambs subjected to various periods o feed withdrawal prior to slaughter as a biological model. This model was selected for two reasons. 1) Feed withdrawal prior to slaughter was observed to increase the rate of myofibrillar proteins turnover and the expression of the ubiquitous calpains at the mRNA level in New Zeala white rabbits (Ilian and Forsberg, 1992). 2) Feed withdrawal prior to slaughter was observed to affect meat tenderness (Fernandez et al., 199

Methods.

Animals. Forty Perendale lambs were used. Animals, with access only to water, were randomly among four treatments; no fast, one d fast, three days fast; and seven days fast. Blood was collected in heparin tubes and then lambs were captive bolt stunned followed by exsanguination. Six major muscles were exposed about 15 minutes postmortem for sampling and probing. Samples were frozen in liquid nitrogen for measuring calpain 3. The carcasses were kept at 15 °C for 4 hours, at 10 °C chiller for 12 hours, and at 2 °C for 56 hours. The pH deep muscle temperature and tenderness were measured as described earlier (Ilian et al., 1997).

Cloning the calpain 3 cDNA and its western analysis. Calpain 3 cDNA of the bovine and ovine were cloned using the Marathon cDN kit (Clonetech, CA, USA). The primers used were; 5' RACE-p94: CTCCTTGTTGCTGTTTGCTCTGTC and 3' RACE-p94: TCCTTCTGGTCTGAACATGGGGGA. The 5' RACE-p94 and 3' RACE-p94 were derived from the conserved IS1 and IS2 regions respectively. The amplicons were gel purified and cloned in pGEMT-Easy (Promega, WI, USA) and sequenced using the dideoxy chain termination method. Based on the calpain 3 cDNA sequences for the bovine (GenBank accession number AF115745) and ovine (GenBank accession number AF115744) species, a mixture of two polypeptides (with an extra COOH-terminal cysteine for coupling to hemocyanin) located in NH_2-side of the IS2 region with the sequence NTISVDRPVKKKKXKPIIFC [where **X** is a mixture of asparagine (as in the bovine and proline (as in the ovine)] was synthesised (Research Genetics, AL, USA) and used to generate the antibody. Preliminary studies indicate that calpain 3 is associated with the myofibrillar proteins. Consequently, we separated the muscle proteins from samples at-death into sarcoplasmic and myofibrillar fractions. Protein concentration of various fractions was determined. Portions of 20 µg proteins together with biotinylated-HRP broad range SDS-PAGE standards (BIO-RAD, CA) were loaded onto discontinuous gels for SDS-PAGE. Proteins on the g were electroblotted on PVDF membranes at 4°C overnight and 30. Immunoprobing of the immobilized proteins was carried out with the Ab diluted 1:500 and anti-chick IgY (HRP) diluted 1:5000 (Promega, WI) in blocking buffer [100 mM Tris.Cl, pH 7.5, 0.9% NaCl, 0.1% Tween 5% nonfat dry milk]. After washing, detection of bound antibodies was performed with chemiluminescence kit (Pierce, IL) and the intensity signal was determined using a Gel Documentation Systems (UVP Inc., CA, USA).

Statistical analysis. Data were analyzed for variance by one way ANOVA using the Minitab Version 11 (State College, PA, USA).

Results and Discussion.

To determine calpain 3 level in bovine and ovine skeletal muscle by western we produced a polyclonal antibody specific against the I region of bovine and ovine. The IS2 region was chosen because it is unique for calpain 3 and not present in the ubiquitous calpains (Sorimach al. 1989) and because Calpain 3 associates with titin via the IS2 region (Sorimachi et al., 1995). We cloned and sequenced the cDNA of calp 3 of bovine (GenBank accession number AF115745) and ovine (GenBank accession number AF115744) as a requisite for designing the antigenic polypeptide. The specificity and the utility of the Ab for the quantitative analysis of ovine calpain 3 level by western assay was established by reacting it with gradations of LD myofibrillar proteins and with solubilised whole liver, heart and lung as non-muscle tissue. T results (Fig. 1) revealed that Ab is specific for calpain 3 as a signal at 94-kDa was observed for the LD (lanes 4-7) but not for the liver, heart lung (lanes 1-3). Results of the calibration curve using 10, 20, 30, and 40 µg myofibrillar proteins showed a linear response of image density gradation in the myofibrillar protein concentrations. Consequently, 20 µg of proteins was used in the analysis of calpain 3 in the muscle samp of the study.

To provide a frame of reference to our work, the effect of feed withdrawal time prior to slaughter on certain body, carcass, and blood parameters usually affected by fasting are listed in Table 1. Analysis of variance of initial weight for various treatments revealed no significa differences. It is well known that fasting causes live weight shrinkage in animals (Ilian and Forsberg, 1992, Fernandez et al., 1996). The weig data indicated significant effect of feed withdrawal time prior to slaughter on lamb size. The lambs lost 1.73 kg per during the first three days fast. Afterward, lambs continued to lose weight at 0.44 kg per day during the last four days of fast. Body weight losses in lambs subjected to fasting were mainly caused by emptying of the alimentary tract at 1d fast and by the losses of edible parts at 3d and 7d fast. This is clear from hot carcass weight data and creatinine level in blood as indexes for muscle wasting. The liver was very sensitive to fasting. Significant losses liver mass were observed at all fasting periods. The shrinkage rate and magnitude of various muscles in response to fasting were not similar. anticipated, Fasting caused significant effect on tenderness of certain muscles. The LD of lambs fasted for one day was significantly more ten

