Calpain and calpastatin activity in porcine longissimus and the red and white portions of the semitendinosus

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Abstract— **The objective of this study was to determine μ-calpain, m-calpain, and calpastatin activity in porcine longissimus (LD) (n=22) and semitendinosus muscles. The semitendinosus was divided into red (RST) (n=19) and white (WST) (n=22) portions. Samples were taken immediately postmortem, and sarcoplasmic proteins were quantitatively extracted and equilibrated on a Q-Sepharose ion exchange column. Protein was eluted with a linear gradient of KCl. Calpastatin eluted in two separate peaks (calpastatin I and II, 50-90m*M* KCl and 120-190m*M* KCl, respectively), followed by μ-calpain (180-240m*M* KCl) and m-calpain (300-400m*M* KCl). Calpastatin I and II inhibition of μ- and m-calpain from the same muscle was determined in one LD. Western blots (1D and 2D) and 2D gels were also used to compare the peaks. The LD had greater μ-calpain activity ($p<0.0001$) and less m-calpain activity ($p<0.001$) than the RST and WST. While the LD had less total calpastatin and calpastatin II than both the RST and WST ($p<0.0001$), LD calpastatin I activity was similar ($p>0.5$) to that of the WST. Both the LD and WST had less ($p<0.05$) calpastatin I activity compared to the RST. Calpastatin II was more active than calpastatin I against both μ- and m-calpain. Each calpastatin was more active against m-calpain than μ-calpain. Results suggest that conditions in the LD may be more favorable for proteolysis than in the semitendinosus, due to greater μ-calpain activity and lesser calpastatin activity. The proportion of calpastatin present as each form indicates a previously undefined source of variation for postmortem proteolysis.**

Keywords— **calpain, calpastatin, pork.**

I. INTRODUCTION

Calpains are a group of calcium dependent cysteine proteases, which are believed to be primarily responsible for increased tenderness during aging of meat [1-2]. In porcine skeletal muscle, two forms of calpain exist, μ- and m-calpain, named for their calcium requirements (3-50 μ*M* and 400-800 μ*M* half-maximal activity, respectively) [3]. In addition to calcium concentration, calpains are regulated by the enzyme calpastatin, an endogenous inhibitor.

Various muscles have different calpain and calpastatin activities due to differing protein turnover requirements. For example, the psoas major in beef and lamb has less desmin degradation during aging than the longissimus dorsi (LD) [4-5]. This could be due either to less calpain activity, greater calpastatin activity, or both. In pork, Wheeler et al. [6] also demonstrated that in the LD, desmin was more degraded than other muscles including the semimembranosus, biceps femoris, semitendinosus, and triceps brachii at 1 d post mortem.

The porcine semitendinosus is unique in that it contains a dark and light portion. These portions contain primarily the oxidative red fiber type and the more glycolytic white fiber type, respectively [7-8]. These fibers may also undergo proteolysis at different rates. Abbott et al. [9] observed that white fibers from the porcine semitendinosus tended to have greater structural degradation at 192 hr post mortem compared to red fibers.

II. MATERIALS AND METHODS

A. Sample Preparation

Gilts (n=22) were euthanized at the Iowa State University Swine Nutrition and Management Farm. Skeletal muscle samples from the longissimus (n=22) and semitendinosus were immediately collected and transported on ice to the lab. The semitendinosus was divided into red (RST) (n=19) and white (WST) (n=22) portions. Sarcoplasmic protein was extracted according to the method of

Melody et al. [10]. Within 45 min post mortem, 10 g muscle samples were finely minced and 3 vol (wt/vol) of pre-rigor extraction buffer containing 100 m*M* Tris-HCl pH 8.3, 10 m*M* EDTA, 100mg/L trypsin inhibitor, 2 μ*M* E-64, and 0.1% 2-mercaptoethanol was added. Samples and buffer were homogenized in a Polytron using 3-30 s bursts. The homogenate was centrifuged at 25,000 x *g* for 20 min, and the supernatant was dialyzed against 40 vol of 40 m*M* Tris-HCl ph 7.4, 1 m*M* EDTA, and 0.1% 2-mercaptoethanol (TEM). The dialyzed sample was centrifuged at 25,000 x *g* for 20 min, and the supernatant was filtered through cheesecloth.