than the control or 3d and 7d fast. However, the most tender SS was found for lambs fasted for 7d. These differences in tenderness in the LD and the SS were not due to differences in the rate or magnitude of pH decline or sarcomere length. Therefore, the major factor contributing to the variation of tenderness in the LD and SS due to fasting may be due to an increase in the degradation rate of structural proteins. This observation is interesting because it offered us a biological model for the studying the role of calpain 3 in postmortem tenderization of meat within the same muscle. For calpain 3 to have a role in meat tenderization we hypothesized that variation in the steady state level of calpain 3 should correlate with variation in tenderness in the LD and SS during fasting. Preliminary studies indicated that calpain 3 is associated with the myofibrillar proteins. Thus to examine our hypothesis, we separated the muscle proteins from samples at slaughter into sarcoplasmic and myofibrillar fractions and determined the steady state level of calpain 3. The steady state level [measured as the average mean of the intensity of the 94-kDa band at slaughter per mg tissue] of calpain 3 was significantly higher in the sarcoplasmic ($P \leq 0.003$) and myofibrillar ($P \leq 0.000$) fractions of LD of 1d fast compared to control (Table 1). Similarly the SS of 7d fast showed significant differences in the levels of calpain 3 compared to control. To evaluate whether variations in the steady state level of calpain 3 underlie the differences in tenderness of the LD and SS due to fasting, we calculated the correlation coefficient between the steady state level of calpain 3 and the associated tenderness score. The results revealed strong and significant correlation between the abundance of calpain 3 and the tenderness of the LD and SS. This is in contrary to Parr et al. (1999) who reported no link between the at-death calpain 3 level of tough and tender porcine LD compared to the control. In sum, the results of this study together with those of Ilian and Bickerstaffe (1999) support a role for calpain 3 in postmortem tenderisation of meat. The discrepancy between our results and those of Parr et al. (1999) may be related to differences in animal species or methodology.

Pertinent Literature.

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Table 1. Effect of feed withdrawal time before slaughter on body and carcass characteristics and blood creatinine level.

Parameter	Control	1-day fasting	3-days fasting	7-days fasting
Initial weight (kg)	28.05^{a*}	27.11^{a}	27.25^{a}	27.11^{a}
Kill weight (kg)	28.47^{d}	26.74^{c}	23.13^{b}	21.38^{a}
Hot carcass weight (kg)	11.29^{c}	11.09^{bc}	10.35^{ab}	9.78^{a}
Liver weight (g)	576^{d}	484^{c}	413^{b}	349^{d}
Tenderness (kg / cm^3)				
LD (1d postmortem)	6.61^{b}	3.84^{a}	5.48^{b}	6.19^{b}
SS (3d postmortem)	5.25^{b}	5.04^{b}	4.93^{b}	3.94^{a}
24h postmortem pH				
LD	5.94a	5.84^{a}	5.88^{a}	5.93^{a}
SS	6.05^{a}	6.1^{a}	6.00^{a}	6.14^{a}
SS Sarc. Length (μm)	1.977^{a}	1.874^{a}	1.920^{a}	1.950^{a}
Blood creatinine (mg / dl)	1.34^{b}	1.46^{b}	1.39^{b}	0.97^{a}
Calpain 3 level (Image density units/mg tissue)				
LD (sarcoplasmic)	60.3^{a}	81.5^{b}	86.5^{b}	82.0^{b}
LD (myofibrillar)	598^{b}	718.1^{c}	576.3^{b}	421.4^{a}
SS (sarcoplasmic)	53.0^{a}	68.3^{b}	81.7^{c}	87.0^{c}
SS (myofibrillar)	522^{a}	569^{ab}	676^{b}	626^{ab}

*Means (average of ten observations) within each row followed by different superscripts are significantly different at $P \leq 0.05$.

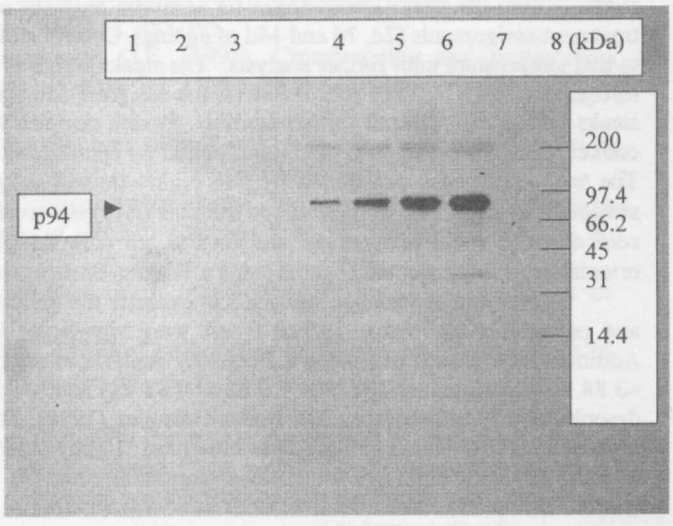

Figure 1. Validation of calpain 3 antisera (Calp3IS2:b/o) for western analysis of ovine calpain 3. Lanes 1, 2, and 3 had 20μg Proteins of whole liver, heart, and lung respectively. Lanes 4-7 had 10, 20, 30, and 40 μg LD myofibrillar proteins. Lane 8 had Bio-Rad broad range protein molecular weight marker.