B. Ion Exchange Chromatography

Samples were loaded onto a 20 ml Q-Sepharose Fast Flow anion exchange column equilibrated with TEM. After washing, calpastatin, μ-calpain, and m-calpain were eluted using a linear gradient of 0-400 m*M* KCl in TEM. Calpastatin eluted in two separate peaks (calpastatin I and II, at 50-90 m*M* KCl and 120-190 m*M* KCl, respectively), followed by μ-calpain (180-240 m*M* KCl) and m-calpain (300-400 m*M* KCl).

C. Calpastatin and Calpain Activities

The activities of μ- or m- calpain or calpastatin-containing fractions were determined using casein as a substrate, using a modified method of Koohmaraie [11]. Protein content of the original sample was determined in order to calculate total activity on a total protein basis. Estimation of crude protein (Nitrogen x 6.25) was done using an Automated LECO Nitrogen Analyser (LECO-TruSpec® N, LECO Corporation, St. Joseph, MI, USA).

Data were analyzed as a complete randomized design using the MIXED procedure in SAS version 9.2.

D. Longissimus - Calpastatin Characterization

In a separate, larger preparation, 300 g of LD was taken from one gilt carcass within 30 min postmortem. The sample was extracted, purified, and assayed as described previously. The calpastatin isolates from this sample were assayed against porcine μ- and m- calpain from the same muscle. Samples were concentrated further using Amicon Centriplus-100. SDS gel samples were prepared according to Lonergan et al. [12]. Gel samples for 2-dimensional (2D) analysis were prepared by rehydrating 7 cm immobilized pH gradient strips (pH 3-10) with 200 μg protein and a Destreak™ solution (Destreak™ plus 20 m*M* dithiothreitol (DTT) and 1.5% IPG buffer). Strips were run using a step-wise gradient totaling 7400 Vh. A 12.5% polyacrylamide separating gel was used for calpastatin determination for one dimensional SDS-PAGE gels (SE 260 Hoefer Mighty Small, 10 cm wide by 8 cm tall) and the second dimension of the 2D gels (SE 280 Hoefer Tall Mighty Small, 10 cm wide by 12 cm tall). One dimensional gels were loaded with 40 μg protein per lane. Gels were transferred to PVDF membranes according to Melody et al. [10] and immunodetection was performed according to Huff-Lonergan et al. [13]. The primary antibody was MA3-945 monoclonal mouse anti-calpastatin IgG2a (Lot 169-106 Affinity Bioreagents) (diluted 1:10,000 in PBS-Tween for 1D and 1:5,000 for 2D), and the secondary antibody was goat anti-mouse IgG(Fc)-peroxidase (Sigma A2554) diluted 1:10,000.

III. RESULTS

Calpain and calpastatin activities for the LD, RST, and WST are reported in Table 1. The LD had the greatest ($P<0.0001$) μ-calpain activity and the least ($P<0.0001$) m-calpain activity. The RST and WST had similar μ-calpain activities, but the RST had greater ($P=0.0065$) m-calpain activity compared to the WST.

Values for calpastatin I were lower in all muscles compared to their values for calpastatin II. The WST was similar in calpastatin I activity to the LD ($P>0.05$); both the LD and WST had lower calpastatin I activity compared to the RST ($P<0.05$); however, the WST and RST were similar in their calpastatin II activity, which was higher than that of the LD ($P<0.0001$). Total calpastatin activity was different for all muscles. The LD had the lowest

(P<0.0001) total calpastatin activity, and the RST had slightly higher (P=0.0494) total calpastatin activity compared to the WST.

Table 1. μ-calpain, m-calpain, and calpastatin activities (units of activity per g of protein) in the longissimus (LD), red semitendinosus (RST), and white semitendinosus (WST).

Enzyme	LD	RST	WST	SEM
μ-calpain	3.52[b]	1.83[a]	1.63[a]	0.134
m-calpain	7.00[a]	9.90[c]	8.87[b]	0.269
Calpastatin I	3.99[a]	5.226[b]	4.40[a]	0.245
Calpastatin II	4.40[a]	11.51[b]	10.63[b]	0.486
Total Calpastatin	8.31[a]	16.74[c]	15.03[b]	0.629

SEM = standard error of the mean

[abc] Values in the same row with different subscripts are significantly different (P<0.05).

In the large preparation, calpastatin I and II were each about twice as active inhibiting m-calpain activity from the same LD compared to μ-calpain. Calpastatin I had 0.17 u/ml of activity against μ-calpain versus 0.33 u/ml against m-calpain. Calpastatin II had 0.63 u/ml of activity against μ-calpain versus 1.18 u/ml against m-calpain.

The Westerns for the 2D blots revealed two similar chains which appeared to be centered at different isoelectric points (Fig. 1). In addition, there were several very large spots detected in calpastatin II which appeared inconsistent with the chains. These spots appeared in identical locations in two separate blots for calpastatin II, so random background was ruled out.

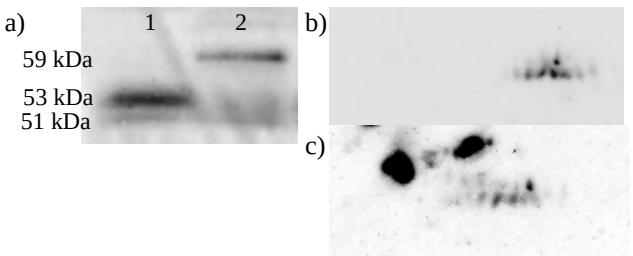

Fig. 1 Calpastatin Western blots. a) Lane 1- calpastatin I, Lane 2-calpastatin II, b) 2D Western blot of calpastatin I, c) 2D Western blot of calpastatin II

Western blots for calpastatin revealed differences between the two isoforms. One dimensional Western revealed three bands (Fig. 1): 59 kDa, 53 kDa, and 51 kDa. Calpastatin II contained primarily the 59 kDa band, but all three bands were present. Calpastatin I only contained the 53 kDa and 51 kDa bands.

IV. DISCUSSION

The LD and the semitendinosus sections had vastly different calpain/calpastatin profiles. The LD had a μ-calpain:total calpastatin ratio which was much more conducive to post mortem proteolysis (1:2.36 compared to 1:9.15 (RST) and 1:9.24 (WST), respectively).

The semitendinosus differed the most from the LD in its calpastatin II activity. To our knowledge, the two isoforms of calpastatin separated using anion exchange chromatography have not been documented in porcine skeletal muscle, although other groups have separated calpastatin from other species and tissues in this manner [14-16]. The conclusions of these other groups are conflicting; some have concluded that the two isoforms are the result of a posttranslational phosphorylation [14-15], while others have documented that they are the result of a molecular weight difference due to alternative splicing [16].

Our results showing that both calpastatin forms are most effective in inhibiting m-calpain are in contrast to those of Pontremoli et al. [15], who found that calpastatin I better inhibited μ-calpain while calpastatin II was more effective against m-calpain.

The one dimensional blots support a molecular weight difference, due to the 59 kDa band in calpastatin II, which is not present in calpastatin I. The 2D blots, however, support the phosphorylation theory; phosphorylation would decrease the isoelectric point and cause a shift to the left in the chain of proteins present on the blot. The large spots on the calpastatin 2D blot may be due to secondary binding; however, they may be analogous to the 59 kDa band present on the one dimensional blot. These spots may be a different isoform of calpastatin which has completely different isoelectric properties from calpastatin I.

V. CONCLUSIONS

The LD has conditions which are much more favorable for proteolysis compared to the semitendinosus. Between the RST and WST, there are differences in calpain and calpastatin activities which may affect post mortem aging as well. The WST has slightly less calpain system activity, but the ratio of protease:inhibitor is similar between the two sections.

The presence of two forms of calpastatin presents a source of variation which may have further implications in post mortem proteolysis. One and two dimensional Western blotting showed biochemical differences between the two calpastatin forms, although whether the differences were due to phosphorylation or different isoforms could not be determined using the methods in this study.

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